IN VITRO CELL-MEDIATED IMMUNE RESPONSES TO THE MALE SPECIFIC (H-Y) ANTIGEN IN MICE

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The male specific antigen H-Y (reviewed by Gasser and Silvers) (1) provides a useful model for the investigation of weak transplantation antigens in mice. It is well known that inbred mouse strains differ in their immune response to syngeneic male skin grafts. Thus C57BL/10 (hereafter "B10") and B10.A (5R) females frequently reject syngeneic male grafts, whereas females of numerous other "nonresponding" strains (e.g. CBA, C3H, BALB/c, and A) usually accept syngeneic male grafts. The factors that determine responsiveness to the H-Y antigen are only partly understood, but evidence exists that the expression of H-Y antigen on male tissues (1–3) and the response by female hosts (1, 4–8) are at least in part determined by genes in the *H-2* major histocompatibility complex (MHC).[1]

Cytotoxic antibody has been demonstrated both in inbred strains which reject and in those which fail to reject syngeneic male skin grafts (9). Thus interstrain differences in female immune responses to H-Y antigen may depend upon cell-mediated immune mechanisms. Goldberg et al. (10) have demonstrated cell-mediated cytotoxicity against C57BL/6 male lymph node cells by spleen cells from syngeneic females previously grafted with C57BL/6 male skin. Maximum cytotoxicity between 20 and 30 days after grafting corresponds roughly with the survival time of male skin grafts on females in this strain.

In this paper we present a method for the study in vitro of cell-mediated cytotoxic responses to the H-Y antigen using the B10 strain in which syngeneic male grafts are regularly rejected by females. The results demonstrate that the cytotoxic activity of female spleen cells sensitized in vitro against syngeneic male target cells is mediated through cytotoxic T lymphocytes (CTL). We were unable to demonstrate an in vitro cellular cytotoxic response in two "nonresponding" strains, CBA and B10.A, which fail to reject male grafts. Additional data show that the target cell specificity of H-Y cell-mediated cytotoxicity is restricted by the *H-2* complex.

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[1] *Abbreviations used in this paper:* A:T, attacking cell to target cell ratio; B10, C57BL/10; BSS, balanced salt solution; CTL, cytotoxic T lymphocytes; LD, lymphocyte defined; MHC, the *H-2* major histocompatibility complex in the mouse; SD, serologically defined.

Materials and Methods

Animals. B10.A (5R), B10.A, B10.BR, and C3H.SW mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. All other inbred mouse strains were obtained from the Animal Division of the Clinical Research Centre, Harrow, England.

Primary Sensitization. Female responding mice were primed in vivo either by grafting with male tail skin (11) or by i.p. injection of isologous male spleen cells. Animals sensitized by spleen cell injections usually received 1×10^7 cells i.p. followed by a second injection of 1×10^7 cells 10–14 days later.

In Vitro Sensitization. The methods and materials employed in setting up cell cultures for primary or secondary in vitro sensitizations have been previously described (12). Briefly, spleens from normal or primed female mice were gently teased apart, washed in balanced salt solution (BSS), treated with Gey's solution to remove erythrocytes, and washed twice more in BSS. T cells were separated by nylon wool filtration (13) when appropriate at this stage. For antigen, a similarly prepared suspension of male spleen cells was given 2,000R from a cobalt 60 source. The concentration of each cell suspension was adjusted to 5×10^6 cells per ml in RPMI medium with 10% fetal calf serum. Cells were dispensed into either flat-bottomed Microtest II (Cooke, Division of Dynatech Laboratories, Ltd., Billingshurst, England) plates containing 0.1 ml (5×10^5) female responding cells and 0.1 ml (5×10^5) irradiated male stimulating cells per well or into 30-ml cell culture flasks (Falcon Plastics, Division of BioQuest, Oxnard, Calif. or Nunc UK Ltd., Stafford, England) containing 5.0 ml (2.5×10^7) responding cells and 5.0 ml (2.5×10^7) irradiated male stimulating cells. Cultures were incubated at 37°C in a humidified 10% CO_2 atmosphere.

Cytotoxicity Assay. The microcytotoxicity assay has been previously described in detail (12). Responding cells were harvested from cell culture, washed once in BSS, and adjusted to 2×10^6 cells per ml (4×10^5 cells per 0.2 ml) in Eagle's minimal essential medium with 10% fetal calf serum. Two or three halving serial dilutions (e.g. 2×10^5 cells per 0.2 ml, 1×10^5 cells per 0.2 ml, etc.) were also made. In initial experiments nonimmune female control cells at the same concentrations were prepared from freshly teased female spleens. In later experiments medium controls consisting of labeled target cells incubated with medium alone served as the only control, since the levels of ^{51}Cr release by nonimmune cells and spontaneous ^{51}Cr release in medium controls were essentially identical within each experiment. 0.05 ml (1×10^5) of ^{51}Cr-labeled target cells was dispensed into each well of the Microtest plate. 0.2 ml of each attacking cell suspension was then added to the appropriate wells, allowing three or four replicates for each attacking cell to target cell (A:T) ratio. Maximum lysis was determined by adding 0.05 ml of target cells and 0.20 ml of 5% Triton to a set of wells. After 5 min of gentle centrifugation at 500 rpm the plates were incubated for 3 h at 37°C in a humidified 10% CO_2 atmosphere. The plates were then centrifuged for 10 min at 1,000 rpm, and 0.1-ml samples were removed for gamma counting.

