Semisyngeneic Hybrid Resistance to Murine Teratocarcinoma Cells

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Abstract. Resistance to two cultured lines of murine embryonal carcinoma was studied in F_1 hybrids constructed between the tumor-syngeneic mouse strain 129/J and several allogeneic strains. Three of four such hybrid strains were significantly more resistant to the multipotent embryonal carcinoma line PCC3 than the tumor-syngeneic 129/J parent strain. All hybrid strains tested showed significantly higher resistance to the nullipotent embryonal carcinoma line F9 than the syngeneic strain.

Hybrid resistance to embryonal carcinoma lines does not require a hybrid *H-2* complex.

Several kinds of evidence indicate that this hybrid resistance has an immunological basis.

Introduction

One of the classic laws of immunogenetics states that F_1 hybrids produced by crossing two inbred strains will grow tumors indigenous to either parent strain (Snell 1953). This law arises from the fact that histocompatibility alleles are expressed in a codominant manner, so a semisyngeneic F_1 hybrid will be tolerant of transplanted tissues originating from either of its two parental strains. However, not long after the formulation of these principles, Hauschka and his colleagues found that (C3H/St × Swiss)F_1 mice were unexpectedly resistant to both a virulent and attenuated line of a lymphosarcoma of C3H/St origin (Hauschka et al. 1956). This was followed by Snell's discovery that semisyngeneic F_1 hybrids show a reduced acceptance of tumor grafts compared to the susceptible parent strain (Snell 1958). These early observations have been repeatedly confirmed in the transplantation of a variety of normal and neoplastic tissues of inbred origin (Cudkowicz 1967, Kiessling

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et al. 1975, Clark et al. 1977, Klein et al. 1978, Sanford and Soo 1971, Schmitt-Verhulst and Zatz 1977). In all cases, hybrid resistance is a quantitative phenomenon. Unlike the stronger allogeneic resistance, hybrid resistance can be observed only when threshold challenges are used; large doses of cells abolish the effect.

Avner and co-workers (1978) found that $(129 \times C57BL/6)F_1$ hybrids showed hybrid resistance to the murine embryonal carcinoma cell line F9. The work we present here examines F_1 hybrid resistance to the nullipotent line F9 and to the multipotent line PCC3. We examine resistance to the tumor cells in several different semisyngeneic F_1 hybrid hosts, and we determine whether such resistance is controlled by factors closely linked to the $H\text{-}2$ complex. Finally, we investigate whether the resistance of the $(C57BL/6 \times 129/J)F_1$ hybrid to F9 is immunological in nature.

Materials and Methods

Mouse strains. The inbred mouse strains 129/J-"129"-, A/J-"A"-, and SJL/J-"S"-, the congenic strain B10.129(6M)-"6M"-, and the F_1 hybrid strain $(C57BL/6 \times 129/J)F_1$-"$(B6 \times 129)F_1$"- were obtained from the Jackson Laboratory, Bar Harbor, Maine. F_1 hybrid mice between strain 129 and strains A, S, and 6M were bred in our own laboratory. At the start of an experiment, all mice were between 6 and 10 weeks old. The sex of the 129 parent had no detectable effect on the tumor incidence of the F_1 offspring.

Teratocarcinoma cell lines. The murine embryonal carcinoma cell lines F9-41-"F9"- and PCC3/A/1-"PCC3"-, syngeneic in 129/Sv mice, were employed in these experiments. Both these lines were isolated from transplantable teratocarcinoma OTT6050, obtained by L.C. Stevens from the grafting of a 129/SvS1^J/+ embryo to the testis of an adult mouse (Stevens 1970). The PCC3 line is developmentally multipotent; the F9 line is nullipotent. Karyotypically, F9 is aneuploid, whereas PCC3 is nearly diploid (Jacob 1977).

Cell culture conditions. Cells were grown in Dulbecco's modified Eagle's medium containing 15% Gibco fetal calf serum. The cultures were maintained at 37° C in a CO_2/humid air mixture. Cultures were harvested and resuspended in fresh medium every 2–3 days.

