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Immunology

Absence of H-2 antigens capable of reacting with cytotoxic T cells on a teratoma line expressing a *T/t* locus antigen

(cell-mediated lympholysis/syngeneic cell-mediated lympholysis)

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ABSTRACT **An established cell line of murine teratoma cells (F9), which lacks serologically detectable H-2 antigens, has a surface antigen structurally similar to H-2 that is determined by a wild-type *T/t* locus gene. These cells are not killed and do not react with cytotoxic T cells sensitized to H-2 antigens in a cell-mediated lympholysis assay. Modification of spleen cells from the strain of origin (129) of this teratoma line with trinitrobenzene sulfonic acid allows the generation of syngeneic killer cells that display a cytotoxic effect against trinitrophenyl-modified splenic targets, but not against trinitrophenyl-modified F9 targets. Thus, the F9 antigen is structurally similar to H-2^b but does not act as a target antigen in the cell-mediated lympholysis assay for anti-*H-2*^{b} cytotoxic T cells, nor does it crossreact with H-2^b antigens at the T cell level.**

A primitive teratoma cell line, F9, was established from a teratocarcinoma isolated from 129/Sv (*H-2*^{bc}) mice (1). These cells appear to lack H-2 antigens by three criteria (2, 3): (*i*) injection of F9 cells into several different strains of mice, including six incompatible at the *H-2* complex, results in death of the host; (*ii*) F9 cells cannot absorb antibody against H-2 from appropriate antisera; and (*iii*) H-2 antigens cannot be precipitated from lysates of enzymatically iodinated F9 cells. These cells express another product of the 17th chromosome of the mouse, known as the F9 antigen. This antigen has been detected by serologic tests and is thought to be a product of the *T/t* locus known as $+^{t12}$ (4). The F9 antigens are similar to H-2 in molecular weight and subunit structure and could be the embryonic equivalent of *H-2* (3).

H-2 antigens on target cells are also recognized by killer thymus-derived (T) cells in cell-mediated lympholysis (CML) assays (5). These effector T cells, generated by the coculturing of lymphocytes from two mouse strains that differ at the *H-2* complex, mediate their effect by cell-to-cell contact with either the sensitizing cell or against target cells from other strains that share either the *K* or *D* region of their *H-2* complex with that of the sensitizing strain (6–9): Whereas, there is some evidence that the target antigenic determinant recognized by the T cell in CML differs from that detected by humoral antibody (10–12), most evidence indicates that the CML target specificity is on the same molecule or controlled by the same gene(s) that is responsible for the expression of the H-2 K or D antigens that have been described serologically and biochemically (13, 14).

In this investigation, we have tested the capacity of F9 cells in CML to act as targets for cytotoxic T cells that have been sensitized to H-2 antigens of 129 strain cells. The data indicate that F9 cells lack CML target specificities detected by the cytotoxic T cell.

Abbreviations: CML, cell-mediated lympholysis; Con A, concanavalin A; Tnp, trinitrophenyl.

MATERIALS AND METHODS

Animals. Animals were obtained from the Jackson Laboratories, Bar Harbor, Me., and maintained in our animal quarters. Strain 129/J mice were used in this study instead of 129/Sv. Both sublines are of common origin and have previously been shown to mutually accept skin grafts (L. C. Stevens, personal communication).

Tumor Cells. F9 cells were obtained from the Institut Pasteur, Paris, France, and were maintained in gelatin-coated 75-cm^2 (250 ml) Falcon flasks or 150-cm^2 (1000 ml) Corning flasks in Dulbecco's modified Eagle's medium containing 15% fetal calf serum. Cells were incubated at 37° in a moist 12% CO_2 incubator. HC-1 cells are a (A × C57Bl)F_1 lymphoma kindly provided by Dr. David Grausz, Dept. of Biology, Univ. of California at San Diego.

