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Abstract

For the purpose of revealing whether AMD inhibits the RNA synthesis of erythroblasts in an effective dose in vivo to eradicate erythroid cells in rabbit bone marrow, the author observed the RNA synthesis by H3-uridine incorporation in vitro and RNA level on the cells from the anemic animals taken at a certain period after a single injection of AMD in a small dose of 50 and 100μg/kg body weight. The data revealed that by such a small dose of injection of AMD the RNA synthesis of erythroid precursors, early basophilic and proerythroblast stages, was successfully suppressed without any suppressing effect on the RNA synthesis of erythroblasts in the later stages of specialization, indicating that there are at least two kinds of RNA synthesis: one seen mainly in the earlier stages of specialization and the other one seen mainly in the later stages, and they can be distinguished from each other by the AMD sensitivity.

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SUPPRESSION OF ERYTHROPOIESIS BY ACTINOMYCIN D
II. THE CHANGE IN RNA METABOLISM BY ACTINOMYCIN D ADMINISTRATION

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In the previous paper it was reported that the sensitivity of hemopoietic organ to actinomycin D (AMD) *in vivo* administration is considerably variable between different species of animal, e. g. mouse and rabbit were highly sensitive but rat was markedly resistant. In the AMD sensitive animals the differentiation of the stem cell to proerythroblast was completely inhibited at a dose of 50μg/kg but the specialization process of the erythroblasts was not suppressed (1). Thus one day after the AMD administration in effective dose, only the erythroblasts in advanced maturation stage were found and after 2 to 3 administrations once a day, the erythroblasts completely disappeared (1, 2. 3). Such a drastic effect of AMD on hemopoiesis of sensitive animal should be due to the specific affinity of AMD to erythropoietin, but it may be expected that RNA metabolism is also affected by the drug, as found originally, AMD binding with DNA molecule and inhibiting the synthesis of DNA-dependent RNA (4).

To settle this problem the author observed the RNA synthesis of the erythroid cells from AMD treated rabbit by flush labeling with H^3-uridine *in vitro* and radioautography. RNA content per cell was also observed by microspectrophotomery on these cells on the smear stained with azure B.

In this paper it is reported that in the early stage of erythroid cell specialization, RNA synthesis was suppressed but not in the later stage. RNA level of each cell reflected the suppressed RNA synthesis in the early stage of cell specialization.

MATERIALS AND METHODS

Eight white adult rabbits of both sexes, weighing about 2kg, were used. They were pretreated with repeated injections of 2.5% neutralized phenylhydrazine, 0.75 to 1 ml/kg subcutaneously daily for consective 3 days. Three days after the last injection of phenylhydrazine, 4 animals received a single subcutaneous injection of AMD (Merck-Banyu), 50 μg or 100 μg/kg. Two of them were

sacrificed 12 hours after AMD administration, 50μg/kg. The other two, 24 hours after AMD administration, 50 μg/kg. The remaining two also 24 hours after AMD administration, 100 μg/kg. RNA synthesis by radioautography was observed on the cells from the animals receiving the injection of 50 μg/kg AMD and RNA content per cell was estimated on those from the animals receiving 100 μg/kg AMD injection. Two rabbits served as anemic control.

For the observation of RNA synthesis by ridioautography, fresh femur bone marrow tissue was added with a small amount of the homologous serum and the cells were freed from the tissue by using glass homogenizer, moving gently. One ml of the cell suspension thus obtained was added with H^3-uridine (specific activity 2.7c/mM, Radiological Centre, England), 10 μc/ml, with cold thymidine, 10 mM/ml. and incubated at 37°C for 1 hour. After incubation the cells were washed twice with Hanks-serum mixture (1 : 1 v/v) by repeated centrifugation and resuspended in the homologous serum. One drop of the cell suspension was smeared, fixed with ethanol and dried. After drying the smears were mounted with nuclear emulsion for radioautography (Sakura NR-M2, diluted with an equal volume of distilled water at 40°C). Then the smears were kept standing vertically in the moist chamber (28°C) for 20 minutes and dried at room temperature for 24 hours. The exposure was carried out for one to two weeks in a silica gel chamber at 3°C. After exposure the samples were developed with "Konidol X" at 20°C for 5 minutes, fixed with "Fuji Fix". After fixation these samples were washed under tap water for two hours, dipped in a phosphate buffer (1/5M, pH 5.0) at 30°C for about 15 minutes and stained with Giemsa (Merck). Grain counts were taken of the individual nucleated erythroblasts and two grains were required as the limiting grain count per labeled cell. The nuclear diameter and the grain number were recorded in each cell.

