Studies on the DNA metabolism of erythroid cell. I. DNA level of erythroblastic nuclei of rabbit bone marrow, observation of normal, blood depleted, and phenylhydrazine anemias, and their recovery by red cell transfusion

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Studies on the DNA metabolism of erythroid cell. I. DNA level of erythroblastic nuclei of rabbit bone marrow, observation of normal, blood depleted, and phenylhydrazine anemias, and their recovery by red cell transfusion

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Abstract

For the purpose to reveal the changes in the metabolism of erythroblast in varied specialization stages the author observed the Feulgen DNA level of rabbit erythroblasts by microspectrophotometry. Observations were made on normal and anemic animals, and those receiving a mass red cell transfusion at the recovery stage of anemia where the early denucleation is stimulated. Observations have revealed that in normal erythropoiesis the DNA contents are kept at n to 2 n level from the proerythroblast to late basophilic stage, but in later stages, polychromatic and orthochromatic, DNA level per cell decreases gradually with advance of the cell specialization reaching the minimum level, nearly haploid level, at orthochromatic stage where most cells are believed to be denucleated. In blood depleted animals nearly the same pattern of DNA level was observed in connection with erythroid specialization as that in normal animal, except a relatively high DNA level in the later specialization. In the cases of the hemolytic anemia a similar tendency has been observed but the minimum level of DNA remains at a higher level, hypo-diploid level, in poly- and orthochromatic stages. Twenty-four hours after the mass red cell transfusion by which severe anemia has been recovered to the original level within one hour, the pattern of the DNA level of the erythroblast returns to the normal one showing a very low DNA level at the polyand the orthochromatic stages. The data indicate that the DNA synthesis of erythroblast kept at n to 2 n levels until the late basophilic stage begins to decline at polychromatic stage and reaches nearly haploid level at orthochromatic stage, but in active hemopoiesis the DNA synthesis is stimulated and the DNA contents are kept at a high level even in the late specialization stages, showing no relation between the denucleation and the low DNA level.
STUDIES ON THE DNA METABOLISM OF ERYTHROID CELL

I. DNA LEVEL OF ERYTHROBLASTIC NUCLEI OF RABBIT BONE MARROW, OBSERVATION OF NORMAL, BLOOD DEPLETED, AND PHENYLHYDRAZINE ANEMIAS, AND THEIR RECOVERY BY RED CELL TRANSFUSION

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The mammalian erythroblast is the only cell that loses nucleus physiologically. The erythroid cells are thought to differentiate from the stem cell, whose morphologic entity is not yet defined. As has been studied by many workers, the stem cell differentiates to erythroblasts by the action of erythropoietin, then they mature to orthochromatic erythroblasts repeating cell divisions 4 times, and the red cells are formed by a peculiar cell division in which a cell is divided into the cytoplasmic and nuclear halves. As pointed out by WEICKER (1, 2, 3,), the nuclear volume reduces itself by about one half at each cell division. The cell volume is also reduced by about one half at each cell division as reported by MIYAHARA and SENO (4, 5). These characters specific to erythroblasts make it easy to recognize the specialization stage of each cell from their nuclear and cell sizes on smear. By the observation on Chinese hamster WEICKER (6) demonstrated clearly, that the chromosome number of erythroblast decreased gradually with the advance of cell specialization reaching haploid level at the terminal stage. KONO (7) also observed the decrease of chromosome number in human erythroblasts in the advanced specialization stages. It has been reported that in erythroblast DNA is replicated at each cell division but synthetic rate decreases with advance of cell specialization (8). This seems to be consistent with the data indicating the decrease in chromosome number. But it is completely obscure why such a peculiar decrease in DNA synthesis and chromosome number occurs in erythroblast and how it relates to the denucleation. DNA level also decreases with erythroid cell specialization. This also reflects the decrease in chromosome number and DNA synthetic rate in more specialized cells.

