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Survival of Murine Hemopoietic Cells in an *in Vivo* Culture System (38096)

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A modification of the diffusion chamber (DC) technique originally described by Algire *et al.* (1) has recently been employed by several investigators for the culture of hemopoietic cells (2–4). When murine bone marrow cells are sealed in DCs and implanted intraperitoneally granulocytic cells, macrophages, and pluripotent stem cells (CFU) proliferate. The rate of proliferation is increased when chambers are implanted in hosts rendered neutropenic by pretreatment with irradiation or cyclophosphamide (4, 5) suggesting that humoral factors generated by the hosts influence growth of the implanted marrow. At cell concentrations between 10^5 and 20×10^5 per DC the growth of all elements is inversely related to the number of cells implanted, suggesting that conditions inside DCs related to cell concentration also affect proliferation (6). Thus, the DC technic appears to have value in the study of both long-range humoral factors and short-range mechanisms, perhaps due to cellular interaction, which may control hemopoiesis.

In order to obtain a more accurate estimate of true cell proliferation in DCs, that is to separate changes in proliferation from possible changes in cell longevity, it seemed desirable to develop a method for measuring cell death in DC cultures. Such a method is reported herein. Murine marrow prelabeled *in vitro* with 2-deoxy-4 ^{125}iodo-uridine (^{125}IUDR) was cultured in DCs. The loss of labeled DNA from cultures implanted into cyclophosphamide pretreated, neutropenic (N) and saline treated control (C) hosts was followed for 5 days. The results indicated that incorporation of ^{125}IUDR into marrow cells did not substantially effect their growth in DCs and that survival of these cells was the same in N as in C hosts.

Materials and Methods. Twelve to 16 wk old virgin female CF_1 mice were used; 350 mg/kg of cyclophosphamide (CY) or saline was injected ip 24 hr before implantation. This dose of CY in CF_1 mice, induces profound neutropenia within 48 hr (6).

^{125}IUDR with a specific activity of 30 Ci/mmole or more was added to suspensions of normal tibial marrow prepared at a concentration of 5×10^6 nucleated cells/ml in Hanks' buffered salt solution modified by the addition of 10% sterile, heparinized mouse plasma and 100 μgm/ml of penicillin and streptomycin. After incubation in a

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TABLE I. Total and Differential Growth in Six Day DC Cultures of 10^5 Mouse Marrow Cells Preincubated with or without IUDR for 60 min.

Preincubation	Cells/DC[a] $\times 10^{-3}$	EPC[b] $\times 10^{-3}$	Myelocytes $\times 10^{-3}$	NPG[c] $\times 10^{-3}$	Macrophages $\times 10^{-3}$
3.0 μCi IUDR	480 ± 60	37 ± 5	85 ± 18	122 ± 22	221 ± 56
No. IUDR	434 ± 89	19 ± 6	100 ± 35	123 ± 28	185 ± 26

[a] Mean ± SEM of 5 or 7 DCs.
[b] Early proliferative cells.
[c] Nonproliferative granulocytes.

shaker bath at 37°C the suspension was placed on ice and then centrifuged (400*g* at 4°C) for 10 min. The supernatant was discarded and the cell button resuspended in fresh medium prior to implantation. One-tenth milliliter of prelabeled marrow containing 10^5 cells was implanted according to previously described methods (4). ^{125}IUDR incorporation was measured on duplicate or triplicate aliquots of the labeled suspension. Each was precipitated in ice cold trichloroacetic acid (TCA). The precipitate was collected and washed on 0.45 μm Millipore filters and counted in an autogamma counter.

Host animals were sacrificed 3 hr to 6 days after implantation. DCs were removed and processed as previously described (4) to obtain a single cell suspension. An aliquot of the suspension from each DC was precipitated in TCA and the retained radioactivity measured. The remainder of each suspension was used for cell counts and smears. Cultured cells were classified morphologically as early proliferative cells (EPC) myelocytes, nonproliferative granulocytes (NPG), and macrophages by previously described criteria (4). Smears for autoradiography were prepared as previously described (6).

Results. In preliminary experiments the incorporation of ^{125}IUDR was evaluated by incubation of a marrow suspension with 0.3 μCi/ml for 15–60 min or with 0.04–4.0 μCi/ml for 30 min. Incorporation increased linearly with time and with increasing concentrations of isotope. ^{125}IUDR did not affect the total growth of marrow cells in DCs (Table I).

