A POPULATION OF MURINE HEMATOPOIETIC PROGENITORS EXPRESSES AN ENDOGENOUS RETROVIRAL gp70 LINKED TO THE *Rmcf* GENE AND ASSOCIATED WITH RESISTANCE TO ERYTHROLEUKEMIA

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Multiple copies of retroviral sequences are stably integrated in the genomes of many higher organisms, and are thus transmitted vertically to offspring via the germline (1). Most of these heritable viral genes are not expressed, and expression, when observed, is commonly limited to envelope (*env*) genes as demonstrated by the presence of cell surface and serum envelope glycoprotein (gp70) in mice. Studies of the mouse have shown that certain tissues such as the reproductive tract and lymphoid organs are common sites for the expression of endogenous *env* genes, suggesting that the transcription of at least some endogenous sequences is tissue specific. The transcription of endogenous viral genes is regulated by both *cis* and *trans* mechanisms (2–5) and their expression can be temporally linked to differentiation and development (6–8).

The consequences to the host of endogenous retroviral genes are varied. At one extreme, expression of endogenous virus can result in the development of leukemia and death. Another potentially detrimental effect is that of insertional mutagenesis, seen when the integration of retroviral sequences interrupts the functioning of a cellular gene (9, 10). However, it is now clear that expression of endogenous retroviral genes may also have a beneficial effect for the host: namely, mediating resistance to retroviral leukemias as has been demonstrated for the *Fv*-4 gene in mice (11) and some *ev* loci in chickens (12). This form of resistance is due to the blockage of cellular viral receptors by the expression of envelope glycoprotein on the cell surface.

The *Rmcf* locus of the mouse is another resistance gene that may exert its effect by the expression of an endogenous *env* gene. A summary of our current state of knowledge concerning the *Rmcf* gene is shown in Table I. The *Rmcf* gene was originally described when it was observed that fibroblast cell cultures derived from certain strains of mice restricted the replication of recombinant mink cell focus-forming (MCF)[1] viruses (13). As detailed in Table I, DBA/2 mice are the prototypic strain

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[1] *Abbreviations used in this paper:* BFU-E, erythroid burst-forming units; CFU-E, erythroid CFU; CFU-GM, granulocyte/macrophage CFU; CFU-S, spleen CFU; DAF, diaminofluorene; F-MuLV, Friend murine leukemia virus; IMDM, Iscove's modified Dulbecco's medium; MCF, mink cell focus-forming virus; RIP, radioimmunoprecipitation; RML, Rocky Mountain Laboratories.

TABLE I

Characteristics of the Rmcf Gene

	DBA/2	C57BL/6	IRW
*Rmcf**	r	s	s
Rmcf-linked gp70‡	MCF	XENOTROPIC	NULL
Cognate antibodies§	514/617	18-6	–

* *Rmcf* is a dominant gene on chromosome 5 of the mouse which restricts infection by MCF viruses of embryo cell cultures derived from strains carrying the resistance (r) allele.

‡ The *Rmcf* gene is tightly linked to an endogenous retroviral *env* gene, which in backcross analysis reveals two allelic forms, xenotropic-like and MCF-like, based on serologic criteria (14).

§ Antibodies 514 and 617 react exclusively with the *Rmcf*-linked gp70 from the *Rmcf*^{r} strains DBA/2 and CBA/N. Antibody 18-6 defines the xenotropic gp70 allele of C57BL/6 and several other *Rmcf*^{s} strains (CBA/J, A/WySn, C57BL/10) (14). Several *Rmcf*^{s} strains, such as IRW, BALB/c, and NFS/N, express neither of these alleles.

exhibiting the *Rmcf* resistance (*Rmcf*^{r}) phenotype. Cell cultures from other strains, such as C57BL/6 and IRW, are permissive for MCF viral replication and are termed *Rmcf* sensitive (*Rmcf*^{s}). Previously, we described two allelic forms of an endogenous *env* gene, whose expression is linked to the *Rmcf* gene (14). Cell cultures from *Rmcf*^{r} mice express gp70 related to that of MCF viruses, whereas cultures derived from *Rmcf*^{s} mice either express no gp70 (IRW) or express an endogenous xenotropic gp70 (C57BL/6). These two gp70 alleles are detectable by type-specific mAbs.

Infection of susceptible strains of mice by Friend murine leukemia virus (F-MuLV) induces an erythroleukemia characterized by a block in erythroid differentiation resulting in an abnormal proliferation of erythroid precursors (15–18). Several lines of evidence support the notion that recombinant MCF viruses are the proximal cause of this erythroleukemia (19, 20). We have previously shown that the gene encoding the *Rmcf*^{r}-linked gp70 derived from resistant DBA/2 mice segregates in genetic backcrosses with restriction of MCF viral replication and resistance to erythroleukemia induced by F-MuLV (21). Currently, however, the in vivo sites of expression of *Rmcf*-linked gp70 are not known. The hypothesis that the *Rmcf* gene functions via an interference mechanism mediated by endogenous gp70 may predict that the cells expressing the *Rmcf*-linked gp70 alleles define the targets for infection and transformation by F-MuLV-induced MCF viruses. Thus, the *Rmcf*-linked *env* alleles might be found on cells resident in hematopoietic organs such as the spleen, and in bone marrow. However, these organs are sites of mature lymphoid cells that in DBA/2 and C57BL/6 mice express abundant endogenous xenotropic gp70 (22), making the identification of *Rmcf*-linked gp70-bearing cells difficult. In the present study, through the use of specific antiretroviral gp70 mAbs and cell sorting techniques, we have identified and separated cells expressing *Rmcf*-linked gp70 molecules and have found that such cells comprise a small population resident in sites of hematopoiesis. Initial expression of this gene occurred in a pool of stem cells with the capacity to differentiate into erythroid, granulocytic, and lymphoid progenitors. No evidence for expression was detected among the mature myeloid or lymphoid cells. These observations provide evidence for a program of expression linked to early lymphohematopoietic differentiation and suggest that the resistance to erythroleukemia mediated by this

gene is a function of its expression within the pool of erythroid progenitors that are the targets of leukemogenesis.

