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Studies on the relation between heme and nucleic acid syntheses in erythroid cell. I. Effects of aminopterin and bromouracil on hemopoiesis and hemoglobin synthesis in anemic rat*

Morio Shigehisa

Abstract

For the purpose to see how the suppression of the nucleic acid synthesis disturbs the cell specialization process the author observed the erythroid cell specialization in anemic rats by treating them with aminopterin (AP) and 5-bromouracil (BU). The observations indicate that the AP injection inhibits the mitosis of erythroblast with the acceleration of hemoglobin synthesis and the denucleation. The bromouracil administration scarcely suppressed the mitosis and the appearance of acidophilicity of erythroblast was retarded. Data indicate that the inhibition of mitosis accelerates the specialization or somatic protein synthesis of erythroblast. The acting mechanisms of the medicaments were discussed from the characteristics of these agents as the analogue of the substances related to DNA metabolism.

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STUDIES ON THE RELATION BETWEEN HEME AND NUCLEIC ACID SYNTHESES IN ERYTHROID CELL

I. EFFECTS OF AMINOPTERIN AND BROMOURACIL ON HEMOPOIESIS AND HEMOGLOBIN SYNTHESIS IN ANEMIC RAT

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As is well known, the development of erythroid cell is very sensitive to the deficiency of folic acid and vitamin B_{12}, the important factors for DNA synthesis. The deficiency of these factors induces the changes in erythroid cell in bone marrow specific to pernicious anemia i. e. the appearance of megaloblasts and megalocytes in circulating blood impressing the distorted development of erythroid cell resulting from the suppressed DNA synthesis.

It is generally believed that the youngest precursor of erythroblast, proerythroblast, is formed by the transformation of the stem cell, whose morphologic entity is not yet defined by the action of inducer, erythropoietin (1, 2). The proerythroblast differentiates to red cell through four stages of specialization (3, 4, 5, 6); early and late basophilic, polychromatic and orthochromatic stages. At each specialization step cell division ensues and the cell reduces its nuclear and cell volumes by about one half at each cell division, respectively (3, 4, 5, 7, 8). Denucleation ensues at the orthochromatic stage in physiologic hemopoiesis but in anemia a number of cells are denucleated at an early specialization stage i. e. at polychromatic, basophilic or even at proerythroblast stage, skipping cell division or divisions (9, 10, 11). Macrocytes and megalocytes are thus formed, because the cell does not divide after denucleation (12). Macrocyte or megalocyte may contain more hemoglobin than normocyte, formed by denucleation at orthochromatic stage (13).

Thus, the symptoms of pernicious anemia or the appearance of megalocyte in circulating blood suggests that the severe arrest of DNA synthesis results in the acceleraiton of early denucleation of erythroid cell with the accelerated hemoglobin synthesis or the erythroid cell specialization. From such a view point the author observed the hemoglobin level of

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erythroid cells from the anemic rat treated with folic acid antagonist and bromouracil. In this paper it is reported that erythroid cell specialization or hemoglobin synthesis is promoted by suppressing DNA synthesis with aminopterin injection indicating a close correlation between nuclear and cytoplasmic functions in the cell specialization.

MATERIALS AND METHODS

Adult male Wistar rats weighing 180—200 g were used. The animals were made anemic by the repeated subcutaneous injections of phenylhydrazine-HCl, 0.5 to 0.7 ml of 1.25% solution once a day for three to four days. By this treatment a marked hemolytic anemia was induced, and three days after the last phenylhydrazine injection a marked erythroid marrow developed. At this stage the animals were divided into three groups; the first group received the administration of aminopterin (AP), the second group 5-bromouracil (BU), and the third group was of the anemic control and received no further treatment. AP was injected subcutaneously 2 mg per 100 g body weight once or daily for two to three days. BU was also injected subcutaneously about 25 mg per 100 g once a day for two to four days, and a few animals received 12 BU-injections daily for 12 days. AP was procured from Tokyo Kasei Kogyo Co. Ltd. and BU from Katayama Kogaku Kogyo Co. Ltd.