Histological analysis of tumors. A sample of tumors from both control and experimental animals was analyzed histologically for each experimental group. Tumors were fixed in Bouin's fixative, paraffin-processed according to Method I described in Luna (1968), and stained with hematoxylin-eosin. F9 tumors contain only embryonal carcinoma with rare endodermal tissue areas. PCC3 tumors contain derivatives of all three germ layers.

PCC3 and F9 cell injections. Cells were harvested by pipetting and resuspended in medium containing 1% fetal calf serum. Cell viability was tested on all samples with the trypan blue dye exclusion test, and all doses are stated as number of viable cells injected. One-milliliter syringes fitted with 22-gauge needles were used for all injections; all injections were completed within 30 min of cell harvesting. Cells were injected subcutaneously, bilaterally, in volumes of 0.2 ml.

Tumor scoring. Animals were scored for tumors regularly by palpation for a period three times as long as that required for tumor production in the 129 control animals. Animals scored as negative throughout this period were necropsied to confirm the lack of tumors. Results were pooled for male and female recipients, since no sex-related differences in tumor incidence were noted.

Sublethal irradiation of mice. Mice were irradiated with a ^{137}Cs source providing a dose rate of 1000 rad/minute. The sublethal dose used for $(B6 \times 129)F_1$ hybrids was 650 rad.

Lymphoid reconstitution of mice. A lethal dose of 900 rad was given to 129 mice from the same ^{137}Cs source. Lymphoid cells were taken from the bone marrow and the spleen of donor $(B6 \times 129)F_1$ mice. Each irradiated animal received 10^7 bone marrow and 10^7 spleen cells intravenously through the tail vein. Reconstituted animals were challenged 4 weeks later.

Immunizations. (B6 × 129)F_1 animals received bilateral subcutaneous injections of 1.2×10^4 viable F9 cells and were challenged with a lethal dose (2×10^5) of F9 cells 14 days later. Animals developing tumors prior to the second challenge (8 of 72) were not included in the results.

Quantitative analysis of results. Dose-response data and comparisons of resistance between hybrid strains were analyzed according to the Wilcoxon signed rank test (Conover 1971); 2×2 contingency tests were performed upon results of experiments testing for the effects of preimmunization with live tumor cells, of sublethal irradiation, or of adoptive transfer of lymphoid tissues. In Tables 2, 3, and 4, $\triangle$ = the number of tumor-positive 129 mice − the number of tumor-positive F_1 mice. Absolute $\triangle$ values were assigned ranks, the lowest rank corresponding to the lowest absolute $\triangle$ value. In the case of ties, each member of a tie was assigned a midrank value. T = the sum of all the negative $\triangle$ values. Given the null hypothesis that the tumor incidence of the F_1 hybrid will not differ significantly from that of the 129/J parent, the probability of T was calculated using a Wilcoxon signed-rank distribution. N = the number of doses tested.

In Tables 5, 6, and 7, data were analyzed by a 2×2 contingency test according to the following formula:

Given a matrix

$$\begin{matrix} a & b \\ c & d \end{matrix}$$

where $a + b + c + d = N$

$$T = \frac{N(ad - bc)^2}{(a+b)(c+d) \times (a+c)(b+d)}$$

The probability of T was calculated using a Chi-square distribution.

Results

Previous experiments have shown that (B6 × 129)F_1 hybrids are resistant to low doses of F9 (Avner et al. 1978). We have extended this observation to examine resistance to both F9 and PCC3 at a number of cell doses in four different F_1 hybrid hosts. F_1 hybrids were constructed between the syngeneic mouse strain 129 and the allogeneic strains B6, 6M, A, and S. These allogeneic strains are all highly resistant to PCC3 (Avner et al. 1978). Strains B6 and 6M are also highly resistant to F9, but A and S mice are susceptible to moderate ($\geq 2 \times 10^5$) doses of F9 (Avner et al. 1978). The *H-2* haplotype of each of these F_1 hybrid strains is given in Table 1. Strain 6M is a congenic strain, possessing the *H-2* region of 129 on the genetic background of C57BL/10 (Klein 1975). The 129 haplotype *H-2*^{bv1} is a minor variant of the *H-2*^{b} haplotype, serologically indistinguishable from *H-2*^{b} (Klein 1975).

