T Cell Determinant Structure: Cores and Determinant Envelopes in Three Mouse Major Histocompatibility Complex Haplotypes

By Guy Gammon, H. Mario Geysen, Raymond J. Apple, Elaine Pickett, Mark Palmer, Ako Ametani, and Eli E. Sercarz

From the Department of Microbiology and Molecular Genetics, University of California, Los Angeles, California 90024; and *Coselco Mimotopes Pty Ltd., Clayton, Victoria 3168, Australia

Summary

T lymphocytes recognize discrete regions on an antigen. The specificity of the T cell responses in three mouse strains of differing major histocompatibility complex (MHC) haplotype to a protein antigen, lysozyme, was analyzed using a series of peptides that walk the antigen in single amino acid steps. These peptide series were synthesized using the pin synthesis system, which was modified to allow the peptides to be cleaved from the pins into a physiological buffer free of toxic compounds. This methodology overcomes many of the problems associated with the production of peptides for screening proteins for antigenic determinants. The T cell determinants for the three strains were markedly different. This result points out the limitations of algorithms predicting determinants without reference to the MHC, and the importance of the empirical methodology. This analysis of the T cell response to lysozyme constitutes the most complete study of reactivity to a foreign protein to date and illustrates many important features of antigen recognition by T cells, e.g., presence of major and minor determinant regions. The outer boundaries of each immunogenic region, the determinant envelope, are difficult to define from recently immunized lymph nodes because of the heterogeneity in T cell recognition. However, core sequences common to all the immunogenic peptides in a continuous sequence can be easily defined.

Unlike B cells, which can recognize free antigen, T cells recognize fragments of denatured antigen bound to cell surface MHC-encoded molecules. All immunogenic peptides can bind an MHC molecule, and non-self peptides, which bind with high affinity, are almost always immunogenic (1). Many regions of an antigen do not contain MHC-binding sequences, and are therefore nonimmunogenic, while some other regions, which appear capable of binding MHC, are not immunogenic because of a deficient T cell repertoire. Therefore, methods for detecting and/or predicting T cell-inducing determinants are needed. Recently, a model of the structure of MHC molecules has been proposed based on crystallographic data (2). In this model, there is no invariant peptide binding surface shared among different MHC alleles. Both the sides and the base of the putative antigen binding groove are polymorphic, which implies that no common MHC-binding motif should exist. Based on this information, we favor the empirical approach for locating T cell determinants rather than predictive algorithms, which make no allowances for MHC polymorphisms (3, 4).

T cell determinant regions on an antigen can be defined experimentally by testing primed T cells for reactivity to different areas of the molecule. The reagents used include homologous proteins from other species, fragments of the whole antigen produced by chemical or enzymatic degradation, and overlapping peptides produced by solid phase synthesis (5). Additionally, DNA sequences corresponding to different parts of the antigen may be expressed and T cells tested on the resulting products (6). The use of a panel of overlapping synthetic peptides is the most comprehensive approach and has been used to analyze T cell responses to several small proteins, including the malarial circumsporozoite protein (7), hen egg-white lysozyme (HEL)1 (8–11), and staphylococcal nuclease (12). Preparation of such a panel, even applying procedures for multiple peptide synthesis (13), is both very expensive and time consuming. To minimize these costs, the number of peptides synthesized is often reduced, so adjacent peptides

