Feline Glucose-6-phosphate Dehydrogenase Cellular Mosaicism

Application to the Study of Retrovirus-induced Pure Red Cell Aplasia

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Abstract

Neoplasms result from the uncontrolled clonal proliferation of abnormal or transformed cells. The early stages of this process are difficult to study because of the lack of sensitive and specific markers of clonal evolution in an experimental system. We have developed a cat model using cellular mosaicism for glucose-6-phosphate dehydrogenase (G-6-PD). Our findings confirm that the structural locus for feline G-6-PD is on the X-chromosome and demonstrate that it is randomly inactivated in somatic cells. Heterozygous cats have balanced ratios of G-6-PD enzyme types in peripheral blood cells and hematopoietic progenitors that remain stable over time. In our initial studies, we used the model to analyze the events surrounding marrow failure experimentally induced by selected strains of feline leukemia virus (FeLV). Two G-6-PD heterozygous cats, one F_1 male hybrid and one domestic cat were infected with FeLV (C or KT) and developed pure red cell aplasia (PRCA). Colonies arising from the more mature erythroid colony-forming cell were not detected in marrow culture of anemic animals although erythroid bursts persisted, suggesting that the differentiation of early erythroid progenitors (BFU-E) was inhibited in vivo. The ratio of G-6-PD types in hematopoietic progenitors and peripheral blood cells from the heterozygous cats did not change when the animals developed PRCA. Thus, the anemia did not result from the clonal expansion of a transformed myeloid stem cell. With this experimental approach, one may prospectively assess clonal evolution and cellular interactions in other FeLV-induced diseases.

Introduction

Environmental toxins, drugs, radiation, and viruses have been implicated in the pathogenesis of human malignancy. Although the precise cellular and molecular events that result in the growth advantage of a transformed cell are unknown, host factors may influence the ability of such cells to proliferate in vivo. At the time of clinical presentation and study, most human neoplasms are derived from a single clone (1). It has therefore been impossible to assess the cellular interactions that permitted the neoplastic clone to expand and dominate. For these reasons, we have developed a model of glucose-6-phosphate dehydrogenase (G-6-PD)[1] cellular mosaicism in cats to study over time clonal evolution in hematologic diseases experimentally induced by feline leukemia virus (FeLV).

FeLV is a horizontally transmitted retrovirus that, in the natural setting, causes a spectrum of hematologic disease in cats: lymphosarcoma with mediastinal mass, acute lymphocytic leukemia, myeloid leukemia, erythroleukemia, nonregenerative anemia including pure red cell aplasia (PRCA), and preleukemic myelodysplastic syndromes (2–4). As these disorders clinically resemble hematologic malignancy in man, the study of FeLV-induced diseases may be relevant to understanding human oncogenesis.

The enzymes of G-6-PD are useful markers of neoplastic growth and differentiation since they occur naturally and are independent of viral infection and cell transformation. Early in embryogenesis, that portion of one X-chromosome that contains the structural locus for G-6-PD is randomly inactivated in somatic cells of females. Once inactivation occurs, the process is fixed and a single parental G-6-PD allele remains active in each cell and all its progeny. Therefore, women heteroxygous for the usual G-6-PD gene (Gd^B) and a variant (such as Gd^A) have some cells that synthesize type B enzyme, while others make type A. No somatic cell makes both G-6-PD types. All tumor cells in a neoplasm originating from a single cell in a G-6-PD heterozygote will have the same single enzyme type, whereas processes arising in multiple cells and normal tissue will have both enzyme types (1).

Cellular mosaicism for G-6-PD has been used extensively to study the clonal nature and level of progenitor involvement of hematologic neoplasms in man. Such studies have shown that the chronic myeloproliferative diseases result from the clonal expansion of a myeloid stem cell common to granulocytic, erythroid, and megakaryocytic differentiation (5). Similar data have demonstrated the clonal and stem cell origin of cases of acquired refractory sideroblastic anemia (6), acute nonlymphocytic leukemia (7), and aplastic anemia (8).

Although G-6-PD has been useful in the study of human disease, information regarding cellular mosaicism in animals is relatively sparse. A variant allele at the X-linked phosphoglycerate kinase (PGK) locus was found in feral mice and has

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1. *Abbreviations used in this paper:* Con A, concanavalin A; ELISA, enzyme-linked immunosorbent assay; BFU-E, erythrocyte burst-forming units; CFU-E, erythrocyte colony-forming units; Ep, erythropoietin; FeLV, feline leukemia virus; ffu, focus-forming unit; G-6-PD, glucose-6-phosphate dehydrogenase; MTT, glucose-6-phosphate,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CFU-GM, granulocyte/macrophage colony-forming units; GM, granulocyte macrophage derived; HBSS, Hanks' balanced salt solution; PGK, phosphoglycerate kinase; PRCA, pure red cell aplasia.