RNA levels of erythroblasts were estimated on smeared cells by microspectrophotometry. For microspectrophotometry the bone marrow smears were prepared on a cover slide, 0.18×25×50mm, with the bone marrow cell suspension as just described, and stained with azure B by the slightly modified method of FLAX and HIMES (5). The smeared cells on cover slide were dried, fixed with methanol and treated with deoxyribonuclease (DNase) solution (crystalized Worthington DNase, 1mg per ml of Gomori's Tris buffer containing 0.2M $MgSO_4 \cdot 7H_2O$, pH 5.7) for 24 hours at 37°C (6) (on one sample the effect of DNase was tested, revealing negative Feulgen reaction). After DNase treatment the smeared cells were washed with tap water for 30 minutes, dried, stained with azure B (National Aniline Division, USA) for 3 hours at 40°C and differentiated in tertiary butyl alcohol for 18 hours at 37°C. The azure B was used as 0.025% solution in McIlvain's buffer at pH 4.0. On these samples RNA level was estimated by using the microspectrophotometer of Olympus Kogoku Co. employing the two-wavelength method deviced by ORNSTEIN (15) and PATAU (16) independently and developed by MENDELSOHN (17) 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 (7).

RESULTS

RNA synthesis of erythroblasts from anemic controls as observed from the grain number per cell after *in vitro* incubation with H^3-uridine and radioautography proved to be the highest in the proerythroblast and to decline exponentially with the advance of cell specialization stages, being minimized at orthochromatic stage. In AMD treated animals the grain count of the cells at more advanced stages were nearly the same as those of the cells in the similar specialization stages in anemic control, but in younger erythroblasts of nuclear diameter of 10 to 13 μ, the grain counts showed a marked or relatively low levels comparing to those of anemic control. Labeling index was also low in these younger precursors from AMD treated animal (Fig. 1).

The cells having the nuclei of 10 to 13 μ in diameter are of early basophilic erythroblast or proerythroblast including those in S and G_2 stages. Therefore, the data indicate that the RNA synthesis in early basophilic and proerythroblast stages is AMD sensitive and that in later basophilic, poly- and orthochromatic stages is AMD resistant.

RNA content per cell of erythroblasts from anemic control decreased exponentially in proportion to the decrease in the nuclear volume. But in those rabbits treated with single injection of AMD 100 μg/kg, the relative amounts of RNA level per cell of the bone marrow erythroblasts showed a markedly low level at early stage of specialization comparing to those from control anemic animal. The RNA level of those at more advanced maturation stages showed no distinct difference from those of anemic controls (Fig. 2). The data are consistent with those obtained by observing the RNA synthesis by the incubation with H^3-uridine and radioautrography and indicate that AMD arrests the RNA synthesis of erythroblast only in their early stage of specialization by *in vivo* administration in a small dose of 50–100 μg/kg body weight.

DISCUSSION

It may be thought that in the erythroid cell specialization AMD acts as to suppress the action of erythropoietin resulting in the inhibition of the transformation of the stem cell to proerythroblast. But the present observation has clearly demonstrated that AMD suppresses the RNA synthesis in the early stage of specialization of erythroblast in such a small dose of 50 μg/kg irrespective of erythropoietin.