Materials and Methods

Mice. Mice used in this study were bred and housed at the animal facilities of the Rocky Mountain Laboratories (RML) or Texas Tech University Health Sciences Center. Parental stocks of DBA/2 and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or the Testing Branch of the National Cancer Institute (Bethesda, MD).

Fetal mice of different gestational ages were obtained by one of two procedures: superovulation or the vaginal plug method. To induce superovulation, female mice were given an injection of 5 IU i.p. of pregnant mare gonadotropin (Sigma Chemical Co., St. Louis, MO) between 4:00 and 5:00 p.m., followed 48 h later by an injection of 5 IU i.p. of human chorionic gonadotropin (Sigma Chemical Co.). After the second injection, male mice were introduced into the cage for a period of 18 h. Time 0 for gestation was considered to be the time of removal of the male mice. For the vaginal plug method, timed pregnancies were obtained by placing females in a cage with a male late in the afternoon and checking for vaginal plugs the next morning. The day of finding a plug was counted as day 0 of gestation.

Adult mice were rendered anemic by treatment with phenylhydrazine HCl (Sigma Chemical Co.) by one of two treatment schedules. Mice were either injected with 1 mg i.p. of phenylhydrazine for five consecutive days (high dosage schedule), or, according to the method of Spivak et al. (23), whereby mice were injected three times with neutralized phenylhydrazine (0.04 mg i.p. per gram of body weight). The first injection was given at 5:00 p.m. on day 1, followed by the second and third treatments at 9:00 a.m. and 5:00 p.m. on day 2.

Hematocrits and Reticulocyte Counts. Blood used for determining hematocrit values was obtained from puncture of the retroorbital sinus with a 70-μl microhematocrit capillary tube. The percentage of circulating reticulocytes was determined by mixing heparinized blood with an equal volume of new methylene blue. After 3–4 h a blood smear was made and the percentage of circulating reticulocytes was determined by counting a minimum of 400 cells for each sample (24).

Antibodies. The derivation and description of the mAbs used to characterize endogenous retroviral gp70 have been previously reported. Briefly, antibody 18-1 has broad specificity, reacting with all retroviral gp70 tested, except Friend ecotropic virus (25). Antibody 18-6 (25) reacts with the gp70 of a subset of xenotropic viruses, while antibody 24-6 (25) reacts with all xenotropic and MCF viral gp70. Antibodies 514 (26) and 617 (27) are specific for the gp70 of recombinant MCF viruses. For technical reasons antibody 617 was used for radioimmunoprecipitation (RIP) and antibody 514 for immunofluorescence.

FACS Analysis. Spleen and fetal liver tissues were processed and stained for FACS analysis as reported previously (14), with the exception that to detect antibody 18-6 reactivity in spleen and bone marrow cell preparations, 0.1 ml of a 1:100 dilution of FITC-conjugated goat anti-mouse IgG3 (Southern Biotechnology Associates, Inc., Birmingham, AL) was used as a second antibody to minimize staining of Ig-bearing B lymphocytes. FACS analysis was performed with a FACS analyzer (Becton Dickinson & Co., Mountain View, CA). The analyzer was operated in log gain, with full scale fluorescence being 3 log decades. Controls consisted of cells reacted with a mAb of irrelevant specificity of the same isotype as the experimental antibody plus the conjugate.

Cell Sorting. Fetal liver cells were incubated at a cell concentration of 10^7 cells/ml in the primary antibody on ice for 20 min and then washed twice with DME (Gibco Laboratories, Grand Island, NY) containing 2% BSA (Boehringer Mannheim Biochemicals, Indianapolis, IN). The cells were incubated in second antibody for 20 min on ice and then washed twice with DME/2% BSA.

The stained samples were first analyzed on a FACSTAR or FACSTAR Plus (Becton Dickinson & Co.) with an argon ion laser emitting 488 nm of light at 200 mW. Sorting windows were then set and cells were sorted into heat-inactivated FCS under sterile conditions. Individual sorting experiments were carried out at least four times for each tissue (fetal liver, phenylhydrazine-treated adult spleen) in C57BL/6 mice using antibody 18-6. At least six in-

dividual sorting experiments were carried out with DBA/2 fetal liver and the 514 antibody. Erythroid colony-forming units (CFU-E), erythroid burst-forming units (BFU-E) and granulocyte/macrophage CFU (CFU-GM) were all assayed (described below) in the experiments enumerated above. The spleen CFU (CFU-S) experiment reported in Fig. 4 was performed twice. The staining of sorted cells for cytological analysis was accomplished by using a modified Wright stain (Accustain; Sigma Chemical Co.).

Precursor Cell Culture. Cells were plated into 35-mm suspension culture dishes (Lux-Miles Scientific, Naperville, IL) as 1-ml cultures of Iscove's modified Dulbecco's medium (IMDM) (Gibco Laboratories) containing the following additions: 1% methylcellulose (Dow Corning Corp., Midland, MI), 20% defined FCS (HyClone Laboratories, Logan, UT), and 1% BSA (Boehringer Mannheim Biochemicals). Triplicate cultures were established for each point. CFU-E cultures were supplemented with 0.3 U/ml of erythropoietin (epo) (HyClone Laboratories). The number of CFU-E was scored by staining with the hemoglobin stain 2,7-diaminofluorene (DAF) (Sigma Chemical Co.), as described previously (28), and by counting positive colonies on the second day of culture. BFU-E cultures were supplemented with 2.5 U/ml of epo and 25 U/ml of IL-3 (Genzyme, Boston, MA). On the eighth day of culture the plates were stained with DAF and the number of bursts was counted. CFU-GM cultures received 50 μl/ml of serum pooled from mice given 10 μg of *Escherichia coli* lipopolysaccharide (Sigma Chemical Co.) 5 h before exsanguination. CFU-GM colonies (>50 cells) were scored on the eighth day of culture at 25× using a dark-field illumination and without any stain. The number of cells cultured varied with the experiment and are given in the text.

