HEMATOPOIETIC STEM CELL REGULATORY VOLUMES AS REVEALED IN STUDIES OF THE bg^J/bg^J:W/W^v CHIMERA*

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Foremost among the open questions of hematopoiesis is the nature of the mechanisms that govern the behavior of pluripotent stem cells. Several types of evidence suggest that stem cell activities are directed by local control systems. Local regulation is apparently expressed in the centripetal movement of active marrow during postnatal growth of many species, in the predilection of hematopoietic foci for endosteal surfaces (1), in the selective distribution of erythroid and granuloid colonies arising from stem cells implanted in X-irradiated mice (2), and in the microenvironmental defect seen in genetically anemic mice carrying the mutant steel locus (3, 4). The effects of adherent cells derived from marrow as seen in heterotopic implants (5) and in bone marrow cultures (6) are also strongly suggestive of a regulatory role of bone marrow-associated elements in the activities of pluripotent stem cells. Because the W/W^v mouse has very few macrocolony-forming stem cells (CFU_S)[1] but an apparently normal marrow microenvironment, this genotype may be useful for studying the interaction between CFU_S and their immediate environment. This follows from the comparative ease with which CFU_S from +/+ littermates or congenic mouse strains can be transplanted to W/W^v mice without irradiation or other treatment (7–9). We are concerned here with the evolution of bone marrow chimerism in the W/W^v mouse as a function of marrow transplantation dose. The results of this study point to the existence of discrete stem cell regulatory vol of about 10^8 μm^3, a dimension consistent with concepts of short-range cell-cell interactions.

Materials and Methods

We chose histocompatible bg^J/bg^J (beige) mouse marrow as the source of CFU_S for the W/W^v mouse because the giant sudanophilic granules (>1 μm) characteristic of bg^J/bg^J neutrophils provide a convenient marker of W/W^v marrow replacement (8). W/W^v and bg^J/bg^J mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. Both male and female bg^J/bg^J mice, 2–4 mo of age and weighing 20–25 g, were used as marrow donors, but only male W/W^v mice of similar age weighing 15–20 g were used as bone marrow recipients because of incompatibility of a male graft in a female host. Donor marrow was obtained from a femur shaft, suspended in Hanks' balanced salt solution for total nucleated cell counts, and then serially diluted with additional Hanks' solution to the desired cell concentrations. Each W/W^v recipient was injected with 0.5 ml of the appropriate bg^J/bg^J marrow cell suspension by tail vein.

* Supported by the U. S. Department of Energy.

[1] *Abbreviation used in this paper:* CFU_S, macrocolony-forming stem cells.

Peripheral blood samples were obtained from each recipient W/W^v mouse at various times up to 720 days. For detection of beige neutrophils, blood smears were fixed in formaldehyde vapor, stained with Sudan black B, and counterstained with Gurr's Giemsa (10). A minimum of 100 neutrophils on each blood smear was scored for the presence of giant sudanophilic granules. To determine whether temporal changes in the concentration of bg^J/bg^J neutrophils mirrored the takeover of W/W^v marrow by the implanted CFU_S, we also performed spleen colony assays in selected mice. For this purpose, 0.5 ml of femoral marrow cell suspensions prepared from bg^J/bg^J:W/W^v chimeras was given intravenously to each of 5–10 CBA/J mice 2 h after their X-irradiation with 1,000 rads. The recipients were killed 7 days later and surface colonies were counted with the aid of a dissecting microscope after fixation of the excised spleen. The number of discrete surface colonies was corrected for endogenous colonies and the basal CFU_S concentration in W/W^v mice (0.4 ± 0.06 per 10^5 nucleated cells).

The fraction of injected bg^J/bg^J CFU_S present in a W/W^v femur at 24 h (seeding efficiency) was determined by secondary transplantation to irradiated recipients and spleen colony assay as described above. Seeding efficiency is thought to be independent of the injected dose up to at least 90×10^6 nucleated marrow cells (11). The initial bg^J/bg^J CFU_S uptake was related to W/W^v marrow volume, determined by agar replacement (12). Femoral medullary cavity volume was estimated by comparison of the weight of a femoral shaft after removal of its marrow content and replacement with 2% agar. It is known that a femur represents 6% of the total marrow in a mouse (13).

