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On the Origin of Hematopoietic Stem Cells after Local Marrow Extirpation (38750)

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The initiation of marrow regeneration in an evacuated medullary cavity does not require the seeding of hematopoietic stem cells from elsewhere in the body even though immigrant cells are known to contribute to later stages of the regenerative process (1–3). Hematopoietic repopulation must, therefore, be activated by stem cells inadvertently left behind in the medullary cavity and/or by stem cells or undifferentiated elements residing in the adjacent bone. We sought an answer to this basic question by comparing the restoration of marrow in a mechanically depleted femur shaft in W/W^v and +/+ mice and in W/W^v mice grafted with +/+ marrow [W/W^v(+/+)]. The W/W^v mouse has very few stem cells, or CFU, as revealed by conventional spleen colony assay (4, 5) and is easily colonized by +/+ CFU without recourse to irradiation or other perturbation (6). The implanted +/+ cells soon become the progenitors of circulating blood cells with resulting complete and permanent cure of the hereditary anemia characteristic of the W/W^v genotype (7). The engrafted animal thus provides an experimental model for studies of the origin of hematopoietic stem cells resulting from local marrow ablation.

Materials and Methods. The experiments were performed with 5-mo-old WBB6 F_1-W/W^v and WBB6 F_1-+/+ litter mates procured from the Jackson Laboratories (Bar Harbor, Maine). The animals were housed in groups of five or six in disposable cages with filter bonnets. Cages were changed twice weekly and water and sterilized lab chow (GIBCO) were provided *ad libitum.* Randomly selected W/W^v mice received 2×10^6 +/+ marrow cells iv at least 8 wk (average, 12 wk) before marrow removal from part of the femur shaft, at which time their hematocrit was the same as that of normal +/+ mice (47.9 ± 0.3, compared to 39.6 ± 0.6 in untreated W/W^v).

The left femurs of mice in each experimental group [W/W^v, +/+, and W/W^v(+/+)] were depopulated *in situ* by a Dextran perfusion–aspiration technique (2). The depopulated area comprised a 4- to 5-mm segment of midfemur shaft. CFU content and hemic cellularity were assayed 7–16 days after marrow removal, the mean sampling time ranging from 10.5 to 12.5 days in the three groups. In each animal a 3-mm (caliper-measured) sample of the repopulating and contralateral control femur shaft was removed at sacrifice and the contents were dispersed in 1 ml of Hanks' balanced salt solution. In a subsequent study, emptied 3-mm segments of femur shaft were dried and weighed before and after being filled with 1% agar to estimate medullary cavity volume.

The number of nucleated cells in the dispersed medullary cavity contents was counted and smears were prepared and treated with Wright's stain for differential counts. Aliquots of the cell suspension were injected iv into young adult CBA mice 2 hr after they were X-irradiated with 850 rads. The cell dose was either 10^5 or 10^6 cells in 0.5 ml depending upon the donor. Recipient mice were sacrificed 7 days later for CFU assay by Till and McCulloch's spleen colony method. Surface colonies were counted with the aid of a dissecting microscope and corrected for endogenous colonies, which averaged 1.6 ± 0.2 in uninjected irradiated recipients. The numbers of CFU and nucleated cells in each repopulating medullary cavity were compared to those in the contralateral control femurs.

Results. Expressed as CFU content per nucleated marrow cell, there were about 18 times more CFU in the +/+ mouse than in the W/W^v and about 10 times more in the W/W^v(+/+) chimera. However, expressed as CFU content per unit length of femur shaft, there were 40 times more CFU

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TABLE I. MARROW CFU AND CELLULARITY IN +/+, W/W^v, AND W/W^v(+/+) MICE.