CFU-S Assay. 1–2 × 10^4 sorted fetal liver cells from 12-d C57BL/6 fetuses were injected intravenously via the lateral tail veins of lethally irradiated (9.5 Gy) syngeneic hosts (10 mice/group). 12 d after transplant, the hosts were killed, and spleens were removed and fixed in Bouins' solution overnight. Macroscopically visible colonies were counted with the unaided eye and results expressed as the mean ± SE of the surviving 9–10 hosts per group.

Immunoprecipitation and SDS-PAGE. ^{125}I surface labeling of cells was performed as described previously (14). Volumes of lysate added to the precipitation reactions were adjusted to contain 5 × 10^6 cpm. Spleen and bone marrow cell lysates were subjected to multiple rounds of preclearing with rabbit anti-mouse Ig serum and formalin-fixed *Staphylococcus aureus* Cowan I strain cells in order to remove iodinated Ig from these lysates. Precipitates were resolved by 10% SDS-PAGE. Dried gels were exposed on Kodak X-Omat AR film with an intensifying screen as reported earlier (14).

Results

Expression of Rmcf-linked env Genes in Hematopoietic Tissues. The role of the endogenous MCF gp70 in resistance to F-MuLV erythroleukemia suggested that cells of the erythroid lineage might express *Rmcf*-linked gp70. Since the murine fetal liver is an active site of erythropoiesis it was chosen for initial study. FACS analysis revealed that a substantial population of fetal liver cells from DBA/2 and C57BL/6 mice expressed the *Rmcf*-linked gp70 allele appropriate for each strain (see Table I) whereas fetal liver cells from B6D2F1 mice expressed both gp70 molecules, each at a lower mean density than the homozygous parents (Data not shown). Thus, the expression of *Rmcf*-linked gp70 was semidominant and identical to the pattern of expression seen for fibroblast cell cultures (14). As expected, (See Table I) fetal liver cells from IRW mice were negative for expression of endogenous gp70.

The percentage of fetal liver cells expressing *Rmcf*-linked gp70 molecules was highest in early fetal liver and decreased with advancing gestational age, a pattern that closely parallels the decline in the number of erythroid cells in this organ (29, 30) (Fig. 1). In addition, cells expressing *Rmcf*-linked gp70 were found almost exclusively in a population of large cells. Thus, >80% of the large cells in 14-d fetal liver expressed *Rmcf*-linked gp70, whereas <10% of the small cells were positive. The relationship

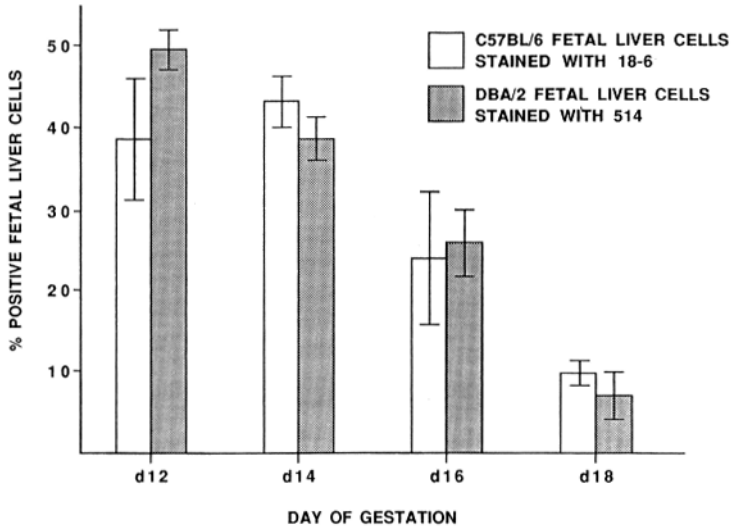

FIGURE 1. Expression of *Rmcf*-gp70 by fetal liver cells from mice of various gestational ages. Fetal liver cells were incubated with either antibody 18-6 or 514, followed by FITC-goat anti-mouse Ig. Controls consisted of cells exposed to the FITC conjugate alone. Percentages of cells bearing cognate antigens were determined by FACS analysis. Fetal liver cells were pooled from individual litters containing from 5 to 10 embryos each. Values represent the mean ± SE from four (day 18), three (day 14 and day 16), or two (day 12) litters.

between *Rmcf*-linked gp70 expression and large cells held for all cell populations examined, including adult tissues. Results of a previous study demonstrated that hematopoietic progenitors are found primarily in a population of large cells resident in hematopoietic organs (31). Thus, our results suggested that *Rmcf*-linked gp70$^+$ cells represented a population of immature progenitors and that smaller more mature forms did not express these proteins.

