

CONTRIBUTION OF ANTIGEN-PRESENTING CELL
MAJOR HISTOCOMPATIBILITY COMPLEX GENE
PRODUCTS TO THE SPECIFICITY OF
ANTIGEN-INDUCED T CELL ACTIVATION*

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It is generally believed (1-3) that T lymphocytes recognize antigen in association with products of the major histocompatibility complex (MHC).¹ If we consider T cell responsiveness in terms of a recognition unit, it would be made up of three components: antigen, a T cell receptor (s), and a surface Ia molecule on an antigen-presenting cell (APC). In such a model, lack of responsiveness (*Ir* gene defects) could be explained by a defect of either antigen-Ia, antigen-receptor, or Ia-receptor interaction, or any combination of these. An understanding of these MHC-associated *Ir* gene defects as well as the mechanism of antigen-induced T cell activation requires the ability to examine the contribution of each component separately by holding the other two components constant. We encountered a unique opportunity to explore this issue using T cell clones specific for cytochrome *c*.

In investigating the T cell responses of B10.A and B10.A(5R) mice to a family of cytochrome *c* peptides, it was found that these two strains, differing only in the *K* region, and *I-A* and *I-B* subregions of the MHC, each had a characteristic pattern of responsiveness to a given set of cytochrome *c* cyanogen bromide cleavage fragments (4-6). When T cell clones were derived from either of these cytochrome *c*-immune animals and tested for antigen specificity, the response profiles of the clones closely resembled those of the whole lymph node population. Remarkably, however, the clones from either strain responded to antigen on both B10.A- and B10.A(5R)-presenting cells. Furthermore, the response pattern of either set of clones resembled that of the whole B10.A lymph node population when B10.A-presenting cells were

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¹ *Abbreviations used in this paper:* Am, acetimidyl; APC, antigen-presenting cell; Con A, concanavalin A; CnBr, cyanogen bromide; ClZ, 2-chlorobenzoyloxycarbonyl; FCS, fetal bovine serum; GLφ, poly(Glu⁵⁶Lys⁸⁶Phe⁹); HAT, hypoxanthine-aminopterin-thymidine; *Ia*, immune response associated; IL-2, interleukin 2; MHC, major histocompatibility complex; NWPLN, nylon wool-passed lymph node; PEG, polyethylene glycol; t-BOC, *t*-butyloxycarbonyl.

used but resembled that of the B10.A(5R) lymph node population when B10.A(5R)-presenting cells were used. These data suggest that the B10.A and B10.A(5R) strains share similar T cell repertoires for certain cytochromes *c* and that at least part of their fine specificity differences derive from differences in the interaction of the MHC gene products with antigen alone or antigen in conjunction with the T cell receptor.

Materials and Methods

Animals. B10.A/SgSn [kkkkkddd]² and B10.A(5R)/SgSn [bbbkkddd] mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.A(2R) [kkkkkddb] mice were obtained from Sprague-Dawley Laboratories, Madison, WI.

Antigens. Cytochrome *c* cyanogen bromide cleavage fragments and their acetimidyl derivatives, which convert each lysine into an analog of homoarginine, were prepared as previously described (4, 6) from the isolated, purified cytochrome *c* protein. Tobacco horn worm moth (moth) cytochrome *c* 81-103 was synthesized by the Merrifield solid-phase method (7) using a Beckman 990B peptide synthesizer (Beckman Instruments, Inc., Fullerton, CA). Chloromethylated polystyrene-1% divinylbenzene copolymer was used as the solid support. A *t*-butyloxycarbonyl (*t*-BOC)-Lys (ClZ) was attached to the solid support (0.397 mmol/g substitution level). The *t*-BOC group was used to protect the α -amino group of each amino acid except arginine, where the more soluble amyloxy derivative was used. The side-chain functional groups were protected as follows: (a) the carboxyl and hydroxyl groups were protected as the benzyl esters and ethers, respectively; (b) the guanidine function of arginine was protected by the toluene sulfonyl group; and (c) the ϵ -amino group of lysine was protected by the 2-chlorobenzoyloxycarbonyl group. Amino acids were attached to the peptide resin by coupling equimolar ratios of (4X) dicyclohexyl carbodiimide (DCC) and the *t*-BOC-amino acid for 120 min. The active ester method and a coupling time of 960 min was used to couple the *p*-nitrophenyl esters of asparagine and glutamine to the peptide chain. Double coupling was used at each step, and the reaction was monitored by the Kaiser ninhydrin test (8). The asparagine at 89 was difficult to couple, and three couplings were needed to get a satisfactory reaction. After the active ester coupling, the peptide-resin was treated with *n*-acetylimidazole to block any unreacted amino group (9).

