coded phosphotransferase signal was also found in the blood. Therefore, the neo^R gene is active in the circulating hematopoietic system of at least some of these animals four months after bone marrow infection and transplantation.

It is not apparent why we have obtained so much higher efficiency (even in the absence of IL-3) of infection and in vivo expression compared to the work of others. One explanation may be stability differences between N2 and the retroviral vectors used by others. A second possibility is that, although little strain difference was found here between the DBA/2J and NFS/N mouse lines, the efficiency of infection of the bone marrow of other mouse strains (for instance, C3H/HeJ[4]) is substantially different. Perhaps the 12- to 14-day foci examined by Williams *et al.* (*4*) have a lower infection frequency than the 10-day foci we studied. A final potential difference is in the reported titers of the vectors. If actual titers do differ significantly between laboratories (*3*), then marked apparent differences in bone marrow infection efficiency could be produced.

To be applicable for gene therapy, vectors such as N2 would carry additional gene sequences. Such additional sequences may have potential detrimental effects on titer. The results here indicate, however, that titers as low as 6×10^4 cfu/ml still infect murine hematopoietic stem cells with high enough efficiency to have possible therapeutic value.

In a complementary study by Keller *et al.* (*10*) it was demonstrated that the N2 vector can be found integrated and expressed in all the blood cell lineages in long-term reconstituted mice, including T and B lymphocytes. Similar studies with a slightly different vector have recently been published by Dick *et al.* (*11*).

In a number of earlier studies it was shown that retroviral vectors can undergo rearrangements and/or deletions. Using a vector derived from Friend spleen focus-forming virus, Joyner and Bernstein reported deletions of either the inserted thymidine kinase sequences or of the viral env gene (*12*). Others have reported similar problems of rearrangement in their vectors (*4, 13*). Although we have not yet sequenced the provirus, analyses such as those presented here, with Southern blots, T1 ribonuclease and neo^R gene product assays suggest an intact vector structure both during initial infection of stem cells and during subsequent in vivo stem cell proliferation and differentiation.

The utility of retroviruses as vectors for the high efficiency transfer of exogenous genetic sequences into hematopoietic cells has potential clinical relevance (*1*). A number of genetic diseases are known where the primary effect is upon the hematopoietic system. This report has established the conditions for the high efficiency transfer and expression of a gene into murine bone marrow using a new retroviral vector.

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Hematopoietic Histoincompatibility Reactions by NK Cells in Vitro: Model for Genetic Resistance to Marrow Grafts

Abstract. *In certain strains of mice, bone marrow grafts from parental donors fail to grow in first-generation hybrid mice. This "hybrid resistance" of nonsensitized F_1 hybrid mice to the engraftment of parental hematopoietic transplants contradicts the classical laws of transplantation and is dependent on a radioresistant but immunogenetically specific effector mechanism. Studies in a new in vitro model reveal that committed hematopoietic precursors of parental origin can be inactivated by direct contact with natural killer–like splenic effectors from F_1 mice. The reaction requires genetically restricted recognition, since only parental competitors syngeneic to the target bone marrow cells partially reversed this inactivation. Models of this type may be useful in studying the possible role of natural resistance in bone marrow transplantation in humans.*

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Transplants of normal and neoplastic hematopoietic cells from parental donor mice to lethally irradiated F_1 hybrid recipients are subject to an unusual host-versus-graft reaction termed hybrid resistance (*1*). This F_1 hybrid reaction to parent cells has been explained by assuming the existence of a class of noncodominant genes designated *Hh* for hematopoietic (or hybrid) histocompatibility, as opposed to the codominant histocompatibility *(H)* genes. Many of the known *Hh* genes are linked to the major histocompatibility complex in the mouse (*2*). The nature of effector cells mediating this reaction has long been a matter of speculation, as they are functional in lethally irradiated mice for at least several days, are as active in congenitally athymic mice as in euthymic littermates, and become functional only between the third and fourth weeks of life (*3, 4*).

