The Effect of Treatment with Cytotoxic Agents on Mouse Spleen Dihydrofolate Reductase Activity

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SUMMARY

Daily treatment of mice with 0.8 mg methotrexate/kg/day for 14 days led to a transient splenic hyperplasia and increased spleen dihydrofolate reductase activity after cessation of treatment. These effects were prevented by the simultaneous administration of 5-formyltetrahydrofolate. A similar increase in spleen size and dihydrofolate reductase activity occurred after treatment with 5-fluorodeoxyuridine or Cytoxan. It was concluded that the increased enzyme levels were not related to the interaction of enzyme and antimetabolite, but to a temporary unbalance of cellular homeostatic mechanisms.

INTRODUCTION

A great deal of interest has been directed toward an understanding of the effect of 4-amino analogs of folic acid on cell metabolism, especially since it was discovered that they are potent inhibitors of dihydrofolate reductase (14). Two reviews have summarized the recent work concerned with this enzyme (2, 18).

The presence of relatively high levels of dihydrofolate reductase in the liver and kidney of the mouse, rat, and guinea pig has been attributed to the fact that these organs are major sites for the synthesis and storage of reduced folate compounds (5). It has also been observed that after administration of the folate acid antagonist methotrexate, increased levels of the enzyme were present in human leukocytes and erythrocytes (4), as well as in guinea-pig erythrocytes (5). An explanation which was proposed for this increased enzyme level was that there has been a transient increase in enzyme synthesis by the cells which permitted them to bypass the drug-induced metabolic blockade (2). Another explanation for the increased enzyme activity which was proposed more recently is that inhibitors of the enzyme have protected the enzyme from degradation in vitro (3). The role of the tetrahydrofolate coenzymes in nucleic acid biosynthesis has been well documented and was recently reviewed (7). It is not surprising, therefore, that significant levels of dihydrofolate reductase have been found in rapidly dividing tissue where considerable DNA synthesis is taking place. Duodenal mucosa and some human and murine leukemic cells have relatively high levels of dihydrofolate reductase (2, 8, 18).

Earlier work in this laboratory has shown that prolonged administration of 0.7 mg methotrexate/kg/day to normal mice resulted in considerable splenic hyperplasia four days after cessation of treatment (17). Accompanying this hyperplasia was a marked increase in purine nucleotide biosynthesis de novo above the pretreatment level, as measured by the incorporation of formate-14C into the acid-soluble adenine of spleen. The present investigation was undertaken in order to determine whether the splenomegaly and increased purine biosynthesis, which followed administration of methotrexate in mice, was also accompanied by an increase level of dihydrofolate reductase and to find out whether the response was specific for folic acid antagonists or a more generalized reaction to suppression of cellular activity by cytotoxic agents. Therefore, FUDR4 and Cytoxan were also examined for their ability to produce splenomegaly and elevated dihydrofolate reductase levels after extended treatment in mice. Neither of these agents has a direct effect on dihydrofolate reductase per se although FUDR, by inhibiting thymidylate synthetase, interferes with the formation of thymidylate acid. This step is also blocked by methotrexate, which prevents the regeneration of tetrahydrofolate from the dihydrofolate formed during the reaction.

MATERIALS AND METHODS

Hybrid male CDBA mice [(BALB/cAn X DBA/2J)F1], 10–12 weeks old and weighing 24–28 gm, were used in all experiments. They were maintained on Purina laboratory chow pellets and given water ad libitum.

Methotrexate and calcium 5-formyltetrahydrofolate (leucovorin) were obtained from the Lederle Laboratories Division of American Cyanamid Company. Cytoxan and 5-fluorodeoxyuridine were obtained through the Cancer Chemotherapy National Service Center, National Cancer Institute. Folic acid, thymidine, and adenosine were obtained from Calbiochem. Methotrexate and folic acid were dissolved in 2% sodium bicarbonate; Cytoxan, FUDR, and adenosine in physiologic saline; and 5-formyl tetrahydrofolate and thymidine in distilled water. The drugs were administered s.c. in the scapular region in a constant volume of 0.01 ml per gm of body weight.

The following abbreviations are used: FUDR, 5-fluorodeoxy uridine; EDTA, disodium ethylenediaminetetraacetate; NADPH, reduced nicotinamide adenine dinucleotide phosphate.
Animal Experiments

In the studies with methotrexate, a group of 100 mice was injected with 0.8 mg methotrexate/kg/day for a total of 14 days. A parallel group of control animals received the vehicle only. Groups of 10 mice were killed by cervical dislocation on each of the 7 days after the last dose of the drug. The spleens were removed, weighed quickly, and frozen at -70°C. The pooled spleens of 8 mice were used for the enzyme studies, whereas one or two spleens were selected randomly and fixed in formalin for histologic examination. Total leukocyte counts were performed on free-flowing blood from a tail incision with a white-cell-diluting pipet. Similar studies were conducted with 40 mg FUDR/kg/day and 20 mg Cytoxan/kg/day. FUDR was administered for 10 days and Cytoxan for 14 days.

