T-LYMPHOCYTE RESPONSE TO CYTOCHROME c

I. Demonstration of a T-Cell Heteroclitic Proliferative Response and Identification of a Topographic Antigenic Determinant on Pigeon Cytochrome c Whose Immune Recognition Requires Two Complementing Major Histocompatibility Complex-linked Immune Response Genes

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The importance of the study of globular proteins as model antigens for analyzing the immune response has been fully appreciated only recently (1, 2). Proteins were eclipsed for many years by the seemingly simpler synthetic polyamino acids (3). Although the latter antigens offer simplicity in composition, they are heterogeneous in molecular weight and spatial conformation and often differ in antigenic properties from one preparation to the next. In contrast, many globular proteins have been sufficiently purified and characterized such that each is a fully homogeneous material of defined amino acid sequence and tertiary structure. Furthermore, because different species produce variants of a given protein, homologous sets of proteins are available that permit exact localization of antigenic determinants by comparing the immunological cross reactivities either of members of the set or of fragments of these proteins obtained by enzymic or chemical cleavages.

Cytochrome c, a heme protein of the mitochondrial respiratory chain, has proven to be an extremely useful antigen for the study of the immune response (1, 4-9). It is a globular protein composed of a single polypeptide chain of a little over 100 amino acids. The amino-acid sequences of >85 cytochromes c from different eukaryotic sources are known (10), and the three-dimensional structures of horse (11), tuna (12), and bonito (13) cytochromes c have been determined at high resolution. The spatial conformations of these three cytochromes c were found to be identical. In fact x-ray crystallographic studies have revealed that the cytochrome c fold (14) is maintained even in c-type cytochromes from prokaryotic organisms (11), which differ from the eukaryotic proteins over most of their amino-acid sequence. It has been concluded from such studies that the conformation of all eukaryotic cytochromes c is essentially the same (11, 14). Thus, this group of proteins represents an excellent set of closely related variants with which to study the effects of diversity of the amino-acid sequence on the antigenicity of a globular protein independent of changes in conformation.

The recent development of a proliferation assay for murine T lymphocytes has
allowed the study of the genetic control of the immune response at the T-cell level (15). In particular, experiments with synthetic polypeptide and protein antigens have demonstrated that immune-response (Ir) genes exert control over antigen-induced T-lymphocyte proliferation. The present study analyzes the murine T-lymphocyte proliferative response to pigeon cytochrome c, identifies and characterizes the major antigenic determinant responsible for the proliferation, and demonstrates that the recognition of this determinant is under the control of dual major histocompatibility complex (MHC)-linked Ir genes.

Materials and Methods

**Animals.** Strains C57BL/10Sn (B10), B10.A/SgSn (B10.A), B10.D2/nSn (B10.D2), B10.A(2R)/SgSn, B10.A(5R)/SgSn, and B10.BR/SgSn were obtained from The Jackson Laboratory, Bar Harbor, Maine. Breeding pairs of the B10.A(3R)/Sg, B10.A(4R)/Sg, B10.M/Sg, B10.RIII/Sg, B10.Q/Sg, B10.S/Sg, and B10.PL/Sg strains were generously provided by Dr. Jack Stimpfling, McLaughlin Research Institute, Great Falls, Mont. B10.WB/Sf breeding pairs were kindly provided by Dr. Donald Shreffler, Department of Genetics, Washington University, School of Medicine, St. Louis, Mo. B10.AKM and B10.AQR/Sx mice were the generous gift of Dr. David Sachs, National Cancer Institute, National Institutes of Health, Bethesda, Md. The (B10 × B10.A) and [B10.A(4R) × B10.A(3R) or (5R)]F1s or their reciprocal hybrids were bred in our laboratory from the parental strains. Mice of both sexes were used between 6 and 18 wk of age.

**Antigens.** All cytochromes c were prepared by the procedure of Brautigan et al. (16) in which careful column chromatography on carboxymethyl cellulose removed any of the polymeric and deamidated artifacts of the protein that commonly contaminate commercial preparations. Peptide fragments of the cytochromes c were prepared by cyanogen bromide cleavage by following the procedure of Corradin and Harbury (17). Final purification of the 1-65 fragment required several rechromatographies to remove the last traces of the 66-104 fragment. Furthermore, rechromatography was routinely employed for all fragments to purify them from traces of other fragments that eluted ahead of them in the same run, even when the peaks in the elution profile were widely separated. Each column used the Sephadex column (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) was washed with 500 ml water, followed by a solution of 4 ml freshly distilled pyridine, 4 ml concentrated ammonium hydroxide, and 16 ml of 50 mM ammonium bicarbonate. Water (2,500 ml) was then passed through the column followed by the re-equilibration solvent of formic acid. This washing procedure was found to be essential to prevent the contamination of the peptide fragments by traces of materials remaining bound to the column from previous chromatographies.

**Immunization.** Cytochrome c was emulsified in complete Freund's adjuvant containing 1 mg/ml of killed *Mycobacterium tuberculosis*, strain H37Ra (Difco Laboratories, Detroit, Mich.). All animals were injected in the hind footpads with various doses of cytochrome c in a total of 0.1 ml of emulsion. The mice were immunized with 0.016, 0.16, 1.6, or 8 nmol (0.2, 2, 20, or 100 μg, respectively) of the various cytochromes c or 1.6 nmol of the CNBr-cleavage fragments of the appropriate cytochrome.

**Preparation of Peritoneal Exudate T-Lymphocyte-enriched Subpopulation (PETLES).** The original and slightly modified procedures for preparing PETLES are described in detail elsewhere (15). In the experiments described in this paper the PETLES population eluted from the column contained an average of 2% macrophages, 43% lymphocytes, and 55% eosinophils, with <2% B lymphocytes or mast cells.

