HEMOPOIETIC SPLEEN COLONY STUDIES

II. ERYTHROPOIESIS*

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Lethally irradiated mice receiving low doses of bone marrow cells intravenously develop discrete nodules of regenerating hemopoietic tissue. In the spleens these are grossly visible and dissectable (1) and are of clonal nature (2), each originating from a single transfused (or endogenously surviving) hemopoietic stem cell. These colonies have provided a useful technique for the study of radiation sensitivity (1), hemopoietic repopulation (3), erythropoiesis (4–8), stem cell kinetics (9), anemic mutants (10), and immune competent cells or their precursors (11). Each of the spleen colonies initially undergoes a single line of hemopoietic differentiation. Erythroid, neutrophilic, megakaryocytic, and eosinophilic hemopoietic cells have each been found in the form of gross or microscopic colonies, each of single differentiated cell type (12). While none of the spleen colonies are lymphoid, they do contain stem cells capable of repopulating the lymphoid system.[1]

We have previously shown (4), and it has been confirmed (6–8, 13), that erythroid spleen colonies are eliminated by polycythemia without significant increase of numbers of colonies of other cell types, and restored in polycythemic animals by exogenous erythropoietin (ESF). This paper seeks to pursue these matters further by more extensive study of the specific erythropoietic stimuli, of the erythroid colony-forming unit (CFU), and of the early influences upon the erythroid CFU.

Materials and Methods

The animals, method of irradiation, technique of marrow collection and injection, and manner of counting and identifying colonies have been described previously (12). Commencing 2 days before irradiation, a daily subcutaneous injection of 0.5 ml isotonic saline containing 1 mg gentamicin sulfate and 4 mg of streptomycin sulfate was given each irradiated animal to

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[1] J. J. Trentin, N. Wolf, V. Cheng, W. Fahlberg, D. Weiss, and R. Bonhag, data in preparation.

prevent early death from *Pseudomonas aeruginosa* (14). Injected-marrow colonies were harvested 8 days after injection in all experiments.

Endogenous colonies were produced by exposing the animals to 580 R whole-body X-irradiation and harvesting the spleens 10 days later. Endogenous colonies were counted grossly after fixation in Bouin's solution and then recounted and typed using 10 longitudinal histological sections at 200 μ intervals through each spleen. In parts 2, 4, and 5 of Experimental Designs the only colonies counted microscopically were those erythroid, granuloid, and mixed colonies judged large enough to be seen grossly at sacrifice (> 500 μ diameter).

Polycythemia was produced by hypertransfusion to suppress endogenous ESF production. Isologous donor mice were heparinized (injected intraperitoneally with 40 units of heparin in 0.2 ml isotonic saline) and exsanguinated by jugular section. The blood was collected aseptically in sterile isotonic saline, and the cells washed twice by centrifugation and decanting the supernatant. The packed cells were finally suspended in two-thirds their volume of isotonic saline to form a 60% suspension. 1 ml of this suspension was given to each recipient animal intraperitoneally on at least two occasions 2 days apart. Controls were injected intraperitoneally with 1.0 ml isotonic saline. In some situations (see Experimental Designs) the hypertransfusions were performed after irradiation, and in these cases irradiated donor mice were used and the 60% cell suspension was given intravenously to avoid possible occult hemorrhage at the site of intraperitoneal injection.

Hematocrits were determined by severing the distal half centimeter of tail, collecting several drops of blood in a microhematocrit capillary tube, sealing one end, and sedimenting the cells in a hematocrit centrifuge.

Erythropoietic stimulation was produced by phlebotomy or by injection of one of three substances: (*a*) purified sheep erythropoietin,[2] 1.0 U/ml in isotonic saline; (*b*) human urinary erythropoietin concentrate,[3] 1.0 U/ml in isotonic saline; or (*c*) cobaltous ion as cobalt chloride, 2.5 μM (0.5 rat erythropoietic units) per ml in isotonic saline. Phlebotomy was performed by removing 0.40 ml of blood from the retro-orbital sinus with a glass capillary pipette. Phlebotomy and cobalt, by stimulating the production of endogenous ESF, utilize the host's own erythropoietic response. The exogenous ESF preparations, on the other hand, require the injection of impure and heterologous protein substances, but the erythropoietic stimulus can be controlled more precisely.

Retransplantation of colonies was performed by dissecting out individual colonies from the spleen with sharp pointed scissors and small curved forceps. The colony was then cut in half; one piece was placed directly into 0.2 ml of minimal Eagle's medium (MEM) without serum, and the other was imprinted lightly, cut surface down, three times on a clean glass slide, and then placed into the MEM. The colony cells were expressed from the colony halves by gentle pressure with a smooth tipped glass rod, and the bits of stroma remaining were removed. The cell suspension was diluted to 0.50 ml with MEM, taken up in a tuberculin syringe, and injected into a single recipient mouse via a lateral tail vein. The imprints were air dried and stained with a conventional blood film technique for identification.

EXPERIMENTAL DESIGNS

1. Duration of Hypertransfusion Polycythemia (Text—figs. 1 a and 1 b).—Three groups of 15 mice were used: (*a*) irradiated (850 R); (*b*) polycythemic (two hypertransfusions); and (*c*) irradiated polycythemic (850 R 2 days following the second of two hypertransfusions). Hematocrits were measured on five animals from each group daily.

[2] Armour Sheep Anemic Plasma Erythropoietin Concentrate, Lot K147- 92A, kindly supplied to us by Dr. L. O. Jacobson and Dr. E. Goldwasser.

[3] Human urinary ESF was extracted from the urine of anemic patients and was kindly supplied to us by Dr. John W. Winkert of New York Medical College.

2. Effects of Erythropoietic Stimulation and Repression of the Donor on the CFU Content of Marrow (Table I).—Four groups of mice were used as bone marrow donors: (*a*) controls, daily subcutaneous injections of saline on the 5 days prior to sacrifice; (*b*) cobalt stimulation, daily injections of 2.5 μM cobaltous ion on the 5 days prior to sacrifice; (*c*) erythropoietin stimulation, daily injections of 1.0 U of ESF on the 5 days prior to sacrifice; and (*d*) polycythemia, injections of isologous erythrocytes 5 and again 3 days prior to sacrifice. On the day of sacrifice, marrow was collected and pooled from three donors in each group and assayed by injecting equal aliquots (4×10^4 or 8×10^4 viable nucleated cells) of each suspension into recipients that had received 850 R whole-body irradiation. 8 days later the spleens were harvested and the number of erythroid, neutrophil, and mixed colonies counted.

3. Effects of Erythropoietic Stimulation and Repression on Endogenous Colonies (Table II).—Four groups of animals were used: (*a*) controls, intraperitoneal injections of saline 4 days and again 2 days before irradiation; (*b*) cobalt stimulation, injections of 2.5 μM cobaltous ion 1, 3, 5, and 7 days after irradiation; (*c*) erythropoietin stimulation, daily injections of 1 U of human ESF from the day of irradiation to the day of sacrifice; and (*d*) polycythemia, injections of isologous erythrocytes 4 and again 2 days before irradiation. All animals were exposed to 580 R whole-body X-irradiation, and spleens were harvested 10 days later. All erythroid, neutrophil, and mixed colonies were counted in sections of each spleen.