It is generally believed that AMD binds with the terminal guanine residue of DNA and inhibits the synthesis of DNA dependent RNA as has

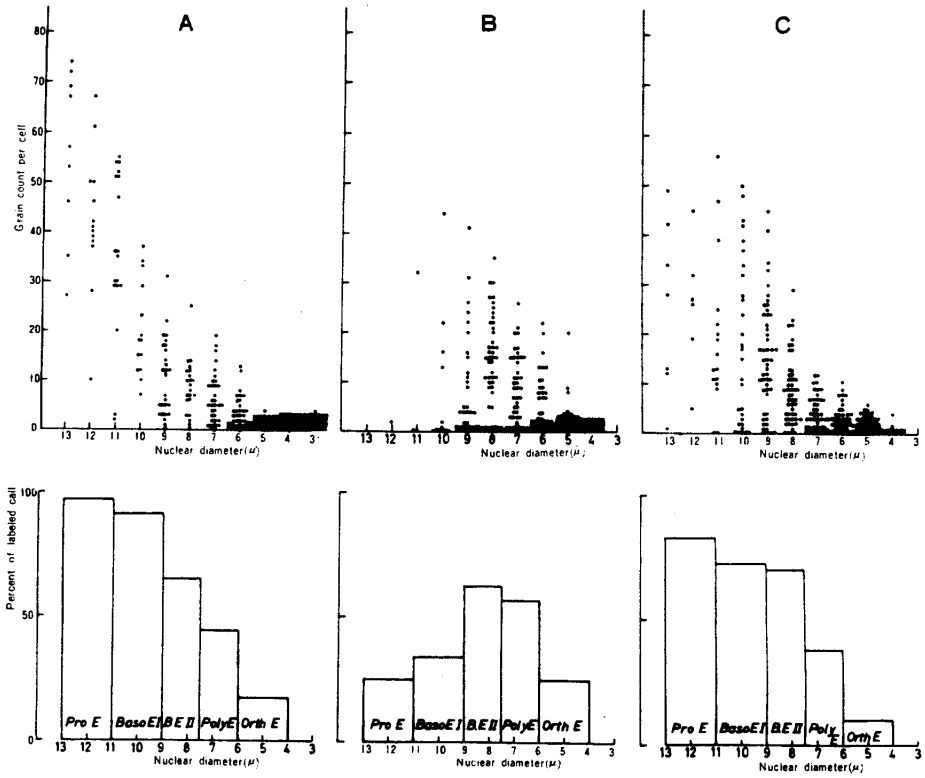

Fig. 1 The incorporation of H^3-uridine into RNA of erythroblasts of anemic rabbits treated with or without actinomycin D (AMD). The upper figures are the scatter diagram for grain counts of labeled erythroblasts as classified by the nuclear diameter and the lower ones are labeling indices of erythroblasts at each specialization stage. Pro E: proerythroblast, Baso E. I: early basophilic erythroblast, B. E. II: late basophilic erythroblast, Poly E: polychromatic erythroblast, Orth E. orthochromatic erythroblast
A: anemic control
B: 12 hours after AMD injection, 50 μg/kg
C: 24 hours after AMD injection, 50 μg/kg

been revealed so far (4, 14). In mammalian cells the similar effect of AMD is expected, but there is a great difference in sensitivity to AMD in the different cell strains. This may be due to the difference in base composition of the DNA chains to be transcribed.

Present observations have revealed that the RNA synthesis of erythroblast of rabbit in the early stage of differentiation is very sensitive to AMD, 50 μg/kg, while that in the later stage of specialization is resistant to the same drug in the same dose. This may suggest that the RNA synthesized in the earlier differentiation stage may differ from that synthesized in the later stages, i. e. mRNA synthesized in the earlier stage may be different one from those synthesized later. HARRIS and coworkers, TORELLI and his associates, and LINGREL reported, using AMD as a tool *in vitro*, of

