Induced Ribotide Reductive Conversion Defect by Hydroxyurea and Its Relationship to Megaloblastosis¹

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SUMMARY

During therapy with an antineoplastic agent, hydroxyurea, early abrupt megaloblastic changes were evident in the marrow. Correlative biochemical studies suggested that the megaloblastosis resulted from an abrupt decrease in reductive conversion of ribotide to deoxyribootide and that hydroxyurea induces defective reductive conversion. Evidence for similar changes in the spleen in considerably less, suggesting that splenic hematopoietic mesenchyme may have a DNA biosynthetic pathway that is different from that of the bone marrow.

INTRODUCTION

The abrupt induction of megaloblastosis during cancer chemotherapeutic clinical trials with hydroxyurea (NSC-32065) has been previously reported (3, 23). Hydroxyurea, a drug under evaluation as a possible antineoplastic agent, has been demonstrated to yield megaloblastic changes in bone marrow aspirates from humans as early as 48 hr following institution of therapy (3). These morphologic changes did not correlate with any evidence of antineoplastic effect of the drug and occur long before any other manifestation of drug toxicity is seen. Previous studies in this laboratory suggested that an early effect of hydroxyurea was to interfere with DNA synthesis by altering the reductive conversion of ribotide to deoxyribootide (6). The present study has extended these previous preliminary observations and has correlated the sequence of morphologic change with the biochemical events.

MATERIALS AND METHODS

The animal studies were performed on female Sprague-Dawley rats (150–180 gm) received 1 week prior to their use, kept in an air-conditioned room, and maintained on Purina rat diet with free access to water. Hydroxyurea was administered in the dosage of 100 mg/kg/day by the i.p. route. Groups of at least 5 animals were killed on each day of continuous therapy.

Reductive Conversion. The in vitro reductive conversion of tritium-labeled cytidine monophosphate (CMP-3H) to deoxycytidine monophosphate (dCMP-3H) was determined in the bone marrow, spleen, and thymus in groups of control and hydroxyurea-treated rats. The bone marrow was removed by forceful expression with cold 0.23 M sucrose. Tissues were weighed and suspended in cold 0.23 M sucrose to a final volume of 2 ml. From this measured volume an aliquot was removed for DNA (10) and protein (11) determinations (the latter with albumin as a standard). The soluble enzyme system was extracted by the method of Reichard et al. (17). The reductive conversion of the labeled ribotide to deoxyribotide was determined as previously described (6, 17) and expressed as mmoles of dCMP formed per mg of DNA. A linear relationship existed in the assay system relative to the amount of protein added.

Morphologic Study. Imprints and sections of all the tissues, as well as smears of the bone marrow, were evaluated as previously described (7).

RESULTS

Table 1 records the mmoles of dCMP-3H formed per mg of DNA from the CMP-3H incubation mixture. The bone marrow demonstrated a sharp decrease in the ability to convert the labeled ribotide to deoxyribootide by the end of 24 hr after the institution of therapy. Correlative morphologic observations done at this time demonstrated only borderline alterations from normal in the erythrocytic series with less nuclear chromatino condensation than anticipated. The granulocytic series did demonstrate enlarged metamyelocytes. By 48 hr the nadir in defective reductive conversion was passed, but at this time megaloblastic morphologic changes were prominent with poor nuclear condensation of the developing erythrocytic series, frequent Howell-Jolly bodies, and evident intermediate megaloblasts. Similarly, the granulocytic series demonstrated poor nuclear condensation. As demonstrated in Chart 1, the ability of the supernate extract to convert ribotide to deoxyribotide reverts to near normal levels during Days 3–4 and then slowly again decreases. The megaloblastic changes were similarly transient and are not regularly demonstrable after Day 5, in spite of the fact that progressively lower levels of reductive conversion were seen beyond that point. The predominant morphologic feature of therapy at this dosage, from Day 8 and beyond, was progressive hypocellularity. The predominant morphologic feature of therapy at this dosage, from Day 8 and beyond, was progressive hypocellularity. Although some nuclear dyspoiesis was present only rare cells demonstrated good morphologic evidence of megaloblastosis.

