GENETIC REGULATION OF THE ANTIBODY RESPONSE TO *H-2D*^{b} ALLOANTIGENS IN MICE

I. Differences in Activation of Helper T Cells in C57BL/10 and BALB/c Congenic Strains*

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In addition to the *H-2I*-associated immune response genes, other genes, independent of the *H-2* complex, have been shown to play an important role in the antibody response to certain antigens (1–3). In particular, C57BL/10 (B10)[1] mice or their *H-2* congenic partners often show a lower or altered antibody response in comparison to mice of other strains which carry the same *H-2* type (4–7). Since the most extensive collection of *H-2* congenic strains consists of the B10 series, these mice are widely used for a variety of investigations, and any influence of the B10 background on the outcome of experiments is of great importance.

After our investigation of the immune response genes that influence the production of antibodies to the *H-2D*^{b} alloantigens carried on the C57BL leukemia EL4 (8), experiments were performed to compare the antibody response of B10.A(5R) mice (5R), a responder strain to *H-2D*^{b} alloantigens on EL4 cells (9), when immunized with various other *H-2D*^{b} carrying cells. The results present the surprising evidence that 5R mice produce anti-*H-2D*^{b} IgG alloantibodies only if the immunizing cell carries additional foreign cell surface antigens. In an *H-2D*^{b} congenic immunization only IgM antibodies are secreted indicating a defect in the T-cell helper function which is responsible for the switch from IgM to IgG production.

In order to ascertain whether the genes that regulate this differing response are linked to *H-2* or are located outside the *H-2* complex, another *H-2* congenic combination of the B10 series (B10.D2 anti-B10.HTG) was compared to an *H-2* identical combination of the BALB/c congenic series (BALB/c anti-BALB.HTG). The evidence suggests that genes, not linked to *H-2*, regulate this differing antibody response to *H-2D*^{b} alloantigens, but do not affect cell-mediated immunity as tested by a T-cell killer assay.

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[1] *Abbreviations used in this paper:* B10, C57BL/10; CML, cell-mediated lympholysis; FCS, fetal calf serum; 5R, B10.A(5R); LMC, lymphocyte-mediated cytolysis; MLC, mixed leukocyte culture; PBS, phosphate-buffered saline; PEC, peritoneal exudate cells; PHA, phytohemagglutinin; 2-Me, 2-mercaptoethanol; 2R, B10.A(2R).

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*^{b} alloantigens. B10.A(5R) mice (2–6 mo old) were immunized with normal spleen cells of strains B10, A.BY, and B10.A(2R) (2R). The *H-2* haplotypes of these mice are shown in Table I. 5R belongs to the series of congenic strains of C57BL/10 origin (10); its *H-2* haplotype (*H-2*^{i5}) (11) is derived from a crossover between *H-2*^{a} and *H-2*^{b} occurring in the *I* region (12). B10 and A.BY both carry the *H-2*^{b} haplotype, B10 on the same background as 5R, A.BY on the genome of the unrelated strain A. 2R differs from 5R in the *H-2K*, *I*, and *D* region. Immunizations of 5R with B10 cells are directed only against *H-2D*^{b}, with A.BY against *H-2D*^{b} and additional non-*H-2* cell surface antigens, with 2R against *H-2K*^{k}, *H-2I*^{k}, and *H-2D*^{b}.

B10.D2 mice were immunized with normal spleen cells of the *H-2* congenic strain B10.HTG, and BALB/c mice were immunized with congenic BALB.HTG normal spleen cells. B10.D2 and BALB/c carry the *H-2*^{d} haplotype; B10.HTG and BALB.HTG carry the *H-2*^{g} haplotype, which is derived from a crossover between *H-2*^{d} and *H-2*^{b} occurring between the *S* and *D* region of the *H-2* complex, as shown in Table I (12). Therefore immunizations of *H-2*^{d} anti-*H-2*^{g} on a congenic background are directed only against the *H-2D*^{b} alloantigens since all other regions of the *H-2* complex are shared by *H-2*^{d} and *H-2*^{g} mice. (Regarding the possible involvement of *I*^{b} genes in the *H-2*^{g} haplotype see ref. 13, 14).

