Site recognition by protein-primed T cells shows a non-specific peptide size requirement beyond the essential residues of the site

Demonstration by defining an immunodominant T site in myoglobin

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In previous studies, six T sites within myoglobin (Mb) were localized. To define precisely the boundaries of the T sites, a new approach is introduced and applied here to the T site residing within residues 107–120 of Mb. Two sets of peptides were synthesized. One set represents a stepwise elongation by one-residue increments of the Mb sequence. The other set represents an identical stepwise addition of one-residue increments of the Mb sequence, but which were extended by additional unrelated (nonsense) residues to a uniform size of 14 residues. The longer peptides (nonsense-extended) usually gave higher proliferative responses than did their shorter counterparts having the same Mb region. Thus a minimum peptide size is required for optimal T-cell stimulation. The T site subtends, in three high-responder mouse strains, residues 109–119 or 110–120, depending on strain, and, in three low-responder strains, maps to residues 108–120. Thus, in this case, the T site coincides with the site of B-cell recognition and resides in a small discrete surface region of the protein chain.

INTRODUCTION

In recent years, there has been considerable speculation on the nature of T-cell recognition sites on protein antigens. The sites of T-cell recognition have been proposed to be more limited in number than sites of B-cell recognition and that, further, the sites of immune recognition are diffuse, approaching a continuum (Benjamin *et al.*, 1984). However, in fact, comprehensive and systematic screenings of entire protein molecules for sites of T-cell recognition by employing well-characterized synthetic peptides have only recently been completed (for review see Bixler & Atassi, 1985). These findings show, contrary to the aforementioned view (Benjamin *et al.*, 1984), that the major T sites of a protein occupy discrete areas of the molecule and that these frequently, but not always, correspond to regions also recognized by B cells.

By the use of a comprehensive synthetic strategy introduced for the localization of continuous (i.e. the constituent residues are directly linked by peptide bonds) antigenic sites of proteins (Kazim & Atassi, 1980, 1982), the sites of T-cell recognition of several proteins [myoglobin (Mb) (Bixler & Atassi, 1983, 1984a), lysozyme (Bixler *et al.*, 1984a,b; Bixler & Atassi, 1984b), haemoglobin (Yoshioka *et al.*, 1986) and ragweed allergen Ra3 (Kurisaki *et al.*, 1986)] have been localized. In addition, by systematic synthetic screening of the surface of influenza-virus haemagglutinin, major sites of T-cell recognition were also mapped (Atassi & Kurisaki, 1984) on this viral protein. In all these proteins, the synthetic peptide strategies employed permitted an evaluation of the entire protein chain and the localization of the continuous T sites to within a few residues of their respective boundaries. The determination of the size and

precise boundaries of the T site, however, would require a more intricate synthetic strategy around the region of each site.

The present study focuses on a major T site of Mb and examines closely the boundaries and size requirements needed for optimal T-cell stimulation. Previous studies (Bixler & Atassi, 1983, 1984a) have shown that, in Mb, a continuous T site resides within region 107–120, which in some strains is immunodominant. From the studies of six mouse strains, both high and low responders to Mb (Okuda *et al.*, 1978), it has been found that the T site has discrete boundaries. In the high-responder H-2^d haplotypes, the T site maps to region 109–119, whereas in the other strains a slight shift of one residue either to the right or left occurs. In addition, there is an unusual peptide-size effect that plays a crucial role in the activation of T cells.

MATERIALS AND METHODS

The major chromatographic component of sperm-whale Mb was prepared by CM-cellulose chromatography of the crystalline protein (Atassi, 1964). The preparation was homogeneous by disc polyacrylamide-gel electrophoresis. Concanavalin A (Con A), purified protein derivative of tuberculin (PPD) and lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Connaught Laboratories (Swiftwater, PA, U.S.A.) and Difco Laboratories (Detroit, MI, U.S.A.) respectively.

The peptides (see Fig. 1) were prepared by solid-phase synthesis (Merrifield, 1963) by using procedures similar to those described for the synthesis of the Mb antigenic sites (Koketsu & Atassi, 1973, 1974a), and the

Abbreviations used: Mb, myoglobin; Con A, concanavalin A; LPS, lipopolysaccharide; PPD, purified protein derivative of tuberculin.

