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THE REGULATION OF NUMBERS OF PRIMITIVE HEMOPOIETIC CELLS

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It is generally accepted that blood cells are derived from a species of primitive undifferentiated cell in the bone marrow which are usually designated as stem cells. Morphological characterization of stem cells is impossible on other than arbitrary, authoritarian grounds, since the stem cell can be identified morphologically only by the absence of any feature characteristic of one of the many cell lines giving rise to mature blood cells. So long as such a negative definition must be employed, any primitive cell tentatively identified as a stem cell may already be a differentiated cell insofar as yet unknown genetic and biochemical processes of cell differentiation have already occurred, although differentiation has not yet reached the point where morphological characteristics can be observed. Investigations of stem cells dependent on morphological features therefore appear destined to be limited and suspect.

The properties, characteristics, and biological features of stem cells are, however, too important to await advances in histological and histochemical techniques, and in recent years, functional characteristics of stem cells have come under investigation. These studies have all been consequences of the monumental experiments of Jacobson *et al.*[1] in which animals receiving otherwise lethal doses of ionizing irradiation survived, provided that the spleen was shielded from injury. It is likely that the mechanism underlying this survival is the recolonization of the system by primitive cells carried in the bloodstream from the protected spleen.[2, 3] A host of radiobiological investigations and a vast literature, culminating in the classic demonstration of spleen cloning, have been direct outgrowths of these experiments. McCulloch and Till[4] first demonstrated that stem cells, or colony-forming units, may be assayed by the number of discrete clones demonstrable in the spleens of lethally irradiated mice ten days after irradiation. Over a wide range, the number of clones is proportional to the number of normal bone marrow cells injected after irradiation.

Another method of investigating primitive marrow cells has been developed in our laboratory.[5] Since morphological evidences of erythropoiesis disappear in the transfusion-induced plethoric mouse, and are manifest again after a single injection of erythropoietin, it was concluded that erythropoietin acts upon stem cells, inducing

differentiation into the erythroid line.[6] This conclusion had previously been reached independently by Alpen and Cranmore[7] and Erslev,[8] who employed different models for their studies. Using the response to a standard dose of erythropoietin as a challenge to the primitive cells of the hematopoietic tissue, it has been possible to quantitate damage and follow patterns of recovery of these cells *in situ* and *in vivo*.[5, 9–11]

Bruce and McCulloch[12] have recently concluded that the primitive marrow cell responsive to erythropoietin is different from the primitive marrow cell capable of proliferating to form a colony after transplantation into the lethally irradiated mouse. The present report describes experiments which indicate that both erythropoietin-sensitive cells (ERC) and colony-forming units or cells (CFU) constitute compartments of cells whose numbers are regulated. This conclusion is based upon evidence interpreted by us as indicating that the rate of proliferation of small protected groups of these cells and their progeny increases after most of these cells in the body have been destroyed by irradiation.

Methods.—CF no. 1 female mice 12–14 weeks of age were used throughout. Rockland Mouse/Rat diet and water were permitted *ad libitum*. X radiation was administered by a 250,000-volt deep X-ray unit with 0.25 mm Cu and 1.0 mm Al filtration; dose rate factor 57 r per minute; target distance 81 cm.

In experiments designed to measure proliferation of ERC, mice were given 900 r total-body irradiation with lead shielding of the right hind limb. Test animals had been given 200 r total-body irradiation with the same leg shielded 4 to 7 days prior to the larger radiation dose. One control group received only the 900 r challenge; another control group was not irradiated. On days 3 and 4 after administration of 900 r, all mice were transfused with 0.6 ml of packed red cells. Six days after the 900 r administration, all mice were given 1 unit of erythropoietin[13] subcutaneously. Forty-eight hours later, 0.5 μc $Fe^{59}Cl_3$ in citrate solution was given intravenously in a total volume of 0.25 ml. Three days after radioiron administration, radioiron was determined in a scintillation well counter on a 0.25-ml sample of blood obtained by cardiocentesis. The percentage of the administered radioiron present in the peripheral blood was calculated from the activity of a dilute standard of the radioiron solution prepared at the time of administration, and assuming a blood volume of 7 per cent of the body weight.[14] Microhematocrit determinations were performed at the end of the experiment, and data from animals with hematocrits below 55 per cent were discarded because such mice were no longer sufficiently plethoric to ensure suppression of endogenously stimulated erythropoiesis.

In experiments designed to measure proliferation of CFU, mice were given 200 or 250 r total-body irradiation followed immediately by 550 or 500 r administered with the left leg shielded. Test groups had received 200 r total-body irradiation with the leg shielded 5, 8, or 13 days previously. Eight days after the larger dose of irradiation, animals were killed, and after spleen fixation in Bouin's solution, the total number of colonies grossly visible on the surface of each spleen was counted. Spleens were supplied in a random fashion to one investigator who counted the colonies without knowing the source of the sample.