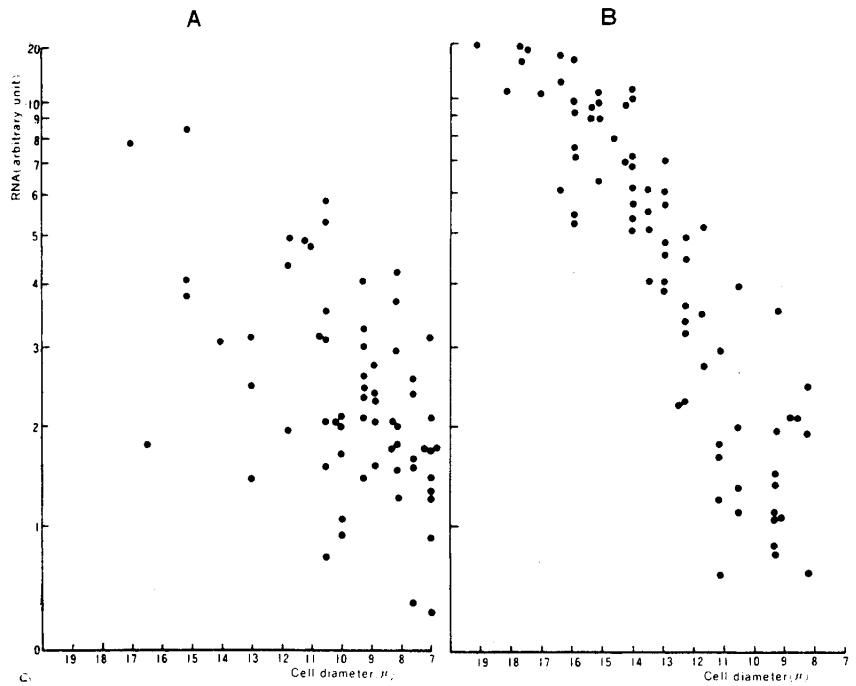

Fig. 2 Relative amount of azure B binding RNA of erythroblasts and reticulocytes of the bone marrow of the anemic rabbit receiving 100 μg/kg actinomycin D, sacrificed 24 hours after actinomycin D injection (A) and of anemic control (B)
7- 8μ: orthochromatic erythroblast
8-11μ: polychromatic erythroblast
11-13μ: late basophilic erythroblast
13-18μ: early basophilic erythroblast
18-19μ: proerythroblast

the rapidly turningover RNA which appears temporarily in the early stage of erythroid cell specialization and disappears soon (8, 9, 13). This RNA found by them may be the same one as the AMD-sensitive RNA found by the author. HARRIS has shown that in cells exposed to AMD the breakdown rate of rapidly labeled nuclear RNA does not proceed in proportion to the decrease in the protein synthesis. On this finding he has suggested that most of the rapidly labeled and short-lived RNA is not functioning as mRNA (8), while AMENTROUT *et al.* have presented evidence in support of a relationship between the breakdown of rapidly labeled RNA and protein synthesis (10). At any rate the failure in detecting the correlation between the breakdown of rapidly labeled RNA and protein synthesis does not necessarily indicate that this RNA is not mRNA, because the protein directed by this small amount of rapidly turningover RNA might be too small

to be detected by the techniques available at present.

On the other hand, the maturation of erythroblasts proceeds with both cell divisions and synthesis of somatic protein (11). Erythroblasts in the stages later than early basophilic one, reduce their cell volumes by about one half at each cell division (7, 11, 12), but it does not necessarily mean the stoppage of protein synthesis. Therefore, the rapidly turning over mRNA may serve for some structural protein but not the mRNA for hemoglobin, because hemoglobin is synthesized only slightly in the nucleated stages, especially in early specialization stages (11).

Besides these, the specialization of proerythroblast to red cells through every maturation steps seems not to be arrested by AMD. During 2 to 3 days after AMD injection the recovery curve of anemia is not severely deviated and no morphological changes of red cells are seen (1). These findings suggest that the rapidly turning over RNA elaborated in early stage will have no close connection with the cell specialization.

In contrast, it is most probable that the mRNA synthsis which appears in the later stages of cell specialization and proves to be AMD resistant would be of stable mRNA for hemoglobin, because hemoglobin synthesis becomes active in later stages, especially after denucleation (11). This is the problem to be settled in the next step of the author's experiment.

