

BRIEF NOTE:

**Effect of Erythropoietin on Polycythemic
Mouse Spleen in Vitro
IV. Response of the Colony-forming
Cells to Erythropoietin**

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RELATIONSHIP BETWEEN the colony-forming cells first described by Till and McCulloch^{1,2} and the erythropoietin-responsive cells³⁻⁷ has been the subject of controversy among several investigators.⁸⁻¹⁴

In the previous report,¹⁵ we demonstrated the existence of erythropoietin-responsive cells in the spleen of polycythemic mice by incubating the spleen in vitro and measuring heme synthetic activity.

The present work has been performed for the purpose of developing an in vitro method to study the relationship of erythropoietin-responsive cells to colony-forming cells. With this in view, the spleens of polycythemic mice, which were subjected to lethal irradiation and bone marrow transplantation, were removed and cultured in the presence of erythropoietin. The number of erythropoietin-responsive cells in the cultured spleens was determined by measuring the heme synthetic activity as described in our previous reports.¹⁵

MATERIALS AND METHODS

Female ddO strain mice, weighing 18 Gm., bred in the institute for Infectious Diseases, University of Tokyo, were used throughout the experiments.

Transfusion-induced polycythemic mice were exposed to lethal doses (900 R) of ⁶⁰Co irradiation and injected intravenously with 10⁷ homologous bone marrow cells which were suspended in NCTC 199 solution. To render the mice polycythemic, they were injected intravenously with 1.5 ml. of 50 per cent homologous red blood cell suspension from 4 to 6 days prior to sacrifice. Only mice with hematocrits over 65 per cent were used. Immediately after, and on the first, second, third, fourth, and fifth day after irradiation and bone marrow transplantation, the animals were sacrificed and their spleens removed.

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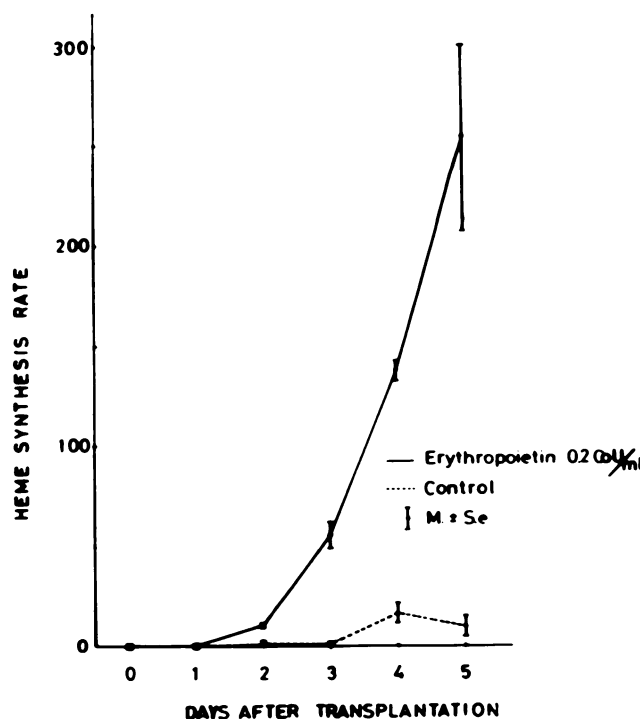


Fig. 1.—In vitro response of transplanted bone marrow cells to erythropoietin in the spleen of irradiated mice.

Tissue cultures of the spleens were performed by the method described in our previous reports.¹⁵ In brief, the extirpated spleen was cut into small cubes of approximately 1 cu.mm. Seven cubes were attached directly on the wall of the sterile culture tube with plasma coagulum. Two ml. of incubation medium consisting of 50 per cent NCTC 109 and 50 per cent inactivated calf serum plus 50 U/ml. penicillin added to each tube. Roller tube culture was performed for 48 hours at 37 C. in an atmosphere of 5 per cent CO₂ and 95 per cent air.