The possible contribution of bg^J/bg^J extramedullary hematopoiesis was examined by determination of neutrophil replacement in W/W^v mice splenectomized 24 h after injection of bg^J/bg^J marrow cells. Redistribution of intravenously injected CFU_S among the various tissues of an X-irradiated mouse is known to be completed by 16–24 h (14).

Results

The concentration of CFU_S in bone marrow is at least 50–100 times greater in the bg^J/bg^J than in the W/W^v mouse. We determined that bg^J/bg^J marrow contains 24 ± 3 CFU_S per 10^5 nucleated cells and that $1.3 \pm 0.4\%$ of the transplanted CFU_S could be recovered from the marrow of a W/W^v femur 24 h later. When the CFU_S concentration is corrected for a seeding efficiency of 10% in the spleen colony assay (15–17), it follows that injection of 10^5 bg^J/bg^J bone marrow cells led to the delivery of about three CFU_S to the marrow in each femur. Because a femur contains 6% of the marrow of a mouse, about 50 CFU_S were therefore delivered to the total marrow. By using the agar replacement technique, we found that the femoral medullary volume in a 20-g W/W^v mouse was 16×10^9 μm^3, which can be extrapolated to a total marrow vol of about 300×10^9 μm^3 or 0.3 ml.

The replacement of W/W^v blood neutrophils by bg^J/bg^J neutrophils after transplantation of bg^J/bg^J marrow cells reflects the growth of CFU_S in the chimeric mice (Fig. 1). The degree of replacement was linearly related to the bone marrow CFU_S concentration over the range of 5–95% bg^J/bg^J neutrophils without a change in overall neutrophil concentration. Therefore, the peripheral blood percentage of bg^J/bg^J neutrophils provides a convenient parameter for assessment of temporal changes in the growth and development of implanted stem cells in a given animal. With marrow cell doses of 0.2×10^5–1×10^5, bg^J/bg^J blood neutrophils were found in only 3 of 20 recipient W/W^v mice even after 300 days: 2 of 5 with 0.2×10^5, 0 of 5 with 0.4×10^5, 1 of 5 with 0.5×10^5, 0 of 5 with 1×10^5. However, with doses of 2×10^5 or more, bg^J/bg^J neutrophils were seen in all of 65 recipient mice.

Beige neutrophils appeared in the peripheral blood after a latency period of

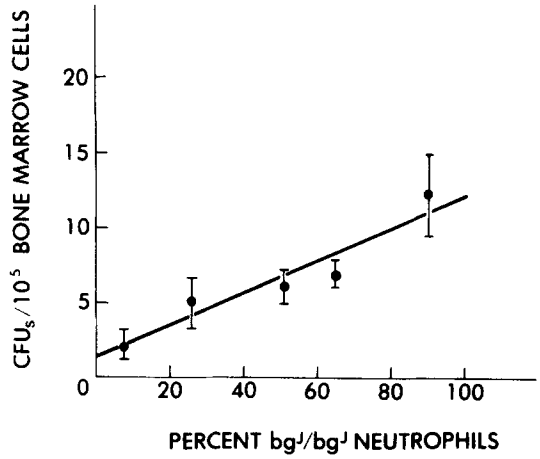

FIG. 1. Relationship between bone marrow CFU_S concentration and bg^J/bg^J blood neutrophil percentage in W/W^v recipients of bg^J/bg^J bone marrow. (Symbols designate mean ± SE for four to seven mice; CFU_S concentration uncorrected for seeding efficiency in spleen colony assay. Correlation coefficient, 0.91; $P < 0.025$.)