1 Abbreviations used in this paper: HEL, hen egg-white lysozyme; LNC, lymph node cells.
move along the sequence in large jumps. A typical panel would consist of peptides corresponding to residues 1–20, 11–30, 21–40, etc.). This has a number of disadvantages. First, determinants may be missed if they span two peptides and are larger than the peptide overlaps. Second, although reactivity to a peptide may be detected, additional peptides need to be synthesized to define the determinant boundaries. Therefore, to reduce the time needed to prepare a complete panel of peptides, we have used an adaptation of the pin synthesis system, which enables the rapid synthesis and cleavage from the solid phase support of small amounts of many different peptides (14). Peptides are synthesized on pin heads inserted into the wells of a 96-well plate. After deprotection, each peptide is cleaved from the pin by exposure to neutral pH PBS, and the peptide solutions can be added to T cell cultures with no further treatment. The relative ease and speed of this methodology allows the synthesis of a peptide series that progresses along the molecule in single amino acid steps, (e.g., 1–10, 2–11, 3–12, etc.), so that determinants will only be missed if the peptides are too short. Also since each peptide is shifted by only one residue, the boundaries of a determinant can be accurately defined in the initial screening. It would be counter productive for the peptides to require purification before use since many are produced. Therefore, the synthesis conditions have been optimized so that the products can be directly used. This is described in detail elsewhere (14). To test the utility of these peptides, series of peptides of 10, 12, and 15 amino acids in length, corresponding to the complete chicken lysozyme (HEL) sequence (Fig. 1), were tested on HEL-primed lymph node cells from different mouse strains, whose HEL response profile was known from earlier studies (8–11).

Materials and Methods

Mice. B10.A/SgSvJ, BALB.B and BALB/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility.

Lymph Node Proliferation Assays. Mice (8–14 wk old) were immunized with 7 nmol (100 μg) of HEL in saline, in a 1:1 emulsion with CFA containing 1 mg/ml Mycobacterium tuberculosis strain H37Ra, (Difco Laboratories, Detroit, MI) in the hind footpads. After 10 d, the popliteal lymph nodes were removed and cell suspensions prepared. The lymph node cells (LNC) were cultured in 96-well plates at 5 × 10^6 cells/well in HL-1 medium (Ventrex Laboratories, Inc., Portland, ME) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml gentamicin, and the indicated concentrations of antigen. For the peptide series, one well per peptide was tested. The results were confirmed by repeat experiments. Proliferation was measured by addition of 1 μCi of [3H]thymidine (International Chemical and Nuclear, Irvine, CA) for the last 18 h of a 5-d culture, and incorporation was assayed by liquid scintillation counting.

T Cell Hybridoma Specificity Assays. The T cell hybridomas were derived by fusion of T cells from the HEL-specific T cell line A0H4 and the thymoma BW5147 as described in detail previously (11). Specificity of hybridomas was tested by culturing 10^5 hybrid cells with 5 × 10^6 irradiated syngeneic spleen cells and antigen in DMEM supplemented with 2 mM glutamine, 1 mM pyruvate, 0.05 mM 2-ME, and 10% FCS. The lysozymes were tested at 7-μM concentration and peptide series tested at 5–10-μM concentration. After 24 h in culture, 40 μl of supernatant was removed and added to 10^6 HT-2 cells. Proliferation was measured by [3H]thymidine incorporation 24 h later.

Peptide Synthesis. Control HEL peptides were synthesized by Dr. J. Young at UCLA or Dr. M. McMillan and L. Williams at USC, using a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA). The methodology has been described previously (15). The peptides were passed over a Sephadex G10 column (Pharmacia Fine Chemicals, Piscataway, NJ), and the peptide fraction was further purified as a single peak by HPLC on a preparative C4 column with a solvent system of 0.1% trifluoroacetic acid and an increasing gradient of acetonitrile. Peptide identity was confirmed by amino acid composition analysis. The 74-96 preparation gave a single major peak on analytical HPLC and, due to poor solubility, was not purified further. The complete series of HEL peptides were synthesized using the pin synthesis technique. The procedure was modified as described in detail elsewhere (14) so that the peptides could be cleaved from the pins. The first amino acid residue added in each case was proline followed by Bodyshine (Fmoc)-OH. The Fmoc protecting group from the ε amino group was removed, and additional Fmoc-protected amino acids were added in a stepwise fashion. 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 PBS, under which conditions the COOH-terminal lysine-proline residues underwent diketopiperazine formation. This methodology, unlike that described previously (16), allows peptides of any sequence to be cleaved. Peptide yield was estimated as described (14). The approximate concentrations of the 10 amino acid series was 50 nmol in 100 μl, and for the 12 and 15 amino acid peptides, 10–20 nmol in 150 μl. The peptides were added to the primed LNC to a final concentration of 10 μM for the 10 amino acid peptides, and 5–10 μM for the 12 and 15 amino acid peptides.