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overlapping peptides were used to localize the sites of T-cell recognition (Bixler & Atassi, 1983, 1984a). After synthesis, the peptides were cleaved from the resin by anhydrous HF (-10°C , 30 min). The dinitrophenyl protecting group on the imidazole ring was removed by treatment with benzenethiol in *NN*-dimethylformamide. The peptides were dissolved in a small volume of trifluoroacetic acid and precipitated by diethyl ether. Although other workers have used unpurified peptides in immunological studies (Green *et al.*, 1982), or have employed synthetic procedures that do not involve cleavage of the peptides from the resin or even minimally its amino acid analysis (Geysen *et al.*, 1984; Geysen, 1985), this practice is not advisable because it leads to erroneous and confusing results. The peptides used here were exhaustively purified by ion-exchange column chromatography by previously established procedures (Koketsu & Atassi, 1973, 1974a; Lee & Atassi, 1977a,b). Peptide purity was determined by high-voltage paper electrophoresis and composition was verified by amino acid analysis.

A/J (H-2^a), C57Bl/10 (H-2^b), Balb/c and DBA/2 (H-2^d), B10.BR (H-2^k) and SJL (H-2^s) mice at 6–7 weeks of age (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were immunized subcutaneously at the base of the tail with 100 μg of sperm-whale Mb. The protein was emulsified in 0.15 M-NaCl/0.01 M-sodium phosphate buffer, pH 7.2, and Freund's complete adjuvant

containing *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories) as previously described (Corradin *et al.*, 1977).

At 7 days after immunization, T-cell responses were assessed by proliferation assay. The inguinal and periaortic lymph nodes were removed and a cell suspension was prepared in RPMI 1640 medium (GIBCO, Grand Island, NY, U.S.A.) containing 1% (v/v) fresh normal mouse serum. The number of viable cells was determined by vital staining with fluorescein diacetate (Rotman & Papermaster, 1966). Viable lymph-node cells (5×10^5 /well) were co-cultured in triplicate with various concentrations of antigens, mitogens or synthetic peptides. Negative controls included peptides and proteins unrelated to Mb. After 3 days' incubation at 37°C in humidified 5% CO_2 in air, the cultures were pulsed (18 h) with 2 μCi of [^3H]thymidine (ICN, Irvine, CA, U.S.A.)/well and then harvested on to glass-microfibre filters (Whatman, Clinton, NJ, U.S.A.) for counting of radioactivity by liquid scintillation.

RESULTS

Characterization of synthetic peptides

The primary structures of the synthetic peptides are shown in Fig. 1. After cleavage, deprotection and purification by column chromatography, the major

(a) Mb peptides having increasing size of natural sequence

107–120	Ile-Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
108–120	Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
109–120	Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
110–120	Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
111–120	Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
112–120	Ile-His-Val-Leu-His-Ser-Arg-His-Pro
113–120	His-Val-Leu-His-Ser-Arg-His-Pro

(b) Natural MB sequences extended by nonsense sequence to a uniform size

N(108–120)	<u>Gly</u> -Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
N(109–120)	<u>Gly-Ala</u> -Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
N(110–120)	<u>Gly-Ala-Ser</u> -Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
N(111–120)	<u>Gly-Ala-Ser-Gly</u> -Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
N(112–120)	<u>Gly-Ala-Ser-Gly-Thr</u> -Ile-His-Val-Leu-His-Ser-Arg-His-Pro
N(113–120)	<u>Gly-Ala-Ser-Gly-Thr-Gly</u> -His-Val-Leu-His-Ser-Arg-His-Pro

Fig. 1. Primary structures of the synthetic peptides employed for the definition of the T site, previously found to be localized within region 107–120 of sperm-whale Mb (Bixler & Atassi, 1984a)

(a) Peptides representing stepwise one-residue extensions of the natural Mb sequence from His-113 to Pro-120. Peptide 113–120 coincidentally corresponds to the previously defined (Atassi, 1975) antigenic (i.e. antibody-binding) site 4 of Mb. (b) Peptides in which the structures shown in (a) are extended by a 'nonsense' sequence to a uniform size of 14 residues by addition of an appropriate segment of the 'nonsense' sequence Gly-Ala-Ser-Gly-Thr-Gly (underlined region).