**Cell Cultures.** The procedure for culturing PETLES has been described elsewhere (15). Briefly, 100 μl of a modified Eagle's-Hank's amino-acids medium supplemented with 10% fetal calf serum and containing 2 × 10⁵ PETLES were placed in each well of a sterile, U-bottom
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polystyrene microculture plate (Cooke, Laboratory Products Division, Dynatech Laboratories, Inc., Alexandria, Va.). Cytochrome c in various amounts was added in another 100 µl to give a total vol of 200 µl. Medium without antigen was used as a nonstimulated control and 20 µg/ml purified protein derivative from M. tuberculosis (purified protein derivative of tuberculin [PPD], Connaught Medical Research Labs., Willowdale, Ontario) was used as a positive control. The cultures were incubated for 5 d at 37°C. Stimulation was assessed by measuring the incorporation of a 1-µCi pulse of tritiated thymidine 16-18 h before harvesting the cultures. Determinations were done in triplicate or for some dose-response curves only in duplicate, and the data are expressed either as arithmetic mean counts per minute plus or minus the standard error of the mean or as the difference between antigen-stimulated and nonstimulated responses (Acpm). The standard errors for the Acpm values were derived from the square root of the sum of the squares of the standard errors of the mean antigen-stimulated and nonstimulated responses. Statistical significance was assessed with a two-tailed Student's t test.

Results

PETLES Response to Pigeon Cytochrome c. Pigeon cytochrome c was one antigen that met our requirements for a potent immunogen that did not prime for a cross-reaction with mouse cytochrome c. It was immunogenic in B10.A mice when administered at 1.6 nmol (20 µg) per mouse (Figs. 1 and 2). The maximal response achieved ranged from a Acpm of 11,700 to a Acpm of 48,200 and averaged 28,000 ± 2,700 for 16 experiments. The mean concentration required to reach one-half the maximal response was 0.52 ± 0.15 µM. The PETLES could not be stimulated by any dose of mouse cytochrome c from 0.08-40 µM. Even PETLES from B10.A mice immunized with 8 nmol of pigeon cytochrome c showed only a negligible cross-reaction with the mouse protein, namely 10% of the response to pigeon cytochrome c at an equivalent dose.

| Source of Cytochrome c | Amino Acid Residue at Position | Proliferative Response (Δ CPM) |
|------------------------|-------------------------------|-----------------------------|
| Mouse                  | Val   Ala   Ala   Gly   Lys   Asn   Glu |  |
| Pigeon                 | Lig   Ser   Glu   Ala   Gin   Ala   Lys |  |
| Human                  | Val   Ser   Pro   Glu   Lys   Asn   Glu |  |
| Neurospora crassa      | Ser   Ala   Asp   Lys   Glu   Ala |  |
| Hippopotamus           | Val   Ala   Pro   Gin   Asn   Glu |  |
| Tobacco Horn Worm Moth | Ala   Ala   Pro   Asn   Gin   Lys |  |
| Chicken                | Lig   Ser   Glu   Ser   Asp   Ser   Lys |  |
| Pekin Duck             | Val   Ser   Glu   Ser   Asp   Alc   Lys |  |

Fig. 1. A list of the seven positions at which pigeon cytochrome c differs from mouse cytochrome c and the amino acid residues at each of these positions in mouse, pigeon, human, Neurospora crassa, hippopotamus, tobacco hornworm moth, chicken, and duck cytochromes c. Underlined amino acid residues are those considered to be part of the pigeon cytochrome c determinant recognized by B10.A T cells. A dash indicates a deletion of that residue. The bar graph at the right shows the T-lymphocyte proliferative response to each of the eight cytochromes by PETLES from B10.A mice primed with 1.6 nmol of pigeon cytochrome c.
little as 0.16 nmol of pigeon cytochrome c was immunogenic, although at this dose the magnitude of the proliferative response was small. Finally, unlike the case with human cytochrome c described previously (7), cross-stimulation with other cytochromes allowed localization of the major antigenic site.

Pigeon cytochrome c differs from mouse cytochrome c at seven amino-acid positions (Fig. 1). When PETLES from B10.A mice immunized to pigeon cytochrome c were challenged in vitro with human or Neurospora crassa cytochrome c, there was no significant proliferative response at any dose tested. Of the seven potential stimulatory sites possessed by pigeon cytochrome c, human cytochrome c shares only the serine at position 15, and neurospora cytochrome c shares only the alanine at position 103. The failure of these two proteins to cross-stimulate suggests that the amino acids at positions 15 and 103 are not the major stimulatory determinants, at least not by themselves. However, such an analysis based entirely on negative data could have other explanations. For example, nonstimulating cytochromes might contain the appropriate substitution for stimulation but possess other nearby substitutions not possessed by pigeon cytochrome c that prevent the stimulation. This could well be the case for a cytochrome c as different as the neurospora protein, which varies at 46 of its residues from pigeon cytochrome c. It was therefore essential to search for cytochromes c which did cross-stimulate to locate the determinant. Positive results were obtained with hippopotamus and tobacco hornworm moth cytochrome c.

Hippopotamus cytochrome c differs from mouse cytochrome c at four positions. Only one of these, the glutamine for lysine substitution at position 100, occurs in pigeon cytochrome c (Fig. 1). PETLES from B10.A mice immunized with pigeon cytochrome c gave a significant proliferative response when challenged with the hippopotamus protein (Figs. 1 and 2). In 14 experiments the maximum stimulation achieved averaged 45% of the maximal response to the pigeon protein. However, in most cases the dose-response curve for the hippopotamus protein was still rising at the
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highest concentration tested (40 \( \mu \)M) (Fig. 2). These results suggested that an antigenic site in pigeon cytochrome \( \epsilon \) recognized by the immune system of the B10.A mouse was located around position 100 and involved the glutamine for lysine substitution. However, the fact that hippopotamus cytochrome \( \epsilon \) gave only a partial cross-reaction suggested that other residues must be involved.

