Acta Medica Okayama

Volume 21, *Issue* 5 1967 *Article* 5

OCTOBER 1967

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Abstract

For the purpose to get the information about the control mechanism of erythropoiesis in bone marrow the author introduced a mass of homologous red cells into anemic animal and observed how the bone marrow cells and circulating blood react against the prompt normalization of the anemic condition. After the red cell transfusion which was enough to restore the anemia promptly the red cell number in the circulating blood continued to increase until 72 hours after the transfusion, reaching an extremely high level in both red cell number and hemoglobin contents. Mitotic index and the DNA synthesis as observed by tritiated thymidine incorporation into DNA proved no actual change even 24 hours after the red cell transfusion, though a marked decrease in labeling index was found in large size precursors. Histologic picture revealed the proliferation of reticulum cells. 48 to 72 hours after the red cell transfusion both mitotic index and DNA synthesis of erythroblasts have largely retarded in all series of specialization with the decreased appearance of the erythroblasts in bone marrow sections. The measurements of red cell size and the RNA contents of erythroblasts and reticulocytes proved the accelerated denucleation at the early stage of erythroid cell specialization, as early as basophilic stage resulting in a marked macrocytosis.

*PMID: 4232097 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 21, 251—265 (1967)

EFFECT OF MASS BLOOD-TRANSFUSION ON ERYTHROID CELL DIFFERENTIATION IN ANEMIC RABBIT

I. AN EVOLUTIONAL CHANGE IN THE CELL SPECIALIZATION PROCESS

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Received for publication, August 8, 1967

The red cell number in the circulating blood is controlled at a certain level by which the tissues are properly supplied with oxygen. The control of the cell number is attained by holding the cells newly formed and the cells to be discarded by aging in balance. Thus, the erythropoiesis in bone marrow is stimulated under anemic condition or some heart failure and greatly suppressed by oxygen inhalation or mass transfusion of red cells[1].

In erythroid hemopoiesis two steps may be distinguished; the first step is the transformation of the stem cell to erythroblast and the second is the specialization of erythroblast to red cell, which proceeds with repeated cell divisions. In the transformation process the stem cell is converted into the proerythroblast probably by the aid of erythropoietin[2-7]. Under normal condition the proerythroblast matures to anucleate red cell through 4 specialization stages and 4 cell divisions[8-10]. There are a number of reports stressing that a humoral mechanism plays a major part in the regulation of red cell production[11,12] and erythropoietin will serve as the inducer for the transformation of stem cell to proerythroblast[13,14]. But it has never been established how the cell division during the specialization process from the pro- to the orthochromatic erythroblast can be controlled.

In this connection, the experiments were undertaken to observe the effect of mass red cell transfusion on the erythropoiesis stimulated by the phenylhydrazine injection. By using standard techniques of autoradiography[15,16] for tritiated thymidine (TD^3H) incorporation into DNA and colchicine injection for obtaining reliable mitotic indices[17] the author tried to reveal how the DNA synthesis and mitosis of the erythroid cell are controlled to keep the proper levels of the red cell number and the hemoglobin content. In this paper it is reported that the transformation of the stem cell to the erythroid young precursor is

suppressed by the mass transfusion of red cells, but cell division in the specialization of erythroblast is hardly suppressed during 24 hours after the red cell transfusion but later on DNA synthesis is suppressed with the accelerated denucleation at early stage of erythroid cell specialization.

MATERIALS AND METHODS

Twenty-one adult male albino-rabbits, weighing about 2.0kg were used. They received the subcutaneous injections of phenylhydrazine hydrochloride, 1.5ml of 2.5 per cent neutralized solution once a day for 3 successive days. For the following 2 days they were kept without phenylhydrazine injection and 6 days after the first injection 15 animals received the intravenous introduction of a mass of packed homologous red cells, 50 to 60ml in one dose. Other 6 anemic rabbits served as controls, and received the injection of saline in comparable volume.

The packed red cells for intravenous injection were prepared as follows; for one injection about 150ml blood were obtained from 3 healthy rabbits by cardiocentesis, 50ml each, by using a syringe containing 5ml of 3.8 per cent sodium citrate. Immediately after drawing the blood was centrifuged at 3,000 rpm for about 10 minutes and the plasma and buffy coat were removed. The precipitated cells were washed twice with 0.85 per cent saline solution at 4°C, and finally resuspended in half volume of the same solution at 37°C and used fresh.

These fifteen rabbits were divided into 3 subgroups, 5 rabbits in each. The animals belonging to the first subgroup and 2 controls were sacrificad 24 hours after the red cell transfusion, another 5 animals 48 hours and the last 5 ones 72 hours after the red cell transfusion with 2 controls in each.

During the period of experiments, red cell number, reticulocyte count, hematocrit value and hemoglobin level of the circulating blood have been observed at certain intervals.