4. Effects of Erythropoietic Stimulation and Repression on Exogenous Colonies (Table III).—Four groups of animals were used; they were given the same treatments as the groups in Experiment 3, except that both human and sheep ESF were used, and all animals were exposed to 850 R whole-body X-irradiation and injected with equal aliquots (6×10^4, 8×10^4, or 12×10^4 cells) of the same isologous marrow suspension from a single donor. 8 days later the spleens were harvested and the erythroid, neutrophil, and mixed colonies were counted in sections of each spleen.

5. Reversal of Polycythemic Inhibition of Erythroid Colonies by Erythropoietic Stimulation (Table IV).—Four groups of animals were used: (*a*) controls, no hypertransfusion and no erythropoietic stimulation; (*b*) polycythemia, hypertransfused 4 and again 2 days before irradiation and a third time 4 days after irradiation; (*c*) polycythemia plus cobalt, hypertransfused as in *b* but given injections of 2.5 μM cobaltous ion 1, 3, 5, and 7 days after irradiation; and (*d*) polycythemia plus erythropoietin, hypertransfused as in *b* but given injections of 1 U of sheep ESF daily until sacrifice. The animals were irradiated and injected and the spleens harvested and examined as in Experiment 4.

6. Microscopic Spleen Colonies Appearing in Polycythemic Mice (Table V).—Two groups of 20 animals each were used: (*a*) controls, no hypertransfusion; and (*b*) polycythemia, hypertransfused 4 and again 2 days before irradiation and a third time 4 days after irradiation. The animals were irradiated with 900 R and injected with equal aliquots (12×10^4 cells) of the same marrow suspension. 8 days later the spleens were harvested and 40 subserial longitudinal sections were made through each spleen, at 50 μ intervals. The sections were carefully examined and all foci of hemopoietic regeneration were tabulated.

7. Effects of Short-Term Exposure to ESF on the Development of Erythroid Colonies in the Spleens of Polycythemic Mice (Table VI).—

Endogenous ESF: Four groups of animals were used: (*a*) no exposure to endogenous ESF, hypertransfused 4 and again 2 days before irradiation and given a third injection of isologous erythrocytes 4 days after irradiation; (*b*) early exposure to endogenous ESF, given one intravenous injection of 1.0 ml 80% isologous erythrocytes 4 days after irradiation; (*c*) late exposure to endogenous ESF, hypertransfused 4 and again 2 days before irradiation, and bled 4 days after irradiation; and (*d*) continuous exposure to endogenous ESF, not hypertransfused but given intraperitoneal injections of saline 4 and again 2 days before irradiation and 4 days after irradiation. The animals were irradiated, injected with bone marrow, the spleens sec-

tioned and the colonies examined as in Experiment 6. The various stages of erythroid colony formation were searched for, described, and counted.

Exogenous ESF: Four groups of polycythemic animals were used: (*a*) no exposure to exogenous (or endogenous) ESF, hypertransfused twice before and 4 days after irradiation; (*b*) early exposure to exogenous ESF, hypertransfused twice before and 4 days after irradiation and given injections of 1 U sheep ESF daily for the first 4 days postirradiation only; (*c*) late exposure to exogenous ESF, hypertransfused twice before and 4 days after irradiation and given injections of 1 U sheep ESF daily on days postirradiation 5, 6, 7, and 8; and (*d*) continuous exposure to exogenous ESF, hypertransfused twice before and 4 days after irradiation and given injections of 1 U sheep ESF daily for all 8 days postirradiation. The animals were irradiated and injected and the spleens harvested and examined as in Experiment 7 (endogenous ESF).

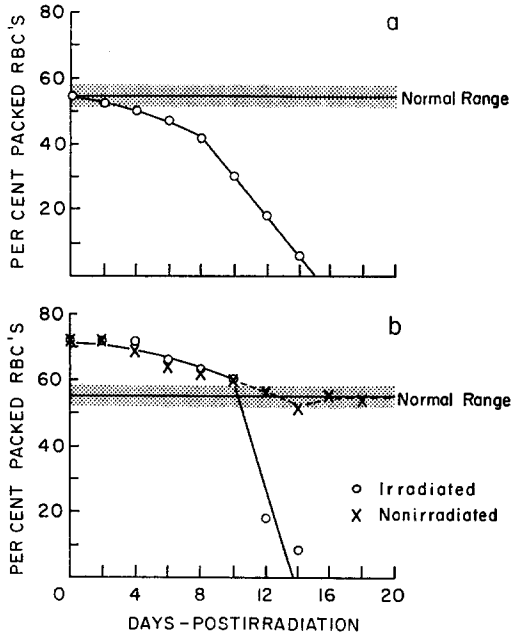
RESULTS

1. Duration of Hypertransfusion Polycythemia.—The hematocrits of nontransfused irradiated mice dropped steadily but slowly until 8 days postirradiation, after which the decline was suddenly much more precipitous (Text-figs. 1 *a* and *b*). Since the doses of irradiation used (850–900 R) have an almost undetectable effect on the life span of the erythrocytes (15), since platelet depletion becomes very severe about 6–7 days postirradiation, and since massive hemorrhages are frequently found in irradiated mice after 9 days postirradiation (16), it seems reasonable to postulate that the early decline was due to the normal cell death rate and that the late drop was due to hemodilution following hemorrhage. In irradiated hypertransfused mice the hematocrits followed the same pattern except that the levels were higher and the sudden drop did not occur until after 10 days postirradiation. Until this time the hematocrits of the irradiated hypertransfused mice remained above the normal range and indistinguishable from those of nonirradiated hypertransfused mice.

On the basis of these results it was felt that the hypertransfusion procedure was adequate for the production of a polycythemia that would suppress the endogenous production of ESF throughout the 8 postirradiation days of the following experiments. Nonetheless, on some occasions a third transfusion was given after irradiation, and in all cases the hematocrits of hypertransfused animals were monitored at sacrifice. Any hypertransfused mouse with a hematocrit less than 53% at sacrifice was discarded.

2. Effects of Erythropoietic Stimulation and Repression on the CFU Content of Marrow.—The purpose of these experiments was to see if alterations in the degree of erythropoietic stimulus would significantly affect the proportional size of the compartment of cells capable of forming erythroid colonies. No significant differences were found between any two groups (Table I). There were changes suggestive of a slight increase in neutrophil colonies as a result of the 5 days of erythropoietic stimuli in both experiments, but this was not statistically significant. It must be concluded that our test system did not demonstrate any effect of the erythropoietic stimulation or repression on the CFU compartment of donor marrow.

3. Effects of Erythropoietic Stimulation and Repression on Endogenous Spleen Colonies.—Cobaltous ion or human ESF failed to make any appreciable difference in either the total colony counts or the distribution of the types of endogenous colonies (Table II). The effect of polycythemia, however, was quite marked. The gross counts showed a reduction to about one-third of the control



TEXT-FIGS. 1 *a* and 1 *b*. Hematocrits in irradiated, polycythemic, and irradiated-polycythemic mice.

TEXT-FIG. 1 *a*. Postirradiation decline in hematocrit of (C57 × Af)F_1 male mice after 850 R whole-body X-irradiation. The increased rate of decline seen after 8 days is probably due to thrombocytopenic hemorrhage.