The growth of marrow prelabeled by incubation with 2 μCi/ml of ^{125}IUDR for 1 hr and implanted into N and C hosts is shown in Fig. 1. Three hours after implantation 20% of the cells was recovered in each group. Thereafter chamber cellularity increased rapidly in both N and C hosts, but the increase was significantly greater in N hosts, so that by the fifth day there were 1.8 times as many cells/DC in N as in C animals. There were significantly more nonproliferative granulocytes in DCs from N than from C hosts on the fifth day (Table II). Granulocyte precursor and macrophage numbers were also greater in N hosts, but the differences were not statistically significant (Table II).

Retention of ^{125}I in the cultured cells is shown in Fig. 2. Three hours after implantation 40–50% of the implanted radioactivity was recovered from each group. During the next 45 hr radioactivity in the cultured

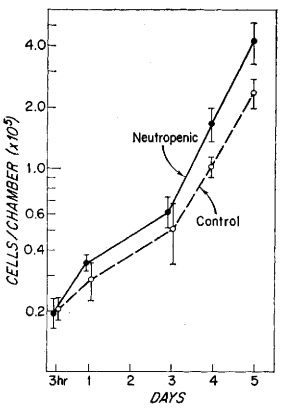

FIG. 1. Total numbers of nucleated cells per DC after culture of ^{125}IUDR prelabeled mouse marrow in neutropenic and control hosts. Values are mean (± SE) of 9-15 DCs from 2 or 3 replicate experiments.

TABLE II. Total and Differential Growth of 10^5 Mouse Marrow Cells Preincubated with 2.0 μCi/ml IUDR and Cultured in DCs.

Day	Experiment	Host	Number of DCs	Cells/DC $\times 10^{-3}$	EPC[d] $\times 10^{-3}$	Myelocyte $\times 10^{-3}$	NPG[e] $\times 10^{-3}$	Macrophages $\times 10^{-3}$
3	M 1100	N[a]	3	46 ± 3[c]	6 ± 1	10 ± 0.6	10 ± 2	19 ± 0.6
		C[b]	4	41 ± 4	5 ± 1	11 ± 0.4	8 ± 1	16 ± 2.0
4	M 1100	N	5	161 ± 44[f]	13 ± 6	59 ± 15	32 ± 10	56 ± 21
		C	5	103 ± 16[f]	7 ± 2	40 ± 5	23 ± 3	45 ± 6
5	M 1100	N	6	371 ± 115	25 ± 10	114 ± 44	108 ± 38	125 ± 31
		C	6	270 ± 57	20 ± 9	100 ± 23	48 ± 8	96 ± 22
5	M 1126	N	5	474 ± 158[f]	32 ± 17	132 ± 85	153 ± 51	144 ± 41
		C	5	205 ± 51[f]	18 ± 7	72 ± 17	35 ± 6	105 ± 21

[a] Neutropenic.
[b] Control.
[c] Mean ± SEM.
[d] Early proliferative cells.
[e] Nonproliferative granulocytes.
[f] This value is the average of 6 DCs, values for the differentials are for 5 DCs.

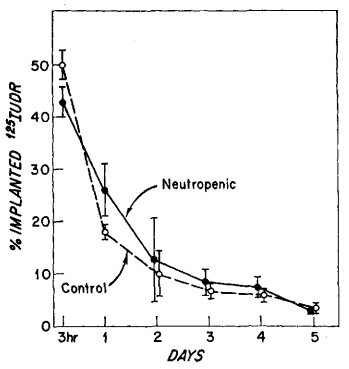

FIG. 2. Retention of IUDR in DCs after culture of marrow prelabeled with 2.0 μCi/ml of IUDR. Numbers of DCs as in Fig. 1.

marrow declined rapidly, but thereafter the rate of decline decreased. There was no overall difference in the retention of ^{125}I in the two groups.

Because of the possibility that chemical or radiotoxicity of the ^{125}IUDR was effecting survival in culture, marrow prelabeled with 0.2 rather than 2.0 μCi was cultured in N and C hosts for 2 days. Survival of labeled DNA was slightly greater than had been previously observed, but the decay curves were similar and again no difference between N and C hosts was seen.