The peptide was cleaved from the solid support with simultaneous removal of the side-chain protecting groups, using anhydrous hydrogen fluoride at 0°C for 30 min (G. W. Tregear, personal communication) in the presence of distilled anisole. Hydrogen fluoride and anisole were then removed under vacuum, and the residue was washed three times with anhydrous ether. The peptide was isolated from the resin mixture by extracting with 1 M acetic acid and was then lyophilized. The crude product was purified successively by gel filtration (G-25 Sephadex, Sigma Chemical Co., St. Louis, MO) in 7% (vol/vol) formic acid and ion-exchange chromatography (Bio-Rad Laboratories, Richmond, CA) (0 → 100 mM NaCl, 0.01 M PO₄, pH 6.0), and fractions were obtained that were homogeneous as judged by reverse-phase high pressure liquid chromatography (0 → 60% acetonitrile, C18- μ Bondapak, Waters Associates, Milford, MA). These fractions were analyzed for amino acid composition and sequence. During the synthesis, a side product was formed that contained the *o*-benzyl tyrosine at residue 97 (*q*-benzyl moth fragment 81-103), identified by mass spectroscopy of the appropriate sequenator fraction. Synthetic and biological peptides were found to be equivalent under our assay conditions and were used interchangeably. The benzyl derivative was found to be antigenically similar to the native molecule, although 10-fold reduced in activity. Its use as an immunogen was dictated by the need to conserve reagents. The intact proteins were not used in this study, and "fragment 81-103" or "fragment 81-104" is sometimes omitted from the text and the figures.

Preparation of T Lymphocytes and Performance of the Proliferation Assay. Animals were immunized with antigen in complete Freund's adjuvant in the hind footpads. Popliteal and inguinal lymph nodes were collected 7 d later, and lymphocytes were passed over nylon wool columns (10) to

² Letters indicate the haplotype source of origin of alleles of the K, I-A, I-B, I-J, I-E, I-C, S, and D regions and subregions of the murine MHC.

purify T cells. The proliferative T cell assay was performed as previously reported (11, 12). Briefly, 4×10^5 nylon wool-purified lymph node (NWPLN) cells plus 1×10^5 x-irradiated (2,000 rad) normal spleen cells were cultured in 0.2 ml of Eagle's Hank's Amino Acids (EHAA) (13) medium plus 10% fetal calf serum (FCS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) and varying amounts of antigen. Cultures were pulsed with $1 \mu\text{Ci}$ of [^3H]thymidine at 84 h and harvested at 96 h. All cultures were done in triplicate, and the arithmetic mean is reported.

T Cell Hybridization. Bulk T cell blasts were prepared by culturing 2×10^6 NWPLN cells and 10^6 x-irradiated spleen (3524, Costar, Data Packaging, Cambridge, MA) in 1.5 ml EHAA plus 10% FCS and antigen. After 3 d of culture, cells were harvested and mixed with the hypoxanthine-aminopterin-thymidine (HAT)-sensitive T cell line BW5147, an AKR thymoma obtained from the Salk Institute Cell Distribution Center, La Jolla, CA, in a ratio of 1:2 to 1:4. Fusion was carried out according to the method of Geffer (14), using 30% (vol/vol) polyethylene glycol 1000 (Baker Chemical Co., Phillipsburg, NJ) for 8 min. Then, 5×10^5 cells were plated out into 96-well Costar plates (3596) containing 5×10^3 x-irradiated (2,500 rad) peritoneal wash-out cells in 0.2 ml of fusion medium. The fusion medium was a DMEM-based medium containing 10% NCTC 109 (Microbiological Associates, Walkersville, MD), 15% FCS, HAT, and other additives, as described elsewhere (15). After 6 d, hybridomas were picked and transferred to 24-well Costar plates, expanded, and tested for antigen-specific growth factor production.

Cloning of T Cell Hybridomas. T cell hybrids were cloned by plating cells at three different concentrations: 1, 0.5, and 0.25 cells/well in the presence of 5×10^5 thymocytes in fusing medium without HAT. The number of wells positive for growth was determined, and clones were picked from the plates containing 0.25 cells/well. These clones were then tested for antigen specificity.

Antigen-specific Growth Factor Assay. In the primary culture, 5×10^4 to 5×10^5 cells from the hybridoma were added to 96-well Costar plates containing 0.2 ml fusing medium without HAT, 5×10^5 x-irradiated spleen cells, and varying concentrations of antigen. After 2 d of culture, supernatants were collected and assayed for growth factor activity in a secondary culture in one of two ways: either using 10^5 thymocytes plus $2 \mu\text{g/ml}$ of concanavalin A (Con A) (Difco Laboratories, Detroit, MI) (16) or 4×10^3 HT-2 cells, an interleukin 2 (IL-2)-addicted T cell line developed by Dr. James Watson, University of Auckland, Auckland, New Zealand and provided by Dr. Phillipa Marrack, National Jewish Hospital, Denver, CO, cultured in 0.2 ml of EHAA and 10% FCS with 25% primary culture supernatant. After 1–3 d, the degree of stimulation was measured by the incorporation of [^3H]thymidine into DNA. Available data indicates that the supernatant activity was the result of IL-2, and it will be so called in the text. The assays were done in duplicate, and the arithmetic means are reported. The cpm obtained in the HT-2 cell assay for IL-2 were shown to be sigmoidally related to the amount of IL-2-containing supernatant added to the secondary culture.

