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RECOVERY OF PROLIFERATIVE CAPACITY OF AGAR COLONY-FORMING CELLS AND SPLEEN COLONY-FORMING CELLS FOLLOWING IONIZING RADIATION OR VINBLASTINE

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### Publication Date

1969-07-01

*cy-2*

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RECOVERY OF PROLIFERATIVE CAPACITY OF AGAR COLONY-FORMING  
CELLS AND SPLEEN COLONY-FORMING CELLS FOLLOWING  
IONIZING RADIATION OR VINBLASTINE<sup>1</sup>

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<sup>1</sup>This research was supported by the United States Atomic Energy Commission and by United States Public Health Service fellowship 5 FO3 CA28902-02 from the National Cancer Institute.

ABSTRACT

The effects of sublethal radiation and the mitotic inhibitor, vinblastine sulphate, on the number of cells in mouse bone marrow capable upon transplantation of forming macroscopic colonies on the surface of the spleens of irradiated recipient mice (CFU) and on the number of cells capable of forming colonies in soft agar after cell culture (ACFU) were studied as a function of time after injury. The results show that ACFU are radiosensitive and vinblastine-sensitive cells, comparable in sensitivity to erythropoietin-sensitive cells. The temporal pattern of recovery following radiation of ACFU, different from that for CFU, is compatible with the concept that these are two distinct but closely related stem cell populations. The relevance of these findings to models of hematopoiesis and to studies on the precursors of macrophages and monocytes in inflammatory exudates is discussed.

Pluznik and Sachs ('65) and Bradley and Metcalf ('66) have independently reported methods for the in vitro culture of mouse spleen or bone marrow cells. Both methods involve growth in solid agar medium supported by a feeder layer of mouse embryonic cells or neonatal kidney cells or by the presence of certain growth factors in the medium itself. Colonies produced by this technique consist of populations of granulocytic cells in varying stages of differentiation or large phagocytic cells indistinguishable from macrophages, or both.

We have studied the relationship between these agar colony-forming units (ACFU) and the spleen colony-forming units (CFU) of Till and McCulloch ('61) after perturbation of the steady state by radiation or by the mitotic inhibitor, vinblastine sulphate, with the particular goal of establishing or ruling out identity between these two measures of hematopoietic stem cells. Evidence is presented that favors two distinct stem cell populations, and these findings are discussed in terms of independent and dependent stem cell criteria (McCulloch, Till and Siminovitch, '65).

#### MATERIALS AND METHODS

##### Mice

Eight- to 11-week-old female  $C_3H/Cum$  mice, obtained from Cumberland Farms, were used as bone marrow donors in all experiments. Neonatal kidneys were obtained from 8- to 10-day-old male and female  $C_3H/Cum$  mice bred from Cumberland stock in this Laboratory.

Three- to 5-month-old female  $C_3H/Cum$  mice were used as recipients in the spleen colony assays.

#### Irradiation

In the radiation studies donor  $C_3H/Cum$  mice were irradiated at various times before sacrifice and collection of bone marrow. Irradiations were at 1.22 meters from a  $^{60}Co$  source with a dose rate in air of 26.6 R/min; measured in air with a Victoreen condenser R-meter under these exposure conditions, the average tissue dose to the mouse was approximately 0.94 rad/R in air. After irradiation, the animals were housed, five to a cage, in sterilized cages and allowed food and chlorinated water ad libitum. For the spleen colony assay, recipient mice received 1100 R under the above conditions. Terramycin (Chas. Pfizer and Co., Inc., New York, N. Y. ), 0.5 to 1.0%, was added to the chlorinated drinking water. The endogenous spleen colony level was approximately 1 colony per spleen.

#### Vinblastine sulphate (VLB)

Vinblastine sulphate (Velban; Eli Lilly and Co., Indianapolis, Indiana) was obtained as lyophilized powder and dissolved in sterile physiological saline to a concentration of 400  $\mu g$  per ml (80  $\mu g$  per 0.2 ml). All vinblastine preparations were injected i.v. as single 0.2-ml injections at various times before sacrifice and collection of bone marrow.