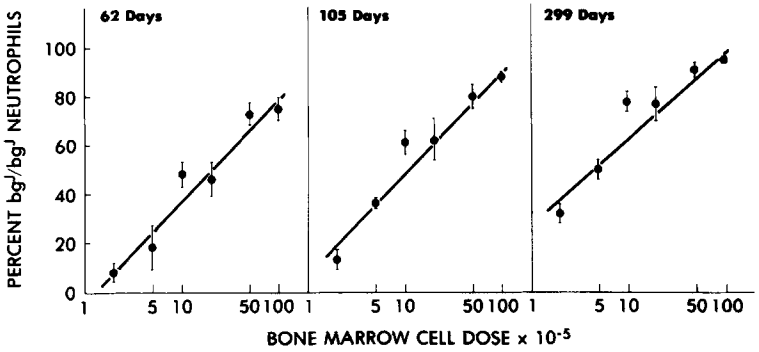

FIG. 2. Replacement of W/W^v blood neutrophils by bg^J/bg^J neutrophils as a function of marrow inoculum dose and time. (Symbols designate mean ± SE for four to eight mice; the same mice were sampled at each time interval. Correlation coefficient at 62 days, 0.97; at 105 days, 0.97; at 299 days, 0.95; $P < 0.005$.)

2–3 wk, the rate of appearance depending on the dose of bg^J/bg^J marrow cells injected. In one experiment, the replacement of W/W^v neutrophils by beige neutrophils was followed in each recipient mouse by sampling at mean times of 62, 105, and 299 days after intravenous bone marrow cell doses of 2×10^5–100×10^5. As shown in Fig. 2, the results are consistent with a linear log dose-response, with 50% replacement doses (D_{50}) of 20×10^5, 10×10^5, and 4×10^5 bg^J/bg^J bone marrow cells at 62, 105, and 299 days, respectively. In a second experiment, W/W^v neutrophil replacement was determined by sampling a few mice repeatedly during a period of 720 days after transplantation of either 2×10^5 or 2×10^6 bg^J/bg^J bone marrow cells. At the lower dose, beige neutrophil concentration increased linearly at a rate of 0.1% per day, the time for 50% replacement (T_{50}) being 450 days (Fig. 3). At the higher dose, beige neutrophils initially increased rapidly and then progressively more slowly, the overall

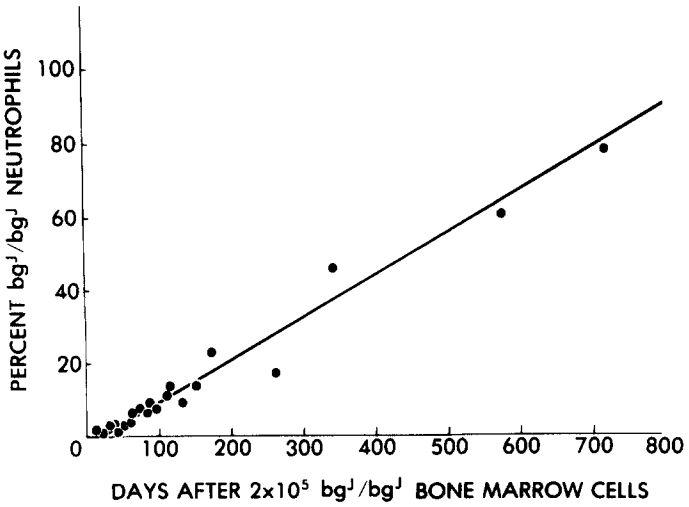

FIG. 3. Blood neutrophil replacement with time after intravenous injection of 2×10^5 bg^J/bg^J bone marrow cells. (Each symbol designates the mean value in two mice sampled repeatedly. Correlation coefficient, 0.98; $P < 0.001$.)

