Conformation-dependent recognition of a protein by T cells requires presentation without processing

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Presentation of a protein antigen to T cells is believed to follow its intracellular breakdown by the antigen-presenting cell, with the fragments constituting the trigger of immune recognition. It should then be expected that T-cell recognition of protein antigens *in vitro* will be independent of protein conformation. Three T-cell lines were made by passage *in vitro* with native lysozyme of T cells from two mouse strains (B10.BR and DBA/1) that had been primed with the same protein. These cell lines responded well to native lysozyme and very poorly to unfolded (*S*-sulphopropyl) lysozyme. The response of the T-cell lines to the antigen was major histocompatibility complex (MHC)-restricted. A line from B10.BR was selected for further studies. This line responded to the three surface-simulation synthetic sites of lysozyme (representing the discontinuous antigenic, i.e. antibody binding, sites) and analogues that were extended to a uniform size by a nonsense sequence. T-cell clones prepared from this line were specific to native lysozyme and did not respond to the unfolded derivative. Furthermore, several of these clones showed specificity to a given surface-simulation synthetic site. The exquisite dependency of the recognition by the clones on the conformation of the protein antigen and their ability to recognize the surface-simulation synthetic sites indicate that the native (unprocessed) protein was the trigger of MHC-restricted T-cell recognition.

INTRODUCTION

The presentation of a protein antigen to T cells is believed to be dependent on a first step in which the protein is internalized and digested down to fragments which reappear on the surface of the antigen-presenting cell (APC) and constitute the signal of immune recognition (Chestnut & Grey, 1985; Bjorkman *et al.*, 1987b; Unanue & Allen, 1987; Allen & Unanue, 1988; Fox *et al.*, 1988). It was proposed (Atassi, 1980) from evidence of protein molecular recognition by B and T cells that antigen presentation of a protein proceeds at the whole-molecule level. Even though evidence that supports this idea has been reported (see Discussion), presentation after processing remains by far the favoured hypothesis. Many consequences of this model can be predicted and tested. One important consequence is that if protein fragments, and not the intact protein, were the presented species which constitute the target of T-cell recognition, then the latter would be expected to be indifferent to protein conformation. This was tested here with the T-cell recognition of hen lysozyme.

Lysozyme and its *S*-alkylated derivatives have been shown to cross-react when lymph node cells are obtained from mice that had been primed with either molecule (Thompson *et al.*, 1972; Sugimoto *et al.*, 1975; Bixler *et al.*, 1984). Previously, workers in this laboratory, by employing a comprehensive synthetic strategy (Kazim & Atassi, 1980), determined the full profile of the continuous regions recognized by lysozyme-primed T cells (Bixler *et al.*, 1984). On the other hand, the regions recognized by antibodies were found to comprise spatially-adjacent surface residues that are mostly distant in sequence (Atassi, 1978). These discontinuous sites were

mimicked by 'surface-simulation' synthesis (Atassi *et al.*, 1976; Lee & Atassi, 1976; Atassi, 1978), in which the binding site residues are linked directly via peptide bonds with appropriate spacing and directionality into a single peptide which does not exist in the protein but mimics a surface region of it. It was fascinating, yet unexpected by present postulates, to find that these surface-simulation synthetic peptides were able to stimulate specific proliferative responses in lysozyme-specific B10.BR (H-2^k) T-cell lines (Bixler & Atassi, 1984).

The demonstration (Bixler & Atassi, 1984) that lysozyme-specific long-term T-cell cultures responded to the discontinuous (surface-simulation) sites indicated that it should be possible to isolate from these lines T-cell clones which should serve as precise reagents that will exhibit total dependency on the presentation of the intact molecule. We have isolated here several T-cell clones obtained by immunization of a mouse strain with native lysozyme and have found that they recognized only the native protein and were essentially unresponsive to its unfolded derivative.

