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I-A Mutation Resulted in a Selective Loss of an Antigen-Specific *Ir* Gene Function

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The immune responses to several antigens were compared in the *I-A* mutant mouse strain B6.C-*H-2*^{bm12} and the wild-type strain C57BL/6. With a lymph node cell proliferation assay, the response to two of these antigens, beef insulin and (TG)A-L, was demonstrated to be controlled by a gene in the *I-A*^{b} region. B6.C-*H-2*^{bm12} mice failed to respond to beef insulin, while their responses to (TG)A-L, DNP-OVA and PPD were comparable with those of the wild-type strain C57BL/6. Taken together with previous studies, these data suggest that the product of a single pleiotropic *I-A* gene, an Ia molecule, functions as a histocompatibility, Ia, and MLR antigen, as well as a necessary component for *Ir* gene function. Furthermore, the data reported here demonstrate that Ia molecules have multiple functional "*Ir* determinants," one of which has been altered in the B6.C-*H-2*^{bm12} mutant. The B6.C-*H-2*^{bm12} mice, therefore, represent a powerful analytical tool for the understanding of the cellular and molecular basis for *Ir* gene control of the immune response.

Key words: insulin, T cell proliferation, Ia molecules

The *I* region of the murine major histocompatibility complex is composed of a series of loci that control a diverse array of immunologic functions, including the *Ir* genes, which control the immune response to a variety of protein antigens [1], and the genes that encode the Ia cell surface glycoproteins [2]. The relationship of *Ir* products and Ia molecules is not precisely defined since the total number of *I*-region gene products and each of their individual functions is still unclear. In addition to *Ir* genes and Ia specificities, several other immunologic phenotypes are controlled by *I* region loci. These determinants include histocompatibility antigens [3], the antigens recognized in mixed lymphocyte reaction (MLR)

Abbreviations: (TG)A-L, multichain poly (L-tyrosine: L-glutamic acid) poly DL-alanine: Poly-L-lysine; DNP-OVA, dinitrophenyl conjugated ovalbumin; CFA, complete Freund's adjuvant; MLR, mixed lymphocyte reaction.

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[4] and graft versus host (GVH) response [5], and the cell membrane antigens, which determine the successful interactions between macrophages and T cells [6] and limit the cooperation between T and B cells in the immune response [7]. Several studies suggest that the above-listed membrane structures are all determinants on Ia molecules.

The selective study of genes encoded in the *I* region has traditionally been conducted by comparing available intra-*H-2* recombinant strains that have subdivided the *I* region into at least five juxtaposed subregions, *A, B, J, E,* and *C* [8]. However, since the number of genes contained within each region is uncertain, the comparisons of allogeneic mouse strains must involve an unknown number of genetic differences. A new approach for the selective study of a single *I-A* region gene has been made possible by discovery of the *I-A* region mutation, B6.C-*H-2*^{bm12} [9,10]. The lesion in this mutant strain is known to result in skin graft rejection, MLR stimulation, and changes in antibody-recognized Ia specificities [11]. This mutation thus provides a new tool for assessing the role of Ia antigens in the immune response.

In this communication we compare the proliferative responses between T cells derived from B6.C-*H-2*^{bm12} and its parental strain, C57BL/6, following immunization with complete Freund's adjuvant containing pork insulin, beef insulin, poly(L-tyrosine:L-glutamic acid)poly DL-alanine:poly-L-lysine [(TG)A-L] and dinitrophenyl conjugated ovalbumin (DNP-OVA). The immune response to antigens (TG)A-L and beef insulin is under *Ir* gene control; ie, the parental strain B6 are responders to both antigens. Our data indicate a selective deletion of the T cell proliferation response of B6.C-*H-2*^{bm12} to beef insulin while responsiveness to (TG)A-L is preserved, as is that to multiple determinant antigens, DNP-OVA, and purified protein derivatives (PPD).

MATERIALS AND METHODS

Animals

C57BL/10Sn, B10.A/SgSn, B10.D2/NSn, and C57BL/6 (synonym B6) were obtained from the Jackson Laboratory, Bar Harbor, ME. Strain B6.C-*H-2*^{bm12} was a gift from Dr. Roger Melvold, Northwestern University. B10.MBR and B10.AKM were gifts of Dr. David Sachs, National Institutes of Health.

Antigens

The following antigens were used for immunizing animals or for immune cell challenging in vitro: beef insulin, (TG)A-L, DNP-OVA. Beef insulin was obtained from Novo Research Institute, Denmark. It contained less than 1 ppm of proinsulin contamination. (TG)A-L (lot MC9; average mol wt 280,000) was purchased from Miles Laboratories. The dinitrophenyl (DNP) conjugate of ovalbumin was prepared as described elsewhere [12]. Purified protein derivative of tuberculin (PPD) was obtained from Connaught Medical Research Laboratory, Toronto, Canada.