The nuclear localization of ^{125}I in the labeled marrow suspension prior to implantation and after 4 or 5 days in culture was checked by autoradiography. Prior to implantation heavy label was seen over the nuclei of immature cells of all types. After 4 or 5 days only NPG and macrophages were labeled. NPG labeling was light, but there were a few macrophages with very heavy label clearly localized to their nuclei. No evidence for cytoplasmic label was seen as might be expected if these phagocytic cells had accumulated nuclear debris in their cytoplasm.

Discussion. The thymidine analog, iododeoxyuridine, appears to be a stable DNA label, persisting until the DNA is metabolized (7, 8). Thymine is preferentially used rather than IUDR for DNA synthesis, so that reutilization of the IUDR salvaged from dead cells is minimal (8, 9). Hence IUDR is preferred over tritiated thymidine as a DNA tracer in experiments where reutilization may occur. IUDR has been used to estimate the extent of thymidine reutilization in rat bone marrow (8) and to follow the death and metastic distribution of tumor cells in mice (10). When labeled with ^{125}I deoxyuridine has been found satisfactory for the quantitative radioautographic study of murine bone marrow kinetics (11).

In the doses used in our experiments there was no evidence that sufficient ^{125}IUDR was incorporated to inhibit proliferation of marrow cells cultured in DCs. Bone marrow preincubated with 3 μCi/ml of ^{125}IUDR produced as many cells after 6 days in culture as a parallel suspension preincubated without ^{125}IUDR. Further, the growth of marrow prelabeled with 2 μCi/ml was similar to that generally seen in our laboratory for freshly prepared unlabeled marrow suspensions. As previously reported for cultures of fresh unlabeled marrow (4, 6), there was a highly significant increase in myelopoiesis when prelabeled marrow was implanted into neutropenic hosts, suggesting a humoral effect on the granulocytic precursors.

Substantial variation in total cell growth and differential counts between individual DCs is evident from our data. This variation appears to be a characteristic of the system, although it was noticeably greater in the present series of experiments than in previous experiments where freshly made marrow suspensions were cultured. The reasons for this variation are not clear, but it emphasizes the need for performing replicate experiments when the diffusion chamber technique is employed.

The survival of labeled DNA was the same in neutropenic as compared to control hosts when the cultured marrow was prelabeled with either 0.2 or 2.0 μCi/ml. Thus the increased number of granulocytic elements in neutropenic hosts was the result of a true increase in cell proliferation rather than improved survival of the cultured cells in a neutropenic millieu. Survival of labeled DNA was slightly greater when marrow was prelabeled with 0.2 rather than 2.0 μCi/ml, suggesting that incorporation of large amounts of ^{125}IUDR was lethal for some of the cells. Similar results have been

reported by Hofer *et al.*, for L1210 cells labeled *in vivo* (10).

Three hours after implantation 40–50% of the labeled DNA but only 20% of the intact, countable cells was recovered. This difference suggests that a greater portion of unlabeled mature cells than of labeled immature cells died and underwent cytolysis during the initial hours after implantation. The lytic action on nonviable cells of pronase would exaggerate this effect.

The nonexponential nature of the DNA survival curve is probably due to different modes of death for the various labeled cell populations. Death of red cell precursors, which disappear from DCs soon after implantation, probably contributed substantially to the rapid initial decline in ^{125}IUDR retention. Loss of label from granulocytic cells and macrophages would in large part be delayed until the labeled cells had matured and died either from senescence or randomly, a combination of which would lead to a nonexponential decay.

Further studies using quantitative autoradiography should help in defining macrophage and neutrophil kinetics in DC cultures. The influences of host neutropenia and of cell concentration within the cultures of these kinetics may also be evaluated.

Summary. Murine bone marrow cells were labeled *in vitro* with ^{125}IUDR and implanted in diffusion chambers into neutropenic and control mice. Proliferation of granulocytic cells and macrophages in the implanted marrow was not affected by ^{125}IUDR. Granulocytic proliferation was enhanced in chambers implanted into neutropenic animals, but the survival of the implanted marrow, judged by the retention of ^{125}I within the chambers, was the same in neutropenic and control hosts. These data suggest that the augmented growth in neutropenic hosts was the result of a true increase in proliferation rather than improved survival of the implanted marrow.

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