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23. Solution A (in millimoles per liter): NaCl, 105; $NaHCO_3$, 25; NaH_2PO_4, 2; $CaCl_2$, 1; KCl, 5; Mg_2SO_4, 1; glucose, 5; sodium lactate, 4; sodium glutamate, 5; sodium malate, 5; alanine; 1, TMA (tetramethylammonium, Eastman Kodak Co.) butyrate, 1; dextran, 0.6 percent; *p*H 7.4. Solution B: choline chloride (Sigma), 105; choline bicarbonate (Sigma), 25; H_3PO_4, 2; $CaCl_2$, 1; KCl, 5; Mg SO_4, 1; glucose, 5; TMA lactate, 4; TMA glutamate, 5; TMA malate, 5; alanine, 1; TMA butyrate, 1; dextran, 0.6 percent; *p*H 7.4. The Na^+ concentration was varied by mixing solution A and solution B in various proportions.
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25. Supported by NIH grants AM26816 and GM29256. We thank A. L. Finn for comments during the preparation of this manuscript and E. J. Cragoe, Jr., of Merck Sharp & Dohme for the gift of amiloride.

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H-2–Linked Resistance to Mastocytoma in Male Mice: Immune Response to a Histocompatibility Antigen on the X Chromosome

Abstract. *Male hybrids from a cross between female mice of strain C57BL/6Kh and males of strain DBA/2J lived longer after injection of P815 mastocytoma cells of DBA/2 origin than did their female siblings. Responses to the histocompatibility antigen on the X chromosome of the DBA/2 strain may be involved in resistance to the tumor. When the female parent was replaced with a C57BL/6Kh carrying one of several mutations in the* H-2 *region, this sex effect disappeared in some of the hybrid combinations. Thus, the* H-2 *complex appears to be involved in the regulation of the immune response to the X-linked histocompatibility antigen in this tumor model.*

In the mouse, the histocompatibility gene on the X chromosome (*H-X*), unlike that on the Y chromosome (*H-Y*) (*1*), has remained relatively obscure. Since the discovery by Bailey (*2*) of an X chromosome–determined skin graft incompatibility between two inbred mouse strains, C57BL/6 and BALB/c, few reports have appeared about the *H-X* locus (*3–8*). The *H-X* histoincompatibility between C57BL/6 and BALB/c was confirmed in skin graft experiments between reciprocal F_1 hybrids (*3*) and has also been shown between the following pairs of strains: A and C57BL/6 (*3–7*), A and BALB/c (*8*), DBA/2 and BALB/c (*8*), and DBA/2 and C57BL/10 (*8*). The *H-X* locus of the mouse is more polymorphic than the *H-Y* locus (*9*) and appears to have at least four alleles.

In studies of the genetic control of the resistance to the DBA/2 mastocytoma P815-X2 in histocompatible F_1 mice (hybrid resistance), we generally used recipients bearing the *H-X* allele of DBA/2 ($H\text{-}X^d$) so that there was no *H-X* incompatibility between tumor and recipients; indeed, no sex differences in resistance were detected (*10*). Recently, however, we observed that male (C57BL/6Kh × DBA/2J)F_1 hybrids (B6D2F_1) ($H\text{-}X^b/H\text{-}X^d$) survived significantly longer than their female siblings ($H\text{-}X^b/H\text{-}X^d$) after injection of 10^6 viable P815-X2 tumor cells (*11*), suggesting that responses to $H\text{-}X^d$ may be involved in the resistance of these hybrids to the tumor cells.