The animals received the injection of the agents were sacrificed from 7 to 10 days after the initiation of the phenylhydrazine injection being accompanied by the sacrifice of anemic control in each. A few animals treated with 12 injections of BU were killed on 13th day. For the observation of mitosis of erythroblast the animals received colchicine injection, 100 μg per 100 g body weight, before sacrifice. After sacrifice the bone marrow cells were smeared two smears in each bone marrow sample; one for Giemsa stain and the other for quantitative estimation of hemoglobin per cell by microspectrophotometry. Organs of each animal, liver, spleen and some lymph nodes were fixed with 10% formol, sectioned and stained with hematoxylin-eosin by routine method for histologic examination.

The estimation of hemoglobin was carried out on methanol-fixed smear by the microspectrophotometer of Olympus Kogaku Co., employing the two-wavelength method of ORNSTEIN (14), PATAU (15) and MENDELSOHN (16) at 405 mμ and 427 mμ.

Mitotic indices were obtained on Giemsa-stained smear by observing 1,000 cells per one bone marrow sample obtained four hours after colchicine injection.

RESULTS

A moderate or severe hemolytic anemia developed in the rats after treating with phenylhydrazine injections, once a day for four days; red

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cell count around 3 millions per cu. mm., hemoglobin content about 30% Sahli (4.8g per dl), and a marked reticulocytosis about 60% (Fig. 1-a). Three days after stopping the phenylhydrazine injections the anemia entered into its recovering phase and a typical erythroid bone marrow developed.

At this stage the injection of the inhibiting agents for DNA synthesis was initiated. The injection of AP suppressed effectively the increase in the red cell number in the circulating blood but did not arrest the recovery of hemoglobin (Fig. 1-b). Consequenty, after two or three iniections of AP the color index reached an extremely high level comparing to that of anemic control. Red cell diameter increased with a marked macrocytosis. With these changes in hemogram the gross appearance of the bone marrow showed a marked change.

The bone marrow of the animals sacrificed 24 hours after one AP injection was soft and hemorrhagic, but after three injections of AP for three successive days it turned rather fluid and reddish purple in color.

Microscopic observations of the Giemsa-stained bone marrow smears taken 24 hours after one AP injection revealed that polychromatic erythroblasts were predominant in number with the decrease in basophilic erythroblasts. The polychromatic erythroblasts were rather large in size, 9—10 μ in nuclear diameter, or 10.5—12 μ cell diameter. Mitotic figures of erythroblasts were rarely encountered (Photo. 1a).

The bone marrow smear taken 24 hours after two AP injections once a days for two days, the cell number was considerably decreased both in erythroid and myeloid cells (pancytopenia), basophilic erythroblasts were rarely encountered, and some large sized cells were polychromatic in their cytoplasm. Besides erythroid and myelogenous cells a number of lymphoid cell appeared. They were 7.5—8 μ in nuclear dimeter, 9—9.5 μ in cell diameter and had pale blue cytoplasm. No mitosis was seen.

In the circulating red cell were anisocytic with distinct macrocytosis and many of them were spherocytic.

Twenty-four hours after three AP injections bone marrow were eradicated with erythroid and myeloid cells and some small lymphoid cells were predominated. All the rats died within a few days after AP administration.

The BU injections, once a day for five days, did not interfere with the recovery of anemia; the red cell number and hemoglobin level increased steadily in the treated animals as well as in anemic control. Body weight was rather increased and the animals had a healthy appearance (Fig. 1-c). In all the animals receiving two to four injections of BU, the

