MAJOR HISTOCOMPATIBILITY COMPLEX-CONTROLLED, ANTIGEN-PRESENTING CELL-EXPRESSED SPECIFICITY OF T CELL ANTIGEN RECOGNITION

Identification of a Site of Interaction and its Relationship to *Ir* Genes

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Ia region gene products have long been postulated to be antigen-binding (recognition) molecules (1); in particular, one set of hypotheses suggests that they are participants along with a T cell product in the antigen-binding complex (2, 3). It has recently been observed that certain T cell clones will react to antigen in association with two different major histocompatibility complex (MHC)[1] haplotypes, but in doing so these clones display different antigen specificities (4, 5). Such results have been interpreted as support for the above hypothesis. In the present work we have subjected the postulate to a further test by attempting to identify the site on a peptide antigen that interacts with the Ia molecule (i.e. controls the specificity changes that accompany the changes of MHC type) and the presumably different site that interacts with the T cell (i.e. contributes to immune memory).

In our initial studies, the nominal antigens were peptides consisting of residues 81–104 or 81–103 of the cytochrome *c* from pigeon or moth, respectively. B10.A mice immunized with low doses of either the pigeon 81–104 or moth 81–103 responded to both moth and pigeon fragments. However, B10.A(5R) mice immunized with low doses of pigeon fragment showed no response at all. When B10.A(5R) mice were immunized with large doses of moth fragment a strong response was seen, but this immune response showed no cross-reactivity with the pigeon peptide. IL2-secreting T cell hybridomas from a moth-primed B10.A(5R) or a pigeon-primed B10.A mouse were prepared and tested with antigen-presenting cells (APC) of these two strains. It was found that the T cell hybridomas from either strain responded to antigen associated with either B10.A or B10.A(5R) APC but to no others (5). Surprisingly, the specificity pattern was determined not by the MHC genes of the T cell hybridoma but by those of the

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[1] *Abbreviations used in this paper:* APC, antigen-presenting cells; EHAA, Eagle's Hanks amino acids medium; HAT, medium containing hypoxanthine, aminopterin, and thymidine; MHC, major histocompatibility complex; PBS, phosphate-buffered saline without Ca^{++} or Mg^{++}; PETLES, peritoneal exudate T lymphocytes; PPD, purified protein derivative.

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APC. Subsequently, Heber-Katz et al. (6) have extended these observations by showing that peritoneal exudate T lymphocytes (PETLES) from pigeon-primed B10.A and moth-primed B10.A(5R) mice behave in a manner indistinguishable from the T cell hybridomas when antigen responses with different APC were tested.

These observations provide the means for identifying two different functional sites on the pigeon cytochrome *c* fragment. They can be used to locate the site wherein moth and pigeon differ structurally, which prevents the pigeon fragment from being presented to these T cells by B10.A(5R) APC. There is a second site, functionally defined by T cell memory and shared by the moth and pigeon fragments, to which this population of T cells has been primed. In the present work synthetic peptides were prepared and tested in order to identify these two sites.

The difference between the moth and pigeon peptides that controlled APC functions was found to be at residue 103. At this position the pigeon sequence contains alanine, which is deleted from the moth peptide. The site of T cell memory was also identified. By introducing changes in the lysines at 99 and 103 and at 99 alone, two additional pairs of antigen peptides were made, each pair consisting of otherwise homologous sequences with and without alanine-103. The immune response pattern of the B10.A(5R) was duplicated for these new antigens; the B10.A(5R) was a low responder to the alanine-103 containing peptides, but a high responder to the des-Ala^{103} peptides. When B10.A(5R) mice were primed with the des-Ala^{103} peptides, the two new antigens did not cross-react with des-Ala^{103}-pigeon 81–104 or with each other. The mothlike (des-Ala^{103}) and pigeonlike forms of these two new antigens were found to behave just as the moth and pigeon fragments themselves behaved when tested for their ability to be presented by either B10.A or B10.A(5R) APC. Thus, both forms were presented by the B10.A APC but only mothlike, des-Ala^{103} peptides were presented to the B10.A(5R) T cells by syngeneic APC. When these experiments were extended by testing T lymphocyte hybridoma clones similar behavior of the Ala^{103} deletion was observed. However, additional residues also appeared to be able to affect the APC's function.

A prominent and interesting feature of our initial studies was that the T cell populations from the B10.A and B10.A(5R) primed to pigeon 81–104 and moth 81–103, respectively appeared to be equivalent. We naturally wished to see whether this equivalence extended to the new antigens. For the new antigen in which lysine-99 was replaced by glutamine, the B10.A and B10.A(5R) repertoires were found not to be symmetrical. Rather the B10.A(5R) APC presented both the Ala^{103}-Gln^{99} and des-Ala^{103}-Gln^{99} antigens to B10.A T cells primed with the Ala^{103}-Gln^{99}. Since the B10.A(5R) was a low responder to the Ala^{103}-Gln^{99} antigen, these data indicate that while Ir differences to some antigens (e.g., pigeon and moth) are expressed solely in the APC, this is not invariably the case.

Materials and Methods

Animals. C57BL10/Sn, B10.A/SgSn, B10.A(4R)/SgSn, and B10.A(5R)/SgSn mice were obtained through contracts provided by the National Institutes of Health, Small Animal Branch, Frederick Cancer Research Center, Frederick, MD. B10.S(9R)/SgSn

were a gift from Dr. C. David, Mayo Foundation, Rochester, MN.