Group	Number of Mice	Number of CFU per 10^5 cells (±SE)	Number of CFU per mm^3 (±SE)	Number of hemic cells $\times 10^{-3}$ per mm^3 (±SE)[a]
+/+	16	26.6 ± 2.2	296 ± 28	874 ± 51
W/W^v	12	1.5 ± 0.3	10 ± 1.4	639 ± 64
W/W^v(+/+)	14	15.8 ± 1.9	127 ± 20	688 ± 62

[a] Excluding lymphocytes and polymorphonuclear leukocytes.

TABLE II. RESTORATION OF CFU AND CELLULARITY AFTER MARROW EXTIRPATION.

Group	Number of mice	Mean sampling time (days)	Recovery of femoral marrow as % of contralateral (±SE)		Ratio of hemic cell to CFU recovery
			CFU	Hemic cells[a]	
+/+	16	10.5	28.6 ± 7.3	37.2 ± 7.9	1.3
W/W^v	12	12.5	22.0 ± 5.5	8.8 ± 2.0	0.4
W/W^v(+/+)	14	11.0	18.5 ± 4.1	34.8 ± 7.5	1.9
			(16.7)[b]	(26.6)[b]	(1.6)[b]

[a] Excluding lymphocytes and polymorphonuclear leukocytes.
[b] Corrected for W/W^v contribution to recovery as described in the text.

in the +/+ and 12 times more in the chimera than in the W/W^v. Yet total cellularity per unit length of shaft differed only by factors of 1.9 and 1.1 for +/+ and W/W^v-(+/+) respectively compared to the W/W^v. It turns out that the medullary cavity volume per unit length of shaft in the W/W^v was 72% of that in the +/+ mouse ($P < 0.01$). Thus, on a unit volume basis there were nearly 30 times as many CFU in the +/+ animal as in the W/W^v with only a 35% difference in marrow cellularity (Table I).

During the second week after local marrow removal, the evacuated medullary cavity regained nearly 29% of the CFU content of the contralateral femoral marrow in +/+ mice, 22% in W/W^v, and 19% in W/W^v(+/+) (Table II). The differences among the groups were not statistically significant. In a 3-mm length of femur shaft, this degree of repopulation corresponds to an average recovery of 373 CFU in the +/+, 7 CFU in the W/W^v, and 75 CFU in the W/W^v(+/+). Therefore, some 90% of CFU repopulation in the chimera can be attributed to +/+ derived CFU. Stated in other terms, if repopulation of the evacuated femoral marrow in the chimera were due solely to its W/W^v background, the expected CFU recovery would be less than 2% of the contralateral marrow CFU content instead of the 19% that we found.

The recovery of hemic cells during the second week after femoral marrow extirpation was, on the average, 37% of the contralateral marrow cellularity in +/+ mice, 9% in W/W^v mice, and 35% in W/W^v-(+/+) chimeras (Table II). The difference in hemic cell repopulation between the +/+ or chimeric mice and the W/W^v was significant ($P < 0.01$). With correction for the W/W^v background as in the CFU analysis, the expected hemic cell repopulation in the chimera that is attributable to +/+ cells corresponds to 27% of the cellularity in the contralateral control femur. Hence, in first approximation, it appears that 77% of the hemic cell recovery occurring in the W/W^v(+/+) mouse from 7 to 16 days after removal of femoral marrow was due to +/+ CFU. After subtraction of the presumed W/W^v contribution to recovery, the ratio of hemic cell repopulation to CFU repopulation was 1.3 in +/+ mice and 1.6 in chimeric mice.

Discussion. The results concerning mar-

row CFU and cellularity in the W/W^v mouse are generally consonant with the findings of others (4, 5). However, the smaller medullary cavity volume of the femur shaft in W/W^v than in +/+ mice was an unexpected observation. When this difference was taken into account, there was comparatively little difference in cellularity per unit volume of marrow space in W/W^v and +/+ mice despite the 30-fold difference in CFU content assayed by the spleen macrocolony technique. Since only colonies visible on the spleen surface are scored, it is possible that W/W^v CFU deficiency is more qualitative than quantitative. This could occur, for example, if W/W^v stem cells form mainly microcolonies, which are unobservable by conventional assay, as suggested by Lewis *et al.* (8). Conceivably, a greatly increased turnover of a very small CFU pool could also contribute to the nearly normal marrow cellularity in the W/W^v mouse, but there is no indication of increased CFU turnover from our preliminary thymidine suicide studies.