been bred into several strains (9). Clonal evolution of lymphoma has been demonstrated in these animals (10, 11). Whether the assay to detect PGK is sufficiently sensitive to analyze small numbers of cells (e.g., hematopoietic colonies) has not been reported. G-6-PD heterozygosity exists in the hare, but there is no established model of hematologic malignancy in this system (12, 13). We have found that domestic cats (*Felis catus*) and Geoffroy cats (*Leopardus geoffroyi*) have electrophoretically distinct G-6-PD types. These cats will interbreed (14) to produce female offspring that are obligate G-6-PD heterozygotes. The results of our studies of F_1 females confirm previous reports that the structural locus for feline G-6-PD is on the X-chromosome (15). The G-6-PD enzyme type d (domestic) or G (Geoffroy) can be reliably assigned not only to tissues and circulating blood cells, but also to hematopoietic colonies growing in culture. In peripheral blood cells, hematopoietic precursors, and other somatic cells tested, the ratios of d/G G-6-PD activities in normal F_1 females are balanced and stable. One may therefore infect cats with FeLV and prospectively assess potential clonal evolution and cellular interaction as hematologic disease develops. Because PRCA can be induced by a biologically cloned strain of FeLV (FeLV-C/Sarma) and has a short latency period (16, 17), we chose to study this disease as the initial application of our feline model of G-6-PD cellular mosaicism.

Methods

Breeding of G-6-PD heterozygous cats. Breeding animals are housed at the School of Veterinary Medicine at Washington State University. A Geoffroy male was bred with naturally cycling domestic females with which he had previously been imprinted. Proesterus in the domestic females was determined by vaginal smear. Pregnancy was confirmed by ultrasound. Delivery was achieved vaginally or by cesarian section following a 63–66-d gestation. When weaned, female kittens were transported to the University of Washington for study.

Obtaining specimens for analysis. After animals were anesthetized with ketamine, marrow cells were aspirated from the femur or humerus into heparinized syringes. Blood samples were obtained by venipuncture.

Heparinized blood was centrifuged at 200 *g* for 10 min at room temperature. The top one-half of the plasma was removed and platelets were centrifuged (8,000 *g* for 2 min) from this fraction. To separate mononuclear cells, the remaining blood sample was diluted 1:1 with Hanks' balanced salt solution (HBSS; Gibco Laboratories, Grand Island, NY), layered over Percoll (density 1:070 g/ml; Pharmacia Fine Chemicals, Piscataway, NJ), centrifuged at 400 *g* for 20 min, and interface cells were collected. A discontinuous density gradient (18) was used in earlier experiments and demonstrated that the best separation of feline granulocytes and monocytes was obtained with a Percoll density of 1.070. To further purify the lymphocyte preparation, mononuclear cells were placed at a concentration of 10^6/ml in alpha medium (Flow Laboratories, McLean, VA) with 10% fetal calf serum (FCS; Reheis Chemical Co., Scottsdale, AZ), with and without concanavalin A (Con A; 25 μg/ml; Pharmacia Fine Chemicals). After a 3-d incubation at 37°C, most monocytes adhered to the plastic flask and residual neutrophils died. 95—100% of the cells appeared to be lymphocytes by Wright-Giemsa and <5% to be monocytes by nonspecific esterase stains. Putative T lymphocytes were further isolated using rat erythrocytes (19). Preliminary experiments showed that rat and guinea pig erythrocytes were equally effective in E-rosetting cat peripheral blood cells (30–40% ER+), whereas mouse and sheep erythrocytes were less effective. Rat erythrocytes were collected in Alsever's solution (Gibco Laboratories) and washed three times in alpha medium. A suspension of 10^8/ml rat erythrocytes was mixed in a 1:1 ratio (vol:vol) with 10^6/ml cat mononuclear cells. The mixture was incubated for 15 min at 37°C in a water bath, then 1 h at 4°C and the cells were then resuspended in alpha medium. The suspended rosettes were separated from nonrosetting mononuclear cells by passage through a Ficoll-Hypaque (Litton Bionetics, Kensington, MD) density gradient (1.077 g/ml). Erythrocytes were lysed with Tris-HCl hemolytic buffer (Sigma Chemical Co., St. Louis, MO) and the remaining cells washed twice in alpha medium. However, cat monocytes were also rosetted by xenogenic erythrocytes, limiting the effectiveness of this technique.

Cells that migrated through the initial Percoll layer were separated into erythrocyte and granulocyte fractions with flotation in a 3% dextran solution for 30 min. Erythrocytes were lysed from the granulocyte, platelet, and lymphocyte preparations. The samples were then frozen as pellets and stored at −70°C for subsequent G-6-PD determination.

When cats died, postmortem samples of nodes, spleen, liver, skeletal muscle, and gastrointestinal tract were obtained. Part of each tissue sample was processed for histologic examination. Adjacent sections were minced and then frozen at −70°C for G-6-PD determination. Mononuclear cells were also isolated from cell suspensions of spleen and nodes.

Colony assay techniques. Heparinized marrow was placed in HBSS containing 5% FCS. Mononuclear cells, obtained as described above, were washed three times with HBSS with 5% FCS. Cultures were established with 10^5 cells suspended in alpha medium containing final concentrations of 1.2% methylcellulose (Fischer Scientific Co., Fairlawn, NJ), 1% bovine serum albumin (BSA; Reheis Chemical Co), 10^{-4} M beta mercaptoethanol, pencillin/streptomycin, and 30% FCS, which optimized feline progenitor growth and colony morphology. Partially purified human urinary erythropoietin (Ep)[2] was added to the cultures; no conditioned medium was used. Cultures were incubated at 37°C in a high humidity, 96% air/4% CO_2 atmosphere.

