Studies on the Effects of Hydroxyurea and Other Anticancer Drugs upon Pyrimidine Metabolism

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SUMMARY

The reversal of azauridine-induced oroticaciduria by hydroxyurea has been studied to further elucidate the control mechanisms involved in the de novo pathways of pyrimidine biosynthesis. No direct effect of hydroxyurea upon partially purified aspartate transcarbamylase and dihydroorotase was observed. Hydroxyurea selectively inhibited the incorporation of 14C-labeled aspartate, orotate, and formate into the DNA bases of human leukemic cells incubated in vitro. No effect of hydroxyurea was observed in the RNA bases isolated from these leukemic cells. Other chemotherapeutic agents, including 5-iododeoxyuridine, 5-fluorodeoxyuridine, Methotrexate, cytosine arabinoside, and cyclophosphamide, were administered to patients receiving 6-azauridine. Only Methotrexate and cyclophosphamide inhibited orotic acid excretion in a manner similar to hydroxyurea. These results suggest that the control of pyrimidine biosynthesis is linked to DNA synthesis or function in an as yet unexplained manner.

INTRODUCTION

Previous studies from this laboratory have shown that hydroxyurea inhibits the oroticaciduria produced in patients treated with 6-azauridine (24). These results suggested that hydroxyurea may inhibit de novo pyrimidine biosynthesis at a site prior to the formation of orotic acid. Other published studies, however, have demonstrated effects of hydroxyurea upon DNA synthesis and the lack of effects of the drug upon RNA synthesis (15, 18, 28). Apparent sites of inhibition of DNA synthesis include ribonucleotide reduction (6, 13) and thymidine incorporation (8, 28).

Since none of these observed effects of hydroxyurea upon DNA synthesis provides a satisfactory explanation of the drug’s effect upon azauridine-induced oroticaciduria, we investigated three possible ways that hydroxyurea inhibition might account for the observed effect upon orotic acid excretion. First, the direct effect of hydroxyurea and purine and pyrimidine compounds, which may serve as feedback inhibitors, was studied utilizing partially purified preparations of aspartate transcarbamylase and dihydroorotase. Second, the effect of hydroxyurea upon the in vitro rate of incorporation of selected RNA and DNA precursors was studied in leukemic cells. Third, other chemotherapeutic drugs which are known to interfere with DNA synthesis were studied for their effects upon orotic acid excretion in patients treated with azauridine.

Since neither direct effects nor feedback effects of hydroxyurea upon orotic acid synthesis were observed in these studies, it appears likely that the control of orotic acid synthesis in man may be mediated by a mechanism which is linked to DNA synthesis in a manner which has not been described. Such a control mechanism is also indicated by the observation that cyclophosphamide and Methotrexate will also inhibit oroticaciduria as is demonstrated below.

MATERIALS AND METHODS

Enzyme Preparations. Mouse adenocarcinoma 755 (Ad 755) ascites and human leukocytes were used as the source of aspartate transcarbamylase and dihydroorotase. The 755 tumor was carried in BDF1 female mice obtained from Cumberland Farms, Clinton, Tenn. The ascites cells were aspirated from the tumor-bearing mice 7—9 days after they had been inoculated with 106 cells. The cells were centrifuged, washed twice with cold saline, and then resuspended in two volumes of 0.01 M Trisbuffer, pH 7.5. The cells were disrupted in the cold by sonication with a Bronson probe model S75 for two 15-second intervals. Human leukocytes were purified according to the method of Skoog and Beck (20). Whole blood was added to one-half volume of 6 percent bovine fibrinogen in 3.8 percent sodium citrate. The suspension was allowed to settle for 30—60 minutes at 2—4°C. The upper leukocyte-rich layer was decanted, and the leukocytes were removed by centrifugation in the cold. The cells were washed twice with 10 volumes of cold saline and disrupted by sonication as above.

