THE CONTRIBUTION OF MUTANT AMINO ACIDS TO ALLOANTIGENICITY

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Recognition of foreign antigens by T lymphocytes involves trimolecular interactions between: (*a*) the receptors present on T cells (TCRs); (*b*) antigenic peptides; and (*c*) the class I or class II MHC molecules on APCs. Recent experiments that demonstrate physical binding of peptides to murine class II MHC molecules (1, 2), analysis of the structural and functional properties of transfected MHC class 2 gene products (reviewed in references 3, 4), and the solution of the crystal structure of a human class I MHC molecule (5) have led to a clearer picture of the nature of these interactions. The putative peptide binding site on MHC molecules is bounded by two a-helical regions. Most allele-specific residues lie on or between these helices, and potentially affect both the shape of the binding site and epitopes exposed to the T cell (6). On the basis of sequence comparison, Brown et al. (7) have argued that the overall structures of human and mouse and class I and class II MHC molecules likely share common features, particularly, the presumptive antigen binding site. This model of class II MHC protein structure is supported by a large collection of data examining the response of T cell hybrids to MHC molecules containing point mutations. These observations explain in part the phenomenon of MHC-restricted recognition of antigens (8). The finding of an electron density in the presumed antigen binding site of the HLA-A2 molecule raises the possibility that this site is always filled, in the absence of foreign antigen, with self peptides and suggests that the moiety recognized by alloreactive T cells is an MHC/peptide complex. Thus, to some degree, the high frequency of alloreactive T cells (9) may be accounted for by the diversity of self-peptide/MHC complexes that constitute the population of alloreactive molecules (10).

Our laboratories have previously studied (11, 12) the spontaneous mutation I-A^{bm12} (13, 14). Using site-directed mutagenesis of the I-A^b molecule, Ronchese et al. (11) have examined in detail the effects of the three amino acid substitutions that constitute the difference between the I-A^b and the I-A^{bm12} class II molecules on the

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MHC-restricted recognition of antigens. In separate experiments, Bill et al. (12) have generated a panel of 178 I-A^{bm12}-reactive T cell hybrids derived from B10 (I-A^b) mice and have studied the TCR repertoire to this mutant I-A molecule. Here we have collaborated to study the effects of the individual amino acid substitutions on the alloantigenicity of the I-A^{bm12} molecule. The results are consistent with our previous conclusions regarding the function and relative importance of the three altered residues and support the Brown et al. (7) model for class II MHC/peptide/TCR interaction. These data also provide insight into the role of TCR/MHC binding and the possible contributions of MHC-bound peptides to the process of allostimulation.

Materials and Methods

Mice. C57BL/6 (B6) mice were purchased from The Jackson Laboratories, Bar Harbor, ME. B6.C-H-2^{bm12} (bm12) mice were bred in the facilities of National Jewish Center for Immunology and Respiratory Medicine, Denver, CO.

T Cell Hybrids. The characteristics of the hybrids used in this study are given in Table I. The I-A^{bm12}-reactive hybrids and the determination of their TCR variable gene expression have been described previously (12). Briefly, 5×10^5 cell equivalents of RNA from each hybrid was spotted onto multiple nitrocellulose filters that were then hybridized to ^{32}P-labeled probes specific for V_b 2-17 and Va 1-8, 10, 11, and 13. All hybridomas were maintained in complete tumor medium as described (15).

I-A Transfectants. Site-directed mutagenesis of the I-A^b b chain to generate all permutations of the I-A^b and I-A^{bm12}-encoded residues at positions 67, 70, and 71 and transfection of these constructs along with the I-A^b a chain into mouse L cells have been described previously (11). The transfectants were maintained in complete medium plus MXH (mycophenolic acid, 6 mg/ml; xanthine, 250 mg/ml; and hypoxanthine, 15 mg/ml) and were grown on bacteriologic petri dishes from which they could be harvested without trypsin.