Evidence that one of the other nearby residues was also part of the antigenic site came from studies with the cytochrome \( \epsilon \) of tobacco hornworm moth. This cytochrome was initially tested for its cross-stimulation because like the pigeon and hippopotamus proteins it has a glutamine at position 100. However, in contrast to the hippopotamus protein, tobacco hornworm moth cytochrome \( \epsilon \) gave a complete cross-reaction (Fig. 1). In fact, throughout the dose-response curve (Fig. 2), it stimulated better than the immunogen, pigeon cytochrome \( \epsilon \). In nine experiments the average maximal response (\( \Delta \text{cpm} \)) to the tobacco hornworm moth protein was 38,300 ± 6,300, which was \( \equiv 1.4 \)-fold greater than that to the pigeon protein. Even more striking, in eight paired experiments the concentration required to achieve a one-half maximal response to tobacco hornworm moth cytochrome \( \epsilon \) was 12.8 ± 3.9-fold lower than that for pigeon cytochrome \( \epsilon \).

These results can most simply be explained by considering the C-terminal amino-acid sequence of tobacco hornworm moth cytochrome \( \epsilon \) (Fig. 1). Like the bird cytochromes \( \epsilon \), this protein has a C-terminal lysine, which constitutes a difference from the C-terminal glutamic acid of the mouse protein. However, the tobacco hornworm moth protein is one amino acid shorter than pigeon cytochrome \( \epsilon \) because residue 103 is deleted. Thus, the heteroclitic nature of the response to the tobacco hornworm moth cytochrome \( \epsilon \) suggests that the alanine (or perhaps any residue) at position 103 in the pigeon protein has a negative influence on the ability of pigeon cytochrome \( \epsilon \) to stimulate a response.

From these experiments it appears that the major determinant of pigeon cytochrome \( \epsilon \) recognized by T cells contains the lysine at position 104 and the glutamine at position 100. These residues are much closer in space than is indicated by their sequence positions, because they are part of the C-terminal \( \alpha \)-helix of the protein (11, 12). The helix puts the \( \beta \)-carbon atom of residue 104 only about 6 \( \AA \) away from that of residue 100. A possible arrangement of the complete side chains of residues 100 and 104 on the surface of the protein is pictured in Fig. 3.

Finally, it should be noted that the closely related cytochromes \( \epsilon \) of chicken and Pekin duck failed to give a substantial cross-reaction, although at very high concentrations, 16 and 40 \( \mu \)M, both occasionally stimulated significant proliferative responses (Figs. 1 and 2). These two cytochromes \( \epsilon \) lack the glutamine at position 100 that occurs in the pigeon protein but have the lysine at 104. It is possible that the terminal lysine accounts for the small cross-reactions. On the other hand, it is also possible that other residues in Pekin duck and chicken cytochromes \( \epsilon \) that are shared by the pigeon but not the mouse protein were responsible for the stimulation. This situation was clarified when we studied the cross-reactions elicited by peptide fragments of the cytochromes \( \epsilon \).

Cross-stimulation of PETLES Immune to Pigeon Cytochrome \( \epsilon \) by Cyanogen Bromide Cleavage Fragments of the Bird Cytochromes \( \epsilon \). To confirm the localization of the antigenic site, cross-stimulation experiments were carried out with fragments of pigeon, duck, chicken, and mouse cytochromes \( \epsilon \). These fragments were produced by cleavage with
Fig. 3. The antigenic determinant in pigeon cytochrome c recognized by T lymphocytes of the B10.A mouse. This is a computer drawn model (18) of the site produced by Richard Feldmann of the Division of Computer Research and Technology, National Institutes of Health. Using the three-dimensional structural coordinates for tuna cytochrome c, the pigeon protein sequence was substituted, keeping the side chain orientations the same as in the tuna protein. The C-terminal lysine was added on to the tuna cytochrome c backbone as an extension of the α-helix. The α-carbon atom was oriented in trans around the nitrogen-carbonyl bond relative to the α-carbon atom of residue 103 and the remainder of the lysine 104 side chain was modeled to maximize potentially favorable side chain interactions with the glutamine 100 side chain. The three residues involved in the antigenic site are shown in black or white whereas the rest of the residues in the molecule are depicted in gray. They are, from top to bottom, the isoleucine at position 3 (black), the glutamine at position 100 (white), and the lysine at position 104 (black). All three amino acids are located as shown on the back face of the molecule. The front of the molecule is defined as the surface containing the exposed edge of the heme along pyrrole rings II and IV.
Stimulation of PETLES from B10.A Mice Immune to Pigeon Cytochrome c with Cyanogen Bromide-cleavage Fragments of Bird Cytochromes c

| Cytochrome source | Fragment residue No. | Proliferative response (Δcpm ± SEM) |
|-------------------|----------------------|-------------------------------------|
|                   |                      | Exp. 1                              | Exp. 2                              |
| Mouse             | 1-104                | 2,400 ± 1,200                       | 200 ± 1,600                         |
|                   | 1-65                 | 2,100 ± 2,100                       | 1,300 ± 2,300                       |
|                   | 66-80                | 1,200 ± 1,100                       | -1,100 ± 1,600                      |
|                   | 81-104               | 2,300 ± 1,100                       | -400 ± 1,600                        |
| Pigeon            | 1-104                | 27,900 ± 1,200                      | 25,700 ± 2,300                      |
|                   | 1-65                 | 8,700 ± 1,100                       | 8,200 ± 2,600                       |
|                   | 66-80                | 1,500 ± 1,100                       | -1,100 ± 1,900                      |
|                   | 81-104               | 43,100 ± 2,900                      | 34,500 ± 6,400                      |
| Pekin duck        | 1-104                | -100 ± 1,100                        | 3,800 ± 1,700                       |
|                   | 1-65                 | 1,300 ± 1,100                       | 1,400 ± 2,100                       |
|                   | 66-80                | 1,800 ± 1,500                       | -1,500 ± 1,700                      |
|                   | 81-104               | 13,700 ± 2,100                      | 6,100 ± 1,800                       |
| Chicken           | 1-104                | 3,400 ± 1,100                       | 2,100 ± 2,200                       |
|                   | 1-65                 | 9,400 ± 1,600                       | 11,500 ± 1,800                      |
|                   | 66-80                | 3,600 ± 1,100                       | -100 ± 1,900                        |
|                   | 81-104               | 16,400 ± 2,800                      | 8,700 ± 2,400                       |

