T-cell recognition and antigen presentation of myoglobin

Protein recognition by site-specific T-cell clones is influenced by amino acid substitutions outside the site

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Six T-cell clones from SJL mice were prepared from T-cell lines that were obtained by passage with synthetic myoglobin (Mb) peptide 107–120. In addition, a T-cell clone, specific to this region of Mb, was isolated from a Mb-passaged T-cell culture. The proliferative responses of these clones to Mb variants from 14 different species were studied. It was found, as expected, that amino acid replacements within the site affected its recognition by the T-cell clones. In addition to these effects, the T-cell recognition site, like the sites recognized by antibodies, was also influenced by substitutions of residues that are close to site residues in three-dimensional structure but are otherwise distant in sequence. This is noteworthy in view of the fact that six of the clones were selected with a free peptide, and thus the environmental residues are clearly not part of the 'contact' residues of the site. These findings are discussed in relation to the presentation of the antigen and are interpreted as indicating that Mb is presented in its intact form to the T-cells *in vitro*.

INTRODUCTION

It is widely believed that the first step in the immune recognition of a protein antigen (i.e. its presentation to the T cell) proceeds via internalization of the antigen, its fragmentation and its re-appearance in smaller fragments on the surface of the antigen-presenting cell (Allen & Unanue, 1984, 1988; Chestnut & Grey, 1985; Simpson, 1986; Bjorkman *et al.*, 1987b; Unanue & Allen, 1987; Fox *et al.*, 1988). There is, however, considerable evidence (see Discussion) indicating that a protein antigen is presented intact both *in vivo* and in the *in vitro* assay (Bixler & Atassi, 1985; Bixler *et al.*, 1985; Klein *et al.*, 1985; Walden *et al.* 1985, 1986a,b; Atassi & Bixler, 1988a; Klein, 1988). The view that antigen presentation is a step subsequent to, and dependent upon, processing remains however the favoured hypothesis. Therefore, the mechanism of this complex phenomenon needs to be further investigated by a variety of different approaches.

We have previously reported (Atassi *et al.*, 1981) that the response to Mb by Mb-primed lymph node cells *in vitro* is influenced by amino acid substitutions in the sites and outside the sites. However, the complexity and multispecificity of the cell mixture in the bulk cultures rendered the interpretations somewhat uncertain. We have shown (Yoshioka *et al.*, 1983) that site-specific T-cell lines and clones with preselected specificity can be readily prepared from protein-primed lymph node cells by passage *in vitro* with a synthetic peptide representing the desired site of T-cell recognition. Thus, the specificity of such T cells will be precisely known. In this communication, using T-cell clones against one Mb site, the dependence of T-cell recognition *in vitro* on the native conformation of the antigen is investigated with Mb variants which have amino acid substitutions within the site and/or at external locations that are close to site residues in the three-dimensional structure but may otherwise be distant in sequence.

MATERIALS AND METHODS

Materials

The isolation and purification of myoglobins of various species (sperm whale, killer whale, finback whale, Atlantic dolphin, horse, cow, red deer, goat, chimpanzee, echidna, sea lion, chicken, dog and rabbit) were performed by previously reported procedures (Atassi, 1964, 1970; Twining *et al.*, 1980). The homogeneity of each Mb was confirmed by polyacrylamide-gel electrophoresis. The T-cell clones used in the present work were isolated from T-cell lines of sperm whale Mb-primed lymph node (LN) cells that were driven into a single preselected specificity by passage *in vitro* with extended site 4 of sperm whale Mb (peptide 107–120) as previously described (Yoshioka *et al.*, 1983). Another clone was isolated from a T-cell line obtained from sperm whale Mb-primed LN cells by passage *in vitro* with sperm-whale Mb. The precise boundaries of the site recognized by each of these clones were defined recently (Atassi *et al.*, 1987).

