In Vivo Effect of Hydroxyurea on Orotic Acid Synthesis

WILLIAM R. VOGLER, JAMES A. BAIN, AND CHARLES M. HUGULEY, JR.

The Department of Medicine, School of Medicine, and the Department of Pharmacology, Division of Basic Health Sciences, Emory University, Atlanta, Georgia

Summary

Hydroxyurea is thought to inhibit DNA synthesis but the site of inhibition has not been established. The urinary excretion of orotic acid was measured in 6 patients, 5 with acute leukemia and 1 with metastatic melanoma, receiving 6-azauridine which is known to inhibit orotidylic decarboxylase. The orotic aciduria observed in these patients was reversed by the addition of hydroxyurea, suggesting inhibition of de novo pyrimidine biosynthesis at a step prior to the formation of orotic acid. No clear-cut potentiation of a chemotherapeutic effect of this combination of drugs was observed.

Introduction

Hydroxyurea is one of several substituted urea compounds with antitumor activity. The finding of megaloblastic marrows in patients receiving doses of 40 mg/kg/day suggested interference with DNA metabolism (1). In bacterial cells hydroxyurea was found to inhibit growth, reduce the DNA/RNA ratio, and inhibit thymidine incorporation into DNA (10, 17). Thymidine incorporation into HeLa monolayer cells (25) and Ehrlich ascites tumor cells (9) was inhibited by hydroxyurea. Thymidine partially reversed the growth inhibition by hydroxyurea in Chinese hamster cell lines (13). In subcellular preparations of HeLa cells (25), hydroxyurea failed to inhibit thymidine kinase or DNA polymerase but caused a 90% inhibition of the incorporation of cytidylic and guanylic acids into DNA. Subcellular extracts of marrow cells from rats and humans pretreated with hydroxyurea formed deoxycoformycin monophosphate from cytidine monophosphate at a reduced rate (8). In Chinese hamster cells hydroxyurea-induced growth inhibition was reversed by pyrimidine deoxyribosides but not by purine deoxyribosides or ribosides of purines and pyrimidines (13). The incorporation of uridine into RNA of HeLa cells and of cytidine into RNA of subcellular extracts of human and rat marrow cells was not inhibited by hydroxyurea indicating an absence of interference with RNA synthesis (8, 25). Leucine incorporation by HeLa cells was uninhibited suggesting no impairment in protein synthesis (25). In a recent study (24) hydroxyurea was shown to be a potent inhibitor of DNA synthesis in regenerating rat liver without measurable effect on RNA or protein synthesis. However, the incorporation of radioactive glycine into nuclear histone was only 61% of the control value, suggesting hydroxyurea may inhibit synthesis of histones in addition to, or as a consequence of, its effect on RNA synthesis.

Thus current evidence suggests that hydroxyurea interferes with DNA synthesis by inhibition of thymidine incorporation into DNA or by inhibition of ribose reduction. We wish to present evidence which suggests that hydroxyurea may block de novo pyrimidine synthesis in vivo.

In our studies of pyrimidine metabolism we became interested in hydroxyurea because the megaloblastic changes suggested interference with DNA synthesis. We measured the carbamyl aspartate excretion in patients receiving hydroxyurea and in some patients it seemed to decrease. To pursue this further we took advantage of the fact that 6-azauridine has very little hematologic toxicity and is known to inhibit specifically the enzyme, orotidylic decarboxylase, resulting in a marked increase in excretion of orotic acid and orotidine in the urine (2, 5, 11). If hydroxyurea did indeed inhibit de novo pyrimidine synthesis at an early stage, the increased orotic acid excretion induced by azauridine should be reversed when hydroxyurea is added. If there is a sequential blockade of pyrimidine synthesis by hydroxyurea and azauridine, a synergistic chemotherapeutic effect might occur, although this concept has been disputed (21, 23). Beneficial results on neoplastic disease with either drug alone have been limited (6, 7, 14, 19).