Immunization

A mixture containing beef insulin, (TG)A-L and DNP-OVA antigens was emulsified in complete Freund's adjuvant (CFA) (0.5 mg/ml killed Mycobacterium

tuberculosis, H37Ra, Difco Laboratories, Inc., Detroit, MI). Mice were immunized by injections of hind foot pads and at the base of the tail subcutaneously with total of 0.2 ml emulsions containing 50 μg of each of the antigens.

Cell Collection and Separation

The lymph node cell proliferation assay described by Corradin and Chiller [13] was followed with some modification. Inguinal, periaortic and popliteal lymph nodes were dissected 11 days after immunization. The nodes were removed aseptically and teased apart by pressing between two glass slides. After two washings, the lymph node cell suspension was applied to a nylon wool adherence column (nylon wool, Fenwall Laboratories, Morton Grove, IL) prepared as described elsewhere [14]. The enriched T cell populations obtained were suspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) supplemented with fresh L-glutamine (0.3 mg/ml), penicillin (100 units/ml), gentamicin (10 μg/ml), 2-mercaptoethanol (5×10^{-5} M), and 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY). Irradiated (2,000 R) spleen cells from normal mice were used as a source of antigen-presenting cells.

Cell Culture and Assay of DNA Synthesis

Aliquots of 0.2 ml of 3×10^5 T-cell suspension with 2×10^5 irradiated spleen cells were pipetted into each well of flat bottom microtiter plates. Ten microliters of antigens at appropriate dilutions were added to the wells. Each experimental point was set up in triplicate. The microtiter plates were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Sixteen to 24 h before harvesting, 1 μCi of tritiated thymidine (6.7 Ci/mM, New England Nuclear Co., Boston, MA) was added to each well. Cells were harvested onto glass fiber filter paper by the use of a microharvester.

Tritiated thymidine incorporation was then determined by liquid scintillation spectrometry, and the results are reported as △cpm (counts per minute above medium control).

RESULTS AND DISCUSSION

The *Ir* gene function of the *I-A* mutant strain, B6.C-*H-2*^{bm12} was compared with its parental strain, B6, using a lymph node cell proliferation assay with five different antigens: beef insulin, pork insulin, (TG)A-L, DNP-OVA, and PPD. For the purpose of having all the necessary controls and for maximal utilization of mice, conditions were established such that all antigens could be tested simultaneously by immunizing the animals with a mixture of all five antigens. With this assay system, it was first established that the immune response to these antigens of restricted heterogeneity was controlled by an *I-A*^{b} region gene. As expected from previous studies [15,16] B10 mice were found to be responders to (TG)A-L and beef insulin, while B10.A mice were nonresponders (see Table I). Since the recombinant strains B10.A(4R) and B10.MBR were both found to be non-responders, the proliferative responses to both (TG)A-L and beef insulin were shown to require a gene coded in the *I-A*^{b} subregion. This conclusion supports the recent data of Markman et al [17], which also used the B10.MBR recombinant to map the (TG)A-L antibody response to the *I-A*^{b} subregion.

TABLE I. Mapping of Genes Controlling Proliferative Response to Beef Insulin and (TG)A-L*

											Δ cpm H^3-thymidine incorporation				
		MCH alleles									Beef insulin	(TG)A-L	DNP-OVA	PPD	cpm Medium
Strain	*H-2*	*K*	*A*	*B*	*J*	*E*	*C*	*S*	*G*	*D*	(100 μg/ml)	(100 μg/ml)	(10 μg/ml)	(10 μg/ml)	control
B10	b	b	b	b	b	b	b	b	b	b	10,425	12,301	35,609	54,337	7,491
B10.A (4R)	h4	k	k	b	b	b	b	b	b	b	1,635	0	61,800	81,267	20,323
B10.MBR	bq1	b	k	k	k	k	k	k	k	q	1,043	0	64,570	66,189	9,406
B10.AKM	m	k	k	k	k	k	k	k	k	q	1,712	42	35,602	42,234	6,127
B10.A	a	k	k	k	k	k	d	d	d	d	943	0	36,807	46,297	9,063
B10.D2	d	d	d	d	d	d	d	d	d	d	10,639	1,671	40,728	58,511	15,339
B6	b	b	b	b	b	b	b	b	b	b	20,370	20,772	48,281	65,861	25,468
B6.C-*H-2*^{bm12}	bm	b	bm	b	b	b	b	b	b	b	0	9,058	34,994	36,227	11,705

*Mice were immunized with 50 μg beef insulin, 50 μg (TG)A-L, and 50 μg DNP-OVA in complete Freund's adjuvant in hind foot pads. Eleven days after immunization T cells from draining lymph nodes were collected and cultured in flat-bottom microtiter plates at 3×10^5 cells/well with the same number of irradiated spleen cells from normal mice of the appropriate strain. Antigens at different dilutions were added to triplicate wells. The cultures were incubated at 37°C, 5% CO_2 in air for 4 days. ^{3}H-thymidine was added 18 h prior to harvesting. Δcpm was calculated by subtracting medium control (right-hand column) from antigen-stimulated cultures. Each experiment point represents the average of triplicates.

