LYMPHOCYTE SPECIFICITY TO PROTEIN ANTIGENS

II. Fine Specificity of T-Cell Activation with Cytochrome *c* and Derived Peptides as Antigenic Probes*

By GIAMPIETRO CORRADIN AND JACQUES M. CHILLER‡

From the Department of Medicine, National Jewish Hospital and Research Center, Denver, Colorado 80206

The knowledge which has accumulated on the specificity and nature of B lymphocytes and their antibody products (1–3) contrasts with the relative paucity of information on the subject of the fine specificity of T lymphocytes. In part, this is due to the fact that T-cell receptors or antigen binding T-cell products remain elusive molecules. As a result, more indirect approaches have been utilized to dissect the problem of the fine specificity of T cells. On the one hand, analyses with anti-idiotypic antibodies have revealed that identical idiotopes can be found on both T and B lymphocytes (4, 5) and furthermore that these antibody probes can specifically modulate both T and B cellular functions (6–8). On the other hand, analyses of T lymphocyte activation with various protocols of antigen induced proliferation have revealed stringent specificity in the capacity of cells to distinguish haptenic moieties on different chemically defined carrier molecules (9, 10) and in the case of recognition of such proteins as insulin (11) and lysozyme (12).

Although the latter protein probes have been of considerable value in approaching the problem of T-cell specificity, the present set of studies undertook similar goals by using the globular protein cytochrome *c* because this well characterized family of antigens offers several positive features. First, the chemical nature, i.e., amino acid sequence of some 90 different cytochrome *c* molecules is known (13). Second, specific peptides from cytochrome *c* are easily obtained after cyanogen bromide treatment which cleaves at the two methionine residues found at position 65 and 80. Third, it is possible to reconstitute the molecule from appropriate peptide fragments and thus obtain hybrid molecules which are indistinguishable from native cytochrome *c* insofar as physicochemical characteristics and function (14, 15). Finally, cytochrome *c* has been used by several groups of investigators as a probe to study the fine specificity of antibody molecules directed to epitopes on globular proteins (16, 17). Resulting observations have demonstrated that a single amino acid substitution can affect the antigen binding capacity of specific antibody.

By using cytochrome *c* from various species, their derived peptides and specifically reconstituted hybrid molecules, the present studies sought to determine the fine specificity of murine T-cell activation in a system of antigen driven lymphocyte proliferation (18). Data to be presented demonstrate that T cells display exquisite

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specificity to the extent that two peptides which differ from each other at a single amino acid position can be discriminated. This conclusion is further substantiated by the pattern of cross-reactivity with cytochrome *c* molecules from different species and in the case where artifical hybrid proteins were tested. Preliminary data on these issues have been previously presented (19, 20).

Materials and Methods

Animals. BDF_1 and SJL mice used were either bred at National Jewish Hospital and Research Center or purchased from The Jackson Laboratory, Bar Harbor, Maine. They were maintained on Lab Blox (Wayne Laboratory Animal Diet) and chlorinated water ad libitum. Animals used were of either sex and ranged in age from 7 to 10 wk.

Antigens. Beef cytochrome *c* was obtained from two different sources. One preparation was purchased from Sigma Chemical Co., (St. Louis, Mo.) and further purified on carboxymethyl (CM)[1] cellulose 52 (Whatman Inc., Clifton, N. J.) using a linear gradient of 0.01–0.5 M ammonium acetate buffer, pH 6.5. A second preparation was obtained by extraction and purification of cytochrome *c* from beef hearts (kindly provided by Piper Co., Denver, Colo.) as described by Margoliash and Vasalek (21), followed by isoelectric focusing using ampholine of pH 8–10 (Brinkman Instruments, Inc., Westbury, N. Y.).

Horse and rabbit cytochrome *c* were purchased from Sigma Chemical Co. and further purified by CM cellulose 52 chromatography. Horse cytochrome *c* was also purified by isoelectric focusing. Mouse cytochrome *c* was extracted from a pool of livers, kidneys, and hearts of A/J and BDF_1 mice and purified according to the method described by Margoliash and Vasalek (21).

