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# Processing of lysozyme by macrophages: Identification of the determinant recognized by two T-cell hybridomas

(tryptic peptides/HPLC/antigen handling)

PAUL M. ALLEN, DANIEL J. STRYDOM, AND EMIL R. UNANUE

Department of Pathology and Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, MA 02115

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ABSTRACT **The purpose of this study was to identify the fragment of the hen egg-white lysozyme (HEL) molecule presented by macrophages to helper T cells. This was investigated by using T-cell hybridomas and macrophages prefixed in paraformaldehyde. We previously had shown that such prefixed macrophages could present a tryptic digest of HEL. The tryptic peptides were separated by HPLC and tested for their ability to stimulate the T-cell hybridomas. Only one tryptic peptide was found to be immunogenic. This immunogenic peptide was identified as the tryptic peptide T-8, containing amino acids 46–61. The precise determinant on the peptide T-8 being recognized was further defined by testing the response of the two T-cell hybridomas to human lysozyme. Neither clone responded to human lysozyme. From the amino acid sequence of human lysozyme, the determinant was localized to the four amino-terminal residues. Cleavage of the immunogenic peptide with either chymotrypsin or protease V-8 completely abolished the immunogenicity. This suggested that the T-cell determinant is located in the hydrophilic amino-terminal residues and that it must be associated with a hydrophobic stretch of amino acids, which allows the peptide to associate with the macrophage plasma membrane.**

The requirement for an antigen-presenting cell in the induction of an immune response has been well established (1–3). Helper T cells must recognize a protein antigenic determinant presented by an accessory cell that also expresses the class II polypeptides encoded in the *I* region of the major histocompatibility complex of the species (the Ia antigens). The nature of the antigenic determinant recognized by T cells is not known, although a substantial number of studies suggest that T cells are recognizing denatured determinants of linear sequences of amino acids rather than native determinants (4–6). In contrast, the majority of the B-cell response is directed against native or conformational determinants (4–8). Thus, the initial study of Gell and Benacerraf showed that guinea pigs immunized with ovalbumin (Ova) were triggered by delayed-type hypersensitivity by either denatured or native Ova, while only native Ova was capable of inducing anaphylaxis (9). This basic phenomenon later was shown to apply to many other antigens and in better defined systems such as T-cell proliferation or helper T-cell activity (5, 6). Recently, the fine studies of Chesnut *et al.* with Ova demonstrated that T cells that recognized denatured Ova were the same cells that recognized native Ova, indicating that the native molecule must eventually be altered and recognized as denatured (4). In contrast, the B-cell product, the antibody molecule, that recognizes native Ova does not crossreact with denatured Ova.

Studies directly testing for antigen processing were performed in our laboratory when we examined the handling by macrophages of the bacteria *Listeria monocytogenes*, using a T-cell binding assay (10, 11). We were able to demonstrate conclusively that an intracellular processing step was required for antigen presentation and that the processing step was inhibited by the lysosomotropic agents chloroquine or ammonia. Furthermore, we showed that the macrophage plasma membrane was the site of recognition of the antigen by T cells: paraformaldehyde-treated macrophages that had processed *Listeria* bound the *Listeria*-specific T cells as effectively as live macrophages (12). Subsequently, these findings involving antigen-processing have been confirmed by using other antigens (13) particularly Ova in Grey's laboratory (14). They demonstrated that an antigen-handling step sensitive to chloroquine applied to Ova. This indicated that a processing event was also required for soluble proteins.