Flow Cytometry. For determination of I-A, TCR, and CD4 cell surface expression, 10^5 cells were suspended in buffer (PBS with 2% FCS and 0.1% sodium azide) and stained with the appropriate mAbs for 20 min at 37°C in a volume of 100 ml. The cells were washed with buffer and were then stained with the appropriate fluorescein- or phycocrythrin-conjugated secondary reagent. After additional washes with buffer, 10,000 cells/sample were analyzed on an EPICS C flow cytometer. The mAbs used were: M5/114 which recognizes I-A^b or I-A^{bm12} (10, 16); H57-597 which recognizes all a/b murine TCRs (17); GK1.5 which recognizes murine CD4 (18); KJ25 which recognizes TCRs containing Vb3 (19); RR4-7 which recognizes TCRs containing Vb6 (20); F23.1 which recognizes TCRs containing Vb8.1, 8.2, or 8.3 (21); F23.2 which recognizes TCRs containing Vb8.2 (22); KJ16 which recognizes TCRs containing Vb8.1 or 8.2 (23); and RR3-15 which recognizes TCRs containing Vb11 (24). M5/114 was used as a culture supernatant and the secondary reagent was FITC-conjugated goat anti-mouse Fc receptor (Rockland, Inc., Gilbertsville, PA). All other antibodies were purified from culture supernatants or ascites and biotinylated. The secondary reagent was phycoerythrin-conjugated streptavidin (Serotec, Oxford, UK). Values given are mean channel fluorescence above background. The scale is logarithmic and 85 channels represent a 10-fold increase.

IL-2 Assay. IL-2 production was assayed by the ability of supernatants from stimulated hybrids to support the growth of IL-2-dependent HT-2 cells as described (15). Briefly, 10^5 T cell hybrids were cocultured with 10^5 transfectants or 10^6 spleen cells in 250 ml CTM for 24 h. Twofold dilutions of the resulting supernatant were used to support the growth of 4×10^3 HT-2 cells in a volume of 100 ml for 24 h. At the end of this time, wells were examined for viability of HT-2 cells using phase-contrast microscopy. 5 U of IL-2 are defined as the minimal amount necessary to maintain the viability of HT-2 cells. HT-2 viability was confirmed and quantitated by the MTT assay as described (25). Briefly, 10 ml of MTT at 5 mg/ml in PBS was added to each well and incubation was continued at 37°C for 4 h. The plates were centrifuged for 3 min at 1,000 rpm in a centrifuge (model TJ-6; Beckman Instruments,

TABLE I

Characteristics of the Hybrids Used in this Study

Hybrid	TCR expression*		CD4 expression*		TCR variable genes‡		Fusion§ partner
	Percent positive	Mean	Percent positive	Mean	α	β	
3 BBM 74	100	145	20	132	2	8.1	BW
4 BBM 17	99	144	0		5	10	BW
5 BBM 98	93	160	50	133	4	6	BW
6 BBM 51	99	170	47	129	2	8.1	BW
6 BBM 131	100	196	95	161	4	6	BW
8 BBM 8	100	165	100	172	4	6	BW
8 BBM 10	100	186	97	163	4	11	BW
15 BBM 82	98	157	21	141	5	14	BW
15 BBM 139	97	132	49	133	2	16	BW
16 BBM 30	100	154	96	156	3	2	BW
16 BBM 34	99	170	0		4	16	BW
16 BBM 53	55	147	43	156	8	12	BW
16 BBM 100	98	145	13	132	2	6	BW
17 BBM 6	100	148	0		4	14	BW/α⁻
17 BBM 43	100	184	98	158	3	8.1	BW
17 BBM 51	100	185	98	157	3	16	BW
17 BBM 64	99	172	30	145	4	15	BW/α⁻
17 BBM 68	96	164	85	144	8	4	BW/α⁻
17 BBM 71	99	165	98	156	3	8.1	BW/α⁻
17 BBM 82	100	183	100	169	3	8.1	BW
17 BBM 136	100	177	98	153	2	6	BW/α⁻
17 BBM 151	99	195	99	165	3	10	BW/α⁻
17 BBM 159	41	120	21	145	8	14	BW/α⁻
17 BBM 179	87	156	87	141	8	4	BW
17 BBM 190	90	146	92	150	9	10	BW
18 BBM 9	100	181	95	158	8	4	BW/α⁻
18 BBM 17	100	166	99	166	8	16	BW/α⁻
18 BBM 19	100	174	100	158	3	6	BW/α⁻
18 BBM 22	100	187	99	167	ND	3	BW/α⁻
18 BBM 23	100	171	100	166	5	6	BW/α⁻
18 BBM 37	100	155	35	135	3	10	BW/α⁻
18 BBM 49	99	155	94	154	4	6	BW/α⁻
18 BBM 68	100	198	99	161	4	8.1	BW/α⁻
18 BBM 78	100	173	64	140	9	8.3	BW/α⁻
18 BBM 98	99	176	93	154	3	2	BW/α⁻
18 BBM 109	100	183	100	163	5	6	BW/α⁻
18 BBM 138	100	158	0		3	14	BW/α⁻
18 BBM 139	100	178	97	155	3	15	BW/α⁻
18 BBM 142	91	163	64	146	3	14	BW/α⁻
18 BBM 153	77	147	0		4	14	BW/α⁻
18 BBM 160	100	157	100	174	2	16	BW/α⁻