Peptides from various cytochromes were obtained by cyanogen bromide (CNBr) cleavage followed by gel filtration chromatography as previously described (22). Briefly, cytochrome *c* at a concentration of 15 mg/ml was incubated with 1:3 or 1:50 molar excess of CNBr to protein for 24 h. The sample was then chromatographed on Sephadex G-50 in 7% formic acid and fractions corresponding to the known peptides collected and lyophilized. Peptides were then further purified on a CM cellulose column using a linear gradient of 0.01–0.5 M ammonium acetate buffer, pH 6.5.

Hybrid molecules were obtained according to the methodology previously utilized by Corradin and Harbury (14). Peptide 1–65 was dissolved in a sodium acetate buffer, pH 7.0, which was thoroughly flushed with nitrogen and reduced by the addition of sodium dithionite. Peptide 66–104 was then added to the solution, which was incubated overnight at room temperature to allow the reconstitution of the peptide bond between residues 65 and 66 (15). The sample was then oxidized with potassium ferricyanide, dialyzed against water, and chromatographed on CM cellulose as described above.

Immunization. Antigens were emulsified in complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, Mich.). Animals were injected subcutaneously at the base of the tail with 100 μg of antigen delivered in 40 μl of emulsion.

Lymph Node Proliferation Assay. This assay has been described in detail elsewhere (18). Briefly, inguinal and periaortic nodes obtained from primed mice were teased into single cell suspensions. Cultures were initiated by incubating 4×10^5 cells in 0.2 ml modified Click's medium with either saline or varying concentrations of antigen in flat bottom microtiter plates (VIS-FB-96, Bellco Glass, Inc., Vineland, N. J.). 4 or 5 d after culture initiation, the degree of proliferation was determined by the addition of a 20–24-h pulse of 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, Mass., sp ac 6.7 Ci/mM). Cultures were then harvested using an Otto Hiller apparatus (Otto Hiller Co., Madison, Wis.).

Cell Separation. Enriched T-cell populations were obtained by passing lymph node (LN) cells through nylon wool (23), or following absorption to Petri dishes coated with rabbit anti-mouse kappa antibody (24) the latter kindly provided by Dr. Howard Grey, National Jewish Hospital. In both cases, less than 1% Ig-positive cells were detectable by treatment of the cell suspensions with fluorescein-conjugated goat anti-mouse Ig.

[1] *Abbreviations used in this paper:* CM: carboxymethyl; CNBr: cyanogen bromide; LN: lymph node; PETLES: peritoneal exudate T lymphocyte-enriched cells.

Results

Stimulation with Related Cytochrome c Molecules of LN Cells from Mice Primed with Either Beef or Horse Cytochrome c. The initial approach to gain insight into the specificity of primed LN cells using cytochrome *c* molecules from various species was as follows: BDF_1 mice were primed with 100 μg beef cytochrome *c* and 8 d later, the cell suspension obtained from a pool of the inguinal and periaortic nodes was challenged in vitro with varying concentrations of either beef, horse, mouse, or rabbit cytochrome *c*. The degree of induced proliferation, assayed on d 4 of culture, revealed that whereas subsequent challenge with either beef or mouse cytochrome *c* induced a marked response, minimal stimulation was observed in those cultures challenged with either horse or rabbit cytochrome *c* (Fig. 1). When such data are viewed from the perspective of the amino acid sequence of these four cytochromes (Fig. 2), it can be seen that the homology restricted to beef and mouse proteins is confined to a single amino acid position, namely position 89, suggesting that this position was critical to the specificity of the proliferative process observed.

An analogous observation was made in the case where cells were obtained from mice primed 10 d previously with 100 μg horse cytochrome *c* and subsequently challenged in vitro with either horse, beef, or rabbit proteins. Thus, in contrast to the observed stimulation with the homologous horse protein, the response to either beef or rabbit cytochrome *c* was marginal (Table I).

