Acta Medica Okayama

Volume 34, *Issue* 4 1980 *Article* 2

SEPTEMBER 1980

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KEYWORDS: parabiosis, stem cell, erythropoiesis, myelopoiesis, irradiation.

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*PMID: 6452026 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **34** (4), 235—244 (1980)

HEMOPOIESIS RECOVERY OF IRRADIATED RATS CONJUGATED WITH NORMO- AND POLYCYTHEMIC ANIMAL BY AORTIC ANASTOMOSES

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Received April 1, 1980

Abstract. The experiment was designed to observe the possible relation between myelopoietic and erythropoietic activities of circulating nucleated cells. Wistar rats were lethally irradiated with ^{60}Co, 1000 r once. Two days after irradiation the bone marrow cells had faded completely. At this stage animals were conjugated with normocythemic or polycythemic rats by aortic anastomoses. After conjugation the aplastic bone marrow of the irradiated animal rapidly regained its hemopoietic activity in cases having normocythemic and polycythemic partners. Active erythropoiesis and myelopoiesis were found 96 h after parabiosis in those having normocythemic partners. In animals having polycythemic partners, however, erythropoiesis was successfully suppressed. An increase in lymphoid cell numbers was found in place of decreased erythroid cells, but there was no change in the myeloid cell proliferation rate. No hemopoietic precursor cells or immature cells were found in circulating blood all through the experimental period before and after parabiosis. The data suggest that circulating nucleated cells have marked erythropoietic activity. Erythropoietic cells may be somehow related to lymphoid cells independent of myelopoietic activity.

Key words: parabiosis, stem cell, erythropoiesis, myelopoiesis, irradiation.

It is generally believed that bone marrow is the sole or major hemopoietic organ in mammals, *i.e.* all the blood cells are formed there from stem cells which originate in the bone marrow tissue and have self-renewal ability. However, several recent reports show the active participation of circulating blood cells in bone marrow hemopoiesis (1-8). Circulating cells of mammals, granulocytes, monocytes and lymphocytes had long been thought to be completely mature cells with no mitotic activity; however, lymphocytes can divide by mitosis after blast formation (9). Other blood cell strains may also have this activity. It has been shown that hemopoietic stem cells exist in the peripheral blood, *i.e.* circulating blood has CFUs (1) and human male-female littermates having common or fused placenta have chimeric bone marrow cells (10). Recently Seno, Fang and

others showed marked hemopoietic activity of circulating nucleated cells in irradiated rats conjugated with healthy animals by aortic anastomoses (11–18). The present study was performed to study the possible correlations between erythro- and myelopoietic activities of circulating nucleated cells.

MATERIALS AND METHODS

Adult male Wistar rats weighing 300–350 g were used (n=112). They were obtained from an animal marketing agency. Before use the animals were kept on general dry food, MF Oriental and water for about one week in the laboratory. Then they were divided into 7 groups; 18 animals for irradiation and parabiosis, 12 healthy non-irradiated partners, 6 polycythemic non-irradiated partners, 30 irradiated and non-parabiosed controls, 5 healthy non-irradiated controls, 5 polycythemic non-irradiated controls and 36 blood and aorta donors.

Eighteen animals for irradiation and parabiosis and 30 animals for irradiated controls were exposed to a single whole body irradiation, ^{60}Co 1000 r.

Two days after irradiation 12 animals were conjugated with healthy partners and 6 animals with polycythemic partners by aortic anastomoses using aortas from healthy donors as bridging vessels (19, 20).

Polycythemia was induced by intraperitoneal transfusion of red blood cell suspensions, 3 times for 2 days before parabiosis and once immediately after parabiosis, 2 ml at one time and 8 ml as total. The RBC suspension was prepared with fresh rat blood drawn from a juglar vein. The RBC were sedimented by centrifugation, 3000 rpm for 10 min, washed with saline for 3 times by repeated centrifugation and resuspended in saline. The final volume of the red cell suspension was adjusted to be the same as that of the original blood. Thus, the Ht value of the RBC-injected animals was maiatained at 75–85% before parabiosis and 65–70% after parabiosis. Animal pairs having low Ht values below 65% at sacrifice were discarded from the polycythemic group.

Peripheral nucleated cell counts, white blood cells classification and Ht value estimation were made with peripheral blood obtained by inserting a heparinized capillary into an orbital sinus just before sacrifice.

