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The Effect of Hydroxyurea on Differentiated Marrow Erythroid Precursors* (33705)

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Hydroxyurea (OHU) an inhibitor of DNA synthesis, is used in the treatment of melanoma and resistant chronic myelocytic leukemia (1, 2). Its action at the cellular level has been studied with several strains of proliferating cells (3–5). It has been amply documented that OHU interferes with DNA synthesis, presumably at the level of conversion of ribonucleotides to deoxyribonucleotides, without influencing the synthesis of RNA and protein. Philips *et al.* (6) reported acute selective cytotoxic effects in rats treated with OHU. Injury was sustained by those tissues with high rates of cellular proliferation. They further concluded that the cytotoxic effects were time related to the effective concentrations of circulating drug. The half clearance in the plasma of the rat was of the order of 1 hr and reflected both excretion and metabolism. The observations of Philips led us to consider that OHU would serve as a useful adjunct in the study of erythroid differentiation. Critical to this consideration was documentation of the selective action of OHU on marrow erythroid precursors in DNA synthesis. Measurement of erythroid cell cycle kinetics after OHU are the subject of this report.

Materials and Methods. Ten-week-old female CF_1[2] mice weighing 20–25 g were used. All injections were given intravenously via the tail vein in the following amounts: OHU, 0.9 g/kg; colchicine, 1 mg/kg; and 2.5 μCi/g of tritiated thymidine (TDR-^{3}H) with a specific activity of 6.7 Ci/mmole. Blood samples were collected in versenate by cardiac puncture from anesthetized animals. Reticulocytes were estimated by the method of Brecher (7). Cellularity of tibial marrow contents was measured by electronic particle counting after saponization as described by Fruhman (8). Bone marrow smears prepared by a brush technic were stained with Wright's and Giemsa. The percentage of erythroid precursors was estimated from differential counts on 500–1000 nucleated cells. The mitotic index was measured in these same preparations. For the studies with TDR-^{3}H, autoradiographs were prepared with Kodak NTB[3] nuclear track emulsion and then processed as described by Ebbe and Stohlman (9). The labeling index was determined on each slide from the observation of a minimum of 250 basophilic normoblasts. For TDR-^{3}H incorporation into DNA, marrow

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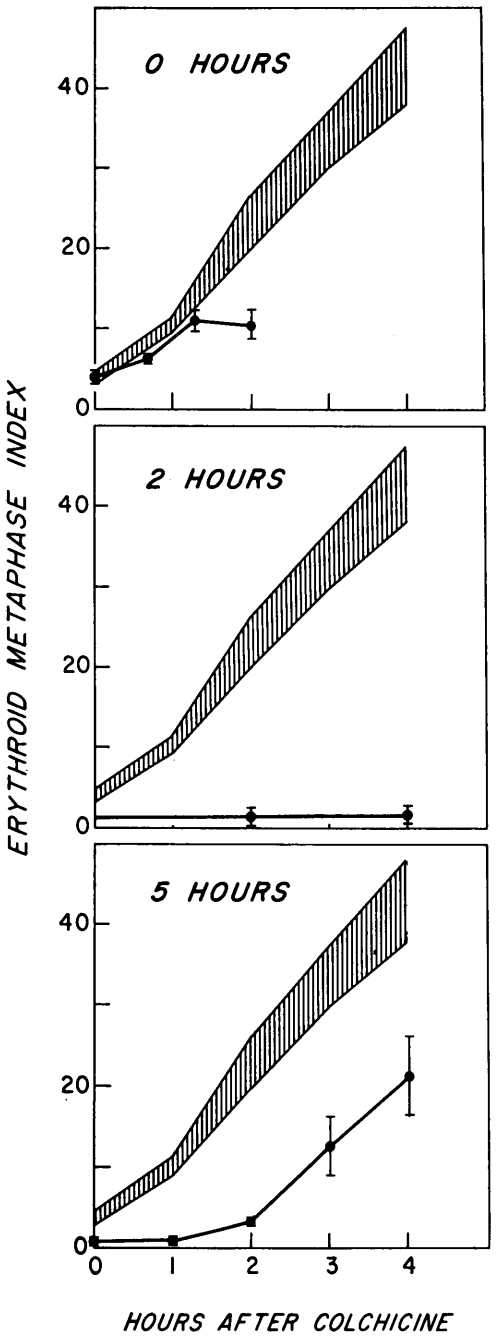

FIG. 1. The effect of OHU on the marrow erythroid stathmokinetic index. The shaded area encloses the mean ± SE of the accumulation of erythroid cells arrested in metaphase following colchicine. OHU and colchicine were given at time 0 in the upper graph; colchicine was given 2 hr after OHU in the middle graph; and colchicine was given 5 hr after OHU in the lower graph. Values are the mean ± SE of 4–8 animals.

