Interaction of Chemotherapeutic Agents with Methotrexate and 5-Fluorouracil and Its Effect on de Novo DNA Synthesis

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SUMMARY

The transport of methotrexate is known to be affected by corticosteroids and vincristine in L1210 leukemia cells. The deoxyuridine suppression test was used to measure the metabolic consequences of using these drugs with the antimetabolites, methotrexate and 5-fluorouracil, in both L1210 leukemia cells and normal human marrow cells. The deoxyuridine suppression test can be utilized as a sensitive measure of methotrexate and 5-fluorouracil biological activity in producing defective de novo DNA synthesis. The deoxyuridine suppression test was found to detect changes in biological activity equal to 20 ng (0.044 nmole) of methotrexate and 200 ng (1.94 nmole) of 5-fluorouracil. Hydrocortisone and prednisone, but not dexamethasone or prednisolone, decreased the methotrexate effect to one-half in both L1210 and human cells as measured by the deoxyuridine suppression test. 5-Fluorouracil biological activity was not affected by any steroid studied. Vincristine produced variable results, but on the average it decreased the methotrexate effect in human marrow. Vincristine consistently decreased the methotrexate effect in L1210 systems. Cephalosporin, 75 μg/ml (0.214 μmole), had no effect. In parallel studies, hydrocortisone decreased the uptake of methotrexate, but not folic acid, in human and L1210 cells. The deoxyuridine suppression test warrants further investigation as a method of screening drugs for interaction with antagonists of de novo DNA synthesis. This study extends earlier evidence of drug interaction with methotrexate in a murine system to human cells and demonstrates that there is a metabolic consequence, reduced potency of methotrexate, as a result of reduced transport produced by certain corticosteroids.

INTRODUCTION

Drug interactions have been recognized as an important factor affecting the therapeutic response of a variety of neoplastic diseases to single agent and combination chemotherapy. Bruckner et al. (2, 24) have described a variety of antibiotics that affect chemotherapy indirectly through their effect on the bowel flora. Recent work has suggested that drug interaction with MTX² may affect its antitumor activity. Capizzi and Nahas (4, 17) reported the antagonism of the antineoplastic effect of MTX by L-asparaginase both in vitro and in vivo. These reactions are highly schedule dependent, and with sophisticated scheduling asparaginase can also be used to improve the therapeutic index of MTX (5). Fyfe and Goldman (9) and Lieberman et al. (16) have made the general observation that drugs that inhibit protein synthesis can change the therapeutic effect of strong (S-phase-specific) DNA inhibitors. Most recently a 3rd mechanism of drug interaction with MTX has been identified when corticosteroids, antibiotics, and L-asparaginase were shown to be inhibitors of MTX transport in the L1210 leukemia system (9, 10, 23). This competitive drug inhibition of MTX transport by corticosteroids was found to be dose related.

The cause of the drug-induced transport inhibition and antagonism of MTX antineoplastic activity in the L1210 system has yet to be fully explained. Moreover, the metabolic consequence of the drug-induced defective MTX transport has not been established.

This report describes the effects of various drugs used during the treatment of neoplastic diseases on the antimetabolic effect produced by MTX on de novo DNA synthesis. A sensitive, reproducible, short-term in vitro assay of MTX effect on dTMP synthesis in L1210 and human bone marrow cells is used for this purpose. The effect of corticosteroids on FU and MTX are compared in the same system because FU is another antimetabolite effecting de novo DNA synthesis. Finally, the effect of steroids on the net influx rate of PGA and MTX are compared because competitive inhibitors may affect close structural analogs. Some of these studies were reported previously in abstract form (3).

MATERIALS AND METHODS

L1210 leukemia cells were harvested into HBSS from the ascitic aspirates of DBA/2J mice 7 days postinoculation with 1 x 10⁶ cells i.p. The cells were washed 3 times to remove the heparin and ascitic fluids and resuspended in

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¹ Supported by NIH Grants CA-14491 and AM-16690, the Chemotherapy Foundation of New York, the United Leukemia Fund, and the Gar Reichman Memorial Fund.

² The abbreviations used are: MTX, methotrexate; FU, 5-fluorouracil; PGA, folic acid (pteroylglutamic acid); HBSS, Hanks’ balanced salt solution; FCS, fetal calf serum; UdR, deoxyuridine; TdR, thymidine; VCR, vincristine.
HBSS containing 33% dialyzed FCS to a concentration of 55 to 65 x 10^6 cells/ml. Friend leukemia cells (745 A) were grown in suspension culture in Roswell Park Memorial Institute Medium 1640 containing 10% FCS, harvested the day of each experiment, washed, and resuspended as for the L1210 studies.

