associated with marked rise of all five fractions but to a different degree. The cationic A_2 fraction increase was greater. This altered isoenzyme distribution resembled the pattern of GOT isoenzymes in the liver homogenate. The implication of these findings with respect to the transfer of the intracellular hepatic GOT into the serum is considered.

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Regulation of Erythropoiesis. XXI. The Effect of Erythropoietin on the Stem Cell* (32797)

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It is generally appreciated that the recognizable elements of the bone marrow are not self-sustaining but are supported by a precursor compartment which is pluripotential, giving rise to erythroid, myeloid and megakaryocytic elements. In significant measure this hypothesis rests upon the observation of chromosomal abnormalities in radiation chimeras (1) and the presence of a pH chromosome in erythroid, myeloid and megakaryocytic elements in patients with chronic myelogenous leukemia (2). More recently a question has been raised as to whether there is, in addition, a committed precursor compartment intermediate between the primitive pluripotential cell and the differentiated or recognizable bone marrow elements. Support for the latter thesis derives from a number of observations: (i) in studies in vincristine-treated rats megakaryocytic differentiation appeared to continue although erythroid differentiation could not be achieved even with massive doses of erythropoietin (EP) (3); (ii) thymidine labeling data on megakaryocytes pointed to a difference in generation time between the precursor cell of the megakaryocyte and that for the erythroid elements (4, 5); (iii) the effect of colchicine on the hematopoietic recovery of mice (6); and (iv) observations of the effect of hypoxia on the pluripotential stem cell (7). In the latter studies Bruce and McCulloch observed that in the mouse the primary erythroid response to hypoxia was splenic and occurred within 2–3 days. This initial response was followed rather than preceded by a decrease in the numbers of splenic colony-forming units (CFU) or pluripotential cells. These observations were interpreted as evidence for an intermediate erythropoietin-sensitive precursor compartment which was not transplantable and served as a buffer between the pluripotential and the differentiated compartments. The slow decline in colony-forming units in the spleen of the hypoxic mice was suggested to be the consequence of a depletion of the pool of erythropoietin-sensitive cells with subsequent dif-

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ferentiation of pluripotential cells (CFU) to restore the intermediate erythropoietin-sensitive compartment. In these experiments, as exposure to hypoxia continued, the CFU declined to levels of 7–13% of control by day 28 after the beginning of exposure.

Previous studies in our laboratory suggested that hypoxia might have an effect on the CFU independent of the erythroid response.[2] We observed significant strain differences between mice, not only in respect to the degree of polycythemia which developed after exposure to hypoxia, but also the extent of changes in the CFU as measured by transplantation. Thus, in the CAF_1 mouse a relatively mild polycythemia, $\sim$ 30% increase in red cell mass, developed after exposure to simulated altitudes of 23,000 feet over a period of 23 days, but in the CF_1 mouse increases of up to 100% in red cell mass were observed. In contrast, the splenic CFU in the CAF_1 mouse decreased to levels as low as 14% of control, whereas in the responsive CF_1 mouse a slight increase in splenic CFU was observed. The difference in the polycythemic response appeared to be due to the relatively poor production of EP in response to hypoxia by the CAF_1 mouse although exogenous EP is effective in inducing polycythemia.[3] In view of these observations suggesting a dissociation between the erythroid response and the numbers of CFU in certain strains of mice after exposure to hypoxia, it appeared important to determine the effect of exogenous erythropoietin on the pluripotential cell in a strain in which a decrease in CFU had been observed after exposure to hypoxia.