Price Jones' curves were drawn on the red cells in wet in each animal before and after the red cell transfusion and also in anemic control.

For the morphologic study and the assay of DNA metabolism of erythroblast, the bone marrow tissue from femurs was used. A small piece of the tissue was taken on an object slide, added with one drop of rabbit serum and mashed gently by pressing with another object slide. Thus, the cells freed from the connective tissues were smeared and stained with Giemsa for morphologic observation. For incubation with TD^{3}H a gross mass of the bone marrow was added to about 10 ml of Hanks' solution and mashed very gently in glass homogenizer moving slowly the inner cylinder up and down 2 or 3 times

for a few minutes. Then the homogenates were centrifuged at 3, 000 rpm for 5 minutes and the supernatant was removed with the floating connective and fat tissues. The precipitated cells were resuspended in an equal volume of Hanks' -serum mixture prepared by adding rabbit serum to Hanks' solution in an equal volume. One ml of the cell suspension was taken into a test tube and added with TD^3H (2 μc in 0.02ml of saline) for the study of DNA synthesis. TD^3H, specific activity 5c per mM, was obtained from the Radiochemical Center in England. Then the cells were incubated for 30 minutes at 37°C. After incubation, the incubation media were removed by centrifugation at 4°C for 5 minutes at 1, 500 rpm and washed twice with Hanks' solution by repeated centrifugation. After washing, the sedimented cells were resuspended in 3 to 4 drops of normal rabbit serum and then smeared. The smears were dried, fixed with methanol, dried, mounted with liquid film emulsion SAKURA NR M-1, and exposed for 15 days at 4°C. After development, the smears were finally stained with May-Grünwald Giemsa at pH 6.0. The labeling indices were obtained by the percentage of labeled erythroblasts in 1, 000 erythroblasts at each specialization stage and the grain number was also recorded on each nucleus. In all slides 2 grains were required as the limiting grain count per labeled cell.

The determination of RNA contents per cell was made by microspectrophotometry on the smeared cells stained by azure B by the slightly modified method of FLAX and HIMES[18]; the cells were smeared on cover slide of 0.18 × 25 × 50 mm, dried, fixed with methanol and treated with desoxyribonuclease solution, crystalized Worthington DNase 1 mg per ml of Gomori's Tris buffer containing 0.2M $MgSO_4 \cdot 7H_2O$ at pH 5.7[19], for 18 hours at 37°C. After the treatment smeared cells were washed with tap water for 30 minutes, dried, stained with azure B for 3 hours at 40°C and differentiated in tertiary butyl alcohol for 15 hours at 37°C. The azure B obtained from National Aniline Division in USA was used as 0.025% solution in McIlvaine's buffer at pH 4.0. The estimation of RNA was carried out by the microspectrophotometer of Olympus Kogaku Co., employing the two-wavelength method of ORNSTEIN[20] PATAU[21] and MENDELSOHN[22] at 590 mμ and 520 mμ.

Identification of the specialization stage of erythroblasts was made by the nuclear diameter according to the principle described by WEICKER[8].

For the observation of mitotic index 6 adult male rabbits were used. They were divided into two groups, 3 animals in each. By the same method as in the cases just described all the animals received the injections of phenylhydrazine. At 2-day interval after the last injection of phenylhydrazine a mass red cell transfusion was made on the animals belonging to the first group and other 3 animals of the second group served as the anemic control. Twenty hours after the red cell transfusion the rabbits received the colchicine injection, 1mg per

kilogram body weight subcutaneously in 0.85 per cent saline solution. Three anemic controls received the same treatment. The animals were sacrificed 4 hours after the colchicine injection and the bone marrow cells were smeared by the method mentioned above. The cells were stained with May-Grünwald-Giemsa by the routine method. The mitotic index was obtained by counting the cells under mitosis in 1, 000 erythroblasts in each specialization stage.

The sequence of specialization process of erythroblasts was described as follows: Proerythroblast (ProE); 15—11 μ in nuclear diameter, early basophilic erythroblast (Baso I); 10.5—8.5 μ, late basophilic erythroblast (Baso II); 8.0—6.5 μ, polychromatic erythroblast (PolyE); 6.0—5.0 μ and orthochromatic erythroblast (OrthE); 5.0—4.0 μ in nuclear diameter.

OBSERVATIONS AND RESULTS

By daily injection of phenylhydrazine in described dose the animals developed a severe hemolytic anemia within a few days, as has been reported by several workers[23,24]. On the third day of the injection the red cell number and hemoglobin content decreased to the level of about 3 million RBC per cu mm and 8.0 g/dl hemoglobin. Even after the cessation of the phenylhydrazine injection the red cell number and hemoglobin content continued to decrease reaching the critical point at three days after the last injection of the agent, 1.5—2.0 million RBC per cu mm and 5—7 g/dl hemoglobin with a marked reticulocytosis and macrocytosis suggesting nearly complete replacement of the damaged red cells by newly formed ones. Observations of the smears and the sections of bone marrow revealed the pictures of typical erythroid marrow being largely occupied by erythroblasts (Fig. 8).

