The Use of Dihydrofolate Reductase as a Signal for Scheduling Optimal Treatment with Antileukemic Agents

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SUMMARY

High levels of dihydrofolate reductase present in an antifolate-resistant leukemia L1210 in mice can be used to assess tissue levels of leukemic cells by measurement of reductase activity. The initial rise in enzyme activity has been used as a signal for initiation of drug treatment, making it possible to administer therapy at an appropriate time late in the course of the disease rather than according to a predetermined schedule.

INTRODUCTION

A biochemical assessment of leukemic cell populations in mice would be a desirable tool for optimizing treatment schedules for selected antitumor agents. An assay of this type would enable one to administer a drug according to the level of disease, rather than following a predetermined schedule, to obtain a maximum chemotherapeutic effect. This would provide a model analogous to the drop in urinary hormone level used by Li et al. (6) as a signal to determine the scheduling of methotrexate in the treatment of choriocarcinoma in women. In this disease, the amount of tumor present was assessed by the output of chorionic gonadotropin hormone in the urine. After successful drug therapy, the quantity of hormone dropped precipitously and reached normal levels; however, tumor recurrence was reflected by a subsequent rise in the amount of hormone.

It has been previously established by Friedkin et al. (2) that greatly increased levels of dihydrofolate reductase activity are present in some antifolate-resistant sublines of mouse leukemia L1210. This finding led to the use of the dihydrofolate reductase level as a biochemical marker. Thus, several workers (4, 5, 10–12) reported that administration of antitumor agents to mice inoculated with the antifolate-resistant tumor caused a reduction in enzyme activity, concomitant with an increase in survival time. Humphreys et al. (5) used this model system to investigate the ability of cyclophosphamide to cross the "blood-brain barrier" and reduce the brain leukemic cell population following intracranial inoculation of the tumor. Narurkar et al. (11) extended this work to include BCNU, 6-mercaptopurine (NSC 755), and other drugs.

The present investigation was undertaken to test the feasibility of using the rise in dihydrofolate reductase activity as a marker for signaling the point of administration for antileukemic agents and for determination of optimum treatment schedules. With this approach, it has been possible to experiment with the use of drug therapy late in the course of the disease and to schedule subsequent treatment when a second rise in the enzyme activity indicates the necessity for it. Because of the nature of the scheduling, drugs known to be effective antileukemic agents following a single dose have been selected for testing. These are cyclophosphamide (NSC 26271), BCNU, BIC, and sangivamycin (NSC 65346).

MATERIALS AND METHODS

Drugs were obtained from Drug Research and Development, National Cancer Institute, Bethesda, Md. Folic acid, NADPH, EDTA, and Cleland's reagent were obtained from Calbiochem, Los Angeles, Calif. Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N. Y. Dihydrofolic acid was prepared by the method of Futterman (3) and stored frozen in 0.001 N HCl containing 0.01 M mercaptoethanol (2, 14).

CDF or BDF male mice, 8 to 10 weeks old and weighing 20 to 25 g, were maintained on Purina chow and water ad libitum. The antifolate-resistant subline of leukemia, L1210/FR-8 (2, 10), was maintained in donor mice of strain CDF. The tumor was maintained in its highly resistant state by continuous exposure to daily injections of 80 mg of dichloromethotrexate per kg. Approximately 5 X 10⁶ splenic cells from moribund donor mice were inoculated s.c. in the right inguinal region. Drugs were administered i.p. in a constant volume of 0.01 ml/g of body weight.

Spleens for dihydrofolate reductase assay were obtained immediately after sacrificing the mice. The tissues were fast-frozen by being placed on Dry Ice and were then weighed. Full enzymatic activity was retained when organs were stored frozen at −10⁶. Enzyme preparations were obtained either by preparing an acetone-dried powder (10) or by homogenizing the tissues in 4 or 9 volumes of cold 0.25 M sucrose solution containing 0.01 M mercaptoethanol and 0.001 M EDTA (8). The acetone-powder method required at least 500 mg of tissue for each preparation, necessitating the pooling of several spleens, except when splenomegaly was well advanced. The sucrose method, however, allowed assays on single spleens. When the former method was used, 2 to 5 preparations were made, and a mean value was obtained. In experiments with the sucrose method, results represent a mean value for a minimum of 4 separate enzyme determinations.
Dihydrofolate reductase activity was determined by a modification (1, 9, 14) of the method of Osborn and Huennekens (13). In a total volume of 3.0 ml, the reaction mixture contained 0.24 μmole of dihydrofolate, 0.1 to 0.5 ml of enzyme extract, and 0.05 M potassium phosphate buffer (pH 7.4) with 0.01 M EDTA. Absorbance readings at 340 nm were made at 1-min intervals for 10 min at 28° 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. (7). Specific enzyme activity (μmoles of dihydrofolate reduced per hr/mg of protein) was based on the combined decrease in absorbance at 340 nm for NADPH ($\Delta_{6200}$ = 6200) and dihydrofolate ($\Delta_{5800}$ = 5800).