The theoretical role for X-linked histoincompatibility in the male B6D2F_1 hybrids is illustrated in Fig. 1. Data reported here (Table 1) led us to the conclusion that we were detecting an anti-$H\text{-}X^d$ response to the tumor. Statistical comparisons between survival of male and female B6D2F_1 hybrids after injection of the tumor cells (*12*) revealed a significant difference in resistance to the tumor, with males surviving longer than females (experiment 1 in Table 1 and Fig. 2). In some cases this anti-$H\text{-}X^d$ response may have resulted in rejection of the tumor, since all survivors of the experiments were males (see footnotes to Table 1). Consistent with our hypothesis was the observation that

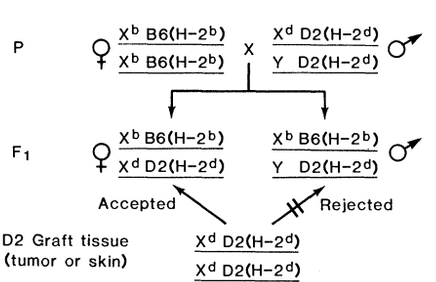

Fig. 1. The theoretical role for incompatibility against $H\text{-}X^d$ in male B6D2F_1 hybrids. The autosomal genome (including *H-2*) is represented as a single chromosome pair.

this sex effect was absent in homozygous DBA/2J controls and in (DBA/2J × C57BL/6Kh)F_1 hybrids (D2B6F_1) (experiment 3 in Table 1). Differences in median survival times between identical strains in separate experiments may reflect numerous variables and therefore cannot be legitimately compared. We report that strains C57BL/6 (B6) and DBA/2 (D2) may be *H-X*–incompatible, as has already been shown for C57BL/10 (closely related to B6) and D2 (*8*).

Hybrid resistance in mice is linked to the *H-2* complex on chromosome 17 (*13*). The introduction of B6 *H-2* mutants with various point mutations of single genes of the $H\text{-}2^b$ haplotype ($B6^m$) provided an opportunity to examine the effects of single genes on tumor resistance. By comparing the survival of $B6^mD2F_1$ females with B6D2F_1 females after injection of P815-X2 cells, we found that differences in a single gene (for example, *H-2K*) could profoundly influence survival, either positively or negatively (see footnotes to Table 1). Since several of the mutant gene products differ from the wild type by only one or a few amino acids (*14*), these small changes in H-2 antigens apparently can alter resistance to a histocompatible tumor, producing an effect similar to that of an immune response gene (*15–17*).

Analysis of the survival of male and female mutant hybrids showed that the anti-$H\text{-}X^d$ phenomenon is not present in all strains. Hybrids with *bm6, bm10, bm11, bm12, bm13*, and *bm16* mutations demonstrated a statistically significant sex effect ($P < .05$; Mantel-Cox logrank test). The male hybrids always survived longer than the females. Hybrids with *bm1, bm4, bm7, bm8*, and *bm14* mutations did not exhibit a statistically significant sex effect (experiments 1 and 2 in Table 1). This apparent variability in the immune response to $H\text{-}X^d$ among mutant hybrids, differing only in *H-2* and with theoretically identical X incompatibilities, suggests that responses to $H\text{-}X^d$ in this system are influenced by the *H-2* genotype of the recipient.

In bm7 and bm8 hybrids the anti-$H\text{-}X^d$ response, if present, may have been simply masked by the strong responses to the tumor (anti-P815-X2 response), as demonstrated by their increased survival over B6D2F_1 controls (footnotes to Table 1). This, however, cannot explain the reduction and perhaps the absence of the sex effect in *bm1, bm4*, and *bm14* hybrids, since they did not exhibit significant differences in survival in comparison with B6D2F_1 controls (*15–17*). Fur-

thermore, a variable sex effect among mutant hybrids *bm1, bm4, bm6, bm13,* and *bm14*, which did not differ significantly from B6D2F$_1$ controls in their resistance to P815-X2 (*15–17*), led us to conclude that independent determinants of the H-2K and H-2D molecules participate in the anti-P815-X2 and the anti-H-X^d responses (Table 1). Of the two *H-2D^b* mutants available, only *bm13* hybrids had an unequivocal response to *H-X^d*, and further studies are required to allow definitive comment on the role of *H-2D*. The *I-A^b* mutant *bm12*, which has a diminished anti-P815-X2 response (*16*), was associated with a significant response to H-X^d (Table 1).