Table 1. Amino acid composition of synthetic peptides

Peptide . . .	Composition (mol of residue/mol)						
	107-120	108-120	109-120	110-120	111-120	112-120	113-120
Thr	—	—	—	—	—	—	—
Ser	1.90 (2)	2.08 (2)	0.90 (1)	1.07 (1)	1.00 (1)	1.12 (1)	0.87 (1)
Glu	1.28 (1)	1.25 (1)	1.38 (1)	—	—	—	—
Pro	0.90 (1)	1.11 (1)	1.00 (1)	1.09 (1)	1.01 (1)	1.07 (1)	0.98 (1)
Gly	—	—	—	—	—	—	—
Ala	1.18 (1)	1.24 (1)	1.19 (1)	1.12 (1)	—	—	—
Val	1.06 (1)	1.22 (1)	1.07 (1)	0.96 (1)	1.01 (1)	1.03 (1)	1.17 (1)
Ile	2.93 (3)	2.00 (2)	2.07 (2)	2.03 (2)	2.08 (2)	0.93 (1)	—
Leu	1.07 (1)	1.25 (1)	1.09 (1)	1.21 (1)	1.21 (1)	1.14 (1)	1.18 (1)
His	3.17 (3)	2.83 (3)	2.93 (3)	2.92 (3)	2.99 (3)	3.17 (3)	3.22 (3)
Arg	0.83 (1)	1.03 (1)	0.93 (1)	0.92 (1)	0.93 (1)	0.89 (1)	0.96 (1)

Peptide . . .	N(108-120)	N(109-120)	N(110-120)	N(111-120)	N(112-120)	N(113-120)
Thr	—	—	—	—	0.96 (1)	0.98 (1)
Ser	1.85 (2)	0.93 (1)	1.90 (2)	1.83 (2)	1.91 (2)	1.84 (2)
Glu	1.22 (1)	1.40 (1)	—	—	—	—
Pro	1.08 (1)	1.05 (1)	0.98 (1)	0.85 (1)	1.17 (1)	0.90 (1)
Gly	1.00 (1)	1.00 (1)	1.17 (1)	2.09 (2)	2.07 (2)	3.23 (3)
Ala	1.07 (1)	2.31 (2)	2.25 (2)	1.17 (1)	1.09 (1)	1.15 (1)
Val	1.17 (1)	1.16 (1)	1.05 (1)	0.96 (1)	1.05 (1)	0.98 (1)
Ile	2.01 (2)	2.03 (2)	2.00 (2)	2.01 (2)	1.01 (1)	—
Leu	1.12 (1)	1.16 (1)	1.09 (1)	0.99 (1)	1.06 (1)	1.03 (1)
His	3.04 (3)	2.61 (3)	2.61 (3)	2.11 (3)	2.70 (3)	3.04 (3)
Arg	1.26 (1)	0.97 (1)	1.03 (1)	1.13 (1)	1.03 (1)	1.04 (1)

chromatographic component of each peptide was homogeneous by high-voltage paper electrophoresis. The amino acid compositions of the purified peptides (Table 1) are in good agreement with those expected from their primary structure.

Definition of the C-terminal end of the T site

Earlier studies on T-cell recognition (Bixler & Atassi, 1983, 1984a; Yoshioka *et al.*, 1983) suggested that the C-terminal end was in close proximity to residue 120, probably residue 120 itself or, at the most, plus or minus one or two residues. In order to define the C-terminal end of this T site, the responses of six Mb-primed mouse strains to the peptides 107-119 and 107-120 over a broad dose range (3-300 µg/ml) were compared. Representative results from these studies are shown in Fig. 2. In each of the strains examined, greater responses were obtained following stimulation with peptide 107-120. The difference in the level of response was most pronounced with cells from SJL and B10.BR mice, but a clear distinction in the level of response was also observed with cells from both A/J and C57BL/10 mice. Although in several experiments with lymph-node cells from Balb/c or DBA/2 mice peptide 107-120 consistently provoked a higher response than did peptide 107-119, the differences between the responses, however, were not substantial. Peptide extension up to residue 125 showed that the addition of the residues 121-125 caused little or no increase, relative to peptide 107-120, in the magnitude of the response. For example, with T cells from Mb-primed SJL mice the responses to peptides 107-120, 109-125 and 107-119 were 391050 ±

10083 c.p.m. (SI 66.77), 320650 ± 21001 c.p.m. (SI 54.76) and 173225 ± 2481 c.p.m. (SI 29.58) respectively. All the strains responded appropriately to the positive controls (Mb, Con A, PPD, LPS) and were unresponsive to unrelated proteins or peptides.