Preparation of Target Cells. Target cells were prepared by incubating spleen cells in RPMI medium with 10% fetal calf serum for 48 or 72 h before assay in the presence of concanavalin A (4 μg/ml) and labeling the harvested cells with sodium ^{51}Cr as previously described (12).

Data Analysis. The corrected percent lysis was computed according to the formula of Wunderlich et al. (14). Linear regression analysis was used to make quantitative comparisons from the data obtained (12).

Results

Cell-Mediated Secondary Cytotoxic Responses to H-Y Antigen. Fig. 1 presents data showing cell-mediated cytotoxicity against H-Y antigen expressed by in vivo primed B10 female spleen cells which were boosted in vitro with B10 male spleen cells. Skin grafting and i.p. spleen cell injections were both satisfactory methods for in vivo priming and could be used in combination to augment the response of female mice grafted 3 or more mo previously. High levels of cytotoxicity were seen at low A:T ratios, similar to responses to *H-2* antigens seen in this assay system (12). We were unable, however, to elicit primary in vitro cytotoxic responses to H-Y antigen.

Sex-Determined Target Cell Specificity. The data for B10 female responding

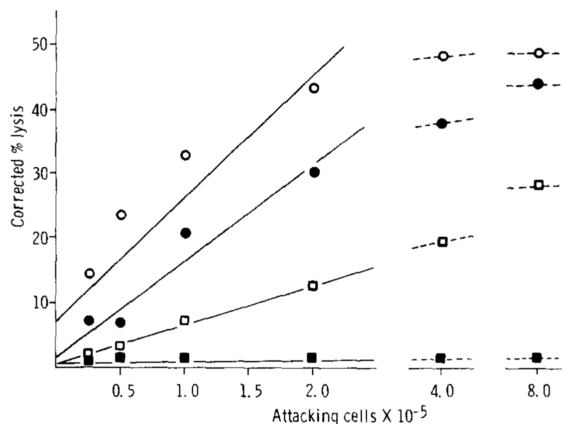

FIG. 1. Cytotoxic response of sensitized B10 female spleen cells against 1 × 10^5 B10 ^{51}Cr-labeled male spleen target cells. All responding cells were cultured for 5 days with an equal number of irradiated B10 male spleen cells. Each culture of responding cells was assayed in quadruplicate for 3 h at six A:T ratios. Linear regression lines were plotted from the origin through A:T = 2:1 (i.e. 2 × 10^5 attacking cells). (■—■) unprimed (normal) B10 female spleen cells (primary in vitro sensitization). (□—□) female responding cells primed in vivo by grafting with B10 male tail skin 44 days before culture. Data fits a linear regression line with slope 6.44±0.16 and r^2 (the coefficient of determination, a measure of the goodness of fit) = 1.00. (●—●) female responding cells primed in vivo by i.p. injection of 1 × 10^7 B10 male spleen cells 14 days before culture. Data fits a linear regression line with slope 15.20±1.95 and r^2 = 0.95. (○—○) female responding cells primed in vivo by two previous male skin grafts applied 5 and 3 mo earlier and boosted with 1 × 10^7 B10 male spleen cells injected i.p. 14 days before culture. Data fits a linear regression line with slope 19.62±4.34 and r^2 = 0.87. Background (spontaneous ^{51}Cr release) was 8.6%. SE were less than 4% of mean counts.

cells in the first section of Table I demonstrates that the in vitro cytotoxic response of sensitized B10 female spleen cells is specific for male target cells. Lysis of female target cells was not significantly above control levels. Similar data is also presented in Table III, Experiment A.

In Vivo Priming with i.p. Spleen Cells. Female B10 mice were given a priming i.p. injection of 1 × 10^4, 1 × 10^5, 1 × 10^6, or 1 × 10^7 B10 male spleen cells and boosted in vitro with irradiated male spleen cells either 7 or 14 days later. Another set of females received second injections of the initial dose of spleen cells 10 days after priming, and cell cultures were set up 7 days after the boosting injection. The results of this experiment are presented in Fig. 2. No cytotoxic responses were observed in mice that received less than 1 × 10^6 male cells regardless of the number of injections or the length of time allowed. 1 wk after a priming injection, females which had received 1 × 10^7 male cells showed a weak cytotoxic response. 2 wk after priming, high levels of cytotoxic activity were seen in females given either 1 × 10^6 or 1 × 10^7 male cells. No additional advantage was achieved by a boosting injection given 10 days after priming and 1 wk before in vitro boosting.

