Daunorubicin Metabolism in Acute Myelocytic Leukemia

By David H. Huffman and Nicholas R. Bachur

Daunorubicin reductase is a cytoplasmic enzyme that converts daunorubicin to a principal metabolite, daunorubicinol, in the presence of NADPH. This enzyme, found in mammalian tissues and studied in rat tissue preparations and normal human blood components, is also present in human leukemic myeloblasts. The enzymes from both normal and leukemic leukocytes require NADPH for activity, have similar kinetics and substrate saturation characteristics, and have a $K_{m}$ of $1.7 \times 10^{-4}$ M. When daunorubicin reductase levels in leukemic myeloblasts are related to the clinical response to daunorubicin therapy, we find that patients with a high enzyme level respond favorably to daunorubicin therapy with either a complete or partial remission, whereas those patients with a lower enzyme level experience either no response or die during therapy. These observations suggest that the level of daunorubicin reductase in the leukemic myeloblast may be important in determining the susceptibility of that cell to daunorubicin. Moreover, the measurement of daunorubicin reductase level may have prognostic value regarding the clinical response to daunorubicin therapy.

A MOST PROMISING DRUG being used for the treatment of acute lymphocytic and acute myelocytic leukemia is the antibiotic, daunorubicin (daunomycin, rubidomycin, rubomycin C). Produced by strains of Streptomyces, this anthracycline glycoside presumably acts pharmacologically through interference with cellular nucleic acid metabolism.

A cytoplasmic enzyme exists in tissues of man and other mammals that metabolizes daunorubicin to the active, principal metabolite, daunorubicinol. Since human blood cells carried out this conversion, we have studied some characteristics of daunorubicin reductase in peripheral blood cells from patients with acute myelocytic leukemia and compared these data within the leukemic group and to data obtained from normal blood cells.

Based on the pharmacokinetic studies of daunorubicin in man, we have studied daunorubicin therapy in these patients with acute myelocytic leukemia employing a high-dose, intermittent regimen. We have attempted to relate the leukocyte levels of daunorubicin reductase in these patients to clinical response to daunorubicin therapy.

MATERIALS AND METHODS

Chemicals

Daunorubicin was obtained from the Drug Development Branch, National Cancer Institute, National Institutes of Health. The drug used for enzyme assay was purified before

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*From the Biochemistry Section, Laboratory of Pharmacology, Baltimore Cancer Research Center, NCI, Baltimore, Md.*

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David H. Huffman, M.D.: Clinical Associate, Baltimore Cancer Research Center, NCI, Baltimore, Md.; presently, Fellow, Clinical Pharmacology Study Unit, University of Kansas School of Medicine, Kansas City, Kansas. Nicholas R. Bachur, M.D., Ph.D.: Head, Biochemistry Section, Laboratory of Pharmacology, Baltimore Cancer Research Center, NCI, Baltimore, Md.
use, whereas daunorubicin formulated for clinical use was purchased from Farmitalia. NADPH was from P. L. Biochemicals, and all other chemicals were reagent grade. Glass-distilled, demineralized water was used for making all solutions.

Patients

Twelve patients with acute myelocytic leukemia, who received daunorubicin as therapy for their leukemia, served as subjects for this study. Their diagnosis of acute leukemia was based on their clinical findings and bone marrow morphology. These patients received daunorubicin as a standard single intravenous dose of 180 mg/sq m body surface area. The details of this high-dose intermittent regimen, as well as results of therapy, are reported separately.

Cell Preparation

Prior to therapy with daunorubicin, approximately 50 ml of heparinized venous blood were obtained and allowed to sediment in the cold without dextran. Leukemic leukocytes were separated from the plasma by differential centrifugation and hypotonic lysis of the contaminating erythrocytes. This procedure resulted in myeloblast preparations that were greater than 95% viable (trypan blue exclusion), and free of erythrocytes (less than 1%) and platelets. Differential counts were performed on the cell preparation prior to homogenization. With the exception of one patient who was subleukemic, all the patients studied had greater than 85% myeloblasts in the analyzed homogenate. For comparison, erythrocyte preparations were made from the erythrocyte-rich sediment for each patient. Similarly, leukocytes and erythrocytes were isolated from the peripheral blood of normal subjects.