Materials and Methods

Studies were carried out in 6 patients, 5 with acute leukemia refractory to other chemotherapy, and 1 with metastatic melanoma who had failed to respond to hydroxyurea alone. The patients were placed on a low purine diet containing 30 mg of purine

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nitrogen for 3–7 days prior to institution of therapy. Four patients with acute leukemia were on continuous i.v. infusions of azauridine. The other 2 patients received triacetyl azauridine in divided p.o. doses for 11 days. After the patients had received azauridine for a minimum of 3 days, hydroxyurea in divided p.o. doses of 40–45 mg/kg was given for 5 days. The azauridine was continued in all but 1 patient for at least 2 days after hydroxyurea was stopped. Five of the 6 patients received more than 1 course of treatment.

Daily 24-hr urine collections were obtained in all patients. Blood counts were done daily. Liver function studies were done once weekly. Blood urea nitrogen, uric acid, and creatinine clearances were done twice weekly and the daily urinary excretion of uric acid and creatinine were measured. Uric acid was determined by the method of Praetorius and Poulsen (15). The orotic acid excretion was determined as previously reported (12), and the spectrophotometric results were checked by an isotope dilution technic.

Urinary orotidine excretion was determined by measurement of orotic acid content before and after acid hydrolysis. An aliquot of urine was evaporated to dryness. Orotic acid-14C was added. The residue was dissolved in 2 ml of concentrated formic acid (88%), transferred to sealed tubes, and heated for 2 hr at 150°C. Orotic acid was measured in the usual manner. The amount of orotidine was equal to the increase in orotic acid following hydrolysis.

Results

Chart 1 shows the effect of treatment on orotic acid excretion in a 61-year-old white female with metastatic melanoma who had previously received hydroxyurea alone without benefit. She was given triacetyl azauridine, 100 mg/kg daily for 11 days. On the 4th day hydroxyurea in doses of 40 mg/kg was added and continued for 5 days. During the control period the orotic acid excretion totaled 39 μmoles/24 hr. After 3 days of azauridine it rose to 1242 μmoles. Three days after hydroxyurea was started the orotic acid excretion had fallen to 565 μmoles. Three days after the hydroxyurea was stopped and while receiving azauridine, the orotic acid excretion again increased to 1056 μmoles. Orotidine excretion paralleled that of orotic acid. Hematologic toxicity was similar to that observed with hydroxyurea alone. The patient received 2 courses of treatment without measurable benefit.

A patient with acute myelomonocytic leukemia who had had a partial remission with methotrexate and a good response to 6-mercaptopurine and prednisone became refractory to treatment. He received azauridine in doses of 220 mg/kg for 14 days without improvement. As shown in Chart 2, his orotic acid excretion rose from a control of 50 μmoles to 2588 μmoles on the 11th day after adding azauridine. After 4 days of hydroxyurea in doses of 40 mg/kg started on the 15th day of azauridine therapy, the orotic acid excretion fell to 1119 μmoles. Both drugs were discontinued for 8 days. At the end of this interval the orotic acid excretion fell to 201 μmoles/24 hr. The WBC fell to 400 but without improvement in differential. Azauridine was resumed in the same dose, and 3 days later hydroxyurea was added. Excretion of orotic acid rose after azauridine and then fell from 5375 to 1284 μmoles after...
TABLE 1

Effect of Azauridine and Hydroxyurea on the Urinary Excretion of Orotic Acid

<table>
<thead>
<tr>
<th>Patient</th>
<th>Day of study</th>
<th>Azauridine (mg/kg)</th>
<th>Hydroxyurea (mg/kg)</th>
<th>Orotic acid (µmol/24 hr)</th>
<th>Orotidine (µmol/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Metastatic melanoma, WF, 61 years old</td>
<td>2</td>
<td>100*</td>
<td>300</td>
<td>1,242</td>
<td>1,905</td>
</tr>
<tr>
<td>2. Acute myeloblastic leukemia, WM, 64 years old</td>
<td>5</td>
<td>100*</td>
<td>40</td>
<td>505</td>
<td>501</td>
</tr>
<tr>
<td>3. Acute myelomonocytic leukemia, WM, 61 years old</td>
<td>14</td>
<td>220</td>
<td>40</td>
<td>2,131</td>
<td>2,131</td>
</tr>
<tr>
<td>4. Acute myelomonocytic leukemia, WM, 49 years old</td>
<td>6</td>
<td>90*</td>
<td>45</td>
<td>1,871</td>
<td>1,871</td>
</tr>
<tr>
<td>5. Blast phase, chronic myelocytic leukemia, NM, 18 years old</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.933</td>
<td>1.933</td>
</tr>
<tr>
<td>6. Acute myeloblastic leukemia, NF, 33 years old</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.383</td>
<td>1.383</td>
</tr>
</tbody>
</table>

* Abbreviations: W, white; N, Negro; M, male; F, female.
* Triacetyl azauridine.

hydroxyurea. There was no significant improvement following 2 courses of combination therapy.

