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Effect of mass blood-transusion on erythroid cell differentiation in anemic rabbits. II. Denucleation in the early stage of erythroid cell specialization, with special reference to RNA- and hemoglobin synthesis*

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Abstract

For the purpose of settling the specialization stage of erythroblast where the transcription for hemoglobin is initiated, the absorption of heme and the incorporation of tritiated uridine into RNA have been observed on the cells from the anemic rabbit after a mass red cell transfusion by which the DNA synthesis of large size precursors is suppressed and the early denucleation of erythroblasts is stimulated. In the erythroblasts obtained 24 to 72 hours after red cell transfusion a distinct absorption of heme appears first in the proerythroblast, followed by a progressive increase with the advance of the specialization. Hemoglobin synthesis is markedly stimulated after the denucleation. The incorporation of tritiated uridine into RNA is most marked in the proerythroblast and decreases with the advance of specialization stage suggesting that the mRNA synthesis for hemoglobin is initiated at the proerythroblast, continuing to the polychromatic erythroblast where. the synthesis is minimized. The volumetric observations indicate a possible denucleation at proerythroblast, but it has been revealed that the maximum RNA level of macrocytes is comparable to that of early basophilic erythroblast and its highest hemoglobin level is only that expected in the cells denucleated at late basophilic stage. From these observations it has been concluded that the transcription for hemoglobin is triggered at the initial step of erythroid cell specialization, proerythroblast, but it is insufficient for the synthesis of the expected amount of hemoglobin and is compensated or completed by the mRNA synthesis in more advanced stage of specialization.

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

II. DENUCLEATION IN EARLY STAGE OF ERYTHROID CELL SPECIALIZATION, WITH SPECIAL REFERENCE TO RNA- AND HEMOGLOBIN SYNTHESIS

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In the preceding paper[1], it was reported that the DNA synthesis and mitosis of erythroblasts, excepting large size precursors, in the bone marrow of anemic rabbit could hardly be suppressed by a mass blood transfusion into the vein even after 24 hours when the red cell number in the circulating blood increased tremendously. But after 48 to 72 hours DNA synthesis and cell division were suppressed with accelerated denucleation in the early stage of erythroid cell specialization. On the other hand, it has been reported by SENO and co-workers[2] that the big reticulocytes denucleated at polychromatic stage skipping one cell division mature to red cells which are double the hemoglobin content and the cell volume of normal ones. It is of interest to note at what stage of specialization the erythroblast acquires the ability of hemoglobin synthesis or transcription of messenger RNAs for hemoglobin. As pointed out in the previous paper[1], the appearance of the enormously large reticulocytes after red cell transfusion into anemic animal was suggestive of the denucleation at basophilic stage. Concerning the specialization stage where messenger RNAs for hemoglobin are transcribed, there seem to be two opinions; one insisting on mRNA synthesis in the early stage of specialization[3—6] and the other only in the later stage[7]. The controversy can be settled by ascertaining the problem at what stage of specialization the erythroblast can synthesize hemoglobin with nuclear RNA synthesis.

In view of this, the present studies were undertaken to observe the hemoglobin level and the RNA synthesis of erythroblasts and reticulocytes after a mass blood transfusion at the critical point of phenylhydrazine anemia of rabbit, because morphologically it was supposed that the cytoplasm of erythroblasts in the early basophilic stage turned somewhat eosinophilic indicative of the hemoglobin synthesis in this stage and the early denucleated big red cells contained a

large amount of hemoglobin.

In this paper it is reported that absorption of heme appears in the proerythroblast with the most active incorporation of uridine into RNA which suggests that the messenger RNAs for heme protein are elaborated at the initial step of the cell specialization.

MATERIALS AND METHODS

Seven male rabbits weighing 2.0—2.5 kg were used. Phenylhydrazine was given subcutaneously in a dose of 20 to 25 mg per kg per day in the concentration of 2.5 per cent for 3 successive days, by which red cell number in the circulating blood reached the level of 1.5—2.0 million per cu mm of blood. Three days after the last phenylhydrazine injection 5 of the 7 animals receiving the transfusion of a mass of homologous red cells suspended in saline (30 ml packed red cells in 30 ml saline) revealed the recovery of the red cell number to the original level before the phenylhydrazine injection. Two controls received no red cell transfusion but were injected only with saline in a comparable volume. Twenty-four hours after the red cell transfusion or saline injection the animals were sacrificed by blood depletion from juglar vein. Immediately after death the femurs were removed and the bone marrow was obtained from the bones whose epiphyseal ends were cut off on both sides and split longitudinally along the diaphysis. The fresh material was divided into three parts; one for histologic observation, another for microspectrophotometric estimation of heme and the third for the RNA synthesis by radioautography. The method of histologic observation and preparation of cell suspension may be referred to the previous paper[1].

