I-A-RESTRICTED T CELL ANTIGEN RECOGNITION

Analysis of the Roles of A_α and A_β Using DNA-mediated Gene Transfer

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The induction of antigen-specific Th responses requires the recognition of antigen in conjunction with Ia (class II) cell surface molecules encoded by genes of the MHC (1). This phenomenon of MHC-restricted antigen recognition was first detected because of the failure of cells bearing antigen in conjunction with nonself, species-variant (allogeneic) MHC molecules to be recognized by primed T lymphocytes (2, 3). Thus, it is clear that intraspecies allelic variation (polymorphism) of the component α and/or β chains of class II molecules plays a central role in the T lymphocyte recognition process.

A variety of experimental approaches has been used to identify the sites of allelic polymorphism, and to explore the manner in which the structural features of Ia molecules determined by such variation influence immune recognition. Nucleic acid sequence analyses of cloned class II A_α and A_β genes have shown a clear concentration of interallelic polymorphism in several hypervariable segments, all within the NH_2-terminal α_1 or β_1 domains (4–7). A limited number of DNA-mediated gene transfer experiments using "exon-shuffled" E_β or A_β genes have confirmed the predicted critical role of the β_1 domain in Ia-restricted T cell recognition (8, 9). Initial dissection of the role of subregions of the β_1 domain has come from studies of the naturally occurring A_β^{bm12} mutation (10), chemically-induced, immunoselected Ia mutants (11, 12), and a small number of transfected cells expressing A_β chains bearing site-directed mutations (13). These early studies suggest the existence of multiple functionally distinct sites important in Th recognition of antigen. This conclusion is consistent with earlier, indirect experiments using monoclonal anti-Ia antibodies as reagents to block T cell activation (14). Finally, the work of Fathman and colleagues (15) studying F_1 specific T lymphocytes has indicated that antigen recognition by at least some T cells is determined by polymorphic contributions of both the A_α and A_β chains of Ia.

The present experiments use the technique of DNA-mediated gene transfer to systematically and broadly explore the contribution(s) of A_α and A_β chain polymorphism to T cell recognition of antigen, and to further dissect the role of the A_β chain with respect to the contribution of subregions within the NH_2-

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terminal domain. This was accomplished by generating a large series of recombinant A_β genes involving the exchange of whole β_1 domains, or halves of the β_1 domain, among the *b*, *d*, and *k* alleles, and transfecting either these hybrid or parental A_β genes together with haplotype-matched or mismatched $A_\alpha^{b,d,k}$ genes into mouse L cells. The resultant Ia-expressing transfectants were used as antigen-presenting cells for stimulation of a large panel of T hybridomas with varying Ia and antigen specificities. The results clearly show that both A_α and A_β chain polymorphisms contribute significantly to Ia recognition by most, if not all Th, and that in most cases residues in both halves of the $A_{\beta 1}$ domain are involved in the specificity of recognition. This analysis suggests that conformational determinants dependent on both A_α and A_β polymorphic residues, and involving distinct regions of the A_β linear sequence, play essential roles in the recognition of antigen by Th, and that multiple such sites exist on any particular Ia molecule.

Materials and Methods

Antigens. Beef insulin (BINS)[1], hen egg lysozyme (HEL), and OVA were purchased from Sigma Chemical Co., St. Louis, MO. GAT (Sigma Chemical Co.) was prepared as previously described (16). KLH (Calbiochem-Behring Corp., La Jolla, CA) was dissolved in 5 M sodium chloride and dialysed against PBS to give a stock solution of ~15 mg/ml; concentration was estimated by measurement of optical density.

Monoclonal Antibodies. The mAbs used in these experiments were 3JP, anti-I-A^b (17), M5/114, anti-I-A^b/I-A^d (18), MKD6, anti-I-A^d (19), and 10.2.16, anti-I-A^k (20). These were all used as cell-free culture supernatants at a 50% dilution for cell surface staining.

Media. RPMI 1640 (Biofluids Inc., Rockville, MD) was supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 5×10^{-5} M 2-ME and 10 μg/ml gentamicin (RPMI-10). Selective media consisted of DME (Gibco Laboratories, Grand Island, NY) supplemented with 10% FCS, 2 mM L-glutamine, and 10 μg/ml gentamicin (DMEM-10), plus hypoxanthine (15 μg/ml), aminopterin (0.2 μg/ml), and thymidine (5 μg/ml) (HAT), or mycophenolic acid (6 μg/ml), xanthine (250 μg/ml), and hypoxanthine (15 μg/ml) (MXH) or a combination of MXH and HAT (MXHAT).

Cells. All the Th hybridomas used in these experiments, their origin, and relevant properties are described in Table 1. All the Th hybridomas, and the B cell hybridomas LK35.2 and LB (28) were grown in RPMI-10 and passed 1:50 twice weekly. The L cell transfectants were all grown in nontissue culture Petri dishes in DMEM-10 with the appropriate drug additions, and passed without trypsinization by 1:10 subculture once weekly. CTL-L cells used for IL-2 quantitation were maintained in 5% Con A-spleen cell conditioned supernatant in RPMI-10 and passaged every 48 h.

Cloned Genes and In Vitro Recombinant Gene Constructs. The A_α^b (Brown, M., and F. Ronchese, unpublished observation) and A_α^k (29) genes were genomic fragments subcloned into the plasmid vectors pcEXV (30) or pBR327, respectively. A_α^d was an expressable cDNA clone (9) in the vector pcEXV. Wild-type A_β^b (6), A_β^d (29), and A_β^k (31) genes were unmodified genomic subclones in the pSV2gpt vector (32). Recombinant A_β genes were generated by exchanging the segments labeled *x*, *y*, and *z* in Fig. 1 among the *b*, *d*, and *k* allelic forms of A_β. All possible combinations were made using conventional recombinant DNA techniques. Expressable forms of these recombinant A_β genes were prepared by reconstituting the missing 3′ half of the intracytoplasmic exon and the 3′ untranslated region with the appropriate portions of the A_β^d gene linked to the pSV2gpt vector. Details of these constructs will be presented elsewhere (Braunstein, N., and R. Germain, manuscript in preparation).

DNA-mediated Gene Transfer and Selection. The thymidine kinase (TK) negative L-cell

[1] *Abbreviations used in this paper:* BINS, beef insulin; HEL, hen egg lysozyme; MXH, mycophenolic acid, xanthine, and hypoxanthine; MXHAT, MXH and HAT; TK, herpes thymidine kinase gene.