MATERIALS AND METHODS

To prepare lysozyme-specific T-cell lines, B10.BR (H-2^k) and DBA/1 (H-2^d) 8–10-week-old mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were immunized subcutaneously at the base of the tail with 100 µg of native lysozyme (3 × crystallized; Sigma Chemical Co., St. Louis, MO, U.S.A.) emulsified in phosphate-buffered saline (PBS; 0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.2) and Freund's complete adjuvant. Long-term cultures of lysozyme-specific T cells were prepared from lymph node cells by periodic (every 10–12 days) passage

Abbreviations used: APC, antigen presenting cell; PBS, phosphate-buffered saline (0.15 M-NaCl in 0.01 M-sodium phosphate buffer, pH 7.2); SSP, lysozyme, *S*-sulphopropyl-lysozyme; MHC, major histocompatibility complex.

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with native lysozyme (100 µg/ml), in the presence of X-irradiated normal spleen cells but without exogenous growth factors, as described (Bixler & Atassi, 1984). Limiting dilution of the lymphocyte long-term culture (Yoshioka *et al.*, 1983), followed by a second limiting dilution gave lysozyme-specific T-cell clones. The specificity of the T-cell lines and clones was established by screening their proliferative response against intact lysozyme and against a lysozyme derivative in which the disulphide bonds were reduced and the resulting -SH groups were sulphopropylated (SSP-lysozyme) (Bixler *et al.*, 1984). Among the useful properties of SSP-lysozyme is its higher solubility as compared to the S-carboxymethylated analogue (Bixler *et al.*, 1984). The surface-simulation synthetic peptides (Fig. 1) have been described (Atassi, 1978). Analogues of these peptides that were extended to a uniform size of 14 residues (Fig. 1) were synthesized, purified and characterized by similar procedures.

RESULTS AND DISCUSSION

Two B10.BR T-cell lines and one DBA/1 line were prepared by passage *in vitro* with native lysozyme of native-lysozyme-primed T cells. These lines responded very well to native lysozyme but showed very low responses to SSP-lysozyme (2–9 % relative to lysozyme, Table 1) indicating that the clones within these three lines are overwhelmingly directed against the native protein.

To determine whether antigen presentation to these T-cell lines was MHC-restricted, APCs of different mouse haplotypes were employed to present lysozyme to these T-cell lines (Table 2). The T cells of B10.BR (H-2^k) recognized lysozyme only when antigen-presenting cells of H-2^k mice (B10.BR or C3H/He) were used. No stimulation was obtained with APCs of the q, d or b

haplotypes. Similarly, T cells of DBA1 (H-2^a) responded only to antigen presented by APCs of H-2^a mice, but did not respond when APCs of k, d or b haplotypes were used. It was therefore concluded that the presentation of antigen to the T-cell lines was under MHC restriction.

Line I.12 of B10.BR exhibited the strongest proliferative response to native lysozyme *in vitro* (Table 1), and was therefore selected for further studies. This line also responded very well to the three surface-simulation synthetic sites (Table 3) that are the target of antibody recognition (Atassi, 1978). It was recently shown that the recognition of peptides *in vitro* by T-cell lines (Bixler *et al.*, 1986) and clones (Atassi *et al.*, 1987) exhibits a non-specific peptide size requirement beyond the essential residues of the site. Since the sizes of the surface-simulation peptides were below the optimum size required for maximum T-cell simulation *in vitro*, the peptides were extended at their N-terminals by the nonsense sequence (Fig. 1). Indeed, these extended surface-simulation sites evoked a higher proliferation response in the T cells than the respective surface-simulation sites. The line mounted a very low response (less than 3 %) to SSP-lysozyme and did not respond to any of the linear consecutive overlapping peptides encompassing the entire protein chain (Bixler *et al.*, 1984) (results not shown).

