T-cell recognition of human haemoglobin

Localization of the full T-cell recognition profile of the β -chain by a comprehensive synthetic strategy

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This paper reports the localization of the regions on the β -chain that are recognized by T cells from mice immunized with haemoglobin. The 14 overlapping peptides encompassing the entire β -chain were examined *in vitro* for their ability to stimulate lymph-node cells from haemoglobin-primed B10.D2 (H-2^d) and SJL (H-2^s) mice. Several regions of the molecule (T sites) were found to stimulate haemoglobin-primed lymph-node cells. This strategy has enabled the localization of the full profile of T-cell recognition of the β -chain by these mouse strains. Some of the regions that stimulated T cells appeared to coincide with those recognized by antibodies (i.e. B cells). It is noteworthy that, in addition to sites recognized by both T and B cells, the protein has other sites that are recognized exclusively by T cells and to which no detectable antibody response is directed.

INTRODUCTION

Although a great deal of knowledge has now been gained about the molecular recognition of proteins by antibodies (for recent reviews see Atassi, 1984*a,b*), our understanding of the molecular features of proteins that are recognized by T cells (i.e. T sites) is just beginning to emerge. The full T-cell recognition profile has, until recently, not been determined for any protein. However, the complete T-cell recognition profiles for two proteins, sperm-whale myoglobin (Bixler & Atassi, 1983, 1984*b*) and hen's-egg lysozyme (Bixler *et al.*, 1984*a,b*) were mapped by application of the comprehensive synthetic peptide strategy previously introduced by this laboratory (Kazim & Atassi, 1980). In the case of lysozyme, the surface-simulation synthetic sites (Atassi, 1978) were also recognized by T cells (Bixler & Atassi, 1984*a*).

In the preceding paper (Yoshioka & Atassi, 1986), from the antibody binding of a set of synthetic overlapping peptides that encompass the entire β -chain of HbA, we reported the full recognition profile of the β -chain by anti-HbA antibodies raised in three different host species. These synthetic overlapping peptides are employed in the present study to define the recognition profile of the β -chain by T cells from mouse strains that had been immunized (primed) with native HbA.

MATERIALS AND METHODS

Materials

The preparation, purification and characterization of HbA and its subunits and the synthesis, purification and characterization of the overlapping peptides encompassing the entire β -chain were described in the preceding paper (Yoshioka & Atassi, 1986). These peptides are:

1–15, 11–25, 21–35, 31–45, 41–55, 51–65, 61–75, 71–85, 81–95, 91–105, 101–115, 111–125, 121–135 and 131–146.

Immunization of mice

B10.D2/oSnJ (H-2^d) and SJL (H-2^s) mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) between 6 and 10 weeks of age were immunized subcutaneously at the base of the tail with 100 μ g of HbA (determined in initial experiments to be the optimum priming dose) emulsified in phosphate-buffered saline and Freund's complete adjuvant containing *Mycobacterium tuberculosis* strain H37Ra (Difco, Detroit, MI, U.S.A.) as described by Corradin *et al.* (1977).

Assay of proliferative response

Lymph-node cells were harvested from HbA-primed mice 7 days after the initial immunization and suspended in RPMI 1640 (Gibco, Grand Island, NY, U.S.A.) containing 1% (v/v) fresh autologous normal mouse serum. The number of viable cells was determined by vital staining with fluorescein diacetate (Rotman & Papermaster, 1966). Viable lymph-node cells (7.0×10^5 cells/well) were co-cultured in triplicate with various concentrations of antigen, mitogen or synthetic peptide. Negative controls included the lysozyme peptides 31–50 and 109–129 as well as an unrelated protein antigen, hen's-egg-white lysozyme. After 3 days of incubation at 37 °C in a humidified air/CO₂ (19:1) atmosphere, the cultures were pulsed (18 h) with 2 μ Ci of [³H]thymidine (Research Products International Corp., Mount Prospect, IL, U.S.A.)/well and then harvested on to glass microfibre filters (Whatman, Clinton, NJ, U.S.A.) for liquid-scintillation counting of radioactivity.

Abbreviations used: HbA, human adult haemoglobin; T sites, protein regions recognized by T cells as defined previously (Bixler & Atassi, 1983).

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Table 1. Proliferative response of human HbA-primed lymph-node cells from H-2^d and H-2^s mice to synthetic peptides

Results are summarized at the optimum stimulating dose for each protein or peptide, although studies were carried out through the dose range 25–100 µg/ml. Cultures containing unstimulated lymph-node cells had the following background radioactivities: B10.D2/OSnJ, 4976 c.p.m.; SJL, 3240 c.p.m. The results are expressed as net label incorporated (Δ c.p.m.) and as stimulation index (S.I.), i.e. c.p.m. of stimulated cultures/c.p.m. of background. Abbreviations: Con A, concanavalin A; PPD, purified protein derivative of tuberculin; LPS, lipopolysaccharide.

