Erythroblastic Differentiation of Stem Cells in Hemopoietic Colonies

By Richard L. DeGowin, John C. Hoak, and Suzanne H. Miller

An early phase of cellular replication precedes the initiation of erythropoiesis in hemopoietic spleen colonies of mice receiving a supralethal split-dose of irradiation, first with the leg shielded and then followed by leg irradiation 3 hr later. Previous studies indicated that this proliferative phase represents the repopulation of a depleted, endogenous stem cell compartment. To label replicating stem cells, tritiated thymidine ($^{3}$HTdR) was injected intraperitoneally in mice on days 1-4 after irradiation. Touch preparations and autoradiograms of splenic colony cells were examined with light and electron microscopy from 2 to 4 days after irradiation and 5-8 days later, when erythroblasts and other differentiated cells first appeared. Replicating mononuclear cells that were pulse labeled during the early proliferative phase of stem cell renewal resembled medium- to large-sized leptochromatic lymphocytes. Electron microscopic examination of autoradiograms demonstrated a labeled undifferentiated cell with a thin rim of nuclear heterochromatin. Sequential studies showed that the injection of $^{3}$HTdR on days 1-4, when only the mononuclear cells were present, resulted in labeled erythroblasts and other differentiated cells on days 5 and 6. These results confirm the presence of an early proliferative phase in endogenous splenic hemopoietic colonies and strongly suggest that mononuclear cells, replicating during this period of self-renewal of the stem cell compartment, transform to erythroblasts and other cells.

Identification of the pluripotential hemopoietic stem cell should facilitate studies of stem cell kinetics in patients with disorders of blood production, including those with leukemia, myeloproliferative syndrome, or aplastic anemia. Such a cell should have the capacity to repopulate hemopoietic tissue by self-renewal and by differentiation to precursors of the various specialized cell lines. Our previous studies$^{1,2}$ and those of others$^{3}$ have shown that replication of stem cells to a certain critical number occurs in hemopoietic colonies before they become responsive to erythropoietin and undergo erythroblastic differentiation. Preliminary studies indicated that it may be possible to label and identify stem cells during this early proliferative phase of recovery from irradiation.$^{2,4,5}$

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We have studied accordingly, the transformation of early proliferating cells to erythroblasts in hemopoietic colonies of mice receiving a split-dose of irradiation, first with a leg shielded, and then followed by leg irradiation 3 hr later (850 R L.S.-3L.I.).

MATERIALS AND METHODS

Irradiation

Split-dose irradiation (850 R L.S.-3L.I. = 850 roentgens Leg Shielded—3-hr interval—850 R Leg Irradiated) was administered to anesthetized 12-14-wk-old female, Carworth Farms No. 1 mice as described previously. A General Electric Maxitron 250 x-ray machine was used to deliver an average output of 56 R/min. Physical factors were: 250 kV, 30 mA (0.25 mm Cu + 1.0 mm Al filter); half-value layer, 1.04 mm Cu; and target distance 80 cm. The technique of split-dose irradiation was used to kill stem cells remaining in the leg 3 hr after the initial radiation. This inhibits continuing stem cell immigration into spleen colonies from the only unirradiated site in the body and, thus, insures the synchronous development of colonies originating from a discrete cohort of colonizing stem cells.

Splenic Cell Counts

The number and types of splenic cells were determined by killing the mice with cervical luxation, splenectomizing them, bisecting their spleens for imprints, and suspending the cells for counting in a hemocytometer, as described before. Imprints for erythroblast counts in Fig. 1 were stained with Wright's stain.

Injections of Tritiated Thymidine

Tritiated thymidine ($^{3}$HTdR = $^{3}$H-methylthymidine, 6.7 Ci/m mole) in isotonic saline was administered intraperitoneally in a dose of 10 $\mu$Ci to mice at 5:00 p.m., 1 day after irradiation. At 1:00 a.m. and 9:00 a.m. on day 2, an additional 10 $\mu$Ci were administered each time, for a total of 30 $\mu$Ci $^{3}$HTdR/mouse. Then the mice were divided into three subgroups. The first group was killed and studied at 10:00 a.m. on day 2; the second group at 10:00 a.m. on day 3, and the third group at 10:00 a.m. on day 6. A similar regimen was followed for a series of experiments on days 3, 4, and 6 and also for days 4, 5, and 6. In the preliminary experiments to study the differentiation of early proliferating cells to megakaryocytes and granulocytes, a single dose of 25 $\mu$Ci of $^{3}$HTdR was given to mice.
DIFFERENTIATION OF STEM CELLS

Fig. 2. Labeling (shaded bars) of 20% of nonerythroblastic cells (NEC) and 22% of erythroblasts (EbI) occurred on day 6 when tritiated thymidine (³HTdR) was administered to irradiated mice on days 1 and 2. No EbI were found on days 2 or 3.