Erythropoietin was obtained from the urine of an anemic patient with 80 per cent acetate precipitation and ammonium sulfate fractionation. Urinary erythropoietin with a specific activity of 14 CoU/mg. was added to the incubation medium in a concentration of 0.2 CoU/ml. Fragments of the same spleen, incubated without erythropoietin, served as the control culture.

Three μ c. of ⁵⁹FeCl₃ (specific activity 7 mCi/mg. in HCl diluted solution) in 0.1 ml. NCTC 109 solution were added to each tube 6 hours prior to the termination of the incubation. Heme was extracted at the 48th hour of incubation from spleen explants by the method already described.¹⁵ In brief, the spleen cells were lysed by the addition of 1.0 ml. of ice-cold diluted hemolysate and 1.0 ml. of Drabkin's solution. The solution was allowed to stand in the cold for 24 hours. The heme was then extracted from the supernatant fluid with methylethylketone by the method of Teale,¹⁶ and the heme solution was dried on steel planchets and its radioactivity was counted with a gas-flow Geiger counter. Preliminary experiments showed that the extracted heme in this series did not cause any quenching effect of scanning β ray from ⁵⁹Fe in the gas-flow Geiger counter.

Morphologic observations were made from the imprint specimen of the spleen fragments at the twenty-fourth and forty-eighth hour of incubation.

Table 1.—*Growth Curve for Erythropoietin-responsive Cells as Detected by Heme Synthesis*

Days After Transplantation	Number of Cultures	Erythropoietin 0.2 U/ml. in the Medium	Heme Synthesis* Rate (M ± Se)		Appearance of† Erythroblasts in Cultures		
					++	+	-
0	4	+	0.9 ± 0.7		0	0	4
		-	0.4 ± 0.1		0	0	4
1	5	+	0.5 ± 0.5		0	0	5
		-	0.0 ± 0.0		0	0	5
2	5	+	10.6 ± 1.0	P < 0.01	0	0	5
		-	3.1 ± 1.3		0	0	5
3	5	+	55.6 ± 6.9	P < 0.001	3	2	0
		-	1.3 ± 0.3		0	0	5
4	4	+	137.0 ± 6.3	P < 0.001	4	0	0
		-	16.0 ± 6.3		0	0	4
5	3	+	254.0 ± 38.0	P < 0.001	3	0	0
		-	10.4 ± 5.0		0	1	2

One specimen consists of spleen explants collected from two different tubes.

*Heme synthesis rate is calculated as follows: Per cent ^{59}Fe uptake into heme $\times 10^{-5}$ /mg. wet weight of spleen explants.

†Erythroblasts were observed in explants after 24 hours of incubation.

- No erythroblasts observed.

+ Few scattered erythroblasts observed.

++ Many clumped erythroblasts observed.

RESULTS

Heme Synthetic Activity in the Cultured Spleen from Transplanted Polycythemic Mice

No heme synthesis was observed in the cultured spleens which had been removed from polycythemic mice immediately or on the first day after transplantation. The presence of erythropoietin in the incubation medium did not induce heme synthesis in the polycythemic mice spleens during this period. (Fig. 1 and Table 1) Spleens removed on the second day after transplantation, however, showed significant heme synthesis in the presence of erythropoietin. As shown in Figure 1 and Table 1, cultures of spleens removed on the third, fourth, and fifth day after transplantation resulted in gradual increase in heme synthetic activity. On the contrary, these polycythemic-mice spleens showed no heme synthesis after 48 hours of incubation in the culture medium containing no erythropoietin.

Morphologic Observations

To confirm that heme synthesis observed in these cultures was due to an appearance of erythroblasts, morphologic observations on each spleen fragment were carried out. As shown in Figures 2, 3, and 4 and Table 1, erythroblasts were first detected in the polycythemic mice spleens which had been removed and cultured with erythropoietin on the third day after transplantation.

