

THE HEME MOIETY OF CYTOCHROME *c* IS AN
AUTOREACTIVE I_r GENE-RESTRICTED
T CELL EPITOPE

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The phenomenon of Th receptor recognition of protein antigens has been intensively studied during recent years, leading to a more comprehensive understanding of the physical and chemical requirements that determine the mode of interaction between antigen, the MHC-restricting element (class II gene products), and the TCR complex (1-4). The current working hypothesis to explain the process of antigen presentation states that the protein antigen must be proteolytically digested (4-6) or at least denatured (7) inside acidic compartments (probably endosomes) within the APC (e.g., B cells, macrophages, dendritic cells) (8-10). The pathways of antigen degradation and subsequent emergence of the immunogenic peptide onto the plasma membrane of the presenting cell in conjunction with the appropriate class II (Ia) heterodimer are still unknown, although it appears most likely that the peptide forms a stable complex with the Ia molecule before the TCR recognition event (11-13). Recent data emerging from several laboratories (14, 15) have provided strong evidence that there exists only a single site on the class II heterodimer that can interact directly with the immunogenic peptide. In the case of the murine system, any given individual may express a limited number of Ia molecules, thus, the peptide-binding site on the class II heterodimer must be of extremely broad specificity in order to accept the large variety of antigenic peptides likely to be encountered.

The learning of MHC restriction is acquired in the thymus where immature thymocytes are selected on the basis of their ability to recognize antigen in the context of self-MHC (16, 17). It is at this stage that tolerance to self antigen is acquired; at least in the case of antigens present in the thymic environment (18-20). However, tolerance to antigens never expressed in the thymus must be established after the mature T cell has arrived in the periphery. Despite evidence that a deletion event for self-reactive T cell clones is operative in the thymus (20), there have been many examples where the existence of self-reactive T cell clones has been demonstrated to both self-MHC antigens (21, 22) and non-MHC self antigens (23, 24). Thus, in these instances, it appears that clonal deletion is not effective. Active regulatory systems, involving suppressor T cells, have been proposed to account for the suppression of such self-reactive T cell clones under normal circumstances (25, 26).

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The study of the immunological recognition of cytochromes *c* (cyt *c*)¹ has played a critical role in elucidating the physical and chemical nature of T cell epitopes derived from protein antigens (2, 27-29). However, as yet, there has been no investigation of the immunological properties of the heme moiety of cyt *c*. The heme group is covalently bound to the polypeptide backbone of the protein through cysteine residues at position 14 and 17. Here we show that the heme moiety of cyt *c* is a dominant T cell epitope that induces a large proliferative response in lymph node T cells derived from SJL and B10.A mice. Not only is this vigorous response observed for cyt *c*-primed T cell populations but also for populations obtained from naive SJL or B10.A mice. In the case of both primed and naive T cell populations, the reactivity to the heme moiety falls under strict MHC restriction, thus behaving as a "classical" T cell epitope. Therefore, these findings require that the current models describing the nature of T cell epitopes be extended to include nonpeptide molecules. Furthermore, as the heme moiety is ubiquitous throughout the organism, although sequestered within proteins, the existence of heme-reactive T cell populations in unprimed animals provides another example of the existence of self-reactive T cell clones.

Materials and Methods

Antigens. Bovine heart cyt *c* (type V) (bov cyt *c*) and pigeon breast muscle cyt *c* (type XIII) were obtained from the Sigma Chemical Co. (St. Louis, MO) and were used without further purification. The chloride salt of the heme moiety, hemin chloride (HmCl), (Calbiochem-Behring Corp., La Jolla, CA) was used in all in vitro proliferation assays. In this form, the iron atom of the penta-coordination complex is in the ferric (Fe³⁺) oxidation state. The CNBr cleavage fragment of bov cyt *c* (1-65) was prepared from the native apo-protein according to the method of Corradin and Harbury (30).

Apo-bov cyt *c* was prepared according to the method of Sano and Tanaka (31). 1 ml of glacial acetic acid and 7.5 ml of 0.8% Ag₂SO₄ (in H₂O) were added to 38 mg of bov cyt *c* dissolved in 5 ml of distilled H₂O. After incubation at 37°C for 6 h, 85 ml of acid acetone (1 ml 5N H₂SO₄/100 ml of acetone) was used to precipitate the protein, which was then pelleted by centrifugation. The protein precipitate was redissolved in the smallest possible volume of 0.2 M acetic acid. To remove the free heme from the protein environment, the solution was then dialyzed overnight under nitrogen, against 1 litre of 0.2 M acetic acid. All reactions until this stage of the procedure were carried out in the dark using light-proof containers. Once the free heme had been removed, the protein solution was dialyzed exhaustively against distilled H₂O. This procedure replaces the heme moiety (covalently bound to the polypeptide backbone of the cyt *c* molecule through cysteines at positions 14 and 17) with Ag ions. As a consequence of this substitution, the Ag-cyt *c* complex is insoluble under nonacidic conditions. To remove the Ag ions, the protein was lyophilized and then redissolved in 10 ml of 0.05 M ammonium acetate and 0.15 ml of pure 2-ME. The solution was incubated at 37°C for 5 h. A white precipitate of silver sulfide was then removed by centrifugation, and the supernatant, containing the apo-protein, was lyophilized. The final purification step was carried out on a Sephadex G-25 column (1 × 30 cm) previously equilibrated with 0.01 M ammonium acetate. The protein peak was collected after elution with 0.01 M ammonium acetate and lyophilized.

Animals. SJL/J, B10.A(4R), A.SW/SgSn, BALB/cByJ, and C57BL/6J were obtained from the breeding colony of Scripps Clinic and Research Foundation (La Jolla, CA). In general, female mice, 6-10 wk old, were used.

