Relationship of Early Proliferating Cells to Erythroblasts in Hemopoietic Colonies[1] (36203)

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Since recovery of the bone marrow after radiotherapy may determine whether or not tumoricidal doses of irradiation may be administered to patients with malignant lymphoma, we have sought to clarify processes leading to the reconstitution of depleted hemopoietic tissues.

Previous studies suggested that a delay in the recovery of erythropoiesis in endocolonized mouse spleens after irradiation (1) was related to the replication of primitive hemopoietic cells to attain a certain critical number before becoming responsive to erythropoietin (2–4). Because this phase of proliferation invariably precedes the initiation of erythropoiesis in our system (1–4), we have attempted to characterize the cells morphologically, and to determine if these early proliferating cells are erythroblast progenitors.

Studies reported in this paper were undertaken to label replicating cells in endocolonized mouse spleens before erythroblasts were believed to be present. We reasoned that if tritiated thymidine, administered before the onset of erythropoiesis, was subsequentlv identified in erythroblasts, the findings would support the hypothesis that some of the replicating primitive hemopoietic cells transform to recognizable erythroblasts.

Methods. Irradiation. Carworth Farms No. 1 female 12–14-week-old mice were maintained in cages of 8 on acidified drinking water (pH 2.5) to prevent infection bv *Pseudomonas* as previously described (4). Thev were given alternate-fraction irradiation (850 R L.S.^{3}—L.I.)[2], as described in detail before (4), by anesthetizing the mice and taping their left hind legs under a lead shield. Three hours after the administration of 850 R to the body while shielding the leg, the body was shielded and the leg given 850 R. Presumably this second dose killed the stem cells remaining in the leg and inhibited emigration of colony forming cells from the only unirradiated site of the body (3). Total body irradiation 850 R tb was carried out by administering 850 R without shielding.

Erythropoiesis. Numbers of erythroblasts per spleen were determined by killing the mice on various days after 850 R L.S.^{3}—L.I., bisecting their spleens, twice-touching each half to a slide for staining with Wright's stain, and then suspending the cells from the spleens in isotonic saline for counting with a hemocytometer, as described before (4).

Autoradiography of progenitors and erythroblasts. Ten microcuries of tritiated thymidine (^{3}HTdR-thymidine-Methyl-^{3}H; 6.7 Ci/mmol) was injected intraperitoneally to each of 25 mice at 5:00 p.m. 3 days after 850 R L.S.^{3}—L.I. At 1:00 a.m. and 9:00 a.m. on day 4, an additional 10 μCi was administered ip at each time for a total of 30 μCi ^{3}HTdR per mouse. At 10:00 a.m. on day 4, 14 mice were weighed, killed and splenectomized. On day 5, the remaining 11 mice were similarly treated. After their spleens were weighed, they were bisected and each half was twice-touched to a subbed slide for autoradiography and staining with McNeal's tetrachrome as de-

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[2] 850 R L.S.—^{3}L.I. = alternate-fraction irradiation in which an anesthetized mouse was given 850 Roentgens with the left hind leg shielded with lead followed by an interval of 3 hours, after which the leg was irradiated while the body was shielded.

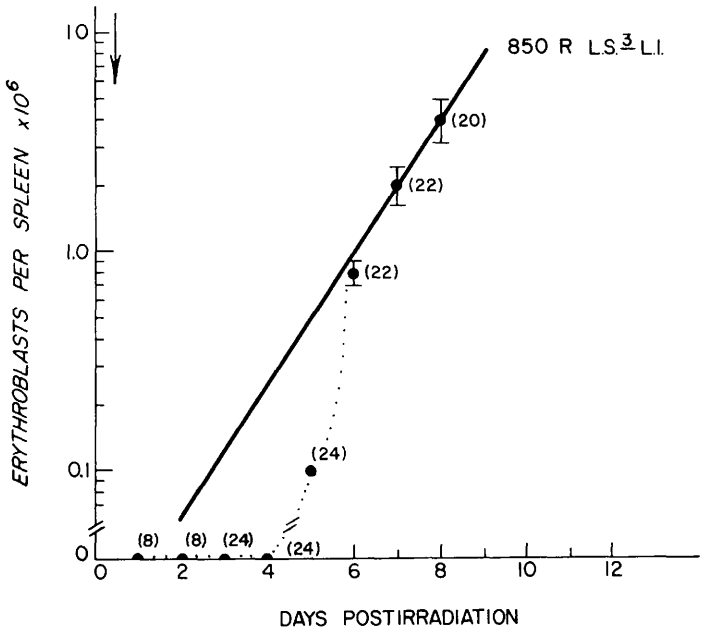

FIG. 1. Mean erythroblasts per mouse spleen in millions on various days after alternate-fraction irradiation (850 R L.S.—³L.I.). Brackets enclose ± SEM. Parenthesis indicate number of mice per point. Arrow indicates time of radiation. Straight line is drawn through points on days 7 and 8 when the population doubled in 24 hr.