Bone marrow culture technique

The method for cloning bone marrow cells in soft agar was essentially that of Bradley and Metcalf ('66). The reagents used in the culture technique had the following composition: E2020 medium (Grand Island Biological Co., Oakland, Calif.): mixture of minimum essential medium with L-glutamine and without sodium bicarbonate (Earle's salts) 10 x, 100 ml; sodium bicarbonate 7.5%, 30 ml; sodium pyruvate 100 x, 10 ml; nonessential amino acids 100 x, 10 ml; penicillin-streptomycin (5000 U of each/ml), 2 ml; 3 x glass-distilled water, 148 ml; fetal calf serum, 100 ml; total volume 400 ml. Trypticase soy broth (TSB): 3 g trypticase soy broth powder (Baltimore Biological Lab., Baltimore, Md.) in 100 ml 3 x glass-distilled water, autoclaved 15 min at 121°C (15 lb pressure) and stored at 4°C for no more than 2 weeks. Bacto agar (Difco Labs., Detroit, Mich.): 1.0 g agar in 100 ml distilled water, boiled for 5 min, thoroughly dissolved, and held at 41°C in a water bath. Agar for bone marrow cell overlay was prepared in the same manner except that 0.6 g agar in 100 ml distilled water was used. Bone marrow-collecting fluid: E2020 medium, 40 ml; trypticase soy broth, 10 ml; distilled water, 50 ml.

Feeder layers were prepared from the kidneys of 8- to 10-day-old C<sub>3</sub>H/Cum mice one day before adding the bone marrow cell overlay. The kidneys were removed aseptically, rinsed in Eagle's minimum essential medium, Spinner (Grand Island Biological Co., Grand Island, N.Y.) with 50 to 70 U/ml penicillin-streptomycin, and minced with fine



irridectomy scissors into a solution containing 0.25% trypsin in solution A (Grand Island Biological Co., Oakland, Calif.), 50 U/ml penicillin-streptomycin, and 0.04% DNase solution (Worthington Biochemical Corp., Freehold, New Jersey, w/v in phosphate buffered saline), 0.3 ml added per 10 ml trypsin solution (after the method of Madden and Burk, '61). The mixture was stirred gently at room temperature for not more than 1 hr and occasionally pipetted up and down to break up larger tissue clumps. The mixture was then centrifuged at about  $500 \times g$  for 7 min and the cell pellet washed twice with bone-marrow collecting fluid. The kidney cells were then counted by hemocytometer after suitable dilution in Turk's diluting fluid; clumps of three or more cells comprised about 10% of an otherwise single-cell suspension. The kidney cells were spun down by centrifugation ( $500 \times g$  for not more than 4 min), and resuspended at appropriate cell concentration in E2020 medium with trypticase soy broth (4 parts E2020: 1 part TSB) previously warmed to  $37^{\circ}\text{C}$ . The suspension was finally mixed with an equal volume of 1% Bacto agar to a final cell concentration of 1 to  $2 \times 10^6$  kidney cells per ml 0.5% agar suspension. Two ml of this suspension was plated in each  $35 \times 100$  mm plastic Petri dish (Falcon Plastics, Los Angeles, Calif.) and allowed to sit undisturbed at room temperature for 15 min; incubation was at  $37^{\circ}\text{C}$  in a humidified incubator with constant flow of 5%  $\text{CO}_2$  in air.

Preparation of bone marrow suspensions

Bone marrow was collected from both femurs by the method of Schooley ('66), and marrow from the four mice (eight femurs) of each group pooled. Marrow was obtained by flushing bone marrow-collecting medium backward and forward through the femoral marrow cavity by means of a 23-gauge needle inserted in the proximal end of the femur. The resulting cell suspension was then passed about 10 times through a 25-gauge needle and finally filtered through a nylon gauze sack. The pooled marrow suspension was made up to a volume equivalent to 1 ml bone marrow suspension per each femur. Cells were counted in a hemocytometer chamber after suitable dilution in Turk's diluting fluid. All cell suspensions were kept at ice water temperature throughout these procedures.