Preparation of Lymph Node T Cells. Mice were primed with either pigeon cyt *c*, bov cyt *c* (100 µg in total), or PBS emulsified 1:1 in CFA (H37 Ra; Difco Laboratories Inc., Detroit,

¹ Abbreviations used in this paper: bov cyt *c*, bovine cytochrome *c*; cRPMI, complete RPMI 1640 medium; cyt *c*, cytochrome *c*; HmCl, hemin chloride; LN, lymph node; Rf, relative mobility.

MI). Injections were given interperitoneally at the base of the tail and into the hind footpads (100 μ l per animal). 8 d later, the inguinal, peri-aortic, and popliteal lymph nodes were removed. The lymph nodes were then ruptured, and the single cell suspension obtained was washed twice in HBSS (Gibco Laboratories, Grand Island, NY). To further enrich the lymph node (LN) T cell population, the single cell suspension was adsorbed onto a nylon wool column as described by Julius et al. (32). The column was incubated for 45 min at 37°C, and the T cells were eluted using complete RPMI 1640 medium (cRPMI) (M.A. Bioproducts, Walkersville, MD) containing 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 10% NCTC-135 medium (Gibco Laboratories), 50 mM 2-ME, 4 mM glutamine (M.A. Bioproducts), and 100 U/ml of penicillin/100 μ g/ml streptomycin (M.A. Bioproducts).

Preparation of APC. Naive spleen cell populations were used as the source of APC in the in vitro T cell proliferation assays. Spleens were removed from unimmunized mice of the same strain as that from which the LN T cell population had been obtained. The spleens were teased into a single cell suspension, washed twice with HBSS, and then irradiated at 3,000 rad, unless otherwise stated, using a ^{137}Cs source.

When appropriate, spleen cells were fixed using 1% paraformaldehyde as follows: spleen cells (5×10^6 /ml) were incubated in HBSS containing 1% paraformaldehyde (J. T. Baker Chemical Co., Phillipsburg, NJ) for 30 min at room temperature. The fixed cells were then immediately diluted ($\times 3$) with RPMI containing 1 M glycine and were then washed three times in RPMI. After a further incubation in cRPMI for 30 min at 37°C, the cells were again washed twice with RPMI before addition to culture wells.

In Vitro T Cell Proliferation Assay. T cell proliferation assays were carried out essentially as described by Corradin et al. (33). Briefly, 100 μ l of antigen of appropriate dilution (in cRPMI) and 100 μ l of cell suspension containing both 4.0×10^6 LN T cells/ml and 4.0×10^6 APC/ml in cRPMI were added to each well of a 96-well flat-bottomed plate. Final cell concentrations were 4.0×10^5 LN T cells and 4.0×10^5 APC per well. The cells were cultured at 37°C and maintained in an atmosphere of 10% CO_2 for 3 d. The culture wells were then pulsed with 0.5 μ Ci of [methyl- ^3H]thymidine (Amersham Corp., Arlington Heights, IL) and harvested 18–20 h later using a Mini-Mash cell harvester (M.A. Bioproducts). Proliferation was quantitated to determine [^3H]thymidine incorporation by proliferating T cells using a liquid scintillation counter (Rack-Beta; LKB Instruments, Inc., Gaithersburg, MD).

In some experiments, the degree of T cell proliferation was expressed as the fraction of the maximum proliferation observed in the presence of 10 μ M Con A (Calbiochem-Behring Corp.).

mAb Blocking Studies. The B cell hybridoma lines MK-S4 and 10-2.16 expressing the anti-I-A^s (IgG2b) mAb and the anti-I-A^k (IgG2b) mAb, respectively, were purchased from the American Type Culture Collection (Rockville, MD). The MK-S4 antibody has been described fully by Kappler et al. (34); Oi et al. (35) have characterized the mAb expressed by the 10-2.16 cell line. The isotype control mAb 10-12 (IgG2b) was raised against the hapten DNP and was a gift from Dr. Norman Klinman (Research Institute of Scripps Clinic). The MK-S4 and 10-2.16 supernatants and the affinity-purified anti-DNP antibody were dialysed, extensively, against PBS before addition to culture wells. For the in vitro blocking studies, 50 μ l of serially diluted antibody supernatant was initially added to each culture well, followed by 100 μ l of appropriately diluted antigen and 50 μ l of cell suspension (8.0×10^6 LN T cells/ml plus 8.0×10^6 APC/ml). Data are expressed as percentage of maximum proliferation where: percent maximum proliferation = (LN T cell proliferation in the presence of blocking antibody)/(LN T cell proliferation in the absence of blocking antibody).

Gel Filtration Chromatography. The heme-protein complex was isolated using gel filtration chromatography. A solution of HmCl (4.0×10^{-4} M) in cRPMI, containing 2% DMSO was applied to a Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ) column (1 \times 40 cm). The material was eluted using PBS. The elution profile was monitored at both 280 and 405 nm.

PAGE. PAGE was carried out as described (36). Briefly, a 15% acrylamide resolving gel, pH 8.8, and a 3% acrylamide stacking gel, pH 6.8, were used. When appropriate, 2% SDS was added to each gel. The relative mobility (Rf) values of the protein and heme bands were calculated as follows: Rf = (distance travelled through gel by heme/protein)/(distance travelled by small molecular weight marker [Bromphenol blue]).