From the above T-cell line, 26 clones were obtained by limiting dilution which were entirely specific to native lysozyme and did not recognize SSP-lysozyme. No clones were obtained that were reactive with both lysozyme and SSP-lysozyme. This frequency is fully expected from the behaviour of the parent line (Tables 1 and 3). Nine of these clones were studied in detail (Table 3). There was a slight possibility that these clones may have recognized continuous regions around the cysteine residues, and the regions were rendered unrecognizable because of the S-alkylation of these residues. This possibility was ruled

(a) Surface-simulation (SS-) sites

SS-Site 1	125 Arg-(Gly-Gly)-	5 Arg(Gly)-	7 Glu(Gly-Gly)-	14 13 Arg-Lys
SS-Site 2	62 Phe-(Gly)-	97 96 93 89 87 Lys-Lys-Asn-Thr-Asp		
SS-Site 3	116 113 114 Lys-Asn-Arg-(Gly)-	34 33 Phe-Lys		

(b) Extended surface-simulation (Ext SS-) sites

Ext SS-Site 1	125 Thr-Glu-Ala-Gly-Arg(Gly-Gly)Arg(Gly)Glu(Gly-Gly)Arg-Lys	5 7 14 13
Ext SS-Site 2	62 96 97 93 89 87 Gly-Ala-Glu-Thr-Glu-Ala-Gly-Phe(Gly)Lys-Lys-Asn-Thr-Asp	
Ext SS-Site 3	116 113 114 34 33 Gly-Gly-Ala-Glu-Thr-Glu-Ala-Gly-Lys-Asn-Arg(Gly)Phe-Lys	

(c) Nonsense peptide Gly-Ala-Glu-Thr-Glu-Ala-Gly-Gly-Ala-Glu-Thr-Glu-Ala-Gly

Fig 1. Surface-simulation synthetic sites of hen lysozyme

Structures of (a) the three surface-simulation synthetic sites of hen lysozyme to which the antibody recognition of the native molecule is directed (Atassi, 1978). Note that these structures do not exist in lysozyme but rather mimic surface areas by linking the respective 'contact' residues whose numbers in the sequence are indicated with appropriate spacing (residues in parentheses represent spacers) and directionality into a single peptide (for details, see Atassi, 1978). In (b), these surface-simulation sites were extended to a uniform size of 14 residues, which is the optimum peptide size for T-cell recognition (Yoshioka *et al.*, 1983; Bixler *et al.*, 1986; Atassi *et al.*, 1987), by the underlined nonsense sequence. (c) A control 14-residue nonsense peptide which represents two segments of the nonsense sequence used to extend site 3.

Table 1. Recognition of lysozyme- and SSP-lysozyme-driven T-cell lines

T-cell lines (1×10^4 cells/well) were cultured in the presence of X-irradiated syngeneic filler cells (5×10^5 cells/well) in RPMI 1640 containing 10% fetal bovine serum. The cells were challenged with several doses of protein or peptide in the dose range 1–200 $\mu\text{g/ml}$, except for concanavalin A (Con A) which was used at 0.5 $\mu\text{g/well}$. Lysozyme, SSP-lysozyme, peptide and controls were dissolved in RPMI media containing 0.05 M-urea (to enhance solubility of SSP-lysozyme). After incubation for 3 days at 37 °C, the cells were pulsed (16 h) with 2 $\mu\text{Ci/well}$ of [^3H]thymidine and then harvested and counted. Values represent means $\pm$ S.D. (c.p.m.) for six replicate wells obtained at optimum dose of antigen (given in parentheses) and have not been corrected for the label bound by control, unstimulated cells (medium). N.D., not done.