Time-Course of In Vitro Sensitization. Fig. 3 shows the level of cytotoxic activity obtained in cell culture as a function of time. Cell cultures were set up on the same day (day 0) and an assay performed on aliquots of cells harvested on

TABLE I

Cell-Mediated Response to H-Y Antigen by B10, B10.A, and CBA Mice

Responding cells	Antigen		A:T	Target cells*		
	In vivo	In vitro				
				B10♂	B10♀	CBA♂
B10♀	B10♂	B10♂	4:1	18.84 ± 0.61	2.50 ± 0.82	3.49 ± 0.55
			2:1	9.11 ± 0.68	0.50 ± 0.93	1.08 ± 0.37
			1:1	4.24 ± 0.45	1.22 ± 0.37	0.13 ± 0.31
				B10♂	CBA♂	CBA♀
B10♀	B10♂	CBA♂	4:1	0.60 ± 0.37	15.11 ± 0.22	17.74 ± 0.61
			2:1	0.80 ± 1.00	6.97 ± 0.47	6.18 ± 0.83
			1:1	1.24 ± 0.42	4.88 ± 0.59	2.84 ± 0.37
				B10.A♂	B10♂	B10♀
B10.A♀	B10.A♂	B10.A♂	4:1	−0.83 ± 0.51	0.22 ± 0.46	
			2:1	0.62 ± 0.34	−0.24 ± 0.40	
			1:1	−0.58 ± 0.24	0.26 ± 0.57	
B10.A♀	B10.A♂	B10♂	4:1	0.38 ± 0.48	22.53 ± 2.61	23.33 ± 2.17
			2:1	−1.13 ± 0.41	14.63 ± 1.34	9.94 ± 1.28
			1:1	0.26 ± 0.24	7.07 ± 0.37	5.99 ± 0.65
				CBA♂	B10♂	B10♀
CBA♀	CBA♂	CBA♂	4:1	0.00 ± 0.36	1.76 ± 0.56	
			2:1	0.33 ± 0.22	0.23 ± 0.28	
			1:1	0.45 ± 0.34	−0.28 ± 0.91	
CBA♀	CBA♂	B10♂	4:1	0.76 ± 0.38	15.72 ± 0.73	18.19 ± 1.56
			2:1	0.60 ± 0.28	7.13 ± 0.85	5.18 ± 0.85
			1:1	0.57 ± 0.22	3.04 ± 1.07	2.52 ± 0.93

Female responding cells were primed in vivo and boosted in vitro with the male antigens shown. Details are explained in the text. All responding cell suspensions were assayed in quadruplicate at three A:T ratios for 3 h. SE were less than 6% of mean counts. Background ^{51}Cr release ranged from 6.2 to 19.0%.

* Corrected percent lysis ± 1 SE.

each day from day 3 to day 7. This permitted use of a single population of female responding cells, but required separate lots of ^{51}Cr-labeled male target cells for each assay. Maximum cytotoxic response was seen at day 5 with detectable activity present as early as day 3 and persisting through day 7. The level of activity of each culture can be expressed as the slope of a linear regression line (Table II, column 3) representing the best straight line fit to the dose response data. If this value is multiplied by the cell yield, expressed in this experiment as cells per well (Table II, column 2), we get a measure of the total cytotoxicity of each culture (Table II, column 4). It can be seen in Table II that, based on the dose response data in Fig. 3, total cytotoxicity is highest at day 5.

We have also performed this experiment using a single lot of ^{51}Cr-labeled target cells by setting up a series of separate responding cell cultures 3–7 days