These and other characteristics of the putative effector cells indicate that they are closely related to the effectors of natural killer (NK) activity in vitro against certain lymphoma targets (*5, 6*) [for a review on NK cells, see Herberman and Holden (*7*)]. However, this correlation between the two types of natural reactivity does not include genetic specificity, the most pertinent property of hybrid resistance (*2*). We showed earlier that the effectors of hybrid resistance in vivo are, unlike NK cells, capable of genetically restricted recognition of parental bone marrow and lymphoma cells

(*8*). Establishment of an in vitro model reflecting the interactions between non-induced NK-like effectors and proliferating normal marrow targets has been a prerequisite for further dissection of the nature of these effectors, their relation to NK cells, and expression of target determinants for *Hh*-incompatibility reactions. This elusive goal appears to be in sight.

Hybrid resistance to parental bone marrow grafts, as detected in vivo by proliferative or spleen colony assays, is directed against pluripotent stem cells (CFU-S), which are detected by spleen colony assay (*1, 9*). To test whether the susceptibility of parental stem cells persists after their commitment to a lineage, we examined the survival of the transplanted parental erythroid burst-forming units (BFU-E) and mixed myeloid granulocyte-monocyte colony-forming units (CFU-GM) in the spleens of irradiated F_1 hybrid recipient mice (Fig. 1). Bone marrow cells from C57BL/6N (B6) mice were injected intravenously into lethally irradiated (C57BL/6N × DBA/2)F_1 (B6D2F_1) hybrid and syngeneic B6 mice, and spleens of the recipients were removed at different times after transplantation to quantitate colony-forming capacity in vitro (see legend to Fig. 1).

Since recipient mice were lethally irradiated, no colonies of recipient origin developed in vitro. In the spleens removed within 3 or 6 hours, no inhibition of parental BFU-E and CFU-GM occurred in B6D2F_1 mice, as compared with syngeneic B6 recipients. After 12 hours of contact with the F_1 environment, however, progressive inhibition of erythroid and myeloid colonies ensued. These data suggest that the committed hematopoietic precursors BFU-E and CFU-GM express target determinants for resistance and represent additional targets for hybrid resistance. Furthermore, the interaction between grafted cells and the host environment must be extended for at least 6 hours before a substantial fraction of the target cells become inactivated or eliminated. This time requirement agrees with that observed for the inactivation of CFU-S in a classical retransplantation study (*3*). The parallel survival curves for myeloid and erythroid progenitors (Fig. 1) suggest a similar kinetics of effector interactions with target cells of the two hematopoietic compartments.

Pretreatment of recipient F_1 mice with rabbit antiserum to asialo GM-1 (AsGM1) confirmed that the resistance to committed hematopoietic precursors depends on NK-like effectors, as does the resistance to CFU-S. Asialo GM-1 is a surface marker expressed on NK cells, and specific antisera are capable of abrogating NK activity (*10*) as well as hybrid resistance (*6*).

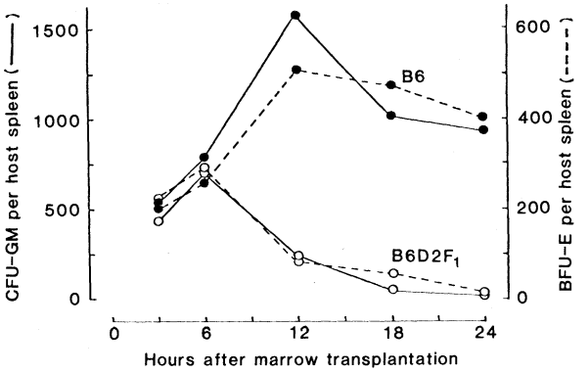

Fig. 1. C57BL/6N (B6) and (C57BL/6N × DBA/2)F_1 (B6D2F_1) mice were irradiated (900 rads) with γ-rays from a ^{137}Cs source and within 3 hours were given intravenous injections of 2×10^6 nucleated B6 bone marrow cells. At each time point from 3 to 24 hours after marrow transplantation, as indicated, spleen cells from four B6 or B6D2F_1 recipients were pooled. Aliquots of cell suspensions were assayed for erythroid (BFU-E) and mixed myeloid (CFU-GM) colony-forming cells in vitro as described (*16*). Cells equivalent to one-tenth of the spleen were plated in each 35 by 10 mm culture dish (Falcon) with 1 ml of culture medium and kept at 37°C in a humidified atmosphere of 5.0 percent CO_2 in air. Colonies were scored 7 days later. No colony-forming cells were recovered from the spleens of control mice, which were irradiated but not injected with bone marrow cells.