Results

The Proliferative Response of SJL Lymph Node T Cells to the Heme Moiety of cyt c. SJL mice can be induced to produce a high titer of anti-mouse cyt *c* antibodies when immunized with mouse cyt *c* emulsified in CFA (Cooper, H. M., N. R. Klinman, and Y. Paterson, manuscript in preparation). While studying the response of SJL LN T cells to mouse cyt *c*, it was observed that the heme moiety of cyt *c*, in the form of HmCl, induced a strong proliferative response in vitro. To characterize this response further, SJL mice were immunized with bov cyt *c*. 8 d later, the lymph nodes were removed, and a single cell suspension of enriched T cells was obtained by nylon wool purification as described in Materials and Methods. Fig. 1 *a* represents the typical dose-response curve observed for SJL LN T cells when responding to bov cyt *c* or HmCl in vitro. It can be seen that the proliferative response to HmCl was significantly greater than that to bov cyt *c*. The maximum response occurred at a HmCl concentration of 25 μ M. The magnitude of this response approached that induced by Con A (10 μ M), a nonspecific polyclonal T cell activator. In fact, in some experiments, the degree of proliferation induced by HmCl was greater than that of Con A. In contrast, the proliferative response to bov cyt *c* was significantly weaker. Stimulation of LN T cell proliferation by 100 μ M bov cyt *c* was only 30% of the response to Con A. Fig. 1 *b* shows the proliferative response of LN T cells from SJL mice that had been immunized with PBS emulsified (1:1) in CFA, as described above. Again, a large proliferative response was observed in the presence of HmCl (in vitro). Both the dose-response curve for HmCl and the maximum proliferation observed were equivalent to that for LN T cells from bov cyt *c* immunized mice. Furthermore, LN T cells from SJL mice that had received no immunization of any kind behaved in an identical manner to PBS/CFA-primed SJL LN T cells when stimulated by HmCl in vitro (data not shown). Overall, for both bov cyt *c*- and PBS-primed SJL LN T cells, the dose-response curve for HmCl was found to be highly reproducible over many experiments. In most cases, the maximum proliferative response was equal to or greater than that to Con A and occurred at an HmCl concentration of 25 μ M. It is also of interest to note that a significant proliferative response to bov cyt *c* was always observed for LN T cells from SJL mice immunized with PBS/CFA only.

As HmCl is insoluble in aqueous solution, it was used as a stock solution (2.0×10^{-2} M) in DMSO. This solution was then serially diluted (100–3,200-fold) across the culture wells to give a final concentration range of 100–3 μ M, and there-

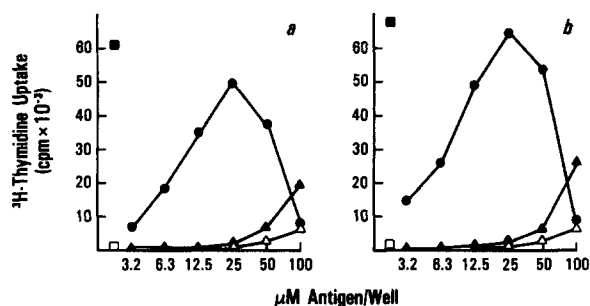


FIGURE 1. The proliferative response of nylon wool-purified LN T cells from SJL mice to the heme moiety of cyt *c*. The response of LN T cells from (a) bov cyt *c*-immunized SJLs and (b) PBS-immunized SJLs, to in vitro stimulation by HmCl (+DMSO) (●), bov cyt *c* (no DMSO) (▲), bov cyt *c* (+DMSO) (Δ), Con A (10 μ g) (■), or no antigen (□).

fore, a corresponding concentration range of 1.0–0.03% DMSO. Fig. 1, *a* and *b* show the dose-response curve to bov cyt *c* in the presence and absence of the appropriate range of DMSO concentrations. It is clear that the presence of DMSO did not enhance, nonspecifically, the proliferative response to antigen *in vitro*. At high concentrations of DMSO (1% in the culture well) the response to bov cyt *c* was significantly reduced, probably due to cell death.

The Proliferative Response to the Heme Moiety of cyt c is Under Class II MHC Restriction. As both LN T cells from PBS- and bov cyt *c*-primed SJLs responded *in vitro* to HmCl, it was possible that the heme moiety may have been acting as a nonspecific T cell activator, analogous in its effects to Con A. Therefore, the ability of HmCl to stimulate a proliferative response to LN T cells from other congenic strains of mice was investigated (Table I). Neither nylon wool-enriched LN T cells from BALB/c (I-A^d, I-E^d) nor C57BL/6 (I-A^b) mice that had been primed with bov cyt *c* or PBS responded to stimulation (*in vitro*) with HmCl. The lack of response to HmCl in these strains could not be attributed to the general inability of these LN T cells to proliferate *in vitro* as their proliferative response to 10 μ M Con A was equivalent to that observed for SJL LN T cells as measured by [³H]thymidine uptake (4×10^4 – 10^5 cpm per 4.0×10^5 T cells). Furthermore, LN T cells from BALB/c mice, immunized with sperm whale myoglobin, did not proliferate to *in vitro* stimulation with HmCl but responded well to stimulation with myoglobin (26% of the Con A-stimulated T cell proliferative response) (data not shown).

TABLE I
MHC Class II Restriction of the LN T Cell Proliferative Response
to the Heme Moiety of Cyt *c*

Stain	Class II expressed	Percent Con A-stimulated T cell proliferation*					
		Cyt <i>c</i> immunized			PBS immunized		
		HmCl		Cyt <i>c</i> [†]	HmCl		Cyt <i>c</i> [†]
12.5 μ M	25 μ M	12.5 μ M	25 μ M				
SJL	I-A ^s	46	56	25	61	56	6
		34	65	17	95	72	8
A.SW	I-A ^s	59	72	2	42	54	– [§]
		23	24	14	38	39	3
B10.A	I-A ^k , I-E ^k	61	74	21	153	125	3
		166	170	56	89	95	–
B10.A(4R)	I-A ^k	131	194	31	162	123	2
C57BL/6	I-A ^b	2	3	–	–	–	1
		–	4	2	4	3	4
Balb/c	I-A ^d , I-E ^d	–	–	3	–	–	–
		–	–	–	–	–	–

* Percent Con A-stimulated T cell response = [³H]thymidine uptake (cpm) in the presence of stimulating antigen/[³H]thymidine uptake (cpm) in the presence of 10 μ M Con A (+ DMSO). Results are shown for two animals from each strain immunized with either cyt *c* or PBS. Assays were generally performed in triplicate. The proliferative response to Con A (10 μ g) for all assays was within the range of 40–100,000 cpm per 4.0×10^5 LN T cells. Background proliferation in the absence of antigen was never observed to be >3–5% of Con A-stimulated proliferation.