The mice received a first inoculation of 0.5 x 10^6 cells injected subcutaneously; 2 wk later 5 x 10^6 cells were injected intraperitoneally, and the immunization was continued with biweekly intraperitoneal injections of 10 x 10^6 cells. The immunized mice were bled from the retroorbital plexus at various intervals after each injection. All antisera were stored at −70°C until tested. Target cells in assays for antibody determination or cell-mediated cytotoxicity were chosen from various *H-2D*^{b} strains: HTH/Go, HTG/Go, BALB/c-*H-2*^{b}, B10.A(4R), D2.GD, and the *H-2D*^{b} strains shown in Table I.

Antibody Determinations. For the complement-mediated cytotoxicity test for antibody activity (15, 16), *H-2D*^{b}-carrying lymph node cells were incubated with 0.1 mC ^{51}Cr (Sodium Chromate 100–300 mC/mg, Amersham/Searle Corp., Arlington Heights, Ill.) for 2-3 h. 1 x 10^5 labeled and washed target cells were mixed with guinea pig complement (Grand Island Biological Co., Grand Island, N.Y.) and twofold dilutions of antiserum in a final vol of 0.3 ml of Medium 199 (Microbiological Associates, Inc., Bethesda, Md.) supplemented with 20% FCS (GIBCO). After 1 h of incubation at room temperature the reaction was terminated by adding 0.3 ml of a cold EDTA-PBS solution, the cells were then centrifuged and 0.3 ml of supernate taken off for determination of ^{51}Cr release. The results are expressed as percent killing, 100% being defined as the counts per minute (minus the spontaneous release) contained in 0.3 ml of supernate of labeled cells that have been frozen and thawed four times.

TABLE I
H-2 Haplotype of the Mouse Strains Used

		H-2 complex			
		K	I	S	D
B10.A(5R)	[*H-2*^{i5}]	b	b |	d	d
B10; A.BY	[*H-2*^{b}]	b	b	b	b
B10.A(2R)	[*H-2*^{h2}]	k	k	d |	b
B10.D2; BALB/c	[*H-2*^{d}]	d	d	d	d
B10.HTG; BALB.HTG	[*H-2*^{g}]	d	d	d |	b

Vertical bars indicate the site of crossover. The crossover in B10.A(5R) occurred inside the *I* region, between *IB* and *IC* (12). The Ia antigens of B10 mice, as known so far, are shared by 5R and therefore do not present any additional cell surface antigens. Regarding the possible involvement of *I*^{b} genes in the *H-2*^{g} haplotype, see ref. 13, 14.

Hemagglutination tests were performed on red blood cells obtained by eye bleeding into isotonic sodium citrate solution; the cells were washed once in saline and resuspended in a mixture of two parts heat-inactivated FCS and one part saline solution at an estimated concentration of 10^9/ml. Antisera were prepared in serial twofold dilutions in dextran (mol wt 115,000) 1.8–2.0% in 6% glucose solution. One drop of antiserum dilution was added to one drop of red blood cell suspension in disposable plastic trays and incubated for 90 min at 37°C. The degree of hemagglutination was determined by examining the extent of clumping by low power microscopy. This method is that of Gorer and Mikulska (17) with minor modifications. Disulfide bond reduction of the *H-2* antibodies with 2-mercaptoethanol (2-Me) was carried out by incubating sera with 0.1 M 2-Me in a 1:1 mixture for 30 min at 37°C as described by Klein et al. (18).

