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Hemopoiesis in Diffusion Chambers in Strontium-89 Marrow-Ablated Mice[1] (40328)

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Hemopoiesis can be evaluated by studying the proliferation of hemopoietic cells in diffusion chambers (DCs) implanted into the peritoneal cavities of animals. During the initial several hours after seeding and implantation, the number of cells recoverable from the inoculum declines by 40 to 60% (1).

The predominant hemopoietic precursor cell responsible for the enlarging hemopoietic cell population in DCs appears to be a granulocyte/macrophage committed precursor (2, 3). Multipotent hemopoietic stem cells (CFU-S), however, also have been shown to play a role in DC hemopoiesis (2, 4–7) and the number of these cells reaches its peak in DC cultures before the total number of hemopoietic elements reaches its maximum (8, 9).

If the hemopoietic cell inoculum consists of steady state cells, such as cells from marrows of normal mice, proliferation in DCs begins after a lag of about 18 hr (1). A number of investigators have found that in DCs the number of cells harvested, at least from Days 4 to 7 after implantation, is related linearly to the number of cells in the inoculum, suggesting that there is little or no significant cell–cell interaction (10–12). Niskanen and his colleagues (13, 14), on the other hand, found that as the numbers of cells seeded in DCs were increased, the growth of both differentiated granulocytes as well as CFU-S was inhibited. In addition, proliferation of cells in DCs is modified substantially by pretreatment of the host animals with agents such as irradiation (8, 10, 15, 16) or cytotoxic drugs (2, 4, 10, 13, 15, 17), both of which perturb the hemopoietic state of host animals.

Elevated levels of colony-stimulating activity (CSA), i.e., glycoproteins required for the growth of granulocyte/macrophage precursors *in vitro*, have been found in the serum of animals treated with whole-body irradiation (13, 16, 18–20). In whole-body-irradiated animals, hemopoiesis in DCs has been found to parallel the increase in serum CSA levels (15, 16); this relationship is expected, as the DC technique primarily assesses granulopoiesis.

The bone-seeking radionuclide, ^{89}Sr, can be used to ablate marrow hemopoiesis selectively (21–24). By 10 days after ^{89}Sr injection (4 μCi/g body wt) the marrows of mice are aplastic and contain less than 2% of the normal number of CFU-S (24). The spleens of ^{89}Sr-treated mice support marked compensatory-hemopoiesis and these mice develop only a mild anemia but a more severe leukopenia; with the passage of time, hemopoiesis is gradually restored in the marrows of these mice (21, 24, 25). In a previous study, we did not detect an elevation in serum CSA levels in ^{89}Sr-treated mice (26).

In an attempt to evaluate the presence of a humoral stimulus for hemopoiesis in ^{89}Sr-treated mice, at various times after ^{89}Sr treatment, we implanted into such mice DCs containing 1×10^6 marrow cells from normal mice. We evaluated the total number of cells, proportions of the various cellular elements, and the number of CFU-S in the DCs 72 hr after implantation.

Materials and methods. Pathogen-free female CAF_1 (Balb/c × A/He) mice (Cumberland View Farms, Clinton, Tenn.), 14 to 16 weeks old, were housed in cages with disposable plastic bottoms; a maximum of 10 mice were housed per cage. The mice were permitted food and acidified (pH 3.2) water *ad libitum*. On Day 0 the mice were given ip injections of $^{89}SrCl_2$ (Oak Ridge National Laboratories, Oak Ridge, Tenn.), 4 μCi/g body wt, in 0.25 ml of a solution buffered to pH 5 to 6; control mice were injected with a comparable amount of cold $^{88}SrCl_2$. On days

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7, 10, 18, and 39 after the Sr injections, DCs were implanted into the peritoneal cavities of anesthetized (sodium pentobarbital) mice and 72 hr later the DCs were removed. Prior to sacrifice, blood was obtained from each host mouse by bleeding it from the lateral orbital plexus into heparinized capillary tubes. A microhematocrit determination, total nucleated cell count (by hemacytometer), and 100-cell differential count were performed on the blood from each mouse. For each time studied, five radio-^{89}Sr-treated and five cold-^{88}Sr-treated mice were studied. Each mouse was implanted with two DCs, one into the right side and the other into the left side of the peritoneal cavity; the chambers were marked for identification prior to implantation.