Preparation and Labeling of Target Cells for the Cytotoxicity Assay. Mice were killed by cervical dislocation. Their spleens were excised, and a cell suspension was made in a balanced salt solution. After washing, the cells were suspended at a concentration of 4.0×10^6 nucleated cells per ml in RPMI 1640 (GIBCO, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum and 30 μM 2-mercaptoethanol. Four ml of this suspension were added to individual Falcon flasks (3013) and placed on a rocking platform at 37°. After 2 days, concanavalin A (Con A, Nutritional Biochemical Co.) was added at a concentration of 5 μg/ml. Three days later the cells were harvested (usually from two such flasks) and centrifuged together in a 17 × 100 mm plastic tube. The cell pellet was resuspended in 0.2 ml of RPMI 1640 with 1% fetal calf serum, and 200 μCi of sodium [^{51}Cr] chromate (Amersham Searle Corp.) was added in a volume of 0.2 ml. The cells were incubated at 37° for 1.5 hr and then washed three times in balanced salt solution. After the third wash, the cells were resuspended in 5.0 ml of RPMI with 10% fetal calf serum and placed at 37° for 1.5 hr. The cells were washed twice more and adjusted to a concentration of 10^5 viable cells per ml. One-tenth milliliter of this target cell suspension was added to petri dishes containing the effector cells. F9 teratocarcinoma cells were labeled by harvesting the cells from flasks using EDTA as described (1), washing them two times in balanced salt solution, and then incubating 5×10^6 cells with ^{51}Cr in RPMI 1640 as described above for the Con A lymphoblasts.

Generation of Effector Cells for CML Assay. Effector cells were generated by coculturing responder cells along with irradiated (3000 R) stimulator cells in 35-mm plastic

Table 1. Inability of F9 cells to act as targets in CML

Responder (genotype)	Stimulator (genotype)	Target (genotype)	No. of targets added	Percent ^{51}Cr release ± SEM[a]			
				Spontaneous release	Autologous release	Immune release	Net release
B10.BR(*H-2*^{k})	B10(*H-2*^{b})	129(*H-2*^{bc})	10^4	25.4 ± 2.6	26.6 ± 0.8	47.7 ± 2.5	22.3
			5×10^4	15.5 ± 0.3	ND[b]	26.6 ± 0.8	11.1
		HC-1(*H-2*^{a}/*H-2*^{b})	10^4	17.1 ± 0.2	24.3 ± 1.0	28.7 ± 2.2	11.6
			5×10^4	9.9 ± 0.6	ND	16.9 ± 1.1	7.0
		F9(*H-2*^{bc})	10^4	26.6 ± 2.9	28.6 ± 2.4	22.2 ± 1.2	−4.4
			5×10^4	15.1 ± 0.5	ND	14.7 ± 0.5	−0.4
B10.A(*H-2*^{a})	129(*H-2*^{bc})	129(*H-2*^{bc})	10^4	25.4 ± 2.6	ND	36.1 ± 1.8	10.7
		F9(*H-2*^{bc})	10^4	26.6 ± 2.9	ND	19.4 ± 0.1	−7.2

[a] Spontaneous release = percent release of ^{51}Cr from target cells alone; autologous release = percent release of ^{51}Cr targets in the presence of syngeneic effectors (stimulators) sensitized to responder strain; immune release = percent release of ^{51}Cr targets in the presence of responders sensitized to stimulators; net release = immune release − spontaneous release.
[b] Not done.

petri dishes in the same media as described for the culture of target cells. Two million responder and stimulator cells were added in a total volume of 1.0 ml to each dish, and each group consisted of three replicates. The cells were cultured in a CO_2 atmosphere for 5 days as described (12), and at this time these cultures contained approximately 5×10^5 viable nucleated cells. Usually 10^4 target cells in a volume of 0.1 ml were then added to the dishes to assay for cytotoxic activity. After 4 hr, the contents of the dishes were harvested with a plastic policeman and transferred to 12 × 75 mm tubes, centrifuged, and half of the supernant was removed. When trinitrophenyl-modified cells were used as the stimulators, 5.0×10^6 responders in a volume of 1.0 ml were added to the dishes along with 0.1 ml of the modified stimulator (10^6 cells per dish).