The parabiotic animals were sacrificed 24, 48, 72, 96 h after parabiosis in those having healthy partners and 72, 96 h in those having polycythemic partners, 3 animal pairs at one time. The 30 irradiated controls were sacrificed on day 1, 2, 3, 4, 5, 6 after irradiation 3 animals at one time. After sacrifice one femoral bone marrow of each animal was taken out, epiphyses were cut off, and bone marrow was pushed out on a watch glass having rat serum. A part of the bone marrow tissues was smashed gently adding one drop of serum and smeared on an object glass. The smears were stained with May-Grünwald-Giemsa for morphologic observation. One part of the remaining tissues of the bone marrow, spleen, lymph nodes and thymus was fixed with 10% formalin in isotonic and phosphate buffered saline. Cell classifications were made on smears by observing 1000 bone marrow nucleated cells. Paraffin sections were prepared with the fixed

tissues and stained with hematoxylin-eosin. They served for histologic observations. To calculate the total cell number of femoral bone marrow cells, another femur was used. By cutting epiphyses bone marrow tissues were pushed out on a watch glass having 5 ml of saline solution. Using a glass homogenizer the tissues were crushed gently and the bone marrow cells were freed from the connective tissues which were removed by filtrating the cell suspension with nylon mesh. The cell count was made by a hemocytometer with the bone marrow cell suspension thus obtained and the total nucleated cell number of one femoral bone marrow was calculated multiplying the cell number in a unit volume by the total volume of cell suspension.

RESULTS

Hematologic mean values of the rats obtained on 5 healthy controls were as follows: In circulating blood, the Ht value was 47 7%, the white blood cell count $6.2 \times 10^3/mm^3$ with lymphocyte predominance (Fig. 1); in bone marrow,

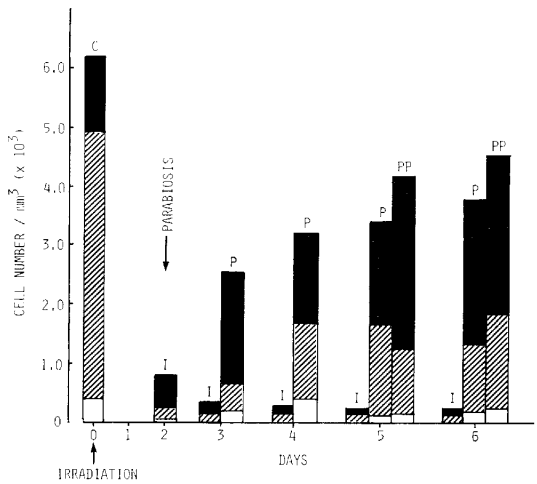


Fig. 1. Daily changes of circulating nucleated cell classification of rats. C; Untreated controls. I; Irradiated and non-parabiosed. P; Irradiated and parabiosed with healthy partner. PP; Irradiated and parabiosed with polycythemic partner. Solid column; Granulocytes. Shadowed column; Lymphoid cells. Open column; Monocytes. Method; See text.

the total nucleated cell count in one femur was 8.7×10^7, myeloid cells including mature granulocytes 45.1%, erythroid cells 23.7%, lymphoid cells 25.4% and other cells 5.8% (Fig. 2, Fig. 3A).

Two days after whole body irradiation, ^{60}Co 1000 r once, the peripheral leukocyte number decreased to about 1/8 of the original level with a predominance of granulocytes. The bone marrow showed an aplastic picture histologically (Fig. 3B). The total nucleated cell numbers in one femur decreased to

