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Abstract

For the purpose of revealing whether the sensitivity of the erythropoiesis to actinomycin D (AMD) differs among different animal species, and to see the acting site of AMD on erythroid cell specialization stage, the author observed the hourly change of the blood cell counts and bone marrow cells after AMD administration to mice, rats and rabbits, and obtained the following results: 1. The data indicated that the erythropoiesis of ra bbit is sensitive to AMD, as much as that of mice, while the rat is resistant to AMD, and its erythropoiesis is not affected by the similar dose of AMD as in the case of mouse and rabbit. 2. The morphologic observations on the eradication process of erythroblasts in the bone marrow of mice and rabbits indicates that AMD acts as to inhibit the transformation of the stem cell to the proerythroblast but not on the erythroblast in the course of specialization. The time required for the eradication coincided with the time of the proerythroblast to the mature red cell. 3. Discussion has been made on the possibility of the common stem cell to erythroid and granulocytic cells in relation to the lymphoid cells in bone marrow and their blast form.

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SUPPRESSION OF ERYTHROPOIESIS BY ACTINOMYCIN D
I. EVOLUTIONAL CHANGE OF HEMOPOIESIS BY ACTINOMYCIN D

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In an adult organism, the cell populations constituting each organ are controlled to stay at a certain level. This means that the cell proliferation and the cell loss are balanced in close relation with the function of each organ. Thus, the red cell number in the circulating blood is controlled corresponding to the oxygenation level of hemoglobin (2, 26). But for the control of cell population itself, the erythropoietin is directly responsible (1, 2, 18). Erythropoietin is believed to trigger the transformation of the stem cell to proerythroblast acting as the inducer for messenger RNA (3, 4, 5), though some other activities in connection with the promotion of specialization process of erythroblast are still in dispute (6, 8). No control mechanism, however, is so far observed in the differentiation process of erythroblasts after the induction (17). That is, in the stages later than proerythroblast the specialization process of erythroblast seems to proceed automatically with the repeated cell divisions, which is destined ultimately to reach red cell by denucleation. This might be achieved by the stable messenger RNA for hemoglobin, which is produced in the early erythroid precursors (4, 7, 9).

It is known that actinomycin D (AMD), a colored antibiotics produced by Streptomyces species (10, 11), forms a tight but reversible complex with DNA and blocks the synthesis of DNA-dependent RNA. For the complex formation, guanine in DNA is believed to be the binding site of AMD. This antibiotics has been widely used as the tool for the analysis of messenger RNA synthesis and related phenomena. The messenger RNA synthesis of erythroid cells has also been studied by using this antibiotics. But it has been shown that AMD totally eradicates the effect of erythropoietin as has been clearly demonstrated *in vitro* experiments (3, 4). REISSMANN and associates also showed the selective eradication of erythropoiesis *in vivo* by the injection of AMD into mice (12).

Using AMD, it became clear that the initial step of the differentia-

tion, the change of stem cell to pronormoblast, includes the synthesis of new messenger RNA rather than the activation of preexisting RNA templates (12, 13, 14). It should also be emphasized that the effect of the drug was brought about at a level far below the lethal dose (15, 19)

The present experiment was undertaken to observe firstly if the eradication of erythropoiesis by AMD is seen generally among different animal species other than mice and then if the acting site of AMD is limited only to stem cell or to all the erythroblasts under specialization.

MATERIALS AND METHODS

Adult male animals, ddN mice weighing about 20 g, Wistar rats about 100 g, and white rabbits about 2 kg were used. Mice and rats were injected AMD without pretreatment but rabbits were divided into two groups, one receiving pretreatment of phenylhydrazine injections and the other without pretreatment. Controls were left without any treatment, though the anemic control of rabbits received phenylhydrazine injection. Phenylhydrazine was injected subcutaneously in 2.5 % neutralized solution, 1 ml/kg successively for 3 days. AMD was dissolved in distilled water in three different concentrations, 5 μg/ml for rats, 2 μg/ml for mice and 25 μg/ml for rabbits. They were kept in freezer at $-20°C$ and used within 10 days after preparation. In all the animals, AMD solution was introduced subcutaneously. Number of circulating blood cells, i. e. red blood cells (RBC), reticulocytes (RC), and white blood cells (WBC), were counted with the blood from orbital plexus in mice and rats, and from ear vein in rabbits by employing Toa Microcellcounter. On the termination of the experiment bone marrow was obtained from the femur of each animal and cells were observed on smeared samples after May-Grünwald-Giemsa stain. AMD used in this experiment was kindly supplied by Japan Merck-Banyu Co., Ltd.