Results

B10.A(2R) mice were immunized with pigeon cytochrome *c* CnBr cleavage fragment 81-104, and a lymph node T cell proliferation assay was performed 7 d later. As shown in Fig. 1, these T cells responded well to pigeon fragment 81-104, less well to tuna fragment 81-103, but gave a heteroclitic response to both screw worm fly (fly) and moth fragments 81-103. We will refer to this as the A pattern. If one immunizes these mice with the moth fragment 81-103 instead of pigeon fragment 81-104, a similar pattern is observed (Fig. 2, upper panel).

When B10.A(5R) mice were immunized with pigeon fragment 81-104, they gave only a minimum response, i.e., they were low responders to this antigen (6). Immunization of B10.A(5R) mice with moth fragment 81-103 yielded a T cell response whose characteristic pattern is shown in Fig. 2, lower panel. B10.A(5R) T cells responded well to high concentrations of both moth and fly fragments 81-103 but did

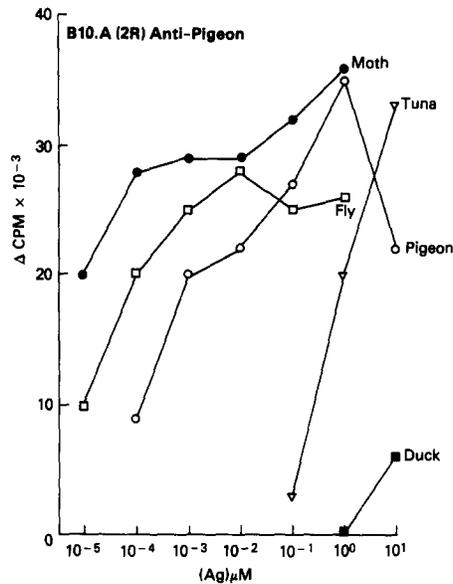


FIG. 1. The response pattern of B10.A(2R) lymph node T cells to various cytochrome *c* peptides after immunization with pigeon cytochrome *c* fragment 81-104. B10.A(2R) mice were immunized with pigeon cytochrome *c* fragment 81-104 (5 $\mu\text{g}/\text{animal}$), and NWPLN cells were tested for their proliferative response in vitro to the following cytochrome *c* fragments: pigeon 81-104 (○); fly 81-103 (□); moth 81-103 (●); tuna 81-103 (▽); and duck 81-104 (■). Medium control, 4,200 cpm.

not respond to either pigeon or tuna fragments, except occasionally at very high concentrations. We will refer to this as the 5R pattern.

We next examined the response of T cell clones obtained from both of these immune T cell populations. Using a slight modification of a technique published by Harwell et al. and Kappler et al. (17, 18), T cell hybridomas were made by fusing BW5147 directly to in vitro restimulated nylon wool column-passed lymph node T cells. The fusion products were then tested for IL-2 production upon stimulation with x-irradiated spleen cells plus antigen. The presence of IL-2 was detected in a thymocyte assay using 2.0 $\mu\text{g}/\text{ml}$ of Con A for stimulation. In Fig. 3, upper panel, the responses to the different cytochrome *c* fragments by a hybridoma from pigeon fragment 81-104-primed B10.A animals is shown. When syngeneic B10.A x-irradiated spleen cells were used as an APC source, the response closely resembled the pattern seen in the whole B10.A lymph node population. Thus, fly and moth fragments 81-103 were both heteroclitic stimulators, and tuna fragment 81-103 gave only a partial cross-reaction. This similarity in responsiveness between the whole lymph node population and the hybridoma indicated that the hybridoma was a representative sample of the B10.A T cell repertoire for pigeon cytochrome *c* fragment 81-104.

To examine the MHC restriction of the B10.A clones, we stimulated the cells with antigen in the presence of B10.A(5R) x-irradiated spleen cells (Fig. 3, lower panel). Although pigeon cytochrome *c* fragment 81-104 plus B10.A(5R) cells did not stimulate, we did observe an antigen-specific response using moth and fly fragments 81-103. Surprisingly, the response profile was that of the 5R pattern, not that of the A pattern. Three out of three B10.A hybridomas tested showed similar responses.

Considering the view that T cells recognize and are restricted to a particular MHC

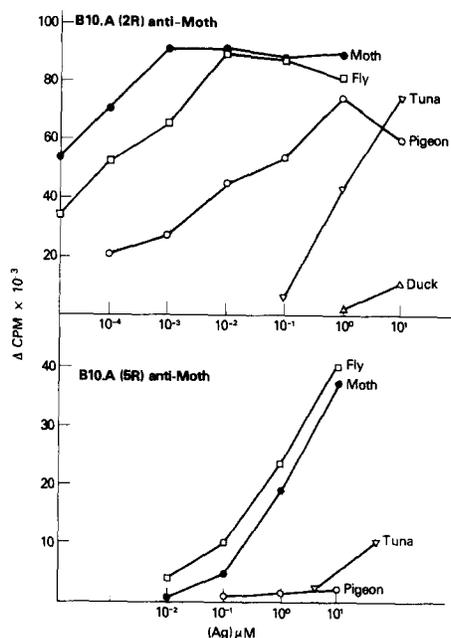


FIG. 2. The response pattern of lymph node T cells to various cytochrome *c* peptides after immunization with moth fragment 81-103. B10.A(2R) mice (upper figure) were immunized with 0.5 μ g of moth fragment 81-103 per animal, and B10.A(5R) mice (lower figure) were immunized with 50 μ g of moth fragment 81-103 per animal. Lymph nodes were removed, and NWPLN cells were tested for their *in vitro* proliferative response to the following cytochrome *c* fragments: moth 81-103 (●); fly 81-103 (□); pigeon 81-104 (○); tuna 81-103 (▽); and duck 81-104 (△). Media controls: B10.A(2R), 11,400 cpm; B10.A(5R), 330 cpm. Biological moth fragment 81-103 was used in the B10.A(5R) assay.

