Polyamine biosynthesis is required for the maintenance of peripheral blood cell elements in the rat
(α-difluoromethylornithine/thrombocytopenia/putrescine)

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ABSTRACT The specific ornithine decarboxylase inhibitor α-difluoromethylornithine, when given to adult rats in vivo for 5 wk, resulted in a decrease in peripheral blood cell elements in normal rats and a marked suppression of marrow recovery in rats with chemotherapy-induced marrow hypoplasia. In normal rats, α-difluoromethylornithine resulted in a reduction of the leukocyte count to 73% of control, erythrocyte count to 61% of control, and platelet count to 24% of control. The bleeding time was increased to twice normal and 67% of the animals had epistaxis and 42% had melena. In rats treated with the 5 phase-specific chemotherapeutic agent 1-β-D-arabinofuranosylcytosine, the simultaneous administration of α-difluoromethylornithine prevented the recovery of the bone marrow. The peripheral blood cell counts remained low—leukocyte count was 10% of control, and erythrocyte and platelet counts were 6% of control. All the animals developed epistaxis and melena and there was a 72% mortality. The administration of putrescine (4 mmol/kg, intraperitoneally, daily), the specific polyamine product of ornithine decarboxylase, reversed these hematologic effects in both normal and recovering marrow and resulted in rapid clinical improvement. Thus, the maintenance of normal, adult rat hematologic parameters, as with the proliferation of neoplastic and transformed cells in culture, is critically dependent on continued polyamine biosynthesis.

The decarboxylation of ornithine via ornithine decarboxylase (EC 4.1.1.17) leads to the formation of putrescine (Put); this initial and rate-limiting enzyme activity in polyamine biosynthesis increases during rapid cell growth and proliferation (1-3). DL-α-Difluoromethylornithine [F2MeOrn (DFMO; MDL 71, 782)] is a potent, enzyme-activated, irreversible inhibitor of ornithine decarboxylase (3, 4). The use of F2MeOrn has served to emphasize the essential role of polyamine biosynthesis in cell proliferation (3). Thus, F2MeOrn has been shown to have marked antiproliferative effects on many cell systems, including HTC hepatoma cells (5, 6), Ehrlich ascites tumor cells (7), mouse L1210 leukemia cells (6, 8, 9), EM67 tumor cells (9, 10), 9L gliosarcoma cells (11, 12), human MA160 prostate adenoma cells (6), and lung cancer cells (13, 14). F2MeOrn also has been shown to have a marked contragestational effect (15) and an antiparasitic activity (16).

Most of the above studies on the polyamine dependence of cell proliferation have been in neoplastic or transformed cells. There has remained a question about whether there is an essential role for polyamines in the proliferation of normal, unperturbed, renewing adult cells. F2MeOrn has now been widely used in animals, including man (17, 18), and few effects on normal tissues have previously been observed. However, the doses of the drug employed have often been lower than the 2–3 g/kg per day dosage currently used, and the administration times were often 2 wk or less. Because of the marked antiproliferative effects seen on almost all neoplastic cell systems studied and on adult rat intestinal epithelium challenged to renew itself through rapid proliferation (19), it is perhaps surprising that effects on normal renewing cell populations have not been observed. Therefore, we studied whether prolonged inhibition of polyamine biosynthesis with F2MeOrn would have any effects on the hematologic system in the rat and if the administration of Put, the polyamine synthesized by ornithine decarboxylase, might abrogate any observed hematologic effects of F2MeOrn.

We also studied whether the requirement for continued polyamine biosynthesis could be even more dramatically defined during periods of rapid growth. We have previously shown that this is the case for normal adult rat intestinal epithelium (19). In these previous studies, F2MeOrn, given for 1 wk, had relatively little effect on normal intestinal epithelial cells but produced a dramatic decrease in their capacity for repopulating the intestine after the intestinal epithelium was denuded by the 5 phase-specific cytotoxic agent 1-β-D-arabinofuranosylcytosine (araC) (19). araC also markedly depletes bone marrow cell elements. Therefore, we administered araC to rats to transiently deplete their marrow of blood cell precursor elements. We then examined whether the concurrent administration of F2MeOrn would have an inhibitory effect on bone marrow recovery and if the administration of Put might again reverse the hematologic effects of F2MeOrn.

