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Hiroshi Tada*

*Okayama University,

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THE ROLE OF CYCLIC NUCLEOTIDE ON FETAL AND NEONANTAL ERYTHROPOIESIS

Hiroshi TADA

Department of Pathology, Okayama University Medical School, Okayama, Japan (Director: Prof. S. Seno)

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Abstract: For the purpose to reveal the changes in stimulatory effect of dibutyryl-cyclic- AMP on erythropoiesis during ontogenetic development, the author studied syntheses of DNA, RNA and protein of erythroid cells in fetal liver, neonatal and adult bone marrows of rats. In the bone marrow of neonatal animals erythropoiesis was stimulated by the intraperitoneal injection of cyclic nucleotide with enhanced DNA, RNA and protein syntheses of erythroid cells. Enhancing effect of dibutyryl-c-AMP on the erythropoiesis decreased gradually with advance of neonatal days. Autoradiographic observations revealed that in erythoid cells isolated from fetal liver and neonatal bone marrows, DNA-, RNA- and protein-snythesis was markedly stimulated by incubating with cyclic nucleotide, but not in those from adult bone marrow. Discussion was made on the changes in the regulatory mechanism of erythropoiesis according to the transition of hematopoietic organs during development.

The regulatory mechanism of cell growth and cell differentiation of individual organism is poorly understood, but it is known that the regulatory processes are affected by cyclic adenosine-3′ : 5′-monophophate (c-AMP) or its derivative, N^6-2′-0-dibutyryl adenosine-3′ : 5′-monophosphate, cyclic (db-cAMP) (1-6). Concerning erythropoiesis, administration of c-AMP or db-cAMP stimulates the incorporation of radio-iron into red cells of spleen but not those of bone marrow in normocytemic adult mice (7), though it stimulates the incorporation of radio-iron into erythroid cells in the bone marrow of polycythemic mice (7, 8, 9, 10). These observations suggest that adult mouse red cells will be composed of two groups, c-AMP sensitive group and c-AMP insensitive one, and this may be related to transition of hematopoietic organs during the ontogenetic development; from yolk sac to liver and spleen and then to born marrow, in each of which the red cells have specific characteristics distinguished from each other biochemically as well as morphologically. SHEPPARD and BURGHARDT reported recetly that adenyl cyclase activity of erythocytes was greater in immature rats than that of mature ones (11).

The findings mentioned above suggest that cyclic nucleotide stimulates only the hematopoiesis of immature type. In view of this the present obser-

vations have been carried out to reveal how db-cAMP is involved in the hematopoiesis at each developmental stage. This paper demonstrates that the stimulatory effects of db-cAMP on the production and differentiation of erythroid cells are marked in early developmental stages but not in adult animals.

MATERIALS AND METHODS

Five adult male rats of Wister strain weighing 200 to 250 g, 150 neonatal rats and 30 fetuses of 14th gestation day of the same strain were used. Among neotal rats 65 animals were used for the observation of the effects of db-cAMP injection on the daily increase of the bone marrow erythroid cells in number after birth, 40 animals for the observation of the mitotic index of bone marrow cells and 30 rats for the observation of DNA-, RNA- and protein-synthesis by injecting the ^{3}H-compounds. Five adult rats, 15 neonatal rats and 30 fetuses were used for the observation of DNA-, RNA- and protein-synthesis of the bone marrow cells *in vitro*. At the termination of experiments all the animals were sacrificed by decapitation and samples of erythroid cells were obtained.

For the observation of mitotic indices 40 neonatal rats of 3 days old were used. They were divided into 8 groups, 5 animals each. They were treated with db-cAMP injection intraperitoneally once, 4.7 mg/g body wt. for first group, 4.7×10^{-1}mg/g for second group, 4.7×10^{-2}mg/g for third group, 4.7×10^{-3}mg/g for fourth group, 4.7×10^{-4}mg/g for fifth group, 4.7×10^{-5}mg/g for sixth group, 4.7×10^{-6}mg/g for seventh group. Those of eighth group were of control and injected with saline intraperitoneally, 0.05 ml/animal. All the animals were injected with colchicine, 1 mg/kg body wt., 5 hours after db-cAMP injection and sacrified one hour later.

For the observation of effects of db-cAMP on the increase in erythroid cell number after birth, 65 neonatal animals were divided into three groups, five, 30 and 30 animals in each. Five animals were sacrificed without the treatment at the initiation of the experiment. Thirty animals in one group were intraperitoneally injected db-cAMP daily, 4.7×10^{-2}mg/g body wt. and the other 30 animals were injected with saline. Ten animals, 5 treated and 5 controls, were sacrificed every day. After sacrifice femur bone marrows were taken out and cells were smeared and stained with May-Grünwald-Giemsa (Merck), and relative number of erythroid cells was counted.