Lymphocyte-Mediated Cytolysis (LMC). The LMC was performed following the method of Berke et al. (19). Peritoneal exudate cells (PEC) from immunized mice were harvested by flushing the peritoneal cavity on the fourth or fifth day after the immunization. As target cells C57BL leukemia cells (EL4, *H-2*^{b}) (20) or phytohemagglutinin (PHA)-induced blasts of different mouse strains were used. PHA blasts were obtained by incubation of mouse spleen cells with PHA (Wellcome Research Laboratories, Beckenham, England) (1:100) for 3 days. 5×10^4 labeled target cells in 0.1 ml were added to 1×10^6 PEC in 1.0 ml and incubated at 37°C on a rocking plate (Bellco Glass, Inc., Vineland, N. J.) at six rocks per minute. All samples were set up in duplicate or triplicate. After various times of incubation the cells were centrifuged and an aliquot of the supernate taken for determination of ^{51}Cr release. Duplicate samples of 5×10^4 target cells in 1.1 ml of medium without any effector cells served to determine the total releasable radioactivity (supernate of cells frozen and thawed four times) and the spontaneous release. The percent killing was calculated as described in the antibody-complement-mediated cytotoxicity test.

To demonstrate the nature of the killer cells in the LMC the PEC were treated with anti-Thy-1.2 serum and complement following the method of Katz and Osborne (21). Anti-Thy-1.2 serum was obtained by immunizing AKR mice with C3H/An thymocytes in a modification of the method described by Cerottini et al. (22). To test the participation of macrophages in the LMC they were removed from the PEC by incubation of the cell suspension in a Sephadex G-10-column (Pharmacia Fine Chemicals, Uppsala, Sweden) for 30 min at 37°C (23).

Results

The Antibody Response of 5R Mice to H-2D^b *Alloantigens.* 5R mice were immunized against *H-2D*^{b} alloantigens carried on three different strains of mice, B10, A.BY, and 2R. When the antibody response was measured in the complement-mediated cytotoxicity test only anti-A.BY and anti-2R yielded detectable levels of anti-*H-2D*^{b} cytotoxicity whereas immunization of 5R mice with normal B10 spleen cells produced either a very low or negative response (Fig. 1).

When the antibodies were tested in the hemagglutination test, a similar but not identical picture evolved as shown in Table II. After five immunizations 5R anti-B10 showed a low titer of 1:128 in the hemagglutination test, comparable to the titers obtained by Stimpfling and Durham in an identical immunization (24) whereas 5R anti-A.BY and anti-2R yielded higher titers (1:1,024 and 1:512).

In order to determine the nature of the antibody involved the sera were incubated with 0.1 M 2-Me for 30 min. This treatment inactivates IgM antibodies but does not inhibit the activity of IgG antibodies. As can be seen in Table II, treatment with 2-Me totally abolished the anti-*H-2D*^{b} response in the 5R anti-B10 combination whereas the titers remained unchanged in the other two combinations.

Cell-Mediated Immunity in 5R Mice Immunized with B10 Spleen Cells. To test the cell-mediated response of 5R mice that had been injected with spleen cells from congenic B10 mice and had not produced detectable cytotoxic antibodies, a ^{51}Cr release assay was used with EL4 cells as targets. Preliminary

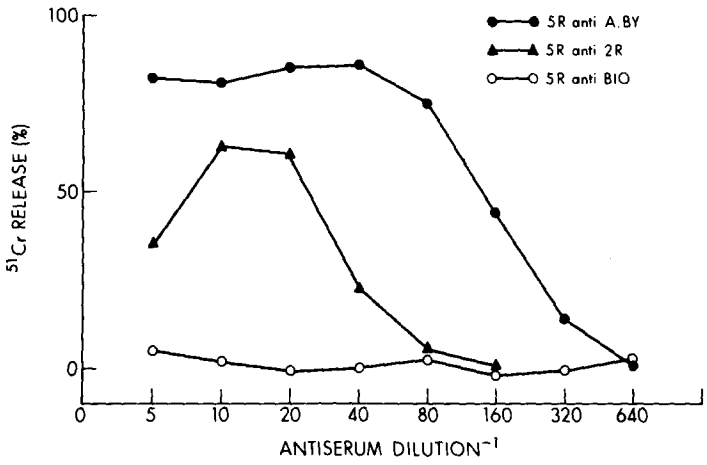

FIG. 1. Antibody levels as measured by a complement-mediated ^{51}Cr release cytotoxicity assay. Titration on *H-2D^b* (B10) lymph node cells of sera collected from 5R mice 7 days after the fifth immunization with spleen cells of A.BY, 2R, and B10 mice.