Antigens. Preparation of antigens has been described elsewhere (7). Briefly, the carboxyl terminal cyanogen bromide fragment from pigeon (*Columbia livia*) was prepared from the isolated protein, kindly provided by Dr. E. Margoliash, Northwestern University, Evanston, IL. The other peptides were synthesized by the method of Merrifield as described previously (5) except that the tyrosine hydroxyls were protected as 2,6-dichlorobenzyl ethers and the amide functions were protected as the 9-xanthenyl amido ester (8). Peptides having 16 or fewer residues could be purified from the hydrogen fluoride-treated solid-phase reaction mixture by a single gel-filtration chromatography step (Sephadex G-25, fine, 2.5 × 100 cm) in 5% (vol/vol) acetic acid. The larger peptides were further purified on CM-BioGel A as previously described. The acetimidyl derivatives were prepared by reaction of the peptide with methyl acetimidate HCl (Aldrich Chemical Co., Milwaukee, WI). The conditions used allowed reaction only with the primary amino groups, thereby converting each lysine into an analogue of homoarginine.

Preparation and Culture of Peritoneal Exudate T Lymphocyte-enriched Cells (PETLES). PETLES were prepared as previously described (9). Animals were immunized in the hind foot pad with antigen and complete Freund's adjuvant followed 10 d later by an injection of 1.0 ml 10% (wt/vol) Brewer's thioglycolate medium intraperitoneally. After 5 d, exudate cells were removed from the peritoneum and passed over nylon wool. 5×10^4 nylon wool passed lymphocytes were cultured in 0.2 ml Eagle's Hanks amino acids medium (EHAA) containing 10% fetal bovine serum and varying numbers of antigen-pulsed presenting cells. After 90 h of culture 1 μCi of [^{3}H]thymidine was added to each culture and 6 h later the cultures were harvested using a MASH II (MA Bioproducts, Walkersville, MD).

Preparation of Antigen-presenting and an Antigen-pulsing Procedure. According to the method of DeFranco (10) spleens were teased, washed twice with phosphate-buffered saline without Ca^{++} or Mg^{++}(PBS) (MA Bioproducts) and then 5×10^7 cells were layered in 3 ml of PBS above 3 ml of a solution (Percoll, 10X PBS, PBS-9:1:10 [vol/vol]) and then centrifuged for 12 min at 4°C at 2,000 *g*. The cells that banded at the PBS/Percoll interface were greatly enriched for antigen-presenting activity (L. Glimcher, unpublished observation). These were washed three times with PBS, and cultured with antigen 15 h at 37°C in complete medium, washed six times and then irradiated (2,000 R).

Preparation of T Cell Hybridomas and Assessment of Antigen-specific Stimulation. T cell hybridomas were made as described previously (5, 11, 12). Briefly, lymph node T cell cultures stimulated with antigen for 3 d were fused with BW5147 and selected by growth in hypoxanthine, aminopterin, thymidine (HAT) medium. Fusion products were cloned by limiting dilution on a thymocyte feeder layer. Antigen specificity was tested by first culturing 5×10^4–10^5 T cell hybrids and 5×10^5 X-irradiated spleen cells in 0.25 ml of fusion medium without HAT for 2 d with varying amounts of antigen. The supernatant from these cultures was harvested and added to an equal volume of complete EHAA in a second culture to assess IL2 production. This culture contained 3×10^3 HT-2 cells, a T cell line developed by Dr. J. D. Watson, Auckland U. Sch. of Med., whose growth is completely dependent on IL2 (a kind gift of Drs. Kappler and Marrack, Natl. Jewish Hosp., Denver, CO). [^{3}H]thymidine was added 24 h later to measure proliferation.

Lymph Node Proliferation Assay. Lymph node proliferation assays were done as previously reported (7). Animals were immunized in complete Freund's adjuvant in the hind footpads. Popliteal and inguinal lymph nodes were collected 7–9 d later, teased, and passed over nylon wool. The 4×10^5 nylon wool passed lymph node cells plus 1×10^5 irradiated (2,000 R) normal spleen cells were cultured with varying amounts of antigen and pulsed with 1 μCi of [^{3}H]thymidine and harvested.

Results

The primary goal of this work was to identify which of the five structural differences between the moth and pigeon cytochrome *c* peptides (see Table I)

TABLE I

Sequences of Peptides Used in this Work

Name	Sequence*																						Code
	81										91										101		
Pigeon 81–104	I	F	A	G	I	K	K	K	A	E	R	A	D	L	I	A	Y	L	K	Q	A	T	A	K	P
des-Ala^{103}-P 81–104																							Δ		DAP_{81}
des-Ala^{103}-P 87–104																							Δ		DAP_{87}
Moth 81–103	V				L			A	N														Δ		M_{81}
Moth 88–103								A	N														Δ		M_{88}
Splice (M 86–90, P 94–104)									K	K	A	N	E												S_p
des-Ala^{103}-Splice									K	K	A	N	E										Δ		DAS_p
Gln^{99}-Splice									K	K	A	N	E						Q						Q^{99}
Gln^{99}-des-Ala^{103}-Splice									K	K	A	N	E						Q				Δ		DAQ^{99}

* The single letter code is used: A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; I, isoleucine; K, lysine; L, leucine; N, asparagine; Q, glutamine; R, arginine; T, threonine; V, valine; Y, tyrosine; Δ, deletion.

prevented B10.A(5R) APC from presenting the pigeon peptide. Earlier analysis (10) of the pigeon cytochrome *c* peptide antigen had shown that changes at residues 100 and 104 could strongly affect antigen function. Thus, we focused initially on the deletion at position 103. In order to examine the contribution of this structural change, we synthesized two des-Ala-pigeon peptides, DAP_{87} and DAP_{81} (see Table I), which were identical in sequence to pigeon except at 103, where they both lacked an alanine.