DCs were constructed by gluing deionized Nuclepore membranes (Nuclepore Corp., Pleasanton, Calif.) which had 0.22-μm pores, to the two sides of plastic rings (Millipore Corp., Medford, Mass.) with Millipore MF cement; the DCs were tested for leaks by injection with air under water and then sterilized in 70° dry heat for 16 hr. They were seeded with 1×10^6 marrow cells pooled from the femurs of three CAF_1 mice; the cells were suspended in 0.1 ml of Hanks' balanced salt solution (HBSS). The holes used to fill the chambers were occluded with plugs of dental wax. Prior to implantation, the DCs were immersed in a solution of penicillin and streptomycin. After 72 hr in the mice, the chambers were removed and placed into a solution containing 0.5% grade B Pronase (Calbiochem, San Diego, Calif.) and 5% Ficoll (Litton Bionetics Lab Products, Kensington, Md.) in which they remained for 90 min at room temperature; they were agitated continuously. The wax plugs were removed and the contents of the DCs were removed by aspiration through the filling hole by means of a 27-gauge needle attached to a tuberculin syringe. The chambers were washed thrice with 0.1 ml of HBSS; the last wash was performed after the removal of one of the Nuclepore membranes. At each time studied, the contents of the five chambers which were implanted into the right sides of the mice were combined to form one suspension of pooled cells, and those from the five chambers from the left sides another. These two suspensions of pooled cells were counted and assayed separately. Cytospin centrifuge (Shandon Southern Instruments, Inc., Sewickley, Penn.) slides were prepared from each suspension of pooled cells and a 400-cell differential count was performed on each of the suspensions. The criteria of Benestad (27) were used to classify proliferative and nonproliferative granulocytic elements. Duplicate nucleated cell counts were performed on each cellular suspension by means of a hemacytometer.

The CFU-S content of each cellular suspension was assayed by the surface spleen colony technique of Till and McCullough (28). The pooled cellular suspensions were diluted so that the equivalent of ⅕th or ⅒th of the contents of a single chamber was contained in 0.5 ml of HBSS which was then injected into a lateral tail vein of an assay mouse which had been exposed to 900 rad of whole-body irradiation provided by a ^{137}Cs source (Gamma Cell 40, Atomic Energy of Canada, Ltd., Ottawa, Canada) within the previous 3 hr. We used 12 to 15 mice to assay each suspension of cells.

The results of the studies performed on the chambers implanted into the right and left sides of the mice were evaluated separately; as the results from the two groups were virtually identical we will report only the pooled data. We had control studies at each time interval and report the results of the cell counts and CFU-S assays individually. The differential counts performed on the contents of the chambers implanted into the control mice were very similar at the four times evaluated; this is to be expected, as the control mice, injected with $^{88}SrCl_2$ were "normal" animals at all times. To simplify the reporting of differential counts of the DC cells, we have reported the differential counts from the cells implanted into the normal mice as means ± SE obtained from all the time intervals studied; the results from the experimental mice are reported separately for each time.

Student's t test was used to evaluate the statistical differences between the results obtained from the ^{89}Sr and ^{88}Sr groups. As there are only two values (obtained from the right and left chamber suspensions) for the total numbers of cells per chamber and for the differential counts of the cells for each group at each time studied, we did not analyze these

statistically (Fig. 1C; Table I).

Results. The ^{89}Sr-treated host mice were significantly anemic only during the period in which the second group of chambers were implanted, i.e., 10 to 13 days after ^{89}Sr injection (Fig. 1A), but these mice were granulocytopenic at all times studied (Fig. 1B).

