Humoral Regulation of Erythropoiesis VI. Mechanism of Action of Erythropoietine in the Irradiated Animal. (26744)

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We previously reported an assay for erythropoietine using the sublethally irradiated animal(1). The results with this assay differ from those using fasted(2) or transfused animals(3); in the irradiated animal there is a longer interval between injection of erythropoietine and a measurable effect. The reason for this difference is unexplained. It has been suggested that erythropoietine promotes differentiation of stem cells(4,5), stimulates division in the erythroid compartment(6), or both(7). In this paper further studies on the response of the irradiated animal to erythropoietine are reported. These data support the concept that erythropoietine promotes stem cell differentiation; a tentative hypothesis is proposed to explain the difference in rate of response of irradiated and transfused animals.

Material and methods. Female Sprague-Dawley rats weighing 135-160 g were used. Irradiation was given with a 3.0 MEV Van de Graaff linear accelerator at a dose rate of 300 r/minute as measured by a Bendix ionization chamber known to be air equivalent at this energy. Transfused animals were given red cells in an amount equivalent to 1.4% of body weight 4 days prior to administration of erythropoietine. Erythropoietine was obtained from the urine of patients with congenital hypoplastic anemia and concentrated by evaporation or precipitated with 80% alcohol; the concentrates were dialyzed against normal saline at 4°C prior to use.

Red cell production was estimated by reticulocyte counts and Fe^{59} incorporation curves. In the Fe^{59} incorporation studies, we injected intramuscularly 5 mg of non-radioactive iron, as Imferon, 3 hours after injection of Fe^{59}. The dose of Imferon was repeated daily thereafter. By diluting out the Fe^{59} the non-radioactive iron minimizes the continued utilization of radioiron recycled through the tissues. Blood volume was measured in each animal by injecting Cr^{51} labelled cells 20 minutes prior to exsanguination; Cr^{51} and Fe^{59} counts were separated using a single channel spectrometer.

Results. 1. *Effect of erythropoietine after 200 r.* Twenty-four hours after 200 r whole body radiation total Fe^{59} incorporation is at a minimum. When Fe^{59} is given 48 hours after irradiation few labeled cells emerge during the following 24 hours but a substantial number emerge between 24 and 48 hours (72-96 hours post-radiation). Administration of 2-4 CS units of erythropoietine(8) immediately after irradiation and 24 hours thereafter affected the cohort of cells emerging between 24 and 48 hours after Fe^{59} (72-96 hours post-radiation) but there was no detectable effect within the first 24 hours after Fe^{59} (72 hours post-radiation) (Table I). A difference in reticulocytes was not detectable until 96 hours after initial injection of erythropoietine (Table II).

2. *Effect of erythropoietine after 400 r.* Erythropoiesis does not resume until several days after 400 r; reticulocyte count rises substantially on the 6th day. Administration of a single dose of erythropoietine immediately after 400 r produces an earlier recovery (Table III). A single dose of erythropoietine given 24 hours after irradiation was no more effective than the dose given immediately after irradiation (Table III). The iron in-

TABLE I. Effect of Erythropoietine (EP) on Recovery of Erythropoiesis in Rats Receiving 200 r.

Treatment	Hr after Fe^{59} 24	48	72
Control	5.2 ± .73*	13.5 ± 1.83	20.2 ± 1
EP × 2†	6.8 ± .83	20.4 ± .52	26.4 ± .51
EP daily	6.8 ± .83	21.5 ± .53	26.9 ± .61

* Iron incorporation as % of inj. dose. Values are mean of 18 animals ± S.E. Fe^{59} given 48 hr after irradiation.

† Erythropoietine given immediately and 24 hr after irradiation.

TABLE II. Effect of Erythropoietine on Reticulocytes after Sublethal Radiation.

Treatment	Hr after 200 r 72	96	120
Control	.3 ± .06†	.8 ± .13	2.0 ± .25
EP × 2*	.3 ± .04	1.3 ± .18	2.7 ± .32
EP daily	.2 ± .05	1.2 ± .13	3.7 ± .36

* Erythropoietine given immediately and 24 hr after irradiation.
† Reticulocyte percentage ± S.E. Each value based on 12 animals.

corporation curves in 4 experiments showed similar changes (Table IV).