Examination of the sites of erythropoiesis in adult mice revealed that, in spite of the presence of abundant endogenous gp70, no *Rmcf*-linked gp70 was detected on adult spleen or bone marrow cells of C57BL/6 or DBA/2 mice (Fig. 2). The anti-gp70 mAbs used in the immunoprecipitation indicated that the endogenous gp70 present on spleen and bone marrow cells was xenotropic (18-1$^+$,24-6$^+$,18-6$^-$,617$^-$) and clearly different from the *Rmcf*-linked gp70 molecules detected on the embryo cell controls. This endogenous xenotropic gp70 was, most likely, the XenCSA antigen described by Morse et al. (22). As the fetal liver is a site of robust erythroproliferation at midgestation, it was possible that the *Rmcf*-linked loci were expressed only by cells in the early stages of differentiation. Because such immature cells are found only in very low frequency in adult spleen and bone marrow (32), we stimulated erythropoiesis in adult mice with the hemolytic agent phenylhydrazine (high dosage). Immunoprecipitation of ^{125}I surface-labeled cells revealed that *Rmcf*-linked gp70 (617$^+$) was expressed in both spleen and bone marrow of anemic DBA/2 mice (Fig. 3). A more quantitative measure of the appearance of *Rmcf*-linked gp70$^+$ cells in sites of hematopoiesis was obtained from C57BL/6 mice. FACS analysis demonstrated

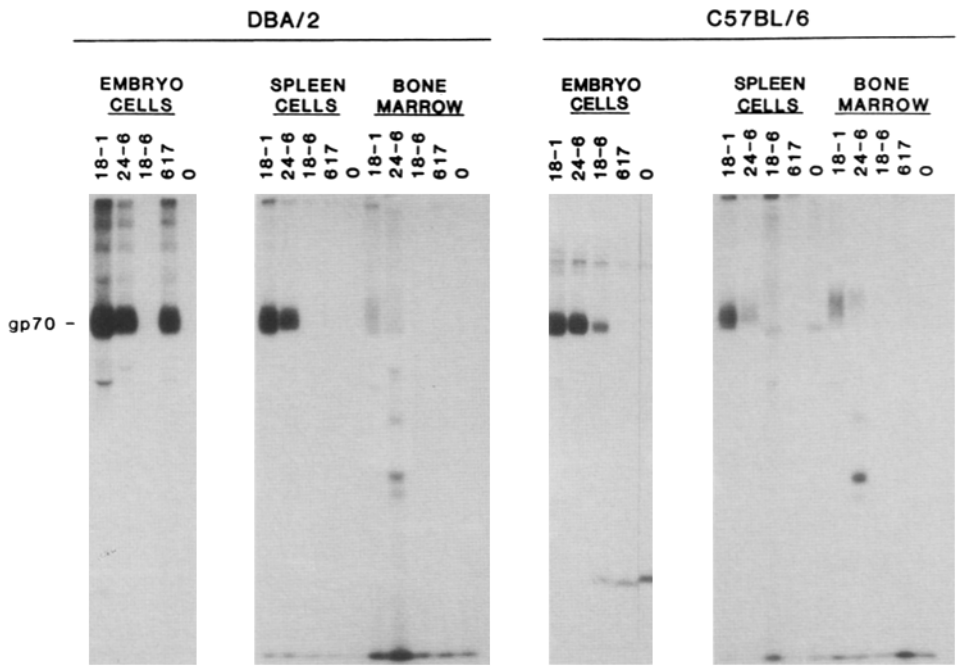

FIGURE 2. Immunoprecipitation of ^{125}I surface-labeled primary embryo fibroblasts, spleen, and bone marrow cells by anti-gp70 mAbs. Antibody 18-1 is broadly reactive for murine retroviral gp70, antibody 24-6 reacts with xenotropic and MCF gp70, antibody 18-6 is specific for a subgroup of xenotropic gp70 and defines the *Rmcf*^{s}-gp70, while antibody 617 is specific for MCF gp70 and reacts with the *Rmcf*^{r}-gp70. The track labeled 0 contains an equal volume of labeled lysate but no mAb. Precipitates were resolved by SDS-PAGE analysis. It was noted the the *env* gene products precipitated from bone marrow lysates sometimes appeared to migrate slightly slower and produce more diffuse bands than those recovered from spleen cell lysates. Although this observation has not been investigated further, it was likely due to differences in gp70 processing as has been reported in other systems (33).

that 18-6$^+$ cells represented 15–20% of both spleen and bone marrow cells after high dose phenylhydrazine treatment.

To determine the relationship between the appearance of spleen cells bearing *Rmcf*-linked gp70 and other hematological parameters of anemia, a kinetic experiment

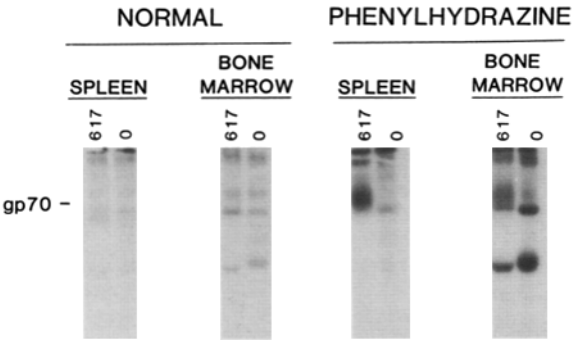

FIGURE 3. Immunoprecipitation of ^{125}I surface-labeled spleen and bone marrow cells of normal and phenylhydrazine-treated DBA/2 mice by anti-gp70 mAbs. Phenylhydrazine-treated mice were given intraperitoneal injections of 1 mg phenylhydrazine/mouse daily for 5 d. Antibody 617 is specific for MCF gp70 and therefore reacts with the *Rmcf*^{r} allele expressed by DBA/2 mice. The track marked 0 contains an equal volume of labeled lysate with no mAb. Precipitates were resolved by SDS-PAGE.

