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Behavior of hematopoietic stem cells in a large animal

(autologous transplantation/clonal hematopoiesis)

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ABSTRACT **To study the behavior of hematopoietic stem cells *in vivo*, we transplanted glucose-6-phosphate dehydrogenase (G6PD) heterozygous (female Safari) cats with small amounts of autologous marrow. The G6PD phenotypes of erythroid burst-forming units and granulocyte/macrophage colony-forming units were repeatedly assayed for 3.5–6 years after transplantation to track contributions of stem cell clones to the progenitor cell compartment. Two phases of stem cell kinetics were observed, which were similar to the pattern reported in comparable murine studies. Initially there were significant fluctuations in contributions of stem cell clones. Later clonal contributions to hematopoiesis stabilized. The initial phase of clonal disequilibrium, however, extended for 1–4.5 years (and not 2–6 months as seen in murine experiments). After this subsided, all progenitor cells from some animals expressed a single parental G6PD phenotype, suggesting that blood cell production could be stably maintained by the progeny of one (or a few) cells. As the hematopoietic demand of a cat (i.e., number of blood cells produced per lifetime) is over 600 times that of a mouse, this provides evidence that an individual hematopoietic stem cell has a vast self-renewal and/or proliferative capacity. The long phase of clonal instability may reflect the time required for stem cells to replicate sufficiently to reconstitute a large stem cell reserve.**

Hematopoiesis is the process by which stem cells differentiate into functional granulocytes, red cells, platelets, monocytes, and lymphocytes. Although certain cell surface determinants and proliferation characteristics of hematopoietic stem cells have been identified, these cells are generally defined by their ability to reconstitute and maintain blood cell production *in vivo.* Studies in mice, using competitive repopulation assays and/or retroviral tagging, have shown that a single donor cell can reconstitute hematopoiesis after lethal irradiation of the host animal (reviewed in ref. 1). It is likely that the proliferative potential of such a cell is sufficient to maintain all hematopoiesis throughout the mouse's lifetime. In other studies, the kinetics by which stem cells reconstitute hematopoiesis after marrow transplantation have been assessed (2–8). When small numbers of syngeneic cells are infused, there is an initial phase, which extends for 2–6 months after transplantation, in which multiple clones contribute sequentially to blood cell production. After that time, one or a few clones can dominate. This has led to the assumption that the unstable phase is brief and that subsequent hematopoiesis can be maintained by the progeny of one or few cells that have restored (perhaps via self-replication) the hematopoietic stem cell reserve (2). Serial transplantation studies also support the concept that the stem cell compartment has recovered by 6 months (but not 1 month) after transplantation (9). Many strategies for clinical transplantation and gene therapy are premised on the concept that similar stem cell kinetics exist in man.

As shown in Table 1, the hematopoietic demand of a mouse is very small. A typical mouse (25 g) makes, in a 2-year lifetime, the same number of blood cells as does man in 1 day, raising the possibility that the stem cell kinetics in large animals are more complex. To investigate the evolutionary adaptation to increased size and longevity, we have studied stem cell behavior in cats heterozygous for the X chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD). A cat, in 8 days, makes as many red cells as the mouse over its lifetime (see Table 1).

Safari cats are the F_1 offspring of Geoffroy cats (a South American wild cat) and domestic cats (of Eurasian origin). These cats have electrophoretically distinct phenotypes of the X chromosome-linked enzyme G-6-PD (13). Because of X chromosome inactivation during embryogenesis, female Safari cats are cellular mosaics, and some of their somatic cells contain domestic-type G6PD, while other cells contain Geoffroy-type G6PD. In previous (control) studies of untransplanted cats, the percentage of progenitors with domestic-type G6PD did not vary significantly during 6 years of observations (P values < 0.05 with χ^2 analyses), suggesting that unperturbed hematopoiesis was polyclonal and stable (14). Similar results have been reported in studies of man and mouse (15, 16).