TEXT-FIG. 1 *b*. Decline in hematocrit of (C57 × Af)F_1 male mice hypertransfused twice with 1 ml of 60% isologous erythrocytes; one group exposed to 850 R whole-body X-irradiation 1 day after the second hypertransfusion. In both groups the hematocrits remain significantly above the normal range for at least 9 days.

counts, whereas the microscopic counts revealed the same number of colonies as the controls. The reason for this was that the erythroid colonies appearing in the polycythemic mice were characteristically much smaller than those appearing in the other groups. Therefore it was necessary to divide erythroid colonies arbitrarily into two groups, large ($> 500\ \mu$ diameter) and small ($<500\ \mu$ diameter), roughly corresponding to the criterion of gross visibility referred to previously. By morphological criteria, the degree of differentiation was approximately the same in both the large and small erythroid colonies. It seems clear,

TABLE I

Mean Counts of 8-Day Spleen Colonies in 850 R-Irradiated Mice Injected With Marrow Of Erythropoietically Stimulated or Repressed Donors

Experiment	Marrow donor pretreatment	No. of cells injected	No. of mice	Mean colonies per spleen*			
				Erythroid	Granuloid	Mixed	Total
1	Controls	8.0×10^4	12	3.2 ± 0.30	1.7 ± 0.31‡	1.3 ± 0.31	6.2 ± 0.51
	Cobalt	"	10	2.7 ± 0.42	3.7 ± 0.33‡	1.5 ± 0.79	7.9 ± 0.43
	ESF (human)	"	11	3.5 ± 0.26	3.5 ± 0.34	2.1 ± 0.34	8.2 ± 0.78‡
	Polycythemia	"	11	2.9 ± 0.77	1.5 ± 0.45	1.7 ± 0.39	6.1 ± 0.81‡
2	Controls	4.0×10^4	11	1.6 ± 0.35‡	0.6 ± 0.24‡	0.6 ± 0.15	2.9 ± 0.31‡
	Cobalt	"	11	2.9 ± 0.29‡	1.1 ± 0.39‡	0.5 ± 0.25	4.5 ± 0.56‡
	ESF (sheep)	"	10	2.9 ± 0.53	1.1 ± 0.33	0.6 ± 0.25	4.6 ± 0.55
	Polycythemia	"	11	3.1 ± 0.29	0.7 ± 0.33	0.7 ± 0.38	4.5 ± 0.41

* ± SE.

‡ $0.20 > P > 0.10$ for difference between groups marked.

TABLE II

Mean Counts of 10-Day Endogenous Colonies in the Spleens of Mice Treated With Erythropoietic Stimulation and Repression After Receiving 580 R Whole-Body Irradiation

Pretreatment	No. of mice	Mean counts*					
		Erythroid		Granuloid	Mixed	Total	
		Large‡	Small			Microscopic	Gross
Controls	9	5.0 ± 2.6	0.2 ± 0.04	0.6 ± 0.27	1.4 ± 0.45	7.0 ± 0.39	6.0
Cobalt	8	5.6 ± 1.8	0.1 ± 0.08	0.9 ± 0.33	1.9 ± 0.76	8.4 ± 0.52	6.0
ESF (human)	8	5.5 ± 0.4	0.2 ± 0.11	1.1 ± 0.52	1.2 ± 0.53	7.8 ± 0.41	7.3
Polycythemia	8	0.0§	5.5 ± 1.7	1.3 ± 0.48	1.9 ± 1.02	8.7 ± 0.79	1.8

* ± SE.

‡ Larger than 500 μ diameter.

§ $P < 0.01$.

therefore, that polycythemia, while not preventing the *formation* of erythroid colonies, markedly inhibited their growth. This experiment with endogenous colonies was not repeated, but the following studies were performed with exogenous (injected marrow) colonies for the following reasons: (*a*) The variation in number of endogenous colonies at a given dose of irradiation is much greater than the variation with transfused marrow colonies at a given cell dose. The use of injected marrow colonies facilitates statistical comparison of groups. (*b*) The exogenous colonies can be read at 7 or 8 days postirradiation, whereas the endogenous colonies require about 2 days longer for growth, under the condi-

tions of this experiment. This 2 day interval occurs at the critical 8–10 day postirradiation period when thrombocytopenic bleeding may cause the production of endogenous ESF even in hypertransfused animals. (*c*) Some endogenous colonies originate from spleen cells and some from migratory marrow CFU; furthermore, there is a continual seeding of the spleen with CFU, giving rise to endogenous colonies of different ages (unpublished observations). With injected marrow colonies, however, one is working with a population of colonies that is uniform at least in age and origin of CFU.

TABLE III

Mean Counts of 8-Day Spleen Colonies in Mice Exposed to 850 R, Injected with Isologous Marrow, and Subjected to Erythropoietic Stimulation or Repression

Experiment	Cell dose	Treatment	No. of mice	Mean colonies per spleen*			
				Erythroid	Granuloid	Mixed	Total
1	6×10^4	Controls	18	3.9 ± 0.44	0.9 ± 0.15	0.5 ± 0.04	5.3 ± 0.09
		Cobalt	5	7.1 ± 1.49‡	0.8 ± 0.32	0.4 ± 0.08	8.3 ± 1.04
		ESF (human)	10	3.0 ± 0.48	1.9 ± 0.25	0.7 ± 0.17	5.6 ± 0.63
		Polycythemia	19	0.6 ± 0.23§	1.2 ± 0.08	0.3 ± 0.13	2.1 ± 0.27
2	8×10^4	Controls	22	5.0 ± 1.08	2.1 ± 0.28	0.5 ± 0.11	7.6 ± 0.47
		Cobalt	23	3.3 ± 0.29	3.1 ± 0.37	0.3 ± 0.11	6.6 ± 0.59
		ESF (sheep)	19	4.8 ± 0.55	3.3 ± 0.28	0.7 ± 0.17	8.8 ± 0.76
3	12×10^4	Controls	12	9.4 ± 0.56	3.4 ± 0.48	1.0 ± 0.38	13.7 ± 0.80
		Polycythemia	12	0.5 ± 0.23§	4.2 ± 0.41	1.1 ± 0.19	5.8 ± 0.57

* ± SE.

‡ $P = 0.05$ when compared to controls.

§ $P = <0.01$ when compared to controls.

4. Effects of Erythropoietic Stimulation and Repression on Exogenous Colonies. —As before, only polycythemia had any appreciable effect, and again this effect was an inhibition of erythroid colonies (Table III). This time, however, instead of having the usual complement of erythroid colonies in a smaller size, there were virtually no erythroid colonies to be found microscopically, and the total number of colonies found in the spleen decreased correspondingly. At first it appeared that the polycythemia had abolished these colonies entirely, but further study showed this impression to be untrue (see part 7 of Results, below). In the first experiment of this series it appeared that the administration of cobalt had caused a significant elevation in the number of erythroid colonies; but this result was not repeatable and was suspect in any case, for 7 of the 12 original mice in that group had died prior to sacrifice and left a presumably nonrepresentative sample.

The conclusion was that polycythemia had a strong and almost absolute inhibitory effect on the development of erythroid colonies. At the same time, there was no significant increase in either granuloid or mixed colonies. The question remained whether this inhibition was mediated through the suppression of endogenous ESF.