The antimetabolic effect of MTX and FU on de novo DNA synthesis was measured by the UdR suppression test as previously described (21, 22). In this system, abnormal de novo DNA synthesis produced by MTX or FU is demonstrable by the reduced ability of UdR to suppress incorporation of subsequently added [3H]TdR into DNA. For culture, 0.2 ml cells (11 to 13 x 10^6) were pipetted into siliconized glass tubes, and the various radioactive and nonradioactive components were added in 0.1-ml aliquots as indicated by the individual experiment. All reactions were performed in triplicate and the final volumes were 1.0 ml HBSS. Incubation times were 60 min at 23° after the addition of UdR, and 3 hr at 37° after the addition of 0.1 μCi [3H]TdR (specific activity, 20 Ci/mM). At the end of the incubation time the cells were washed twice with cold 0.9% NaCl solution. Two ml of 10% trichloroacetic acid were added and the resultant precipitate was washed once with trichloroacetic acid. The acid-precipitable material was dissolved in 1.0 ml NCS reagent (Amersham/Searle Corp., Arlington Heights, Ill.) and counted in 15 ml of a scintillation mixture (toluene containing 30% ethanol, 0.6% PPO, and 0.03% dimethyl POPPOP in a Beckman LS250 liquid scintillation system to a counting error of 2% or less. Reactions were performed in triplicate and the reproducibility is within 2%.

RESULTS

The effect of preincubation with various concentrations of UdR on [3H]TdR incorporation into DNA in L1210 leukemia cells is shown in Chart 1. The UdR enters the dUMP—dTMP—DNA thymine pathway, so that the incorporation into DNA of the labeled (tritiated) dTMP derived from the subsequently added [3H]TdR is diminished. The degree of blockaded [3H]TdR uptake is thus a measure of the amount of dUMP incorporated into DNA. The [3H]TdR uptake decreased as the concentration of UdR was increased. This suppression of [3H]TdR into DNA could be detected by changes in the concentration of UdR of as little as 0.2 μg. The curve represents a composite of triplicate experiments and the reproducibility is within 2%.

The conversion of UdR to DNA thymine requires the methylation of dUMP to dTMP. This step requires the availability of N-5,10-methylene tetrahydrofolate acid and dTMP synthetase. Normal UdR suppression of [3H]TdR will be inhibited by the addition of MTX (a dihydrofolate reductase inhibitor) and/or FU (an inhibitor of dTMP synthetase). Increasing amounts of MTX added to the appropriate experiment at room temperature for 15 min prior to the addition of UdR.

[3H]MTX (12.3 Ci/mM) and [3H]PGA (52 Ci/mM) were purchased from Amersham/Searle, Arlington Heights, and diluted to a concentration of 5 ng/ml in HBSS. For measurement of MTX and PGA uptakes, L1210 cells were incubated for 15 min at 23° with hydrocortisone or HBSS. Incubation volumes were brought to 0.9 ml with HBSS and placed at 37°. The reactions were started with the addition of 0.1 ml (0.5 ng) isotope and stopped at intervals ranging from 0 to 180 min by the addition of cold 0.9% NaCl solution. Cells were centrifuged and washed 2 times with cold 0.9% NaCl solution, and the pellet was dissolved in 1.0 ml NCS reagent and counted. All experiments were run in triplicate.

Dihydrofolate reductase levels in L1210 cells were measured by the reduction of [3H]PGA to [3H]tetrahydrofolate utilizing the method of Rothenberg (18).

These studies were performed according to the guidelines of the Declaration of Helsinki and informed consent was obtained for the collection of bone marrow specimens.
in vitro culture of either L1210 or human bone marrow inhibited the UdR suppression of [3H]Tdr into DNA giving detectable changes in biological activity equal to that of 20 ng of MTX (Chart 2). Similarly, increasing amounts of FU added to the L1210 culture inhibited UdR suppression of [3H]Tdr into DNA. Changes in biological activity equal to that of 200 ng FU were detected by this method (Chart 3).