Erythroid colonies (from CFU-E), containing 8-50 hemoglobinized cells, were counted on day 3 or 4 of culture. Erythroid bursts (from BFU-E), containing ≥200 hemoglobinized cells, and granulocyte/macrophage (GM) colonies (from CFU-GM), containing ≥50 cells, were enumerated on day 9 or 10 on the same plates as erythroid colonies.

Determination of G-6-PD activity. Cell lysates were prepared by freezing and thawing separated blood samples. Cellular debris was sedimented with centrifugation. 3 μl of lysate that had been prepared from a standardized number of cells (5×10^4) or by spectrophotometric assay for G-6-PD (7.8×10^{-4} dehydrogenase units), were placed on a filter paper wick (no. 1, Whatman, Inc., Clifton, NJ) near the cathode end of a polyacrylamide gel (100 ml × 125 mm × 0.8 mm). The photopolymerized gel contained pH 3–10 ampholytes (Biolyte 3/10; Bio-Rad Laboratories, Richmond, CA). It was subjected to electrophoretic focusing with constant power (3 W) for 2.5 h at 4°C (LKB Instruments, Gaithersburg, MD). It was then incubated for 20 min at 37°C with a solution containing NADP, glucose-6-phosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and phenazine methosulfate in Tris-HCl buffer (pH 8.0) (20) to visualize the G-6-PD activity. The relative intensities of the single domestic G-6-PD band and the minor Geoffroy G-6-PD bands (Fig. 1) were estimated visually by comparison with a standard mix of domestic and Geoffroy preparations. Since hemoglobin comigrated with feline G-6-PD on cellulose acetate at alkaline pH, the amount of G-6-PD activity in nonerythroid cell lysates was confirmed by cellulose acetate electrophoresis. This electrophoresis technique was identical to that reported for the determination of human G-6-PD (20), except that NADP was omitted from the feline lysate.

To analyze tissue specimens, 20 μl of distilled water were added to the stored samples. After refreezing and thawing, specimens were processed as described above.

For G-6-PD analysis of hematopoietic progenitors, colonies were

2. Ep was obtained from urine pooled from a patient with PRCA and processed in our laboratory. The partial purification procedure included ion exchange and affinity chromatography, and yielded a specific activity of 200 U/mg.

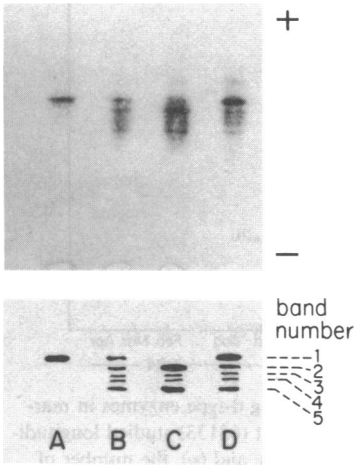

Figure 1. Isoelectric focusing of feline G-6-PD. A, domestic. B, a 1:1 mix of equal numbers of domestic (d) and Geoffroy (G) cells. C, Geoffroy. D, female heterozygote. The samples, lysates containing a total of 5 × 10^4 granulocytes, were placed near the cathode of the polyacrylamide gel. After isoelectric focusing for 2.5 h, the gel was stained for G-6-PD activity. A single band (1) characterized the domestic pattern. The Geoffroy has four bands, with 2 and 5 most prominent. The heterozygote in this sample would be read as 70:30, d:G. The percent of Geoffroy activity is best estimated by band 5, which never overlaps the domestic range. More accurate estimations of activity ratios are obtained with cellulose acetate electrophoresis as only one domestic and one Geoffroy band are visible with that technique.

individually harvested by micropipette under direct vision with an inverted microscope. The cells of the colonies were lysed by freezing and thawing before each sample was placed directly on a thin-layer polyacrylamide gel for isoelectric focusing.

Virus preparation. FeLV-KT, containing A, B, and C viral subgroups (21, 22), was obtained from Dr. Edward Hoover, Colorado State University, Fort Collins, CO. The virus was initially derived from FL74, a lymphosarcoma cell line, and has been repeatedly passed in vivo. A 20% weight-to-volume suspension in alpha medium was prepared from homogenized splenic tissue of an infected animal and was stored at −70°C.

Feline embryonic fibroblasts that were infected with biologically cloned FeLV-C/Sarma were obtained from Dr. David Onions and Dr. Os Jarrett, University of Glasgow, Scotland, and have been maintained in our laboratory. Cells were grown in McCoy's medium (Gibco Laboratories) with 15% FCS. At the time of virus harvest, a small quantity of medium was incubated for 24 h with fibroblasts grown to near-confluence. Aliquots of virus-rich supernatant were frozen for later use. The amount of virus in the specimens was quantitated by Dr. Edward Hoover using the 8C cell line focus-forming unit (ffu) assay described (23).

FeLV infection protocol. Cats received 5 mg methylprednisolone/kg subcutaneously on days −4, 0, 1, 4, 7, and 10, or 10 mg/kg on days 0 and 7. FeLV was given on days 0 and 1. Various dose schedules were used empirically to determine the optimal procedure to produce PRCA in these hybrid animals. Disease was induced most reliably with a total dose of 0.5–1.0 × 10^5 ffu/kg. As cats became anemic they were transfused with 50 ml of littermate blood when clinically indicated.