TABLE II

Antibody Response After Five Immunizations of B10.A(5R) Mice Immunized With Cells Carrying H-2D^b Alloantigens

Immunizing cells	Cell surface antigens in addition to *H-2D^b*	Anti-*H-2D^b* antibody response in the cytotoxicity test	Titers in the hemagglutination test	Titers in the hemagglutination test after treatment of the sera with 2-Me
B10	—	Low	1:128	0
A.BY	Non-*H-2* cell surface antigens	High	1:1,024	1:1,024
B10.A(2R)	*H-2K*; *H-2I*	High	1:512	1:512

experiments had shown that PEC were more effective as killer cells than spleen or lymph node cells, confirming results described by Berke et al. (19). Therefore PEC were used for all experiments. PEC were harvested 4 or 5 days after each immunization, and the mice were bled the same day. The sera were tested for cytotoxic antibody activity and were consistently found to be negative. Fig. 2 represents the results of the LMC tests performed after each immunization. No cell-mediated killing during 6 h of incubation was observed after the first immunization. After the second inoculation a positive cytotoxic response was seen; subsequent to the fourth immunization this response increased to 70–80% lysis in 4 h.

To determine the specificity of the reaction for *H-2D^b* LMC tests were performed with PHA blasts of various mouse strains carrying *H-2D^b* alloantigens. No killing reaction of PEC from 5R mice, immunized with B10 cells, could be obtained on any PHA blasts used. These negative results confirm in vivo the experiments performed in vitro by Festenstein et al. (25); these authors presented evidence that 5R cells responding in MLC to B10 cells do not kill B10

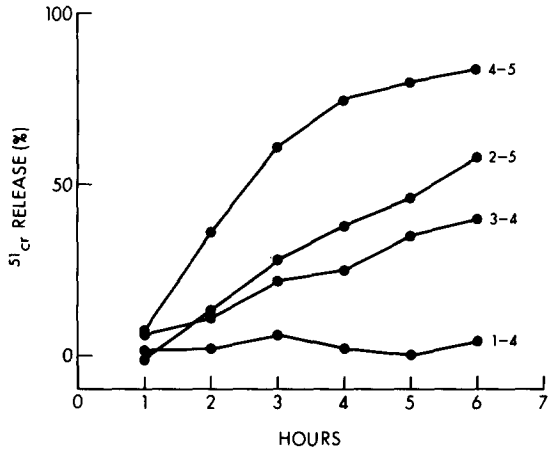

FIG. 2. Lymphocyte-mediated cytolysis during 6 h of incubation of peritoneal exudate cells from 5R mice immunized intraperitoneally with 20 × 10^6 B10 spleen cells. (LMC assays performed after the immunization schedule described in Materials and Methods gave the same pattern.) Target cells: EL4 leukemia cells. Killer to target cell ratio, 20:1. The first number refers to the number of immunizations, the second number to the day after each immunization on which the PEC were harvested.

PHA blasts in a subsequent CML. We therefore tested the specificity of 5R anti-B10 PEC on a tumor cell line of DBA/2 (*H-2*^{d}) origin (P815; a gift from Dr. B. Bloom, Albert Einstein College of Medicine, Bronx, N.Y.). P815 cells were not lysed by PEC from 5R mice immunized with normal B10 spleen cells. Experiments performed with PEC from unimmunized mice were also negative.

To examine the participation of lymphocytes and macrophages in this cell-mediated cytotoxicity two experiments were performed. PEC were incubated in a Sephadex G-10-column for 30 min to remove the macrophages. The reaction remained unchanged, which suggests that macrophages are not the basis for the cytotoxicity. Similar results were obtained by Berke et al. (19). In another experiment PEC were treated with anti-Thy-1.2 serum (AKR anti-C3H normal thymus cell) or normal AKR serum and complement before incubation with the target cells. Treatment with normal AKR serum only slightly diminished the number of PEC and did not change the cell-mediated reaction, whereas treatment with anti-Thy-1.2 serum lysed approximately 40% of the PEC and completely abolished the killing reaction (Fig. 3). This indicates that the cells reactive in this assay are Thy-1-carrying T cells (19).

