Non-specific peptide size effects in the recognition by site-specific T-cell clones

Demonstration with a T site of myoglobin

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Six regions (T sites) of myoglobin (Mb) were found by a comprehensive synthetic strategy to stimulate Mb-primed lymph-node cells. To define precisely the *N*-terminal boundary of the immunodominant T site (residues 107–120) with site-specific T-cell clones and to determine the effects of peptide size on their stimulation, two sets of peptides were employed. In one set, the peptides were elongated to the left from His-113 by one-residue increments of the Mb sequence. The other set represented an identical stepwise elongation by one-residue increments of the Mb sequence, but which were extended by additional unrelated ('nonsense') residues to a uniform size of 14 residues. Examination of the proliferative responses of eight T-cell clones, derived from Mb-primed DBA/2 (H-2^d) or SJL (H-2^s) mice, revealed a dramatic non-specific size requirement. In every clone, the longer nonsense-extended peptides achieved maximum stimulating activity at a lower optimum peptide dose than its natural-sequence, but shorter, analogue. In addition, slight (one-residue) differences in the *N*-terminal boundaries among the clones was observed. Thus, the fine specificity of each clone was mapped to the region from residue 111 or 112 to about residue 120 of Mb, which coincides with the site of B-cell recognition and resides in a small discrete surface region of the protein chain.

INTRODUCTION

Knowledge of the submolecular features of a protein that are recognized by antibodies (i.e. antigenic sites) is now very advanced (for reviews see Atassi, 1980, 1984*a,b*). However, the understanding of the submolecular features recognized by T cells has, until recently, lagged behind, and has been the subject of a great deal of speculation. The problem stemmed primarily from the fact that, other than fragmentary information, the complete submolecular T-cell recognition profile was not known for any protein. The last few years, however, have witnessed the localization of the full T-cell recognition profiles of several proteins (Bixler & Atassi, 1983, 1984*a,b*, 1985; Bixler *et al.*, 1984*a,b*; Yoshioka *et al.*, 1986; Kurisaki *et al.*, 1986), and this information has permitted, for the first time, the comparison on a given protein of the sites recognized by T cells (i.e. the T sites) with the sites recognized by antibodies.

Myoglobin (Mb) is one such protein for which both the antibody (Atassi, 1975) and T-cell recognition profiles (Bixler & Atassi, 1983, 1984*b*) are presently known. The definition of the boundaries of the T sites is complicated because previous findings have suggested that peptide size may play a role in the stimulation by peptides of T-cell responses *in vitro* (Young & Atassi, 1982, 1983). More recently, while defining the boundaries of an immunodominant T site in myoglobin (Bixler *et al.*, 1986), a dramatic non-specific size effect in the recognition of the T site was observed. In the present work, T-cell clones of predetermined specificity are employed as precise reagents to examine unambiguously the aforementioned unusual size effect.

MATERIALS AND METHODS

Materials

Sperm-whale Mb used in these studies was the major chromatographic component (no. 10, MbX) obtained by CM-cellulose chromatography of the crystalline protein (Atassi, 1964), and it was homogeneous by polyacrylamide-disc-gel electrophoresis. DBA/2 (H-2^d) and SJL (H-2^s) mice (6–7 weeks of age) were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.). Concanavalin A was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), purified protein derivative of tuberculin from Connaught Laboratories (Swiftwater, PA, U.S.A.) and lipopolysaccharide from Difco Laboratories (Detroit, MI, U.S.A.).

Synthetic peptides

The synthesis, purification and characterization of the peptides used in the present studies (Fig. 1) have been reported previously (Bixler *et al.*, 1986). In one set the peptides were elongated to the left by one-residue increments of the natural Mb sequence, whereas in the other set these incrementally lengthened Mb sequences were brought to a uniform size of 14 residues by the addition of a 'nonsense' sequence that bears no relationship to any part of Mb.

Preparation of site-specific T-cell clones

Site-specific T-cell clones were prepared in two ways, as follows. (1) By limiting dilution of long-term cultures of lymphocytes (from Mb-primed mice) (Infante *et al.*, 1981) that were passaged *in vitro* with optimum doses of Mb (determined *in vitro* by proliferation assay at various

Abbreviation used: Mb, myoglobin.