Fig. 1*A* shows that moth-primed B10.A(5R) lymph node cells responded to both these synthetic peptides, DAP_{81} and DAP_{87}, almost as well as they responded to the moth fragment immunogen and 10,000-fold better than they responded to the pigeon fragment. Thus, the secondary response of moth-primed B10.A(5R) T-cells showed this strain to be a high responder to DAP_{81} and DAP_{87} but not to pigeon. Fig. 1*B* shows the results of the test of B10.A(5R) DAP_{81}-primed lymph node cells. A strong secondary response to DAP_{81} was seen that cross-reacted fully with moth 81–103 (M_{81}) and moth 88–103 (M_{88}) but not with AmM_{88} or pigeon 81–104 (P). These results therefore show that it is solely the presence of the Ala^{103} that prevents the pigeon peptide from being a strong antigen or immunogen in B10.A(5R) mice.

Previously it has been demonstrated (6) that there is an antigen-specific defect in B10.A(5R) APC in that they present the pigeon fragment 81–104 poorly to pigeon-primed B10.A peritoneal exudate T lymphocytes (PETLES) but can present M_{81} to this population. We naturally wished to determine whether this antigen-specific defect in presentation to B10.A T cells also depended on the Ala^{103} deletion. The data in Table II showed this to be the case. Pigeon-primed B10.A PETLES were stimulated with APC-enriched B10.A and B10.A(5R) splenocytes that had been precultured with either moth, pigeon, or DAP_{81} fragments (antigen pulsed) or with medium control. The B10.A APC caused marked stimulation with all three of the test peptides. B10.A(5R) APC caused proliferation with DAP_{81} and moth; however, there was no B10.A(5R)-pigeon response above the medium control. Thus, the Ala^{103} residue prevented the effective presentation of the pigeon peptide by B10.A(5R) APC in this case to

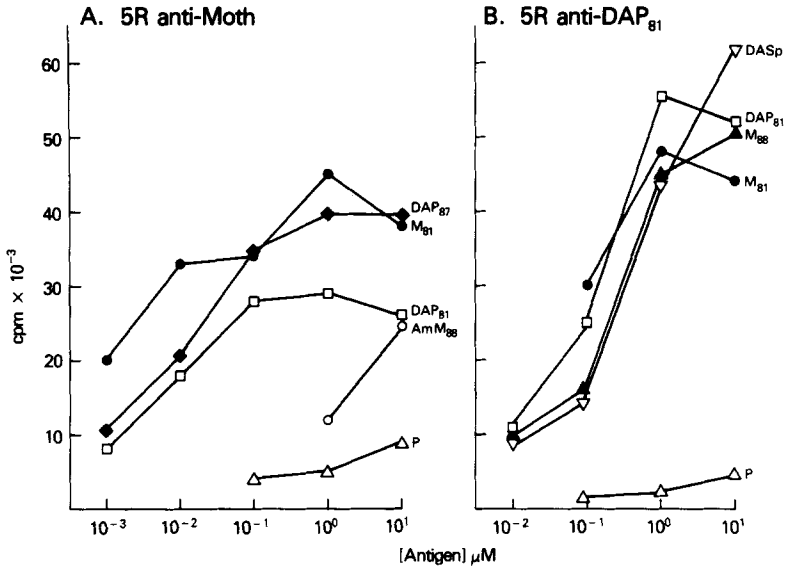

FIGURE 1. The specificity of the immune response of B10.A(5R) mice. B10.A(5R) mice were immunized with 50 μg of moth 81–103(A) or des-Ala^{103}-pigeon (DAP_{81}) (B). After 9 d nylon wool passed lymph node cells were cultured with varying concentration of the antigen peptides for 72 h. During the last 12 h 1 μCi of [^{3}H]thymidine was present. Antigens: des-Ala^{103}-pigeon 81–104 (DAP_{81}, □), moth 81–103 (M_{81}, ●), Am moth 88–103 (AmM_{88}, ○), moth 88–103 (M_{88}, ▲), des-Ala^{103}-splice (DASp, ▽), des-Ala^{103}-pigeon 87-104, (DAP_{87}, ♦), pigeon 81–104 (P, △). Media controls: *A*, 8,300 ± 3,500; *B*, 1,900 ± 800; PPD stimulation: *A*, 67,700 ± 5,800; *B*, 81,600 ± 3,600 (cpm ± SEM).

TABLE II

*B10.A(5R) APC Can Present DAP_{81} but Not Pigeon to B10.A PETLES**

APC‡	Antigen§:	M_{81}	P	DAP_{81}	Medium
B10.A		90.6 ± 3.6‖	41.1 ± 3.8	69.8 ± 13.7	10.1 ± 2.0
B10.A(5R)		113.9 ± 7.3	17.1 ± 3.0	73.5 ± 7.7	17.3 ± 0.7

* B10.A mice were primed with 5 μg of pigeon 81–104 and peritoneal exudates induced. Nylon wool passed peritoneal exudate T lymphocyte-enriched cells (PETLES) were cultured for 96 h. During the last 12 h 1 μCi of [^{3}H]thymidine was present.

‡ The antigen-presenting cell (APC) enriched fraction of Percoll separated spleen cells was precultured with antigen (10 μM), extensively washed and then co-cultured with PETLES lymphocytes. A titration of APC (1.6×10^5, 8×10^5, 4×10^4/well) were tested. The data from the point which gave optimum antigen-dependent stimulation with syngeneic APC is reported, in this experiment 8×10^5/well.