Materials and Methods. Eleven-week-old virgin CAF_1/J hybrid females (BALB/cJ x A/J)[4] were randomized into control and erythropoietin-treated groups. The latter received 3 units of erythropoietin[5] every 8 hours and were sacrificed on days 2, 5, 8, 16, and 23 in the first experiment and on 1, 2 and 8 in the second experiment. Control groups of animals received saline and were sacrificed at the indicated time intervals in each experiment. Four hours prior to sacrifice ^{59}Fe-labeled plasma was given intravenously. Twenty minutes prior to sacrifice ^{51}Cr-labeled cells were given intravenously. Animals were bled by cardiac puncture. The red cell mass was measured by isotope dilution. The spleen was immediately removed and placed in Puck's solution. Splenic iron incorporation was measured, the spleen was weighed, then finely minced, and a suspension was prepared by repeatedly drawing it gently through a needle. All suspensions were kept at ice-water temperature (7). Total nucleated and differential counts were made from the suspension and the appropriate dilution was made with ice-cold Puck's medium. The distal epiphysis of one femur was removed and the adjacent shaft was cut to a distance of 9 mm; the marrow was blown into ice-cold Puck's media and the suspension made. From isotope dilution studies this represents $\sim$ 60% of the total femoral marrow.[6] Total nucleated counts were made on the suspension and the appropriate dilution with ice-cold Puck's medium was made immediately before injection. The ^{59}Fe uptake was measured in the opposite femur and differential counts were made on the tibial marrow. In previous experiments it was determined that there were no significant differences in the total nucleated counts or iron incorporation between femora nor in the differential between tibia and femora.[6] Total red counts and reticulocyte counts were made on the peripheral blood; the total body reticulocyte count was derived from this and the blood volume. Bone marrow (7.5×10^4 cells) and splenic (1×10^6 cells) suspensions were injected into heavily irradiated syngeneic recipients (total dose 850 R; dose rate 48 R/mm; 250 kV, 15 mA, with 1.0 mm Cu and 1.0 mm Al filtration). Eight days after transplantation the animals were sacrificed, the spleens fixed in Bouin's solution, and the spleen colonies were counted independently by two observers.

Results. The erythropoietic response of the

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[4] Jackson Laboratories, Bar Harbor, Maine.

[5] Human urinary erythropoietin, kindly furnished by the Erythropoietin Committee of the National Heart Institute.

[6] Kubanek, B., Ferrari, L., Tyler, W. S., and Stohlman, F., Jr., unpublished observations.

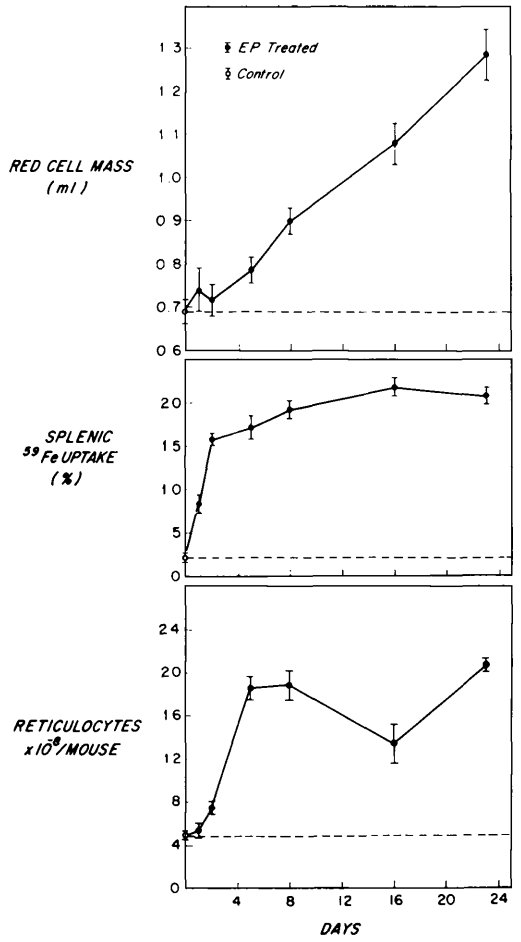

FIG. 1. The effect of erythropoietin on the peripheral blood parameters and splenic iron incorporation of the CAF_1 mouse. Each point represents the mean ± SE of 4–10 animals (cf. Table I); — — — —, mean value for 20 control animals.