5. Reversal of Polycythemic Inhibition of Erythroid Colonies by Erythropoietic Stimulation.—The effect of polycythemia was almost completely reversed by either of the erythropoietogenic agents used, i.e., cobalt or ESF (Table IV). In

TABLE IV

Mean Counts of 8-Day Colonies in Polycythemic Mice Exposed to 850 R, Injected with Isologous Marrow, and Subjected to Erythropoietic Stimulation

Experiment	Cell dose	Treatment	No. of mice	Mean colonies per spleen*			
				Erythroid	Granuloid	Mixed	Total
1	6×10^4	Controls	12	4.8 ± 0.52	2.3 ± 0.33	0.5 ± 0.22	7.7 ± 0.44
		Polycythemia	17	0.5 ± 0.17	3.1 ± 0.42	0.3 ± 0.14	3.8 ± 0.42
		Pc + Cobalt	8	2.6 ± 0.80‡	3.0 ± 0.57	0.3 ± 0.16	5.9 ± 0.83
		Pc + ESF (sheep)	8	3.3 ± 0.21‡	2.6 ± 0.40	0.3 ± 0.16	6.1 ± 0.67
2	12×10^4	Controls	12	9.4 ± 0.56	3.4 ± 0.48	1.0 ± 0.38	13.7 ± 0.80
		Polycythemia	12	0.5 ± 0.23	4.2 ± 0.41	1.1 ± 0.19	5.8 ± 0.57
		Pc + Cobalt	10	7.4 ± 1.64§	2.9 ± 0.73	1.4 ± 0.44	11.7 ± 2.04
		Pc + ESF (sheep)	10	8.8 ± 0.99§	3.5 ± 0.40	1.1 ± 0.34	13.4 ± 1.09

Pc, Polycythemia.

* ± SE.

‡ $P < 0.01$ when compared to polycythemia alone.

§ $P < 0.001$ when compared to polycythemia alone.

polycythemic mice the number of erythroid and total colonies were returned virtually to normal values by these stimuli, while the other colony types were not affected. The size and degree of differentiation of the erythroid colonies found in the erythropoietically stimulated polycythemic animals did not vary appreciably from that of the erythroid colonies in the nonhypertransfused controls.

The conclusion was drawn, therefore, that the action of polycythemia was through the specific repression of endogenous ESF and that the irradiated mouse is secreting amounts of ESF sufficient for maximal erythroid colony differentiation and growth.

6. The Microscopic Appearance of Presumptive Suppressed Erythroid Colonies.—The experiments done with endogenous colonies (Results, part 3 above) sug-

gested that in the presence of polycythemia the erythroid colonies were not totally absent, but present in a severely underdeveloped form. Therefore, a careful search was carried out to find and identify every colony in the spleens of polycythemic mice. Subserial sections (about 40 per spleen) were taken at 50 μ intervals, and every identifiable colony was counted. The data make it apparent that erythropoiesis is almost completely shut off by polycythemia and that the decrease in erythroid colonies is matched by an equally significant increase in small undifferentiated colonies (Table V). The changes in the other types of colonies are either unremarkable or can be explained by the disappearance of erythropoiesis from various types of mixed colonies.

The small undifferentiated type of colony (see Fig. 1) is ostensibly the form in which the "erythroid" colony appears after 8 days' growth in the polycythe-

TABLE V

Survey of All Types of Microscopically Identifiable 8-Day Colonies in the Spleens of Control and Polycythemic Mice Receiving 900 R and 12 × 10^4 Isologous Marrow Cells

Group	No. of spleens	Mean gross counts	Ery.	Neutr.	Eos.	Meg.	Undiff.	Mixed				Total
								Ery. + N	Ery. + M	N + M	Ery. + N + M	
Controls	20	11.5	193	74	1	51	18	23	9	6	3	378
Polycythemic	20	4.2	6	83	2	68	207	2	0	9	0	377

Ery. = erythroid; neutr. = N = neutrophilic; eos. = eosinophilic; meg. = M = megakaryocytic; and undiff. = undifferentiated.

mic spleen. This type of colony is characteristically composed of 100–200 cell[S] (estimated from sections) of the primitive or undifferentiated type and is usually found in the red pulp. The cells are quite distinctive, having abundant cytoplasm, large pale nuclei with peripheral chromatin, and large prominent nucleoli; because of the small colony size and the lack of any eye-catching features these colonies may be overlooked in a cursory examination of a slide or may be missed entirely if too few sections are taken from each spleen.

7. *Stages of Erythroid Colony Development and the Effects of ESF.*—The following morphological types of erythroid and presumptive erythroid colonies were found: (*a*) *Very small undifferentiated* colonies, containing 100–200 cells as described above; (*b*) *Intermediate undifferentiated* colonies, containing 5,000–10,000 cells, although still not grossly visible (Fig. 2); (*c*) *Small diffuse mature erythroid* colonies, containing diffusely scattered mature erythroid elements only (Fig. 3). Because of the small colony size and diffuse distribution these could not be seen grossly; (*d*) *Intermediate panerythroid* colonies, containing up to 100,000 cells in all stages of erythropoiesis (Fig. 4). Grossly these colonies

could usually be seen as tiny spots (about 0.5 mm diameter) after fixation in Bouin's fluid. (*e*) *Large panerythroid colonies* were the normal, easily visible erythroid colonies (Fig. 5) containing up to a million cells in all stages of development, but with polychromatic erythroblasts somewhat more numerous than in the intermediate panerythroid colonies.

Effects of early or late exposure to "endogenous" ESF on erythroid colony development: The four experimental groups had virtually the same number of total and "erythroid" colonies, if one includes the undifferentiated colonies as presumptive erythroid colonies (Table VI), but the distribution of the stages of

TABLE VI

Mean Microscopic Colony Counts in Polycythemic Mice Exposed to Endogenous or Exogenous Erythropoietin for Various Periods After Receiving 900 R and 12 × 10^4 Isologous Marrow Cells

Source of erythropoietin	Period of exposure	No. of mice	Erythroid and Presumptive Erythroid						Granuloid	Megakaryocytic	Mixed	Total
			Small undiff.	Intermediate undiff.	Small diffuse mature erythroid	Intermediate pan-erythroid	Large pan-erythroid	Erythroid subtotal				
Endogenous (mouse)	None	9	5.7	0.9	0.0	0.0	0.0	6.6	7.2	4.2	1.5	19.5
	Early	10	2.2	0.3	4.6	0.0	0.3	7.4	7.6	4.4	1.3	20.6
	Late	10	1.8	0.6	3.3	1.4	1.1	8.2	5.9	3.7	1.7	19.5
	Continuous	12	0.6	0.5	0.3	2.3	5.5	9.2	5.8	3.3	1.1	19.4
Exogenous (sheep)	None	10	7.2	1.7	0.0	0.3	0.0	9.2	4.3	2.7	0.9	17.1
	Early	16	2.9	5.1	0.0	0.8	0.2	9.0	3.4	2.5	0.7	15.6
	Late	12	0.5	2.8	0.0	5.5	0.6	9.4	4.3	2.9	1.1	17.7
	Continuous	17	0.3	1.8	0.0	4.6	4.1	10.8	3.1	3.3	0.9	18.1

colony development was different in each group. The predominant colony type in the group which received no exposure to endogenous ESF was the very small undifferentiated colony. Early exposure (first 4 days) brought out the small diffuse mature erythroid colony; late exposure (last 4 days) produced some of each colony type with a predominance of small diffuse mature erythroid colonies; continuous exposure resulted in a predominance of the larger differentiated colonies. Each period of exposure to erythropoietic stimulation, therefore, produced characteristic stages of erythroid colony development.