An in vitro model for hybrid resistance was constructed on the basis of the foregoing experiments. Spleen cells from B6D2F_1 mice were passed through nylon wool columns and then fractionated on discontinuous Percoll density gradients (*11*). Large granular lymphocytes were most enriched (20 to 25 percent) in fraction 2. Cells from each fraction were irradiated, tested for NK activity against the susceptible YAC-1 target cells, and cocultivated in a semisolid medium with parental (B6) or syngeneic (B6D2F_1) bone marrow cells for quantitation of hematopoietic colonies. The potential effector cells and bone marrow target cells were either preincubated in liquid medium for 6 to 8 hours to allow cell-to-cell contact or mixed immediately before being embedded in the semisolid culture medium. When a preincubation in liquid medium preceded coculture, F_1 hybrid cells from the NK-enriched fraction 2, but not NK-depleted fraction 4, inhibited both BFU-E and CFU-GM of B6 parental origin at a 4:1 ratio of effector to target cells (Table 1). B6D2F_1 bone marrow–derived colonies were also often

Table 1. Effects of interactions in vitro between F_1 hybrid spleen cells and syngeneic (B6D2F_1) or parental (B6) bone marrow–derived hematopoietic progenitors. Spleen cells from normal B6D2F_1 mice, nonadherent to nylon wool, either unfractionated or fractionated by Percoll density gradients (*11*), were exposed to 900 rads of γ-rays. Aliquots of the cell suspensions were tested for NK activity and cultured with syngeneic or parental B6 bone marrow cells after an 8 hour incubation in Iscove's modified Dulbecco's medium supplemented by 2 percent bovine serum albumin at 37°C in 5.0 percent CO_2 in humidified air. The culture condition was as described (*16*). The ratio of splenic effectors to bone marrow target cells was 4:1. The colonies were scored on day 7 of culture; each number represents the mean ± standard error of the mean of two plates containing initially 2×10^5 bone marrow cells per plate. No colonies developed when irradiated effector cells were cultured alone. As in these and other experiments reported in this paper, pure macrophage colonies were not included in CFU-GM. NK activity is expressed as the percentage of specific lysis of YAC-1 targets in a 4-hour ^{51}Cr release assay at an effector to target cell ratio of 12.5:1. For the antibody plus complement treatments, fraction 2 cells were resuspended at 1×10^7 cells per milliliter in RPMI 1640 medium supplemented by heat-inactivated fetal bovine serum. Culture supernatant from hybridoma 30-H12 was the source of anti-Thy-1.2 antibody. Rabbit antiserum to AsGM1 was used at a 1:100 dilution. Rabbit complement (C) dilution was 1:16.

Effector	NK activity (%)	BFU-E per plate		CFU-GM per plate	
		B6D2F_1	B6	B6D2F_1	B6
		Experiment 1			
None (medium)		21 ± 2	18 ± 3	146 ± 4	100 ± 3
Unfractionated	7	16 ± 1	16 ± 0	149 ± 4	67 ± 6
Fraction 2	33	45 ± 2	5 ± 1	112 ± 2	48 ± 2
Fraction 4	5	16 ± 2	20 ± 2	149 ± 4	102 ± 3
		Experiment 2			
None (medium)		31 ± 3	26 ± 2	109 ± 4	113 ± 0
Fraction 2	48	42 ± 2	12 ± 1	105 ± 6	50 ± 3
Fraction 2 +anti-AsGM1 + C	1	28 ± 4	30 ± 3	111 ± 5	104 ± 6
Fraction 2 +anti-Thy-1.2 + C	54	44 ± 3	8 ± 2	121 ± 8	38 ± 4

inhibited by syngeneic effector cells but to a lesser degree than parental colonies. Syngeneic inhibition of CFU-GM was more evident at higher effector to target cell ratios (data not shown). The suppression of autologous and allogeneic CFU-GM (*12*) and BFU-E (*13*) by human NK cells in vitro, as reported previously, probably represents an analogous phenomenon, even though genetically controlled target cell selectivity was not examined.