scribed before (4). The spleen halves were then fixed in 3% glutaraldehyde. Then they were postfixed in osmium tetroxide, dehydrated in osmium tetroxide, dehydrated in graded alcohol solutions and propylene oxide and embedded in epon–araldite. After thin sections were cut on a Reichert ultramicrotome, they were stained for 5 min with uranyl acetate and then for 3 min with lead citrate. A Denton vacuum evaporator DV-502 was used to give the sections a thin coating of carbon. Then Ilford L4 emulsion was applied by the method of Budd and Pelc (5). After an exposure of 8 weeks, the autoradiographs were developed in Microdol-X and fixed in 20% sodium thiosulfate. The coded sections were examined in a Philips EM 300 electron microscope.

Results. Erythroblast counts. Erythroblasts in Wright's-stained touch preparations diminished from normal levels of about 12 million per spleen to undetectable numbers one day after alternate-fraction irradiation. None were seen until day 5 when suddenly they appeared at a mean level of 100,000 erythroblasts per spleen (Fig. 1). Levels of 2 and 4 million erythroblasts per spleen were attained on days 7 and 8. If simple doubling of the population every 24 hr had occurred immediately after irradiation to attain levels recorded on days 7 and 8, 100,000 erythroblasts per spleen should have been detected 2 or 3 days before that many were actually counted. In fact, erythroblasts increased about 8 times in the 24-hr period from days 5–6. No erythroblasts were found after the first day in spleens of mice that received 850 R tb.

Autoradiography. About ⅓ of the nonerythroblastic cells were labeled in autoradiograms of spleens taken from irradiated mice

TABLE I

	Days After 850 R L.S.—³L.I.	
	4 ($n = 14$)	5 ($n = 11$)[b]
Mouse Weight (g)	24 ± 0.4	26 ± 0.7[c]
Spleen Weight (mg)	34 ± 2.1	40 ± 3.0
Nonerythroblastic cells (%)	100.0 ± 0.0	99.5 ± 0.1[d]
³HTdR-labeling index (%)[a]	31.2 ± 0.9	70.9 ± 2.9[d]
Erythroblasts (%)	0.0 ± 0.0	0.5 ± 0.1[d]
³HTdR-labeling index (%)	0.0 ± 0.0	91.4 ± 1.8[d]

[a] HTdR was administered on days 3 and 4; [b] n = number of mice; [c] mean ± standard error of mean. [d] $P < .001$ for values on day 4 vs. day 5.