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(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	
X ₁ (108-120)	<u>Gly</u> -Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
X ₂ (109-120)	<u>Gly-Ala</u> -Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
X ₃ (110-120)	<u>Gly-Ala-Ser</u> -Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
X ₄ (111-120)	<u>Gly-Ala-Ser-Gly</u> -Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro
X ₅ (112-120)	<u>Gly-Ala-Ser-Gly-Thr</u> -Ile-His-Val-Leu-His-Ser-Arg-His-Pro
X ₆ (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 localize within region 107-120 of sperm-whale Mb (Bixler & Atassi, 1984a)

(a) Peptides representing stepwise extensions by one-residue increments 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. [From Bixler *et al.* (1986).]

Mb doses). After a second limiting dilution, the specificity of the clones was established by screening their proliferative responses against a panel of overlapping peptides encompassing the entire Mb chain (Bixler & Atassi, 1983, 1984b) and the extended Mb antigenic sites (Yoshioka *et al.*, 1983). (2) By limiting dilution of site-specific T-cell lines that were prepared by passage *in vitro* of Mb-primed T cells with extended site 4 (Yoshioka *et al.*, 1983). In this procedure, protein-primed T cells that recognize a given immunogenic site are driven into proliferation by periodic (12 days) stimulation with optimum doses of the synthetic site. Usually after two or three such passages *in vitro* the cell line will be entirely specific to the driving peptide (Yoshioka *et al.*, 1983). After a second limiting dilution, the specificity of the clones for extended site 4 was verified by proliferative assay.

Proliferation assay of the T-cell clones

The responses of the T-cell clones to various proteins and peptides were monitored by thymidine incorporation. T-cell clones (5×10^3 cells/well) and X-ray-irradiated (3300 R) syngeneic filler cells (5×10^5) were co-cultured (72 h at 37 °C), in triplicate, 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 μ Ci of [³H]thymidine (ICN, Irvine, CA, U.S.A.), and then harvested on to glass-microfibre filters (Whatman, Clinton, NJ, U.S.A.) and counted for radioactivity by liquid scintillation. Each experiment, which included unrelated proteins and peptides as controls, was done at least twice.

RESULTS

Responses to peptides of DBA/2 T-cell clones derived by passage with extended site 4

Two T-cell clones were isolated from Mb-primed DBA/2 T-cell lines that were made specific for extended site 4 (region 107-120). The response of each clone to the various peptides and to Mb was studied at doubling antigen concentrations from 1.5 μ g/ml to 200 μ g/ml of culture. In both DBA/2 T-cell clones the peptides that were increased in size by the addition to the left of one-residue increments of natural Mb sequence caused lower plateau proliferation *in vitro* at a higher peptide concentration than did their longer peptide analogues that had the same Mb sequence but were kept to a uniform size by the addition of nonsense sequence. The dose-response curves for one DBA/2 T-cell clone (clone E4.15.15) are shown (Fig. 2) as an example. It can be seen that peptides X₁(108-120) and X₂(109-120) achieved optimal responses at lower peptide concentrations than did the Mb peptides 108-120 and 109-120 that were not extended by nonsense sequence. Removal of residues 107 and 108 (i.e. peptide 109-120) caused a small loss of proliferative activity, which was completely retained by the addition of nonsense residues [i.e. peptide X₂(109-120)]. Similarly, peptides 110-120 and 111-120 had very little activity, which was completely retained in peptides X₃(110-120) and X₄(111-120). Peptide 112-120 was inactive, whereas its analogue that was extended by nonsense sequence [peptide X₅(112-120)] possessed most of the original activity. The plateau stimulation values for the two DBA/2 T-cell clones are summarized in Fig. 3. It can be seen that for clone E4.15.15 the major loss in activity in the peptides of the natural Mb sequence

Fig. 2. Representative dose-response curves to peptides of the natural Mb sequence (○) or peptides extended by nonsense sequence to a uniform size (●) of a site-specific DBA/2 T-cell clone (clone E4.15.15)

The responses, which were examined over a broad dose range, are shown in the panels as follows: (a) peptides 108–120 and X_1 (108–120); (b) peptides 109–120 and X_2 (109–120); (c) peptides 110–120 and X_3 (110–120); (d) peptides 111–120 and X_4 (111–120); (e) peptides 112–120 and X_5 (112–120); (f) peptides 113–120 and X_6 (113–120). Stimulation index is the ratio c.p.m. of stimulated cells/c.p.m. of unstimulated cells. The background response of unstimulated cells was 535 c.p.m.