The total number of nucleated cells harvested from the DCs implanted into the ^{89}Sr-treated mice was greater than that harvested from the DCs housed in the control mice at all times studied (Fig. 1C); the largest differences occurred at the 10- to 13-day and 18- to 21-day time periods when the ratios between the cell contents of the DCs from the ^{89}Sr and those from the ^{88}Sr control mice were 1.8 and 2.4, respectively (Fig. 1C). In addition, at the first three times studied, the numbers of cells harvested from the chamber housed in the ^{89}Sr-treated mice exceeded the numbers (1×10^6 cells) in the original inoculum.

In general, the proportion of the various cellular elements in the DCs of the ^{89}Sr and ^{88}Sr mice were quite similar (Table I). There was, however, a slight increase in the proportion of blasts in the DCs from the ^{89}Sr mice during the first three times studied (Table I). In addition, in the 10- to 13-day DCs from the ^{89}Sr-treated mice there was a modest increase in the proportion of nucleated red blood cells (Table I); this was the only time during which the ^{89}Sr-treated mice were significantly anemic (Fig. 1A).

The inoculum contained about 340 CFU-S. The numbers of CFU-S harvested from the DCs housed in the ^{89}Sr-treated mice were significantly greater than those from the DCs housed in the ^{88}Sr control mice. The greatest difference between the numbers of CFU-S in the two groups occurred in those chambers implanted during the second (10–13 days) and third (18–21 days) intervals studied (Fig. 1D); these were the same times during which the largest differences were found in total numbers of nucleated cells per chamber. The second-interval-chambers, implanted 10 days after ^{89}Sr injection, contained more than

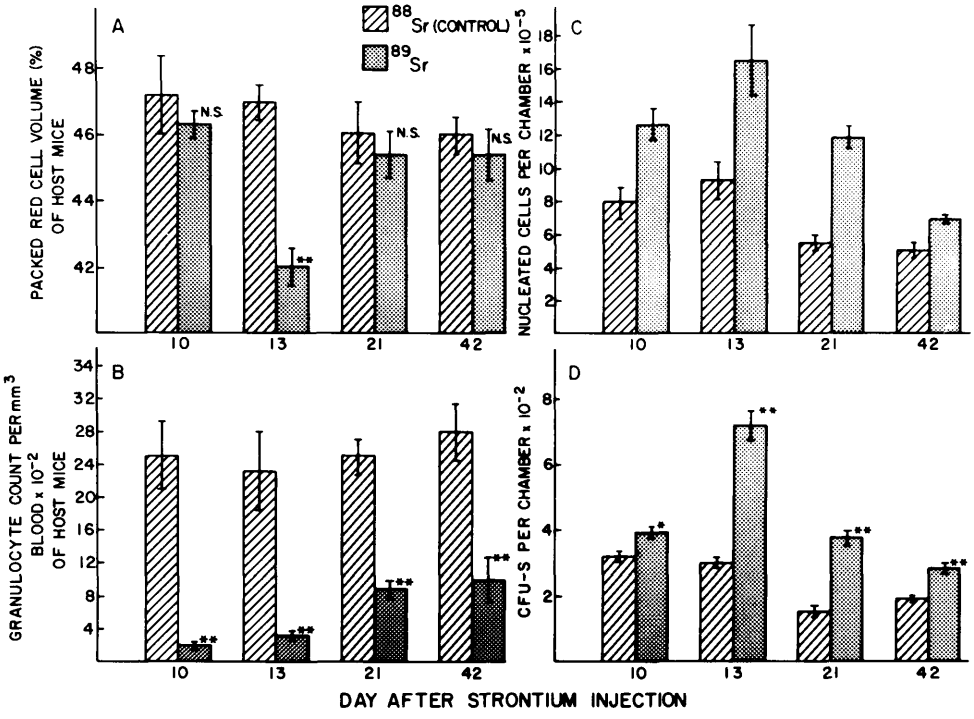

FIG. 1. (A) Packed red cell volume (as a percentage) and (B) granulocyte counts per cubic millimeter of blood from diffusion chamber (DC) host mice; and (C) nucleated cell counts and (D) numbers of CFU-S of DCs. All chambers were in mice for 72 hr. Days indicated are numbers of days after Sr injections which also were days on which blood counts were performed and DCs harvested. Means ± SE. N.S., not significant; $*p < 0.01$; $**p < 0.001$.

twice as many CFU-S as did the inoculum.