§ Codes as given in Table I.

‖ PETLES cultures were harvested on to glass filters and counted. Mean ± SEM ($\times 10^{-3}$) of triplicate cultures are given.

allogeneic B10.A T cells.

We next considered whether this APC defect represents a change in specificity of the immune response or merely a change in its sensitivity. This latter possibility was suggested by the observations that higher doses of moth fragment are required to prime B10.A(5R) than the B10.A (data not shown) and that lymph

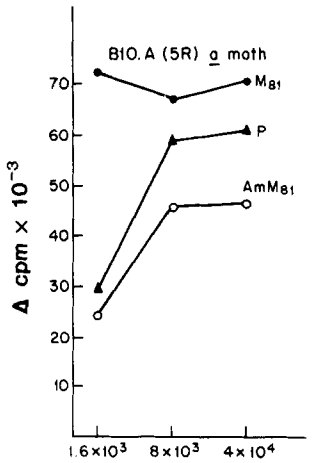

FIGURE 2. The response of B10.A(5R) anti-moth 81–103 PETLES to varying numbers of antigen precultured (pulsed) B10.A Percoll-enriched APC. Experimental details as in Table III. Antigens: moth 81–103 (M_{81}, ●); pigeon 81–104 (P, ▲); acetimidyl moth 81–103 (AmM_{81}, ○). Medium controls 1.6 × 10^3 APC/well: 6,300 ± 670; 8 × 10^3 APC/well: 18,500 ± 2,270; 4 × 10^4 APC/well: 26,900 ± 3,640.

node cells and hybridomas respond to moth fragment at 100-fold lower doses on B10.A-presenting cells then B10.A(5R) APC (5). That is, even those peptides to which both B10.A and B10.A(5R) are responders, are presented more effectively by B10.A APC.

Whether there was also a specificity change could be considered by noting that in moth-primed B10.A(5R) mice the acetimidyl derivative of moth 81–103 (Am-moth) is a weak antigen but clearly stronger than pigeon (Fig. 1*A*); while in a pigeon or moth-primed B10.A, the pigeon 81–104 peptide is a stronger antigen than Am-moth (data not shown). If a change of relative strengths of these antigens were found when B10.A and B10.A(5R) antigen-pulsed APC were compared, this would be evidence of a change in specificity. Fig. 2 reports the response curves of moth-primed B10.A(5R) PETLES to antigen-pulsed B10.A APC. There is clearly a reversal of the relative strengths of pigeon 81–104 (P) and acetimidyl moth between Fig. 1*A* and Fig. 2. Results from this type of experiment are summarized in Table III. Shown in Table III, Exp. I is a comparison of the response of moth-primed PETLES to Am-moth and pigeon-pulsed B10.A and B10.A(5R) APC and illustrates the reversal of pigeon and Am-moth. The reciprocal experiment using Am-moth primed B10.A(5R) and Am-pigeon primed B10.A PETLES was performed and yielded an identical result (Table III, Exps. II and III). In these experiments B10.S(9R) APC were also tested. These APC failed to present the Am-pigeon fragment to any appreciable extent, indicating that the altered specificity was not the result of an allogeneic effect. These results made clear that there was a difference in the specificity of the B10.A(5R) PETLES response to B10.A and B10.A(5R) APC.

The results in Table III further suggested that the role of Ala^{103} in governing the ability of B10.A and B10.A(5R) APC remained unchanged when two

TABLE III

Antigen Specificity Depends on the MHC of the APC

Exp. I: B10.A(5R) anti-moth PETLES*

APC	Medium	M_{81}	AmM_{81}		P
B10.A(5R)	1.0 ± 0.4	77.4 ± 2.5	22.6 ± 0.8	>	8.0 ± 0.6‡
B10.A	4.5 ± 4.0	76.7 ± 4.1	55.9 ± 0.3	≤	68.3 ± 0.5
B10.A(4R)	1.7 ± 0.1	3.8 ± 3.4	NT		3.3 ± 0.1

Exp. II: B10.A(5R) anti Am-moth PETLES

	Medium	AmM_{88}	M_{88}		AmP	$AmDAP_{81}$
B10.A(5R)	0.7 ± 0.1	43.1 ± 1.5	11.0 ± 2.1	>	3.4 ± 0.7	37.1 ± 2.3
B10.A	5.6 ± 0.3	31.7 ± 0.8	8.1 ± 1.9	<	32.5 ± 3.1	29.9 ± 2.6
B10.S(9R)	8.5 ± 0.5	13.8 ± 0.9	8.8 ± 1.0		10.7 ± 3.3	12.8 ± 1.1

Exp. III: B10.A anti Am-pigeon PETLES§

	Medium	AmM_{88}	M_{88}		AmP	$AmDAP_{81}$
B10.A(5R)	2.1 ± 0.4	33.1 ± 1.6	12.7 ± 0.6	>	6.9 ± 1.5	40.1 ± 4.5
B10.A	1.3 ± 0.2	27.1 ± 2.9	10.8 ± 0.4	<	30.0 ± 1.2	27.9 ± 3.9
B10.S(9R)	4.1 ± 0.3	11.8 ± 1.2	4.9 ± 1.1		6.2 ± 1.1	16.5 ± 1.4

* B10.A(5R) mice were immunized with 50 μg of either moth 81–103 or acetimidyl moth 81–103. Otherwise as in Table II.

‡ The changes in rank-order of antigen strengths are boxed and indicated.

§ B10.A mice were primed with 10 μg of pigeon 81–104.