determinant, these findings were quite unexpected. It is generally believed, based on a large body of data using radiation-induced bone marrow chimeras and thymic chimeras (19), that T cells acquire their MHC restriction specificity in the thymus and that this event in turn affects their antigen specificity. The clones studied here appeared to be restricted in the conventional sense in that only B10.A APC could present the immunizing antigen, pigeon fragment 81-104. However, they were unrestricted in the sense that both B10.A and B10.A(5R) spleen cells could present moth and fly fragments 81-103.

Several mechanisms can be envisioned to describe this antigen-specific breakdown in MHC restriction. First, the T cell receptor(s) might only recognize an interaction between antigen and the presenting cell Ia; e.g., an altered self type antigen-Ia interaction. In this case the B10.A Ia might form relevant complexes with both pigeon and insect cytochromes *c*, whereas the B10.A(5R) Ia might only form complexes with the insect cytochromes *c*. Second, a single T cell receptor with two separate combining sites might recognize both antigen and presenting cell Ia in a ternary complex. In this case, B10.A Ia would fit into the receptor with either pigeon or insect cytochromes *c*, whereas B10.A(5R) Ia would only fit with the insect cytochromes *c*. Finally, in a model in which there is independent recognition of Ia and antigen, such as a dual receptor model, the reduced affinity of the anti-self receptor for the allogeneic 5R Ia

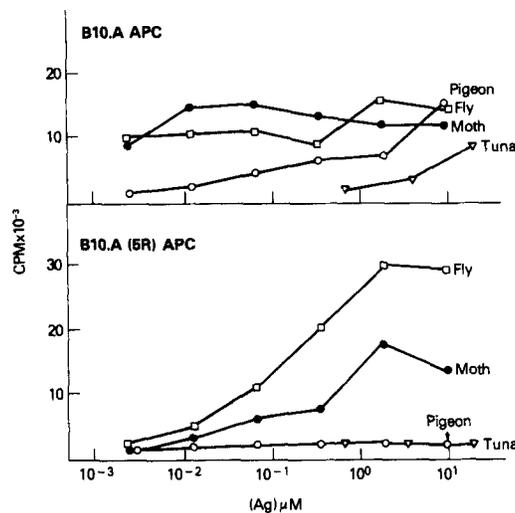


FIG. 3. The effect of different APC on the cytochrome *c* response of a B10.A T cell hybridoma. B10.A mice were immunized with 5 μ g of pigeon cytochrome *c* fragment 81-104, and NWPLN cells were stimulated in vitro with pigeon fragment 81-104, fused to BW5147, and selected for antigen-specific IL-2 production. The results seen in this figure represent a secondary assay that measures ability of supernatants (25% vol/vol) from primary cultures containing 5×10^4 hybridoma cells, 2.5×10^5 x-irradiated spleen, and varying concentrations of different cytochrome *c* fragments to stimulate the proliferation of thymocytes (10^6 cells/well) in the presence of 2 μ g/ml Con A. The results are expressed in cpm of [3 H]thymidine incorporation. The primary cultures were stimulated with the following cytochrome *c* fragments: pigeon 81-104 (○), fly 81-103 (□), moth 81-103 (●), and tuna 81-103 (▽), in the presence of B10.A x-irradiated spleen cells (upper panel) or B10.A(5R) x-irradiated spleen cells (lower panel) as APC.

might be compensated for by the very high affinity of the antigen-specific receptor for the insect cytochromes *c*.

To explore these various interpretations and to confirm the observations by doing a reciprocal experiment, hybridomas were made from B10.A(5R) mice primed to a synthetic analogue of moth fragment 81-103. The use of syngeneic x-irradiated B10.A(5R) spleen cells as the presenting cell population for this hybrid (Fig. 4, lower panel) resulted in a response pattern very similar to the B10.A(5R) whole lymph node population. Fly cytochrome *c* fragment 81-103 stimulated slightly better than moth fragment 81-103, and pigeon and tuna fragments did not stimulate at all. However, when the B10.A(5R) hybridoma was tested with B10.A x-irradiated spleen cells, it displayed a B10.A pattern of responsiveness, i.e., it could now respond to pigeon fragment 81-104 and tuna fragment 81-103. Here we have a case in which the B10.A(5R) T cells respond better to the moth cytochrome *c* fragment 81-103 with B10.A-presenting cells, even though the T cells were selected to see B10.A(5R) Ia molecules. Furthermore, with the B10.A spleen cells, the clones respond to pigeon cytochrome *c* fragment 81-104, an antigen they cannot recognize in association with their own B10.A(5R) spleen cells.