For the observation of effects of db-cAMP injection on DNA, RNA and protein syntheses of bone marrow erythroid cells in neonatal rats, 30 animals were divided into two groups, 15 animals each, and those in one group were injected intraperitoneally 4.7×10^{-2}mg/g body wt. db-cAMP daily for three days and the other 15 animals were treated with saline injection, 0.05 ml/animal for three days as control. Two hour after the last injection each animal received the intraperitoneal injection of isotopes; (methyl-^{3}H)-thymidine, 1 μCi/g body wt. for 10 animals, 5 db-cAMP treated and 5 controls; ^{3}H-uridine, 5 μCi/g for animals 5 treated and 5 controls; and ^{3}H-leucine, 1 μCi/g for re-

maining 10 animals. One hour after the injection of isotopes all the animals were sacrificed, bone marrow cells were smeared, fixed with methanol and mounted with nuclear emulsion. After three weeks' exposure samples were developed, stained with May-Grünwald-Giemsa and observed under microscope. Three grains were required as the limiting grain counts per labeled cell.

For the observation of the effects of db-cAMP on the DNA-, RNA- and protein-synthesis of erythroid cells *in vitro*, femoral bone marrow cells of adult and 3-day old neonatal rats were obtained without any pretreatments. The fetal hepatic erythroid cells were separated by the method of KOVACH J. S. and coworkers (12). The cells were suspended in Hanks-serum mixture (1 : 1 v/v) and divided into two parts, the one was incubated with db-cAMP, 10^{-4}M for 40 min at 37°C and the other incubated without the agent under the same conditions. Then each part was further divided into 3 parts and incubated again with ^{3}H-compounds, (methyl-^{3}H)-thymidine, 1 μCi/ml; ^{3}H-uridine, 5 μCi/ml, for 20 min at 37°C, respectively. After incubation the cells were washed three times with cold Hanks-serum mixture by repeated centrifugation, suspended in homologous serum, smeared and fixed with methanol. After drying the smears were mounted with sensitive nuclear emulsion, exosed, developed, stained and grain counts were taken on each erythroid cells as described above. Serum used was obtained from normal adult Wistar rats.

Dibutyryl-cAMP used was a product of Boehringer-Mannheim Ltd. in the form of saline solution, 4.7 mg/ml. ^{3}H-compounds were obtained from The Radiochemical Centre, Amersham and the specific activities were 27.7 Ci/mmol in (methyl-^{3}H)-thymidine, 18.5 Ci/mmol in ^{3}H-uridine and 19.0 Ci/mmol in ^{3}H-leucine. Nuclear emulsion (NR-M2) was a product of Konishiroku Syashin Kogyo Co., Ltd.

RESULTS

The observation of erythroid cells on metaphase in the bone marrow smearr from three day old neonatal rats treated with db-cAMP injection revealed that a slight suppresion of the cell division at 4.7 mg/g of db-cAMP and stimulation in the range from 4.7×10^{-1} mg/g to 4.7×10^{-6} mg/g, most marked at 4.7×10^{-2}mg/g, moderate at 4.7×10^{-1}mg/g and 4.7×10^{-3}mg/g, slight at 4.7×10^{-4} mg/g, 4.7×10^{-5} mg/g and 4.7×10^{-6} mg/g. At the dose of 4.7×10^{-2}mg/g the mitotic index was as high as 2-fold that of control (Fig. 1). On the basis of this result the dose of 4.7×10^{-2}mg/g db-cAMP was used in the further observations *in vivo* and *in vitro*.

Daily observation on the increase of erythroid cell population in relative number after birth indicated that the percentage of the erythroid cell number after birth indicated that the percentage of the erythroid cell number increases by about 10% per day until 5 days after birth without any treatment, while in the animals administered cyclic nucleotide daily, 4.7×10^{-2}mg/g, the