For this report, female Safari cats were transplanted with small numbers of autologous marrow cells. In this way, hematopoiesis was reestablished from a limited stem cell pool. By studying the G6PD phenotype of progenitor cells, the varying contributions of stem cells to this compartment were observed for 3.5–6 years after engraftment.

MATERIALS AND METHODS

Transplantation Procedure. F_1 female offspring of Geoffroy male × domestic female cat matings were used for these studies. After marrow harvest, the cats (n = 6) received 920 centigrays of total body irradiation, and $1–2 \times 10^7$ autologous buffy coat cells (i.e., nucleated marrow cells) were reinfused. Prophylactic antibiotics were provided from day −7 until the time of engraftment, and transfusions of irradiated blood were given as needed for platelet support. Transplanted animals engrafted by week 3–4 (granulocytes > 500/μl), whereas irradiated control animals failed to regenerate hematopoiesis, and these animals were sacrificed while persistently pancytopenic. After hematologic recovery, repeated marrow aspirates were obtained for analysis of the G6PD phenotype of erythroid burst-forming units (BFU-E) and granulocyte/

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Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; SCF, stem cell factor; rh, recombinant human; rc, recombinant canine; CFU-GM, granulocyte/macrophage colony-forming unit; BFU-E, erythroid burst-forming unit; Ep, erythropoietin.

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Table 1. Hematopoietic demand of different species

Species	RBC per ml	Weight	Blood volume, ml	RBC lifespan, days	RBC per day	Life expectancy, years	RBC per life of animal
Mouse	9.0×10^9	25 g	1.8	50	3.2×10^8	2	$\underline{2.4 \times 10^{11}}$
Man	6.0×10^9	70 kg	4900	120	$\underline{2.5 \times 10^{11}}$	80	7.3×10^{15}
Cat	7.5×10^9	4 kg	280	70	$\underline{3.0 \times 10^{10}}$	15	1.6×10^{14}

Data for this chart were derived from refs. 10–12. A typical adult Safari female cat also weighs 4 kg. RBC, red blood cells. The underlined values indicate that a mouse during its lifetime makes as many red blood cells as a man in 1 day or a cat in 8 days.

macrophage colony-forming units (CFU-GM). Aspirates were obtained from the humerus, femur, or pelvic brim after ketamine and azepromazine anesthesia. Since cats are genetically variable (in contrast to congenic mice), these data provide six independent *in vivo* analyses of hematopoietic stem cell kinetics.

Data presented from cat 40005 are from a second transplantation (using 2×10^7 cells cryopreserved prior to the initial radiation exposure per kg). As cat 40005 received irradiation on two occasions, it is especially likely that the clone or clones that maintain its hematopoiesis derive from transplanted cells (6, 8) and not endogenous cells resistant to the preparative regimens. In other circumstances (cats 40004, 40006, 40665, 40821, and 40823), freshly harvested cells were transplanted. These were unmanipulated and spent at maximum 4 hours *ex vivo* during which time the cat was irradiated. Additional methodologic details are in ref. 14.

Laboratory Analysis. The procedures of the methylcellulose culture of feline hematopoietic progenitor cells are in refs. 9 and 17. For later studies, the cultures were supplemented with recombinant human (rh) erythropoietin (Ep; 0.5 unit/ml; Amgen) and recombinant canine (rc) stem cell factor (SCF; 2 ng/ml; Amgen) and not 5% FEF-A cell-conditioned medium. BFU-E- and CFU-GM-derived colonies were enumerated after 10 days of culture at 37°C in 5% CO_2/95% air. Individual colonies were aspirated into micropipettes. Freeze/thaw lysates were placed on polyacrylamide gels and subjected to isoelectric focusing (3 W per gel for 2.5 hr). The gels were then stained for G6PD activity, and the phenotypes of progenitors were deduced from the locations of the bands (13, 14). This technique is sufficiently sensitive that the G6PD phenotype of 8 granulocyte/macrophage cells or 50 erythroid cells can be assessed (13). In all determinations in all animals, the percentage of BFU-E with domestic-type G6PD was similar to the percentage of CFU-GM with domestic-type G6PD, confirming that lineage commitment is a stochastic event. This ratio was also equivalent to that seen among the red cells, granulocytes, and lymphocytes in the peripheral blood of individual animals (13).