Aspartate Transcarbamylase. Activity in the crude Ad 755 sonicate was purified as follows: The sonicate was centrifuged at 1500 rpm in an International clinical centrifuge for 20 minutes. The supernate was centrifuged at 40,000 rpm in a Spinco ultracentrifuge for 40 minutes. The supernate from this high speed centrifugation was adjusted slowly to 25% saturation with (NH4)2SO4. After standing in the cold for 15 minutes,
the precipitate was collected by centrifugation at 40,000 rpm for 20 minutes as above. Each precipitate was dispersed or dissolved in 0.01 M Tris buffer, pH 7.5, and (NH₄)₂SO₄ was removed by dialysis for 12 hours in the cold against a large volume of 0.01 M Tris buffer, pH 7.5. The fraction which was precipitated with (NH₄)₂SO₄ was used for the studies reported here.

The sonicate from the human leukocytes was centrifuged at 40,000 rpm for 40 minutes as above, and the supernate was used as the source of aspartate transcarbamylase.

Dihydroorotase. Activity in the Ad 755 sonicate was obtained after centrifugation in a Sorvall RC-2 centrifuge for 10 minutes at 10,000 rpm. The supernate was used as the source of dihydroorotase.

Enzyme Assays. Aspartate transcarbamylase from Ad 755 was determined in a reaction mixture which contained 100 μmoles of Tris buffer, 10 μmoles L-aspartic acid-U-¹⁴C (8.4 X 10⁴ cpm/μmole), 7.0 moles of carbamyl phosphate, and enzyme preparation in a final volume of 1.2 ml. The enzyme activity from human leukocytes was assayed in the same manner except that 15 μmoles of L-aspartic acid-U-¹⁴C (1.1 X 10⁵ cpm/μmole) and 10 μmoles of carbamyl phosphate were added. The reaction mixtures were incubated for 30 minutes at 37°C and the reactions were stopped by the addition of 0.15 ml of 2 N perchloric acid. After centrifugation in the cold the supernatants were adjusted to pH 8.0 with 1.0 N KOH and brought to a volume of 2.5 ml. To remove unreacted aspartate-¹⁴C, a modification of the method of Smith and Baker (21) was employed. The neutralized supernatants were passed through Dowex-50H formate columns (1 x 3 cm). The columns were eluted with water until two 2.5-ml fractions were collected. An aliquot from each fraction was assayed for carbamylaspartic acid by the method of Koritz and Cohen (11). A second aliquot was also assayed for carbamylaspartic acid by determining the ¹⁴C content by liquid scintillation counting.

In general, both methods yielded similar results except when hydroxyurea was present in the incubation mixture. Hydroxyurea gives a positive color reaction in the Koritz and Cohen method for carbamylamino acids. Therefore, the data reported for aspartate transcarbamylase is based on the isotope assay. Controls without enzyme preparation and controls without aspartic acid were included in each group of assays.

Dihydroorotase was assayed according to the method of Yates and Pardee (27). Each incubation mixture contained 100 μmoles potassium phosphate buffer, pH 6.5, 6 μmoles carbamylaspartic acid, and enzyme in a total volume of 1.0 ml. The mixture was incubated for 30 minutes at 37°C, and the reaction was stopped by the addition of 0.15 ml of 2 N perchloric acid. After centrifugation the concentration of dihydroorotic acid was measured spectrophotometrically at 240 μm by mixing 0.5 ml of the perchloric acid supernate with 0.5 ml of 1 N NaOH. Each assay mixture was read against a control incubation mixture in which carbamylaspartic acid was omitted.

Intact Leukocyte Incubations. Leukocytes were harvested from the blood of patients with acute leukemia and purified as described above. The cells were then suspended in Kребs-Ringer medium, buffered with 0.154 M glycyl-glycine solution, pH 7.8, and containing 1 mg/ml fructose. The cells were incubated at 37°C under oxygen in a rotary shaker at 120 rpm in siliconized 50-ml flasks for periods of 1—4 hours. The rate of incorporation of aspartate-U-¹⁴C, orotate-6-¹⁴C, and formate-¹⁴C was measured in the presence or absence of hydroxyurea. After incubation the purines and pyrimidines of RNA and DNA and the total cellular protein were isolated and purified. The specific activity of each isolated compound was determined as previously reported (9).