Proliferation assay of the T-cell clones

The proliferative activity of the T-cell clones in response to each of the 14 myoglobins and to peptide 107–120 was determined by ³[H]thymidine incorporation. T-cell clones (5×10^3 cells/well) and the X-ray-irradiated (3300 R) syngeneic filler cells (5×10^5) were co-cultured for 3 days at 37 °C with various doses of antigens or mitogens in 0.2 ml of RPMI medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with 1% (v/v) syngeneic normal mouse serum or 5% (v/v) fetal bovine serum. The cells were pulsed (18 h) with 2 µCi of [³H]thymidine (ICN, Irvine, CA, U.S.A.), then harvested onto glass-microfibre filters (Whatman, Clifton, NJ, U.S.A.) and their radioactivity counted by liquid scintillation. Each Mb and the peptide 107–120 were tested at varying concentrations in the range 2.5–100 µg/ml of

Abbreviations used: Mb, myoglobin; APC, antigen-presenting cell; LN cells, lymph node cells.

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culture. In addition, the responses of the clones to positive controls (concanavalin A) and to negative controls [purified protein derivative of tuberculin, lipopolysaccharide, hen egg lysozyme, bovine serum albumin, and sperm-whale Mb peptide 141–153 (extended site 5)] were determined to establish the viability of the cells and the specificity of the responses. Each protein and peptide was tested in this dose range at least twice with a given clone, each time in triplicate.

RESULTS

Proliferative responses to various myoglobins of T-cell clones preselected with extended site 4

T-cell clones were obtained from T-cell lines that were made entirely specific to extended site 4 (peptide 107–120) of sperm-whale Mb by passage with the peptide *in vitro* of LN cells from sperm-whale Mb-primed SJL mice. Six such clones (from two different cell lines) were examined for their proliferative responses *in vitro* to the passage

peptide 107–120, to sperm-whale Mb and to Mb from 14 other species as well as appropriate controls (Table 1). Each clone gave the expected response to the peptide and also responded to sperm-whale Mb. The response to the latter was, however, considerably lower than the response to the passage peptide. The responses to killer-whale Mb were higher than those obtained with sperm-whale Mb (Table 1), being either comparable to (clones E4.5.1, E4.4.4, E4.5.6 and E4.4.8) or somewhat lower than (clones E4.5.4 and E4.5.10) the response to the passage peptide. The response of a given clone to finback-whale Mb was either slightly higher than, or equal to, the response to Atlantic-dolphin Mb, with both responses being higher than the response to sperm-whale Mb but lower than the response to killer-whale Mb. On a molar basis, the optimum dose of the peptide was 3- to 20-fold higher than that of any stimulating Mb (depending on the clone and the Mb, see Table 1). This is not unusual and, in all likelihood, is caused by the conformational freedom of the peptide in solution (Atassi, 1975) and by

Table 1. Proliferative responses of T-cell clones obtained from long-term cultures that were passaged with synthetic extended site 4 (peptide 107–120) of sperm-whale Mb

The boundaries of the T site recognized within region 107–120 have been delineated previously (Atassi *et al.*, 1987) for these clones (except clone E4.4.4). The region recognized by clone E4.4.4, however, must reside within residues 107–120 because the specificity of the clone was preselected by driving the cultures *in vitro* with peptide 107–120. All myoglobins and extended sites 4 and 5 were tested at the same time, at least twice, in the range of 2.5–100 µg/ml, employing triplicates at each dose. The s.d. values (which are deleted in order not to overcrowd the table) were $\pm 10\%$ or less. n.d., not determined, PPD, purified protein derivative of tuberculin; LPS, lipopolysaccharides.