Results.—Effect of previous radiation damage on response to erythropoietin following a second exposure to irradiation: Table 1 illustrates two separate experiments in which the recovery of response to one unit of erythropoietin after 900 r total-body

irradiation with one leg shielded was determined in control plethoric animals and in plethoric mice which had received 200 r total-body irradiation with one leg shielded 5 days previously. In both experiments a substantial reduction in erythropoietic response, as measured by radioiron incorporation into newly found erythrocytes, is seen in mice which had received a massive dose of irradiation 6 days before administration of the challenging injection of erythropoietin. Since very few mice survive 900 r for 8 days unless the leg is shielded, and the few survivors show no response to erythropoietin, we attribute the 4.3 per cent response to the effect of erythropoietin on the progeny of primitive cells that were in the leg at the time of irradiation, and that had been distributed between the leg and the rest of the body by the time of erythropoietic challenge. The essential feature of this experiment is that mice which had been given 200 r with the leg shielded 5 days before the larger irradiation responded to erythropoietin much more vigorously than did animals not receiving the initial irradiation. Indeed, in both experiments the twice-irradiated animals showed a response that was approximately normal.

Effect of previous radiation damage on colony-forming units following a second exposure to irradiation: The initial experiment in this series measured the effect of previous irradiation on the clone-forming ability of nonplethoric mice receiving 750 r total-body irradiation with one leg protected. With complete protection of the leg, so many totipotential cells colonized the spleen that hematopoietic tissue in this organ was confluent in 8 days. After 250 r was administered to the leg, the average number of spleen colonies was reduced to 4 (15 mice). In thirteen mice which had received 200 r total-body irradiation with leg shielded 5 days before 500 r leg shielded–250 r total-body irradiation, the average number of spleen colonies was 10. This study is summarized in Table 2.

The final experiment was designed to test whether increasing the time between first and second doses of radiation had any effect on the number of colonies appearing in the spleen. In an effort to increase the numbers of colonies in both control and test animals, the dose of radiation administered to the leg was reduced to 200 r. Table 3 shows that mice given 550 r leg shielded–200 r total-body radiation had an

TABLE 1

EFFECT OF PREVIOUS RADIATION DAMAGE ON RESPONSE TO ERYTHROPOIETIN AFTER A SECOND EXPOSURE TO RADIATION

Expt. no.	5 Days: 200 r, L.S.	5 Days: 900 r, L.S.	6 Days: E	2 Days: Fe^{59}	3 Days: t	% Fe^{59} ± 1 S.E.
			×	×		9.0 ± 1.8
1		×	×	×		4.3 ± 0.9
	×	×	×	×		11.0 ± 1.6
			×	×		13.5 ± 1.6
2		×	×	×		4.3 ± 0.5
	×	×	×	×		10.4 ± 1.0

L.S. = leg shielded; E = 1 unit erythropoietin; t = time of sacrifice.

TABLE 2

EFFECT OF PREVIOUS RADIATION DAMAGE ON SPLEEN CLONE FORMATION AFTER A SECOND EXPOSURE TO RADIATION

5 Days: 200 r, L.S.	500 r, L.S. 8 Days: 250 r, t.b.	8 Days: t	Spleen clones ± 1 S.E.
	×	×	4 ± 0.7
×	×	×	10 ± 0.6

L.S. = leg shielded; t.b. = total-body; t = time of sacrifice.

TABLE 3

EFFECT OF PREVIOUS RADIATION DAMAGE ON SPLEEN CLONE FORMATION AFTER A SECOND EXPOSURE TO RADIATION

200 r, L.S.		550 r, L.S. 200 r, t.b.	8 Days t	Spleen clones ± 1 S.E.
		×	×	8 ± 2.3
×	— 5 Days—	×	×	17 ± 4.0
×	— 8 Days—	×	×	21 ± 3.4
×	—13 Days—	×	×	25 ± 2.9

L.S. = leg shielded; t.b. = total-body; t = time of sacrifice.

average of 8 colonies per spleen after 8 days. Animals pretreated with 200 r total-body with leg shielding 5, 8, or 13 days before being treated with 550 r leg shielded–200 r total-body irradiation had respectively 17, 21, and 25 colonies per spleen.

Discussion.—The experiments described were prompted by our interest in the postulation that the numbers of primitive hematopoietic cells are regulated. It is reasonable to speculate that a compartment of cells of such critical importance to an animal would have a definite mechanism for preservation of its numbers. Evidence for rapid growth of primitive hematopoietic cells is found in experiments where as few as 8×10^5 marrow cells will protect a lethally irradiated mouse.[15] Some of the transplanted primitive cells proliferate rapidly in a few days, and weeks later the marrow is completely repopulated. Were this initial rate of proliferation to continue, the mass of primitive cells would reach abnormal proportions in a short time. Since a depleted compartment of cells expands rapidly, presumably attaining equilibrium without further net growth once near-normal numbers are present, we conclude that a mechanism for regulation of cell numbers does indeed exist.

Another experimental approach, employing sublethal irradiation, has demonstrated that a primitive compartment of cells recovers from damage quickly. Following 150 r total-body irradiation, the response to erythropoietin recovers rapidly after an initial lag period of 4 days,[10] and indeed even overshoots the normal level by a substantial margin.[11] These experiments were conducted in transfusion-induced plethoric mice where no red cell formation occurs until the challenging dose of erythropoietin is administered. Hence recovery of a characteristic property of the compartment of cells, namely, the response to erythropoietin, occurs even when this process is in abeyance. Again the rapidity of recovery, once regeneration begins, suggests to us a mechanism for regulation of primitive cell numbers.