It should be pointed out that even though both the B10.A and the B10.A(5R) hybridomas could respond to antigen with either B10.A or B10.A(5R) APC, they were otherwise Ia restricted. As shown in Table I, both hybridomas could not respond to any of the cytochrome *c* peptides in association with B10.A(4R), B10, or B10.S(9R)

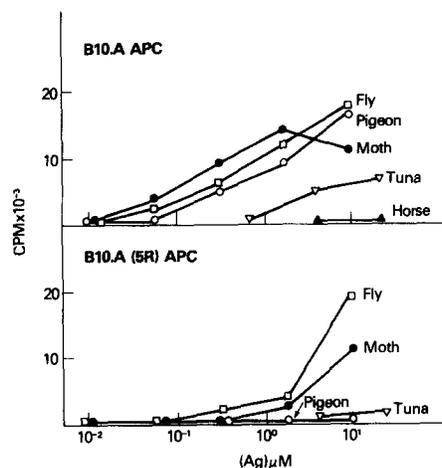


FIG. 4. The effect of different APC on the cytochrome *c* response of a B10.A(5R) T cell hybridoma. B10.A(5R) mice were immunized with 50 μg per animal of *g*-benzyl moth fragment 81-103, and hybridomas were made and tested as in Fig. 3. The primary cultures were stimulated with the following cytochrome *c* fragments: pigeon 81-104 (○); fly 81-103 (□); moth 81-103 (●); tuna 81-103 (▽); and horse 81-104 (▲). In the upper panel, B10.A-irradiated spleen cells, and in the lower panel, B10.A(5R)-irradiated spleen cells, were used to furnish APC.

TABLE I
T Cell Hybridomas Do Show MHC Restriction

Presenting cell§	B10.A hybridoma*			B10.A(5R) hybridoma		
	None	Pigeon‡	Moth	None	Pigeon	Moth
B10.A (kkkd)	0.09	6.5	8.4	0.11	5.3	5.7
B10.A(5R) (bbkd)	0.49	0.10	2.3	0.32	0.30	3.4
B10.A(4R) (kkbb)	0.05	0.07	0.09	0.07	0.05	0.08
B10 (bbbb)	0.18	0.12	0.51	0.20	0.43	0.40
B10.S(9R) (sskd)	0.13	0.16	0.18	0.09	0.12	0.10

* 5×10^4 hybridoma cells per well.

‡ The final concentration of the cytochrome *c* fragments used was 8 μM .

§ 2.5×10^6 spleen cells per well.

|| Letters indicate the haplotype source of the K, I-A, I-E, and D subregion alleles of the murine MHC.

¶ Supernatants from the primary culture were assayed for support of thymocyte proliferation in the presence of Con A as a measure of IL-2 content. The data are expressed as $\text{cpm} \times 10^{-3}$ of [^3H]thymidine incorporated during a 12-h pulse 72 h after the start of the assay.

spleen cells. The failure of the B10.A(4R) and B10 cells to present antigen suggests that the $A_\epsilon:E_\alpha$ Ia molecule, which these cells are lacking, is required for activation. The actual involvement of this Ia molecule in stimulation was demonstrated by completely blocking IL-2 release from the T cell hybrids with the monoclonal antibody, 17.3.3., directed against the Ia.22 determinant on the $A_\epsilon^{k/b}:E_\alpha^k$ molecule (data not shown). The failure of the B10.S(9R) spleen cells to present pigeon and moth cytochrome *c* C-terminal CnBr fragments (Table I) unequivocally shows that the T cell hybridomas are MHC restricted. This strain expresses an $A_\epsilon:E_\alpha$ molecule as well as being a responder to pigeon cytochrome *c*, yet the T cell hybrids could not respond to the antigen when A_ϵ^s had replaced $A_\epsilon^{k/b}$. Thus, the B10.A(5R)-B10.A degeneracy in MHC restriction observed for these hybridomas is highly selective.

Although these results strongly suggest the idea of an antigen-Ia interaction, it is possible to maintain an independent recognition model by explaining the change in the response pattern of the B10.A(5R) T cell clone, with an assumption of an unusual T cell anti-self receptor that can recognize B10.A MHC determinants better than B10.A(5R) determinants. An antigen-Ia interaction can only be conclusively demonstrated when changes in the APC result in a change in the order of relative strengths of the different cross-reacting antigens.