NT, Not tested.

different T cell specificities were examined, moth and Am-moth. This possibility was explored in more detail utilizing an observation reported elsewhere,[2] which indicated that T cell memory was directed primarily toward residue-99. Two new immunogens in addition to the Lys^{99} specificity, were synthesized and tested: AmDASp, which shares Am-Lys^{99} with Am-moth, and $AmDAQ^{99}$ (Gln^{99}). Fig. 3 shows that for B10.A(5R) mice these two peptides cross-reacted neither with each other nor with the Lys^{99} containing DAP_{81} peptide. Interestingly $AmDAQ^{99}$ and DAQ^{99} cross-reacted well (compare Fig. 2*B* and Fig. 1*A*), indicating that the antigenically significant site of acetimidation was position 99. Also shown in Fig. 3 are the failure of the Ala^{103}-containing analogues of these immunogens, AmSp and AmQ^{99}, to stimulate. The ability of DAQ^{99} to stimulate moth-primed B10.A(5R) lymph node cells was assessed and found to be negligible (data not shown). Thus, AmDASp/AmSp (Am-Lys^{99}) and $AmDAQ^{99}$/AmQ^{99} (Gln^{99}) are two pairs of peptides, antigenically distinct from moth or DAP and from each other, whose antigenicity in the B10.A(5R) exactly parallels those of the DAP/pigeon pair.

To test whether the B10.A(5R) T cells could respond to the Ala^{103}-containing peptides in association with B10.A APC, the response of B10.A(5R) PETLES to syngeneic and allogeneic antigen-pulsed APC was tested. These experiments, (Table IV), show that the Ala^{103}'s roles in the presentation of pigeon and of the two new antigens were identical. Thus, in each case the B10.A APC presented

[2] Hansburg, D., T. Fairwell, R. H. Schwartz, and E. Appella, 1983. The T lymphocyte response to cytochrome *c* IV. Distinguishable sites on a peptide antigen which affect antigenic strength and memory. *J. Immunol.* In press.

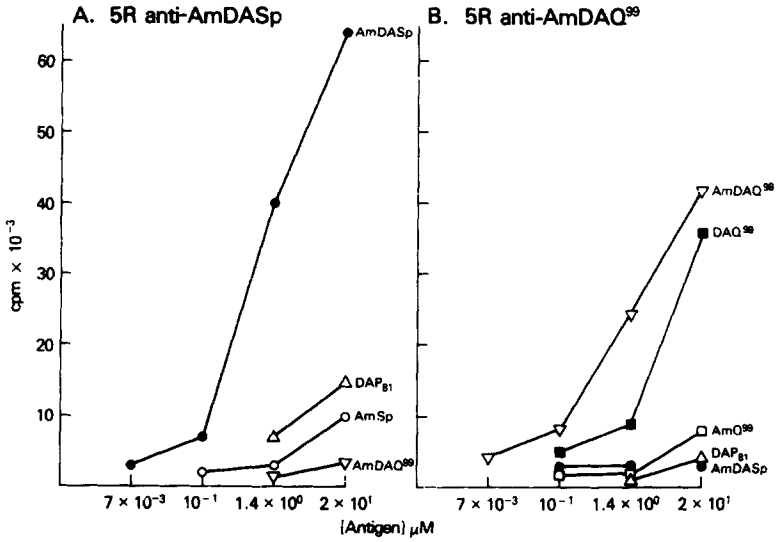

FIGURE 3. B10.A(5R) mice distinguish acetimidyl-des-Ala^{103}-splice (AmDASp) and acetimidyl-Gln^{99}-des-Ala^{103}-splice (AmDAQ^{99}) from each other and from des-Ala^{103}-pigeon (DAP$_{81}$). B10.A(5R) mice were primed with 50 μg of AmDASp(A) or AmDAQ^{99}(B) and then tested as in Fig. 1. Antigen identification is given in Table I. Media controls: *A*, 2,000 ± 100; *B*, 2,900 ± 600; PPD stimulation: *A*, 72,500 ± 16,500; *B*, 106,900 ± 10,000.

TABLE IV

*B10.A(5R) PETLES Respond to Ala^{103}-Containing Peptides with B10.A but Not B10.A(5R) APC**

B10.A(5R) anti Am-des-Ala^{103}-Splice (AmDASp) PETLES			
	Medium	AmDAS$_p$	AmSp
B10.A(5R)	1.9 ± 0.2	42.0 ± 1.0	3.6 ± 0.2
B10.A	6.2 ± 0.4	55.4 ± 1.3	24.3 ± 1.4
B10	3.2 ± 0.8	4.4 ± 0.5	4.3 ± 0.5
B10.S(9R)	10.9 ± 3.3	16.9 ± 1.5	12.0 ± 0.6
B10.A(5R) anti-Am-Gln^{99}-des-Ala^{103}-Splice (AmDAQ^{99}) PETLES			
	Medium	AmDAQ^{99}	AmQ^{99}
B10.A(5R)	1.5 ± 0.2	34.1 ± 3.3	2.3 ± 0.1
B10.A	28.5 ± 3.9	63.2 ± 9.3	58.1 ± 3.7
B10	7.5 ± 0.9	10.1 ± 2.0	NT
B10.A(4R)	34.9 ± 3.7	35.0 ± 1.8	NT

* Experiments performed as in Tables II and III.

both Ala^{103}-containing and des-Ala^{103}-peptides while the B10.A(5R) APC were able to stimulate only with the des-Ala^{103} form. C57Bl/10 and B10.A(4R)/(IE^b) APC were not able to stimulate at all, indicating that the A$_e$E$_\alpha$ Ia molecule was utilized. Likewise B10.S(9R)(IE^k) APC failed to present, indicating that even among mice expressing an A$_e$E$_\alpha$ molecule antigen recognition was MHC restricted. Taken together, the data presented above indicate that for three separate T cell specificities (Lys^{99}, Am-Lys^{99}, Gln^{99}) there was a consistent

antigen-specific defect in the ability of the B10.A(5R) APC's function, namely its disruption by the Ala^{103} insertion. That this defect lay in the APC was established by the B10.A APC's ability to present the Ala^{103}-containing peptides to the same T cell population. These data suggest that there is an interaction between the restriction element (the A_eE_α Ia molecule in this case) and nominal antigen.