Data Analysis. The complete series of HEL peptides was tested with one well for each peptide. The background proliferation to peptide synthesis byproducts was assayed on the non-HEL peptides PLRQGGGGGG or GLAQGGGGGG with additional COOH-terminal glycine residues for 12 and 15 amino acid sets. A peptide was considered to have generated a positive response if, either on its own it reproducibly stimulated a response of greater than the background + 3 x SD, or it and an adjacent peptide induced a response above this level. This low threshold was chosen to maximize sensitivity. No false positives were detected using these criteria.

Results

Specificity of Response by B10.A and BALB.B Mice. There are numerous T cell determinants on HEL recognized by B10.A (H-2b) and BALB.B (H-2b) T cells, although the level of reactivity to each after HEL priming is unequal. Responsiveness to major determinant regions can readily be detected after priming with native HEL but responses to minor ones are often very weak and may only be detected after peptide immunization. The determinants recognized by B10.A mice, in approximate order of importance, are contained within the following HEL peptides: 46–61, 20–35, 116–129, 34–45, 95–109, 17–58, and for BALB.B, 74–90, 30–53, 81–96, 20–35, and 46–61 (8–11). The data in Fig. 2 show reactivity detected by the peptide series in B10.A mice to the major determinant regions within 46–61 and 20–35, with lesser reactivity to the minor determinant regions within
Chicken (HEL)  
Guinea Fowl (NEL)  
Bobwhite quail (BEL)  
Japanese quail (JEL)  
Turkey (TEL)  
Ring-necked pheasant (REL)  
Mouse  

34–45, 1–17, 116–129, 85–96, and 74–82. Responses to three other HEL peptides, 25–43, 90–110, and 105–120, have been reported in the H-2^k mouse strain C3H (9), and therefore, B10.A mice would probably respond to immunization with these peptides. However, these peptides contain minor determinant regions only, requiring direct peptide priming to stimulate a response, and so the failure of the peptide series to recall reactivity in these regions was not unexpected.

BALB.B mice can be seen to respond mainly to determinants within the 30–53 and 74–96 peptides, and to a lesser extent to determinants within 13–35 and 46–61. In both strains reactivity was usually detected using peptides of all three lengths, although the 10-mer series barely detected reactivity with 20–35 in B10.A mice and stimulated only weak reactivity to the 74–96 region in BALB.B mice.

The data shown in Fig. 2 were obtained by testing one well per peptide. The assays were performed in this way to minimize the size of each experiment, which would be of critical importance in screening long sequences. Rather than set up replicate wells, complete experiments were repeated using different groups of mice. The results obtained in repeat experiments were highly concordant (for example, see Fig. 3). The significance of reactivity in a single well is reinforced by reactivity in adjacent wells. This is seen especially with the series of 15 mers, since more than one adjacent peptide in a determinant region is capable of recalling proliferation. It should also be noted that the data obtained with either the 10, 12, or 15 amino acid–long peptide series, which were synthesized independently, are consistent with each other.

Figure 1. Amino acid sequences of lysozymes from different species. The lysozyme sequences were obtained from the National Biomedical Research Foundation database. The peptide series synthesized corresponded to the HEL sequence shown except for the substitution of aspartic acid for asparagine at position 103.
This not only shows the reproducibility of T cell proliferation between experiments but also demonstrates that different lots of peptides yield reproducible results.

