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Partitioning of bone marrow into stem cell regulatory domains

(spleen colony-forming unit seeding/spleen colony-forming unit growth)

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ABSTRACT **To examine the hypothesis that bone marrow consists of discrete stem cell regulatory volumes or domains, we studied spleen colony-forming unit (CFU-S) population growth kinetics in unirradiated WBB6F$_1$-W/W^v mice receiving various doses of +/+ bone marrow cells. Assay of femoral marrow CFU-S content in the eight recipient dose groups revealed a family of growth curves having an initial dose-independent exponential phase and a subsequent dose-dependent deceleration phase. CFU-S content at the growth transition (inflection point) was not a simple linear function of inoculum dose but was shown rather to reflect a random distribution of initially seeded donor CFU-S in discrete volumes of recipient bone marrow. The inoculum dose resulting in a mean of 1 CFU-S per bone marrow sampling unit was estimated to be 17 × 10^6 bone marrow cells, corresponding to a total marrow uptake of ≈5100 CFU-S (based on a seeding efficiency factor of 10%). If we assume single-hit kinetics, it follows that the recipient W/W^v bone marrow may contain ≈5100 domains in which stem cell proliferation is geared to the density of the stem cell population. When the various inocula were corrected for multiple seeding in a given domain, the mean inflection point per domain was similar and indicative of five or so divisions before departure from exponential growth at ≈20% of final CFU-S content 8 days after bone marrow injection. The partitioning of bone marrow into highly localized functional units is consistent with the putative regulatory role of short-range interactions between stem cells and essential stromal elements.**

Local mechanisms are known to play a key role in the activation and direction of hematopoietic stem cells (1–5). Hence, the question arises whether intrinsic control of the stem cell population is vested in discrete bone marrow domains. There is some indication that this may indeed be so. In an earlier study, using B6-bg^j/bg^j (beige) bone marrow inocula in unirradiated WBB6F$_1$-W/W^v mice, we observed a hyperbolic pattern of beige neutrophil appearance in peripheral blood as a function of inoculum dose (6). Because of the relative slowness of the apparent marrow replacement in this parental F$_1$ chimera, it was possible to discern that the dose–response relationships were reasonably consistent with random seeding of stem cells into discrete volumes of the bone marrow. We report here an analysis of stem cell [spleen colony-forming unit (CFU-S)] population growth after injection of various doses of WBB6F$_1$-+/+ bone marrow into unirradiated WBB6F$_1$-W/W^v mice. The analysis sought to determine whether the nonlinear asymptotic regression characteristic of CFU-S population growth can be interpreted in terms of a Poisson distribution of the initial seeding of putative bone marrow domains, which was inferred from our earlier studies (6, 7). The results are consonant with this expectation and, therefore, provide direct indication of the functional partitioning of bone marrow into stem cell regulatory units.

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MATERIALS AND METHODS

Normal +/+ male mice, 2–4 mo old and weighing 20–25 g, were used as bone marrow donors for male W/W^v mice of similar age weighing 15–20 g. Donor marrow was obtained from a femur shaft, suspended in Hanks' balanced salt solution for nucleated cell counts, and then serially diluted with additional Hanks' solution to the desired cell concentrations. The W/W^v recipient groups received various doses of +/+ nucleated bone marrow cells in 0.5 ml by tail vein. Femoral marrow samples were taken at specified times from three to nine W/W^v recipients in each dose group for conventional CFU-S assay. For this purpose, 0.5 ml of femoral marrow cell suspension prepared from each recipient was injected intravenously into 5–10 CBA/J mice 2 hr after their x-irradiation with 1000 rads (1 rad = 1.0 × 10^{-2} gray). The number of bone marrow cells given ranged from two femur-equivalents downward, depending on the +/+ bone marrow cell dose used initially and the time of assay. Surface spleen colonies were counted with a dissecting microscope after fixation of the excised spleen at 7 days. The CFU-S concentration of the donor +/+ bone marrow inocula [uncorrected for seeding efficiency (f in the spleen colony assay)] varied in replicate experiments from 16 to 26 per 10^5 nucleated cells. The corresponding bone marrow cell inoculum doses were, therefore, normalized for data analyses to the mean CFU-S concentration of 22.5 ± 1.4 per 10^5 cells. The eight inoculum groups ranged from 0.6 × 10^6 to 36 × 10^6 bone marrow cells.