Further experiments were undertaken in an attempt to reverse the phenomena seen after treatment with methotrexate. Metabolites known to reverse the growth-inhibitory effects of folic acid antagonists under various circumstances were administered simultaneously with methotrexate for 14 days. A group of 10 animals was treated with 0.8 mg methotrexate/kg for 14 days. In similar groups, the same dose of methotrexate was combined with 20 mg folic acid/kg, 20 mg 5-formyltetrahydrofolate/kg, 20 mg adenosine/kg, or 25 mg thymidine/kg. Groups of 10 mice each were treated with the metabolites alone, and 10 mice were used in a saline control group. The spleens were removed on the 4th day following the last treatment.

Preparation of Enzyme Extracts and Assay of Dihydrofolate Reductase

The frozen spleens were homogenized in about 50 ml of chilled acetone (-40°C to -60°C) with a VirTis "45" homogenizer at maximum speed for about 1 min. The acetone-dried powders were collected on Whatman s 54 filter paper by suction, washed with acetone (-20°C), and air-dried rapidly. They were stored in a freezer at -20°C. Enzyme extracts were prepared from the powders with 10-20 volumes of 0.05 M potassium phosphate buffer (pH 7.4) containing 0.01 M mercaptoethanol and 0.001 M EDTA at 3°C and centrifuged at 35,000 X g before the clear supernatant solution was assayed. Dihydrofolic acid was prepared by the method of Futterman (9) and stored frozen in 0.001 N hydrochloric acid containing 0.01 M mercaptoethanol (8, 16).

Dihydrofolate reductase activity was determined by a modification (13, 16) of the method of Osborn and Huennekens (15). The reaction mixture contained 0.1 to 0.5 ml enzyme extract, 0.24 μmole NADPH, 0.08 μmole dihydrofolate, and 0.05 M potassium phosphate buffer (pH 7.4) with 0.01 M mercaptoethanol and 0.001 M EDTA (total volume, 3 ml). Absorbance readings at 340 nm were made at 1-min intervals for 10-20 min at 28°C in a Zeiss PMQ-2 spectrophotometer against a reference cuvet from which dihydrofolate was omitted. Protein was determined by the method of Lowry et al. (12). Specific enzyme activity (μmole dihydrofolate reduced per hr/mg protein) was based on the combined decrease in absorbance at 340 nm for NADPH (Δε = 6200) and dihydrofolate (Δε = 5800).
CHART 2. Effect of extended treatment with cytotoxic agents on the posttreatment spleen weight and spleen dihydrofolate reductase activity of normal mice. Mice treated with 40 mg 5-fluorodeoxyuridine/kg/day for 10 days.

CHART 3. Effect of extended treatment with cytotoxic agents on the posttreatment spleen weight and spleen dihydrofolate reductase activity of normal mice. Mice treated with 20 mg Cytoxan/kg/day for 14 days.
RESULTS AND DISCUSSION

The results shown in Chart 1 indicate that there was approximately a 2.5-fold increase in spleen size 4 days after the last dose of a 14-day treatment course of methotrexate at a dose level of 0.8 mg/kg. It is also apparent that the splenic hypertrophy was paralleled by a 5- to 6-fold increase in dihydrofolate reductase activity as compared to the untreated control level. These responses are comparable in magnitude to the increase in purine biosynthesis that was observed previously under similar experimental conditions (17). It can be seen that by the 8th day after cessation of treatment, the spleen size and the enzyme level had returned to normal values. Histologic examination of the spleen at the time of maximum enlargement showed that there was a marked increase in the amount of both the red and white pulps as well as increased numbers of megakaryocytes. However, this response was not associated with a change in the peripheral leukocyte count, which did not differ from that of control values (10-16 × 10^6 cells/cu mm) even at the time of maximum response in the spleen. Thus, it would appear that the increase in spleen size can be accounted for by a transient hyperplasia of hematopoietic precursor cells. The parallel changes in dihydrofolate reductase activity and the previously recorded increase in purine biosynthesis de novo strongly suggests that a close metabolic relationship exists between these two biochemical phenomena. Charts 2 and 3 show that changes similar to those resulting from administration of methotrexate take place after prolonged treatment with FUDR or Cytoxan. Maximal response for FUDR was on the 10th day, and for Cytoxan about the 11th day after treatment was stopped. The longer period required for maximal response in these cases may be related to the fact that FUDR and Cytoxan produced a greater degree of toxicity in the mice than did methotrexate at the respective dose levels used. The mice treated with FUDR and with Cytoxan suffered some body weight loss whereas the animals treated with methotrexate did not. The weight lost during treatment with FUDR and Cytoxan was regained after treatment was discontinued and had returned to at least the pretreatment weight at the time of maximum splenomegaly and increase in dihydrofolate reductase. It was apparent from the results in Charts 2 and 3 that the increased enzyme level was not a specific response to 4-amino analogs of folic acid. A pyrimidine antimetabolite and an alkylating agent both elicited a similar response in terms of splenic hypertrophy and increased dihydrofolate reductase activity. Although there is no information on the effect of treatment with these agents on purine nucleotide biosynthesis, it would not be unreasonable to expect that there was a concomitant increase.