Before infection, peripheral blood and marrow were obtained on two occasions for complete blood count, reticulocyte count, leukocyte differential, quantitation of progenitors in methylcellulose culture, and G-6-PD analysis of erythrocytes, platelets, granulocytes, lymphocytes, erythroid bursts and GM colonies. Marrow morphology was assessed by Wright-Giemsa stain of marrow cells aspirated in EDTA. Similar studies were conducted at 1–3-wk intervals.

Viremia was detected by an enzyme-linked immunoabsorbent assay (ELISA) (Pittman-Moore & Co., Washington Crossing, NJ), which assays gag-related antigens or by immunofluorescence staining of peripheral blood cells (24). Heterologous goat antiserum to gag (predominantly p27)-related antigens, provided by Dr. Edward Hoover, and fluorescein-labeled anti-goat immunoglobulin (Biological Carcinogenesis Branch, NCI, Bethesda, MD) were used in the indirect immunofluorescence assay.

Results

Characterization of feline hematopoietic progenitors. Marrow cells from 22 hybrid cats were cultured in vitro for progenitor cell growth. The morphology of feline erythroid colonies, erythroid bursts, and GM colonies was comparable to that described (17). Dose-response curves with Ep showed a plateau growth of erythroid colonies and bursts at 0.1 and 0.3 U/ml, respectively. Normal values for the various progenitors per 10^5 mononuclear marrow cells were: erythroid colonies: 202±102 (SD); erythroid bursts: 40±21; GM colonies: 57±18 (n = 51).

Sensitivity of the G-6-PD assay. Domestic (d) cat G-6-PD migrated as a single band on isoelectric focusing to pH 6.7. Geoffroy (G) G-6-PD formed several bands with more basic isoelectric points (Fig. 1). With cellulose acetate electrophoresis, one d (fast) and one G (slow) band were visible (data not shown). Varying ratios of d and G erythrocytes and granulocytes were assayed for G-6-PD activity to determine the lower limit of detection of one G-6-PD type in a mixture of the two. With isoelectric focusing, one could detect as little as 5% d and as little as 10–15% G type G-6-PD. With cellulose acetate electrophoresis, one could detect as little as 5–10% of each G-6-PD type. Since hemoglobin comigrates with G-6-PD on cellulose acetate, one cannot analyze erythrocyte preparations with this procedure.

The isoelectric focusing technique was sufficiently sensitive such that the G-6-PD type of individual bursts with 50–100 erythroblasts or GM colonies with more than 8 monocytes or 20 granulocytes could be reliably determined.

Demonstration that the structural locus of feline G-6-PD is on that part of the X-chromosome that is inactivated and that the ratio of G-6-PD activities remains stable. To demonstrate that feline G-6-PD is X-linked, blood cells from the hybrid offspring of a Geoffroy male and multiple female cats were examined. All F_1 females (n = 20) had both d and G banding. The five F_1 male cats tested had single d bands, the maternal banding pattern. Of 1,557 hematopoietic colonies tested, 779 were d and 758 were G. Both d and G bands were seen in 20 colonies (1.3%). That the G-6-PD type of hematopoietic colonies was either d or G, but rarely both, demonstrates that only one G-6-PD locus was active in each BFU-E or CFU-GM and its differentiated progeny (Fig. 2). These observations also prove that feline hematopoietic colonies originate from a single precursor.

Table I shows the percentage of colonies containing d-type G-6-PD from 20 F_1 heterozygotes. The mean is 50%±11% (SD) and the median is 49%. In each heterozygous cat tested, the ratio of G-6-PD enzyme types in erythroid bursts and GM colonies was similar to the ratios of d/G G-6-PD activities determined for erythrocytes (n = 10), platelets (n = 5), granulocytes (n = 19; Table I), and mononuclear peripheral blood cells (n = 12). Three heterozygous cats have been studied repeatedly for over 1 yr and the d/G ratios in peripheral blood cells and hematopoietic colonies have remained unchanged. Fig. 3 shows colony data in one cat. In a killed control cat, balanced G-6-PD d and G activities were detected in spleen and node. Diffuse bands with equivalent activity in d and G regions were seen with G-6-PD analysis of liver and muscle.

Natural history of FeLV-C/Sarma and KT infection. Two heterozygous cats were infected with FeLV-C/Sarma and one male F_1 received FeLV-KT. Progressive anemia with reticulocytopenia developed 9–21 wk after infection. Granulocyte and

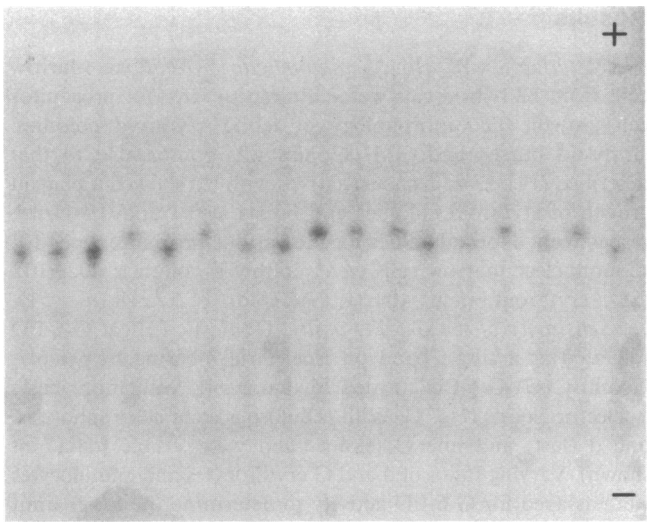

Figure 2. G-6-PD determination of individual GM colonies. GM colony lysates were placed near the cathode of a polyacrylamide gel. After isoelectric focusing and staining for G-6-PD, bands were visualized. This cat was a balanced heterozygote with similar numbers of more anodic (domestic or d) and more cathodic (Geoffroy or G) bands. Colonies were either d or G, not both, confirming X-inactivation of feline G-6-PD. Similar results were obtained with erythroid bursts.

