NATURAL RESISTANCE OF LETHALLY IRRADIATED F_1 HYBRID MICE TO PARENTAL MARROW GRAFTS IS A FUNCTION OF H-2/Hh-RESTRICTED EFFECTORS

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Grafts of normal and neoplastic hemopoietic cells fail or grow poorly in certain lethally irradiated allogeneic mice, including F_1 hybrid recipients of parental cells (1–4). The manifestation of histoincompatibility in the latter host/donor combination has been referred to as hybrid resistance, an apparent contradiction to the classic laws of transplantation. A similar resistance to allogeneic bone marrow cell (BMC)[1] grafts has been shown to occur in other mammalian species (5–7), and may contribute to clinical marrow graft failure in humans (8). The resistance is effected by an unusual host-vs.-graft reaction that is radioresistant and independent of the thymus (9–12). The sharing of numerous characteristics between this resistance and natural cytotoxic activity in vitro against a broad range of target cells suggests that the natural resistance to hemopoietic cells is mediated by a subset of natural killer (NK) cells (13–15). Treatment of mice with antisera with selective reactivity against NK cells indeed abrogates hemopoietic resistance as well (16, 17). To date, however, the growing list of properties shared between such resistance and NK cell activity does not include the single most pertinent property of hemopoietic resistance, i.e., its immunogenetic specificity.

Hybrid resistance in mice is controlled by the hemopoietic histocompatibility (Hh) loci, most of which are linked to the major histocompatibility complex (MHC) of the mouse, the H-2 (reviewed in 18). One of the best known Hh loci, Hh-1, is tightly linked to the H-2D region. It has been hypothesized that these genes are noncodominantly expressed so that donor cells homozygous for a given allele of an Hh locus are recognized as nonself and are rejected by F_1 hybrid recipients heterozygous for the same allele (9). In spite of its unorthodox assumption of noncodominant, or corecessive, inheritance of Hh genes (19), the hypothesis successfully explains the genetic constraints of both hybrid and allogeneic resistance (18). Direct evidence is still lacking, however, for the existence of effector cells capable of specifically recognizing the putative Hh

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[1] *Abbreviations used in this paper:* BMC, bone marrow cell(s); CTL, cytotoxic T lymphocyte(s); FUdR, 5-fluoro-2′-deoxyuridine; HBSS, Hanks' balanced salt solution; Hh, hemopoietic histocompatibility; IFN, interferon; IL-2, interleukin 2; IUdR, 5-iodo-2′-deoxyuridine; MHC, major histocompatibility complex; NK, natural killer; p-I:C, polyinosinic:polycytidylic acid.

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antigens, thereby mediating parental marrow graft rejection. In fact, some recent observations (20–22, 23) tend to support an alternative hypothesis that the genetic specificity of hemopoietic resistance rests on regulatory mechanisms rather than on the postulated recognition specificity of effector cells. These observations include the findings that bone marrow cells from normal mice or healthy humans are susceptible to NK cell-mediated cytolysis in vitro (20–22), and that resistance to bone marrow allografts can be reconstituted by an NK-like cell line (23). Moreover, the level of NK activity is in part controlled by genes linked to the MHC (24–28).

Thus, definitive information on the recognition specificity of the effectors mediating hybrid resistance is a prerequisite for the further understanding of this and other natural resistance phenomena. To directly test the recognition specificity of the effectors, an in vivo competitive inhibition assay has been developed. The experiments described in this initial report used a panel of tumor cells as inhibitors, several of which were chosen on the basis of susceptibility to cytolysis by NK cells in vitro. The results provide the first direct evidence for the existence of radioresistant effectors that specifically recognize cellular determinants controlled by gene(s) within, or closely linked to, the H-2D region. These effectors are reactive only with the H-2D homozygous tumor cells, an indication of Hh-1 restriction of recognition.

Materials and Methods

Animals. Female C57BL/6N (B6), C57BL/10ScSn (B10), C3H/HeN (C3H), DBA/2, (C57BL/6 × DBA/2)F_1 (B6D2F_1), and B10.A mice were obtained from the Animal Genetics and Production Branch, Division of Cancer Treatment, National Cancer Institute. Female (C57BL/10 × C3H)F_1 (B10C3F_1) mice used were obtained from Health Research Inc., West Seneca, NY. All other strains of mice used were bred in our own colony, including B10.D2, B10.A(2R), B10.A(4R), B10.A(5R), [B10.A × B10.A(2R)]F_1, and (BALB.B10 × BALB/c)F_1. Bone marrow donors were 5–8 wk old and recipients were 12–20 wk old.

Tumor cells. The following cell lines were maintained in vivo as ascitic tumors by serial passage in syngeneic mice: benzpyrene-induced lymphoma EL-4 (H-2^b, C57BL/6), radiation-induced lymphoma L5MF-22 [H-2^b, B10.129(5M)], methylcholanthrene-induced leukemia L1210 (H-2^d, DBA/2), lymphosarcoma GL-1 (H-2^k, C3H), erythroleukemia HFLb/d [H-$2^{b/d}$, (BALB.B10 × BALB/c)F_1] (29), and Friend virus-induced myeloid leukemia 427E (H-$2^{b/d}$, B6D2F_1) (30). The following radiation-induced lymphomas were maintained by serial passage in syngeneic mice as splenic tumors: LAF-17 (H-2^a, B10.A), RDNM-27 (H-2^d, B10.D2), LHM-14 [H-2^{h2}, B10.A(2R)], L4RM-73 [H-2^{h4}, B10.A(4R)], LIM-65 [H-2^{i5}, B10.A(5R)], and LBA × 2RM-80 (H-$2^{a/h2}$, [B10.A × B10.A(2R)]F_1). The Friend virus-transformed bone marrow stem cell line 416B (H-$2^{b/d}$, B6D2F_1) (31), Moloney virus-induced lymphoma YAC-1 (H-2^a, A/Sn), Rauscher virus-induced lymphoma RBL-5 (H-2^b, B6), radiation-induced leukemia RL♂-1 (H-2^d, BALB/c), and a subline of EL-4 (not an interleukin 2 [IL-2] secreter) were maintained in vitro. The cell line HFLb/d was originally a gift from Dr. T. V. Rajan. Both the 427E and 416B lines were a gift of Dr. S. K. Ruscetti.