Values in the figures are presented as the percent of progenitors (BFU-E and CFU-GM) with domestic-type G6PD ± SE of the observation. Sixty to 80 colonies were typically analyzed at each observation time. Because BFU-E and CFU-GM present soon after transplantation could represent reconstitution by progenitor cells present in the marrow inoculum (as well as the differentiation of infused stem cells), all analyses were performed using data beginning 10 weeks after transplantation. By this time, it appeared that the entire progenitor cell compartment was stably reconstituted because frequencies of BFU-E and CFU-GM, and their cell cycle kinetics (as determined by [^{3}H]thymidine suicide experiments), were normal (14).

Methods for the separation of feline red cells and granulocytes from blood are in ref. 13. To assay the percentage of cells with a domestic G6PD phenotype, lysates of 8×10^4 red cells or 10^4 granulocytes were placed in each lane, respectively. After isoelectric focusing and staining for G6PD activity, the relative intensities of bands were visually compared to that obtained when mixes of red cells or granulocytes of Geoffroy and domestic origin containing 0%, 1%, 2%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, and 100% Geoffroy cells were studied. For all blood T-cell analyses, mononuclear (density < 1.070 g/ml) blood cells were exposed to concanavalin A (5 μg/ml) and then expanded in the presence of human recombinant interleukin 2 (100 units/ml; Hoffmann–La Roche) for 4 days. Over 95% of resulting cells were CD5-positive (with fluorescence-activated cell sorting analysis using a monoclonal antibody obtained from Southern Biotechnology Associates). Lysates of 12×10^4 cells were analyzed per lane, and band intensities were quantitated by comparison to simultaneously studied mixtures of concanavalin A/interleukin 2-expanded Geoffroy and domestic T cells.

For the long-term marrow culture of feline progenitor cells, 20×10^6 buffy coat marrow cells were cultured in 8 ml of medium (14) supplemented with rcSCF (5 ng/ml) and 10 μM hydrocortisone. The 25-cm^2 flasks were incubated at 37°C in 5% CO_2/95% air, fed twice each week, and demidepopulated once weekly. At these times, nonadherent cells were cultured in methylcellulose, and GM colonies were analyzed for the G6PD phenotype (13). Alternatively, low-density (<1.060 g/ml) marrow mononuclear cells were placed on preformed irradiated allogeneic feeder layers using the long-term marrow culture methods of ref. 17. To determine the dose–response of progenitors to Ep and SCF, cultures were done in the presence of 0, 0.01, 0.025, 0.05, 0.1, 0.3, 0.6, 1.0, and 5.0 units of rhEp per ml or 1 unit of rhEp per ml with 0, 0.1, 0.5, 1.0, 2.0, 5.0, and 10.0 ng of rcSCF per ml. CFU-E-, BFU-E- and CFU-GM-derived colonies were enumerated after 3, 10, and 10 days of incubation, respectively.

RESULTS

Clinical Observations. After irradiation and transplantation, all cats became pancytopenic but recovered normal peripheral blood counts by 4–6 weeks. Complete blood counts were generally monitored every 3–4 weeks after hematologic reconstitution, and all animals remained hematologically normal throughout the duration of the study (3.5–6 years).

G6PD Analyses of Progenitor Cells. The data for three animals (40004, 40005, and 40006) that were obtained during the first 1–1.5 years after transplantation have been reported (14). Wide fluctuations in G6PD phenotypes of progenitors were seen between repeated samples, and P values were $<10^{-7}$ (when the hypothesis that proportions were constant over time was tested with χ^2 analyses) (Fig. 1*A* and *B* and ref. 14). As the variation was patterned (and did not appear sporadic or random), these data supported the hypothesis that hematopoiesis was maintained by small and changing populations of stem cell clones (14). Significant fluctuations were also seen during the initial studies of cats 40628, 40629, and 40665 (Fig. 2).