CAF_1 mouse to erythropoietin is shown in Fig. 1 and Table I. There was a prompt reticulocytosis which was detectable on day 2 after initiation of treatment and reached levels of 4–5-fold that of control by day 5; this degree of reticulocytosis was maintained throughout the period of treatment. The red cell mass rose progressively throughout the 23-day period of treatment, reaching a value of 1.23 ± 0.06 cc on day 23 as compared with the average control value of 0.69 ± 0.03 cc.

The erythroid response to EP in these animals appeared to be primarily splenic as judged from evaluation of the nucleated erythroid cells in the pooled suspensions used for transplantation and the 4-hour ^{59}Fe incorporation in the spleen and marrow. Splenic iron uptake rose from ~ 2% to 17–22% of the injected dose (Table II). Concomitant with this rise in splenic iron uptake was an increase in the number of nucleated erythroid elements (Table II). The numbers of bone marrow red cell precursors initially showed a slight increase but thereafter decreased and appeared to be no different than the controls (Table II). A significant decrease in iron incorporation of about 50% was observed even though there appeared to be no significant difference in the number of erythroid elements in the marrow.

An increase in the splenic CFU was observed within 24 hours (Fig. 2). The increase in splenic CFU occurred prior to the development of peripheral reticulocytosis and concomitant with the increase in splenic iron uptake (Tables I–III). The numbers of CFU continued to increase over the next several days and achieved levels of ~ 5-fold that of the average control between days 8 and 16 after the initiation of EP treatments (Fig. 2, Table III). The CFU in the femur remained within the range observed in normal animals

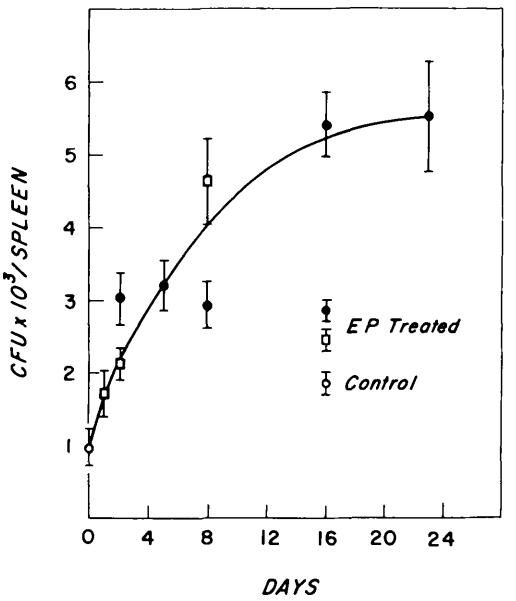

FIG. 2. The effect of erythropoietin on the splenic CFU in the CAF_1 mouse. Values are the mean ± SE of 9–11 animals; (○), pooled mean of control groups.

TABLE I. Peripheral Blood Parameters.

Day	No. of animals	Treatment	HCT. (%)	Body reticulocytes ($\times 10^{-8}$)	Red cell mass (cc)
8	5	NaCl	47 ± 1.4	4.5 ± 0.5	0.61 ± 0.05
23	5	NaCl	47 ± 0.9	5.8 ± 0.6	0.67 ± 0.32
2	5	EP	49 ± 1.3	8.3 ± 0.6	0.76 ± 0.05
5	5	EP	54 ± 0.2	18.5 ± 1.0	0.79 ± 0.03
8	3	EP	57 ± 1.5	16.3 ± 0.9	0.83 ± 0.03
16	4	EP	62 ± 1.4	13.3 ± 1.7	1.08 ± 0.05
23	6	EP	62 ± 1.7	20.8 ± 0.6	1.23 ± 0.06
1	5	NaCl	46 ± 1.5	3.9 ± 0.4	0.82 ± 0.04
8	5	NaCl	47 ± 0.6	3.4 ± 0.5	0.66 ± 0.04
1	5	EP	46 ± 0.8	5.5 ± 0.6	0.74 ± 0.05
2	5	EP	49 ± 1.7	6.6 ± 0.8	0.66 ± 0.03
8	7	EP	56 ± 0.7	20.2 ± 2.0	0.94 ± 0.04

TABLE II. Erythropoietic Parameters.