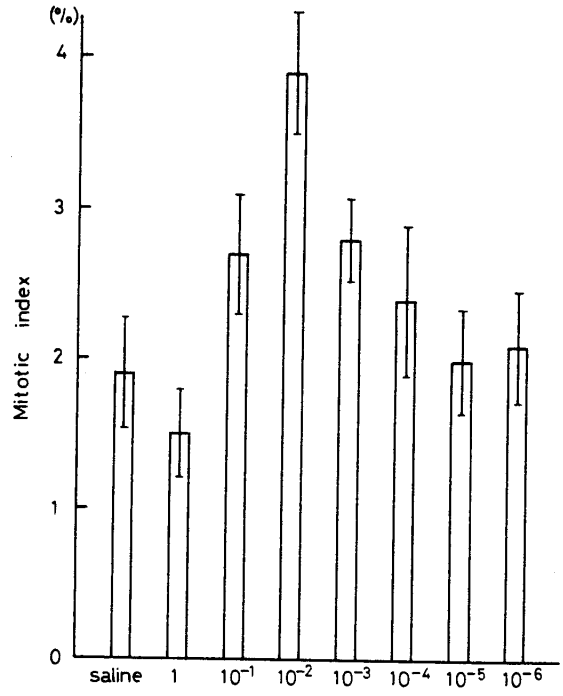

Fig. 1. Effect of db-cAMP on mitosis of bone marrow hematopoietic cells in neonatal rats

Five hours after the intraperitoneal injection of db-cAMP, 1 mg/kg body wt. of colchicine was administered intraperitoneally. Animals were sacrificed one hour after colchicine injection and smeared bone marrow cells were stained with May-Grünwald-Giemsa. Each data shows mean values from 5 animals, respectively and the vertical bars in the column indicate the standard error.

increasing rate of erythroid cells in percentage becomes higher comparing with those of untreated animals till 5 days after birth, the highest difference is seen 2 days after birth and more than 150% of controls (Fig. 2).

Radioautographic observations on the DNA, RNA and protein syntheses of the bone marrow erythroid cells from the new-born animals receiving ^{3}H-compounds injections indicated that the cells from the animals treated with cyclic nucleotide for 3 days showed higher grain counts and higher labeling indices in the cases given labeled uridine and leucine as compared with those from the non-treated animals (Fig. 3 and Table 1).

In animals injected with ^{3}H-thymidine without pretreatment by db-cAMP, grains on the nuclei were observed most markedly in proerythroblasts, moderately in basophilic erythroblasts but no grain in orthochromatic ones

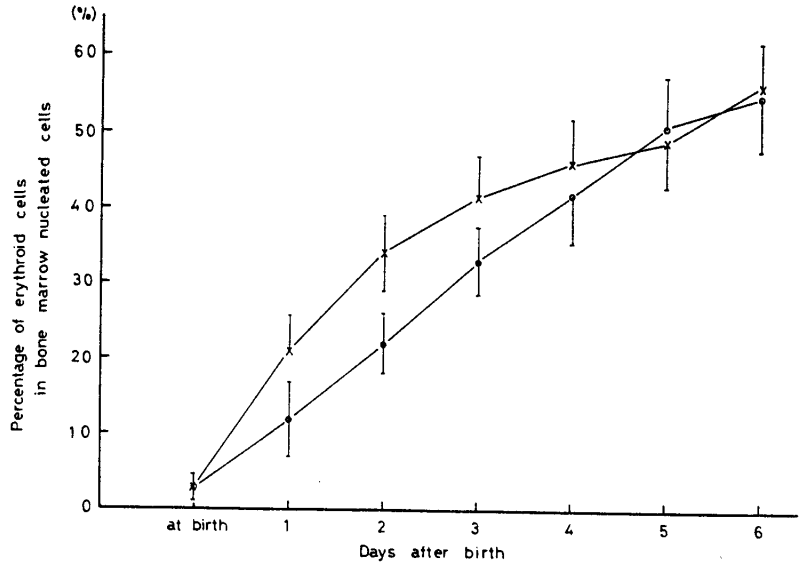

Fig. 2. Stimulation of erythroid cell appearance in the bone marrows of neonatal rats treated with db-cAMP *in vivo*

Dibutyryl-cAMP, 4.7×10^{-2}mg/g body wt., was intraperitoneally injected daily after birth for 6 days. Bone marrow cells were prepared on each day and smeared, and then erythroid cells were classified after May-Grünwald-Giemsa staining. The data shows mean values from 5 animals and vertical bars in the figure indicate standard error.