Specificity of Response by BALB/c Mice. Previous investigation of the HEL-induced response in BALB/c mice showed dominant reactivity to a single region within peptide 108–120 (17, 18). However, because of the limitations of using an arbitrarily chosen panel of peptides, other major determinant regions could have been overlooked. Therefore, we examined the specificity of the T cell response in BALB/c (H-2b) mice using the HEL peptide series. The data shown in Fig. 4 clearly confirm the presence of a dominant T cell determinant region within the previously defined area, and show that no additional major determinant regions are recognized. Thus, the response appears to be localized to one dominant region, a part of the HEL molecule that BALB.B mice apparently do not recognize. Comparison of the results from the three mouse strains shows a remarkable degree of variability in response specificity. This reflects the genetic polymorphisms in the MHC, which is presumably the result of strong evolutionary pressure for diversity in T cell responses. These and similar data on the response to λ repressor peptides (19) demonstrate that different peptides are immunogenic in individual strains and, consequently, that the only secure approach to identify all determinant regions is an empirical one.

Specificity of HEL-reactive T Cell Hybridomas. The limitations of the previous methodology for defining determinant regions are apparent in that a high frequency of T cell hybridomas isolated even from well-studied strains do not react to the known immunogenic peptides. For example, although the peptide specificity of HEL-induced responses in B10.A mice has been extensively studied, three out of nine B10.A T hybridomas derived in this laboratory fail to react to any of the known HEL determinants (11). Two of these hybrid-
Figure 3. Reproducibility of the in vitro proliferative response of HEL primed B10.A LNC to pin-synthesized lysozyme peptides. LNC from four to eight HEL-immunized B10.A mice were pooled and tested for in vitro reactivity against 12 (a and b) and 15 (c and d) amino acid series, synthesized independently, covering the whole lysozyme molecule. a and c represent a different experiment from b and d. The peptide number at the bottom of each panel corresponds to the position of the NH2-terminal residue of the peptide in the HEL sequence. The average non-HEL peptide background response (cpm x 10^-3) of four to six wells ± SD was: a, 7.6 ± 1.3; b, 8.9 ± 0.8; c, 6.2 ± 0.6; and d, 7.8 ± 0.7. The non-HEL peptide background has been subtracted in each case. The responses to control conventional HEL peptides (cpm x 10^-3) in Exp. 1 (a and c) were: medium, 1.0; HEL, 142.8; peptide (p)20-36, 61.5; p30-53, 11.0; p46-61, 18.0; p74-96, 3.0; and p116-129, 7.9. The responses to conventional HEL peptides (cpm x 10^-3) in Exp. 2 (b and d) were: medium, 0.8; HEL, 162.3; p20-35, 98.4; p30-53, 16.4; p46-61, 32.3; p74-96, 7.9; and p116-129, 15.8.

Figure 4. The peptide specificity of the HEL-induced proliferative response in BALB/c mice. Four BALB/c mice were primed with HEL and the draining lymph nodes removed after 10 d. The LNC were pooled and tested for in vitro reactivity against the 12 amino acid (a) and the 15 amino acid (b) peptide series. The peptide number shown at the bottom of each panel corresponds to the position of the NH2-terminal residue of the peptide in the HEL sequence. The average non-HEL peptide background response (cpm x 10^-3) ± SD was: (a) 8.4 ± 1.1 and (b) 8.5 ± 0.8. The dashed line in each panel indicates three times the standard deviation of the background. The background has been subtracted. The responses to control peptides (cpm x 10^-3) were: medium, 8.1 ± 0.5; HEL, 134.2 ± 24.1; and p108-120, 136.3 ± 7.5.

The data in this paper clearly show the value of peptide series produced by the modified pin synthesis technique in

omas, A04H.H9.1 and A04H.H5.3, which were HEL specific and (I-A^k) restricted but did not react to any of the previously defined (I-A^k)-restricted determinants within peptides 13-35, 30-53, 46-61, 74-96, and 116-129, were examined further. Initially, we completed the conventional approach and tested the hybridomas on lysozymes from several different bird species. One hybrid, A04H.H9.1, responded to HEL, Japanese quail, bobwhite quail, and guinea fowl lysozymes but not to turkey or ring-necked pheasant lysozymes (Table 1 a). Comparison of the sequences of these lysozymes indicates three possible locations for this determinant, which would include residues 15, 41, or 73. No putative sites for the determinant recognized by the other hybrid could be defined since it reacted strongly to each lysozyme. The data in Table 1 b show that A04H.H9.1 reacted to the 10 amino acid peptide 73-82, which includes the polymorphic residue 73. The second