METHODS

Female Wistar–Lewis rats, 12 wk old, weighing 180–200 g (Charles River Breeding Laboratories), were allowed to acclimate to our animal facilities for 1 wk prior to studies. The animals were kept four to a cage, housed with 12-hr light and 12-hr dark (7:00 a.m. to 7:00 p.m.) cycles, and were given regular Purina laboratory rat chow ad libitum. Animals were studied for 42 days in all experiments.

F2MeOrn was given as a 3% solution in the drinking water continuously. The mean daily intake of the drug was 3.3 g/kg and was relatively constant (daily intake variation was ±8%). Putrescine dihydrochloride (Sigma) was given to the Put-treated groups at 322 mg (2 mmol)/kg in sterile water intraperitoneally (i.p.) twice a day throughout the entire course of study, and control animals received (i.p.) an equivalent amount of sterile water. araC (Upjohn) was given at a dose of 300 mg/kg sub-
cutaneously every 8 hr for a total of 6 doses during the first 2 days of the experiments. Control animals were given an equivalent amount of 0.9% sodium chloride solution subcutaneously over the same time schedule.

Blood was obtained from the animals via retroorbital sinus puncture daily between 8:00 and 10:00 a.m. Peripheral blood counts were determined on a Coulter Counter, model ZBI (Coulter). Peripheral erythrocyte counts and leukocyte counts were done with a 100-μm aperture and a 500-μl manometer. Erythrocyte counts were done at a 1:50,000 dilution and leukocyte counts at a 1:500 dilution. Platelet counts were done by using a 70-μm aperture and a 100-μl manometer, at a dilution of 1:500, and confirmed by visual counting on a hemocytometer.

Bleeding times were done by a modification of the Ivy bleeding time technique. An incision, 1 cm long by 0.5 mm deep, was made in an avascular area of the shaved abdomen, and the time for bleeding to cease was measured. The bleeding times were always done concurrently on untreated controls and were expressed as percent of control.

For bone marrow studies, the animals were sacrificed between 8:00 and 10:00 a.m., both tibiae were removed and marrow cells were flushed out with RPMI 1640 cell culture medium (GIBCO). Aliquots of 50,000 bone marrow cells were then cytopsins onto microscope slides, air dried, fixed in methanol, stained with Wright’s stain, and examined under light microscopy.

RESULTS

In the present study, the possible contributory roles of water intake, food intake, and body weight on peripheral blood counts were examined. F₂MeOrn-treated animals, Put-treated animals, and control (no drug) animals all had a daily water intake of about 110 ml/kg. However, daily food intake was about 5% less for the F₂MeOrn-treated animals. The total weight gain at the end of the 5-wk study for the F₂MeOrn-treated animals was also 5% less than the controls. Therefore, another set of normal controls that were pair-fed to have their body weights match the F₂MeOrn-treated animals were also studied. These weight-matched animals had no significant changes in their peripheral blood counts from control after 5 wk. Another group of normal animals received Put i.p. for 35 days; these animals showed no appreciable changes in water intake, food intake, weight gain, or in peripheral blood counts.

Hematologic Effects in Normal Animals. In rats treated with oral F₂MeOrn (3.3 g/kg per day) for 5 wk, a distinct effect on circulating blood cell elements was seen (Fig. 1). The peripheral leukocyte counts began to decrease by day 14, were decreased to 73% of control by day 20, and remained at that level thereafter. The erythrocyte counts began to decrease within 3–4 days after treatment and had dropped to 61% of control by the fourth week. Platelet counts also began to decrease promptly within the first week of treatment and were decreased to 24% of control at the end of the third week (Fig. 1). The rats suffered biological effects from the decrease in circulating platelets. Assessment of clotting studies showed that bleeding times were prolonged to about twice normal. Accordingly, 67% of the F₂MeOrn-treated animals had epistaxis (easily bloodied noses) and 42% had melena (loose fecal pellets containing occult blood). During surgical procedures or during sacrifice, the tissues from F₂MeOrn-treated animals were extremely friable and many bleeding points were noted. Despite these signs of decreased platelet function, all of the animals survived the 6-wk experiment. Their bone marrow smears appeared morphologically normal under light microscopy, with precursor cells of all cell lineages present, including megakaryocytes, the platelet precursor. There was a suggestion that the number of early precursor cells of the granulocytic series was increased and the number of megakaryocytes was decreased, but no statistically significant difference could be documented. Furthermore, when F₂MeOrn was stopped after 5 wk, the peripheral blood cell counts of all the animals increased within 2 days and returned to normal limits within 1 wk (Fig. 1).

