Evidence for the maintenance of hematopoiesis in a large animal by the sequential activation of stem-cell clones

(sequential activation of stem-cell clones/cats)

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ABSTRACT To test if hematopoiesis can be maintained by the sequential activation of stem-cell clones, we performed autologous marrow transplantations with limited numbers of cells in cats heterozygous for the X chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) and observed the G6PD phenotypes of erythroid and granulocyte/macrophage progenitors over time. The animals were the female offspring of Geoffroy male and domestic female cats. In repeated studies of marrow from control animals (n = 5) or experimental animals prior to transplantation (n = 3), the percent of progenitors with domestic-type G6PD did not vary. After transplantation, the peripheral blood counts, marrow morphologies, frequencies of progenitors, and progenitor cell cycle kinetics returned to normal. However, abrupt and significant fluctuations were seen in the G6PD type of progenitors from each cat during the 1–1.5 years of observation. These data cannot be explained if there were either a large or constant population of active stem cells and thus imply, in a large-animal system, that hematopoiesis was maintained through clonal succession. A stochastic model was developed to estimate the numbers of active clones and their mean lifetimes.

Two theories describe early hematopoietic stem-cell differentiation. The first states that stem cells may contribute indefinitely to hematopoiesis. The second theory, the theory of clonal succession, initially proposed by Kay (1), states that hematopoiesis is maintained by a subset of active stem cells and a dormant reserve. As clones are depleted, perhaps through terminal differentiation, reserve cells become active. These theories have been difficult to test experimentally and transplantation studies in mice with enzymatically or retrovirally marked cells present evidence both for (2–5) or against (6–8) clonal succession. Many murine experiments are limited by difficulties obtaining samples for repeated analysis over time. Also, when the infection of marrow cells with retroviral vectors is used to mark clonal origin, it is difficult to distinguish the contribution of the earliest stem cells from that of cells with a more restricted proliferative potential. In addition, the growth characteristics of a stem cell may change during in vitro incubation (9). For these reasons and to extend the observations to a large-animal system, we studied cats heterozygous for isotypes of the X chromosome-linked enzyme glucose-6-phosphate dehydrogenase (G6PD).

Geoffroy cats (Leopardus geoffroyi, South American origin) and domestic cats (Felis catus, Euroasian origin) have evolved independently for 12 million years (10) and have electrophoretically distinct G6PD types (11). These animals were bred to produce female F₁ (Safari) cats that were obligate G6PD heterozygotes. In previous studies, we demonstrated that individual colonies derived from erythroid progenitors (burst-forming units-erythroid; BFU-E) or granulocyte/macrophage progenitors (colony-forming units-granulocyte/macrophage, CFU-GM) contained domestic-type G6PD (d-G6PD) or Geoffroy-type G6PD (G-G6PD), and not both (11), confirming that the structural locus for feline G6PD was on the X chromosome. The ratio of BFU-E with d-G6PD to BFU-E with G-G6PD from each cat was equivalent to ratios of G6PD phenotypes among CFU-GM, erythrocytes, granulocytes, lymphocytes, platelets, and skin fibroblasts (11), which suggested that X chromosome inactivation occurred during embryogenesis. In this study, we show that there is no change in the percent of hematopoietic progenitors with d-G6PD when marrow cells from individual cats are analyzed repeatedly over >5 years. Thus, the locus for feline G6PD is randomly and stably inactivated in somatic cells, a necessary prerequisite for studies of clonal evolution.

These initial observations further suggest that either many stem-cell clones or a small, yet constant, population of clones must support hematopoiesis and, in this setting of uncompromised marrow reserve, neither support nor deny clonal succession. Therefore, we performed autologous marrow transplantations in three female Safari cats to observe the behavior of normal stem cells under the circumstance of a more limited reserve. We reasoned that if hematopoiesis was maintained by a small number of stem-cell clones, the percent of marrow progenitors with d-G6PD might change. In addition, if active stem-cell clones were replaced periodically, the percent of domestic-type progenitors could cycle or vary with time.

MATERIALS AND METHODS

Marrow Culture and G6PD Analysis. Marrow cells, aspirated from the femur or humerus of G6PD heterozygous cats, were cultured in methylcellulose, by using methods described (11–14). Erythroid bursts and GM colonies were enumerated, individually transferred to polyacrylamide gels with micropipets (11), subjected to isoelectric focusing (3 W per gel for 2.5 hr), and then stained for G6PD activity. The G6PD phenotype of each colony (and thus of the BFU-E or CFU-GM from which it was derived) was determined by the location of bands on the gels (11). Methods for tritiated thymidine suicide studies have been reported (13).

Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; BFU-E, burst-forming units-erythroid; CFU-GM, colony-forming units-granulocyte/macrophages; d-G6PD, domestic-type G6PD; G-G6PD, Geoffroy-type G6PD.

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**Transplantation Protocol.** After repeated baseline studies and marrow harvest, each cat received 920 cGy of total body irradiation (7 cGy/min from opposing Cobalt-60 sources) and 1–2 × 10¹⁰ nucleated cells per kg. Marrow cells were reinfused intravenously within 4 hr of collection and were not manipulated *in vitro*. Buffy-coat cells were prepared from the excess harvested marrow and, after hypotonic lysis of erythrocytes, were cryopreserved [1 × 10⁶ cells/ml of fetal calf serum with 5% (vol/vol) dimethyl sulfoxide; controlled rate freezer, Union Carbide, Indianapolis; liquid nitrogen storage]. Infection prophylaxis included neomycin (5 mg/kg) and polymyxin (5000 units/kg), administered orally every 8 hr beginning 5 days prior to transplantation and continuing until hematologic recovery. After transplantation, the animals also received broad-spectrum antibiotics (s.c. ampicillin (6.6 mg/kg, every 12 hr) and gentamicin (4 mg/kg, every 12 hr for 1 day and then every day)). Transfusions of irradiated blood were given as needed for platelet support. If animals failed to engraft at 4 or 5 weeks (i.e., leukocytes, <0.5 × 10⁹ cells per liter; 0 polymorphonuclear leukocyte; platelets, <5 × 10⁹ cells per liter; and no precursor cells on marrow aspirates), additional (cryopreserved) marrow cells were infused. After hematologic recovery, marrow aspirates were obtained every 2 or 3 weeks to culture hematopoietic progenitors. As the percent of BFU-E with d-G6PD was similar to the percent of CFU-GM with d-G6PD in all analyses, the results are expressed as the percent of progenitors (BFU-E and CFU-GM) with d-G6PD (mean ± SEM).

**Stochastic Model.** A stochastic model was developed to estimate the number of active stem cells in each animal and the mean lifetimes of derivative clones. The model assumes that there is a stem-cell reserve with cells containing d- or G-G6PD. The proportion of cells with d-G6PD is p (thus the proportion of cells with G-G6PD is 1 − p). Under the model, N active stem-cell clones equally contribute to hematopoiesis throughout independent lifetimes in which the risk of **‘death’** is constant (λ). Progeny may enter and exit the G₀ phase of the cell cycle during the life span of each clone. When a stem cell **‘dies,’** a randomly selected previously dormant reserve cell is activated. Thus, the probability that a domestic-type cell is activated is p. The stochastic model has two components. One is unobserved [the number X(t) of N active stem-cell clones that have d-G6PD at time t (a finite state-space continuous-time Markov process)]; the other is observed [the numbers (Y(t)] of BFU-E and CFU-GM with d-G6PD in samples of nₙ progenitors at times tᵢ (i = 1, 2, . . . , k)]. Although the steady-state distribution of X(t) is binomial with parameters N and p, values at different times are dependent, with the strength of dependency a function of λ and N. For a given X(tᵢ), Yᵢ is a binomial random variable with parameters nₙ and Xᵢ/N. In a preliminary analysis by examining the proportions Yᵢ/nₙ, we confirmed that G6PD phenotype is a neutral marker that confers no selective advantage or disadvantage to a cell (15). Therefore, p was defined as the percent of domestic-type progenitors prior to transplantation, and likelihood surfaces for parameters N and mean lifetime (L = 1/λ) were generated by recursive updating (for technical details, see Guttrop et al. (15, 16)).

**RESULTS**

**Baseline Studies.** Baseline studies established that the X chromosome-linked locus for feline G6PD was randomly and stably inactivated in somatic cells. Among 44 cats, the percent of progenitors with d-G6PD ranged from 25 to 72% [49 ± 11% (mean ± SD)]. The percents of BFU-E and CFU-GM with d-G6PD were 47 ± 13% and 49 ± 11% (mean ± SD), respectively. When marrow cells from individual cats (n = 5) were analyzed repeatedly for >5 years, there was no significant change in the percent of hematopoietic progenitors with d-G6PD. A representative study is shown in Fig. 1a.