Irradiation. Recipient mice were exposed to 800–950 rad of ^{137}Cs gamma radiation from a Gammacell 20 Small Animal Irradiator (Atomic Energy of Canada, Ltd., Ottawa, Canada) at a dose rate of 95 rad/min, 2–3 h before the intravenous injection of either tumor or normal BMC. Tumor cells to be used as inhibitors were suspended in ice-cold Hanks' balanced salt solution (HBSS) and exposed to 10,000 rad of ^{137}Cs gamma radiation using an Isomedix irradiator (model M-38-2; Isomedix Inc., Parsippany, NJ) at the dose

rate of 1,000 rad/min. Irradiated tumor cells were injected into mice within 30 min of irradiation.

Treatment with Polyinosinic:Polycytidylic Acid (p-I:C). Lyophilized p-I:C (Sigma Chemical Co., St. Louis, MO) was dissolved in HBSS at a concentration of 1 mg/ml. Both lyophilized and dissolved p-I:C were stored at −20°C. Mice were injected intraperitoneally with 100 μg of p-I:C 18 h before injection with BMC or [^{125}I]5-iodo-2′-deoxyuridine ([^{125}I]-IUdR)-labeled tumor cells (see below).

Splenic [^{125}I]IUdR Uptake Assay for Marrow Cell Proliferation. The standard assay for proliferation of grafted hemopoietic cells in lethally irradiated recipients has been previously described (32, 33). The assay was modified, as required, to include an intravenous injection(s) of irradiated tumor cells. Briefly, lethally irradiated recipient mice were injected intravenously with inhibitor cells suspended in 1.0 ml of HBSS 3 h before the intravenous injection of BMC, unless otherwise stated; in some experiments the time interval between tumor and BMC injections was the variable to be tested. Viable cell counts were made with a hemocytometer, based on trypan blue dye exclusion. The proliferation of donor cells in the spleens of recipients was assessed 5–7 d later by measuring the incorporation of [^{125}I]IUdR. 30 min before the intraperitoneal injection of 0.25 μCi [^{125}I]IUdR (Amersham Corp., Arlington Heights, IL), each animal was injected with 10^{-7} mol i.p. of 5-fluoro-2′-deoxyuridine (FUdR) to reduce competition by endogenous thymidylate. All animals were sacrificed 18 h after isotope injection and their spleens removed and placed in glass counting vials. [^{125}I]IUdR retention was measured with a well-type gamma scintillation counter. The retention of [^{125}I]IUdR in the spleens was expressed as a percentage of the injected isotope dose (% injected dose). Irradiated syngeneic recipients served to measure the full growth potential of the grafted BMC. The spleens of lethally irradiated mice not injected with BMC or injected with irradiated tumor cells alone retained 0.01–0.03% of the injected isotope.

In Vitro [^{125}I]IUdR Labeling of Tumor Cells for Localization Studies. Tumor cells to be labeled were suspended at the concentration of 10^6/ml in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. FUdR (2×10^{-8} mol) was added to 50 ml of cell suspension in each plastic petri dish and incubated for 20 min at 37°C in a 5% CO_2 incubator. 15 μCi of [^{125}I]IUdR was then added to each culture dish and the cells were incubated for an additional 18 h. The cells were harvested and washed extensively with HBSS. They were resuspended in HBSS at a concentration of 10^6/ml and injected intravenously into lethally irradiated recipients. At selected times after injection, the animals were sacrificed, their spleens removed, and the amount of retained ^{125}I activity in each spleen determined as described above.

Statistical Analysis. The data are expressed as geometric means of the percent of injected [^{125}I]IUdR dose. Group comparisons were made using Student's t test for geometric means. Differences between groups were considered significant when P was ≤ 0.05.

Results

Inhibition of Hybrid Resistance to Parental Bone Marrow Grafts by Irradiated Lymphoma Cells. Two examples of hybrid resistance are shown in Fig. 1. The growth of parental B6 (H-2^b) BMC in irradiated B6D2F$_1$ (H-2$^{b/d}$) recipients was impaired as compared with that of the same cells grafted into irradiated syngeneic hosts (Fig. 1*A*). In this F$_1$ hybrid recipient vs. parental donor combination, growth of parental BMC was completely suppressed when graft size was below $1–2 \times 10^6$. When the graft size was increased, proportionally more BMC escaped from rejection and proliferated in the F$_1$ host spleen. Similarly, grafts of parental B10 (H-2^b) BMC were resisted by irradiated B10C3F$_1$ (H-2$^{b/k}$) hosts (Fig. 1*B*). However, resistance by B10C3F$_1$ mice was somewhat weaker than that by B6D2F$_1$ mice, as judged by the partial escape from rejection of 2×10^6 or larger

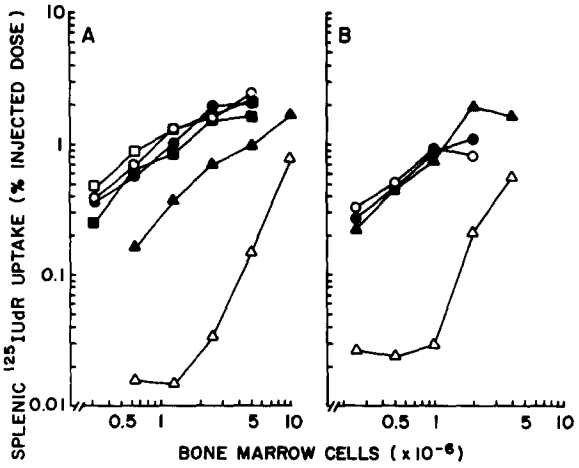

FIGURE 1. Inhibition of hybrid resistance against parental H-2^b BMC grafts by irradiated H-2^b lymphoma cells. (*A*) Graded numbers of B6 BMC were injected into irradiated B6D2F$_1$ (△, ▲) or B6 (○, ●) mice, either without additional treatment (△, ○) or after inoculation of 5×10^7 irradiated EL-4 cells 3 h earlier (▲, ●). Graded numbers of B6D2F$_1$ BMC were injected into irradiated B6D2F$_1$ mice otherwise untreated (□) or after inoculation of 5×10^7 irradiated EL-4 cells 3 h earlier (■). [^{125}I]IUdR uptake in the recipient spleens was tested 5 d later. (*B*) Graded numbers of B10 BMC were injected into irradiated B10C3F$_1$ (△, ▲) or B10 (○, ●) mice, either without additional treatment (△, ○) or after inoculation of 5×10^7 L5MF-22 cells 3 h earlier (▲, ●). [^{125}I]IUdR uptake by the recipient spleens was measured 5 d later. Each point represents the mean of three to six mice.

grafts in B10C3F$_1$ hosts. In B6D2F$_1$ mice, a comparable degree of escape from rejection occurred only with 5×10^6 or larger grafts.