For assay of the spleen colony-forming unit (CFU) content of each sample (Till and McCulloch, '61), an aliquot was taken and diluted to a suitable concentration with minimal essential medium, Eagle (Grand Island Biological Co., Grand Island, New York); 0.5 ml of this dilution was then injected into the lateral tail veins of recipient mice which had received 1100 R whole-body irradiation less than 3 hr earlier. Groups of 15 to 20 recipient mice were used for each dilution, and a group of irradiated but uninjected control mice was included in each experimental run in order to determine endogenous CFU levels. Seven to 10 days later the mice were sacrificed, and their spleens were removed and fixed in Bouin's solution. Counts of macroscopically visible colonies on the surface of the spleen were

performed under 10 × magnification.

For assay of agar colony-forming unit (ACFU) content, an aliquot of the original bone marrow suspension was taken (usually 100 to 250  $\mu$ l) and diluted with 20 to 50 volumes of a 4:1 solution of E2020 medium and trypticase soy broth; two dilutions were usually prepared. The cell suspensions were held at 37°C, and then mixed with an equal volume of 0.6% agar held at 40°C. One-ml aliquots of this mixture were pipetted over the kidney feeder layers prepared the day before, and left at room temperature for 15 min to allow gelling. Incubation was at 37°C in a humidified incubator with a constant flow of 5% CO<sub>2</sub> in air. Colony counts were performed routinely, after 10 days of incubation, under 10 × magnification. A schematic summary of the experimental procedures is depicted in figure 1.

## RESULTS

### Recovery patterns of ACFU and CFU

#### following 80 $\mu$ g vinblastine

The data on the recovery of proliferative capacities of ACFU and CFU as a function of time after intravenous injection of 80  $\mu$ g VLB, simultaneously measured in femoral bone marrow, are summarized in table 1 and figure 2. Twenty-four hours after the drug, both ACFU and CFU were at a minimum. The ACFU were about 20% of control after this dose of mitotic inhibitor; preliminary dose-response studies have shown an exponential decrease in ACFU survival with a D<sub>37</sub> value of 50  $\mu$ g. Over the next 48 hr, recovery to supranormal levels was exponential, with a doubling time of about 18 hr, and survival remained at normal levels (except for days 7 and 8) for the remainder

of the experiment. The pattern of recovery of ACFU is similar to that for recovery of erythropoietin responsiveness following 80  $\mu$ g VLB reported by Schooley ('69) and for recovery of numbers of  $H^3$ -thymidine labeling peritoneal macrophage precursors after the same dose of the drug (Chen and Schooley, '69b).

Recovery of CFU followed a similar time course, and a doubling time of 24 hr during the exponential phase of recovery was found. Smith, Wilson and Fred ('68) have reported qualitatively similar data with less initial depression at 24 hr and recovery to normal levels by 60 hr; the greater survival at 24 hr and slightly later recovery to normal levels may be due to the different mode of injection of the drug (intraperitoneal) and different strain of mouse. Valeriote, Bruce and Meeker ('66) reported approximately 20% survival at 24 hr compared with the 40% found in this experiment. The doubling time of 24 hr for the CFU recovering from the 80  $\mu$ g VLB is identical to the doubling time of transplanted marrow CFU in the spleens of lethally irradiated normal and polycythemic mice (McCulloch and Till, '64; Schooley, '66).

Recovery patterns of ACFU and CFU following 200 R  
whole body  $\gamma$ -irradiation

The data on the recovery of proliferative capacities of ACFU and CFU as a function of time after irradiation, simultaneously assayed in femoral bone marrow, are summarized in table 2 and figure 3. As in the previous experiment, minimum values of ACFU and CFU were attained within 24 hr after injury. The striking finding, however,

TABLE 1.