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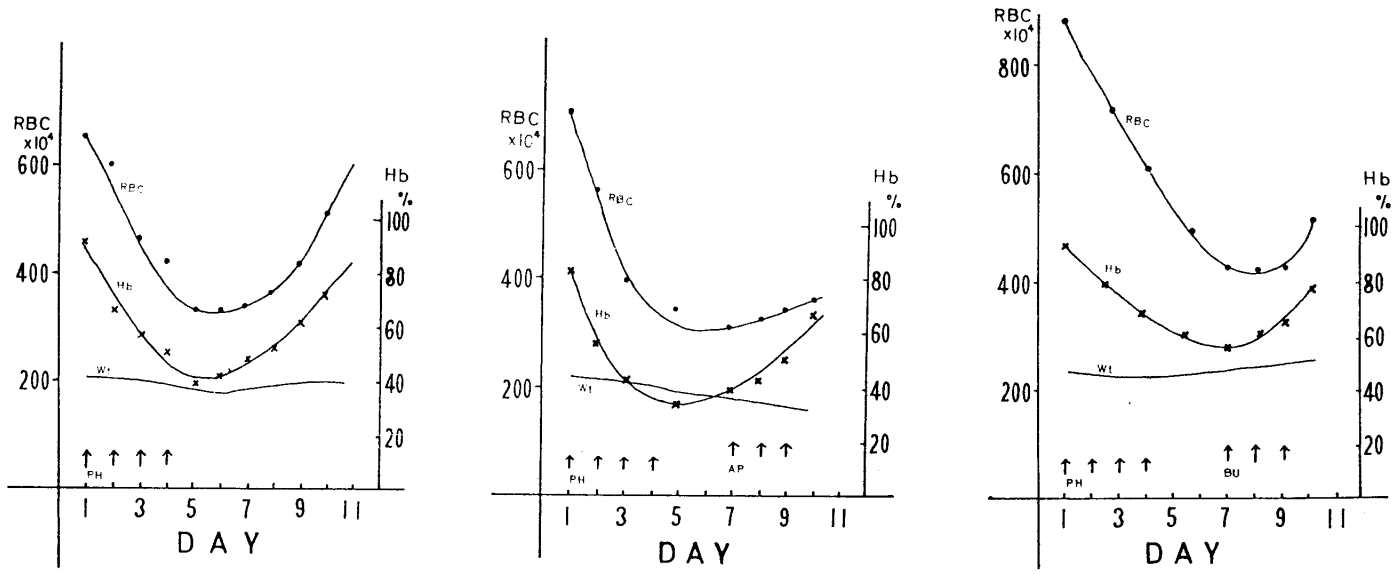

Fig. 1-a Fig. 1-b Fig. 1-c

Fig. 1 Daily changes in circulating blood of rats treated with phenylhydrazine followed by aminopterin and bromouracil injections

Fig. 1-a Anemic control, solely receiving phenylhydrazine injections

RBC: red blood cell Hb: hemoglobin, Sahli

Wt: body weight (g) PH: phenylhydrazine injection

Fig. 1-b Hemogram of the anemic animals treated with aminopterin

AP: aminopterin injection

Fig. 1-c Hemogram of the anemic animals treated with bromouracil

BU: bromouracil injection

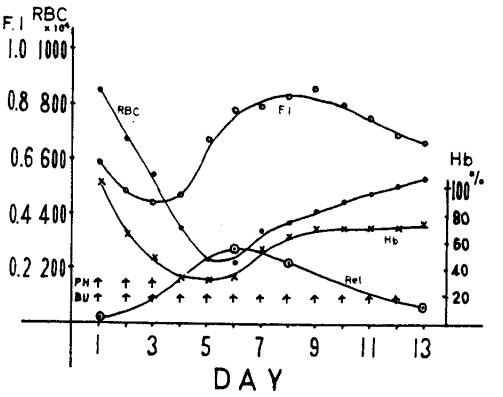

Fig. 2 Daily changes in hemogram of circulating blood of an anemic rats treated with bromouracil for a long period. BU was injected starting from the first day of 3 phenylhydrazine injections and continued to the last day. Arrows are phenylhydrazine and bromouracil injecion.

bone marrow showed no abnormalities in gross appearance, it was reddish grey in color and solid but not fluid. Microscopic observations revealed an erythroid marrow as in anemic control but most erythroid cells were basophilic in appearance. Small erythroblasts comparable to the poly- to orthochromatic cells in size had the basophilic cytoplasm, and most of the denucleated red cells in the bone marrow appeared bluish (Fig. 2). Repeated injections of BU, more than 10 times during 12 days, showed some retardation in the recovery of anemia. but the red cell proliferation was not so severely arrested. The erythroblasts retained their cytoplasmic basophilicity till later stage of specialization. Some basophilic erythroblasts in the advanced stage of specialization had clear nucleoli (Photo. 1-b).

The mitotic indices of erythroblasts decreased markedly in the animals treated with AP injections but not in those treated with BU injections which gave nearly the same index as anemic control (Fig. 3).