Even after this demonstration of an obligatory intracellular processing step, the precise steps involved in antigen-processing are largely unknown. To further delineate the steps, we chose to examine the handling and processing of a single antigenic determinant on the hen egg-white lysozyme (HEL) molecule. HEL is a well-defined protein both biochemically and immunologically, which makes it an ideal antigen to study (15–17). Initially, we generated T-cell hybridomas reactive against HEL to use as our functional probes of a single determinant. With live macrophages as antigen-presenting cells, two clones responded to all three forms of antigen—the native HEL, the intact denatured carboxymethylated HEL (CM-HEL), and a tryptic digest (TD). Shimonkevitz *et al.* have just reported the ability of prefixed antigen-presenting cells to present fragmented Ova, but not native Ova to Ova-specific T-cell hybridomas (18). We confirmed and extended these findings, using the two HEL-specific T-cell hybridomas (19). When prefixed macrophages were used, neither clone responded to native HEL, while both clones responded to the TD. A differential response was seen using CM-HEL with the T-cell line 2A11 not responding, in contrast to the line 3A9, which did respond. These results with prefixed macrophages precisely correlated with the processing requirements for the three forms of HEL as determined by sensitivity of the live macrophages to treatment with chloroquine, (i.e., the live macrophages treated with chloroquine would not present HEL but would present the TD).

In this paper we report on the identification of a single determinant on HEL recognized by these two T-cell hybrids. We first identified the tryptic fragment that stimulated both clones and then further defined the determinant by using lysozymes from other species and subfragments of the tryptic peptide.

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Abbreviations: Ova, ovalbumin; HEL, hen egg-white lysozyme; CM-HEL, carboxymethylated hen egg-white lysozyme; IL-2, interleukin 2; TD, tryptic digest of HEL; T-8, tryptic fragment of HEL containing amino acids 46–61.

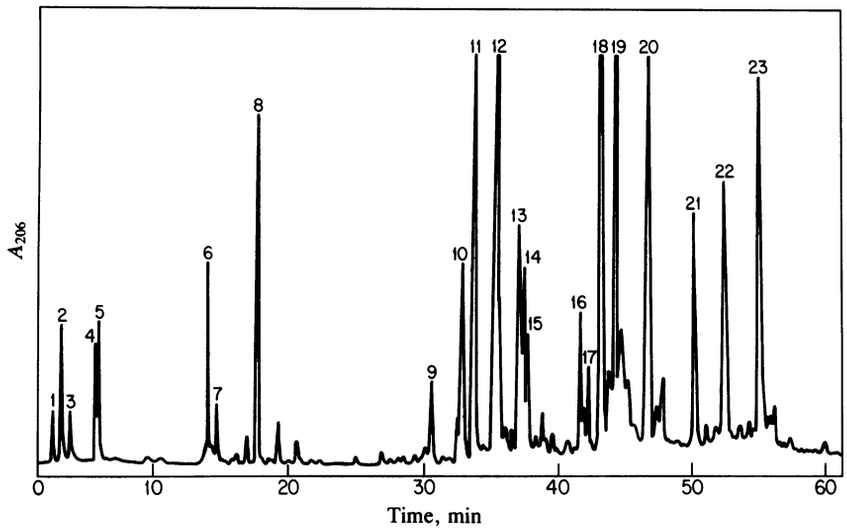


FIG. 1. The separation of the tryptic fragments of HEL by HPLC. Samples from the 23 indicated peaks were then tested for their immunogenicity.

## MATERIALS AND METHODS

**Mice.** CBA/J mice (The Jackson Laboratory) were used between ages 12 and 20 wk.

**Antigens.** The preparation of HEL, CM-HEL, and TD has been described (19). Human lysozyme was purchased from Alpha Therapeutics (Los Angeles, CA) and was used without further purification.

**Enzymatic Digestions.** Digestion of the immunogenic tryptic peptide T-8 was done as follows: 100 μg of T-8 was resuspended in 0.05 M Tris (pH 8.0), TLCK-treated chymotrypsin (Sigma) was added (1 μg), and the solution was incubated at 37°C for 20 hr. Also in another digestion, 100 μg of T-8 was resuspended in 0.1 M sodium phosphate buffer (pH 7.8). Protease from *Staphylococcus aureus* strain V-8 (Miles) was added (1 μg), and the solution was incubated at 37°C for 20 hr.