To determine whether this occurred, we used the observation that this B10.A(5R) clone responded not only to native cytochrome *c* fragments but also to the acetimidyl (am) derivatives of these peptides. The data in Fig. 5 demonstrate that the order of antigenic reactivity can be changed solely by changing the presenting cell MHC. Looking at the response to the amidinated peptides first, one can see that the B10.A(5R) clone responded with syngeneic B10.A(5R)-presenting cells to only the am-fly fragment 81-103. With the B10.A-presenting cell not only was there now a response to am-moth fragment 81-103 and am-pigeon fragment 81-104, but these responses were better than that to am-fly fragment 81-103, i.e., the order of the antigenic reactivity was reversed. If one looks at the response to both sets of antigens

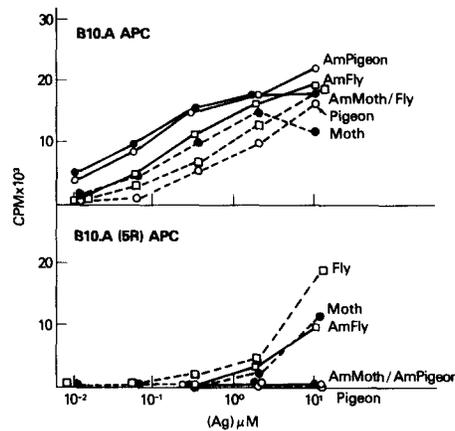


FIG. 5. The effect of different APC on the response of a B10.A(5R) T cell hybridoma to native and acetimidyl cytochromes *c*. The response of the B10.A(5R) hybridoma to pigeon (○---○), moth (●---●), and fly (□---□) fragments are reproduced from Fig. 4. The responses to their acetimidyl derivatives, am-pigeon (○—○), am-moth (●—●), and am-fly (□—□) fragments, were simultaneously assayed and are also shown. The upper panel shows the responses in the presence of B10.A-irradiated spleen cells; the lower panel shows the responses in the presence of B10.A(5R)-irradiated spleen cells.

TABLE II
Rank Order of Antigen Strength for Stimulating the B10.A(5R) Moth Cytochrome *c*-specific T Cell Hybridoma

	B10.A APC	B10.A(5R) APC
Strongest	Am-pigeon, am-moth Am-fly Moth, fly, pigeon Tuna	Fly Am-fly, moth
None	Horse	Am-pigeon, pigeon, am-moth, tuna

TABLE III
*Progeny Test of B10.A(5R) Hybridoma**

Subclone	B10.A APC‡				B10.A(5R) APC			
	None	Am-pigeon§	Fly	Pigeon	None	Am-pigeon	Fly	Pigeon
1	0.8¶	15.3	5.7	19.2	0.5	0.5	2.8	0.4
2	0.3	50.5	24.5	47.0	0.2	0.3	18.7	0.3
3	0.09	24.8	6.7	19.3	0.1	0.1	3.7	0.1
4	0.1	10.0	0.3	1.4	0.2	0.3	2.9	0.1
5	0.3	110.0	36.9	81.1	0.3	0.3	12.5	0.3
6	0.1	61.7	11.8	43.5	0.1	0.1	3.4	0.2
7	0.07	16.7	1.9	7.9	0.1	0.2	8.0	0.2
8	0.3	27.2	7.6	15.1	0.2	0.1	10.0	0.4
9	0.1	59.2	13.4	32.0	0.2	0.3	22.1	0.6
10	0.2	19.7	3.3	19.5	0.1	0.2	14.3	0.1
11	0.2	20.3	2.6	21.4	0.4	0.6	13.4	0.3

* The B10.A(5R) anti-*q*-benzyl moth fragment 81-103 hybridoma was subcloned at 0.25 cells per well. 11/96 wells were positive for growth.

‡ 2.5×10^5 spleen cells per well.

§ Final concentrations used were 8 μ M for pigeon and am-pigeon fragment 81-104 and 4 μ M for fly fragment 81-103.

|| 5×10^4 cells per well.

¶ Supernatants from the primary culture were assayed for support of HT-2 cell growth as a measure of IL-2 content. The data are expressed as cpm $\times 10^{-3}$ of [3 H]thymidine incorporated during a 12-h pulse 36 h after the start of the assay.

together, native and acetimidyl, the many differences in the overall pattern using the two different APC can be clearly seen (Fig. 5). Table II summarizes the differences in the antigen hierarchies. Note, for example that am-pigeon fragment 81-104, which is one of the strongest antigens with B10.A APC, gave no response with B10.A(5R). Conversely, fly fragment 81-103, which is the strongest stimulator with B10.A(5R) APC, was 25-fold less effective than am-pigeon fragment 81-103 and am-moth fragment 81-103 when used with B10.A APC. Thus, by changing the presenting cell, a change in the apparent antigen specificity of this T cell clone has resulted. This implies that the T cell receptor can accommodate a variety of antigen-Ia configurations but that these must be considered as pairs, i.e., the T cell specificity is affected by Ia-antigen interactions.

These conclusions are based on the assumption that the response of the B10.A(5R) hybridoma is the response of a single clone. To demonstrate this unequivocally, subcloning was carried out by limiting dilution. 96 wells were plated at densities of 1, 0.5, and 0.25 cells/well, and the number of wells showing positive growth were 44, 16, and 11, respectively. All 11 subclones that grew out at 0.25 cells/well were tested for their antigen reactivity in the presence of either B10.A or B10.A(5R) irradiated spleen cells. As shown in Table III, the fine specificity of all these subclones was similar to the original hybridoma. Thus, although the amount of IL-2 produced by each subclone varied enormously, in all cases syngeneic B10.A(5R) APC could only present fly fragment 81-103, whereas allogeneic B10.A APC could present pigeon and am-pigeon fragments 81-104 in addition to fly fragment 81-103. We conclude that the lack of MHC restriction and the change in fine specificity with a change in APC are

the properties of a single T cell and, therefore, that the interaction of Ia and antigen must be responsible for the observed change in specificity of T cell activation.