For the microspectrophotometry of hemoglobin content per cell the bone marrow cell suspension was smeared on the cover slide ($0.18 \times 25 \times 50$ mm), dried and fixed with methanol. The measurements were made on the smeared cells at 409 mμ from xenon arc (Ushio UXL-150D) attached to the microspectrophotometer of the Olympus Kogaku Co. and employing the method devised by SENO and UTSUMI[8–12], i. e. using a spot light of 0.7 μ in diameter scanning was made along the cell diameter on the slide and the hemoglobin level per cell (C_{Hb}) was estimated by the following formula.

$$C_{Hb} = k \cdot \frac{S}{d} \cdot \frac{d^2\pi}{4} \text{ or } = k\frac{\pi dS}{4}$$

Where k = constant, d = cell diameter, $S = \int_0^d f(x)dx$, $f(x)$ = the curve obtained by connecting each extinction (E), $E = \log I_0/I_1$, I_0 = transmission at

blank, I_1 = transmission at the cell.

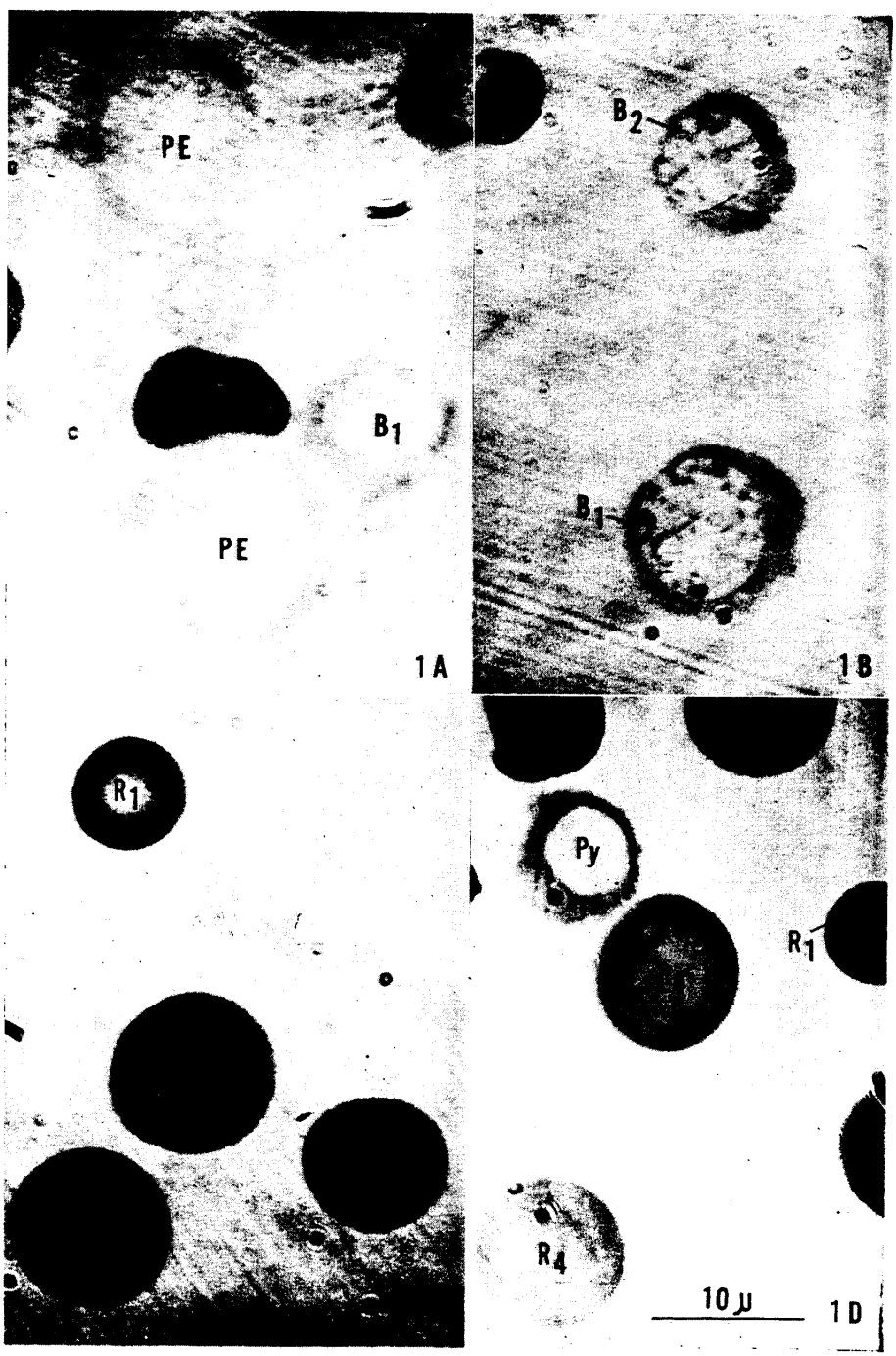
Photographs of the smeared cells were also taken at 409 mμ only for morphologic observation.