Comparison of the Antibody Response of B10.D2 to B10.HTG and of BALB/c to BALB.HTG. In order to determine whether the low response of 5R mice immunized with congenic B10 cells is a feature specific for 5R or is shared by other strains of the C57BL/10 series, an immunization of another B10 congenic strain, B10.D2 anti-B10.HTG, was performed. B10.D2 carries the same *H-2S* and *H-2D* regions as 5R; it differs from B10.HTG at the *H-2D* region only. This immunization was compared to an *H-2* identical combination on the BALB/c background, BALB/c anti-BALB.HTG. Any difference between the B10.D2 and BALB/c sera cannot be attributed to the *H-2* complex.

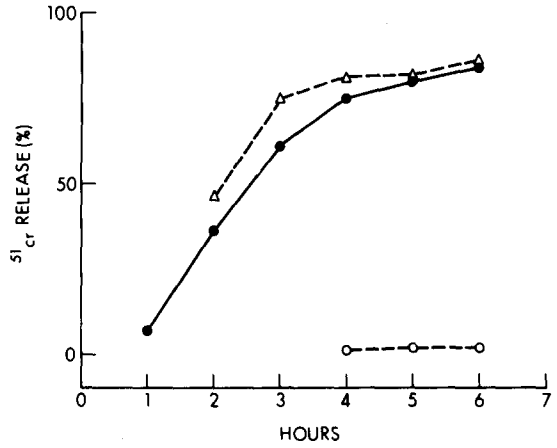

FIG. 3. Lymphocyte-mediated cytolysis 5 days after the fourth immunization of 5R mice with B10 cells. Target cells: EL4 leukemia cells. Killer to target cell ratio, 20:1. (●---●), untreated PEC; (Δ---Δ), PEC treated with normal AKR serum and complement; (O---O), PEC treated with anti Thy-1.2 serum and complement.

In the complement-mediated cytotoxicity test only very low or negative antibody titers were found in the B10.D2 anti-B10.HTG sera, whereas the titers in the BALB/c anti-BALB.HTG sera were significantly higher. Titers of the bleedings obtained after three and five injections are shown in Fig. 4. 7 days after the third immunization the B10.D2 anti-B10.HTG sera showed no cytotoxic antibodies, whereas the sera of the BALB/c combination exhibited 60% killing at a 1:5 dilution. 7 days after the fifth immunization the situation was virtually unchanged: 50% killing with the BALB/c sera and only 13% at a 1:5 dilution with the B10.D2 sera. Sera tested after several further injections showed an identical pattern.

When tested by hemagglutination, the same pattern could be seen as in the cytotoxicity test: high titers in the BALB/c sera and low titers in the B10.D2 sera (Table III). 7 days after the fifth immunization the BALB/c sera reached a titer of 1:512 in comparison to 1:128 in the B10.D2 sera.

Treatment of the sera with 2-Me completely abolished the reaction in the B10.D2 anti-B10.HTG sera, as in the case of 5R anti-B10, but 2-Me treatment only decreased the level of response from 1:512 to 1:128 in the BALB/c anti-BALB.HTG sera. This strongly suggests that the anti-*H-2D*^{b} antibodies of the B10.D2 sera are exclusively of IgM type whereas the BALB/c sera contain IgG antibodies to *H-2D*^{b} alloantigens. Thus the antibody response of B10.D2 is similar to the response of 5R, whereas the immunization of BALB/c mice yields an antibody reaction different from B10.D2.

