Lack of Recovery of Murine Hematopoietic Stromal Cells After Irradiation-induced Damage

By W. Chamberlin, J. Barone, A. Kedo, and W. Fried

The rates of recovery of colony-forming units (CFU) and of hematopoietic stroma following x-irradiation were compared. Hematopoietic stromal function of femurs and spleens was assessed by assaying the number of CFU in these sites 6 wk after implanting them subcutaneously into iso-
geneic hosts. Whereas the number of CFU returned to preirradiation levels in 6 wk, the hematopoietic stroma did not recover significantly in 9 wk. The reasons for regeneration of CFU in a defective stroma were next investigated. Two contributing factors were identified: (1) Stromal damage following 950 rads is such as to re-
tard the rate of regeneration of CFU without significantly limiting the eventual number of CFU in the site, and (2) Radi-
ated mice contain factor(s), possibly humoral, which accelerate the rate of regeneration of CFU.

The localization of hematopoiesis to the marrows and spleens of adult mammals indicates that the stroma of these sites contain certain specialized factors which are essential for supporting the growth and differenti-
tation of hematopoietic stem cells. Studies of the anemia of mutant S1/S1 mice indicate that these factors are probably cellular and are radiosensitive (although less so than the hematopoietic stem cells). Furthermore, whereas hematopoietic stem cells circulate in the blood stream, the stromal cells are fixed in the spleen and marrow.

Murine hematopoietic stem cells are assayed by suspending cells aspirated from the site under study, injecting the suspended cells intravenously into mice with a depleted hematopoietic stem cell compartment, and measuring the number of these which land in the spleen to form macroscopic hematopoietic colonies. Hematopoietic stromal cells, being fixed in nature, must be assayed by transplantation of intact hematopoietic organs. Tavassoli and Crosby, Maniatis et al., and others have shown that after implantation of femoral marrow or of femurs subcutaneously, the hematopoietic cells in the implanted marrow disappear; this is followed by proliferation of a primitive mesenchymal tissue, formation of sinusoids, and reconstitution of hematopoiesis. The re-
constituted hematopoietic tissue is derived primarily from host CFU, whereas the stroma is probably of donor origin. The extent to which CFU grow in sub-
cutaneously implanted femurs and spleens is determined by the integrity of the stroma in these sites, and accordingly this model is considered to provide an assay for hematopoietic stromal function.
There is evidence both in experimental animals and in man that high-dose x-irradiation (3000-5000 rads) to hematopoietic sites damages the stroma in such a way that hematopoietic recovery in the irradiated site occurs only very slowly if at all.\textsuperscript{11,13} The studies to be reported here show that hematopoietic stromal function recovers slowly, if at all, after exposure to 950-rad x-irradiation, whereas the number of CFU in irradiated sites eventually regenerates to near normal levels. Studies were then carried out to help explain why regeneration of hematopoietic stem cells proceeds in irradiated sites in spite of residual stromal damage.

**MATERIALS AND METHODS**

Ten- to twelve-week-old CAF\textsubscript{1} mice were used. X-irradiation was delivered by a Cobalt 60 source at a dose range of 50 rad/min. Colony-forming units (CFU), which are considered to be multipotential hematopoietic stem cells, were assayed by the method of McCulloch and Till\textsuperscript{5} as follows: cells were aspirated from the femoral marrow cavity into Hanks’ solution; or spleens were strained through a fine wire mesh into Hanks’ solution. The cells from five organs (no more than one per mouse) were pooled in 5 ml of Hanks’ solution to make each sample for assay. The cell suspensions were then flushed several times through a 25-gauge needle to disperse any cell aggregates. Additional Hanks’ solution was then added until \( \frac{1}{3} \) ml contained the cells from that aliquot of femur or spleen estimated (on the basis of results from previous experiments) to contain 3-20 CFU (linear portion of the dose-response curve). One-half milliliter of each suspension was then injected into each of 15 mice which had been exposed to 950 rads 1 day previously. The mice were sacrificed 8 days later, and the number of macroscopic colonies on each spleen surface was counted. The number of CFU per femur or spleen was then calculated, presuming each spleen colony to arise from one CFU.

Femurs and spleens were implanted as follows: The organs were removed, and the connective tissue and the fat was stripped from them. The two ends of the femurs were cut off, and the spleens were cut in half. The host mice were anesthetized with nembutal, a 1-cm skin incision was then made, and the skin was undermined by blunt dissection for a distance of about 2 cm. The organ to be implanted was placed into the subcutaneous space as far from the incision as possible, and the incision was closed with skin clips. The clips were removed 5 days later.

**Specific Experiments**

**Recovery of CFU and of stromal function in femurs of mice exposed to 950 rad.** Mice were exposed to 950 rads after which they received \( 10^6 \) marrow cells from nonirradiated donors. Another group (control) was not irradiated. At intervals of 1, 2, 3, 5, and 6 wk afterwards, batches of five mice per group were sacrificed. The number of CFU in the right femur was determined, and the left femur of each was implanted subcutaneously into an unirradiated host. Six weeks later the number of CFU in the implants was determined. In a second experiment these same parameters were studied 1, 6, and 9 wk after x-irradiation.