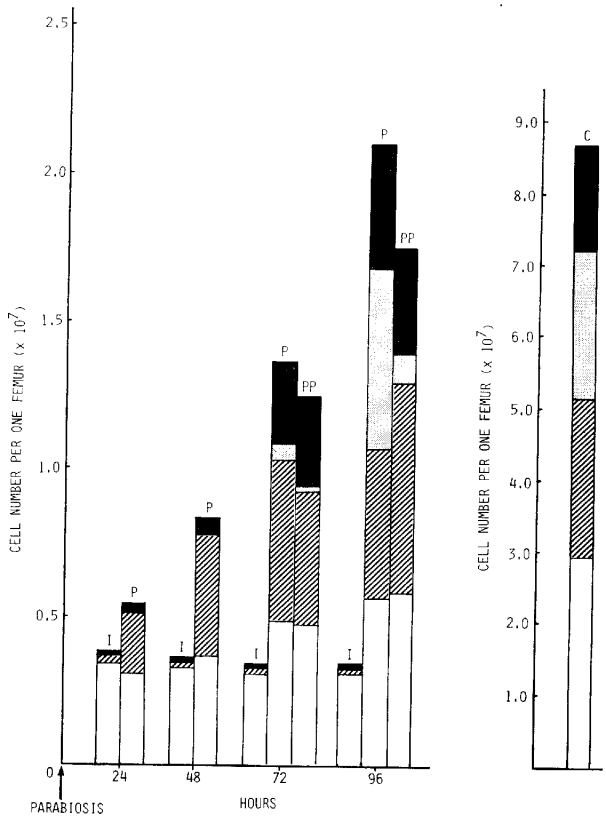
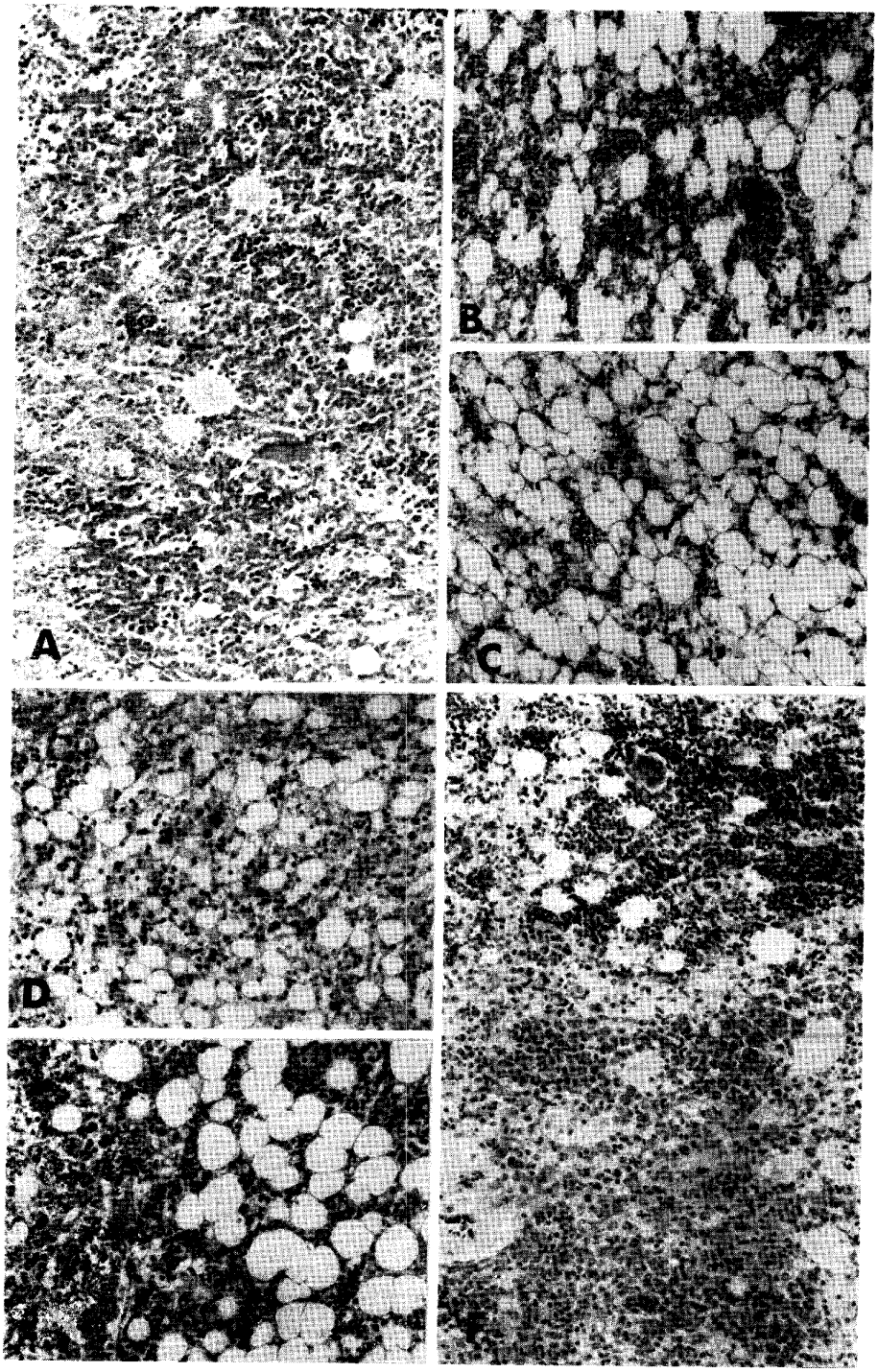
