Coordinated Changes in Biochemical Patterns: The Effect of Cytosine Arabinoside and Methotrexate on Leukocytes from Patients with Acute Granulocytic Leukemia

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SUMMARY

Cytosine arabinoside and Methotrexate alter in different ways the metabolic patterns of leukocytes from patients with acute granulocytic leukemia. The variations in the incorporation into DNA of deoxyuridine and thymidine during the first twenty-four hours after cytosine arabinoside administration were correlated with one another but not with the variation in activity of thymidylate synthetase, thymidine kinase, or uridine kinase. The activity of these three enzymes varied as a related unit. Dihydrofolate reductase activity varied independently of the other five parameters. The response of these parameters to cytosine arabinoside varied from stimulation for some patients to inhibition of activity in others. After the administration of Methotrexate, an inhibition of dihydrofolate reductase was observed, but this was not correlated with the inhibition of deoxyuridine or the general stimulation of thymidine incorporation into DNA nor to a general variation in the activity of thymidine kinase and uridine kinase. A correlation after Methotrexate administration was observed between the inhibition of deoxyuridine incorporation into DNA and a stimulation of thymidylate synthetase activity. A dissociation was observed after Methotrexate administration of the relationship between thymidylate synthetase, thymidine kinase, and uridine kinase which followed cytosine arabinoside administration.

INTRODUCTION

The incidence and duration of remission of acute leukemia have been increased by treatment with combinations of drugs (7, 8, 14, 27). As a result of these improvements in therapy, the differences in response between the various types of acute leukemia are being reduced.

The well-demonstrated variation in response of individual patients and in different types of leukemia to individual agents indicates biochemical differences in leukemic cells. Patterns of correlations in the levels of thymidylate synthetase, thymidine kinase, uridine kinase, dihydrofolate reductase, and in vitro deoxyuridine and thymidine incorporation into DNA with leukocytes from untreated patients have emerged which provide a greater understanding of a drug sensitive area of the metabolism of leukemic cells (21). These observations led to the examination of the changes that occur after drug administration.

The incorporation of thymidine into DNA was used as a measure of inhibition of DNA synthesis. Thymidylate synthetase activity was examined because of its relationship to the de novo biosynthetic pathway of a precursor of DNA. Deoxyuridine incorporation measured DNA synthesis, but more importantly, the activity of thymidylate synthetase in the intact cell and the changes in a specific metabolite, N⁵,N¹⁰-methylene tetrahydrofolate. Dihydrofolate reductase has been proposed as the target of Methotrexate activity although the relationship of the therapeutic response to the inhibition of this enzyme has become less well understood as study has progressed (18, 23). Thymidine kinase was studied as a measure of the effect of the activity of this enzyme on the utilization of deoxyuridine and of thymidine for DNA synthesis. Although data have not accumulated to test the hypothesis, the possibility was considered that insensitivity to Methotrexate could result from the ability of the cell to circumvent the action of the drug by drawing on the body's small stores of thymidine. Uridine kinase catalyzes the conversion of uridine to uridine 5'-monophosphate.

Changes in the levels of activity of these enzymes following drug administration could be important in modifying the response of the cell to drugs. The relationship between these biochemical parameters and Methotrexate or cytosine arabinoside has been examined in the present report with leukocytes from patients with acute granulocytic leukemia. The basis of Methotrexate action is interference with one-carbon metabolism (9, 29), presumably as a result of the inhibition of dihydrofolate reductase (1, 30). Cytosine arabinoside interferes
with the utilization of cytidine, and the inhibition of thymidine incorporation into DNA can be reversed by deoxycytidine (6, 25). A preliminary report of the present study has been presented (19).