Thereafter, in the control animals left to survive receiving no red cell transfusion the red cell number in the circulating blood increased steadily by about 0.3 to 0.5 million daily and the anemia recovered completely by 10 to 14 days (Fig. 1).

In the animals receiving the red cell transfusion, 30 ml of packed red cells suspended in 30 ml of saline at one time at the maximal stage of anemia, the red cell number and the hemoglobin level reached the normal value 30 minutes after the red cell transfusion. Twenty-four hours after the transfusion, however, a marked increase in red cell number was found, 0.7 to one million per cu mm for 24 hours. The rate of increase in red cell number was extremely high comparing to the anemic control left without red cell transfusion (Fig. 2). Histologic sections gave the picture of proliferation of reticulum cells with a marked decrease in erythroblasts (Fig. 9). In the second and the third day of transfusion the red cell number still continued to increase, though the rate of increase

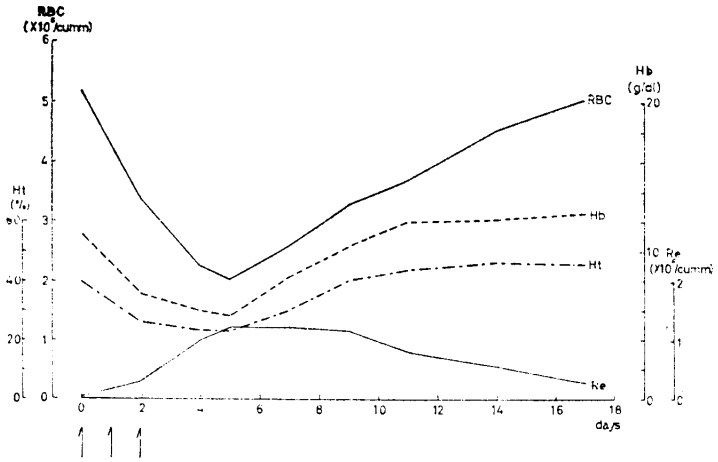


Fig. 1 Changes in the hemogram of circulating blood after the phenylhydrazine injection. RBC: Red cell number, Hb: Hemoglobin content, Ht: Hematocrit value, Re: Reticulocyte number, Arrows: Phenylhydrazine injections.

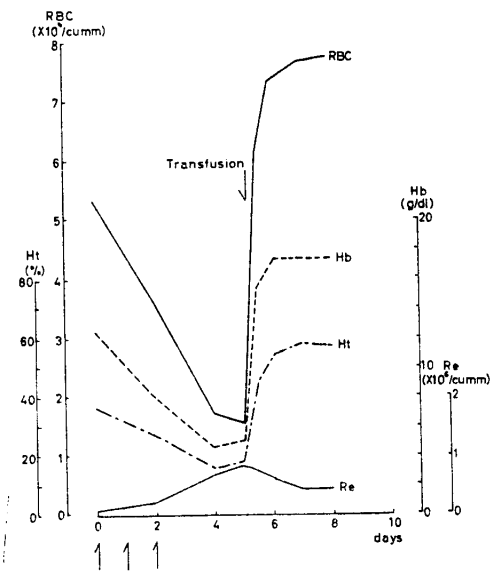


Fig. 2 Evolutional changes in hemogram of the circulating blood after a mass red cell transfusion into an anemic rabbit. Note an abnormal increase in red cell number after the red cell transfusion. Arrows: Phenylhydrazine injections.

lowered day by day and at the fourth day it reached the equilibrium, where it stopped increasing and thereafter showed a tendency to decrease, though very slowly. Histologic picture of the bone marrow 72 hours after the transfusion demonstrated the much retarded proliferation of both reticulum cells and erythroblasts.

Mitotic indices of erythroblasts from the bone marrow taken 24 hours after red cell transfusion gave nearly the same level as the anemic control (Fig. 3). Observations dividing into four groups (the large precursor cells, early basophilic, late basophilic and polychromatic erythroblasts) revealed a slightly lowered mitotic index in large size precursor cells but no actual difference in other three groups (Fig. 10). The large size precursor cells included proerythroblasts and probably stem cells (Fig. 11).