In 16 of 24 experiments, an enhancement of BFU-E production occurred after coculture with syngeneic fraction 2 cells (Table 1). Production of parental B6 BFU-E were also enhanced if no preincubation with F_1 cells preceded coculture. Therefore, the BFU-E enhancement appears nonspecific and independent of direct contact with fraction 2 spleen cells. A similar observation was made earlier in a human system (*14*). When the coculture experiments were performed without effector-target cell preincubation or with shorter preincubation, no significant inhibitory activity was observed. This suggests that several hours of effector-target cell contact is a prerequisite for the manifestation of inhibitory activity in vitro as well as in vivo. The similarity of effectors for in vivo and in vitro reactions was confirmed by pretreatment of Percoll fraction 2 effectors with antiserum to AsGM1 plus complement; this in vitro treatment of effectors led to a complete abrogation of the colony suppression as well as of NK activity, whereas treatment with a monoclonal antibody to Thy-1.2 and complement did not substantially modify suppressive activity (Table 1, experiment 2).

These results lend further support to the widely accepted notion that NK cells and effectors of hybrid resistance to parental bone marrow grafts are similar or identical (*5*). The colony suppression in vitro may require a recognition of *Hh* determinants, as in resistance in vivo. Alternatively, nonspecific effectors may appear to react selectively with parental target cells, if a contact with parental cells led to activation of the F_1 hybrid effectors. To test whether the effector-target cell interactions in vitro are genetically restricted as they are in vivo, we performed a competitive inhibition experiment. Since target determinants for hybrid resistance are expressed on differentiating hematopoietic precursors (Fig. 1), regenerating splenic hematopoietic cells were used as a source of competitors (see Table 2). A parallel in vivo experiment was also carried out with the same pools of competitor cells. Both the in vivo resistance and the in vitro colony suppressive activity against parental B6 ($H\text{-}2^b$) bone marrow cells were reduced in the presence of irradiated B6 ($H\text{-}2^b$) hematopoietic competitors, but not of similar cells from B6D2F_1 ($H\text{-}2^{b/d}$) or DBA/2 ($H\text{-}2^d$) donors (Table 2). These data suggest that the effector-target cell interactions in vitro depend on recognition of *Hh* antigens.

In conclusion, these data indicate that (i) the differentiating precursors of erythropoietic and myelopoietic compartments are subject to elimination by noninduced effectors in vivo and in vitro in a specific fashion; (ii) progenitors of the two lineages have comparable kinetics of inactivation; and (iii) the effectors of this resistance are coenriched with, and bear a surface marker of, typical NK cells cytotoxic to YAC-1 lymphoma target cells in vitro. Moreover, syngeneic interactions in vitro between these hematopoietic precursors and NK-like cells led to contact-dependent suppression of myeloid precursors and contact-independent enhancement of erythroid bursts. The data raise the possibility that these effector cells not only mediate natural resistance to foreign hematopoietic grafts but also function as regulatory cells for hematopoiesis. In clinical bone marrow transplantation, the reduced incidence of graft-versus-host disease in patients receiving marrow grafts depleted of donor T lymphocytes may be associated with an increase in graft rejection (*15*). An in vitro model such as the one discussed in this report may be useful for studies of the mechanisms underlying bone marrow graft rejection in man.