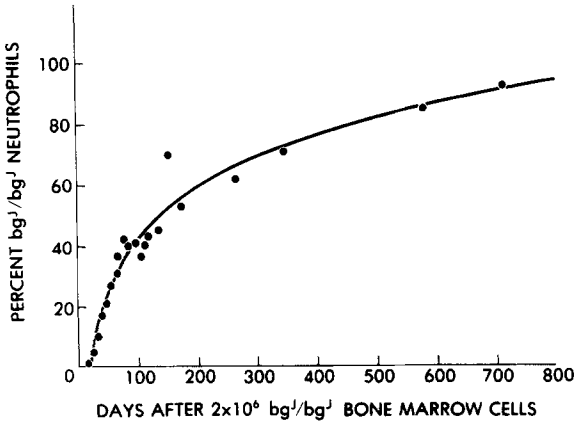

FIG. 4. Blood neutrophil replacement with time after intravenous injection of 2×10^6 bg^J/bg^J bone marrow cells. (Each symbol designates the mean value in two mice sampled repeatedly. Correlation coefficient, 0.99; $P < 0.001$.)

hyperbolic relationship being consistent with a linear log time-response (Fig. 4); the T_{50} was 140 days, a reduction by only a factor of three despite a 10-fold increase in the transplantation dose.

The replacement of W/W^v neutrophils was not altered significantly by splenectomy 24 h after injection of 50×10^5 bg^J/bg^J bone marrow cells. At 10 wk, the mean bg^J/bg^J blood neutrophil percentage was 86 ± 2 in six splenectomized W/W^v recipients compared to 91 ± 2 in five control mice. Because the spleen is known to be an important repository of intravenously injected CFU_S, this finding suggests that essentially all of the relevant stem cells were delivered to the marrow within a day after bg^J/bg^J bone marrow cell injection.

Discussion

The W/W^v mouse presents a macrocytic anemia, apparently related to a deficiency of CFU_S along with a deficiency of a "theta-sensitive" regulatory cell (9, 19). Nevertheless, this genotype has a fairly cellular bone marrow with normal concentrations of early erythroid (CFU_E) and granuloid (CFU_C) progenitors (20, 21), a reasonably normal neutrophil reserve (22), and normal blood neutrophil concentration (22–24). Despite the anemia, overall rates of replacement of W/W^v erythrocytes and neutrophils by bg^J/bg^J erythrocytes and neutrophils are generally similar after bone marrow transplantation (8). The correlation described here between the increase of donor marrow stem cells (CFU_S) and the replacement of W/W^v neutrophils by neutrophils containing the bg^J/bg^J cytoplasmic marker provides further evidence of the appropriateness of the marker as an indicator of the takeover of W/W^v marrow by the implanted bg^J/bg^J stem cells.

The present studies, covering a 50-fold range of bg^J/bg^J inoculum doses and a 2-yr period of observation, reveal a hyperbolic pattern of W/W^v blood neutrophil replacement that conforms to a linear log dose-response. To interpret the hyperbolic relationships observed in the takeover of W/W^v marrow by bg^J/bg^J CFU_S, it is necessary to consider the competency of the injected CFU_S and their initial distribution and secondary colonization within the marrow volume. CFU_S assays are reported to be similar in X-irradiated and unirradiated W/W^v mice (17, 18); hence, competency of the bg^J/bg^J CFU_S would seem to be independent of the presence of disadvantaged W/W^v stem cells. Although our low-dose studies thus far might signify that all of the marrow-implanted CFU_S were not competent with respect to W/W^v blood neutrophil replacement, further work is necessary to determine the extent to which this may reflect neutrophil counting statistics and the observation period. Extrapolation of the log dose-response slopes to the X (dose) axis (Fig. 2) points to a limiting bone marrow cell dose of 1.1×10^5 (55 marrow-implanted CFU_S) at 62 days, 0.7×10^5 (35 marrow-implanted CFU_S) at 105 days, and 0.14×10^5 (7 marrow-implanted CFU_S) at 299 days. The probability of activation of one or another implanted CFU_S no doubt depends on various factors, e.g., its location in relation to bone surfaces (25) and the number deposited within a specified volume. However, despite any heterogeneity of discrete regions within the marrow as a whole, the supposition that a single bg^J/bg^J CFU_S can eventually overcome proximate W/W^v stem cells would appear to provide a reasonable starting point for analysis of the bone marrow cell dose-response relationships in the development of bg^J/bg^J:W/W^v chimerism.