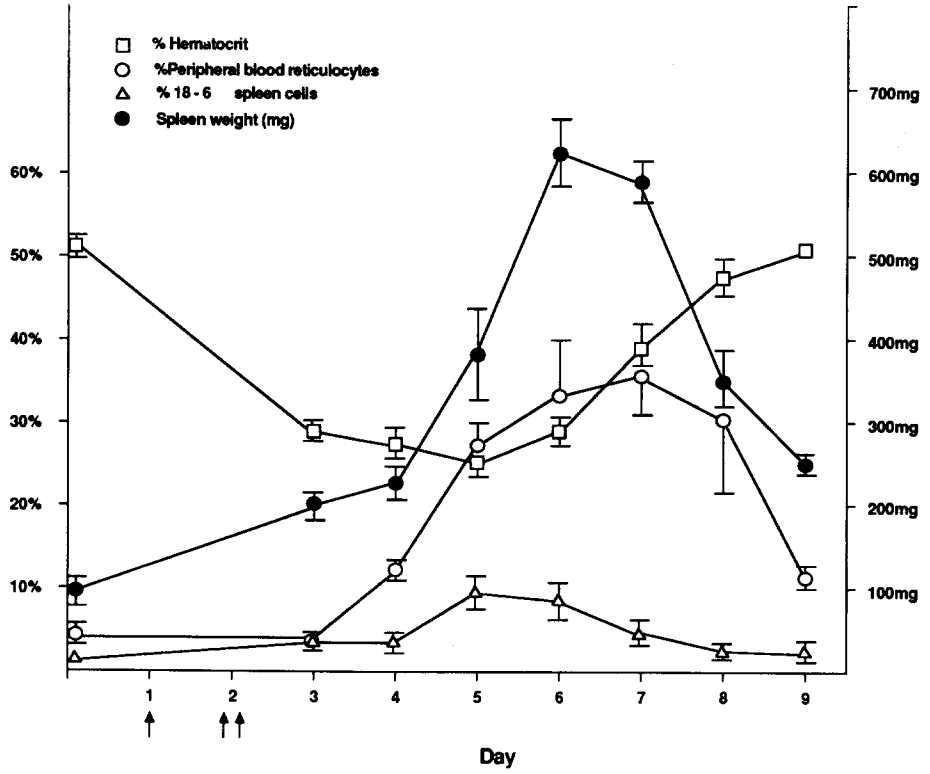

FIGURE 4. Changes in hematological parameters and the detection of 18-6 reactive spleen cells in phenylhydrazine-treated C57BL/6 mice. Mice were given three 0.04 mg i. p. injections of phenylhydrazine per gram body weight on days 1 and 2. Hematological parameters and the percentage of cells expressing gp70 reactive with antibody 18-6 were determined on days 3-9 as described in the Materials and Methods. Each point represents the mean ± SE of five mice.

was performed. C57BL/6 mice were given a total of three injections of phenylhydrazine on days 1 and 2. After the final injection, spleen weight, hematocrit, peripheral blood reticulocytes, and the percentage of *Rmcf*-linked gp70$^+$ (18-6$^+$) spleen cells were monitored daily for 7 d (Fig. 4). As expected from the immunoprecipitation data, spleens from normal mice did not contain a detectable number of 18-6$^+$ cells. Production of anemia, with accompanying splenomegaly and reticulocytosis, resulted in the transient appearance of a subpopulation of cells expressing 18-6$^+$ gp70 in the spleens of C57BL/6 mice. The percentage of these cells rose to a maximum (~10%) 3 d after the final treatment with phenylhydrazine and then gradually returned to control levels. This short burst of 18-6$^+$ cells coincided with the minimum hematocrit reading and the ascending portion of the peripheral reticulocytosis curve. This relationship was consistent with the postulate that the *Rmcf*-linked gp70-bearing cells represented an early step in erythroid differentiation.

Cell Sorting and Functional Analyses of Fetal Liver Cells and Spleen Cells from Anemic Adults. To functionally characterize the cells expressing *Rmcf*-linked gp70, cells from 12-d fetal liver and anemic adult mouse spleens were sorted. Since hematopoietic colony-forming cells are virtually all found in the large cell population, as defined

by forward angle light scatter, and the large-cell pool also contained the majority of *Rmcf*-lined gp70$^+$ cells, only the large cell population was sorted into *Rmcf*-linked gp70$^+$ and gp70$^-$ fractions. *Rmcf*-linked gp70$^+$ and gp70$^-$ cells from DBA/2 fetal liver were analyzed for early (BFU-E) and late (CFU-E) erythroid precursors. The clonogenic assay results (Table II) demonstrated that the gp70$^+$ population contained the majority of the erythroid precursors (1 of 4 large gp70$^+$ cells was a CFU-E, and 1 of 192 was a BFU-E). Thus, 93% of the BFU-E and 96% of the CFU-E populations in day 12 DBA/2 fetal liver expressed *Rmcf*-linked gp70. A similar analysis of C57BL/6 fetal liver cells using mAb 18-6 revealed that the partition of both CFU-E and BFU-E into the large stained population was virtually 100%. In this experiment small cells were also sorted and assayed for CFU-E and BFU-E. No erythroid precursors were present in the small cell population (data not shown). Similarly, spleen cells from phenylhydrazine-treated (low dose) C57BL/6 mice stained with antibody 18-6 and sorted into large stained and unstained cells revealed that essentially all of the erythroid precursors were found among the large stained cells. These results indicated that erythroid precursors in DBA/2 and C57BL/6 mice expressed *Rmcf*-linked gp70.

We were interested in determining whether the expression of *Rmcf*-linked gp70 was limited to the erythroid lineage or whether it was expressed by other myeloid progenitors. Therefore, GM precursors (CFU-GM) were also examined. Assaying stained and sorted day 12 fetal liver cells from DBA/2 and C57BL/6 mice revealed that a large proportion of CFU-GM also expressed the *Rmcf*-linked gp70 molecules (Table II). The segregation was, however, not as dramatic as that observed for erythroid precursors (80–90% CFU-GM were gp70$^+$).

The expression of *Rmcf*-linked gp70 by early fetal liver cells in two distinct myeloid differentiation pathways suggested that these endogenous gp70 species may have been expressed on a less mature multipotential stem cell. To investigate this possibility, day 12 fetal liver cells from C57BL/6 mice were sorted as before and the cells were analyzed for the frequency of CFU-S, a primitive multipotent cell capable of giving rise to mixed spleen colonies in irradiated mice. After sorting, the majority of CFU-S cells (81%) segregated into the large, *unstained* population (Table II). However, 19% of the CFU-S were found among the 18-6$^+$ cells, indicating a heterogeneity of expression within this pool of cells.

