

GENETIC REGULATION OF THE ANTIBODY RESPONSE TO  
*H-2D<sup>b</sup>* ALLOANTIGENS IN MICE

II. Tolerance to Non-*H-2* Determinants Abolishes the Antibody  
Response to *H-2D<sup>b</sup>* in B10.A(5R) Mice\*

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Several genetic systems have been reported that regulate the antibody response to *H-2D<sup>b</sup>* alloantigens in mice (1-5). Some of the genes described are linked to *H-2* (2, 3), whereas other genes are located in the extra-*H-2* genome (1, 4, 5).

In concentrating on the genetic regulation by genes not linked to *H-2* we have tried to determine the site of gene action in the sequence of immunological phenomena that leads to antibody production. Most experiments have been performed with B10.A(5R) [5R] mice immunized with spleen cells from congenic or noncongenic *H-2<sup>b</sup>* mice (6). Repeated immunizations with congenic C57BL/10 (B10) cells induces the production of antibodies of IgM type only, whereas immunization with noncongenic cells induces both IgM and IgG antibodies to *H-2D<sup>b</sup>*. The hypothesis was proposed (4) that, to mice with the genetic background of the B10 series, an *H-2* antigen on a congenic cell is analogous to a hapten on a nonimmunogenic carrier (7, 8) which fails to induce the helper T-cell function and thereby fails to stimulate the switch from IgM to IgG in B cells.

In order to support this hypothesis by experimental data we have rendered 5R mice tolerant to gene products of the extra-*H-2* genome of the BALB/c mouse strain and have compared the noncongenic immunization with BALB.B cells in these tolerized mice to the congenic immunization with B10 cells. The data obtained confirm our hypothesis that non-*H-2* cell surface antigens act as carrier determinants in an anti-*H-2* immunization.

**Materials and Methods**

*Mice and Immunizations.* All animals used in the experiments reported here were from our own colonies of inbred mouse strains. All immunizations were directed against *H-2D<sup>b</sup>* alloantigens. 5R mice (2- to 6-mo old) were immunized with normal spleen cells from either B10, A.BY, or BALB.B mice or with EL4 leukemia cells, carried by serial passage in B6 or B10 mice. The *H-2* haplotypes of all strains used are shown in Table I. 5R belongs to the series of congenic-resistant strains of B10 origin; its *H-2* haplotype (*H-2<sup>ib</sup>*) is derived from a crossover of *H-2<sup>a</sup>* and *H-2<sup>b</sup>* occurring between *I-B* and *I-C* (9). B10, EL4, A.BY, and BALB.B all carry the *H-2<sup>b</sup>* haplotype; B10 and EL4 on the same genetic background as 5R, and A.BY and BALB.B on the background of the

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unrelated strains A and BALB/c, respectively. Immunizations of 5R mice with *H-2<sup>b</sup>* cells are directed against gene products coded for by the *I-C<sup>b</sup>*, *S<sup>b</sup>*, and *D<sup>b</sup>* region of the *H-2* complex. In the case of B10 cells, no other foreign cell surface antigens should be recognized; in the case of EL4 cells, tumor-specific antigens should be recognized in addition to *H-2D<sup>b</sup>*; and in the case of A.BY and BALB.B cells, several different non-*H-2* antigens are present on the immunizing cell.

To tolerize 5R mice to cell surface antigens of the BALB/c background, 5R mice were injected intravenously during the first 24 h after birth with  $8 \times 10^6$  bone marrow cells from adult BALB.K (*H-2<sup>k</sup>*) mice (Table I). According to Law et al. this treatment leads to humoral and cellular tolerance to any cell from the injected strain (10). As controls, bone marrow cells from B10.BR mice were injected, which also carry *H-2<sup>k</sup>* but on the B10 background (Table I). Another set of control mice was injected with saline.

For immunizations, groups of 7-10 mice received a first inoculation of  $0.5 \times 10^6$  cells injected subcutaneously; 2 wk later  $5 \times 10^6$  cells were injected intraperitoneally, and the immunization was continued with fortnightly intraperitoneal injections of  $10 \times 10^6$  cells. The immunized mice were bled from the retroorbital plexus 7 days after the fourth, fifth, and sixth immunization. All antisera were stored at  $-70^\circ\text{C}$  until tested. As target cells in the antibody complement (C)-mediated cytotoxicity test B10.HTG lymph node cells were chosen (Table I); this way only antibodies against *H-2D<sup>b</sup>* were assayed.