Homogenates of the isolated cells were made in two volumes of 0.05 M Tris-Cl (pH 7.44) and frozen until assayed. Only once-thawed homogenates were assayed for enzyme activity. Protein was estimated by the method of Lowry et al.

Daunorubicin Reductase Assay

The level of daunorubicin reductase in the blood cell homogenates was estimated by a previously described method. The reaction mixture contained 0.583 μmole daunorubicin, 1 μmole NADPH, 50 μmoles Tris-Cl (pH 7.44), and 0.4–0.8 mg homogenate protein in a final volume of 0.5 ml incubated at 37°C for 30 min. In this assay, one enzyme unit equaled 1 μmole × 10⁻⁹ daunorubicinol produced/mg protein per 30 min. Both leukocyte and erythrocyte homogenates from each patient were assayed for daunorubicin reductase activity at the same time. Since there was no difference in daunorubicin reductase between erythrocytes isolated from normal and leukemic patients, the daunorubicin reductase level was expressed as the ratio of specific activity of the isolated myeloblast or leukocyte homogenate to the specific activity of the erythrocyte homogenate. This minimized any day to day variation in enzyme assay that might have occurred.

Thin Layer Chromatography

Thin layer chromatography of the extracted drug and metabolite was carried out as previously described. This was done routinely to assess metabolite formation by the cell homogenates.

RESULTS

When homogenates of leukemic myeloblasts were analyzed for daunorubicin metabolism, the only fluorescent metabolite found was daunorubicinol. This was similar to what we observed with normal blood components. Since no interfering reactions were present, we used the myeloblast homogenate to study several aspects of daunorubicin reductase.

Like rat tissue and normal human blood homogenates, myeloblast homo-
DAUNORUBICIN METABOLISM IN LEUKEMIA

Fig. 1. Effect of NADPH concentration on daunorubicinol production by leukemic homogenate. Leukemic cells were obtained from patient with acute myeloblastic leukemia who responded to daunorubicin therapy (group A, Table 1). Preparation of the cells is previously described. Each reaction mixture contained 0.583 μmoles D1, 50 μmoles Tris-Cl (pH 7.44), and 0.05 ml myeloblast homogenate (11.0 mg protein/ml), in addition to the NADPH that was varied in final volume of 0.5 ml. Reaction was for 30 min at 37°C.

Fig. 2. Effect of daunorubicin concentration on daunorubicinol production by normal and leukemic leukocyte homogenates. Preparation of myeloblast cells from patient who responded to daunorubicin therapy (group A) are described in Methods. Each reaction mixture contained 1 μmole NADPH, 50 μmoles Tris-Cl (pH 7.44), and 0.05 ml homogenate (myeloblast, 13.4 mg protein/ml; normal leukocyte, 11.5 mg protein/ml), in addition to daunorubicin that was varied in final volume of 0.5 ml. Reaction was for 30 min at 37°C.

Leukemic homogenate was dependent on NADPH as a cofactor for the reductase. However, unlike those tissues, the undialyzed myeloblast homogenate was absolutely dependent, and no activity was observed without added NADPH (Fig. 1). For comparison, erythrocyte homogenates usually show about 10% of their maximal activity without added NADPH, suggesting an endogenous level of NADPH. The saturating characteristics of NADPH and lack of substrate inhibition to 3 mM with the myeloblast daunorubicin reductase are similar to that seen in normal leukocytes. Other nucleotides were tested for cofactor activity. Of those tried, 2mM NADH had about 15% of the activity of 2 mM NADPH, whereas both NAD and NADP at 2 mM were inactive. These levels of pyridine nucleotide were about six times saturating as is seen in Fig. 1.