A comparison of bone marrow spleen colony-forming units (CFU) and  
 agar colony-forming units (ACFU) as a function of time  
 after 80 µg vinblastine i.v. per mouse

Day post-VLB injection	Nucleated cells per femur $\times 10^6$	CFU per femur	CFU per $10^5$ nucleated cells	ACFU per femur	Percent of control No. of ACFU
0	13.5	4830 $\pm$ 570*	35.8	14,200 $\pm$ 420	100
1	3.8	1940 $\pm$ 210	51.0	4,150 $\pm$ 65	29 $\pm$ 1
2	1.9	4070 $\pm$ 330	214	7,660 $\pm$ 840	54 $\pm$ 6
3	3.7	7130 $\pm$ 950	194	18,200 $\pm$ 1900	130 $\pm$ 10
4	8.7	6050 $\pm$ 1100	69.5	12,100 $\pm$ 870	85 $\pm$ 7
5	12.5	4060 $\pm$ 260	32.5	16,600 $\pm$ 1600	120 $\pm$ 10
6	10.5	4860 $\pm$ 740	46.3	11,000 $\pm$ 910	77 $\pm$ 7
7	15.4	-	-	9,070 $\pm$ 720	64 $\pm$ 5

\* Standard error of the mean

TABLE 2.

A comparison of bone marrow spleen colony-forming units (CFU) and  
 agar colony-forming units (ACFU) as a function of time  
 after 200 R whole-body gamma irradiation

Day post- 200 R	Nucleated cells per femur $\times 10^6$	CFU per femur	CFU per $10^5$ nucleated cells	ACFU per femur	Percent of control No. of ACFU
0	13.6	4700 $\pm$ 850*	34.6	8,930 $\pm$ 930	100
1/4	9.4	367 $\pm$ 36	3.9	1,100 $\pm$ 89	12 $\pm$ 2
3	9.1	533 $\pm$ 51	5.9	9,530 $\pm$ 460	110 $\pm$ 10
5	9.6	433 $\pm$ 46	4.5	6,560 $\pm$ 420	73 $\pm$ 9
8	14.7	450 $\pm$ 26	3.1	2,780 $\pm$ 330	31 $\pm$ 5
12	13.8	980 $\pm$ 120	7.1	1,790 $\pm$ 150	20 $\pm$ 3
15	10.2	1630 $\pm$ 180	15.9	7,710 $\pm$ 190	86 $\pm$ 9
21	12.0	3670 $\pm$ 450	30.6	12,300 $\pm$ 380	140 $\pm$ 10

\*Standard error of the mean

was the rapid (if abortive) recovery of ACFU to near control values during the first week after irradiation, while the CFU remained suppressed at values 10% of control. The pattern of recovery of ACFU is similar to but more rapid than that reported for recovery of erythropoietin responsiveness after 150 to 200 R (Gurney, Lajtha and Oliver, '62; Schooley, Cantor and Havens, '66). The pattern of recovery of CFU confirms similar findings by several groups (McCulloch and Till, '61; Schooley, Cantor and Havens, '66; Smith, Wilson and Fred, '68).

A minimum value of 14% of control was found for ACFU 24 hr after 200 R; dose-response studies in this Laboratory, measuring ACFU 24 hr after various doses of whole-body  $\gamma$ -irradiation, have shown an exponential decrease in survival of ACFU with a  $D_{37}$  value of approximately 95 rads. Robinson, Bradley and Metcalf ('67) have reported a  $D_{37}$  value of 85 rads for ACFU measured 48 hr after various doses of x-rays to C57Bl mice. In this experiment a doubling time of about 12 hr was observed for ACFU during the exponential recovery phase.

#### DISCUSSION

The experimental results presented in this paper are consistent with the view that agar colony-forming cells (ACFU) are a population of cells distinct from spleen colony-forming cells (CFU). This was clearly demonstrated by the recovery patterns of each type of stem cell after 200 R: the rate and timing of recovery of the ACFU were faster and earlier than that of CFU. There was also resemblance to

the pattern of recovery after 200 R of erythropoietin-sensitive cells (ESU) in relation to CFU as demonstrated by Schooley, Cantor and Havens ('66), which suggests that ACFU may exist in a similar relationship to CFU.