TABLE I

T Cell Hybridomas Used in This Study

Name	Antigen specificity	I-A restriction	Reference
AF3	BINS	$A^b_\beta A^b_\alpha$	21
DA3	BINS	$A^b_\beta A^b_\alpha$	*
DC3	BINS	$A^b_\beta A^b_\alpha$	*
2B2.F5	KLH	$A^b_\beta A^b_\alpha$	22
BDK11.1	KLH	$A^b_\beta A^b_\alpha$	23
3D08.2	OVA	$A^d_\beta A^d_\alpha$	24
3D011.10	OVA	$A^d_\beta A^d_\alpha$	24
3D018.3	OVA	$A^d_\beta A^d_\alpha$	24
3D054.8	OVA	$A^d_\beta A^d_\alpha$	24
RF9.140	GAT	$A^d_\beta A^d_\alpha$	25
A2	HEL	$A^k_\beta A^k_\alpha$	26
A4A2	HEL	$A^k_\beta A^k_\alpha$	26
A6A2	HEL	$A^k_\beta A^k_\alpha$	26
C10	HEL	$A^k_\beta A^k_\alpha$	26
C8.A3	Auto.	$A^k_\beta A^k_\alpha$	12
SKK9.11	KLH	$A^k_\beta A^k_\alpha$	27
SKK45.10	KLH	$A^k_\beta A^k_\alpha$	27
BDK44.5	KLH	$A^d_\beta A^b_\alpha$	28
BDK65.2	KLH	$A^d_\beta A^b_\alpha$	‡
FS9-6.3	Allo.	$A^b_\beta A^d_\alpha$	‡
FS11-4221	Allo.	$A^b_\beta A^d_\alpha$	‡

* Wassmer, P., and E. M. Shevach, unpublished observations. The method used to isolate these hybridomas was as for AF3 (21).

‡ Kappler, J., and P. Marrack, unpublished observations. These cells were generated as described for BDK 44.5 (28).

subline, DAP.3, was transfected with pairs of A_β and A_α genes using the standard calcium phosphate precipitation technique, as described previously (33). In some cases the TK gene was cotransfected and successfully transfected cells selected in HAT-containing medium throughout. Other TK cotransfected cells were initially selected in HAT medium, and subsequently doubly selected in MXHAT medium. Finally, some cells transfected with A_β and A_α alone were selected from the outset in MXH medium. Colonies of transfectants were pooled, stained with appropriate mAb, analyzed by microfluorimetry, and repeatedly sorted by preparative flow microfluorimetry to achieve high levels of cell surface I-A expression.

Flow Microfluorimetry Analysis. For analysis by flow microfluorimetry 5×10^5 cells were incubated with 100 μl of the indicated mAb for 30 min on ice in round bottom 96-well microtiter plates. After washing twice in PBS with 5% FCS the cells were incubated for a further 30 min on ice with 50 μl of a 1:50 dilution of fluoresceinated-$F(ab')_2$ goat anti-mouse IgG [FITC-GAMIg] (Cappel Laboratories, Cochranville, PA). After two additional washes stained cells were analyzed using an EPICS V Coulter flow microfluorimeter with log amplifier (Coulter Electronics, Inc., Hialeah, FL).

T Cell Assay. Evaluation of the APC activity of L cell transfectants was carried out essentially as described previously (34). In brief, 5×10^4 responder T hybridoma cells were incubated for 24 h in 200 μl RPMI-10 in the wells of 96-well microtiter plates, either alone or together with the indicated number of APC and either no antigen, or 250 μg/ml KLH, 100 μg/ml HEL, 1 mg/ml OVA, 100 μg/ml BINS, or 200 μg/ml GAT. The

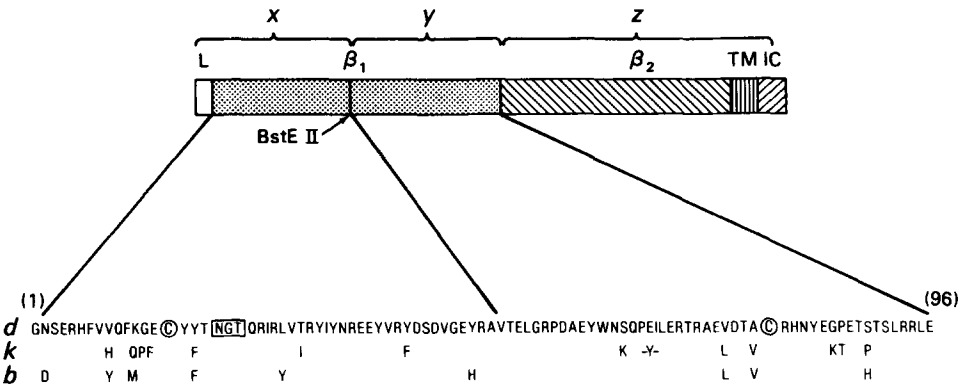

FIGURE 1. Schematic diagram of the A_β chain showing protein domain boundaries. Segments *x*, *y*, and *z* represent the portions of the encoded protein that were exchanged as the result of generating the A_β recombinants as described in Materials and Methods. The conserved BstEII restriction endonuclease site (arrowed) is indicated in the protein sequence at the position corresponding to its position in the $A_{\beta 1}$ exon. This conserved site permitted the genetic sequences encoding the hemi-domain segments *x* and *y* to be recombined. Comparative protein sequences of $A_{\beta 1}$ from *d*, *k*, and *b* haplotypes are shown below, using the one-letter amino acid code.

supernatant was harvested and assayed for IL-2 activity on CTL-L cells, as reported (35). A unit of IL-2 is defined as that amount of IL-2 required to achieve 50% of the maximal [^{3}H]TdR incorporation shown by CTL-L cells in the presence of a saturating amount of IL-2-containing conditioned medium.