It must be considered that all the residues on the nominal antigen that participate in this interaction cannot be defined with present techniques. The identification of the site of this interaction depends on the availability of an MHC degenerate T cell. Even so, only the differences in antigen-MHC interaction between the degenerate haplotypes can be identified. The previous data make clear that there was a difference between B10.A and B10.A(5R) in their ability to tolerate the insertion of Ala^{103}. The B10.A was permissive for this change. The B10.A(5R) was restrictive. In contrast acetimidylation of the ϵ-amino groups had in these experiments a pronounced effect on the T cell clones activated, i.e., on T cell memory, but did not appear to affect the antigen-MHC interaction.

We next considered the possibility that different T cell clones might reveal different APC polymorphisms. This possibility was explored using two hybridoma clones. The first, labeled *A* in Table V, is derived from the fusion of a B10.A(2R) Am-pigeon primed lymphocyte and BW5147. Clone *A* behaved as would be predicted from the data reported above for lymph node and PETLES T cells. It responded to AmM_{88} and AmDAP on both B10.A and B10.A(5R) APC but not to any nonacetimidated peptide. It responded to Am-pigeon on B10.A alone. The second hybridoma, labeled *B*, is the B10.A(5R) anti-moth fragment 81–103 clone previously investigated (5). Clone *B* showed the same specificity as the

TABLE V

*B10.A(5R) APC are Blocked by Ala^{103} Uniformly but by Am-Lys Variably**

Antigen	Clone:	"A"(B10.A anti-AmP)		"B"(B10.A(5R) anti-B-Moth)‡	
	APC:	B10.A(5R)	B10.A	B10.A(5R)	B10.A
Medium		0§	0	0	0
M_{88}‖		0	0.1/0.1	11.5/9.9	103.8/94.6
DAP_{81}		0	1.0/2.5	47.3/66.1	73.2/84.2
Pigeon		0	0	0	107.0/132.9
AmM_{88}		32.8/36.5	82.5/661.1	0	80.5/85.6
$AmDAP_{81}$		97.8/101.5	85.3/81.6	0	83.5/78.4
AmPigeon		0	89.8/107.8	0	112.7/101.9
DAQ^{99}		NT	NT	0	1.4/6.5

* Hybridomas between BW5147 and antigen-stimulated T cells as indicated were cultured with 2×10^5 spleen cells and antigen in 0.25 ml of medium as previously described (5). IL2 production was induced by some antigen/APC combinations and detected by a secondary culture with 50% fresh medium and 3×10^5 HT-2 cells/0.2 ml.

‡ Clone "B" is subclone of the B10.A(5R) anti-q-benzyl-moth hybridoma previously reported (5).

§ After 24 h of culture 1 μCi [^{3}H]thymidine was added to the secondary HT-2 cultures which were harvested 4 h later. Cpm $\times 10^{-3}$ of duplicates are given except when each are <100 cpm which is reported as 0.

‖ 20 μM of each peptide.

B10.A(5R) anti-moth fragment 81–103 PETLES when it was tested with M_{88}, pigeon, and DAP fragments. However, there was an additional difference between B10.A and B10.A(5R) APC that was revealed by testing of the acetimidyl derivatives of the M_{88} and DAP peptides. The APC from B10.A presented both acetimidyl and native M_{88} and DAP_{81} while B10.A(5R) APC function was limited to only the unmodified peptides. The differences between B10.A and B10.A(5R) APC's stimulation of clone *B* illustrates once again the relative stringency of the B10.A(5R) antigen-MHC interaction compared with the B10.A. Yet, the ability of B10.A(5R) to present the acetimidyl peptides to some T cells, e.g., our clone *A*, but not clone B indicates that the MHC-controlled, APC-expressed specificity of the antigen-MHC interaction might be determined, in part, by the T cell population.

This question, i.e., the relationship between the T cell and the antigen-MHC interaction site that can serve that T cell, can perhaps be better answered by knowing the correlation between the MHC-controlled APC-expressed antigen specificity and immune responsiveness. If one assumes that the interaction between antigen and MHC was for the most part independent of the T cell population, then, since B10.A(5R) APC were unable to present the Ala^{103} containing peptides to des-Ala^{103}-primed B10.A(5R) T cells, one would expect that the B10.A(5R) should be a low responder to all these peptides. The data presented in Table VI test this hypothesis. It shows that B10.A(5R) responded, in general, very poorly to the Ala^{103}-containing peptides. The largest response, to the $moth_{86-90}$, $pigeon_{94-104}$ splice peptide (Sp) was roughly two-fifths that of PPD, while responses to the des-Ala^{103}-peptides were usually twice that. Further, no response to the Ala^{103}-containing peptides was seen at 1.0 μM while responses to mice primed to the des-Ala^{103}-peptides were evident as low as 10^{-1} μM (Figs. 1 and 3). Lastly, the low level of the B10.A(5R) anti-Sp response has not allowed