For the study of RNA synthesis in the nuclei of erythroblasts at each specialization stage, the incorporation of tritiated uridine into RNA was observed by radioautography. One ml of the bone marrow cell suspension was incubated with tritiated uridine (10 μc) and cold thymidine (1 mM) at 37°C for 30 minutes, and the cells were smeared, fixed with methanol, treated with DNAase or perchloric acid and then mounted with nuclear emulsion SAKURA NR M-1. After 2 weeks' exposure the films were developed and the cells were stained with Giemsa. The tritiated uridine, specific activity 2.7 c per mM, was obtained from the Radiochemical Center in England. The details of the methods for radioautography and others may be referred to the previous publication[1].

OBSERVATIONS AND RESULTS

The morphologic observations of the smeared unstained cells at 409 mμ revealed the absorption of heme in the cytoplasm and interchromatin space in the nuclei of erythroblasts (Plate 1-A, B), and a marked absorption on the denucleated cells (Plate 1-C, D). The largest erythroblast showing the absorption of heme in the cytoplasm as well as in the nucleus was the proerythroblast and the intensity of absorption increased with the advance of specialization stage or with decrease in cell diameter. Among the proerythroblasts those indicating the heme absorption were rather small in percentage but frequently encountered in the materials taken after red cell transfusion and hardly encountered in those from anemic controls. They were 12—13 μ in nuclear diameter and had a distinct Golgi zone on one side of perinuclear area, where heme absorption was lacking (Plate 1-A).

Microspectrophotometric estimation of hemoglobin content per cell revealed that hemoglobin level of erythroblasts was higher in larger cells and showed the tendency to decrease with the advance of specialization stage or with the decrease in cell diameter. In the denucleated cells very high values were obtained in the larger cells and minimized values in smaller cells. The highest hemoglobin level of the denucleated cells 10—11 μ in diameter was nearly 4 times that of normal red cells 6—7 μ in diameter (Fig. 1-A). The highest hemoglobin level of the denucleated red cells at each group of macrocytes and normal size cells was about 4 times that of the erythroblasts from which the denucleated red cells were formed, indicating about 3/4 of the expected amount of hemoglobin is synthesized after denucleation. A similar tendency was also found in anemic controls (Fig. 1-B) and there was no significant difference among these taken at


PE
B1
PE
1A
B2
B1
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R1
Py
R1
R4
10 μ
1D