MATERIALS AND METHODS

The procedures for isolation of leukocytes from blood, for measuring the incorporation into DNA of deoxyuridine and thymidine, and for the assay of thymidylate synthetase, thymidine kinase, uridine kinase, and dihydrofolate reductase have been described in detail in earlier publications (22). Incorporation of the deoxyribosides into DNA was measured immediately after the isolation of the cells. The cells were disrupted by sonic oscillation, and, after centrifugation at 30,000 X g for 25 minutes at 4°C, the supernatant fluid was removed and frozen at —20°C until assayed. The enzyme reactions were assayed simultaneously. Protein determination was on the sonically disrupted cells by the method of Lowry et al. (15).

Drugs were administered by an intravenous pulse in the range accepted for treatment of patients on a once- or twice-a-week regimen (24, 26). All of the patients receiving cytosine arabinoside had previously received other drugs. A few of the patients received Methotrexate as the first drug in the treatment of their disease.

Several of the patients received a single dose of cytosine arabinoside. Other patients died of complications to their disease before an adequate course of treatment, which would permit evaluation of the patient's clinical response, could be administered. Methotrexate, for optimal therapeutic effectiveness, was administered in combination with other drugs. Therefore, the relationship of changes in biochemical parameters to clinical response was not ascertainable.

RESULTS

Response to Cytosine Arabinoside. Examples of the changes in deoxyuridine and thymidine incorporation into DNA and in the activity of thymidylate synthetase, thymidine kinase, uridine kinase, and dihydrofolate reductase at 1, 4, and 24 hours after the administration of cytosine arabinoside are presented in Chart 1. Although the changes observed for these two patients are representative of the patterns of other patients, they do not indicate the full range of variation observed for the six parameters with the eleven patients included in the present report. The incorporation of deoxyuridine and thymidine was inhibited for more than twenty-four hours after administration of the drug. A slight stimulation of thymidine kinase, uridine kinase, and thymidylate synthetase occurred in leukocytes from G. C., while a pronounced inhibition in these same three parameters was observed for R. W. Dihydrofolate reductase activity was inhibited in both patients, although the pattern of inhibition was different. Patterns of changes appear to emerge when the effect of cytosine arabinoside on these parameters is examined. Thymidine and deoxyuridine change together for each patient. Thymidine kinase, uridine kinase, and thymidylate synthetase change as a unit. Dihydrofolate reductase appears to change independently of the two other groups.

The apparent coordinated change of these parameters in independent units has been examined. In order to use the data from all the patients in a statistical analysis, the activity observed after cytosine arabinoside administration was normalized on the basis of the percent change with respect to the pretreatment level.

A plot of the percent change of the in vitro incorporation of deoxyuridine and thymidine into DNA after cytosine arabinoside administration is presented in Chart 2. The changes at 1, 4, and 24 hours have been incorporated into this study as well as in the studies which follow. A wide variation in the response of these parameters was observed. A pronounced inhibition was observed in some patients, as was shown in Chart 1, while other patients had an actual stimulation of the incorporation of deoxyuridine and thymidine into DNA. This variation in response was not time related in the sense that inhibition was routinely observed at one hour and stimulation at twenty-four hours. A correlation, r = 0.615 with P < 0.01, was observed between the changes in deoxyuridine and thymidine incorporation into DNA, indicating that the change in the in vitro incorporation of deoxyuridine and thymidine occurred as a unit and that thymidylate synthetase, the single enzymatic
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In order to present the pattern of changes observed in dihydrofolate reductase activity following the administration of cytosine arabinoside, the relationship between this enzyme and the change in thymidylate synthetase activity is presented in Chart 7. No significant correlation was observed in the changes in the activity of these two enzymes, $r = 0.091$ with $P > 0.05$. Analyses of the relationship between the change in dihydrofolate reductase activity and the four other parameters did not reveal any correlations in response to cytosine arabinoside (Table 1).