Results

Analysis of Ia Structure-Function Using L Cell Transfectants. In order to address the issues outlined above, a large panel of recombinant A_β genes was generated by the exchange of segments encoding whole β_1 domains, or the first or second half of the β_1 domain, between *b*, *d*, and *k* allelic forms of A_β. All the possible recombinant genes were made between each pair of haplotypes. The boundaries of the segments exchanged among A_β genes are shown in Fig. 1, together with the amino-acid sequences of the $A_{\beta 1}$ domains of the three haplotypes. These "domain and hemi-domain shuffled" genes were transfected into L cells with a haplotype-matched or mismatched A_α gene. Each L cell APC is referred to in the Tables and in the text by listing the haplotype origin of the three exchangeable portions of the A_β gene in the order: (*a*) NH_2-terminal half of the β_1 domain, (*b*) COOH-terminal half of the β_1 domain, and (*c*) β_2, transmembrane, and intracytoplasmic domains. The haplotype of the A_α chain is given by a single letter following the A_β designation. Thus, the L cells transfected with a hybrid A_β gene consisting of the NH_2-terminal half of the β_1 domain of *k* haplotype, and the remainder of the A_β gene of *b* haplotype, cotransfected with A^b_α would be represented as *kbb/b*. These experiments also showed the expression of both A_β and A_α chains on the surface of all the transfectants used in this study.

Cell surface expression of certain A_β and A_α pairs could not be achieved despite the presence of high levels of β and α mRNA and repeated attempts at preparative cell sorting. Thus, certain gaps will be noted in the Tables, in particular, *ddd/k*, *dkd/k*, and *dkk/k*, as well as *bdd/d* and *bdb/d*. The subject of control of I-A

expression and a more detailed analysis of the allelic constraints on chain pairing are reported elsewhere (reference 36; Braunstein, N., and R. Germain, manuscript in preparation).

The responder cells used in these experiments comprised a panel of 21 Th hybridomas with a range of antigen specificity and I-A restriction. When single concentrations of antigen were used they were chosen to be high enough to elicit strong, near plateau responses in the context of wild-type I-A, yet slightly below the maximal noninhibitory antigen concentration, in order to detect less than all-or-none shifts in antigen sensitivity due to the modifications introduced into the I-A molecule. Nonetheless, some subtle quantitative effects of recombinant Ia chains on T cell reactivity have probably been missed. These could have been detected by analyzing full dose-response curves for all responder-stimulator combinations. Therefore, only three categories of response are shown in Tables III, IV, and V: (+++), (+), and (−). Responses were recorded as (+++) when the amount of IL-2 release stimulated by a particular L cell APC was comparable to that induced by the wild-type transfectant expressing the parental restriction element for the T cell in question. Responses recorded as (+) indicate situations in which the IL-2 release was < 50% of that detected using APC expressing the parental restricting element, and the lower amount of IL-2 could not be accounted for by differences in levels of cell surface I-A expression. Finally, results were scored as (−) when no IL-2 was detected. Such negative results were validated in two ways. First, the negative combinations of APC and Th cells were further tested by doubling the APC number and by using a maximal noninhibitory antigen concentration. In no case did this alter the results shown in Tables II–V. Second, in order to exclude a simple quantitative explanation due to inadequate levels of APC I-A expression, at least one L cell APC bearing a stimulatory I-A molecule and expressing the same or a lower level of I-A than the nonstimulatory transfectant was included in the same experiment.

Role of A_α and A_β Chains in T Cell Recognition. To determine whether I-A-restricted T cell recognition of antigen is dependent on contributions from polymorphic regions of one or both of the chains of I-A, the Th cells were tested with L cell transfectants expressing either both A_α and A_β chains of parental type, or expressing one parental and one nonparental chain. Detailed results are presented in Table II for two Th cells stimulated with antigen and L cell APC expressing either the parental I-A molecule, or I-A molecules composed of one parental chain, either α or β, with the complementary chain from one of the other two haplotypes used here. The patterns shown by the two responder cells are very different. DC3 (BINS-specific, I-A^b-restricted) only responds to the transfectant expressing both A^b_α and A^b_β chains of the parental haplotype. In contrast 3D054.8 (OVA-specific, I-A^d-restricted) is able to use A^b_β paired with A^d_α (*bbb/d*) as a restriction element. In addition, the F_1 molecule $A^b_\beta A^d_\alpha$ is recognized by this T cell as an allodeterminant, inducing a weak response that is unaltered by the presence of antigen.

The results of testing all the T cells with transfectants expressing either the parental $A_\beta A_\alpha$ restricting element, or expressing one parental chain and a complementary chain from a different haplotype, are summarized in Table III. First, it is clear that L cell APC expressing "wild-type" parental I-A molecules

TABLE II

Evaluation of A_β and A_α Chain Contributions to I-A-restricted Antigen Recognition by DC3 and 3D054.8

T Hybridoma	Specificity	Restriction	Antigen*	A_β:A_α genotype of L cell APC						
				bbb/b	*ddd/d*	*kkk/k*	*bbb/d*	*bbb/k*	*ddd/b*	*kkk/b*
DC3	BINS	I-A^b	+	90‡	<1	<1	<1	<1	<1	<1
			−	<1	<1	<1	<1	<1	<1	<1
				ddd/d	*bbb/b*	*kkk/k*	*ddd/b*	*bbb/d*	*kkk/d*	
3D054.8	OVA	I-A^d	+	32	<1	<1	<1	6	2	
			−	<1	<1	<1	<1	<1	2	

* 100 μg/ml BINS or 1 mg/ml OVA were included in the indicated cultures.
‡ Data are presented as units of IL-2, calculated as described in Materials and Methods.

TABLE III

Evaluation of A_β and A_α Chain Contributions to I-A-restricted T Cell Antigen Recognition by Diverse T Hybridomas

