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Studies on erythropoiesis Ⅱ In vitro studies on red cell proliferation under varied oxygen tension*

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Abstract

For the purpose to reveal the mechanism of the stimulated erythropoiesis in anemic condition, the author observed the numerical changes of the erythroblasts from normal rabbit bone marrow cultured under the environment of varied oxygen tensions, and revealed the following: 1. The erythroblasts incubated with air are increased after 24 to 48 hours and decreased gradually disappearing by 120 hours with a corresponding increase of erythrocytes. But no active proliferation of the stem cells or proerythroblasts is observed, all the cells have differentiated to erythrocytes. Hyperoxygen tension suppresses the increase of erythroblasts slightly, while hypoxygen tension stimulates the increase. Data suggest that the cell number destined to be ineffective erythropoiesis is regulated by oxygen tensions of the environment. 2. Basophilic erythroblasts are reduced in number from the beginning showing not any increasing tendency. The reducing rate is almost the same among those cultured under the hypo- and hyperoxygen tension, comparable to that incubated with air. 3. The hypoxygen tension brings about a marked increase in the number of orthochromatic erythroblasts with a decrease in polychromatic erythroblasts suggesting an accelerated cell differentiation, while the hyperoxygen tension elicits the suppression in the formation of orthochromatic erythroblasts with suppressed differentiation. Data also show the lack of denucleation mechanism in polychromatic stages in vitro differing from the case of the bone marrow of anemic animal.

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STUDIES ON ERYTHROPOIESIS

II. *IN VITRO* STUDIES ON RED CELL PROLIFERATION UNDER VARIED OXYGEN TENSION

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In the previous paper[1] the author reported the changes in red cell volume in phenylhydrazine anemia of rabbits and revealed macrocytosis in recovery stage. The numerical distributions of the erythroblasts in each differentiation stage were also studied. The data suggested the denucleation at polychromatic erythroblast skipping one cell division, supporting the view of BORSOOK and coworkers[2], BRECKER and associates[3] and of SENO and collaborators[4]. This should be one of the adaptation mechanisms in emergent erythropoiesis. The erythroid bone marrow of the anemic animal showed an increase in the percentage of basophilic erythroblasts and polychromatic erythroblasts suggesting the stimulated cell division from pro- to baso- and baso- to polychromatic erythroblasts. On the basis of these findings the author aimed to observe whether or not these changes in erythropoiesis in anemic animal can be seen *in vitro* in cultured bone-marrow cells where oxygen tension is easily controlled. In the present paper it is demonstrated that the increase in the number of erythroblasts, which can be seen within a certain period of incubation, is suppressed under hyperoxygen tension, and stimulated under hypoxygen tension but not accompanied by the proliferation of basophilic erythroblasts and stimulated denucleation at polychromatic stage.

MATERIALS AND METHODS

The bone-marrow cells were obtained from normal rabbits sacrificed by blood depletion. The bone marrow tissues taken aseptic from the femurs were put in a watch glass and crushed gently dipping in the warm (about 30°C) Ringer solution containing heparin, 1/20,000 in concentration and connective tissues were removed after centrifugation, 1,000 r. p. m. for 5 minutes. The precipitated cells were suspended in the warm medium, around 30°C, which was composed of one volume of Tyrode's solution and one volume of normal rabbit serum, so as to contain about 5,000 to 6,000 of nucleated cells per cu mm. Each one ml of this suspension was poured into a test tube, TD 15 of 2 ml in capacity.

The air in the space of each test tube was changed with the gas, pure oxygen gas, 40% oxygen gas in nitrogen gas, air, 10% oxygen gas in nitrogen gas or pure nitrogen gas, by the method devised by MATSUOKA[5]. And these were incubated at 37°C. For one series of observations five test tubes were employed, and three observations were made of one material. Observations have been made at selected intervals: prior to the morphologic observation the number of the nucleated cells and red cells in the suspension have been counted by the routine methods. After this the cell suspension in the tube is centrifuged and the precipitated cells are stained supravitally by mixing with one drop of 0.1% Nile blue solution in saline, and they are smeared and stained with May-Grünwald Giemsa. On the stained samples the classification of the nucleated cells is made. The numerical observations of erythroid cells have been carried out dividing into 3 classes; basophilic, polychromatic, and orthochromatic erythroblasts. Reticulocytes and matured red cells are also counted from the total number of nucleated cells and red cells and percentage of each cell species.