Data Processing. The formula used to calculate percent release of isotope is:

$$\% \text{ release } {}^{51}\text{Cr} = \frac{(\text{cpm supernatant} - \text{bk}) \times 2}{(\text{cpm supernatant} + \text{pellet}) - (2 \times \text{bk})} \times 100$$

where cpm = counts per minute and bk = background cpm. A Student's t test was performed on the data, and values of $P > 0.025$ are considered not significant.

Inhibition Assay of CML Activity. Con A lymphoblasts were prepared in the same manner as the target cells for the CML assay except that they were not labeled with ^{51}Cr. The cells were adjusted to 10 times the concentration required for the assay, and a 0.1 ml volume of these inhibitors was added to the effector cell mixtures at the same time as the target cells.

Modification of Stimulator and Target Cells with 2,4,6-Trinitrobenzene Sulfonic Acid. To modify the stimulator cells with trinitrobenzene sulfonic acid, approximately 30×10^6 spleen cells were incubated in phosphate-buffered saline containing 1.0 mM trinitrobenzene sulfonic acid. After 10 min at 37°, the cells were washed twice in balanced salt solution containing 10% fetal calf serum and then adjusted to a concentration of 10×10^6 cells per ml. To prepare ^{51}Cr labeled target cells that were trinitrophenyl (Tnp) modified, lymphoblasts or teratoma cells that were already prelabeled with isotope were resuspended in phosphate-buffered saline containing trinitrobenzene sulfonic acid for 10 min at 37°. The cells were washed twice in balanced salt solution along with 10% fetal calf serum, incubated for 1.5 hr at 37° in RPMI with fetal calf serum, and then washed and adjusted to the appropriate concentration before adding to the petri dishes.

RESULTS

Ability of F9 cells to act as targets in CML

Irradiated C57Bl/10Sn (*H-2*^{b}) or 129/J [a minor variant of the *H-2*^{b} haplotype designated *H-2*^{bc} (15)] spleen cells were cocultured with *H-2* allogenic responders to generate cytotoxic T cells. After 5 days, labeled target cells were added to the cultures and the cytotoxic effect was assayed. In Table 1 is presented a representative experiment where B10.BR (*H-2*^{k}) and B10.A (*H-2*^{a}) effector cells, that are *H-2* congeneic strains with C57Bl/10, displayed a specific cytotoxic effect against 129 strain lymphoblasts as well as HC-1 (*H-2*^{a}/*H-2*^{b}) tumor cell targets. However, when the same effectors were tested against labeled F9 cells, no increase in percent isotope release was observed. These data demonstrate that specific cytotoxic cells generated against components of the *H-2*^{b} complex are incapable of lysing F9 cells in the CML test.

Ability of F9 cells and spleen lymphoblasts to inhibit cell-mediated lysis *in vitro*

The data in Table 1 do not exclude the possibility that there still may be H-2 CML specificities present on F9, but the cell line may be resistant to cell-mediated lympholysis due to a low density of target specificities. Therefore, we generated effector cells in the CML assay and then added various unlabeled target cells to block the effect. If F9 cells are resistant to lysis but have H-2 antigens that can be recognized by killer T cells, then this cell line should inhibit specific cytotoxicity.

C57Bl/10 cells were cocultured with B10.A stimulators. The C57Bl/10 (*H-2*^{b} anti-*H-2*^{a}) responders displayed a specific cytotoxic effect against 10^4 B10.A (*H-2*^{a}) targets (Fig. 1). When a 10- to 100-fold excess of unlabeled C57Bl/10 Con A lymphoblasts were added to the effector cells along with the labeled targets, no inhibition of isotope release was observed. On the other hand, a 50- to 100-fold excess of unlabeled specific targets (B10.A) almost completely reduced the percent of ^{51}Cr release to background levels. When unlabeled F9 cells were added at a 10- to 100-fold excess, no inhibition of CML activity occurred. Thus, F9 cells do not nonspecifically suppress T cell killer activity in this assay.