Effects of early or late exposure to "exogenous" ESF on erythroid colony development: The results are quite similar to those of the preceding experiment, with one notable exception: the diffuse mature erythroid colonies were not found at all, and the characteristic colony type in the group receiving early exposure was the intermediate undifferentiated colony (Table VI).

Other than size, there is no apparent difference between the very small and the intermediate undifferentiated colonies, suggesting that mitosis alone is the key factor. The diffuse mature erythroid colony, on the other hand, is well differentiated and gives the impression of being "burned out"; i.e., of being a remnant left after the majority of the cells have differentiated and departed. Very few undifferentiated cells may be found.

TABLE VII

Number and Type of Progeny (Second Generation Colonies) Formed by the Transplantation of Individual Colonies of a Single Differentiated Cell Line into Single 850 R-Irradiated Recipients

Age of colony	Colony type	No. of colonies	No. with no progeny	Per cent with progeny	No. of progeny		Progeny predominantly erythroid	Overall mean progeny per colony‡
					E*	N*		
days							%	
7	Erythroid§	12	12	0	—	—	—	—
9	Erythroid§	47	21	55	74	21	79	2.0 ± 0.37
10	Erythroid§	31	8	74	108	32	76	>4.8‖
9	Neutrophilic§	13	4	69	36	24	60	4.7 ± 1.01¶
10	Neutrophilic§	19	0	100	89	50	63**	>17.4‡‡

* E, colonies that are predominantly (>75%) composed of erythroid cells. N, colonies that are predominantly (>75%) composed of neutrophilic cells. Megakaryocytic and undifferentiated foci were not counted.

‡ ± SE.

§ "Erythroid" implies less than 10% of the identifiable cells were of a nonerythroid differentiated cell line. "Neutrophilic" implies less than 10% of the identifiable cells were of a nonneutrophilic differentiated cell line.

‖ 2 of the 31 colonies in this group had too many progeny to count accurately (>20).

¶ $0.20 > P > 0.10$ for difference between mean progeny per colony of 9-day erythroid and 9-day neutrophilic colonies.

** $P < 0.01$ for significance of proportion of predominantly erythroid and predominantly neutrophilic progeny of erythroid and neutrophilic colonies. X^2 test used.

‡‡ 11 of the 19 colonies in this group had too many progeny to count accurately.

8. Colony Retransplantation Studies.—The CFU content of transplanted spleen colonies increased from undetectable levels in 7-day erythroid colonies, to a maximum in 10-day neutrophilic colonies. Neutrophilic colonies had a higher CFU content than erythroid colonies of the same age (Table VII). Interestingly, both erythroid and neutrophilic primary spleen colonies gave rise to both erythroid and neutrophilic secondary colonies. The percentage of erythroid secondary colonies was slightly but significantly higher among the progeny of erythroid primary colonies than among the progeny of neutrophilic primary colonies.

DISCUSSION

In an earlier paper (12) it was shown that: (*a*) each of the types of hemopoiesis normally occurring in the bone marrow (erythroid, neutrophilic granuloid,

megakaryocytic, eosinophilic granuloid) may appear in the form of gross or microscopic discrete spleen colonies; (*b*) each of the colonies initially undergoes a single line of hemopoietic differentiation, but retains some undifferentiated cells, with the less differentiated cells characteristically on the periphery; (*c*) in the spleen the erythroid colonies are by far the most numerous, followed by neutrophilic, then megakaryocytic, with undifferentiated colonies being uncommon, and eosinophilic colonies rare; (*d*) as each colony enlarges with time, it inevitably undergoes differentiation into one or more additional cell lines to become a "mixed" colony, such that early colonies are typically of a single differentiated cell type and late colonies are typically of mixed cell types; and (*e*) at any one time the size of the colonies in decreasing order is mixed, erythroid, granuloid (all grossly visible), megakaryocytic, eosinophilic, and undifferentiated (not grossly visible).

The data of the present paper indicate that the erythrogenic CFU requires ESF for erythroid morphological differentiation and rapid proliferation, but not for maintenance of a slow replication rate and undifferentiated state. In the face of polycythemia-induced ESF repression the erythrogenic CFU gives rise to small colonies of undifferentiated cells. Such colonies do not shift to granuloid colonies and other types, but only the normal number of granuloid and other types of colonies develop. In polycythemic mice there often appears an apparent slight increase in the relative number of neutrophilic granuloid colonies. However, since polycythemia suppresses erythropoiesis in mixed colonies as well as in erythroid colonies, the apparent increase in neutrophilic colonies is usually matched by an equivalent decrease in mixed colonies, and the total number of colonies exhibiting granulopoiesis (neutrophilic plus mixed) is not significantly altered by polycythemia. Therefore, no detectable fraction of the presumptive erythroid colonies, freed of erythropoietic stimulation, divert to granulopoietic differentiation. This suggests that the erythroid colony precursor cells are not pluripotent but rather committed to the erythroid line. Contrarily, the following facts indicate that neither the erythroid nor the granuloid colony precursor cell is monocommitted, but is rather pluripotent: (*a*) as either erythroid or granuloid colonies enlarge they inevitably develop also differentiated cells of the other cell line, as well as megakaryocytes, to become mixed colonies (12); (*b*) if erythroid colonies are retransplanted to new irradiated hosts, they give rise to the usual spectrum of early spleen colony types produced by marrow cells, with approximately the same percentage of erythroid, of granuloid, of megakaryocytic, and of mixed colonies (Table VII and reference 17); (*c*) if granuloid colonies are retransplanted, they give rise to approximately the same proportion of erythroid and of granulocytic spleen colonies as obtained with normal marrow (Table VII); (*d*) if granuloid colonies are removed from the spleens of hypertransfused mice and retransplanted they give rise to erythroid, granuloid, megakaryocytic, and mixed spleen colonies in approximately the same per-

centages as given by transplanted marrow cells (V. Cheng and J. J. Trentin, data in preparation); (*e*) if the remainder of such spleens (containing no gross colonies but containing microscopic hypertransfusion-repressed, undifferentiated-presumptive-erythroid colonies, and megakaryocytic colonies) is retransplanted, it too gives the same types and percentages of spleen colonies as in (*d*) above (V. Cheng and J. J. Trentin, data in preparation); and (*f*) regeneration of the various types of hemopoietic and lymphoid cells can occur from a common stem cell as indicated by the presence of the same distinctive irradiation-induced chromosome aberration(s) in almost all such cell types of some endogenously repopulating irradiated animals (18), and other evidence.

Actually the data of Table VII indicate that transplanted erythroid colonies give rise to a slightly higher percentage of erythroid secondary colonies than do transplanted granuloid colonies, which difference is statistically significant. This suggests that perhaps some colonies may be formed by cells already monocommitted to either the erythroid or the granuloid cell line. If such colonies do indeed exist, the magnitude of the above mentioned difference is sufficiently small as to indicate that such colonies are in the minority as compared to colonies formed by apparently pluripotent CFU's.