In two separate experiments B6.C-*H-2*^{bm12} and B6 mice were compared for their ability to respond to these same four soluble protein antigens. The first experiment (Table I) indicated that mutant mice respond comparably with the wild-type to (TG)A-L, DNP-OVA and PPD. However, the same mutant mice failed to respond to beef insulin as the wild type mice did, thus indicating that in the B6.C-*H-2*^{bm12} mutation there is a selective loss of the *I-A*^{b} region-controlled recognition of only a single protein antigen. Data from the second experiment, which are shown in Figure 1, focus on the comparisons between the responses to (TG)A-L and beef insulin, both of which were tested under conditions showing *I-A*^{b} gene control. At antigen concentrations of both 10 and 100 μg/ml, B6.C-*H-2*^{bm12} mice failed to respond to beef insulin, but generated a response to (TG)A-L similar to that of the wild-type, B6 mice.

Limited biochemical studies of several spontaneously derived mutations of *H-2K*^{b} genes have previously found them all to be "simple" mutations in structural genes, ie, mutations that result in changes of a restricted portion of a single gene product [18]. Therefore, it seems reasonable to speculate that the spontaneously derived *I-A* mutation, B6.C-*H-2*^{bm12}, will also be found to be a simple mutation. Recent biochemical studies suggest that the antigenic changes in B6.C-H-2^{bm12} mice are pleiotropic manifestations of a simple mutation in the Aβ gene [19].

Our present studies thus suggest that in the B6.C-*H-2*^{bm12} mouse a restricted alteration of a single *I-A*^{b} gene product has resulted in both changes in Ia specificities [10,11] and the selective loss of *Ir* gene function (this communication). The data presented here also show that the Ia^{b} molecule(s) has at least two functional sites, one determining recognition of (TG)A-L and the other for beef insulin, and

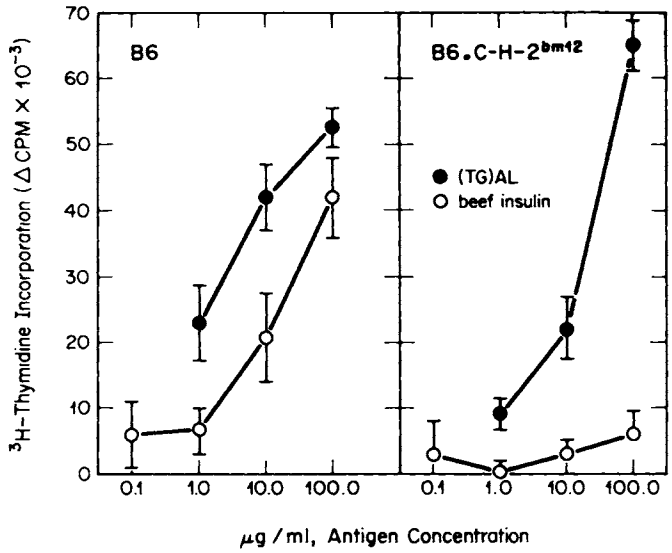

Fig. 1. Proliferative responses of B6 and B6.C-*H-2*^{bm12} mice to (TG)A-L and insulin. Mice were immunized with 50 μg each of beef insulin and (TG)A-L as well as DNP-OVA (data not shown). As in the experiment shown in Table I, control mouse strains were tested in parallel, which demonstrated the *I-A*^{b} gene control of the (TG)A-L and beef insulin responses.

only the latter being deleted in the B6.C-*H-2*^{bm12} mutation. Subject to biochemical verification, our studies represent functional and genetic evidence that determinant selection in genetic control of the immune response is dependent directly on the molecular fine structure of the the Ia molecule.

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NOTE ADDED IN PROOF

Recent studies by M. Michaelides et al (Journal of Experimental Medicine, 153:464, 1981) demonstrated that B6.C-*H-2*^{bm12} mice fail to respond to the male-specific antigen, H-Y, corroborating that there is an *Ir* gene defect in these mutant mice.