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Immunology

T cells specific for α–β interface regions of hemoglobin recognize the isolated subunit but not the tetramer and indicate presentation without processing

(subunit-interacting surfaces/site-specific T cells/T-cell clones/synthetic peptides)

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ABSTRACT **Processing of a protein antigen into fragments is believed to be a prerequisite for its presentation by the antigen-presenting cell to the T cell. This model would predict that, in oligomeric proteins, T cells prepared with specificity for regions that are buried within subunit association surfaces should recognize the respective regions *in vitro* equally well on the isolated subunit or on the oligomer. Three hemoglobin (Hb) α-chain synthetic peptides, corresponding to areas that are situated either completely [α-(31–45)] or partially [α-(41–45) and α-(81–95)] within the interface between the α and β subunits of Hb, and a fourth peptide representing a completely exposed area in tetrameric Hb were used as immunogens in SJL/J (*H-2*^{s}) mice. Peptide-primed T cells were passaged *in vitro* with the respective peptide to obtain peptide-specific T-lymphocyte lines. T-cell clones were isolated from these lines by limiting dilution. T-cell lines and clones that were specific for buried regions in the subunit association surfaces recognized the free peptide and the isolated subunit but not the Hb tetramer. On the other hand, T cells with specificity against regions that are not involved in subunit interaction and are completely exposed in the tetramer recognized the peptide, the isolated subunit, and the oligomeric protein equally well. The responses of the T-cell lines and clones were major histocompatibility complex-restricted. Since the same x-irradiated antigen-presenting cells were employed, the results could not be attributed to differences or defects in Hb processing. The findings indicate that *in vitro* the native (unprocessed and undissociated) oligomeric protein was the trigger of major histocompatibility complex-restricted T-cell responses.**

The presentation of a protein antigen to T lymphocytes is believed to be dependent on a first step in which the protein is internalized and processed into fragments that reappear on the surface of the antigen-presenting cell (APC) and are presented in association with major histocompatibility complex (MHC) molecules to the T cell (1, 2). Although there have been reports that a protein molecule is presented intact by the APC (see *Discussion*), the idea that processing is a prerequisite for presentation is by far the most widely accepted model. Many consequences of this model can be predicted and tested. If protein fragments and not the intact protein are presented by the APC to the T cell, it would be expected that T cells that are specific for the subunit interface in an oligomeric protein (if such T cells could be made) should recognize the isolated subunit or the oligomer equally well. Obviously, the T-cell recognition site cannot remain buried (in the interface between the subunits) after the oligomer is processed into fragments. If, on the other hand, these T cells recognize the peptide and the isolated subunit but not the oligomer, then this would be indicative that the subunit interface has remained buried in an intact oligomeric protein. In this paper we report the role in T-cell recognition of the quaternary structure of human hemoglobin A (Hb) resulting from the association of its α and β subunits.

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MATERIALS AND METHODS

The major chromatographic component of adult Hb was prepared as previously described (3). The α and β chains of Hb were prepared as described (4). The α- and β-chain preparations were homogeneous by polyacrylamide gel electrophoresis, and their purity was further confirmed by subunit-specific monoclonal antibodies (5). Synthesis, purification, and characterization of the α-chain peptides α-(31–45), α-(41–55), α-(51–65), and α-(81–95) have been reported (6, 7). These four peptides correspond to areas in Hb that are completely exposed [α-(51–65)], completely buried [α-(31–45)], or partially buried [α-(41–55) and α-(81–95)] within the α–β interface (Table 1). Immunization of SJL (*H-2*^{s}) mice (The Jackson Laboratory), 6–8 weeks old, was done subcutaneously at the base of the tail with 50 μg of each peptide emulsified in 50 μl of 0.15 M NaCl/0.01 M sodium phosphate buffer, pH 7.2, and an equal volume of complete Freund's adjuvant (9). The lymph node cells (LNCs) were driven into a single preselected specificity by passage *in vitro* with the peptide (10), and the T-cell clones were isolated from T-cell lines by two limiting dilutions (10). The responses of LNCs, T-cell lines, and clones were determined by their proliferative activity *in vitro* on challenge with peptide or protein (10, 11) as described in the respective tables.

RESULTS

Proliferative Response of Peptide-Primed LNCs. (*i*) *Region completely buried in the α–β interface.* Mice immunized with peptide α-(31–45), representing a region completely buried within the α–β interface, gave T cells that exhibited comparable responses to the peptide and to the α chain but did not respond to Hb (Table 2).