Discussion

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Table 1. Response of HEL-specific T Cell Hybridomas to Species Variant Lysozymes and HEL Peptides

| Antigen in vitro | Response of T cell hybridomas |
|------------------|------------------------------|
|                  | A04H.H9.1 | A04H.H5.3 |
| a. -             | 1.3 ± 0.8 | 1.1 ± 0.2 |
| HEL              | 96.6 ± 11.1 | 83.5 ± 11.5 |
| BEL              | 134.8 ± 4 | 95.0 ± 8.3 |
| NEL              | 170.3 ± 10 | 96.9 ± 6.4 |
| JEL              | 107.5 ± 7.3 | 202.2 ± 10.4 |
| REL              | 1.5 ± 0.3 | 87.6 ± 5.5 |
| TEL              | 1.5 ± 0.4 | 194.0 ± 3.0 |
| 74–96            | 0.9 ± 0.4 | 1.1 ± 0.0 |
| b. -             | 1.6 ± 0.4 | 0.5 ± 0.1 |
| HEL              | 48.5 ± 9.4 | 64.0 ± 3.8 |
| 71–80            | 1.3     |
| 72–81            | 1.6     |
| 73–82            | 8.3     |
| 74–83            | 1.4     |
| 46–60            | 0.5     |
| 47–61            | 0.4     |
| 48–62            | 53.2    |
| 49–63            | 73.1    |
| 50–64            | 65.5    |
| 51–65            | 71.6    |
| 52–66            | 68.9    |
| 53–67            | 0.5     |
| 54–68            | 0.4     |

The data in Fig. 2 show that the adjacent 15 amino acid peptides, from 31–45 through 38–52, recall a response in HEL-primed BALB.B LN. These peptides share residues 38–45, and this defines a “core” sequence essential for immunogenicity. Examination of the responses to the 10 and 12 amino acid peptide series (Figs. 2a, d, and e) confirms the importance of residues 38–45. The two determinants within peptide 74–96 recognized by BALB.B mice are difficult to define because they overlap but appear to have core sequences corresponding to 77–85 and 83–90. Several determinant core sequences can be defined for BALB.C mice. The size of the critical core sequence within the peptide 20–35 varies, with a core sequence of 23–32 in the 10-mer series (Fig. 2a), 24–31 in the 12-mer series (Fig. 2b), and 25–30 in the 15-mer series (Fig. 2c). Variation in the core sequence also occurs for the other major determinant region seen by BALB.C mice and contained within peptide 46–61. However, in this case it varies regardless of the size of the peptides used and the critical sequence fluctuates from 52–58 to 56–58, with all intermediate results. We have arbitrarily designated 54–58 as the core sequence for this determinant region. Core residues for determinants that recall weaker and more variable responses are difficult to define. This problem can be overcome by direct immunization with the appropriate peptide, which will induce strong responses even to minor determinants.

It is particularly interesting that the determinants recognized by the two hybridomas studied share all but one amino acid residue with known (I-Ak)-restricted determinants; A04H.H9.1 required residue 73 in addition to 74–82, and A04H.H5.3 required residue 62 in addition to the previously defined 52–61. This shows that the peptide products derived from processing of the native antigen are not the same as the experimental peptides that have been used. In fact, the natural peptides may be quite large compared with the size of the individual determinants they contain. Much attention has been focused on defining minimal determinants, but clearly this only has meaning for individual T cell clones. The most immunologically relevant parameter is the functional size of the “determinant envelope”, which includes all the critical residues related to each of the determinants recognized by individual T cells in a continuous region.