Comparison of the Cell-Mediated Immunity of B10.D2 to B10.HTG and of BALB/c to BALB.HTG. The LMC assay was performed to investigate the cell-mediated immunity of B10.D2 and BALB/c mice immunized with *H-2*^{g} cells of the corresponding congenic series. In contrast to the 5R response, PEC from both combinations tested here were able to lyse *H-2D*^{b} carrying PHA blast cells. Therefore in addition to EL4 leukemia cells PHA blasts of various mouse strains

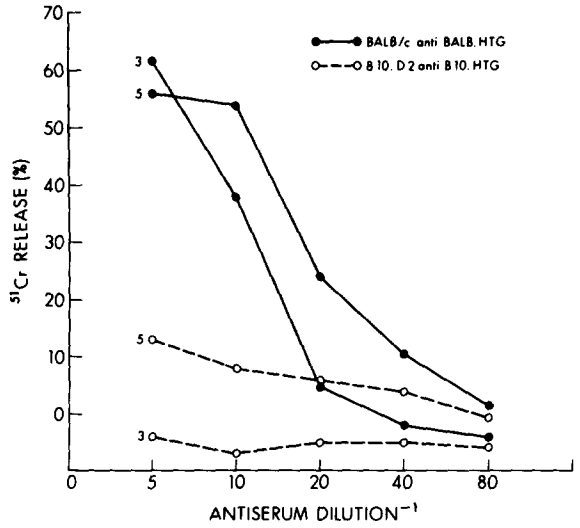

FIG. 4. Antibody levels as measured by a complement-mediated ^{51}Cr release cytotoxicity assay. Titration on *H-2D^b* (2R) lymph node cells of sera collected 7 days after the third (3) and fifth (5) injections.

TABLE III

Comparison of the Antibody Response of B10.D2 anti-B10.HTG and BALB/c anti-BALB.HTG after 5 Immunizations

	Anti-*H-2D^b* antibody response in the cytotoxicity test	Titers in the hemagglutination test	Titers in the hemagglutination test after treatment of the sera with 2-Me
B10.D2 anti-B10.HTG	Low	1:128	0
BALB/c anti-BALB.HTG	High	1:512	1:128

were used as target cells. The results of LMC tests after two, three, five, and seven injections are shown in Table IV. After the second immunization PEC from neither B10.D2 nor BALB/c mice lysed ^{51}Cr labeled PHA blasts with *H-2D^b* alloantigens, but PEC from the BALB/c immunization did lyse EL4 target cells up to 40%. This confirms the findings in 5R mice that tumor cells are more readily killed than PHA blasts. After three immunizations both EL4 cells and PHA blasts were killed by PEC from either B10.D2 or BALB/c mice although the B10.D2 response was weaker. After further injections there was virtually no difference in the killing capacity of cells from B10.D2 and BALB/c mice immunized with *H-2^g* cells of the corresponding congenic series.

Using PHA blasts from various strains of mice carrying the *H-2D^b* alloantigens (HTH/Go, HTG/Go, C57BL/6) it was shown that the T-cell response was specific for *H-2D^b*; the PEC did not lyse *H-2^k* (AKR) PHA blasts as shown in Table IV. To confirm that the effector cells in the LMC are still T cells, even after seven immunizations, half of the PEC were treated with anti-Thy-1.2 serum and complement before incubation with the target cells. As can be seen in the last

TABLE IV
Lymphocyte-Mediated Cytolysis of PEC Harvested 4 Days After Each Immunization (Killer to Target Cell Ratio, 20:1)

	% ^{51}Cr release									
	No. of immunizations									
	2		3		5		7		7*	
Target cells	a‡	b	a	b	a	b	a	b	a	b
EL4 (C57BL leukemia)	−3.6	40.1	30.8	52.3	73.8	66.6	74.2	62.4	−2.5	5.8
H-2D^b PHA blasts	−17.2	3.0	16.1	31.9	ND	ND	38.6	45.0	−13.6	−17.4
H-2^a PHA blasts	−4.8	−4.3	−6.7	−5.7	ND	ND	ND	ND	ND	ND

* PEC treated with anti Thy-1.2 + C′.
‡ a = B10.D2 anti-B10.HTG; b = BALB/c anti-BALB.HTG.

column of Table IV the killing capacity of the effector cells was abolished by this treatment, indicating that after seven immunizations the killer cells in both strains were still of T-cell origin.