In connection with these facts, the author aimed to reveal the exact stage where the revolutionary changes occur in DNA metabolism of erythro-
blast to get a reliable information of the possible relation between DNA metabolism and denucleation of erythroid cells. This paper deals with the Feulgen DNA level of the erythroid cells in varying specialization stages from normal, blood depleteted and phenylhydrazine anemias and those receiving a mass red cell transfusion on the recovery stage of anemia, and indicates that DNA level per cell is kept at the diploid level until late basophilic stage and then it begins to decrease at the polychromatic stage reaching nearly haploid level at orthochromatic cells, with some higher DNA level in anemic condition. Observations suggested no close correlation between the decrease in the DNA level and the denucleation.

MATERIALS AND METHODS

Eighteen male adult rabbits, 2.5 to 3.0 Kg. were used: 3 animals in each of 6 series of experiments: (1) normal, (2) blood depleted anemia, (3) phenylhydrazine anemia, (4) 24 hours (5) 48 hours and (6) 72 hours after red cell transfusion in the early recovery stages of phenylhydrazine anemia. Blood depleted anemia, about 2 million R. B. C. per cumm of peripheral blood, was induced by drawing blood from ear vein, 20 to 25 ml/day for 9 days. In all animals the whole blood drawn from ear vein was immediately returned to the depleted animal by injecting it subcutaneously to prevent the suppressed hemopoiesis by the deficiency of protein and iron. Red cell number in the circulating blood was counted, at certain intervals to check the grade of anemia. Animals were sacrificed on the day after the last blood depletion, and the fresh bone marrow tissue was taken.

For haemolytic anemia phenylhydrazine chloride was injected subcutaneously, 0.5 ml of 2.5 % solution per kg of body weight, daily for 3 days. Through this treatment a severe anemia, less than 2 million R. B. C. per cumm, developed. Two days after the last injection these animals were sacrificed to obtain bone marrow cells. Nine animals of remaining 3 groups were also treated with phenylhydrazine by the same method as just described and two days after the last injection they received the intravenous transfusion of homologous red cell suspension, red cells contained in 150 ml of whole blood to one animal within 30 minutes, by which the red cell count recovered to the original level within one hour. The animals were sacrificed 24, 48 and 72 hours after the red cell transfusion, 3 animals each.

Homologous blood for transfusion was obtained from normal rabbit, generally two animals for one anemic rabbit. About 150 ml of blood was obtained from the carotid of two animals with sterilized anticoagulant, 3.8 % sodium citrate solution. Immediately after drawing the blood was centrifuged at 3,000 r. p. m. for 10 minutes and the precipitated cells were washed with cold saline (4°C) three times by repeated centrifugation and the cells were resuspended in the saline of the same quantity as the decanted plasma. The freshly prepared red cell suspension was warmed at 37°C and transfused slowly into the ear vein of the animal of phenylhydrazine anemia. By sacrificing the animal after 24, 48, and 72 hours
the fresh bone marrow tissue, approximately 1 g, was obtained from the femur. The fresh tissue was taken into a glass homogenizer, added with about 5 ml of rabbit serum and the cells were freed from tissue by removing the core gently up and down 2 to 3 times. The cell suspension thus prepared was filtered with gauze, centrifuged at 1,000—2,000 r. p. m. for five minutes and the precipitate was resuspended in 3 ml of rabbit serum. This bone marrow cell suspension was smeared on a coverslide of 24×60 mm and 0.13—0.17 mm in thickness. The smears were dried, fixed with formol gas for about 20 minutes at room temperature and stained with Feulgen reaction by the method of Shibatani (9, 10) and the DNA level of each cell was estimated by microspectrophotometry (MSP) employing two-wave length methods (11, 12, 13, 14, 15, 16, 17, 18) at 480 mμ and 560 mμ (Fig. 1.)

The apparatus used was Olympus MSP, type 2001. The method for the estimation may be referred to the paper of Seno and Utsumi (10, 20, 21, 22). In each sample DNA contents per cell were measured on more than 250 cells, about 50 cells in every specialization stage, proerythroblasts (13—14 μ in diameter), early basophilic erythroblasts (10—12 μ), late basophilic ones (8—10 μ), polychromatic (6—8 μ) and orthochromatic ones (4—6 μ). Besides these, a general smear was made with each sample. These were fixed with methanol and stained with Giemsa for the morphologic observation of erythroid cells.