Antigen	[³ H]Thymidine incorporation					
	B10.D2 T cells			SJL T cells		
	Optimum dose (µg/ml)	Δ c.p.m.	S.I. $\pm$ S.D.	Optimum dose (µg/ml)	Δ c.p.m.	S.I. $\pm$ S.D.
HbA	100	41 219	9.28 $\pm$ 0.60	100	53 480	17.51 $\pm$ 1.18
β -Chain	100	27 467	5.52 $\pm$ 0.23	100	55 779	18.22 $\pm$ 1.92
1–15	100	1 889	1.38 $\pm$ 0.10	100	4 484	2.38 $\pm$ 0.44
11–25	100	11 610	3.33 $\pm$ 0.17	100	10 930	4.37 $\pm$ 0.58
21–35	100	1 347	1.27 $\pm$ 0.16	100	1 032	1.32 $\pm$ 0.24
31–45	25	1 152	1.23 $\pm$ 0.04	50	862	1.27 $\pm$ 0.00
41–55	100	254	1.05 $\pm$ 0.11	25	351	1.11 $\pm$ 0.15
51–65	50	2 393	1.48 $\pm$ 0.11	100	3 702	2.14 $\pm$ 0.77
61–75	100	2 150	1.43 $\pm$ 0.10	100	19 005	6.87 $\pm$ 0.06
71–85	100	6 277	2.26 $\pm$ 0.22	100	11 386	4.51 $\pm$ 0.56
81–95	100	139	1.03 $\pm$ 0.07	25	612	1.19 $\pm$ 0.05
91–105	50	1 264	1.25 $\pm$ 0.11	25	511	1.16 $\pm$ 0.56
101–115	25	839	1.17 $\pm$ 0.02	25	0	0.87 $\pm$ 0.18
111–125	100	1 382	1.28 $\pm$ 0.12	100	39 149	13.08 $\pm$ 0.69
121–135	100	4 977	2.00 $\pm$ 0.17	100	3 790	2.17 $\pm$ 0.12
131–146	100	28 990	6.82 $\pm$ 0.33	25	7 540	3.33 $\pm$ 0.26
Controls						
Con A	1	137 604	28.65 $\pm$ 1.26	1	78 752	25.31 $\pm$ 1.26
PPD	100	153 220	31.79 $\pm$ 1.74	100	168 903	53.13 $\pm$ 1.39
LPS	500	152 144	31.57 $\pm$ 1.91	500	115 025	36.50 $\pm$ 0.58
Lysozyme	100	151	1.03 $\pm$ 0.06	100	199	1.06 $\pm$ 0.16

RESULTS

Proliferative response to peptides of haemoglobin-primed lymph-node cells from B10.D2 mice

The proliferative responses to HbA, β -chain and synthetic peptides of lymph-node cells from B10.D2/OSnJ mice that had been primed with HbA were examined over a wide dose range (25–100 µg/ml). The results are summarized in Table 1. The highest proliferative responses, relative to the background of unstimulated cultures, were obtained with peptides 131–146 and 11–25. Intermediate proliferative responses were observed with peptides 71–85 and 121–135. Minimal, but nonetheless reproducible, positive responses were induced by peptides 51–65, 61–75 and 1–15. None of the remaining peptides was found to induce proliferation. As expected, the cells responded to HbA and to β -chain. The cells were viable, as evidenced from their response to the mitogens. Finally, no responses were obtained to unrelated proteins or peptides and, conversely, the synthetic β -chain peptides listed in Table 1 did not activate lymph-node cells from B10.D2 mice that had been primed with an unrelated protein, hen's-egg lysozyme (results not shown).

Proliferative responses of lymph-node cells from HbA-primed SJL mice

The proliferative responses of HbA-primed SJL/J lymph-node cells to challenge *in vitro* with synthetic

peptides were also examined (Table 1). The highest proliferative responses were induced by peptides 111–125, 61–75, 71–85 and 11–25. Peptides 1–15, 51–65 and 121–135 provoked intermediate responses. The remaining peptides did not induce an increase in proliferative activity. The cells responded to HbA, β -chain, concanavalin A, purified protein derivative of tuberculin and lipopolysaccharide but did not respond to unrelated protein (hen's-egg lysozyme) or peptides (lysozyme peptides 31–50 and 109–129). In addition, the β -chain peptides did not stimulate or suppress the response of T cells from SJL mice that had been primed with lysozyme (results not shown).