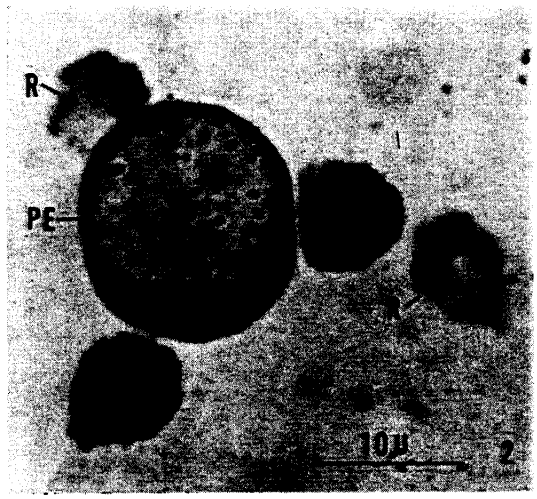


Plate 1-A, B, C, D The macrocytes and erythroblasts from anemic rabbit 24 hours after red cell transfusion, photographed at 409 mμ. PE: Proerythroblast. B_1: Early basophilic erythroblast. B_2: Late basophilic erythroblast. Py: Polychromatic erythroblast. R_1: Normal size red cell. R_2 and R_4: The macrocytes resulting from the early denucleation of erythroblasts.

Plate 2. The radioautographic picture of tritiated uridine incorporated into the nuclei of erythroblasts. The nucleus of proerythroblast (PE) is most heavily labeled and the grain appears diffusely on the nucleus. No RNA synthesis in the reticulocytes (R).

24, 48 and 72 hours after red cell transfusion.

RNA synthesis observed by *in vitro* incubation with tritiated uridine and radioautography proved to be the highest in the proerythroblast and to decline successively with the advance of cell specialization, being minimized at orthochromatic stage (Fig. 2-A). No RNA synthesis was observed in reticulocytes though they had some RNA (Plate 2). Such a pattern of RNA synthesis of erythroblasts showed no appreciable change after the red cell transfusion comparing to the anemic control (Fig. 2).

Histologic change of the bone marrow and the Price Jones' curve have been also checked in this experiment and actually the identical results were obtained as in the former experiment[1], i.e. the reticulum cells of special type became predominant in the bone marrow after red cell transfusion and the Price Jones' curve gave 4 peaks indicating the anisocytosis composed of the cells denucleated at orthochromatic, polychromatic and late basophilic stages. Some cells were especially large in size and suggested denucleation at early basophilic stage or even at proerythroblast, whose volume was comparable to the cytoplasmic