Challenge antigen	Clone... Specificity...	[³ H]Thymidine incorporation in c.p.m. (and optimum dose in µg/ml in parentheses)					
		E4.5.1 112–120	E4.4.4 n.d.	E4.5.4 111–120	E4.5.6 112–120	E4.4.8 112–120	E4.5.10 112–120
Sperm-whale Mb		10 100 (10)	138 000 (10)	7090 (5)	18 890 (10)	51 470 (40)	37 200 (20)
Pept. 107–120 (Ext. site 4)		20 520 (10)	276 460 (5)	22 410 (20)	41 610 (20)	105 800 (20)	80 150 (20)
Pept. 141–153 (Ext. site 5)*		470	1580	690	660	560	690
Killer-whale Mb		22 050 (20)	262 010 (20)	14 830 (20)	36 450 (20)	106 030 (20)	62 180 (20)
Finback-whale Mb		15 040 (5)	169 340 (5)	10 210 (5)	25 160 (10)	60 080 (20)	44 640 (20)
Atlantic-dolphin Mb		14 140 (5)	142 240 (5)	8720 (5)	20 210 (10)	59 190 (10)	43 900 (5)
Horse Mb		340	1280	630	440	620	600
Beef Mb		370	970	560	410	500	480
Red deer Mb		280	1000	950	1050	620	620
Goat Mb		n.d.	1200	610	n.d.	620	n.d.
Chimpanzee Mb		370	900	660	490	640	510
Echidna Mb		510	1050	460	470	620	690
Sea lion Mb		410	760	890	970	600	460
Chicken Mb		390	1010	630	630	520	490
Dog Mb		390	1200	620	640	530	600
Rabbit Mb		380	520	610	560	850	640
Concanavalin A		41 950	227 990	46 500	59 380	80 250	76 520
PPD		630	860	1050	790	1570	790
LPS		330	1440	340	480	430	n.d.
Bovine serum albumin		460	820	690	660	810	530
Hen lysozyme		640	1390	540	530	700	550
None (media)		410	760	720	780	990	570

* Extended site 5 (sperm-whale Mb peptide 141–153) is used here as an internal negative control because it is not expected to be recognized by site 4-specific T-cell clones.

Table 2. Proliferative response of T-cell clone (VII.2.2) obtained from a long-term culture that was passaged with sperm-whale Mb

Note that the specificity of this clone which has been precisely defined (Atassi *et al.*, 1987) is directed to the region 112–120 of sperm-whale Mb. Extended site 5 (sperm-whale Mb peptide 141–153) is used here as an internal control because it should not be recognized by this site 4-specific T-cell clone. Results were obtained from the same experiment and represented the mean of three replicate analyses which had an S.D. of $\pm 8\%$ or less. [^3H]Thymidine incorporation by controls was as follows: concanavalin A, 112 790 c.p.m.; purified protein derivative of tuberculin, 680 c.p.m.; no antigen (medium), 580 c.p.m.

Challenge antigen	Dose ($\mu\text{g/ml}$)	[^3H]Thymidine incorporation (c.p.m.)			
		2.5	5.0	10.0	20.0
Sperm-whale Mb		17 150	17 550	39 130	33 870
Pept. 107–120 (Ext. Site 4)		26 750	35 060	38 670	39 400
Pept. 141–153 (Ext. Site 5)		980	750	820	990
Killer-whale Mb		12 560	23 840	43 160	51 640
Finback-whale Mb		26 250	44 110	43 280	35 650
Atlantic-dolphin Mb		13 150	32 400	35 880	37 980
Horse Mb		860	1050	1160	940
Beef Mb		1040	980	930	900
Red deer Mb		360	480	300	330
Goat Mb		850	860	810	660
Chimpanzee Mb		710	980	930	1120
Echidna Mb		890	940	800	930
Sea lion Mb		430	380	350	350
Chicken Mb		660	550	730	610
Dog Mb		970	1000	970	1040
Rabbit Mb		1320	1130	1120	930
Hen lysozyme		710	760	680	790
Bovine serum albumin		750	690	580	710

the proteolysis of the peptide by the cells *in vitro*. None of the clones responded to Mb of horse, cow, red deer, goat, chimpanzee, echidna, sea lion, chicken, dog or rabbit (Table 1). The clones were viable and responded to concanavalin A and, as expected, did not respond to purified protein derivative of tuberculin, lipopolysaccharide or control, unrelated proteins and peptides. Only syngeneic antigen-presenting cells (APCs) could present antigen to the T cells. None of the clones responded to antigen stimulation when APCs from C57BL/6 (H-2^b), C3H/He (H-2^k), SWR (H-2^q) or RIIS (H-2^r) mice were used.