TABLE VI

*B10.A(5R) Was a Low Responder to Ala^{103}-Containing Peptides**

Strain	2° Antigen	[Ag] in vitro	Immunogen		
			Splice	AmSplice	AmQ^{99}
B10.A(5R)	Medium		2.5 ± 0.2‡	3.2 ± 0.1	4.5 ± 0.3
	Purified protein derivative (PPD)		59.2 ± 1.0	82.2 ± 6.4	84.9 ± 1.8
	Immunogen	15 μM	23.2 ± 1.0	7.1 ± 1.0	5.8 ± 0.8
		1.0 μM	3.5 ± 0.6	3.3 ± 0.4	5.5 ± 1.0
	des-Ala^{103}-	15 μM	8.0 ± 1.1	9.3 ± 1.9	5.6 ± 1.8
	Immunogen	1.0 μM	6.6 ± 0.3	4.1 ± 0.7	5.3 ± 1.4
B10	Medium		1.0 ± 0.3	0.8 ± 7.8	1.1 ± 0.1
	PPD		94.8 ± 4.6	83.5 ± 7.8	85.9 ± 4.0
	Immunogen	15 μM	1.1 ± 0.5	1.5 ± 0.1	2.1 ± 0.8

* B10.A(5R) and B10 mice were immunized with 50 μg of the peptides indicated. Nylon wool passed lymph node cells were placed in culture on day 9. Cultures were stimulated with 15 μM or 1.0 μM of the immunogen, similar concentrations of the immunogen's des-Ala^{103}-analogue, 50 μg/ml PPD, or the medium control.

‡ At 72 h 1 μCi [^{3}H]thymidine was added to the cultures and harvested 16 hrs later. The mean cpm ± SEM. ($\times 10^{-3}$) of triplicates are reported.

TABLE VII
*B10.A(5R) APC Can Present AmQ^{99} to B10.A PETLES**

APC	Medium	AmDAQ^{99}	AmQ^{99}
Exp. I			
B10.A	2.0 ± 0.7	85.4 ± 1.5	99.8 ± 16.3
B10.A(5R)	18.4 ± 3.0	36.2 ± 2.4	39.4 ± 13.3
B10.A(4R)	2.6 ± 0.6	5.8 ± 2.0	3.4 ± 1.6
Exp. II			
B10.A	3.9 ± 0.9	91.7 ± 8.0	160.7 ± 7.8
B10.A(5R)	7.9 ± 0.5	21.5 ± 2.9	30.8 ± 3.3
Exp. III			
B10.A	6.8 ± 0.9	114.6 ± 10.2	111.3 ± 8.6
B10.A(5R)	13.2 ± 1.0	32.2 ± 3.4	40.7 ± 0.5
B10.A(4R)	NT	NT	4.0 ± 0.5

* B10.A mice were primed with 50 μg of acetimidyl-Q^{99} (Exp. I and II) or Q^{99} (Exp. III). Experiments performed as in Table II.

us to test its specificity beyond noting that there is no heteroclitic stimulation by DASp. In particular, there is no evidence that the site of T cell memory contains residue-99. Thus, there is an excellent correlation between the low immune responsiveness of B10.A(5R) to the Ala^{103}-containing peptides and the poor ability of its APC to present them to des-Ala^{103}-primed T cells.

A marked response to the AmQ^{99} peptide in B10.A and B10.S(9R) mice could be obtained.[2] If immune response differences do in fact reflect only the capacity of the APC to interact with antigen, and this capacity were independent of the T cell test population, one would excpect that B10.A(5R) APC would present AmDAQ^{99} but not AmQ^{99} to AmQ^{99}-primed B10.A PETLES. The data presented in Table VII show three experiments in which this was not the case. Rather, there was significant and largely equivalent presentation of both AmQ^{99} and AmDAQ^{99} to some fraction of the B10.A PETLES even though the B10.A(5R) was essentially a nonresponder to AmQ^{99} and a strong responder to AmDAQ^{99}. Again B10.A(4R) failed to present, indicating a requirement for the A_eE_α Ia molecule. Note particularly that Table II and Table VII present comparable experiments examining the effects of the Ala^{103} insertion on the ability of B10.A(5R) APC to present two different antigens to B10.A T cells. The results found were, in fact, opposite: B10.A(5R) APC can present AmQ^{99} but not the pigeon 81–104 fragment. These data suggest that there may be two mechanism of Ir gene effects. For one type, of which the B10.A(5R) poor response to pigeon 81–104 is an example, low responsiveness is explicable solely on the basis of the APC-expressed antigen specificity. For the second type, e.g., B10.A(5R) anti-AmQ^{99}, both repertoire and APC specificity appear to be involved.

Discussion

The data presented in this work address two questions: Does the B10.A(5R)'s low responsiveness to the Ala^{103}-containing peptides correlate with some limitation of its APC function? If so, is this the only cause of the B10.A(5R) low

responsiveness?