OBSERVATIONS AND RESULTS

The morphologic observations of bone-marrow cells incubated and stained by the method just mentioned proved that the cells well retained both their staining characteristics and morphologic appearances. The numerical observations made prior to incubation showed that among about 6,000 bone-marrow nucleated cells in normal rabbit the erythroblasts were around 2,300 in total, basophilic erythroblasts about 700, the polychromatic about 1,000 and the orthochromatic 550. And the myelogenous cells together with lymphatic cells were around 4,000, i. e. about 2/3 of the total nucleated cells were consisted of erythroblasts. Red cell number was about 8,500 including reticulocytes of about 1,000 in number.

After 24 hours' incubation under air the total number of erythroblasts increased by about 1/5 of the original level reaching nearly 3,000 (Fig. 1). After 48 hours the total number increased further by about 1/10 of the original value and then decreased reaching nearly zero by 120 hours of incubation. Reticulocytes increased by 72 hours and then decreased, while red cells increased gradually, though the count slightly decreased after 120 hours' incubation comparing to the value at 96th hour. Morphologic appearance of the cells by 96 hours was kept almost normal showing the cell maturation to proceed in the medium without any abnormal degeneration. The red cells increased by about 1,500 at 96th hour which is comparable to the reduced number of the nucleated erythroid cells during the same period. The basophilic erythroblasts were reduced by one half at 24th hour, by 3/4 at 48th hour and disappeared at 72th hour, showing that

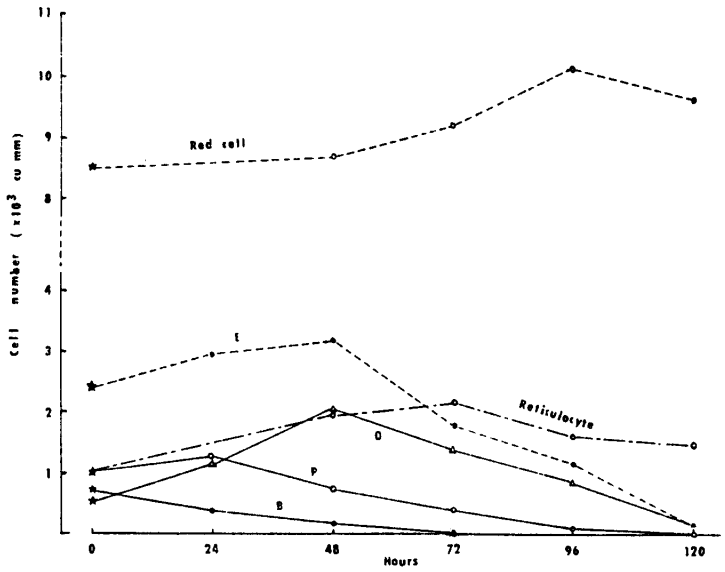

Fig. 1 Changes in erythroid cell number as observed during 120 hours' incubation of normal rabbit bone marrow under air.
E: Total number of erythroblasts, B: Basophilic erythroblasts, P; Polychromatic erythroblasts, O: Orthochromatic erythroblasts. Method: see text.

the division cycle of them is about 24 hours in a given environment, provided the two steps of cell division at basophilic stage as suggested by LAJTHA[6]. The polychromatic erythroblasts increased slightly after 24 hours, by about 500 in number, and then reduced progressively reaching 0 at 120 hours. Orthochromatic ones increased reaching twice the original level after 24 hours and 4 times after 48 hours. Thereafter it decreased gradually reaching the minimum value at 120 hours. The reticulocytes increased reaching more than twice the original value at 72 hours, then decreased (Fig. 1). The slight decrease in red cell number by the incubation of over 96 hours is probably due to the destruction of old cells, though there was not any recognizable sign for hemolysis.