Proliferative responses of T-cell clone obtained from Mb-specific T-cell line

The T-cell clone VII.2.2 was obtained from a long-term T-cell culture that was maintained by passage with sperm-whale Mb *in vitro*. The specificity of the clone was found by comprehensive screening with overlapping uniform peptides encompassing the entire sperm-whale Mb chain (Bixler & Atassi, 1983, 1984a). The precise boundaries of the T site recognized by this clone were recently defined (Atassi *et al.*, 1987). The responses of

this clone to various antigens in the region of the optimum dose are summarized in Table 2. The responses, at the optimum doses, to the passage antigen (sperm-whale Mb, net c.p.m. 39 130 at 10 $\mu\text{g/ml}$) and to extended site 4 (peptide 107–120, net c.p.m. 39 400 at 20 $\mu\text{g/ml}$) were equal. On the other hand, the response to killer-whale Mb was substantially higher (net c.p.m. 51 460 at 20 $\mu\text{g/ml}$) while that to finback-whale Mb was only slightly higher than the response to sperm-whale Mb. The response to Atlantic-dolphin Mb was somewhat lower than the response to sperm-whale Mb (Table 2). The other myoglobins (horse, cow, red deer, goat, chimpanzee, echidna, sea lion, chicken, dog and rabbit) did not stimulate a response in this clone. The clone responded as expected to concanavalin A but not to any unrelated control proteins and peptides (Table 2). Antigen stimulation was obtained only when syngeneic APCs were used. No stimulation was obtained with APCs of the b, k, q or r haplotypes,

DISCUSSION

It has been established that the antibody response to native protein antigens is dependent on their conformation (Atassi, 1967, 1975, 1978, 1984; Habeeb, 1977). In addition, charge-transfer and steric effects of substitutions outside protein antigenic sites can influence the binding properties of the site, even in the absence of a conformational change (Atassi & Kazim, 1980; Kazim & Atassi, 1980; Twining *et al.*, 1980). Because of its important implication to antigen presentation, we have investigated here, employing site-specific T-cell clones, whether T-cell recognition at the clonal level is dependent on conformation.

The T-cell clones possessed preselected and precisely known specificities. It was shown recently (Atassi *et al.*, 1987) that four of these clones had specificities directed to Mb region 112–120, and the specificity of another clone was directed towards region 111–120. The precise boundaries of the region recognized by the sixth clone (E4.4.4) have not been determined, but it must reside within the passage peptide 107–120. Another clone, isolated from a sperm-whale Mb-passage T-cell line, possessed specificity against the region 112–120 (Atassi *et al.*, 1987). Therefore, these clones will be highly suited to determine the effects of amino substitutions within the 'contact' residues of the site as well as the effects, if any, of substitutions in residues outside the site.

The present findings show that myoglobins having substitutions within the region 112–120 lose their ability to stimulate T cells that are specific to sperm-whale Mb-region 112–120. It has been proposed (Berkower *et al.*, 1982, 1984) by study of protein mutants using T-cell clones of unknown specificity, that in the S haplotype the site centres around Glu-109 and does not extend as far as residue 118. The results reported here (Tables 2 and 3) show that replacement of Glu-109 by aspartic acid in finback-whale Mb does not cause any loss in proliferative activity, whereas substitution of Arg-118 in sperm-whale Mb by lysine in rabbit Mb abrogates the stimulating activity. This is consistent with the recent finding (Atassi *et al.*, 1987) that the site encompasses the area 111–120 or 112–120, depending on the T-cell clone, and therefore superimposes well with the site of antibody recognition (residues 113–119) (Atassi, 1975). Other than the three whale and the dolphin myoglobins, all the remaining