Discussion

In the present work, we found, in the B10.A and B10.A(5R) strains, similar sets of T cells specific for the pigeon and tobacco hornworm moth cytochrome *c* C-terminal CnBr fragments, as determined by the study of T cell hybridomas from these animals. First, all the clones examined from both strains closely resembled in their response to various cytochromes *c* the whole lymph node population from which they derived. Thus, three out of three B10.A clones from animals immunized to pigeon cytochrome *c* fragment 81-104 responded best to moth and fly fragments 81-103, less well to pigeon fragment 81-104, and least well to tuna fragment 81-103 when syngeneic B10.A APC were used. The one B10.A(5R) clone from animals immunized with the synthetic *o*-benzyl tyrosine derivative of moth fragment 81-103 responded to fly and moth fragments 81-103 but not to pigeon or tuna fragments when syngeneic B10.A(5R)-presenting cells were used. Thus, it appears that these clones are a representative sample of the whole population. From this observation, we feel it is very unlikely that significant artifacts are introduced by the use of the BW5147 fusion technique to obtain cloned antigen-specific T cells. We may conclude, then, that this population of T cells is relatively homogenous because the pattern of cross-reactivity to various cytochrome *c* fragments showed little heterogeneity not only in repeat assays of animals of the same strain but also when examined at a clonal level.

Second, in testing the MHC restriction of the clones, all clones from both strains and six out of six (A × B)_{F1} clones (E. Heber-Katz et al., manuscript in preparation) responded to moth and fly cytochrome *c* fragments 81-103 on both the B10.A- and B10.A(5R)-presenting cells. This is, to our knowledge, the first report of a T cell response to a non-MHC antigen (cytochrome *c*), where the predominant clonotype is competent to recognize the same antigen in association with not only self-MHC but also an allo-MHC. In previous reports of T cells competent to recognize antigen in association with allo-MHC, the T cells' competence with self-MHC either could not be ascertained (20) or was absent (21–25).

It was very surprising that the B10.A(5R) clone responded better to moth fragment 81-103 with the B10.A Ia molecule than with its own Ia molecule; that is, the B10.A(5R) clone appeared to be heteroclitic for the B10.A restriction element. This is not a general finding for all B10.A(5R) clones because PPD and GL ϕ -specific B10.A(5R) clones cannot be stimulated with antigen in the presence of B10.A spleen cells (26). It is also not related to the responder status of the donor of the presenting cells because B10.S(9R) (sskkddd) spleen cells would not present any of the cytochrome *c* fragments to the B10.A(5R) clone (see Table I), even though the B10.S(9R) is a high responder to both moth and pigeon cytochrome *c*.³ Thus, the Ia heteroclicity appears to be a specific degeneracy associated with the receptor(s) on moth cytochrome *c*-reactive B10.A(5R) and B10.A clones. This observation argues strongly in favor of determinant selection or a one-receptor T cell model, unless there exist multiple anti-

³Matis, L. A., S. A. Hedrick, C. Hannum, M. E. Ultee, D. Lebwohl, E. Margoliash, A. M. Solinger, E. A. Lerner, and R. H. Schwartz. The T lymphocyte response to cytochrome *c*. III. Relationship of the fine specificity of antigen recognition to major histocompatibility complex phenotype. Manuscript in preparation.

self receptors and a genomic or somatic association between the anti-moth cytochrome *c* receptor and the heteroclitic anti-self receptor.

There is the possibility that the fusing partner for the hybridomas, BW5147, which is of AKR origin, contributes an anti-Ia^k-specific self receptor. This might explain how the B10.A(5R) hybridoma could see antigen plus B10.A APC, but it cannot explain how the B10.A hybridomas could see antigen plus B10.A(5R) APC. Furthermore, at the whole T cell population level, where no extra receptors could possibly be introduced, we found that the majority, if not all, of the T lymphocytes respond as the hybridomas; that is, normal T cells from B10.A or B10.A(5R) respond to moth cytochrome *c* fragment 81-103 on either B10.A or B10.A(5R) APC (E. Heber-Katz, D. Hansburg, and R. Schwartz, manuscript in preparation).

The third point made from the data is that the apparent fine specificity of the cytochrome *c* response was dependent on the MHC haplotype of the presenting cell used. When we examined the response of the B10.A(5R) clone to six different cytochromes *c*, derivatized and native, we found a clear difference in the fine specificity of the response when using the B10.A or B10.A(5R) APC, as determined by the change in the response profile. These results strongly indicate that an interaction between the MHC-encoded Ia molecule and the antigen occurs during T cell activation.