Microscopic Examination of Fetal Liver Cells Sorted on the Basis of Rmcf-linked gp70 Expression. In addition to functional assays, sorted fetal liver cells were examined by light microscopy. Distinct cytological differences between *Rmcf*-linked gp70$^+$ and gp70$^-$ cell populations were observed. Cells in the gp70$^-$ population (Fig. 5 *A*) were heterogeneous, consisting predominantly of erythroid cells exhibiting features of more mature forms, including normoblasts and orthochromic normoblasts. Occasional myelocytes and metamyelocytes as well as mature monocytes were also seen. Similar mature cell types were generally not identifiable in the gp70$^+$ population, the majority of these cells having the appearance of immature blasts with scant basophilic cytoplasm and large nuclei (Fig. 5 *B*). Microscopic examination, therefore, confirmed that *Rmcf*-linked gp70 expression was confined to a population of primitive hematopoietic progenitors.

Identification of a Small Subpopulation of Thymocytes Expressing Rmcf-linked gp70. Because *Rmcf*-linked gp70 molecules were expressed by myeloid progenitors, we also

TABLE II

Frequency of Hematopoietic Progenitors in Cell Populations Sorted on the Basis of Rmcf-linked gp70 Expression

	Rmcf-linked* gp-70	CFU-E‡	1/F§	BFU-E‡	1/F	CFU-GM‡	1/F	CFU-S‖	1/F
DBA/2 fetal liver	+	2,785 ± 106	4	52 ± 4.2	192	198 ± 7.1	50	ND	–
	–	105 ± 7.1	95	4 ± 0	2,500	44 ± 8.5	227	ND	–
C57BL/6 fetal liver	+	2,170 ± 141	5	15 ± 1.4	667	47 ± 6.4	213	4.3 ± 1.5	23,256
	–	0	–	0.3 ± 0.4	33,333	6 ± 0.7	1,667	18.8 ± 4.2	5,319
C57BL/6 anemic spleen	+	452 ± 80	22	ND		ND		ND	–
	–	0.3 ± 0.4	33,333	ND		ND		ND	–

* Cells were stained with antibody 18-6 or 514 followed by FITC goat anti-mouse Ig. Sorting windows were set based on forward angle light scatter and fluorescence. The forward angle light scatter parameter was set to include only the large cell population known to contain hematopoietic precursor cells. The break-off point for positive fluorescence was drawn empirically based on the cell sample stained only with the conjugate. After staining, the cells were sorted into large stained ($gp70^+$) and large unstained ($gp70^-$) populations. The in vitro functional assays were performed as described in Materials and Methods.

‡ Colony counts are expressed as the mean ± SE from triplicate cultures of 10^4 cells. Frequencies of CFU-E, BFU-E, and CFU-GM in stained and unstained sorted populations differ significantly ($p < 0.001$) for each source of cells.

§ 1/F = 1/frequency.

‖ Counts represent colonies/10^5 cells injected. Spleen colony counts made on day 12 after transplantation are expressed as the mean ± SE of 9–10 spleens per group. The frequency of CFU-S in the stained and unstained populations differs significantly ($p < 0.01$).