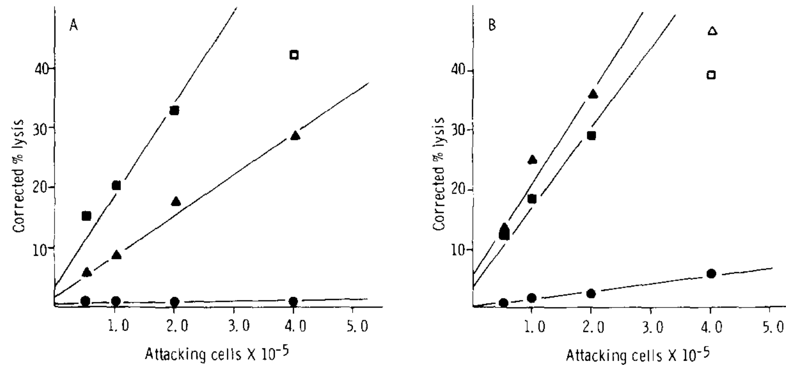

FIG. 2. Immunization of B10 female mice to H-Y antigen by i.p. injection of B10 male spleen cells. (A) (●—●) female mice received 1 × 10^6 male cells 7 days before culture. (■—□) female mice received 1 × 10^6 male cells 14 days before culture. Closed symbols (■) fit a linear regression line with slope 15.41±2.67 and r^2 = 0.94. (▲—▲) female mice given a boosting injection of 1 × 10^6 male cells 7 days before culture and 10 days after a priming injection of 1 × 10^6 male cells. Closed symbols (▲) fit a linear regression line with slope 6.97±0.52 and r^2 = 0.98. (B) (●—●) female mice received 1 × 10^7 male cells 7 days before culture. Closed symbols (●) fit a linear regression line with slope 1.29±0.10 and r^2 = 0.98. (■—□) female mice received 1 × 10^7 male cells 14 days before culture. Closed symbols (■) fit a linear regression line with slope 13.63±2.00 and r^2 = 0.96. (▲—△) female mice given a boosting injection of 1 × 10^7 male cells 7 days before culture and 10 days after a priming injection of 1 × 10^7 male cells. Closed symbols (▲) fit a linear regression line with slope 17.38±2.90 and r^2 = 0.95. Responding cells in (A) and (B) were cultured for 5 days before assay with an equal number of irradiated B10 male spleen cells. All cultures were assayed in quadruplicate for 3 h. Backgrounds (spontaneous ^{51}Cr release) ranged from 5.9 to 12.7%. SE were less than 5% of mean counts.

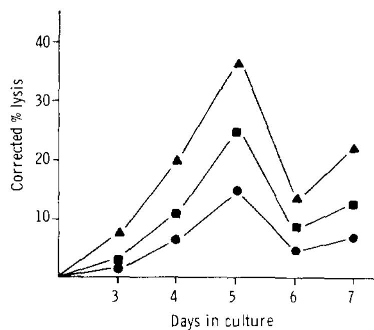

FIG. 3. Effect of length of in vitro sensitization on the expression of cytotoxic activity against the H-Y antigen. Cultures were set up on the same initial day and assayed on successive days against separate lots of ^{51}Cr-labeled B10 male target cells. Linear regression data and total cytotoxicity for this experiment are given in Table II. Cultures were assayed in quadruplicate for 3 h at three A:T ratios: (●) 1:1 (■) 2:1, and (▲) 4:1. Backgrounds (spontaneous ^{51}Cr release) were 6.9 to 11.9%. SE were less than 5% of mean counts.

before assay. The results (not shown) were similar to the experiment presented in Fig. 3 and Table II.

In addition we have studied the effects of time on the generation of primary cytotoxic responses to H-Y antigen by unprimed female spleen cells. We have

TABLE II
Comparison of Corrected Percent Lysis and Total Cytotoxicity in Cultures from Day 3 to Day 7 of Sensitization

Days in culture	Yield of sensitized cells per well ($\times 10^{-5}$)	Corrected percent lysis*	Total cytotoxicity‡
3	1.23	1.82 ± 0.16 (r^2 = 1.00)	2.24
4	1.47	4.81 ± 0.28 (r^2 = 0.99)	7.07
5	2.46	8.63 ± 1.45 (r^2 = 0.95)	21.23
6	4.13	3.30 ± 0.34 (r^2 = 0.98)	13.63
7	2.34	5.33 ± 0.34 (r^2 = 0.99)	12.47

Data shown in this table is from the experiment also presented in Fig. 3.

* Slope of the linear regression line (± 1 SE) representing the best straight line fit to the dose response curve obtained by assaying at three A:T ratios (4:1, 2:1, and 1:1). r^2 is the coefficient of determination, a measure of the goodness of fit.

‡ Slope of the linear regression line × yield of sensitized cells.