Clinical Studies. Fifteen clinical studies were carried out in 13 patients. There were 6 patients with acute myeloblastic leukemia refractory to other modes of therapy, 3 patients with metastatic breast cancer, 2 patients with metastatic melanoma, 1 patient with metastatic adenocarcinoma, and another with metastatic fibrosarcoma. The patients were hospitalized on the Clinical Research Facility and fed a low purine diet containing 30 mg of purine nitrogen for 5 days prior to initiation of therapy. The patients were begun on a 24-hour infusion of 6-azauridine in a dose of 5 to 10 gm daily which was continued in all but one patient for 10 to 12 days. During the middle days of the 6-azauridine infusion, a second drug was added. These drugs consisted of 5-iododeoxyuridine (IUDR) in a dose of 20 mg/kg/day or 30 mg/kg/day; 5-fluorodeoxyuridine (FUDR) in doses of 0.5 mg/kg/day or 1 mg/kg/day; Methotrexate in a divided oral dose totaling 5 or 7.5 mg/day for 5 days; 1-β-D-arabinofuranosylcytosine (cytosine arabinoside) in doses of 20 mg/kg/day for 5 days or 30 mg/kg/day for 5 days; and cyclophosphamide in doses of 4 mg/kg/day for 5 days. One patient received 5-fluorouracil in doses of 1 gm daily from Day 2 to 10 of the 6-azauridine infusion, and hydroxyurea in doses of 2 gm daily from Days 4 to 9. Except for Methotrexate, cyclophosphamide, and hydroxyurea, which were given orally, all other drugs were given by continuous infusion along with the 6-azauridine. Daily 24-hour urine collections were obtained in all patients. Blood counts were done at least every two days. Liver function studies were done before and at the end of treatment. Creatinine clearances were done twice weekly, and daily urinary excretion of creatinine and orotic acid was measured. Orotic acid was determined as previously reported (10). Results were corrected for grams of creatinine excreted per day.

Reagents. All of the compounds tested as possible inhibitors were obtained from Calbiochem except for hydroxyurea which was supplied by E. R. Squibb & Sons. The potential inhibitors were dissolved in 0.01 Tris buffer, pH 7.0, and readjusted to pH 7.0 with KOH as necessary. All of the ¹⁴C-labeled compounds were purchased from New England Nuclear Corporation. Fibrinogen was supplied by Armour & Co. Azauridine was supplied by the National Cancer Institute.

RESULTS

Purification of Aspartate Transcarbamylase from Ad 755 Ascites. Most of the activity of the sonicate was recovered in the nonparticulate soluble fraction which was obtained after centrifugation at 40,000 rpm. The activity was precipitated by the addition of (NH₄)₂SO₄ to 25% saturation. A six-fold purification was obtained (Table 1).

Aspartate Transcarbamylase Activity from Ad 755 Ascites Cells. Under the conditions described, synthesis of carbamyl
Aspartate was linear for at least 60 minutes. Control values for the partially purified aspartate transcarbamylase yielded 1.34 μmoles of product per 30 minutes per mg of protein at 37°C. No inhibition was observed by hydroxyurea in concentrations up to 0.005 M. Pyrimidines (bases, mono-, di-, and triphosphoribonucleosides and deoxyribonucleosides) and purines (mono- and triphosphoribonucleosides and deoxyribonucleosides) in concentrations of 0.001 M to 0.02 M failed to inhibit enzyme activity. In a few experiments the enzyme was prepared from an homogenate (instead of a sonicate) of the tumor cells. This homogenate was prepared in an all-glass, conically shaped tube and pestle (Kontes). The same lack of inhibition was observed. The pH was carefully maintained in all experiments, and measurements were made of both the initial and final pH. In a few experiments significant inhibition was observed with several of the compounds, but this was demonstrated to be due to a decrease in pH during incubation. No inhibition was observed when the pH was maintained in potassium phosphate buffer at pH 7, 8, or 9.