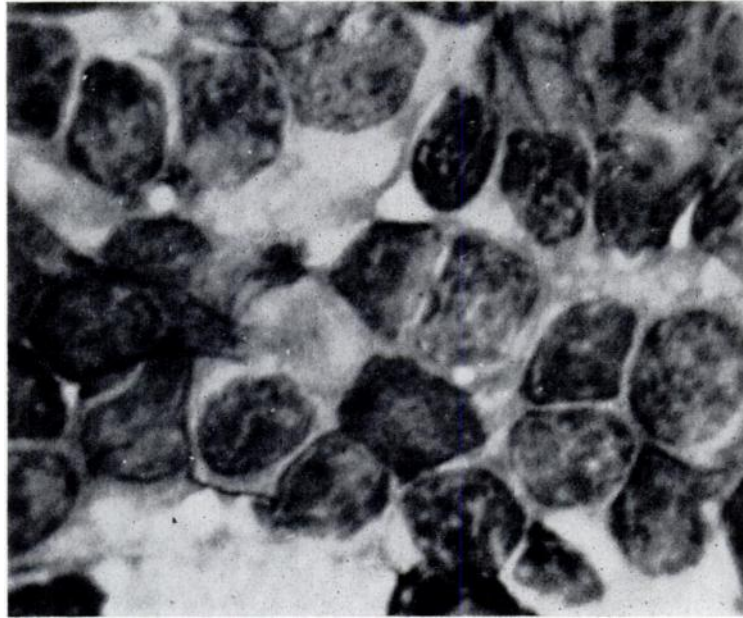


Fig. 2.—Spleen of transfusion-induced polycythemic mouse three days after irradiation and bone marrow transplantation. No erythroblasts are observed.

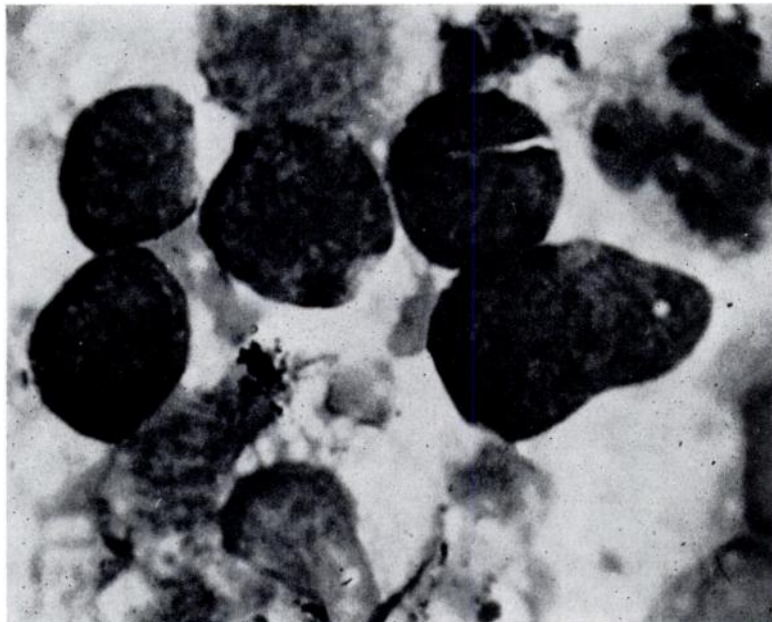


Fig. 3.—Spleen three days after irradiation and transplantation, and 24 hours incubation with erythropoietin. Large immature basophilic erythroblasts are seen.

