Ir GENE FUNCTION IN AN *I-A* SUBREGION MUTANT B6.C-*H-2*^{bm12}*

By MARY MICHAELIDES,‡ MAURO SANDRIN,‡ GILLIAN MORGAN,‡ IAN F. C. McKENZIE,‡ ROBERT ASHMAN,§ AND ROGER W. MELVOLD‖

From the Department of Medicine, Austin Hospital, Heidelberg, Victoria; and The Department of Microbiology, John Curtin School of Medical Research, Australian National University, Australian Capital Territory, Australia; and the Medical Oncology Section, Department of Medicine, Northwestern University, Chicago, Illinois 60611

We have recently defined a mouse strain, B6.C-H-2^{bm12} (bm12), which carries a mutation in the I-A subregion of the *H-2* complex (1). The mutation has resulted in: (*a*) an alteration in the serologically detected specificities Ia-3,8,9,15, and 20, (coded for by *Ia-1*); (*b*) skin graft rejection (*H-2IA*) between parent and mutant; and (*c*) a strong mixed lymphocyte reaction between parent and mutant (*Lad-1*) (1, 2). As all three moieties were altered by the same mutation, the presumption was that the three genes were identical. The occurrence of this mutant strain provides an opportunity to examine the relationship between different phenomena coded for by *I* region genes—in particular to determine whether *Ir* genes and genes coding for the Ia specificities are the same. We have therefore examined the response to several defined antigens: poly(LTyr, LGlu)-poly(DLAla)--poly(LLys) [(T,G)-A--L], poly(Phe,Glu)poly(DLAla)--poly(LLys) [(Phe,G)-A--L], poly(His,Glu)-poly(DLAla)--poly(LLys) [(H,G)-A--L], and to the male-specific H-Y antigen—all antigens being totally or partially under control of *Ir* genes mapping in the *I-A* subregion (3, 4). In brief, the antibody responses to the synthetic polypeptide antigens were the same in C57BL/6 and in bm12; by contrast, bm12 female mice were unable to generate cytotoxic T cells (Tc) in response to the male H-Y antigen, whereas C57BL/6 females reproducibly made a good response.

Materials and Methods

To generate anti-H-Y cytotoxic cells, female mice were primed by an intraperitoneal injection of 10^7 male spleen cells (in 0.2 ml phosphate-buffered saline [PBS]), then boosted in vitro 2 wk–2 mo later (4, 5). Spleen cells (10^8) from primed female mice were stimulated with x-irradiated (1,500 rad) male spleen cells in 20 ml culture medium in upright Costar 3050 flasks (Costar, Data Packaging, Cambridge, Mass.) for 5 d at 37°C in a humidified 10% CO_2 atmosphere. The culture medium consisted of RPMI-1640 medium (Flow Laboratories, Inc., Rockville, Md.), supplemented with 2% heat-inactivated human serum, 20-mM Hepes buffer, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 5×10^{-5} M 2-mercaptoethanol. Target cells used in the cell-mediated lysis (CML) assay were prepared by stimulating spleen cells (5×10^6/ml) with 2 μg/ml concanavalin A (ConA) (Pharmacia Fine Chemicals, Uppsala,

* Supported by grants obtained from the National Health and Medical Research Council of Australia.

‡ Department of Medicine, Austin Hospital, Heidelberg, Victoria, Australia.
§ Department of Microbiology, John Curtin School of Medicine, Australian National University, Australian Capital Territory, Australia.
‖ Medical Oncology Section, Department of Medicine, Northwestern University, Chicago, Ill.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/81/02/0464/06 $1.00
Volume 153 February 1981 464–469

Sweden) in 50 ml of culture medium in 75-cm^2 flasks (Lux Scientific Corp., Newbury Park, Calif.). After an incubation time of 2 d at 37°C in 10% CO_2, the blast cells were isolated on Ficoll-Paque (1.077 g/ml), washed twice, and resuspended in RPMI-1640 medium with 10% fetal calf serum (FCS). 10^6 target cells in 0.5 ml RPMI-FCS were labeled with 300 μCi $Na_2[^{51}Cr]O_4$ (Radiochemical Centre, Amersham, England) for 90 min at 37°C, then washed three times, and resuspended to 2×10^5/ml. For the CML assay, Tc generated in vitro were harvested, washed twice, and resuspended in RPMI-FCS. Graded numbers of Tc in 100 μl were mixed with 100 μl of target cells (2×10^4) in round-bottomed Cooke microtiter plates (Cooke Engineering Co., Alexandria, Va.). After a 4-h incubation at 37°C in 10% CO_2, 100 μl of the supernate was harvested from each well and the radioactivity measured in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). All experiments were set up in triplicate. The specific ^{51}Cr release was calculated as:

$$\text{percent specific release} = \frac{\text{percent } ^{51}\text{Cr release of test} - \text{percent spontaneous release}}{\text{percent maximum release} - \text{percent spontaneous release}}$$

The spontaneous and maximum ^{51}Cr release were determined by incubating target cells with medium or 1% Nonidet P-40, respectively.