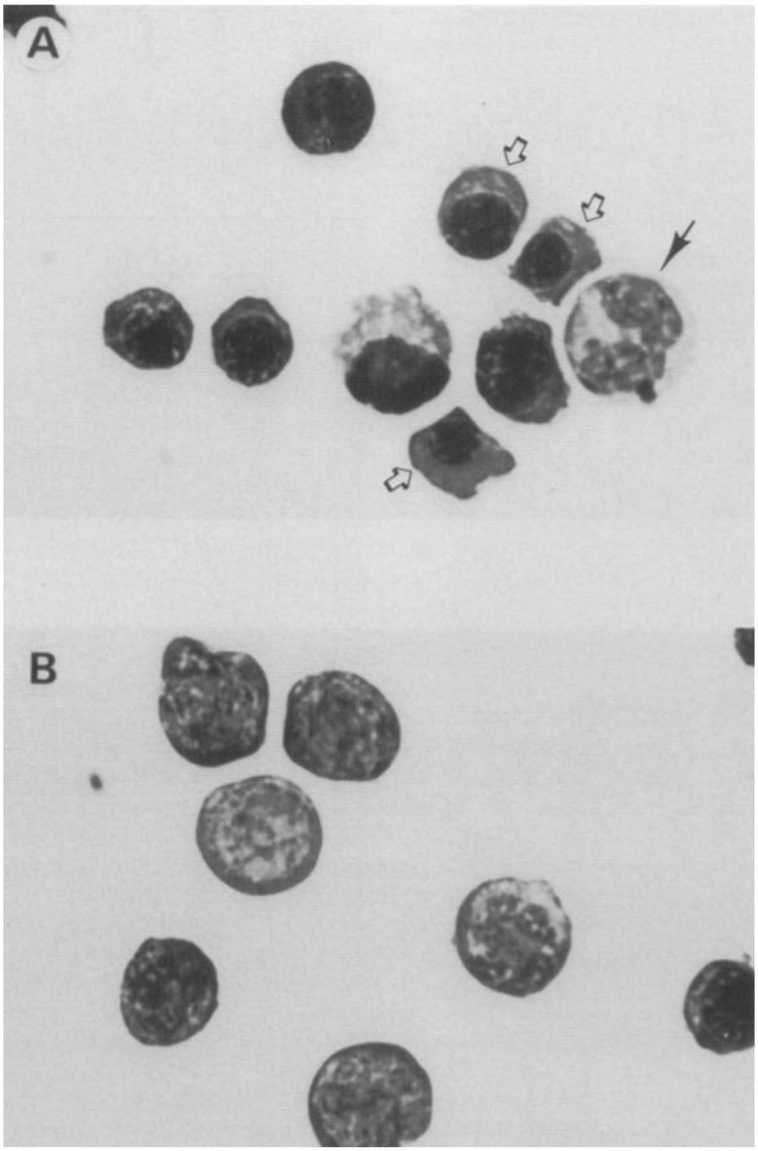

FIGURE 5. Cytologic characteristics of C57BL/6 12-day fetal liver cells sorted on the basis of large size and expression of *Rmcf*-linked gp70. Cells were stained and sorted as described for Table II. Samples of $gp70^+$ and $gp70^-$ cells were fixed and stained with a modified Wright stain and examined microscopically. (*A*) $gp70^-$ cells, (*B*) $gp70^+$ cells. Note the heterogeneity of cell types in the $gp70^-$ population compared with the $gp70^+$ population. Also, observe the lower nuclear-to-cytoplasm ratio and the presence of a mature granulocyte (*solid arrow*), as well as several late nucleated erythroid cells (*open arrows*), in the $gp70^-$ population.

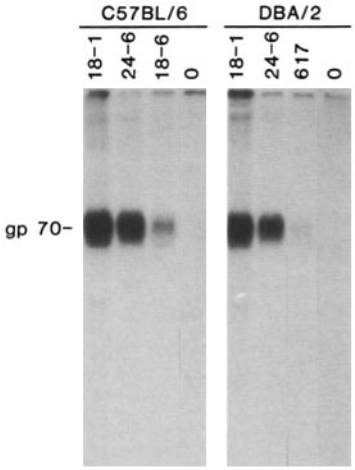

FIGURE 6. Immunoprecipitation of ^{125}I surface-labeled thymocytes with anti-gp70 mAbs. Antibody 18-1 is broadly reactive for murine retroviral gp70, antibody 24-6 is reactive with xenotropic and MCF gp70, antibody 18-6 is reactive with a subgroup of xenotropic gp70 and defines the *Rmcf*^{s}-linked gp70, and antibody 617 is specific for MCF gp70 reacting with the *Rmcf*^{r}-linked gp70. The track marked 0 contains an equal volume of labeled lysate with no mAb. Precipitates were resolved by SDS-PAGE.

looked for these *env* gene products on lymphoid progenitors resident at a site of lymphoid development. The thymus was selected for study as it is known to contain lymphoid progenitors while being devoid of myeloid cells. Immunoprecipitation of ^{125}I surface-labeled DBA/2 and C57BL/6 thymocytes by gp70-specific mAbs revealed, as has been reported previously, expression of abundant endogenous gp70 (22, 33) (Fig. 6). As in the spleen, the pattern of reactivity indicated that the majority of the gp70 was related to that of xenotropic viruses (18-1$^+$, 24-6$^+$). Clearly, however, a small proportion of this gp70 was encoded by the *Rmcf*-linked *env* gene (18-6$^+$ in C57BL/6, 617$^+$ in DBA/2), a finding supported by FACS analysis which revealed that 5–10% of thymocytes from 3-wk-old C57BL/6 mice expressed *Rmcf*-linked gp70. Unlike the spleen, however, no increase in the frequency of *Rmcf*-linked gp70$^+$ cells was observed after phenylhydrazine treatment. Microscopic examination of sorted thymocytes revealed that the *Rmcf*-linked gp70-bearing cells were a homogenous population of large blasts having a 10-fold higher frequency of mitotic figures when compared with the *Rmcf*-gp70$^-$ cells. These observations suggest that the *Rmcf*-linked gp70$^+$ cells in the thymus were lymphoid progenitors.

Discussion

These studies demonstrate that cells expressing *Rmcf*-linked gp70 are committed progenitors of two or possibly three hematopoietic lineages. Expression of these retroviral *env* genes by erythroid progenitors was supported by several experimental results. First, the frequency of cells bearing *Rmcf*-linked gp70 increased in the spleen in response to anemia. Second, the frequency of fetal liver cells expressing *Rmcf*-linked gp70 decreased with increasing gestational age, paralleling a similar decrease in the frequency of erythroid cells in this organ (29, 30). Third, cell sorting revealed that *Rmcf*-linked gp70$^+$ cell populations in fetal liver and anemic adult spleen were highly enriched for the erythroid precursors BFU-E and CFU-E. Cell sorting revealed that a second committed myeloid progenitor (CFU-GM) also expressed *Rmcf*-linked gp70. *Rmcf*-linked gp70 expression by committed progenitors from two lineages led us to examine cells in the more primitive multipotential CFU-S compartment.

The differential expression of *Rmcf*-linked gp70 found among CFU-S may be a function of the heterogeneity of this compartment. Evidence indicates that CFU-S represent a hierarchy of cells with differing capacities for differentiation and self-renewal (34). The approximately one-fifth of CFU-S expressing the *Rmcf*-linked gp70 may represent a subpopulation whose phenotype heralds imminent differentiation into progenitor pools of several lineages. Examination of the lymphoid cells of the spleen and thymus indicated that mature T and B lymphocytes in the spleen did not express *Rmcf*-linked gp70. Similarly, a majority of the cells in the thymus of young adult mice were negative for the expression of these particular retroviral proteins. However, RIP and FACS data demonstrated that a small (~5–10%) population of thymocytes from young mice expressed *Rmcf*-linked gp70, suggesting that lymphoid progenitors also express these endogenous retroviral proteins. In support of this interpretation, other investigators have reported that the adult thymus contains a low percentage (1–5%) of cells having the characteristics of lymphoid progenitor cells found in the fetal thymus (35). It is interesting that some of the cells initially responsible for populating the fetal thymus appear to originate in the fetal liver (35).