**Autologous Transplantations.** Autologous marrow transplantations were performed in three female Safari cats. Prior to transplantation, 46 ± 3% (mean ± SEM) of progenitors from the first cat (40004) expressed d-G6PD (Fig. 1b). As seen in control cats, the variability in the percent of progenitors with d-G6PD was consistent with binomial variation about a common mean and thus was not significant (P = 0.7). In contrast, after transplantation with presumably small numbers of autologous stem cells, there were wide and significant fluctuations in the percent of progenitors with d-G6PD (18–68%, P < 10⁻⁵). More importantly, the variation was not sporadic but rather was patterned over time. To confirm this variation represented temporal variation in stem-cell activity and not geographic **‘patches’** of clonal hematopoiesis at different sites, marrow aspirates, obtained at week 41 from all four limbs, were independently analyzed. The percents of progenitors with d-G6PD were 32 ± 8%, 29 ± 4%, 42 ± 7%, and 29 ± 8% (mean ± SEM) from the right humerus, left humerus, right femur, and left femur, respectively. As P values were >0.1, with paired χ² tests, marrow obtained at a single site was equivalent to that throughout the animal (17). Therefore, in contrast to autologous transplantation from the second cat (40005), there was no significant variation in gG6PD with irradiation and transplantation, 35 ± 2% of progenitors in this animal expressed d-G6PD. After transplantation, this cat was pancytopenic for <1 week and required no blood product support. Although the mean percent of progenitors expressing d-G6PD changed (19 ± 1%, P < 10⁻³), variation about the posttransplantation mean was not significant (P = 0.16). At 54 weeks, more marrow was harvested, the cat was irradiated again, and 1 × 10⁷ nucleated cells per kg were infused. The cat failed to engraft with this inoculum or with an additional 1 × 10⁷ cryopreserved cells per kg from this second harvest, suggesting that the marrow had a greatly reduced content of cells capable of hematopoietic reconstitution. The cat then received 2 × 10⁷ cells per kg, cryopreserved prior to the first radiation exposure, and recovered normal blood counts. Subsequent marrow aspirates contained rare (4–10%) BFU-E or CFU-GM with d-G6PD. At week 18, however, there was an abrupt change with 53 ± 3% of progenitors expressing d-G6PD, followed by a progressive increase to 84 ± 4% at week 40. Variation about the posttransplant mean was thus significant (P < 10⁻⁶). The outlying value (15 ± 3% domestic-type progenitors) observed in marrow from the left humerus at week 20 was thought to represent a **‘patch’** effect during the abrupt transition time. At 28 weeks, marrow was obtained from both the left humerus and the right femur. Analyses showed 56 ± 6% and 66 ± 6% domestic-type progenitors, respectively (P > 0.1), demonstrating that a **‘patch’** effect, if previously present, did not persist.

The third cat (40006) received 7.5 × 10⁹ nucleated marrow cells per kg, failed to engraft, and then received 2 × 10⁷ cryopreserved cells per kg. Variation in the G6PD phenotype of progenitors after transplantation was again significant (P = 0.007, Fig. 1d), consistent with clonal succession.

In each transplanted cat, the percent of progenitors with d-G6PD during the 10 weeks directly after marrow transplantation was unchanged from that observed prior to transplantation (Fig. 1 b–d). For this reason, all data were analyzed beginning 10 weeks after transplantation. Also, by this time, the peripheral blood counts, the frequencies of BFU-E and CFU-GM in marrow aspirates, and their cell cycle kinetics, as determined by tritiated thymidine suicide studies, were normal. These values remained normal throughout the period of observation. For instance, the percents of BFU-E and CFU-GM in DNA synthesis were 34 and 24%, 39 and 12%, 35 and 19%, before transplantation and 14 and 28 weeks after transplantation, respectively, in cat 3 (40006). Thus, there
were no discernible abnormalities of the committed progenitor cell pool.

Estimation of the Number of Active Stem-Cell Clones and Their Mean Lifetimes. A birth and death model was developed to estimate the number of active stem cells \( (N) \) and the mean lifetime \( (L) \). For data from the first cat, the model predicted 7 active stem cells with an average life span of 26 weeks (Fig. 2b). Limits of two log likelihoods, \( \pm 95\% \) confidence limits, were 6–26 active stem cells and a mean lifetime of 7–60 weeks (Fig. 2b). Likelihood surfaces for data from cats 2 and 3 had similar topography (Fig. 2 c and d). Cumulative results are shown in Table 1.