The hematologic effects of F₂MeOrn in normal rats appeared to result specifically from a block in polyamine biosynthesis, because secondary effects from changes in body weight or food intake, or both, were not observed in the pair-fed control animals. Most important, when the F₂MeOrn-treated animals were simultaneously given Put at 4 mmol/kg per day (which had no effect on the peripheral blood counts when given alone), the hematologic effects were reversed. The peripheral leukocyte count remained at normal levels throughout the course of the study, and the erythrocyte count showed only a mild (15%) decrease during the second and third weeks. The thrombocytopenia was only partially reversed; the platelet count in Put-rescued animals returned to 62% of control. This represented a 250% improvement of the platelet count compared to F₂MeOrn-treated animals (Fig. 1). This partial improvement in platelet number did result in a return of platelet function because the bleeding time in the Put-rescued animals was prolonged only by 30%. Also, the incidence of epistaxis was 16%, and none of

![Fig. 1. Hematologic effects of F₂MeOrn and reversal by Put. Female Wistar-Lewis rats, weighing 180–200 g, were given F₂MeOrn as a 5% solution in their drinking water (3.3 g/kg per day as measured by average water intake) and 2 mmol of Put per kg i.p. twice a day in the reversal studies throughout the entire course of the study. The blood counts of animals receiving Put only were similar to those in control animals, and the results for the normal controls and Put controls were therefore combined. ●, Controls and Put controls; ○, F₂MeOrn; and □, F₂MeOrn and Put. Each point represents the mean from at least five animals; bars denote SEM.](image-url)
the animals had melena. Their bone marrow was normal when compared to controls.

**Hematologic Recovery After Cytotoxic Therapy.** Animals that received araC alone had a marked but transient decrease in all peripheral blood cell elements for 10–14 days. The leukocyte count decreased to 50% of control by day 4 and returned to normal by day 10. The erythrocyte count decreased to 56% of control by day 6 and returned to normal by day 16. Platelet counts also decreased to 50% of control by day 4, then returned to 135% of control on day 10, and were within normal limits by day 14 (Fig. 2). Although 40% of these animals developed loose stools, the stools did not contain blood, and none had epistaxis.

When these araC-pretreated animals were simultaneously given F2MeOrn, the decrease in peripheral blood cell elements was more severe and did not recover even after 35 days. The peripheral blood in these animals was markedly devoid of cellular elements, with leukocyte count decreased to 10% of control, and erythrocyte and platelet counts to 6% of control (Fig. 2). Bone marrow smears showed markedly decreased granulocytic precursor elements throughout the study. Bleeding times were prolonged to four times normal. All of the animals developed epistaxis and melena and appeared clinically debilitated. Seventy-two percent of the animals died, with most of the deaths occurring during the second and third weeks. The hematologic effect of the combination of araC and F2MeOrn was easily reversed. When F2MeOrn was stopped at the end of 5 wk, all peripheral blood cell counts in the surviving animals began to increase within 2 days and returned to normal limits within 1 wk (Fig. 2).

Again, the inhibitory effects of F2MeOrn on hematologic recovery after araC marrow hypoplasia appeared to result specifically from a block in polyamine biosynthesis. The administration of Put, which had no effect on the transient hematologic effect of araC alone, reversed the decrease in bone marrow and peripheral blood cell elements introduced by the combination of araC and F2MeOrn. With Put administration, the bone marrow showed a gradual recovery beginning in 1 wk and was within normal limits in 2 wk. Leukocyte counts began to increase by day 6 and were within normal limits by day 14. Erythrocyte counts began to increase from the nadir by day 10 and had returned to normal by the end of the third week. The effect on the platelet count was less complete than the effect on leukocyte or erythrocyte counts, and the platelet count remained at 60% of control throughout the course of this study (Fig. 2). This partial improvement in the platelet count resulted in a partial correction of the bleeding times, which were only prolonged to 150% of control.