Discussion. The larger number of CFU-S in DCs cultivated in ^{89}Sr-treated mice as compared to that in DCs from control mice suggests that in ^{89}Sr-treated mice there is a humoral mechanism(s) which effects either more rapid proliferation of CFU-S or a shortening of the preproliferative lag period or both. The early (by 72 hr) substantial increase in the number of differentiated blood elements in DCs from ^{89}Sr-treated mice suggests that there also is a stimulus for the proliferation of committed precursor cells.

In Table II we have summarized studies from the literature on the growth of cells in DCs implanted into hemopoietically stressed mice in which both cell numbers and CFU-S were studied early after DC implantation. The increase in DC contents above that in the inoculum in our ^{89}Sr-treated mice occurred as early (Day 3) as a similar increase in 800-rad whole-body-irradiated (WBI) mice (8) (Table II); on Day 3, the magnitude of the increase in DCs from ^{89}Sr-treated mice may even have been slightly greater than that in DCs from 800-rad WBI mice (8). Moreover, if cell density does influence the growth rate of cells in DCs, the increase noted in our

TABLE I. DIFFERENTIAL COUNTS, AS PERCENTAGES, OF CELLS FROM MICE USED TO INOCULATE DIFFUSION CHAMBERS (DCs) AND OF CELLS HARVESTED FROM DCs CULTIVATED IN ^{88}Sr- OR ^{89}Sr-TREATED MICE.

	Blasts	Proliferative granulocytes	Nonproliferative granulocytes	Monocytes and macrophages	Red cell precursors	Lymphocytes	Other[a]
Inoculum[b]	2.1 ± 0.3	11.1 ± 1.3	30.3 ± 3.0	0.6 ± 0.2	30.0 ± 1.4	20.6 ± 0.6	5.3 ± 1.2
^{88}Sr[c]	1.0 ± 0.7	19.6 ± 3.3	42.0 ± 2.1	26.1 ± 1.6	1.0 ± 0.9	7.2 ± 1.7	3.1 ± 1.8
^{89}Sr[d]							
Day[e]							
10	2.3	18.7	39.2	33.8	0.5	4.7	0.8
13	2.2	19.5	43.7	25.3	5.5[f]	2.5	1.3
21	2.0	18.8	48.2	23.8	1.0	4.0	2.2
42	1.5	19.0	38.5	27.5	1.3	8.3	3.9

[a] This category includes: basophils, eosinophils, plasma cells, megakaryocytes, and cells in mitoses.

[b] For each interval, cells pooled from the femurs of three normal CAF_1 mice were used to inoculate the DCs.

[c] Data for DCs cultivated in ^{88}Sr-treated mice are values pooled from all four times studied; means ± SE.

[d] Data for DCs cultivated in ^{89}Sr mice are averages of data obtained from two groups (right and left) of chambers at each time.

[e] Day after injection of ^{89}Sr; this was the day on which chambers were harvested. All chambers were in mice for 72 hr.

[f] There were more red cell precursors in DCs implanted into ^{89}Sr-treated mice on Day 10 and harvested on Day 13; Day 13 was the only time at which ^{89}Sr mice were substantially anemic (Fig. 1A).

TABLE II. REVIEW FROM THE LITERATURE OF STUDIES IN WHICH BOTH NUMBERS OF CELLS AND CFU-S WERE ASSAYED IN DCs IMPLANTED INTO HEMOPOIETICALLY STRESSED MICE: (A) THE FIRST DAY AFTER DC IMPLANTATION ON WHICH THE CELL POPULATION (TOTAL AND CFU-S) EXCEEDED THAT OF THE INOCULUM AND (B) THE MAGNITUDE OF THIS VERY EARLY INCREASE.