Responder cell			A_β:A_α genotype of L cell APC							
Name	Specificity	Restriction	bbx*/b	ddx/d	kkx/k	bbx/d	bbx/k	ddx/b	kkx/b	kkx/d
AF3	BINS	$A^b_\beta A^b_\alpha$	+++‡	−	−	−	−	−	−	NT§
DA3	BINS	$A^b_\beta A^b_\alpha$	+++	−	−	−	−	−	−	NT
DC3	BINS	$A^b_\beta A^b_\alpha$	+++	−	−	−	−	−	−	NT
2B2.F5	KLH	$A^b_\beta A^b_\alpha$	+++	−	−	−	−	−	−	NT
BDK11.1	KLH	$A^b_\beta A^b_\alpha$	+++	−	−	−	−	−	−	NT
3D08.2	OVA	$A^d_\beta A^d_\alpha$	−	+++	−	−	NT	−	NT	−
3D011.10	OVA	$A^d_\beta A^d_\alpha$	−	+++	−	+++(allo)	NT	−	NT	−
3D018.3	OVA	$A^d_\beta A^d_\alpha$	−	+++	−	−	NT	−	NT	−
3D054.8	OVA	$A^d_\beta A^d_\alpha$	−	+++	−	+++	NT	−	NT	+(allo)
RF9.140	GAT	$A^d_\beta A^d_\alpha$	−	+++	−	−	NT	+	NT	−
A2	HEL	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
A4A2	HEL	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
A6A2	HEL	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
C10	HEL	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
C8.A3	Auto	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
SKK9.11	KLH	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
SKK45.10	KLH	$A^k_\beta A^k_\alpha$	−	−	+++	NT	−	NT	−	−
BDK44.5	KLH	$A^b_\beta A^b_\alpha$	−	−	NT	−	NT	+++	NT	NT
BDK65.2	KLH	$A^b_\beta A^b_\alpha$	−	−	NT	−	NT	+++	NT	NT
FS9-6.3	Allo	$A^d_\beta A^d_\alpha$	−	−	−	+++(allo)	NT	−	NT	NT
FS11-4221	Allo	$A^d_\beta A^d_\alpha$	−	−	−	+++(allo)	NT	−	NT	NT

* The same results were obtained throughout when the $A_{\beta 2}$ domain was from any of the three haplotypes (x).
‡ Data shown as +++, +, or − according to classification described in Results section. Results given are for assays in the presence of specific antigen. When responses were seen without antigen they are indicated as (allo).
§ NT, not tested.

act as effective APC for all the Th hybridomas used in these experiments. The second and more striking observation is that 19 out of the 21 responder cells showed an absolute requirement for both A_β and A_α chains to be of parental haplotype, failing to exhibit any response if either parental chain was replaced by that from either of the other haplotypes. Only one cell, RF9.140 (GAT-specific, I-A^d-restricted) responded to an I-A molecule composed of the parental A_β chain with a nonparental A_α chain. Similarly, 3D054.8 was the only hybridoma to show a detectable response when a nonparental A_β chain was present. In both of these cases the degeneracy only extended to one of the other two haplotypes

used in these experiments, showing that even in the presence of this degeneracy both chains contribute to the process of antigen-specific activation. It should be noted that the panel of T cells includes four cells, isolated from F_1 animals, that are restricted to F_1 (b × d) I-A molecules. In these cases, there is the same requirement for maintaining the original stimulatory $\alpha\beta$ pair, as for cells recognizing "parental" I-A molecules. The responses of these cells also confirm the functional expression of these F_1 I-A molecules following transfection, and extend the range of restriction specificities included in the T cell panel. From these results, it would appear that restricted recognition by most, if not all, I-A restricted T cells involves contributions from the polymorphic regions of both A_β and A_α chains.

In addition, two of the I-A^d restricted cells (3D011.10 and 3D054.8), recognized F_1 I-A molecules composed of $A^b_\beta A^d_\alpha$ and $A^k_\beta A^d_\alpha$, respectively, as allodeterminants. No augmentation of the allo-specific response was seen following the addition of antigen, suggesting that these F_1 I-A molecules were not also functioning as restriction elements for these T cells.

Importance of the Allelically Highly Polymorphic $A_{\beta 1}$ Domain in T Cell Recognition. Results from the use of APC expressing the products of exon-shuffled A_β genes have strongly suggested that the polymorphism within the $A_{\beta 1}$ domain is responsible for the contribution of the A_β chain to the specificity of I-A-restricted recognition by T cells (9). This finding has been extended here by exchanging the NH_2-terminal domains between *b*, *d* and *k* A_β allelic genes and testing the L cells transfected with these hybrid A_β and wild-type A_α genes with 17 T hybridoma cells. The results are included in Table III, and show that polymorphisms within the NH_2-terminal domain of the A_β polypeptide can entirely account for the restriction imposed on Th responses by the entire A_β chain. All the transfectants expressing a parental β_1 domain joined to an allogeneic β_2 domain and paired with a parental A_α chain induced T cell activation as efficiently as wild-type $A_\beta A_\alpha$ transfectants.

Complex Role of the $A_{\beta 1}$ Domain in I-A-Restricted T Cell Recognition. Important polymorphic residues within the $A_{\beta 1}$ domain were more finely mapped by further subdividing the $\beta 1$ domain into two halves (see Fig. 1) and creating "hemi-domain–shuffled" A_β genes by exchanging NH_2-terminal or COOH-terminal halves of the $\beta 1$ domain between the *b*, *d*, and *k* allelic A_β genes. All the T cell hybridomas were then tested with transfectant APC expressing the product of hemi-domain–shuffled A_β and wild-type parental A_α genes. As an example of the actual data generated from this type of experiment, the results obtained from one T cell, BDK11.1 (KLH-specific, I-A^b restricted) are shown in full in Table IV. The microfluorimetry profiles of all the stimulatory, as well as some nonstimulatory transfectants for this responder cell are portrayed in the accompanying figure (Fig. 2).

Several points are illustrated by the data in Table IV; first, as evidenced by the difference in amounts of IL-2 released when two wild-type $A^b_\beta A^b_\alpha$ transfectants (Fig. 2, APC 1 and 2) differing only in their level of cell surface I-A expression were used as APC, I-A density per cell is an important variable leading to marked quantitative effects that need to be considered when interpreting these data (34). A difference of 30 in the channel number on the relative fluorescence intensity

TABLE IV
Responses of BDK11.1 (KLH, I-A^b) to L Cell APC Bearing I-A Molecules Involving Recombinant A_β Chains