Bennett, Cudkowicz, Foster and Metcalf ('68) have shown in genetically anemic mice of the W series that ACFU content of bone marrow was normal in numbers and in vitro colony-forming ability, while CFU were severely deficient in numbers and in the ability to produce normal-sized spleen colonies. They suggest that ACFU are an intermediate stage of differentiation between CFU and myeloblasts. McCulloch ('68) has also proposed this concept with the reservation that the differences in proliferative and differentiative potential expressed in the two assay systems may be a reflection of the influence of environment on the expression of potential rather than the existence of two different classes of stem cells with different potentials.

We feel the results of this investigation provide physiological evidence for the concept that these are two functionally distinct stem cell populations. Other evidence of this type has come from 14-day serial transplantation studies in this Laboratory, which show increased numbers of ACFU per spleen during the first four passages, whereas CFU per spleen are below normal levels at the second passage and continue to decline after subsequent passages (Chen and Schooley, '69a). Using physical techniques for fractionation of bone marrow suspensions, Worton, McCulloch and Till ('69) and independently, Schooley, Borsook and Chen (unpublished observations) have found



significant differences in the ratio of ACFU to CFU in several of the fractions.

Wu, Siminovitch, Till and McCulloch ('68) examined the relationship between ACFU and CFU in a study of the ability of individual chromosome-marked spleen colonies to produce progeny capable of clonal proliferation in vivo (CFU) and in vitro (ACFU). Their findings demonstrated that many spleen colonies possessed such capabilities and that a remarkable correlation existed between the numbers of each type of colony-forming cell in each spleen colony. Both findings are compatible with the idea that a close parent-progeny relationship exists between CFU and ACFU respectively. To our knowledge, no one has shown that ACFU can produce spleen colonies upon transplantation of agar colonies into a suitable recipient animal (i.e., form CFU).

In 1965, McCulloch, Till and Siminovitch proposed the idea of independent and dependent stem cells in order to explain data from studies on the action of erythropoietin on primitive hematopoietic cells (Bruce and McCulloch, '64); their work and that of Schooley ('66) showed that erythropoietin did not act on CFU, but rather on a different cell population, called erythropoietin-sensitive cells (ESU). These ESU were classified as dependent stem cells, characterized by a more committed pattern of differentiation, different rate and capacity for proliferation, sensitivity to specific inductive agents, and a close ontogenic relationship to cells of the independent stem cell class (e.g., CFU). In light of studies reviewed here and

accumulating evidence for hormonal agents in granulocytopoiesis and monocytopoiesis, it is appropriate that ACFU should also be included in this class of dependent stem cells.

We have been interested in ACFU as progenitors of monocytes and inflammatory macrophages (Chen and Schooley, '69b). As assayed in the present experiments (colonies counted on day 10 of incubation), the colonies in agar are composed almost entirely of phagocytic mononuclear cells indistinguishable from inflammatory macrophages; this has been reported by Ichikawa, Pluznik and Sachs ('66) and Metcalf, Bradley and Robinson ('67). Volkman and Gowans ('65a, '65b) have described a rapidly proliferating bone marrow cell population, called macrophage precursors, identifiable after  $^3\text{H}$ -thymidine labeling in skin window or peritoneal exudates as labeled macrophages. Macrophage precursors in the steady state must include progeny of ACFU differentiating along monocytic lines and new daughter ACFU resulting from self-renewal divisions within the ACFU compartments. After radiation or vinblastine injury, the rapidly dividing CFU compartment survivors must also contribute to this  $^3\text{H}$ -thymidine-labeled pool of cells. For this reason and because of several other problems involved with the use of  $^3\text{H}$ -thymidine-labeling as a measure of cell proliferation (e.g., changing endogenous thymidine pool sizes or altered transport of the labeled nucleoside after injury), ACFU provide a more accurate measure of specifically committed monocyte-macrophage progenitor cells. Finally, the macroscopic agar colony represents a measure of reproductive integrity after

injury, not only an assay for reproductive capacity, as has been emphasized by Puck and Marcus ('56) in their studies on irradiated mammalian cells in clonal culture.