3. *Effect of erythropoietine in the transfused rat.* A single dose of erythropoietine produced a small increase in reticulocytes within 48 hours with a very pronounced effect by 72 hours (Table V). Multiple injections produced an even greater effect. Fe^{59} data were consistent with reticulocyte values.

TABLE III. Effect of Erythropoietine after 400 r.

Treatment	Reticulocytes Day post-radiation 6	7	8
EP immediately after irrad.	1.94 ±.3*	4.67 ±.8	6.79 ±1.23
Control irradiated	.31 ±.09	1.9 ±.34	5.01 ± .66
EP 24 hr after irrad.	.6 ±.19	3.45 ±.91	6.61 ± .45

* Mean of reticulocytes of 6 animals ± S.E.

Discussion. Previously we proposed a model for erythropoiesis(9) in which the erythroid compartment is replenished from a primitive or stem cell compartment. After differentiation into erythroid elements, maturation and division are asynchronous. Some cells and their progeny will divide more than others; some may mature without dividing. Cell death may occur during maturation and under some circumstances may be substantial. The sequence of events in the erythroid compartment following hypertransfusion suggests that the suppression results primarily from a decreased input from the stem cell compartment(10). Irradiation affects erythropoiesis by destruction of cells within the erythroid compartment as well as in the stem cell compartment.

TABLE IV. Effect of Erythropoietine on Recovery of Erythropoiesis in Rats Receiving 400 r.

	Hr after Fe^{59} 24	48	72
Control	8 ± 1.21†	18 ± 2.13	20 ± 1.93
EP*	15 ± 1.73	30 ± 1.86	26 ± 2.05

* Erythropoietine given immediately after irradiation; Fe^{59} labeled plasma given 5 days after irradiation.
† Percentage of inj. iron incorporated in red cells; mean of 24 animals ± S.E.

TABLE V. Reticulocyte Response of Hypertransfused Rats to a Single Injection of Erythropoietine.

Hr after treatment	Erythropoietine	Control
24	.13 ± .04*	.21 ± .04
48	.40 ± .09	.25 ± .05
72	1.78 ± .32	.15 ± .03
96	3.10 ± .7	.17 ± .06
120	1.91 ± .11	.14 ± .02

* Avg reticulocyte percentage in 5 animals ± S.E. of mean.

The response of the hypertransfused animals to erythropoietine seems best explained by the differentiation of large numbers of stem cells. The emergence of some cells as early as 24-48 hours after a strong stimulation suggests either a shortening of generation times or skipped divisions (*i.e.* maturation without division). Alpen(4) has presented evidence to suggest that even after severe stimulation the generation time of the erythroblast is unchanged. Whether this applies to later stages remains moot. A primary effect on later stages of development is unlikely since there are few such cells remaining and certainly not enough to explain the magnitude of the response unless the generation times are incredibly short. Jacobson's studies in the mouse(3) where erythroid elements are reported to be completely absent after hypertransfusion lend further support to the notion that erythropoietine promotes stem cell differentiation.

The data clearly demonstrate a difference between the effect of erythropoietine in irradiated and hypertransfused animals. The effect was delayed after 200 r but much more so after 400 r. It seems unlikely that this could result from continued availability of

erythropoietine for several days during which time the stem cell compartment regenerated. Estimates of a circulating half time for erythropoietine of 5-10 hours in irradiated animals seem incompatible with such a thesis(11). Also, these estimates represent a maximum since they were derived from studies in which there was undoubtedly an overlap between production and destruction of erythropoietine. Furthermore, administration of erythropoietine 24 hours after irradiation was no more effective than when given immediately after irradiation, contrary to what would be expected if there were continued availability.