(*ii*) *Partially exposed and completely exposed regions.* T cells obtained after immunization with peptide α-(41–55), α-(81–95), or α-(51–65) recognized the respective immunizing peptide as well as the α chain and Hb (Table 2).

The cells were viable and responded to Con A and purified protein derivative of tuberculin (PPD). Their specificity was confirmed by absence of response to unrelated antigen (lysozyme).

Abbreviations: APC, antigen-presenting cell; Hb, human hemoglobin A; LNC, lymph node cell; MHC, major histocompatibility complex; PPD, purified protein derivative of tuberculin.

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Table 1. Residues on the α chain involved in α–β subunit contacts in Hb

Interactions and α-chain residues involved		Synthetic α-chain peptide	No. of subunit-interface contact residues on peptide
α_1–β_1	α_1–β_2		
Arg-31			
Met-34			
Ser-35			
Phe-36			
	Pro-37		
	Thr-38		
	Lys-40		
	Thr-41		
	Tyr-42		
	Pro-44	α-(31–45)	10
		α-(41–55)	3
		α-(51–65)	0
	Leu-91		
	Arg-92		
	Asp-94		
	Pro-95	α-(81–95)	4
	Val-96		
	Asn-97		

This table does *not* list all the α-chain contact residues that are involved in the α–β subunit interactions; it lists only the region encompassed by the peptides studied in the present work. The identity of the contact residues was obtained from Fermi (8).

Response of T-Cell Lines and Clones Directed Against Partially Buried α-Chain Regions. Since LNCs against the partially buried regions α-(41–55) and α-(81–95) were unable to distinguish between free α chain and Hb, they were passaged with the respective peptide to enrich for site-specific T cells. The T-cell line specific for peptide α-(41–55) responded equally to the peptide and to the free α chain and gave only a partial (about 50%) response to Hb (Table 3). From this line, 12 T-cell clones were prepared. Five clones responded to both α chain and Hb, and 7 clones responded to α chain but not to Hb. An example of a clone that recognized the peptide and free α chain but not Hb is shown in Table 3. The T-cell line obtained by passage *in vitro* with peptide α-(81–95) gave comparable responses to the peptide and free α chain, while its response to Hb was extremely poor (4% relative to peptide) (Table 4). In view of the restricted specificity of this line and its ability to distinguish free α chain from that in tetrameric Hb, it was felt that cloning of this line would not be necessary.

Response of T-Cell Lines and Clones That Are Specific for a Completely Exposed α-Chain Region. The T-cell line obtained by passage with peptide α-(51–65) gave comparable responses to the peptide, free α chain, and Hb (Table 5). Nine clones were prepared from this line. They possessed the same specificity as the parent line (example in Table 5), each responding equally to the peptide, free α chain, and Hb.

DISCUSSION

To examine the role of site accessibility in the T-cell recognition of an oligomeric protein, we prepared T cells possessing precisely defined specificities to preselected regions of the molecule. The peptides selected for the present work correspond to areas having three different levels of exposure in the 3-dimensional structure of tetrameric Hb (8, 12). Table 1 summarizes the residues in the N-terminal two-thirds of the α chain that are involved in the α–β subunit contacts. Table 1 also summarizes the number of contact residues on each of the peptides. Peptide α-(31–45) corresponds to a region on the α chain that is entirely buried within the α–β interface, having 10 contact residues. Peptides α-(41–55) and α-(81–95) correspond to regions that are partially buried, carrying 3 and 4 contact residues, respectively. Finally, peptide α-(51–65) was selected to represent a region that is not involved in any α–β contacts and is also well exposed on the surface of the tetramer.

The results reported here show that LNCs whose specificity is directed against a region [α-(31–45)] that is completely buried within the subunit association surfaces in Hb recognize the free synthetic peptide and the isolated subunit but not the intact tetramer. LNCs against regions that are only partially involved in subunit association or against exposed regions that are not involved at all in subunit contacts recognize the peptide and the isolated subunit as well as intact Hb.