* TCR and CD4 surface expression was determined by indirect immunofluorescent staining using H57-597 and GK1.5 as described in Materials and Methods.

‡ TCR variable gene expression was determined by hybridization of radiolabeled probes tc total cellular RNA. Vα probes for Vα 1-8, 10, 11, 13, and Vβ probes for Vβ 2-17 were used. Surface expression of Vβ 3, 6, 8, and 11 was confirmed by indirect immunofluorescence as described in Materials and Methods. ND indicates lack of hybridization to any of the radiolabeled Vα probes used.

§ BW indicates BW5147 was used as the fusion partner. BW/α⁻ is a variant of BW5147 selected for deletion of the Vα1 gene (26).

Inc., Fullerton, CA). The medium was discarded and the cell pellet was redissolved in 70% (vol/vol) isopropanol, 2% (vol/vol) 1 N HCl. OD_{590} was then read on an ELISA reader, and IL-2 values were determined by comparison with a standard curve. Each hybrid was tested on at least three occasions and the values given are from representative experiments.

Results

Cell Hybridomas. We have previously generated a panel of 178 I-A^{bm12}-specific T cell hybrids derived from C57BL/10 mice (12). Table I lists 41 of these hybrids that were selected for the present study. To correlate TCR a and b variable gene expression with fine specificity, hybrids were chosen that express only one Va and one Vb mRNA. All hybrids express surface TCR as determined by indirect immunofluorescent staining with the pan-TCR-reactive mAb 597 (17). The majority of hybrids were >90% positive at the time of analysis and the expression of TCR, as assessed by the mean channel of the positive cells, differed by up to 8-fold (a difference of 85 channels corresponds to a difference of 10-fold). Indirect immunofluorescent staining for CD4 surface expression similarly demonstrated that the majority of hybrids express this accessory molecule; however, with 14 hybrids, only 50% or less of the TCR^+ cells were also $CD4^+$, while five hybrids showed no detectable CD4 on the cell surface. TCR V gene expression by these hybrids was previously determined by hybridization of radiolabeled V-specific probes to total cellular RNA. One hybrid, 18BBM22, most likely expresses a Vα for which we did not have a probe. TCR Vβ 3, 6, 8, and 11 surface expression was confirmed by indirect immunofluorescent staining with specific mAbs. Vβ8 family members were determined by comparison of staining with F23.1, F23.2, and KJ16 as described (22). Also listed is the fusion partner used to generate the hybrid. BW/α^- has deleted the Vα1 gene expressed in BW5147 (23).

Transfected Fibroblast-presenting Cells. As the I-A^{bm12} mutation involves only three amino acid substitutions in the I-A^b β chain (Ile→Phe^{67}, Arg→Gln^{70}, and Thr→Lys^{71}), one can examine the importance of individual amino acid substitutions by changing the residues one or two at a time, resulting in eight permutations of the I-$A^{b/bm12}$ β chain. Site-directed mutagenesis of the β chain of the I-A^b molecule and transfection of the resulting constructs along with the I-A^b α chain into mouse L cells has been previously described (11). For reference, phenotypic data are reproduced in Table II along with indirect immunofluorescent staining data of the transfectants using the mAb M5/114, which does not discriminate between I-A^b and I-A^{bm12} (11). Values given are the mean channel fluorescence above background determined on two occasions 1 mo apart during the course of this study. On both occasions virtually 100% of the cells were positive and the intensity of staining was relatively constant. With the exception of FT 6.7, none of the transfectants differed among themselves by more than twofold in their level of I-A expression. Table III shows that all transfectants containing an I-A^{bm12}-derived residue at position 67, 70, or 71 could stimulate at least one I-A^{bm12}-reactive T cell hybrid. A panel of six I-A^b (autoreactive) T cell hybrids were used to confirm presentation of I-A^b by FT7.1 and B6 spleen (data not shown).