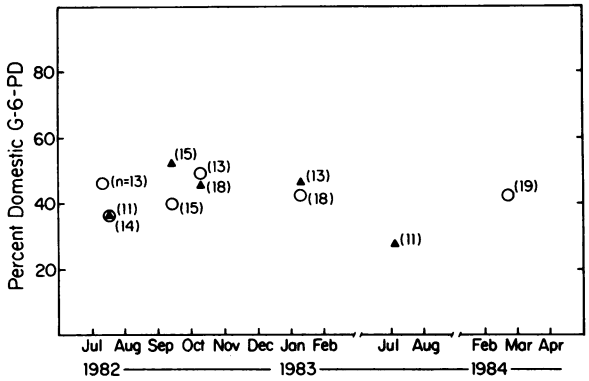

Figure 3. Percentage of colonies containing d-type enzymes in marrow cultures of a G-6-PD heterozygous cat (63133) studied longitudinally. O represents GM colonies; ▲, bursts, and (*n*), the number of colonies analyzed for G-6-PD type each time. The percent of bursts with d G-6-PD is similar to the percent of GM colonies with d G-6-PD. The ratio of d to G type colony forming cells and peripheral blood granulocytes and erythrocytes remained stable during 18 mon of study.

platelet counts remained normal. Bone marrow morphology was diagnostic of PRCA and showed erythroid aplasia, normal numbers and maturation of granulocyte precursors, and normal megakaryocytes. A few cells with deeply basophilic cytoplasm characteristic of proerythroblasts were noted (Fig. 4). These cells did not stain with benzidine.

In cats infected with FeLV, viremia was detectable within 2–3 wk. All animals that remained persistently viremic developed PRCA. Of note, most cats developed transient cytopenias and a diarrheal illness ~4–5 wk after infection. Two cats died during this time, while two other cats cleared their FeLV infection and remained hematologically normal and virus negative by ELISA and immunofluorescence assays.

Table I. G-6-PD Type of Colonies and Granulocytes from Heterozygous Cats

Cat	Bursts			GM colonies			Total colonies		Granulocytes
	d	*G*	*%d*	*d*	*G*	*%d*	*%d*	*95% Confidence limit*	*%d Cellulose acetate*
63131	53	35	60	103	76	58	58	±6	50
63132	60	19	76	57	37	61	68	±7	50
63133	25	32	44	38	52	42	43	±8	40
63134	19	39	33	32	59	35	34	±8	50
63179	4	3	57	28	20	58	58	±14	60
63689	13	15	46	19	14	58	52	±13	40
63690	20	16	56	24	19	56	56	±12	50
63691	28	52	35	35	46	43	39	±8	50
63775	8	13	38	11	18	38	38	±14	40
63776	3	9	25	14	18	44	39	±16	40
63846	11	10	52	8	27	23	34	±13	40
63848	5	5	50	16	10	62	58	±18	60
63851	10	16	38	16	17	48	44	±13	50
64013	9	13	41	17	15	53	48	±14	—
64029	13	14	48	13	17	43	46	±14	40
64030	6	3	67	19	15	56	58	±16	50
64031	17	5	77	17	17	50	61	±14	50
64146	3	7	30	12	12	50	44	±18	50
64147	4	8	33	17	13	57	50	±16	50
64337	1	1	50	12	4	75	72	±24	50
Mean			48			51	50±11 (SD)		