2 × 10⁶ PETLES from B10.A mice primed with 1.6 nmol of pigeon cytochrome c were stimulated in vitro with various concentrations of pigeon, mouse, Pekin duck, or chicken cytochrome c (residues 1-104) or the three cyanogen bromide cleavage fragments (residues 1-65, 66-80, and 81-104) from either pigeon, mouse, Pekin duck, or chicken cytochrome c. Only the maximum stimulation from a dose-response curve is shown for each antigen. The medium control was 3,400 ± 1,064 in exp. 1 and 4,300 ± 1,600 in exp. 2. The data are expressed as the difference between antigen-stimulated and medium control cultures (Δcpm). The standard errors listed are the square root of the sum of the squares of the standard errors of the antigen-stimulated and medium control means. Statistically significant (P < 0.05) differences are in italics.

cyanogen bromide and the three nonoverlapping peptides (residues 1-65, 66-80, and 81-104) were isolated. To demonstrate that the cyanogen bromide treatment by itself did not affect the stimulating ability of intact cytochromes c in the assay, uncleaved pigeon and duck cytochromes c were recovered from the cyanogen bromide reaction mixtures and compared to the original native preparations for their ability to stimulate a proliferative response. No differences were observed (7).

PETLES from B10.A mice immunized with intact pigeon cytochrome c were challenged in vitro with equimolar concentrations of the whole molecule or the various fragments. As shown in Table I, no response was seen to any of the mouse cytochrome c fragments. Of the three pigeon cytochrome c peptides only the C-terminal fragment (residues 81-104) elicited a strong response, confirming the localization of the major antigenic site. In fact, this fragment stimulated better than the intact molecule throughout the entire dose-response curve (Fig. 4). In 12 experiments, the average maximal response was 1.38 ± 0.1-fold higher than that obtained with the whole molecule, and the concentration required to attain one-half maximal stimulation was 7.1 ± 1.9-fold lower. In contrast, fragment 66-80 did not stimulate significantly at any concentration tested.

Pigeon fragment 1-65 gave a significant cross-reaction (Table I) but usually only at
high concentrations (dose-response curve not shown). This stimulation could have resulted from a 1% contamination of fragment 1-65 by the minor yielding fragment 66-104, which contains the two stimulating residues at 100 and 104 and which immediately follows fragment 1-65 in elution from the chromatography column. However, three rechromatographies of fragment 1-65 failed to eliminate its ability to cross-stimulate, suggesting the presence of a third stimulating residue. This was confirmed in studies with fragments of chicken and Pekin duck cytochromes c. Fragment 1-65 of chicken cytochrome c, with a sequence identical to that of pigeon cytochrome c fragment 1-65, elicited a similar small response even though the chicken fragment 81-104 was not strongly stimulatory (Table I and Fig. 4). In contrast, fragment 1-65 from Pekin duck cytochrome c did not elicit any response, similar to mouse fragment 1-65, despite the fact that duck fragment 81-104 was as stimulatory as chicken fragment 81-104 (Fig. 4). These findings make it very unlikely that the stimulatory properties of pigeon fragment 1-65 could be caused by a minor contamination with a fragment containing the 100 and 104 residues of pigeon cytochrome c. Instead, these results appear to localize a third stimulating residue of pigeon cytochrome c to the isoleucine at position 3, as the duck fragment 1-65 differs from the pigeon and chicken fragments 1-65 only at this site (Fig. 1). Furthermore, the stimulation with chicken fragment 81-104 confirms the earlier finding that the lysine at position 104 is part of the antigenic determinant, because this lysyl residue is the only difference in amino acid sequence from mouse cytochrome c in the 81-104 region that both chicken and pigeon cytochromes c have in common (Figs. 1 and 4). Finally, the similarity of the responses to fragments 81-104 from chicken and duck cytochromes c (Fig. 4) confirms that the alanine at position 103 of the pigeon protein is not a critical part of the determinant because the chicken protein has a serine in this position.

The spatial relationship of the three residues of pigeon cytochrome c involved in the stimulation of T cells from B10.A mice is shown in Fig. 3. Although the glutamine at 100 and the lysine at 104 are located in the C-terminal α-helix, whereas the isoleucine at position 3 is located in the N-terminal α-helix, when the molecule folds...
Table II
The T-Lymphocyte Proliferative Response to Pigeon Cytochrome c by PETLES from C57BL/10 H-2 Congenic Strains