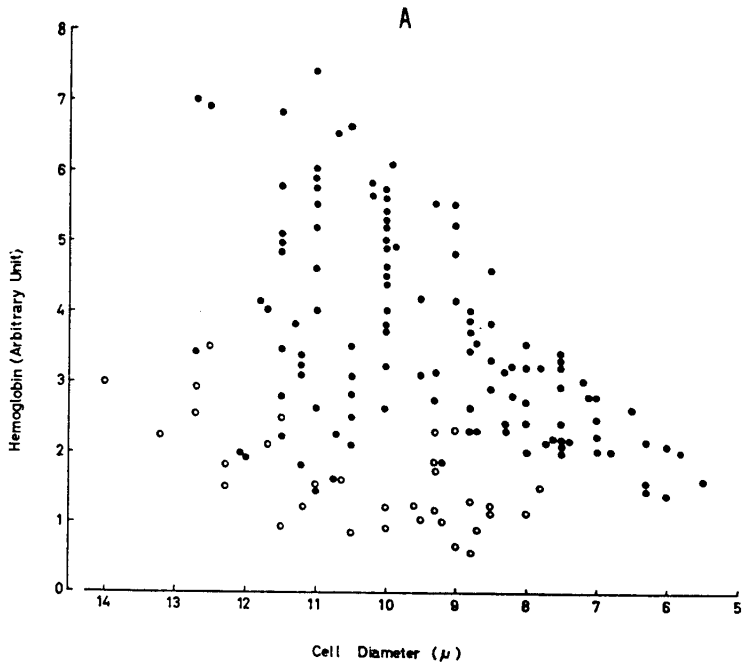


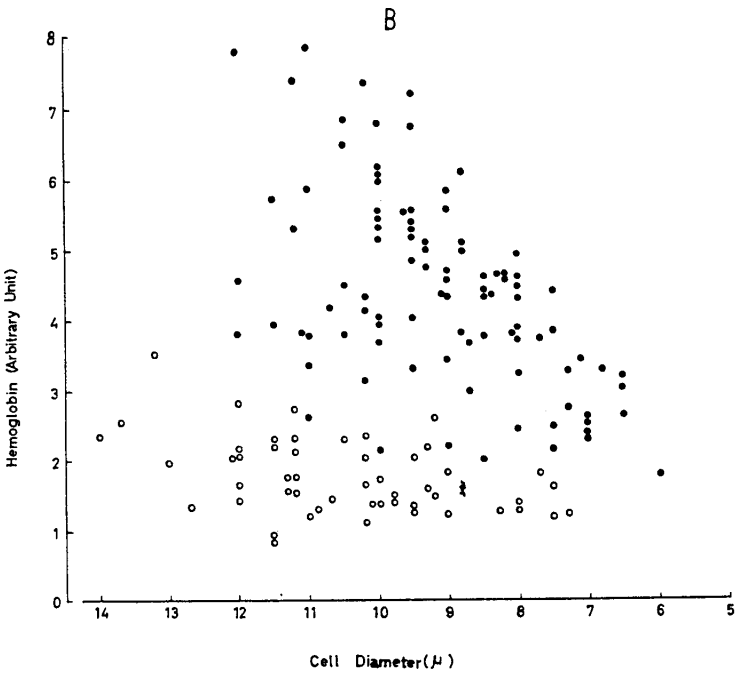
Fig. 1-A. B The hemoglobin contents of erythroblasts, reticulocytes and red cells in the bone marrow from the anemic rabbit 24 hours after red cell*

volume of pro- and early basophilic erythroblasts.

DISCUSSION

Through this experiment it has been revealed that the erythroblast acquires the capacity to synthesize heme or hemoglobin in its initial step of specialization, the stage of proerythroblast. This has been clearly demonstrated on the cells from the bone marrow taken after a mass transfusion of red cells to the anemic rabbit, where DNA synthesis and cell division of the erythroblasts were suppressed and the denucleation was accentuated at an early stage of specialization, producing a large amount of macrocytes with decrease in the number of erythroblasts, especially in those of early stage of specialization, as is obvious from the histologic section and bone marrow smear[1].

As the proerythroblast having the heme absorption can hardly be encountered in phenylhydrazine anemia, it seems that the mass transfusion of red cells stimulates the hemoglobin synthesis which will be suppressed to some extent in



*transfusion (A) and from the anemic control (B). Solid circles: Red cells and reticulocytes. Open circles: Erythroblasts.

general anemic erythropoiesis. The mechanism may be related to the level of oxygen tension in the blood as the hemoglobin synthesis is largely dependent on the energy liberated by oxidative phosphorylation[6,13].

Thus, it is obvious that the transcription for hemoglobin synthesis is made at the initial step of specialization. The most marked incorporation of tritiated uridine into RNA in the nucleus is found in the initial stage of specialization and decreases with the advance of cell specialization. The synthesized RNA will largely be composed of mRNAs for hemoglobin[14–16]. The data are consistent with that of GRANICK[17] and associates[18] clarifying that the δ-ALA synthetase for heme is activated when the differentiation of stem cell is induced by erythropoietin.

Hemoglobin content per cell is higher in the erythroblasts in an early stage of specialization and decreases with the advance of the specialization stage. In order to obtain the accurate information for initiation of transcription for heme or hemoglobin synthesis it may be helpful to observe the relation between the early denucleation and the hemoglobin level of early denucleated cells. As