The araC- and F2MeOrn-treated animals that received Put had a rapid clinical improvement. Melena disappeared by the end of the second week, and only one death occurred, accounting for a mortality of 14% (P < 0.05, compared to araC + F2MeOrn).

**DISCUSSION**

The present study emphasizes that polyamine biosynthesis probably plays a critical role in the growth processes of normal, unperturbed renewing adult cells. It provides strong evidence that the polyamines are essential for proper maintenance of peripheral blood cell elements. Because the only documented effect of F2MeOrn is the specific inhibition of ornithine decarboxylase and subsequent polyamine biosynthesis and because Put reversed the hematologic effects of F2MeOrn, we conclude that continued polyamine biosynthesis is necessary for adult hematopoietic cell proliferation or differentiation, or both.

Recent studies from our laboratory with cultured human promyelocytic leukemia cells suggest that the polyamines may be more critical for proliferation rather than differentiation in the granulocytic cell series (20). We, as well as other investigators, have observed that after a short-term (5–12 days) exposure of animals to F2MeOrn, the hematopoietic progenitor cells are increased (21, 22). This apparent discrepancy between progenitor growth and the hematologic effects reported in this study might be explained by a maturation block of progenitors after long-term (21–35 days) exposure. This might be expressed as a decrease in peripheral blood cell elements. Furthermore, depletion of stem cell compartments by increased proliferation during the first 2 wk could result in the decreased availability of mature cells during the subsequent 3 wk. The exact mechanisms underlying the observed hematologic effects of F2MeOrn remain to be elucidated.

Our current results also suggest that the in vivo biologic effects of F2MeOrn are manifested only after complete polyamine depletion has been allowed to occur. This probably explains why little effects on many normal adult renewing tissues are apparent after short durations or low doses of F2MeOrn (or both). When F2MeOrn was given alone in the present study, hematologic effects were not fully developed until 3 wk after administration. These results are consistent with previous in vitro studies, which showed that F2MeOrn effects on cell growth
are seen only after several cell divisions have taken place, with resultant depletion of endogenous cellular polyamines (5, 13, 14, 20).

Our study is also important from a clinical standpoint in considering future therapeutic uses of the drug F2MeOrn. Recently, F2MeOrn has been shown to have activity in vivo, when used either as a single agent or in combination with other chemotherapeutic agents, against several tumors, such as murine L1210 leukemia (8, 9), EMT6 mammary sarcoma (9, 10) and 9L gliosarcoma (12), and human acute leukemias (23, 24). European clinical studies of F2MeOrn in acute human leukemia have shown promising results (23, 24), and a phase I trial of oral F2MeOrn has recently been completed at our institution (25). Our results emphasize the need for consideration of hematologic parameters when using F2MeOrn alone and especially in combination with other agents. When F2MeOrn is administered alone, the reduction in peripheral leukocyte and erythrocyte counts, even though significant, are still within the broad range of normal limits and probably pose no clinical problem. However, the effects of F2MeOrn on platelet counts may bear closer monitoring, because the peripheral platelet counts were decreased to 24% of control, the bleeding times were twice normal, and the animals developed epistaxis and melena. Preliminary results from our phase I trial suggest F2MeOrn-induced thrombocytopenia is also observed in humans and may be the dose-limiting toxicity of the drug in about 50% of the patients (25).

The hematologic effects of F2MeOrn become much more important when the drug is administered in combination with other chemotherapeutic modalities. F2MeOrn, in combination with araC, contributes to a severe and prolonged bone marrow hypoplasia, with a resultant high mortality in rats. Thus, the timing of F2MeOrn administration will be important in avoiding the critical recovery period of a depleted bone marrow. Our data also suggest potential maneuvers for optimizing F2MeOrn effect while minimizing the hematologic effects seen. Dramatic and rapid reversal of the hematologic effects of F2MeOrn can be afforded by acutely withdrawing the drug or by administering Put, or both. The addition of Put, as a means to protect the bone marrow from the combined effects of F2MeOrn and agents that result in depletion of bone marrow cell elements, merits consideration during therapeutic trials.

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