The antibody responses of the parental C57BL/6 and the mutant bm12 mice were examined by immunizing mice with 10 μg/ml (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L (Miles-Yeda Ltd., Rehovot, Israel) in Freund's adjuvant (6, 7). Individual mice were bled and tested in an indirect antibody binding radioiummune assay for antibody activity. Polyvinylchloride plates (Cooke Engineering Co.) were coated with 25 μl of antigen (at 100 μg/ml in PBS) at room temperature for 1 h, after which the plates were washed four times with PBS that contained 2% bovine serum albumin and 0.06% sodium azide. Doubling dilutions of 25 μl of antiserum were added to the plates, and these were incubated at 37°C for 1 h, then at 4°C overnight, and subsequently washed four times. I^{125}-protein A (Pharmacia Fine Chemicals) in 25 μl was added to each well, the plates incubated for 1 h at 4°C, washed five times, dried, and then cut using a hot wire. The individual wells were counted for radioactivity. Protein-A was iodinated by the chloramine T method.

Results

Antibody Response to (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L. Groups of 5–10 mice were immunized with the above-mentioned antigens and their antibody response measured by the protein-A binding assay. The results are shown in Table I for bm12, C57BL/6, and an appropriate responder or nonresponder strain for each antigen. With (T,G)-A--L, both C57BL/6 and bm12 showed a high response with mean titers of 1:320 and 1:640, respectively. Similarly, both strains gave a high response to (Phe,G)-A--L, with mean titers of 1:256, for C57BL/6 and 1:432, for bm12. Neither C57BL/6 nor bm12 showed any response to (H,G)-A--L (Table I). There was clearly no difference in the response of C57BL/6 and bm12 to any of the antigens.

Comparison of Tc to H-Y in C57BL/6 and bm12. C57BL/6 females produced an effective cytotoxic response against male ConA blast cells (Fig. 1), with 30% cell lysis at a 25:1 effector:target cell ratio; no lysis of C57BL/6 female cells was detected, indicating that the response was H-Y specific. By contrast, bm12 females were unable to make any H-Y cytotoxic response, and the results of Fig. 1 have now been reproduced on several occasions. The defect lay in the ability of bm12 to respond to H-Y and not in the expression of the antigen, as female C57BL/6 mice, primed with C57BL/6 male cells could also lyse bm12 male cells and C57BL/6 male cells (Fig. 1). Furthermore, there is no intrinsic problem in the bm12 strain in the proliferation and generation of Tc, for bm12 can respond to an *H-2* or *I-A* difference as indicated elsewhere ([1]; K. Melief, personal communication; and our unpublished results). For the experiments presented, bm12 was primed by an intraperitoneal injection of male

TABLE I

*Antibody Response to (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L**

Antigen	C57BL/6	B6.C-H-2^{bm12}	B10.BR	B10.S
(T,G)-A--L	80, 80, 160, 320, 320, 320, 320, 320, 640, 640	160, 160, 320, 320, 320, 640, 640, 1,280, 1,280, 1,280	<5, <5, <5, <5, <5, <5, <5, <5, <5, <5	NT
	(320 ± 62)	(640 ± 149)	(0)	
(Phe,G)-A--L	160, 160, 160, 320, 320	80, 160, 640, 640, 640	NT	(0)
	(256 ± 39)	(432 ± 128)		
(H,G)-A--L	<5, <5, <5, <5, <5	<5, <5, <5, <5, <5	160, 320, 320, 320 624	NT
	(0)	(0)	(352 ± 78)	

The titers were calculated as the dilution of serum which gave greater than or equal to four times the background counts (400 cpm). The background was ~90 cpm and the maximum cpm ranged from 8,000 to 26,000 in the different mice. The reciprocal titers are shown and the mean ± SEM in parentheses. NT, not tested.

cells as described above. In addition, priming was also done by skin grafting, grafting plus lymphocyte injection and by cell injection into the footpads—all with the same result: bm12 was unable to generate Tc to the H-Y antigen.