Both normal leukocyte homogenates and myeloblast homogenates attain maximal daunorubicinol production at a daunorubicin concentration of about $3.4 \times 10^{-4} M$, with no substrate inhibition observed to $1.7 \times 10^{-3} M$ (Fig. 2). However, the myeloblast homogenates were more active than comparable...
normal leukocyte homogenates. This difference in activities of the preparations was analyzed further by constructing double reciprocal plots of the substrate concentration data. From this plot, a derived $K_m$ for both the normal leukocytes and the myeloblasts was estimated to be $1.7 \times 10^{-4}$ M (Fig. 3).

Under standard incubation conditions, daunorubicinol production by myeloblast homogenates was linear for both protein concentration and time (Fig. 4). Linearity for time was seen up to 30 min, which was the standard incubation period.

Although the myeloblasts contained more activity than normal leukocytes, erythrocyte preparations from both leukemic and normal sources contained similar enzyme levels. The average value of daunorubicin reductase from 14 normal subjects was $1.84 \pm 0.44$ SEM, while that from 13 leukemics was $1.88 \pm 0.49$ SEM. The characteristics of the erythrocyte daunorubicin reductase from these leukemic patients were similar to what we had found for erythrocytes from normal subjects.11

The myeloblast daunorubicin reductase levels of the patients studied are given in Table 1. Conditions and substrate concentration described in Methods were used, which yielded linearity in the enzyme assay. Except for the sub-

![Fig. 3. Lineweaver-Burk plot of daunorubicin reductase data from normal and acute myeloblast leukocytes. Details are same as in Fig. 2.](image1)

![Fig. 4. Effects of protein concentration and time on daunorubicinol production by acute myeloblastic leukemic homogenates. Conditions for incubation and assay are previously described. Leukemic cells were obtained from patient who responded to daunorubicin therapy (group A).](image2)
leukemic preparation, all of the myeloblast preparations contained higher levels of daunorubicin reductase than the normal leukocyte preparations. In the patients who responded to daunorubicin therapy, the levels of daunorubicin reductase were highest and were significantly greater than enzyme levels in groups B or C \((p < 0.001 \text{ and } p < 0.01)\), respectively. No difference existed between the reductase level in patients who died in therapy and those who did not respond to daunorubicin. This suggested that the low levels of daunorubicin reductase observed in the patients who died in therapy would have been associated with clinical failure to daunorubicin therapy.

Since the responders were younger than nonresponders, the daunorubicin reductase was compared with age by a simple regression analysis of the least-squares method for the leukemic patients. The \(R\) value for 12 patients was 0.538 and significant \((p < 0.05)\). In contrast, there was no correlation for normal subjects between enzyme level and subject age \((R=0.099, p <0.05)\). There was also no relationship of leukocyte daunorubicin reductase level to sex.

**DISCUSSION**

Leukemic myeloblasts contain the enzyme daunorubicin reductase at levels four- to ninefold greater than the concentration of enzyme in normal mature leukocytes. Despite the diverse levels, enzyme from myeloblasts has properties similar to enzyme from normal blood components with respect to cofactor requirements and substrate and cofactor saturation. In addition, kinetic studies indicate that the \(K_m\) for both the myeloblast enzyme and the normal leukocyte enzyme is \(1.7 \times 10^{-4} \text{ M}\). This suggests that the same enzyme is present in both cell types, but that the concentration of the reductase is higher in periph-