When similar analyses were applied to data obtained from normal cats, the likelihood curves were flat (Fig. 2a and Table 1) and predicted \( N > 40 \) active stem cells or \( L > 300 \) weeks.

**DISCUSSION**

Clonal succession, though studied extensively in mice (2–8), has been less frequently evaluated in large animal systems. Studies by Nash et al. (18) and Turhan et al. (19) analyzed granulocytes from patients who had received allogeneic marrow with distinct restriction fragment length polymorphisms of the X chromosome. After transplantations with large numbers of normal donor cells (>2 \( \times 10^8 \) buffy-coat cells per kg), oligoclonal reconstitution occurred infrequently (0/20 and 2/12 patients studied) and data were insufficient to evaluate clonal succession.

In previous experiments, we treated G6PD heterozygous cats with dimethylbusulfan (20). This led to a significant variation in the percent of domestic-type progenitors about the postchemotherapy mean in three of six animals. As dimethylbusulfan, an alkylating agent, could damage as well as deplete stem cells, we proceeded with studies of autologous transplantation.

For the first 10–12 weeks directly after transplantation, the percent of progenitors with d-G6PD was unchanged from that observed prior to transplantation in each cat (Fig. 1 b–d). These data are thus consistent with observations in mice (3–8, 21) and suggest that many cells with a restricted potential were present in the marrow inoculum and initially reconstituted hematopoiesis (21).

After this time, the percents of progenitors with d-G6PD fluctuated widely. The variation, however, was not random but rather was patterned over time (Fig. 1 b–d), suggesting the numbers of stem-cell clones successively rise and decline. Because the variation was in excess of that explained by a binomial distribution about a common mean \( (P \text{ values } 7 \times 10^{-3} \text{ to } <1 \times 10^{-7}) \), the data implied that a small and changing population of stem-cell clones contributed to hematopoiesis.
The kinetics of early stem-cell differentiation was further explored with mathematical analyses. A stochastic model was feasible because all progenitor cells carried the label of the earliest active stem cell from which they derived and because precise values for the percents of progenitors with d-G6PD could be determined at sequential time intervals. Log likelihood curves predicted small numbers of active stem cells after transplantation in each cat. As the observed data from cats 1 and 2 (after the second transplantation) could not be explained with mean lifetimes of >3 years (log likelihood units of less than −4; Fig. 2 b and c), this analysis required a finite lifetime for active stem cells and hence clonal succession.

In addition, the maximum likelihood estimates (Table 1) may collectively suggest that stem-cell clones have a defined proliferative potential (e.g., number of divisions or number of offspring per lifetime) (22). When 6–8 clones contributed to hematopoiesis [cats 1, 2 (after the second transplantation), and 3] each clone supported 1/8 to 1/6 (13–17%) of the total blood cell production and the mean lifetimes were short (<80 weeks) (Fig. 2 and Table 1). When there were 11 active clones (cat 2 after the first transplantation), each supported 9% of hematopoiesis and \( L \) was 150 weeks. This interpretation might also be extended to observations in control cats (Fig.

![Diagram](image-url)

Fig. 2. Log-likelihood surfaces for the number of active stem cells (\( N \)) and the mean lifetimes (\( L \)). On these topographical maps of log likelihood minus its maximum, less-negative numbers represent more likely results. Modes are denoted by solid circles, with larger solid circles corresponding to higher peaks. (a) Normal cat (63848). The curve is flat with increasing likelihood as \( N \) and \( L \) increase. (b) Cat 1 (40004). Peak likelihood is \( N = 7 \) and \( L = 26 \) weeks. This contour map differs from that for normal cats and has well defined ridges near the coordinate axes. It is topographically similar to maps in c and d. (c) Cat 2 (40005) after the second transplantation. Peak likelihood is \( N = 6 \) and \( L = 7 \) weeks. When the week-20 outlier value is removed prior to mathematical analysis, the map is nearly equivalent and peak values occur at \( N = 8 \) and \( L = 17 \) weeks (15). The contour of the likelihood surface for data obtained after the first transplantation is also similar. Although \( N \) is well defined (peak \( N = 11 \), log likelihood units greater than −2 for \( N = 6 \) to 33), there is a wide range of possible lifetimes (peak \( L = 150 \) weeks, log likelihood units greater than −2 for \( L > 28 \) weeks) (15). (d) Cat 3 (40006). Peak likelihood occurs at \( N = 7 \) and \( L = 80 \) weeks.