Resistance of F_1 hybrids to PCC3 and F9. Results of experiments using PCC3 are presented in Table 2. Each hybrid strain was challenged with doses ranging from

Table 1. *H-2* haplotypes of F_1 hybrid mouse strains

Mouse strain		*H-2* haplotype
C57BL/6	× 129/J	*b/bv1*
B10.129(6M)	× 129/J	*bv1/bv1*
A/J	× 129/J	*a/bv1*
SJL/J	× 129/J	*s/bv1*

Table 2. Tumor incidence of F_1 hybrids challenged with PCC3

(a)	B6 × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	△	Rank of |△|
4 × 10⁵	10/10	10/10	0	1
2 × 10⁵	8/10	10/10	+2	2.5
1 × 10⁵	7/10	10/10	+3	4
5 × 10⁴	0/20	12/20	+12	6
2.5 × 10⁴	12/30	16/30	+4	5
1.25 × 10⁴	5/20	3/20	−2	2.5

Sum of ranks of negative △'s = T = 2.5. For N = 6, P = 0.0625.

(b)	6M × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	△	Rank of |△|
1 × 10⁶	10/10	10/10	0	1
3.3 × 10⁵	5/10	10/10	+5	4.5
2 × 10⁵	4/10	8/10	+4	2.5
1 × 10⁵	4/10	10/10	+4	2.5
5 × 10⁴	0/10	5/10	+5	4.5

Sum of ranks of negative △'s = T = 0. For N = 5, P = 0.031.

(c)	A × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	△	Rank of |△|
3.3 × 10⁵	10/10	10/10	0	1
2 × 10⁵	9/10	10/10	+1	2.5
1 × 10⁵	7/10	9/10	+2	4
5 × 10⁴	4/10	8/10	+4	5
3.3 × 10⁴	0/10	1/10	+1	2.5

Sum of all ranks of negative △'s = T = 0. For N = 5, P = 0.0313.

(d)	S × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	△	Rank of |△|
3.3 × 10⁵	19/19	19/19	0	1
2 × 10⁵	10/10	9/10	−1	2.5
1 × 10⁵	6/10	9/10	+3	5
5 × 10⁴	7/10	9/10	+2	4
3.3 × 10⁴	0/10	1/10	+1	2.5

Sum of ranks of negative △'s = T = 2.5. For N = 5, P = 0.125.

one that yielded 100% tumors to another that yielded virtually 0% tumors in the hybrid hosts. All hybrids tested, with the exception of the $(S \times 129)F_1$, showed significantly higher resistance to PCC3 than the tumor-syngeneic 129 parent.

Results of experiments using F9 are presented in Table 3. Hybrid strains were tested again over a dose range yielding from 100% to 0% tumors. All four of the hybrid strains showed significant F_1 resistance to F9.

Table 3. Tumor incidence of F_1 hybrids challenged with F9

(a)	B6 × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	$\triangle$	Rank of $|\triangle|$
2 × 10^5	10/10	10/10	0	1.5
1 × 10^5	9/10	10/10	+1	3
5 × 10^4	2/10	10/10	+8	8.5
2.5 × 10^4	0/10	10/10	+10	10
1.25 × 10^4	2/10	10/10	+8	8.5
6 × 10^3	0/10	6/10	+6	6
3 × 10^3	2/10	9/10	+7	7
1.5 × 10^3	0/10	2/10	+2	4
7.5 × 10^2	0/10	3/10	+3	5
3.75 × 10^2	0/10	0/10	0	1.5

Sum of ranks of negative $\triangle$'s = T = 0. For N = 10, P = 0.001.

(b)	6M × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	$\triangle$	Rank of $|\triangle|$
5 × 10^5	10/10	10/10	0	1
2 × 10^5	7/10	10/10	+3	2
1 × 10^5	4/10	10/10	+6	3
5 × 10^4	2/10	10/10	+8	5
2.5 × 10^4	2/17	13/17	+11	6
1.25 × 10^4	0/10	7/10	+7	4

Sum of ranks of negative $\triangle$'s = T = 0. For N = 6, P = 0.0156.