To test the ability of H-2^b lymphoma cells to inhibit resistance to parental H-2^b BMC, EL-4 cells of B6 origin or L5MF-22 cells of B10.129(5M) origin were exposed to 10,000 rad of γ irradiation and injected at a dose of 5×10^7 cells/mouse into irradiated B6D2F$_1$ or B10C3F$_1$ mice, respectively, 3 h before transplantation of graded numbers of parental BMC. Partial (Fig. 1*A*) or complete (Fig. 1*B*) inhibition of hybrid resistance by irradiated lymphoma cells ensued, as indicated by higher levels of splenic [^{125}I]IUdR uptake 5 d later in the lymphoma-injected hosts as compared with the hosts grafted with BMC alone. Residual DNA synthesis by irradiated lymphoma cells, if any at all, did not contribute to the increase in [^{125}I]IUdR uptake for the following reasons. First, irradiated mice injected with these lymphoma cells but not BMC failed to incorporate [^{125}I]IUdR above the levels of incorporation (0.01–0.03% splenic uptake) in irradiated control mice not grafted with cells (data not shown). Second, aliquots of the same irradiated lymphoma cells had no effect on [^{125}I]IUdR uptake by irradiated hosts given syngeneic BMC grafts (Fig. 1, *A* and *B*). The relative effectiveness of the inhibition of rejection, as measured by the increase in [^{125}I]IUdR uptake, was greatest for the BMC dose at the inflection point of resistance. The data also indicate a higher inhibition efficiency by the same number of lymphoma cells against the weaker resistance of B10C3F$_1$ than that of B6D2F$_1$ mice. Fifty million inhibitor cells allowed the growth of B6 BMC in B6D2F$_1$ mice to an extent substantially below that in syngeneic B6 hosts (Fig. 1*A*), but the growth of B10

BMC in B10C3F$_1$ was raised by the same number of inhibitor cells to a level comparable to that in syngeneic B10 hosts (Fig. 1*B*). The two H-2^b lymphomas EL-4 and L5MF-22 were indistinguishable in their inhibitory capacity when simultaneously tested in the same F$_1$ recipient/parental BMC donor combinations (data not shown). Irradiated EL-4 cells from the same pool were also injected into irradiated B6 and B6D2F$_1$ recipients of syngeneic BMC grafts. The effect of EL-4 cells on syngeneic BMC growth, if any, was a slight, statistically insignificant depression rather than an enhancement of growth. Presumably, this was due to a nonspecific crowding effect, since the injection of unrelated lymphoma cells resulted in a similar degree of depression (data not shown). Similarly, injection of L5MF-22 cells had no effect on the growth of B10 BMC in syngeneic hosts.

Dependence of Inhibition on the Number of Irradiated Lymphoma Cells and the Time of their Injection. Lethally irradiated B6D2F$_1$ mice were inoculated with graded numbers of irradiated EL-4 cells 3 h before the injection of either 5×10^5, 2×10^6, or 7×10^6 B6 BMC. B6D2F$_1$ recipients given only the B6 BMC served as controls. The three doses of B6 BMC were chosen to cover the three identifiable levels of resistance indicated by the data in Fig. 1*A*, i.e., complete suppression of the growth of 5×10^5 parental BMC, the inflection point at 2×10^6 cells, and the partial escape of 7×10^6 B6 BMC from rejection. The inhibition of BMC rejection by EL-4 cells was dependent on both the BMC dose and the EL-4 cell dose (Fig. 2). With 2×10^6 or 7×10^6 B6 BMC, inhibition was a linear function of the EL-4 cell dose. However, with 5×10^5 BMC, substantial inhibition occurred only when 5×10^7 or higher numbers of EL-4 cells were injected, an indication that irradiated EL-4 cells compete with B6 BMC as targets for rejection. If this interpretation is correct, it is expected that the time of inhibitor

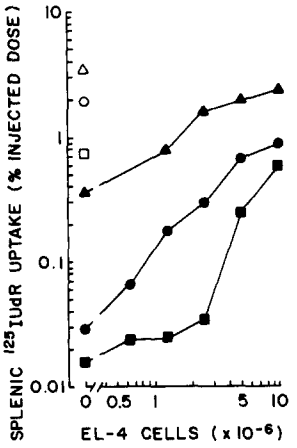

FIGURE 2. Dependence of the inhibition of hybrid resistance on both the bone marrow graft size and the inhibitor cell dose. Parental B6 BMC were injected into irradiated B6 (open symbols) or B6D2F$_1$ (closed symbols) mice at the dose of 5×10^5 (□, ■), 2×10^6 (○, ●), or 7×10^6 (△, ▲) cells. F$_1$ mice receiving B6 BMC were either not treated otherwise or given graded numbers of irradiated EL-4 cells 3 h before B6 BMC transplantation. All mice were tested for splenic [^{125}I]IUdR uptake 5 d later. Each point represents the mean of four to six mice.

