A Comparison of the Biological Effects of Dichloromethotrexate and Methotrexate on Human Leukemic Cells in Culture

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INTRODUCTION

The antitumor activity of MTX has now been well established in humans (16). The main mechanism of action of MTX is thought to be the inhibition of dihydrofolate reductase resulting in the inhibition of both pyrimidine and purine biosynthesis (4, 16). An increase in dihydrofolate reductase and decrease in the transport of MTX have been regarded to be the major mechanisms of resistance (5) and constitute a rationale for using MTX at a high dose. The high-dose MTX and LV rescue regimen. The differences in the effects of DCM and MTX on cell growth in a culture medium containing HA warrant a further modification of the in vitro drug-screening system.

ABSTRACT

The effects of human serum albumin (HA) on the cell growth inhibition produced by dichloromethotrexate (DCM) and methotrexate (MTX) and the reversibility by leucovorin (LV) of the cell growth inhibition induced by both agents were examined in vitro using a malignant human lymphocyte line, MOLT 3. The biological activity of DCM, which was slightly higher on an equimolar basis than that of MTX in HA-free culture medium, was reduced to a much lower level than that of MTX in the presence of HA (2.5 g/dl) in the culture medium. Thus, on a basis of the dose causing 90% inhibition, the equitoxic concentration of MTX increased only about 2-fold in the presence of HA, while that of DCM increased about 5-fold. Ultrafiltration and bioassay revealed that DCM and MTX were bound to HA about 85 and 50%, respectively. HA binding with these antifolics and their consequent loss of biological activity both appeared to be reversible. The present findings explain, at least in part, why a higher equitoxic dose of DCM than of MTX is required in humans. The cell growth inhibition induced by equitoxic concentrations of both agents was reversed by an equimolar concentration of LV. This finding suggests that the LV dose required to protect or rescue DCM-induced toxicity in humans may not need to be higher than that used in the high-dose MTX and LV rescue regimen. The differences in the effects of DCM and MTX on cell growth in a culture medium containing HA warrant a further modification of the in vitro drug-screening system.

MATERIALS AND METHODS

Tumor Cells. A human acute lymphocytic leukemia cell line, MOLT 3, with T-cell characteristics (25, 28) was maintained in our laboratory. The cells were maintained in suspension in culture flasks (Falcon Plastics, Oxnard, Calif.; No. 3024) containing RPMI Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 10% (v/v) heat-inactivated FCS (Grand Island Biological Co.) and antibiotics (penicillin [100 units/ml] and streptomycin, [100 μg/ml]; Pfizer Laboratories Division, New York, N. Y.) and fed with fresh medium 3 times per week. Cells in stock culture were counted daily and used when they were in an exponential growth phase.

Drugs. DCM (Lot UI-77-108) was obtained from the Na-
Determination of Effects of DCM and MTX on Cell Growth. The initial cell number in individual culture tubes (Falcon No. 3033) was adjusted to 1.5 x 10⁵/ml. One-tenth ml of each drug solution at different concentrations was added to 10 ml of the cell suspension in individual culture tubes on Day 0. Cells were not fed during the period of the experiment. Viable cells were determined by the trypan blue dye exclusion method, and dose-response curves were obtained by calculating the percentage of viable cells on Day 3 as compared to those in the control tubes containing no drug.

The effects of DCM and MTX on cells pretreated with HA were determined as follows. Cells (1.5 x 10⁵/ml) were grown in RPMI Medium 1640 containing 10% FCS and antibiotics with or without HA (2.5 g/dl; 3.78 x 10⁻⁴ M) at 37°C from Day −1 to Day 0. After 3 washings with fresh culture medium, the cells were reincubated in culture medium containing equimolar concentrations (5 x 10⁻⁸ M) of DCM or MTX. The percentage of viable cells was determined on Day 3.

To determine the effects of various concentrations of HA in the inhibition of the biological activities of DCM and MTX, HA concentrations were the same in the control tubes as in the experimental tubes.

Determination of Binding of DCM and MTX to HA. The binding of both drugs to HA was determined by the degree of inhibition of the cell growth produced by the ultrafiltrates. Equimolar concentrations (5 x 10⁻⁸ M) of DCM and MTX were incubated at 37°C from Day −1 to Day 0 in 10 ml of RPMI Medium 1640 containing HA (2.5 g/dl). On Day 0, 5 ml of each sample were transferred into Centriflo type 50A membrane cones (Amicon Corp., Lexington, Mass.) and centrifuged at 1000 x g for 30 min (8). The ultrafiltrate and the parent medium were sterilized by passage through a 0.45-μm Millipore filter membrane, and 0.1 ml of each material was added to each culture tube containing 10 ml of HA-free culture medium and cells. Drug solutions, which contained no HA, were processed similarly and used as controls. The percentage of viable cells on Day 3 was determined, and the biologically active molar concentration of each drug was calculated from the dose-response curve in HA-free culture medium. The percentage of drug binding to HA was determined by the following formula:

\[
\% \text{ binding} = \frac{X - Y}{X} \times 100
\]

where \( X \) is the biologically active molar concentration of ultrafiltrate obtained after incubation without HA, and \( Y \) is the biologically active molar concentration of ultrafiltrate obtained after incubation with HA.