A similar decrease in reductive conversion capacity was seen in the spleen and thymus (Table 1, Chart 1). The initial nadir of suppression reached was of lesser degree than that seen in the bone marrow. Correlative morphologic observations of thymus gland failed to reveal any significant structural changes by light

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Hydroxyurea-induced Reductive Conversion Defect

**CHART 1.** The relationship of the changes in reductive conversion of ribotide to deoxyribotide (dCMP) correlated with the morphologic changes in the bone marrow.

**TABLE 1**

<table>
<thead>
<tr>
<th>Test day (5 rats per day)</th>
<th>Bone marrow</th>
<th>Spleen</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (9 rats)</td>
<td>0.22 ± 0.10</td>
<td>0.28 ± 0.10</td>
<td>0.43 ± 0.15</td>
</tr>
<tr>
<td>1</td>
<td>0.04 ± 0.005</td>
<td>0.16 ± 0.04</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.14 ± 0.10</td>
<td>0.20 ± 0.10</td>
<td>0.11 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>0.19 ± 0.09</td>
<td>0.27 ± 0.06</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>4</td>
<td>0.13 ± 0.06</td>
<td>0.18 ± 0.09</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>0.15 ± 0.04</td>
<td>0.18 ± 0.10</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>0.13 ± 0.08</td>
<td>0.11 ± 0.01</td>
<td>0.17 ± 0.09</td>
</tr>
<tr>
<td>8</td>
<td>0.09 ± 0.02</td>
<td>0.11 ± 0.10</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.07 ± 0.04</td>
<td>0.08 ± 0.05</td>
<td>0.07 ± 0.009</td>
</tr>
</tbody>
</table>

*P* values determined from the changes in the first 72 hr (prior to hypocellularity).

DISCUSSION

The morphologic phenomena designated megaloblastosis is characterized by structural changes in developing cells recognizable by the increase in size of the cell with visual evidence of poor nuclear condensation and an apparent increase in the cytoplasmic mass. Although these changes are most apparent in the developing erythrocytic series, the same defect is apparent in almost all cells undergoing active maturation. Structural nuclear alterations, often termed dyspoiesis, also occur and are identifiable as nuclear lobulations and even DNA fragments (e.g., the Howell-Jolly bodies) present in the cytoplasm (4).

The present data demonstrate that the drug hydroxyurea rapidly induces structural changes that are characterized as megaloblastic both in humans (3, 23) and in animals (5) suggesting that at least some aspect of the drug's action is related to an interruption of DNA synthesis. Of interest is that these changes occur very early following the institution of the drug and then become progressively less apparent, and, in fact, often disappear in spite of chronic administration to the point of serious toxicity (3, 23).

The megaloblasts have been demonstrated by Glazer et al. (8) to have markedly increased RNA to DNA ratios over normal cells. In reviewing the data of at least one clinical form of microscopy. The spleen demonstrated only very minimal megaloblastic changes during the entire course of observation and these changes were patchy. A moderate amount of indeterminate proteinaceous precipitate was apparent in both organs beyond Day 6 as well as nuclear pyknosis and some apparent necrosis.

Although marrow hypocellularity ensued, the mean spleen and thymus weights did not significantly decrease during the course of the study, in spite of the morphologic evidence of cellular destruction.

**MEGALOBLASTOSIS:**

\[ - 0.2 \]

\[ - 0.1 \]

\[ 0.1 - 0.2 \]

\[ 0.3 - 0.5 \]
megaloblastosis (that associated with a deficiency of vitamin B12), Beck (1) presents evidence of defective deoxyribonucleotide synthesis as the basis for the altered DNA synthesis in these cells.

Considerable evidence has accumulated to suggest that the so-called reductive sequence, i.e., the conversion of a ribonucleotide to a deoxyribonucleotide, is a major source of deoxyribose in the biosynthesis of DNA (9, 14, 17-20, 22). Bacterial, mammalian, and avian enzyme systems convert cytidylic acid to deoxyctydyllic acid, and isolated systems of enzyme activity have demonstrated that the conversion occurs at the ribonucleoside 5'-diphosphate level (13, 15, 17).

We have examined the conversion of ribotide to deoxyribotide in relationship to the induced megaloblastosis in rats. The morphologic evidence of rapidly induced, although transient, megaloblastic changes in the bone marrow correlated with the rapid decrease in the ability of the marrow cells to convert reductively the labeled cytosine ribotide to the deoxyribotide. The suggestive evidence that the megaloblastic state may be due, in part, to the inhibition of reductive conversion is in accord with a variety of previous indirect studies.