The effect of various steroids on the biological activity of MTX was studied by the UdR suppression system (Chart 4: Table 1). Hydrocortisone (5 to 20 μg) significantly decreased the MTX antimetabolic effect on DNA synthesis at all levels of MTX (10 to 80 ng) studied in L1210 leukemia cells (Chart 4). Hydrocortisone prevented MTX effect even when 80 ng MTX were added to the L1210 culture which, in the absence of hydrocortisone, has a striking effect on de novo DNA synthesis. The addition of dexamethasone (5 to 20 μg) or prednisolone (8 to 64 μg) did not significantly inhibit, whereas prednisone (5 to 20 μg) inhibited the antimetabolic effect of MTX as measured by the UdR suppression test. Similar corticosteroid effects on MTX effect were observed in Friend leukemia cells (Table 2) and human bone marrow (Table 3). Depo-Provera and testosterone did not significantly alter the MTX effect on UdR suppression (Table 1). Unlike their effect on MTX, the corticosteroids at several concentrations did not affect the biological activity of FU (6 μg/ml) as measured by the UdR suppression test in L1210 or human bone marrow cells (Table 4). None of the corticosteroids added alone to either L1210 or human marrow significantly affected the incorporation of [3H]Tdr or the UdR suppression of [3H]Tdr into DNA. VCR consistently slightly decreased the MTX effect in the L1210 system (Table 1). VCR (0.8 to 8 μg/ml) produced variable results, but the average was to decrease the MTX effect in human marrow (Table 3). Cephalosporin (75 μg/ml) had no effect on UdR suppression by MTX.

In parallel studies, hydrocortisone (20 μg/ml) decreased the uptake of [3H]MTX into L1210 cells. This inhibition of

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug concentration (μg/ml)</th>
<th>% Δ MTX inhibition of UdR suppression of [3H]Tdr into DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>5</td>
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<tr>
<td></td>
<td>10</td>
<td>-33</td>
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<td>Aqueous testosterone</td>
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<td>+2</td>
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<td>VCR</td>
<td>8</td>
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</table>

*a Average of 3 experiments done in triplicate.

*b Control UdR (0.1 μmole/ml) + MTX (1.2 x 10^-7 M).
MTX uptake was initially evident at 45 min following incubation and continued to be present for a 3-hr period. Hydrocortisone did not appear to cause cell death as measured by the trypan blue dye exclusion test. The addition of dexamethasone (20 μg/ml) did not significantly inhibit the uptake of [3H]MTX into the L1210 cell culture at any time over a 3-hr period (Chart 5). The uptake of [3H]PGA was similarly studied in the L1210 leukemia system (Chart 6). In contrast to that of MTX, the addition of either hydrocortisone or dexamethasone did not significantly inhibit the uptake of [3H]PGA at any time during a 3-hr incubation period in this system.

The effect of various corticosteroids on the dihydrofolate reductase activity in the L1210 cell was studied (Table 5). A 3-hr incubation with hydrocortisone, prednisone, prednisolone, dexamethasone, or ethanol did not significantly change the measurable dihydrofolate reductase activity in the L1210 leukemia cell system.

**DISCUSSION**

Zager et al. (23) have shown that hydrocortisone decreases the normal rate of MTX influx into the L1210 leukemia cell by competitive inhibition in vitro. Their in vivo experiments show that hydrocortisone decreases the antitumor effect of MTX. However, more than drug-drug transport inhibition is thought to be responsible for the decreased antitumor effect of MTX because the inhibition may have been dependent on the schedule of treatment with hydrocortisone. These studies have not examined the effect of hydrocortisone-MTX interaction on the metabolism of the L1210 cell (23).

This report describes the in vitro metabolic effect of drugs on the capacity of MTX to inhibit the suppression of [3H]TdR incorporation into DNA thymine by UdR. Hydrocortisone and prednisone decrease the potency of MTX at the biochemical level as determined by this assay. Other
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corticosteroids produce no effect or a qualitatively different effect. Thus, the choice of dosage and steroid may sometimes determine the potency of MTX. The only clinical trial comparing the steroids was designed to evaluate their effect on the incidence of infection in the therapy of acute lymphocytic leukemia and involved a complex drug combination rather than MTX alone.

Hydrocortisone and prednisone produce quantitatively comparable dose-dependent inhibition of MTX in the UdR suppression test in L1210, Friend leukemia cells, and human bone marrow. Dose dependence would be expected if competitive inhibition is, as postulated, the mechanism of drug-drug interaction. Dexamethasone does not inhibit the transport or metabolic effect of MTX in L1210 cells throughout a range of in vitro drug concentrations that exceed conventional clinical therapeutic requirements. Surprisingly, prednisolone had no inhibitory effect despite its close structural relationship to prednisone. The dose-response experiments demonstrate that there is no inhibitory effect when the molar concentration of prednisolone is 3-fold greater than the concentration of prednisone which produces 50% inhibition. This suggests that an all or none difference between prednisolone and prednisone exists.