Generation of Tc in an F_1 Hybrid Using the Two Nonresponder Strains bm12 and B10.A. As bm12 was clearly a nonresponder to the H-Y antigen—a response which must now be considered to be influenced by *Ia-1* gene, as this is the site of the mutation in bm12—we determined whether there were any circumstances under which bm12 could respond to the H-Y antigen, particularly as other studies had indicated the presence of complementary *Ir* genes for H-Y in several *H-2* haplotypes. Thus, two nonresponder strains, bm12 and B10.A, were examined in the F_1 hybrid for the generation of Tc to the H-Y antigen. Female F_1 hybrid mice, primed in vivo and then stimulated in vitro with bm12 male cells, were able to lyse both bm12 and C57BL/6, but not B10.A male ConA blast cells (Fig. 2A). The response was specific for the H-Y antigen as bm12 female cells could not be lysed (Fig. 2). C57BL/6 and bm12 could be lysed as they share the appropriate associative target H-2 antigen—H-$2D^b$ in this case—with the priming cell. B10.A (H-$2K^k$,D^d) does not have the appropriate associative antigen and was not lysed (8, 9), nor could it stimulate an anti-H-Y cytotoxic response in F_1 female mice that had been primed with bm12 male cells (Fig. 2B). The results demonstrate the presence of complementary *Ir* genes in bm12 and B10.A.

Discussion

The results demonstrate, unequivocally, that a mutation in the *Ia-1* gene in the *I-A* subregion has affected one immune response (*Ir*) gene, but has not affected those *Ir* genes responsible for the antibody response to (T,G)-A--L and (Phe,G)-A--L. Thus, the preliminary analysis has led us to conclude that the gene(s) coding for the Ia specificities (detected serologically) and at least one *Ir* gene may be the same, but that this does not apply to all *Ir* genes and under all circumstances. (T,G)-A--L, (Phe,G)-A--L, and (H,G)-A--L were selected for initial study as they are well-characterized antigens, the responses to which are under the control of *Ir* genes that map in the *I-A* subregion (3, 6, 7). Furthermore, anti-Ia sera have been noted to block the proliferative response to (T,G)-A--L (10) and this and other evidence has led to much speculation on the relationship of *Ir* genes and Ia antigens—in particular of *Ir-1* [which controls the response to (T,G)-A--L] and *Ia-1^b* [which codes for the Ia^b specificities Ia-

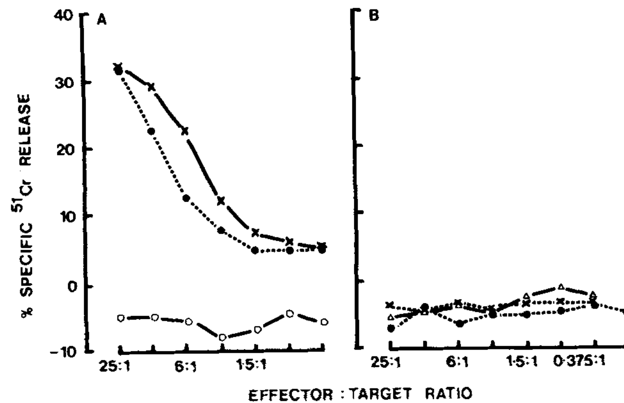

FIG. 1. Anti-H-Y cyytotoxic responses of C57BL/6 (A) and B6.C-H-2^{bm12} (B) female mice, primed in vivo and boosted in vitro with syngeneic male spleen cells. The target cells used were: C57BL/6 male (×) and female (○) cells; and B6.C-H-2^{bm12} male (●) and female (△) cells.