Aspartate Transcarbamylase Activity from Leukemic Leukocytes. Several compounds were tested as inhibitors of aspartate transcarbamylase activity found in crude sonicate extracts of human leukemic leukocytes. Cells were obtained from patients with acute leukemia. The leukocytes were all essentially immature. The same lack of inhibition of aspartate transcarbamylase by hydroxyurea and pyrimidine derivatives was observed. The aspartate transcarbamylase preparation used for these studies was capable of synthesizing 0.28 mmole of carbamylaspartate in 30 minutes per mg protein at 37°C.

Dihydroorotase Activity from Ad 755 Ascites Cells. Under the conditions employed in the studies enzyme activity in the crude sonicate extract was proportional to the amount of extract added. Synthesis of dihydroorotase was linear for a period of at least 30 minutes. In control samples 0.53 μmole of dihydroorotase was synthesized. As shown in Table 2, hydroxyurea had no effect. Various pyrimidine derivatives in large concentrations showed varying degrees of inhibition.

Effect of Hydroxyurea upon in Vitro Leukocyte Metabolism. The synthesis of RNA and DNA purines and pyrimidines was studied by measuring the rate of incorporation of 14C-labeled aspartate, formate, and orotate into the appropriate bases of human leukemic leukocytes. The results of these experiments are shown in Table 3. Under the conditions employed a linear rate of incorporation was obtained for the data presented in this table. Shorter incubation periods of 1 and 2 hours gave proportionately lower specific activities in all the RNA and DNA bases than is presented here for the 4-hour incubation period. It is evident from the data that hydroxyurea was a highly specific inhibitor of DNA synthesis. No significant inhibition of RNA purine or pyrimidine synthesis was obtained in the presence of 5.5 x 10⁻⁴ M hydroxyurea regardless of the precursors employed. Marked inhibition by hydroxyurea of the incorporation of all labeled precursors was observed, however, in the DNA purines and pyrimidines. Although not shown here, a similar lack of inhibition by hydroxyurea of the incorporation of 14C-labeled aspartate, glycine, and formate into the proteins was also observed in the 4-hour incubation of human leukocytes.

Clinical Results. The effect of various drugs on orotic acid excretion is shown in Charts 1–6. Unless 6-azauridine is given, orotic acid excretion is less than 100 μmoles per day (24). As shown in Chart 1, FUDR administration caused no fall in orotic acid excretion. Two patients with acute leukemia who had an increasing leukocyte count during treatment showed an increased excretion. Orotic acid excretion was unchanged in two patients with metastatic cancer who failed to show a therapeutic effect. In a patient with blast phase of chronic myelocytic leukemia given 6-azauridine plus 5-fluorouracil (Chart 2), no change in orotic acid excretion occurred until hydroxyurea was added. This resulted in a marked drop in orotic acid excretion. When hydroxyurea was discontinued, a rise in orotic acid excretion occurred despite the fact that 5-fluorouracil was continued. This treatment resulted in a fall in leukocyte count from 55,000 to 3,200 by the 5th day of hydroxyurea therapy.

The effect of IUdR on orotic acid excretion is shown in Chart 3. In one patient with metastatic melanoma, no change occurred in orotic acid excretion in two studies on two different dose levels of IUdR. However, in a patient with acute myeloblastic leukemia, a 64 percent reduction in orotic acid excretion occurred. This did not correlate with the clinical response as his leukocyte count rose from 34,000 to 90,000 during the interval.

