of collagen and its export from the odontoblast into the extracellular organic matrix was completed. Both proline and hydroxyproline exhibit similar specific activities in the insoluble collagen fractions throughout the experimental period. The developing rodent molar was found to be an excellent experimental model for the study of rapid collagen synthesis and its role in the formation of the dentine organic matrix.

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Effect of Endogenous Erythropoietin on Replicating Hemopoietic Stem Cells. (32471)

RICHARD L. DEGOWIN AND SHIRLEY JOHNSON
(Introduced by L. O. Jacobson)
Department of Medicine, University of Chicago and the Argonne Cancer Research Hospital, Chicago, Ill.*

Cells of most neoplastic tissues fail to differentiate normally. Although elevated levels of erythropoietin have been detected in patients with leukemia(1), inappropriately diminished erythroid differentiation(2) of hemopoietic stem cells leads to anemia in many of them. It remains unclear how the target cell for erythropoietin has become refractory to the hormone that usually induces its differentiation.

We have examined the suggestion that replication, *per se,* might render hemopoietic stem cells refractory to erythropoietin (3). Previous autorepopulation experiments in mice indicated that stem cells in the leg, shielded from a supralethal dose of x-rays, colonize the spleen and synthesize deoxyribonucleic acid (DNA) during the first 5 days after irradiation(4). Injections of human urinary erythropoietin failed to induce erythropoiesis during this proliferative phase(4). We wondered whether the exogenous erythropoietin had been inactivated or if the stem cell had become temporarily refractory to it.

Studies described here were undertaken: (1) to demonstrate by a radiobiological method that replication of stem cells occurs within the first five days after irradiation; and (2) to see whether erythropoietin secretion produced by hypoxia would induce erythroid differentiation in the "shielded mouse" during this postulated proliferative phase.

Methods. Mice. Carworth Farms No. 1, 10-to-12-week-old female mice were used in these studies. They were housed, 8 to a cage, on a bed of sterilized ground corn cobs, and fed Rockland complete mouse diet. Drinking water, provided *ad libitum,* was acidified to pH 2.5 with HCl to control Pseudomonas infections.

Irradiation. Irradiation procedures were identical to those previously described(4). Total-body x-ray, from a General Electric Maxitron, 250 x-ray machine delivering an average output of 60 R/min, was administered to the mice which were confined during irradiation in perforated lusteroid tubes on a

* Operated by University of Chicago for U. S. Atomic Energy Commission.

lucite turntable that rotated at 3.5 rpm. Physical factors were: 250 kv, 30 ma, 0.25 mm Cu plus 1.0 mm Al filter; HVL 1.04 mm Cu: target distance 79.5 cm.

"Shielded mice" (850 R L.S.) were placed on the turntable and had their entire left hind legs restrained by a rubber band under a tunnel-shaped, 3 mm lead shield during the partial body irradiation. 850 R x-ray was delivered to these shielded mice in a single dose. Mice were not anesthetized during irradiation.

Splenic uptake of ^{59}Fe. The splenic uptake of ^{59}Fe was determined by the method of Smith(5). Approximately 0.1 μC of ^{59}Fe chloride in 0.25 ml of isotonic saline solution was injected intraperitoneally. Six hours later, spleens were removed, fixed in Bouin's fluid, and their radioactivity was measured in a welltype scintillation counter. A standard prepared at the time of injection was counted, and splenic uptake of ^{59}Fe was expressed as percentage of the injected dose.

The splenic uptake of ^{59}Fe was regarded as a valid estimate of erythropoiesis, because those conditions that suppress erythropoiesis, like plethora, decrease uptake of iron; and those that enhance erythropoiesis, like exposure to hypoxia or injection of erythropoietin, increase the splenic uptake of radioiron(4). Moreover, after a lag of 24 hours, reticulocytosis paralleled the splenic uptake of ^{59}Fe.

Resistance to irradiation. Stem cell replication was estimated by noting increased resistance to irradiation. Shielded mice were given an initial dose of 850 R L.S. Each day thereafter, a sublethal dose of total-body x-ray (100 R, 200 R, or 300 R) was administered to a different group of previously irradiated, shielded mice. Six days after the total-body irradiation, $^{59}FeCl_3$ was administered intraperitoneally and splenic uptake in 6 hours was measured as described above (5). Increasing splenic uptake of ^{59}Fe, despite exposure to identical doses of x-rays, was interpreted as evidence that stem cell replication had occurred, giving rise to more daughter erythroblasts, which incorporated more radioiron.