3,8,9,15,20]. In addition, the response to (H,G)-A--L was examined; this was done as it was considered possible that an *Ir* gene mutation could lead to the conversion of a nonresponder to a responder strain, and also as it has been suggested (3) that the (T,G)-A--L and (H,G)-A--L responses are governed by allelic genes at the one locus. However, both C57BL/6 and bm12 were good responders to both (T,G)-A--L and (Phe,G)-A--L and nonresponders to (H,G)-A--L. In addition, the T cell proliferative responses to these antigens appear to be similar (data not shown) and preliminary studies have not shown any differences in either the antibody or proliferative response to ovalbumin or to glutamine-alanine-tyrosine (GAT). Can we therefore conclude that *Ir-1* for (T,G)-A--L and *Ir-* (Phe,G)-A--L genes are not the same as *Ia-1*? The answer is yes, but with several qualifications: First, the immunizing dose should be considered, as sufficiently low doses of antigen are critical in demonstrating the presence of *Ir* genes (12). Although it cannot be excluded that other doses of antigen may have given different results, the doses used could clearly differentiate between high and nonresponders for the three antigens used (Table I). Secondly, it is possible that *Ir* gene products and Ia alloantigens are expressed on different sites of the one glycoprotein chain and the mutation has altered one site but not the other. This is possible, but unlikely as there appears to be a gross disturbance in the Ia-bearing chain, as demonstrated serologically, and biochemically with both alloantisera and xenoantisera (M. Sandrin, I. F. C. McKenzie, and R. W. Melvold. Manuscript in preparation.). In addition, the response to the H-Y antigen has been altered (Fig. 1). At this time, with the evidence presented herein (Table I), we must conclude that the *Ia-1* and *Ir-1*[(T,G)-A--L],[Ir-(Phe,G)-A--L] genes are separate structures. Whether this applies to other soluble polypeptide antigens is currently under investigation with a large series of antigens.

By contrast to the foregoing, the immune response to *H-Y* is clearly altered in the bm12 mutant strain and at least for this antigen we can state that it is likely that *Ir-H-Y* and *Ia-1* are the same. However, the immune response to the H-Y antigen is complex and different aspects of the immune response appear to be under separate (unrelated?) gene control. For example, skin graft rejection and delayed-type hypersensitivity responses are effected by a gene mapping in the *I-B* subregion ([12]; and F.

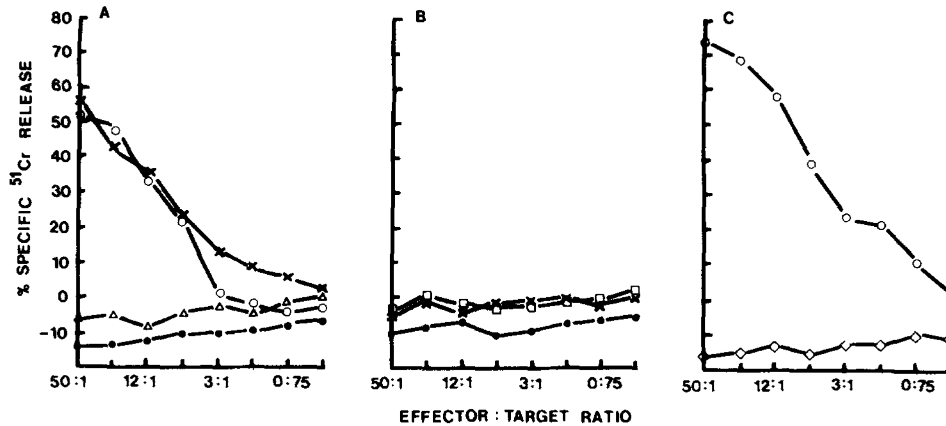

FIG. 2. Anti-H-Y cytotoxic responses of C57BL/6 and (B10.A × B6.C-H-2^{bm12})F_1 mice. (B10.A × B6.C-H-2^{bm12})F_1 female mice, primed in vivo with B6.C-H-2^{bm12} male cells, were stimulated in vitro with the priming cells (A) or with B10.A male cells (B). C57BL/6 female mice (C) were primed and boosted as described in Fig. 1. Cytotoxic effector cells were assayed against various target cells: B6.C-H-2^{bm12} male (×) and female (△) cells: C57BL/6 male (○) and female (◇) cells; and B10.A male (●) and female cells (□).

Liew and E. Simpson. Personal communication.), whereas the generation of Tc is under the control of an *I-A* subregion gene (4) (which now appears to be identical with *Ia-1*). There are also other genes, (i.e., complementary *Ir* genes), which in other haplotypes have been found elsewhere within *H-2*, e.g. in IC^k (8). In addition the appropriate associative *H-2K* or *H-2D* antigen must be present for Tc to be detected. When these conditions are satisfied, bm12 is clearly a nonresponder and has an alteration in the *Ir-H-Y* gene. The defect produced by the mutation is in the response, and is clearly not present in the expression or presentation of the H-Y antigen. But how this defect is manifest or how the *Ir-H-Y* gene product is expressed is not clear at present. The ability of the two nonresponder strains bm12 and B10.A to complement each other to generate Tc is of interest and indicates, in bm12, that other *Ir* genes exist which also effect the generation of Tc to H-Y.