Reactivity of I-A^b- and I-A^{bm12}-specific T Cell Hybrids to Transfected Fibroblasts Expressing the Partially or Fully Reconstituted I-A^{bm12} Mutation. Table III (*A–F*) shows the IL-2

TABLE II

Transfectant	Sequence* 67	70	71	I-A surface staining (mean channel fluorescence)‡
FT 6.3	bm§	b	b	140/144
FT 6.4	b	bm	b	162/134
FT 6.5	b	b	bm	127/138
FT 6.7	bm	b	bm	98/114
FT 6.8	b	bm	bm	147/131
FT 7.7	bm	bm	b	135/133
FT 7.1	b	b	b	120/134
FT 7.2	bm	bm	bm	145/124

* Numbers refer to residue position in the mature Aβ polypeptide chain.
‡ Cells were stained with M5/114 on 2 occasions as described in the Results section.
§ b, amino acid from A^{b}_{β}; bm, amino acid from A^{bm12}_{β}.

production of I-A^{bm12}-specific T cell hybrids in response to the panel of transfected fibroblasts that express all possible permutations of the I-A^{b} or I-A^{bm12} amino acids at residues 67, 70, and 71 of the I-A β chain. Several distinct patterns of reactivity are apparent. Table III *A* shows six hybrids that produce IL-2 when stimulated by spleen cells from B6.C-H-2bm12 but that fail to produce IL-2 in response to the I-A^{bm12}-bearing fibroblast FT7.2. The remaining hybrids in Table III, *B–F*, are all capable of responding to I-A^{bm12} on fibroblasts, though they differ in their requirements for specific I-A^{bm12} residues. The 12 hybrids in Table III *B* require that all three residues of the I-A β chain be derived from the I-A^{bm12} sequence. Some hybrids respond with approximately equal strength to either spleen cells or fibroblasts while others demonstrate a clear preference for one or the other. Table III *C* shows six hybrids that are dependent only on the presence of bm12 residues at positions 67 and 70. Hybrid 15BBM139 can respond weakly when only residue 70 is mutated and hybrid 17BBM68 can respond weakly if either residue 67 or 70 is mutated, but both respond more vigorously when both residues are bm12 derived. Table III *D* shows four hybrids that require the bm12 phenotype only at position 70 and respond to all four transfected fibroblasts that express this mutated residue either alone or in combination with altered residues 67 and/or 71. The nine hybrids shown in Table III *E* require bm12-derived residues at positions 70 and 71. Finally, the four hybrids shown in Table III *F* display unusual reactivity patterns. For example, 18BBM142 reacts with FT6.3 (bm^{67},b^{70},b^{71}) and FT7.2 (bm^{67},bm^{70},bm^{71}) transfectants but not with the (bm^{67},b^{70},bm^{71}) or (bm^{67},bm^{70},b^{71}) transfectants. Thus, the bm12 residue at position 67 can be recognized by this hybrid but not in all contexts. Hybrid 17BBM136 reacts with FT6.3 (bm^{67},b^{70},b^{71}) or with FT6.8 (b^{67},bm^{70},bm^{71}), but more vigorously with FT7.2 (bm^{67},bm^{70},bm^{71}). The hybridoma 17BBM71 is most dependent on the presence of the bm12-derived glutamine at position 70 as it reacts with the FT6.4 (b^{67},bm^{70},b^{71}), FT6.5 (b^{67},bm^{70},bm^{71}), and FT7.2 (bm^{67},bm^{70},bm^{71}) transfectants. Surprisingly, this hybrid is not stimulated with the FT7.7 (bm^{67},bm^{70},b^{71}) transfectant. Finally, 4BBM17 inexplicably reacts much more vigorously with the FT6.7 (bm^{67},b^{70},bm^{71}) transfectant than with the transfectant that carries the complete bm12 mutation.