Methotrexate (Chart 4) reduced orotic acid excretion by 74 percent in a patient with acute myeloblastic leukemia who showed a drop in leukocyte count and by 60 percent in a patient with metastatic melanoma. No orotic acid excretion was detected in these two patients during the interval.
Table 3

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<th>Hydroxyurea</th>
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<th>% inhibition</th>
<th>Orotate-14C</th>
<th>% inhibition</th>
<th>Formate-14C</th>
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Effect of hydroxyurea on the incorporation of various precursors into RNA and DNA bases of human leukemic cells. Results are expressed as cpm/µmole base. Washed leukemic leukocytes, suspended in Krebs-Ringer medium, buffered with 0.154 M glycyl-glycine solution, pH 7.8, containing 1 mg/ml fructose, and 14C-labeled compounds, were incubated at 37°C under oxygen for 4 hours. Hydroxyurea was added at a final concentration of 5.5 × 10⁻⁴ M. Addition of radioactive precursors was as follows: aspartate-U-14C, 2.5 µc; orotate-6-14C, 1 µc; formate-14C, 5 µc. Final incubation volume, 2 ml; 0.1-0.2 ml packed cells added per flask.

Chart 1. Effect of 5-fluorodeoxyuridine (FUDR) upon orotic acid excretion. FUDR was added to a 24-hour intravenous infusion of 6-azauridine (6-AZUR). AML, acute myeloblastic leukemia; Ca, carcinoma.
Effects of Hydroxyurea upon Pyrimidine Metabolism

Chart 2. Effect of 5-fluorouracil (FU) and hydroxyurea (HU) on orotic acid excretion in a patient with acute myeloblastic leukemia (AML). 6-Azauridine (6-AZUR) intravenous infusion was begun on Day 1 and given continuously to Day 12. FU was given by continuous infusion from Day 2 to 10. HU was given by mouth in a dose of 0.5 gm every 6 hours from Days 4 to 9.

Patient with metastatic breast cancer who was responding to the antifolate compound.

Since both cytosine arabinoside and hydroxyurea apparently block the enzymatic reduction of ribonucleotides to deoxyribonucleotides, the effect of cytosine arabinoside on orotic acid excretion was measured. As shown in Chart 5, cytosine arabinoside caused only a 23 percent to 39 percent reduction in orotic acid excretion in two patients with breast cancer and one with metastatic melanoma. In contrast to hydroxyurea no reversal was observed in two patients after cytosine arabinoside was discontinued. Patient C. E. showed some reversal but developed infection with leukocytosis during the second week of study, and this may have accounted for this difference.

Chart 6 illustrates the effect of the alkylating agent, cyclophosphamide, on orotic acid excretion. In a patient with acute myeloblastic leukemia, there was a 96 percent reduction of orotic acid excretion. This patient failed to obtain a remission, but the white count fell from 17,800 to 2,450. In a patient with fibrosarcoma, cyclophosphamide resulted in a 49 percent fall in orotic acid excretion. There was no apparent clinical change.

DISCUSSION

The normal excretion of orotic acid in man is negligible. Following 6-azauridine therapy a prompt increase in orotic acid excretion occurs. It was observed that orotic acid excretion was higher in leukemic patients than in patients with solid tumors. This may reflect a greater dependence upon the de novo pyrimidine pathway in acute leukemia. The partial reversal of this effect by hydroxyurea, both in leukemic and non-leukemic patients, presented this laboratory with an intriguing problem.

Yates and Pardee (27) were the first workers to describe the inhibition of aspartate transcarbamylase by cytosine 5'-phosphate in E. coli. Since that original report, many studies have demonstrated feedback inhibition by pyrimidine derivatives of aspartate transcarbamylase. These studies have included Ehrlich ascites cells (2), rat liver and hepatomas (1), HeLa cells (4), and lettuce seedlings (14). Smith and Baker (22), however, were unable to demonstrate any inhibition of this enzyme with either cytosine 5'-phosphate or uridine 5'-phosphate in human erythrocytes or leukocytes. A preliminary report has appeared (23) presenting evidence that uridine triphosphate inhibits carbamyl-phosphate synthetase in mouse spleen cells. Some mechanism of control of de novo pyrimidine synthesis must exist in humans as a patient with hereditary oroticaciduria showed a fall in orotic acid excretion following the administration of a mixture of cytidylic and uridylic acids (10).