Transfectant number	Genotype	IL-2 (U)
1	*bbb/b*	90
2	*bbb/b*	19
3	*ddd/d*	<1
4	*kkk/k*	<1
5	*bbb/d*	<1
6	*bbb/k*	<1
7	*ddd/b*	<1
8	*kkk/b*	<1
9	*bbd/b*	35
10	*bbk/b*	95
11	*ddb/b*	<1
12	*kkb/b*	<1
13	*bdb/b*	18
14	*bdd/b*	9
15	*bkb/b*	<1
16	*bkk/b*	<1
17	*dbb/b*	24
18	*dbd/b*	12.5
19	*kbb/b*	<1
20	*kbk/b*	<1

scale represents a twofold difference in I-A expression; thus, the two wild-type I-A^b transfectants differ by approximately twofold in their level of I-A expression, and this is reflected as a fourfold difference in the amount of IL-2 released by BDK11.1 in the presence of 250 μg/ml KLH. Second, the specific responses reported in Table IV provide further evidence consistent with the conclusion that polymorphism in the $A_{\beta 2}$ domain is unimportant for I-A-restricted T cell recognition of antigen. Thus, pairs of transfectant APC that express the same A_α chain and $A_{\beta 1}$ domain and differ only in the β2 domain (i.e., APC 1 and 10, 13 and 14, and 17 and 18) induced very comparable levels of IL-2 secretion, when differences in I-A cell surface expression are taken into account. The third and most striking observation from Table IV is that although replacement of the whole $A^b_{\beta 1}$ domain with $A^d_{\beta 1}$ sequence abolishes the response of BDK11.1 (APC 11), BDK11.1 is permissive of the substitution of *d* haplotype sequence in either half of the β1 domain. APC 13, 14, 17, and 18 generate a readily detectable response. In contrast, no response is seen when *k* haplotype sequence is introduced in either half of the β_1 domain. Furthermore, though the APC with *d* haplotype sequence introduced into the NH_2-terminal half of the β_1 domain does stimulate BDK11.1, there is a marked reduction in efficiency. As shown in Table IV, the amount of IL-2 release induced is several-fold lower than that seen when wild-type I-A^b expressing L cells were used as APC, and this cannot be accounted for by differences in I-A density per cell. A more detailed analysis of this effect is given below. Taken together, all these observations strongly suggest that although some degeneracy exists in the restricted recognition of antigen by

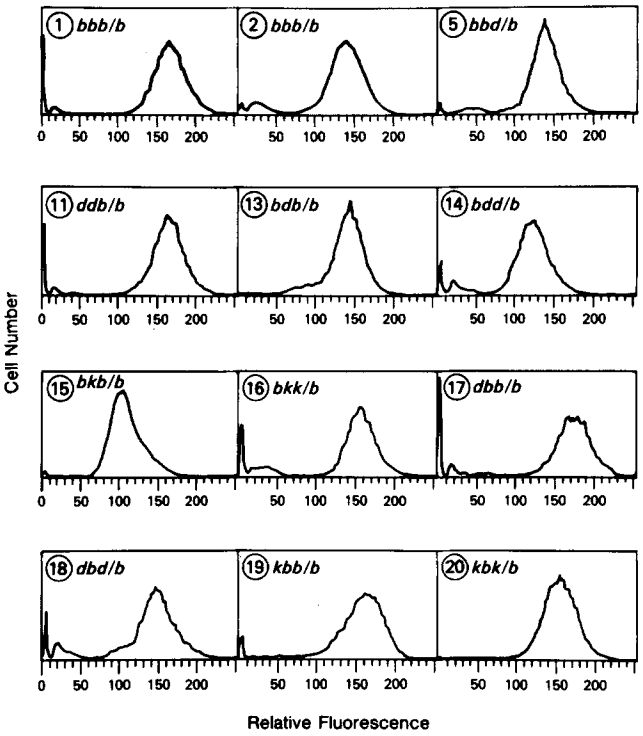

FIGURE 2. Microfluorimetry profiles of I-A transfected L cells used as APC for the I-A^b-restricted Th cells. The numbering corresponds to the APC designations in Table IV, and the genotype of each transfectant is shown. APC numbers 15 and 16 were stained with the 10.2.16 (anti-A^k_β) mAb, the remainder with M5/114 (anti-$A^{b,d}_\beta$). Abscissa, relative fluorescence (channel number is shown); ordinate, cell number.

BDK11.1, residues in both halves of the β_1 domain are contributing in some way to the T cell receptor-Ia/antigen interaction.

The results of testing the whole panel of T hybridoma cells with all the L cell APC transfected with hemi-domain–shuffled A_β and wild-type A_α genes are presented in Table V. The responder cells can be divided into four groups according to their patterns of reactivity.

Group I. For the nine T hybridomas in this set, effective I-A-restricted stimulation was abolished by any substitution of the first or second halves of the β_1 domain with the same region from either of the other haplotypes. Three of these cells were BINS-specific and I-A^b restricted; because DA3 and DC3 arose in the same fusion with BW5147 and share identical patterns of T cell receptor β-chain rearrangements (data not shown), it is likely they are siblings derived from the same Th clone. The remaining six cells in this group are *k* restricted, four specific for HEL, and two for KLH.

Group II. Cells in this set required the first half of the β_1 domain of the parental haplotype, while permitting the substitution of the second half of the domain with that from at least one of the other two haplotypes tested. Four OVA-specific, I-A^d-restricted, one HEL-specific, I-A^k-restricted, and one auto-I-A^k-reactive cell comprised this group. Within Group II, A2 was the only cell

TABLE V

Patterns of Th Hybridoma Responses to L Cell APC Expressing Wild-Type A_α with Recombinant (Hemi-Domain–shuffled) A_β Genes

Group	Hybridoma	Specificity	Restriction	A_β:A_α genotype of L cell APC				
				bbb/b	*bdb/b*	*dbb/b*	*kbb/b*	*bkb/b*
I	AF3	BINS	$A^b_\beta A^b_\alpha$	+++*	–	–	–	–
I	DA3	BINS	$A^b_\beta A^b_\alpha$	+++	–	–	–	–
I	DC3	BINS	$A^b_\beta A^b_\alpha$	+++	–	–	–	–
III	2B2.F5	KLH	$A^b_\beta A^b_\alpha$	+++	–	+++	+	–
IV	BDK11.1	KLH	$A^b_\beta A^b_\alpha$	+++	+++	+++	–	–
				ddd/d	*dbd/d*	*kdd/d*	*dkd/d*	
II	3D0 8.2	OVA	$A^d_\beta A^d_\alpha$	+++	+++	–	–	
II	3D011.10	OVA	$A^d_\beta A^d_\alpha$	+++	+++	–	–	
II	3D054.8	OVA	$A^d_\beta A^d_\alpha$	+++	+++	–	+++(allo)	
II	3D018.3	OVA	$A^d_\beta A^d_\alpha$	+++	+	–	–	
IV	RF9.140	GAT	$A^d_\beta A^d_\alpha$	+++	+++	+++	–	
				kkk/k	*kbk/k*	*bkk/k*	*kdk/k*	
I	A4A2	HEL	$A^k_\beta A^k_\alpha$	+++	–	–	–	
I	A6A2	HEL	$A^k_\beta A^k_\alpha$	+++	–	–	–	
I	3A9	HEL	$A^k_\beta A^k_\alpha$	+++	–	–	–	
I	C10	HEL	$A^k_\beta A^k_\alpha$	+++	–	–	–	
I	SKK9.11	KLH	$A^k_\beta A^k_\alpha$	+++	–	–	–	
I	SKK45.10	KLH	$A^k_\beta A^k_\alpha$	+++	–	–	–	
II	A2	HEL	$A^k_\beta A^k_\alpha$	+++	+++	–	+	
II	C8.A3	Auto	$A^k_\beta A^k_\alpha$	+++	+++	–	–	

* See footnote to Table III.

responsive to APC bearing I-A molecules with substitutions of either of the two allogeneic sequences in the COOH-terminal half of the $A_{\beta 1}$ domain.