Summary

The B6.C-*H-2*^{bm12} (bm12) strain has a mutation in the *I-A* subregion of the murine *H-2* complex and is characterized by a loss of serologically detected Ia antigens and a strong graft rejection and mixed lymphocyte response between parent and mutant. It was presumed that the mutation affected the *Ia-1* gene and to determine the relationship of Ia antigens and *Ir* genes, the immune responses of mutant and parent were compared. The immune responses to poly(LTyr,LGlu)-poly(DLAla)--poly(LLys), poly(Phe,Glu)-poly(DLAla)--poly(LLys), and poly(His,Glu)-poly(DLAla)--poly(LLys) in parent and mutant were the same, indicating that *Ia-1* and the *Ir* genes for these antigens are not identical. By contrast, although C57BL/6 gave a good response, the mutant strain was unable to generate cytotoxic T lymphocytes to the male-specific H-Y antigen—a response under *I-A* subregion *Ir* gene control, which now must be considered to be the *Ia-1* gene. In addition, complementary *Ir* genes in the H-2^b haplotype for the H-Y immune response could be detected when the bm12 mutant was used.

We thank Dr. Elizabeth Simpson for the helpful discussion on the H-Y studies.

Received for publication 10 October 1980.

References

1. McKenzie, I. F. C., G. M. Morgan, M. S. Sandrin, M. M. Michaelides, R. W. Melvold, and H. I. Kohn. 1979. B6.C-H-2^{bm12}. A new *H-2* mutation in the *I* region in the mouse. *J. Exp. Med.* **150:**1323.
2. McKenzie, I. F. C., M. S. Sandrin, G. M. Morgan, M. Henning,and R. W. Melvold. 1980. Mutation in the I-A subregion of the murine *H-2* complex. *Immunogenetics.* **11:**103.
3. Klein, J. 1975. Biology of the Mouse Histocompatibility-2 Complex. Springer-Verlag New York, Inc., New York. 424.
4. Hurme, M., C. M. Hetherington, P. R. Chandler, and E. Simpson. 1978. Cytotoxic T-cell responses to H-Y: mapping of the *Ir* genes. *J. Exp. Med.* **147:**758.
5. Simpson, E., L. Mobraaten, P. Chandler, C. Hetherington, M. Hurme, C. Brunner, and D. Bailey. 1978. Cross-reactive cytotoxic responses. H-2 restricted are more specific than anti-H-2 responses. *J. Exp. Med.* **148:**1478.
6. McDevitt, H. O., and M. Sela. 1965. Genetic control of the antibody response. I. Demonstration of determinant-specific differences in response to synthetic polypeptide antigens in two strains of inbred mice. *J. Exp. Med.* **122:**517.
7. McDevitt, H. O., and M. Sela. 1967. Genetic control of the antibody response. II. Further analysis of the specificity of determinant-specific control, and genetic analysis of the response to (H,G)-A--L in CBA and C57 mice. *J. Exp. Med.* **126:**969.
8. Hurme, M., C. M. Hetherington, P. R. Chandler, R. D. Gordon, and E. Simpson. 1977. Cytotoxic T-cell responses to H-Y: *Ir* genes and associative antigens map in *H-2*. *Immunogenetics.* **5:**453.
9. Simpson, E., and R. D. Gordon. 1977. Responsiveness to HY antigen. Ir gene complementation and target cell specificity. *Immunol. Rev.* **35:**59.
10. Lonai, P., D. B. Murphy, and H. O. McDevitt. 1978. Inhibitory effect of anti-Ia sera on proliferative responses to (H,G)-A--L and (Phe,G)-A--L. *In* Ir Genes and Ia Antigens. H. O. McDevitt, editor. Academic Press, Inc., New York. 433.
11. Vaz, N. M., and B. B. Levine. 1970. Immune responses of inbred mice to repeated low doses of antigen: relationship to histocompatibility (H-2) type. *Science. (Wash. D. C.).* **168:** 852.
12. Hurme, M., P. R. Chandler, C. M. Hetherington, and E. Simpson. 1978. Cytotoxic T-cell responses to H-Y: correlation with the rejection of syngeneic male skin grafts. *J. Exp. Med.* **147:**768