PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 171, 151–157 (1982)

# Macromegakaryocytosis After Hydroxyurea[1] (41490)

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*Abstract.* A single injection of hydroxyurea (OHU) produced transient megakaryocytopenia in mice. An increase in the average mean size of mature, stage III megakaryocytes coincided with their depopulation. This was due to a selective reduction in numbers of smaller cells. In contrast, the macromegakaryocytosis of immunothrombocytopenia showed substantial increases in numbers of larger cells and reductions in smaller. Further reduction in numbers of smaller cells occurred when OHU was given to mice with immunothrombocytopenia, and the megakaryocytopenia was somewhat more severe than that produced by OHU in normal mice. OHU produced mild thrombocytopenia in normal mice and compromised recovery of the platelet count from immunothrombocytopenia. The most likely explanation for the increase in mean megakaryocyte size in the hypomegakaryocytic state produced by OHU is that the temporary imbalance between a low rate of influx and a normal rate of maturation produced a shift of the age distribution of the cells due to a deficiency of immature cells.

Megakaryocyte size adjusts in response to perturbations of platelet count, demonstrating that it is one of the variables in the regulation of platelet production (1, 2). When megakaryocytopoiesis is stimulated or suppressed by perturbations of the platelet count, ploidy and size of megakaryocytes are, respectively, increased or decreased (2, 3). Size is determined not only by ploidy but also by the level of maturation in the compartment of recognizable megakaryocytes (4). Within any maturation stage, megakaryocyte size is proportional to ploidy, and within any ploidy group, size is proportional to maturity (5, 6). Megakaryocyte maturation is subject to some degree of variability being accelerated in response to thrombocytopenia (4) but remaining normal in transfusion-induced thrombocytosis (7). However, instances in which changes in the age distribution of megakaryocytes alone may have effected changes in mean cell size have not been described.

In addition to the megakaryocyte size changes that can be produced by manipulation of the platelet count, mean megakaryocyte size may be increased in hypomegakaryocytic states in which thrombocytopenia does not appear to be causative (8). The present studies were done to analyze the macromegakaryocytosis that accompanies the transient hypomegakaryocytic state following a single injection of hydroxyurea (OHU).

**Materials and Methods.** Female mice of the $CF_1$ strain (Charles River) were used at the age of 12–14 weeks. Each mouse was sampled only once; sequential studies were done with cohorts of mice. Blood for platelet counts was obtained by cardiac puncture under ether anesthesia and anticoagulated with dry $K_2$EDTA; platelets were counted by phase microscopy (9). Cells were flushed out from each tibia with 1 ml of 1% $Na_2$EDTA in saline for counts of nucleated cells by Coulter counter and megakaryocytes by microscopy with new methylene blue stain.

Bone marrow smears were made from split femurs with a paint brush technique

[1] Supported, in part, by Grant R01-AM21355 from the National Institutes of Health and, in part, by the Office of Health and Environmental Research of the U.S. Department of Energy under Contract DE-AC03-76SF00098.

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0037-9727/82/100151-07$01.00/0

and stained with Wright's and Giemsa stains. Megakaryocytes were classified according to morphological criteria; all megakaryocytes were larger than myeloid and erythroid cells. Stage I megakaryocytes had basophilic cytoplasm without visible granules and a high nucleus/cytoplasm ratio. Stage III had azurophilic granules throughout the cytoplasm and a low nucleus/cytoplasm ratio. Stage II had intermediate morphology. Differential counts were done to classify 100 megakaryocytes from each mouse as stage I, II, or III. Sizes of stages I and III megakaryocytes were determined by measuring the areas of images enlarged from negatives of black and white photomicrographs; the areas were expressed as planimeter units.

Anti-mouse platelet serum (APS) was produced in rabbits or guinea pigs; before use it was heat inactivated and absorbed three times with equal volumes of washed mouse red cells. It was injected ip in 0.1 ml volumes after appropriate dilution with saline.

OHU was freshly dissolved in saline before injection. It was injected iv (tail vein) in a dose of 900 mg/kg body weight.