RESULTS

In the case of non-anemic rabbit, the erythroblasts in the early specialization stages had DNA at n to 2n level through the stages of proerythroblast, early and late basophilic ones, but in the cells smaller than 7 μ in nuclear diameter or polychromatic and orthochromatic erythroblasts, DNA contents decreased gradually with the advance of maturation reaching the minimum level, nearly the haploid level, at orthochromatic stage (Fig. 2).

In blood depleted anemias the distribution pattern of the DNA per cell was the same as that of non-anemic animals from the period of proerythroblast to that of late basophilic ones, but somewhat higher DNA level was observed in poly- and orthochromatic stage, except only in a few cells which showed the DNA at haploid level (Fig. 3).

In phenylhydrazine anemias, the DNA contents of proerythroblasts
Fig. 2 DNA contents of erythroblasts from a normal rabbit bone marrow.

Fig. 3 DNA contents of erythroblasts from blood depleted anemic rabbit bone marrow.
and early and late basophilic ones were found again in the range between n and 2n, showing no abnormalities, and yet DNA contents of polychromatic and orthochromatic erythroblast showed a level higher than those of blood depleted anemia. It stayed nearly at the diploid level even in orthochromatic erythroblast and the cells having haploid DNA were hardly encountered (Fig. 4).

Fig. 4 DNA contents of erythroblasts from a phenylhydrazine anemic rabbit bone marrow

The transfusion of a mass of red cells, about 150 ml of blood, into the animals of phenylhydrazine anemia, 1.5—2.0 million R. B. C. cumm, resulted in prompt recovery of anemia to the original R. B. C. level, but thereafter the red cell number increased by about 1 to 2 million incessantly for 72 hours of the red cell transfusion, and then reached an equilibrium at an extremely high level of red cell number. The distribution of DNA level of erythroblasts 24 hours after the red cell transfusion gave a quite similar one to that from non-anemic (Fig. 4) showing a distinct difference from that of anemic animal. That is the DNA of erythroblasts of the red cell transfused animals showed a very low level at polychromatic and orthochromatic stages, though DNA contents of pro- and basophilic eryth-
Erythroblasts remained always unchanged (Fig. 4).

Observations were also made on the erythroblasts from the animals 48 and 72 hours after the red cell transfusion, but the distribution pattern of DNA level of erythroblast from these animals showed always a similar tendency (Fig. 5), and gave patterns quite similar to those obtained 24 hours after the red cell transfusion.

Morphologic observations on the Giemsa stained bone marrow and peripheral blood smears of anemic animals revealed a marked macrocytosis with many big red cells of around 8 μ in diameter. This was quite distinct in phenylhydrazine anemia, where normal sized cells were rarely encountered, suggesting that most red cells were denucleated at polychromatic stage skipping one cell division (5, 23, 24, 25). Twenty-fours after the red cell transfusion into phenylhydrazine anemia the macrocytosis was accentuated with appearance of large sized cells of 12 to 13 μ in cell diameter, which should be denucleated in the early stage of basophilic erythroblast (26, 27).
DNA Level of the Erythroblastic Nuclei

Fig. 6 DNA contents of rabbit erythroblasts in phenylhydrazine anemia 48 hours after red cell transfusion.