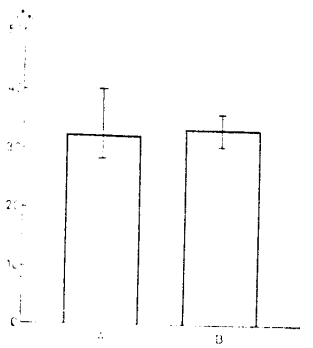


Fig. 3 Mitotic indices of the erythroblasts in the bone marrow of the anemic rabbit 24 hours after red cell transfusion (A) and of the anemic control (B). Each value shows the mean of those from three rabbits.

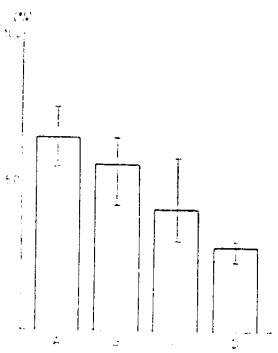


Fig. 4 Labeling indices of erythroblasts 24 hours after the mass red cell transfusion (B) showing no actual change comparing to the anemic control (A), a marked drop after 48 hours (C) and extremely low level after 72 hours (D).

Incorporation of TD^{3}H into DNA of erythroblast, taking both the grain count per cell and the labeling index into account, observed at the recovery stage of anemia was most marked in the large size erythroid precursors and lowered with the advance of specialization stages, high in early basophilic stage, moderate in late basophilic stage and scarce at polychromatic stage. On orthochromatic erythroblasts the incorporation of TD^{3}H into DNA was hardly recognized (Fig. 12). Twenty-four hours after red cell transfusion the labeling index of erythroblasts showed no actual difference comparing with anemic control, but a marked drop after 48 hours and extremely low level after 72 hours (Fig. 4). Observations on erythroid cells divided into 4 groups as just mentioned

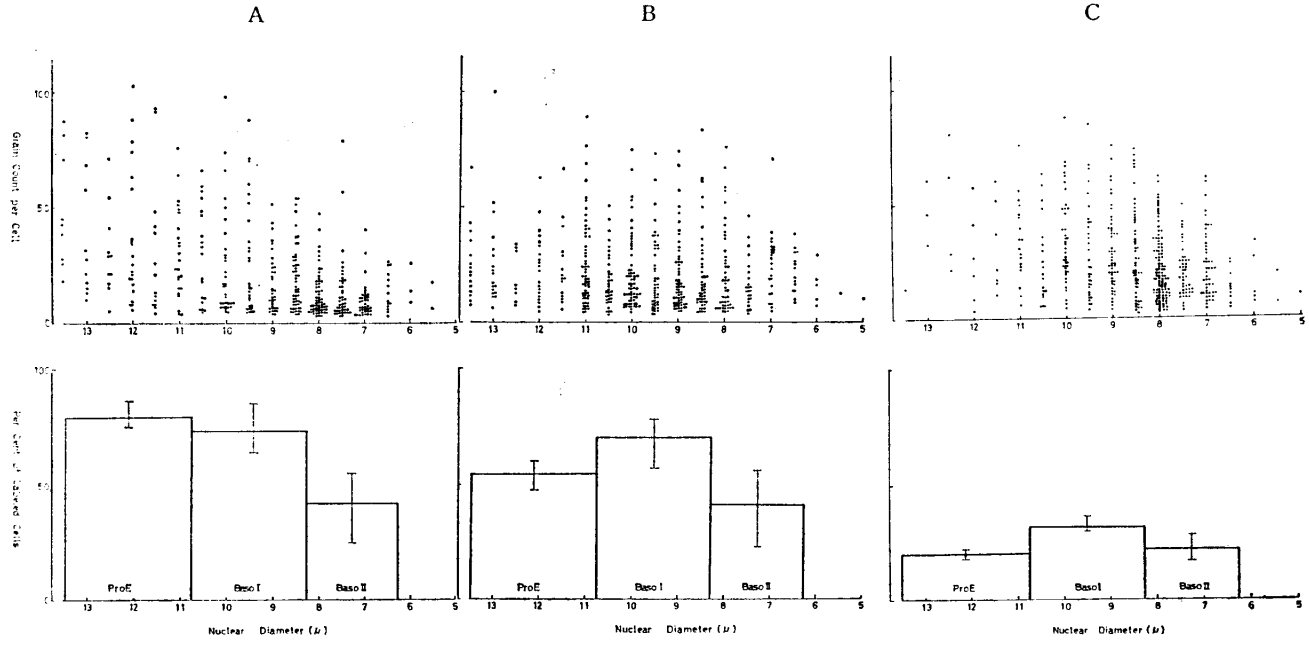


Fig. 5 A, B, C The incorporation of tritiated thymidine into DNA of erythroblasts in each specialization stage. (A) Anemic control. Labeling index was greatest in the proerythroblast, less in early basophilic and least in late basophilic erythroblast. (B) 24 hours after red cell transfusion. Labeling index was reduced in the proerythroblast and greatest in basophilic erythroblast. (C) 72 hours after red cell transfusion. Labeling index was decreased in the entire erythroid compartment. Each value shows the mean of those from 5 rabbits.

revealed that slightly lowered level of the index is due to lowered incorporation rate in the group of large size precursors with the no actual changes in those of advanced stages (Fig. 5A). After 48 hours the index of the basophilic cells also lowered with the minimized level of the labeling index of the large size precursors, and after 72 hours the indices of all the cell series from pro-to polychromatic erythroblasts showed extremely low level indicating suppressed DNA synthesis of all the cells at varied stages of specialization (Fig. 5C). Even after the red cell transfusion, however, mean grain number per cell stayed unchanged giving nearly the same value as in anemic control, in which the grain number per cell decreased with the advance of specialization stages.