*C-Mediated Cytotoxicity Test.* The C-mediated cytotoxicity test for antibody activity was performed on  $^{51}\text{Cr}$ -labeled lymph node cells as previously described (4) with the following modification to allow for greater sensitivity and for the use of 2-mercaptoethanol (2-Me) to test for 2-Me-sensitive and 2-Me-resistant antibodies: Instead of a one-step cytotoxicity assay with an incubation time of 1 h at room temperature, a two-step test was used in which the labeled target cells were incubated with antiserum in the cold, centrifuged, and then incubated at  $37^\circ\text{C}$  with guinea pig C. Disulfide bond reduction of the *H-2* antibodies with 2-Me was carried out by incubating equal volumes of serum and 0.1 M 2-Me for 30 min at  $37^\circ\text{C}$  as described by Klein et al. (11) assuming that 2-Me-sensitive antibodies are of IgM type, whereas 2-Me-resistant antibodies are of IgG type.

*Lymphocyte-Mediated Cytolysis.* The lymphocyte-mediated cytolysis assays were performed as previously described (4). As target cells EL4 leukemia cells (*H-2<sup>b</sup>*), or P815 mastocytoma cells (*H-2<sup>d</sup>*, control) were used. Tests were performed 4 days after the fourth, fifth, and sixth immunization with an incubation time of 5 h.

## Results

*Antibody Response of 5R Mice to Congenic and Noncongenic H-2<sup>b</sup> Cells.* 5R mice were immunized with congenic B10 normal spleen cells, with EL4 leukemia cells, or with noncongenic A.BY and BALB.B normal spleen cells. Part of

TABLE I  
*H-2 Haplotype of All Mouse Strains Used*

	Haplotype	<i>H-2</i> complex						Non- <i>H-2</i> background	
		<i>K</i>	<i>I-A</i>	<i>I-B</i>	<i>I-C</i>	<i>S</i>	<i>D</i>		
Responding strain									
5R	<i>H-2<sup>b</sup></i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>I</i> <sup>*</sup>	<i>d</i>	<i>d</i>	<i>d</i>	B10
Cells used for immunization									
B10	<i>H-2<sup>b</sup></i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	B10
EL4	<i>H-2<sup>b</sup></i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	C57BL
A.BY	<i>H-2<sup>b</sup></i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	A
BALB.B	<i>H-2<sup>b</sup></i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	<i>b</i>	BALB/c
Bone marrow cells injected into new-born 5R mice									
B10.BR	<i>H-2<sup>k</sup></i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	B10
BALB.K	<i>H-2<sup>k</sup></i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	<i>k</i>	BALB/c
Target cells in antibody C-mediated cytotoxicity tests									
B10.HTG	<i>H-2<sup>b</sup></i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>d</i>	<i>b</i>	<i>b</i>	B10

\* Vertical lines indicate site of crossover.

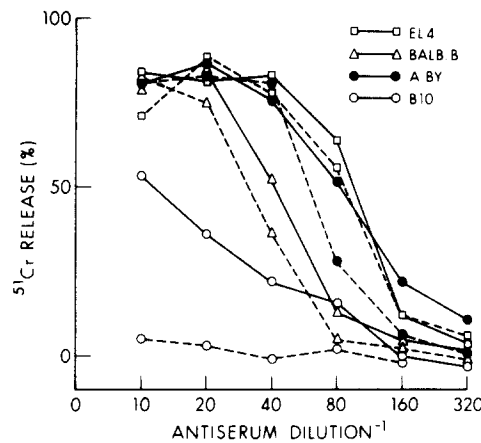


Fig. 1. Antibody levels as measured by a C-mediated  $^{51}\text{Cr}$ -release cytotoxicity assay. Titration on B10.HTG target cells of sera collected 7 days after the sixth injection of 5R mice with EL4 leukemia cells, or normal spleen cells of strains BALB.B, A.BY, or B10. Solid lines, sera not treated with 2-Me. Broken lines, sera after 2-Me treatment.

these results have been described earlier (6). When tested in a C-mediated cytotoxicity test on B10.HTG cells in which only antibodies against cell surface antigens governed by the  $H-2D^b$  region should be detected, the anti-B10 sera showed either a weak IgM response (Fig. 1) or no detectable antibody response at all. In contrast, all immunizations with  $H-2^b$  cells bearing additional antigenic differences, tumor antigens on EL4 and non-H-2 antigens on A.BY and BALB.B, induced a stronger response, consisting mostly of 2-Me-resistant antibodies of IgG type (Fig. 1). Since the IgG antibody response to H-2 antigens is thymus dependent (11), we concluded that foreign cell surface antigens in addition to  $H-2D^b$  are needed in 5R mice to induce the T-cell helper function which is responsible for the switch from IgM to IgG production.