Table 3. Structure of the peptide 107–120 (extended site 4) used for *in vitro* passage of Mb-primed T cells from SJL mice and amino acid replacements within this region in other Mb variants

The table shows the primary structure of region 107–120 of sperm-whale Mb and the amino acid replacements that occur within this region in Mb from other species. The specificity of the present T-cell clones has been determined (Atassi *et al.*, 1987). Five clones recognize the region 112–120 and one clone has specificity for the region 111–120. The area underlined (residues 113–119) represents antigenic site 4 (i.e. a site recognized by anti-protein antibodies) of sperm-whale Mb. The sequences of the myoglobins were obtained from Romero-Herrera *et al.* (1978).

	Residue number													
Myoglobin	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Sperm whale	Ile-	Ser-	Glu-	Ala-	Ile-	Ile-	<u>His</u>	<u>Val-</u>	<u>Leu-</u>	<u>His-</u>	<u>Ser-</u>	<u>Arg-</u>	<u>His-</u>	Pro
Killer whale														
Finback whale			Asp											
Atlantic dolphin														
Horse			Asp									Lys		
Beef			Asp								Ala	Lys		
Red deer			Asp								Ala	Lys		
Chimpanzee				Cys		Gln						Lys		
Echidna										Gln		Lys		
Sea lion										Gln		Lys		
Chicken				Val		Lys		Ile		Ala	Glu	Lys		
Dog			Asp			Gln				Gln		Lys		
Rabbit												Lys		

myoglobins have a substitution either at Arg-118 only (replaced by lysine) (rabbit and horse Mb) or at Arg-118 as well as a number of other substitutions within the region 111–120 (Table 3) and therefore show no activity towards any of the T-cell clones.

Whereas substitutions within the site can explain fully the presence or absence of activity in a given Mb, they will not rationalize the differences in the levels of these activities among the active myoglobins that have no substitutions within this region. To explain these differences, the environmental residues of the site must be considered. An analysis of the residues which constitute the environment of each of the antigenic sites of Mb has been reported (Kazim & Atassi, 1980). Table 4 summarizes the environmental residues that are neighbouring [within 7 Å (0.7 nm)] to the residues of antigenic site 4 in sperm-whale Mb, together with their replacements in the other myoglobins.

For the clones, obtained by peptide passage, the activity was highest to the passage peptide 107–120, being in fact considerably higher than the response to sperm-whale Mb. In the peptide used for cell passage, the critical 'contact' residues of the site are in a water-salt environment and thus the charge distribution surrounding each side chain is relatively unencumbered by steric obstruction or by other side chains that are either electron-acceptors or electron-donors. Any water molecules or ions associated with the peptide are readily displaced upon its binding to the cell receptor. Thus, the T-cell clones that were obtained by passage *in vitro* with the peptide would respond best to that peptide. In sperm-whale Mb, environmental residues that exert their influence cannot be readily removed but only somewhat adjusted. In contrast to the aforementioned clones, the T-cell clone VII.2.2, isolated from a cell line that was obtained through passage with sperm-whale Mb, responded equally well to sperm-whale Mb and to peptide 107–120. The free peptide, in an aqueous medium, has no

rigid electron donors or acceptors in its environment and can readily adapt to the charge requirement of the T cell that was educated with intact sperm-whale Mb.