Fig. 3. Approximately 15% of NEC and 12% of EbI were labeled on day 6 when ³HTdR was administered on days 2 and 3. No EbI were found on days 3 or 4.

Light Microscopic Autoradiography

After splenectomy the spleens were weighed and bisected, and each half was touched twice to a subbed slide for autoradiography and for staining with MacNeal's tetrachrome stain, as described before. Since the background was considerably less than 1% and zero in most areas, a cell was considered labeled if two or more silver grains were seen over its nucleus. At least 200 cells were counted to determine labeling indices, labeling by nuclear size, and mean grain counts. Nuclear diameters were measured with a calibrated micrometer eyepiece. Medium lymphocytes had a nuclear diameter of 9–12 μ. Small lymphocytes were less than 9 μ and large lymphocytes were greater than 12 μ.

Electron Microscopic Autoradiography

Bisected spleens were fixed in 3% glutaraldehyde. They were postfixed in osmium tetroxide, dehydrated in graded alcohol solutions and propylene oxide, and embedded in Epon-Araldite. Thin sections were cut on a Reichert Ultramicrotome and stained for 5 min

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with uranyl acetate and then for 3 min with lead citrate. A thin coating of carbon was applied to the sections using a Denton vacuum evaporator, DV-502. Ilford L4 emulsion was then applied. The autoradiograms were developed in microdol-X and fixed in 20% sodium thiosulfate after an exposure of 8 wk. A Phillips EM-300 electron microscope was used to examine the coded sections.4

RESULTS

Splenic Cellularity

Splenic cellularity diminished to 3% of normal during the first 3 days after \(850\) R L.S.→\(L.I.\)} (Fig. 1). From its nadir of \(4.3 \pm 0.2 \times 10^6\) nucleated cells/spleen (mean \(\pm\) SEM) on the third day, splenic cellularity increased about 1–2 million cells over the next 2 days. Then erythroblasts, which had disappeared from splenic touch preparations from days 1 to 4, suddenly reappeared on the fifth day. Normal numbers of erythroblasts were attained between days 10 and 12.

Erythroblastic Differentiation of Early Proliferating Cells

Injections of \(3HTdR\) in irradiated and shielded mice before the reinitiation of erythropoiesis on days 5 and 6 labeled spleen colony cells that resembled medium to large lymphocytes with leptochromatic nuclei in autoradiograms examined with the light microscope. On day 4, about 75% of these mononuclear cells were medium sized, and about half were labeled 1 hr after an injection of \(3HTdR\) at 9:00 a.m. on that day. When autoradiograms were examined with electron microscopy, cells with a thin rim of heterochromatin identical to those previously reported were seen. They had numerous polyribosomes, a moderate number of mitochondria, but few other cytoplasmic organelles. Results of these experiments indicated that detailed sequential studies on days 4, 5, and 6 were required, because that was the time the proliferating cells transformed into recognizable erythroblasts.

Injections of \(3HTdR\) on Days 1–2 (Fig. 2)

When injections of \(3HTdR\) were given to a group of mice on the evening of day 1 and the morning of day 2, approximately \(0.7 \pm 0.1 \times 10^6\) (mean \(\pm\) SEM) nonerythroblastic cells (NEC) per spleen were found to be labeled when the mice were killed at 10:00 a.m. on day 2. A similar number were found in a group of mice injected at the same time but killed and splenectomized 24 hr later. No erythroblasts were found on either the second or the third day. A third group of mice, which received injections of \(3HTdR\) on days 1–2, as before, was killed and examined on day 6. A mean of \(0.9 \pm 0.4 \times 10^6\) erythroblasts/spleen was found, and \(0.2 \pm 0.0 \times 10^6\) were labeled with \(3HTdR\). Over twice as many nonerythroblastic cells were labeled on day 6 as compared to day 2.

Injections of \(3HTdR\) on Days 2–3 (Fig. 3)

Injections of \(3HTdR\) on the evening of day 2 and the morning of day 3 resulted in labeling of about \(0.6 \pm 0.1 \times 10^6\) NEC by 10:00 a.m. on day 3. In another group of mice injected at the same time, but examined 24 hr later,
Fig. 4. All of the EbI and 50% of the NEC were labeled on day 5 when $^3$HdR was administered on days 3 and 4. On day 6, 52% of the NEC and 54% of the EbI were labeled. No EbI were found on day 4.