*Induction of Tolerance to the BALB/c Background Gene Products in 5R Mice.* To test this theory we devised the following experiment: 5R mice were made tolerant to the extra- $H-2$  genome of BALB/c by injecting bone marrow cells into newborn mice. Instead of using BALB/c mice, bone marrow cells from the BALB.K strain ( $H-2^k$  on the BALB/c background) were chosen which differ from 5R mice at the whole  $H-2$  complex and not at only part of it as BALB/c. Two controls were performed: 5R newborns were injected with either saline or congenic B10.BR bone marrow cells ( $H-2^k$ ). These groups of mice are referred to as 5R(SALINE), 5R(BALB.K), and 5R(B10.BR). 5R(SALINE) mice should represent normal controls, 5R(BALB.K) and 5R(B10.BR) should be tolerant to antigens of the  $H-2^k$  haplotype, and 5R(BALB.K) in addition should be tolerant to all relevant non-H-2 cell surface antigens of the extra- $H-2$  portion of the BALB/c genome.

At 2 months of age the mice were immunized with BALB.B normal spleen cells. Fig. 2 gives the results of sera collected 7 days after the sixth injection. As expected 5R(SALINE) showed a cytotoxicity curve identical to 5R mice that had not been handled as newborns. In contrast, in the 5R(BALB.K) anti-BALB.B immunization no cytotoxic antibodies to  $H-2D^b$  were detected (Fig. 2). In some

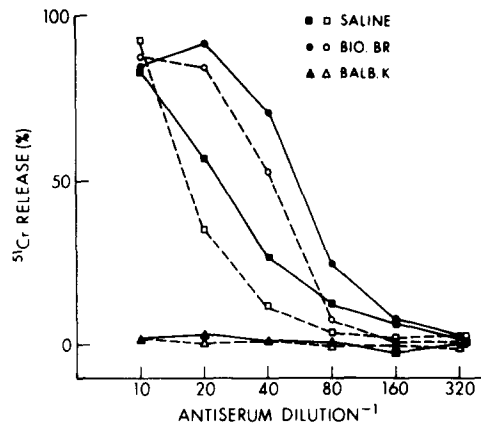


FIG. 2. Antibody levels as measured by a C-mediated  $^{51}\text{Cr}$ -release cytotoxicity assay. Titration on B10.HTG target cells of sera collected 7 days after the sixth injection of 5R mice with BALB.B normal spleen cells. 5R mice had been injected as newborns with either saline or B10.BR bone marrow cells or BALB.K bone marrow cells. Solid lines, sera not treated with 2-Me. Broken lines, sera after 2-Me treatment.

bleedings a low IgM response of 15% cytotoxicity was seen. This evidence closely resembles the results obtained in the congenic 5R anti-B10 immunization. The 5R(B10.BR) control exhibited a cytotoxicity curve similar to 5R(SALINE), and this indicates that the induction of tolerance to the  $H-2^k$  haplotype does not interfere with the antibody response to  $H-2D^b$ . The implications of this experiment are that tolerance to non-H-2 cell surface antigens abolishes the antibody response to  $H-2D^b$  in 5R mice.

*Cell-Mediated Immunity to  $H-2D^b$  of 5R Mice, Rendered Tolerant to BALB.K.* The cell-mediated immunity was investigated in a  $^{51}\text{Cr}$ -release killer assay. We have previously shown that killing against  $H-2D^b$  under the conditions used is T-cell mediated (4). 5R mice, injected as above as newborns, were immunized with BALB.B cells. 4 days after the sixth injection peritoneal exudate cells were tested as effector cells on  $^{51}\text{Cr}$ -labeled EL4 cells ( $H-2^b$ ) and control P815 cells ( $H-2^d$ ). As can be seen in Table II peritoneal exudate cells from 5R(SALINE) and 5R(B10.BR) killed over 80% of EL4 cells in 5 hours, whereas 5R(BALB.K) showed a considerably lower cytotoxicity of at most 30%. The cells of all three groups of mice failed to kill P815, indicating specificity of the reaction for  $H-2D^b$ . These experiments suggest that tolerance induction to the BALB/c background influences, but does not abolish the cell-mediated immunity to  $H-2D^b$  in an immunization with BALB.B cells.