The application of functional criteria to characterization of primitive hematopoietic cells, as pioneered by McCulloch, Till, Siminovitch and their co-workers in in vivo systems, has relevance to in vitro studies as well. With recognition of other dependent stem-cell populations and agents regulating their behavior and with development of new in vivo and in vitro techniques, a more complete picture should emerge of an important but poorly understood area of hematopoiesis.

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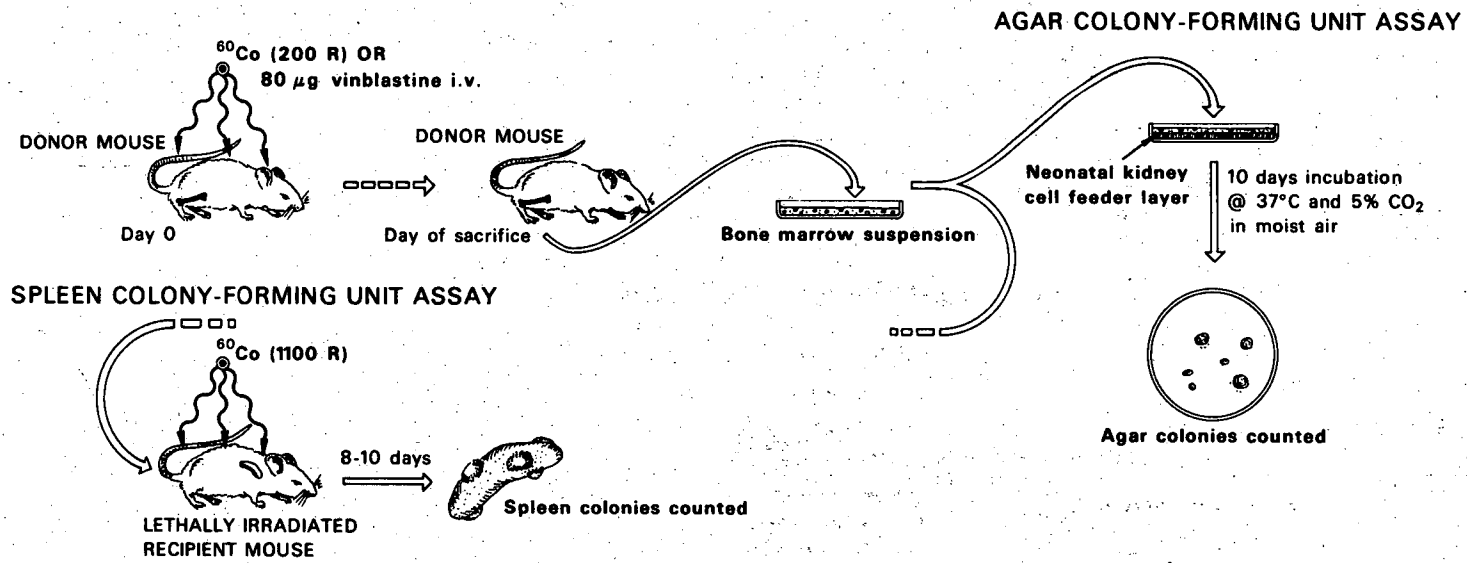
FIGURE LEGENDS

Fig. 1. Schematic summary of experimental procedures employed in the parallel determination of spleen colony-forming units/femur and agar colony-forming units/femur after 200 R whole-body  $^{60}\text{Co}$  gamma rays or 80  $\mu\text{g}$  vinblastine i.v.

Fig. 2. Comparison as a function of time of agar colony-forming units and spleen colony-forming units in the bone marrow of mice treated with 80  $\mu\text{g}$  vinblastine i.v. on day 0. Agar colony-forming units are expressed as percent of control values from corresponding groups of untreated mice. Standard errors of the mean of 2 to 6 separate experiments are shown for agar colony-forming units, and for spleen colony-forming units, standard error of the mean for an average of 12 replicate determinations.