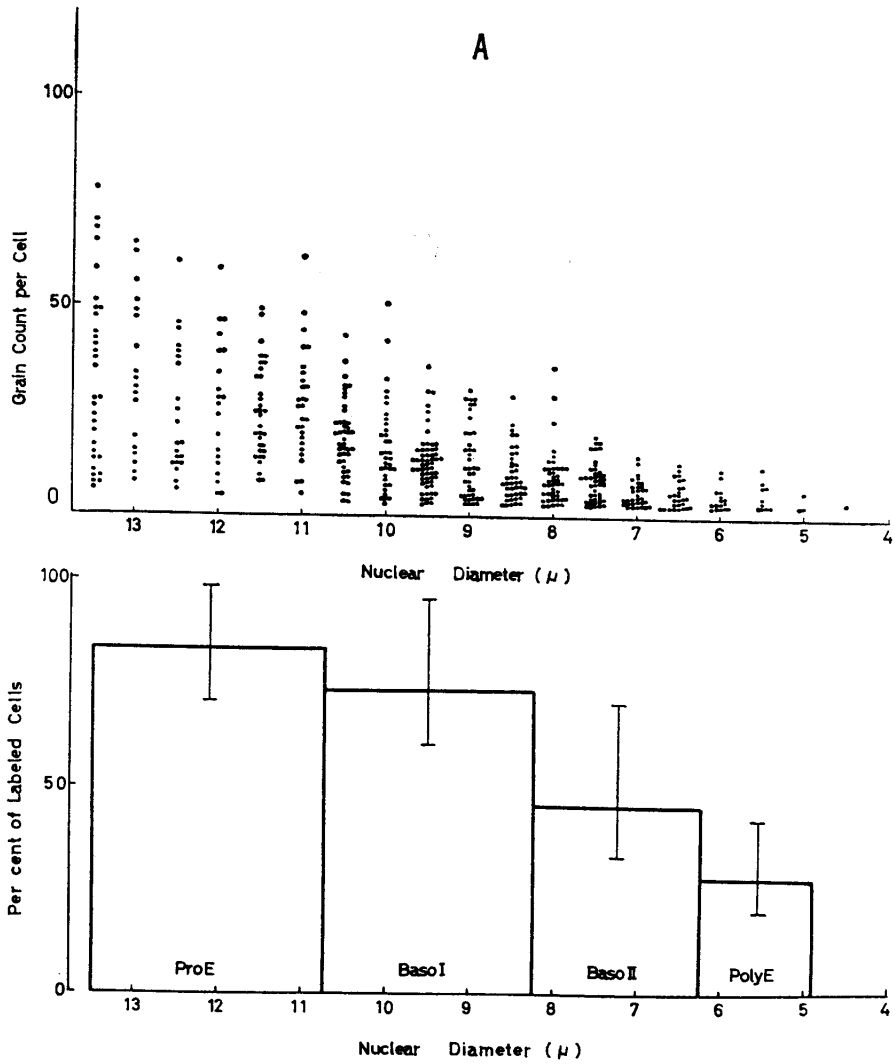
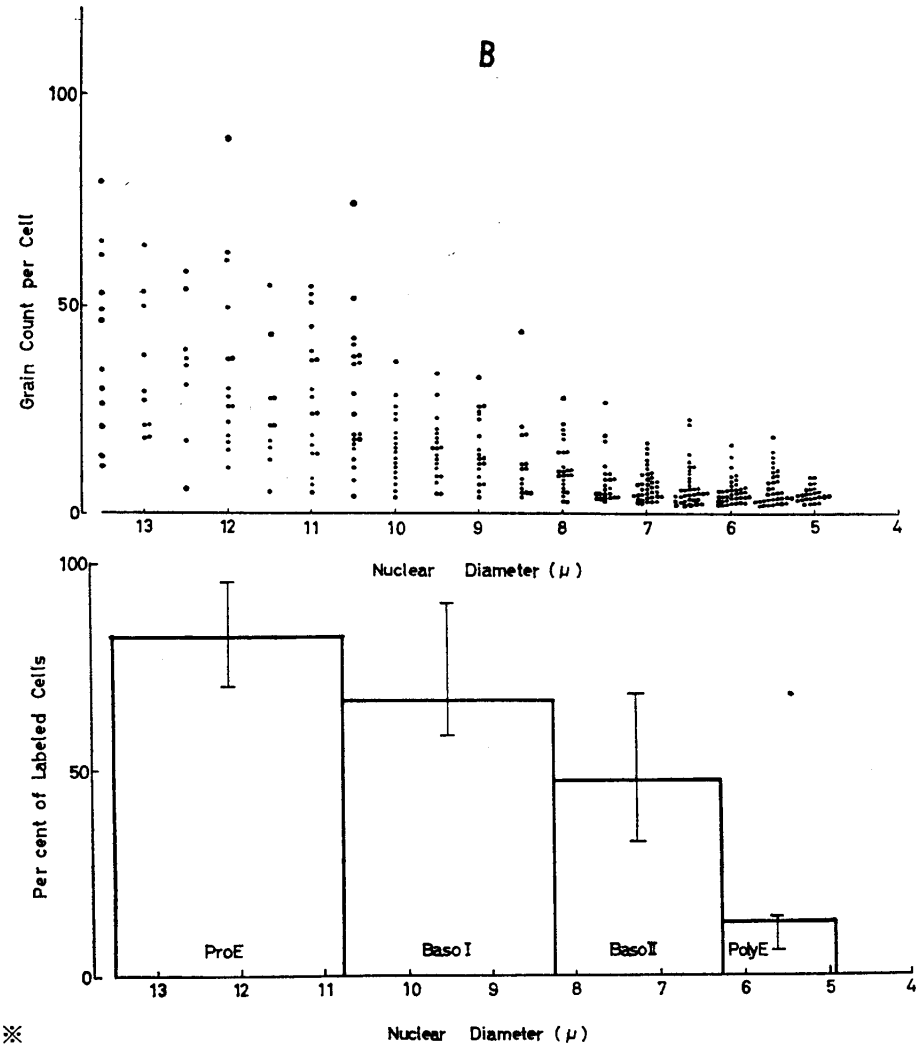