**Culture Conditions.** The generation, characterization, and assay conditions of HEL-specific T-cell hybridomas have been described (19). Briefly, $10^5$ peritoneal macrophages elicited by peptone from *Listeria monocytogenes*-infected mice were adhered in a microtiter well (Costar, 3596). The macrophages were left viable or fixed with 1% paraformaldehyde. T-cell hybridomas ($10^5$) were added to the macrophages along with antigen and cultured at 37°C for 22–24 hr. The culture supernatant was then assayed for the presence of interleukin 2 (IL-2) by tritiated thymidine incorporation into the IL-2-dependent CTLL cell line.

**Amino Acid Analysis.** Samples were hydrolyzed in 6 M HCl containing 0.1% phenol at 105°C for 24 hr (20). The amino acid content was then determined by using a Durrum D-500 automated amino acid analyzer.

**HPLC.** The tryptic fragments of HEL were separated by reverse-phase HPLC by using a Waters HPLC system consisting of model 6000A pumps, WISP automatic sample injector, and model 720 system controller. A 250 mm × 4.6 mm Altex Ultrasphere IP Column (5 μm, $C_{18}$) was used with buffers A (0.1 M $NaClO_4$/0.1% $H_3PO_4$, pH 2.3) and B (75% $CH_3CN$) (21); 20 nmol of the TD was chromatographed by using a 60-min linear gradient from 3%–70% B. The column

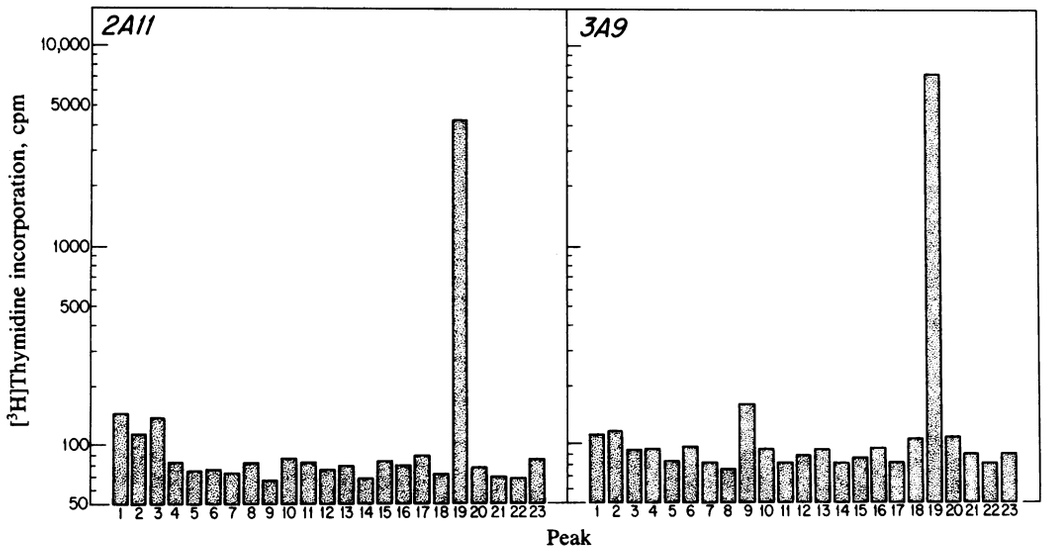


FIG. 2. The testing of the 23 HPLC peaks for their ability to stimulate the T-cell hybridomas. Peritoneal exudate cells ($10^5$) were adhered in a microtiter well and then fixed with 1% paraformaldehyde. Samples from each of the peaks were then added to the wells along with either 2A11 or 3A9 T cells. At the end of 24 hr, supernatants were assayed for the presence of IL-2. The values represent the mean of duplicate values. The standard deviation of these values never exceeded 20%.

effluent was monitored at 206 nm (LKB 2138 Uvicord S) and 254 nm (Waters M440) and 0.5-min fractions were collected. [We also separated the tryptic fragments by using volatile buffers for buffers A (0.1% trifluoroacetic acid) and B (75% $CH_3CN$/0.09% trifluoroacetic acid). We could identify a single peak that contained the reactivity; however, further examination of this peak showed that it contained at least three different peptides.]