Hypoxia. Hypoxia was produced by placing mice in a chamber evacuated to 1/2 atmosphere for 16 hours (overnight) on 3 consecutive nights. The mice were removed on the morning of the third day. Splenectomy or exsanguination for assay of plasma erythropoietin was carried out at that time.

Assay of plasma erythropoietin. Plasma from groups of 32 experimental mice was pooled and frozen immediately after bleeding. After thawing at room temperature, 1.0 ml of plasma was injected subcutaneously into a plethoric mouse prepared for assay by the methods described previously(6,7).

Results. Stem cell replication in the spleen, as shown by increasing radioresistance, increased markedly during the first 4 days after irradiation, an interval devoid of erythroid differentiation. Intermittent periods of hypoxia produced elevated plasma levels of endogeneous erythropoietin, but did not induce erythropoiesis during the early proliferative phase of stem cell replication.

Stem cell replication. Fig. 1 shows that incremental sublethal doses of total-body x-ray administered to mice one day after they had received partial body x-ray (850 R L.S.) resulted in a corresponding dose-response when

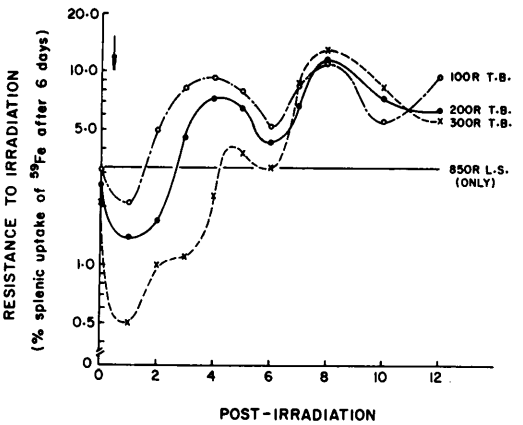

FIG. 1. Resistance to irradiation, a measure of stem cell replication, was estimated by the splenic uptake of ^{59}Fe given 6 days after a sublethal dose of total-body (TB) x-ray was admininstered to shielded (850 R L.S.) mice. Days post-irradiation refers to the day after 850 R L.S. on which the sublethal dose of x-ray was administered. Each point represents the mean splenic uptake of a group of at least 12 mice. The horizontal line is the mean value for 53 mice that received no total-body x-ray. The arrow indicates the administration of 850 R L.S. The ordinate is a logarithmic scale.

the splenic uptake of ^{59}Fe was measured 6 days later. After day 1, radioresistance increased nearly 5 times, reaching a peak on day 4. After falling to a nadir on day 6, the dose-response disappeared, and another peak, higher than the first, was attained on day 8.