During the long-term follow-up, the fluctuation in the G6PD phenotype of progenitors stabilized in all animals. Stabilization occurred between 1 and 4.5 years after transplantation (Table 2). Complete data for cats 40004, 40005, and 40665 are presented in Figs. 1*C*, 1*D*, and 2, respectively.

FIG. 1. Clonal contributions to hematopoiesis after the autologous marrow transplantations of cats 40004 and 40005. Data obtained during the first 1.5 years after transplantation for cats 40004 and 40005 are presented in *A* and *B*, respectively. The percentage of progenitors with domestic-type G6PD ± SE of the determination is plotted. Variations about the mean values were highly significant in the posttransplantation periods (e.g., $P < 10^{-7}$, with χ^2 analyses). Extensions of studies of cats 40004 and 40005 are in *C* and *D*, respectively. The variation in the G6PD phenotype of progenitors about its mean subsided during these extended observations. After week 240, 90–100% of progenitors from cat 40005 contained domestic-type G6PD (*D*).

Observations in cat 40005 (Fig. 1*D*) are particularly instructive. During the first 78 weeks after transplantation, the percentage of progenitors with domestic-type G6PD varied between 4% and 98%. From week 78 to week 236, this percentage fluctuated less widely, but the variations still exceeded variability that would be consistent with a binomial distribution. After 236 weeks, however, no significant variation was seen, and 90–100% of progenitors (i.e., essentially all progenitors) contained domestic-type G6PD. These results suggest that the stem cell reserve had been reconstituted by the progeny of one (or a few) stem cells, each of which contained domestic-type G6PD.

A second cat (40006) reconstituted mostly with cells containing the Geoffroy-type G6PD (10% ± 4% of its progenitors expressed domestic-type G6PD in 38 separate observations during weeks 77–335 after transplantation), whereas other animals have stabilized with intermediate values (e.g., cat 40004, Fig. 1*C* and cat 40665, Fig. 2). In each cat studied, the mean percentage of progenitors with domestic-type G6PD in the observations obtained after the clonal disequilibrium subsided was different than the mean percentage observed prior to transplantation ($P < 10^{-6}$ using a z test for comparing binomial proportions).

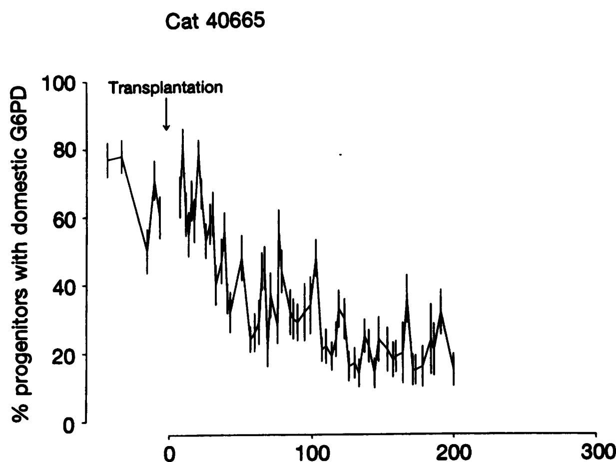

FIG. 2. Clonal contributions to hematopoiesis after the autologous marrow transplantation of cat 40665. The variation in the G6PD phenotype of progenitors around its mean subsided after week 107 following transplantation.