RESULTS

One day after injection of +/+ bone marrow, femoral CFU-S content in the W/W^v recipients was found to be linearly related to the inoculum dose, with a slope corresponding to 0.8 CFU-S per 100 CFU-S injected (Fig. 1). The intercept of 10 CFU-S can be taken to represent the average femoral marrow background level in the W/W^v mice used for these studies, and all values were corrected accordingly for analysis of +/+ CFU-S population growth. Representative growth curves are shown in Figs. 2 and 3. For all +/+ bone marrow inocula, increase of femoral CFU-S content conformed to an exponential curve during the first week, with a mean doubling time of 1.65 ± 0.15 days. Because the doubling times derived from measurements on days 1, 4, and 7 did not differ significantly for the eight inoculum groups, the initial exponential growth for all of the groups can be displayed on a common time axis by placing the means of the natural logarithms of the CFU-S numbers on a line with the mean slope (8).† This transformation is shown in Fig.

Abbreviation: CFU-S, spleen colony-forming unit(s).

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† Time axis transformation in days $T = [(\bar{Y} - a)/b] - \bar{X}$, where $\bar{Y}$ = mean ln CFU-S, a = log arbitrary intercept = 0, b = slope = 0.371, and $\bar{X}$ = mean of sampling times in days = 4. T ranged from 2 days for the 0.6 × 10^6 marrow cell dose to 10.7 days for the 36 × 10^6 marrow cell dose.

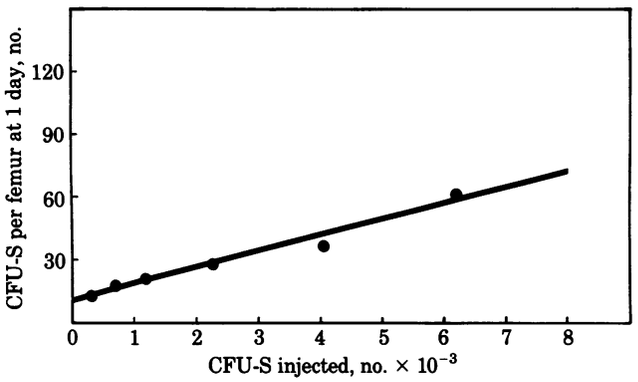

FIG. 1. CFU-S content of W/W^v femoral marrow 1 day after injection of +/+ bone marrow. Data are expressed as a three-point moving average (correlation coefficient, 0.985).

4. The slope of the resulting common-time-axis regression was 0.393 ± 0.015, compared with the mean slope of 0.371 ± 0.012 derived from the 90 individual determinations made during the first week after injection of the various bone marrow doses.

The inoculum dose-independent initial growth rate was followed by a progressively decelerating growth rate toward an asymptote. Significant CFU-S growth deceleration ($P < 0.01$) could be shown to occur between 7 and 10 days after bone marrow inoculation by comparison of common-time-axis regressions based on values obtained at 1, 4, and 7 days (doubling time, 1.76 ± 0.07 days) and at 4, 7, and 10 days (doubling time, 2.38 ± 0.08 days). Although CFU-S content at the onset of growth deceleration can thus be approximated by the mean of the 7- and the 10-day values, the inflection point was estimated

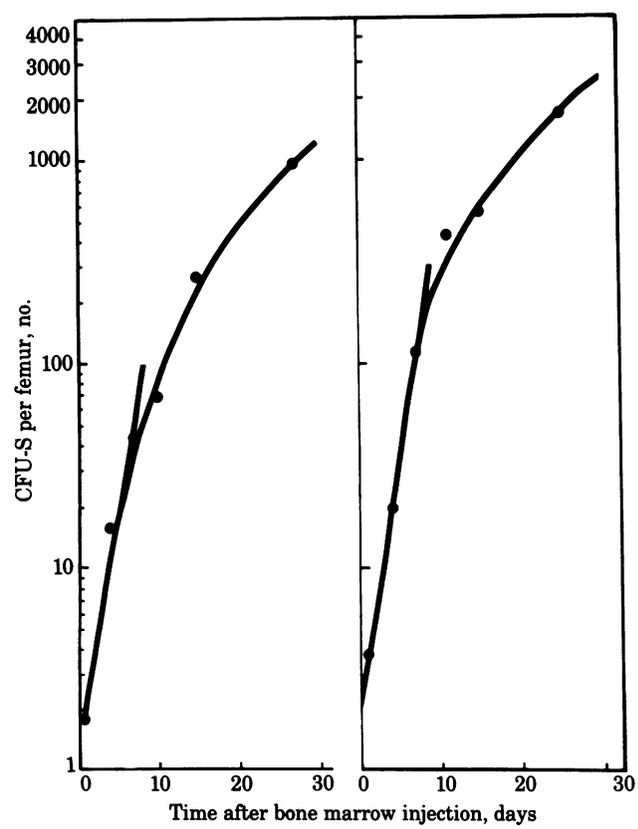

FIG. 2. CFU-S population growth in femoral marrow of W/W^v mice injected with 0.6 × 10^6 (*Left*) or 3 × 10^6 (*Right*) +/+ bone marrow cells.