**Assay of CFU in irradiated and control femurs and spleens at various times after subcutaneous implantation into unirradiated hosts.** Mice were exposed to 950 rads after which they received \( 10^6 \) marrow cells. Controls were not irradiated. At intervals of 1, 2, 4, 6, 12, and 18 wk afterwards, batches of five mice per group were sacrificed. The number of CFU in the right femur was determined, and the left femur of each was implanted subcutaneously into an unirradiated host. Six weeks later the number of CFU in the implants was determined. In a second experiment these same parameters were studied 1, 6, and 9 wk after x-irradiation.

**Assay of CFU in femurs and spleens from normal donors implanted into irradiated as compared to unirradiated hosts.** Twenty mice were exposed to 950 rads and injected with \( 10^6 \) marrow cells. Controls were not irradiated. Two days later a spleen and a femur was implanted subcutaneously into each. One, two, four, and six weeks afterwards five mice from each group were sacrificed, and the CFU content of the spleen and femur implants was assayed. This experiment
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was replicated with the exception that irradiated hosts were exposed to 700 rads (and did not receive $10^6$ marrow cells).

RESULTS

Figure 1 shows the results of two experiments designed to measure the recovery of CFU and of stromal function in femurs of mice exposed to 950 rads. (The number of CFU present in a femur 6 wk after it is subcutaneously implanted into an isogeneic host is considered in this and subsequent experiments to be a measure of the functional integrity of the femur's hematopoietic stroma.) In both experiments, the number of femoral CFU recovered to the preirradiation level within 6 wk. The stromal function, on the other hand, remained defective (<20% of that in unirradiated femurs) for at least 9 wk after x-irradiation.

The next experiment, the results of which are presented in Fig. 2, is designed to determine whether subcutaneously implanted femurs and spleens from irradiated (950 rads) donors are actually incapable of supporting as many CFU as are those from nonirradiated donors; or whether CFU merely accumulate in these implanted organs at a slower rate. Femurs and spleens were obtained from mice exposed 6 wk previously to 950 rads and from nonirradiated mice (control). The number of CFU were assayed and the results are shown in Table 1. There is no significant difference between the number of CFU in the femurs and spleens of irradiated and control mice. The organs of others were implanted subcutaneously into isogeneic hosts and, at various intervals up to 18 wk later, the number of CFU in these implants was determined. The number of CFU in control femur implants rose at a near logarithmic rate to a value of approximately 500 in 6 wk after which they did not increase significantly in
number. The number of CFU in irradiated femur implants rose at a much slower rate but reached levels which are approximately 80% as great as in control implants after 18 wk. Control spleen implants contained only about 20–60 CFU (5%–10% of that in femur implants) at all time intervals studied from 2 to 18 wk postimplantation; whereas irradiated spleen implants contained an insignificant number of CFU at all times up to 18 wk postimplantation.

The next two experiments were performed to determine whether the relatively slow rate of regeneration of the CFU population in femurs and spleens of irradiated donors implanted into nonirradiated hosts, as compared to that in femurs and spleens of irradiated mice left in situ, could be attributed to the existence of factors present only in the irradiated host which enhance the rate of CFU proliferation. Femurs and spleens from nonirradiated mice were implanted subcutaneously into either irradiated (950 rads + 10⁶ cells in experiment 1 and 700 rads in experiment 2) or control hosts. The number of CFU in these implants was measured at various times after implantation. The results of the determinations of CFU in the spleen implants are shown in Fig. 3. At time intervals between 2 and 6 wk after implantation, spleens implanted into irradiated hosts contained several times more CFU than did those implanted into control hosts.

Figure 4 shows the number of CFU in femurs implanted into both irradiated and control hosts. Although the differences are less marked than those ob-

| Table 1. CFU in Femurs and Spleens Six Weeks After 950 rad and in Controls |
|-----------------------------|-----------------------------|-----------------------------|
|                             | Control | 950 rad + 10⁶ Cells | Δ* |
| Femur                       | 3010 ± 213 | 2700 ± 208 | N.S. |
| Spleen                      | 2010 ± 130 | 1740 ± 121 | N.S. |

*Significance.
CFU, primarily of host origin, migrate into and proliferate in femurs and spleens implanted subcutaneously into isogeneic hosts. The number of CFU in femur implants increases at a logarithmic rate for 6 wk after implantation to a value of about 500, after which it remains nearly constant. The number of CFU in spleen implants rises in the first 2 wk after implantation to about 40 and does not increase significantly in the subsequent 16 wk.

The stromal function of implanted femurs has been shown to be significantly impaired by exposure to 950-rad x-irradiation. The data reported here indicate, in addition, that this function does not recover detectably for at least 9 wk after x-irradiation. Although the stromal function of femurs from irradiated mice (as assessed by recovery of CFU in implants) remains impaired, the number of CFU in the femoral marrow recovers to nearly normal within 6 wk. This

served in the case of spleen implants, femurs implanted into irradiated hosts contained significantly more CFU than did those implanted into control hosts ($p < 0.01$) both 2 and 4 wk after implantation. Six weeks after implantation, the number of CFU in both groups was comparable.