TABLE I

Temporal Requirement for Inhibition, by Irradiated EL-4 Cells, of the Resistance of B6D2F$_1$ Mice Against Parental B6 Bone Marrow Grafts

Group	Recipient* (*n*)	EL-4‡			Mean percent [^{125}I]IUdR uptake (95% confidence limits)
		−24 h	−3 h	+24 h	
1	B6 (4)	−	−	−	2.3019 (1.5878–3.3372)
2	B6D2F$_1$ (5)	−	−	−	0.1254 (0.0865–0.1817)
3	B6D2F$_1$ (4)	+	−	−	0.0773 (0.0419–0.1426)
4	B6D2F$_1$ (6)	−	+	−	0.3555 (0.1447–0.8735)§
5	B6D2F$_1$ (6)	−	−	+	0.1382 (0.0854–0.2238)
6	B6D2F$_1$ (5)	+	+	−	0.8787 (0.5416–1.4255)§
7	B6D2F$_1$ (5)	−	+	+	0.4788 (0.2546–0.9001)§
8	B6D2F$_1$ (4)	+	+	+	1.1333 (0.7059–1.8195)§

* Irradiated recipients received 5×10^6 B6 BMC at 0 h.
‡ Each inoculum consisted of 10^7 irradiated cells per recipient.
§ Statistically significant ($P < 0.05$) inhibition of graft rejection as compared to group 2.

TABLE II

Specificity of Inhibition of Hybrid Resistance by Tumor Cells

Exp.	Recipient* (*n*)	Inhibitor‡		Mean percent [^{125}I]IUdR uptake (95% confidence limits)
		Tumor line	H-2	
I	B6 (5)	—	—	2.2422 (1.0576–4.7538)
	B6D2F$_1$ (6)	—	—	0.3153 (0.1752–0.5677)
	B6D2F$_1$ (6)	EL-4 (in vivo)	b	0.8872 (0.6316–1.2465)§
	B6D2F$_1$ (5)	L1210	d	0.1907 (0.1214–0.2995)
	B6D2F$_1$ (6)	GL-1	k	0.0625 (0.0196–0.1993)
	B6D2F$_1$ (6)	416B	b/d	0.2408 (0.1142–0.5077)
	B6D2F$_1$ (5)	427E	b/d	0.1032 (0.0332–0.3208)
	B6D2F$_1$ (6)	HFL b/d	b/d	0.1709 (0.0665–0.4395)
II	B6 (5)	—	—	2.7621 (1.9257–3.9617)
	B6D2F$_1$ (5)	—	—	0.6071 (0.3996–0.9224)
	B6D2F$_1$ (6)	EL-4 (in vitro)	b	1.6490 (1.1576–2.3490)§
	B6D2F$_1$ (5)	RBL-5	b	1.3202 (0.9324–1.8692)§
	B6D2F$_1$ (4)	RLδ-1	d	0.7799 (0.5757–1.0566)
	B6D2F$_1$ (6)	YAC-1	a	0.7400 (0.3734–1.4666)
III	B10 (3)	—	—	1.3752 (0.6248–3.0270)
	B10C3F$_1$ (5)	—	—	0.0468 (0.0264–0.0828)
	B10C3F$_1$ (4)	L5MF-22	b	0.5455 (0.3846–0.7736)§
	B10C3F$_1$ (5)	GL-1	k	0.0458 (0.0207–0.1016)
	B10C3F$_1$ (6)	YAC-1	a	0.0778 (0.0341–0.1779)

* Irradiated recipients received either 5×10^6 B6 BMC (Exps. I & II) or 10^6 B10 BMC (Exp. III).
‡ In all experiments, 5×10^7 irradiated tumor cells were given to recipients 3 h before BMC grafts.
§ Statistically significant ($P < 0.05$) inhibition of graft rejection.

cell injection relative to BMC grafting should critically influence the effectiveness of inhibition. To test this, 5×10^6 B6 BMC were injected into irradiated B6D2F$_1$ mice and 10^7 irradiated EL-4 cells were injected once, twice, or three times within 24 h before or after BMC grafting. The relatively low dose of EL-4 cells was selected to facilitate comparisons among the different experimental groups.

The results are summarized in Table I. A single dose of EL-4 cells was effective only when it was given 3 h before BMC transplantation (cf., groups 3, 4, and 5). When mice were given EL-4 cells 3 h before BMC grafting, an additional dose 24 h before BMC grafting augmented inhibition but one 24 h after BMC injection did so only slightly (cf., groups 6 and 7 with group 4). The regime of three doses of inhibitor cells was the most effective, but relatively little was gained over the two doses at −24 and −3 h (cf., groups 6 and 8). These data suggest that EL-4 cells must be present in the spleen simultaneously with the target BMC for inhibition to take place, and that the critical phase of BMC rejection is complete within 24 h of grafting. Additional data presented later (Fig. 3) lend further support to these conclusions.

Specificity of Inhibition of Hybrid Resistance by Irradiated Tumor Cells. The kinetic data presented thus far strongly suggest that the inhibition of hybrid resistance by H-2^b lymphoma cells is due to the competitive inhibition of resistance at the level of target cell recognition by the effector cells mediating this resistance. To substantiate this interpretation and to analyze the recognition specificity of the effectors, lymphoma and other tumor cells of standard H-2 haplotypes were tested for their inhibitory capacity. Among the tumor cell lines tested were NK-susceptible YAC-1, RBL-5, and RL♂-1, and three H-2 heterozygous lines. One of the latter, 416B, is highly susceptible to NK-mediated cytolysis in vitro (unpublished observation). As shown in Table II, cells of the H-2^d (L1210 of DBA/2, RL♂-1 of BALB/c), H-2^a (YAC-1 of A/Sn), and H-2^k (GL-1 of C3H) haplotypes were unable to inhibit the resistance of B6D2F_1 mice to parental B6 BMC, but another H-2^b lymphoma, RBL-5 (B6 origin), was nearly as inhibitory as EL-4 cells. Since the NK-susceptible lymphoma cells were maintained by in vitro passage, an in vitro subline of EL-4 was used in experiment II. The H-$2^{b/d}$ heterozygous "stem cell" line 416B and myeloid leukemia 427E of B6D2F_1 bone marrow origin were noninhibitory, as were erythroleukemic cells HFLb/d of (BALB.B10 × BALB/c)F_1 origin. These data indicate that the inhibitory capacity of tumor cells is associated with the H-2^b haplotype, that the H-2^b haplotype must be in the homozygous state to be inhibitory, and that susceptibility to NK-mediated cytolysis in vitro is irrelevant for inhibitory capacity in vivo.