TABLE III

Alloantigen Presenting Cell

	Hybrid	FT6.3 bm b b	FT6.4 b bm b	FT6.5 b b bm	FT6.7 bm b bm	FT6.8 b bm bm	FT7.7 bm bm b	FT7.1 b b b	FT7.2 bm bm bm	B6 spleen	BM12 spleen
A	1 8 BBM 8										376*
	2 17 BBM 6										174
	3 17 BBM 51										46
	4 17 BBM 151										>1,000
	5 18 BBM 78										618
	6 18 BBM 19										322
B	1 8 BBM 10								135		128
	2 17 BBM 43								267		22
	3 17 BBM 179								66		357
	4 3 BBM 74								154	5	400
	5 6 BBM 51								98		347
	6 18 BBM 68								91	35	>1,000
	7 16 BBM 53								286		424
	8 17 BBM 64								323		236
	9 18 BBM 37								178		185
	10 18 BBM 109								596		443
	11 18 BBM 153								462		564
	12 18 BBM 22								316	6	16
C	1 18 BBM 139						149		30		308
	2 6 BBM 131						60		111		100
	3 5 BBM 98						20		9	11	19
	4 17 BBM 190						17		81		66
	5 15 BBM 139		24				105		174		277
	6 17 BBM 68	11	12				312		450		618

TABLE III (*continued*)

	Hybrid	FT6.3 bm b b	FT6.4 b bm b	FT6.5 b b bm	FT6.7 bm b bm	FT6.8 b bm bm	FT7.7 bm bm b	FT7.1 b b b	FT7.2 bm bm bm	B6 spleen	BM12 spleen
D	1 15 BBM 82		92			125	87		412		83
	2 18 BBM 9		195			67	57		>1,000		>1,000
	3 18 BBM 17		904			501	100		656		430
	4 18 BBM 23		34			>1,000	390		>1,000		992
E	1 16 BBM 34					>1,000			>1,000	16	>1,000
	2 16 BBM 30					28			411	8	522
	3 16 BBM 100					390			486		317
	4 17 BBM 82					366			572		110
	5 17 BBM 159					137			513		553
	6 18 BBM 98					163			162		>1,000
	7 18 BBM 138					>1,000			>1,000		956
	8 18 BBM 160					199			700		130
	9 18 BBM 49					9			21		97
F	1 18 BBM 142	>1,000		6					>1,000		>1,000
	2 17 BBM 136	79				267			952		>1,000
	3 17 BBM 71		656			>1,000			717		>1,000
	4 4 BBM 17			158	531				20		260

* Units of IL-2 present in supernatants of hybrids stimulated by the indicated presenting cells were determined by the ability of the supernatants to support the growth of the IL-2-dependent cell HT-2.

Discussion

The three amino acid substitutions, Ile→Phe^{67}, Arg→Gln^{70}, and Thr→Lys^{71}, which distinguish the β chain of I-A^b from that of I-A^{bm12}, were originally detected by the ability of mice that differed at only this locus to reciprocally reject skin grafts. It has also been shown that these mice differ in their ability to present and respond to exogenous antigen (27, 28). Understanding in molecular terms how three amino acid substitutions so dramatically perturb the T cell repertoire of these mice should provide important information about the relationship between MHC molecule structure and function regarding both thymic selection and antigen presentation. Although the bm12 mutation is the sole spontaneous class II mutation discovered so far (vs. 20 spontaneous class I mutations), it seems likely that the mutation is biologically effective because it involves residues that are critical to class II MHC function. Sequence comparisons of the mutated I-A^{bm12} β chain with those of other I-A β chains show the mutated residues to lie in the third hypervariable region. We have previously demonstrated the importance of the hypervariable regions to antigen presentation and allostimulation using exon-shuffling techniques to localize relevant structures to the NH_2-terminal 97 residues (29–31). Others have also demonstrated the importance of this region using in vitro mutagenesis to change specific amino acids in the hypervariable regions including residues 65, 66, and 67 of I-A^b, a change that overlaps with the bm12 mutation (32). In previous experiments (11), we have investigated the relative contribution of each of the three amino acid substitutions that constitute the bm12 mutation to the observed serologic differences and to the changes in antigen presentation. mAbs that distinguish I-A^b from I-A^{bm12} are primarily or solely dependent on the change at residue 70 (Arg→Gln), implying that this residue is uniquely accessible to antibody (and presumably to TCRs). In the same experiments, we also demonstrated that all three residues were important to antigen presentation to T cells, suggesting that amino acids 67 and 71 affected quantitative and/or qualitative aspects of peptide binding to Ia. Using the existing structure/function data and sequence similarity with the human class I molecule HLA-A2 for which the crystal structure has been solved, Brown et al. have proposed a physical model for class II molecules. Consistent with the observed importance of the bm12 mutation, this model places the mutated, bm12 residues on one of the helical portions of the molecule that form the presumed antigen binding site. According to the model, residues 67 and 71 point down into the binding site, and thus, are ideally suited to affect binding of antigen, i.e., peptides. Residue 70 points up and away from the helix and thus is accessible to responding T cells and their receptors. Thus, both structural modeling and functional results of experimental manipulation of the I-A^b molecule yield a congruent picture of the probable roles of residues β67, 70, and 71 in peptide or TCR binding.