Price Jones' curves drawn on the wet samples of red cells from anemic animals 3 days after the last injection of phenylhydrazine showed a right shift indicating nearly the complete disappearance of normal size red cells, being replaced by large size red cells of about 2 times in volume. After the red cell transfusion the macrocytosis was accentuated, being accompanied by the appearance of the extremely large size cells (Fig. 13). The Price Jones' curve gave 3 peaks each of which represented the cells of normal, twice and 4 times normal volume, respectively (Fig. 6). However, the largest denucleated cells found in the bone marrow were as large as 8 times normal volume. Such a phenomenon was observed from 24 to 72 hours after the red cell transfusion.

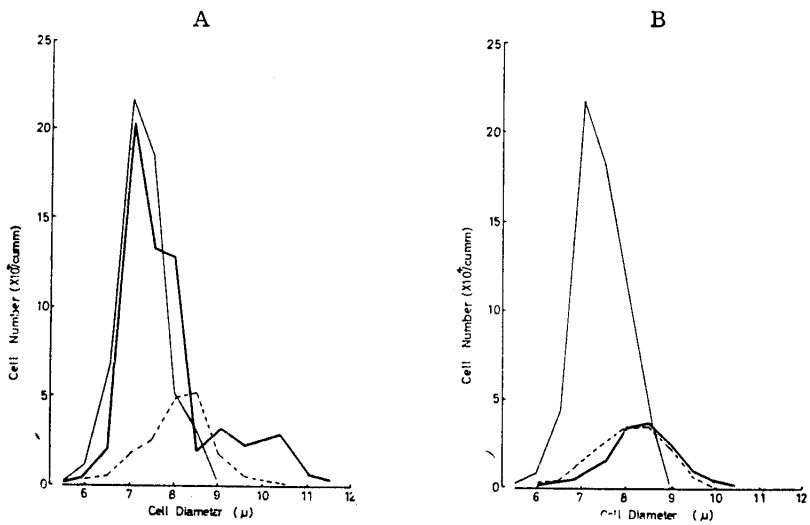


Fig. 6 Price Jones' curves of red cells in the circulating blood taken 72 hours after the red cell transfusion (A) and of the anemic control (B). Solid thin-line: The curve drawn before the phenylhydrazine injection. Broken line: Anemic stage after the phenylhydrazine injection. Solid thick-line: 72 hours after transfusion. Estimated on wet preparation.