<table>
<thead>
<tr>
<th>Clinical Result</th>
<th>Number of Patients</th>
<th>Average Age</th>
<th>Enzyme Ratio Leukocyte/Erythrocyte*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Remission</td>
<td>5</td>
<td>41</td>
<td>46.5 ± 5.4</td>
</tr>
<tr>
<td></td>
<td>1 partial</td>
<td>66</td>
<td>20.0 ± 5.17†</td>
</tr>
<tr>
<td>B. Died in therapy</td>
<td>4</td>
<td>53</td>
<td>19.5 ± 9.7†</td>
</tr>
<tr>
<td>C. No response</td>
<td>3</td>
<td>(45-60)</td>
<td></td>
</tr>
<tr>
<td>D. Subleukemic</td>
<td>1</td>
<td></td>
<td>3.2</td>
</tr>
<tr>
<td>E. Normal subjects</td>
<td>14</td>
<td></td>
<td>5.3 ± 2.0</td>
</tr>
</tbody>
</table>

*Enzyme ratio is specific activity of myeloblast homogenate or normal leukocyte homogenate divided by specific activity of erythrocyte homogenate for each patient. Value represents mean ± SD.
†A is significantly different from B, \(p <0.001\).
‡A is significantly different from C, \(p <0.01\).

Statistics are T distribution comparing means of two small samples. Values for comparison of B and A, \(T = 7.44, n = 7\); comparison of C and A, \(T = 5.18, n = 6\).

Patients were divided according to clinical response to daunorubicin therapy by Acute Leukemia group B criteria. Enzyme levels were determined without knowledge of clinical response.
eral circulating leukemic myeloblasts. Because of the difficulties in obtaining sufficient quantities of normal myeloblasts, we were unable to compare enzyme levels of normal myeloblasts to leukemic myeloblasts.

A favorable clinical response to daunorubicin therapy in the acute myelocytic leukemic patients was associated with highest daunorubicin reductase levels, and an intermediate enzyme level was associated with death during therapy or failure to respond to the high-dose intermittent regimen. Although limited by the small number of patients, this study evaluated patients at the limit of clinical dosage based on the associated toxicity.13

The demonstration of daunorubicin metabolism by leukemic cells is an unusual example of drug metabolism by the cells that are the therapeutic target of daunorubicin. Daunorubicinol, the principal product of this metabolism, has a longer plasma and urinary half life than daunorubicin in man.17 From these observations, we conclude that daunorubicinol is retained longer by tissue than the parent compound, daunorubicin. This is particularly important, since daunorubicinol is cytotoxic18 and inhibits L1210 nucleic acid metabolism similar to the parent drug.7 Since there is a correlation between enzyme level and clinical response, it appears that the greater concentration and longer duration of exposure of daunorubicinol in those cells with higher enzymatic activity may explain their increased susceptibility.

It was unclear why such divergent enzyme levels existed in leukemic myeloblasts from this patient population. To evaluate the effect of patients' age on daunorubicin reductase levels, a regression analysis was performed and demonstrated a significant relation between these parameters. It appeared, therefore, that the poorer response rate for this older group of patients was at least in part related to the tendency of this older group of patients to have lower daunorubicin levels. No correlation existed between the sex of the patients and either the daunorubicin reductase level or clinical response to daunorubicin. It could be possible that the level of daunorubicin reductase was related to the degree of cellular differentiation, with less mature cells having higher enzymatic activity, but this must await confirmation.

Clinical response to daunorubicin in acute myelocytic leukemia appears to be multifactorial.19-23 Accordingly, an accurate prediction of clinical response is not possible on the basis of the daunorubicin reductase analysis alone. It appears, however, that it may be possible to identify potential nonresponders to daunorubicin therapy and to avoid giving this very toxic drug to those patients. The level of daunorubicin reductase activity could be used to adjust drug therapy to the individual patient. By adjusting the dosage of administered drug to the level of daunorubicin reductase, the therapeutic index might be improved for those patients with high enzymatic activity. For example, patients with the highest enzyme level would be given less drug. Alternatively, patients with intermediate levels would receive more drug, unless the values of daunorubicin reductase were low enough to anticipate clinical failure. In the latter case, the drug should be avoided entirely during the initial attempts to achieve a clinical remission.

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DAUNORUBICIN METABOLISM IN LEUKEMIA

643

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Daunorubicin Metabolism in Acute Myelocytic Leukemia

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