| H-2 type | Strain | Medium | Pigeon | Mouse | PPD |
|---------|--------|--------|--------|-------|-----|
| a       | B10.A  | 300    | 23,900 | 600   | 42,200 |
|         |        | (± 25) | (± 1,300) | (± 30) | (± 2,000) |
| b       | C57BL/10 | 500    | 1,300  | 1,300  | 32,800 |
|         |        | (± 100) | (± 200) | (± 400) | (± 3,000) |
| d       | B10.D2 | 3,100  | 4,700  | 4,300  | 42,500 |
|         |        | (± 400) | (± 700) | (± 1,200) | (± 2,100) |
| f       | B10.M  | 1,700  | 8,600  | 3,200  | 107,100 |
|         |        | (± 300) | (± 1,400) | (± 500) | (± 6,100) |
| ja      | B10.WB | 1,700  | 3,100  | 2,100  | 19,700 |
|         |        | (± 30) | (± 150) | (± 500) | (± 2,800) |
| k       | B10.BR | 4,100  | 43,300 | 3,100  | 59,900 |
|         |        | (± 1,000) | (± 1,500) | (± 700) | (± 7,000) |
|         | B10.BR | 4,800  | 16,900 | 6,100  | 28,000 |
|         |        | (± 1,700) | (± 4,200) | (± 3,200) | (± 5,600) |
| p       | B10.P  | 600    | 1,700  | 800    | 50,100 |
|         |        | (± 100) | (± 400) | (± 100) | (± 2,700) |
| q       | B10.Q  | 1,800  | 3,400  | 1,900  | 23,600 |
|         |        | (± 500) | (± 700) | (± 100) | (± 800) |
| r       | B10.RIII | 3,300 | 11,200 | 4,700  | 47,300 |
|         |        | (± 200) | (± 800) | (± 800) | (± 2,100) |
| s       | B10.S  | 1,400  | 6,900  | 4,300  | 25,300 |
|         |        | (± 300) | (± 2,300) | (± 300) | (± 3,200) |
| u       | B10.PL | 1,100  | 2,200  | 3,400  | 162,900 |
|         |        | (± 100) | (± 300) | (± 1,900) | (± 6,900) |

2 × 10^5 PETLES from mice primed with 1.6 nmol of pigeon cytochrome c were stimulated in vitro with various concentrations of pigeon cytochrome c, mouse cytochrome c, or PPD. Only the maximum stimulation from each dose-response curve is shown.

to the three-dimensional structure of native cytochrome c, these three residues lie next to each other on the back surface of the protein. Thus, the antigenic determinant appears to be a topographic linear array of the three amino acids, isoleucine, glutamine, and lysine. If only these residues are involved, the determinant spans a distance of ≈ 12 Å.

Genetic Control of the T-Lymphocyte Proliferative Response to Pigeon Cytochrome c. To determine whether MHC-linked Ir genes were involved in the recognition of the major antigenic determinant, mice of the B10.H-2 congenic series were immunized with 1.6 nmol of pigeon cytochrome c and their PETLES assayed for responsiveness to pigeon cytochrome c, mouse cytochrome c, or PPD (Table II). This series of strains includes different MHC haplotypes on the same C57BL/10Sn non-H-2 background. Thus, differences in responsiveness can be directly attributed to differences in MHC genes. As shown in Table II, PETLES from only two of the 11 B10-congenic strains tested responded well to pigeon cytochrome c, namely, those from mice of the H-2^a and H-2^k haplotypes. The responses to pigeon cytochrome c ranged from 12,000–48,000 Acpm as shown by the two experiments listed for B10.BR. The responses to PPD showed a corresponding proportional variation. The responses to pigeon cytochrome c were specific for pigeon cytochrome c in that no significant
### Table III

Genetic Mapping of Immune Response Genes Controlling the T-Lymphocyte Proliferative Response to Pigeon Cytochrome c

| H-2 Strain | MHC alleles* | Thymidine incorporation in response to Medium Pigeon Mouse PPD |
|------------|--------------|---------------------------------------------------------------|
|            | KABJECGSD    | cpm ± SEM                                                    |
| a B10.A    | kkkkkkkdddd  | 1,100 ± 300 (26,000) ± 200 (1,800) ± 200 (32,700) ± 200     |
| b B10      | bbbbbb      | 400 ± 50 (1,700) ± 1,200 (2,300) ± 1,200 (38,100) ± 1,200 |
| m B10.AKM  | kkkkkkq     | 400 ± 50 (25,900) ± 100 (2,800) ± 100 (28,800) ± 100       |
| y B10.AQR  | qkkkkkdd    | 400 ± 50 (16,200) ± 200 (600) ± 200 (27,600) ± 200         |
| h B10.A(2R)| kkkkkdk    | 2,600 ± 200 (52,000) ± 190 (3,400) ± 190 (55,200) ± 190   |
| i B10.A(5R)| bbbkkkdd   | 400 ± 50 (900) ± 100 (500) ± 100 (25,100) ± 100            |
| i B10.A(18R)| bbbbbbd.b | 1,100 ± 50 (7,600) ± 500 (7,800) ± 500 (57,500) ± 500 |
| b/a B10(5R)F1| bbbbb   | 2,200 ± 200 (21,900) ± 500 (3,400) ± 500 (60,000) ± 500 |
| i5/h4 B10.A(4R)F1| bbbkkkdd | 4,400 ± 200 (17,600) ± 100 (1,900) ± 100 (32,100) ± 100 |
| h4/i5 B10.A(5R)F1| bbbkkkdd | 1,300 ± 200 (15,500) ± 200 (1,200) ± 200 (53,400) ± 200 |
| h4/i3 B10.A(3R)F1| bbbkkkdd | 1,300 ± 200 (31,700) ± 200 (5,800) ± 200 (33,600) ± 200 |

* Letters indicate the haplotype source of the genetic information for each region of the MHC. A, B, J, E, and C designate the I-A, I-B, I-J, I-E, and I-C subregions, respectively.

† See legend of Table II.

stimulation was observed with mouse cytochrome c. All the other strains were either nonresponders: H-2b, H-2d, H-2k, H-2p, H-2q, or low responders: H-2r, H-2q, H-2s. The nonresponder and low-responder strains were clearly selective poor responders since in all cases their PETLES responded well to PPD. It would appear from these results that the Ir gene control of the response to pigeon cytochrome c is as restricted as the Ir gene control of the response to certain synthetic polypeptides such as poly (Tyr, Glu)-poly d,L-Ala—poly Lys [(T,G)-A—L] in that only a few haplotypes are good responders (15).