RESULTS

In the four ddN mice, receiving the administration of AMD, 60 μg/kg daily for 6 days, the number percent of RC in the peripheral blood fell below 10 ‰ in two of them and 0 % in the rest (Table 1A). In the nine mice receiving the injection of AMD, 120 μg/kg every other day for 6 days, all the animals showed 0 % RC, reconfirming Reissmann's result (Table 1). In both series of experiments RBC count showed little change before and after the AMD treatment indicating no destructive effect on mature RBC. A slight decreasing tendency was observed in both hemoglobin (Hb) and hematocrit (Ht) values, but mean corpuscular hemoglobin remained almost unchanged (Tabe 1).

Bone marrow films from the animals treated with AMD injection, 60

Table 1. Blood counts of mice before and after the treatment with actinomycin D (AMD) injection. (A) mean values from 4 mice treated with daily injection of AMD, 60 μg/kg for 6 days, and (B) mean from 9 mice treated with AMD, 120μg/kg every other day for 6 days and 360 μg/kg as total. RBC, RC, WBC, Hb and Ht; see text, MCH; mean corpuscular hemoglobin

	Determination	RBC (10^6/mm^3)	Hb (g/dl)	Ht (%)	RC (‰)	WBC (10^3/mm^3)	MCH (μμg)
(A)	Before AMD administration	7.13±0.83	11.3±0.5	42±49	34±18	4.1±0.65	15.8
	After AMD administration	6.54±1.15	9.7±2.6	36±8.6	7	8.3±1.3	14.9
(B)	Before AMD administration	6.54±0.72	11.4±1.1	43±3.7	56±22	4.8±1.5	17.2
	After AMD administration	6.18±1.05	9.4±1.2	35±4.6	0	6.7±2.5	15.2

μg/kg daily for 6 days, showed a picture predominant in myeloid cells of about 90 % with some lymphoid cells of about 10 %, and no erythroblasts except one case which retained some erythroblasts of about 10 %.

In the animals receiving 120 μg/AMD/kg every other day for 6 days also showed the similar change: no erythroblasts in the bone marrow with predominant myeloid cells of 90 to 99 % and some lymphoid cells of 1 to 10 % except one case in which the lymphoid cells predominated in the bone marrow: 80.7 % lymphoid cells and 19.2 % myeloid cells (Table 2).

Table 2. Individual effects of AMD on the myelogram of 13 mice appearing in Table 1. (A); 360 μg/kg in 6 divided doses for 6 days, (B); 360 μg/kg in 3 divided doses, every other day for 6 days, (C); mean of 6 controls

Animals	(A) E1	H1	H2	H3	(B) I1	J1	J2	K1	K2	L1	L2	MI	M2	(C) No
Total erythroid	0	10	0	0	0	0	1	0	0	0	0	0	0	21.0
Myeloid, young	35	23	36	40	42	41.5	29	34	9.6	58	16	29	18	26.8
mature neutrophils	53	54	54	53	53	48	62	57	9.6	41	80	77	78	36.2
eosinophils	1	2	2	1	0	0	0	0	0	0	0	0	0	3.8
basophils	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphocytes	9	11	7	6	5	9	7	9	80.7	1	4	1	3	11.0
Plasma cells	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Monocytes	2	0	0	0	0	0	0	0	0	0	0	0	0	0
Reticulum cells	0	0	0	0	0	1.5	1	0	0	0	0	0	0	0

To observe the process of eradication of erythroblasts in detail after being exposed to AMD, 28 mice were injected with AMD, 120 μg/kg once and couple of animals each were sacrificed successively every two hours

up to 24 hours after the AMD injection. The remaining four animals received the additional AMD injection, 120 μg/kg 24 hours after the first injection, and these two couples were sacrificed at 48 and 72 hours after the first AMD injection.