[†] Bov cyt *c* (100 μ M + 1% DMSO) was used as stimulating antigen for all mice strains except B10.A and B10.A(4R) where pigeon cyt *c* (50 μ M, 0.5% DMSO) was used.

[§] No response above background.

In contrast to the H-2^b and H-2^d strains LN T cells obtained from A.SW (I-A^s, only), B10.A (I-A^k, I-E^k) and B10.A(4R) (I-A^k only) strains responded vigorously to HmCl (Table I). Thus, it appears that the T cell proliferative response to the heme moiety of cyt *c* was not due to a nonspecific polyclonal activating event. Instead, the recognition of HmCl was restricted to those T cells derived from mouse strains expressing class II molecules of the "s" or "k" haplotype. Since both SJL and A.SW mice express only I-A^s on their APC, and were high responders, it seems likely that the I-A^s molecule was the restricting element in the SJL and A.SW T cell recognition of HmCl. Similarly, the I-A^k molecule also appears to present the heme moiety, since both B10.A (I-A^k, I-E^k) and B10.A(4R), which only express I-A^k, were also high responders.

mAbs against the I-A Heterodimer Block the Proliferative Response of LN T Cells to HmCl. To confirm that the heme moiety of cyt *c* is presented to the SJL LN T cells in conjunction with the I-A^s heterodimer, the mAb MK-S4 (anti-I-A^s) was used to block the specific proliferative response. From Fig. 2, *a* and *b*, it is clear that the MK-S4 antibody could totally inhibit the ability of both bov cyt *c* and HmCl to stimulate the in vitro proliferation of LN T cell populations derived from either cyt *c*-primed (Fig. 2 *a*) or PBS-primed (Fig. 2 *b*) animals. Similarly, the anti I-A^k (10-2.16) could inhibit the heme-specific proliferative response of LN T cells derived from B10.A mice primed with either pigeon cyt *c* (Fig. 2 *d*) or PBS (Fig. 2 *e*). Also, partial inhibition of pigeon cyt *c* stimulated proliferation was observed for LN T cells from pigeon cyt *c*-primed B10.A mice (Fig. 2 *d*). LN T cells from PBS-primed B10.A mice did not respond to pigeon cyt *c*. Fig. 2, *c* and *f* show that both mAbs, MK-S4 and 10-2.16, respectively, did not interfere with the ability of KLH-primed BALB/c LN T cells (H-2^d) to respond to in vitro stimulation by KLH. Thus, the inhibition observed in Fig. 2, *a*, *b*, *d*, and *e* is not due to nonspecific inhibitory effects of these antibody preparations. Furthermore, the isotype-matched anti-DNP mAb had no effect on the ability of either cyt *c* or HmCl to induce LN T cell proliferation in vitro (data not shown). Therefore, the inhibition of proliferation observed in the presence of the anti-I-A^s and anti-I-A^k mAbs suggests that HmCl stimulates T cell proliferation in association with the I-A^s or I-A^k heterodimer.

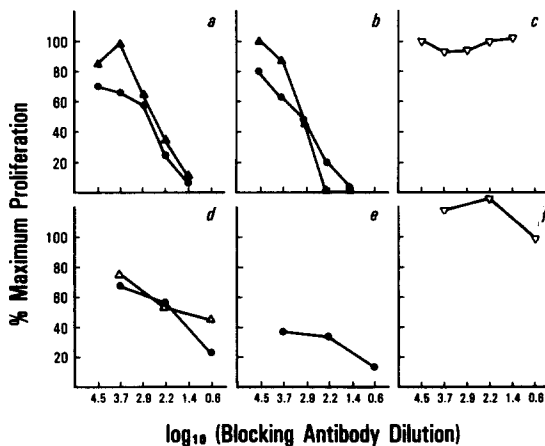


FIGURE 2. Blocking of the proliferative response of SJL LN T cells by an antigen-I-A^s mAb (MK-S4) (*a* and *b*) or B10.A LN T cells by an anti-I-A^k mAb (10-2.16) (*d* and *e*). The ability of LN T cells from bov cyt *c*- or pigeon cyt *c*-primed mice (*a* and *d*, respectively) or PBS-primed mice (*b* and *e*) to proliferate in response to the appropriate cyt *c*; bov cyt *c* (50 μM) (▲); pigeon cyt *c* (50 μM) (Δ); or HmCl (25 μM) (●) in the presence of blocking antibody. The mAbs MK-S4 and 10-2.16 did not inhibit the KLH-specific proliferative response of BALB/c LN T cells (*c* and *f*, respectively).

Paraformaldehyde-fixed Spleen Cells Can Present the Heme Moiety of cyt c to SJL LN T Cells. To define more clearly the mechanism by which the heme moiety of cyt *c* is recognized when presented in conjunction with the I-A^s heterodimer on the surface of the APC, splenic cell populations obtained from SJL mice were fixed with 1% paraformaldehyde as described in Materials and Methods. The ability of fixed and nonfixed spleen cells to present bov cyt *c*, HmCl, and the heme-containing fragment 1-65 of bov cyt *c* (bov [1-65]) to LN T cells obtained from SJL mice primed with either bov cyt *c* or PBS were compared (Fig. 3, *a* and *b*, respectively). Firstly, it can be seen that the pattern of the proliferative responses to the various antigens tested was very similar for bov cyt *c*- and PBS-primed mice. Secondly, as expected, bov cyt *c* induced a significant proliferative response when presented by nonfixed APC (Fig. 3 *solid lines*) but not when paraformaldehyde-fixed APC were used (Fig. 3 *broken lines*). In contrast, the heme moiety of cyt *c* was presented efficiently by the fixed APC such that a strong proliferative response was observed for LN T cells from both bov cyt *c*- and PBS-primed mice. The magnitude of the proliferative response, however, was not as great as that observed in the presence of nonfixed APC. The reduced ability of fixed APC compared with nonfixed APC to present peptide antigens has previously been observed (37, 38, 39). This decrease could be the result of the paraformaldehyde fixation procedure, which may reduce the number of functional Ia heterodimers on the cell surface of the presenting cell due to crosslinking.