TABLE I.

Hr	Nucleated cells (× 10^6/tibia)	Nucleated RBC (× 10^6/tibia)	Reticulocytes (%)
Control	9.59 ± 0.93[a]	2.20 ± 0.16	2.6 ± 0.2
12	6.57 ± 0.61	0.90 ± 0.16	
24	5.79 ± 0.63	0.65 ± 0.09	1.4 ± 0.2
48	6.64 ± 0.38	1.10 ± 0.12	0.5 ± 0.1
72	7.47 ± 0.25	2.12 ± 0.29	1.2 ± 0.1
144	9.52 ± 0.32	2.47 ± 0.16	5.0 ± 0.4

[a] Mean ± SE, 4–8 mice/point.

contents were expelled from a femur into cold saline. The cell suspension was washed in saline and then homogenzied in cold 0.5 *M* perchloric acid. The resultant precipitate was washed with water, dried in a cellulose casing, and combusted in oxygen-filled flasks. Tritiated water thus formed was absorbed in a dioxane solution containing 0.7% PPO, 0.03% POPOP, and 10% naphthalene (w/v). The samples were counted for 10-min intervals in a liquid scintillation detector at 8° at an efficiency of 16%.

Results. Bone marrow smears prepared from animals treated with OHU revealed considerable cellular fragmentation and karyorrhexis. These changes were noted after the second hour and persisted for the next 12 hr during which time phagocytosis of cellular debris was observed. The eythroid precursors were more severely damaged than other marrow cell lines. Twenty-four hr after OHU, tibial cellularity had decreased by 40% whereas total nucleated erythroid elements decreased by 70% (Table I). By the third day after OHU, the marrow erythroid cellularity had returned to control values. Peripheral blood reticulocytes decreased from 2.5 to 0.5% 2 days after OHU (Table I). On the following day the reticulocyte count began to increase and control values were exceeded by the sixth day.

After the administration of colchicine to normal mice, marrow smears revealed a linear accumulation of erythroid metaphase forms for 4 hr (Fig. 1). Extrapolation of this curve

to 100% provided an estimate of ~10 hr for the generation time. When OHU and colchicine were administered simultaneously, erythroid mitoses accumulated for 80 min. Thereafter no further increase was observed (Fig. 1). When colchicine was administered 2 hr after OHU, no accumulation of erythroid mitoses were observed for the next 4 hr. Lastly when colchicine was given 5 hr after OHU, there was no appreciable accumulation of erythroid mitoses for the first 2 hr. Thereafter there was a linear accumulation for the next 2 hr with a slope parallel to that observed in the normal mouse (Fig. 1).

Thirty min after the administration of TDR-^{3}H to normal mice, the labeling index of marrow basophilic normoblasts was ~60%. From these data a 6 hour t_s was obtained. In contrast when OHU and TDR-^{3}H were given simultaneously, the labeling index at 30 min was less than 1%. Comparable results were obtained when the incorporation of TDR-^{3}H into bone marrow DNA was measured. Thirty-min TDR-^{3}H incoporation into DNA as determined by both labeling index and DNA extraction remained at near zero levels for 2 hr. Although recovery began after the second hour, the incorporation of TDR-^{3}H into DNA was 40% of control values at the eighth hour. The labeling index, in contrast, at this time was 70% control.