(c)	A × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	$\triangle$	Rank of $|\triangle|$
2 × 10^5	10/10	10/10	0	1
1 × 10^5	7/10	10/10	+3	4
5 × 10^4	16/20	17/20	+1	2
2.5 × 10^4	5/10	8/10	+3	4
1.25 × 10^4	12/20	16/20	+4	6
6 × 10^3	0/10	3/10	+3	4

Sum of ranks of negative $\triangle$'s = T = 0. For N = 6, P = 0.0156.

Table 3. Continued.

(d)	S × 129			
Cell dose	Tumor incidence-F_1	Tumor incidence-129	$\triangle$	Rank of $|\triangle|$
2×10^5	10/10	10/10	0	1.5
1×10^5	9/10	10/10	+1	4
5×10^4	7/10	10/10	+3	7
2.5×10^4	6/10	7/10	+1	4
1.25×10^4	6/10	10/10	+4	8
6×10^3	8/10	9/10	+1	4
3×10^3	3/10	5/10	+2	6
1.5×10^3	4/10	4/10	0	1.5

Sum of ranks of negative $\triangle$'s = T = 0. For N = 8, P = 0.0039.

When the magnitude of hybrid resistance to F9 was examined, it was discovered that the strains fall into two groups. Strains $(B6 \times 129)F_1$ and $(6M \times 129)F_1$ belong to a high resistance category, whereas strains $(A \times 129)F_1$ and $(S \times 129)F_1$ belong to

Table 4. Comparison of strength of hybrid resistance to F9 between F_1 strains

(a)	$(B6 \times 129)F_1$ vs. $(6M \times 129)F_1$			
Cell dose	% Tumor-negative F_1 mice —	% Tumor-negative 129 controls	$\triangle$	Rank of $|\triangle|$
	$(B6 \times 129)F_1$	$(6M \times 129)F_1$		
2×10^5	0	0	0	1.5
1×10^5	10	60	−50	5
5×10^4	80	80	0	1.5
2.5×10^4	100	64	+36	4
1.25×10^4	80	70	+10	3

Sum of ranks of negative $\triangle$'s = T = 5.
For N = 5, P = 0.3125.

(b)	$(A \times 129)F_1$ vs. $(S \times 129)F_1$			
Cell dose	% Tumor-negative F_1 mice —	% Tumor-negative 129 controls	$\triangle$	Rank of $|\triangle|$
	$(A \times 129)F_1$	$(S \times 129)F_1$		
2×10^5	0	0	0	1
1×10^5	30	10	+20	3
5×10^4	5	30	−25	5
2.5×10^4	30	10	+20	3
1.25×10^4	20	40	−20	3

Sum of ranks of negative $\triangle$'s = T = 8.
For N = 5, P = 0.59.

Table 4. Continued.

(c)	"C57"F_1 vs. "A, S" F_1			
Cell dose	% Tumor-negative F_1 mice	% Tumor-negative − 129 controls	$\triangle$	Rank of $\lvert\triangle\rvert$
	"C57" F_1	"A, S" F_1		
2×10^5	0	0	0	1
1×10^5	35	20	+15	2
5×10^4	80	13	+65	5
2.5×10^4	78	20	+58	4
1.2×10^4	75	27	+48	3

Sum of ranks of negative $\triangle$'s $= T = 0$. For $N = 5$, $p = 0.031$.

a low resistance group (Table 4, parts a and b). Data from each group were pooled and compared (Table 4, part c). It was found that hybrid resistance to F9 was significantly stronger in the "C57" group than in the "A, S" group ($P = 0.03$). This may indicate the presence of a strong factor for hybrid resistance in the C57BL background (common to the B6 and 6M strains) that is absent in the A and S strain backgrounds.