These seemingly contradictory facts can be rationalized by a working hypothesis proposed earlier (4, 12) and reaffirmed by the present data, i.e., that pluripotent bone marrow stem cells (CFU) nevertheless require both systemic ESF *and* the proper hemopoietic-inductive microenvironment (HIM) in order to undergo erythroid differentiation. Such a theory explains why CFU do not normally form erythroid colonies in sites other than spleen and bone marrow, since presumably these latter sites exclusively are rich in erythroid HIM. The fact that some stem cells that lodge elsewhere in the spleen differentiate first into granuloid or megakaryocyte colonies suggests that there are separate and discrete HIM for each of these lines of differentiation. A different distribution of erythroid versus granuloid HIM within the spleen is indicated by the fact that erythroid spleen colonies are found throughout the red pulp, whereas granuloid colonies are found primarily adjacent to the capsule and trabeculae and, less frequently, in the center of atrophic lymphoid follicles (12). A richer supply of erythroid HIM than of granuloid HIM in spleen and the reverse in bone marrow is indicated by the fact that erythroid colonies predominate in spleen whereas granuloid colonies predominate in the marrow (N. Wolf and J. J. Trentin, to be published). The theory also explains why early colonies are of single differentiated cell types, but as they enlarge and encroach on other areas of the spleen (containing other types of HIM), the undifferentiated cells, which are preferentially on the periphery, undergo other lines of differentiation (12). This furthermore explains why presumptive erythroid colonies, in the absence of ESF, do not differentiate into nonerythroid cell lines, yet erythroid colonies on retransplantation give rise to all of the nonerythroid spleen colony

types. If one accepts the pluripotential nature of most if not all CFU's, then the fact that undifferentiated colonies, freed from erythropoietic differentiation by polycythemia, do not develop into other cell lines is one of the strongest pieces of evidence supporting the concept of microenvironmental influence.

Possible Nature of the Hemopoietic-Inductive Microenvironment (HIM).—One can only speculate as to the exact nature of the erythroid HIM and wait for future experimentation to define it. Since it survives supralethal irradiation, it must be relatively radioresistant compared to the CFU, and may consist of reticular cells or other radioresistant stromal cells of the spleen or bone marrow. These sites probably act in an inductive fashion, similar to the reported mesenchymal induction of thymic lymphocyte differentiation from endoderm (19), and may relate to the finding that bone marrow fails to develop in the cartilaginous femoral anlage unless mesenchyme is present (20). There is some indication that the CFU may not itself be the erythropoietin-responsive cell (ERC) (5, 21–23). While the data in this regard are open to alternate interpretations, it seems logical that a pluripotent stem cell capable of differentiation into each of the hemopoietic cell lines (the CFU) should not be ESF sensitive, or else all such stem cells might be triggered irrevocably into the erythroid line by a strong ESF stimulus, to the detriment of the nonerythroid lines of differentiation. The fact that strong ESF stimulation (exogenous ESF, cobalt, or endogenous ESF) of the recipient did not reduce the number of granuloid colonies produced by either endogenous or transfused CFU (Tables II, III, IV, and VI) may, indeed, be taken as direct evidence that the pluripotent CFU is not erythropoietin sensitive, in the sense of being irrevocably shunted by ESF into the erythroid line. The function of the erythroid HIM could possibly be to render the pluripotent stem cell sensitive to ESF, in which case the CFU would be ESF responsive only by virtue of being associated with an erythroid HIM. If so, not all stem cells of a given erythroid colony are shunted into the erythroid line, for erythroid colonies can later develop other cell lines either *in situ* or on transplantation. Hormones in general stimulate mitosis and/or secretion by target cells that have been "induced" into a state of hormone responsiveness by a prior local induction phenomenon classically involving specific mesenchyme as the inducer.

The undifferentiated cells of the intermediate undifferentiated colony (Fig. 2) probably are largely ESF sensitive, for these colonies were at one time (4 days) morphologically erythroid, but under polycythemic repression became undifferentiated, while continuing to proliferate. The pluripotent CFU must have its own feedback control mechanism for homeostasis in the presence of drain-off by HIM and ESF, as indicated by the fact that an erythropoietic stimulus of the donor produced no depletion of marrow CFU (Table I and references 5 and 23). Bessis has reported that within the bone marrow, erythroblastic islands exist around centrally placed reticular cells packed with ferritin, which is trans-

ferred to the erythroblasts (24). It is possible that these reticular cells represent the erythroid HIM.

Postirradiation Anemia.—The observed hematocrit decline in irradiated animals (Text-figs. 1 *a* and 1 *b*) corresponds well to the observations of O'Grady et al. (13), who maintain, however, that in nonpolycythemic mice the circulating levels of endogenous ESF are not significant until 9 days postirradiation, and that erythropoiesis in spleen colonies before that time is not dependent on elevated levels of endogenous ESF. Schooley, however, has found significantly elevated plasma levels of ESF as early as 3 days postirradiation (6). The fact that polycythemia blocks erythroid differentiation of spleen colonies (Tables II, III, IV, V, VI, and references 4, 6, 7, 13), which effect is reversed by ESF (Table IV and references 4, 8, 13), indicates that erythroid differentiation of spleen colonies is dependent on endogenous ESF. This view is further verified by the fact that injections of antierythropoietin antisera (in nonpolycythemic mice) similarly suppresses the appearance of erythroid colonies (6). Since exogenous ESF given to nonpolycythemic mice does not increase the proportion of erythroid colonies (Tables II, III), one must assume that the level of endogenous ESF in irradiated mice is sufficient to induce maximal erythroid differentiation of transfused CFU in the spleen.

SUMMARY

The polycythemic repression of erythropoiesis and the restoration of erythropoiesis by specific stimulation were studied in the spleen colony system in irradiated mice.

1. A 5 day period of erythropoietin stimulation (exogenous erythropoietin) or repression (polycythemia) of the bone marrow donor only, does not significantly alter the number or type of colonies formed by the transplanted marrow cells.

2. Erythropoietin stimulation did not alter the number or type of endogenous colonies formed in mice receiving 580 R. Erythropoietin repression (polycythemia) markedly reduced the growth but not the number of erythroid colonies, while not affecting the other types of colonies formed endogenously.

3. Erythropoietin stimulation of the irradiated recipient during colony growth did not alter the number or type of spleen colonies formed by transplanted marrow. Erythropoietin repression by polycythemia during colony growth completely suppressed the appearance of morphologically erythroid colonies without significantly altering the incidence of the other colony types. This effect of polycythemia was completely prevented by exogenous erythropoietin. Irradiated mice are therefore presumed to be secreting sufficient erythropoietin for maximal erythroid colony development.

4. The erythroid colonies suppressed by polycythemia were recognizable as microscopic foci of undifferentiated cells. Exposure of these foci to erythro-

poietin stimulation at different periods in their development was manifested by different degrees of growth and differentiation, from which it is apparent that erythropoietin stimulates not only morphological differentiation but also rapid mitosis.