TABLE 1 STIMULATION OF DNA, RNA AND PROTEIN SYNTHESES IN BONE MARROW ERYTHROID CELLS OF NEONATAL RATS TREATED WITH db-cAMP *in vivo*

stage	db-cAMP	labeling index (%)		
		^{3}H-thymidine	^{3}H-uridine	^{3}H-leucine
pro	−	85	90	94
	+	95	95	97
BI	−	75	78	86
	+	88	87	90
BII	−	57	63	73
	+	75	78	87
poly	−	40	49	62
	+	59	71	80
orth	−		28	41
	+		41	63

Labeling indices show mean values from 5 animals. For details see Fig. 3 and text. The signs + and − indicate db-cAMP injection and no injection, respectively. Pro: proerythroblast, BI: early basophilic erythroblast, BII: late basophilic erythroblast, Poly: polychromatic erythroblast, Orth: orthochromatic erythroblast

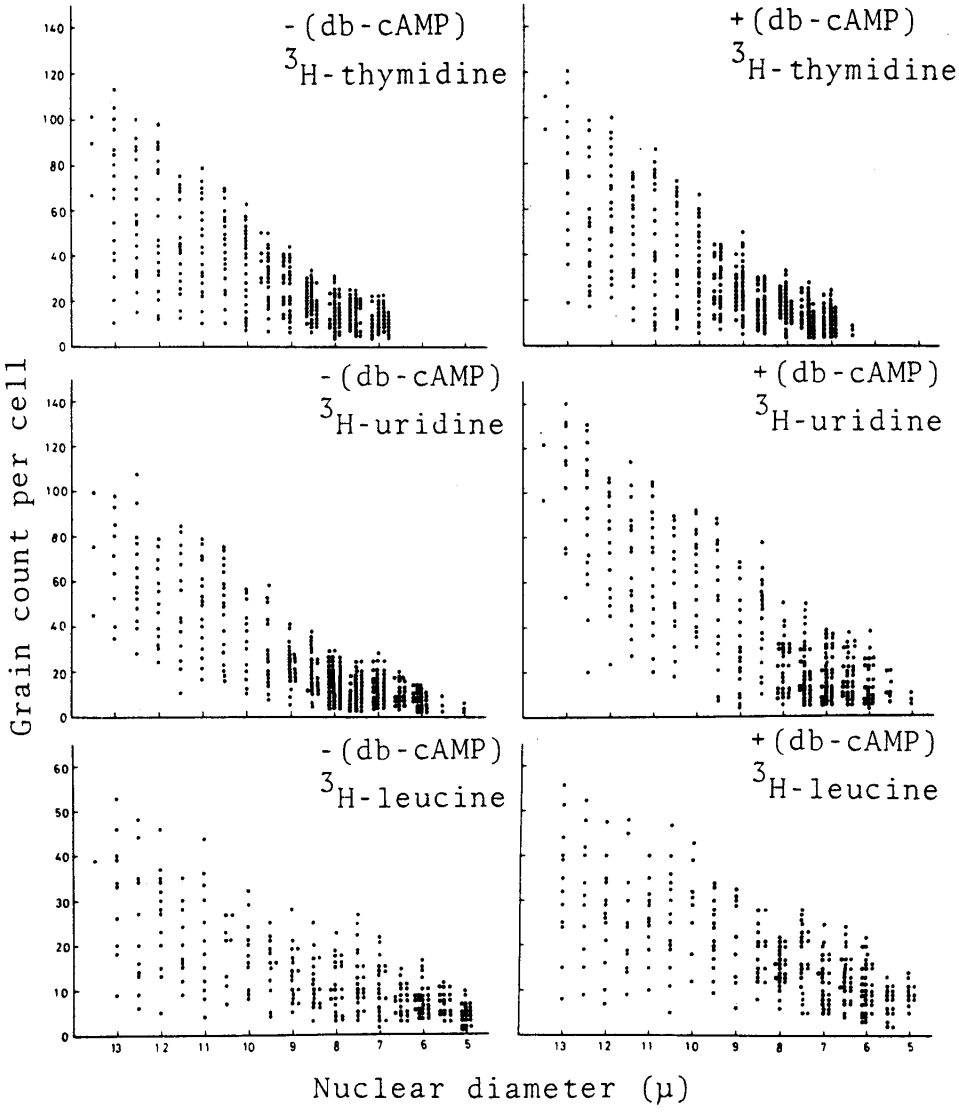

Fig. 3. Effects of db-cAMP *in vivo* on the DNA, RNA and protein syntheses in neonatal rat bone marrow

Dibutyryl-cAMP, 4.7×10^{-2}mg/g body wt., was injected peritoneally for three days and 1 μCi/g body wt. ^{3}H-thymidine for DNA synthesis, 5 μCi/g ^{3}H-uridine for RNA or 1 μCi/g ^{3}H-leucine for protein was intraperitoneally injected, repectively. Animals were sacrificed one hour after each precursor injection and used for radioautographic observations

(Fig. 3). The similar findings were also obtained on the erythroblasts from the animals receiving pretreatment of db-cAMP (Fig. 3), though the labeling indices appeared higher than those of controls (Table 1).