## RESULTS AND DISCUSSION

We first wanted to determine which tryptic fragment of HEL was recognized by each of the clones. The tryptic fragments of HEL were separated by using reverse-phase HPLC. A typical separation is shown in Fig. 1. Samples from 23 of the peaks were then tested for their ability to stimulate either the 2A11 or 3A9 T-cell hybridomas. Aliquots from each peak were dried, resuspended in culture medium, and then added to microtiter wells containing $10^5$ prefixed macrophages and $10^5$ T-cell hybridoma cells. The results (Fig. 2) were unequivocal in that peptide 19 was the only fragment capable of stimulating the T-cell hybridomas. To identify the tryptic fragment contained in peak 19, a sample was subjected to amino acid analysis (Table 1). The amino acid composition of peak 19 fits exactly with the known composition of tryptic fragment T-8, first described by Canfield, which consists of amino acid residues 46–61 (22). Thus, using reverse-phase HPLC, we were able to purify in one step and to identify a single tryptic fragment containing residues 46–61, which was recognized by both T-cell hybridomas 2A11 and 3A9.

To further identify the determinant that the T-cell hybridomas were recognizing, we compared the sequence of the HEL peptide 46–61 to the corresponding sequence in mouse lysozyme. Peritoneal macrophages synthesize and secrete large amounts of lysozyme (23). Neither of the cell lines responded to peritoneal macrophages in the absence of antigen. This indicated that the cell lines were not autoreactive, and they must be recognizing a determinant unique to HEL and not found in mouse lysozyme. A comparison of the sequence between HEL and mouse lysozyme reveals that there are five amino acid differences in the T-8 peptide: Asn-46, Thr-47, a deleted Gly between positions 47 and 48, Gly-49, and Leu-56 (24) (Fig. 3). The known amino acid sequence of human lysozyme shows that it differs from HEL in the T-8 peptide region in four residues, three of which are located in the amino terminus (Fig. 3) (25). We then tested the ability of both cell lines to respond to human lysozyme, using live macrophages (Table 2). No detectable response was seen with human lysozyme even when a 10- to 100-fold excess of antigen was used.

To further define the precise determinant recognized by the T-cell hybridomas, the T-8 peptide was cleaved with either chymotrypsin or protease V-8. The fragments were separated by using reverse-phase HPLC, identified by amino acid analysis, and then tested for their ability to stimulate the T-cell hybridomas with prefixed macrophages. Fig. 4 depicts the fragments that were purified and subsequently tested. The fragments failed to stimulate the T-cell hybridomas, even when used at concentrations in excess of those necessary to see stimulation with the whole TD (Table 3). The failure of any of these fragments to stimulate either clone indicated that the intact peptide of 16 residues, or at least a peptide larger than 8 residues, was required to stimulate the T-cell hybridomas when prefixed macrophages were used.

Table 1. Amino acid analysis of peak 19

| | No. of residues | Tryptic peptide T-8 (46–61) |
|---|---|---|
| Asp | 3.58 | 4 |
| Thr | 1.88 | 2 |
| Ser | 1.73 | 2 |
| Glu | 1.04 | 1 |
| Gly | 1.99 | 2 |
| Ile | 1.85 | 2 |
| Leu | 1.00 | 1 |
| Tyr | 0.98 | 1 |
| Arg | 1.00 | 1 |

The number of residues was based on one arginine residue.

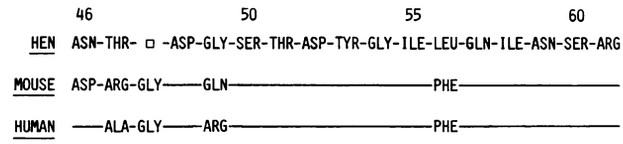


FIG. 3. The amino acid sequence of the T-8 tryptic fragment of HEL and the corresponding peptides in mouse and human lysozymes. The amino acid numbers refer to the residues in the HEL. Mouse and human lysozyme have an additional Gly between residues 47 and 48. Homologous residues are indicated by a solid line.