In the next series of experiments, B10C3F_1 mice were grafted with parental B10 BMC and tested for inhibition of resistance by radiation-induced lymphoma cells from congenic strains of mice with informative H-2 haplotypes. Since the lymphoma lines used were splenic rather than ascitic, the L5MF-22 cells used as the positive control for inhibition were from the splenic line from which the ascitic subline used in earlier experiments was derived. Growth characteristics of the splenic lymphomas were comparable. The results of three representative experiments are shown in Table III. Though the efficiency of inhibition by these splenic tumors was lower than that by ascitic tumors, the presence or absence of inhibitory activity was always clearly demonstrable. The H-2^a (LAF-17 of B10.A) and H-2^d (RDNM-27 of B10.D2) cells were ineffective as inhibitors. Cells with a recombinant haplotype carrying the b allele in the D region only [LHM-14 of B10.A(2R)] or in the D end regions [L4RM-73 of B10.A(4R)] were inhibitory, but those cells carrying the b allele in the K end only [LIM-65 of B10.A(5R)] were noninhibitory. LBA × 2RM-80 lymphoma cells that were derived from a

TABLE III

Mapping of Gene(s) Controlling Inhibitory Activity of Lymphomas

Exp.	Recipient* (*n*)	Inhibitor‡		Mean percent [^{125}I]IUdR uptake (95% confidence limits)
		Tumor line	H-2 (KAESD)	
I	B10 (5)	—	—	1.6633 (1.3068–2.1169)
	B10C3F$_1$ (4)	—	—	0.0747 (0.0357–0.1563)
	B10C3F$_1$ (5)	L5MF-22	bbbbb	0.5534 (0.3913–0.7827)§
	B10C3F$_1$ (5)	LAF-17	kkkdd	0.0638 (0.0360–0.1129)
	B10C3F$_1$ (5)	LBA × 2RM-80	kkkdd / kkkdb	0.0408 (0.0238–0.0698)
II	B10C3F$_1$ (6)	—	—	0.0606 (0.0450–0.0815)
	B10C3F$_1$ (7)	L4RM-73	kkbbb	0.2349 (0.1629–0.3386)§
	B10C3F$_1$ (5)	RDNM-27	ddddd	0.0700 (0.0371–0.1320)
	B10C3F$_1$ (3)	LIM-65	bbkdd	0.0444 (0.0209–0.0945)
III	B10 (4)	—	—	2.5682 (1.5829–4.1666)
	B10C3F$_1$ (5)	—	—	0.2603 (0.1777–0.3814)
	B10C3F$_1$ (8)	LHM-14	kkkdb	0.6724 (0.3629–1.2457)§
	B10C3F$_1$ (6)	LAF-17	kkkdd	0.2950 (0.1606–0.5420)
	B10C3F$_1$ (6)	LIM-65	bbkdd	0.1408 (0.0688–0.2881)

* Irradiated recipients received either 10^6 (Exp. I) or 1.5×10^6 (Exps. II & III) B10 BMC. Splenic [^{125}I]IUdR uptake was tested on day 5 (Exp. I), day 6 (Exp. II), or day 7 (Exp. III).

‡ Either 5×10^7 (Exp. I) or 10^8 (Exps. II & III) irradiated tumor cells were given 3 h before BMC grafts.

§ Statistically significant ($P < 0.05$) inhibition of graft rejection.

[B10.A × B10.A(2R)]F$_1$ mouse were not inhibitory. Thus, the inhibitory capacity of tumor cells is dependent on the homozygosity of a gene or genes within or on the telomeric side of the H-2D region.

Effect of the Interferon Inducer Poly-I:C on Hybrid Resistance and the Specificity of Inhibition. Since interferon (IFN) is known to have an antiproliferative effect on hemopoietic bone marrow-derived cells (34), the effect of a single injection of p-I:C 18 h before BMC grafting was tested in syngeneic transplantation (Table IV, Exps. I and II). Although the growth of syngeneic BMC in p-I:C-treated recipients had a tendency to be slightly less than that in untreated recipients, a statistically significance difference was not observed under the experimental conditions in the strains of mice used. In contrast, growth of parental BMC was markedly reduced in p-I:C-treated F$_1$ hosts as compared with untreated F$_1$ hosts, indicating an amplification of resistance by p-I:C treatment (Exp. III). At this dose of parental BMC (5×10^6), the resistance in untreated recipients was partial and, hence, amenable to augmentation. Since p-I:C or IFN augments the cytolytic activity (35–37) and the target-binding capacity of NK cells for some targets (38), the specificity as well as the extent of inhibition of hybrid resistance by various tumor cells was tested. The specificity of inhibition was unaltered by p-I:C treatment of recipients, including the inability of NK-susceptible YAC-1 cells to inhibit parental BMC graft rejection (Exp. III). The extent of the inhibition by EL-4 cells was reduced, presumably because the inhibition is a function of the strength of resistance (Fig. 1).

TABLE IV

Effect of the Interferon Inducer p-I:C on Hybrid Resistance and the Effector Specificity