Certain additional parameters to these observations should be noted here. First, the fact that cell proliferation was temporally evaluated 8–10 d after in vivo priming reflects the kinetic profile of the response to beef or horse cytochrome *c* which reveals maximal stimulation between 7 and 12 d after injection (data not shown). Second, the pattern observed with LN cells held when enriched T cells were tested. This can

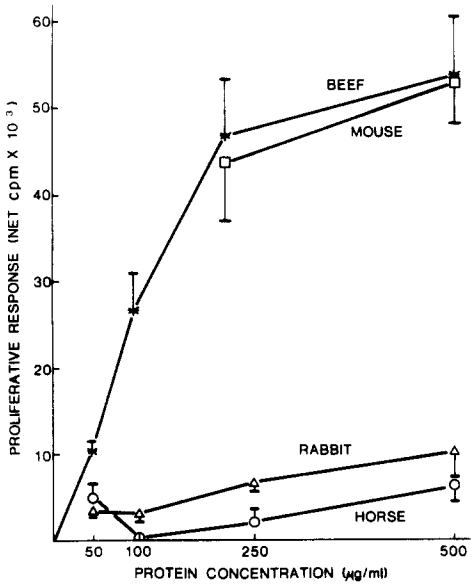

FIG. 1. BDF_1 mice were injected with 100 μg of beef cytochrome *c*. 8 d later, draining LN were removed and cell suspensions tested for proliferative response to various concentrations of beef cytochrome *c* (*), mouse cytochrome *c* (□), horse cytochrome *c* (○), or rabbit cytochrome *c* (△). Bars represent standard error of the mean values. Background (saline) response was 4,599 ± 1,071 cpm.

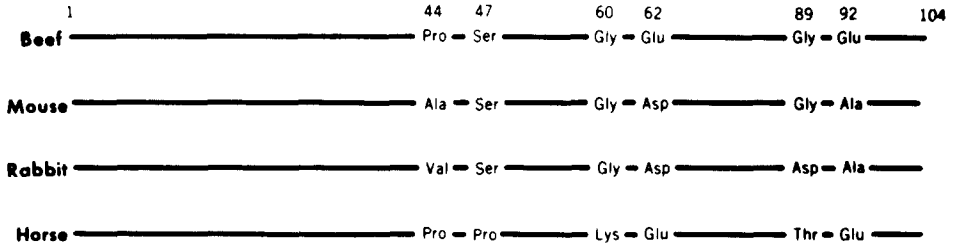

FIG. 2. Amino acid variations among beef, horse, mouse, and rabbit cytochromes *c*. Positions explicitly indicated represent amino acid variations among the four proteins.

TABLE I

*Antigen Induced Proliferative Responses of LN Cells from BDF_1 Mice Primed with Horse Cytochrome c**

Challenge antigen (cytochrome)	Concentration‡	Response§
	μg/ml	*net cpm ± SE × 10^{-3}*
Horse	100	19.3 ± 3.8
Horse	250	25.0 ± 0.3
Horse	500	19.9 ± 4.1
Beef	100	2.1 ± 0.3
Beef	250	2.1 ± 0.8
Beef	500	2.6 ± 1.0
Rabbit	100	0
Rabbit	250	0
Rabbit	500	0

* BDF_1 were primed with 100 μg horse cytochrome *c* and 10 d later, draining LN cells were challenged and assayed for proliferative responses as described in the text.

‡ Final concentration in culture.

§ Net proliferative responses were obtained by subtracting background (saline) responses of 3,744 ± 730.

be seen in Fig. 3 which depicts the response of T cells from LN of beef cytochrome *c* immunized animals and in Fig. 4 which shows the response of T cells from LN of horse cytochrome *c* immunized mice. In each case, the specificity pattern of stimulation is identical to that observed in Fig. 1 and Table I, respectively. Third, the pattern of cross-reactivity observed in the present studies appeared to be unique to BDF_1 mice and not seen in a limited number of other strains tested. This point is exemplified in Table II where it can be seen that LN cells from SJL mice immunized with beef cytochrome *c* responded not only to the homologous protein but also to horse and rabbit cytochrome *c*.