The answer to the first question is emphatically yes. The correlation between the B10.A(5R)'s poor responsiveness to the Ala^{103}-containing peptides as immunogens and the poor response of des-Ala^{103}-primed B10.A(5R) PETLES to the Ala^{103}-containing peptides on B10.A(5R) but not B10.A APC is the fundamental observation of the present work. As has been reported previously (7, 13) and in Table VI of this report, B10.A(5R) is a low but not a complete nonresponder to the five Ala^{103}-containing peptides tested (pigeon, Am-pigeon, splice, AmSp, and AmQ^{99}). In each case the B10.A(5R) T cells that have been primed with a des-Ala^{103}-peptide are able to respond well to the Ala^{103}-containing peptides when B10.A APC are used, but poorly with its own B10.A(5R) APC. From the examination of the B10.A(5R) PETLES we might conclude that there is an antigen-specific defect of the presenting cell with an antigen structure-function relationship that is independent of the specificity of the T cell clone used to detect it. Although most of our data indicated that the APC-expressed antigen specificity did not depend on the test T cell population, analysis of the hybridoma clone *B* yielded an apparent exception. However, this exception is difficult to interpret because it was revealed by peptides that had been modified by acetimidate at two sites: residue-99, the site of T cell memory, and the carboxyl terminus. The resulting specificity pattern is fairly complex. Although clone *B* responds to neither AmDAP or Am-moth fragment 81–103 on B10.A(5R) APC, it is not completely unresponsive to peptides with Am-Lys at 99 and 103. For example, it responds well to Am-fly fragment 81–103 in association with B10.A(5R) APC (5). Because, in this case, changes in specificity and sensitivity can not be carefully separated, we feel the question of whether additional residues affect APC-expressed antigen specificity should be deferred until further data can be obtained.

Examination of the immune response to the peptides with Gln^{99} addresses the second question: whether the APC-expressed antigen specificity is the exclusive cause of Ir gene effects. As is shown by the comparison of Table VII and Table IV, the T cell repertoires to the Gln^{99}-containing peptides appear to differ between B10.A and B10.A(5R). The B10.A possesses clones that can respond to AmQ^{99}-B10.A(5R) but the B10.A(5R) does not. This result is completely analogous to the earlier reports of allogeneic T cells being able to respond to antigen on low responder APC (14–18). The difference is that previously low responder APC, allogeneic to the T cell source, were used for both priming and the secondary assay; whereas, in the present case, the degeneracy of the B10.A anti-AmQ^{99} T cells allowed syngeneic priming. In either case, the data indicate that differences in T cell repertoires can be the cause of Ir gene effects.

In our opinion, the significant points to be made from the present work are as follows. Duplicate experiments have been performed with two different pairs of antigens: (*a*) pigeon/des-Ala^{103}-pigeon; (*b*) $AmDAQ^{99}/AmQ^{99}$. In the first case the immune response differences between B10.A and B10.A(5R) appear entirely attributable to APC-expressed antigen specificity. The second case is clearly more complex. Examination of the B10.A(5R) anti-$AmDAQ^{99}$ response reveals

the same APC-expressed antigen specificity as seen in (1). However, examination of the B10.A anti-AmQ^{99} response indicates additional clones are present for this antigen in the B10.A that are absent in the B10.A(5R). Thus APC-expressed antigen specificity is demonstrable and correlates with differences in immune responsiveness, but is not the sole cause of immune response polymorphisms.

Lastly, some brief speculation on the mechanism of MHC-controlled, APC-expressed specificity may be useful. The simplest, and therefore most attractive, hypothesis is that the nominal antigen and the Ia region gene products physically interact through a site that contains the Ala^{103}-deletion (6) and that the site on the nominal antigen of this interaction depends only on the location of the site of T cell memory (the T cell epitope), not on its particular structure. In the present case the putative site of interaction would be characterized by the B10.A's ability to accept Ala^{103} or its deletion while the B10.A(5R) is apparently able to present only the des-Ala-peptides well. The hypothesis is in good agreement with all the data except the AmQ^{99}-primed B10.A T cell subpopulation, which recognizes AmQ^{99} and $AmDAQ^{99}$ equally well with B10.A(5R) APC. Further investigations to determine which residues, in addition to 99, contribute to the site of T cell memory will allow us to compare the location and extent of the T cell epitope for clones both of the B10.A anti-Lys^{99} and the B10.A anti-Gln^{99} responses that recognize antigen in association with B10.A(5R). These data should provide a further test of the hypothesis.

Summary

In previous work (5, 6), we have reported studies on a T lymphocyte hybridoma clone and the peritoneal exudate T cells (PETLES) from B10.A(5R) mice primed with the cytochrome *c* carboxyl terminal peptide (residues 81–103) of the tobacco horn worm moth (*Manducca sextus*). As expected, since B10.A(5R) is a low responder to pigeon fragment 81–104, it was found that the B10.A(5R) lymphocytes were unable to respond to the pigeon cytochrome *c* 81–104 fragment presented on syngeneic B10.A(5R) antigen-presenting cells (APC). However, these same T lymphocytes did respond to the pigeon fragment when presented on B10.A APC. Thus, some structural difference between the pigeon and moth peptides had prevented B10.A(5R) APC from effectively presenting the pigeon fragment to moth-primed B10.A(5R) lymphocytes. This structural difference was found to be the deletion of an alanine at position −103 (Ala^{103}) from the pigeon sequence in the moth peptide. Two additional T cell specificities were created by changing residue-99. These T cell populations from the B10.A(5R) showed an identical dependence on the Ala^{103} deletion when B10.A and B10.A(5R) APC were compared.

The relationship of APC-expressed antigen specificity and MHC-linked immune responsiveness differences was also examined. The B10.A(5R) was found to be a high responder to each of three peptides that lack Ala^{103} but not to the Ala^{103}-containing analogues. B10.A mice, in contrast, respond to both types of peptides. Utilizing allogeneic antigen-presentation to B10.A PETLES by pulsed APC, it was shown that the poor response of the B10.A(5R) to the Ala^{103}-

containing peptides was, in two of three cases, not associated with any differences in T cell repertoires but due to two different APC capabilities of B10.A and B10.A(5R). The exception apparently represents a case of T cell repertoire polymorphism between B10.A and B10.A(5R) that can also affect immune responsiveness.

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