Because medullary sites in a mouse are completely hematopoietic, the injected CFU_S should be distributed within the total marrow volume. In contrast to the report by Lord et al. (25) of a normally occurring CFU_S gradient from the bone surface to marrow axis, we have found a quite uniform distribution throughout the femoral marrow (26). Although there may be some selectivity of the initial marrow distribution when CFU_S are introduced rather abruptly into the peripheral circulation, as a first approximation it seems reasonable to assume an overall random distribution. Thus, the initial seeding of marrow by bg^J/bg^J CFU_S might be expected to conform to statistics of random

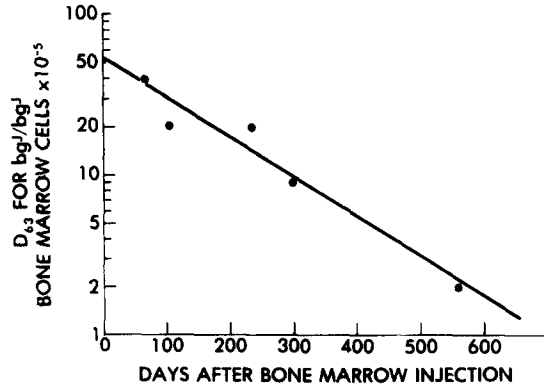

FIG. 5. The bg^J/bg^J bone marrow cell dose for 63% replacement of W/W^v blood neutrophils. (Correlation coefficient, 0.97; $P < 0.005$.)

sampling in which the probability of no seeding in a sampling unit or target volume will be given by $e^{-\lambda}$ where λ represents the mean occurrence of CFU_S per target volume after a particular bone marrow cell dose. The probability of seeding in a target volume $(1 - e^{-\lambda})$ will equal 0.63 when λ corresponds to a mean of one CFU_S per volume. Accordingly, the bg^J/bg^J bone marrow cell dose required for 63% replacement of W/W^v neutrophils (D_{63}) should correspond to the seeding of an average of one bg^J/bg^J CFU_S per target volume after correction for subsequent (secondary) colonization.

The temporal decrease in D_{63} derived from the data presented in Figs. 2–4 provides a basis for distinguishing the immediate seeding of CFU_S from the subsequent migration. The D_{63} decreased exponentially with a half-time of 120 days as a reflection of secondary colonization by the progeny of initially deposited CFU_S (Fig. 5). Hence, the Y axis intercept of 52×10^5 bg^J/bg^J bone marrow cells should approximate the theoretical D_{63} before bg^J/bg^J CFU_S migration to neighboring marrow microenvironments. This D_{63} corresponds to an uptake by bone marrow of about 2,600 CFU_S. If single-hit kinetics prevail, the sampling unit or target volume when $\lambda = 1$ will be 1.1×10^8 μm^3 (bone marrow volume, or 300×10^9 μm^3 divided by D_{63}-equivalent CFU_S, or 2,600). A Poisson plot derived from a target vol of 1.1×10^8 μm^3 is shown in Fig. 6 in relation to intercepts of least squares regressions computed for bg^J/bg^J isoeffect doses (D_{20}, D_{30}, etc.) as a function of time. The various isoeffect dose-time intercepts are in reasonable agreement with the Poisson prediction, which does not, of course, take into account biological variability. Target volumes may differ, for example, in the number of hits required for successful repopulation and thus a model based on a single hit with variable target size may provide a more precise representation.

Our analysis leads to the hypothesis that mouse bone marrow is compartmentalized into essentially self-contained stem cell regulatory volumes or domains equivalent on the average to about 50 cell diameters. This dimension is consistent with the presumptive role of short-range cell-cell interactions in the regulation of pluripotent stem cells as seen, for example, in studies with the Sl/Sl^d mouse (4). Among the factors determining the dimensions of the stem cell domains may be the distribution of putative regulatory cells of the marrow