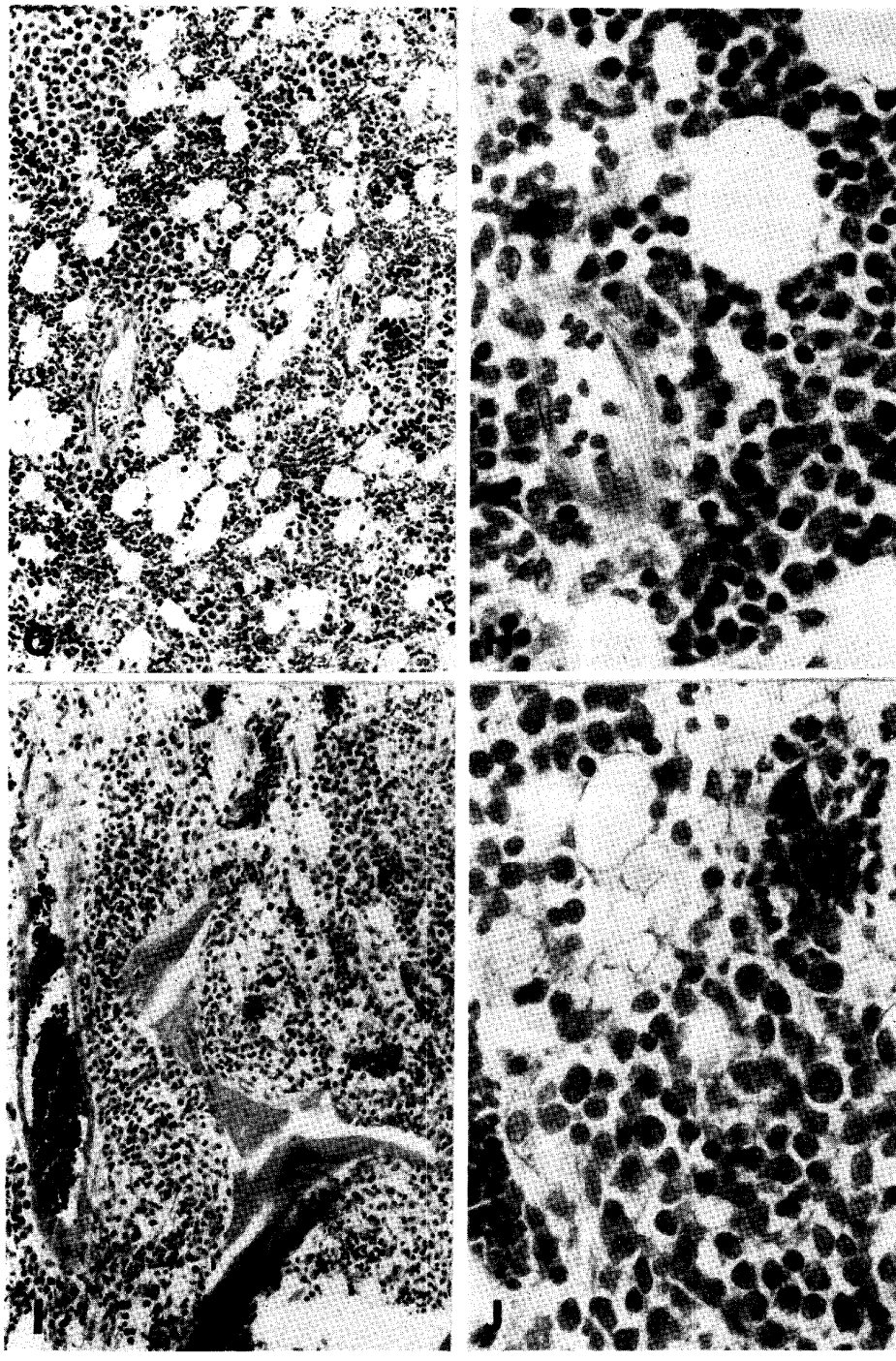