**Results.** One day after a single injection of OHU, tibial megakaryocytes and total nucleated cells declined to about 80% of normal (Fig. 1). Megakaryocytes decreased further to about half of normal on Days 2 and 3 before beginning to recover on Day 4. Total cellularity was less severely diminished and recovered about one day sooner than megakaryocytes. Cell counts returned to, but did not exceed, normal numbers during the week following administration of OHU.

A plot of average stage III megakaryocyte size (Fig. 2) was a mirror image of the plot of megakaryocyte numbers; size increased as number decreased, and both returned to normal at the same time. The maximum increase in mean size of stage III megakaryocytes occurred on Days 2 and 3, and it was not preceded by an increase in mean size of stage I cells on Day 1. This finding confirmed earlier unpublished observations in which rats were given 900 mg OHU/kg; for 3 days there was a gradual increase in average size of stage III cells to about 140% of normal with no change in average size of stage I cells.

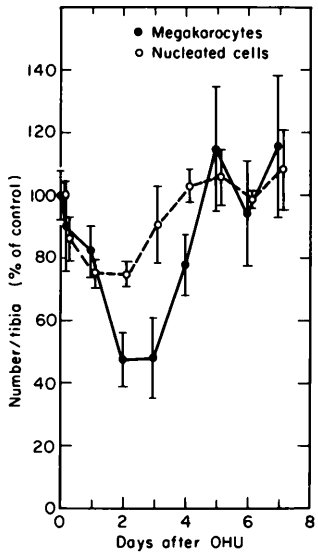


FIG. 1. Numbers of tibial megakaryocytes and nucleated cells, expressed as percentage of control values, for 7 days after administration of OHU to mice. Mean ± SEM for 33 controls is shown at 0 time; other points represent 11–12 mice.

To evaluate the changes in mean size of stage III cells, it was necessary to determine the relative number of stage IIIs at each sampling time after OHU. Differential counts of stages I, II, and III were done (Fig. 3, left); multiplication of percentage of each by the relative total number of megakaryocytes (Fig. 1; controls = 100) yielded the relative number of each stage in the marrow (Fig. 3, right). Megakaryocyte depletion occurred first in stage I cells and progressed to involve all three stages. Normal numbers of stage III cells were maintained through the first post-treatment day after which they decreased. On Days 2 and 3, the 50% reduction in total megakaryocytes was associated with a normal differential count, and therefore, equal reduction in all stages.

Size distribution curves for stage III megakaryocytes were prepared by determining the percentage of the cells that were in each 500-unit segment of the size range

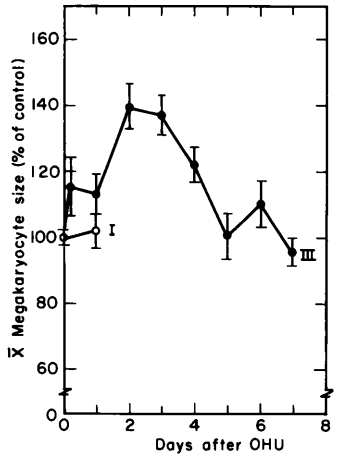


FIG. 2. Mean megakaryocyte size (±SEM), expressed as percentage of control values, after administration of OHU to mice. Stage III control value represents 1087 megakaryocytes from 33 mice; subsequent points, 376–386 megakaryocytes from 12 mice. Stage I control value represents 474 megakaryocytes from 33 mice and, at one day, 118 megakaryocytes from 12 mice.

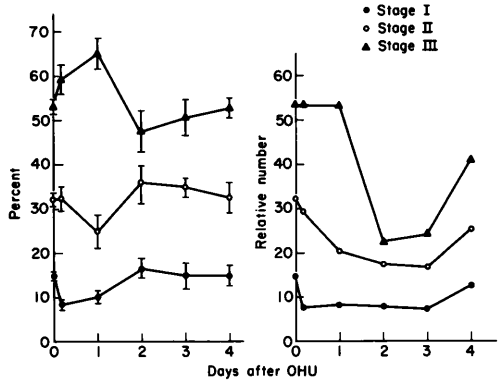


FIG. 3. Left panel: differential count of stages I, II, and III megakaryocytes after administration of OHU to mice; 100 megakaryocytes from each of 33 controls and 12 mice at each time after OHU were classified. Results are means ± SEM. Right panel: relative number of each maturation stage was calculated by multiplying mean percentage (left panel) by relative mean total number of megakaryocytes (Fig. 1).