In the present studies none of the demonstrable metabolic control points involved in de novo pyrimidine biosynthesis has been shown to be involved in the mechanism of hydroxyurea inhibition of mammalian cell metabolism. Hydroxyurea did not inhibit partially purified preparations of aspartate transcarbamylase and dihydroorotase from both animal and human cells. Hydroxyurea did not inhibit the incorporation of 14C-labeled aspartate, orotate, and formate into the RNA pyrimidines of intact animal and human cells.

It has been considered that the in vivo effect of hydroxyurea upon oroticaciduria may be due to a metabolite of hydroxyurea. Fishbein and Carbone (5) found that from one to 10 percent of the administered hydroxyurea was converted to acetohydroxaminic acid which is converted to hydroxylamine. Hydroxylamine has been shown to inhibit the synthesis of RNA, DNA, and protein in HeLa cells. Although the effect of hydroxylamine on orotic acid excretion in man has not been...
Chart 3. Effect of iododeoxyuridine (IUDR) on orotic acid excretion. IUDR was added to a continuous intravenous infusion of 6-azauridine (6-AZUR). AML, acute myeloblastic leukemia.

Chart 4. Effect of Methotrexate given by mouth in a divided daily dose for 5 days on orotic acid excretion. 6-Azauridine (6 AZUR) was given by continuous intravenous infusion. AML, acute myeloblastic leukemia. Ca, carcinoma.
Chart 5. Effect of cytosine arabinoside (Cyt. Arab.) on orotic acid excretion. Cyt. Arab. was added to a continuous intravenous infusion of 6-azauridine (6 AZUR). Ca, carcinoma.

Chart 6. Effect of cyclophosphamide on orotic acid excretion. 6-azauridine (6 AZUR) was given by continuous intravenous infusion. Cyclophosphamide was given orally in a divided daily dose. AML, acute myeloblastic leukemia.
investigated, the fact that other drugs, particularly cyclophosphamide, mimic the effect of hydroxyurea upon orotic acid excretion, argues against the involvement of hydroxycyphilus as the active agent in our studies.

In confirmation of numerous other reports (15, 17—19, 28, 29) DNA synthesis in the animal and human cells was found to be inhibited by hydroxyurea. Frenkel et al. (7) have presented in vivo evidence that hydroxyurea inhibits ribonucleotide reduction. Pyrimidine deoxyribonucleosides reverse the inhibitory effects of hydroxyurea in Chinese hamster cells (12). Moore (13) has demonstrated an inhibitory effect of hydroxyurea on partially purified ribonucleotide reductase. In the present studies, however, inhibition of ribonucleotide reduction did not correlate with inhibition of oroticaciduria, since cytosine arabinoside, a potent inhibitor of ribonucleotide reduction (3), did not significantly affect orotic acid excretion. In addition, cyclophosphamide and Methotrexate inhibit orotic acid excretion but have no reported effects upon ribonucleotide reduction.

There is evidence that hydroxyurea has more than one site of action. Pollack and Rosenkranz (16) failed to reverse hydroxyurea inhibition of DNA synthesis in polyoma transformed kidney cells by the administration of deoxyribonucleosides. Similar results have recently been obtained in 6C3HED ascites cells by Yarbro (25). Hydroxyurea inhibits the incorporation of glycine-14C into nuclear histones (26).

From the present studies we have concluded that hydroxyurea inhibition of orotic acid excretion cannot be explained either by its demonstrable effect upon ribonucleotide reduction or by a direct upon de novo pyrimidine biosynthesis. Therefore, hydroxyurea (and possibly cyclophosphamide and Methotrexate) may have an inhibitory site(s) which controls orotic acid metabolism and which is linked to DNA synthesis or function in a manner yet to be defined.

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