In the Mb variants, the steric effects of replacements by hydrophobic side chains would necessitate some readjustment (and hence some energy expenditure) upon binding of the site to the T cell. In addition, replacement of those environmental side chains that perturb the charge distribution around, and those that form hydrogen bonds with, 'contact' residues would also have remarkable effects (Atassi & Kazim, 1980; Kazim & Atassi, 1980). Thus, the introduction of a carboxylate ion within a few Angstroms of Arg-118, His-113 and His-119 (Table 4) would cause a lowering of the pK of these side chains in Mb, relative to the free peptide, which should have an effect on the binding activity. This would explain the decreased activity exhibited by sperm-whale Mb relative to that of the free passage peptide. In killer-male Mb, Asp-122 (3.5 and 7.0 Å from site residues His-119 and Arg-118, respectively) is replaced by glutamine. Electrons are no longer donated to His-119 and the charge distribution within the region 112–120 approximates that of the free peptide 107–120, and so does the activity of killer-whale Mb. In finback-whale Mb, like sperm-whale Mb, residue 122 is again an aspartic acid and donates electrons to His-119. On the other hand, the environment of His-113 is altered. Arg-31 and Glu-109 in sperm-whale Mb, which are 3.6 and 6.0 Å respectively from His-113, are replaced by serine and aspartic acid respectively in finback-whale Mb. The replacement of arginine by serine would in itself tend to bring the pK of His-113 closer to its value in the free peptide, while the replacement of glutamic by aspartic would displace the negative charge of the carboxyl group further away from His-119 and thus increase its effective basicity. All these factors would be expected to increase somewhat the T-cell stimulating activity of finback-whale Mb, relative to sperm-whale Mb. However, because of the negative

Table 4. Environmental residues of antigenic site 4 of sperm-whale Mb that undergo substitution in the other myoglobins and the nature of the substitution

The table is compiled from the reports of Kazim & Atassi (1980) and Twining *et al.* (1980). Values in parentheses refer to the minimum distance (in Å) of approach between the closest atoms of the environmental residue and the indicated site residue (1 Å = 0.1 nm). For the identity of the atoms of closest approach, see Kazim & Atassi (1980). Note that the environment of site 4 comprises at least 24 residues and only those that undergo substitution in these myoglobins and whose distance of separation from a site residue is less than 6.0 Å are listed here. For a more detailed analysis of this and the other Mb antigenic sites, see Kazim & Atassi (1980).

Substitutions of the environmental residues													
(I) Environ- mental residue	(II) Neighbouring site residues (distance from I in Å)	Killer whale	Finback whale	Atlantic dolphin	Horse	Beef	Red deer	Chimpanzee	Echidna	Sea lion	Chicken	Dog	Rabbit
Val-13	Leu-115, His-119 (3.7) (4.5)		Ile	Ile		Ala	Ala			Ile	Ile	Ile	
Ala-19	His-119 (6.2)								Thr			Thr	
Asp-27	Val-114, Arg-118 (3.9) (2.8)				Glu	Glu	Glu	Glu		Glu	Glu	Glu	Glu
Ile-28	Val-114, Leu-115 (3.9) (5.2)			Val	Val	Val	Val	Val	Val	Val	Val	Val	Val
Arg-31	His-113, Val-114 (3.6) (4.6)		Ser										
Glu-109	His-113 (6.0)		Asp		Asp	Asp	Asp					Asp	
Ala-110	His-113, Val-114 (4.2) (5.3)							Cys		Val	Val		
Pro-120	His-116, Arg-118 (5.4) (4.8)								Ser		Ala	Ser	
	His-119 (2.8)												
Gly-121	His-119 (5.7)	Ala	Ala	Ala		Ser	Ser		Ala	Ala			
Asp-122	His-119 (3.5)	Gln		Glu		Asn	Asn						
Gly-124	His-116 (5.7)					Ala						His	
Gln-128	His-113, His-116 (6.2) (4.4)								His			Glu	

charge at position 122 in finback-whale Mb, this increase would be lower in magnitude than expected (and found) for killer-whale Mb. Atlantic-dolphin Mb again has a negatively charged carboxylate side chain (glutamate) at position 122 and, therefore, its activity is expected to be comparable to that of sperm-whale Mb. The slight differences observed between these two myoglobins can be attributed to steric effects arising from the replacements in the environmental residues at position 13, 28 and 121 (Ile, Val and Ala).