Author (method used to stress mouse hemopoiesis) and size of inoculum	Nucleated cells/chamber[a] Day; magnitude of increase over input[b]	CFU-S/chamber Day; magnitude of increase over input[b]
Niskanen *et al.* (13) (Cyclophosphamide, 350 mg/kg)		
1×10^5 nucleated cells	5; 2×	4; 2×
5×10^5 nucleated cells	5; 1.6×	Not done
Shulman and Robinson (9) (500 R WBI[c])		
1×10^5 nucleated cells	4; 3.4×	6; 1.4×
Boyum *et al.* (8) (800 R WBI)		
7×10^4 granulocytes	3; 1.2×	3; 1.2×
Adler and Trobaugh (present study) (4 μCi/g of ^{89}Sr)		
1×10^6 nucleated cells	3; up to 1.7×	3; up to 2×

[a] For the study of Boyum *et al.* (8) data are granulocytes/chamber rather than nucleated cells/chamber.

[b] In some cases the magnitude of increase had to be approximated from data supplied in the publications.

[c] WBI, whole body irradiation

studies becomes even more striking as we seeded the DCs with 1×10^6 cells, substantially more cells than were used in the other studies cited in Table II. The contents of the DCs exceeded the input levels earlier in our ^{89}Sr-treated mice (Day 3) than they did in cyclophosphamide-treated mice (Days 4–5) (13) even though the latter had a lower neutrophil nadir ($250/mm^3$ vs $1/mm^3$). Thus, it seems that DC growth is influenced not only by the degree of neutropenia but also by the modality used to induce it. This confirms the finding of Brevik and Benestad (7) who have noted that irradiation provides a stronger stimulus for DC chamber than does cytoxan treatment; we might add that ^{89}Sr irradiation may provide even a stronger stimulus than external WBI.

Although we did not assay the committed granulocyte/macrophage precursor cells (CFU-C), this cell is one of the primary cells which proliferates and differentiates in DCs (9, 14, 29, 30). Beran (15) has shown that the increase in mature cells in DCs implanted into hemopoietically stressed mice from the third day onward is not due to variations in survival times of the cells implanted, rather it is related to proliferative characteristics of the cells and Quesenbery *et al.* (14) have shown that granulocyte production correlates well with CFU-C proliferation. Based on this knowledge it seems reasonable to assume that the larger population of differentiated white cell elements in DCs in ^{89}Sr-treated mice as compared to that in DCs in control mice results from increased CFU-C proliferation in the DCs implanted into the ^{89}Sr-treated mice. In spite of the augmented granulopoiesis in ^{89}Sr-treated mice as measured by the DC assay, in a previous study (26) we were not able to detect any elevated levels of CSA in ^{89}Sr-treated mice. It may be that for the ^{89}Sr model, the DC technique is more sensitive to CSA than is the *in vitro* assay for CSA. Alternatively, a factor other than CSA may be responsible for the enhanced granulopoiesis in DCs. Although some investigators have found support for the role of CSA in DC growth (15, 16), Rothstein *et al.* (31) have adduced experimental evidence which casts doubt on the role of CSA in DC hemopoiesis. In any event, the studies reported here underscore the importance of employing multiple experimental systems before excluding the presence of a humoral factor in states of hemopoietic stress.

Summary. The numbers of pluripotent stem cells (CFU-S) and of the more differentiated granulocyte/macrophage elements in diffusion chambers (DCs) implanted into the peritoneal cavities of radio-^{89}Sr-marrow-ablated mice are increased as compared to those in DCs implanted into cold-^{88}Sr-marrow-ablated mice. These findings suggest that there is a systemic humoral response capable of stimulating hemopoiesis even in mice with aplastic marrows and whose hemopoiesis is localized to their spleens. The magnitude of this response and the promptness with which the response is manifest in DC growth suggests that marrow aplasia induced by ^{89}Sr provides a stronger stimulus for proliferation of cells in DCs than does either cyclophosphamide or lethal external whole-body irradiation.

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