Animal Experiment. After inoculation of the tumor, mice were sacrificed at frequent intervals, and spleens were removed, weighed, and assayed for dihydrofolate reductase. Starting with Day 7 after tumor inoculation, spleens were assayed daily, until a 2- to 3-fold rise in specific enzyme activity signaled a progressive increase in tumor infiltration; the drug under study was administered at this time. This occurred on Days 8 to 10 after leukemic inoculation. A single dose of the drug, approximately equal to the 10% lethal dose, was administered to one group of animals, while another group was retained for enzymatic assay as untreated controls. Treated animals, selected at random, were sacrificed and assayed at frequent intervals. In experiments in which a 2nd dose of the drug was used, the same criterion was used to determine the time of administration, i.e., a 2- to 3-fold rise in the splenic reductase level. Again, a 2nd group, which received only 1 dose of drug, was retained for reductase assay. In addition, separate groups of mice (10 animals/group) were maintained for each of the conditions described, to obtain survival time data. In the last experiment to be described, sangivamycin at 2 dose levels was administered on Day 1 only, rather than according to the preceding protocol, since sangivamycin was shown to be ineffective either in reducing activity or in prolonging the life-span of leukemic mice when administered late in the course of the disease.

RESULTS AND DISCUSSION

The data shown in Chart 1 indicate the 2- to 3-fold rise in dihydrofolate reductase activity and the administration of 200 mg/kg of cyclophosphamide on Day 8. The MST's for the untreated and treated groups were 12 and 25 days, respectively, indicating an increase in life-span of over 100% after drug therapy was initiated on Day 8 of the disease. Furthermore, in the treated group, the increase in life-span was concomitant with a drop in reductase activity, and death occurred only after the leukemic cell population had again risen as indicated by a 2nd rise in the enzyme level. Attempts to administer a 2nd dose of cyclophosphamide at the time of the 2nd rise in enzymatic activity have so far been unsuccessful, as the animals do not survive the 2nd dose. However, in the period of survival there was no recurrence of the disease as measured by dihydrofolate reductase activity.

Chart 2 shows the effect of treatment with 20 mg/kg of BCNU on Day 8 only and on Days 8 and 21. The MST's for the untreated mice and for the 2 treated groups were 8, 21, and 51 days, respectively. Again, the signal for both treatments was the initial rise in enzyme activity, and the effectiveness of the drug is evident from the return of reductase levels to normal splenic values. After the 2nd treatment there was no further rise in enzyme levels. Also, among the mice receiving a 2nd dose of drug, 30% of the
long-term survivors (more than 100 days) showed no evidence of tumor regrowth.

Chart 3 presents the data from an experiment in which 400 mg/kg of BIC was administered on Day 10 after tumor implantation. The MST's for the treated and untreated groups were 12 and 20 days. In this experiment, there was a lag in the response of the treated group; the enzyme activity continued to rise for 1 day and then fell slowly over several days to levels somewhat above normal. After a single treatment with BIC, there was no systemic tumor regrowth in animals that survived (40% long-term survivors), although reductase activity remained slightly elevated above normal. The evidence suggests that delayed toxicity, rather than systemic overgrowth of leukemic cells, may have been the cause of death in this instance. However, the possibility remains that sequestered foci of cells, in the central nervous system for example, might have contributed to the deaths of some animals.

Chart 4 shows the effect of sangivamycin, 6 mg/kg, administered on Day 9. In this study, there was no difference in the MST's of the 2 groups of mice; it can be seen that the rates of tumor growth, as indicated by reductase activity, were identical. These data indicate that sangivamycin, unlike the other 3 drugs studied, is not effective in a single dose against late leukemia L1210/FR-8, whereas it does have antileukemic activity in a single dose against early L1210 (Cancer Chemotherapy National Service Center Screening Data). The possibility remained that its therapeutic effect was the result of a drug-induced lag in the initial growth of the tumor. This hypothesis is supported by the data in Chart 5. Sangivamycin, given in a single dose of 4 or 8 mg/kg injected on Day 1, caused a delay in the initial rise in reductase activity in the treated groups of animals compared with the controls; after the initial drug-induced lag of enzyme activity, the rate of tumor growth was the same for all groups.

The present studies indicate that the level of dihydrofolate reductase activity in spleens of mice inoculated with leukemia L1210/FR-8 can be used as an index of the amount of disease present, and, therefore, a rise in enzyme activity can serve as a signal for initiating treatment with chemotherapeutic agents. This approach to chemotherapy could serve as a model for scheduling treatment according to the level of the disease, rather than relying on an arbitrary predetermined schedule.
This would be especially useful in assessing the value of drugs that might be effective in the later stages of disease. After therapy, the enzyme activity serves as a direct index of the extent of leukemic cell kill. Thus, it can be seen that the first 3 agents tested produced an appreciable cell kill when administered late in the course of disease.

One is also able to gain insights into the course of events when the drug is not effective under our standard conditions of administration. Thus, it is possible to state that sangivamycin administered on Day 9 was ineffective because it did not produce a drop in leukemic cell population and not for any other of several reasons, for example, toxicity. This conclusion, in conjunction with the observation drawn from Chart 5 that sangivamycin is able to depress the rise of a cell population only when the original population is small, suggests that it might be worthwhile to consider the use of a drug such as sangivamycin for remission maintenance rather than remission induction.

In addition, the use of dihydrofolate reductase as a parameter for optimal scheduling of drug treatment makes it possible to gain considerable insight into the cause of death, whether from the disease (in which case one might see a subsequent rise in enzyme level) or from drug toxicity (in which case animals eventually die with no increase in splenic enzyme activity). For these reasons, the use of dihydrofolate reductase as a biological marker promises to be useful both as adjunct to the study of drugs already in use and for gaining useful insights into the properties of experimental antileukemic drugs.

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