Under the environment of hypoxygen tension, 10 per cent oxygen in nitrogen gas, the number of the total erythroblasts increased markedly by 24 hours' incubation comparing to that of those incubated with air. Thereafter they showed a decreasing tendency. The decreasing tendency seen after 48 hours of incubation was also marked comparing to those cultured under air (Fig. 2). Under the environment of higher oxygen tension, 40 per cent oxgen in nitrogen gas, the initial increase and the subsequent decrease of the total number of erythroblasts were less marked comparing to those cultured under air (Fig. 2).

Observations on the erythroblasts in different maturation stages revealed

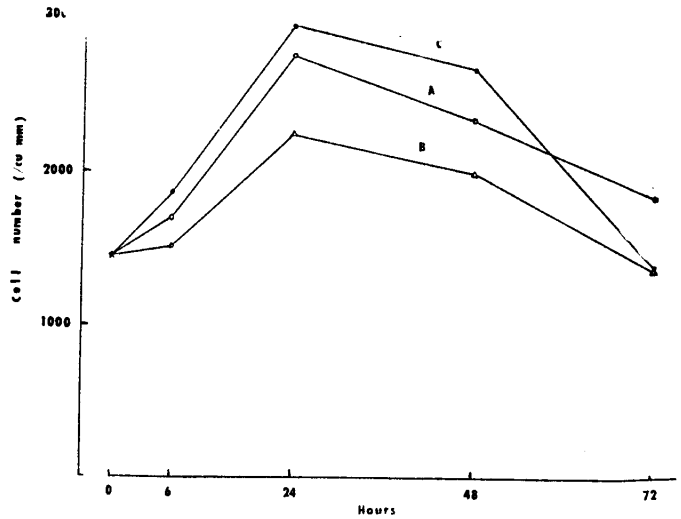

Fig. 2 Changes in total number of erythroblasts from normal rabbit 72 hours' culture under the environments of varied oxygen tension. A: cultured under air, B: under hyperoxygen tension, 40% of O_2, 59.7% of N_2 and 0.3% of CO_2, C: under hypoxygen tension, 10% of O_2, 89.7% of N_2 and 0.3% CO_2.

that the basophilic erythroblasts showed not any difference in the reducing tendency in number between those incubated with gas containing 10 per cent oxygen, and 40 per cent oxygen and those incubated with air (Fig. 3). However, in polychromatic erythroblasts a remarkably stimulated cell proliferation was observed on those living under low oxygen environment and a remarkably susppressed increase on those under high oxygen environment at 6th hour of incubation, comparing to that of those under air (Fig. 3). After 24 hours the number of polychromatic ones became much less comparing to those incubated with air and 40% oxygen. In contrast, the orthochromatic cells increased markedly in lower oxygen tension, while under higher oxygen tension they showed a suppressed increase by the time of 24th to 48th hour of incubation, about one half of the former which reduced abruptly at 72th hour of incubation (Fig. 3). The numerical changes in reticulocytes and mature red cells showed not any difference between those cultured under higher and lower oxygen tension and those under air (Fig. 4).

Under pure oxygen or nitrogen gas environment the cells did not increase (Fig. 5). The basophilic erythroblasts decreased showing a similar tendency as those under air by 24 hours but under pure oxygen gas they were not reduced in number after 24 hours (Fig. 6). Morphologic observations revealed a severe cell damage. Polychromatic cells showed a decreasing tendency from the beginn-