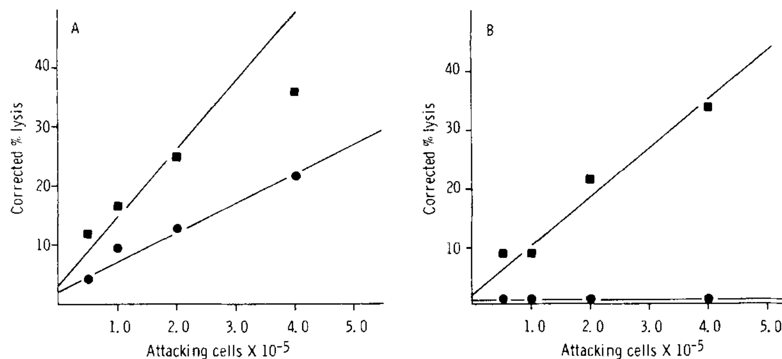

FIG. 4. Cytotoxicity of unseparated female spleen cells and female splenic T cells against ^{51}Cr-labeled male spleen target cells. (A) cytotoxic activity of unseparated (■) and nylon wool filtered (●) B10 female spleen cells against ^{51}Cr-labeled B10 male targets. Female spleen cells from mice primed in vivo by i.p. injection of isologous male spleen cells were cultured for 5 days in vitro with equal numbers of irradiated male spleen cells. One portion of female cells was filtered through a nylon wool column before culture to remove nearly all the B lymphocytes. (B) Unseparated female spleen cells primed in vivo and cultured in vitro for 5 days with male spleen cells were treated with anti-Thy 1.2 serum, preabsorbed with Thy 1.1 thymocytes, with (●) and without (■) complement just before assay against ^{51}Cr-labeled male target cells. All responding cell cultures were assayed in quadruplicate for 3 h at four A:T ratios. Background (spontaneous ^{51}Cr release) was 19.6%. SE were less than 4% of mean counts.

been unable to detect in vitro primary cytotoxic responses, even in cultures extended to 6 or 7 days. These time-course data are similar to results we have previously reported for sensitizations involving *H-2* differences (12), except for the inability to elicit primary in vitro responses in the H-Y system.

Anti-H-Y Responses are Mediated by CTL. Two methods were used to demonstrate that in vitro cytotoxic responses to the H-Y antigen are mediated by CTL. Fig. 4A shows that if before cell culture spleen cells from a sensitized female B10 mouse are filtered through a nylon wool column to remove nearly all

B lymphocytes, high levels of cytotoxic activity are still elicited after in vitro sensitization. If sensitized B10 female cells are treated with anti-Thy 1.2 serum, preabsorbed with Thy 1.1 thymocytes, and complement just before assay, the cytotoxic response is abolished (Fig. 4B). Preabsorption of the anti-Thy 1.2 serum with Thy 1.2 mouse brain tissue preserves the cytotoxic activity, thus confirming the specificity of the anti-Thy 1.2 serum (data not shown).

Cytotoxic Responses by "Nonresponding" Strains. CBA and B10.A are among the strains classified as "nonresponders" to the H-Y antigen. A large proportion of male to female isografts within these strains survive indefinitely. CBA and B10.A females were given two i.p. injections of 1×10^7 syngeneic male spleen cells 2 wk apart. After another 2 wk these primed female spleen cells were cultured for 5 days with irradiated syngeneic male cells or allogeneic B10 male spleen cells. A cytotoxicity assay was then done against syngeneic (CBA or B10.A) male target cells and allogeneic (B10) male and female target cells. In addition, B10 female cells from animals primed in vivo with B10 male spleen cells were boosted in vitro with either B10 male or CBA male spleen cells and assayed against B10 and CBA male and female spleen cells. The results are presented in Table I. Neither CBA nor B10.A female cells demonstrated cytotoxic responses against syngeneic male target cells (H-Y response) regardless of whether boosted with syngeneic or allogeneic male cells, but both strains showed appropriate *H-2* responses against allogeneic (B10) male and female target cells when boosted with allogeneic male cells. B10 female spleen cells showed the expected male specific cytotoxic response when primed and boosted with B10 male cells, but responded against CBA male and female target cells (*H-2* response) only when boosted with CBA male cells.

H-2 Determined Target Cell Specificity. A series of experiments presented in Table III was performed to determine whether the *H-2* complex affects the specificity of target cell lysis in the H-Y system. In each of these experiments B10 female responding cells from mice primed in vivo with either B10 male isografts or i.p. injections of B10 male spleen cells were boosted in vitro with irradiated B10 male spleen cells and assayed against a panel of selected ^{51}Cr-labeled target cells. Each panel of target cells included B10 male cells to provide a basis for comparison. All assays were done at four A:T ratios (4:1, 2:1, 1:1, and 0.5:1) for 3 h. The corrected percent lysis given in Table III is the kill of target cells at A:T = 4:1 as determined from a four point linear regression curve. As expected, male specific cytotoxic activity was seen against B10 target cells (Experiment A). Moreover, congenic recombinant male target cells expressing $H\text{-}2D^b$, such as B10.A (2R) and B10.A (4R), were also lysed, but not B10.A (5R) which expresses only $H\text{-}2K^b$ (Experiments A and B). B10.C (47N) differs from B10 only at the *H-7* locus (15), and male target cells from this strain are killed at levels equal to B10 male targets (Experiment B). D2.GD is an $H\text{-}2^{d/b}$ recombinant on a DBA/2 background, and C3H.SW is $H\text{-}2^b$ on a C3H background. Both of these target cells were killed as well as B10 male targets (Experiments B and C). Target cells expressing haplotypes other than $H\text{-}2^b$, whether on or off a B10 background, were not lysed. Thus lysis of B10.D2, B10.BR, and B10.A, as well as BALB/c, CBA, C3H, and A male target cells, was not significantly above control levels.