Evidence that hybrid resistance to F9 is an immunological response. The $(B6 \times 129)F_1$ hybrid strain, which demonstrated strong F_1 resistance to F9 and significant resistance to PCC3, was chosen for further studies. The first of these involved an attempt to increase the hybrid resistance by preimmunizing with sublethal doses of viable F9 cells. The immunization procedure is described in *Materials and Methods*. The results, presented in Table 5, show that preimmunized F_1 were significantly more resistant to challenge with lethal doses of F9 than their nonimmunized counterparts ($X^2 = 4.5$; $P = 0.02$). A more conservative estimate of the level of significance would classify the eight animals that incurred tumors from the preimmunization dose as tumor-positive in the set of challenged animals. This estimate gives $X^2 = 2.56$, $P = 0.04$.

Total-body irradiation with sublethal levels of X-rays eliminates many immunological responses. Avner and co-workers (1978) found that sublethal total-body irradiation diminished the resistance to F9 of $(B6 \times 129)F_1$ hybrids. Our results (Table 6) confirm this observation. Sublethally irradiated $(B6 \times 129)F_1$ mice were unable to resist a sublethal dose of F9 cells to which unirradiated control hybrids showed a high degree of resistance ($X^2 = 25.1$; $P < 0.001$).

Adoptive transfer of immunocompetent cells provides a positive test for the immunological basis of a response. Thus, the capacity of the $(B6 \times 129)F_1$ hybrid to transfer its resistance to the tumor-permissive 129 parent strain was tested. Construction of 129←F_1 radiation chimeras is described in *Materials and Methods;* results are presented in Table 7. The chimeras showed a highly significant increase in resistance to F9 (part a; $X^2 = 11.75$; $p < 0.001$); this resistance approached that of the F_1 hybrid itself (part b; $X^2 = 1.2$; $p > 0.16$).

Table 5. Immunization of $(B6 \times 129)F_1$ mice

	Total	Tumor-negative	Tumor-positive
Control hybrids	72	19 (26%)	53 (74%)
Immunized hybrids	64	28 (44%)	36 (56%)

Hybrids were immunized with sublethal doses of F9, as described in *Materials and Methods*. These animals and their age- and sex-matched controls were challenged at the same time with 2×10^5 F9 cells from the same preparation.

Discussion

The present experiments extend previous observations on F_1 hybrid resistance to murine embryonal carcinoma cells (Avner et al. 1978). We have studied resistance to both multipotent and nullipotent cell lines of a murine teratocarcinoma in four strains of F_1 mice semisyngeneic to the strain of tumor origin.

Most of the F_1 hybrid strains showed significant resistance to both embryonal carcinoma cell lines PCC3 and F9. Our results concerning the *H-2* control of this hybrid resistance fit the pattern described by Klein and co-workers (1978), who reported that F_1 hybrid resistance to three carcinomas and two sarcomas failed to show significant linkage to the *H-2* complex. In the present studies, the hybrid $(6M \times 129)F_1$ showed undiminished resistance to both embryonal carcinoma cell lines tested, although the *H-2* haplotype of this hybrid is homozygous and identical to that of the inbred syngeneic 129 parent. Resistance of the $(6M \times 129)F_1$ hybrid was equal in strength to that of the *H-2* heterozygote $(B6 \times 129)F_1$. Thus, we conclude that heterozygosity of the *H-2* region is not necessary to account for hybrid resistance to the embryonal carcinoma cell lines PCC3 and F9.

Avner and co-workers (1978) showed that B6 carries strong allograft rejection character(s) for F9, whereas A and S do not. It is possible that the same character(s) responsible for this difference in allograft rejection continues to operate in the semisyngeneic hybrid host. This could account for the greater hybrid resistance of the "C57BL" group (Table 4). In principle, such a character could be either a dominant immune-response factor in the B6 genome or else a strong histocompatibility difference for which the 129 allele is incompletely expressed in the F_1 hybrid.