In addition to the above relationships, the data on these patients have also been analyzed for the correlation between the level of activity observed immediately prior to cytosine arabinoside administration and the percent of the original activity observed at 1, 4, or 24 hours after drug administration. The number of specimens for each time period was small, and the probability that we have achieved an accurate sampling of the total patient population is low. A single statistically significant correlation was observed. The incorporation of deoxyuridine at twenty-four hours was related to the level of deoxyuridine incorporation prior to cytosine arabinoside administration, $r = 0.777$; D.F. 5; $0.05 > P > 0.01$ with a negative slope. The negative slope of the regression line suggests that recovery of the cell's capacity to utilize deoxyuridine may be uniform. Those patients with the lowest initial incorporation level which was not in common on this anabolic pathway, was not rate limiting. A poor correlation was observed if the incorporation of one or both of the deoxynucleosides exceeded the value observed for the control. Although the stimulation may be related to a recovery of DNA synthesis after inhibition by cytosine arabinoside, the increases were observed as early as one and four hours after drug administration. Rather than use an arbitrary grouping of only those specimens for which an inhibition of DNA labeling was observed, all the values were included in the statistical analysis (Chart 2). This decision resulted in large variance.

A wide variation ranging from severe inhibition to stimulation in the level of thymidylate synthetase and thymidine kinase activity was also observed after cytosine arabinoside (Chart 3). A relationship with a high degree of correlation, $r = 0.926$ with $P < 0.01$, was observed between these two enzymes. A similar relationship is also apparent in Chart 4 between thymidylate synthetase and uridine kinase, $r = 0.876$, with $P < 0.01$. As would be expected from the preceding statistical analyses, thymidine kinase activity and uridine kinase activity were also related (Chart 5). The coefficient of correlation was $r = 0.964$ with $P < 0.01$.

The administration of cytosine arabinoside has resulted in correlated changes of deoxyuridine and thymidine incorporation into DNA as well as in thymidylate synthetase, thymidine kinase, and uridine kinase activity. Chart 6 presents the results of an analysis of the relationship between one member of each of these two groups. The changes in thymidine kinase activity and thymidine incorporation into DNA were not related, $r = 0.195$ with $P > 0.05$. The other relationships between the changes in these five parameters were not correlated (Table 1).
of deoxyuridine into DNA recover their initial capacity more rapidly than those patients with a high rate of incorporation. Other correlations will probably develop with further study.

Response to Methotrexate. A different pattern of biochemical relationships was observed following the administration of Methotrexate. An inhibition of deoxyuridine incorporation was observed in nine of the thirteen specimens studied (Chart 8). Thymidylate synthetase activity was elevated in twelve of the thirteen specimens studied after Methotrexate administration. A significant correlation was observed between the percent change in deoxyuridine incorporation into DNA and the elevation of thymidylate synthetase activity, \( r = 0.706 \) with \( P < 0.01 \). This is the first indication that Methotrexate alters the level of thymidylate synthetase activity.

The changes in the level of thymidine kinase activity and uridine kinase activity were less pronounced than for other parameters (Chart 9). An inhibition of both of these parameters was only observed in three of the eleven specimens studied. In earlier studies with fewer specimens (19), we reported a correlation between these two enzymes which was not confirmed here, \( r = 0.706 \) with \( P > 0.05 \).

The changes in dihydrofolate reductase activity and thymidine incorporation into DNA were not related, \( r = 0.025 \) with \( P > 0.05 \) (Chart 10). A pronounced inhibition of dihydrofolate reductase, which would be expected from our knowledge of Methotrexate action, was observed, as was the stimulation of thymidine incorporation which would occur if Methotrexate were not interfering with purine biosynthesis and thus preventing base-pairing of thymidine in new DNA.

The various other possible correlations were analyzed and are summarized in Table 2. The only statistically significant correlation, \( P < 0.05 \), after Methotrexate was between the elevation of thymidylate synthetase activity and the incorporation of deoxyuridine into DNA. The expected relationship between an inhibition of dihydrofolate reductase and the incorporation of deoxyuridine was not observed. Yet, under these in vitro conditions for measuring deoxyuridine incorporation into DNA, the cells were probably dependent upon the intracellular level of tetrahydrofolate for one-carbon metabolism. Although folate was present in the media, 0.6 \( \mu g/ml \), our present knowledge of the interrelationship between Methotrexate and the reduction of folate with the exception of one study (5) suggests that this folate would not be available to the cell. Hence, we are at a loss to explain the lack of correlation between the inhibition of dihydrofolate reductase and deoxyuridine incorporation unless, as has been suggested, there are other biochemical sites sensitive to the action of Methotrexate (23).
Table 1