DISCUSSION

In previous studies (Krco *et al.*, 1981a) the mouse strain B10.D2 (H-2^d) was a high responder to HbA and to β -chain, and an H-2^s haplotype (B10.S) was an intermediate responder. In the present work, another H-2^s haplotype (SJL) was a high responder to HbA and to β -chain. The difference in the levels of responses between the two H-2^s haplotypes is indicative of non-H-2 influences, which are known to participate in the immune responses to protein antigens (Okuda *et al.*, 1978, 1979a,b; Krco *et al.*, 1981a,b,c; Twining *et al.*, 1981; David & Atassi, 1982). Thus the two strains selected for this work, B10.D2 and SJL, are high responders to the β -chain.

The antigenic sites of proteins occupy conformationally sensitive areas (for reviews see Atassi, 1975, 1978, 1980, 1984a,b) comprising residues that are either directly linked by peptide bonds (e.g. the continuous antigenic sites of sperm-whale myoglobin; Atassi, 1975) or distant in sequence but, through folding of the polypeptide chain, come in close spatial proximity (e.g. the discontinuous antigenic sites of lysozyme; Atassi, 1978). Since both of these architectures (for discussion of appropriate terminology see Atassi & Smith, 1978) are recognized by antibodies (B cells), it is reasonable to assume that T cells can also recognize both types of antigenic sites. Indeed, the T-cell recognition of the three discontinuous antigenic sites of lysozyme has been reported (Bixler & Atassi, 1984a). The present study was aimed at determining the full profile of the continuous regions of T-cell recognition for the β -chain of HbA in two high-responder mouse strains, thus providing a unique opportunity for comparison of the molecular parameters involved in the immune recognition of an oligomeric protein by both B and T cells. The rationale and design of the comprehensive overlapping peptide strategy have been discussed previously in detail (Kazim & Atassi, 1980, 1982; Bixler *et al.*, 1984a) and also in the preceding paper (Yoshioka & Atassi, 1986).

Earlier studies from this laboratory (Okuda *et al.*, 1979b) showed that the synthetic antigenic sites of myoglobin (six or seven amino acid residues) are able to stimulate T-cell proliferation *in vitro*. However, in subsequent studies it was found that extension of the sites by additional five or six residues improved the proliferative activity of lymph-node cells and could in fact be used to drive the culture into a T-cell population (T-cell lines) of a single desired specificity, and from these T-cell lines T-cell clones possessing the same predetermined specificity are prepared (Yoshioka *et al.*, 1983). Accordingly, the peptide size of 15 residues chosen for the present work is appropriate for mapping the T-cell recognition profile.

It should be noted that the strategy is not designed to give the precise boundaries of the sites. A broad localization of the major T sites recognized by the two strains (Fig. 1) was according to the procedure previously described in detail (Kazim & Atassi, 1980, 1982) and takes into consideration activities associated with individual

peptides as well as activities residing in the overlap regions of adjacent peptides. It is not implied that the T sites comprise the entire regions shown in Fig. 1, but rather that they reside within those regions. Indeed, the regions containing the sites are made intentionally larger than the expected size of the site in order to avoid errors arising from close delineation at this stage.

Examination of the three-dimensional structure of haemoglobin (Perutz *et al.*, 1968; Fermi, 1975) reveals that the T site residing within the region 112–124 constitutes almost entirely residues involved in the β_1 - α_1 contacts (i.e. residues 112, 115, 116, 119, 122, 123 and 124). Also, the last T site, residing within the region 133–146, carries residues 145 and 146, which are normally involved in the β_2 - α_1 contact. Since such residues would be buried in the tetramer, they would not be expected to participate in immune recognition by T cells unless this recognition takes place at the subunit level. Similar conclusions were made from the locations of the antigenic sites of the α -chain (Kazim & Atassi, 1982) and the β -chain (Yoshioka & Atassi, 1986).

The present work reveals that the β -chain contains several T-cell recognition regions (T sites) (Fig. 1), four of which coincide with the antigenic (antibody-binding) sites that are recognized by mouse antibodies (Yoshioka & Atassi, 1986). There is, however, at least one T site (residing within region 60–69 for the H-2^s haplotypes) that is exclusively T-cell-specific and is not recognized by B cells. Previously we have found in hen's-egg lysozyme that the three sites recognized by antibodies (Atassi, 1978) are also recognized by lysozyme-specific T cells (Bixler & Atassi, 1984a). In addition, lysozyme has four T sites that are recognized exclusively by T cells and to which no antibody responses are detectable (Bixler *et al.*, 1984a,b). Similarly, myoglobin contains sites that are recognized by both B and T cells as well as sites that are recognized exclusively by T cells. The roles of the various protein T sites in immune recognition and regulation have yet to be determined, but it has been shown that inter-site influences play an important role in the immune response to the whole protein (Atassi *et al.*, 1981; Krco *et al.*, 1981a). Inter-site T-cell influences could either be 'help' or 'suppression' in nature (Atassi *et al.*, 1981).