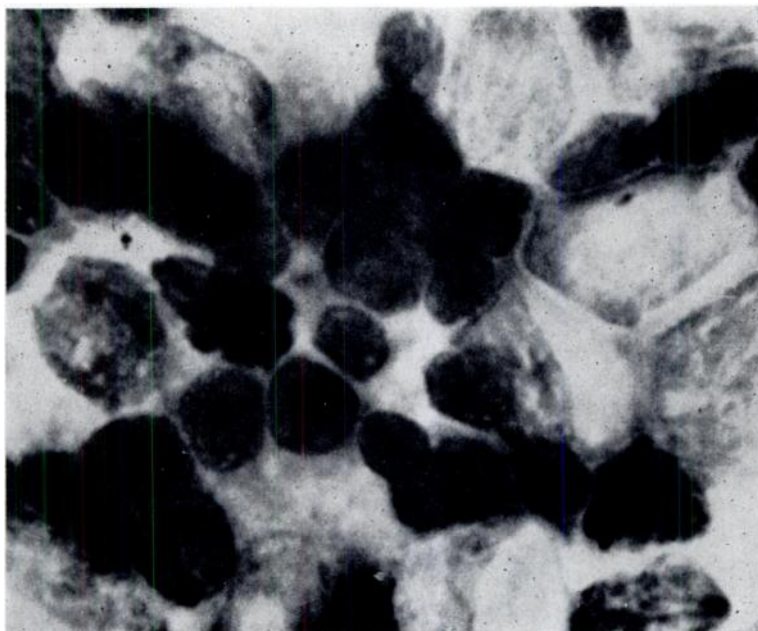


Fig. 4.— Spleen three days after irradiation and transplantation, and after 48 hours incubation with erythropoietin. Small basophilic erythroblasts are seen.

DISCUSSION

An *in vitro* system to assay the size of erythropoietin-responsive stem cell pool has been described in our previous report,¹⁵ in which spleen fragments from the transfusion-induced polycythemic mice had been cultured in the presence of erythropoietin.

In the present study, this system has been applied to assay the size of erythropoietin-responsive cell pool in the spleens of polycythemic mice which received lethal irradiation and bone marrow transplantation. Numbers of transplanted cells in our experiments are far exceeding the usual values reported by Smith¹⁷ or Hodgson.¹⁸ However, considering the short period of time between the bone marrow transplantation and sacrifice, we had chosen these large numbers of bone marrow cells. Spleens of irradiated and transplanted Swiss or C₃H mice are reported to contain colony-forming cells within several hours after transplantation.^{10,19} With our present *in vitro* tissue culture system, the presence of erythropoietin-responsive cells in the spleen was first detected on the second day after transplantation. Therefore, these irradiated and transplanted mice spleens may be considered to contain only colony-forming cells immediately after and on the first day after transplantation. Our results support the ideas that the colony-forming cells and the erythropoietin-responsive cells are at different stages of maturation⁸⁻¹² or cell cycle^{13,14} as suggested by many investigators.

It is possible that the present difference between the colony-forming cells

and the erythropoietin-responsive cells could be the result of different methods of detection. Tissue deterioration occurring in the irradiated mice spleen could have prevented stem cell response to erythropoietin; or, erythropoietin-responsive cells could be too few to be detected by our present in vitro system on the first day of transplantation.

Furthermore, necessity of erythropoietin for further differentiation of transplanted stem cells into erythroblasts is also suggested.

SUMMARY

It is shown that an in vitro system of assaying the size of an erythropoietin-responsive stem cell pool could be applied to the spleens of polycythemic mice after irradiation and bone marrow transplantation.

With this method, the presence of erythropoietin-responsive cells in the spleen was first detected on the second day after transplantation. Therefore, it is considered probable that colony-forming cells and erythropoietin-responsive cells are at different stages of maturation or cell cycle.

Furthermore, necessity of erythropoietin for further differentiation of transplanted stem cells into erythroblasts is also suggested.

SUMMARIO IN INTERLINGUA

Esseva demonstrate que un systema de essayage in vitro del magnitudine de un pool de erythropoietino-responsive cellulas primordial es applicabile al splens de muses polycythemic post irradiation e transplantation de medulla ossee.

Con iste methodo le presentia de erythropoietino-responsive cellulas in le splen esseva primo detegite le secunde die post le transplantation. Iste constatation suggestiona le possibilitate e mesmo probabilitate que cellulas colonisante e cellulas erythropoietino-responsive representa non differente cellulas sed differente stadios de maturation in le mesme cyclo.

Le datos etiam suggestiona que erythropoietina es requirite pro le differentiation subsequente de transplantate cellulas primordial ad in erythroblastos.

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