Definition of the N-terminal end of the T site

Since the T site does not extend beyond residue 120, the N-terminal end of the site was defined, with the C-terminal end being fixed at Pro-120. The capacity of each of the synthetic peptides in Fig. 1 to evoke a response from Mb-primed lymph-node cells was examined over a broad dose range (1.5-300 µg/ml). The dose-response curves from a representative experiment, with cells from DBA/2 mice, are shown in Fig. 3. In this strain peptides 108-120 and 109-120 stimulated substantial responses, whereas peptides 110-120 and 111-120 had intermediate stimulatory activity. Peptides 112-120 and 113-120 were weakly stimulatory or negative. In comparison, synthetic peptides that were extended to a uniform size by nonsense sequence (Fig. 1b), i.e. peptides N(108-120), N(109-120) and N(110-120), stimulated much greater proliferative responses than peptides representing the same Mb region (i.e. peptides 108-120, 109-120 and 110-120) without the extension. Similarly, peptides N(111-120) and N(112-120) stimulated intermediate responses that were also greater than the responses obtained with the unextended counterparts (peptides 111-120 and 112-120). Peptide N(113-120), however, was not stimulatory at any dose examined. The cells from DBA/2 mice, known to be a high responder to Mb (Okuda *et al.*, 1978), responded well to Mb and to

Fig. 2. Summary proliferative responses of lymph-node cells obtained from six mouse strains to challenge *in vitro* with peptides representing the natural Mb sequences 107-119 (▨) and 107-120 (▧)

The responses were tested over a wide dose range (3-300 $\mu\text{g/ml}$) and the maximum responses are shown for both the high responders (Balb/c at an optimum dose of 50 $\mu\text{g/ml}$ for both peptides, DBA/2 at an optimum dose of 300 $\mu\text{g/ml}$ for both peptides and SJL at optimum doses of 300 and 200 $\mu\text{g/ml}$ for peptides 107-119 and 107-120 respectively) and low responders (A/J at optimum doses of 300 and 200 $\mu\text{g/ml}$ for peptides 107-119 and 107-120 respectively, B10.BR and C57BL/10 at an optimum dose in both strains of 200 $\mu\text{g/ml}$). The background responses of unstimulated cells ranged from 4999 c.p.m. to 7166 c.p.m., except for cells from Balb/c mice, which had a background response of 10880 c.p.m.

extended site 4 (residues 107-120), Con A, PPD and LPS, but were unresponsive to unrelated proteins or peptides (results not shown).

Similar experiments were performed in five additional mouse strains, both high and low responders to Mb. Representative experiments that give the maximal response for each peptide are summarized in Fig. 4. In

the three high-responder mouse strains, the highest T-cell stimulation was given by peptide N(109-120) with cells from Balb/c and DBA/2 mice and by peptide N(110-120) with cells from SJL mice. However, when these cells were challenged with the unextended peptides containing the corresponding Mb regions, maximal responses were obtained with peptides 107-120 (in Balb/c), 108-120 (in DBA/2) and 109-120 (in SJL). In the three low-responder strains, the maximal responses were obtained with peptide N(108-120). For the peptides of the natural sequence, the strongest response was obtained with peptide 107-120. In all six strains examined, the expected responses were obtained to Mb (low in the low-responder strains) and to Con A, PPD and LPS (results not shown). The cells from all strains were unresponsive to unrelated proteins or peptides.