(0–4000 units) at each sampling time. Each percentage was then multiplied by the relative number of stage III's at the same sampling time to develop size distribution curves that were representative of the actual number of stage III megakaryocytes and thus accounted for changing numbers of cells (Fig. 4). The 40% increase in mean size on Days 2 and 3 was due to a disproportionately great reduction in numbers of smaller megakaryocytes (<1000 units) and retention of normal numbers of larger cells (>1250 units); intermediate cells were diminished in proportion to the whole stage III population. Partial recovery on Day 4 was due to influx of intermediate size cells rather than those at either extreme of the curve.

Platelet counts after OHU are shown in Fig. 5; they were normal for 3 days, but declined to about 85% of normal on Days 4 and 5.

The effects of OHU on APS-treated mice were similar when the OHU was given after 1 day or after 4 days of APS-induced thrombocytopenia, so only the results of OHU after 1 day of thrombocytopenia are presented. Because of variability in platelet and megakaryocyte counts, two experiments are presented individually; however, tibial cell counts were almost identical in all four experiments done with APS and OHU, thus indicating that there was a consistent response to the chemical in all experiments.

Recovery from APS-induced thrombocytopenia was associated with variable degrees of rebound thrombocytosis and increases in numbers of tibial megakaryocytes, but there was a consistent increase in mean size of stage III megakaryocytes (Fig. 6). Administration of OHU retarded recovery of platelet counts and prevented rebound thrombocytosis. Maximal reductions in megakaryocyte numbers occurred 2 days after administration of OHU and were about one-third to one-half of the concomitant values in mice treated only with APS. Mean stage III megakaryocyte size was greater after combined treatment with APS and OHU than after APS alone, and the first appearance of a difference in size coincided with the reduction in number.

Size distributions of stage III megakaryocytes for the Day 2 samples were corrected for changing numbers of cells from the differential count of stages I, II, and III and total numbers of megakaryocytes

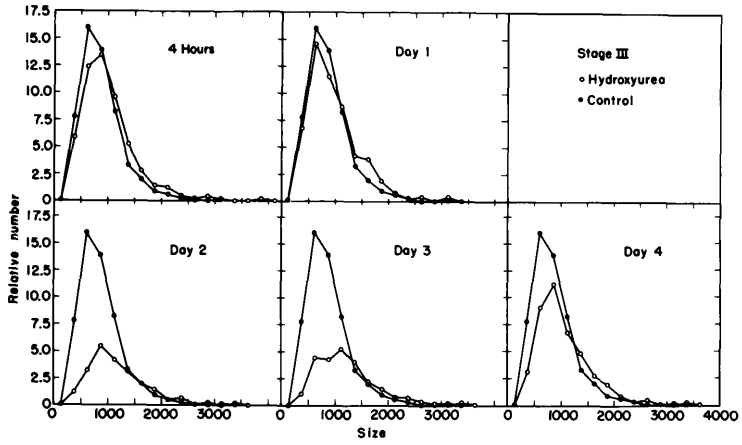


FIG. 4. Size distribution curves for stage III megakaryocytes from control mice and at intervals after administration of OHU. Abscissa expresses size in arbitrary units; ordinate is proportional to actual numbers of cells per tibia. Controls represent 1087 megakaryocytes from 33 mice; each curve after OHU represents 376–386 megakaryocytes from 12 mice.

as described above. Size distribution curves which are, therefore, representative of the actual numbers of stage III megakaryocytes are presented for the two experiments in Fig. 7. APS-induced thrombocytopenia alone was associated with reduced numbers of smaller megakaryocytes (<1000 units), substantial increases in larger ones (>1250 units), and inconsistent appearance of a few cells larger than any normally found in the marrow. OHU had the same effect on the size

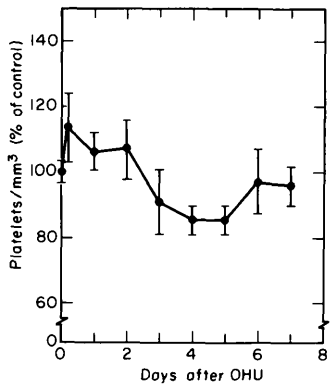


FIG. 5. Platelet counts, expressed as percentage of control, of 33 control mice and 11–12 mice at each sampling time after administration of OHU. Each point is the mean ± SEM.