Retransplantation of either erythroid or of neutrophilic primary spleen colonies gave rise to both erythroid and neutrophilic secondary spleen colonies. The percentage of erythroid secondary colonies was slightly but significantly higher among the progeny of transplanted erythroid primary colonies than among the progeny of transplanted neutrophilic primary colonies.

On the basis of these and other results, a working hypothesis is proposed for factors controlling the growth and differentiation of spleen colonies from transplanted bone marrow. It is postulated that most but perhaps not all spleen colony-forming units are pluripotent hemopoietic stem cells. It is further postulated that hemopoietic-inductive microenvironments (HIM) of different kinds exist in both the spleen and the bone marrow, and that these determine the differentiation of pluripotent stem cells into each of the lines of hemopoietic differentiation. Erythropoietin therefore may "induce" erythroid differentiation of only those stem cells under the influence of an erythroid HIM. Alternatively erythropoietin may act only as a growth and function stimulant of those stem cells that have been "induced" by an erythroid HIM into a state of erythropoietin responsiveness. In the latter case morphological differentiation presumably results from the functional activity stimulated by ESF.

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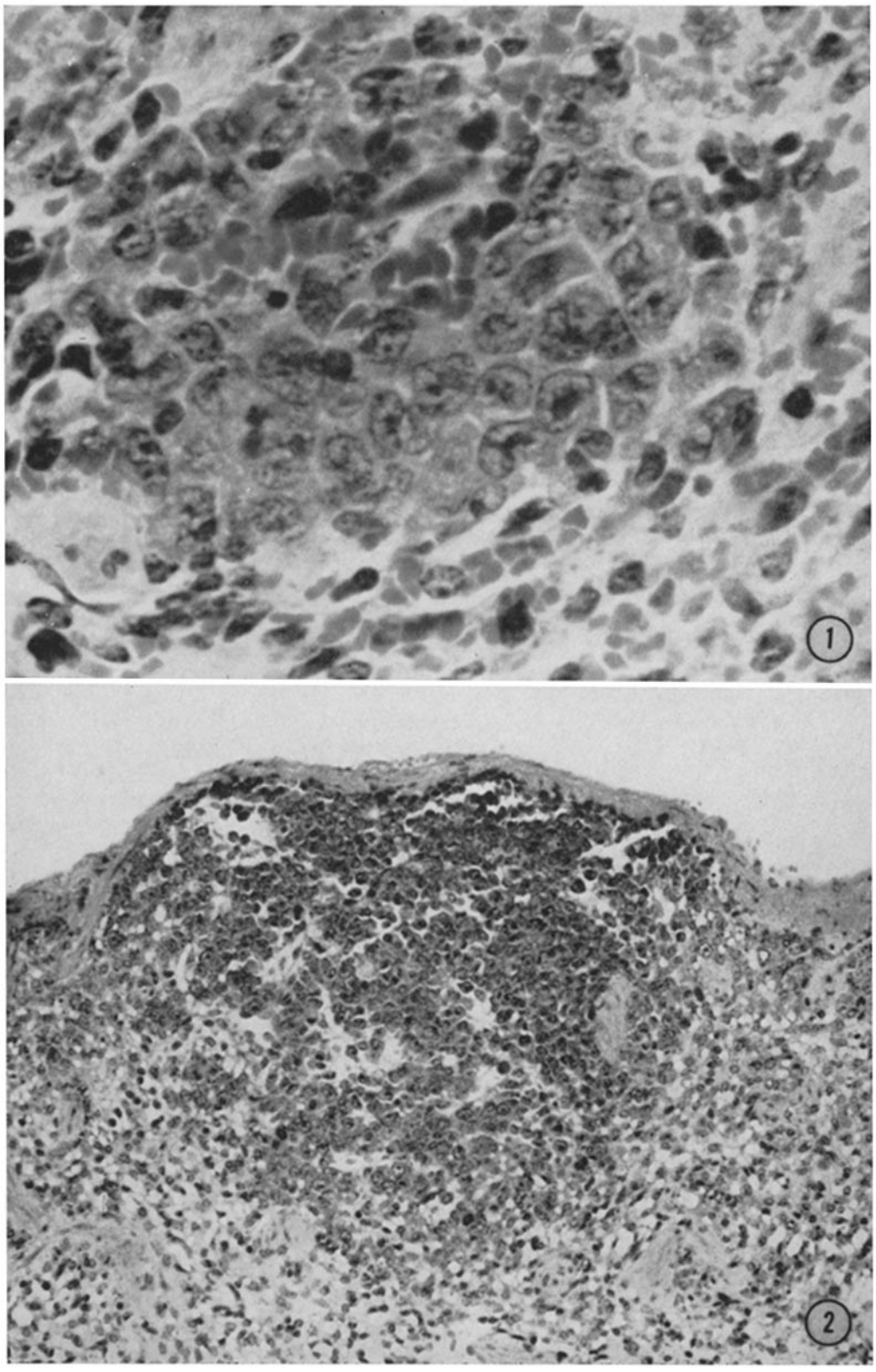
EXPLANATION OF PLATES

PLATE 87

FIG. 1. Small, undifferentiated colony, appearing as the predominant colony type in the spleens of polycythemic mice, 8 days postirradiation (900 R whole-body) and isologous marrow injection (6×10^4 viable nucleated cells). Because of the small size and fairly nondescript characteristics, these colonies are difficult to find at low magnification. The cells are characterized by abundant, pale, reticulated cytoplasm, large nuclei with a peripheral chromatin distribution, and one or several very prominent nucleoli. Generally they fit the description of the hemohistioblast traditionally believed to be the marrow "stem cell." Hematoxylin and eosin, × 1975.

FIG. 2. Intermediate size undifferentiated colony; microphotograph of the spleen of a polycythemic mouse receiving 900 R whole-body X-irradiation, daily injections of sheep ESF for the first 4 days postirradiation, and a third hypertransfusion 4 days postirradiation; sacrificed after 8 days. This type of colony is characteristic of such a treatment schedule, is significantly larger than the very small undifferentiated colony, yet shows no differentiated elements. It is postulated that the four doses of ESF caused both an increased rate of proliferation and waves of differentiation, but during the 4 days of polycythemia without ESF stimulation the differentiating elements completed the maturation process and entered the circulation, leaving only the proliferating, undifferentiated, but ESF-sensitive cells in the colony. Hematoxylin and eosin, × 380.