Antigen added	Strain (haplotype) ... T-cell line ...	[^3H]Thymidine incorporation (c.p.m. $\pm$ S.D.) at optimum dose ($\mu\text{g/ml}$, in parentheses)		
		B10.BR (H-2 ^k)		DBA/1 (H-2 ^q)
		I.12	II.13	II.7
Lysozyme		109 014 $\pm$ 3530 (50)	11 076 $\pm$ 820 (100)	12 578 $\pm$ 1052 (50)
SSP-lysozyme		2753 $\pm$ 570 (50)	1268 $\pm$ 156 (50)	2070 $\pm$ 115 (50)
Con A		133 733 $\pm$ 5662	74 300 $\pm$ 2183	47 930 $\pm$ 4810
Ribonuclease		310 $\pm$ 10	330 $\pm$ 77	1100 $\pm$ 30
Nonsense peptide		406 $\pm$ 75	N.D.	N.D.
None (medium)		283 $\pm$ 17	332 $\pm$ 95	1153 $\pm$ 56

Table 2. MHC-restriction of antigen presentation to lysozyme-specific T-cell lines

The T-cell lines were obtained from the lines in Table 1 after cryopreservation and two more passages with native lysozyme. T-cell lines were cultured (1×10^4 cells/well) as described in Table 1 in the presence of X-irradiated filler cells (5×10^5 cells/well) obtained from spleens of non-immune mouse strains representing k, q, d and b haplotypes. The cell lines were challenged with the appropriate optimal dose of native lysozyme, Con A (0.5 $\mu\text{g/well}$) as a positive control and unrelated protein (negative control). Results (given in mean c.p.m.) had a standard deviation of 7.5% or less, and have not been corrected for label bound by control, unstimulated cells.

Antigen-presenting cell		[^3H]Thymidine incorporation (c.p.m.)		
		B10.BR (H-2 ^k) T cells	DBA/1 (H-2 ^q) T cells	
Strain	Haplotype	I.14	II.15	II.9
B10.BR	k	24 510	14 168	919
C3H/He	k	28 463	11 672	859
DBA/1	q	780	445	14 632
Balb/c	d	765	410	839
C57/BL6	b	1813	380	890

out by the finding that these clones did not respond (results not shown) to the consecutive uniform synthetic overlapping peptides that encompass the entire lysozyme chain (Bixler *et al.*, 1984).

The responses of the T-cell clones to the surface-simulation sites and their extended analogues were also investigated (Table 3). Two clones (2D5 and 2H8) responded to surface-simulation site 3 and its extended

analogue, with the latter clone giving a better response to the extended analogue. Two clones (2F10 and 2A4) responded to surface-simulation site 2 and gave a slightly greater response to its extended analogue. The response of a given clone to the synthetic site was lower than its response to native lysozyme (Table 3). The remaining clones did not respond to the surface-simulation sites or their extended analogues, suggesting that they either recognized different discontinuous regions on the surface of native lysozyme or, alternatively, these peptides lack one or more contact residues that are essential for the recognition by these clones. When T and B cells recognize the same site on a protein molecule, T cells can recognize a region that is somewhat larger than that recognized by B cells (Bixler *et al.*, 1986; Atassi *et al.*, 1987). The absence of one contact residue can often render a peptide non-stimulatory towards T-cell clones, or drastically decrease its activity *in vitro* (Atassi *et al.*, 1987). This would also explain the lower response of clones D5, D12, F10 and H8 to the surface-simulation peptides relative to their lysozyme response. Because the surface-simulation sites mimic discontinuous surface features, the identification of any missing residue(s) from the peptide constructs is not so straightforward.

The recognition of the surface-simulation synthetic sites by T-cell lines and clones confirms the observation (Bixler & Atassi, 1984) that T-cell recognition is not restricted to continuous parts of the primary structure. It also rules out the possibility that the observed differences in the responses to native lysozyme and to SSP-lysozyme may be due to differences in the processing of the two molecules. It should be emphasized that the surface-simulation synthetic sites do not exist in native lysozyme and cannot possibly be generated by APC 'processing'. On the contrary, because of their architecture (Fig. 1), processing would lead to their disassembly on the surface.