Several lines of experimental evidence support the efficacy of the UdR suppression test as a method for measuring the metabolic effect of MTX. Earlier experiments have shown that the UdR suppression test measures a number of predictable and independently confirmed defects in the de novo DNA pathway, most importantly those related to folate deficiency (14), B12 deficiency (7), abnormal amino acid metabolism (20), and well-studied metabolic antagonists such as MTX, FU, and pyrimethamine (22). There is also evidence that the UdR suppression test has some, but not absolute, value in predicting the therapeutic efficacy of MTX against specific lines of human leukemic cells (13, 20).

This study defines the sensitivity and reproducibility of the dose-response relationship with this assay for MTX (Charts 2 and 4) and FU (Chart 3). The concentrations at which the assay is most useful are in the same range as those that have clinical therapeutic significance in man.

The UdR suppression test measures what is believed to be the major metabolic effect of MTX on the dihydrofolate reductase pathway. The effect of MTX is completely corrected by the addition of reduced folates (22). Reduced folates, however, do not correct the effect of FU on the UdR suppression test (21). This is to be expected since FU does not directly block the folate reductase pathway or produce a deficiency of reduced folate.

This study also defines the suitability of the UdR suppression test for the measurement of the specific drug interactions under discussion because the steroids in the dosage used in these studies do not effect [3H]Tdr incorporation into DNA. UdR suppression of [3H]Tdr into DNA, or the effect of FU on the UdR suppression test. These new experiments provide the 1st evidence that the UdR suppression test is a sensitive and valuable research tool applicable to studies of drug-drug interaction.

The transport of MTX and natural folates have been compared by several investigators (1, 6, 8, 10). Transport characteristics are sometimes a function of the cell line used in the study (8). In the L1210 leukemia system, there are several analogies between the transport of MTX and natural folates that there is a physiologically important influx and efflux mechanism which is energy, temperature, and pH dependent (15). MTX apparently inhibits net folate influx as well as the intracellular metabolism of folates (12). It has been concluded that MTX and natural folates share, at least in part, the same transport mechanism (8, 15). The kinetics of competitive inhibition is not consistent with an entirely identical transport system (12).

The current experiments provide further indirect evidence that the mechanism for influx of MTX and natural folates may not be entirely identical. Steroids had no inhibitory effect on the net influx of PGA but do decrease influx of MTX. The cell does not appear to use the site at which hydrocortisone produces competitive inhibition of MTX influx as a measurable means of transporting PGA in our system.

These experiments avoid theoretical objections to the earlier experiments because they bypass the simultaneous use of MTX and folates. MTX apparently inhibits net folate influx but also inhibits the intracellular metabolism of folate, normal cellular energy processes, and metabolism (15). This may indirectly affect folate transport (9, 15).

Fyfe and Goldman (9), using Ehrlich ascites cells in vitro, found that VCR decreased the normal rate of MTX efflux. This result in a net increase in MTX within the cell. They hypothesize that VCR damages energy-dependent efflux in a manner analogous to those of other metabolic poisons, which decrease MTX efflux and produce a net increase in MTX within the cell (9). They also found that although VCR had no effect on DNA formation, VCR plus MTX produced greater inhibition of DNA than MTX alone (11). In vivo experiments by Zager found that VCR increased net influx of MTX into L1210 cells, but the increased antitumor effect in vivo produced by combining MTX and VCR appeared to be schedule dependent (23).

VCR did not enhance and usually slightly diminished the effect of MTX on de novo DNA synthesis as measured by the UdR suppression test in L1210 cells and human bone marrow. The lack of VCR enhancement of MTX effect in this system may be possibly be explained by the presence of glucose (1000 mg/liter) in the culture media used in this study. Fyfe and Goldman (9) have shown that glucose addition (5 mm) will reverse the VCR augmentation of MTX uptake. The lack of VCR enhancement of MTX antimitabolic effect in our physiological system suggests that VCR does not directly affect MTX metabolism in vivo and that the enhanced antitumor effect of the combination is the result of other mechanisms (19).

ACKNOWLEDGMENTS

The expert technical assistance of Eugene Gagliardi is gratefully acknowledged. The authors also wish to thank Dr. George Bekesi for maintaining the L1210 line used in these studies.
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