Another patient with acute myeloblastic leukemia received azauridine in doses of 300 mg/kg/day for 3 weeks by continuous i.v. infusion and had a fall in WBC from 80,000 to 20,000. Increasing the dose of azauridine to 400 mg/kg/day produced nausea, vomiting, and diarrhea without further fall in WBC. The dose was reduced to 300 mg/kg/day, and hydroxyurea was started. On the 7th day the WBC was 3800 and by the 11th day it was 3500. There was some improvement in differential. The orotic acid excretion fell from 621 µmoles on azauridine alone to 2131 µmoles after the addition of hydroxyurea. He received 3 courses of combination therapy which maintained his WBC at neutropenic levels without further benefit. Subsequently, he was treated with hydroxyurea alone without benefit.

A 4th patient with acute myelomonocytic leukemia excreted 49 µmoles/24 hr of orotic acid. After the addition of triacetyl azauridine it rose to 18,071 µmoles but dropped sharply to 136 µmoles after the addition of hydroxyurea and rose to 1385 µmoles after it was discontinued. The patient died 2 weeks later of a myocardial infarction.

A 5th patient was an 18-year-old Negro male with a “blast” phase of chronic myelocytic leukemia. He was treated with a modification of the POMP program: allopurinol, 300 mg daily; vincristine, 3 mg; prednisone, 150 mg daily for 7 days; methotrexate, 1.25 mg every 6 hrs for 16 doses; and 6-mercaptopurine, 200 mg daily for 5 days. He received 3 courses of treatment without improvement. He was then given azauridine 180 mg/kg for 8 days. On the 3rd day of azauridine administration, hydroxyurea in doses of 45 mg/kg was given in a divided daily dose schedule. Orotic acid excretion was 65 µmoles/24 hr prior to institution of the POMP program, 1903 µmoles prior to azauridine, and increased to 16,352 µmoles after administration of azauridine. On the 2nd day of hydroxyurea administration, the orotic acid excretion fell to 1043 and rose to 54,246 µmoles after hydroxyurea was discontinued. A similar fall in orotic acid excretion occurred following a 2nd course of hydroxyurea. Orotidine excretion decreased but not to the same degree as orotic acid. The WBC fell from 76,500 to 1450 on the 12th day. His spleen shrank from 11 to 4 cm. Three courses of treatment were given. The WBC was kept within normal range, but the differential did not significantly improve. He remained anemic and thrombocytopenic. He was given methotrexate in doses of 1.25 mg every 6 hr for 20 doses during a 3rd course of azauridine. There was no clinical benefit. It is interesting that the drop in orotic acid excretion as seen with hydroxyurea was not observed with methotrexate. The patient subsequently received 5-fluorouracil, azauridine, and hydroxyurea without further benefit. He was discharged on hydroxyurea alone but relapsed and died of infection.

A 6th patient, a 33-year-old Negro female with acute myeloblastic leukemia, achieved a complete remission on a combination of methotrexate, 6-mercaptopurine, prednisone, and vincristine lasting 5 months. She was given 2 courses of azauridine, 150 mg/kg, and hydroxyurea, 45 mg/kg, with a fall in leukocytes from 83,000 to a low of 1850 without improvement in differential. There was a similar fall in urinary orotic acid and orotidine when hydroxyurea was added. A 2nd course of hydroxyurea and azauridine was given. At this time the patient developed pneumonia and received chloramphenicol, penicillin, and analgesics. The orotic acid did not fall after adding hydroxyurea but this may have been due to technic since other materials were eluted from the column with the orotic acid. There was no essential change in hematologic status when the patient died of pneumonia 5 days later.