The basis of resistance in the $(B6 \times 129)F_1$ mouse to F9 appears to be an immune response to an antigenic determinant (or determinants) present on the embryonal carcinoma cell. This conclusion stems from the observations that: (a) F_1 hybrid

Table 6. Sensitivity of sublethally irradiated $(B6 \times 129)F_1$ mice to F9

	Tumor-negative mice	Tumor-positive mice
Irradiated hybrids	1	20
Nonirradiated hybrids	19	5

Mice were irradiated as described in *Materials and Methods*. Irradiated mice and their age- and sex-matched controls received a sublethal challenging of F9 cells (2.5×10^4) subcutaneously bilaterally using the same cell suspension.

Table 7. Resistance of 129←F_1 chimeras to F9

	Tumor-negative mice	Tumor-positive mice
129	4	25
F_1	22	9
Chimeras	16	12

Construction of 129←F_1 chimeras is described in *Materials and Methods*. Chimeras, 129 controls, and F_1 controls were age-matched and were challenged at the same time from the same cell suspension. All animals received 2.5×10^4 F9 cells subcutaneously bilaterally, a dose that is lethal for the 129 strain but sublethal for the F_1 strain.

resistance is eliminated by sublethal doses of total-body irradiation, (b) hybrid resistance is adoptively transferred via lymphoid cells from a naive F_1 mouse, and (c) the resistance of the hybrids is heightened by preimmunization using live tumor cells. The positive effect of preimmunization, although not large, gives important evidence that hybrid resistance is immunological.

Does preimmunization heighten the same immune-response channel as that operating in hybrid resistance? Or, does it act in animals protected by hybrid resistance to heighten other channels? We have not tested whether syngeneic hosts can be preimmunized by live tumor cells; the doses sublethal for F_1 hosts would be lethal in syngeneic hosts. We note with interest the report of Boon and Van Pel (1978) that syngeneic hosts can be preimmunized by live attenuated (*tum⁻*) embryonal carcinoma variants. It is possible that F_1 resistance operates on the same channel as that elicited in the syngeneic host by Boon and Van Pel's procedure.

Our experiments do not permit identification of the embryonal carcinoma antigen(s) to which the F_1 hybrid responds. It is unlikely to be encoded at the *H-2* locus because the (6M × 129)F_1 is fully syngeneic at this locus but shows unabated hybrid resistance.

Hybrid resistance is also unlikely to be directed against the male-specific antigen H(Y). PCC3 lacks the Y chromosome (Nicolas et al. 1976), and we have observed no difference in the level of resistance between male and female hybrids (data not shown).

A third unlikely candidate for the transplantation antigen against which hybrid resistance is directed is the F9 antigen (Artzt et al. 1974, Kemler et al. 1976, Marticorena et al. 1978). This embryonic antigen is immunogenic in the syngeneic host, as judged by serological response; for it to form the basis of graft rejection in hybrids and not in the syngeneic host would thus require an additional hypothesis.

One interesting candidate for the transplantation antigen for hybrid resistance is the antigen associated with the Gross leukemia virus, G_{IX}. This antigen is found on adult lymphoid cells and in some malignant tissues; 129 is a positive strain, whereas B6 is negative (Klein 1975). Obata and his co-workers (1976) have studied the genetic control of the serological response to G_{IX}. Although the syngeneic strain 129 is antigen-positive and seronegative, and the antigen-positive congenic strain B6.129 is also seronegative, the F_1 hybrids (129 × B6) and (129 × B6.129) are seropositive. These workers have suggested that B6 and 129 carry dominant immune-response factors that cooperate to permit a serological response to the G_{IX} auto-antigen encoded by the 129 genome.

Though embryonal carcinoma cells in culture show no evidence for virus growth (Jacob 1977), it is not known whether F9 or PCC3 carry the G_{IX} antigen. Its involvement in hybrid resistance is, therefore, only conjectural at this time.

The studies reported here demonstrate that resistance of (129 × B6) hybrids to F9 teratocarcinoma transplants is mediated by lymphoid tissue and does not depend upon heterozygosity at the H-2 locus. The multipotent line PCC3 is also susceptible to hybrid resistance. Semisyngeneic hybrids involving A/J or SJL/J as the allogeneic parent also display hybrid resistance to F9 and PCC3. The target antigen(s) and the immune mechanisms(s) of transplant rejection remain to be determined.

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