<table>
<thead>
<tr>
<th>Coefficient of correlation</th>
<th>Number of specimens</th>
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<tbody>
<tr>
<td>Thymidine vs thymidylate synthetase</td>
<td>17</td>
</tr>
<tr>
<td>Thymidine vs uridine kinase</td>
<td>17</td>
</tr>
<tr>
<td>Deoxyuridine vs thymidylate synthetase</td>
<td>17</td>
</tr>
<tr>
<td>Deoxyuridine vs thymidine kinase</td>
<td>16</td>
</tr>
<tr>
<td>Deoxyuridine vs uridine kinase</td>
<td>17</td>
</tr>
<tr>
<td>Dihydrofolate reductase vs thymidine kinase</td>
<td>14</td>
</tr>
<tr>
<td>Dihydrofolate reductase vs uridine kinase</td>
<td>15</td>
</tr>
<tr>
<td>Deoxyuridine vs dihydrofolate reductase</td>
<td>15</td>
</tr>
<tr>
<td>Thymidine vs dihydrofolate reductase</td>
<td>15</td>
</tr>
</tbody>
</table>

Summary of the statistically nonsignificant changes in the metabolic pattern of leukocytes following cytosine arabinoside.

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% PRETREATMENT LEVEL AT 1, 4 OR 24 HOURS AFTER CYTOSINE ARABINOSIDE

Chart 6. The lack of correlation between the percent change in thymidine incorporation into DNA and thymidine kinase activity at 1, 4; 4, 0; and 24, 0, hours after cytosine arabinoside administration.

% PRETREATMENT LEVEL AT 1, 4 OR 24 HOURS AFTER CYTOSINE ARABINOSIDE

Chart 7. The lack of correlation between the percent change in thymidylate synthetase and dihydrofolate reductase activities at 1, 4; 4, 0; and 24, 0, hours after cytosine arabinoside administration.

Chart 8. The correlation between the percent change in thymidylate synthetase activity and deoxyuridine incorporation into DNA at 1, 4; 4, 0; and 24, 0, hours after intravenous Methotrexate, Circa 25 mg, administration.
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...in sensitivity from patient to patient may have masked the time relationship. Thymidine kinase activity has been shown to be subject to allosteric effectors (3, 13, 17). Variation in response by different patients may reflect the intracellular concentration of these effectors and would permit elevation or decrease in enzyme activity depending upon whether the enzyme was being inhibited or activated prior to drug administration. *In vivo* stabilization of proteins or RNA templates for several enzymes (2, 4, 12, 20, 28) was observed.

The changes in enzyme activity appear to be drug specific since only a single relationship was observed following Methotrexate. Variations in the metabolic pattern resulting from shifts in the leukocyte population, following drug administration, were apparently masked by the "drug induced" changes; otherwise, a similar pattern, with the exception of specific sites of drug inhibition, would have been observed for both drugs. Or, it becomes necessary to postulate two large populations of cell types, e.g., Methotrexate- and cytosine arabinoside-sensitive populations with different metabolic patterns. The pretreatment levels of activity are a summation of both populations and the posttreatment levels are representative of the less sensitive population of leukocytes. Methotrexate inhibition of the de novo pathway of thymidine 5'-monophosphate synthesis would result in an increased utilization of thymidine if purine deoxynucleoside triphosphate pools were not severely inhibited. This was the general pattern observed.

**DISCUSSION**

These studies show that changes occur in the level of activity of thymidylate synthetase, thymidine kinase, uridine kinase, dihydrofolate reductase, and the incorporation into DNA of deoxyuridine and thymidine in response to drug administration. Of this group, only dihydrofolate reductase has been shown to be inhibited by either Methotrexate or cytosine arabinoside, i.e., the former.