In the present set of experiments, we have extended our observations to a large panel of previously established (12) alloreactive T cell hybridomas, specific for I-A^{bm12}. We examined the response of 41 I-A^{bm12}-specific hybridomas (see Table I) to a panel of eight fibroblast cell lines transfected with constructs encoding various permutations of the I-A$^{b/bm12}$ molecule (see Table II). Surprisingly, the reactivities of these hybrids segregated into a small number of patterns. Six hybrids (see Table III *A*) do not respond to the I-A^{bm12} molecule expressed on the surface of L cells but do respond when I-A^{bm12} is expressed on the surface of splenocytes. This is dis-

cussed further below. 12 of the 35 hybrids that can recognize I-A^{bm12} on transfected fibroblasts require the "complete" I-A^{bm12} phenotype (I-A^{bm12}-encoded aminoacids at positions 67, 70, and 71). Of the 23 hybrids that can be stimulated by less than the "full" bm12 mutation, four hybrids (see Table III *D*) are capable of responding when position 70 represents the only I-A^{bm12}-encoded amino acid, six hybrids (see Table III *C*) require the presence of I-A^{bm12}-encoded amino acids at positions 67 and 70, and nine hybrids (see Table III *E*) require I-A^{bm12}-encoded residues at positions 70 and 71 for the production of IL-2. Finally, four hybrids (see Table III *F*) have reactivities that are complex and not easily categorized. These hybrids generally recognize a specific I-A^{bm12} residue but not in all contexts. Interestingly, the hybridomas in each category do not express a particular set of Vα or Vβ gene segments. Thus, even when these alloreactive hybrids are grouped by their fine specificity, no simple pattern of Vα or Vβ expression emerges. Apparently, alloreactive T cells with the same fine specificity express a diverse array of V gene segments.

The most striking feature of our data is the importance of the residue at position 70. Only 4 of the 35 hybrids that are capable of responding to the I-A^{bm12} molecule expressed on the surface of L cells can tolerate the loss of the I-A^{bm12}-encoded mutant residue at position 70 and two (17BBM68 and 17BBM136) of these are 10-fold less reactive when this amino acid is I-A^{b} derived. It is remarkable that a single substitution at this position should interfere with the majority of I-A^{bm12}-reactive T cell hybrids, and this finding strongly suggests that this residue directly interacts with TCRs. Such an interaction would be consistent with the proposed model of class II structure that places the Gln^{70} of the I-A^{bm12} β chain on one of the α-helical regions pointing up and away from the presumed Ag binding site, i.e., toward the T cell. While we favor the interpretation that Gln^{70} is directly involved in binding to alloreactive TCRs, it is also possible that this altered residue sterically inhibits a separate TCR-MHC interaction or a peptide-MHC interaction.