Fig. 2-A, B The incorporation of tritiated uridine into RNA of erythroblasts. The upper figures are the scatter diagram for grain counts of labeled erythroblasts as classified by the nuclear diameter, and the lower ones are labeling indices ※

the volume of round living cell is proportional to the diameter of smeared cell as revealed on lymphocytes by SHIGEHISA[19], the cell volume of erythroblast and reticulocyte, both of which are round, [20,21] can be estimated from the diameter of smeared cell. The volume of cytoplasm can also be calculated by subtracting the nuclear volume from the cell volume, because the denucleation means the extrusion of the nucleus as a whole[22–26]. In the present experiment, the volume of the largest denucleated cell (11—12 μ) was comparable to that of the cytoplasm



of erythroblasts at each specialization stage. A: Anemic control. B: 24 hours after a mass red cell transfusion.

of proerythroblast, as supposed by LAJTHA and others[27,28]. But the highest hemoglobin level attained by the denucleated cells is 4 times the normal and just the amount to be expected of the cells denucleated at the late basophilic stage. The highest RNA level of the reticulocyte is comparable to that of early basophilic stage as reported in the previous paper[1]. As the reticulocyte does not synthesize RNA[2,29], this value indicates that the earliest denucleation will occur at an early basophilic stage.

Thus the volumetric estimation suggests that the earliest denucleation will take place at the stage of proerythroblast, the studies on RNA level of reticulocyte indicate that the denucleation can be induced at an early basophilic stage at the earliest and the studies on hemoglobin content per cell indicate the late basophilic stage to be as the possible earliest stage of denucleation. Among these, the results obtained by the volumetric estimation may be most reliable, because the denucleation mechanism indicates that the volume of reticulocyte is just identical with that of cytoplasm of erythroblast from which the reticulocyte was formed. Some RNAs may be lost on the extrusion of the nucleus and then a lower level of RNA than expected may be found in the reticulocyte. The lower level of the hemoglobin in the macrocyte, 4 times the normal at the highest, may be related to the incompleteness in transcription for the synthesis of the expected amount of hemoglobin at the final maturation stage, or a shorter life-span of mRNA than the period required for the maturation of proerythroblasts to red cells through 4 cell divisions. The fact that RNA synthesis continues to the later stage of specialization of erythroblast seems to indicate that it is essential to compensate the decayed mRNA or supplement the incomplete mRNA synthesis in the early stage of specialization.

SUMMARY

For the purpose of settling the specialization stage of erythroblast where the transcription for hemoglobin is initiated, the absorption of heme and the incorporation of tritiated uridine into RNA have been observed on the cells from the anemic rabbit after a mass red cell transfusion by which the DNA synthesis of large size precursors is suppressed and the early denucleation of erythroblasts is stimulated.

In the erythroblasts obtained 24 to 72 hours after red cell transfusion a distinct absorption of heme appears first in the proerythroblast, followed by a progressive increase with the advance of the specialization. Hemoglobin synthesis is markedly stimulated after the denucleation.

The incorporation of tritiated uridine into RNA is most marked in the proerythroblast and decreases with the advance of specialization stage suggesting that the mRNA synthesis for hemoglobin is initiated at the proerythroblast, continuing to the polychromatic erythroblast where the synthesis is minimized.

The volumetric observations indicate a possible denucleation at proerythroblast, but it has been revealed that the maximum RNA level of macrocytes is comparable to that of early basophilic erythroblast and its highest hemoglobin level is only that expected in the cells denucleated at late basophilic stage.

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