Because the H-2d haplotype (kdkkddddd) is felt to be a natural recombinant between the high responder H-2b and the nonresponder H-2d haplotypes, it would appear that only B10-congenic strains possessing k alleles in the K and I regions of the MHC can respond to pigeon cytochrome c. The responsiveness of the B10.AKM and B10.A(2R) strains is consistent with this conclusion (Table III). The responsiveness of the recombinant strain B10.AQR (qkdkkddd) further restricts the genetic locus to the right of the K region, completely in the I region (Table III). An interesting complexity...
was revealed when genetic mapping studies within the I region were attempted. As shown in Table III, PETLES from both the B10.A(4R) and B10.A(5R) recombinant strains did not respond to pigeon cytochrome c. The failure of these recombinants to respond could not be attributed to the acquisition of a suppressive gene product from the nonresponder B10 parent, because responsiveness was dominant over nonresponsiveness as shown by the strong proliferative response to pigeon cytochrome c of PETLES from (B10 × B10.A)F1 mice (Table III). Thus, the failure of the B10.A(4R) (kbbbbbbb) to respond must be the result of a loss of H-2k genetic material to the right of the I-A subregion and the failure of the B10.A(5R) (bbbkdddd) to respond must be the result of a loss of H-2k genetic material to the left of the I-J subregion. These observations suggest either that a single Ir gene controls the proliferative response to pigeon cytochrome c and is located in the I-B subregion, or that more than one Ir gene controls the response.

To distinguish between these two alternatives [(B10.A(4R) × B10.A(5R)]F1 mice and the reciprocal hybrid were bred and examined for their response to pigeon cytochrome c. A failure to respond would indicate a single gene in I-B, because the (4R × 5R)F1 is still homozygous for the low-responder b allele in I-B. On the other hand, a response by this strain would indicate gene complementation and suggest that at least two genes control the response (19, 20). The results in Table III demonstrate that the cross between the two nonresponders produced a responder strain. Therefore, the T-lymphocyte proliferative response to pigeon cytochrome c is controlled by at least two MHC-linked Ir genes, one mapping to the left of the I-B subregion in I-A, and the other to the right of the I-B subregion in I-J or I-E/I-C. The responsiveness of the [B10.A(3R) × B10.A(4R)]F1 maps the second gene to the right of the I-J subregion in the I-E/I-C subregion.

Immunogenicity of the Cyanogen Bromide-cleavage Fragments of Pigeon Cytochrome c. The immunogenicity of the fragments of pigeon cytochrome c was studied by immunizing B10.A mice with 1.6 nmol of fragment in CFA. Fragments 1-65 and 66-80 were not immunogenic as determined by stimulating the PETLES with either the fragments or intact cytochrome c. However, pigeon fragment 81-104 was strongly immunogenic. PETLES responded not only to the immunizing fragment but also to the whole pigeon protein, tobacco hornworm moth cytochrome c, hippopotamus cytochrome c, and the 81-104 fragments of chicken and duck cytochromes c (Fig. 5). There was no response to mouse cytochrome c. The pigeon protein fragment 81-104 elicited a better response than the whole molecule, with a twofold higher maximal stimulation and a twofold lower concentration for one-half maximal stimulation. The large response to the tobacco hornworm moth protein and the partial cross-reactions with Pekin duck cytochrome c fragment 81-104 and hippopotamus cytochrome c resembled the pattern seen on immunization of B10.A mice with the whole pigeon protein (compare Fig. 5 with Figs. 2 and 4). These results suggest that the fragment was priming clones of T cells with similar specificity to those primed by the whole molecule.

As shown in Fig. 6, the genetic mapping results with the fragment as immunogen were also similar to those obtained with the whole protein. PETLES from B10.A(4R) and B10.A(5R) mice did not respond when immunized with pigeon cytochrome c fragment 81-104, whereas PETLES from the [B10.A(4R) × B10.A(5R)]F1 did respond. These results demonstrate that two separate MHC-linked Ir genes are also required for immune recognition of the pigeon cytochrome c fragment 81-104.
Fig. 5. The T-lymphocyte proliferative response to pigeon (○), tobacco hornworm moth (●), hippopotamus (●), and mouse (△) cytochromes c, and pigeon (81-104) (*) and Pekin duck (81-104) (▲) fragments by PETLES from B10.A mice primed with 1.6 nmol of pigeon fragment (81-104).

Fig. 6. The T-lymphocyte proliferative response to pigeon cytochrome c fragment (81-104) by PETLES from B10 (●—●), B10.A (●—●), B10.A(4R) (●—●), B10.A(5R) (●—●), (B10.A × B10)F1 (●—●), and (B10.A(4R) × B10.A(5R))F1 (●—●) mice primed with 1.6 nmol of pigeon fragment (81-104). Note that the (B10.A × B10)F1 dose-response curve has been presented in two forms. One curve is the actual Δcpm, the other is adjusted with reference to a PPD standard because only PETLES from this strain had a twofold larger PPD response.
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Table IV
The Same T-Cell Clones Primed to Pigeon Cytochrome c Fragment 81-104 Recognize Hippopotamus Cytochrome c and Chicken or Duck Cytochrome c Fragment 81-104

| Antigens added to the cultures | Proliferative response (Δcpm) |
|-------------------------------|-------------------------------|
| Hippopotamus cytochrome c     | +                             | 7,100 |
| Chicken or duck fragment 81-104 | -                           | 5,100 |
| Chicken or duck fragment 81-104 | +                           | 9,400 |
| Exp.                          | Proliferative response (Δcpm) |
| 1                             | 4,700                         | 9,700 |
| 2                             | 6,600                         | 4,300 |
| 3                             | 11,000                        | 5,300 |
| 4                             | 4,500                         | 7,900 |

Arithmetic mean ± SEM

6,700 ± 1,500
6,700 ± 1,200
6,900 ± 800

B10.A mice were primed with 1.6 nmol of pigeon cytochrome c fragment 81-104 and 3 wk later PETLES prepared. 2 × 10⁶ cells were stimulated with 24 or 40 pM of hippopotamus cytochrome c, chicken or duck (Exp. 4) cytochrome c fragment 81-104, or an equimolar mixture of the two types of molecules. Stimulation was assessed 2-5 d later by measuring the incorporation of a 1 µCi pulse of tritiated thymidine administered 16 h before harvesting the cultures.