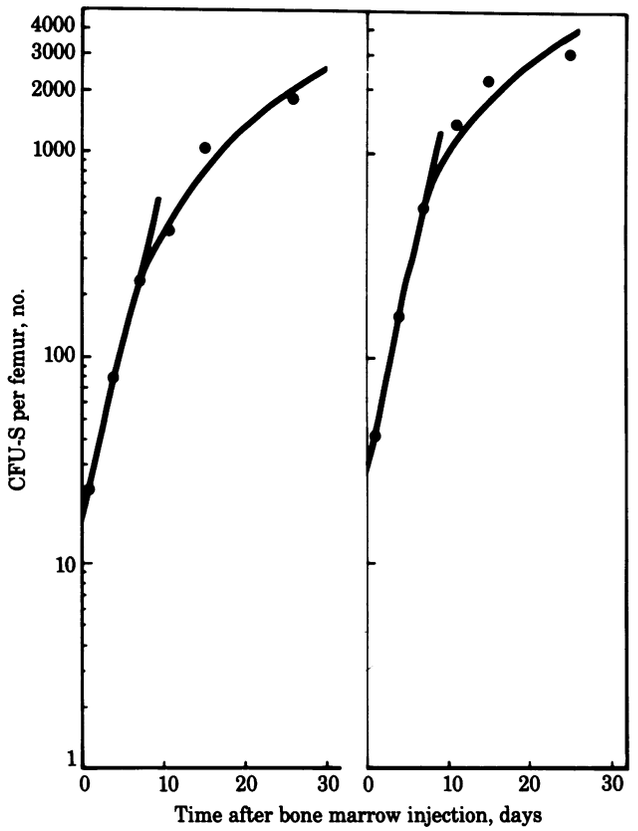

FIG. 3. CFU-S population growth in femoral marrow of W/W^v mice injected with 7.2 × 10^6 (*Left*) or 30 × 10^6 (*Right*) +/+ bone marrow cells.

by determining the intersection of the initial exponential for each inoculum group and the curve obtained by plotting the natural logarithm of the femoral CFU-S content against the natural logarithm of the time from 7 to 25 days. Correlation coefficients for these curves ranged from 0.946 to 0.997 with a mean of 0.977 ± 0.007. Inflection points determined in this way (see Figs. 2 and 3) increased with bone marrow cell dose, giving a curve that was convex upward and became asymptotic at the higher doses (Fig. 5).

The growth inflection data can be shown to conform to a linear regression with an acceptable (i.e., near zero) intercept by matching the inflection points to Poisson distributions of the initially implanted stem cells, assuming various bone marrow cell doses for seeding of 63% of hypothetical marrow volumes [$(1 - e^{-\lambda}) \times 100$, where $\lambda = 1$ for D_{63}—i.e., a mean of 1 CFU-S per sampling unit]. When λ is taken as 1 for an inoculum of 17 × 10^6 bone marrow cells (injection of 3825 CFU-S uncorrected for f), the zero dose intercept of the regression of inflection point on occupancy is essentially zero [−1.3 (Fig. 6)]. The correspondence of CFU-S growth inflection to a Poisson distribution with a D_{63} of 17 × 10^6 bone marrow cells is highly significant (correlation coefficient 0.999; 95% confidence interval of $D_{63} = \pm 1 \times 10^6$). When the various bone marrow cell inocula are adjusted for the probability of multiple CFU-S seeding in a given sampling unit, the CFU-S content at growth inflection is about the same for each inoculum, the mean being 28.3 ± 1.7 CFU-S per initially seeded volume, which corresponds to 4.8 ± 0.1 doublings (Table 1).