Finally, as was observed previously in the case of bov cyt *c* stimulation of the PBS-primed SJL LN T cell population (Fig. 1 *b*) response to bov cyt *c*, the heme-containing fragment 1-65, could also induce a significant response in T cells derived from PBS/CFA-immunized mice. Furthermore, from Fig. 3, *a* and *b* it can be seen that this fragment is recognized only when nonfixed APC were used. No specific T cell proliferation to bov (1-65) was observed in the presence of paraformaldehyde-fixed APC. These data would suggest that the heme-containing fragment of bov cyt *c* requires further processing before a specific T cell proliferative response is observed.

Removal of the Heme Group from cyt c Results in the Loss of its Ability To Induce T Cell Proliferation. Since the CNBr cleavage fragment bov (1-65), as well as the whole cyt *c* molecule, were not presented by fixed APC, it was likely that a processing event

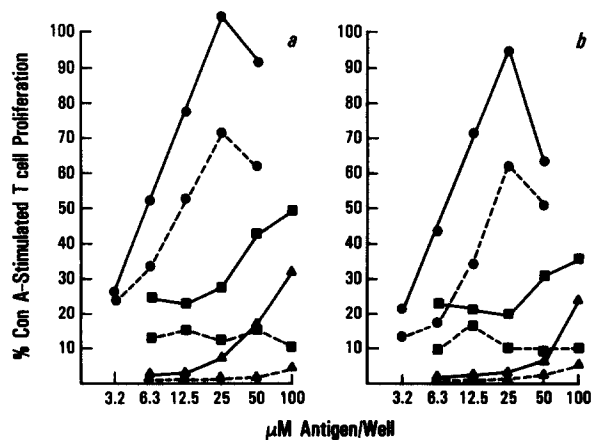


FIGURE 3. The proliferative response of SJL LN T cells to the heme moiety of cyt *c* presented by fixed APC. The ability of (a) bov cyt *c*-primed and (b) PBS-primed SJL LN T cells to proliferate in response to HmCl (●), bov cyt *c* (▲), or bov (1-65) (■) when presented by normal SJL APC (—) or APC fixed with 1% paraformaldehyde (-----).

was required before presentation of the correct epitope could take place. In the light of the above data, it was possible that the epitope being recognized within the framework of the polypeptide chain of the cyt *c* molecule was in fact the heme moiety alone. To address this question, the heme group was chemically removed from the cyt *c* molecule as described in Materials and Methods. The apo-bov (1-65) fragment was then cleaved from apo-bov cyt *c* using CNBr (see Materials and Methods). These modified antigens were then tested for their ability to induce both bov cyt *c*- and PBS-primed SJL LN T cells to proliferate in vitro. Fig. 4 *a* and *b* clearly demonstrate that removal of the heme prosthetic group from both the whole molecule and the CNBr cleavage fragment, bov (1-65), resulted in the abrogation of T cell recognition of these antigens. The loss of T cell reactivity could not be attributed to T cell death due to chemical contamination of the apo-cyt *c* preparation since the degree of Con A-induced T cell proliferation was not reduced in the presence of 100 μ l apo-bov cyt *c* or apo-bov (1-65) (data not shown). Therefore, these results strongly suggest that the heme moiety, per se, or perhaps in conjunction with a small number of amino acid residues deriving from the cyt *c* molecule, is the T cell epitope responsible for the bov cyt *c*-specific SJL LN T cell proliferation observed in our in vitro assays (Figs. 1, 3, and 4). It is quite possible that a population of T cells from bov cyt *c*-primed SJLs would be specific for epitopes residing within the polypeptide backbone of the molecule. Such populations would go undetected in our assay if their frequency was relatively low. Fig. 4 *a* demonstrates some residual stimulating activity of apo-bov (1-65) for bov cyt *c*-primed LN T cells.

Enhanced Presentation of the Heme Moiety by Radio-sensitive APC. Resting B cells are known to be radiosensitive such that their ability to act as APC is inhibited when they are irradiated at 3,300 rad but not 1,000 rad. This radiosensitivity allows the antigen-presenting function of resting B cells to be distinguished from that of macrophage and dendritic cells which are radioresistant to 3,300 rad (39, 40). Thus, to examine the nature of the presenting cell on which the heme moiety of cyt *c* is recognized in conjunction with the appropriate class II heterodimer, an SJL splenic cell suspension was subjected to either 1,000 or 3,000 rad before addition to culture wells. The ability of the differentially irradiated splenic cell suspensions to stimulate the in vitro T cell proliferative response to HmCl is shown in Table II. From the

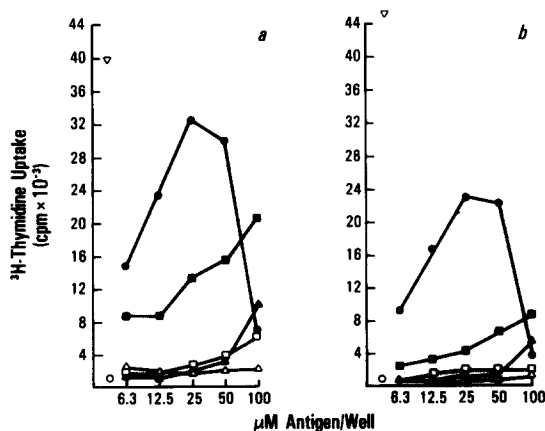


FIGURE 4. The proliferative response of nylon wool-enriched LN T cells from SJL mice to apo-bov cyt *c* and apo-bov (1-65). The response of LN T cells from (a) bov cyt *c*-immunized SJLs and (b) PBS-immunized SJLs to in vitro stimulation by HmCl (●), bov cyt *c* (▲), apo-bov cyt *c* (△), bov (1-65) (■), apo-bov (1-65) (□), Con A (10 μ g) (▽), or no antigen (○).

