Brief Definitive Report

A CLASS II GENE CONVERSION EVENT DEFINES AN ANTIGEN-SPECIFIC *Ir* GENE EPITOPE

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The Ia glycoproteins, encoded by the *I-A* and *I-E* regions of the murine major histocompatibility complex (MHC), are the immune response (*Ir*) gene products. As a result, theories of *Ir* gene control can be analyzed by comparing immune responses of mice that have a defined mutation in an I region gene with those made by wild-type animals. The *I-A*^{b} mutant B6.C-*H-2*^{bm12} (bm12) and their wild-type counterpart C57BL/6 (B6) mice provide such a model system. Biochemical studies (1, 2) reveal that the mutant mouse has three peptide substitutions in the A^b_β chain, while the A^b_α chain seems to be unchanged. DNA sequencing of the *I-A*$^{bm12}_\beta$ gene shows (3) three nucleotide differences in the first domain which are clustered within a stretch of 14 nucleotides coding for amino acid residues 67–71 of the mature polypeptide.

The reactivity profile of bm12 mice to antigens that are known to be under *Ir* gene control mapping to *I-A*^{b} has been studied. As shown in Table I, despite the mutation, the general *Ir* phenotype characteristic of *I-A*^{b} mice was retained. However, bm12 mice are unresponsive to H-Y antigen (4) and beef insulin (5), compared with the B6 wild-type. Our results suggest that the mutation affected a discrete functional domain on the A^b_β chain. Our recent data (6) revealed that bm12 mice possess an insulin-specific immune potential. Therefore, we considered that the alteration of the nucleotide sequence may not have resulted in complete unresponsiveness, but rather in a change in the fine specificity of the immune response to these antigens. Heterologous insulins are useful because *H-2*^{b} mice respond with strong T cell proliferation and antibody responses only to beef insulin, whereas *H-2*^{k} mice respond to sheep and horse insulin (Table II; references 7–11). These types of insulin differ at a single amino acid in position 9 of the A chain loop (Table II).

Materials and Methods

Mice. C57BL/6 and B10.A mice were purchased from The Jackson Laboratory, Bar Harbor, ME. B10.A(4R) and B10.A(5R) mice, and B6.C-*H-2*^{bm12} breeding pairs were kindly supplied by Dr. M. Dorf, Harvard Medical School and Dr. J. Forman, University of Texas Southwestern Medical School, respectively. Mice were used at 5–7 wk of age.

Antigens. Insulins (Sigma Chemical Co., St. Louis, MO) were dissolved in 0.5% sodium carbonate and adjusted to 1 mg/ml with phosphate-buffered saline. Purified protein

This work was supported by grant AI-14910 from the National Institutes of Health and grant PCM-8109166 from the National Science Foundation. B. Huber is the recipient of Research Career Development Award AI 00527; P. Hochman is a fellow of the Charles A. King Trust, Boston, MA.

derivative of mycobacteria was purchased from Connaught Research Laboratories, Toronto, Canada.

Immunizations. Mice were injected in the hind footpads and in the base of the tail with an emulsion of insulin in complete Freund's adjuvant (H37Ra) (Difco Laboratories, Detroit, MI) containing 0.5 mg/ml killed *Mycobacterium tuberculosis.* 50 μg of insulin in 0.1 ml of emulsion were injected into the three sites.

Antigen-specific T Cell Proliferation Assays. 7–14 d after immunization, the draining popliteal, paraaortic, and inguinal lymph nodes, or peritoneal exudate cells induced with thioglycollate, were collected. Cells were placed in single-cell suspension and T lymphocytes were enriched in a nylon wool–nonadherent fraction. 2×10^5 T cells were then incubated in 96-well microtiter plates for 96 h (37°C incubator, 5% CO_2 in air) with 10^5 irradiated (2,000 rad) syngeneic spleen cells (serving as antigen-presenting cells [APC]) in a final volume of 0.2 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2-mercaptoethanol, glutamine, penicillin, streptomycin and Hepes buffer. Antigens were present at a final concentration of 100 μg/ml. During the final 18 h of culture, each well was pulsed with 1 μCi of tritiated thymidine (30 Ci/mM; ICN Pharmaceuticals, Inc., Irvine, CA). Cells were harvested on glass fiber filter paper using a microharvester (MASH II). Thymidine incorporation is reported as the mean counts per minute of triplicate samples. Underlined values in the tables are significant ($P < 0.01$) according to the Student's *t* test.