Group III. One hybridoma, 2B2.F5, falls into Group III, which is the converse of Group II. Inclusion in the second half of the $A_{\beta 1}$ domain of *d* or *k* sequence abolishes the responsiveness of this I-A^b restricted cell; however, it will respond to *b*, *d* or *k* in the NH_2-terminal half of $A_{\beta 1}$.

Group IV. This set includes two hybridomas, the I-A^b-restricted and KLH-specific cell BDK11.1, and the GAT-specific and I-A^d-restricted cell RF9.140. The pattern for BDK11.1 has already been presented in Table IV. This cell responds to APC expressing I-A^d sequence in either the first or the second half of the $A_{\beta 1}$ domain, provided that the parental (*b*) sequence is retained in the other half of the domain. The I-A^d restricted cell, RF9.140, exhibits even greater degeneracy in that it permits substitution of *b* haplotype sequence in the COOH-terminal half, and *k* sequence in the NH_2-terminal half of the domain.

Since APC expressing detectable levels of the I-A molecules *bdd/d* or *bdb/d* could not be generated, it cannot be excluded that RF9.140, and the other I-A^d restricted hybridomas, might also respond to substitution of *b* for *d* sequence in the NH_2-terminal half of the $A_{\beta 1}$ domain. Similarly, since *dkk/k* could not be made, some of the *k* restricted cells in Group I could belong in Group II, and some of the Group II cells could belong in Group IV.

In addition to retaining or losing the ability to be triggered by APC expressing

these modified I-A genes, two of the OVA-specific I-A^d-restricted T cells exhibited a cross-reactive allo-specificity unique to the F_1 I-A molecules $A^b_\beta A^d_\alpha$ (3D011.10) and $A^k_\beta A^d_\alpha$ (3D054.8) (see Table III). The results presented in Table V show that allo-recognition by these two T cells is controlled by different regions of the β_1 domain. For 3D011.10 the I-A molecule *dbd/d* functions as a parental restricting element while stimulating no alloresponse. This implies that allogeneic residues from the *b* haplotype in the NH_2-terminal half of the β_1 domain are necessary for the induction of an allo-response. In contrast, the $A^k_\beta A^d_\alpha$ allodeterminant recognized by 3D054.8 is effectively expressed when the β chain is encoded by the modified gene *dkd*. This provides evidence that, as for the control of antigen-specific restricted T cell recognition, control of allo-recognition can be mapped to different regions of the A_β chain for different T cells.

Quantitative Effects of Changes in $A_{\beta 1}$ Structure on T Cell Activation. A more detailed comparison of the stimulatory potential of several of the transfectants expressing the product of hemi-domain–shuffled A_β genes was carried out by generating dose-response curves using wild-type $A_\beta A_\alpha$ transfectants and cells transfected with A_α plus a hemi-domain–shuffled A_β gene. Pairs of APC with matched levels of I-A expression were compared to avoid major shifts in the dose-response curve due to mismatched I-A density. In some instances the dose-response curves were practically superimposable, while in others the cell expressing the shuffled gene product was clearly a less potent stimulator. One example of each pattern is shown in Fig. 3. Using 2B2.F5 (I-A^b-restricted, KLH-specific), the wild-type transfectant *bbb/b* was compared with cells expressing *dbb/b*. As can be seen in Fig. 3 *a*, the L cell expressing *dbb/b* acts as a slightly more efficient APC despite the substitution of *d* sequence in the NH_2-terminal half of the β_1 domain. The wild-type I-A^b transfectant requires approximately a twofold increment in antigen concentration in order to stimulate equivalent amounts of IL-2 production. This can be attributed to the slightly greater density of cell surface

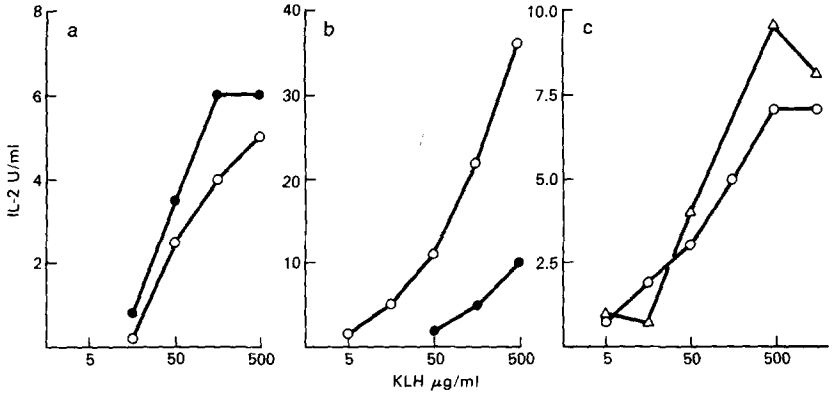

FIGURE 3. Antigen dose responses of two KLH-specific, I-A^b-restricted Th hybridomas stimulated using pairs of I-A^+ L cell APC matched for levels of I-A surface expression. ○, APC *bbb/b*, ●, APC *dbb/b*, △, APC *dbd/b*. *a*, Th hybridoma 2B2.F5; b and c, Th hybridoma BDK11.1. Responses measured as units of IL-2 quantitated as described in Materials and Methods.