Fig. 5. Light microautoradiograph of several labeled mononuclear cells in a touch preparation from a colonized mouse spleen 4 days after 850 R L.S.-$^3$L.I. $^3$HdR had been injected on days 3 and 4. Cells resemble medium to large lymphocytes with leptochromatic nuclei. × 1200.

about $0.9 \pm 0.1 \times 10^6$ NEC/spleen were labeled. No erythroblasts could be found on either the third or fourth day. About $0.8 \pm 0.4 \times 10^6$ labeled erythroblasts (EbI)/spleen were found in the group injected on days 2–3 and examined on day 6. Approximately $0.1 \pm 0.0 \times 10^6$ EbI/spleen were labeled.

**Injections of $^3$HTdR on Days 3–4**

Injections of $^3$HTdR on the evening of day 3 and the morning of day 4 labeled approximately $2.1 \pm 0.2 \times 10^6$ NEC/spleen on day 4 (Fig. 4). These mononuclear cells resembled medium to large leptochromatic lymphocytes (Fig. 5). Electron microscopic examination of radioautograms of the splenic colonies revealed the characteristic undifferentiated cell, as described previously (Fig. 6). No erythroblasts were found in spleens on day 4. In a group of mice injected simultaneously with the first group on days 3 and 4 and examined on day 5, approximately $3.6 \pm 0.6 \times 10^6$ labeled NEC/spleen were counted (Fig. 7). About $0.2 \pm 0.1 \times 10^6$ EbI/spleen, all of which were labeled, were found on day 5. In the third group, injected at the same time as the other two groups, about $5.5 \pm 0.8 \times 10^6$ labeled NEC/spleen and $0.7 \pm 0.2 \times 10^6$ labeled EbI/spleen were counted on day 6 (Fig. 8).

Most of the labeled cells had mean grain counts of 1–10 grains/cell, but
Fig. 6. Electron micrograph showing a relatively undifferentiated cell labeled with \( ^{3} \text{HTdR} \) (black curly lines) on day 4. Injections of \( ^{3} \text{HTdR} \) had been administered on days 3 and 4. Mitochondria, polyribosomes, and rough endoplasmic reticulum can be seen in cytoplasm, but no ferritin bodies were found. \( \times 12,000 \).

32% of the cells had from 11 to greater than 31 grains per cell on the fourth day (Fig. 9). Of the 5-day mice that had been injected 24 hr previously, about 95% of the labeled erythroblasts had ten grains or less. Nearly 100% of the erythroblasts in the 6-day mice, injected 48 hr previously, had ten grains or less. Seven percent of the 6-day NEC had more than ten grains, as compared to 24% on day 5 and 32% on day 4. Repetition of the experiment yielded similar results.

Myeloid and Megakaryoblastic Differentiation of Early Proliferating Cells

Preliminary experiments were undertaken in which mice that had received 850 R L.S.--\( ^{3} \text{L.I.} \) were injected with a single dose of \( ^{3} \text{HTdR} \) on day 4 and were subsequently divided into five subgroups for examination on days 4, 5,
Fig. 7. Electron micrograph showing a typical cell labeled on day 5 after injections of $^3$HTdR on days 3 and 4. It resembles those cells noted on day 4 but has slightly more heterochromatin within a rounder nucleus. $\times$ 5586.
Fig. 8. Electron micrograph of a $^3$HTdR-labeled mature erythroblast found in a splenic colony on day 6. Injections of $^3$HTdR had been administered on days 3 and 4. Note condensed nuclear chromatin characteristic of normoblast. $\times$ 18,000.
Fig. 9. Mean grain counts of cells from spleens for which labeling indices were depicted in Fig. 4. Injections of ³HtdR were made on days 3 and 4. Repeated divisions of nonerythoblastic cells (NEC = open bars) is suggested by finding progressively diminished grain counts with the passage of time. No labeled erythroblasts (EbI) were found on day 4. At least 5% of labeled EbI on day 5 had more than ten grains, but the lower grain counts in EbI on day 6 suggest the divisions have occurred in erythron, as well as in NEC population.