**DISCUSSION**

Fig. 3. Assay of CFU in spleens from normal donors implanted into irradiated as compared to unirradiated hosts. Closed circles show results from experiment 1. Open circles show results from experiment 2. Solid lines connect mean results of assaying pooled cells from five spleens implanted into irradiated hosts (experiment 1, 950 rads + $10^6$ cells; experiment 2, 700 rads). Interrupted lines connect mean results of assaying pooled cells from five spleens implanted into unirradiated (control) hosts. Each point represents mean results from 15 assay mice. Vertical bars indicate ±1 SEM.

Fig. 4. Assay of CFU in femurs from normal donors implanted into irradiated as compared to unirradiated hosts. Closed circles show results from experiment 1. Open circles show results from experiment 2. Solid lines connect mean results of assaying pooled cells from five femurs implanted into irradiated hosts (experiment 1, 950 rads + $10^6$ cells; experiment 2, 700 rads). Interrupted lines connect mean results of assaying pooled cells from five femurs implanted into unirradiated (control) hosts. Each point represents mean results from 15 assay mice. Vertical bars indicate ±1 SEM.
is reminiscent of what has been observed to occur in mice with a congenital stromal defect (Sl/Si\textsuperscript{d}S).\textsuperscript{4} In these mice, the number of CFU in the femoral marrow is about half of that in nonmutants of the same strain, whereas the number of CFU in femur implants from Sl/Si\textsuperscript{d} donors is less than 15% of that in implants from nonmutants (6 wk after implantation). This apparent discrepancy, wherein a near normal number of CFU reside in a hematopoietic site with a severely impaired stroma, can be explained by the concept that a sub-maximal stromal defect reduces the rate of accumulation or of proliferation of CFU in the site, but not necessarily the eventual number that it is capable of supporting. In accord with this is the observation of McCulloch et al.\textsuperscript{1} that CFU transplanted into irradiated Sl/Si\textsuperscript{d} mice regenerate slowly, and that made in the present study that femur implants from unirradiated donors accumulate 500 CFU in 6 wk, whereas those from irradiated donors contain this many CFU only after 18 wk.

The interpretation proposed above does not explain why recovery occurs more rapidly in femurs of irradiated mice when left in situ than in those which have been implanted subcutaneously into an unirradiated host. Several factors are probably responsible for this difference. The irradiation-damaged stroma is susceptible to further injury during exposure to the ischemic condition which occurs before vascularization of the implant occurs. The degree and duration of ischemia is likely to be particularly prolonged in the irradiated implant, since the splenic and marrow microvasculature is damaged by x-irradiation. The relationship between the hematopoietic stroma essential for supporting hematopoiesis and the microvasculature has been suggested by the studies of Knospe et al.\textsuperscript{11} and of McCuskey et al.\textsuperscript{14}

Another factor(s) which probably contributes to the more rapid regeneration of CFU in irradiated femurs left in situ as compared to those implanted into nonirradiated mice is the existence of conditions in CFU-depleted, irradiated mice which enhance the rate of regeneration of CFU. This concept is supported by the observation that the number of CFU in femurs, and particularly in spleens implanted into irradiated hosts, increases more rapidly than does that in organs implanted into nonirradiated hosts. It is tempting to speculate that this is caused by the presence of a humoral factor, elaborated after x-irradiation in response to the decrease in the number of CFU, which stimulates surviving stem cells to proliferate. The existence of such a humoral factor has been proposed on the basis of a variety of experiments.\textsuperscript{15-19} However, the experiments reported here have not excluded the effect of irradiation-induced anemia of the host mice on the number of CFU in spleen implants. Another possible mechanism which is under investigation is that conditions in the irradiated host somehow promote the more rapid egress of cells from the implant and consequently provide a more suitable microenvironment in the implant for CFU proliferation. Finally, the growth of CFU in implanted organs is dependent on the rate of revascularization of the implants. It is possible that the vascularity of organ implants is established more rapidly in irradiated than in nonirradiated hosts. To determine the role of these factors, histologic studies including differential cell counts of the organ implants are underway, and the results will be reported later.
Although murine spleens in situ contain approximately as many CFU as do femurs, they support the growth of only about 20% as many CFU as do femurs after subcutaneous implantation. This cannot be explained by postulating that splenic implants vascularize slower, since the number of CFU present in spleens 2 wk after they are implanted into irradiated hosts is approximately ten times that present in spleens 2 wk after implantation into control hosts. We propose the following explanation: the stroma of spleens is less favorable than that of femurs for supporting the growth of CFU. Accordingly, a greater stimulus is required to encourage CFU growth in spleens than in femurs. Under normal conditions, in situ, the need for CFU is sufficient to saturate the femoral marrow and support the growth of as many CFU in the less favorable splenic microenvironment. However, after implanting spleens into the CFU-saturated, unirradiated organism, the stimulus for CFU growth is insufficient to encourage significant CFU growth in the spleen implant. Irradiation of the host prior to implantation of spleens, on the other hand, provides a sufficient stimulus to promote CFU growth in the spleen implant.

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