TABLE III
H-2 Target Cell Specificity of Cell-Mediated Responses to H-Y Antigen

Target cell	*H-2* haplotype						Corrected percent lysis	r^2
	K	IA	IB	IC	S	D		
A B10♂	b	b	b	b	b	b	22.17 ± 0.85	0.99
B10♀	b	b	b	b	b	b	2.03 ± 0.23	0.98
B10.A(4R)♂	k	k	b	b	b	b	20.74 ± 0.72	1.00
B10.A(4R)♀	k	k	b	b	b	b	5.06 ± 1.19	0.88
B10.A(5R)♂	b	b	b	d	d	d	3.30 ± 0.22	0.99
B B10♂	b	b	b	b	b	b	33.48 ± 2.95	0.97
B10.A(2R)♂	k	k	k	d	d	b	30.56 ± 2.10	0.97
B10.A(2R)♀	k	k	k	d	d	b	2.24 ± 0.17	0.97
B10.A(5R)♂	b	b	b	d	d	d	3.92 ± 0.34	0.97
B10.C(47N)♂	b	b	b	b	b	b	33.26 ± 2.45	0.98
B10.C(47N)♀	b	b	b	b	b	b	2.52 ± 0.21	0.99
C3H.SW♂	b	b	b	b	b	b	38.48 ± 3.80	0.96
C3H.SW♀	b	b	b	b	b	b	1.51 ± 0.11	0.98
C3H♂	k	k	k	k	k	k	7.30 ± 1.25	0.88
C B10♂	b	b	b	b	b	b	16.74 ± 0.70	0.99
B10.D2♂	d	d	d	d	d	d	2.78 ± 0.45	0.93
BALB/c♂	d	d	d	d	d	d	1.14 ± 0.20	0.91
D2.GD♂	d	d	b	b	b	b	15.41 ± 0.54	1.00
B10.BR♂	k	k	k	k	k	k	1.32 ± 0.27	0.94
CBA♂	k	k	k	k	k	k	3.30 ± 0.42	0.88
B10.A♂	k	k	k	d	d	d	2.80 ± 0.29	0.98
A♂	k	k	k	d	d	d	2.60 ± 0.49	0.92
	Parental haplotypes							
D B10♂	$H\text{-}2^b/H\text{-}2^b$						33.48 ± 2.95	0.97
(B10 × BALB/c)F_1♂	$H\text{-}2^b/H\text{-}2^d$						22.49 ± 2.33	0.96
(B10 × BALB/c)F_1♀	$H\text{-}2^b/H\text{-}2^d$						2.29 ± 0.16	0.99
(BALB/c × B10)F_1♂	$H\text{-}2^d/H\text{-}2^b$						23.05 ± 3.14	0.93
(CBA × B10)F_1♂	$H\text{-}2^k/H\text{-}2^b$						24.29 ± 1.34	0.99
(B10 × A)F_1♂	$H\text{-}2^b/H\text{-}2^{k/d}$						21.20 ± 0.90	0.99
(B10.D2 × BALB/c)F_1♂	$H\text{-}2^d/H\text{-}2^d$						3.18 ± 0.25	0.99

B10 ♀ spleen cells from mice primed in vivo and boosted in vitro with B10 ♂ spleen cells were assayed in quadruplicate for 3 h with ^{51}Cr-labeled target cells at A:T = 4:1, 2:1, 1:1, and 0.5:1. Corrected percent lysis is the percent kill of target cells at A:T = 4:1 as determined from a four point linear regression curve ± 1 SE. r^2 is a measure of the goodness of fit. Background ^{51}Cr release ranged from 5 to 20%. SE were less than 5% of mean counts. Maternal parents are listed first in describing the origins of F_1 animals, e.g., (B10 × A)F_1 means (B10♀ × A♂)F_1.

Finally, a panel of F_1 target cells which were the products of crosses between B10 males or females and mice from other selected strains is presented in Table III, Experiment D. All of these F_1 male target cells were killed at levels only moderately lower than lysis of B10 male targets. (B10.D2 × BALB/c)F_1 male target cells, which express only $H\text{-}2^d$ antigens, were not lysed.

We conclude that recognition and lysis of target cells expressing the H-Y

antigen is determined by the *H-2* complex. Non-*H-2* genetic background did not have any significant effect.