The hourly changes in erythroblast number in bone marrow are shown in Fig. 1. As seen in the figure, the bone marrow before AMD

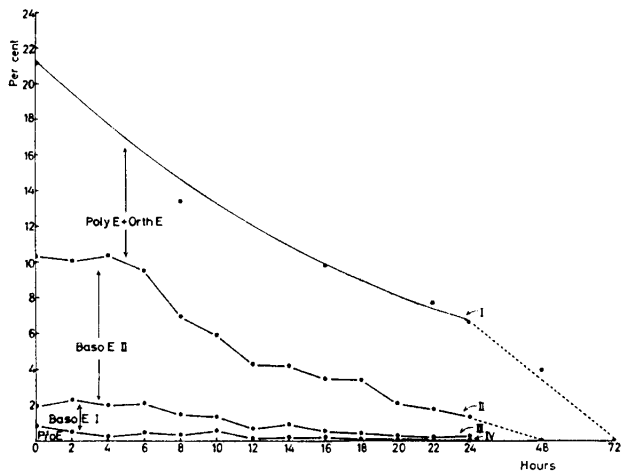

Fig. 1. Hourly changes in the number percent of erythroid cells in mouse bone marrow after a single dose of actinomycin D, 120 μg/kg. Curve I: the erythroblasts to all the nucleated cells in bone marrow. Curve II: total number of the younger erythroblasts having basophilic cytoplasm. Curve III: total number of early precursors. Curve IV: number of the youngest precursors, proerythroblasts. Number percent of erythroblast in individual groups, i.e. poly- and orthochromatic (poly E+Orth E), late basophilic (Baso E II), early basophilic (Baso E I) and proerythroblast (Pro E) are presented as the difference between two curves; I-II; Poly E+OrthE, II-III; Baso E II, III-IV; Baso E I and IV; Pro E

injection has as many erythroid cells as 21.0 % of all the nucleated cells. They decreased to 5.7 % in 24 hours after AMD administration, 4.0 % after 48 hours and completely disappeared after 72 hours. Proerythroblasts disappeared first from the bone marrow and later the more mature ones became predominant comprising larger part of the erythroid compartment as the time proceeded.

Seven rats administered with AMD, 60 μg/kg daily or 120 μg/kg every other day for more than 4 weeks, also showed the decrease in RC number in the circulating blood after one week of AMD administration but they never disappeared. Differing from mouse, rats proved to be resistant to

Table 3. Cell counts of circulating blood of Wistar rats treated with actinomycin D injection, 120 μg/kg every other day (A) and 60 μg/kg every day (B) for 29 days, data obtaiued on two representative cases
RBC, Hb, Ht, RC and WBC; see text

	Day	RBC (10^6/mm^3)	Hb (g/dl)	Ht (%)	RC (‰)	WBC (10^3/mm^3)	Weight (gm)
(A)	0	6.67	8.7	25	50	6.8	90
	6	8.67	9.5	37	12	10.8	
	19	8.49	11.0	41	37	12.2	90
	29	8.22	10.4	41	62	13.1	136
(B)	0	6.10	7.4	33	29	8.9	92
	6	6.81	7.0	32	3	10.3	
	13	8.67	9.5	38	22	14.3	
	26	8.36	10.1	41	36	24.0	110
	29	8.80	10.2	43	36	16.2	110

the drug. Unexpectedly, the RC recovered to the initial level gradually 3 to 4 weeks of AMD administration (Table 3). The change of the Price-Jones' curve, obtained on the photographed wet preparation from the circulating erythrocytes, was very slight. Erythroblasts were 20 to 40 % of all the nucleated cells in the bone marrow even after 20 to 30 days' administration of AMD. Peripheral blood count, RBC, Hb, and Ht did not decrease but they increased as the days proceeded, differing from other animals (Table 3).