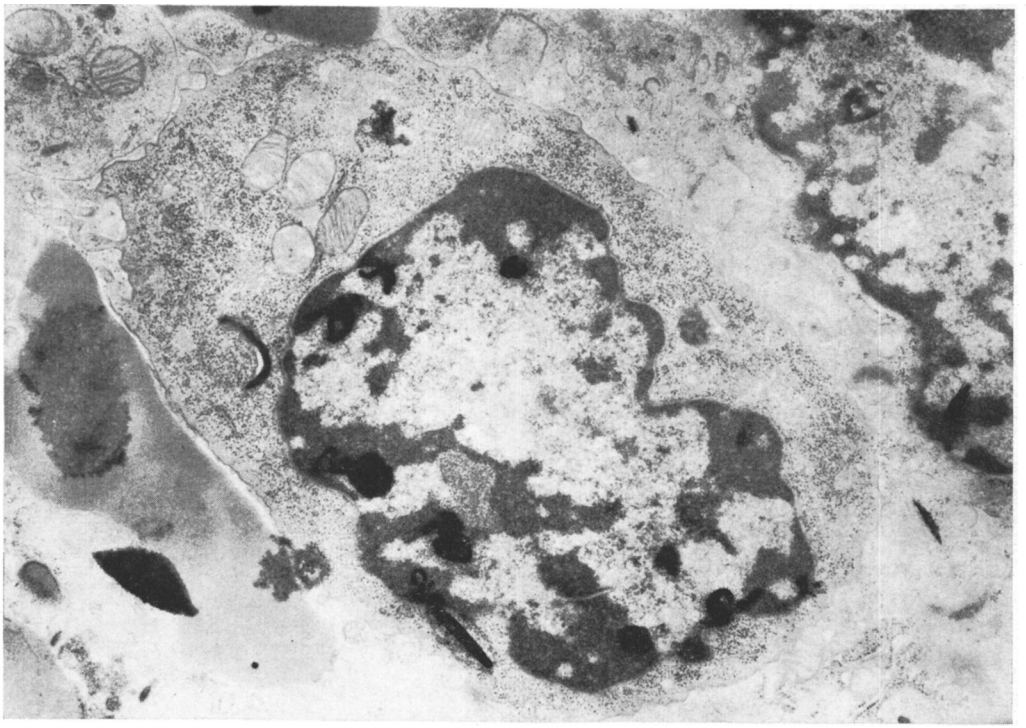

FIG. 2. Electron microscopic autoradiograph of an early proliferating cell 1 hr after the last dose of ^{3}HTdR; day 4. Black curly lines represent labelling with ^{3}HTdR (14,400×).

at 10:00 a.m. on day 4, one hour after the last dose of ^{3}HTdR had been administered (Table I). The light microscopic appearance of these early proliferating cells resembled that of medium to large lymphocytes containing leptochromatic nuclei. Examination of autoradiograms with the electron microscope revealed ^{3}HTdR-labeled cells with numerous mitochondria, moderate amounts of rough endoplasmic reticulum and large numbers of polyribosomes (Fig. 2). Heterochromatin was distributed centrifugally on the inner nuclear membrane. A total of 37 erythroblasts were found among the thousands of cells in the 4 touch preparations from one spleen on day 4. No erythroblasts could be found in the other 52 touch preparations made from the remaining 13 spleens on day 4.

In the group of mice that were killed and examined on day 5, twice as many nonerythroblastic cells were labeled then compared to those labeled on day 4 (Table I). The appearance of these early proliferating cells in autoradiograms on day 5 differed somewhat from that noted on the previous day; the label was found marginally distributed in a dense broader rim of heterochromatin on the inner nuclear membrane (Fig. 3). Incomplete intranuclear bridging of heterochromatin was observed in some cells. Erythroblasts constituted 0.5% of the cells in autoradiograms prepared for light microscopy, and 91% of these erythrocyte precursors were labeled with ^{3}HTdR.

Mean mouse and spleen weights were not significantly different on days 4 and 5 (Table I).

Discussion. The results of studies reported in this paper show that the injection of ^{3}HTdR on the fourth day after administration of alternate-fraction irradiation, when erythroblasts could not be found in endocolonized spleens, is followed by the appearance on the fifth day of labeled erythroblasts. There are several explanations for this. The most likely is that the labeled proliferating cells, as observed on the fourth day in the first group of mice, retained their label and transformed to the erythroblasts that were found on the fifth day after irradiation. Our previous studies have demonstrated the absence of erythroblasts and