Exp.	Recipient (*n*)	BMC		Inhibitor*	p-I:C‡	Mean percent [^{125}I]IUdR uptake (95% confidence limits)
		Donor	Dose ($\times 10^{-6}$)			
I	B6 (4)	B6	4.0	–	–	1.9501 (1.3350–2.8488)
	B6 (3)	B6	4.0	–	+	1.6650 (1.1685–2.3726)
	B6 (4)	B6	8.0	–	–	3.0558 (2.4151–3.8666)
	B6 (3)	B6	8.0	–	+	2.9389 (2.6758–3.2279)
II	B6D2F$_1$ (3)	B6D2F$_1$	2.0	–	–	1.1251 (0.5366–2.3589)
	B6D2F$_1$ (3)	B6D2F$_1$	2.0	–	+	0.9555 (0.3964–2.3037)
	B6D2F$_1$ (4)	B6D2F$_1$	8.0	–	–	1.9578 (1.7153–2.2346)
	B6D2F$_1$ (4)	B6D2F$_1$	8.0	–	+	2.0248 (1.6647–2.4629)
III	B6 (6)	B6	5.0	–	–	2.3339 (2.1201–2.5691)
	B6D2F$_1$ (6)	B6	5.0	–	–	0.1770 (0.0548–0.5722)
	B6D2F$_1$ (5)	B6	5.0	–	+	0.0455 (0.0200–0.1036)§
	B6D2F$_1$ (7)	B6	5.0	EL-4	–	1.4967 (1.1558–1.9382)‖
	B6D2F$_1$ (7)	B6	5.0	EL-4	+	0.6989 (0.4899–0.9972)§,‖
	B6D2F$_1$ (5)	B6	5.0	L1210	–	0.1099 (0.0811–0.1489)
	B6D2F$_1$ (5)	B6	5.0	L1210	+	0.0322 (0.0247–0.0419)§
	B6D2F$_1$ (5)	B6	5.0	GL-1	–	0.0575 (0.0280–0.1174)
	B6D2F$_1$ (6)	B6	5.0	GL-1	+	0.0261 (0.0191–0.0357)§
	B6D2F$_1$ (4)	B6	5.0	YAC-1	–	0.0860 (0.0282–0.2626)
	B6D2F$_1$ (6)	B6	5.0	YAC-1	+	0.0390 (0.0271–0.0562)§

* Irradiated inhibitor cells were given at the dose of 10^8 per recipient 3 h before BMC grafting. For H-2 haplotype of tumor cells used, see Table II.

‡ Prospective recipients were given 100 μg of p-I:C i.p. 18 h before BMC grafting.

§ Statistically significant ($P < 0.05$) enhancement of graft rejection as compared with p-I:C-untreated control.

‖ Statistically significant ($P < 0.05$) inhibition of graft rejection as compared with the recipients not receiving tumor cells, with or without p-I:C treatment.

Splenic Localization of Inhibitory and Noninhibitory Lymphomas. It was conceivable that the inhibitory capacity of certain lymphoma cells and its absence in others depended on the ability to localize in the host spleen, rather than on the H-2 haplotype. In addition, the reduced efficiency of inhibition in p-I:C-treated hosts could reflect a lower efficiency of splenic localization of lymphoma cells in the treated than in the untreated hosts. To test these possibilities, the frequency of splenic localization was studied for selected inhibitory and noninhibitory tumor cells in untreated as well as p-I:C-treated F_1 recipients. Between 1 and 2% of [^{125}I]IUdR-labeled tumor cells localized in the spleen 1–2 h after injection, irrespective of their capacity to inhibit hybrid resistance (Fig. 3). Thus, noninhibitory L1210 (Fig. 3*B*) and YAC-1 and GL-1 cells (Fig. 3*C*) localized in F_1 spleens at about the same time and frequency as inhibitory EL-4 or L5MF-22 cells (Fig. 3*A*). Pretreatment of recipient mice with p-I:C did not substantially alter this pattern, although there was a trend for a higher initial localization frequency and a more rapid clearance of tumors from the spleens of treated than untreated mice. These characteristics were remarkably reproducible in independent experiments included, or not included, in Fig. 3. It was also apparent,

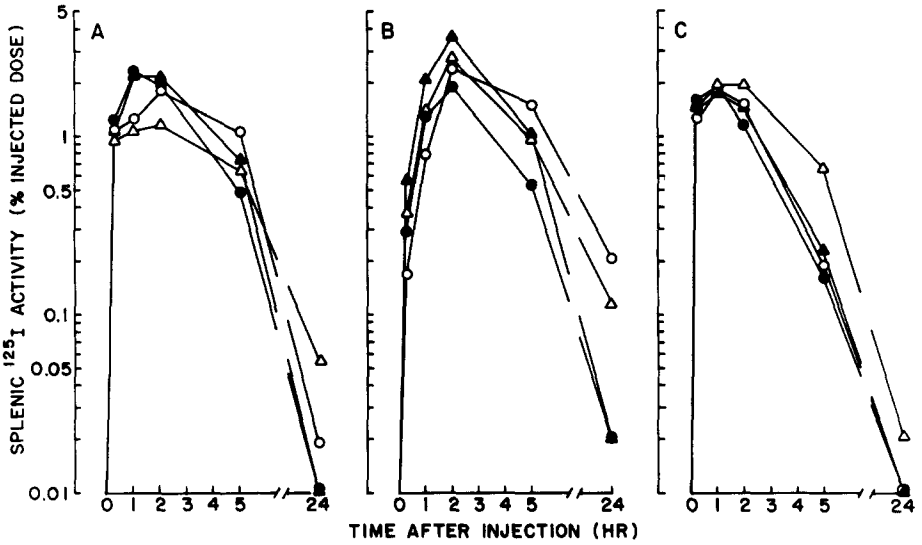

FIGURE 3. Splenic localization of lymphoma cells. One million [^{125}I]IUdR-labeled lymphoma cells were injected intravenously into irradiated B6D2F$_1$ or B10C3F$_1$ mice with (closed symbols) or without (open symbols) p-I:C treatment 18 h earlier; the spleens were removed at intervals between 15 min and 24 h after injection and the ^{125}I activity in the spleens was measured. (*A*) EL-4 cells injected into B6D2F$_1$ mice (○, ●), and L5MF-22 cells injected into B10C3F$_1$ mice (△, ▲). (*B*) L1210 cells injected into B6D2F$_1$ mice (○, ●) or, in another experiment, into B10C3F$_1$ mice (△, ▲). (*C*) YAC-1 cells (○, ●) or, in a separate experiment, GL-1 cells (△, ▲) injected into B6D2F$_1$ mice. Each point represents the mean of three to five mice.

not unexpectedly, that some minor differences exist among different tumor cells. For example, L1210 cells seemed to reach the spleen more slowly than EL-4, L5MF-22, YAC-1, or GL-1 cells, while both YAC-1 and GL-1 cells seemed to be cleared more rapidly from the spleen than either EL-4, L5MF-22, or L1210 cells. However, these characteristics did not obviously correlate with either the ability to inhibit parental H-2^b BMC rejection or susceptibility to NK-mediated cytolysis, at least within the 24 h observation period.