Fig. 2. Daily changes in bone marrow cell classification of rats. C; Untreated controls. I; Irradiated and non-parabiosed. P; Irradiated and parabiosed with healthy partner. PP; Irradiated and parabiosed with polycythemic partner. Solid column; Immature myeloid cells (myeloblast, promyelocytes, myelocytes). Dotted column; Erythroid cells. Shadowed column; Lymphoid cells. Open column; Other cells. Method; See text.

about 1/23 of the original level (Fig. 2). Observation of bone marrow smears revealed no hemopoietic precursor cells but some plasma cells and reticulum cells. Thereafter the animals showed no recovery of hemopoiesis and 6 to 10 days after irradiation most animals died from severe dehydration with aplastic bone marrow (Fig. 3C).

Fig. 3. Microscopic pictures of bone marrow tissue sections stained by H.E. A; Femoral bone marrow of a control animal, 10×10. B, C; Femoral bone marrow of rats 2 and 6 days after ^{60}Co irradiation, 1000 r once, respectively, 10×10. D; The bone marrow of an animal irradiated 1000 r once and conjugated with a healthy rat. Picture taken 24 h after parabiosis. 10×10. E; Bone marrow of an animal treated similarly to that in D but 48 h after parabiosis, 10×10. F; The bone marrow of an animal treated as in D but 72 h after parabiosis, 10×10.


A
B
C
D



By parabiosis with a healthy animal via an aortic anastomoses 2 days after irradiation, however, irradiated rats survived, recovering their bone marrow hemopoiesis. After conjugation with healthy animals, irradiated rats had an immediate increase in the leukocyte count of circulating blood to about one half of the control level (Fig. 1). Thereafter peripheral white blood cell numbers increased gradually, but no hemopoietic precursor cells appeared in the circulating blood.

The nucleated cell numbers in femoral bone marrow increased from 3.8×10^6 to 5.5×10^6 after 24 h, 8.3×10^6 after 48 h, 1.37×10^7 after 72 h and 2.11×10^7 after 96 h (Fig. 2). The initial increase in bone marrow cell numbers from 24 to 48 h after parabiosis was mainly made by lymphoid cell immigration 2.1×10^6 cells, 37.5% of total nucleated cells at 24 h and 3.7×10^6 cells, 44.8% at 48 h (Fig. 3. D, E). Bone marrow cell classification made 72 h after conjugation, however, revealed a marked increase in immature myeloid cells reaching 2.7×10^6 cells, 19.9% of total nucleated cells. At this stage lymphoid cells also increased markedly, 5.6×10^6 cells, 41.0% of total nucleated cells, but only a few erythroblasts at varied maturation stages appeared, 5×10^5 cells, 4.0%. Histologic observation of bone marrow revealed active myelopoiesis in areas surrounding large vessels and endosteum and poor erythropoietic foci in deep reticular tissues apart from the myelopoietic area (Fig. 3 F). Ninety-six h after parabiosis immature myeloid cell numbers increased further to 4.2×10^6 cells, 20.1% of the total number of nucleated cells. At this period erythroblasts increased abruptly giving an erythroid marrow 6.8×10^6 cells, 32.2% of total nucleated cells. Thus the E/G ratio became 1.1, a high value 2 times normal. Histologically hemopoiesis appeared diffusely in bone marrow tissues with irregularly jumbled myelo- and erythropoietic foci (Fig. 3 G, H).