The effect of AMD was also observed in six rabbits. These animals received AMD injection daily for 3 days, 100 μg/kg/day for 3 animals, 50 μg/kg/day for two, and 25 μg/kg/day for the remaining one. In the cases receiving AMD 100 μg/kg daily, RC number in the circulating blood became less than 1.0 % 4 days after the first administration of AMD, but even in a dose of 25 and 50 μg/kg, a marked decrease in RC number was observed. No actual change coccurred in RBC number, Hb level and Ht value before and after the AMD treatment in all the cases (Table 4). Myelogram of the May-Grünwald-Giemsa stained smears proved that 3 days were sufficient to minimize the erythropoiesis in rabbit bone marrow (Table 5). The levels of erythroblast number were very low in the animals receiving 50 μg/kg AMD daily for 3 days, and the granulocytic cells were 50 to 60 % of all the nucleated cells. In the cases receiving 100 μg AMD/kg daily for 3 days, lymphoid cells were predominant in the bone marrow nucleated cells, 90 % in one case and nearly 50 % in another one case, showing an occasional severe suppression of granulopoiesis by the dose of 100 μg

Table 4. Blood counts of rabbits before and after the treatment with actinomycin D, daily subcutaneous injection for 3 days B: values before treatment A: values after treatment RBC, Hb, Ht, RC and WBC; see text

Animal	T1		T3		T4		T5		T6		T7	
Actinomycin D (μg/kg/day)	100		50		25		100		100		50	
Weight (kg)	2.7		2.3		2.2		2.5		2.2		2.4	
	B	A	B	A	B	A	B	A	B	A	B	A
RBC (10^6/mm^3)	5.10	5.62	4.35	4.08	4.98	5.30	5.23	5.01	5.68	6.03	5.68	6.03
Hb (g/dl)	10.5	12.8	9.8	9.7	10.2	10.7	10.2	9.5	11.0	11.5	11.0	11.0
Ht (%)	38	50	38	42	40	48	38	34	38	41	41	41
RC (‰)	43	0	31	8	28	4	18	1	21	0	10	1
WBC (10^3/mm^3)	3.6	7.8	4.3	11.0	7.8	10.1	6.2	15.4	7.2	11.5	7.6	12.1

AMD/kg/day (Table 5).

Then in the 9 anemic rabbits treated with phenylhydrazine injection, the recovery of anemia was observed after treating with AMD, 50 μg/kg/day for 3 days, with one anemic control. Blood counts during 20 experimental days of the representative rabbit are shown in Fig. 2. As seen in

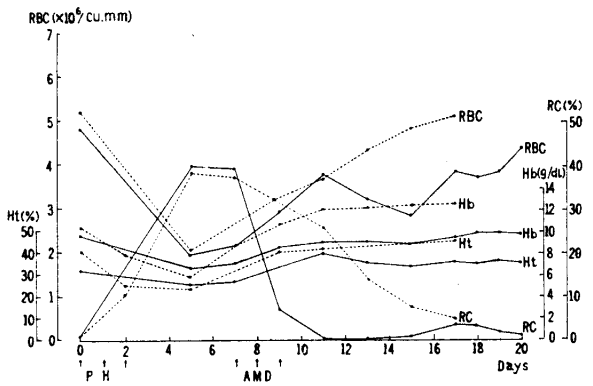

Fig. 2. Daily changes in blood counts of anemic rabbits treated with phenylhydrazine followed by the administration of actinomycin D 50 μg/kg/day for 3 days, solid lines; animal receiving actinomycin D injections on the recovery stage of phenylhydrazine anemia, broken lines; anemic control receiving only the phenylhydrazine injection. Data obtained on one representative case RBC, Hb, Ht, RC; see text, PH; phenylhydrazine injections, AMD; actinomycin D injections

Table 5. Myelogram of the rabbits appearing in Table 4, animals treated with actinomycin D for 3 successive days in various doses without phenylhydrazine pretreatment