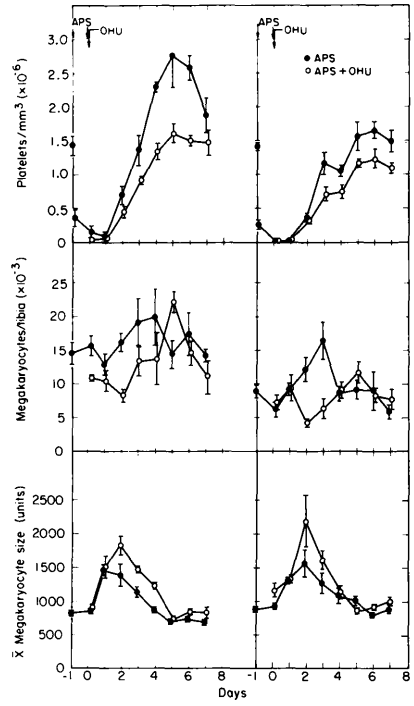


FIG. 6. Platelet counts, tibial megakaryocytes, and mean stage III megakaryocyte sizes in mice given APS on Days −1 and 0 with or without OHU on Day 0. Two experiments are shown. Each control is shown at 0 time and represents eight mice; each other point represents three to five mice. Each point is the mean ± SEM.

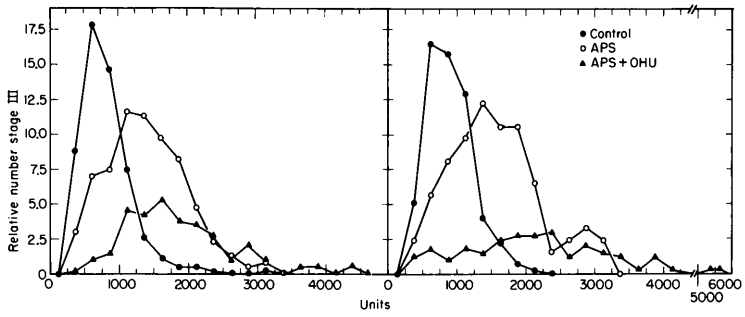


FIG. 7. Size distribution curves for stage III megakaryocytes from the same mice depicted in Fig. 6. Abscissa is size units; ordinate is proportional to actual numbers of cells per tibia. Controls represent 289 and 160 megakaryocytes from seven and eight mice. APS treatment represents 174 and 94 megakaryocytes from five mice in each experiment. APS + OHU treatment represents 175 and 88 megakaryocytes from five mice in each experiment. Values for treated mice were obtained 2 days after the treatment.

distribution of megakaryocytes in APS-treated mice as it had had in normals, i.e., retention of the largest cells, reduction in number of intermediate cells proportional to the reduction in the whole population, and disproportionately great reduction in numbers of smaller cells.

**Discussion.** The present results help to explain how macromegakaryocytosis develops in the hypomegakaryocytic state produced by OHU (8).

The number of stage I megakaryocytes was reduced as early as 4 hr after administration of OHU, indicating that the drug destroyed either stage I cells directly or precursors that should have matured rapidly to become stage I's. Long *et al.* (10) concluded that stage I megakaryocytes are actually a part of a population of smaller cells with lobed nuclei that could be identified histochemically as megakaryocytes. They did not find a cytocidal effect of OHU on this population 3 hr after its injection; rather, they found a 57% reduction in small precursor cells with round nuclei. Transit time for stage I rat and mouse megakaryocytes has been estimated to be 6–14 hr (11–13). Thus, the drop of about 50% at 4 hr could have been due to cessation of influx from a drug-sensitive compartment, but it seems more likely that some of the stage I cells were directly killed by OHU in these experiments.

The deficiency of megakaryocytes was apparent in stages II and III 1 to 3 days after administration of OHU as the requisite time elapsed for the damaged populations to mature into those compartments. Persistence of low numbers of megakaryocytes for 3 days indicated that OHU destroyed cells in an important precursor compartment, probably a dividing 2N cell population. The delay in recovery could not be attributed to prolonged action of the drug, as Morse *et al.* (14) have shown that DNA synthesis resumes as soon as 2 hr after 900 mg OHU/kg in the bone marrow of $CF_1$ mice.