B10.A cells were cocultured with irradiated C57Bl/10

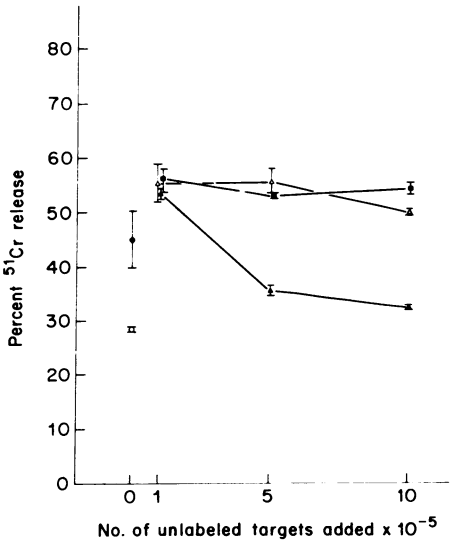

FIG. 1. Percent release of ^{51}Cr from 10^4 B10.A target cells in the presence of C57Bl/10 anti-B10.A effectors. Various numbers of unlabeled B10.A (▲) or C57Bl/10 (△) Con A lymphoblasts or F9 cells (■) were added along with the labeled target cells. ●, Isotope release in the absence of unlabeled inhibitors; O, isotope release in the absence of effector cells (spontaneous release).

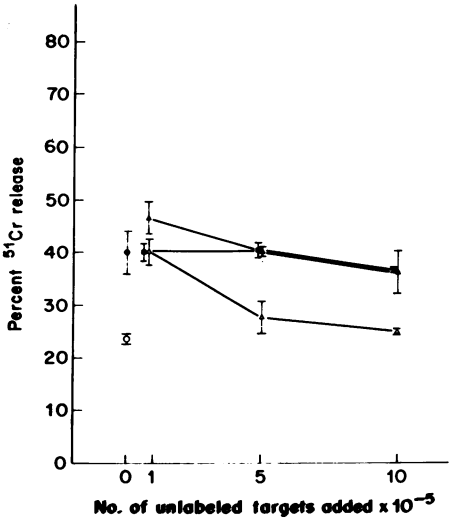

FIG. 2. Percent release of ^{51}Cr from 10^4 target cells in the presence of B10.A anti-C57Bl/10 effectors. Various numbers of unlabeled B10.A (▲) or C57Bl/10 (△) Con A lymphoblasts or F9 cells (■) were added along with the labeled target cells. ●, Isotope release in the absence of unlabeled inhibitors; O, isotope release in the absence of effector cells (spontaneous release).

stimulators so that specific cytotoxic cells (*H-2*^{a} anti-*H-2*^{b}) were generated against 129 (*H-2*^{bc}) targets (Fig. 2). This effect was not reduced by a 10- to 100-fold excess of B10.A lymphoblasts, but was completely abrogated by a 50- to 100-fold excess of C57Bl/10 lymphoblasts. When a 10- to 100-fold excess of unlabeled F9 cells was added, no inhibition of cytotoxicity was observed. These data demonstrate, therefore, that there is no H-2, or an insufficient number of H-2 antigens on F9 cells to block cytotoxic cells directed against *H-2*^{b} targets.

Tnp-modified 129 or F9 cells do not sensitize syngeneic spleen cells to display a cytotoxic effect against Tnp-modified F9 targets

It has recently been observed that modification of spleen cells with reactive haptens allows them to sensitize syngeneic cells so as to induce cytotoxic T cells specific for the syngeneic haptenated targets (16, 17). Further, the specificity of the response is dependent on the *H-2* genotype of the target cell; i.e., hapten modified targets from an unrelated *H-2* haplotype to the sensitizing strain are not killed (16). If the specificity of these effector cells is directed against new determinants created on a given H-2 glycoprotein by the haptenation procedure, then one may be able to detect such determinants on F9 cells by this approach. The data in Table 2 demonstrate that 129 cells respond to 129-Tnp stimulators by inducing lysis of 129-Tnp targets (group 1 compared with group 2). However, when the same effector cells were tested against F9-Tnp modified targets, no significant lysis was observed (group 3 compared with group 4). Further, when the Tnp-modified F9 cells were used as stimulators, no significant lysis of Tnp-modified F9 targets occurred (group 5 compared with group 6).