In general, cytosine arabinoside lowered the incorporation of deoxyuridine and thymidine as would be expected if the cells were lacking or unable to utilize deoxycytidine triphosphate from de novo synthesis of DNA. The effect of cytosine arabinoside on thymidylate synthetase, thymidine kinase, and uridine kinase varied from patient to patient, and this variation appears to be unrelated to time during the first twenty-four hours after drug administration. Dihydrofolate reductase may either be inhibited or stimulated by the administration of cytosine arabinoside.

This variation in the enzymatic pattern of the cells could result from changes in the rate of enzyme synthesis, in the concentration of allosteric effectors (16), or interference with enzyme turnover (2, 12). Drug effects on changes in enzyme synthesis might possibly be expected to be more closely related to time than we have observed in this study. Variations in sensitivity from patient to patient may have masked the time relationship. Thymidine kinase activity has been shown to be subject to allosteric effectors (3, 13, 17). Variation in response by different patients may reflect the intracellular concentration of these effectors and would permit elevation or decrease in enzyme activity depending upon whether the enzyme was being inhibited or activated prior to drug administration. *In vivo* stabilization of proteins or RNA templates for several enzymes (2, 4, 12, 20, 28) was observed.

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with isolated leukocytes from patients with granulocytic leukemia. A variable degree of inhibition of deoxyuridine incorporation was observed after Methotrexate as could be expected with variation in patient response. The stimulation of thymidylate synthetase which existed in the elevation by the present sampling at 1, 4, and 24 hours, such a relationship may develop with variation in patient response. The stimulation of thymidylate synthetase, uridine kinase, and thymidine kinase presumably treating a different metabolic pattern than mitotic changes after cytosine arabinoside, or a derivative, and deamination of cytosine arabinoside, or even 5-fluorouracil, might be most effective as the cell starts to recover the capacity to incorporate deoxyuridine into DNA (11).

The relationship of changes in metabolic pattern of leukocytes after the administration of Methotrexate to patients with acute leukemia. A variable degree of inhibition of deoxyuridine incorporation was observed after Methotrexate as could be expected with variation in patient response. The stimulation of thymidylate synthetase was unexpected, and, although no temporal relationship was observed in the elevation by the present sampling at 1, 4, and 24 hours, such a relationship may develop with more extensive study. The complete dissociation of thymidylate synthetase, uridine kinase, and thymidine kinase (the latter two were inhibited as well as stimulated) emphasize the differences which exist in the response of enzymes on the same pathway for thymidine 5'-monophosphate synthesis. This difference in response after Methotrexate and cytosine arabinoside, particularly the observation of coordinated changes after cytosine arabinoside, is difficult to reconcile with deamination of cytosine arabinoside, or a derivative, and the potentiality of isosteric effects by the derivatives on these enzymes.

The clinical implications, although unproven, appear to be of importance for combination chemotherapy. The changes reported here were observed after the administration of the first dose of cytosine arabinoside or Methotrexate or, in some instances with the latter drug, several months after the preceding dose. If the drug-induced changes in metabolic pattern continue to occur after several doses of drug, then the clinician is presumably treating a different metabolic pattern than initially existed. The observation that patients vary in their patterns of response suggests that chemotherapy should be individualized for each patient. The change in metabolic pattern suggests that the metabolism of the cell may in some instances, e.g., increase in thymidylate synthetase after Methotrexate, favor recovery from the effect of the drug on DNA synthesis. If this does occur and the inhibition of DNA synthesis is the basis of the oncolytic action of Methotrexate, then the properly timed administration of other drugs with different spectrums could supplement or prolong the action of Methotrexate. As an example, 5-bromodeoxyuridine might be given simultaneously with Methotrexate (10), whereas 5-fluoro-deoxyuridine, or even 5-fluorouracil, might be most effective as the cell starts to recover the capacity to incorporate deoxyuridine into DNA (11).

REFERENCES

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