DISCUSSION

Results of studies reported in this paper confirm the presence of a proliferative phase in endogenous hemopoietic spleen colonies during the first 4 days after the administration of 850 R L.S.-³L.I. to mice. If the recovery of erythropoiesis on days 5 and 6 is dependent on the prior attainment of a certain critical number of stem cells, as indicated by other studies,¹-⁷ then this proliferative phase undoubtedly represented early self-renewal of the stem cell population. When ³HtdR is administered during the proliferative phase (days 1-4), silver grains can be found over erythroblasts after the initiation of erythropoiesis on days 5 and 6. Since only mononuclear cells resembling lymphocytes are replicating during that early proliferative phase,⁴.⁶ it seems highly probable that such a cell has transformed to an erythroblast. There are several reasons that this early proliferating cell might appropriately be called a stem cell. First, it is replicating during the time the stem cell compartment is undergoing self-renewal.¹-² It is not responsive to the elevated levels of endogenous erythropoietin known to be present after irradiation in these mice.¹.⁸.⁹ Finally, the preliminary labeling studies, begun on day 4 when only
mononuclear cells were present, resulted in the subsequent finding of silver grains over representatives of the other cell lines, namely megakaryocytes and granulocytes on days 7 and 8.

Our previous studies\textsuperscript{1,2,9,10} and those of other investigators\textsuperscript{11-18} led us to favor a concept that the stem cells must replicate until they attain a certain critical number before they become responsive to the erythropoietin known to be present at increased levels. This concept coincided with that predicted by Kretchmar on the basis of his computer model of the mechanism of action of erythropoietin.\textsuperscript{7} Chervenick and Boggs\textsuperscript{3} and Boggs et al.\textsuperscript{17} have reached similar conclusions on the basis of their studies. They note that self-replication of stem cells must occur until at least 10% of the population is attained, and then both self-replication and differentiation can occur.\textsuperscript{3,17}

We reasoned that the administration of $^{3}$HdR during this period of self-replication ought to label a rather homogenous population of cells in S-phase that were destined to give rise to erythropoietin-responsive cells as soon as they had attained a certain critical number. Since the onset of erythropoiesis could be detected morphologically and radioisotopically, we could expect to label stem cells in the interval after irradiation and before the appearance of erythroblasts. The subsequent appearance of the $^{3}$HdR label in erythroblasts on the fifth and sixth days is highly suggestive evidence that a mononuclear cell in DNA synthesis on days 1–4 differentiated to an erythroblast. Preliminary experiments demonstrated the labeling of young megakaryocytes and granulocytes several days after $^{3}$HdR was injected on day 4. The replicating mononuclear cells on day 4 again resembled lymphocytes and not megakaryoblasts or myeloblasts. This suggests that the cells that are labeled with $^{3}$HdR before the onset of erythropoiesis are truly pluripotential.

Several comprehensive reviews have cited the results of numerous studies that implicate a bone marrow cell resembling the lymphocyte as the pluripotential hemopoietic stem cell.\textsuperscript{18,19} The first reviewer\textsuperscript{18} has regarded the lymphocytes as a heterogenous class of cells impossible to separate in terms of function by morphologic criteria. However, neither reviewer believed that convincing evidence for transition of a lymphocytelike stem cell to an erythroblast had previously been presented.\textsuperscript{18,19} Reutilization of $^{3}$HdR was considered a possible mechanism for the subsequent labeling of differentiated daughter cells in some of the experiments.\textsuperscript{18} However, reutilization of $^{3}$HdR via the salvage pathway in the bone marrow is thought to occur by the scavenging of labeled erythroblast nuclei extruded prior to the reticulocyte stage of maturation.\textsuperscript{20} In our experiments, $^{3}$HdR is administered before the appearance of erythroblasts in hemopoietic colonies, so the scavenging of labeled erythroblast nuclei could not explain our results. Moreover, over 5% of the labeled erythroblasts examined on day 5 had more than ten grains (Fig. 9), militating against reutilization of $^{3}$HdR as an explanation for the labeling of those cells. Without detailed analyses of grain counts from more frequent samplings, we cannot be absolutely certain that reutilization of $^{3}$HdR has not occurred, however.

When autoradiograms of the $^{3}$HdR-labeled, early proliferating cells are
examined with the electron microscope, the differences between them and mature small lymphocytes become more apparent. The early proliferating cell is larger, and there is a thinner rim of heterochromatin on an indented nuclear membrane. There is a striking resemblance between these mononuclear cells and those regarded as likely candidates for the stem cell by Orlic and more recently by Van Bekkum et al. and by Murphy et al. Morphologic features that will clearly distinguish this cell from lymphoid cells are currently under investigation.

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