Specificity of Stimulation Obtained with Cytochrome c Peptides. To test the possibility that the observed T-cell activation could be so radically influenced by sequential variations restricted to a single amino acid, the next series of experiments sought to confirm the previous observations with specific peptides obtained after CNBr cleavage of either beef or horse cytochrome *c*. The advangage of using such peptides stems from the fact that it is possible to obtain fragments consisting of amino acids 81–104 which differ from each other at either a single amino acid residue in the case of beef

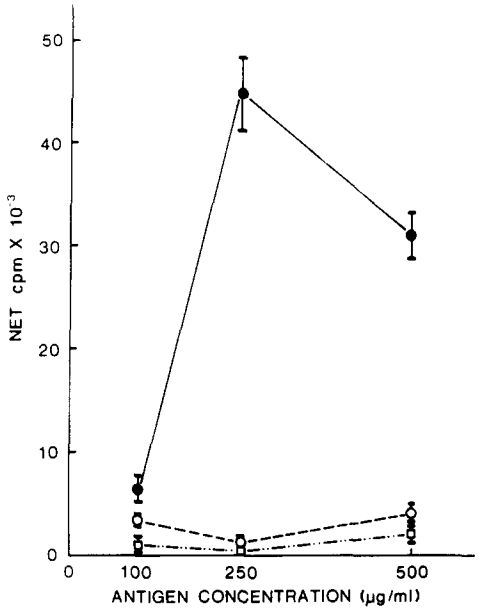

FIG. 3. BDF_1 mice were injected with 100 μg of beef cytochrome *c*. 8 d later, draining LN were removed, cell suspensions depleted of Ig-positive cells with anti-mouse light (κ) chain treatment and tested for proliferative response to various concentrations of beef cytochrome *c* (●), horse cytochrome *c* (○) or rabbit cytochrome *c* (□). Bars represent standard error of the mean values. Background (saline) response was 3,559 ± 394 cpm.

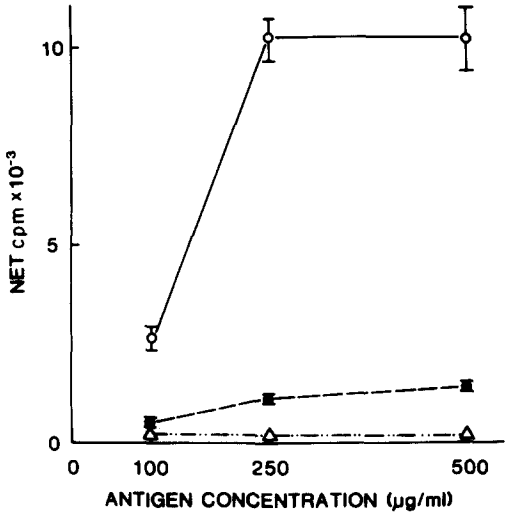

FIG. 4. BDF_1 were injected with 100 μg of horse cytochrome *c*. 8 d later, draining LN were removed, cell suspensions fractionated through nylon wool, and the passed cells tested for proliferative response to various concentrations of horse cytochrome *c* (○), beef cytochrome *c* (●), or rabbit cytochrome *c* (△). Bars represent standard error or the mean. Background (saline) response was 250 ± 30 cpm.

and horse peptides or 2 amino acid residues in the case of horse and rabbit peptides (Fig. 2).

Mice were primed with beef cytochrome *c* as before and their LN cells assessed for subsequent in vitro responses to intact cytochrome *c* or peptides derived therefrom. It

TABLE II

*Antigen Induced Proliferative Response of LN Cells from SJL Mice Primed with Beef Cytochrome c**

Challenge antigen‡	Response§
	net cpm ± *SE* × 10^{-3}
Beef	69.1 ± 4.9
Horse	43.7 ± 4.7
Rabbit	33.1 ± 7.1

* SJL mice were primed with 100 μg of beef cytochrome *c*. 8 d later, cells from draining lymph nodes were cultured and processed as described in Materials and Methods.

‡ At a final culture concentration of 500 μg/ml.

§ Net proliferative response was obtained by subtracting background (saline) responses of 3,100 ± 425.

TABLE III

*Comparison of the Capacity of Peptides 81-104 from Beef or Horse Cytochrome c to Stimulate Lymphocytes from BDF_1 Mice Primed with Beef Cytochrome c**

Challenge antigen‡	Response§
	net cpm ± *SE* × 10^{-3}
Beef cytochrome	55.5 ± 4.8
Beef 81-104	57.5 ± 2.3
Horse 81-104	5.7 ± 4.8

* BDF_1 mice were primed with 100 μg of beef cytochrome *c*. 8 d later, cells from draining lymph nodes were cultured and assayed for proliferative responses as described in text.

‡ At a final concentration of 500 μg/ml in culture.