Discussion

Previous investigations suggest that the male specific antigen in mice is a sex-limited antigen which may be the product of a gene or genes on the short, nonpairing segment of the Y chromosome. Earlier in vivo experiments did not demonstrate strain specificity for the H-Y antigen. In fact, experiments by Billingham and Silvers (16) showed that adult C57BL/6 female mice given neonatal injections of adult male cells from nonresponding strains such as A, AU, C3H, or CBA are tolerant of C57BL/6 male skin grafts. Moreover, Eichwald et al. (17) and Zaalberg (18) showed that F_1 males produced by reciprocal pairings between responding and nonresponding strains (e.g. B10 and CBA) will accept each other's skin and the skin of either male parent. These results suggest that at least part of the H-Y antigen is shared by all the strains investigated.

We mentioned earlier that the *H-2* complex influences both the expression of H-Y antigen on male tissues and the responsiveness of female hosts. The exact nature of the relationship between H-Y and *H-2* remains unclear. We have demonstrated that B10 female spleen cells sensitized to B10 male cells display T-cell mediated cytotoxic activity restricted to male target cells which are *H-2* compatible with B10 over at least a portion of the MHC. Compatibility at the D end [B10.A(2R)] of the MHC appears to be required. B10.A (5R) male cells which express $H\text{-}2^b$ only at the K end of the MHC were not lysed. Non-*H-2* background appears to play no significant role since D2.GD, C3H.SW, and B10.C (47N) male target cells were lysed but not B10.A, B10.BR, or B10.D2 male cells. F_1 male targets produced by reciprocal pairings of B10 males and females with BALB/c, CBA, or A strain males and females were lysed whether the H-Y gene was inherited from a responding (B10) or nonresponding (BALB/c, CBA, or A) parent and whether the *H-2* compatible haplotype ($H\text{-}2^b$) came from a female or male B10 parent.

Cell-mediated cytotoxic responses to hapten and virus-induced cell surface antigens have been recently shown to be *H-2* restricted (19–22). Two operational models have been proposed by Zinkernagel and Doherty (23) to explain the *H-2* restricted cytotoxic responses to mouse target cells infected with lymphocytic choriomeningitis virus. These are applicable to the H-Y system as well. In the first model, the "altered self" hypothesis, the H-Y gene may code for an intracellular product affecting the *H-2* genome or an *H-2* gene product resulting in an altered *H-2* specificity *H-2 (Y)*. In a B10 system this would be $H\text{-}2\ (Y)^b$, indicating a male specific antigen derived from an $H\text{-}2^b$ determined specificity. This version of the model defines H-Y as a gene coding for a change in the MHC. H-Y is not itself a histocompatibility antigen. One could also envision an H-Y gene coding for an antigen expressed in intimate proximity to an *H-2* product resulting in a complex antigen *H-2* plus H-Y. The altered self hypothesis predicts that *H-2* compatibility between antigen-bearing cells (e.g. male skin grafts, male spleen cells) during sensitization and target cells during assay is

essential. Thus, in our experiment the *H-2*^{b} female T cell has been sensitized to *H-2 (Y)*^{b} bearing male cells and will lyse any male target cell expressing *H-2 (Y)*^{b} at the D end of the MHC.

A second model proposed by Zinkernagel and Doherty is the "intimacy" hypothesis. This states that a close association between attacking cell and target cell is essential if cytolysis is to occur. A process of self recognition between attacking cell and target cell, dependent upon the mutual interaction of compatible *H-2* specificities, is necessary to establish the close association between cells required for recognition of the H-Y antigen. In this model H-Y would be itself an antigen and would not be strain specific. The model predicts that *H-2* compatibility between attacking cell and target cell is essential for cytotoxic response. Thus, in our experiment, B10 female CTL sensitized to B10 male cells affiliate only with male target cells bearing identical *H-2* specificities over a portion of the MHC. Once the required degree of intimacy between attacking cell (*H-2*^{b}) and target cell (*H-2*^{b}) is established, cytotoxic activity against the target cell male antigen (H-Y) is possible.

It is not possible from the present data on *H-2*-restricted H-Y responses to determine whether *H-2* compatibility between responding (attacking) cell and target cell (intimacy model) or between stimulating (sensitizing) cell and target cell (altered self model) is the essential feature. However, using appropriately selected semiallogeneic F_1 female responding cells and parental and congenic recombinant male stimulating cells and target cells, it may be possible to test these requirements in the H-Y system.