These data demonstrate an *H-2*-linked host control of the immune response to H-X, a minor histocompatibility antigen, and implicate more than one *H-2* gene in this control. Control of immune responsiveness by *H-2* genes has been previously reported (*18–21*) for responses to other minor histocompatibility loci.

In addition to genetically determined differences in immune response, *H-2*–controlled differences in the penetrance and expressivity of the H-Y antigen have been observed (*22*). There may be similar factors in the "donor" tissue affecting the antigenicity of H-X in our tumor model. The H-X antigen, in fact, appears to be more immunogenic on P815-X2 than it is on normal DBA/2 skin, as evidenced by the failure of B6D2F$_1$ male hosts to reject DBA/2J tail skin grafts. Of 11 B6D2F$_1$ males (*H-X^b*), given D2 male grafts (*H-X^d*), all accepted the grafts permanently (more than 176 days). The H-X antigen on P815-X2 may react with tumor-associated antigens that are not present on normal DBA/2 cells in such a way that the H-X antigen becomes more immunogenic. It is also possible that the antigen defined as H-X^d in the context of this B6 anti-DBA/2 response is not identical to the antigen defined by the C57BL/10 anti-DBA/2 response (*8*) or that the concentration of H-X^d is much greater on tumor cells than on skin. Tumor grafts may be more sensitive than skin grafts for studies of *H-X* or other minor histocompatibility loci. For example, Kohn (*3*) reported that only about 50 percent of *H-X*–incompatible allografts were rejected in the tail skin graft system.

The availability of *H-2* mutant strains with differential influences on the response to H-X^d and detailed data on the molecular nature of the relevant gene products (*14*) makes the model presented in this report a particularly useful system to study the *H-X* locus of mice. The use of tumor grafts enables us to study the X-linked histocompatibility gene of the mouse in a model pertinent to the regulation of autochthonous tumor growth. Because spontaneous tumors are poorly immunogenic, their antigens acting like weak transplantation antigens, the minor histocompatibility gene *H-X*, as it becomes more clearly defined, may serve as a potentially informative model for the weak tumor antigens.

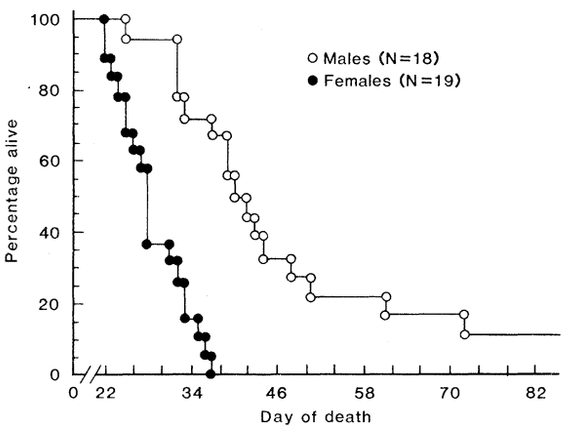

Fig. 2. Survival of B6D2F$_1$ hybrids after injection of P815-X2 mastocytoma cells in experiment 1. Male hybrids survived significantly longer than female siblings, suggesting that responses to H-X^d may be detectable in this model of hybrid resistance to P815-X2.

Table 1. Sex effect in F$_1$ hybrid mice from normal and mutant parents in resistance to P815-X2 mastocytoma. Probabilities of male-female differences are based on the Mantel-Cox logrank test (*12*).