Results

bm12 Mutation Alters the Specificity of Insulin Recognition. B6, bm12, and B10.A mice were injected with beef or sheep insulin, and the ability of their primed T cells to proliferate in vitro upon challenge with insulin was assessed. One proliferation study with beef insulin–primed cells (Table III) confirms previous data (7–11) indicating that B6, but not bm12 or B10.A, mice respond to beef insulin (summarized in Table II). Proliferation studies with sheep insulin–primed T cells are represented by three experiments (Tables III and IV). Sheep insulin–immune T cells from bm12 mice proliferated upon challenge with sheep insulin. As expected, such a response was obtained with B10.A but not with B6 mice. Thus, the bm12 mice, like B10.A mice (which are *H-2*^{k}), are responders to sheep insulin. It should be noted that T cells of sheep insulin–primed B6 and bm12 mice responded to challenge with beef insulin in vitro, although the immune T cells from the B10.A mice did not. Such priming by "forbidden" insulin variants has been reported previously (9) for *H-2*^{b} mice. This is another example of the general retention of the *I-A*^{b} *Ir* phenotype by bm12 mice.

Ir Gene for the Sheep Insulin Response of H-2^k *Mice Maps to the I-E Subregion.* Having shown that bm12 mice, like B10.A mice, respond to sheep but

TABLE I

Immune Response Pattern of H-2^b *Wild-type and Mutant Mice, and H-2*^{k} *Recombinant Mice*

	MHC haplotype				(PHE,G)–A––L	(T,G)–A––L	(H,G)–A––L	H-Y antigen	Beef insulin	Sheep insulin
	A_β	A_α	E_β	E_α						
B6 H-2^b	b	b	b	b	High	High	Low	High	High	Low
bm12 H-2^{bm12}	bm12	b	b	b	High	High	Low	Low	Low	High*
B10.A H-2^k	k	k	k	k	High	Low	High	+/–	Low	High
B10.A(4R) H-2^{b4}	k	k	k	b	High	Low	High	+/–	Low	Low*

Data are compiled from references 6–16.

* As determined in this paper.

‡ The B10.A(5R) mice express the E^b_β first domain and the E^k_β second domain.

TABLE II

Ir Gene Control of Murine Response to Insulins

MHC haplotype				Species	Amino acid sequences of heterologous insulins						
					A CHAIN				B CHAIN		
$H\text{-}2^{bm12}$	$H\text{-}2^{b}$	$H\text{-}2^{k}$	$H\text{-}2^{d}$		A4	A8	A9	A10	B4	B10	B31
–	–	–	–	Mouse	Asp	Thr	Ser	Ile	Lys	Pro	Ser
+*	–	+	+	Sheep, Horse	Glu	Ala	Gly	Val	Asn	Ser	Ala
–	+	–	+	Beef	Glu	Ala	–	Val	Asn	Ser	Ala
–	–	–	+	Pork	Glu	–	–	–	Asn	Ser	Ala

Data are compiled from references 9–12.
* As determined in this paper.

TABLE III

Response of the I-A^{b} Mutant bm12 and Wild-type B6 Mice to Beef and Sheep Insulin

Antigen	Beef insulin–primed T cells		Sheep insulin–primed T cells			
			Experiment 1		Experiment 2	
	B6	bm12	B6	bm12	B6	bm12
—	4,332	2,187	4,073	5,773	2,661	3,798
Sheep	5,292	3,487	5,334	18,334	5,284	12,361
Beef	12,731	4,332	15,724	16,279	12,453	28,105
Pork	ND	ND	3,433	8,684	3,124	3,712
PPD	21,349	33,979	35,438	62,139	34,114	30,145

Data represent cpm tritiated thymidine incorporated. ND, not determined.