I-A expression by the *dbb/b* APC (see Fig. 2, *1* and *17*). In striking contrast, using the same pair of transfectants to stimulate BDK11.1 (I-A^b-restricted, KLH-specific), the *dbb/b* APC is much less efficient than the wild-type transfectant, requiring a tenfold increase in KLH concentration in order to induce the same level of IL-2 release (Fig. 3*b*). The transfectant expressing the reciprocal hemi-domain–shuffled A_β gene *bdb* with A^b_α, which also functions as an APC for BDK11.1, was compared in the same way with an expression-matched wild-type I-A^b transfectant. As can be seen in Fig. 3*c*, the transfectant expressing the modified A_β gene acts as a more efficient APC than the wild-type transfectant. Again, this can be accounted for by slight differences in the levels of I-A expression by the two cells, as the *bdb/b* APC expressed slightly more I-A molecules per cell than the wild-type transfectant used for comparison (Fig. 2, *2* and *13*). Thus, while substitution of *d* for *b* sequence in the NH_2-terminal half of the $A_{\beta 1}$ domain leads to a 10- or 20-fold loss of efficiency of triggering of BKD11.1, the reciprocal modification of the $A_{\beta 1}$ domain has no detectable effect on the efficiency of interaction of the I-A molecule with the BDK11.1 receptor and/or with specific antigen.

Discussion

The experiments reported here used recombinant DNA methods and DNA-mediated gene transfer to explore the contributions of the α- and β-chains of I-A to restricted T cell recognition, and to dissect the A_β chain to define critical sites involved in the interaction of I-A with T cells and antigens.

Our first conclusion is that restricted recognition by most, if not all, of the Th cells used in these studies requires contributions from polymorphic residues in both the α and the β chain of I-A. As mentioned above, T cells restricted by allelic polymorphisms in both A_α and A_β have been previously described by Beck et al. (15). However, the findings presented here provide the first indication that the norm, if not the rule, is for I-A-restricted recognition to be controlled by polymorphic residues in both chains. It is not yet clear whether this finding reflects the separate, specific recognition of A_α and A_β chains by the T cell receptor(s), the recognition of a combinatorial site formed by the interaction of the two chains of I-A, or T cell recognition of determinants expressed on one or both chains of the molecule which are conformationally dependent on pairing with the complementary parental chain.

The second conclusion from these studies is that polymorphisms in the $A_{\beta 1}$ domain can entirely account for the restriction imposed on Th cell responses by the entire A_β chain. This is shown by experiments using L cell APC expressing a domain-shuffled A_β chain with a wild-type A_α chain and emphasizes that not all the external domains of I-A are equally important in controlling the restricted recognition of antigen by Th cells. No effect on the stimulatory capacity of transfectant APC was seen as the consequence of introducing a haplotype-mismatched $A_{\beta 2}$ domain, thus extending earlier observations involving transfectants expressing single examples of domain-shuffled A_β and E_β chains (8, 9).

These conclusions are entirely consistent with recent observations on the control of CTL recognition examined using target cells transfected with hybrid, domain-shuffled class I genes (37–41). The majority of these studies show that

CTL recognition is, for most T cells, dependent on allele-specific contributions from both $\alpha 1$ and $\alpha 2$ domains, while $\alpha 3$ polymorphisms have no detectable effects. These results have been taken to indicate that class I determinants recognized by CTL are located in the two external domains and are conformational structures generated by the specific interaction of the α_1 and α_2 domains. As these two external domains of class I molecules, and the α_1 and β_1 domains of class II molecules are structurally analogous, it appears that T cell recognition of class I and class II molecules follows similar rules, consistent with the recent data on $V\alpha$ and $V\beta$ receptor gene usage in subsets of T lymphocytes (42, 43).

The recent results of Golding et al. (44) contrast with both the observations reported here and the findings described concerning the recognition of hybrid class I gene products by CTL. Using a gene construct composed of the $A^k_{\beta 1}$ domain joined to the α_3, transmembrane, and intracytoplasmic portions of the class I molecule H-2D^d, they reported the recognition and lysis of L cells transfected with this hybrid gene by CTL raised against the native $A^k_\beta A^k_\alpha$ molecule. However, the L cell transfectant expressing this hybrid gene fails to induce a measurable response from any of the I-A^k-restricted Th hybridomas used in the experiments reported here (Lechler, R., and J. McCluskey, unpublished observations), providing further evidence that contributions from both α and β chains are generally required in I-A-restricted antigen recognition by Th cells. The reason for this apparent difference in Ia recognition between allospecific CTL and antigen-specific Th cells is unclear at present, and is under investigation.

A third conclusion from these results is that, for a given T cell, multiple sites in both halves of the polymorphic $A_{\beta 1}$ domain are important in controlling I-A-restricted recognition by Th cells. This is illustrated by the exhibition of all four possible patterns of reactivity by the panel of Th hybridoma cells. Of all the examples of degeneracy shown in groups II, III, and IV (Table V) only two cells (2B2.F5 and A2) permitted the introduction of $A_{\beta 1}$ sequence from either of the other haplotypes. Thus, even when parental sequence was not strictly required, in most cases only partial degeneracy was seen, implying that both halves of the domain are contributing to T cell recognition of I-A/antigen. Working with a series of in vitro generated I-A-mutant B cell tumors or transfectants, Glimcher and co-workers (11–13) have also recently described evidence to suggest that multiple sites in the $A_{\beta 1}$ domain contribute to I-A-restricted Th cell recognition.

It may also be noted that degeneracy in I-A restriction observed in these experiments corresponds to predictions arising from comparison of the amino acid sequences of the three haplotypes tested. Thus, degeneracy was much more frequently observed with cells restricted to I-A^b and I-A^d, and APC bearing *d/b* recombinant A_β chains, than with cells restricted to either of those haplotypes and APC expressing recombinant genes involving portions of $A^k_{\beta 1}$. The deletion of two amino acids between position 65–67 in the second half of the $A^k_{\beta 1}$ domain (Fig. 1) may result in critical conformational differences between *k* and *b* or *d* A_β chains, or directly affect the structure of a particularly important segment of the β chain (see below) and hence account for the lack of degeneracy when this region of the gene is exchanged. Conversely, the degeneracy in the COOH-terminal half of the $A_{\beta 1}$ domain seen between *b* and *d* haplotypes may reflect their high level of homology in the region surrounding the site of the bm12

mutation (i.e., residues 65–70), a region clearly important in T cell restricted recognition of antigen, as evidenced by the rare nature of T cells able to see antigen presented by both $A^b_\beta A^b_\alpha$ and $A^{bm12}_\beta A^b_\alpha$. However, of the nine cells responding to nonparental sequence in either half of the $A_{\beta 1}$ domain, cells requiring the NH_2-terminal half of the domain to be of parental type (Group II) predominated. This contrasts with expectations generated by these studies of the bm12 mutation which stress the role of the COOH-terminal half of $A_{\beta 1}$ (45). Nonetheless this result is consistent with recent results showing a critical role for the NH_2-terminal β_1 region in $A_\beta A_\alpha$ assembly/expression and recognition by mAb, which suggests that this segment of the β chain contributes significantly to Ia conformation (Braunstein, N., and R. Germain, manuscript in preparation).