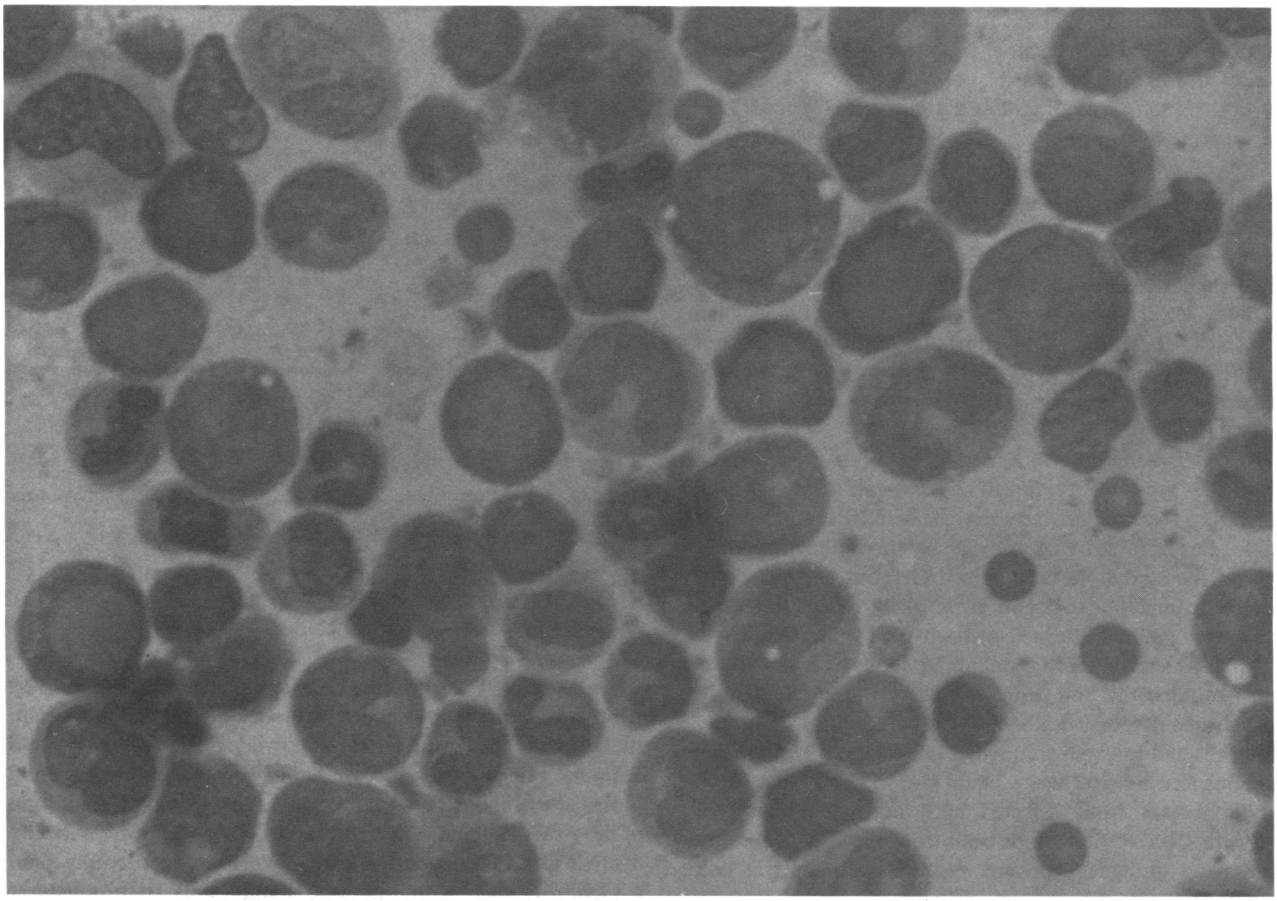

Figure 4. Marrow aspirate from cat #63775 with PRCA stained with Wright-Giemsa (× 100). Several cells with dark cytoplasm characteristic of proerythroblasts are seen.

The results of assays for colony-forming units in three cats that developed PRCA are shown in Fig. 5. At a time when the hematocrit was decreased, CFU-E-derived colonies were undetectable, although virtually normal numbers of BFU-E- and CFU-GM-derived colonies were found. Similar results were obtained in an adolescent domestic cat given FeLV-C/

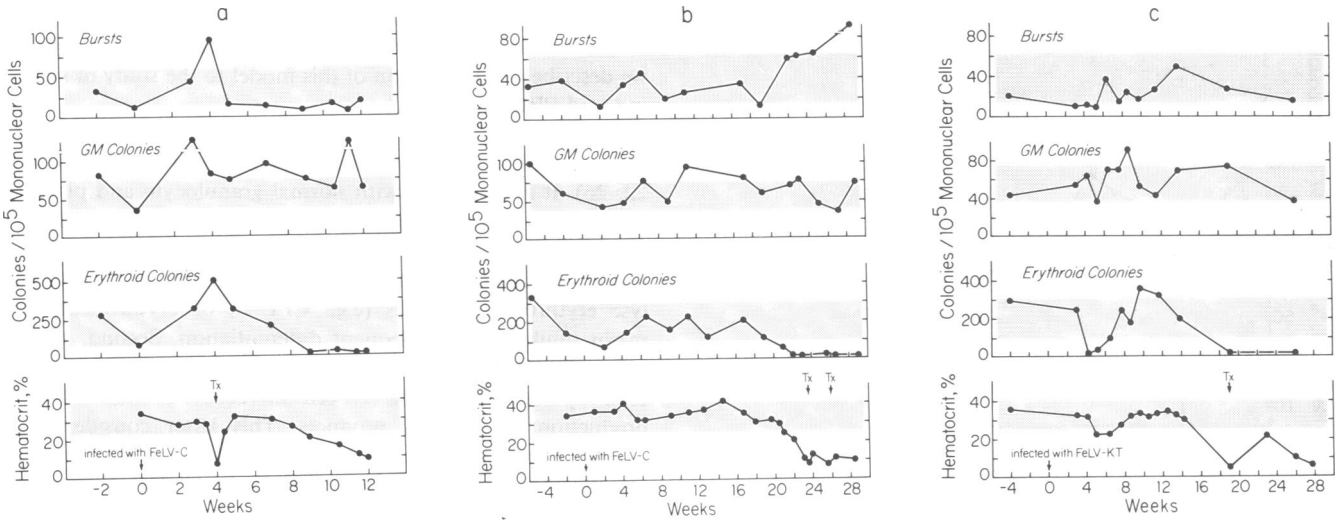

Figure 5. Experimentally induced PRCA in hybrid cats. Individual points represent the mean colony counts of three identical methylcellulose plates with 10^5 mononuclear marrow cells. 0.5 U/ml Ep was used in all cultures. Tx indicates transfusions. In each cat studied, erythroid colonies became undetectable in vitro coincidentally with the fall in hematocrit. Normal numbers of erythroid bursts persisted. (*a*) cat 63775, F_1 female infected with FeLV-C/Sarma, (*b*) cat 63776, F_1 female infected with FeLV-C/Sarma, (*c*) cat 63530, F_1 male infected with FeLV-KT.