	Animal		T1	T3	T4	T5	T6	T7	Control
	Dose (μg/kg)		100	50	25	100	100	50	
Erythr.	ProE								1.4
	Mk	B							
		P							
		O							
	No	B				0.8	0.2	0.2	9.2
		P		0.2	1.4	0.8		0.4	13.8
		O	1.4	1.0	3.2	2.4		1.0	19.4
	Mitosis								
Leuk.	Mybl			7.2	5.6	0.6	0.2	0.6	3.0
	Nt	Pr		24.6	39.8	5.4	0.8	5.8	5.8
		My		10.4	15.0	21.4	2.4	14.8	7.6
		Mt		11.8	11.0	24.6	4.6	19.6	10.8
		I	5.2	3.8	1.2	2.6	7.4	6.6	7.8
		II		0.4	0.2	0.8	2.4	2.0	2.4
		III			0.2	2.0	1.4	1.0	
		IV		0.2		1.0	0.4		
		V				0.6	0.2		
	Eo	Pr	0.2	1.8		0.2			
		My		0.4		0.2			
		Mt		1.0					0.6
		Bd			0.8	0.6		0.4	1.0
		Sg		0.2	1.0	0.4	0.6	0.8	0.2
	Ba	Pr							
		My							
		Mt				0.2	0.2	0.2	0.2
		R				0.2	0.2	0.4	0.2
	Mo	Bl		0.2			0.4	0.2	
		Pr		3.2	1.4	0.2	0.8	0.8	
		Mo	0.5	6.6	1.8	1.2	8.6	3.4	
	Ly	Bl		1.6	0.2	1.0	2.0	0.6	
		L	90.0	23.4	17.0	30.6	45.4	37.0	1.6
		S							13.8
	Mitosis								
Th	Mgk		0.3	0.2	0.4	0.2	0.2		
	Pl		0.6	0.6	0.6	0.2	1.6		0.6
	Ret		1.7	1.2		1.6			0.6
	unidentified					0.2			

the figure, during the first 4 days of AMD administration the recovery rate of anemia showed no difference from the control except that RC count became below 3 % in all the cases 4 days after the first injection of

AMD and 0 % in the following 24 hours. At this stage red cell number was at a lower level than that of anemic control. Hb nad Ht values showed also slightly lower levels than control. RC stayed at 0 % for 2 days but they reappeared thereafter in the circulating blood, showing a slight recovery of erythropoiesis by this time. In the bone marrow no erythroblast was found 48 hours after the last AMD injection but after 72 hours proerythroblast reappeared (Fig. 3).

Figure 3. A proerythroblast which appeared first in the rabbit bone marrow 72 hours after cessation of actinomycin D injection. 50 μg/kg daily for 3 days

DISCUSSION

From the observations on mouse hemopoietic tissue by several authors, it has been elucidated that AMD selectively suppresses or eradicates the erythroid cells from the bone marrow, but the present observations revealed that sensitivity of erythroid cells to the drug varies considerably depending on animal species. In sensitive animals like mouse and rabbit, younger precursors of the erythroid cells in bone marrow were eradicated selectively by injecting a small dose of AMD, but in resistant animals like rat erythropoiesis was not so severely affected even with repeated administration of AMD in a large dose. For example in mice, already 24 hours after the single injection of the drug, 60 μg/kg, younger precursors were hardly encountered on the bone marrow smear, while in rats nearly normal hemopoiesis was observed even after 29 injections, 60 μg/kg/day. Obser-

vations proved that the eradication is achieved by the inhibition of the transformation of the stem cell to proerythroblast with the maturation of the erythroblast to red cell but not by the cell degeneration. It has also been elucidated that the stem cell in itself is not affected by AMD because proerythroblasts reappear at a certain period after cessation of the AMD administration with complete recovery of erythropoiesis. Data are consistent with those of REISSMANN and ITO (12) and of GURNEY and HOFSTRA (16). The similar results were obtained on anemic rabbits i.e. the activated erythropoiesis by phenylhydrazine injection was suppressed by AMD: erythroblast disappeared from the bone marrow after 3 injections of AMD 60 μg/kg/day. In this instance eradication of erythroid cells was attained by the disappearance of younger precursors first and then the more mature cells diminished, indicating inhibition of the transformation of the stem cell to proerythroblast. The mechanism may suggest some congestive hyperplasia of stem cells or of undifferentiated primitive cells in the bone marrow. Actually, in the rabbits of phenylhydrazine anemia treated with the injection of 50 μg/kg AMD the bone marrow smears obtained 24 to 72 hours after the last AMD injection had a number of "undifferentiated" cells of large size with many granulocytic precursors. Morphologically, they showed some pattern of young reticulum cells or blast form of lymphocytes, though not clearly identified (Fig. 4), but the degree of hyperplasia of undifferentiated cells was not so marked as would be expected. These undifferentiated cells were morphologically indentical with the cells observed by TAKEBAYASHI in the marrow of the anemic rabbits