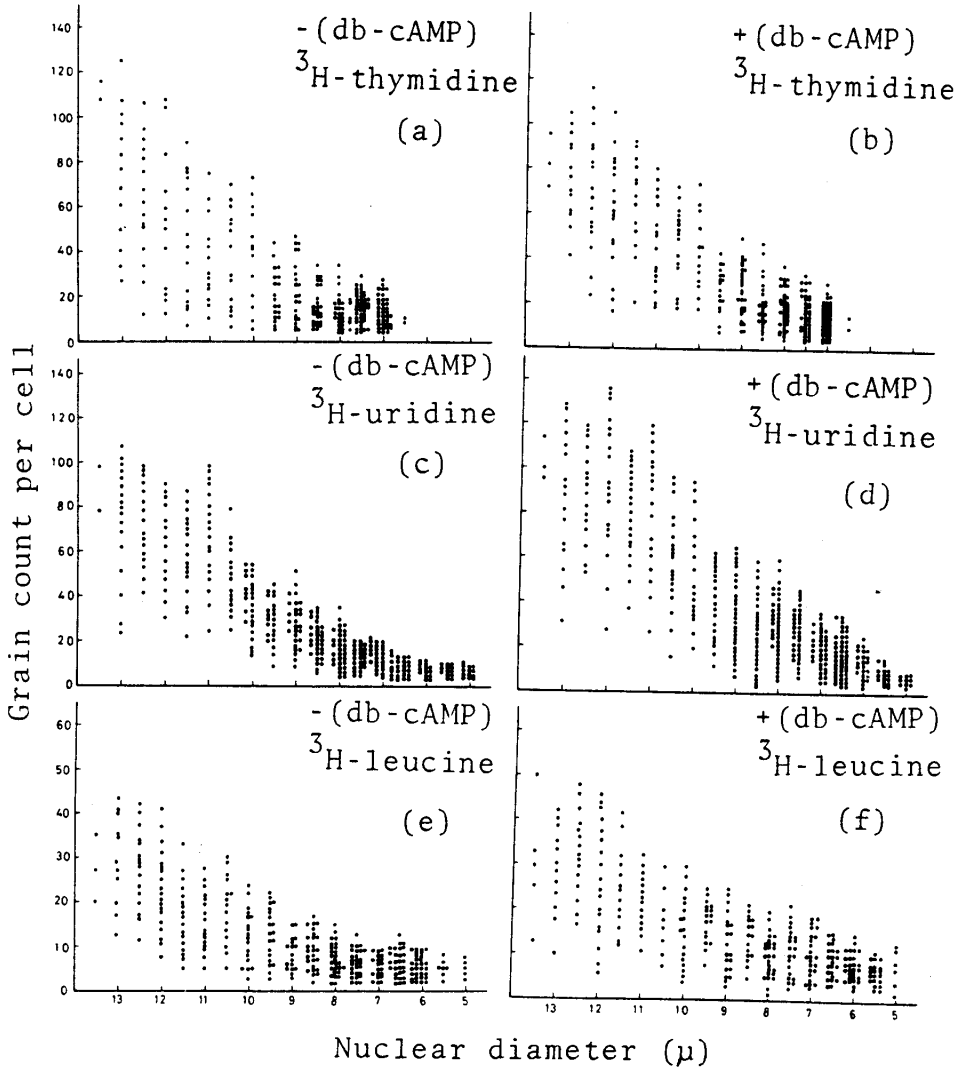

Fig. 4. Effect of db-cAMP *in vitro* on DNA, RNA and protein syntheses in erythroid cells isolated from neonatal rats

Bone marrow cells isolated from 3-day old neonatal rats were preincubated with db-cAMP, 10^{-4}M, for 40 min at 37°C and then incubated further for 20 min in the presence of ^{3}H-thymidine, 1 μCi/ml, ^{3}H-uridine, 5 μCi/ml and ^{3}H-leucine, 1 μCi/ml, respectively. For details see text.

TABLE 2 VARYING EFFECTS OF db-cAMP ON THE DNA SYNTHESIS IN ERYTHROID CELLS ISOLATED FROM FETAL LIVERS, NEONATAL AND ADULT BONE MARROWS *in vitro*

stage	db-cAMP	labeling index (%) by ^{3}H-thymidine erythroblasts origin		
		fetal liver	neonatal bone marrow	adult bone marrow
pro	−	91	90	92
	+	93	93	95
BI	−	75	77	80
	+	89	90	82
BII	−	62	58	62
	+	80	80	58
poly	−	39	38	29
	+	60	57	27

The data show mean values of labeling indices from 5 experiments. For details see Table 1.