The hemoglobin content of each erythroblast measured by microspectrophotometry showed a high level in the cells from the animals treated with AP and a rather low level in those treated

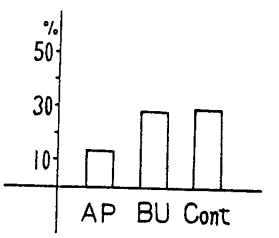

Fig. 3 Mitotic indices of erythroblasts from anemic animals treated with aminopterin, bromouracil and anemic control
AP: 24 hours after AP injection, 2mg per 100g body weight.
BU: 24 hours three BU injections, 25mg per 100g body weight daily for three days.
Cont: anemic control
Method; see text.

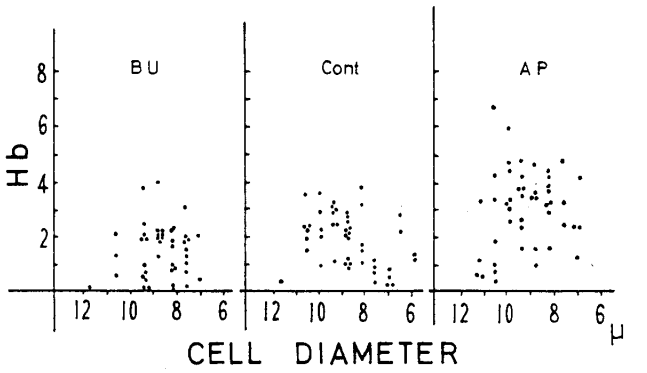

Fig. 4 Hemoglobin contents (arbitrary unit) of polychromatic erythroblasts, estimated by microspectrophotometry
AP: The cells from the animals treated with aminopterin
BU: The cells from the animals treated with bromouracil
Cont: anemic control receiving phenylhydrazine injections only

with BU (Fig. 4).

Histological observation showed a marked decrease in cellularity in the bone marrow treated with AP but not any change in those treated with BU. Besides these severe ulcerative changes have been observed in the alimentary tracts of those treated with AP.

DISCUSSION

As can be seen clearly from the experimental results just presented the administration of AP suppresses the erythroid cell proliferation in anemic rats. In the early stage of AP administration the Hb level of peripheral blood rised as in anemic control but the increase of red cell number stoped, resulting in the increase of color index, the red cell showed a marked macrocytosis indicating early denucleation. In bone marrow the mitosis of erythroblasts stops and the cell specialization seems to proceed without cell division showing the increase in acidophilicity of the cytoplasm of erythroblasts or in heme level. All these phenomena indicate that the process of the cell specialization including the denucleation at early stage should closely be correlated to the lowered activity of erythroblast.

Aminopterin is a structural analogue of folic acid, and inhibits the methylation catalysed by folic acid as coenzyme (17, 18, 19, 20, 21, 22), i. e. the purine ring formation and the change of uracil to thymine (23). Thus, AP administration inhibits the DNA and RNA syntheses. But it has been evident that the most prominent biochemical effect of AP observed in some cellular system is a diminution of thymidine synthesis (35). Thymi-

dine synthesis is of higher requirement for a folic acid coenzyme (33). Observations by WAHBA and FRIEDKIN revealed that the folic acid antagonist inhibit the activity of thymidilate synthetase directly (36). Consequently, the main effect of aminopterin will be on the synthesis of thymidilate from uridilate and on DNA synthesis. Therefore, the hemoglobin synthesis and the denucleation of erythroblast at early specialization stage seen after AP treatment possibly be primed by the suppression of DNA synthesis. As the Hb contents per cell are increased by AP administration, the translation process for hemoglobin is not interfered by the disturbances of the DNA synthesis. The AP administration results in the complete suppression of hemopoiesis. This indicates the transformation of stem cell to proerythroblast is suppressed. In the early stage of AP administration the younger precursors of bone marrow cells disappears and then all the erythroid cells are eradicated by the continued injections of AP. Data also suggest that the inhibition of DNA replication suppresses the transcription for the specialization of stem cell to proerythroblast. These facts indicate again that the physiological specialization step of erythroid cell is dependent upon the retained DNA synthesis. The data may be referred to the report of TAKEBAYASHI (24), indicating that erythroid cell specialization is accelerated by a mass red cell transfusion where the DNA synthesis and mitosis is suppressed only in later stage, i. e. the cell specialization or the synthesis of heme in erythroblast preceeds and the suppression of DNA synthesis is followed. It may reasonably be thought that the cell specialization and cell division, or DNA synthesis and somatic protein synthesis are the matter of close association and the inhibition of DNA replication may accelerate the protein synthesis or vice versa.