The response of a T-cell line is a reflection of the specificity distribution of the clones within that line. The T-cell line against peptide α-(81–95) could not respond to intact Hb, but it mounted strong responses to the isolated α subunit and to the free peptide. Thus, the clonal distribution was predom-

Table 2. Proliferative response of peptide-primed lymphocytes to the immunizing peptide, α chain, and Hb

	[^{3}H]Thymidine incorporation							
	α-(31–45)		α-(41–55)		α-(51–65)		α-(81–95)	
Challenge antigen*	cpm	Opt. dose,† μg/ml	cpm	Opt. dose,† μg/ml	cpm	Opt. dose,† μg/ml	cpm	Opt. dose,† μg/ml
Immunizing peptide	10,950	27.9	14,522	55.8	27,582	55.8	85,375	55.8
α Chain	9,233	6.3	13,432	6.3	32,970	6.3	72,311	6.3
Hb	1,095	1.6	18,624	1.6	39,772	1.6	74,249	1.6
None (medium)	444		1,793		2,208		1,113	
Lysozyme	725		1,893		ND		1,389	
Con A	63,220		189,160		234,520		142,250	
PPD	55,640		206,200		271,344		128,310	

SJL (*H-2^s*) mice were immunized subcutaneously at the base of the tail with peptide (50 μg per mouse) emulsified in phosphate-buffered saline and Freund's complete adjuvant. Seven days later, LNCs (7×10^5 per well) were cocultured with various concentrations of antigen, mitogen, or synthetic peptide in RPMI 1640 containing 1% (vol/vol) fresh autologous normal mouse serum. Stimulation with hen-egg lysozyme was used as a negative control. After 3 days at 37°C in a humidified air/CO_2 (19:1) atmosphere, the cultures were incubated (18 hr) with [^{3}H]thymidine (2 μCi per well; 1 Ci = 37 GBq) and then harvested onto glass microfiber filters. The [^{3}H]thymidine incorporated was determined by liquid scintillation spectroscopy. Results have *not* been corrected for nonspecific incorporation of label into unstimulated cells and represent the average of triplicates, which varied by ±8% or less. ND, not determined.

*Challenge antigens were used in the dose range 1–100 μg /ml except for Con A and PPD, which were used at 1 and 100 μg/ml, respectively.

†Optimum dose of challenge antigen.

Table 3. Response of a T-cell line and a clone specific for peptide α-(41–55) to challenge with peptide, α chain, and Hb

Challenge antigen	[³H]Thymidine incorporation			
	T-cell line		T-cell clone	
	cpm	Opt. dose,* μg/ml	cpm	Opt. dose,* μg/ml
Peptide α-(41–55)	60,897	25	27,153	12.5
α Chain	62,688	100	31,086	100
Hb	33,103	100	3,056	50
None (medium)	1,110	—	3,178	
PPD	599	100	2,573	100
LPS	1,028	500	3,480	500
Con A	104,167	1	43,113	1

The T-cell line was obtained by five *in vitro* passages with peptide α-(41–55) of peptide-primed T cells. The T-cell clone was isolated from this line. The T-cell line and clones (1×10^4 cells per well) were cultured in the presence of x-irradiated (3300 R) syngeneic filler cells (5×10^5 cells per well). The cells were challenged with peptide or protein in the dose range 1–100 μg/ml, except for Con A, PPD, and lipopolysaccharide (LPS), which were used at 1, 100, and 500 μg/ml, respectively. Values represent the mean cpm of six replicate analyses. The standard deviation was ±9% or less. The experiment was carried out at the same time with the same preparation of APCs employed for the lines and clones in Tables 4 and 5 to ensure that any differences were not due to defects in antigen presentation by various APC preparations.
*Optimum dose of challenge antigen.

inantly against the part of the peptide residing within the α–β interface. Cloning of this line was, therefore, considered unnecessary. In the T-cell line against peptide α-(41–55), about half the clones recognized the part of the peptide involved in the α–β interface while the other half recognized the exposed part of this region (to the right of residue 45). Finally, all the T-cell clones against the region α-(51–65), which is fully exposed in tetrameric Hb, responded equally to Hb, the α chain, and the immunizing peptide.

T-cell recognition clearly requires an APC that must present antigen in the context of the MHC to the T cell (for review, see refs. 1, 2, and 13). This concept, which is universally accepted by immunologists, however, is distinct from the concept of antigen processing, which imposes fundamental limitations on the form of the antigen perceived by T cells.