§ Net proliferative response was obtained by subtracting background (saline) responses of 9,635 ± 1,147.

can be seen that whereas a proliferative response was observed in cultures challenged with either beef cytochrome *c* or a beef cytochrome *c* peptide containing amino acids 81–104, horse peptide 81–104 failed to stimulate (Table III). The relevance of this observation is linked to the fact that the horse cytochrome *c* fragment differs from that obtained from beef cytochrome *c* at a single amino acid position, namely residue 89, where there exists a threonine to glycine interchange. Therefore, the data obtained with peptide fragments confirm what was more circumstantially evidenced by using the intact proteins, namely that the specificity of T-cell activation can discriminate proteins or peptides which differ at a single amino acid position. The fact that these observations are reflective of T-cell specificity can be seen in Fig. 5 which shows that LN cells from beef cytochrome *c* immunized mice which were depleted of Ig-positive cells can respond to beef peptide 81–104 but not to the corresponding horse peptide. The data presented in Table IV reinforce this interpretation since the reciprocal situation reveals an analogous pattern of stimulation, namely that LN cells from mice primed with horse cytochrome *c* can be induced to proliferate with peptide 81–104 from horse but not with beef or rabbit peptides 81–104.

Specificity of Stimulation Obtained with Hybrid Cytochrome c Molecules. Because it is possible to reconstitute cytochrome *c* from its peptide elements without altering its physicochemical properties (14, 15) and in this manner obtain hybrid molecules composed of cytochrome fragments derived from different species, a further test of the specificity and restriction of primed LN cell stimulation was performed by using

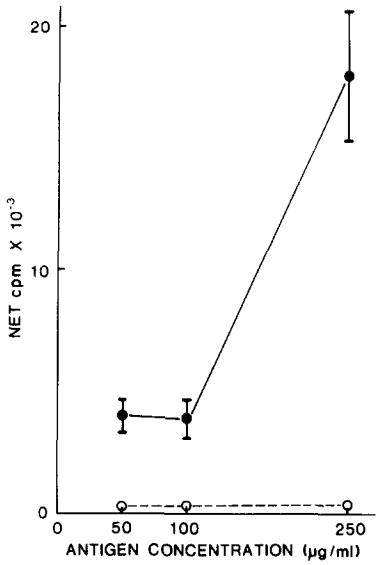

FIG. 5. BDF_1 mice were injected with 100 μg of beef cytochrome *c*. 8 d later, draining LN were removed, cell suspensions depleted of Ig-positive cells by rabbit anti-mouse light (κ) chain treatment and tested for proliferative response to various concentrations of beef cytochrome *c* peptide 81–104 (●) or horse cytochrome *c* peptide 81–104 (○). Bars represent standard error of the mean. Background (saline) response was 3,559 ± 394 cpm.

TABLE IV

*Specificity of the Proliferative Response of LN Cells from Mice Primed with Horse Cytochrome c Stimulated with Various Cytochromes or Their Derived Peptides**

Challenge antigen	Concentration‡	Response§
	μg/ml	*net cpm ± SE × 10^{-3}*
Cytochrome c		
Horse	250	18.3 ± 3.8
Beef	250	4.8 ± 0.1
Rabbit	250	−1.7
Peptide 81-104		
Horse	500	28.5 ± 8.1
Beef	500	−0.5
Rabbit	500	−0.9

* BDF_1 mice were primed with 100 μg horse cytochrome *c* and draining LN cells were challenged and assayed for proliferative responses as described in the text.

‡ Final concentration in culture.

§ Net proliferative responses were obtained by subtracting background (saline) responses of 2,415 ± 315.

hybrid molecules for in vitro challenges. These molecules offer the advantage of presenting the relevant peptide within the context of the tertiary structure of cytochrome *c* and thus obviate the possibility that the previous observations are ones restricted to the use of peptides which assume a three dimensional structure different from that present in the content of the intact molecule.