TABLE II
 Comparison of the Ability of Spleen Cells Irradiated at 1,000 or 3,000 rad
 to Present the Heme Moiety of cyt *c* to SJL LN T Cells

Exp.	rad	Percent Con A-Stimulated T cell proliferation*			
		cyt <i>c</i> immunized		PBS immunized	
		HmCl		HmCl	
		12.5 μ M	25 μ M	12.5 μ M	25 μ M
1	1,000	54	83	62	72
	3,000	36	42	29	43
2	1,000	57	95	126	158
	3,000	38	60	112	138

* The values given are the average of duplicate cultures within each experiment.

two experiments represented, it is clear that the HmCl-specific SJL LN T cell proliferation was significantly enhanced in the presence of splenic cells irradiated to 1,000 rad. These data indicate that a subpopulation of APC are radiosensitive and are functional only after a light irradiation dose. Thus, it is likely that the heme moiety of cyt *c* can be presented to T cells of the appropriate specificity by resting B cells as well as macrophage and/or dendritic cells within the splenic cell population.

The Heme Group Forms a Stable Complex with BSA in Culture. As stated previously, the heme moiety of cyt *c*, when in the oxidized form of HmCl, is insoluble in aqueous solution. Therefore, the HmCl was initially dissolved in DMSO to give a stock solution of 2.0×10^{-2} M. For use in in vitro proliferation assays, the stock solution was then diluted 100–3,000-fold in cRPMI. However, when these dilutions were made in the absence of FCS, the heme moiety was observed to precipitate immediately. Thus, an interaction between the heme group and a component of FCS appeared to be required to maintain the solubility of HmCl. As the major component of FCS is protein, it seemed likely that one or more protein species were interacting with the heme group. Therefore, an HmCl solution in cRPMI (4.0×10^{-4} M) was chromatographed on a Sepharose 6B gel filtration column as described in Materials and Methods. The elution profile is shown in Fig. 5. The eluant was monitored at 280

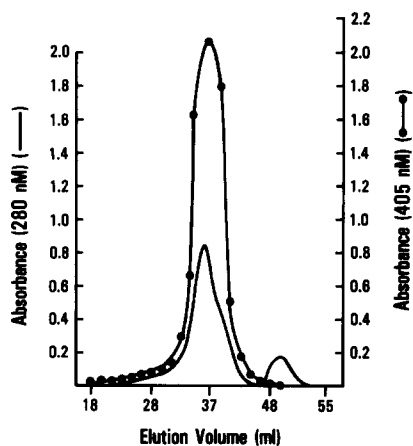


FIGURE 5. Elution profile of heme-protein complex. 2 ml of a 4.0×10^{-4} M solution of HmCl dissolved in cRPMI was applied to a Sepharose 6B column (1×40 cm). The column was eluted in PBS and monitored at both 405 and 280 nm.

nm and also at 405 nm. The latter wavelength corresponds to the wavelength of maximum absorption in the Soret absorption band characteristic of the heme moiety. It can be seen that the heme group eluted with the major protein peak indicating that a stable heme-protein complex was present in the HmCl/cRPMI solution which remained complexed throughout the chromatographic procedure (unbound heme would elute in the salt peak).

PAGE was then carried out under nonreducing conditions on a solution of HmCl/cRPMI to identify the protein species to which the heme moiety was complexed. In the absence of SDS, the heme moiety (which could be visualized without staining due to its red color) was observed to have an Rf value of 0.29. After staining with Coomassie Blue R, a protein band (Rf value of 0.29) was apparent in the exact position at which the heme was observed. In addition, a pure preparation of standard BSA run on the same gel also was observed to have an RF value of 0.29. Therefore, as BSA is the major protein species in FCS, it seems likely that the heme moiety forms a stable complex with BSA when dissolved in media containing FCS. Furthermore, this complex does not appear to be covalently bound as the heme-protein complex was observed to dissociate in polyacrylamide gels containing SDS.

Discussion

The immunogenicity of cyt *c* has been extensively studied in many laboratories leading to the identification of dominant T cell epitopes on the protein backbone of cyt *c* from several species. The region 88-104 of pigeon cyt *c* has been shown to be an immunodominant epitope when presented in the context of the I-E^k MHC heterodimer (1, 27). Corradin and co-workers (29) have shown that distinct I-A^b restricted T cell epitopes lie within the regions 1-39 and 39-53 of apo-horse cyt *c*. Also BALB/c T cells (H-2^d) recognize epitopes within the regions 13-38 and 1-25 of apo-bov cyt *c* (28). Until this report, however, there has been no investigation into the immunological properties of the heme moiety of cyt *c*. As it is not a protein and bears no amino acid residues (see Fig. 6), one would predict, on the basis of current models of T cell epitopes (1, 3, 41) that such a small molecule would be poorly immunogenic, if at all. However, data presented here clearly demonstrate that the heme moiety can induce a vigorous and specific proliferative response (in vitro) in nylon wool-enriched LN T cell populations from SJL mice primed with bov cyt *c* (Fig. 1 *a*) or B10.A mice primed with pigeon cyt *c* (Table I). More unexpected was the observation that an equivalent response was induced by HmCl in LN T cell populations derived from SJL and B10.A mice immunized only with PBS/CFA (Fig. 1 *b* and Table I, respectively), suggesting the existence of a heme-specific auto-reactive T cell population within these mouse strains.