The RNA synthetic activity observed by injecting ^{3}H-uridine indicated that the grain counts of erythoid cells were higher in the animals treated with db-cAMP than those of controls in all the differentiation stages compared, especially marked in the cells at earlier differentiation stages (Fig. 3). The labeling indices were also higher in the db-cAMP treated animals comparing with those of non-treated animals (Table 1). The results indicated the stimulation of RNA synthesis by db-cAMP injection.

In the cells from the animals treated with ^{3}H-leucine the immature cells had a larger of grains, which decreased with the advance of differentiation, as in the cases treated with ^{3}H-thymidine and ^{3}H-uridine. The grain counts were slightly higher in the animals receiving the treatment of db-cAMP injection comparing with those of controls (Fig. 3). The labeling indices of erythroid cells were also higher in the db-cAMP treated animals than those of controls (Table 1).

In the *in vitro* observations on the cells incubated with ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine, db-cAMP stimulated hepatic erythroid cells from embryo and neonatal bone marrows in their nucleic acid and protein syntheses but did not stimulate the bone marrow cells from adult animals (Figs. 4-6 and Tables 2-4).

The bone marrow cells of neonatal rats showed a marked incorporation of ^{3}H-compounds in the presence of db-cAMP *in vitro*, as those of rats treated with the cyclic nucleotide injection (Figs. 3 and 4). The grain counts of erythroid cells incubated with ^{3}H-thymidine were nearly the same both in the cases of those incubated in the presence and absence of db-cAMP but the labeling indices were higher in those incubated with db-cAMP (Fig. 4 a, b, and Table 2). The grain counts of the cells incubated with ^{3}H-uridine were much

higher than those incubated without db-cAMP (Fig. 4 c, d). Labeling indices were also higher in those incubared with db-cAMP (Table 3). In the cells

TABLE 3 VARYING EFFECTS OF db-cAMP ON THE RNA SYNTHESIS IN ERYTHROID CELLS ISOLATED FROM FETAL LIVERS, NEONATAL AND ADULT BONE MARROWS *in vitro*

stage	db-cAMP	labeling index (%) by ^{3}H-uridine erythroblasts origin		
		fetal liver	neonatal bone marrow	adult bone marrow
pro	−	93	92	93
	+	95	94	95
BI	−	83	74	82
	+	92	85	89
BII	−	60	57	60
	+	82	78	59
poly	−	44	41	35
	+	69	57	37
orth	−	19	23	16
	+	31	34	14

For details see Table 1.

incubated with ^{3}H-leucine the grains appeared more in those incubated with db-cAMP comparing with those incubated without db-cAMP (Fig. 4 e, f) and also higher labeling indices in the former than the latter (Table 4).

TABLE 4 VARYING EFFECTS OF db-cAMP ON THE PROTEIN SYNTHESIS IN ERYTHROID CELLS ISOLATED FROM FETAL LIVERS, NEONATAL AND ADULT BONE MARROWS *in vitro*

stage	db-cAMP	labeling index (%) by ^{3}H-leucine erythroblasts origin		
		fetal liver	neonatal bone marrow	adult bone marrow
pro	−	94	94	95
	+	97	98	95
BI	−	86	93	84
	+	92	95	80
BII	−	62	68	70
	+	81	82	73
poly	−	48	55	44
	+	61	65	45
orth	−	35	36	28
	+	45	47	25

For details see Table 1.

The erythroid cells of embryonic rat liver showed characteristics as those of the bone marrow of neonatal rats. Dibutyryl-cAMP markedly stimulated their DNA, RNA and protein syntheses (Fig. 5 and Tables 2, 3 and 4). In