One Family of T-Cell Clones in B10.A Mice Responds to the Pigeon Cytochrome c Determinant. The ability of tobacco hornworm moth cytochrome c and pigeon cytochrome c fragment 81-104, which both lack the isoleucine at residue 3, to stimulate strong proliferative responses, suggested that the glutamine at site 100 and the lysine at site 103 and 104 were immunodominant residues in the antigenic determinant. Furthermore, the pattern of immunogenicity of pigeon fragment 81-104 suggested that two separate Ir genes control the response to these two residues. Thus, it was formally possible that two families of T-cell clones were responding to pigeon cytochrome c, one to the glutamine at 100 and the other to the lysine at 104, with each response controlled by only one of the two Ir genes. To examine this possibility, B10.A PETLES immune to pigeon fragment 81-104 (Table IV) were stimulated with fragment 81-104 of chicken or duck cytochrome c (to stimulate clones specific for the lysine at 104), hippopotamus cytochrome c (to stimulate clones specific for the glutamine at 100), or mixtures of these two types of molecules. As shown in Table IV, no additive effect was observed with any of the equimolar mixtures. Similar results were observed when whole pigeon cytochrome c was used as the immunogen (Fig. 7). Thus, it appears that the T-cell receptors on all the primed B10.A cells have sufficient affinity for both the hippopotamus protein and the bird cytochrome c fragments 81-104 to allow the cells to be stimulated by either antigen alone. These results support the conclusion that there is only one family of T-cell clones responding to both residues 100 and 104 and that, therefore, two complementing Ir genes control the proliferative response to a single antigenic determinant.

Discussion

The usefulness of the cytochrome c family of proteins in the study of the immune system has been amply demonstrated over the past 15 yr (1, 4-9). Because of the ability of this family to accurately delineate the nature of antigenic determinants, unusual antibody cross-reactions have been readily understood, and the single-site antibody populations that comprise the totality of the B-cell response have been isolated. In the present paper we demonstrate that this set of proteins can also provide

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a powerful tool for probing T-lymphocyte immune responses. Pigeon cytochrome c was chosen for this study because in B10.A mice it primed for a strong PETLES proliferative response to pigeon but not mouse cytochrome c. In addition, the response was analyzable by cross-stimulation with other cytochromes c that share in common with the pigeon protein one or more of the seven differences in amino-acid sequence from the mouse protein. These essential features were not found for other cytochromes c previously tested as immunogens (7).

Analysis of the B10.A PETLES proliferative response to pigeon cytochrome c revealed that three of the seven amino-acid residues that differ from mouse cytochrome c were involved in the antigenic stimulation of the T cells: the isoleucine at position 3, the glutamine at position 100, and the lysine at position 104. These three residues lie in close proximity to each other on the outer surface of the back face of the molecule (Fig. 3). This close spatial relationship suggests that all three residues comprise a single topographic determinant. However, because all of the cytochromes (or their peptide fragments) possessing only one of the three residues were capable of stimulating the T cell population (Figs. 2 and 4; Table I), it is formally possible that more than one determinant exists, each stimulating its own clone of T cells. On the other hand, it is clear from both the dose-response curves (Fig. 4) and the immunogenicity (Fig. 5) of pigeon fragment 81-104 that two residues were required for strong stimulation, namely, the glutamine and the lysine. Furthermore, mixing experiments with hippopotamus cytochrome c and chicken or duck cytochrome c fragments 81-104, each of which contains only one of the two immunodominant amino acids, did not reveal additive stimulatory effects (Table IV and Fig. 7). These results suggest that at least the T cells responding to the immunodominant portion of the proposed antigenic determinant comprise a single family of clones reacting equally well to the
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glutamine 100 and the lysine 104. Because the proliferative responses to both pigeon fragment 81-104 and the whole protein were shown to be under the control of two MHC-linked Ir genes (Fig. 6; Table III), the data suggest that two complementing Ir genes can control the proliferative response to a single antigenic determinant. It is clear, however, that a clonal analysis will be necessary to demonstrate unequivocally whether all three residues are recognized by the same receptor.

The conclusion that two Ir genes control the response to a single determinant is in disagreement with a model recently proposed by Keck (21) that suggests that each Ir gene controls the response to a separate antigenic determinant. Although it is possible that Keck's model will hold in some situations, the two gene-single determinant example of pigeon cytochrome c is clearly substantiated by results obtained in another two-gene system, namely, the immune response to poly (Glu<sup>53</sup>Ly<sup>3</sup>Ph<sup>9</sup>) [GL<sub>b</sub>] in this system, experiments with radiation chimeras and antigen-presentation by non-immune spleen cells have suggested that both Ir gene products must be expressed in the same antigen-presenting cell to generate a T-lymphocyte proliferative response to GL<sub>b</sub> (22). Even though it has not been possible to localize the determinants in GL<sub>b</sub> as has been done for pigeon cytochrome c, these results of the GL<sub>b</sub> experiments are inconsistent with a synergistic two-cell recognition model (21). Thus, most Ir gene complementation probably involves intracellular events such as the formation of hybrid molecules on the surface (22) or in the cytoplasm (23) of B cells and antigen-presenting cells.