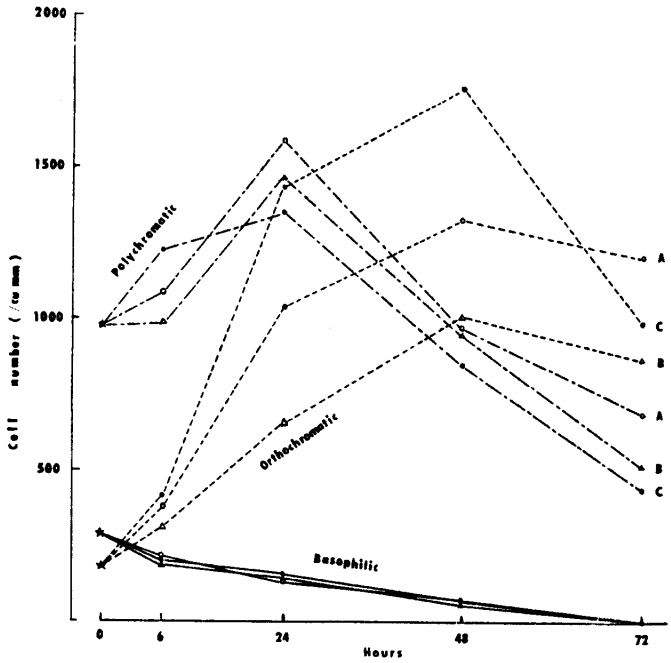

Fig. 3 Changes in the number of the erythroblasts in each maturation stage as calculated on smeared and stained cells from the same blood samples as in Fig. 2, A, B, and C: refer to Fig. 2.

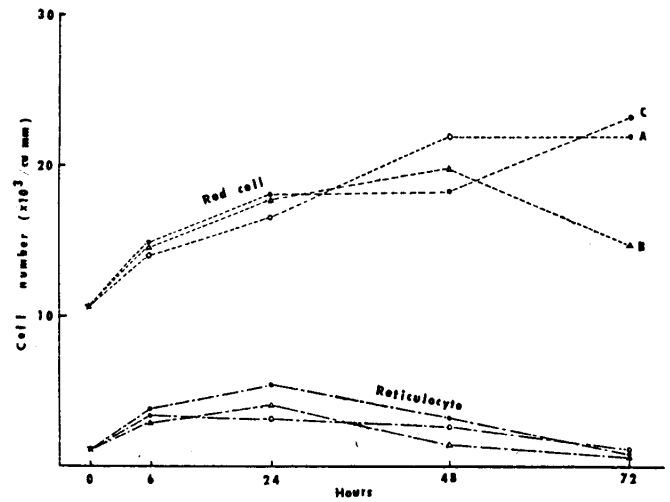

Fig. 4 Changes in the number of the reticulocytes and red cells in the same experiment as in Fig. 2. A, B and C: Refer to Fig. 2.

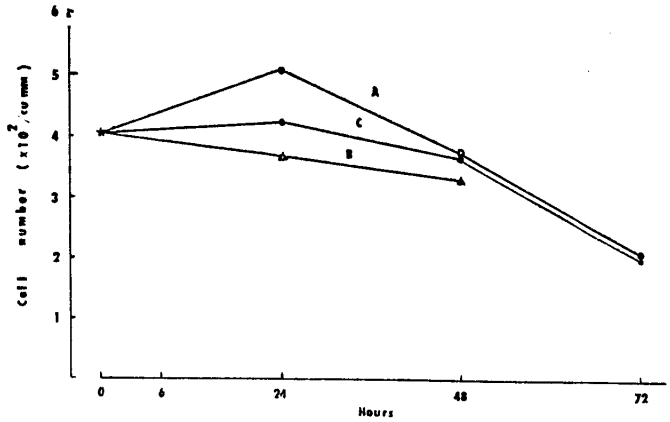

Fig. 5 Changes in the total number of erythroblasts from normal rabbits in culture under air (A), pure oxygen gas (B) and pure nitrogen gas (C).

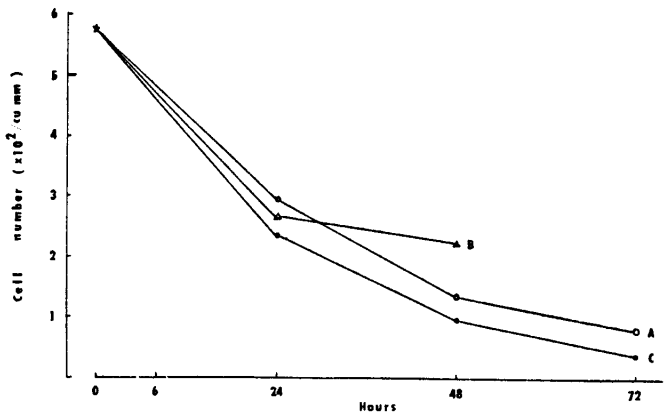

Fig. 6 Changes in the number of basophilic erythroblasts in the same experiment as in Fig. 5. A, B, and C: Refer to Fig. 5.

ing (Fig. 7), but the orthochromatic ones showed some increasing tendency (Fig. 8). This signifies that the maturation of the polychromatic to orthochromatic cells is somehow proceeding, though the cell damage is severe. These experiments show that under the destruction of mitochondria in pure oxygen gas environment[7] or the suppressed respiration in pure nitrogen gas environment the hemoglobin synthesis is proceeding to a certain extent in the polychromatic stage.