As before, mice were primed with beef cytochrome *c* and cells from draining LN were challenged in vitro with various intact cytochrome *c* or hybrid molecules. It can

TABLE V

*Specificity of the Proliferative Response of LN cells from Mice Primed with Beef Cytochrome c and Stimulated In Vitro with Various Cytochrome c or Hybrid Molecules**

Challenge antigen‡	Response§
	net cpm ± *SE* × 10^{-3}
Exp. 1	
Beef (1-104)	22.2 ± 0.3
Horse (1-104)	5.2 ± 2.1
Horse–Beef (1-65)-(66-104)	33.3 ± 4.3
Exp. 2	
Beef (1-104)	47.3 ± 6.8
Rabbit (1-104)	6.6 ± 0.4
Rabbit–Beef (1-65)-(66-104)	44.0 ± 4.1

* BDF_1 mice were primed with 100 μg beef cytochrome *c* and draining LN cells were challenged and assayed for proliferative responses as described in the text.

‡ Antigens tested were either beef, horse, or rabbit cytochrome *c* denoted as (1-104), or hybrid molecules composed of either horse peptide 1-65 and beef peptide 66-104 or rabbit 1-65 and beef 66-104 prepared as described in the text. Each antigen was used in culture at a final concentration of 500 μg/ml.

§ Net proliferative responses were obtained by subtracting background (saline) responses of 5,125 ± 620.

be seen in Table V that primed LN cells which responded to beef cytochrome *c* but not to horse or rabbit cytochrome *c* were nevertheless responsive to either horse-beef or rabbit-beef hybrid molecules. These data thus extend the observations made with isolated peptides and demonstrate that it is possible to obtain cell activation using the beef peptide 66–104 even when it is linked to peptides of proteins from either horse or rabbit cytochrome *c*, neither of which are stimulatory in this system.

Discussion

The data presented indicate that recognitive processes leading to T-cell proliferation are discriminatory to the extent that differences of a single amino acid residue in the primary sequence of proteins or peptides can lead to an all or none effect in the capacity of T cells to be activated. This conclusion was most forcibly reached in experiments where T lymphocytes obtained from mice primed either to horse or beef cytochrome *c* responded to peptide 81–104 from horse or beef cytochrome *c*, respectively, but not vice versa although those peptides differ from each other at a single amino acid position, namely position 89 where there is a threonine to glycine interchange. These results are complementary to those obtained with native cytochrome *c* from which it could be concluded that the pattern of cross-reactivity in the response of BDF_1 mice primed with beef or horse cytochrome *c* is linked to a sequence homology restricted to amino acid 89. Similarly, when artificial hybrid molecules were tested with cells from BDF_1 mice primed with beef cytocrhome *c*, it was observed that beef fragment 66–104 covalently linked to horse or rabbit peptide 1–65 led to hybrid molecules which were capable of stimulation although native horse or rabbit cytochrome *c* were inactive. It is important to point out that the pattern of cross-reactivity observed appears to be circumscribed by the haplotype of individual strains of mice inasmuch as it was seen with cells from primed BDF_1 mice but not with SJL or some other strains tested. On the other hand, the pattern of cross-reactivity

observed in other strains also lead to the conclusions that restricted amino acid substitutions, in some cases, other than at position 89, can be critical for T-cell activation.

The conclusion that T-cell specificity is highly discriminatory has been previously considered in studies by others. For example, Stashenko and Schlossman (10) reported that primed guinea pig T lymphocytes could distinguish DNP-oligolysines in which the hapten was coupled to the amino group of either the amino or carboxyl terminal lysyl residue of different length oligopeptides. Barcinski and Rosenthal (11) reported that stimulation of strain 2 guinea pig T lymphocytes is sensitive to changes in three amino acid positions of the insulin A chain. In addition, using gallinaceous lysozymes, Sercarz et al. (12) have recently postulated that the degree of murine T-cell (PETLES, 25) proliferation responses may be influenced by substitutions of two amino acid positions, an effect which was seen within restricted genetic background. Finally, while the present manuscript was in preparation, Wolff and Reichlin (26) reported that primed T cells from guinea pig peripheral blood appear to distinguish donkey from horse cytochrome *c* which differ from each other at the single amino acid position 47 (27).