TABLE IV

I Region Restriction of the Immune Response to Sheep Insulin

Antigen	Sheep insulin–primed T cells:				
	Experiment 1		Experiment 2		
	B10.A(4R)	B10.A	B6	bm12	B10.A(4R)
—	3,174	3,057	4,604	8,776	5,819
Sheep	2,906	11,793	7,329	28,284	4,682
Beef	3,452	3,713	23,649	40,242	6,113
PPD	37,527	36,296	37,320	38,222	41,385

Data represent tritiated thymidine incorporated.

not beef insulin, we considered that the bm12 mutation arose by an intergenic exchange of $H\text{-}2^{b}$- and $H\text{-}2^{k}$-like sequences. To define the origin of the putative donor sequence, we compared the sheep insulin response of $H\text{-}2^{k}$ recombinant B10.A ($A^{k}_{\beta}A^{k}_{\alpha}E^{k}_{\beta}E^{k}_{\alpha}$) and B10.A(4R) ($A^{k}_{\beta}A^{k}_{\alpha}E^{k}_{\beta}E^{b}_{\alpha}$) mice. As shown in Table IV, T cells from sheep insulin–primed B10.A(4R) mice did not proliferate upon challenge in vitro with sheep insulin. Since B10.A(4R) mice express only $A_{\alpha,\beta}$ chains, responsiveness to sheep insulin in $H\text{-}2^{k}$ mice must map to the $E_{\alpha,\beta}$ complex. This conclusion was confirmed by studies (data not shown) in which the proliferation of sheep insulin–primed T cells derived from $H\text{-}2^{k}$ CBA and A/J mice was blocked by the addition of monoclonal antibodies directed against the E^{k} but not the A^{k} molecule. The E^{k}_{β} gene is likely to control the response of $H\text{-}2^{k}$ mice to sheep insulin, since the E^{k}_{α} gene is virtually nonpolymorphic.

Sheep Insulin Recognition Maps to the Ia Epitope 67-71, Expressed by $A^{bm12}_{\beta}/E^{k}_{\beta}/E^{b}_{\beta}$

TABLE V

$E_\beta^b/E_\beta^k/A_\beta^{bm12}$ Ia Epitope Controls the Immune Recognition of Sheep Insulin

APC	Insulin	Sheep insulin–primed T cells		
		B10.A	bm12	
			Exp. 1	Exp. 2
B10.A	—	2,581	9,031	13,842
	Sheep	7,593	13,419	21,116
	Pork	1,904	7,586	8,203
bm12	—	4,201	5,170	7,029
	Sheep	9,031	15,129	13,365
	Pork	2,554	7,545	10,627
B10.A(5R)	—	3,910	8,933	ND
	Sheep	14,465	17,294	ND
	Pork	ND	6,492	ND

Data represent counts per minute tritiated thymidine incorporated. ND, not determined.

67-71. The mapping of the sheep insulin response of *H-2^k* mice to the *I-E_β^k* gene suggests that, if the bm12 mutation arose by a gene conversion event, the donor sequence was derived from the second exon of the *I-E_β^b* gene (12), which is homologous at the bm12 mutation site, and its flanking regions, to the *I-A_β^{bm12}* (3) and *I-E_β^k* (13) genes. To assess whether the amino acid cluster 67-71 controls recognition and/or presentation of sheep insulin, and whether bm12 and *H-2^k* T cells recognize the same sheep insulin determinant in the context of this Ia epitope, sheep insulin–immune bm12 and B10.A peritoneal T cells were challenged in vitro with sheep insulin presented by bm12, B10.A, or B10.A(5R) APC. These APC express $A_\beta^{bm12}A_\alpha^b,E_\beta^kE_\alpha^k$, and $E_\beta^bE_\alpha^k$ Ia molecules, respectively. As shown in Table V, sheep insulin–immune bm12 and B10.A T cells responded to sheep, but not pork, insulin presented by either of these three APC.

Discussion

We have shown that the mutation in the *I-A_β^b* gene results in the expression of an *Ir* gene epitope that allows for an *I-A*-restricted immune response to sheep insulin (since the bm12 mice do not express an E molecule). The bm12 mutation then defines the actual nucleotide sequence controlling the specificity of the immune response to this nominal antigen. Moreover, the insulin system provides for a very precise correlation between such nucleotide sequences and various insulin determinants, dependent upon a single amino acid change (Table I).

The observation that three substitutions in the first domain of the A_β^b chain result in an alteration of the specificity of immune recognition, such that bm12 mice respond to sheep but not beef insulin, suggests that the association of beef insulin–unique determinants with A_β^{bm12} resembles self, while the association of sheep insulin–specific determinants is significantly different from self, so as to allow T cell activation. We have shown previously (6) that such "self-reactive", beef insulin–specific T cells can be rescued by hybridization from beef insulin–immune bm12 mice.