Fig. 3. Summary of proliferative responses of two site-specific DBA/2 T-cell clones to challenge *in vitro* with peptides of the natural Mb sequence (○) or peptides extended by nonsense sequence to a uniform size (●)

The maximum proliferative responses obtained are plotted against the corresponding residue added. The clones correspond to (a) clone E4.15.15 and (b) clone E4.8.19. Background responses for the unstimulated cells were 557 and 535 c.p.m. respectively.

occurs upon removal of residue 109. On the other hand, for the analogues that were extended by nonsense sequence to a uniform size, the loss of activity occurs very sharply upon nonsense substitution of residue 112. With clone E4.8.19 the activity shows very little decline upon decrease of peptide size of natural Mb sequence from 107–120 down to 111–120, and then falls sharply at peptide 112–120. However, for the peptides that had stepwise extensions up to a uniform size by a nonsense sequence, the activity remains constant until the replacement of residue 112 [peptide X_6 (113–120)], upon which it falls very sharply (about 85%) (Fig. 3). Finally, it should be noted that both clones responded well to Mb (stimulation index: clone E4.15.15, 242.0 at 11.2 nmol of Mb; clone E4.8.19, 78.0 at 1.4 nmol of Mb) and to concanavalin A (stimulation index 483 and 81 respectively), but did not respond to lipopolysaccharide, purified protein derivative of tuberculin, unrelated proteins or unrelated peptides.

Responses to peptides of SJL T-cell clones from extended-site-4 passage or Mb passage

Five T-cell clones from SJL mice were studied. These were isolated from Mb-primed cell lines that were specific for extended site 4 (peptide 107–120). Another clone was isolated from a Mb-driven long-term culture and was entirely specific for peptide 107–120. The responses of the clones were studied with peptide doses from 0.3 to 200 µg/ml. As was observed with the DBA/2 clones, each of the SJL clones achieved maximum response with a given peptide of natural Mb sequence at a higher optimum dose than with its analogue that was extended to a uniform size with a nonsense sequence. The dose-response curves of these clones are not shown, but in general their behaviour was very similar to those shown in Fig. 2 in that the nonsense-extended peptides always plateaued at a much lower dose than did the

corresponding natural Mb-sequence analogues. The responses of each clone to the peptides at the plateau are shown in Fig. 4.

With clone E4.5.1 decrease of the size of the Mb peptides from 107–120 down to 112–120 caused a gradual decrease in activity, which fell sharply upon removal of residue 112 (i.e. peptide 113–120). On the other hand, the activity of the nonsense-extended peptides was higher, and very similar to that of peptide 107–120, up to peptide X₅(112–120), and then it fell sharply upon nonsense-substitution of residue 112 [i.e. peptide X₆(113–120)]. With clone E4.5.4 the activity decreased slowly upon decrease in peptide size from 108–120 to 112–120, then it fell sharply upon removal of residue 112 (peptide 113–120). With the nonsense-extended peptides, the activity was only very slightly affected up to peptide X₅(112–120). The nonsense-replacement of residue 112 [peptide X₆(113–120)] caused a greater decline in proliferative activity.

The SJL T-cell clones E4.5.6, E4.4.8 and E4.5.10 from extended-site-4-driven cell lines and clone V11.2.2 from Mb-driven lines showed, in general, similar behaviour. The response remained constant (clone E4.4.8) or showed minor fluctuations (clones E4.5.6, E4.5.10 and V11.2.2) on decrease of Mb peptide size from 107–120 to 112–120, and declined sharply upon removal of residue 112 (peptide 113–120). The nonsense-extended peptides exhibited, with a given clone, a level of activity that remained essentially unchanged (and compared well with that of peptide 107–120) from peptides X₁(108–120) to X₅(112–120). Upon nonsense-replacement of residue 112 [i.e. peptide X₆(113–120)] a decline in activity was observed, but this decline was not as sharp as that obtained with the unextended counterpart 113–120. Each of the SJL clones responded equally as well to Mb relative to its response to extended site 4. The clones were viable and responded very well to concanavalin A, but

not to lipopolysaccharide or purified protein derivative of tuberculin. Also, the specificities of the above clonal responses were confirmed from the absence of responses to any unrelated proteins and peptides.