A fundamental question raised by the present studies and others which have used globular proteins to determine the specificity of T lymphocytes is the type of determinant structure recognized by T cells. Whereas B cell or antibody specificity has been linked to determinants which can be considered conformational (28, 29), i.e., dependent on the native spacial conformation of the protein, a substantial body of evidence suggests that T cells may recognize sequential determinants, namely those which arise from amino acid sequences that exist in a random coil form (20, 30–32). In this regard, it is germane that in the present studies T-cell stimulation can be obtained with cytochrome *c* fragments which display little α-helical conformation in aqueous solution (G. Corradin, unpublished observations) although the same segment of the protein can be seen to be in a helical structure within the context of the native molecule (33). In a similar vein, other data not presented here reveal that apocytochrome *c*, obtained by cleaving the heme group from the peptide chain, is fully capable of stimulating T lymphocytes from animals primed with the native protein although the molecule in aqueous solution assumes a conformation strikingly different from native cytochrome *c* (34). The possibility that T-cell specificity to globular proteins may be more dependent on the recognition of sequential rather conformational determinants is enhanced by the observations which have demonstrated a macrophage presentation step as a requirement for T-lymphocyte activation (35). In this light, it could be assumed that macrophage processing involves degradation of antigen to fragments which display epitopes markedly different from those revealed by the constraints of tertiary configuration, in fact epitopes whose antigenicity may be radically altered by substitution at a single amino acid position.

At least two general possibilities could account for the observations that a single amino acid change can lead to an all or none effect in terms of cell activation. On the one hand, the problem could be related to T-cell recognition. The more elemental interpretation would be that T cells recognize a linear sequence of a processed fragment and that substitution of a given amino acid position can be distinguished by receptors responsible for T-cell specificity. As a variation on that theme, it could be that fragments exhibited on macrophages assume a conformation specifically influenced by the amino acid variation of a single residue and therefore that the lack of

cross-reactivity would be mainly due to a conformation effect of the residue on the overall structure of the peptide. On the other hand, it is possible that the amino acid interchange of glycine with threonine directly affects the processing of antigen by macrophages in terms of altering the enzymatic cleavage pattern of proteins or peptides. This consequence could yield different peptides ultimately presented to T lymphocytes. In this case the specificity would be macrophage directed in the sense that sensitization would be a direct function of macrophage handling and display, a process which may well involve haplotype restriction as suggested by Rosenthal et al. (36).

A point of some interest in the present studies was the observation that murine cytochrome *c* could elicit a response with cells from mice primed with beef cytochrome *c*. While this apparent autoreactivity is somewhat surprising, it may be that cytochrome *c* as a membrane bound mitochondrial protein never reaches sufficient concentration in the circulation to impose self tolerance (37). On the other hand, it is equally possible that active suppression to self cytochrome *c* may be operational in mice and that cross-immunization can circumvent the homeostatic balance. It will be of obvious interest to determine whether murine cytochrome *c* can itself be immunogenic in mice.

Finally, it should be emphasized that although studies determining the specificity of T-cell proliferation do not directly shed light on specificity of functional T-lymphocyte subsets, preliminary data looking at the carrier effect using beef and horse cytochrome *c* reveal similar cross-reactive restriction in BDF_1 mice as that observed for T-cell proliferation. That is, mice primed with beef cytochrome *c* form antibody to a subsequent challenge with beef but not horse cytochrome *c*. Similarly, horse cytochrome *c* primed mice do respond to a subsequent horse challenge but not to beef cytochrome *c*. On the other hand, antibody raised to beef cytochrome *c* reacts similarly with both beef and horse cytochrome *c*, suggesting that observations on the carrier effect may well be attributable to the specificity of T helper function and that cytochrome *c* may be another case in which an antigen may elicit different pattern of immunological specificity in B and T cells (38–40).

Summary

Murine T-lymphocyte specificity was determined in a system of antigen driven in vitro T-cell proliferation using cytochrome *c* molecules from different species, their derived peptides and reconstituted hybrid proteins. It was observed that primed T cells could discriminate between peptide fragments which differed from each other at a single amino acid residue. These conclusions were substantiated by the pattern of cross-reactivity noted in the response of closely related cytochrome *c* proteins as well as when artificial hybrid molecules reconstituted by the covalent linkage of peptide fragments were analyzed. The pattern of specificity observed appeared to be haplotype (BDF_1) dependent although similar conclusions about the fine specificity of T cells in the response to cytochrome *c* have been obtained in other strains but associated with different residues.

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