Table 2. Genetically restricted inhibition of in vivo hybrid resistance and of in vitro suppression of parental B6 colony-forming cells by irradiated competitors. For both in vivo and in vitro inhibition experiments, the competitor cells were splenocytes from B6, DBA/2, or B6D2F_1 mice that had been lethally irradiated (750 rads) and reconstituted with 7×10^6 syngeneic bone marrow cells 15 to 18 days earlier. The regenerating hematopoietic cells were irradiated (2000 rads) and used without additional treatment as competitors for in vivo resistance. The prospective recipient mice were lethally irradiated (900 rads) and given intravenous injections of 1×10^8 irradiated competitor cells each, 24 hours and 3 hours before transplantation of 5×10^6 B6 bone marrow cells. These conditions were based on previous titration experiments (*8*). The splenic ^{125}IdUrd incorporation was assessed 5 days later by the standard assay as described (*8*); *n* represents the number of animals receiving bone marrow transplants. Inhibition of resistance results in grafted cells escaping rejection and hence increased proliferation and ^{125}IdUrd uptake. For the experiments in vitro, regenerating hematopoietic cells were fractionated on a three-step gradient of 47.6, 56.6, and 70.1 percent Percoll in RPMI 1640 medium to enrich colony-forming progenitors. The fraction recovered from the 47.6-56.6 percent interface contained more than 95 percent of all the colony-forming cells and therefore was used as the source of competitors. This fraction represented 20 to 30 percent of nucleated cells. These competitors were irradiated (1000 rads) and incorporated into the preincubation mixtures of irradiated (1000 rads) fraction 2 effectors and parental B6 bone marrow targets to allow cell-to-cell contact for 8 hours before being plated in the colony-forming assay. The ratio of effector, target bone marrow, and competitor cells was 4:1:4. Control groups consisted of target bone marrow cells without addition of effectors or competitors, all other conditions being equal. Irradiated regenerating cells alone did not produce colonies in vitro, nor increase background levels of splenic ^{125}IdUrd uptake in vivo.

Recipient or effector cell donor	Competitor		CFU-GM per plate	^{125}IdUrd uptake		
	Strain	*H-2*		Geometric mean	95 percent confidence interval	*n*
			216 ± 8			
	B6D2F_1	*b/d*	172 ± 6			
	B6	*b*	169 ± 8			
	DBA/2	*d*	190 ± 3			
B6D2F_1			71 ± 5	0.189	0.065–0.549	5
B6D2F_1	B6D2F_1	*b/d*	54 ± 6	0.071	0.021–0.243	4
B6D2F_1	B6	*b*	112 ± 2	1.398	0.955–2.045*	5
B6D2F_1	DBA/2	*d*	63 ± 10	0.233	0.121–0.449	5
B6			145 ± 5	1.978	1.062–3.684	5
B6	B6	*b*	159 ± 7	1.895	1.033–3.475	6

*Significant growth ($P < 0.03$, *t*-test with Welch and Bonferroni corrections) over that in F_1 recipients not injected with competitor cells.

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Detection of DNA Sequences in Nuclei in Suspension by in Situ Hybridization and Dual Beam Flow Cytometry

Abstract. *This report describes the fluorescence hybridization of DNA sequence probes to interphase nuclei in suspension and the quantification of bound probe by dual beam flow cytometry. Nuclear proteins were first cross-linked with dimethylsuberimidate to prevent disintegration of the nuclei during denaturation and hybridization. To demonstrate that in situ hybridization can be performed in suspension, stabilized mouse thymocyte nuclei were hybridized with a probe for mouse satellite DNA sequences. The DNA probes were labeled with 2-acetylaminofluorene. After hybridization, an indirect immunofluorescent labeling procedure was used to visualize the target sequences. With dual beam flow cytometry, both the amount of hybridized probe and the DNA content of individual nuclei were determined. Thus, the specificity of DNA hybridization can be combined with the speed and quantitative analysis provided by flow cytometry.*

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Flow cytometry is widely used for the analysis of cell nuclei and chromosomes. With this technique, the binding of fluorescent markers to individual cells or chromosomes in a heterogeneous population can be determined rapidly. After labeling with fluorescent dyes, chromatin can be analyzed quantitatively for DNA content (*1–5*), base composition (*6*), and structural proteins (*7, 8*). A logical extension of the application of flow cytometry is the detection and quantification of specific DNA sequences by fluorescence DNA hybridization techniques.