Dependence of T-cell recognition on conformation would necessitate re-examination of the present understanding of antigen presentation. The surface-simulation synthetic peptides, representing the discontinuous antigenic (i.e. antibody-binding) sites of lysozyme (Atassi, 1978), were found to be recognized by lysozyme-specific T-cell lines (Bixler & Atassi, 1984b). Surface-simulation synthesis is a procedure by which residues that are spatially close in three-dimensional structure and might otherwise be distant in sequence are linked together, with appropriate spacing and directionality, into a single peptide that does not exist in the protein but mimics a surface region of it (Lee & Atassi, 1976; Atassi, 1978). These peptides, because they are constructed by linking together spatially adjacent site residues that are otherwise distant in sequence, cannot be generated, but on the contrary, their assembly will be destroyed by processing. We have recently shown, by employing synthetic overlapping peptides representing entire proteins as immunogens, that antigen presentation *in vivo* is independent of processing (Bixler & Atassi, 1985; Bixler *et al.*, 1985; Atassi & Bixler, 1988a). A T-cell clone was recently reported (Cohly *et al.*, 1988) which did not recognize native Mb but was specific to its altered conformational state in apomyoglobin. Antigen presentation to T lymphocytes has been obtained with liposomes carrying MHC and protein antigens (Walden *et al.*, 1985, 1986a,b; Klein *et al.*, 1985; Klein, 1988). These findings and the localization of the T-cell recognition sites of Mb (Bixler & Atassi, 1983, 1984b) and several other proteins (for recent review, see Atassi & Bixler, 1988b) are consistent with the proposal (Atassi, 1980, 1984) that the critical aspects in the presentation of antigen *in vivo* involve predominantly recognition of an intact protein, and that processing is not the triggering signal for presentation, but is rather an attendant phenomenon concerned with clearance.

It is necessary to address whether the observations reported here can be explained by differences in the processing of the various myoglobins. This, however, will not offer a satisfactory explanation, for the following reasons. (1) Previous conformational studies (Atassi, 1970) on several of these myoglobins by optical rotatory dispersion, circular dichroism and measurements of hydrodynamic parameters revealed no detectable conformational differences among these myoglobins. More importantly, on proteolysis by trypsin (Atassi 1970), 22 or 23 peptides were obtained from each Mb (except monkey Mb which gave 27 peptides), indicating that they are processed similarly (at least by trypsin). (2) Pronounced replacement effects are observed with residues that are quite distant in sequence from site residues and these effects would be expected to be lost by proteolysis (e.g. the following replacement effects on site residues: effect of residue 27 on site residue 118; effect of residue 13 on residue 119; effect of residue 128 on

residues 113 and 116, etc., see Table 4). (3) Once the protein is processed to a stimulating peptide, then the effect of residues that were in the environment of the site in the intact protein becomes irrelevant. With these clones, synthetic peptides containing the site behaved similarly even when they contained, outside the sites, segments comprising sequences that did not exist in Mb (Atassi *et al.*, 1987). We have therefore concluded that the protein is recognized intact by the T cells.

The finding that only APCs from the s haplotype presented antigen to the T cells indicated that the recognition by the T cells was H-2 restricted. However, it is generally believed that T cells recognize relatively short peptides that can bind in the groove of the MHC molecule (Bjorkman *et al.*, 1987a,b; Brown *et al.*, 1988). How an intact protein is presented in a manner that does not fit this hypothesis (i.e. at the molecular level with site specificity and MHC restriction) is not clear at present.

In conclusion, the results reported here clearly show that recognition and responses *in vitro* of T-cell clones whose specificity is preselected to a given site are dependent on the conformational integrity of the protein. Obviously, if presentation *in vitro* of Mb to the T cells were to involve processing of the protein as a prerequisite step, then changes in amino acid side chains that are close to site residues in the three-dimensional structure but are otherwise distant in sequence would be totally irrelevant and would have no effect. We suggest that the protein is presented *in vitro* in its intact form to each T-cell clone.

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