Mother	Mutated gene	Father	Number tested		Median survival (days)		Probability (*P*) of female-male differences
			Female	Male	Female	Male	
			Experiment 1				
DBA/2J		D2	14	5	22	22	.29
B6		D2	19	18*	28	42	<.001
B6.C-*H-2^{bm1}*	K^b	D2	15	7†	31	39	.05
B6-*H-2^{bm6}*	K^b	D2	12	24*	29	44	<.001
B6.C-*H-2^{bm7}*‡	K^b	D2	14	20†	33	33	.51
B6-*H-2^{bm8}*‡	K^b	D2	12	17†	33	38	.22
B6.C-*H-2^{bm10}*§	K^b	D2	23	22†	23	32	<.001
B6.C-*H-2^{bm14}*	D^b	D2	20	19	29	37	.06
			Experiment 2				
DBA/2J		D2	23	3	22	22	.71
B6		D2	15	0	31		
B6.C-*H-2^{bm4}*	K^b	D2	10	10†	30	32	.15
B6.C-*H-2^{bm11}*§	K^b	D2	19	9	27	33	<.01
B6.C-*H-2^{bm12}*§	I-A^b	D2	24	22†	27	35	<.001
B6.C-*H-2^{bm13}*	D^b	D2	14	20†	30	34	.03
B6-*H-2^{bm16}*§	K^b	D2	8	15†	27	31	.02
			Experiment 3				
DBA/2J		D2	7	8	22	23	.32
DBA/2J		B6	8	8	33	29	.99

*Two survivors remaining after termination of the experiment at 80 days. †One survivor remaining after termination of the experiment at 80 days. ‡Hybrids with *bm7* and *bm8* mutations showed longer survival times than did B6D2F$_1$ controls ($P < .05$) (*15, 16*). §Hybrids with *bm10, bm11, bm12,* and *bm16* mutations showed shorter survival times than did B6D2F$_1$ controls ($P < .05$) (*15–17*).

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11. A tumor transplantation protocol was developed that minimized the variability of tumor growth after transplantation into histocompatible mice.

A single large batch of P815-X2 was prepared in DBA/2J mice and preserved at low temperature in 10 percent dimethyl sulfoxide and P815-X2 ascites fluid by controlled rate freezing. A single vial was thawed and washed twice in RPMI 1640 medium with penicillin and streptomycin added and 5 million cells were injected into a female DBA/2J mouse. Tumor for experimental studies was harvested after 13 days. By this protocol, the variability of tumor weights of individual mice after 10 days did not differ from the variability in the means of numerous experiments.

12. Mantel-Cox logrank test was used as described by R. Peto and J. Peto [*J. R. Stat. Acad. A* **135**, 185 (1972)].
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Mutagenicity of Glutathione and Cysteine in the Ames Test

Abstract. *Postmitochondrial supernatant from rat liver and kidney homogenates transformed cysteine into a mutagen that reverted bacteria of the strain* Salmonella typhimurium *TA100 to histidine independence. Glutathione was also activated by kidney postmitochondrial supernatant but not by liver preparations. Hence, important endogenous compounds of mammals are positive in the most commonly used short-term test for carcinogenicity and mutagenicity. Glutathione is positive in the test even at concentrations found in mammalian tissues.*

Bacterial tests of mutagenicity mediated by mammalian enzymes have demonstrated that numerous compounds, including substances from plants and microorganisms, are mutagenic (*1*). We now report that even compounds that are physiologically important for mammals and present in these organisms in large concentrations, such as the tripeptide glutathione or the amino acid cysteine, can exert mutagenic effects. Biological functions of glutathione include transport of amino acids through membranes, protection of lipids against autoxidation, inactivation of electrophilic intermediates, and modulation of proteins by the formation of mixed disulfides (*2*). The sulfhydryl group of cysteine, one of the three amino acids that form glutathione, is of primary importance in most functions and dominates its biochemistry.

Mutagenicity was determined in bacteria in the presence of mammalian subcellular preparations. The Ames test (*3*) was used. In some experiments the S9 fraction of liver [the postmitochondrial supernatant fraction supplemented with a system generating reduced nicotinamide adenine dinucleotide phosphate (NADPH)] was replaced by different metabolizing systems. Postmitochondrial supernatant, microsomal, and cytosolic fractions were prepared from homogenates of kidney and liver from untreated (*4*) adult male Sprague-Dawley rats. Histidine-dependent bacteria of the strain *Salmonella typhimurium* TA100, the subcellular fraction from 100 mg of tissue with or without an NADPH-generating system, a neutralized solution of the test compound in water, and histidine-poor soft agar were mixed and added to culture plates containing minimal agar. The colonies reverting to histidine independence were counted after incubation for 2 days in the dark.