These various considerations relate directly to the data obtained with BDK-11.1. Although replacing the COOH-terminal half of the $A^b_{\beta 1}$ domain with *d* haplotype sequence has no observable effect on the response of this hybridoma, and though *d* for *b* substitution in the NH_2-terminal half of the domain causes only a moderate shift in the antigen dose response, no response can be elicited to the APC expressing *ddb/b*. Thus, both the NH_2-terminal and COOH-terminal portions of the β_1 domain affect BDK11.1 response, and the roles of these two regions are not independent of one another. In other words, with respect to T cell recognition, an A_β polypeptide is more than the sum of its parts. Clearly, therefore, in the analysis of I-A structure-function correlations it is not reasonable to assume that the association of a particular change in the linear sequence of an A_α or an A_β gene with a functional effect implies that the residue or residues that were changed are directly involved in the physical interactions measured functionally.

Several lines of evidence have recently been reported in support of the concept that, in addition to interacting with the T cell receptor, Ia molecules also physically interact with processed forms of antigen in order to generate a trimolecular complex (25, 46–48). This raises the possibility that two mechanisms may be responsible for the loss of, or diminution of, T cell responsiveness to mismatched α:β pairs or to hemi-domain–shuffled A_β chains. On the one hand, the affinity of the I-A:T cell receptor interaction may be reduced. Alternatively, the recombinant I-A molecule may interact less well with the antigenic peptide recognized by the cell in question, giving rise to a requirement for higher antigen concentrations in order to achieve the same level of stimulatory ligand. Recently described analytic methods employing functional tests of T cells (49) may permit a distinction to be made between these two possible effects in individual cases. Ultimately, direct binding studies with T cell receptors and Ia plus antigen should enable a more precise resolution of this issue.

Two caveats should be considered in evaluating the present results. The first is that the presence or absence of L3T4 expression by the Th cells used in these experiments could have influenced the patterns of degeneracy seen. However, analysis of the panel of hybridomas for L3T4 revealed no clear correlation between the expression of L3T4 and the ability to respond to APC expressing modified I-A molecules. The second is that T cell receptor α and/or β chains from the BW5147 T cell lymphoma could have paired with the chains derived from the T cell parent and contributed to some of the observed alloreactivity

and/or degeneracy in restricted recognition (50). However, it is highly unlikely that, if such chain pairing does occur, it would lead to degeneracy on more than one haplotype. Further, any contribution BW5147 receptor chains make to the observed degeneracy only serves to strengthen the conclusion that polymorphic sites in α and β chains of I-A, and in both halves of the $A_{\beta 1}$ domain, are important in controlling restricted recognition.

Taken as a whole, the data presented here support the concept that Ia-restricted T cell recognition of antigen involves multiple distinct conformational epitopes unique to a particular combination of α and β chains. Although this conclusion makes direct physical interpretation of future mutagenesis and gene transfer experiments problematic, such studies should nonetheless prove extremely useful in at least two ways. First, it is possible to directly test for the existence of functionally distinct regions of Ia (e.g., histotopes and desetopes, interacting with the T cell receptor and with antigen, respectively [46]) by looking for mutations that affect only one and not the other process. This can be done in well-defined model systems such as that involving pigeon cytochrome *c* and $E^k_\beta E^k_\alpha$, without knowledge of the actual structural effects of such mutations. Second, it is possible to accumulate a large body of correlative data which will be of great value when the requisite structural (crystallographic) data ultimately become available. Such investigations, combined with the use of mutant MHC genes in transgenic mouse models, should ultimately provide a detailed view of how Ia structure subserves immunologic function.

Summary

The contributions of A_α and A_β chains, and of subregions of A_β, to Ia-restricted recognition of antigen by Th lymphocytes were analyzed using a panel of L cells transfected with various pairs of $A_\alpha^{b,d,\text{ or } k}$ genes and recombinant or wild-type $A_\beta^{b,d,\text{ or } k}$ genes. The A_β genes included all possible exchanges of the whole NH_2-terminal ($\beta 1$) domain or halves of the $\beta 1$ domain among these three allelic A_β genes. The Ia^+ L cells derived from such transfections were used as antigen-presenting cells with a 21 member panel of responding Ia-restricted T hybridoma cells of differing nominal antigen specificity and Ia-restriction. Special care was taken to account for quantitative variation in levels of Ia expression throughout the experiments.

The results of this analysis reveal that (*a*) only 2 of the 21 Th cells recognized Ia molecules involving either a nonparental A_α or a nonparental A_β chain, and in both cases the degeneracy extended to only one of the two other alleles tested. This suggests that allele specific contributions from both A_α and A_β chains are important in restricted recognition for most, if not all I-A-restricted Th cells. (*b*) In no case did substitution of the $A_{\beta 2}$ domain from either of the alternative haplotypes lead to any functionally detectable effects, demonstrating that polymorphisms in the $A_{\beta 1}$ domain can entirely account for the restriction imposed on Th cell responses by the entire A_β chain. (*c*) For 90% of the cells tested, replacement of the NH_2-terminal portion of the $\beta 1$ domain with an allogeneic segment led to Ia molecules unable to elicit Th responses. Furthermore, of all the cells permissive of the substitution of one or other half of the $\beta 1$ domain, only two permitted the substitution of sequence from both alternative haplotypes.

Taken together, these data strongly suggest that antigen recognition by most, if not all, I-A-restricted Th cells involves contributions from both halves of the $A_{\beta 1}$ domain.

These data suggest that the role of I-A molecules in restricted Th cell recognition of antigen depends on conformational determinants unique to a particular combination of polymorphic α and β chains, and that multiple such sites exist on a single Ia molecule. This conclusion suggests caution in attempting to physically interpret the result of MHC mutagenesis experiments, and a need for testing numerous T cells in several antigenic systems before general conclusions about Ia structure-function relationships can be drawn.

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