In the past it has been difficult to generate cytotoxic cells to antigens other than those expressed by the MHC. The altered self hypothesis is attractive in that it provides an explanation for the generation of CTL to weak histocompatibility antigens if these can induce changes in the MHC or an MHC product. The in vitro cell-mediated response to H-Y antigen is similar in many features to anti-*H-2* responses. The relative numbers of responding cells and stimulating cells required in cell culture, the time-course of the sensitization in vitro, and the A:T ratios at which a linear dose response is seen in the microcytotoxicity chromium release assay are similar in both systems. However, the altered self model predicts that H-Y is strain specific. As discussed above, this has not been found to be the case in previous in vivo experiments. It should be stressed, however, that the target cell specificity of the *H-Y* response, which we have demonstrated, concerns only the end phase of the immune response, namely, the cytotoxic response of sensitized T lymphocytes directed against antigens expressed on target cells. We are not yet certain if in vitro cytotoxic activity against H-Y can be produced by priming B10 females with allogeneic male cells and whether any activity so produced is cross-reactive. However, we have found that females primed with syngeneic male cells but boosted with allogeneic cells show only *H-2* responses and fail to kill syngeneic male target cells (*vide* Table I). The antigens involved in the generation of CTL during primary sensitization may be more complex than those involved in secondary responses or target cell lysis. It has recently been found that, in addition to serologically-defined (SD) target cell antigens, lymphocyte-defined (LD) antigens are important for primary anti-*H-2* cytotoxic responses (24). For secondary cytotoxic responses and

target cell lysis, however, SD antigens alone are effective[2]; in other words, no amplifier determinants seem required. Recent evidence based on Ly markers has revealed the existence of three subpopulations of T lymphocytes (25, 26). One population, Ly 1 positive, gives rise to both helper cells for antibody responses and mixed lymphocyte-reactive (LD) amplifier cells, whereas another population, Ly 2,3 positive, gives rise to cytotoxic effector cells. Co-operation between these two populations is necessary to obtain optimum cytotoxic responses. In the H-Y system it is possible that amplifier determinants, perhaps coded by a gene or genes linked either to H-Y or *H-2*, play a role in the generation of amplifier and/or cytotoxic T cells during primary sensitization.

The difficulty in obtaining primary immune responses to H-Y antigen in vitro may be a function of low levels of antigen reactive cells either to amplifier determinants or to target cell antigens or both. Expansion of clones of T cells reactive to one or both of these antigens by immunization in vivo may be necessary before a measurable level of cytotoxicity occurs. This may also be the mechanism for the development of secondary cytotoxic responses to LD determinants which normally elicit no, or very low, cytotoxic responses (24). If amplifier determinants for H-Y responses are shared by various strains of mice, this may in part explain the lack of strain specificity of H-Y antigen seen in previous experiments.

We have been unable to generate cytotoxic responses to H-Y antigen in two nonresponding strains, CBA and B10.A. One limitation of the present study is that we have only looked at animals primed by multiple injections of syngeneic male spleen cells. It has been reported that second male skin grafts are occasionally rejected by nonresponding strains (9), but it does appear difficult to raise CTL against H-Y antigen in nonresponding strains either in vivo or in vitro. From the viewpoint of the altered self hypothesis, $H\text{-}2\ (Y)^{k}$ and $H\text{-}2\ (Y)^{k/d}$ may not be sufficiently different from self to elicit a response, and these strains may lack necessary amplifier determinants. In addition to differences in antigenicity there is evidence that an immune response gene, Irl, within the MHC (5, 27) and, to a significantly lesser extent, the non-*H-2* genetic background (8, 27) influence the ability to respond to H-Y antigen.

The regulation of immune responses to weak histocompatibility antigens could be relevant to both clinical transplantation and tumour immunotherapy. It is now possible to study both proliferative (MLR) and cytotoxic (CML) immune responses in parallel using in vitro micro techniques (12). Methods are nw becoming available for the characterization and separation of functional subpopulations of T lymphocytes (25, 26, 28). It is hoped that continued application of these methods to the study of the H-Y system will further clarify the relationship between H-Y and the *H-2* major histocompatibility complex and the nature of both the antigen and the lymphocyte subpopulations involved in each phase of the immune response to H-Y antigen.

Summary

C57BL/10 female mice were primed to the male specific antigen H-Y, either by grafting with syngeneic male tail skin or by i.p. injection of syngeneic male

[2] Bach, F. H. Personal communication.

spleen cells. Primed female spleen cells, either unseparated or filtered through nylon wool to remove most of the B lymphocytes, were then cultured for 5 days in vitro with irradiated syngeneic male spleen cells and assayed against ^{51}Cr-labeled target cells. Both unseparated and nylon wool filtered female cells displayed significant cytotoxic activity restricted to male target cells. Pretreatment of sensitized female cells with antitheta serum and complement just before assay abolished cytotoxic responses. We were unable to demonstrate cell-mediated cytotoxic responses in two nonresponding strains, CBA and B10.A, which fail to reject male isografts. The cytotoxic activity of C57BL/10 female cells was restricted to male target cells histocompatible with C57BL/10 over at least a portion of the major (*H-2*) histocompatibility complex. We conclude that secondary in vitro cytotoxic responses against the H-Y antigen are mediated by cytotoxic T lymphocytes, and that the H-Y target cell antigen may be specified by the *H-2* complex.

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