DISCUSSION

Previous studies from this laboratory (Young & Atassi, 1982, 1983; Yoshioka *et al.*, 1983) suggested that peptide size (other than the specific contact residues) may play a role in the stimulation of T cells by peptides. The idea was confirmed by a different approach that employed two sets of peptides to define, with lymph-node cells, the boundaries of an immunodominant T site in Mb (Bixler *et al.*, 1986). In one set the peptides were decreased in size stepwise by one residue at a time. In the other set the incremental decrease in size was compensated by the addition of a nonsense sequence to make peptides of a uniform size of 14 residues. This strategy showed unequivocally that the responses of the lymph-node cells were dependent on the size of the peptide and that, in appropriate peptides, extension of the natural Mb sequence by a nonsense sequence enhanced the activity of the peptide. The nonsense sequence by itself has no antibody-binding (Twining & Atassi, 1979; Twining *et al.*, 1981; Allen *et al.*, 1985) or T-cell-stimulating activity (Okuda *et al.*, 1979; Young & Atassi, 1983).

The enhancing effect on T-cell activity observed by addition of a nonsense sequence to maintain peptide size was unexpected and most dramatic. These observations, however, were made with lymph-node cells, and because of the heterogeneity of such a cell population some degree of uncertainty remained whether this peculiar phenomenon is indeed associated with T-cell recognition or is caused by some unknown parameters in the complex culture of lymph-node cells. The question must

Fig. 4. Summary of proliferative responses of six site-specific SJL T-cell clones to challenge *in vitro* with peptides of the natural Mb sequence (○) or peptides extended by nonsense sequence to a uniform size (●)

The maximum proliferative responses obtained are plotted against the corresponding residue added. The clones correspond to: (a) T-cell clone E4.5.4; (b) T-cell clone E4.5.1; (c) T-cell clone V11.2.2; (d) T-cell clone E4.4.8; (e) T-cell clone E4.5.6; (f) T-cell clone E4.5.10. Background responses for the unstimulated cells were 329, 474, 2392, 2915, 4817 and 5708 c.p.m. respectively.

then be answered with precise reagents. For this purpose T-cell clones of pre-determined specificity were used in the present studies.

It should be noted that the two DBA/2 and five of the SJL T-cell clones were isolated from T-cell lines derived from Mb-primed lymph-node cells that were driven in culture by periodic passage with peptide 107–120. These cell lines were entirely specific to the driving peptide and recognized no other regions of Mb (Yoshioka *et al.*, 1983). The recognition was imparted to the cells by priming the mouse with Mb, and the specificity desired was selected by driving with the peptide. It would then be expected that the driving peptide, and hence its structural features, would be best for stimulation of the T-cell clones. Thus these clones would be the most precise reagents to examine whether size effects of a non-specific and otherwise non-recognizable structure (at least by these T-cell clones) play a role in T-cell recognition. In addition, one clone that was obtained from a Mb-driven long-term T-cell culture was also specific to region 107–120. The peptide design (Fig. 1) is such that the incremental one-residue addition of the nonsense sequence would be capable of answering the above question and also of defining the left-hand boundary of the T site for each of the T-cell clones.

The results reported here have shown that peptide size exerted a dramatic effect on T-cell recognition at the clonal level. In every clone, a nonsense-extended peptide achieved maximum stimulating activity at a lower optimum peptide dose than did its natural-sequence (but shorter) analogue. In addition, removal of natural sequence by one-residue increments caused loss of proliferative activity, whereas the nonsense-extended analogues retained full activity until a critical 'contact' residue was removed. Thus in the DBA/2 clone E4.15.15 the activity of the natural-sequence peptides decreased steadily upon shortening and was drastically lost upon removal of residue 109. However, the nonsense-extended peptides retained full activity until residue 112 was removed, thus indicating that Ile-112 defines the left-hand boundary of the T site. Similarly, the DBA/2 clone E4.8.19 revealed a left-hand boundary at Ala-110 or Ile-111 with the natural-sequence peptides, whereas with the nonsense-extended peptides its boundary falls very sharply at Ile-112. With the five SJL clones obtained from cell lines passaged with peptide 107–120, the left-hand site boundary, as summarized in Fig. 5, was precisely defined with the nonsense-extended peptides at Ile-112 (clones E4.5.1, E4.5.6, E4.4.8 and E4.5.10) or Ile-111 (clone E4.5.4). Even with the clone derived with Mb passage, the left-hand boundary of the site was at Ile-112, thus indicating a comparable structural requirement for the recognition by this clone. In contrast, with the natural-sequence peptides the site boundary was very poorly defined (and more of the natural Mb sequence was required) for most of the clones.