Determination of Reversibility by LV. LV (0.1 ml) and drug solution (0.1 ml) were added to individual culture tubes, and the reversibility of cell growth inhibition was determined by calculating the percentage of viable cells on Day 3 as compared to the controls containing no LV.

To minimize effects in the condition of the cells, DCM and MTX were used simultaneously in each of the comparative experiments. All experiments were carried out in triplicate and repeated at least twice.

RESULTS

In Vitro Cell Growth. When cells at an initial concentration of 1 to 2 x 10⁵/ml were transferred on Day 0 to a culture tube containing 10 ml of RPMI Medium 1640 with 10% FCS and antibiotics and incubated at 37°C, the cells grew exponentially from Days 1 to 4, and then the growth rate declined. High concentrations of HA in the medium with 10% FCS produced inhibition of cell growth. Thus, cell growth was inhibited in culture medium containing HA (4.5 and 5 g/dl). In the presence of HA (2.5 g/dl) in the medium, however, cells grew exponentially with minimal inhibition of cell growth by Day 4 (Chart 1).

Inhibitory Effects of DCM and MTX on Cell Growth. The relationship between the concentration of the antifolics and inhibition of cell growth in RPMI Medium 1640 containing 10% FCS and antibiotics, with and without HA (2.5 g/dl) is illustrated in Chart 2. In the absence of HA, DCM had slightly better inhibitory effects than did MTX on an equimolar basis; the ID₅₀'s of DCM and MTX were 3 and 4 x 10⁻⁸ M, respectively. The presence of HA (2.5 g/dl) in the culture medium resulted in DCM and MTX ID₅₀'s of 1.5 x 10⁻⁷ and 8.4 x 10⁻⁸ M, respectively.
× 10⁻⁸ M, respectively. On an ID₅₀ basis, the equitoxic concentration of MTX increased 2-fold in the presence of HA, while the effects of HA on DCM were more pronounced, requiring a 5-fold increase in the DCM concentration to produce the same degree of inhibition.

Effects of DCM and MTX on Cells Pretreated with HA. Pretreatment of cells with HA (2.5 g/dl) did not influence the inhibitory effects of DCM and MTX (Table 1). These findings strongly suggested that drug-HA interaction was the basis of the effects shown in Chart 2 rather than the production of resistance to the cells.

Influence of HA Concentration of Biological Activities of DCM and MTX. Chart 3 shows the effects of different concentrations of HA on the inhibitory activities of equimolar concentrations (5 × 10⁻⁸ M) of DCM and MTX. The biological activities of DCM and MTX were essentially unchanged in a culture medium containing HA (less than 0.25 g/dl), in which DCM exhibited slightly but definitely higher inhibitory effects on cell growth than did MTX. The activity of DCM began to decrease at about 0.25 g HA per dl; at HA (2.5 g/dl) 80 to 90% of the activity was lost. Similarly, MTX lost its activity with increasing concentrations of HA, but retained nearly one-half its activity at 2.5 g/dl.

Binding of DCM and MTX to HA in Vitro. Passage of DCM and MTX through a Centrifo type 50A membrane cone or a 0.45-μm Millipore filter membrane produced no loss of biological activity.

The results of the experiments on the binding of DCM and MTX to HA are shown in Table 2. When 0.1 ml of the ultrafiltrates obtained from an incubation mixture of DCM (5 × 10⁻⁸ M) and HA (2.5 g/dl) was added to individual culture tubes, the viable cells on Day 3 were 91 ± 7% (S.D.) of the control, and the binding of DCM to HA was calculated to be 83.5 ± 5.6%. In a similar study with MTX, viable cells were 9 ± 1% of the control and binding was 49 ± 0.6%. These results are consistent with the reversal experiments shown in Chart 3 and clearly demonstrate that DCM bound to HA more extensively (nearly 2-fold) than did MTX.

Chart 4 illustrates cell growth curves in the presence of an equal volume (0.1 ml) of ultrafiltrates or parent solutions. Cell growth in culture medium containing the parent solutions was completely inhibited by Day 3.