Whatever the precise nature of the underlying hematopoietic abnormality in the W/W^v mouse, its easily identifiable deficit of macroscopic marrow CFU and its ready acceptance of +/+ CFU without irradiation or other treatment provide a basis for tracing the origin of hematopoietic stem cells that initiate repopulation of a mechanically depleted medullary cavity. Although circulating stem cells can contribute to the later stages of marrow regeneration, their traffic is not a requirement and seems, moreover, to be a negligible component of recovery during the first 2 wk after marrow extirpation (1–3). The time chosen for analysis in this study ranged from 7 to 16 days with mean times of 10.5–12.5 days. Under these conditions, comparison of CFU recovery in +/+, W/W^v, and W/W^v(+/+) animals should reveal the extent to which residual CFU contribute to the initiation of hemic cell repopulation and thereby whether it is necessary to consider the possible transformation of a more primitive cell to a hematopoietic stem cell in the overall regenerative program.

The substantial restoration of macroscopic colony-forming capacity in +/+ engrafted W/W^v mice clearly indicates that residual CFU play a predominant role in the onset of hematopoietic regeneration. Indeed, it appears that about 90% of CFU repopulation in the chimeric mice is due to residual +/+ cells. When allowance is made for the W/W^v contribution to recovery, the relationship of CFU repopulation to cellularity in the W/W^v(+/+) approaches that seen in the +/+. This points to a similar pattern of +/+ CFU differentiation irrespective of genotype.

Although the microenvironment immediately after perfusion or curettage may not be conducive to stem cell survival, it is possible that a few cells remaining in the medullary cavity could be responsible for hematopoietic regeneration. Still, since microscopic marrow foci are usually not evident at this time (9), the significant residual cells might reside in a more intimate connection with bone, e.g., within the endosteum and haversian canals. A few CFU have been detected in mouse bone cell suspensions (10) and while this might reflect the presence of marrow in endosteal crevices, it might also signify the presence of CFU within the bone itself. Such CFU may constitute a residue of cells derived during the formation of embryonic marrow or, in the case of the W/W^v(+/+) chimera, they may constitute a residue of cells deposited in the perivascular connective tissue of the haversian canals during the original injection of +/+ marrow. In this connection, it is of interest to recall the apparent nonuniform distribution of CFU in marrow of the mouse femur with about twice as many CFU adjacent to bone as in the center of the shaft (11).

In view of the overwhelming contribution by +/+ cells to CFU repopulation in the W/W^v(+/+) chimera, it does not seem necessary to invoke the transformation of more primitive mesenchymal cells to hematopoietic stem cells. There is, however, reason to believe that mesenchymal cells in bone play an essential role in the regeneration of stromal tissue (9).

Summary. The early hematopoietic regeneration in a depopulated segment of

femur shaft is compared in +/+ and W/W^v mice and in W/W^v mice previously treated with +/+ marrow. Since the W/W^v mouse has an intrinsic CFU deficiency on spleen colony assay and since immigrant cells play a negligible role in the onset of regeneration after marrow extirpation, the W/W^v(+/+) chimera provides a model for evaluation of the contribution of residual cells to the regenerative program. There was little difference in the relative recovery of CFU in +/+, W/W^v, and W/W^v-(+/+). Moreover, +/+ derived CFU were responsible for nearly all of the CFU repopulation in chimeric mice. Thus, recovery of hemic cellularity must be due to residual stem cells rather than to stem cells derived by transformation of more primitive mesenchymal elements. The residual CFU are probably intimately associated with bone, most likely within the endosteum and haversian system.

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