G6PD Analysis of Peripheral Blood Cells. Fig. 3 contains an analysis of peripheral blood cells from cat 40005, obtained at week 280 after transplantation. Eighty to 90% of T cells contained domestic-type G6PD. This is an expected observation because of the long life span of circulating T cells *in vivo*. Samples of red cells (data not shown) and of granulocytes (Fig. 3*B*) contained only domestic-type G6PD, confirming clonal

Table 2. Length of the unstable phase of stem cell contributions to hematopoiesis

Cat	Time when clonal disequilibrium stops, weeks
40004	52
40005	236
40006	77
40628	98
40629	103
40665	107

These data were computed with repeated χ^2 analyses beginning at 10 weeks after transplantation and including data from all subsequent observations. The time when clonal disequilibrium stops was established as the timepoint where subsequent determinations showed nonsignificant variation about the mean value for the remaining data. To assure the validity of this assessment, all calculations were repeated excluding the computed binomial variation rejection point from the data set. In all circumstances (except cat 40004), this manipulation identified the preceding data point as the new binomial variation rejection point. In studies of cat 40004 (see Fig. 1*C*) when week 273 was excluded from sequential χ^2 analyses, it appeared that the clonal disequilibrium ceased at week 52. Week 52 is therefore recorded in the table as the time when criteria were met. Data from the control (untransplanted) cats ($n = 6$) never exceeded binomial variability with sequential χ^2 analyses.

dominance. Only 5–15% of T cells, red cells, and granulocytes from cat 40006, obtained at weeks 305–316, contained domestic-type G6PD (data not shown).

Other Studies. Because clonal dominance often reflects neoplasia (e.g., a myeloproliferative disorder), studies were done to independently confirm that the clones maintaining hematopoiesis in cats 40005 and 40006 were normal. First, the behavior of marrow cells from cats 40005 (obtained at weeks 296 and 303 after transplantation) and 40006 (obtained at week 322) was assessed in long-term marrow culture to determine if progenitors that derived from a nondominant clone preferentially expanded in this circumstance. Only 4–10% of nonadherent CFU-GM sampled at weeks 1, 2, 3, and 4 from cultures of marrow cells from cat 40006 had domestic-type G6PD (25–53 colonies analyzed per data point). Similarly, in cultures of marrow cells from cat 40005, 95–100% of nonadherent CFU-GM contained domestic-type G6PD (10–62 colonies analyzed per data point). In addition, the frequencies of BFU-E (e.g., 33 of 10^5 and 43 of 10^5 marrow mononuclear cells at weeks 292 and 331, respectively) and CFU-GM (22 of 10^5 and 31 of 10^5 marrow mononuclear cells) from cats 40005 and 40006 were normal. Similarly, the dose–response of progenitors to Ep and SCF were equivalent to controls (data not shown). The morphology of Wright–Giemsa-stained marrow aspirates was normal. Cat 40005 developed a neck mass and hypercalcemia and was sacrificed at week 303. Postmortem examination revealed a malignant fibrous histiocytoma, an uncommon finding in cats, thought to be secondary to radiation exposure. There was no metastatic disease and specifically no involvement of the marrow or spleen with this tumor. The morphology of cells in this marrow biopsy was normal, and the marrow cellularity was normal. Taken together, these data suggest that the clones maintaining hematopoiesis in cats 40005 and 40006 were of normal, and not of neoplastic, origin (18–20).

DISCUSSION

A few instances of skewed clonal contributions to hematopoiesis have been reported after human allogeneic marrow transplantation [i.e., four cases among the 40 allogeneic transplantations studied (21–24)]. Although these cases raise the possibility that a single or few normal (nonneoplastic) stem cell