The shifts observed in the locations of the T sites from strain to strain is not unexpected. Similar frame shifts were observed in the boundaries of the sites recognized by their respective antibodies in myoglobin (Koketsu & Atassi, 1973, 1974; Atassi, 1975, 1984b) and haemoglobin (Kazim & Atassi, 1982; Yoshioka & Atassi, 1983, 1986) as well as in the sites of T-cell recognition in lysozyme (Bixler *et al.*, 1984a,b) and myoglobin (Bixler & Atassi, 1983, 1984b). The levels of responses to the T sites were different in the two haplotypes. For example, the H-2^s haplotype mounted a high response to the T site residing within residues 112–124, whereas in the H-2^d haplotype this site provoked little or no response. Similarly, the T site near the C-terminus of the β -chain stimulated a high response in the H-2^d haplotype but only a moderate response in the H-2^s haplotype. These results are consistent with our previous findings that the immune responses to the individual antigenic sites in a protein are each under separate *Ir*-gene control (Okuda *et al.*, 1979b; Twining *et al.*, 1981; David & Atassi, 1982; Bixler & Atassi, 1984b; Bixler *et al.*, 1984a,b). The immune response to HbA and its subunits has been shown (Krco *et al.*, 1981a,b,c, 1984) to be under genetic control. The

Fig. 1. Schematic diagram showing the full profile of the regions of the β -chain of HbA that carry the continuous sites of T-cell recognition (T sites) in two mouse strains

The T-cell recognition sites are also compared with the sites of mouse antibody (B-cell) recognition (i.e. antigenic sites), which were localized in the preceding paper (Yoshioka & Atassi, 1986). It is not implied that the entire regions shown comprise the T site, rather that the sites reside within these regions. Indeed, in the localization by the overlapping peptide strategy, the T sites are localized within intentionally larger regions than their expected size.

present findings reveal that the responses to each antigenic site are under separate genetic control.

It has been postulated that the sites recognized by T cells may be different from, and more limited in number than, the sites recognized by antibodies (Benjamin *et al.*, 1984). This speculation, which has no experimental basis, overlooked the fact that the myoglobin sites recognized by B cells had been well documented to be recognized by T cells as well (Okuda *et al.*, 1979b; Young & Atassi, 1982, 1983; Yoshioka *et al.*, 1983; Bixler & Atassi, 1983). Similarly, as shown in the present paper and in studies of the T-cell recognition of myoglobin (Bixler & Atassi, 1983, 1984b) and lysozyme (Bixler & Atassi, 1984a; Bixler *et al.*, 1984a,b), T cells can indeed recognize the same sites that B cells recognize as well as other regions to which no antibody responses have been detected. Thus the fine specificity of T cells, when examined systematically, is neither limited nor necessarily different from that of B cells.

The regions of T-cell recognition that have been localized reflect the responses of numerous subpopulations or clones of T cells, each of which perceives the T site somewhat differently. Thus various cell clones recognize different overlapping parts of the site (i.e. shifting to the right or to the left), and the maximal effective response, which is the average of all of these activities, will be directed towards the centre of the region, i.e. the T site. This would be consistent with a heterogeneous nature of the T-cell response to a given region at the clonal level, as has been found in the B-cell recognition of myoglobin (Atassi, 1975) and other proteins (Atassi, 1978, 1980, 1984a,b). In view of the heterogeneity, the possibility exists, as found in B-cell recognition, that T-cell clones with specificity for a region outside of a known T site and that are present in traces would not be detected in T-cell proliferative assays of bulk cultures but could be isolated. It remains uncertain whether such trace amounts of T-cell and B-cell clones, whose specificity is directed towards non-dominant regions (termed background responses; Atassi, 1980) have any biological significance.

In conclusion, the full profile of the regions within the β -chain recognized by T cells has been localized by employing synthetic overlapping peptides accounting for the entire chain. The regions varied in their activity from strain to strain, as expected from independent *Ir*-gene control for each antigenic site. Some of these regions coincide with known antigenic (antibody-binding) sites, and additional regions are recognized exclusively by T cells.

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