The aforementioned size effects are remarkable in view of the fact that seven of the eight clones were obtained by driving the parent T-cell lines with peptide 107–120. Yet, clearly, not all the residues of the peptide are needed for T-cell recognition. Previously we had observed (Yoshioka *et al.*, 1983) that peptides that were shorter than peptide 107–120 were not capable of maintaining and driving long-term cultures to obtain monospecific cell lines. The findings reported in the present paper

Fig. 5. Boundary shifts of T-site recognition by several T-cell clones

The site-specific T-cell clones were isolated from peptide (region 107–120) or myoglobin-driven long-term T-cell cultures of T cells obtained from mice that were immunized with intact Mb.

provide a rational explanation for this observation. 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), 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 association 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 & Scheraga, 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 (Koketsu & Atassi, 1974; Atassi & Pai, 1975; 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.

The recognition of the T site at the clonal level revealed, for a given clone, a sharper boundary for the site than was obtained with bulk cultures (Bixler *et al.*, 1986). Also, it is apparent from the present work that the boundary of the T site shifts slightly from clone to clone. So, it should be expected that the response registered with bulk cultures will naturally define an 'average' boundary recognized by the numerous clones in the cultures (Bixler *et al.*, 1986). The C-terminal boundary of the T site recognized by each of these clones was not determined. However, it has been shown (Bixler *et al.*, 1986) that the C-terminal 'average' boundary of the T site with T-cell bulk cultures was His-119 for DBA/2 and Pro-120 for the SJL strain. Therefore the peptides studied here were all terminated at residue 120 in order

to stimulate the activity of both sets of clones. It should be noted that the T site defined here at the clonal level coincides with antigenic (i.e. antibody-binding) site 4, which occupies residues 113–119.

It has been proposed, from studies with Mb mutants, that the T-cell recognition of Mb involves a determinant 'centring' on residue 109 (Berkower *et al.*, 1982) that is different from the antigenic (i.e. antibody-recognition) site and does not extend as far as residue 118 (Berkower *et al.*, 1984). On the other hand, previous studies from this laboratory (Bixler & Atassi, 1983, 1984b; Yoshioka *et al.*, 1983) have shown that peptide 107–120 exhibited strong proliferative activity with Mb-primed T-cell bulk cultures and/or clones and a T site was localized to reside within, but not including all of, region 107–120. Further, Berkower and colleagues (Cease *et al.*, 1986) have examined the responses of two T-cell clones by the use of synthetic peptides rather than protein variants and reported T-cell responses to peptide 106–118. Although extensions to right of residue 118 were not tested to determine whether Arg-118 was in fact the C-terminal boundary for their clones, the results obtained by Cease *et al.* (1986) clearly confirm our previous reports that a T site is localized within the region 107–120 (Bixler & Atassi, 1983, 1984b) and includes the residues 113–119 (Okuda *et al.*, 1979). The present work with clones and studies with T-cell cultures (Bixler *et al.*, 1986) have shown that the T site superimposes very well with antigenic site 4 (residues 113–119) of Mb (Atassi & Pai, 1975; Atassi, 1975).

Previous speculations (Benjamin *et al.*, 1984) have advanced the views that the sites of T-cell recognition are different from, and more diffuse than, the sites of B-cell recognition. Although these views have remained popular (Simpson, 1986; Unanue & Allen, 1987), they are not confirmed by the present and previous studies (Okuda *et al.*, 1979; Atassi & Young, 1982; Yoshioka *et al.*, 1983; Bixler & Atassi, 1983, 1984a,b), which show that the major T sites of a protein occupy discrete areas that very frequently [but not always (Bixler *et al.*, 1984a,b; Bixler & Atassi, 1984b)] coincide with sites of B-cell recognition.

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