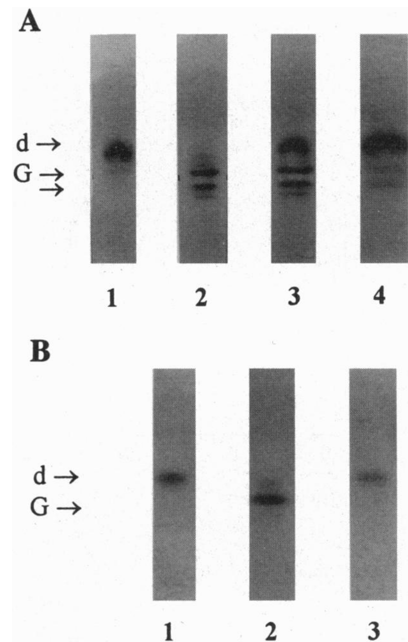

FIG. 3. G6PD analyses of peripheral blood cells. (*A*) Peripheral blood T cells from a control domestic cat (lane 1), a Geoffroy cat (lane 2), and a 1:1 mixture of these cell populations (lane 3). Lane 4, T cells from cat 40005 obtained 280 weeks after transplantation. Between 80% and 90% of these cells contained domestic-type G6PD. For these determinations, cells were expanded with concanavalin A and recombinant human interleukin 2. (*B*) Comparable studies of granulocytes. Lane 1, granulocytes from a domestic cat; lane 2, granulocytes from a Geoffroy cat; lane 3, granulocytes from cat 40005 obtained 280 weeks after transplantation. These cells were tested directly [after separation with dextran flotation (10)]. No Geoffroy band is visible. In mixing experiments with granulocytes of Geoffroy and domestic cat origin, 2–5% Geoffroy-type cells can be detected (data not shown). With similar analyses, all red cells from cat 40005 at 280 weeks after transplantation contained domestic-type G6PD (data not shown).

clones can dominate hematopoiesis in man, coexistent clinical issues (e.g., failed engraftment, graft-vs.-host disease, and recurrent leukemia) and the lack of longitudinal studies over long time intervals make their interpretation difficult. In addition, the low incidence of monoclonal or oligoclonal hematopoiesis may result from the relatively large numbers of cells (>2 × 10^8 buffy coat cells per kg) infused in clinical transplantations (22).

To study stem cell contributions to hematopoiesis in a controlled experimental setting, six G6PD heterozygous cats received autologous transplantations containing small numbers of marrow cells (1–2 × 10^7 buffy coat cells per kg). In this way, animals were forced to reconstitute their hematopoiesis from a limited number of transplanted cells. Because observations could be performed repeatedly and over long periods of time, the kinetics by which stem cells repopulated and then maintained steady-state hematopoiesis was assessed. By 10 weeks after transplantation, the peripheral blood counts, the frequencies of marrow progenitor cells, and their cell cycle kinetics were normal (13), suggesting that the progenitor cell compartment was entirely regenerated. However, the contributions of stem cells to hematopoiesis varied, as significant fluctuations were seen in the G6PD phenotypes of BFU-E and CFU-GM in all animals. In cat 40005, the percentage of progenitors with a domestic G6PD phenotype varied as widely as 4% to 98%. After 1–4.5 years (Table 2), however, this unstable phase stem cell kinetics ceased, and stable contributions of stem cell clones were observed throughout the remaining study period. Because the average percentage of progenitors with domestic-type G6PD in individual cats was significantly different from that observed prior to transplan-

tation, it is likely that hematopoiesis was maintained by the progeny of relatively few cells, as anticipated from the experimental design.

These observations suggest that the pattern of stem cell kinetics after transplantation in cats is analogous to the pattern of stem cell kinetics after transplantation in mice. There is an initial phase of clonal disequilibrium followed by a second phase in which stem cell contributions to hematopoiesis remain stable. The time frame, however, is disparate. Clonal disequilibrium extends for 1–4.5 years when cats are transplanted with small numbers of autologous cells and not 2–6 months as seen in murine experiments (2–4, 6, 7). We hypothesize that this is the length of time that is required for transplanted stem cells to replicate (i.e., self-renew) and completely reconstitute the large hematopoietic stem cell reserve. These kinetics, when extrapolated to man, could complicate strategies for gene therapy targeting hematopoietic stem cells.

As importantly, data from cats 40005 (Fig. 1*D*) and 40006 suggest that a single (or few) clones can stably maintain all hematopoiesis in cats. Given the large hematopoietic demand in cats (vs. mice, see Table 1), these data imply that a normal hematopoietic stem cell has an enormous self-renewal and/or proliferative potential.

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