The data on all patients studied is presented in Table 1. No sustained clinical improvement occurred in these patients, but they received only 1–3 courses of treatment, and all were or had become refractory to other drugs including methotrexate, 6-mercaptopurine, and prednisone.

In contrast to the marked reduction in orotic acid excretion, hydroxyurea had a variable effect on urate clearance. Azauridine has been shown to have a uricosuric effect (5), and this was observed in our patients. The addition of hydroxyurea reduced the

* See Proc. Am. Assoc. Cancer Res., 6: 34, 1965. POMP: prednisone, 1.0 gm/sq m; vincristine, 2 mg/sq m; methotrexate, 7.5 mg/sq m; and 6-mercaptopurine, 0.5 gm/sq m.
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Urate clearance approximately 25–30% 4 out of 6 times in 4 patients and had no effect in 2 patients. The more pronounced effect was observed in those patients in whom leukocyte counts dropped significantly.

Blood urea nitrogen levels remained within normal range in 5 patients during treatment. Patient 5 developed acute pyelonephritis during therapy with a rise in blood urea and creatinine. In the remaining patients creatinine clearances remained constant or improved. Liver function studies were unaltered.

Discussion

Our data indicate that hydroxyurea can reverse the orotic aciduria produced by 6-azauridine. Orotidine excretion was decreased proportionately in the patient with melanoma and although decreased in 2 patients with leukemia, it was not as marked a fall as in orotic acid. Thus in contrast to results expected from previous studies showing inhibition of DNA synthesis by hydroxyurea, it appears to inhibit de novo pyrimidine synthesis in man. It may be that hydroxyurea or a metabolite of hydroxyurea directly inhibits an enzymatic step in orotic acid synthesis. There is no other evidence to support this thesis. The administration of 6-azauridine forces the body cells to rely upon salvage synthesis of pyrimidine nucleotides. The relatively mild toxicity of the drug suggests that salvage synthesis is quite effective. If hydroxyurea inhibits the incorporation of pyrimidine nucleotides into DNA, they might accumulate rapidly enough to produce the observed reduction of orotic acid synthesis by feedback inhibition. Such feedback inhibition has been demonstrated by Bresnick and Hitchings (3) in rat liver and hepatomas and by Bresnick and Hitchings (4) in Ehrlich ascites cells. Further studies are underway to delineate these observations more clearly.

It is unlikely that hydroxyurea interferes with the measurement of orotic acid. It has no absorption at 260 μm. Under the conditions in our laboratory, it is not adsorbed as is orotic acid to the Dowex 1-chloride resin. Furthermore, there was good agreement between spectrophotometric determination and isotope dilution measurements of orotic acid.

It is not likely that hydroxyurea produces alterations in the renal transport mechanism for orotic acid which would account for the marked fall in its excretion. The clearance of orotic acid in the normal approaches that of creatinine (22). Following orotate infusion, clearance of orotic acid exceeds the glomerular filtration rate indicating an active renal tubular transport system in man (16). Data from fowl (20) indicate that orotic acid excretion may be via the same mechanism as that of uric acid as both are blocked by probenecid. In our patients there was only a slight and inconsistent fall in urate clearance after the addition of hydroxyurea, and if the same mechanism were operative, only a slight fall in orotic acid would be expected. This was not the case. Plasma concentrations of orotic acid would be necessary to prove the point. This was not done.

The question of a therapeutic advantage of the combination of 6-azauridine and hydroxyurea remains. Our patient with melanoma had previously been treated with hydroxyurea and had failed to respond. The patients with leukemia had all been previously treated and were refractory to more conventional modes of therapy. In 1 leukemic patient the combination reduced the leukocyte count below the level achieved by azauridine alone.

Extension of these studies to a larger number of patients will be necessary to clearly determine if a therapeutic advantage exists. In vitro studies with a particle-free supernatant fraction of rat liver have shown a lack of synergistic effect on de novo pyrimidine synthesis using 5-azaurorotate and 6-azauridine (18). A similar lack of potentiation of inhibition was observed in intact dog leukocytes (18). Further investigation is necessary to determine whether these findings can be substantiated in the intact animal.

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