In contrast to the expression of this locus observed in immature hematopoietic cells, evidence indicated that their expression was shut down as a consequence of differentiation to more mature forms. Thus, in late fetal liver, cells expressing *Rmcf*-linked gp70 were barely detectable in spite of the abundance of mature erythroid forms present at this time. Additionally, we found by cytologic criteria that *Rmcf*-linked $gp70^-$ cells from early fetal liver contained numerous mature nucleated erythroid forms as well as clearly recognizable late granulocytic and monocytic cells. In contrast, few such mature forms were observed in the *Rmcf*-linked $gp70^+$ population. These lines of evidence provide compelling support for the interpretation that expression of *Rmcf*-linked *env* genes is limited to immature hematopoietic progenitors and that expression is downregulated as a function of maturation.

Expression of *Rmcf*-linked gp70 by lymphoid and myeloid progenitors, as well as the heterogeneity seen in the multipotent stem cell pool, suggests that this gene may be simultaneously activated in three different hematopoietic lineages in response to early differentiation signals. Preliminary experiments indicate that the frequency of cells expressing the *Rmcf*-linked gp70 of C57BL/6 mice was not increased in the bone marrow after treatment with 5-fluorouracil, a regimen that has been shown to increase the frequency of primitive hematopoietic stem cells (36). This suggests that expression of this gene is initiated, coordinately, within a population of committed progenitors feeding both the myeloid and lymphoid lineages.

The linkage of the *env* genes described in this study with the *Rmcf* locus suggests that the tissue specific pattern of expression of this gene may help define its role in resistance to leukemia. Based on in vitro studies, the presumed mechanism of the restriction of MCF viral replication is blockage of the cell surface MCF viral receptor by endogenous cell surface gp70 (viral interference) (13, 14, 37). Erythroleukemia induced by F-MuLV can be divided into two stages (38–40). The initial step appears to involve hyperplasia of at least two hematopoietic compartments followed by a block in erythroid differentiation. The block in erythroid differentiation occurs at an early stage, most likely at the level of BFU-E (17, 18). Our findings suggest that the expression of *Rmcf*-linked gp70 protects a specific cell population of hematopoietic progenitors from infection by oncogenic MCF viruses. Thus, al-

though *Rmcf*-linked MCF gp70 was expressed by lymphoid, granulocytic, and erythroid progenitors of DBA/2 mice, it appears to protect only the erythroid lineage from transformation as DBA/2 mice commonly succumb to late-onset lymphoid and granulocyte leukemias after infection by F-MuLV (18, 41). Because MCF viruses appear not to be involved in these nonerythroid leukemias (41), our results indicate a high level of specificity of F-MuLV-generated MCF viruses for cells of the erythroid lineage. However, the expression of *Rmcf*-linked gp70 by lymphoid progenitors may afford protection from transformation by lymphotropic MCF viruses. In fact, Rowe and Hartley (42) have suggested that the *Rmcf* gene functions in resistance to AKR thymomas.

Multiple copies of retroviral sequences reside in murine chromosomes, the majority of which are related to the genomes of MCF and xenotropic viruses. A question raised by our results concerns the mechanism controlling the expression of the *Rmcf*-linked *env* genes. Because expression of *Rmcf*-linked gp70 is limited to a few strains of mice, the expression of this locus does not appear to be requisite for normal development. However, the association of expression of endogenous retroviral genes with differentiation and development is well documented (6–8). Work from the laboratory of Jaenisch has indicated that endogenous retroviruses are acquired via infection of germ cells and that their subsequent expression (or lack of expression) is a function of the chromosomal site of integration and the methylation of proviral DNA (4, 43, 44). The control of endogenous proviral expression envisioned in this model is a *cis*-control mechanism regulated by flanking cellular sequences (2–4). Thus, it is possible that the *Rmcf*-linked *env* genes are expressed in hematopoietic cells simply as a consequence of their integration into a chromosomal site that is transcriptionally active in hematopoietic cells. Cloning of the endogenous sequences encoding the *Rmcf*-linked gp70s should shed light on the mechanism of the control of their expression and should also prove useful in identifying chromosomal sites that are transcriptionally active during hematopoiesis. In this regard it is intriguing that the W^v locus, a gene responsible for a defective stem cell compartment, and the *Rmcf* locus appear to be in close proximity on chromosome 5.

The *Rmcf* gene restricts the replication of recombinant mink cell focus-forming (MCF) viruses in fibroblast cell cultures derived from mice carrying the resistance allele ($Rmcf^r$). mAbs that define specific endogenous retroviral *env* products previously revealed that the expression of two alleles of an endogenous *env* gene is tightly linked to the *Rmcf* locus. Cell cultures from $Rmcf^r$ mice express the MCF gp70 allele, which appears to be responsible for the restriction of MCF viral replication, presumably by a mechanism of viral receptor blockade (viral interference). Cell cultures derived from some $Rmcf^s$ mice express the xenotropic gp70 allele, which does not interfere with MCF viral replication. Recently we reported that in backcross analyses the endogenous MCF gp70 of the $Rmcf^r$ strain, DBA/2, slows the tempo of erythroleukemia induced by neonatal inoculation of nondefective Friend murine leukemia virus (F-MuLV), a disease believed to be mediated by MCF viruses. To better understand the mechanism of this resistance we have identified the sites of expression of the *Rmcf*-linked *env* gene in uninoculated mice. Using mAbs specific for the MCF gp70 of DBA/2 mice and the xenotropic gp70 of C57BL/6 mice, these gp70 molecules were detected on a population of primitive myeloid cells within the multipotential stem cell pool. Expression persisted in the more differentiated erythroid

progenitors (BFU-E and CFU-E) as well as in granulocytic progenitors (CFU-GM). However, no evidence for expression of this gene was seen among mature erythroid or granulocytic cells. Similarly, the gene was also expressed by a lymphoid progenitor resident in the thymus, but the proteins were not detected on mature lymphocytes in either the thymus, bone marrow, or spleen. The relevance of this pattern of expression in resistance to leukemia mediated by the *Rmcf* gene in DBA/2 mice is discussed.

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