Day	No. of animals	Treatment	Iron incorporation		Nucleated erythroid cells ($\times 10^{-6}$)	
			Spleen	Marrow	Spleen	Marrow
8	5	NaCl	2.2 ± 0.54	2.5 ± 0.20	15.2	3.7 ± 0.4
23	5	NaCl	2.4 ± 0.56	2.1 ± 0.60	8.2	2.8 ± 0.2
2	5	EP	17.2 ± 0.95	1.6 ± 0.09	57.3	4.1 ± 0.2
5	5	EP	17.2 ± 1.39	1.0 ± 0.13	116.5	3.9 ± 0.1
8	3	EP	18.7 ± 1.30	1.1 ± 0.25	90.4	3.3 ± 0.2
16	4	EP	21.9 ± 0.90	1.0 ± 0.58	63.7	1.9 ± 0.2
23	6	EP	20.9 ± 1.10	0.8 ± 0.07	85.8	2.6 ± 0.1
1	5	NaCl	1.3 ± 0.27	2.3 ± 0.08	14.6	3.5 ± 0.2
8	5	NaCl	2.9 ± 0.49	2.3 ± 0.10	8.3	3.6 ± 0.1
1	5	EP	8.4 ± 1.14	2.5 ± 0.09	23.6	3.8 ± 0.4
2	5	EP	14.4 ± 0.55	1.6 ± 0.07	32.6	4.2 ± 0.05
8	7	EP	20.0 ± 1.63	1.1 ± 0.04	147.7	5.7 ± 0.02

in these experiments. Although no individual point was significantly different from the average of 3.51 ± 0.55 × 10^3 CFU observed in the 4 control groups, there appeared to be the suggestion of a decrease in CFU during the first week after initiation of EP treatment (Table IV).

Discussion. In these studies an intense erythropoietic response was induced in the CAF_1 mouse by the daily administration of 9 units of EP given in equal doses at 8-hour intervals. The red cell mass doubled and the total body reticulocyte pool increased 4–5-fold and was maintained at this level. The difference between the magnitude of increase in red cell mass (2-fold) and the degree of reticulocytosis (4–5-fold) suggested that the cells produced in response to large doses of erythropoietin were short-lived, as has been previously reported (8). The response was primarily splenic as judged from the iron uptake and the numbers of erythroid elements. The iron incorporation of the femora was reduced but the numbers of erythroid precursors were not importantly affected. The difference between the iron incorporation and the numbers of nucleated erythroid precursors in the marrow may have reflected changes in bone marrow transit time of the erythroid precursors or more likely a competitive change in

TABLE III. Results of Assay for CFU in the Spleen.

Day	Treatment	Nucleated cells $\times 10^{-8}$/spleen[a]	Av no. of colonies in recipients[b]	CFU/spleen $\times 10^{-3}$
8	NaCl	1.52	3.4 ± 0.35	0.52 ± 0.05
23	NaCl	1.49	5.2 ± 0.55	0.77 ± 0.08
2	EP	2.47	12.3 ± 1.47	3.03 ± 0.36
5	EP	2.67	12.0 ± 1.28	3.21 ± 0.34
8	EP	2.91	10.1 ± 1.11	2.94 ± 0.32
16	EP	2.33	23.1 ± 2.03	5.40 ± 0.47
23	EP	3.27	16.9 ± 2.31	5.56 ± 0.76
1	NaCl	1.68	5.5 ± 0.88	0.92 ± 0.15
8	NaCl	2.14	7.7 ± 0.97	1.69 ± 0.21
1	EP	2.05	8.3 ± 1.48	1.70 ± 0.30
2	EP	2.05	10.3 ± 1.03	2.10 ± 0.21
8	EP	4.32	10.7 ± 1.4	4.63 ± 0.6

[a] Total number of nucleated cells in the spleen suspension.

[b] Average number of colonies in 9–11 recipients; 1×10^6 spleen cells were given to each recipient.