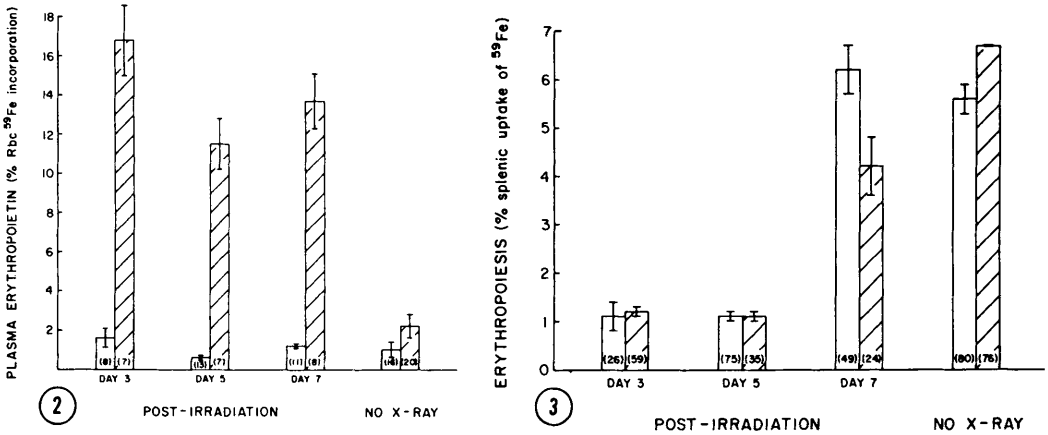

FIG. 2. Plasma erythropoietin was estimated by measuring the erythrocyte (Rbc) radioiron incorporation in plethoric assay mice. Time elapsed after administration of 850 R L.S. to shielded mice is indicated by "post-irradiation." Cross-hatched bars denote mean values of pooled plasma from mice exposed to intermittent hypoxia; open bars depict plasma activity from mice at ambient pressure. Brackets enclose ± 1 standard error of the mean. Numbers in parentheses indicate number of assay mice.

FIG. 3. Erythropoiesis in mouse spleens was estimated by measuring the splenic uptake of radioiron. Numbers in parentheses indicate number of experimental animals from which the mean values (bars) were obtained. Cross-hatching, brackets and titles are explained in the legend for Fig. 2.

Plasma erythropoietin levels. Fig. 2 shows that plasma from hypoxic mice 3, 5, and 7 days after 850 R L.S., contained levels of erythropoietic activity 5 to 8 times that of plasma from unirradiated mice after a similar exposure to intermittent hypoxia. Plasma levels in shielded mice at ambient pressure did not differ significantly from those of unirradiated mice.

Erythropoiesis. Fig. 3 demonstrates that although the splenic uptake of radioiron was increased in normal mice exposed to intermittent hypoxia, there was no increase in shielded mice on days 3 and 5 after 850 R L.S., whether they had been exposed to hypoxia or not. Moreover, hypoxia did not enhance erythropoiesis on day 7, when splenic uptake of ^{59}Fe had increased about 5-fold in shielded mice kept at ambient pressure.

Discussion. Data reported here indicate that elevated plasma levels of endogenous erythropoietin elicited by hypoxia, fail to induce erythroid differentiation during the 5 days immediately after partial body irradiation. This suggests that the erythropoietin target cell is temporarily refractory to the hormone and not that the hormone is inactivated, an alternative proposal that was made in a previous paper(4). The radiobiological method of demonstrating cellular replication supports conclusions from earlier work which suggested that endogenous hemopoietic stem cell proliferation occurred during the first 5 days after partial body x-ray (850 R L.S.)(4).

Because of the discrepancies encountered when comparing results obtained with exogenous colony-forming cells(8) and endogenous stem cells(9), a review of the evidence for early stem cell replication is necessary. Our initial work(4) showed that previous shielding of the left hind leg of mice subsequently exposed to supralethal doses of x-ray effected 100% survival. Unlike spleens of unshielded mice, which died of bone marrow failure following total-body x-ray, spleens of shielded surviving mice developed macroscopic hemopoietic colonies 4 or 5 days after 850 R L.S. Increasing splenic uptake of ^{131}I-labeled iododeoxyuridine (^{131}I-UdR) during

those first 5 days indicated accelerated DNA synthesis. There was, however, no evidence of differentiation until after day 5. At this time, splenic uptake of ^{59}Fe increased, and later, the reticulocyte, leukocyte, and platelet concentrations began to increase. Thus, during the first 5 days after 850 R L.S., the spleens of shielded mice contained cells that were capable of forming hemopoietic colonies and synthesizing DNA before their differentiation, in this way preventing death from radiation-induced marrow failure(4).

Results reported here confirm the idea that there is early replication of hemopoietic stem cells after irradiation. The method is based on the evidence that erythroblasts, which incorporate radioiron, arise from hemopoietic stem cells(10,11). Other workers have considered the amount of radioiron incorporated by erythroblasts, and hence their number, to be a function of the number of stem cells available for erythroid differentiation(12,13). The diminishing ability of incremental doses of radiation to produce a measurable lesion has been used as an estimate of cellular replication(9). When, therefore, sublethal doses of x-ray delivered to colonized spleens of shielded mice progressively failed to suppress splenic uptake of ^{59}Fe, we concluded that more stem cells were present on each succeeding day after 850 R L.S.