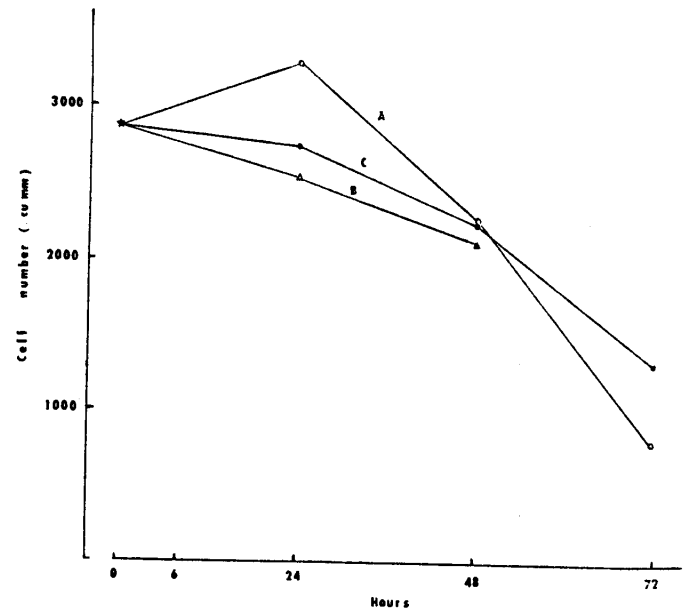

Fig. 7 Changes in the number of polychromatic erythroblasts in the same experiment as in Fig. 5.

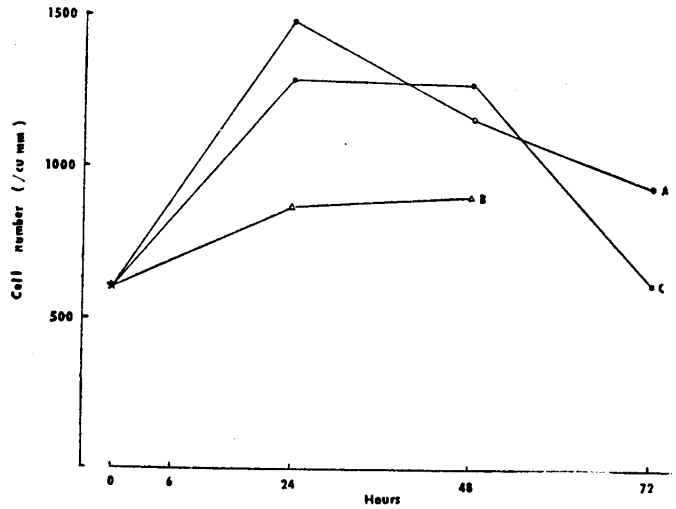

Fig. 8 Changes in the number of orthochromatic erythroblasts in the same experiment as in Fig. 5.

DISCUSSION

As just demonstrated in the present observation, the total number of nucleated erythroid cells increases in the culture media by being incubated with air showing the peak at 24 or 48 hours of incubation, and gradually decreases reaching nearly zero by 120 hours. This shows that all the nucleated cells differentiate to form erythrocytes, but the cell division of younger erythroblasts or stem cells by which the erythroblasts continue to proliferate in the bone marrow *in vivo*, does not occur *in vitro*. In the papers of LAJTHA and others[8,9,10], likewise, the erythroblasts can only mature *in vitro* in all the given environments tested. Therefore, with the erythroblasts cultured *in vitro* the differentiation-division only can be studied. In the previous report the author revealed that in the anemic animal, i. e. under oxygen deficient environment of bone marrow, the basophilic erythroblasts increased in number per cent, suggesting that the cell division from proerythroblasts to basophilic erythroblasts is stimulated. This does not, however, occur *in vitro* probably due to lack of some unknown mechanism to stimulate the cell division of proerythroblasts, which is supposed to be present *in vivo* and lost in the *in vitro* culture environment.