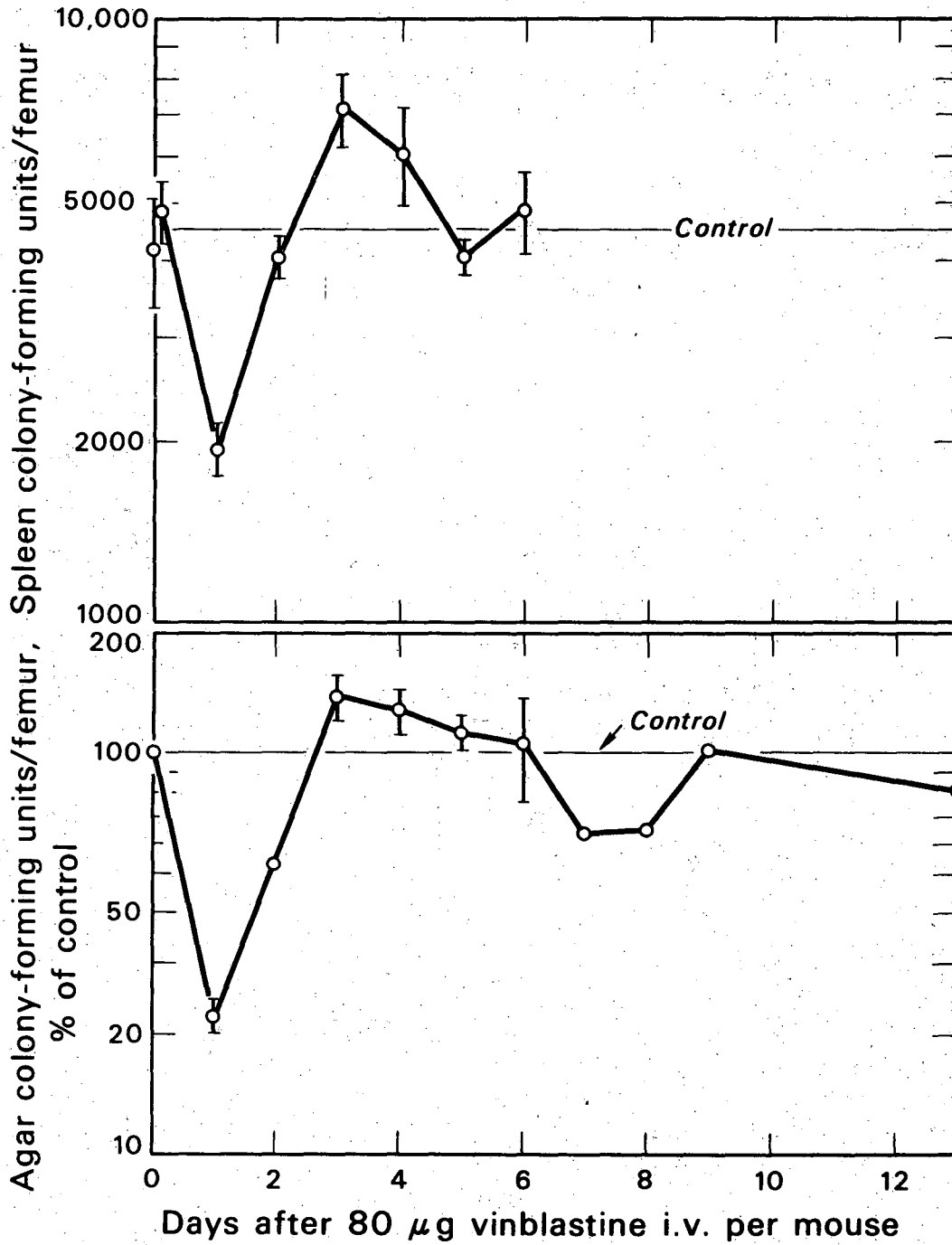
Fig. 3. Comparison as a function of time of agar colony-forming units and spleen colony-forming units in the bone marrow of mice irradiated with 200 R whole-body  $^{60}\text{Co}$  gamma rays on day 0. Agar colony-forming units are expressed as percent of control values from corresponding groups of unirradiated mice. For agar colony-forming units, standard errors of the mean of two experiments are shown, and for spleen colony-forming units, standard error of the mean for an average of eight replicate determinations.