DISCUSSION

This study was undertaken to test the hypothesis that bone marrow consists of multiple functional domains for intrinsic reg-

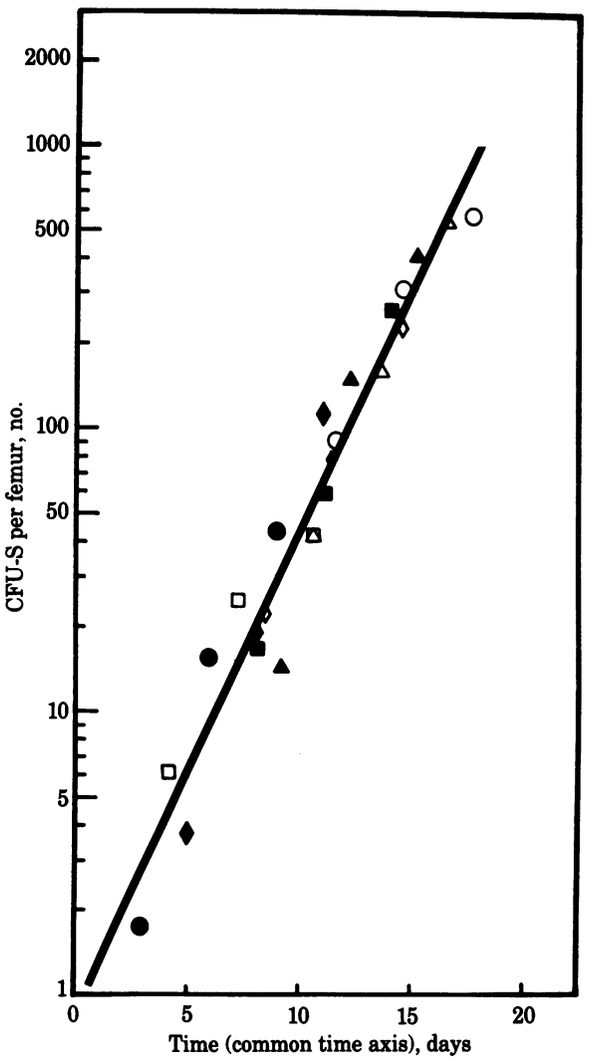

FIG. 4. Exponential phase of CFU-S population growth in W/W^v femoral marrow after injection of +/+ bone marrow (common-time-axis transformation; correlation coefficient, 0.974). Doses: ●, 0.6 × 10^6; □, 0.9 × 10^6; ◆, 3 × 10^6; ■, 5.8 × 10^6; ◇, 7.2 × 10^6; ▲, 17.5 × 10^6; △, 30 × 10^6; ○, 36 × 10^6.

ulation of its stem cell content. If this is so, random dispersal of CFU-S into such fairly autonomous units should lead to a family of growth curves that have similar initial slopes but different inflection points, depending on inoculum size. This proposition differs from the growth pattern in a single functional domain as exemplified by murine ascites tumors, in which growth inflection is independent of inoculum size and occurs when a given tumor cell content is reached (8).

For this analysis, CFU-S from normal +/+ littermates were given to W/W^v mice, which are known to accept congeneic marrow without recourse to irradiation or other treatment. The slope of the dose–response regression curve 1 day after injection of +/+ bone marrow showed that ≈0.8% of donor CFU-S appeared in the femoral marrow of the recipient. Significantly, the zero-dose intercept was consistent with the presence in W/W^v marrow of only a few tenths of a percent of CFU-S found in normal marrow (9). By correcting for this basal level, we observed that the initial growth rate of +/+ CFU-S in unirradiated W/W^v femoral bone marrow was essentially the same over a 60-fold range of inoculum sizes (from 0.6 × 10^6 to 36 × 10^6 bone marrow cells, normalized to a CFU-S concentration of 22.5 per 10^5). A similar dose independence for the early CFU-S growth rate in femoral bone marrow was inferred by others using syngeneic transplants in x-irradiated recipients (10).

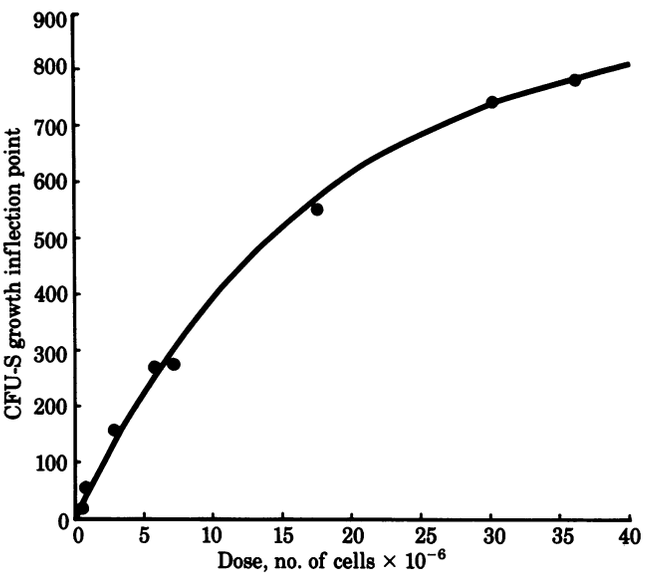

FIG. 5. Relationship between CFU-S content of W/W^v femoral marrow at growth inflection and +/+ bone marrow dose.