Sarma to induce PRCA (Fig. 6). In this domestic cat, however, the number of BFU-E detectable in culture decreased late in its disease course.

At postmortem examination, thymic atrophy and mild diffuse adenopathy were evident. Histopathology demonstrated marrow hypocellularity, depletion of cells in the paracortical and cortical regions of lymph nodes, and less developed follicles in the spleen. The causes of death, feline infectious peritonitis (cat 63530) and nonbacterial pneumonitis (cat 63775), were consistent with immunologic suppression induced by FeLV (3, 25). The third hybrid cat (63776) was killed when anemic (Hct = 10) and while clinically healthy. Except for mild atrophy of villi and hyperplasia of the crypt epithelium in the small bowel, nonhematopoietic tissues from this cat were normal.

G-6-PD determinations in F_1 heterozygous cats with PRCA. The G-6-PD types of erythroid bursts and GM colonies obtained from the two heterozygous cats with PRCA are shown in Table II. Both cats remained balanced heterozygotes throughout the study. Variations in d/G ratios are not statistically significant ($P > 0.4$) and most likely reflect the small numbers of colonies examined at each data point. Ratios of G-6-PD d and G activities in serial preparations of erythrocytes, platelets, granulocytes, and peripheral blood mononuclear cells ranged between 40:60 and 50:50.

ER+ cells and mitogen-stimulated nonadherent lymphocyte preparations also showed nearly equal G-6-PD activities. However, lymphocytes from infected cats did not numerically expand with Con A. Therefore, following the 3-d adherence procedure and mitogen exposure, residual cells were at best 95% lymphocytes by morphologic criteria.

Postmortem specimens were obtained from one cat (63776) for G-6-PD study. Balanced activity of d and G were seen in spleen, spleen mononuclear cells, lymph nodes, liver and muscle.

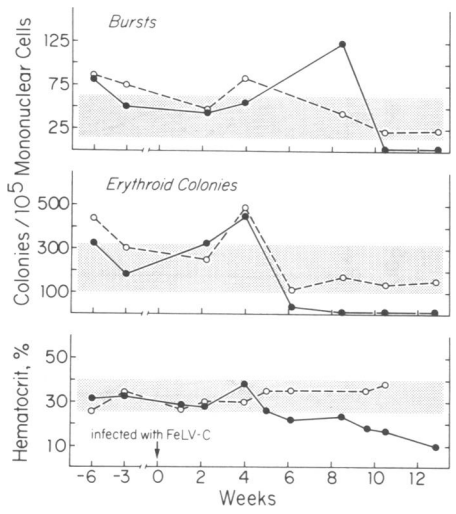

Figure 6. Experimentally induced PRCA in an adolescent domestic cat (●). Colony growth and hematologic parameters were compared with a littermate control (○). When the hematocrit began to fall, erythroid colonies became undetectable though erythroid bursts were present. Later in this cat's disease course, however, erythroid bursts were not seen.

Table II. G-6-PD Analysis of Hematopoietic Precursors in Heterozygous Cats with PRCA

	Week	BFU-E	CFU-GM
		%d	%d
Cat 63775			
Baseline (Hct 33)	−2–0	38 (n = 21)	38 (n = 29)
Anemic (Hct 10–21)	9–12	50 (n = 2)	32 (n = 50)
Cat 63776			
Baseline (Hct 37)	−6–0	25 (n = 12)	44 (n = 32)
Anemic (Hct 7–20)	22–29	44 (n = 27)	49 (n = 47)

n, Number of individual colonies tested.

Discussion

To prospectively analyze the cellular events that precede overt neoplasia, we have developed a model of cellular mosaicism in the cat. The South American wildcat (*L. geoffroyi*) and domestic cat (*F. catus*), species that have evolved independently during the millions of years following the geographic separation of the American and Asian continents, have electrophoretically distinct G-6-PD enzyme patterns. We have successfully bred a Geoffroy male with domestic female cats to produce F_1 females, which are obligate G-6-PD heterozygotes. We have confirmed that the structural locus for feline G-6-PD is X-linked and extended this observation to demonstrate that it undergoes random inactivation. We have shown that hematopoietic colonies growing in cultures have a single-cell origin and that somatic cells from G-6-PD heterozygous cats have balanced ratios of domestic and Geoffroy enzymes that are stable over time. The model can thus be applied to many issues concerning clonal evolution in neoplasia and, for example, to the study of FeLV-induced disease. In this report, we describe the application of this model to the study of virus-induced PRCA.