Figure 1 shows the results of experiments in which glutathione and cysteine were tested for mutagenicity in the absence of mammalian enzymes and in the presence of liver or kidney S9. In the absence of S9 neither compound increased the number of revertants. In fact, glutathione led to a small decrease, probably due to toxicity. With the addition of liver S9, cysteine increased the number of revertants. Under the same conditions glutathione did not increase the number of revertants. However, when liver S9 was replaced by kidney S9, both glutathione and cysteine increased the number of revertants severalfold above the spontaneous level. Batches of glutathione and cysteine from different manufacturers (Boehringer, Sigma, and Merck) did not show any quantitative differences, strongly suggesting that the compounds rather than impurities were responsible for the effects. Moreover, glutathione formed enzymatically in situ from glutathione disulfide also acted as a mutagen (*5*).

From Fig. 1 it can be calculated that the number of revertants was twice the control number with 6 m*M* glutathione in the top layer of agar (5 mg per plate) or with 8 m*M* cysteine (3 mg per plate). Although these concentrations are high compared to those of many other mutagens, physiological levels of glutathione in mammalian cells are also high (*2*); values from 2 to 10 m*M* are typical for rat liver and kidney. Concentrations of glutathione in *S. typhimurium* (*6*) and of cysteine in mammalian tissues (*2*) are much lower. Meijer *et al.* (*7*) reported 0.15 m*M* (150 nmole per gram wet weight) glutathione in *S. typhimurium* TA100. Cysteine is present in rat liver and kidney at concentrations of 0.1 to 0.5 m*M*. Hence, whereas glutathione was clearly mutagenic at concentrations that are physiological for mammals, cysteine

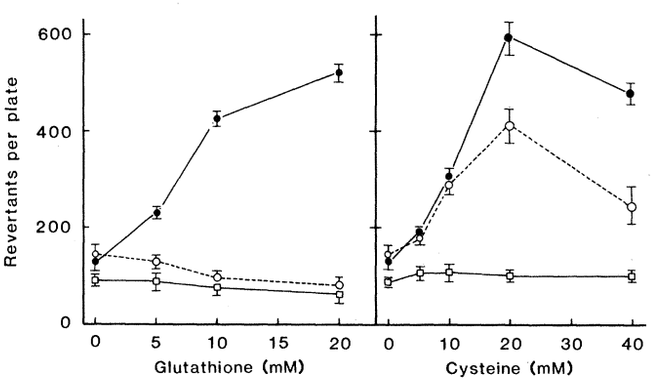

Fig. 1. Effect of glutathione and cysteine on the reversion of histidine-dependent *S. typhimurium* TA100 directly (□) and in the presence of liver (○) or kidney (●) postmitochondrial supernatant. Kidneys and livers from adult male Sprague-Dawley rats were homogenized in three volumes of 150 m*M* KCl with 10 m*M* sodium phosphate buffer (*p*H 7.4). The homogenate was centrifuged for 10 minutes at 9000*g* to yield the postmitochondrial supernatant fraction. For each incubation the following were mixed in a test tube and poured onto plates containing minimal agar: 0.97×10^8 bacteria; 330 μl of postmitochondrial supernatant fraction from liver (containing 8.2 mg of protein) or kidney (5.6 mg of protein); an NADPH-generating system (2 μmole of $NADP^+$ and 2.5 μmole of glucose-6-phosphate) in 330 μl of a solution containing 50 m*M* KCl, 12 m*M* $MgSO_4$, and 75 m*M* sodium phosphate buffer (*p*H 7.4); the test compound in 400 μl of neutralized aqueous solution; and 2 ml soft agar (0.55 percent NaCl, 0.55 percent agar, 50 μ*M* biotin, 50 μ*M* histidine, and 25 m*M* sodium phosphate buffer at *p*H 7.4 and 45°C). We incubated the cultures for 2 days in the dark at 37°C and then counted the colonies that had reverted to histidine independence. Values are means ± standard deviations for three replicate incubations.