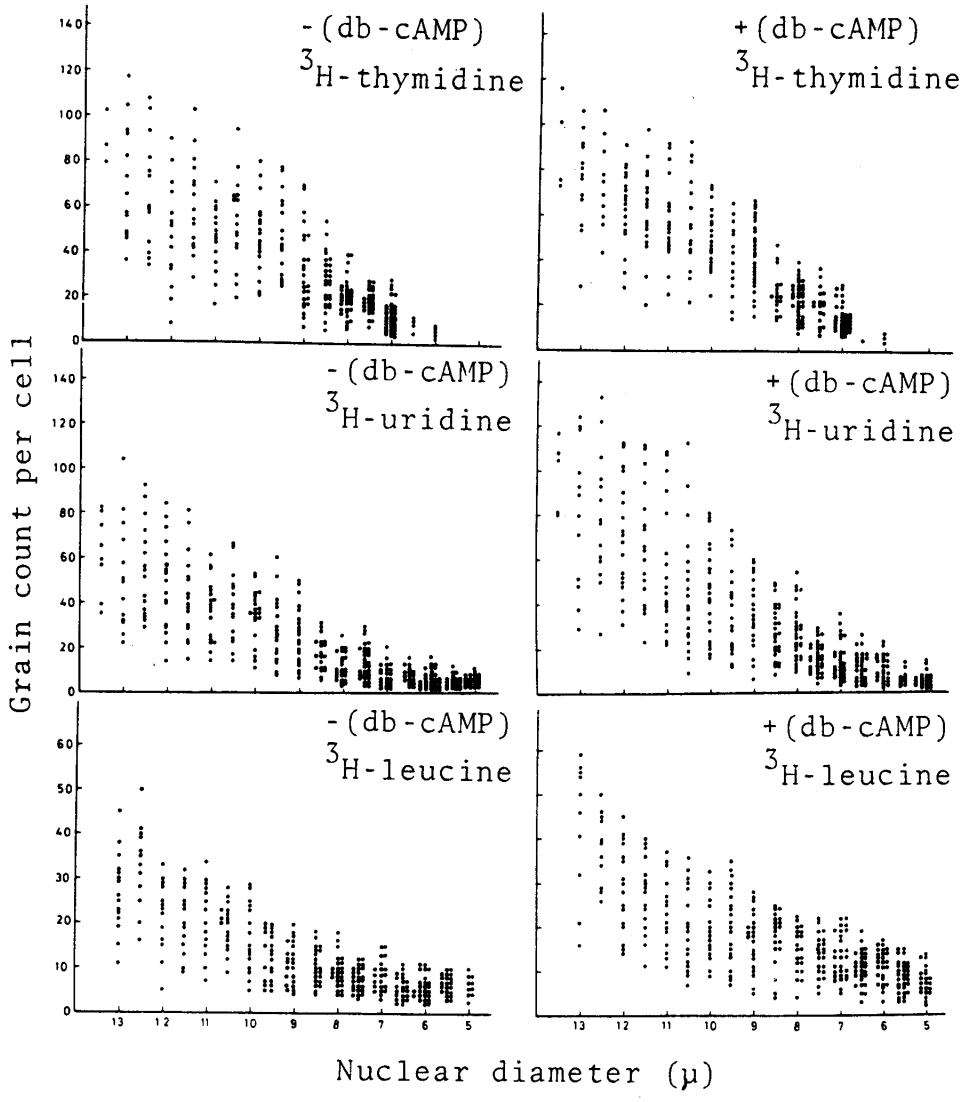

Fig. 5. Effect of db-cAMP *in vitro* on DNA, RNA and protein syntheses in erythroid cells isolated from fetal livers

Hematopoietic cells isolated from fetal livers of 14-gestation day rats were performed as indicated in Fig. 4.

contrast, the cyclic nucleotide not give any stimulatory effects on the bone marrow erythroids from adult rats in their nucleic acid and protein syntheses. Any increase in grain counts were not observed in all the cases observed as compared with those of controls (Fig. 6 and Tables 2, 3 and 4).