DISCUSSION

Several laboratories (Thomas *et al.*, 1980, 1981; Singh *et al.*, 1980; Hansburg *et al.*, 1981) have previously examined some aspects of T-cell recognition of either proteins or small peptide hormones by using synthetic peptides. Mb, however, was the first protein that was systematically screened by a comprehensive synthetic strategy for the localization of the full profile of its continuous T-cell recognition sites (Bixler & Atassi, 1983, 1984a). This strategy enabled the mapping of the entire protein chain for potential T sites. When Mb-primed lymph-node cells or Mb-specific long-term T-cell cultures were examined, six major regions of T-cell recognition were localized within the entire polypeptide chain of the protein (Bixler & Atassi, 1983, 1984a). Five of the six regions correspond closely to the previously defined antigenic (antibody-binding) sites of this protein (Atassi, 1975, 1977) but the sixth T site resides in an area to which no antibody response has been detected. In addition, the T-cell and antibody responses to the sites were found to be under individual genetic control (Okuda *et al.*, 1979; Twining *et al.*, 1981; Bixler & Atassi, 1984a). Thus the major antigenic (antibody-binding) sites (Atassi, 1975) and the major T sites (Bixler & Atassi, 1983, 1984a) have been shown to occupy discrete regions of the molecule. In the current work, with Mb as a model, the boundaries of a T site have been defined and the effect of peptide size on its ability to activate T cells was evaluated.

The obvious approach to the definition of the boundaries of a T site is through the stepwise addition (or deletion) of residues on one end of the indicated region while keeping the other end constant. The resulting set of peptides would represent various lengths of the natural sequence of the protein (Fig. 1a). Since earlier studies on B-cell recognition showed that deletion of a single residue at the boundary end of an antigenic site can cause complete abrogation of the binding activity (Koketsu & Atassi, 1973; Atassi, 1975), this approach would seem to be promising. In 1983, Young & Atassi (1983) showed that extension of a synthetic Mb site by incremental additions of the natural sequence had an effect on the ability of a given peptide to stimulate T-cell responses in protein-primed lymph-node cells. Other studies (Yoshioka *et al.*, 1983) also showed that longer peptides appeared to improve the response of peptide-driven long-term T-cell lines. Schwartz *et al.* (1985) applied a similar approach to cytochrome *c* and

Fig. 3. Representative dose-response curves to peptides of the natural sequence (○) or peptides extended by nonsense sequence to a uniform size (●) in lymph-node cells obtained from Mb-primed DBA/2 mice

The responses, which were examined over a broad dose range, are shown in the panels as follows: (a) peptides 108–120 and N(108–120); (b) peptides 109–120 and N(109–120); (c) peptides 110–120 and N(110–120); (d) peptides 111–120 and N(111–120); (e) peptides 112–120 and N(112–120); (f) peptides 113–120 and N(113–120). The background response of unstimulated cells was 3412 c.p.m.

reported similar results with synthetic peptides representing incremental additions of the natural sequence of cytochrome *c*. Collectively, these findings suggest the possibility that a peptide containing the essential 'contact' residues for T-cell stimulation might possess higher activity if it were lengthened. However, since the peptides employed in these studies represent segments of the natural sequences of their respective proteins, it is difficult to distinguish between contributions made to the T-cell response by size alone versus those contributions related to required additional natural sequence residues. To circumvent this ambiguity, a different approach was introduced.

Focusing on the T site residing within (but including all of) the segment 107–120 (Bixler & Atassi, 1983, 1984a), peptides increasing in size by one-residue increments from the known N-terminal boundary of the antigenic (i.e. antibody-binding) site (His-113; Atassi & Pai, 1975) were synthesized. The increase in size was done in two ways: (1) by addition of residues (one at a time) corresponding to the natural Mb sequence up to residue 107 and designated by the sequence locations of the first

and last residues, or (2) by addition of one-residue increments corresponding to the natural Mb sequence plus further extension of the peptide up to a uniform size of 14 residues by a sequence that is not in any way related to Mb in this region or anywhere else in the molecule (nonsense sequence; Twining & Atassi, 1979). These peptides are also designated by the location of the first and last natural sequence residues but, in addition, are prefixed by 'N' to denote the nonsense extension. The nonsense structure has been shown not to bind anti-Mb antibodies (Twining & Atassi, 1979; Twining *et al.*, 1981; Allen *et al.*, 1985) and also has no effect on Mb-primed T cells (Okuda *et al.*, 1979; Young & Atassi, 1983). Thus, regardless of whether or not peptide size influenced T-cell responses, the precise definition of the boundaries of the site should be possible. Further, this approach would distinguish between those residue additions that contribute size effects alone and those that are essential contact residues of the site.