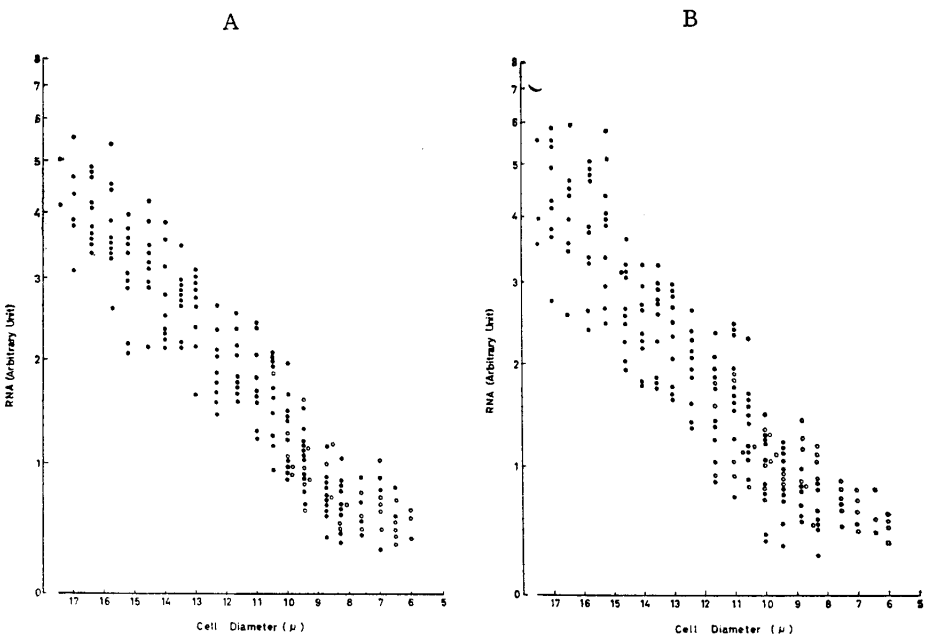
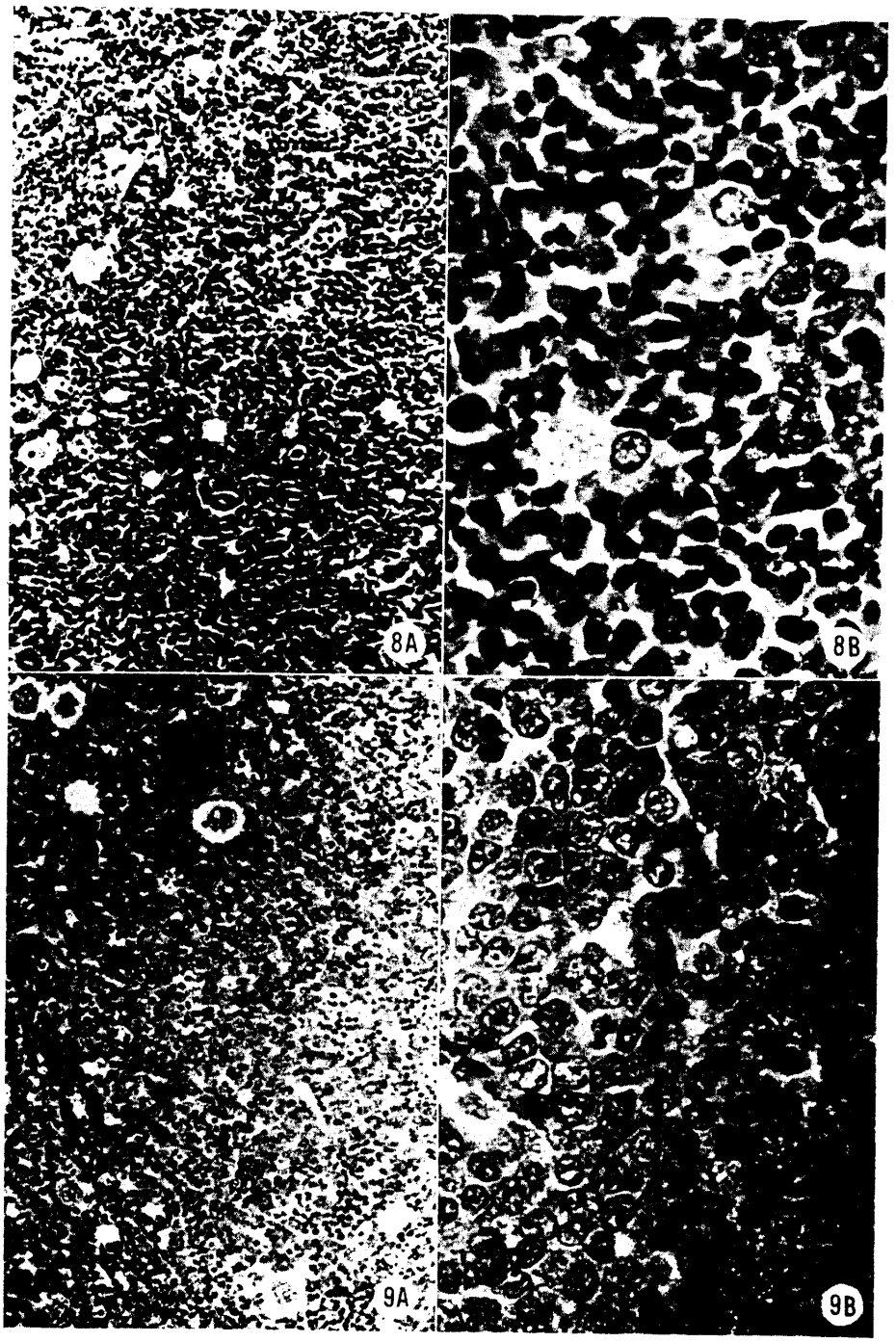


Fig. 7 A, B Relative amount of azure B binding RNA of erythroblasts and reticulocytes in the bone marrow of the anemic rabbit 24 hours after the red cell transfusion (B) and of the anemic control (A). Note the high RNA contents of some reticulocytes. Solid circles: Erythroblasts. Open circles : Reticulocytes.

The relative amount of RNA of erythroblast per cell observed on the bone marrow smears of anemic rabbits proved to decrease exponentially with the advance of the stages of cell specialization or the decrease in cell diameter (Fig. 7A). Such a decrease in RNA contents per cell with the advance of cell specialization was found to be unchanged even after the red cell transfusion (Fig. 7B). The RNA level of reticulocytes was found to be rather low but some higher ones were comparable to those of basophilic erythroblasts and the highest value to the level of early basophilic erythroblasts. These reticulocytes enormously high in RNA contents increased in number after the red cell transfusion.