The observations are consistent with the general concept that the cells replicating DNA are poor in cell specialization or the synthesis of somatic protein.

In contrast, the administration of BU did not result in any inhibitory effect on the mitosis of erythroblast. The red cell number in the circulating blood increased as in the case of anemic control. In spite of several reports on bacteria including some cultured animal cells and plant cells, it seems that BU does not induce so severe disturbances in the nucleic acid metabolism of both DNA and RNA of erythroid cells *in vivo*. According to the past reports, 5-bromouracil is incorporated into DNA (25, 26, 27, 28, 29, 30, 31), and can replace 26—27% of DNA thymine under an appropriate condition (27). Competition with thymine can occur at the level of phosphorylation to the triphosphate or at the stage of DNA polymerization. Besides these, BU may act as a tautomeric mutagen to produce

errors in DNA nucleotide sequence; pairing with guanine in its enol form and replacing cytosine whereas it pairs with adenine in its keto form like thymine (23, 32). BU may also be incorporated into RNA as well as 5-fluorouracil as revaled in *C. utilis* adapted to grow on uracil (23, 30). The DNA formed by mistake in replication may cause the synthesis of nonfunctional proteins (27, 33, 34). These characteristics of BU may give an information for the analysis of the results obtained by BU. In spite of little suppression of mitosis or cell replication, morphologic observations on smeared bone marrow from the animals received BU treatment suggested the suppression of hemoglobin synthesis; i. e. the bone marrow smears showed a numbers of small erythroblast just comparable to orthochromatic ones in size and having a strong basophilic cytoplasm. The heme contents per cell estimated by microspectrophotometry showed only a slight decrease compared with that of anemic control. Data seem to indicate a possible error at transcription, leading to some defect in globin synthesis rather than the enzymes involving the heme synthesis, though the problem is left to be settled in future.

SUMMARY

For the purpose to see how the suppression of the nucleic acid synthesis disturbs the cell specialization process the author observed the erythroid cell specialization in anemic rats by treating them with aminopterin (AP) and 5-bromouracil (BU). The observations indicate that the AP injection inhibits the mitosis of erythroblast with the acceleration of hemoglobin synthesis and the denucleation. The bromouracil administration scarcely suppressed the mitosis and the appearance of acidophilicity of erythroblast was retarded. Data indicate that the inhibition of mitosis accelerates the specialization or somatic protein synthesis of erythroblast. The acting mechanisms of the medicaments were discussed from the characteristics of these agents as the analogue of the substances related to DNA metabolism.