SUMMARY

For the purpose of revealing whether AMD inhibits the RNA synthesis of erythroblasts in an effective dose *in vivo* to eradicate erythroid cells in rabbit bone marrow, the author observed the RNA synthesis by H^3-uridine incorporation *in vitro* and RNA level on the cells from the anemic animals taken at a certain period after a single injection of AMD in a small dose of 50 and 100μg/kg body weight.

The data revealed that by such a small dose of injection of AMD the RNA synthesis of erythroid precursors, early basophilic and proerythroblast stages, was successfully suppressed without any suppressing effect on the RNA synthesis of erythroblasts in the later stages of specialization, indicating that there are at least two kinds of RNA synthesis: one seen mainly in the earlier stages of specialization and the other one seen mainly in the later stages, and they can be distinguished from each other by the AMD sensitivity.

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REFERENCES

1. OKADA, S.: Inhibition of erythropoiesis by actinomycin D, I. Evolutional change of the bone marrow cells during the drug administration, *Acta Med. Okayama* 23, 165, 1969
2. REISSMANN, K. R. and ITO, K.: Selective eradication of erythropoiesis by actinomycin D as the result of interference with hormonally controled effector pathway of cell differentiation. *Blood* **28**, 201, 1966
3. SCARO, J. L.: Suppression of erythropoiesis by actinomycin D in normal and erythropoietin treated mice. *Acta Physiol. Lat. Amer.* **17**, 88, 1967
4. REICH, E.: Biochemistry of actinomycin. *Cancer Res.* **23**, 1428, 1963
5. FLAX, M. H. and HIMES, H.: Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* **25**, 207, 1952
6. AMANO, M.: Improved techniques for the enzymatic extraction of nucleic acids from tissue sections. *J. Histochem. Cytochem.* **10**, 297, 1962
7. WEICKER, H.: Zellteilung und Zellteilungsstörungen, in "Handbuch der gesamten Hämatologie", 2 Auflage, Band I (ed. L. HEILMEYER and A. HITTMAIR) München, Berlin, Wien, Urban and Schwarzenberg. 1957, pp. 148
8. HARRIS, H.: Function of the short-lived ribonucleic acid in the cell nucleus. *Nature* **201**, 863, 1964
9. TORELLI, U., ARTUSI, T., GROSSI, G. EMILIA, G. and MAURI, C.: Unstable ribonucleic acid in normal human erythroblasts. *Nature* **207**, 755, 1965
10. ARMENTROUT, S. A., SCHMICKEL, R. D. and SIMMONS, L. R.: Actinomycin effect on erythroid messenger ribonucleic acid. *Arch. Biochem. Biophys.* **112**, 304, 1965
11. SENO, S.: Differentiation of erythroid cell. *Acta Path. Jap.* **16**, 457, 1966
12. WEICKER, H.: Morphologie und Kinetik der normalen und pathologischen Erythropoese. *Folia Haemt. N. F.* **9**, 153, 1964
13. LINGREL, J. B.: Studies on the rapidly labeled RNA of rabbit bone marrow. *Biochem. Biophys. Acta.* **142**. 75, 1967
14. REICH, E., CERAMI, A. and WARD, D. C.: Actinomycin, in "Antibiotics" vol. 1, (ed. D. GOTTLIEB and P. D. SHAW) Springer-Verlag, Berlin, Heidelberg, New York, 1967, pp. 714
15. ORNSTEIN. L.: The distributional errors in microspectrophotometry. *Lab. Invest.* **1**, 250, 1959
16. PATAU, K.: Absorption microphotometry of irregular shaped object. *Chromosoma* **5**, 341, 1952
17. MENDELSOHN, M. L.: The two-wave-length method of microspectrophotometry I. A microspectrophotometer and tests on model system, II. A set of tables to facilitate the calculations. *J. Biophys. Biochem. Cytol.* **44**, 407, 1958