The ability of tobacco hornworm moth cytochrome c to stimulate a significantly better proliferative response than pigeon cytochrome c from PETLES which were immune to pigeon cytochrome c (Fig. 2) provides the first clear-cut example of a functional heteroclitic T-cell proliferative response that is under the control of MHC-linked Ir genes. The heteroclicity was seen most dramatically in the 13-fold shift in concentration required to stimulate one-half the maximal response. This suggests that the receptors expressed by the B10.A T-cell clones have a higher affinity for the determinant if the terminal lysine is at position 103 rather than at 104. However, we cannot be certain of this as tobacco hornworm moth cytochrome c has numerous other differences in sequence from pigeon cytochrome c that might be influencing the antigenicity of the molecule. The one other published example of heteroclicity at the T-cell level is the (4-hydroxy-3-nitrophenyl)-acetyl and (4-hydroxy-4-iodo-3-nitropheryl)-acetyl hapten-binding system described by Mäkelä and co-workers (24). In this case, both the B-cell and the T-cell receptors (25) and responses (24, 26) show a similar heteroclitic nature. Whether the antibody response to pigeon cytochrome c will reveal the same phenomenon remains to be determined.

Although the observations discussed so far suggest that the major antigenic determinant is three dimensional in nature, one paradoxical result remains to be explained. Why is it that the cyanogen bromide-cleavage fragment 81-104 of pigeon cytochrome c stimulates better than the whole molecule? Studies on the structure of the 81-104 fragment (9, 27) have shown that only a small percentage of the molecules in aqueous solution are in an α-helical conformation. This, coupled with the loss of isoleucine 3, might be expected to decrease the stimulatory capacity of the fragment. However, knowing that tobacco hornworm moth cytochrome c stimulates better than whole pigeon cytochrome c, one could argue that the increased flexibility of the fragment more than compensates for the loss of the isoleucine by allowing the lysine to rotate...
into the proper orientation for optimal stimulation. In fact, many of the fragments stimulate better than the whole molecules they come from, such as fragments 1-65 and 81-104 of chicken cytochrome c (Table I). This has also been shown to be true for T-cell responses to other protein antigens (28-30), even when the fragments have been shown to contain little of the tertiary structure of the intact molecule (31). Similarly, chemically modified and/or denatured proteins will often prime for T-cell responses to the native protein, although antibodies against the two forms are highly discriminatory (31-34).

The biologic basis for this T-cell phenomenon is not clear. One possibility is that the whole protein molecules contain inhibitory amino-acid residues oriented in the proper direction such that they block binding to the T-cell receptors. In the fragments these might be missing or displaced. A second possibility is that suppressor T cells recognize other determinants on cytochrome c and consequently dampen the response to the whole molecule. On fragmentation of the molecule, such suppressor determinants could become separated from the stimulatory determinants, thus eliminating the suppression. Studies on β-galactosidase and lysozyme have demonstrated the existence of such suppressor determinants (30).

A third possibility is that the T-cell repertoire is highly restricted such that only a few clones of T cells have receptors that can bind pigeon cytochrome c at the concentrations used for immunization. The actual fine specificity of these receptors, however, might be such that they possess higher affinity for the relative spatial positions of the glutamine and lysine found in pigeon fragment 81-104. This might more than compensate for the absence of the isoleucine and allow the fragment to stimulate better than the intact protein. Finally, a fourth possibility is that fragmentation of the molecule is the normal mechanism by which antigen-presenting cells process antigen before priming T lymphocytes. In fact, several theories of Ir gene function are based on the possibility that T cells recognize antigen fragments (35, 36). However, if this idea were true, then one would predict in the present experiments that the T-cell clones stimulated by pigeon cytochrome c fragment 1-65 should be independent of the T-cell clones stimulated by pigeon fragment 81-104. The failure of pigeon fragment 1-65 to act as an immunogen as well as preliminary results with fragment mixing experiments suggest that this is not the case. On the other hand, if the T-cell repertoire is highly restricted, then fragment 81-104 might only be able to prime clones that can also recognize fragment 1-65. This possibility is currently being tested.

Summary

The T-lymphocyte proliferative response to pigeon cytochrome c was studied in the mouse. H-2a and H-2d strains were responders to this antigen whereas H-2b, H-2s, H-2f, H-2m, H-2p, H-2r, H-2z, H-2s, and H-2u strains were low or nonresponders. Genetic mapping demonstrated that two major histocompatibility complex (MHC)-linked Ir genes control the response, one in I-A, the other in I-E/I-C. The major antigenic determinant recognized in this response was localized by cross-stimulations with species variants and cyanogen bromide cleavage fragments of cytochrome c. It was found to be a topographic surface determinant composed of an isoleucine for valine substitution at residue 3, a glutamine for lysine substitution at residue 100 and a lysine for glutamic acid substitution at residue 104. Tobacco hornworm moth
cytochrome c, which contains a glutamine at residue 100 but a terminal lysine at residue 103 (one amino acid closer to the glutamine), stimulated pigeon cytochrome c immune T cells better than the immunogen. This result demonstrates for the first time a functional T-cell heteroclitic proliferative response in a system under Ir gene control.

Immunization with the cyanogen bromide cleavage fragments revealed that only pigeon cytochrome c fragment 81-104 was immunogenic. This fragment primed for a T-cell proliferative response whose specificity was nearly identical to that of the T-cell response primed for by the whole molecule, suggesting that the glutamine at 100 and the lysine at 104 form the immunodominant portion of the antigenic site. Furthermore, mixing experiments using the two cross-reacting antigens, hippopotamus cytochrome c and Pekin duck or chicken cytochrome c fragment (81-104), each of which contains only one of the two immunodominant substitutions, demonstrated that the T lymphocytes responding to the major antigenic determinant comprise a single family of clones that recognize both amino acids as part of the same determinant. Thus, two complementing MHC-linked Ir genes can control the immune response to a single antigenic determinant.

The authors would like to thank Richard Feldmann and Vincenzo Pavone for the many hours of hard work and intellectual input required to develop the computer model for the antigenic site on pigeon cytochrome c. In addition, we would like to thank Dr. William E. Paul for many helpful and stimulating discussions during the entire course of this work and Dr. Louis Matis for his generous contribution of his data on the pigeon cytochrome c response of PETLES from [B10.A(3R) × B10.A(4R)]F1 mice.

Received for publication 4 June 1979.

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