Discussion

The experiments described in this paper were undertaken to answer one of the most basic questions regarding the enigmatic phenomenon known as hybrid resistance, i.e., whether the immunogenetic specificity of resistance reflects the recognition specificity of putative effector cells. We addressed this issue through a new approach. The results show that inhibition in situ of hybrid resistance by irradiated tumor cells depends on (*a*) the strength of resistance, (*b*) the parental BMC graft size, (*c*) the inhibitor cell dose, (*d*) the time of inhibitor cell inoculation relative to bone marrow transplantation, and (*e*) the inhibitor cells' H-2 haplotype and homozygosity. It is unlikely that characteristics of tumor cells not associated with the H-2 haplotype, such as the cell size, splenic localization frequency, possible lymphokine or cytotoxin secretion, or the presence of alloreactive T lymphocytes accompanying in vivo-derived tumor cells that could be activated to secrete lymphokines, played a substantial role. Although not all of the 16

tumor lines used were tested for their effect on BMC transplantation in syngeneic donor/recipient combinations, there was no evidence for nonspecific growth enhancement or suppression other than a slight depression in growth attributable to initial physical crowding. The frequency of splenic localization was also comparable so far as those cell lines tested were concerned. The H-2 haplotype and homozygosity were consistently the most critical factors determining the inhibitory capacity, regardless of whether the tumor cells in question were derived from in vivo or in vitro lines. The latter tumors were unlikely to contain immunocompetent cells, since all of them had been passaged in vitro for a number of years before being tested in this study. The tumor lines used in this study were chosen primarily for their availability in our laboratory, convenience, H-2 haplotype of the strain of origin, and NK susceptibility; no other tumor lines were tested or excluded for unexpected behavior. In addition, the genetic specificity of inhibition observed in this study has been confirmed and extended by using normal hemopoietic cells as inhibitors (Daley and Nakamura, manuscript in preparation).

The inhibition of hybrid resistance by irradiated tumor cells could result from direct competition with parental BMC at the level of the effectors. Alternatively, inhibition could conceivably occur via determinant-specific regulatory cells modulating a nonspecific effector mechanism. For example, inhibitory cells may stimulate the reactive host cells to release nonspecific growth factors for transplanted hemopoietic cells, or in some way cause suppression of nonspecific rejection, cell-mediated or otherwise. The data provided in the present study suggest, however, that the inhibition is unlikely to have a two-step mechanism. Firstly, inhibitory tumor cells had no effect on the growth of F_1 BMC grafted into syngeneic F_1 hosts. The release of nonspecific growth factor(s) or the suppression of nonspecific effectors should have caused enhanced growth of F_1 BMC if either of these mechanisms was operative. Secondly, NK-susceptible tumor cells not bearing homozygous H-2D^b alleles had absolutely no detectable influence on the growth of parental BMC in F_1 hosts. If nonspecific effectors with NK-like reactivity played a role in hybrid resistance, one would expect NK-sensitive tumor cells to have at least some impact. It seems reasonable to conclude, therefore, that the inhibition of hybrid resistance observed in this study reflects specific competitive inhibition at the level of the effectors mediating the resistance.

The present data have several interesting implications for the nature of the effector cells and the target antigens recognized in natural hemopoietic resistance. Most importantly, the results have confirmed the presence, in responder F_1 mice, of radioresistant effector cells that specifically recognize H-2D^b/Hh-1^b homozygous BMC and promptly eliminate them or suppress their proliferation, as proposed in the original hypothesis (9). The effector cell activity could not be blocked by H-2D^b/Hh-1^b-negative tumor cells, including NK-susceptible YAC-1 (H-2^a), RL♂-1 (H-2^d), or 416B that is heterozygous for H-2^b. Some alloreactive cytotoxic T lymphocyte (CTL) clones have been shown to have a dual reactivity, one of which is MHC-restricted and the other, nonrestricted but directed to cell surface structures of NK-sensitive lymphomas (39). The coexistence of two independent recognition structures on some of these CTL clones has recently

been demonstrated, but the binding of CTL to target cells through one type of receptor was found to block the binding through the other type of receptor (40). The effectors mediating hybrid resistance did not exhibit such competitive binding, even under conditions that could have increased their binding capacity for NK-susceptible target cells, i.e., p-I:C pretreatment of the hosts. Therefore, barring the possibility that the effectors for resistance somehow manage to bind NK-sensitive lymphoma cells without impeding their ability to bind parental BMC, these effectors do not appear to have a dual recognition specificity. The relationship between the effectors of natural hemopoietic resistance and NK cells requires reassessment in the light of these data. Some of the shared properties of natural hemopoietic resistance and NK cell activity may only reflect a requirement for the same accessory functions. It is possible that the effectors of hemopoietic resistance do belong to a subset or a differentiation stage of NK cells, adding another dimension to the known heterogeneity of this class of effector cells. Alternatively, hemopoietic resistance may be mediated by a new class of effector cells whose function depends in some way on interactions with NK cells. Further studies are needed to distinguish these possibilities and establish the identity of the resistance-mediating effectors. The augmentation of hybrid resistance by p-I:C is mediated by IFN, since semipurified IFN-β is also effective and anti-IFN-β antiserum can abrogate the augmentation (Cudkowicz et al., unpublished data). Xenogeneic hemopoietic resistance has been shown to be potentiated by p-I:C (41).