Several laboratories have developed fluorescent labeling procedures that allow the visualization of hybridized DNA sequence probes in cells fixed to slides (*9–14*). Nuclei, isolated into suspension with a procedure developed for the isolation of chromosomes (*15, 16*), fall apart rapidly under the conditions required for DNA hybridization. This can be prevented by cross-linking the nuclear proteins with dimethylsuberimidate (DMS) (*17*). Nuclei that have been treated with DMS can withstand a variety of denaturation and hybridization conditions involving incubation at high temperatures in the presence of chelating agents and high concentrations of salt. After DNA-staining with Hoechst 33258, these nuclei show normal fluorescence distributions when they are measured in a flow cytometer. The distributions consist only of a narrow peak, corresponding to the expected DNA content of the nuclei. Without DMS treatment, the peak is broad or is replaced by a debris continuum. Isolated and DMS-treated chromosomes can also be denatured by these procedures with preservation of a normal karyotype as measured in a flow cytometer (*17*).

To demonstrate in situ hybridization in suspension, DMS-treated mouse thymocyte nuclei were hybridized with a probe for mouse satellite DNA sequences. These sequences comprise approximately 10 percent of the total DNA in mouse nuclei (*18*). Total human DNA served as a control; this material shows no cross-hybridization to mouse DNA on filters or slides. Approximately 20 percent of the guanine residues in the DNA probes were chemically modified with *N*-acetoxy-2-acetylaminofluorene (N-AcO-AAF) (*9, 19*). Details of the hybridization procedure are as described (*20*). Briefly, nuclei, suspended in hybridization buffer in the presence of AAF-labeled probe, were denatured and incubated at the hybridization temperature overnight. After hybridization, the bound probe was visualized with a rabbit anti-AAF antibody (*21*) and a goat–anti-rabbit immunoglobulin conjugated to rhodamine (TRITC). Hoechst 33258, a DNA-specif-

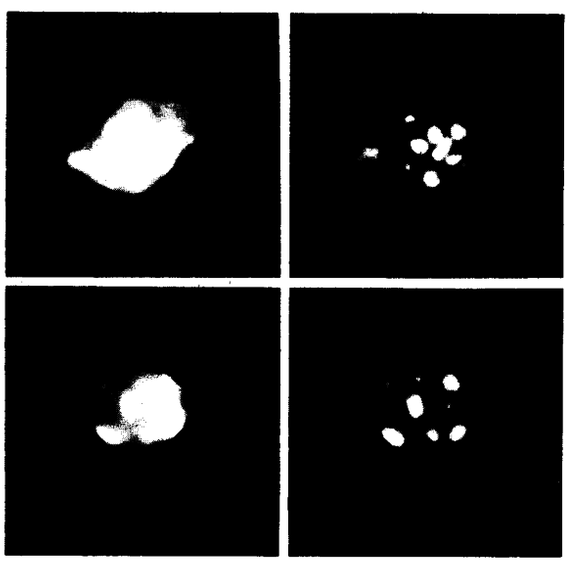

Fig. 1. Hybridization of mouse satellite DNA to mouse thymocyte nuclei in suspension. After denaturation, hybridization, and immunofluorescent labeling in suspension (*20*), a portion of the nuclei was spun onto microscope slides in a cytocentrifuge (Shandon, Cheshire, England), with the following protocol. Fetal calf serum (50 μl) was first spun onto the glass slide (10*g*, 20 seconds), followed by 250 μl nuclei suspension (300*g*, 3 minutes), and 100 μl 96 percent ethanol (300*g*, 1 minute). Microscopic observation and photography were as described (*9*). Exposure times were 15 to 30 seconds for Hoechst fluorescence and 2.5 to 3.0 minutes for rhodamine fluorescence. The two photographs on the left show examples of the blue Hoechst 33258 fluorescence, indicating the more intensely staining, A-T–rich heterochromatin areas in the nuclei. The panels on the right show the red rhodamine (TRITC) fluorescence that indicates bound probe. The actual size of a nucleus is approximately 5 μm. The original magnification was ×630.