On the other hand, we also observed that inflection from the initial exponential slope depended on the amount of bone marrow injected. However, the point of inflection was not a simple linear function of the inoculum size. Although the number of seeded CFU-S increased in strict proportion to the inoculum dose, seeding was apparently random and thus the number of marrow sampling units or discrete volumes that were occupied increased more slowly with dose because of the increasing probability of multiple occupancy. In contrast to the example of murine ascites tumors noted above, we suggest that the CFU-S content corresponding to the onset of growth inflection is independent of the number of CFU-S seeded initially in a given volume and is determined mainly by the number of volumes

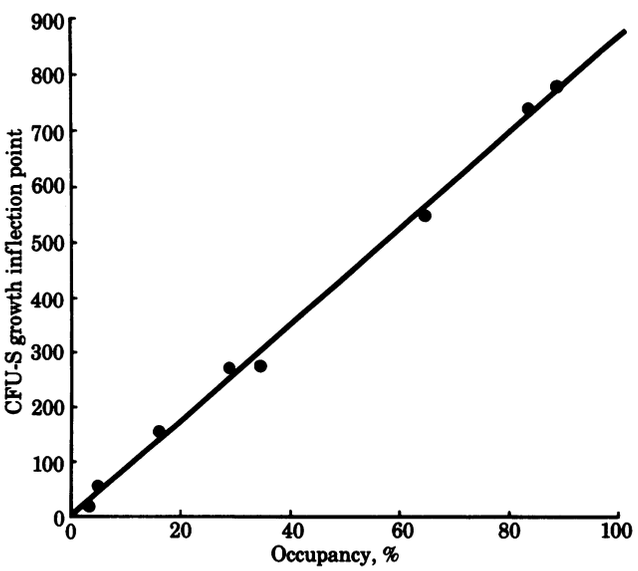

FIG. 6. Relationship between CFU-S content of W/W^v femoral marrow at growth inflection and percent occupancy of discrete volumes or domains [$(1 - e^{-\lambda}) \times 100$, where $\lambda = 1$ for a +/+ bone marrow inoculum of 17 × 10^6 cells; slope, 8.821; intercept, −1.32; correlation coefficient, 0.999)].

Table 1. Relationship of CFU-S growth inflection to a Poisson distribution of injected CFU-S in femoral bone marrow

CFU-S to femur*	Mean CFU-S in seeded volumes†	CFU-S dose adjusted for multiplicity‡	Femoral CFU-S at growth inflection	CFU-S inflection point per CFU-S per volume§	Doublings to growth inflection¶, no.
1.0	1.0	1.0	18	18.0	4.2
1.6	1.0	1.6	55	34.0	5.1
5.4	1.1	4.9	155	31.6	5.0
10.4	1.2	8.7	270	31.0	5.0
13.0	1.2	10.8	275	25.5	4.7
31.4	1.6	19.6	550	28.1	4.8
53.6	2.1	25.5	740	29.0	4.9
64.4	2.4	26.8	780	29.1	4.9

* 0.8% of injected CFU-S (uncorrected for f in spleen colony assay).
† Calculated by using $\lambda/1 - e^{-\lambda}$, where $\lambda = 1$ for 30.6 CFU-S to femur ($D_{63} = 17 \times 10^6$ bone marrow cells).
‡ Calculated by dividing column 1 by column 2.
§ Calculated by dividing column 4 by column 3; mean = 28.3 ± 1.7.
¶ Calculated by dividing the natural logarithm of column 5 by 0.693; mean = 4.8 ± 0.1.

that have been seeded. Indeed, when the various inocula were corrected for multiple seeding using a D_{63} value of 17×10^6 bone marrow cells, the inflection point per initially seeded volume was quite constant. The mean inflection point of 28 CFU-S per volume specifies about five divisions before obvious departure from early exponential growth at ≈8 days, based on doubling times computed from either individual or common-time-axis growth slopes. It should be noted that the inflection point per volume derived for the lowest inoculum dose, which corresponds on the average to the delivery of only 1 CFU-S to a femur, is evidently an underestimate attributable to background noise. Nevertheless, the points of inflection from exponential growth for all of the eight inoculum dose groups conform closely to a Poisson distribution (see Fig. 6).