Our observations may seem at odds with the results of several recent studies. For instance, lysis of human BMC by autologous or allogeneic NK cells in vitro can be inhibited by NK-susceptible K562 erythroleukemic cells (21), and lysis of NK-sensitive YAC-1 lymphoma targets was blocked by regenerating murine hemopoietic cells syngeneic to NK cells (22). Assuming that the blocking was due to competitive binding with effector cells, the results would indicate that the same NK cells are reactive with normal hemopoietic cells as well as genetically unrelated lymphoma cells. In our view, the target structures recognized in these studies are unrelated to the determinants controlled by Hh genes or their human counterpart. The transferrin receptor has recently been suggested as a candidate for such structures (42). The ability of a cell line with NK-like reactivity to reconstitute resistance in vivo to allogeneic marrow grafts seems to contradict the present data, since these NK-like cells do not show immunogenetic specificity in vitro but appear to mediate specific allograft rejection in vivo (23). It remains to be seen, however, whether these NK-like cells directly function as effectors in vivo, or these cells secrete IFN-γ and activate or recruit endogenous effectors. Although the recognition specificity of effector cells mediating allogeneic marrow graft resistance has not been investigated in detail, our preliminary data suggest that these effectors, too, have immunogenetic specificity. In another study (43), lysis of EL-4 cells in vitro by NK-like splenic effectors of an F_1 hybrid origin was inhibited by YAC-1 cells, and vice versa. However, unlike the hemopoietic resistance in vivo (44), the in vitro activity was not dependent on bone marrow integrity (43, 45), suggesting that the in vitro cytotoxicity is mediated by effectors distinct from those analyzed in the present study.

It is often implicitly assumed that hybrid resistance to parental BMC and

lymphoma cells is mediated by the same effectors, based on a similar genetic control over resistance to these cells. It is probable that hybrid resistance against tumor cells involves a variety of mechanisms (46–48). In some cases, however, the assumption of identical effectors seems valid (9). The present data indeed provide the first direct demonstration that the effectors mediating resistance to parental BMC are reactive with parental or other tumors of the H-2D^b/Hh-1^b genotype. This observation may seem to contradict the reported failure to block hybrid resistance against a parental H-2^b lymphoma by inoculation of irradiated normal spleen cells from H-2^b mice (49). The disagreement is probably more apparent than real, since several simple explanations are possible, such as higher density of target antigens on tumor cells than on normal splenocytes, relatively low frequencies of antigen-bearing cells among splenocytes of unmanipulated mice, or an insufficient inhibitor cell dose.

The results of this study offer some initial insight on the nature of the cell surface antigens recognized by the effectors of hybrid resistance. The consistent failure to inhibit parental marrow graft rejection by four independent lines of H-2D^b heterozygous cells indicates that the target antigens are either not expressed on these cells or expressed at such low levels as to be undetectable by the assay. These data are in contrast with those obtained with F_1 antiparental H-2^b CTL. In the latter, direct cytolysis is detectable only on H-2D^b homozygous target cells, but the lysis of such targets can be effectively and specifically inhibited by H-2D^b heterozygous cells, including F_1 hybrid cells syngeneic with the CTL (50). These data and the fact that monoclonal antibodies specific for D^b molecules block the lysis of parental target cells (Nakamura, unpublished observations), indicate that F_1 antiparent CTL recognize determinants controlled by class I genes. The present results with H-2D^b heterozygous cells are consistent with the hypothesis that the effectors of resistance recognize Hh-1^b antigens expressed only on homozygous cells, rather than self antigens restricted by H-2D^b. However, a note of caution is necessary for this interpretation. Since the present study used intact F_1 hosts, the effectors were surrounded by an excess of endogenous H-2 heterozygous cells. Should the endogenous cells express small amounts of target antigens, exogenous H-2 heterozygous inhibitors might not exert additional effects over preexisting partial autoinhibition. It is conceivable, therefore, that hybrid resistance represents a form of autoreactivity that becomes detectable in intact F_1 hybrid mice only when homozygous target cells are tested. Further comparisons between the recognition specificity of F_1 antiparent CTL and that of the effectors mediating hybrid resistance will be useful in determining whether the putative Hh-1^b antigen is distinct from the known class I gene products. Discordant genetic patterns between the two F_1 hybrid antiparent reactivities have already been pointed out (51). In a series of ongoing studies, normal hemopoietic cells from a panel of inbred strains of mice have been used as inhibitors for hybrid resistance against parental H-2^b BMC. The results so far indicate that the effector specificity is consistent with the known strain distribution and properties of Hh-1 alleles and not with class I H-2 specificities defined by serological reagents, allogeneic anti-H-2D^b, or F_1 antiparental H-2D^b CTL (Daley and Nakamura, manuscript in preparation). Analyses of effectors responsible for hybrid resistance against parental BMC from various H-2^d strains of

mice are also in progress. Since the Hh loci defined by H-2^d strains of mice have been identified either at some distance from the D end of the H-2 complex or not linked to the H-2 (18), such studies should be particularly informative.

Summary

The natural resistance of F_1 hybrid mice against parental bone marrow grafts is thought to be mediated by natural killer (NK)-like effector cells. However, unlike the NK cell activity against a wide range of tumors and normal cells, hybrid resistance is characterized by the immunogenetic specificity controlled by a set of unique noncodominant genes denoted as Hh. Two alternative hypotheses can account for the specificity. Thus, the specificity may reflect either the Hh restriction of effectors or the Hh gene control of mechanisms regulating non–Hh-restricted effector activity. In this study, therefore, we tested the recognition specificity of putative effectors mediating hybrid resistance in lethally irradiated H-$2^{b/d}$ and H-$2^{b/k}$ F_1 hybrid mice to the engraftment of parental H-2^b bone marrow. As a direct means of defining the effector specificity, rejection of parental bone marrow grafts was subjected to competitive inhibition in situ by irradiated tumor cells. Of the 16 independent lines of lymphoma and other hemopoietic tumor cells tested, the ability to inhibit hybrid resistance was the exclusive property of all tumors derived from mice homozygous for the H-$2D^b$ region, regardless of whether the tumor cells were susceptible or resistant to NK cell-mediated cytotoxicity in vitro. Four cell lines heterozygous for the H-$2D^b$ were noninhibitory, including one that is susceptible to natural killing. Pretreatment of the F_1 hosts with an interferon inducer augmented the resistance with no alteration in the recognition specificity of effector cells. Therefore, natural resistance to parental H-2^b bone marrow grafts was mediated by effectors restricted by the H-$2D^b$/Hh-1^b gene(s), and not by the nonrestricted NK cells detectable in conventional in vitro assays.

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