Active proliferation of erythroblasts, especially of basophilic ones composing of erythroblastic marrow of anemic animal, is clearly due to the stimulated cell division of stem cells and stimulated cell differentiation as well. But *in vitro* experiment it is rather ambiguous whether or not the period of division cycle of each step later than that of basophilic erythroblast is shortened or the cell division for differentiation is stimulated.

The present *in vitro* study revealed that under hypoxygen tension the number of total erythroblasts increased remarkably with the incubation but under hyperoxygen tension the increasing rate was rather low as compared to that incubated under air. The stimulated increase in erythroblast number under hypoxygen tension and the suppressed increase under hyperoxygen tension are rather slight, by about one tenth of the control under air in both cases. This stimulated or suppressed increase will probably be due to a decrease or an increase of those of ineffective erythropoiesis[6]. Because, if the cell division in some cells is stimulated or suppressed, microcytic red cells in hypoxygen tension and macrocytic red cells under hyperoxygen tension should result. This is not so in this experiment; no microcytic and macrocytic erythroblasts have been observed in both environments.

Observation on the numerical changes of the erythroblasts in each maturation step revealed that the population of basophilic erythroblasts received no influence by the changed oxygen tension in environment, while under hypoxygen tension the population of polychromatic ones increased markedly in the

beginning and diminished rapidly with prolonged incubation being accompanied by a marked increase in number of orthochromatic ones. The data show clearly that the differentiation of the cells is stimulated under hypoxygen tension, in contrast to those under hyperoxygen tension where the increase in the orthochromatic population is much less with a moderate increase in polychromatic cells.

The result is rather inconsistent with that obtained from the bone marrow study of animal where orthochromatic ones were reduced extremely or disappeared completely in severe anemia. The difference will be due to the lack of some stimulated denucleation mechanism *in vitro* culture as just mentioned. As there is not any difference in red cell size between those from the tubes of hypo- and hyperoxygen tension, stimulated denucleation skipping cell division does not occur under hypoxygen tension *in vitro*.

Under pure oxygen as well as pure nitrogen gas the cell proliferation is extremely suppressed but the differentiation seems to proceed in the polychromatic to the orthochromatic stages. The increase of the orthochromatic erythroblasts in number shows that this terminal differentiation is accompanied by the cell division. Thus under an extremely low oxygen tension, in pure nitrogen gas, the stimulated denucleation at polychromatic stage does not occur *in vitro*.

SUMMARY

For the purpose to reveal the mechanism of the stimulated erythropoiesis in anemic condition, the author observed the numerical changes of the erythroblasts from normal rabbit bone marrow cultured under the environment of varied oxygen tensions, and revealed the following:

1. The erythroblasts incubated with air are increased after 24 to 48 hours and decreased gradually disappearing by 120 hours with a corresponding increase of erythrocytes. But no active proliferation of the stem cells or proerythroblasts is observed, all the cells have differentiated to erythrocytes. Hyperoxygen tension suppresses the increase of erythroblasts slightly, while hypoxygen tension stimulates the increase. Data suggest that the cell number destined to be ineffective erythropoiesis is regulated by oxygen tensions of the environment.

2. Basophilic erythroblasts are reduced in number from the beginning showing not any increasing tendency. The reducing rate is almost the same among those cultured under the hypo- and hyperoxygen tension, comparable to that incubated with air.

3. The hypoxygen tension brings about a marked increase in the number of orthochromatic erythroblasts with a decrease in polychromatic erythroblasts suggesting an accelerated cell differentiation, while the hyperoxygen tension elicits the suppression in the formation of orthochromatic erythroblasts with

suppressed differentiation. Data also show the lack of denucleation mechanism in polychromatic stages *in vitro* differing from the case of the bone marrow of anemic animal.

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