TABLE IV. Results of Assay for CFU in the Bone Marrow.

Day	Treatment	Nucleated cells[a] $\times 10^{-7}$	Av no. of colonies in recipients[b]	CFU/femur $\times 10^{-3}$
8	NaCl	1.65	15.3 ± 1.99	3.36 ± 0.44
23	NaCl	1.73	9.4 ± 1.56	2.16 ± 0.36
2	EP	1.83	12.3 ± 2.16	2.99 ± 0.53
5	EP	1.61	13.2 ± 1.8	2.82 ± 0.38
8	EP	1.51	12.4 ± 1.4	2.49 ± 0.27
16	EP	1.64	18.1 ± 1.3	3.97 ± 0.29
23	EP	1.75	13.8 ± 1.6	3.22 ± 0.38
1	NaCl	2.10	14.8 ± 2.01	4.14 ± 0.56
8	NaCl	1.81	18.3 ± 1.84	4.42 ± 0.44
1	EP	1.55	13.2 ± 1.08	2.72 ± 0.22
2	EP	1.51	13.9 ± 1.18	2.79 ± 0.24
8	EP	1.92	12.2 ± 1.3	3.11 ± 0.33

[a] Total number of nucleated cells in the bone marrow suspension.

[b] Average number of colonies in 9–11 recipients; 7.5×10^4 marrow cells were given to each recipient.

iron availability to the marrow due to the striking increase in splenic and peripheral blood iron uptake. In view of the maintenance of the nucleated precursors, however, this decrease in iron incorporation is not thought to represent a true decrease in red cell production by the bone marrow. The reason for the failure of the marrow to participate to a greater extent in the response to erythropoietin is unknown, but studies on the effect of erythropoietin in splenectomized animals may provide further insight into this problem.

The CFU of the spleen in EP-treated animals did not decrease as might have been anticipated from previous observations on the response of mice to hypoxia (7),[2] but began to increase within 24 hours and reached levels of ~ 5 times those observed in the controls. The extent to which migration of CFU from the marrow to the spleen may have accounted for the marked increase of splenic CFU cannot be answered with certainty. It is estimated from ^{59}Fe-dilution studies that the total marrow pool of CFU in the CAF_1 mouse is

$\sim$ 60,000.[6] The average splenic CFU in control animals in these experiments was 975 and the average that we have observed in 10 control studies on this strain of mouse is approximately 1000 CFU/spleen. Thus, migration of less than 10% of the marrow CFU to the spleen could account for the observed increase in splenic CFU. There was a suggestion of a decrease in the numbers of CFU in the femoral marrow during the early phase of EP treatment which may have reflected migration. The assay technic, however, is not sensitive enough to detect with certainty changes in marrow CFU of this magnitude. It is clear from a number of studies that migration of stem cells does occur. Although most of these have been carried out after irradiation, the studies of Brecher and Cronkite (9), Swift *et al.* (10) and Goodman and Hodgson (11) clearly indicate that pluripotential cells are normally present in peripheral blood. It seems entirely possible, therefore, that under normal physiologic stimulation such as the administration of erythropoietin, an increase in the migration of stem cells may occur. Although it is not possible to draw any firm conclusions as to the extent that migration and/or replication of CFU *in situ* contributed to the increase in splenic CFU during the course of treatment with EP, it is clear that the erythroid response to erythropoietin is accomplished without depletion of the pluripotential stem cell compartment as might have been inferred from experiments on the response of hypoxic animals.