Table 4. Response of LNCs and a T-cell line specific for peptide α-(81–95) to challenge with peptide, α chain, and Hb

Challenge antigen	[³H]Thymidine incorporation			
	LNCs		T-cell line	
	cpm	Opt. dose,* μg/ml	cpm	Opt. dose,* μg/ml
Peptide α-(81–95)	16,802	100	49,096	10
α Chain	18,905	50	42,198	200
Hb	19,399	50	2,480	200
None (medium)	472	—	580	—
Lysozyme	546	100	522	100
PPD	121,130	100	619	100
Con A	116,520	1	52,083	1

The response of LNCs from mice that were primed with peptide α-(81–95) to peptide, α chain, and Hb was first examined as described in Table 2. These cells were then passaged *in vitro* six times with peptide α-(81–95). The responses of the T-cell line were determined as described in Table 3. The experiment here was done at the same time as that shown in Tables 3 and 5 with the same preparation of filler cells to ensure that the results were not due to differences in the ability of the APCs to present Hb, but rather due to the ability of the T cells to recognize their respective site on the antigen.
*Optimum dose of challenge antigen.

Table 5. Response of T-cell line and a clone specific for peptide α-(51–65) to challenge with peptide, α chain, and Hb

Challenge antigen	[³H]Thymidine incorporation			
	T-cell line		T-cell clone	
	cpm	Opt. dose, μg/ml	cpm	Opt. dose, μg/ml
Peptide α-(51–65)	12,583	12.5	7,081	3.1
α Chain	13,821	25	7,670	100
Hb	14,638	50	6,560	50
None (medium)	1,683	—	1,783	—
PPD	1,333	100	1,617	100
LPS	1,560	500	1,815	500
Con A	25,573	1	31,467	1

The T-cell line was obtained by five *in vitro* passages with peptide α-(51–65) of peptide-primed T cells. The T-cell clone was isolated from this line. The rest of the details are as in Table 3. The experiment was carried out at the same time with the same preparation of APCs employed in Tables 3 and 4. LPS, lipopolysaccharide.

For the purpose of this discussion, antigen processing is classically defined as the intracellular degradation of antigen into small, immunogenic fragments and excludes, in our view, the simple conformational alteration or denaturation of a protein, which may occur in the presence or absence of any cellular activity. The small fragments generated by processing are thought to associate with the MHC molecule by binding in its groove (14–16). The bound fragment constitutes the species presented on the surface of APCs to T cells. That processing is a prerequisite for antigen presentation in the early events leading to T-cell recognition is the favored view held by the majority of immunologists.

Several lines of evidence are considered to be supportive of the concept of processing. It has been noted that a lag phase occurs between antigen uptake by APCs and their capacity to present antigen (17, 18). It has also been shown that paraformaldehyde fixation of APCs interferes with the presentation of proteins but not of protein fragments or synthetic peptides (19, 20). Aldehydes, however, in addition to effecting cross-links, cause modification of many other amino acid side chains (21). Also, it cannot be excluded that some aldehyde may be internalized and thus cause disruption of cell function. Several agents, including lysosomotropic agents or carboxylic ionophores (18, 22–24), inhibitors of protein synthesis (25), and soluble or particulate molecules (26), have been shown to interfere with presentation. In the latter study (26), it is of interest to note that, although soluble or particulate antigens interfered with presentation, catabolism and Ia expression were unaltered. This suggested the presence of other crucial, but hitherto unknown, events that intervene between antigen encounter and presentation. It is obvious that the effects of these agents are far more involved than has been perceived and, therefore, caution should be exercised in their application to study antigen presentation. In light of the complex intracellular events that occur during presentation, the interpretation that the only crucial event affected must be lysosomal, prelysosomal, or endosomal processing of antigen should be reconsidered.

Several lines of evidence favor an alternative interpretation of the early events of immune recognition in which the protein is presented intact (27, 28). Several years ago, studies from this laboratory showed that T-cell epitopes on a protein very frequently overlap or coincide with B-cell epitopes that occupy exposed surface regions of the protein (10, 11, 29–35). These regions, therefore, are prime targets for proteolysis and would be expected to be readily destroyed by processing (27, 36). Further, it was shown that the pattern of T-cell recognition following priming with peptides differs from the

pattern observed when the native protein is the priming antigen (9, 37).