Fig. 7 DNA contents of rabbits erythroblasts in phenylhydrazine anemia 72 hours after red cell transfusion.
As pointed out by SENO and others (28, 29, 30), it is clear that in erythroblast the nucleus controls the somatic protein synthesis in some way. By denucleation or by cell division into nuclear and cytoplasmic halves, the maturation of the cytoplasmic half is accelerated with enhanced hemoglobin synthesis and the disintegration of cytoplasmic organelles, mitochondria, ribosomes, and degradation of some chemical components like RNA. Thus, the mechanism of maturation should not be independent of the function of nucleus, and will be reflected in the DNA metabolism. As just mentioned briefly, the decrease in the chromosome number of erythroblast with the advance of cell specialization has been elucidated in hamster (6) and in man (7, 31, 32). And it has been also shown that the rate of DNA synthesis as observed by the incorporation of H³-thymidine decreases with advance of the cell specialization (33, 27). These processes, the cell division with insufficient DNA synthesis, inevitably results in the decrease in DNA level by cells in advanced specialization stage. WEICKER (6), and KINOSITA and OHONO (34) reported a gradual decrease in the chromosome number during the erythroid cell specialization, and SENO, MIYAHARA and others (33) reported the exponential decrease in the incorporation rates of H³-thymidine in specialization. These facts seem to suggest the gradual decrease of DNA contents through whole stages of cell specialization. But, as demonstrated in the present experiments the DNA level seems to be kept at n to 2n levels till the late basophilic stage in every animal, and only in normal or non-anemic hemopoiesis the DNA level declines steadily up to maturation during poly- and orthochromatic stages, where DNA of some cells reaches near haploid level. That is, solely in non-anemic hemopoiesis of rabbit, abnormality in the DNA level occurs in the poly- and orthochromatic stages. This means that incomplete DNA replication or the irregularity in chromosome number will appear first at late basophilic stages. However, there seems to be no sharp distinction between the diploid cells and haploid ones.

DNA decreases gradually giving varying values between diploid and haploid levels. The data are consistent with those reported by WEICKER (6) in man and by KINOSITA and OHONO (34) in rat erythroblast where chromosome number showed a wide variety between diploid and haploid levels.

The mechanism is not clear but there seem to be two possibilities. One is the segregation of some chromosomes during mitosis and other is the failure in DNA replication in S-phase resulting in the formation of
unpaired chromosomes. As the reticulocyte, or the cytoplasm of erythroblast does not have any Feulgen positive substance, the segregation of chromosome during mitosis is unlikely, but the incomplete DNA synthesis followed by mitosis will possibly result in such an irregular way in chromosome number. This means that the nuclei of the erythroblast at poly-and orthochromatic stages will be incomplete in genetic information.

In this sense, the nuclei of these cells may have not a function like the general cell nucleus as pointed out by Joseph (25), but these cells will need nuclei for their cell division, by which the cell size becomes smaller and the surface for unit hemoglobin increases making the gas metabolism efficient. After denucleation the cells do not divide, as revealed by Lewenstein (35). One of the important biological problems concerning erythroid cell specialization is the denucleation. The mechanism has been completely revealed by the work of Awai, Seno and others (36). That the denucleation is attained by the cell division which divides the cell into nuclear and cytoplasmic halves without being accompanied by any karyolytic process but it is quite obscure why such a peculiar cell division ensues in erythroblasts. One of the purposes of the present work is to find a possible relation between the denucleation and the abnormality in DNA metabolism, as the decrease in DNA level occurs at poly- and orthochromatic stages where denucleation ensues in normal hemopoiesis.

As reported by Takabayashi, Matsuoka, Seno and collaborators (22, 26, 27) early denucleation ensues in anemia. Therefore, some decrease in DNA level in the early stage of erythroid specialization would be expected in anemia, but the estimation gave an unexpected result as indicated in Figs. 3 and 4, DNA levels staying at higher level in severe anemia than in non-anemic cases, showing that DNA synthesis of erythroblast is stimulated in anemic individuals. The data have clearly indicated that there is no relation between the denucleation and the decrease in DNA level at the terminal stage of erythroid cell specialization.

**SUMMARY**

For the purpose to reveal the changes in the metabolism of erythroblast in varied specialization stages the author observed the Feulgen DNA level of rabbit erythroblasts by microspectrophotometry.

Observations were made on normal and anemic animals, and those receiving a mass red cell transfusion at the recovery stage of anemia where the early denucleation is stimulated. Observations have revealed that in normal erythropoiesis the DNA contents are kept at n to 2n level from the...
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The data indicate that the DNA synthesis of erythroblast kept at n to 2n levels until the late basophilic stage begins to decline at polychromatic stage and reaches nearly haploid level at orthochromatic stage, but in active hemopoiesis the DNA synthesis is stimulated and the DNA contents are kept at a high level even in the late specialization stages, showing no relation between the denucleation and the low DNA level.

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