The contribution made by peptide size to the stimulation of Mb-primed T cells in these studies is apparent. From the dose-response curves (Fig. 3) the

Fig. 4. Summary of proliferative responses of lymph-node cells obtained from six mouse strains to challenge *in vitro* with peptides of the natural sequence (○) or peptides extended by nonsense sequence to a uniform size (●)

The maximum proliferative responses obtained are plotted against the corresponding residue added. Mouse strains were characterized as either high (Balb/c, DBA/2 and SJL) or low (A/J, B10.BR and C57BL/10) responders to Mb. Background responses of unstimulated cells ranged from 1094 c.p.m. to 4680 c.p.m.

larger peptides consistently provoked a detectable proliferative response from protein-primed cells at lower concentrations and also elicited greater maximal responses than did the corresponding peptides containing only the natural Mb sequence. Similarly in five of the six strains examined (Fig. 4) the peptides extended by nonsense sequence elicited higher proliferative responses than did their counterparts that contain only natural Mb sequence. Clearly the greater stimulatory capacity is not related to non-specific stimulation by the nonsense sequence extension, since those peptides containing the largest segment of nonsense residues (and, correspondingly, the shortest segment of natural sequence) provoked only weak or no responses above baseline. These observations are consistent with earlier reports (Yoshioka *et al.*, 1983; Young & Atassi, 1983) that initially indicated the potential role of size in the T-cell recognition of free peptides. However, the increase in peptide activity associated with the nonsense-extended peptides, when compared with the appropriate natural sequence peptides, appears to be for the most part attributable to a requirement for a minimal peptide size rather than the need for additional 'contact' residues in the natural sequences (Yoshioka *et al.*, 1983; Young & Atassi, 1983) or required conformational effects imparted by the additional natural sequence (Young & Atassi, 1983; Schwartz *et al.*, 1985).

These synthetic peptides, which dissect the region 107–120 of Mb, allow the definition of the boundaries of a major T site. In the three low-responder mouse strains, A/J, C57BL/10 and B10.BR, the T site consists of 13 residues extending from Ser-108 to Pro-120. In two of the three high-responder mouse strains, Balb/c and DBA/2, the site consists of the 11 residues Glu-109 to His-119. In the remaining high-responder mouse strain, SJL, the T site occupies the region Ala-110 to Pro-120. Thus the T site in this region was mapped (for summary see Fig. 5) to a relatively small (11–13 residues) continuous discrete region of the polypeptide chain of Mb.

From studies with Mb variants (Berkower *et al.*, 1982) the site residues His-113 and His-116 identified by Atassi (1975) have been tenuously linked in T-cell recognition with Glu-109 in B10.S (H-2^s) mice. Results with SJL T-cell clones that were obtained by driving the cultures *in vitro* with synthetic peptide (Yoshioka *et al.*, 1983) strongly indicated that Glu-109 was not involved in T-cell recognition in the H-2^s haplotype, since these clones were completely indifferent to substitutions at position 109 in Mb variants (G. S. Bixler, Jr., M. Bean & M. Z. Atassi, unpublished work). These observations were confirmed here since SJL (H-2^s) mice only required the residues 110–120 to achieve optimal T-cell proliferative responses. In the other strains residue 109 is in or near the margin of the T site, and although it contributes

Fig. 5. Summary of the precise boundaries of the T site within region 107–120 of sperm-whale Mb as defined in six mouse strains that were either high or low responders to Mb

to T-cell activity its removal does not drastically diminish the response. Therefore, contrary to the conclusion that recognition of sperm-whale Mb involves a determinant 'centring' on residue 109 (Berkower *et al.*, 1982), this T site focuses on the previously defined region of antibody binding (Atassi, 1975).

The slight displacement of the T site by one or two residues that was observed among the various mouse strains examined was not unexpected. In earlier studies that localized the major sites of T-cell recognition in Mb (Bixler & Atassi, 1983, 1984a) and also in lysozyme (Bixler *et al.*, 1984a,b) shifts in the locations of the T site were observed. These shifts are reminiscent of those previously observed in antibody binding to some of the antigenic (antibody-binding) sites of Mb and other proteins (Koketsu & Atassi, 1973, 1974a; Atassi, 1975; Kazim & Atassi, 1982; Yoshioka & Atassi, 1986).