This analysis does not necessarily signify that only a single CFU-S can be activated in each volume but rather that the seeding of only one is required. Although growth deceleration might occur sooner in volumes that have multiple seeding, it was not possible to discern this because the mean number of CFU-S seeded per occupied volume was only 2.4, even with the largest inoculum used. It is of interest to relate the inflection from exponential growth in each initially seeded domain as seen in Table 1 (total femoral marrow domains = 30.6, uncorrected for f) to the plateau value of 4240 ± 354 CFU-S found in W/W^v femoral marrow within 60 days after injection of $8–40 \times 10^6$ +/+ bone marrow cells (7). It turns out that departure from exponential growth occurred when each regulatory unit had ≈20% of its final CFU-S content. The computed transition of CFU-S population growth at ≈8 days is in agreement with the observation of a significant increase in doubling time from 4 to 10 days, compared with 1 to 7 days after bone marrow inoculation. It is noteworthy that the means of the 7- and 10-day values can also be used to reveal a Poisson distribution of stem cell seeding, even though they may overestimate the graphically determined growth inflection by 20% or more.

If single-hit kinetics prevail, the matching of inflection points to a Poisson distribution anchored to a D_{63} value of 17×10^6 bone marrow cells (95% confidence interval, $\pm 1 \times 10^6$) provides an estimate of the number of putative stem cell regulatory domains. This D_{63} value corresponds to 3825 CFU-S, of which about 510 will be delivered to the bone marrow [0.8% of injected dose to femoral marrow, which is 6% of total marrow (11)]. If spleen colony assay seeding efficiency (f) is taken as 10% (12), then 5100 CFU-S will seed the marrow after injection of 17×10^6 bone marrow cells. Because this dose represents a mean of 1 CFU-S per sampling unit, it follows that the bone marrow contains ≈5100 CFU-S or domains with 95% confidence limits of ±300, a number somewhat greater than that inferred by us previously from an indirect criterion of CFU-S population growth (6). If the present estimate of the number of stem cell domains is related to the total bone marrow volume of 300×10^9 μm^3 in the W/W^v mouse (6), the average volume of each domain would be $\approx 5.9 \times 10^7$ μm^3, corresponding to some 40–50 cell diameters. Although medullary sites in a mouse are completely hematopoietic, stem cell domains may, of course, vary in size and, moreover, their total volume need not be equivalent to the total volume of marrow.

Our analysis of the number of stem cell regulatory units in the W/W^v mouse is sensitive to several variables: CFU-S seeding efficiency and multiplicity in the spleen colony assay and CFU-S self-renewal heterogeneity. The present estimate is based on a generally accepted f value of 10%, which is intermediate between reported values [5–20% (13)]. It is also based on the conventional inference that each spleen colony is derived from a single CFU-S, but there is karyotypic evidence of an average multiplicity of ≈1.25 (14), which, if applicable, would decrease the estimate by some 20%. Finally, the present estimate is predicated on the assumption that all CFU-S can generate CFU-S. Although prolonged self-renewal may be restricted to a small subset of the CFU-S population (15, 16), our analysis revolves around the point of inflection from exponential growth, which occurs after only five or so divisions. Apropos this, a majority of spleen colonies have been shown to contain multiple CFU-S despite great pressure for differentiation in x-irradiated mice (17, 18) and such pressure should be considerably less in unirradiated W/W^v recipients. Hence, the number of domains as influenced by this variable could be somewhat lower, but not much lower, than we have estimated.

Whether the presumptive regulatory domains contain specific anatomic sites or "niches" that must be occupied for stem cell self-renewal to occur (19) or whether they merely represent functional volumes governed, for example, by short-range interactions of essential stromal elements (1, 3–5) remains to be determined. Regardless of the underlying mechanism(s), our analysis of CFU-S population growth suggests that (*i*) the exponential increase in CFU-S content of W/W^v femoral bone marrow after injection of +/+ cells reflects activity in initially seeded discrete volumes, (*ii*) the CFU-S content corresponding to the onset of growth deceleration is independent of the number of CFU-S seeded initially in a given volume, and (*iii*) the subsequent asymptotic growth pattern is likely to be determined by a density dependency in occupied volumes coupled with loss by differentiation and by migration to other sites.

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