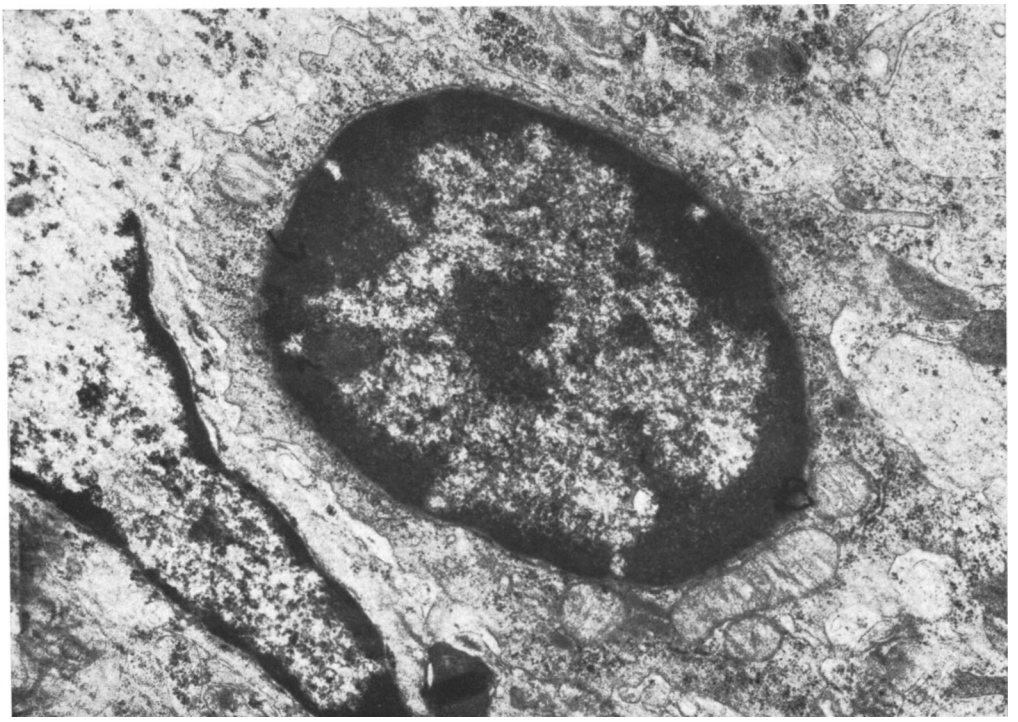

FIG. 3. Electron microscopic autoradiograph of an early proliferating cell 25 hr after the last dose of ^{3}HTdR; day 5. Note centripetal extension of heterochromatin (17,600×).

erythropoietic activity in endocolonized mouse spleens and in bone marrow on the fourth day after 850 R L.S.[3]—L.I. (4), so it is highly unlikely that erythroblasts, *per se*, were labeled in the spleen or elsewhere in the body to migrate into the colonies. Studies by other investigators have demonstrated a delay in initiation of erythropoiesis to support that view (6, 7). To conceive that the labeled replicating cells observed on the fourth day were destroyed and a significant amount of their label reutilized, is unlikely in light of a lack of evidence for cell destruction, increasing cell counts, and the absence of labeled orthochromic erythroblasts, the extruded nuclei of which are believed to be the major source of thymidine reutilized by bone marrow cell (8).

It has been well substantiated that shielding a portion of bone marrow, during the administration of an ordinarily supralethal dose of X-rays to a mouse enhances survival from radiation-induced bone marrow failure (1, 9). Circulating stem cells (10) promote the formation of hemopietic colonies in the spleen (11) which precedes the recovery of peripheral blood counts (1). A phase of primitive cell proliferation to a certain critical mass, in turn, precedes the initiation of erythropoiesis (1–4). Erythroblastic differentiation is delayed for about 5 days after irradiation despite the presence of elevated levels of erythropoietin (2, 3). Because of the interest in the morphological identification of the pluripotential hemopoietic stem cell, investigators have questioned whether some of the nonerythroblastic cells proliferating before the initiation of erythropoiesis transformed to erythroblasts or simply replicated first following a predetermined sequence of proliferation of various cell lines. If these replicating nonerythroblastic cells transform, they are previously unrecognized erythroblast progenitors and conceivably, daughters of pluripotential hemopoietic stem cells or the stem cells themselves.

Extremely interesting, is the observation that these early proliferating cells, which play such an important role in hemopoietic colony formation, resemble medium to large leptochromatic lymphocytes when examined with the light and electron microscope (12). Other

investigators have found that enriching an inoculum with bone marrow lymphocytes increases the number of colony forming units when measured with exocolonization stem cell assay techniques (13, 14). Moreover, when erythropoiesis is suppressed in the posthypoxic or transfused plethoric mouse, similar cells become more abundant in bone marrow (15). The electron microscopic studies of Orlic and Gordon (16, 17), in which erythropoiesis in mouse spleens is suppressed by plethora and reinitiated with erythropoietin, demonstrated the incorporation of ^{3}HTdR by "stem cells" resembling those that we found when we examined labeled early proliferating cells in autoradiograms of endocolonized spleens with the electron microscope (12).

Summary. Labeled erythroblasts were found in the hemopoietic colonies of endocolonized mouse spleens 5 days postirradiation after ^{3}HTdR had been administered 24–40 hr previously. No erythroblasts could be found in the spleens of other mice at the time of the administration of ^{3}HTdR. About one third of the nonerythroblastic cells, which resembled medium to large leptochromatic lymphocytes, were labeled on day 4. One explanation for the findings is that some replicating cells resembling lymphocytes on day 4, transformed to erythroblasts by day 5.

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