Experimental infection with FeLV-C or KT causes PRCA in neonatal kittens and immune suppressed adult cats (16, 17, 22, 26). It is associated with normal granulocyte and platelet counts and, in some animals, myelofibrosis (22). Although the mechanism by which erythropoiesis is inhibited is not clear, several possibilities are apparent. First, virus might infect and lyse erythroid stem cells (e.g., CFU-E) or erythroblasts or might inhibit their subsequent differentiation. Second, virus-infected accessory cells (e.g., lymphocytes) might interfere with erythropoiesis directly via cell-cell interaction or indirectly via production of humoral products. This latter consideration would be consistent with the tropism of this retrovirus for lymphocytes and postulated mechanisms of PRCA in man (27–29). In addition, infection with FeLV might result in the clonal proliferation of a hematopoietic stem cell that has lost its capacity for erythroid differentiation. Consistent with this, human PRCA has been found as a preleukemic illness and as a manifestation of myelodysplastic syndromes (30). Recent

studies of a G-6-PD heterozygous patient with pancytopenia confirm that marrow failure may result from the clonal expansion of the myeloid stem cell (8). Alternatively, an infected and transformed accessory cell might clonally expand. Retroviruses cause clonal lymphomas in cats (31), birds (32), mice (33), rats (34), and man (HTLV) (35) as demonstrated by hybridization probes to viral and flanking sequences following restriction endonuclease digestion of tumor DNA. In addition, clonal lymphoid malignancies in man such as chronic lymphocytic leukemia and thymoma, have been associated with PRCA (27).

To investigate the possible role of clonal expansion in the pathogenesis of PRCA we analyzed the enzyme type of cells from G-6-PD heterozygous cats with FeLV-C-induced disease. If feline PRCA were a clonal disorder of the myeloid stem cell, we would predict that in granulocytes, platelets, erythrocytes, BFU-E and CFU-GM either d or G type G-6-PD would predominate over time. In contrast, if PRCA were caused by a humoral or cellular suppression of erythroid progenitors, both G-6-PD types would be found among myeloid cells.

In two cats studied longitudinally, no significant change was seen in the ratio of d to G G-6-PD activities in blood cells or in erythroid bursts or GM colonies. PRCA in the cat is thus not a clonal disease of a myeloid stem cell. Although no change was seen in the d to G G-6-PD ratios in lymphocyte preparations, we cannot exclude the possibility that FeLV infection resulted in the expansion of a clone of T or B lymphocytes. As reported by others (36), lymphocytes from our cats infected with FeLV did not proliferate with Con A. Preparations of mononuclear cells exposed to mitogen during a 3-d adherence procedure were, at best, 95% lymphocytes by morphologic criteria and presumably contained both T and B cells. Preparations of ER+ mononuclear cells contained some monocytes. As the G-6-PD activity per lymphocyte is 1/10–1/20 that of a monocyte or granulocyte, results suggesting balanced activity in the lymphocyte population are difficult to interpret.

In addition to determining whether feline PRCA is a clonal disease, we analyzed the effect of in vivo FeLV infection on the number of hematopoietic progenitors. As cats developed anemia, CFU-E were no longer detected in marrow cell culture. However, essentially normal numbers of erythroid bursts remained in three F_1 hybrid cats and one domestic cat with experimentally induced PRCA. These data suggest that erythropoiesis is inhibited in vivo subsequent to the BFU-E. That BFU-E are able to differentiate to erythroid bursts in vitro implies that the in vivo inhibition is potentially reversible. Inhibition at the CFU-E or proerythroblast level of differentiation would be consistent with the morphology of marrow aspirates that suggests persisting proerythroblasts. As feline CFU-E are detectable as well-hemoglobinized colonies by day 3 in culture, it is likely that there are few cell divisions between CFU-E and erythroblasts in cats. This implies that CFU-E may not be expressed in vitro as recognizable erythroid colonies if the hemoglobinization of developing proerythroblasts were impaired.

Our observations contrast the results of other groups. In studies by Onions et al. (16) and Testa et al. (17), the number of BFU-E detected in marrow cultures from neonatal cats decreased 2–3 wk following infection with FeLV-C/Sarma and the hematocrit decreased at 6–7 wk. Similar results were obtained by Gasper et al. (26) in studies of PRCA in steroid-suppressed adolescent domestic cats infected with FeLV-KT. These authors postulated a direct effect of virus on early erythroid progenitors that resulted in a decline in subsequent erythropoiesis and produced severe anemia. The reasons for the differences between our results and those previously reported are presently unknown.

More importantly, however, we have described an experimental system in which issues such as clonal dominance in neoplasia and the influence of virus on hematopoietic precursor number and differentiation may be addressed. As the G-6-PD type of a cell is not related to a viral disease process or transforming event, but is fixed in embryogenesis, the analysis of G-6-PD type independently evaluates clonal evolution. Hybrid cats become viremic with FeLV and develop clinical disease similar to naturally and experimentally infected domestic cats. G-6-PD heterozygous animals may be studied repeatedly over time so that each animal serves as its own control. As an initial application of this model of G-6-PD cellular mosaicism, we have shown that FeLV-induced PRCA does not result from the clonal expansion of an abnormal myeloid stem cell. This model should be applicable to the study of clonal evolution in vivo and the interactions of normal and neoplastic cells in vitro in other experimental disease states.

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