Sheep insulin recognition by bm12 mice and the mapping of the *Ir* gene for the sheep insulin response of *H-2^k* mice to the *I-E* subregion correlate with reports of DNA sequence homology of the *I-A_β^{bm12}* (3), *I-E_β^b* (12), and *I-E_β^k* (13) genes at the sites where the *I-A^{bm12}* differs from the *I-A^b* gene. These functional

and sequencing data, therefore, strongly suggest that the bm12 mutation arose by a gene conversion event in which there was a transfer of $I\text{-}E^b_\beta$ nucleotide sequences to the $I\text{-}A^b_\beta$ gene, resulting in a sheep insulin–specific *Ir* gene epitope on the A^{bm12}_β molecule. Indeed, our observation of sheep insulin recognition by B10.A and bm12 sheep insulin–specific T cells in the context of B10.A, bm12, and, most notably, B10.A(5R) APC that express the A^{bm12}_β, E^k_β, and E^b_β epitopes, respectively, substantiates this hypothesis. Since there is very little sequence homology between A^b_α and E^k_α molecules, or between A^{bm12}_β and E^k_β molecules except for the conserved region 67-71, the bm12 mutation site forms the actual *Ir* gene epitope. Furthermore, the antigen cross-presentation by the three different APC strongly suggests that the bm12 mutation defines a "histotope"; i.e., a T cell–Ia interaction site. This is further substantiated by our previous findings (6) that the majority of B6 and bm12 T cell clones, regardless of their antigen specificity, respond to antigen in the context of their own APC only.

It is of particular interest that, although APC of B10.A(5R) mice phenotypically express the sheep insulin–specific E^b_β epitope, their sheep insulin–primed T cells do not proliferate when challenged in vitro with sheep insulin, but rather, like sheep insulin–primed T cells of B6 mice, proliferate upon challenge with beef insulin (data not shown). The failure of the B10.A(5R) mice to respond to sheep insulin may reflect preferential recognition of insulin in the context of $I\text{-}A^b$ rather than $I\text{-}E^b$ gene products upon priming in vivo and/or subsequent challenge in vitro, as has been reported (15) for the response of $H\text{-}2^k$ mice to L-tyrosine-*p*-azobenzenearsonate. Alternatively, the failure of B10.A(5R) mice to respond to sheep insulin may reflect a hole in their insulin-specific T cell repertoire or epitope-specific suppression. These points are currently being investigated.

Finally, not all functional Ia recognition units were altered by the bm12 mutation, which is reflected in the apparent retention of the $I\text{-}A^b$ reactivity profile by the mutant. Our observation that sheep insulin–primed bm12 T cells proliferate upon challenge, not only with sheep insulin, but also with beef insulin, as do sheep insulin–primed B6 T cells, supports the existence of multiple functional domains on Ia molecules (16).

Summary

To assess the role of Ia epitopes in conferring specificity for the immune response to nominal antigen, we compared the insulin response of mice with a defined mutation in the $I\text{-}A^b_\beta$ gene, the B6.C-$H\text{-}2^{bm12}$ (bm12), with that of wild-type $H\text{-}2^b$ C57BL/6 (B6) mice. We report that the bm12 mutation resulted in a selective alteration of the specificity of insulin recognition, such that bm12 mice responded upon immunization with sheep but not beef insulin, which differ by only one amino acid at position 9 of the insulin A chain. Thus, the bm12 mutation allows for the definition of the actual nucleotide sequence coding for an Ia epitope that is responsible for controlling the specificity of immune recognition of insulin. Furthermore, we show that the sheep insulin response of $H\text{-}2^k$ mice is controlled by the E molecule and that sheep insulin can be recognized by primed bm12 and $H\text{-}2^k$ T cells in the context of either bm12, B10.A, or B10.A(5R) antigen-presenting cells. Our data suggest that the mechanism for

the bm12 mutation was the intergenic transfer of a hypervariable region in the first domain that is identical in the $I\text{-}A_\beta^{bm12}$, $I\text{-}E_\beta^b$, and $I\text{-}E_\beta^k$ genes.

Received for publication 20 August 1984 and in revised form 26 September 1984.

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