First, previous in vivo studies in this laboratory (6) failed to demonstrate that the block in DNA synthesis occurred beyond the point of initial formation of deoxyribotide since isotopically labeled deoxyctydine and thymidine incorporation into DNA was not altered. In fact, increased incorporation of labeled thymidine occurred during drug administration (6). Secondly, in vitro tissue culture studies by Mohler (16) employing a Chinese hamster cell line (V79H) demonstrated that the addition of thymidine reversed part of the growth-inhibiting and cytotoxic effects of hydroxyurea, again suggesting that the block occurs at an early stage of deoxyribonucleic acid synthesis. That some variability exists in at least in vitro systems is suggested by his evidence that thymidine did affect the growth of a human cell line (HeLa 53) (16). Young and Hodas (24) employing the HeLa cells in culture further demonstrated that hydroxyurea inhibited the incorporation of labeled thymidine under the conditions of their study. Finally, in the in vivo system of man and rat (3, 6) a rapidly induced megaloblastosis seems related to the defect in ribotide conversion to deoxyribotide.

The previous observation of the altered reductive conversion (6) suggested that the initial hydroxyurea-induced block was quickly overcome yielding only transient megaloblastic changes (3, 6, 23). Longer serial morphologic observations in our animals again suggested that the block was transient, in that the megaloblastosis was transient. Yet, measurement of reductive conversion late in the course of therapy continued to reveal reduced reductive conversion. Several possible explanations may be the basis for the transient aspects of the morphologic changes. Since cellular destruction does occur, at least in the bone marrow, during the course of hydroxyurea therapy, it is possible that the cells so destroyed serve as a readily available pool of deoxyribotide (or deoxyriboside). The transient nature of the megaloblastic changes under such a circumstance could be related to "salvage" reutilization of these breakdown products with resultant reversal of the block of DNA synthesis similar to the events in tissue culture with the addition of thymidine (9). Also, it is possible that the large pool of breakdown products available from the cytotoxically injured adjacent cells may serve to inhibit the reductive conversion enzyme system. This could explain the absence of megaloblastic changes beyond Day 6 when decreased reductive conversion again is apparent. This explanation, however, does not clarify the initial pattern of depressed reductive conversion followed by abrupt recovery (Days 1-4) despite continued drug administration. However, the progressive hypopcellularity of the marrow that supervenes may mask the more subtle morphologic changes of megaloblastosis, or may represent an induced metabolic abnormality that is very early in the sequence of cellular maturation, prior to the potential development of the megaloblastic change.

The above observed biochemical or morphologic changes do not necessarily imply that these effects are related to the supposed cytotoxicity that led to the investigation of hydroxyurea as an antineoplastic drug. Bendich et al. (2, 5) have presented evidence that the hydroxyurea cytotoxicity is related to alteration of the agent to an intermediate product such as hydroxylamine or hyponitrite with the subsequent effect on DNA bonds.

The paucity of morphologic changes in the thymus could be predicted from the cellular character of the gland. The decrease in reductive conversion here suggests this is a fairly general result of the drug hydroxyurea.

The meaning of the morphologic changes in the spleen is not clear. The spleen in the rat has the potential to serve as a general hematopoietic organ with all cell lines recognizable, yet only minimal morphologic changes were demonstrated. Similarly, only a slight decrease in reductive conversion resulted. We have no evidence to suggest any difference in the reductive conversion enzyme system in splenic tissue of the rat. It is of interest that the evidence of ribotide-reductive conversion as the major source for DNA biosynthesis should then presume a normal tissue capability of converting the formed deoxyctydyllic acid to thymidylic acid via the deamination step to deoxyuridylic acid and subsequent methylation to thymidylic acid (21). Such a conversion then presumes the presence of dCMP deaminase activity. Maley and Maley (12) in fact have demonstrated that significant dCMP deaminase activity is present in the bone marrow and thymus but not in the spleen. Thus, although we have no major morphologic evidence of a difference between splenic and marrow hematopoietic tissue the significant biosynthetic pathways may be different.

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