Conjugation of irradiated animals with polycythemic partners having leukocytosis resulted in a more marked increase in peripheral leukocyte count with granulocyte predominance compared with those parabiosed with healthy ones. The proliferation rate of bone marrow nucleated cells, however, was lower than those having healthy partners, i.e. total nucleated cell number in one femur was 1.25×10^7 at 72 h of parabiosis and 1.76×10^7 cells at 96 h. Erythropoiesis was suppressed markedly, 2×10^5 cells, 1.8% of total nucleated cells at 72 h and 8×10^5 cells, 4.5% at 96 h after parabiosis. Immature myeloid cells occupied 3.0×10^6 cells, 24.3% after 72 h and 3.8×10^6 cells, 21.5% after 96 h. The

Fig. 3. Continued. G; The bone marrow of an animal treated as in D but 96 h after parabiosis, 10×10. H; An enlarged picture of the bone marrow appearing in G, 10×40. I; The bone marrow of an animal irradiated as in D but conjugated with polycythemic partner. Picture taken 96 h after parabiosis, 10×10. J; An enlarged picture of the bone marrow in I, 10×40.

E/G ratio was 0.15 at 96 h. The bone marrow showed a picture of myeloid marrow with poor erythropoietic foci (Fig. 3 I, J). But the actual immature myeloid cell number was essentially the same as that of those conjugated with healthy animals.

DISCUSSION

The aplastic bone marrow of lethally irradiated rats recovered its hemopoietic activity after conjugating the host animal with healthy or polycythemic animals by aortic anastomoses. In the animals left without parabiosis, the aplastic bone marrow did not show any hemopoiesis recovery, and all animals died from dehydration 6 to 10 days after irradiation. Thus, the present experiment confirms the results obtained by Seno, Fang, Nakashima *et al.* on X ray irradiated animals conjugated with healthy animals by aortic anastomoses (11–18).

Cells proliferating in the bone marrow of irradiated animals after parabiosis should be those from the non-irradiated partner as revealed by the sex chromosome analysis made on irradiated male—non-irradiated female rat parabionts by Fang *et al.* (16).

The experiments of Fang *et al.* revealed that myeloblasts appear in the aplastic bone marrow as early as 24 h after parabiosis. Erythroblasts appear after some delay, but 42–48 h after parabiosis (11–17). Nakashima, however, observed that erythroblasts appeared first 96 h after parabiosis and an erythroid marrow 120 h after parabiosis with suppressed myelopoiesis in an experiment similar to that of Fang *et al.* (18). The present experiment shows that, in contrast to Nakashima's results, erythroblats appeared first 72 h after parabiosis and active erthropoiesis and myelopoiesis 96 h after parabiosis.

In irradiated animals having polycythemic partners, erythropoiesis was extremely suppressed, but myelopoiesis became prominent 72 h after parabiosis and myelopoietic activity equivalent to those having healthy partners was maintained even later. The total femoral bone marrow cell number was somewhat smaller in rats having polycythemic partners than in those having normocythemic ones, but in the former the bone marrow lymphoid cells were increased compared to the latter.

Yamashita observed the chimeric rate of bone marrow cells of polycythemic and anemic male rats conjugated with female animals (21). He showed a very low chimeric rate in the myeloid marrow of polycythemic animals, nearly 4%, in contrast to a very high chimeric rate in erythroid marrow of anemic animals, 18–35%, 5 to 10 days after parabiosis. This shows that erythropoiesis is actively conducted by the circulating nucleated cells, while myelopoiesis is mainly conducted by *in situ* proliferation of cells having self-renewal activity.

The present experiment, the r[illegible]al observation of bone marrow cell proliferation in aplastic bone marrow after parabiosis revealed clearly that erythropoiesis proceeds independently of myelopoiesis. Myelopoiesis is not affected by suppression of erythropoiesis. Erythroid precursor cells, which develop mainly from circulating stem cells, may differenciate from lymphoid cells in the bone marrow which increase in number by suppressing erythropoiesis. This is is inconsistent with the modern concept that all hemopoietic cells develop from the common stem cells, CFUs (22-24). But it is clearly indicated that committed stem cells have self-renewal activity (25, 26). It is reasonable to suppose that very rapid recovery of hemopoiesis *i.e.* within 24 to 96 h can be made by committed stem cells. Cell proliferation by CFUs, which may require 5 to 10 days for differentiation to hemopoietic precursor cells, may be triggered mainly in emergency cases. In the present experiment, no undifferenciated cell colonies developed in the spleen or bone marrow.

Acknowledgment. The author is much indebted to Professor Satimaru Seno for valuable advice through this work and painstaking revision of the paper. Many thanks are also due to Dr. Keiki Hayashi, Dr. Chao Liang Hsueh and Dr. Syoichi Yamashita for their help with this work.

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