Fig. 8 Histologic pictures of bone marrow from anemic rabbit showing typical erythroid marrow with many erythroblastic islets. Hematoxylin-eosin stain. A: ×100. B: Enlarged picture of a part of A, ×400.

Fig. 9 Bone marrow from anemic rabbit 24 hours after receiving a mass red cell transfusion. Note the increase in the number of reticulum cells of special type. Hematoxylin-eosin stain. A: ×100, B: Enlarged picture of a part of A, ×400.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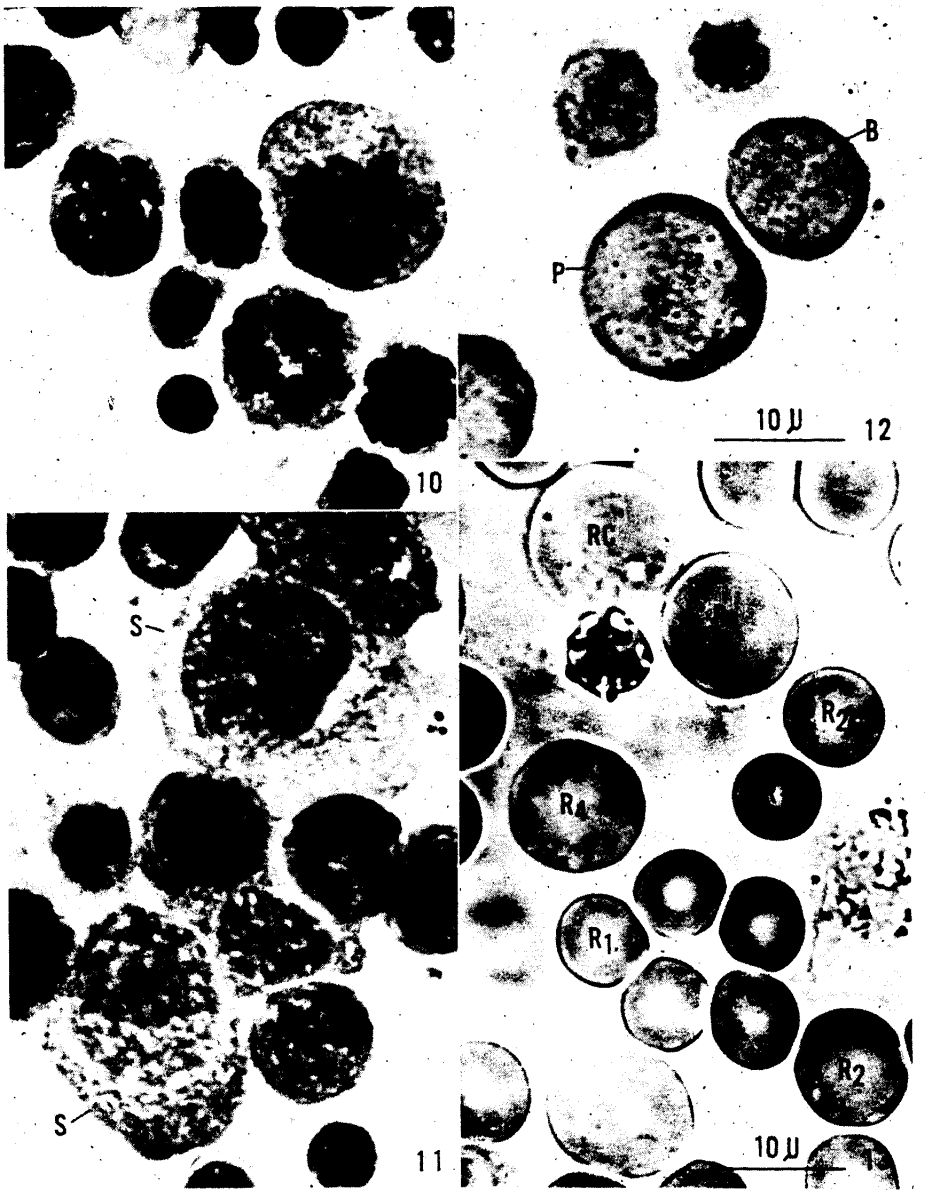

Fig. 10 Mitotic figures of basophilic erythroblasts 24 hours after red cell transfusion.

Fig. 11 The large size precursor cells (S) from the bone marrow of anemic rabbit. Smeared 24 hours after a mass red cell transfusion.

Fig. 12 The autoradiographic picture of TD^3H into the nuclei of erythroblasts in the pro-(P) and basophilic (B) stages.