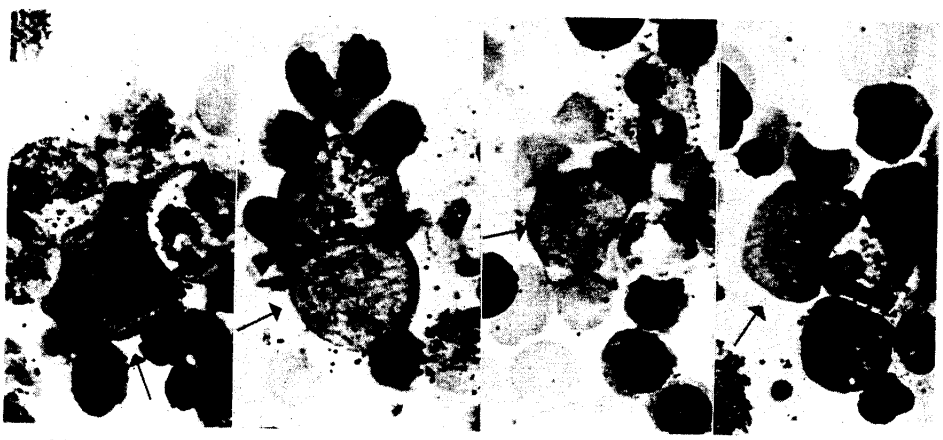

Fig. 4. Four "undifferentiated" cells which appeared in the rabbit bone marrow 24 hours after actinomycin D injection 50 μg/kg. The animal was preliminarily treated with phenylhydrazine, and actinomycin D was injected at the recovery stage of anemia.

receiving the excessive transfusion of red cells (17). Even under AMD influence, the differentiated erythroblasts mature to red blood cells. This differentiation is uncontrollable by any substances so far known.

In contrast to the erythroid cells, the granulocytic cells invariably increased after AMD injection. The myelocytic differentiation was not inhibited by the drug in dose used in this experiment. These facts seem to suggest the possibility that the erythroid and granulocytic cells would have the common stem cells, which is sometimes suggested by other authors (20). Another important finding in this experiment is a marked proliferation of lymphoid cells in the bone marrow, induced by the AMD treatment, which rarely occurs in the hematopoietic marrow of untreated animals. At present we have no direct evidence to show the relationship between the blast form cells, lymphoid cells, granulocytes and erythroid cells, but the data suggest that the lymphoid cells will have a cyclic change with blast form cells (21) and they may be transformed to erythroid or granulocytic cells being affected by the specific inducers, which will be erythropoietin for erythroid cells and some unknown substance for granulocytic cells.

Besides these, it is possible to calculate roughly the mean generation time from the decreasing curve of erythroblasts in the marrow after AMD administration. For example, for the complete disappearance of erythroid cells from the bone marrow of mice, it took 72 hours. As the proerythroblasts mature to erythrocytes through 4 cell divisions and 4 steps of specialization (22, 23, 24), the mitotic time at each step will be around 18 hours, though the generation time of the younger cells would be shorter than those of more mature ones (25).

SUMMARY

For the purpose of revealing whether the sensitivity of the erythropoiesis to actinomycin D (AMD) differs among different animal species, and to see the acting site of AMD on erythroid cell specialization stage, the author observed the hourly change of the blood cell counts and bone marrow cells after AMD administration to mice, rats and rabbits, and obtained the following results:

1. The data indicated that the erythropoiesis of rabbit is sensitive to AMD, as much as that of mice, while the rat is resistant to AMD, and its erythropoiesis is not affected by the similar dose of AMD as in the case of mouse and rabbit.

2. The morphologic observations on the eradication process of

erythroblasts in the bone marrow of mice and rabbits indicates that AMD acts as to inhibit the transformation of the stem cell to the proerythroblast but not on the erythroblast in the course of specialization. The time required for the eradication coincided with the time of the proerythroblast to the mature red cell.

3. Discussion has been made on the possibility of the common stem cell to erythroid and granulocytic cells in relation to the lymphoid cells in bone marrow and their blast form.

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