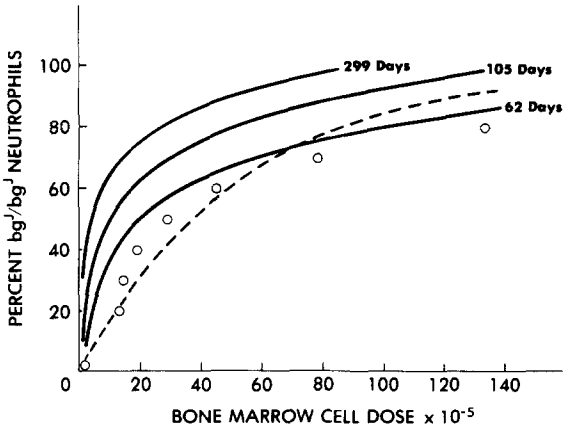

FIG. 6. Bone marrow cell dose and time parameters for blood neutrophil replacement in the bg^J/bg^J:W/W^V chimera in relation to Poisson expectation. (Solid lines are derived from data in Fig. 2; dashed line depicts Poisson plot with symbols designating intercepts of iso-effect dose-time linear regressions.)

stroma and the nature of the marrow vasculature, about which much more needs to be learned. Although each stem cell regulatory volume is undoubtedly subject to extrinsic influences, the concept of a discrete functional unit in which stem cell proliferation is geared to the density of the stem cell population provides a framework for understanding control of the stem cell population in marrow as a whole. On a broader scale, we have shown that the cellularity of distinct marrow areas is also locally controlled (27).

Our analysis of blood neutrophil replacement patterns in the bg^J/bg^J:W/W^V chimera also suggests that the local traffic of stem cells is ordinarily quite limited and restricted to proximate target or regulatory volumes, as in relays. If colonization were a fairly rapid event, only a week or so would have been required for the six doublings necessary to overcome the 50-fold difference in the number of transplanted bg^J/bg^J CFU$_S$. Yet secondary colonization by migrant CFU$_S$ was clearly a slowly evolving process in the W/W^V marrow; only about 80% of the W/W^V blood neutrophils were replaced 2 yr after transplantation of 2×10^5 bg^J/bg^J bone marrow cells, whereas a similar degree of replacement occurred 2 mo after transplantation of 100×10^5 bg^J/bg^J bone marrow cells. Significantly, this limitation of CFU$_S$ migration is not seen in a severely hypocellular marrow. Marrow regeneration in a radiation chimera is also a hyperbolic function of the marrow transplantation dose (28), but the rate of regeneration is much faster than the rate of replacement of W/W^V marrow by implanted bg^J/bg^J stem cells. Apparently, the more rapid expansion of the donor stem cell population and the decreased constraint to stem cell movement in a radiation-induced hypocellular marrow facilitates secondary colonization from the initially seeded sites. In a preliminary study, we have observed a similar result in bg^J/bg^J:W/W^V chimeras after treatment with hydroxyurea. The discontinuous nature of local stem cell migration would appear to be an important consideration in the marrow transplantation dose required for various clinical applications.

Summary

The kinetics of bone marrow replacement was studied in W/W^v mice implanted with bg^J/bg^J (beige) stem cells, with the characteristic beige neutrophil marker as a criterion of the takeover of host marrow by donor marrow. A hyperbolic pattern of W/W^v marrow replacement conforming to a log dose-response was observed in experiments encompassing a 50-fold range of bg^J/bg^J inoculum doses and a 2-yr period of observation. The dose-response relationships were consistent with random seeding of stem cells in the host marrow coupled with a decreasing efficiency of secondary colonization by local migration. Application of single-hit Poisson sampling statistics to the dose-response data led to the hypothesis that mouse bone marrow is compartmentalized into essentially self-contained stem cell regulatory volumes or domains. We estimate that W/W^v marrow contains about 2,600 stem cell regulatory units with an average volume of about 10^8 μm^3, a dimension consistent with the presumptive role of short-range cell-cell interactions in the regulation of pluripotent stem cells. Our analysis of the dose-response data is also indicative of the discontinuous and limited nature of local stem cell migration in a cellular marrow, a consideration that may be of practical as well as theoretical interest.

Received for publication 17 October 1977.

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