It is important to differentiate between the terms T cell-inducing determinant, determinant envelope, and core sequence. A T cell determinant is the shortest peptide that stimulates a response of normal magnitude in a T cell clone. This minimal peptide contains MHC-binding residues, which form the agretope, and T cell contact residues, which form the epitope (21). The determinant envelope includes all the overlapping determinants recognized in a continuous stretch of the sequence. The core sequence consists of the residues shared by all the immunogenic peptides in a region. Nevertheless, the core itself may not be immunogenic. Although this sequence contains residues absolutely required for antigenicity by all clones, other residues on either side of the core sequence may be needed for activation of individual T cell clones.
The determinant recognized by two (I-A^k)-restricted T cell clones has been previously defined as 52-61 (20, 22). Amino acids 52, 58, and 61 were defined as MHC contact residues, and 53, 56, and 57 as T cell contact residues. However, the T cell hybridoma, AO4H.H5.3, recognizes a different determinant, 52-62, within the determinant envelope. Analysis of the response to this region by HELprimed B10.A LNC defined a core sequence of 54-58. The small size of this core does not indicate a restricted diversity in the response. In fact, it indicates the opposite, since given a core of 13-35, shown as a crosshatched bar, is observed. This peptide contains two major determinant regions in the related strain B10.GD (I-A^k, I-Ed^-) mice and the core sequences defined by the peptide series in the latter are 13-21 and 22-32 (unpublished data). The determinant regions predicted by the methods of Rothbard and Taylor (3) and Margalit et al. (4) are also shown. In the case of the latter, only regions with a high amphipathic score above 8 are shown, in accord with the authors recommendation. For the former, seven core regions are predicted, 1-4 (KVFG), 54-57 (GILQ), 61-64 (RWWC), 87-91 (DITAS), 97-100 (KIVS), and, in reverse order, 30-33 (CAAK) and 121-125 (QAWIR).

The outer boundaries of the determinant envelope are difficult to delineate, but for all practical purposes, knowledge of the core sequence, which can easily be defined, is sufficient. A peptide centered on the core sequence will have maximal reactivity for its length. Determinant envelopes can be named according to their core sequence, rather than what is done at present, after a peptide that fortuitously contains some or all of the region. Thus, the (I-A^k)-restricted determinant envelope with core sequence 54-58 was previously defined by the tryptic peptide 46-61, which misses some of the determinant envelope. From the vaccinologist’s point of view, it should be easy to compare the position of the core sequences of determinant envelopes recognized by T cells from MHC-disparate individuals. This will enable regions of the antigen to be synthesized that will have the best chance of being immunogenic in the largest number of individuals.

A summary of the data obtained in this study is shown.
in Fig. 5. The filled bars show the determinant cores detected using the peptide series. The open bars show peptides found previously to be immunogenic in the indicated strains. The analysis using the peptide series clearly yields much more information on the exact location of the determinant region than the previous analyses. The sites of determinant regions predicted by the methods of Margalit et al. (4) and Rothbard and Taylor (3) are also shown. These predictive algorithms were only designed to detect regions of a protein likely to be immunogenic. Each predictive algorithm accurately defines some determinant regions, but misses others. Since neither algorithm was designed to take into account variability in MHC haplotype in the prediction of T cell determinants, peptides synthesized on the basis of these predictive methods may be immunogenic in one or more strains, but not in every strain.

Peptides produced by the modified pin synthesis technique clearly have great potential for detecting and defining T cell determinants, especially the dominant ones in any MHC haplotype. The data in this paper were obtained in a short period of time and, as shown in summary form in Fig. 5, not only correlate accurately with the previous data generated over several years in many laboratories but provide additional information on the determinant regions. These data constitute the most complete analysis of T cell reactivity in several MHC-disparate mouse strains to a model protein antigen. Individual determinants can be examined further using this same synthesis technology by producing peptide series containing multiple single substitutions at each site. The analysis of peptide recognition by T cells is just one example of a protein-protein interaction whose consequences can be studied using a peptide series; this technology should be applicable to protein-protein interactions in many biological systems.

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Address correspondence to Eli E. Sercarz, Department of Microbiology and Molecular Genetics, 5304 Life Sciences Building, UCLA, 405 Hilgard Avenue, Los Angeles, CA 90024. G. Gammon's present address is Xenova Limited, 545 Ipswich Road, Slough SL1 4EQ, Berkshire, UK.

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