These boundaries reflect the average of the maximal responses of a polyclonal T-cell population whose specificity is directed to this region. Further, the boundary residues define the end of the minimum continuous segment of Mb that elicits an optimum proliferative response. In addition, although the residues that define the boundaries are apparent, the differences in activity between peptides changing by one-residue increments are not always dramatic. This is clearly a reflection of the polyclonal nature of the lymph-node cell response. In contrast, T-cell clones may be expected, in future studies, to yield, for each clone, sharply demarcated boundary residues.

Interestingly, the T site, which was originally localized by using overlapping synthetic Mb peptides (Bixler & Atassi, 1983, 1984a) and is defined in the current work, coincides with antigenic (antibody-binding) site 4, which was previously defined to occupy the residues 113–120 in

several species (Atassi, 1975) including both outbred and congenic mice (Twining *et al.*, 1981). The T and B sites were localized and defined by entirely different strategies and are separated in time by more than 10 years. Although the same region of Mb is recognized by both B and T cells, the T site was found to be somewhat larger, extending several residues towards the *N*-terminal end. In those mouse strains examined, the *C*-terminal ends for T and B cells, however, are virtually identical since both map to residues 119 and 120.

The slightly larger size of the T site, in comparison with its B-cell counterpart, may be attributed to many factors. The size effects observed show that, in addition to the essential 'contact' residues, there is a decided advantage in increasing peptide size, even by an unrelated sequence. The larger peptide size (by addition of unrelated residues) may prolong its survival in the culture during T-cell stimulation. Furthermore, it may have been a reflection of the lower affinity of the T-cell receptor (relative to antibody affinity; for a review on T-cell receptor see Collins & Owen, 1985), since the 'contact' region required for optimal T-cell recognition is somewhat larger than that needed for binding to antibody. Should the binding energy of the T-cell receptor be less than, or even the same as, that of the antibody, the additional residues may assist in increasing the binding energy of the receptor-peptide complex and the known association (Lafuse & David, 1984) with the Ia molecule. Alternative or additional influences of size may be through conformational effects. The added residues may assist the peptide to approximate more closely the shape of its corresponding region in the native protein (Atassi & Saplin, 1968; Epand & Scherga, 1968; Singhal & Atassi, 1970; Atassi & Singhal, 1970). It should be noted, however, that the addition of large bulky side chains at an end of a site may result in steric interference with the binding of the site (Atassi & Pai, 1975; Koketsu & Atassi, 1974b; Young & Atassi, 1983). Therefore attention was paid in the present studies to the unrelated 'nonsense' sequence, which, while maintaining solubility, carried no bulky side chains that may potentially exert a steric hindrance on the 'binding part' of the peptide.

Although it is tempting to conclude that the observations reported here may portray a general property of T-cell recognition of proteins, it must be cautioned that, at the present time, it is premature to generalize to other T sites of Mb or other proteins. Clearly, additional detailed submolecular studies of other Mb T sites, at both the clonal and polyclonal level, and similar studies with other proteins must be performed before a general picture will emerge.

Note added in proof (received 19 September 1986)

Berkower and colleagues (Cease *et al.*, 1986) have examined the responses of two T-cell clones using a panel of synthetic peptides rather than protein variants, as they did in their earlier work, and reported T-cell responses to peptide 106–118. Thus the localization of an immunodominant region of T-cell recognition by synthetic peptides around region 107–120 of sperm-whale myoglobin (Okuda *et al.*, 1979; Yoshioka *et al.*, 1983; Bixler & Atassi, 1983, 1984a) previously rejected by Berkower *et al.* (1984) has now been confirmed by these workers (Cease *et al.*, 1986).

This work was supported by Grant AI-21336 from the National Institutes of Health, U.S. Public Health Service, and in part by the Robert A. Welch Foundation from Grant Q994 and the award to M.Z.A. of the Robert A. Welch Chair of Chemistry. M.Z.A. is a Robert A. Welch Professor of Chemistry, a chair endowed by the Welch Foundation.

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Received 4 April 1986/10 June 1986; accepted 21 July 1986