The large majority (28 of 35) of I-A^{bm12}-reactive hybrids that recognize I-A^{bm12} on transfected fibroblasts show an absolute requirement for I-A^{bm12}-encoded amino acids at position 67 and/or 71 as well. Since the amino acids at these two positions are thought to point down and into the peptide binding groove, their effects on allorecognition may be indirect. One obvious explanation is that the actual ligand recognized by alloreactive T cells is a self-peptide/MHC complex and that the amino acids at positions 67 and 71 affect what peptides can actually be bound by the I-A^{bm12} glycoprotein. Permuted I-A^{bm12} molecules may not bind the same proteolytic fragments as the "complete" I-A^{bm12} molecule, accounting for the observed patterns of reactivity. Further support for this idea comes from the finding that six of the I-A^{bm12}-specific hybrids (Table III *A*) do not respond to the "complete" I-A^{bm12} molecule expressed on the surface of L cells but do respond when this molecule is expressed on the surface of splenocytes. Given the presence of identical I-A^{bm12} genes in the two cell types, it is possible that fibroblasts lack a particular proteolytic fragment that contributes to the peptide/MHC ligand; this potentially explains the behavior of the hybridomas listed in Table III *A*. This phenomenon has been observed for I-E-reactive T cell hybrids that respond to I-E expressed on splenocytes but not to I-E expressed on fibroblasts (33). Conversely, we have identified two hybrids (17BBM43 and 18BBM22) that weakly recognize I-A^{bm12} expressed on spleen cells but secrete 5–10-fold more IL-2 when stimulated with I-A^{bm12}-transfected fibro-

blasts. Fibroblasts may express a peptide that contributes to the ligand recognized by the TCRs on these two hybridomas that is lacking or poorly expressed in splenocytes. Another mechanism by which the mutations at positions 67 and 71 may have their effect is that while they still allow binding of certain peptides, they impose an altered conformation on the resultant peptide-MHC complex and thus change the epitope available to T cells.

From these experiments, we have been able to determine the relative contribution of the individual amino acid replacements towards the alloantigenicity of the I-A^{bm12} molecule. Clearly, the amino acid at position 70 has the most pronounced effect on the alloantigenicity of I-A^{bm12} in that the reversion of Gln^{70}→Arg^{70} interferes with allorecognition by 33 of 35 I-A^{bm12}-reactive hybrids. This amino acid most likely participates in a direct interaction with the majority of I-A^{bm12}-specific TCRs. Our data also provide experimental support for the Brown et al. (7) model of class II MHC structure, which predicts that the amino acid at position 70 of the I-A β chain points up and away from the surface of the MHC molecule and is accessible to relevant TCRs. Reversion of the amino acids at positions 67 and 71 affects a significant but smaller fraction of I-A^{bm12}-reactive T cells. The effects of substitutions at these positions may be mediated through the effects of binding of self-peptides on the antigen binding site.

Summary

The I-A^{bm12} mutation has been used extensively to study the relationship between structure and function of murine class II major histocompatibility molecules. I-A^{bm12} differs from I-A^b by three amino acid replacements in the Aβ chain, and the proposed structural model of the I-A^{bm12} molecule places these three amino acid substitutions along one of the α-helices where they may affect both antigen and TCR binding. Two of the substitutions, Ile→Phe^{67} and Thr→Lys^{71}, are thought to point into the binding site, whereas the third substitution, Arg→Gln^{70}, is thought to point up and hence, be available for binding to the TCR. These predicted orientations are consistent with serologic analysis of the bm12 molecule, which demonstrates that residue 70 is uniquely accessible to mAbs distinguishing I-A^b from I-A^{bm12}. In this study we have determined the influence of each of these amino acid substitutions on the ability of the resulting molecules to stimulate a panel of I-A^{bm12} (allo) reactive T cell hybridomas. Our experiments indicate that reversion of the amino acid at position 70 from Gln (I-A^{bm12}) to Arg (I-A^b) interferes with allorecognition by 33 of 35 I-A^{bm12}-reactive hybridomas. On the other hand, many hybrids can tolerate amino acid substitutions at positions 67 or 71. Single amino acid substitutions at position 67, 70, or 71 are recognized by only a minority of I-A^{bm12}-specific hybrids and usually the reactivity is greatly diminished. These data are most consistent with the idea that the amino acid at position 70 directly interacts with the TCR during allorecognition. The additional effects of residues 67 and 71 are consistent with a contribution by bound peptide to the allorecognition process.

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