We have suggested that the decrease in splenic CFU which has been observed in some strains of mice after exposure to hypoxia is not directly related to the EP-induced erythroid response.[2] In both the CAF_1/J mouse,[2] which we have used, and the C57 BL/Ha and C57 BL/J strains, which Bruce and McCulloch studied (7), the increase in red cell mass was relatively modest but there was a marked decrease in the numbers of splenic CFU. In contrast, the CF_1 mouse which developed a striking polycythemia after exposure to hypoxia had, if any change, a slight increase in splenic CFU. Further, the erythropoietin-induced polycythemia of the CAF_1 mouse herein reported was associated with a stricking increase in the splenic CFU. It might be suggested, then, that hypoxia has a secondary perhaps "toxic" effect on the stem cell. There are studies, both clinical and experimental, which point to the fact that hypoxia, when severe, may have a limiting effect on the erythroid response. Talbot (12) observed in 1936 during the course of a mountain expedition that after ascent above 20,000 feet the hemoglobin of the mountaineers began to decline. In a patient with segmental hypoxia and polycythemia secondary thereto, we observed intense erythropoiesis in normally oxygenated areas with an M/E ratio of 1:1.7 whereas in the hypoxic area there was relative hypoplasia with an M/E ratio of 2.3:1 (13). It has also been demonstrated in sublethally irradiated animals that the response to a given level of erythropoietin is reduced when the animals are exposed to a pO_2 of less than 0.5 atmospheres (14). Finally, it has been reported that hypoxia has a toxic effect on bone marrow explants (15).

It is perhaps worthy of emphasis that the present studies do not provide a cogent argument either for or against the existence of an intermediate committed stem cell compartment. They do, however, indicate that the pluripotential stem cell participates in the response to a severe erythroid stimulation. Further, it is clear that the stem cell participates early in the erythroid response, within the first 24 hours after the beginning of treatment, rather than after several days as had been previously postulated from studies of the response to hypoxia. Studies of the splenic CFU during the first 24 hours after administration of EP to normal and hypertransfused animals will be necessary to gain insight into the interrelationship between the postulated committed stem cell and the pluripotential compartment in the physiologically stimulated animal. Finally, we can draw no conclusions as to the extent of participation of the stem cell after lesser degrees of stimulation and, indeed, there may be strain differences in this respect with the levels of erythropoietin used in these experiments.

Summary. Nine units of erythropoietin given in equally divided doses at 8-hour intervals for 23 days to the CAF_1 mouse produced a significantly greater polycythemia than had been observed in this strain when

exposed to 23,000 feet for a similar period of time. The splenic CFU began to increase within 24 hours after the beginning of treatment and reached levels of 5-fold those of controls between days 8 and 16. The extent to which migration of CFU from the marrow and/or replication of CFU *in situ* is responsible for the increase in splenic CFU is discussed. It is concluded that the stem cell participates in the physiologic response to erythropoietin but without depletion of this compartment. The possibility that the previously reported decrease in CFU in hypoxic animals represents a secondary perhaps "toxic" effect of hypoxia unrelated to erythropoietin is considered.

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Reabsorption of Albumin-Evans Blue Via Membrane Vesicles of Proximal Kidney Tubule Cells* (32798)

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Small amounts of foreign and natural blood plasma protein filter into proximal tubule of kidney. Tissue electron micrographs of labeled protein followed in time after injection reveal the following path of reabsorption into tubule cells. Label becomes lodged inside invaginations of tubular plasma membrane from its surface between the base of microvilli of the brush border. Small apical vacuoles are presumed to bud off the invaginations. These fuse into large apical vacuoles slightly deeper in the tubule cell. Ultimately protein label becomes enclosed as cytoplasmic bodies or granules which lie surrounding the nucleus as though related to the Golgi apparatus, which may supply proteolytic enzymes. Probably proteins are split to constituent amino acids and are absorbed as such rather than as intact macromolecules into peritubular blood. This pathway is followed by all proteins (1) that can be visualized by electron microscope or histochemistry (hemoglobin, ferritin, horse radish peroxidase, albumin-^{125}I by electron autoradiograph). Evans blue T1824, a firmly bound label for blood albumin, may follow a similar pathway into the tubular cell (2). This interpretation is strengthened by our present freeze-dry histology, which is far superior to that previously published. We can immobilize and detect albumin–Evans blue in free solution. Travel across tubular epithelium is not as free molecules but inside the vesicle system. Recent literature provides more complete interpretation than possible when earlier publication on T1824 appeared.

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