That the heme moiety behaved as a classic MHC, class II-restricted T cell epitope was shown in Table I, where high responsiveness was correlated with congenic mouse strains expressing either the I-A^s or I-A^k heterodimers. H-2^b- and H2^d-bearing strains displayed no capacity to recognize HmCl. The ability of the anti-I-A^s mAb (MK-S4) to specifically block the T cell proliferative response to HmCl (Fig. 2, *a* and *b*) provided strong evidence that the heme group must interact directly with the I-A^s heterodimer in order to promote recognition by the appropriate TCR and induce the subsequent proliferation event. Similarly, the anti I-A^k antibody (10-2.16) specifically inhibited the heme-induced proliferative response in B10.A LN T cell

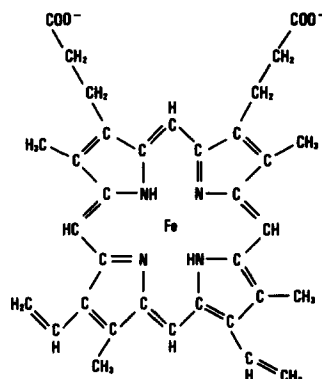


FIGURE 6. The molecular structure of the heme moiety of cyt *c*.

populations (Fig. 2, *c* and *d*). The inability of the B10.A T cell hybridoma, 2H10, specific for the pigeon cyt *c* sequence 88-104, to produce IL-2 in response to HmCl, provides convincing evidence that the heme moiety did not achieve its effect by an indirect and/or nonspecific interaction with T cells from the high responder haplotypes. As it was shown that in culture, the heme group formed a stable complex with BSA present in FCS, it was possible the immunogenic species recognized in our assays was the heme moiety coupled to a peptide fragment of BSA produced by intracellular processing. However, the heme moiety could be presented by paraformaldehyde-fixed presenting cells (Fig. 3). Therefore, no processing event was required for heme presentation. In addition, the removal of the heme moiety from both bovine cyt *c* and bovine cyt *c* (1-65) resulted in the abrogation of the ability of these antigens to stimulate proliferation in LN T cells from either bovine cyt *c*- or PBS-primed SJLs (Fig. 4, *a* and *b*) indicating that the heme moiety per se was the stimulating epitope within these antigens. Thus, it seems unlikely that BSA or BSA peptides play a role in the T cell recognition of the heme moiety.

The structure of the heme group is shown in Fig. 6. It is a flat molecule comprised of four pyrrole groups linked by methene bridges to form a tetrapyrrole ring having the dimension of $9\text{\AA} \times 9\text{\AA}$. To the ring structure are attached four methyl (CH_3), two vinyl ($-\text{CH}=\text{CH}_2$) and two propionyl ($-\text{CH}_2-\text{CH}_2-\text{COO}^-$) groups. When the heme group forms the prosthetic group of cyt *c*, the central iron atom of the heme forms a hexa-coordination complex. Four of the coordination positions around the iron atom are occupied by the nitrogen atoms of the pyrrole rings. The sulphur atom from methionine 80 occupies the fifth site, while the sixth position is taken up by the ring nitrogen from histidine 18 of the polypeptide backbone. In the assays described here, the free heme moiety was used in the oxidized form (Fe^{3+}) with Cl^- occupying the fifth coordination site.

Recently, the X-ray crystallographic structure of a human class I antigen (HLA-A2) has been determined (42, 43). A large groove at the top of the molecule comprising both the $\alpha 1$ and $\alpha 2$ domains of the class I molecule was tentatively identified as the site of recognition for processed peptides. The groove was found to be 25\AA long, 10\AA wide, and 11\AA deep. As both class I- and II-restricting elements are recognized by the same TCR structure and share a number of sequence homologies, it is likely that the three dimensional structure of the class II heterodimer will

be similar to that described for the class I complex. If that is the case, then the heme moiety ($9\text{\AA} \times 9\text{\AA}$) could fit into a binding site of the dimensions described for the proposed antigen-binding site of HLA-A2. The propionyl groups of the heme would be available to form hydrogen bonds with adjacent side chains within the groove. Hydrophobic interactions are also possible between substituent groups of the heme and amino acid side chains bearing ring structures (i.e., tryptophan, histidine, and tyrosine). In addition, the fifth and sixth coordination sites of the central iron atom could also interact with appropriately positioned side chains. Obviously, since the heme/I-A complex can be recognized by TCRs of correct specificity, the heme moiety must be able to interact with amino acid residues from both I-A-binding site and TCR simultaneously.

The interaction of a small, nonprotein molecule with a restricted set of class II heterodimers, resulting in the formation of a complex capable of being recognized by TCRs of correct specificity, has also been observed in the tyrosine-azobenzenearsonate response by class II-restricted T cell clones (44). This example, together with the observations reported here require that the current models describing the nature of T cell epitopes be modified. Such models propose that the immunogenicity of a given peptide fragment is governed by the ability of that peptide to interact simultaneously with the appropriate MHC-restriction element and the specific TCR complex. The structural constraints of this interaction have been dissected for a series of immunogenic peptides derived from protein antigens such as cyt *c* (1, 27, 39), hen egg-white lysozyme (4), and myoglobin (41). Amino acid residues that have been identified as either TCR or Ia molecule contact residues are interspersed along the immunogenic fragment whose minimum length for immunological activity appears to be 10–12 amino acid residues. Nonconservative substitutions at such residue positions result in either lack of TCR recognition or an inability of the peptide to interact with the appropriate Ia molecule, respectively. On the basis of these experimental observations, several models have been put forward to generalize the physical nature of a T cell epitope. Thus, Berzofsky and colleagues (41, 45) contend that immunogenic peptides have a strong propensity to form an amphipathic α helix such that the residues contacting the TCR lie on the hydrophilic face of the helix, while the Ia contact residues are arranged on the hydrophobic face. Alternatively, Rothbard et al. (46) propose that the T cell epitopes contain a four-amino acid motif common to many immunological peptides so far studied. Clearly, the heme moiety of cyt *c* does not comply with the above requirements. Although, if more than one heme group occupied the same Ia-binding site, then the array of substituent groups of the ring structures may mimic the display of amino acid residue side chains of a peptide.