Depopulation of the stage III compartment was associated with an increase in the mean cell size, due to selective loss of smaller stage III's without an increase in numbers of larger cells. Thus it contrasted with the macromegakaryocytosis produced in response to immunothrombocytopenia in which larger megakaryocytes were increased in number at the apparent expense of smaller ones. The macromegakaryocytosis produced in response to peripheral thrombocytopenia is known to be associated with increased ploidy (2, 3). Endoreduplication is completed and final ploidy determined before the cells leave stage I (11, 12, 15), and macrocytosis of stage I cells precedes that of stage III cells when stimulated by platelet depletion (1). Failure of stage I's to

show increases in size after OHU shows that the mechanism responsible for stage III macrocytosis after OHU differs from that seen after thrombocytopenia.

Megakaryocytes increase progressively in size as they mature from stage I through stage III (1); since stage III occupies 1–2 days of the 2- to 3-day total transit time (12, 13), it can be assumed that they continue to grow within this morphological group. Reduced influx in the presence of a normal transit time would account for the presence of fewer immature, and smaller stage III's during the period when their number was dropping. The somewhat greater reduction in megakaryocytes produced by OHU in APS-stimulated mice than in normals could then be attributed to the shorter transit time that occurs in thrombocytopenic animals (4, 16). The uniqueness of the OHU-treated mice is the increase in mean megakaryocyte size while their number is dropping. The mere reduction in smaller megakaryocytes has been observed (17) in irradiated mice during periods of stable megakaryocyte counts in which, therefore, an imbalance between rate of influx and rate of maturation does not appear to be involved.

Levin *et al.* (18) and Paulus *et al.* (19) have observed an inverse relationship between numbers of cells and their ploidy in megakaryocyte colonies cultured from hemopoietic cells. In these *in vitro* systems, therefore, the more a precursor cell divides, the less it appears to endoreduplicate. If this principle applies *in vivo,* the precursors of the smaller stage III's might have divided more than the precursors of larger ones and, therefore, have been more heavily damaged by OHU. This notion, however, would imply that the larger cells that persisted after OHU would have a higher degree of polyploidy than those that disappeared, and, as noted above, the failure of stage I cells to enlarge speaks against there being significant shifts in ploidy distribution.

OHU produced minor changes in platelet counts, probably because of the transient nature of the reduction in megakaryocytes. Considering that mouse platelets survive for about 4 days (20), the 15% drop in platelet count on Day 4 is consistent with a platelet production rate of about 50% for the precedent 24 hr, which, in turn, is consistent with the megakaryocyte number having been 50% of normal on Day 3. However, platelet counts on Days 3 and 4 were somewhat greater than would have been expected if production had been reduced to 50% for 2 days as might have been expected from the numbers of stage III megakaryocytes. It could be proposed that, even though total cell number was reduced on Day 2, those that remained represented a nearly normal complement of the most mature, platelet-forming, stage III cells.

Recovery from immunothrombocytopenia was delayed, and rebound thrombocytosis was prevented, demonstrating a somewhat different effect for OHU than was seen when vincristine (VC) was administered to acutely or chronically thrombocytopenic rats (21). VC inhibited recovery from acute, but not chronic, thrombocytopenia whereas OHU inhibited recovery from both. The two drugs produced comparable reduction in megakaryocytes, so the reason for the difference is not clear. However, a difference in ability to recover from acute immunothrombocytopenia has also been found between genetically anemic mice of the $W/W^v$ (22) and $S1/S1^d$ (23) strains. Both strains are comparably hypomegakaryocytic, but the $W/W^v$ responds normally to acute platelet depletion whereas the $S1/S1^d$ fails to develop rebound thrombocytosis. Neither the drugs nor the genetic abnormalities interfered with the development of macromegakaryocytosis. These variable responses to thrombocytopoietic stimulation under different conditions indicate that platelet production can not be accurately predicted from just the number of megakaryocytes and their mean size and that other factors must also be important.

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Received May 17, 1982. P.S.E.B.M. 1982, Vol. 171.