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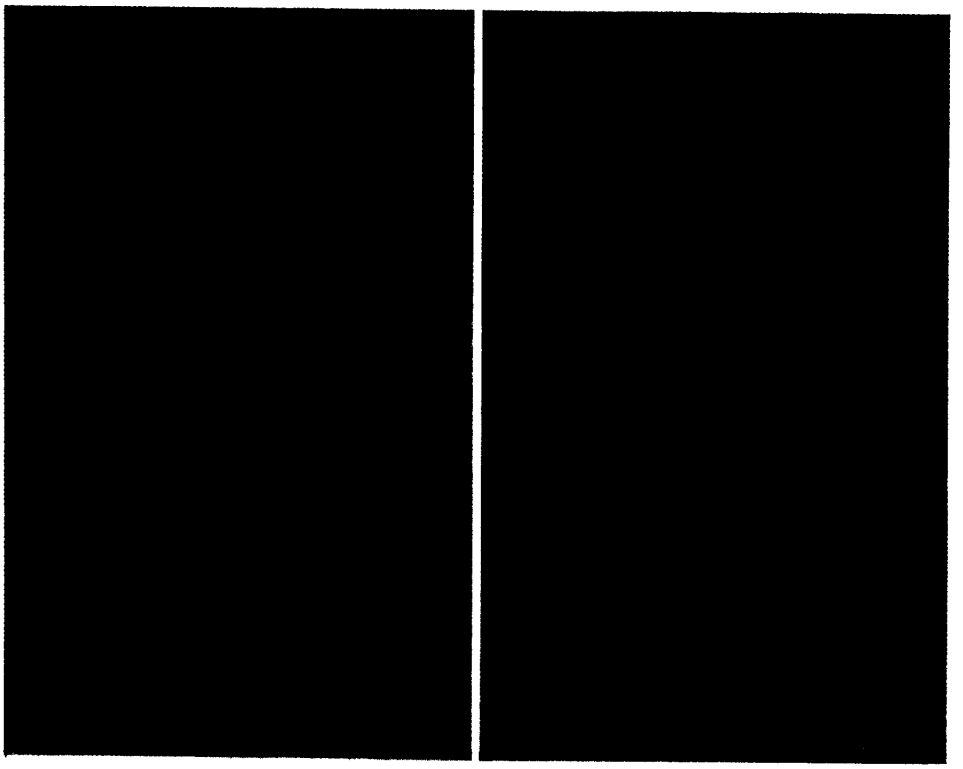
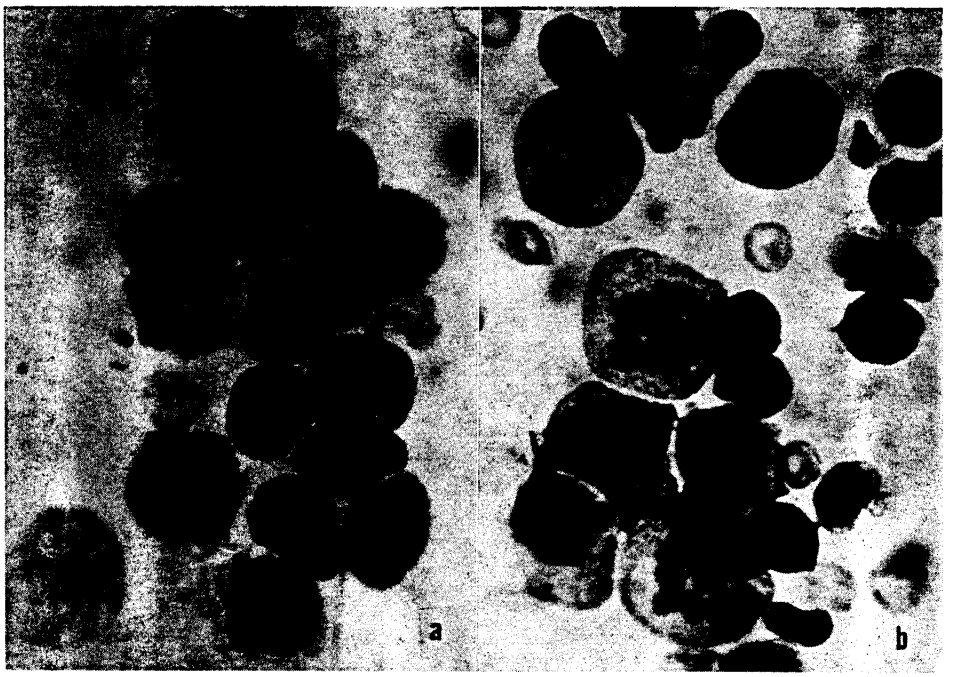


(a) (b)

Photo. 1 Photographs of bone marrow cells

(a) Bone marrow smear of the anemic animal treated with aminopterin, 24 hours after AP injection, 2 mg per 100 g body weight. Note that the cells comparable to basophilic erythroblast in their size have polychromatic cytoplasm.

(b) Bone marrow cells of the anemic animal treated with bromouracil, 24 hours after three BU injections, 25 mg per 100 g body weight. Note the strong basophilicity of erythroblasts through all the specialization stages.



(a) (b)

Photo. 2 Bone marrow smear of the animals treated with aminopterin and bromouracil. Twenty four hours after the last injection of AP (a) or BU (b) colchicine was injected and 4 hours later bone marrow was obtained. Note actually no mitosis in AP treated animal, while active mitosis in BU treated animal.