T-cell recognition, like B-cell recognition, is sensitive to the conformation of the protein antigen. T-cell lines and clones are able to distinguish between the native and unfolded protein molecule (31, 38) [for example, between myoglobin and its altered conformational state, apomyoglobin (39), and between lysoyme and its unfolded derivative (38)]. Similarly, it has been demonstrated that the discontinuous surface-simulation synthetic sites of lysozyme (40) are recognized by T cells (31, 38). It is difficult to reconcile the recognition of epitopes where the precise spatial relationship of the contact residues is critical for the integrity of the sites with a process that would be expected to destroy a 3-dimensional structure.

It has been shown that site-specific T-cell recognition of myoglobin is influenced by amino acid substitutions outside of the site itself (41, 42). These substitutions were distant in sequence from the site but close to it in the 3-dimensional structure. Since the site-specific clones were obtained by repeated passage with peptide (42), it is difficult to visualize how environmental residues, which themselves are not directly involved in binding, could influence recognition unless the intact protein was the presented species.

Several studies from other laboratories also favor the alternative interpretation (27, 28) that presentation does not require processing. Liposomes bearing MHC and intact protein antigens are able to present the antigen to T cells only when the two molecules are on the same liposome, whereas mixtures of liposomes bearing either antigen or MHC molecules do not present antigen (43–47). T-cell clones recognize the native conformation of cytochrome *c* (48) or the hemagglutinin of influenza virus (49, 50). Indeed, it has been shown that recognition of conformational features extends to cytotoxic T cells, which have been reported to recognize the intact influenza nucleoprotein (51). More recently, it has been concluded that the A α chain of fibrinogen was recognized by T cells without processing (52). In the latter report, however, presentation without processing was attributed, with little supporting evidence, to conformational flexibility of the C-terminal region of the A α chain. Collectively, these studies strongly indicate that a more open interpretation of the early events of immune recognition must be considered in which T-cell recognition involves the presentation of the whole protein routinely rather than as a spurious exception to the rule.

Finally, as shown here in the case of Hb, the quaternary structure of a protein may also be preserved during presentation *in vitro*. T cells whose specificity is directed at regions hidden within the α–β interface can recognize synthetic peptides representing the interface regions and the isolated subunit but not Hb. In contrast, T cells whose specificity is directed at exposed regions recognize the synthetic region and the subunit as well as intact Hb. The results cannot be explained by competition for Ia between homologous regions on the α and β chains of Hb. The buried region α-(31–45) shows very poor homology to the β chain. In fact the homology of the exposed region α-(51–65) to the β chain (residues 56–70) is quite high (9 of 15 residues, with one segment of 5 residues). Therefore, if there were to be competition for Ia, it will more likely happen with region α-(51–65) and not with α-(31–45). Therefore, the lack of recognition of Hb by anti-[α-(31–45)] T cells is not caused by the region's homology to the β chain, but rather by its inaccessibility in the Hb tetramer. Also, the results could not be due to a defect in the presentation of Hb by some APC preparations because all the experiments summarized in Tables 3–5 were performed with the same APC preparation. These findings indicate that the regions in the subunit interface of Hb remain buried when the protein is presented *in vitro* to T cells. If processing of Hb occurs, how is it possible to retain the integrity of the tetramer?

These findings strongly suggest that the concept of antigen processing be critically reappraised. It is clearly necessary, for whatever mechanism is ultimately found to occur during presentation, to allow the recognition or presentation of the conformationally sensitive T-cell epitopes before such information is destroyed by catabolic processes. This would require the recognition by the T cell of a protein molecule that is presented predominantly in its intact form (9, 27, 28, 37). Or alternatively, the conformational information may be preserved by an antibody whose variable region enters the APC with the antigen. The T cells recognize the antibody idiotopes in context of the MHC (N. K. Jerne, ref. 53 and personal communication). This would be consistent with specific antigen presentation by nonimmune B-cell clones (54). Further, we previously suggested (9, 37) that antigen presentation bears striking similarities to other biological, membrane-mediated cellular activities involving both chemical mediators and membrane receptors and that the protein–receptor complex is internalized after recognition in order to recycle receptor and dispose of (degrade) the antigen. Indeed, recent studies have demonstrated the potential involvement of Ia recycling (55) and regulation of presentation by interferon-γ (56).

This brief treatment should serve to emphasize the complexity of antigen presentation and the need to reevaluate the concept of antigen processing.

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