We have shown that the intact 16-residue peptide is required for stimulation of the two T-cell hybridomas. This 16-amino-acid peptide has two distinct regions. The amino-terminal region, residues 46–50, contains hydrophilic amino acids and the residues that we have identified as being different from mouse and human lysozyme, which we believe are recognized by the T cells. A second region, residues 53–58, is hydrophobic, containing the hydrophobic residues Tyr-53, Ile-55, Leu-56, and Ile-58. Rose has calculated the hydrophobicity profile for HEL, and these two regions are easily identified (26). The region 46–50 is one of the most hydrophilic regions of the molecule and actually corresponds to a peptide chain turn, while the profile for the region 51–61 contains a hydrophobic peak centering on Leu-56. When the 16-amino-acid peptide is cleaved in half at residue Tyr-53 by chymotrypsin, neither fragment is able to stimulate the T-cell hybridomas (Table 3). We suggest that the determinant recognized by the T cells is present in the fragment 46–52, but that the hydrophobic region is also required. The presence of this hydrophobic region could allow the intact peptide to associate with the macrophage plasma membrane, and then the amino-terminal hydrophilic region could be available to be recognized by the T cells. An alternative explanation is that the amino acid differences in peptide 46–53 between mouse/human lysozyme and HEL are reflected in changes in peptide 54–61. These differences may influence the reactivity of the entire T-8 peptide with the T-cell receptor and/or membrane-anchoring structure.

Table 2. Lack of reactivity against human lysozyme

| T-cell line | Antigen concentration, μg/ml | [$^3$H]Thymidine incorporation, cpm | |
|---|---|---|---|
| | | HEL | Human lysozyme |
| 2A11 | 2.5 | 146 (27) | 100 (4) |
| | 7.9 | 702 (218) | 139 (46) |
| | 25 | 2,939 (112) | 100 (25) |
| | 79 | 7,278 (941) | 104 (14) |
| | 250 | 9,678 (382) | 62 (4) |
| 3A9 | 2.5 | 8,390 (2490) | 57 (24) |
| | 7.9 | 11,517 (3864) | 62 (13) |
| | 25 | 11,666 (2169) | 62 (19) |
| | 79 | 10,677 (2040) | 47 (5) |
| | 250 | 11,815 (2299) | 75 (19) |

Peritoneal macrophages ($10^5$) were adhered in a microtiter well. Either HEL or human lysozyme were added along with 2A11 or 3A9 T cells. After 24 hr the supernatant was removed and assayed for the presence of IL-2 by [$^3$H]thymidine incorporation into the CTLL cell line. The values are the mean (standard deviation) of triplicate wells.

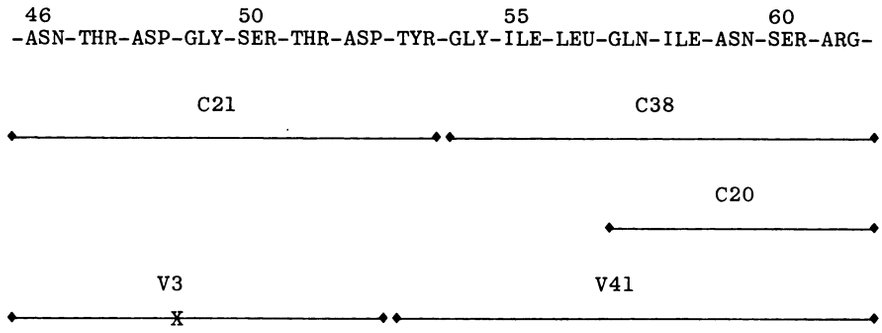


FIG. 4. The tryptic fragment T-8 was treated with either chymotrypsin or protease V-8. The resulting fragments were purified by HPLC and identified by amino acid analysis. The fragment V-3 contained the residues 46–52. Most likely the protease V-8 cleaved after the Asp-48, yielding the two fragments 46–48 and 49–52; however, we did not separate these two fragments.