The experiments recorded here utilize methods by which the mechanism of regulation of primitive cells may be demonstrated and investigated. If the conclusion of Bruce and McCulloch[12] is correct, the two series of experiments are directed at two different cell lines—the stem cell or colony-forming unit, and the erythropoietin-responsive cell. If the cell which responds to erythropoietin is distinct from the stem cell, then the two cell lines respond similarly to the different stimuli employed. Such an observation is probably not surprising since, if the ERC is different from the CFU, the former is undoubtedly an outgrowth of, or a differentiated product of, the latter.

We interpret the results of our experiments as follows. When one leg is spared an initial radiation injury, the primitive cells within that leg are stimulated to proliferate at a rate in excess of normal. Undoubtedly, many cells emerge from the protected leg and 5 days later have colonized the hematopoietic tissues elsewhere in the

body. The progeny of such cells are destroyed by 900 r, but the shielding of the leg again protects the primitive cells within its marrow. In the same manner, control mice not previously irradiated received 900 r with one leg shielded. All animals are then made polycythemic by transfusion in order that the erythropoietin tolerance test[16] could be used for quantitation of the numbers of erythropoietin-sensitive cells. We realize that several cell cycles elapse between the final irradiation and the administration of erythropoietin, and that the number of ERC, both those within the leg and those having migrated elsewhere, is probably substantially increased. However, we believe that response to erythropoietin on the sixth day after irradiation is related to the viable number of CFU or ERC immediately after irradiation, and that the greater response to erythropoietin in twice-irradiated animals represents enhanced proliferation of primitive cells within the leg between the first and second radiation exposure.

A similar enhancement of CFU proliferation within the leg following shielding of that leg and 200 r total-body irradiation is demonstrated by colony counts. Animals receiving 750 r total-body irradiation have no spleen clones 8 days later. If one leg is shielded, too many stem cells are protected and the spleen shows contiguous masses of hematopoietic tissue in 8 days. When, however, the protected leg is partially damaged by 200 or 250 r, only enough stem cells survive to give rise to a small number of discrete clones. Animals so damaged after having previously received 200 r total-body irradiation with leg shielding from 5 to 13 days earlier have increased numbers of clones in comparison to animals not receiving the initial irradiation. Again we interpret these findings as indicating that there are increased numbers of stem cells in the leg at the time of the second irradiation, as a consequence of the initial damage to stem cells outside the leg.

If the interpretations presented are correct, attention is focused on the mechanism by which the number of ERC and CFU are regulated. Since primitive cells in one area respond when similar cells in another area are damaged, a humoral mechanism is suggested. While factors capable of stimulating cell proliferation may arise elsewhere in the body and exert an effect on stem cells in the protected portion of the marrow, an equally plausible humoral mechanism based upon reduction of inhibition may be postulated. If it is further postulated that inhibitors are natural products of the cells whose proliferation is inhibited, it follows that a decrease in the number of these cells would temporarily suppress inhibition, through reduction of the titer of hypothetical inhibitor, until cell division had restored the population to a normal number, at which time a normal titer of inhibitor would be restored. Such a concept, first presented by Weiss,[17] is attractive because it offers a theoretical basis accounting for tissue growth without invoking the existence of a separate monitoring organ for each tissue capable of regeneration after partial ablation or destruction. The most delicately regulated system might rely upon both an inhibitor and a stimulator of cell replication, and evidence arising from other model systems for the existence of inhibitor or "retine," and promotor, or "promine," has recently been reviewed by Szent-Györgyi.[18]

The control of populations of primitive cells and the nature of abnormal states arising from breakdown of such control are problems of great interest. It is hoped that the methods, results, and interpretations presented here are sufficiently informative and provocative to attract the attention and efforts of fellow investigators,

and that modifications of the model described will enable us to determine whether specific stimulators or reduction in titer of specific inhibitors account for the results here recorded.

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*CYCLIC BIREFRINGENCE CHANGES IN PSEUDOPODS OF CHAOS CAROLINENSIS REVEALING THE LOCALIZATION OF THE MOTIVE FORCE IN PSEUDOPOD EXTENSION**

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The extension of pseudopods appears to be the fundamental mode of movement of many cells. This applies not only to sarcodine protists such as amebas, testaceans, foraminiferans, radiolarians, and heliozoans, but also to all or nearly all embryonic metazoan cells. Despite numerous descriptive and experimental studies of ameboid movement, there is no universally accepted general theory to account for pseudopod extension and the cytoplasmic streaming that inevitably accompanies it. In fact, there are even strong indications that the details of ameboid movement in different organisms, even those now classified within the same genus, may be so diverse as to demand different models.[1]

A starting point for several investigations into the mechanism of ameboid movement[1–8] and cytoplasmic streaming[9] has been a search for fibrils that might serve as the structural basis for cytoplasmic contractility, for a majority of workers have