### Discussion

5R mice appear to be capable of mounting an IgG response to immunization with cells bearing  $H-2D^b$  alloantigens only if the cells also bear other foreign antigens (e.g., non-H-2 or tumor-specific antigens). Thus immunization with congenic B10 cells ( $H-2^b$ ), which differ from 5R essentially only at the  $H-2D^b$  subregion, elicits only an IgM response, whereas immunization with BALB.B cells ( $H-2^b$ ) elicits both IgM and IgG antibodies. These results appear analo-

TABLE II  
*Lymphocyte-Mediated Cytolysis by Peritoneal Exudate Cells from 5R Mice Immunized with BALB.B Spleen Cells*

Target cells	<sup>51</sup> Cr release		
	5R(SALINE)	5R(BALB.K)	5R(B10.BR)
	%	%	%
EL4	89	30	81
P815	4	3	1

5R mice had been injected as newborns with either saline or BALB.K bone marrow cells or B10.BR bone marrow cells. Peritoneal exudate cells were harvested 4 days after the sixth immunization. Killer to target cell ratio 80:1.

gous to those from immunizations of mice with haptens coupled to syngeneic erythrocytes as nonimmunogenic carriers (7, 8). Therefore, to 5R mice the *H-2D<sup>b</sup>* alloantigen on B10 cells would represent a hapten which does not activate helper T cells and therefore cannot induce the switch from IgM to IgG production.

This hypothesis was confirmed by the data presented here. 5R mice were made tolerant to cell surface antigens of the BALB/c background by injecting BALB.K bone marrow cells (*H-2<sup>k</sup>*) into newborn 5R mice. In the subsequent noncongenic immunization with BALB.B cells, the mice responded as if the immunization had been with congenic B10 cells. No IgG antibodies to *H-2D<sup>b</sup>* could be detected. The cell-mediated immunity to *H-2D<sup>b</sup>* was impaired when compared to two control groups, but was still detectable.

Induction of tolerance by injecting allogeneic cells into newborn mice may lead to clonal deletion or clonal inactivation of lymphocytes committed to the allogeneic cells (12, 13) or may lead to long-lasting suppression of the antigen-specific cells by serum-blocking factors (14) or suppressor cells. In any case no cells recognizing gene products of the extra-*H-2* BALB/c background could be activated in 5R mice injected as newborns with BALB.K cells and thus the BALB.B cells behaved as nonimmunogenic carriers. Under these circumstances the *H-2D<sup>b</sup>* antigen represents a single haptenic site which does not lead to activation of helper T cells but which is sufficient to stimulate IgM-producing B cells and killer T cells.

These experiments confirm our hypothesis that in a 5R anti-B10 immunization the *H-2* antigen is analogous to a hapten on a nonimmunogenic carrier. Comparative immunizations have shown that this phenomenon is not unique to 5R but is demonstrated by other mice of the B10 series (5) and may be shared by mice carrying the genome of strain A (3). BALB/c mice do not show this phenomenon in a congenic immunization (5). Analysis of the genetic regulation of the failure in T-cell helper activation is currently underway.

### Summary

B10.A(5R) mice (*H-2<sup>is</sup>*), immunized with spleen cells from congenic B10 mice (*H-2<sup>b</sup>*), responded to alloantigens of the *H-2D<sup>b</sup>* region by producing antibodies of only IgM type. In contrast, they produced both IgM and IgG antibodies when immunized with noncongenic *H-2<sup>b</sup>* cells that carry other foreign cell surface antigens (non-*H-2*) in addition to *H-2D<sup>b</sup>*. A hypothesis was proposed comparing

the *H-2D<sup>b</sup>* antigen on a congenic cell to a hapten on a nonimmunogenic carrier which fails to induce T-cell helper function responsible for the switch from IgM to IgG secretion in B cells. Data presented here confirmed this hypothesis. 5R mice rendered tolerant to the relevant non-H-2 antigens were unable to mount the anti-*H-2D<sup>b</sup>* IgG response in a noncongenic immunization. Tolerance induction did not lead to abrogation of the T-cell-mediated cytotoxicity.

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