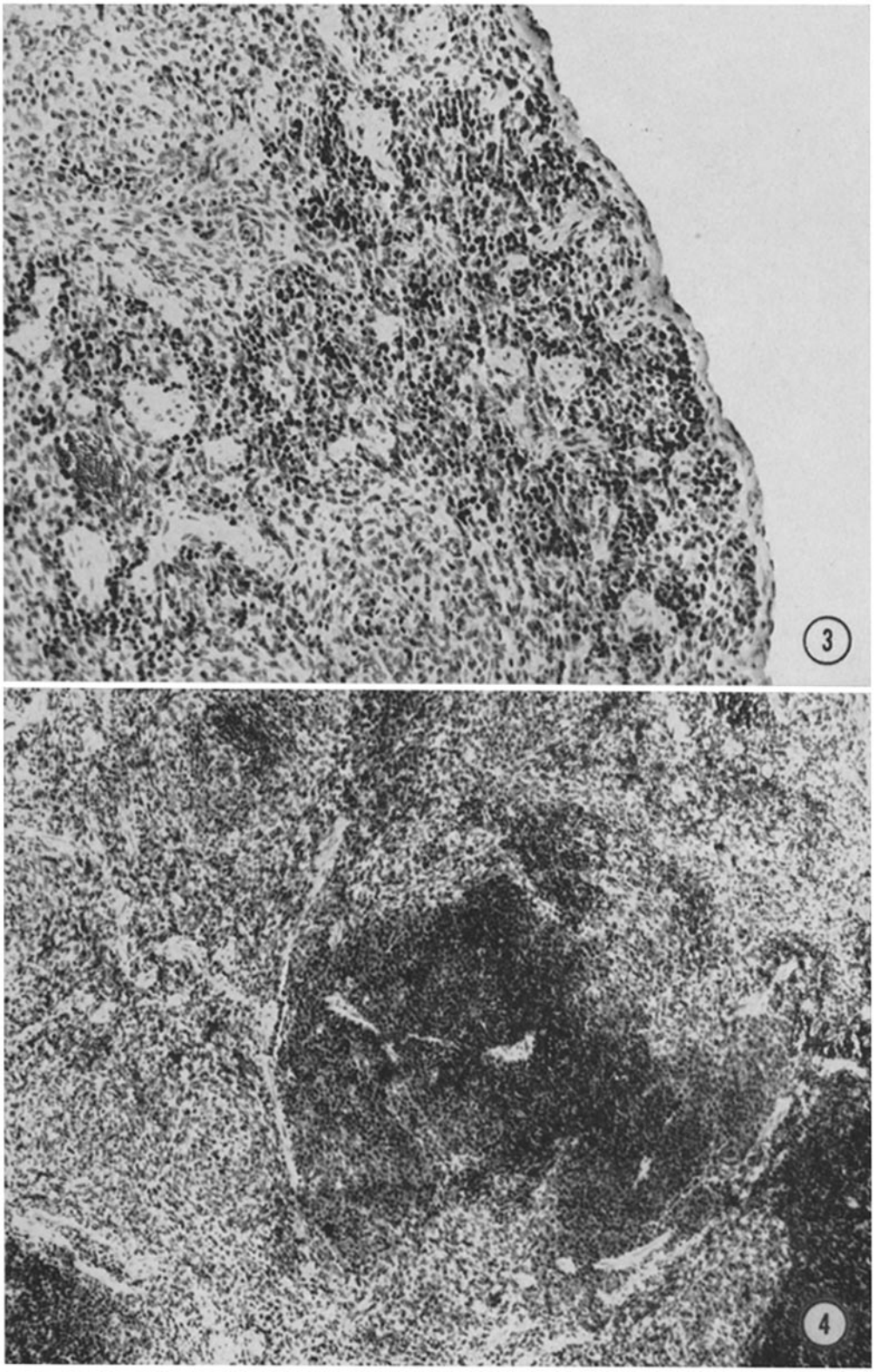


(Curry et al.: Hemopoietic spleen colony studies. II)

PLATE 88

FIG. 3. Diffuse mature erythroid colony. A sparsely populated focus of differentiated erythroid elements appearing in the spleen of a mouse hypertransfused 4 days after 900 R whole-body X-irradiation. The colony cells were exposed to 4 days of endogenous erythropoietic stimulation followed by 4 days of polycythemia. It is postulated that the colony was more densely populated earlier, but, at the cessation of the erythropoietic stimulation, depletion of the more mature elements was no longer matched by the differentiation and proliferation of less mature cells, and the colony cell population diminished. Hematoxylin and eosin, × 255.

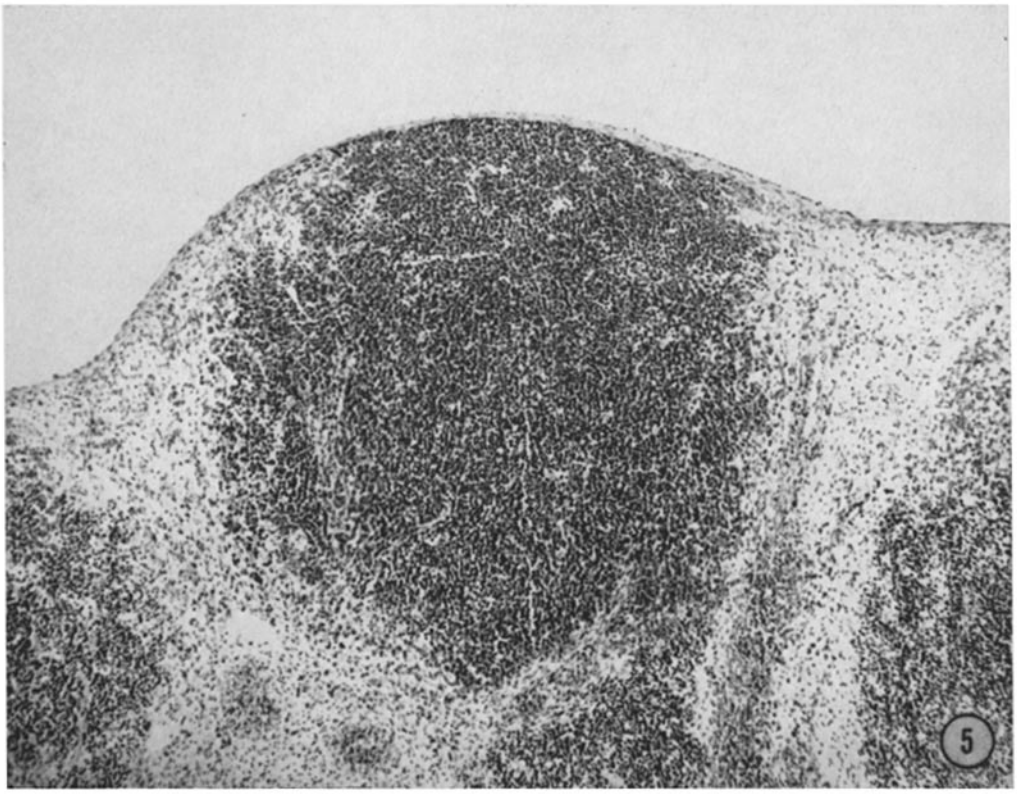
FIG. 4. A small, well differentiated erythroid colony, appearing in the spleen of a polycythemic mouse irradiated with 900 R whole-body X-irradiation and given daily injections of ESF for the last 4 days of 8 days' colony growth. A spectrum of erythroblastic forms is seen, but the colony is only on the borderline of visibility grossly. In lower left and lower right hand corners are seen two additional erythroid colonies. Hematoxylin and eosin, × 128.



(Curry et al.: Hemopoietic spleen colony studies. II)

PLATE 89

FIG. 5. Large mature panerythroid colonies appearing in the spleen of a polycythemic mouse irradiated with 900 R whole-body X-irradiation and given daily injections of ESF throughout the entire 8 days of colony growth. These colonies are grossly visible as nodules on the spleen and are in all ways similar to erythroid colonies appearing in nonpolycythemic-irradiated mice 8 days after the injection of marrow. Hematoxylin and eosin, × 94.



(Curry et al.: Hemopoietic spleen colony studies. II)