In Table 1, the effect of various metabolites on the splenic hypertrophy and the increased dihydrofolate reductase activity resulting from treatment with methotrexate is shown. Of all the metabolites used, only 5-formyltetrahydrofolate was able to counteract the increase in spleen size and enzyme activity. It has been previously reported that hypoxanthine and thymidine are able to render cultured mammalian cells insensitive to methotrexate (10). At the doses employed, adenosine and thymidine had no effect on the splenic or enzyme response to methotrexate. Folic acid was likewise inactive. Previous studies have shown that 5-formyltetrahydrofolate does not prevent the inhibition of dihydrofolate reductase by methotrexate (13). However, it can serve as a source of tetrahydrofolate derivatives and thus circumvent the metabolic block introduced by methotrexate. Therefore, since the splenic hypertrophy and increased enzyme activity produced by methotrexate were diminished by 5-formyltetrahydrofolate, it would appear that they were caused by the inhibitory effect of methotrexate on cellular activity rather than by its specific interaction with dihydrofolate reductase.

An attempt was made to take advantage of the increased enzyme activity in the spleen in order to determine whether the elevated enzyme level might be able to afford the animals some degree of protection against a lethal dose of methotrexate. Accordingly, mice were treated for 14 days with 0.8 mg methotrexate/kg/day and then, 4 days after treatment was stopped, they were injected with a single dose of the drug ranging from 100 to 800 mg/kg (at 100 mg/kg intervals). Control animals were injected similarly, but without pretreatment. All the animals that died did so on the 4th and 5th day following the single high dose. There was not a significant difference in mortality between the pretreated and control groups at any of the dose levels. The 50% lethal dose in both cases was between 300 and 400 mg/kg. It was concluded that the splenic hyperplasia and elevated enzyme levels had not protected the mice from the lethal effects of methotrexate.

The heterotopic development of hematopoietic tissue has been produced by repeated bleeding and also by s.c. injections of phenylhydrazine (1). Certain spontaneous and transplantable tumors in mice are also capable of inducing extramedullary hematopoietic changes in liver and spleen (11). It remains to be determined whether the underlying mechanism for the transient hyperplasia described in this report is similar to the above responses. The present findings do establish that the splenic response of mice in terms of cellular proliferation and the associated increase in dihydrofolate reductase levels is not a specific response to inhibitors of the enzyme but rather reflects the biologic state of hyperplasia and its increased requirement for folate.

<table>
<thead>
<tr>
<th>Drug administered s.c. daily for 14 days</th>
<th>Spleen weight and specific activity of dihydrofolate reductase 4 days after cessation of treatment</th>
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<tbody>
<tr>
<td>Untreated controls and metabolites alone</td>
<td>Untreated controls and metabolites alone</td>
</tr>
<tr>
<td>MTX⁴</td>
<td>MTX+ folate acid⁵</td>
</tr>
<tr>
<td>MTX⁴ + thymidine⁶</td>
<td>MTX⁴ + adenosine⁷</td>
</tr>
<tr>
<td>MTX⁴ + 5-formyltetrahydrofolate</td>
<td>136</td>
</tr>
</tbody>
</table>

* Average weight of pooled spleens from 10 mice in each group.  
× Specific activity of pooled spleens from 10 mice in each group.  
† Folic acid, 20 mg/kg; thymidine, 25 mg/kg; adenosine, 20 mg/kg; and 5-formyltetrahydrofolate, 20 mg/kg, were each administered alone or simultaneously with methotrexate (MTX).  
‡ 0.8 mg/kg.
coenzymes. It would be of interest to know whether the increased leukocyte dihydrofolate reductase activity which has been observed in some clinical situations in response to treatment with methotrexate (2,4) would occur after treatment with compounds other than the 4-amino analogs of folic acid.

The rebound phenomena that followed when treatment with cytotoxic agents was stopped may have their basis in the homeostatic mechanisms which control cellular proliferative activity in normal spleen. After imposing an external restraint on this cellular activity, the homeostasis will presumably be adjusted in an attempt to restore normalcy. With the abrupt removal of the external agent, it is quite conceivable that there could be an overreaction in terms of cellular proliferation before the reestablishment of normal controls. This rebound effect does not appear to result in any serious physiologic consequences in normal mice. The possibility exists, however, that in leukemic mice any therapeutic benefit which might accrue from intermittent treatment with cytotoxic agents might be nullified to some extent by the rebound phenomenon promoting an exacerbation of the disease during the interval between treatments.

REFERENCES
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