### Table 1. Peak likelihood values predicted by the stochastic model

<table>
<thead>
<tr>
<th>Animal</th>
<th>Active stem-cell clones, ( N )</th>
<th>Mean lifetime, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td></td>
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<tr>
<td>a</td>
<td>&gt;40</td>
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<td>b</td>
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<td>Cat 1</td>
<td>7</td>
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<tr>
<td>Cat 2</td>
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<tr>
<td>T1</td>
<td>11</td>
<td>150</td>
</tr>
<tr>
<td>T2</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Cat 3</td>
<td>7</td>
<td>80</td>
</tr>
</tbody>
</table>

Maximum values for normal controls a–e are outside the grid of \( N = 0 \) to 40 by \( L = 0 \) to 300 weeks. T1 and T2, first and second transplants, respectively.
would imply that clonal contributions to normal unperturbed hematopoiesis were constant for long periods of time, consistent with murine (23) and human (24) data. If true, the detection of clonal succession in this setting would be difficult, or impossible, with any methodology, and approaches such as transplantation or chemotherapy to reduce stem-cell reserve would be required. The hypothesis also predicts that in an animal with a small blood volume, such as a mouse, one or few reconstituting clones could continuously maintain hematopoiesis throughout its 1–2 year life span, as suggested by others (3, 7, 8). With studies from additional cats we will determine if there is a direct correlation between estimated values for \( N \) and \( L \).

Our data are also compatible with the hypothesis that the activation of a dormant hematopoietic stem cell, like its subsequent differentiation (25, 26), is a stochastic event. This may be testable with repeated studies 2–5 years after transplantation. If activation occurs with a probability that is an intrinsic property of a stem cell, and not its environment, and should the number of dormant cells comprising the stem-cell reserve increase through self-renewal or through recovery over time, \( N \) must increase. In these circumstances, there should be less fluctuation in the percent of progenitors with d-G6PD during follow-up studies. In addition, if radiation-damaged stem cells recover sufficiently to contribute to hematopoiesis, the mean lifetime estimated from the follow-up data could decrease.

A mathematical model requires simplifying assumptions and its validity depends on the strength of those assumptions. With the assumption that \( N \) is constant from 10 weeks to 1.5 years after transplantation, we have simplified the more complex concept that the chance that a reserve cell is "born," or begins contributing to hematopoiesis, approximates the chance that an active cell "dies," or stops contributing to hematopoiesis. \( N \), however, could fluctuate widely, could increase, or could decrease over the time interval, necessitating a different approach. A modification of the model where \( N \) fluctuates slowly yields equivalent shorter values for \( L \) with data from cats 1–3 (16). One alternate analysis, a "constant death" model in which changes in the percent of progenitors with d-G6PD reflect "cell death" without activation of additional cells, cannot explain data in cat 2 (after its second transplantation).

The second assumption of the model is that all clones contribute equally to hematopoiesis. Although individual progeny of a stem cell may enter and exit the Go phase during the lifetime of the stem-cell clone, a "birth/death" model does not account for "infancy" or "old age" in which clones produce fewer BFU-E and CFU-GM. This concern, however, should not substantially impact the likelihood determination for \( N \) or \( L \) with \( N > 3 \) or with short intervals (e.g., <4 weeks) in which the relative contribution of an individual stem cell is decreased (16).

Given these caveats, the mathematical model provides a methodology to analyze the behavior of cells that cannot be observed directly and thus an insight into the process of early stem-cell differentiation. G6PD-heterozygous cats may be studied at baseline and after perturbations of hematopoiesis. In studies of normal cats, the G6PD phenotype of committed progenitors did not change over time. Either stem cells continuously contributed to hematopoiesis or there was a large and changing population of active clones. In contrast, a direct interpretation of the G6PD data after autologous transplantation and the mathematical analyses suggest that hematopoiesis in the setting of a limited marrow reserve is maintained through increasing and decreasing the numbers of stem-cell clones, which is evidence for clonal succession in a large-animal system. These observations have implications for strategies for gene transfer into autologous hematopoietic progenitor cells, for aggressive drug and/or radiation therapies, and for allogeneic or autologous transplantation of marrow compromised by prior therapy, purging techniques, or perhaps hematopoietic growth factor (e.g., granulocyte/macrophage or granulocyte colony-stimulating factor, or interleukin 3) administration.

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