Fig. 13 Macrocytes appeared in the circulating blood 24 hours after red cell transfusion. R_1: Normal size red cell. R_2 and R_4: Macrocytes resulting from the early denucleation of erythroblasts. RC: A reticulocyte just at denucleation. N: The extruded nucleus of erythroblast.

DISCUSSION

Through the observations introduced above it has been clearly demonstrated that the red cell proliferation stimulated in anemia can not be suppressed by the intravenous transfusion of a mass of red cells enough to bring promptly the complete recovery of anemia. Even after the transfusion the red cell number in the circulating blood continued to increase for a few days largely exceeding the original level.

Observations of the smears of hemopoietic tissue 24 hours after the red cell transfusion revealed a typical picture of erythroid marrow as in the case of anemic control, though the histologic picture of the bone marrow showed an enormous increase of reticulum cells of special type, which will be the stem cells inhibited to transform to proerythroblasts. The findings indicate that the prompt recovery of anemia renders it possible to stop the differentiation of the stem cell to proerythroblast but it does not give any suppressing effect on the cell division of erythroblasts, the cells on specialization.

Mitotic indices of erythroblasts and the incorporation of TD^3H into DNA observed 24 hours after the red cell transfusion reflected the morphologic picture showing the reduced rate of DNA synthesis and mitotic indices at the stage of large size erythroid precursors but no changes in the cells of advanced specialization stages, basophilic and polychromatic stages.

During 48 to 72 hours after the red cell transfusion, however, the increasing rate of the red cell number in the circulating blood dropped markedly as compared with that found 24 hours after the red cell transfusion. The mitotic indices and the synthetic rate of DNA also lowered markedly in all series of erythroid cells, pro- to polychromatic erythroblast[25]. The phenomenon may imply the elongated G_1 stage, the resting phase, of the erythroblasts, but it was found that the number of erythroblasts in the bone marrow decreased, especially in the tissue taken 72 hours after red cell transfusion. This fact suggested that under the suppressed DNA synthesis and cell division the erythroblasts did not stay in the resting stage but by denucleation they matured to red cells. That is, it means the denucleation of cells in immature state, or early denucleation.

As has been revealed by WEICKER[8] and SENO *et al.*[10,23,26] the proerythroblast matures to red cells through 4 cell divisions and at each cell division the erythroblasts reduce their nuclear and cell volumes by one half respectively. There fore, the early denucleation means the formation of macrocytes.

The Price Jones' curves drawn on the wet samples of red cells obtained before and after the transfusion of red cells gave the peaks indicating the appearance of macrocytes whose volume was 2 and 4 times as much as normal. The biggest ones observed 24 hours after red cell transfusion were as large as 8

times normal, suggesting the denucleation at early basophilic stage.

Cytophotometric estimation of RNA contents in each cell revealed that the RNA level of the largest reticulocytes is comparable to that of basophilic erythroblast in the early stage. The fact confirms that the biggest reticulocytes as large as 8 times normal volume are actually formed by the denucleation at early basophilic stage of erythroblast, because after denucleation reticulocyte does not synthesize RNA and its RNA contents are solely decreased with the maturation of the cells[23].

Thus, it has been clarified that the rapid increase in red cell number in the circulating blood after a mass transfusion of red cells into anemic animal is largely due to the uncontrolled mitosis of erythroblasts, but later they are suppressed in DNA synthesis and cell division with accelerated denucleation in the early stage of erythroid cell specialization.

SUMMARY

For the purpose to get the information about the control mechanism of erythropoiesis in bone marrow the author introduced a mass of homologous red cells into anemic animal and observed how the bone marrow cells and circulating blood react against the prompt normalization of the anemic condition.

After the red cell transfusion which was enough to restore the anemia promptly the red cell number in the circulating blood continued to increase until 72 hours after the transfusion, reaching an extremely high level in both red cell number and hemoglobin contents. Mitotic index and the DNA synthesis as observed by tritiated thymidine incorporation into DNA proved no actual change even 24 hours after the red cell transfusion, though a marked decrease in labeling index was found in large size precursors. Histologic picture revealed the proliferation of reticulum cells. 48 to 72 hours after the red cell transfusion both mitotic index and DNA synthesis of erythroblasts have largely retarded in all series of specialization with the decreased appearance of the erythroblasts in bone marrow sections. The measurements of red cell size and the RNA contents of erythroblasts and reticulocytes proved the accelerated denucleation at the early stage of erythroid cell specialization, as early as basophilic stage resulting in a marked macrocytosis.

ACKNOWLEDGMENT

The author is indebted much to Professor Satimaru SENO, for valuable advices throughout this work and painstaking proof reading of the paper. Many thanks are also due to Dr. MIYAHARA and the members of the Pathology Department for their helpful suggestions.

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