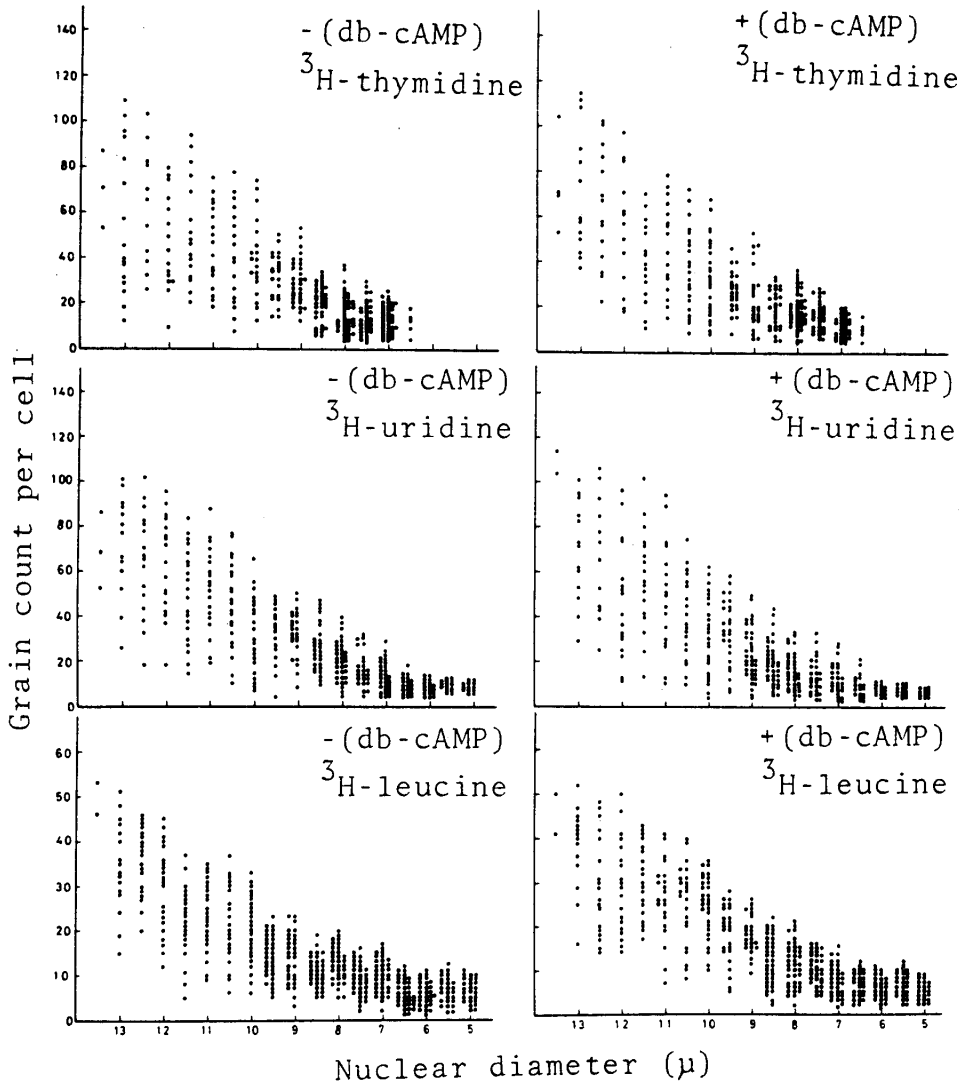

Fig. 6. No effects of db-cAMP *in vitro* on DNA, RNA and protein syntheses in erythroid cells isolated from adult bone marrows
For details see Fig. 4.

DISCUSSION

The present data indicate that in erythropoiesis of fetal and neonatal rats nucleic acids and protein syntheses are stimulated by db-cAMP while that of adult bone marrow is not affected by cyclic nucleotide. The observations

suggest that in early developmental stages the erythropoiesis is under a specific control mechanism different from that of adult. The fact that the enhancing effect of db-cAMP on erythroid cell number in neonatal rat diminishes 5 days after birth suggests that the changes in the control mechanism from embryonic type to adult type occur relatively rapid after birth. CARMENA and associates showed that in rat neonates erythropoietin is very low at birth and increases with advance of age (13). LUCARELLI and his associates reported that in newborn rats the erythropoiesis was scarcely affected by nephrectomy and starvation, which suppress the erythropoiesis of adult rats (14). CARMENA and coworkers also reported that in newborn rats hypoxia failed to enhance the rate of erythropoiesis (15). All these findings support the view that in early developmental stages the erythropoiesis is controlled by cyclic nucleotide.

As is well known, the erythroid differentiation from precursor cells is controlled by erythropoietin in adult. It is uncertain whether the cyclic nucleotide acts on erythroid cells of embryo and newborn in connection with erythropoietin, but the present experiment *in vitro* indicates that the cyclic nucleotide stimulates the syntheses of nucleic acids and proteins of erythroid cells from fetal livers and neonatal bone marrows. As the effect of erythropoietin is thought to be minimal in Hanks-serum solution used in the present experiment, the cyclic nucleotide should affect directly the syntheses of nucleic acids and proteins of erythroid cells of embryo and newborn. It has been reported that in adult mice the cyclic nucleotide stimulates erythropoiesis in the spleen and that of bone marrow of polycythemic animals, but the effect is suppressed by antierythropoietin (7, 10). This indicates that in the foregoing cases the cyclic nucleotide acts in connection with erythropoietin, suggesting that in adult the erythroid proliferation is somehow controlled by erythropoietin. Thus it will be deduced that the erythropoiesis is controlled by cyclic nucleotide in immature animals and by erythropoietin in adults. This may be related to the control of erythoid cells having fetal hemoglobin produced in the immature individuals and those having adult hemoglobin produced in mature animals.

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