Tnp-modified stimulator cells do not generate cytotoxic effector cells against Tnp-modified F9 targets in an allogeneic system

Tnp-modified cells have a new H-2 dependent CML determinant as detected in a syngeneic system. However, since syngeneic responders did not kill Tnp-modified F9 cells (Table 2), we cocultured Tnp-modified 129 or F9 cells with H-2 allogeneic responders, Balb/c (*H-2*^{d}), in order to determine if these cells could detect a new determinant on F9-Tnp targets.

Balb/c cells cultured with 129 cells that were or were not Tnp-modified were cytotoxic against both 129 and 129 Tnp-modified targets, as expected (Table 3, groups 1, 3, and 4). However, these same effector cells had almost no detectable activity against F9 or F9-Tnp targets (groups 2, 5, and

Table 2. Ability of Tnp-modified stimulator cells to generate cytotoxic effector cells against Tnp-modified targets in a syngeneic system

Group	Responder	Stimulator	Target	Percent ^{51}Cr release ± SEM	*P* Value
1	129	129-Tnp	129-Tnp	43.9 ± 3.2	gr 1 vs gr 2[a]
2		129	129-Tnp	28.0 ± 2.2	
3		129-Tnp	F9-Tnp	28.7 ± 1.2	gr 3 vs gr 4[b]
4		129	F9-Tnp	25.5 ± 0.7	
5		F9-Tnp	F9-Tnp	26.7 ± 1.0	gr 5 vs gr 6[b]
6		F9	F9-Tnp	29.1 ± 0.3	

[a] $0.02 > P > 0.01$.
[b] $P > 0.025$.

6). Further, F9-Tnp stimulators failed to generate cytotoxic cells against F9-Tnp targets (groups 7 and 8). Therefore, cell surface modifications, presumably dependent on H-2 plasma membrane antigens, resulted in generation of cytotoxic cells with killer activity against similarly modified target cells that had H-2 antigens on their membrane, but not against the F9 teratocarcinoma.

DISCUSSION

Spontaneous teratomas arise in 129 strain male mice from germ cells, and can be serially transplanted (18). *In vivo*, these teratomas have the potential to differentiate into derivatives of all three embryonic germ layers (19), and *in vitro*, both primitive and differentiated cell types can be established (20, 21). One undifferentiated cell line maintained *in vitro*, F9, was found to induce the production of an antiserum when injected into syngeneic hosts (1). The specificity of this antiserum as determined by quantitative absorption studies, indicates that it defines an antigen controlled by the $+^{t^{12}}$ allele of the *T/t* locus, which is also expressed on sperm and morulae (4).

In this study, using several approaches we demonstrated that there is no detectable H-2 antigen on F9 cells as recognized by cytotoxic T cells. Thus, *H-2* specific anti-$H\text{-}2^b$ killer cells were cytotoxic for $H\text{-}2^b$ targets as well as 129 targets, but were not able to kill F9 cells. This lack of cytotoxicity could not be attributed to a resistance of the cell line to cell-mediated lysis or a low density of H-2 target determinants (22, 23), since unlabeled F9 cells could not block the cytotoxic activity of anti-$H\text{-}2^b$ killer cells in the CML test while unlabeled $H\text{-}2^b$ lymphoblasts could. Further, Tnp-modified 129 spleen cells induced cytotoxicity against Tnp-modified 129 targets, but Tnp-modified 129 or F9 cells did not induce cytotoxicity against Tnp-modified F9 cells. Finally, allogeneic responder cells demonstrated no cytotoxic effect when sensitized with Tnp-modified cells and tested against Tnp-modified F9 targets. Since the specificity of the effector cells sensitized against Tnp-modified syngeneic stimulators is directed against altered H-2 controlled products (24, 25), these data are consistent with the F9 cell line being H-2 negative and suggest that syngeneic killing, as defined by the Tnp-modification model system, requires the presence of H-2 antigens on the target cell.