The isolation of T-cell clones whose recognition *in vitro* is dependent so profoundly on native conformation would lead to the conclusion that the protein is presented

Table 3. Specificity of native lysozyme-selected T-cell line and clones

T-cell line or clones (1×10^4 cells/well) were cultured in the presence of X-irradiated syngeneic filler cells (5×10^5 cells/well). The cells were challenged with peptide or protein in the dose range 1–100 $\mu\text{g/ml}$, except for Con A which was used at 0.5 μg per well. The values represent the mean c.p.m. of six replicate analyses. The standard deviation (usually $\pm 8\%$ or less) is omitted to avoid overcrowding the table. Values in parentheses represent the optimum antigen dose at which the indicated response is obtained. (Ext) SS-site, (extended) surface-simulation synthetic site.

Antigen added	[^3H]Thymidine incorporation (c.p.m.) at optimum dose ($\mu\text{g/ml}$)									
	Line I.12	Clone 2A4	Clone 2A8	Clone 2A12	Clone 2D5	Clone 2D12	Clone 2F10	Clone 2G2	Clone 2H4	Clone 2H8
None (medium)	2860	1820	1100	1325	6447	2883	2952	472	260	4820
Lysozyme	90810 (50)	18840 (100)	75780 (100)	50048 (100)	18145 (100)	11524 (50)	106000 (50)	15970 (50)	8254 (100)	30877 (100)
SSP-lysozyme	5020 (50)	2374	1550	1440	6684	2990	3124	565	252	5534
SS-Site 1	10010 (10)	1790	1660	1275	6525	3130	3202	653	248	5270
SS-Site 2	13260 (10)	2910	1650	1743	6900	4720	6293 (50)	708	384	5525
SS-Site 3	18560 (20)	2450	1320	2408	11640 (20)	3060	3777	507	315	9363 (20)
Ext SS-Site 1	19350 (50)	2590	1630	1293	7347	3322	3120	769	350	5568
Ext SS-Site 2	20720 (50)	9500 (50)	2110	1541	7100	4695 (5)	10210 (10)	798	584	5498
Ext SS-Site 3	29330 (50)	2780	1440	1712	12863 (10)	3180	2965	680	418	12286 (20)
Con A	70180	18190	68030	31170	22756	18814	157875	13157	9960	23085
Ribonuclease	2960	2110	1060	1393	7115	2875	2938	489	280	5543
Nonsense peptide	3430	2140	1180	1310	6430	2850	2945	486	282	5275

in vitro by APC in its intact form to these T cells. Since these T cells were obtained by expansion *in vitro* of T lymphocytes that were educated *in vivo*, it can be concluded that native lysozyme was also the signal for priming of these T cells *in vivo*. Therefore, both *in vivo* and *in vitro*, the native protein was the molecular species presented by APC to these T cells.

Results of immunization with synthetic overlapping peptides encompassing entire proteins indicated that antigen presentation to T cells is independent of processing (Bixler & Atassi, 1985a; Bixler *et al.*, 1985; Atassi & Bixler, 1988a). A conformation-dependent T-cell clone which recognized apomyoglobin but not myoglobin was recently reported (Cohly *et al.*, 1988). Myoglobin recognition by site-specific T-cell clones is influenced by amino acid substitutions outside the site (Yoshioka & Atassi, 1989). Liposomes carrying MHC and protein can present the latter to T cells (Walden *et al.*, 1985, 1986a,b; Klein *et al.*, 1985; Klein, 1988). These findings, together with the location of the T-cell recognition sites in the three-dimensional structure of proteins (for reviews, see Atassi, 1984; Bixler & Atassi, 1985b; Atassi & Bixler, 1988b) and other reports (Mills *et al.*, 1986; Naquet *et al.*, 1987; Lee *et al.*, 1988) are consistent with the proposal that the presentation of antigen to T cells involves predominantly recognition of an intact protein and that processing is an accompanying phenomenon concerned with clearance (Atassi, 1980). Nevertheless, it is generally assumed that T cells recognize relatively short peptides bound in the groove of the MHC molecule (Bjorkman *et al.*, 1987a,b; Brown *et al.*, 1988). It is not clear at present

how a protein is presented intact, with site specificity and MHC restriction, in a model that does not fit this hypothesis.

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