A more attractive hypothesis is that erythropoietine when given immediately after irradiation differentiates a portion of the stem cell compartment, which after one or 2 abortive attempts at division dies. The resultant depopulation of the stem cell compartment serves as a stimulus for earlier regeneration of the stem cell compartment(12). This explanation entails certain assumptions, which, while unproved, are compatible with current knowledge. The erythroid compartment is not self-sustaining but is constantly replenished from a stem cell pool, which turns over at a slower rate than the erythroid compartment. Rate of turnover of the stem cell compartment is determined by a feedback mechanism governed by rate of removal of stem cells; cells may be removed by death within the compartment or by differentiation. After irradiation cells in both the erythroid and stem cell compartment are lost either directly or upon entering mitosis. The damage to the erythroid compartment is greater than to the stem cell compartment due to differences in sensitivity perhaps reflecting differences in generative cycle. Following radiation injury the number of cells killed directly, dying during abortive division, and surviving is dependent on dose. It is further suggested that at higher doses some surviving cells for a period are viable but temporarily "infertile," and others permanently sterile, the proportion being dose dependent. These cells at least temporarily retain the capacity to differentiate. The stage of "repair" involves restoration of the capacity of division of those cells "temporarily infertile." Alternatively, the latter might be considered as "undamaged"(12) but this seems unlikely at high doses. The period for repair is also dose dependent. Once the capacity for division has been renewed, rate of division and regeneration will again be governed by the feedback mechanism and be dependent on the numbers of cells in the compartment, *i.e.*, surviving but sterile cells would influence rate of regeneration. Erythropoietine by reducing the number of the latter cells would permit a more rapid regeneration.

Summary. Erythropoietine produces an increase in reticulocytes in transfused animals within 48-72 hours. In irradiated animals the response is delayed, length of delay being in part dose dependent. A change in reticulocytes is not observed until 96 hours when erythropoietine is given immediately after 200 r; after 400 r the effect is first noted at 6 days. A tentative hypothesis is proposed to explain these results. It is suggested that erythropoietine promotes differentiation of stem cells into erythroid elements and that depopulation of the stem cell compartment stimulates division within that compartment.

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Binding of Radioactive Sodium, Potassium, and Bromide in Guinea Pig Brain Homogenates.* (26745)

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Binding of various electrolytes in the brain has been described over the years since Fremy in 1841 first noted the presence of fixed mineral substances in brain tissue. Thudichum found potassium to be the chief inorganic constituent present in protagon and demonstrated that calcium is attached to cephalin. Koch and Pike(7) showed that cephalin contains more potassium than sodium, but that in lecithin the reverse is true.

Christensen and Hastings(3) found that cephalin has the ability to combine with sodium and potassium with equal affinity in amounts increasing with pH. At neutrality they found that 0.5-0.6 equivalents of alkali were bound per mole of cephalin.

Folch(4) reported 30% excess of cations over anions in brain, accounting for this "anion deficit" by the binding of approximately this proportion of brain cations to cerebron sulfuric acid and phosphatidyl serine. Twenty per cent of the brain sodium (10 mEq) was bound to phosphatidyl serine.

Stone and Shapiro(13) filtered homogenates of brain and muscle under negative pressure at 3-4°C and found that 20% of brain and muscle potassium did not diffuse through cellophane, while 100% of sodium was freely diffusible. Bergen, Stone and Hoagland(2) used the ultrafiltration technic of Stone and Shapiro to demonstrate binding of about 30% of rat brain potassium not influenced by adrenalectomy. Their results confirmed earlier reports of a slowly exchanging brain potassium compartment which develops with age in rats. On the other hand, Leiderman and Katzman(9) demonstrated a significant difference in radio-potassium uptake by the brains of normal and adrenalectomized rats. They(8) found 20 mEq of radio-potassium per kilogram of wet brain to be non-exchangeable in normal adult rats, as determined by comparison of brain and plasma specific activities up to 72 hours after intraperitoneal injection. This non-exchangeable potassium compartment was not present in rats younger than 35 days and was abolished by adrenalectomy in adults.

Shaw and Simon(12) found that the potassium in toad nerve and muscle, allowed to die naturally or poisoned with iodoacetate or cyanide, did not equilibrate with the surrounding medium. They postulated potassium binding to explain this effect.

Shanes and Berman(11) studied diffusion out of toad sciatic nerve. They found that a small but significant proportion of radioactive sodium did not come to diffusion equilibrium with the surrounding medium.

Pappius and Elliott(10) noted that potassium content of rat cerebral cortex slices in a Warburg apparatus under anaerobic conditions failed to come to equilibrium with the medium when potassium concentration of the medium was high. They suggested that this disequilibrium might be due to binding of a large portion of intracellular potassium.

Recapitulation of the reported literature seems to indicate that there is probably

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