The location of the determinant on HEL that we have described is consistent with the findings of Sercarz and his colleagues (6). They have shown that *H-2*$^k$ strain T cells recognize determinants on HEL that are present in the large cyanogen bromide fragment L2, which is comprised of amino acids from 13 to 105. The determinant we have identified is clearly contained in this L2 fragment. Katz *et al.* have reported mapping the determinant recognized by a long-term T-cell clone (27) by using live antigen-presenting cells. They ascertained that the tryptic fragment T-11, residues 74–96, contained the determinant. Thus, it appears that in *H-2*$^k$ mice, several determinants on HEL are recognized by helper T cells, including one in the T-8 peptide and one in the T-11 peptide.

It was surprising that both T-cell hybridomas appeared to recognize the same tryptic peptide of HEL. We had previously shown that the two T-cell hybridomas differed in their reactivity to CM-HEL when prefixed macrophages were used (19). The most likely explanation for this paradox involves the nature of the T-cell receptors on the two T-cell hybridomas. Perhaps the explanation lies in the affinity of the receptor of the two clones for the 46–61 peptide, reflecting a difference in recognition of critical amino acids of the T-8 peptide. The precise conformation of CM-HEL is not known, but it has been determined that it is unfolded in water and has no enzymatic activity (28). Perhaps, in this unfolded state, the residues 46–50 are in a conformation that allows them to be recognized by T cells with high-affinity receptors, such as 3A9 cells, while T cells with lower affinity receptors could not be stimulated by the determinant in its present conformation. When the determinant is generated by live macrophages or extracellular processing by trypsin, the determinant is in a conformation that then can be recognized by both clones. There is some evidence that the 3A9 T-cell receptor may be of higher affinity than the 2A11 T-cell receptor because 3A9 cells require a lower concentration of antigen for maximal stimulation (Table 2). (It was possible that, in our CM-HEL preparation, there were some low molecular weight breakdown products and that the differential response to CM-HEL of the two T-cell hybridomas was merely a reflection of the response to the contaminating peptides. We have analyzed our CM-HEL preparation by $NaDodSO_4$/PAGE, dialysis, and column chromatography and have found no low molecular weight products. Therefore, we believe that the 3A9 clone is recognizing a determinant present on the intact denatured CM-HEL molecule.)

Our current model for the processing of HEL has HEL entering the macrophage, where it is then processed in a step sensitive to chloroquine in an endosome or lysosome. During the processing, the native HEL is proteolytically cleaved, and fragments that include the critical determinants are then transported to the plasma membrane, where they are then recognized in the context of an Ia molecule by a T cell. Very little is known about the shuttling of the immunogenic peptides to the plasma membrane, but the system we have developed with functional and biochemical probes against HEL will allow us to examine this transport process in detail.

Table 3. Fragments of T-8 fail to stimulate either T-cell hybridoma

| Antigen | Amount, pmol | [$^3$H]Thymidine incorporation, cpm 3A9 | | 2A11 | |
|---|---|---|---|---|---|
| T-8 fragments | | | | | |
| C20 | 20 | 35 | (4) | 40 | (1) |
| C21 | 54 | 41 | (7) | 38 | (5) |
| C38 | 61 | 49 | (23) | 32 | (2) |
| V3 | 34 | 31 | (1) | 32 | (9) |
| V41 | 18 | 55 | (13) | 31 | (1) |
| TD | 1.1 | 70 | (8) | 43 | (2) |
| | 3.5 | 582 | (24) | 55 | (7) |
| | 11.1 | 2,606 | (109) | 543 | (246) |
| | 35 | 7,762 | (740) | 2,622 | (405) |
| | 111 | 16,637 | (3,917) | 19,134 | (4,269) |
| | 350 | 21,281 | (1,454) | 23,734 | (5,792) |

To a macrophage monolayer, fragments of the T-8 peptide or TD in the number of picomoles indicated were added along with either 2A11 or 3A9 T cells. After 24 hr, the amount of IL-2 in the supernatant was determined by [$^3$H]thymidine incorporation into the CTLL cell line. The values represent the mean (standard deviation) of triplicate cultures.

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