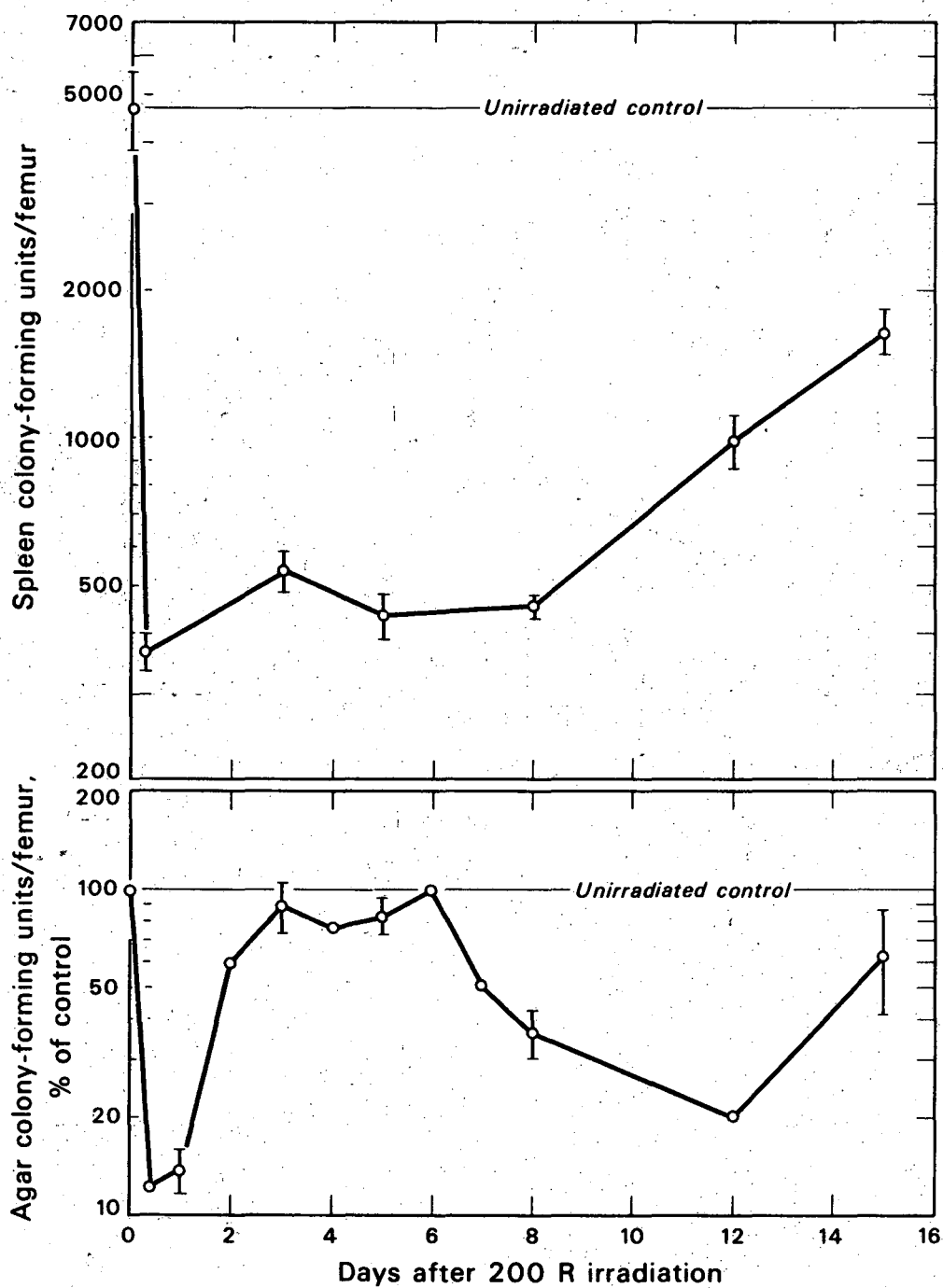
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Fig. 1



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Fig. 2



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Fig. 3

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