In a previous study, Artzt and Jacob (2) demonstrated that humoral allo-antibody against $H\text{-}2^b$ specificities of 129 cells ($H\text{-}2^{bc}$) could not be absorbed by F9 cells. Recently (4), using both anti-$H\text{-}2^b$ and syngeneic anti-F9 sera, it was demonstrated by the sensitive technique of enzymatic radioiodination and immunoprecipitation, that there is no detectable $H\text{-}2^b$ antigen on F9 cells or sperm, whereas the F9 antigen is detected on both. While $H\text{-}2^b$ and 129 ($H\text{-}2^{bc}$) are not identical at the *H-2* complex, the variation is minor and may be related to a gene more closely linked to the *Tla* locus and to the right of *H-2D*.

Table 3. Ability of Tnp-modified stimulator cells to generate cytotoxic effector cells against Tnp-modified targets in an allogeneic system

Group	Responder	Stimulator	Target	Net release[a]
1	Balb/c	129	129	25.7
2		129	F9	−0.6
3		129-Tnp	129-Tnp	32.0
4		129	129-Tnp	28.7
5		129-Tnp	F9-Tnp	4.4
6		129	F9-Tnp	3.0
7		F9-Tnp	F9-Tnp	1.4
8		F9	F9-Tnp	−2.2

[a] Net release = % ^{51}Cr released from targets in the presence of specific immune cells − % ^{51}Cr released from targets in the presence of nonimmune cells.

The results of this study indicating that this cell line has no detectable H-2 CML target specificities, as well as the fact that this line lacks serologically detectable H-2 antigens, probably accounts for its transplantability *in vivo* across the *H-2* barrier (2). Another teratoma line has also been described that is unable to sensitize *H-2* allogeneic strains for second-set graft rejection. This teratoma does, however, express antigens shared by several *H-2* positive tumor and transformed cell lines which cocaps with the H-2 antigens on these lines (26, 27).

Although there is no alloantigenic crossreactivity between the products of the $+^{t^{12}}$ allele and $H\text{-}2^b$, there are some structural and perhaps functional similarities between them. For example, in a previous paper, it was demonstrated that the cell surface antigen recognized by anti-F9 serum has structural similarities to $H\text{-}2^b$ glycoprotein (3) in that both molecules show peaks on sodium dodecyl sulfate/polyacrylamide gels of 150,000, 120,000, 90,000, and 12–14,000 daltons when unreduced; and 44,000, 22,000, and 12–14,000 daltons when reduced. It has been proposed that alleles at the *T/t* locus are involved in cell recognition during morphogenesis (28), since mutation of different alleles at this locus affect development at certain stages of embryogenesis (29). A recognition role for cell-cell interaction in the immune response has also been suggested for antigens controlled by the *H-2* complex since (*i*) T cells do not collaborate efficiently with H-2 allogeneic B cells in their humoral response to antigens (30), (*ii*) antigens under control of *Ir* genes require sharing of the *H-2 I* region in order for optimal T-B cell collaboration to occur (31), and (*iii*) the ability of T cells to undergo optimal DNA synthesis *in vitro* in both guinea pig and mouse requires that they share the major histocompatibility locus with antigen pulsed macrophages (32, 33).

If products determined by gene(s) at the *T/t* locus share structural or functional similarities with H-2 antigens, then the fact that no antigenic crossreactivity is shared by this wild-type *T/t* allele product and $H\text{-}2^b$ antigens does not preclude the possibility that products of other *T/t* alleles, perhaps expressed at a later stage of ontogeny, may crossreact with H-2 antigens.

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