Discussion

The experiments reported here have shown that 5R mice immunized with B10 spleen cells respond to *H-2D^b* alloantigens with low levels of antibodies of IgM type only. T cells from these mice reveal a normal cell-mediated immunity; they are able to recognize the *H-2D^b* difference and kill EL4 (*H-2D^b*) leukemia cells. On the other hand, 5R mice immunized with cells carrying alloantigen specificities in addition to *H-2D^b* (non-*H-2* alloantigens in the case of A.BY, *H-2K*, and *H-2I* antigens in the case of 2R) produce IgG antibodies to *H-2D^b*.

McKenzie and Snell (5) have reported that B10.A anti-B10.AKM x B10 and B10.AKM anti-B10.A yield no or only a very weak antibody response to *H-2D* antigens whereas similar congenic combinations on a background different from B10 give a good antibody response. This is confirmed in our findings that BALB/c mice immunized with BALB.HTG cells yield IgG antibodies to *H-2D^b* whereas B10.D2 anti-B10.HTG does not produce anti-*H-2D^b* IgG antibodies. These findings indicate that the low response of 5R anti-B10 and B10.D2 anti-B10.HTG is not due to the *H-2* complex itself but is governed by a gene or genes separate from *H-2* on the B10 background. Under the influence of these genes there is no IgG response to *H-2D^b* antigens unless the immunizing cells carry additional foreign cell surface antigens.

It has been shown by Klein et al. (18) that the IgM response to *H-2* is thymus independent whereas the IgG response is thymus dependent. On the basis of these findings the following hypothesis is proposed: for mice of the B10 series *H-2D^b* alloantigens, presented on a congenic background without additional foreign cell surface antigens, constitute a "hapten" on a nonimmunogenic carrier. This "hapten" elicits a thymus-independent IgM response only, as has been demonstrated in another system with autologous red blood cells as nonimmunogenic carriers (26-27). The nonimmunogenic carrier fails to activate the T-cell

helper function which switches the initial IgM antibody production to the IgG response, whereas additional foreign cell surface antigens convert the nonimmunogenic carrier into an immunogenic carrier that elicits an IgG response. Genes separate from *H-2* regulate the carrier recognition of T cells and thereby influence the activation of the T-cell helper function.

In BALB/c mice these genes enable the T cells to recognize cells with only an *H-2D*^{b} difference; the T-cell helper function is activated and leads to the switch from IgM to IgG production. In mice of B10 origin either there is no recognition by T helper cells of cells that differ only at *H-2D*^{b}, or there is recognition by some subset of T cells that does not lead to activation of T-cell helper function.

Our experiments clearly show activation of T-cell killer function in all mice immunized with *H-2D*^{b} bearing cells. Therefore the genes that are responsible for the low response of IgM type in mice of the B10 series are not involved in the regulation of the T-cell-mediated cytotoxicity to *H-2D*^{b} antigens on congenic cells.

In the light of this hypothesis our experiments suggest separate genetic regulation and activation of T-cell helper function and T-cell-mediated cytotoxicity. A separation of humoral and cellular immunity has been described by several other groups in different experimental systems (28-30).

Summary

B10.A(5R) mice immunized with C57BL/10 spleen cells demonstrate a normal T-cell-mediated cytotoxicity to *H-2D*^{b} tumor cells but they do not mount any IgG antibody response to *H-2D*^{b} alloantigens. B10.A(5R) mice do show a high titered IgG response when immunized with A.BY cells, which differ at *H-2D*^{b} plus non-*H-2* cell surface antigens, or with B10.A(2R) cells, which differ at *H-2D*^{b}, *H-2K*^{k}, and *H-2I*^{k} cell surface antigens. These findings indicate a failure of the T-helper cells to induce the switch from IgM to IgG when the *H-2D*^{b} alloantigens are the only difference on the immunizing cell.

In immunizing *H-2*^{d} mice with congenic *H-2*^{g} cells which differ only in the *H-2D*^{b} region, mice of the C57BL/10 background made only IgM antibodies whereas mice of the BALB/c background made IgG antibodies. This comparison confirms that genes separate from *H-2* regulate the T-cell helper function. The genes that influence the T-cell helper function do not regulate the T-cell-mediated cytotoxicity.

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