The low point of the curves on day 6 in Fig. 1, coincides with the onset of erythroid differentiation in the spleen indicated by splenic uptake of radioiron(4). Thereafter the dose-response phenomenon disappears. Presumably, this is because erythroblasts are more radioresistant than stem cells(14). After day 6, the curve closely corresponds to the splenic uptake of radioiron which precedes a reticulocytosis in the peripheral blood(4). Thus, in the absence of splenic erythropoiesis during the first 5 days after 850 R L.S., the curves in Fig. 1 must depict an increase in undifferentiated cells that will later give rise to erythroblasts. After day 5, the curves undoubtedly reflect the increase in radioiron incorporation which accompanies hemoglobin synthesis by differentiated cells.

Erythropoietic activity of plasma from shielded mice made hypoxic was markedly increased over the activity of plasma from mice maintained at ambient pressure, and greatly exceeded the activity of plasma from normal mice exposed to hypoxia. The site where erythropoietin is inactivated has not been ascertained, but some evidence suggests that it is consumed by an erythropoietically active bone marrow(15). Immediately after irradiation, only a small fraction of active marrow cells remains in shielded mice, and therefore, decreased utilization of erythropoietin because of this depleted marrow may conceivably explain the higher levels of the hormone detected in irradiated animals. If erythropoietin derepresses the gene for hemoglobin synthesis to induce erythroid differentiation of stem cells(16), one might predict that erythropoietin would be cleared from the blood during erythropoiesis. It seems unlikely that the irradiation alters the kidney so that hypoxia causes it to secrete more erythropoietin than normal, but this remains a possibility.

Finally, these studies on increasing radioresistance, supported by previous results with splenic colony formation and increasing splenic uptake of ^{131}I-UdR(4), permit the observation that stem cell replication occurs during the first 5 days after irradiation. During this early proliferative phase of autorepopulation, stem cells are refractory to elevated levels of either exogenous or endogenous erythropoietin; erythroid differentiation, as indicated by splenic uptake of radioiron and reticulocytosis, does not occur until after the fifth day.

How does the replicating stem cell transform to the erythropoietin responsive cell? Kretchmar postulates that erythropoietin enters the cell during the G_1 phase of the cell cycle(17). During marrow repopulation, however, most stem cells are in the S phase of the cycle, and G_1 is too short to permit the entry of the erythropoietin that would induce differentiation. His computer model relates the duration of G_1 as a function of the number of cells in the stem cell compartment. Once this is replete, G_1 lengthens, and erythroid differentiation may proceed in the presence of erythropoietin. His predictions

(17) are strikingly similar to our experimental results.

Summary. Autorepopulation studies using a radiobiological method provide evidence that endogenous hemopoietic stem cell proliferation in the shielded mouse occurs during the first 5 days after irradiation. Exposure to hypoxia, sufficient to produce elevated levels of endogenous erythropoietin, failed to induce erythroid differentiation during the early proliferative phase of marrow repopulation. The data support the concept that the replication of stem cells, *per se*, renders them refractory to the induction of erythroid differentiation by erythropoietin.

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Effect of Saline Infusion and Norepinephrine on Response of the Kidney to Bacterial Endotoxin.* (32472)

JACK ZELLER, WILLIAM MARGARETTEN, AND DONALD G. MCKAY†
(Introduced by H. Stoerk)
Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City

Exposure of the blood stream to endotoxin derived from gram negative bacteria results in a progressive elevation of circulating catecholamines(1,2,3). One of the early indications that the adrenergic system is involved in the pathogenesis of the generalized Shwartzman reaction came from the experiments of Palmerio *et al*(4) who prevented the reaction in rabbits in the kidney which had been subjected to sympathetic denervation of the renal pedicle. Further support came from the experiments of Müller-Berghaus, when he showed that two alpha adrenergic blocking agents, dibenamine and dibenzyline, significantly reduced the incidence of glomerular thrombosis in pregnant rats exposed to a single small dose of bacterial endotoxin(5).

These experiments indicate that stimulation of the alpha adrenergic receptor sites in the kidney are necessary for localization of fibrin thrombi in the glomeruli in the evolution of the generalized Shwartzman reaction.

If this is true then it follows that it should be possible by the appropriate experiment to use alpha adrenergic stimulation to localize the thrombotic process in the glomeruli after triggering the clotting mechanism with bacterial endotoxin(6) in an animal not

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† Present Address: Dept. of Pathology, Univ. of California School of Medicine, San Francisco, Calif.