In the entire lymph node population, B10.A is a high responder to pigeon fragment 81-104, whereas B10.A(5R) is a low responder at very high immunizing doses. Surprisingly, the B10.A(5R) clone we isolated could respond to pigeon fragment 81-104 when tested with B10.A-presenting cells. This result could not be accounted for by postulating a haplotype-specific suppressor T cell in the APC population, as experiments using anti-theta and anti-Lyt-1.2 plus complement-treated spleen cells gave identical results (E. Heber-Katz, unpublished observations). This raises the question of what role, if any, the MHC has in the development of the T cell repertoire for cytochrome *c* in these two strains. The striking similarity in the patterns seen with the clones from either the B10.A(5R) or B10.A suggests that the major effects of the MHC genes on specificity might be determined by the APC during antigen contact and not through thymic selection. However, subtle differences in specificity, such as the reduced heteroclicity for the insect cytochromes *c* over pigeon cytochrome *c*, shown by the B10.A(5R) clone with B10.A APC, might be the result of thymic selection. Alternatively, this difference might be secondary merely to the large (100×) differences in antigen dose used to prime the B10.A and B10.A(5R) animals. Examination of more B10.A(5R) clones as well as chimera experiments are now in progress to investigate this question.

The concept of specificity has had a particular meaning in terms of the interaction of antibody and antigen, and this understanding has been extrapolated to the interaction of B cell receptors and antigen. The same concepts have been used for the T cell, even though the relevant experiments do not detect antigen binding, but only cell activation, which is presumed secondary to binding. In light of the present results, one now has to consider that there is, in addition to T cell receptor interactions, an interaction between presenting cell Ia molecules and antigen. Thus, T cell specificity must be a description of the way all three components, the T cell receptor, the antigen, and the Ia molecule, interact.

The requirement for Ia antigen interaction during T cell recognition can be

visualized in a number of different ways. In the determinant selection model (27-30), Ia molecules are postulated to possess binding sites for antigen. Different Ia molecules have different binding specificities. As a consequence of this binding, only certain epitopes on the antigen remain to be presented to the T cell. These will vary for the same antigen, depending on which Ia molecule is involved. In this model, the T cell receptor may bind only to the antigen or it may also recognize the Ia molecule. This could be accomplished with either one or two receptor molecules. The unique feature of this model is that Ia and antigen interact independent of the T cell receptor.

The alternative point of view would postulate that Ia and antigen only interact when they both bind to the T cell receptor. This is most easily visualized in a single combining site model in which the receptor has separate contact regions for antigen and Ia. In the simplest version of this model, the Ia and antigen would be brought into close proximity when the ternary complex of receptor, Ia, and antigen is formed (31, 32). Thus, direct physical interaction between Ia and antigen could either stabilize or disrupt the ternary complex. In a more complicated version of this model involving two separate combining sites, it is possible to imagine an indirect interaction between Ia and antigen via the receptor that would obviate the need for physical contact between the two moieties. When Ia binds to the T cell receptor, it could induce an allosteric change in the receptor that in turn would affect the affinity of the receptor for antigen. Different Ia molecules would be postulated to have different allosteric effects and thus to have different influences on antigen specificity.

In all of these models, the T cell receptor still contributes a substantial component to the antigen specificity of the binding. It is this component that imparts the clonal nature to the response. However, what the data in this paper have shown is that the T cell receptor is not the only component that influences specificity. Using T cell clones, we demonstrated for the first time that the Ia molecule also contributes to the specificity of an antigen-induced T cell response.

Summary

Previous studies from our laboratory showed that B10.A mice are high responders to pigeon cytochrome *c* fragment 81-104, whereas B10.A(5R) mice are low responders. In the present studies, the C-terminal cyanogen bromide cleavage fragment and homologous synthetic peptides of tobacco horn worm moth cytochrome *c* were shown to be immunogenic in both B10.A and B10.A(5R) mice. These strains, however, showed different patterns of cross-reactivity when immune lymph node T cells were stimulated with cytochrome *c* fragments from other species. To examine the two patterns of responsiveness at a clonal level, cytochrome *c* fragment-specific T cell hybridomas were made and found to secrete interleukin 2 in response to antigen. The patterns of cross-reactivity of these B10.A and B10.A(5R) clones were similar to that seen in the whole lymph node population. Surprisingly, when these clones were tested for major histocompatibility complex (MHC)-restricted antigen recognition, they were all found to respond to antigen with both B10.A and B10.A(5R) antigen-presenting cells (APC). Furthermore, the cross-reactivity pattern appeared to be largely determined by the genotype of the APC, not the genotype of the T cell clone. That is, a given T cell clone displayed a different fine specificity when assayed with B10.A or B10.A(5R) APC. This observation indicates that the APC MHC gene product and antigen interact during the stimulation of the T cell response and that

as a consequence the specificity of antigen-induced T cell activation is influenced by these MHC gene products.

(During the preparation of this manuscript it has come to our attention that results similar to our own, concerning the fine specificity of cytotoxic T cell clones, have been obtained by Dr. T. R. Hünig and Dr. M. J. Bevan, Massachusetts Institute of Technology, Boston, MA. T. R. Hünig and M. J. Bevan. 1981. Specificity of T-cell clones illustrates altered self hypothesis. *Nature*. **294**:460.)

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