Further evidence to support the contention that the free heme moiety, per se, induces the vigorous proliferative response observed comes from experiments using paraformaldehyde-fixed APC (Fig. 3). In contrast to the whole bovine cyt *c* molecule, the heme moiety could be effectively presented by fixed APC indicating that it could interact directly with the I-A heterodimer without further intracellular processing or modification. Since it has now been well established that in most cases protein antigens in their native conformation cannot be presented to T cells in a recognizable form by fixed APC (5), one would expect to observe the loss of T cell proliferation to bovine cyt *c* when using paraformaldehyde-fixed APC (Fig. 3). However, we

were surprised to find that the heme containing peptide bov (1-65) could not be presented by fixed APC, indicating that this fragment needed to be further processed before the T cell epitope was revealed.

Apo-bov cyt *c* and apo-bov (1-65) were then used as stimulating antigens to determine whether the actual T cell proliferative responses induced by the whole cyt *c* molecule and by bov (1-65) were derived from the direct recognition of the heme moiety within these antigens, or, were directed to epitopes on the polypeptide backbone. Fig. 4, *a* and *b* clearly show that the removal of the heme moiety from these antigens resulted in the abrogation of the cyt *c*-specific, *in vitro*, proliferative response by LN T cells from both bov cyt *c*- and PBS-primed SJLs. Therefore, this data provides convincing evidence that the immunologically active epitope of bov cyt *c* recognized by SJL LN T cells in our *in vitro* assay is the heme moiety *per se*. As the prosthetic group of cyt *c*, the heme moiety is covalently bound to the polypeptide backbone via cysteine residues at positions 14 and 17. The inability of the heme to be presented by fixed APC, in the more exposed context of the denatured CNBr cleavage fragment, bov (1-65) (Fig. 3) further supports the contention that the heme moiety, in the absence of an amino acid residue, is the stimulating epitope. Thus, it seems likely that the heme molecule alone is capable of interacting with the peptide binding domain of the I-A^s heterodimer and that the remaining, free substituent groups of the pyrrole ring are recognized by appropriate amino acid residues of the TCR with a significant affinity. It is possible that after intracellular processing of the cyt *c* molecule the heme moiety is recognized by the appropriate TCR in conjunction with a small number of amino acid residues deriving from the polypeptide chain of the protein. However, we believe that this is unlikely since free heme can be presented by fixed APC (Fig. 3, *a* and *b*). Furthermore, the proliferative response induced by either bov cyt *c* or bov (1-65) did not achieve the magnitude of the response stimulated by an equimolar concentration of free heme (Figs. 1, 3, and 4). This significant reduction in the immunological activity of the heme moiety when sequestered within the framework of the polypeptide chain may be due to an inability of the APC to efficiently process the protein such that the free heme group is available to interact with the class II-restricting element. Alternatively, only a small fraction of the heme may enter the correct intracellular compartment for interaction with Ia, after the processing event has taken place.

The heme moiety of cyt *c* is also the prosthetic group for the oxygen-binding proteins myoglobin and hemoglobin and, therefore, is a ubiquitous self molecule. As has been demonstrated, the heme moiety induces a vigorous proliferative response (*in vitro*) in LN T cells obtained from naive animals of the higher responder haplotypes demonstrating the existence of an auto-reactive heme-specific T cell population. Furthermore, the magnitude of the response suggests that the frequency of T cells within the polyclonal population able to recognize the heme moiety is surprisingly high. Since the heme molecule is small, one would expect that very few reactive groups would be available for interaction with the TCR once the heme has complexed with the appropriate Ia molecule, thereby significantly reducing the structural constraints for such an interaction. It is possible that the heme molecule is a highly permissive structure for T cell recognition and would be able to fit the binding site from TCRs of quite diverse specificity, analogous to hapten recognition by Igs.

As a consequence of the limited number of reactive groups on the heme molecule,

one might predict that the affinity of the TCR for the heme-Ia complex may be relatively low. Therefore, only under conditions where the concentration of the free heme moiety and, therefore, the heme-Ia complex, is high could activation of heme-specific T cell clones occur. Such conditions are met in our in vitro culture system. In vivo, however, the heme moiety is sequestered within the framework of the protein molecule and, hence, high concentrations (in vivo) of free heme would never be attained. We anticipate, therefore, that the heme-specific T cell population described in this study is quiescent in vivo even though this T cell population has escaped tolerance induction. In addition, an active regulatory mechanism such as a suppressor T cell network may also be operative. It has been shown that the phenomenon of B cell tolerance is affinity dependent (47). Thus, the hypothesis has been put forward that those immature B cells whose affinity for self antigen falls below the threshold for tolerance induction would escape functional deletion (47). A similar mechanism has also been proposed to account for the existence of self-reactive T cell populations. Wood et al. (24) have demonstrated that tolerogen-specific cytotoxic T cells present in neonatally tolerant mice are low avidity cells that have preferentially escaped clonal deletion. Thus, the auto-reactive T cell population demonstrated here may have escaped tolerance induction due to its low affinity for the heme moiety.

Summary

In these studies, we have shown that the heme moiety of cyt *c* is a dominant T cell epitope that induces a large proliferative response in lymph node T cells derived from SJL and B10.A mice when presented on either unfixed or fixed syngeneic APCs. Not only is this vigorous response observed for cyt *c*-primed T cell populations but also for populations obtained from naive SJL or B10.A mice. The reactivity to the heme moiety falls under strict MHC restriction, in that it is present only in murine strains bearing either the I-A^k or I-A^s molecule and can be blocked by antibodies specific for these class II molecules. Therefore, these findings require that the current models describing the nature of T cell epitopes be extended to include nonpeptide molecules. Furthermore, as the heme moiety is ubiquitous throughout the organism, although sequestered within proteins, the existence of heme-reactive T cell populations in unprimed animals provides another example of the existence of self-reactive T cell clones.

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