Discussion. The data reported herein are consistent with previous reports that the major effect of OHU is directed against cells in DNA synthesis (3–5). This has been clearly demonstrated by the virtually complete eradication of TDR-^{3}H incorporation into DNA as judged by both extraction and autoradiography. Since the duration of suppression of TDR-^{3}H incorporation lasted about 2 hr, it may be inferred that effective concentrations of OHU were present for a maximum of 2 hr. Philips *et al.* (6) showed that the concentration of OHU in the plasma and tissues of rats decayed exponentially with a half-life of 65 min further substantiating a limited duration of action. The prompt repair observed in this and other studies (6) also favors the short duration of action. Recovery of TDR-^{3}H incorporation began after the second hour. The rates of incorporation however remained considerably below control values for the next several hours. Even at the eighth hour, a time at which nucleated erythroid cells were shown to enter mitosis at a normal rate, the incorporation of TDR-^{3}H was only 40% of control values. Although this low rate of incorporation may at first suggest a depressed rate of DNA synthesis, a more likely explanation would appear to be that reutilization of available DNA breakdown products limited the amount of injected TDR-^{3}H utilized for DNA synthesis. It has been amply documented that breakdown products of DNA are normally reutilized for DNA synthesis (10–12). Ebbe and Stohlman (9) showed that the mean grain count halving time of immature megakaryocytes was increased in erythropoietin-treated rats and decreased in hypertransfused rats. They implicated the extruded red cell nucleus as a probable source of reusable DNA components. The rather impressive degree of cellular fragmentation and karyorrhexis observed in the marrow of mice treated with OHU would appear to provide a substantial source of DNA and its components for local reutilization. Under these conditions TDR-^{3}H incorporation would underestimate the actual rate of DNA synthesis.

The transient marrow erythroid depopulation and reticulocytopenia suggested that those erythroid cells in DNA synthesis were killed. This notion was more clearly established from the measurement of cell cycle kinetics with colchicine. In the normal mouse there was a linear accumulation of erythroid mitoses for 4 hr after colchicine which provided an estimate of ~10 hours for the generation time of mouse erythroid precursors. When OHU and colchicine were administered simultaneously erythroid cells entered mitosis at a normal rate for approximately 80 min, consistent with unaffected cells in G2 entering mitosis. Further influx of cells into mitosis was not observed until 7–8 hr after OHU at which time they again accumulated in mitosis at a normal rate. These findings are consistent with the hypothesis that OHU kills cells in DNA synthesis thus

creating a 6-hr void in the generative cell cycle. From the data at hand, it may be adduced that the 6-hr void represents the portion of the generative cycle occupied by cells in DNA synthesis. Cells progressing through cycle require 6 hr to complete DNA synthesis and an additional 60–90 min to enter mitosis. If cells in DNA synthesis were selectively removed and flux rate was constant, a 7–8 hour delay would be anticipated before cells in G1 at the time of drug administration progressed into mitosis. Such a delay was in fact observed and lends strong support to the contention that OHU kills those erythroid cells in DNA synthesis without appreciably influencing cells in other phases of cell cycle. In tissue culture, however, it has been reported that cells in G1 were temporarily prevented from beginning DNA synthesis when exposed to OHU (3). If such a block exists *in vivo* in the case of the erythroid cell, it must be of short duration. It might be argued that the delay in resumption of DNA synthesis as evidenced by the failure to detect TDR-^{3}H uptake for a period of up to 2 hr after OHU administration would in part be accounted for by a holdup of G1 cells similar to that reported in tissue culture. If this be the case then one must infer some acceleration in the rate of DNA synthesis once resumed. It should be noted that as was reported with other agents, *e.g.*, actinomycin D(13), the erythroid elements appeared to be more uniformly affected than did other bone marrow elements. The return to normal marrow erythroid cellularity required 72 hr in this study. The erythropoietin-treated plethoric mouse manifests peak marrow erythroid cellularity by 48 hr. In view of this difference one may infer some degree of damage to the immediate erythroid precursor cell. Preliminary studies in erythropoietin–OHU-treated plethoric mice indicate this to be the case.

Summary. A single i.v. dose of hydroxyurea (0.9 g/kg) produced transient marrow erythroid depopulation and reticulocytopenia in the mouse. Within minutes after drug administration there was a virtually complete erradication of marrow DNA synthesis which persisted for approximately 2 hr. Erythroid cell cycle kinetics, estimated with colchicine, revealed an entry of erythroid cells into mitosis for 80 min followed by no further accumulation of metaphase forms until the seventh hour. Thereafter erythroid cells accumulated in mitosis at a normal rate. These findings are consistent with the hypothesis that hydroxyurea kills erythroid cells in DNA synthesis; the initial 80-min accumulation of erythroid mitoses represents the progression of G2 cells into mitosis. Unaffected cells in G1 at the time of drug administration require 7–8 hr to progress through DNA synthesis, G2, and enter mitosis. From these data it would appear that hydroxyurea may prove a useful agent in the study of erythropoiesis because of its selective effect on DNA synthesis in the erythroid series.

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