Reversibility of Cell Growth Inhibition by LV. Chart 5 shows the alterations of the DCM dose-response curve by various concentrations of LV. The dose causing 50% inhibition and ID₅₀ of DCM increased 7- and 14-fold, respectively, in the presence of 10⁻⁸ M LV, and 25- and 50-fold, respectively, in the presence of 10⁻⁹ M LV. LV reversals of the cell growth inhibition produced by equitoxic concentra-
DISCUSSION

Biological and pharmacological studies on DCM and MTX, carried out earlier by Davidson and Oliverio (6) and Henderson et al. (13, 14), have explained significant differences in metabolic alteration, protein binding, and drug elimination. Unlike MTX, a majority of DCM is excreted in bile and is extensively metabolized by human liver aldehyde oxidase to 7-hydroxydichloromethotrexate, a biologically inactive form (22, 26). More than 90% of DCM and about 50% of MTX were shown to bind to human serum protein, whereas both agents were 50% bound in mouse serum. These differences in ability to bind with albumin were, in part, regarded to be the reasons for the lack of superiority of DCM in humans. We confirmed in this study that DCM bound more extensively to HA than did MTX. Furthermore, we clearly demonstrated that the albumin-bound DCM was biologically inactive. Higher equitoxic doses of DCM in humans, in comparison with MTX, can readily be explained from our findings.

The culture medium that we used contained 10% dialyzed FCS, which corresponds to 0.3 to 0.4 g of calf serum albumin (21). It is undeniable that calf serum albumin interacts with MTX or DCM in our system; however, the facts that all the experiments were done in the presence of 10% FCS and that there was no major difference in the shape of the antifolic-produced dose-response curves with or without HA indicate that the displacement of the dose-response curve in the presence of HA is clearly due to the interaction produced by HA alone.

The binding of drugs to human albumin has been studied extensively in vitro and in vivo, particularly in antimicrobial antibiotics, anticoagulants, and cardiovascular agents (1, 24, 30, 31). The interaction between HA and drug is influenced by the concentrations of each of the agents. The dose effect of HA on the biological activities of DCM and MTX in vitro, presented here, is consistent with this principle. Thus, we observed that the parent solution, which was diluted 100 times for both DCM and albumin (final DCM concentration, 5 x 10^{-6} M; HA, 0.025 g/dl) was inhibitory, while the ultrafiltrate lost most of its activity. This difference can be interpreted to mean that all the DCM bound to HA was removed by ultrafiltration. The ultrafiltration of the DCM solution containing no HA had biological activity similar to the parent solution, indicating no absorption of DCM by the ultrafiltration membrane. The equilibrium between free and bound drug is reported to be maintained, and the HA-drug complex may serve as a circulatory reservoir (18). Therefore, the inhibitory effect of the parent solution seems to be caused by dissociation of the HA-drug complex.

The HA-drug complex has been shown to be readily displaced by unrelated drugs (11, 19). It is important to consider this phenomenon in DCM because of its high HA binding. Small changes in binding have great effects on the proportion of free drug and may produce severe toxicity in humans. Therefore, concomitant administration of drugs that displace DCM from HA requires close clinical monitoring in patients treated with a high-dose regimen.

Decreased glomerular filtration in highly HA-bound antimicrobial antibiotics is well known (20). The passive diffu-
sion process in drug elimination is usually limited by the extent of binding to HA (18, 29). The relatively less extensive urinary excretion of DCM in humans may be partly explained by the high binding to HA. On the other hand, albumin, which is known to be catabolized by the liver, may act as a carrier to transport DCM into the hepatic cells for elimination by this route.

LV acts as an antidote for MTX by bypassing the MTX-induced block in the reduction of folic acid and also by competing with MTX in transport into the cell (27). The present work revealed that DCM-induced inhibition of cell growth could be reversed by LV in vitro. Furthermore, the biological activity of equitoxic concentrations of DCM and MTX was reversed by equimolar concentrations of LV, not only in an HA-free medium but also in a medium containing HA (2.5 g/dl). Borsa and Whitmore (3), using L-cells in vitro, demonstrated that cell growth inhibition induced by 72-hr exposure to MTX (10^-6 g/ml) was enhanced by LV (10^-7 g/ml). They concluded that the observation was due to the ready recovery of MTX-induced inhibition of purine synthesis, preventing effective cell killing by MTX, and not of thymidylate synthesis. In our study of a human malignant cell (MOLT 3) in vitro, this paradoxical LV phenomenon could not be recognized with MTX or DCM.

The reasons for the inhibitory effects of a large concentration of HA on MOLT 3 cells are not clear, but the concentration of HA (2.5 g/dl) which we used in these experiments is close to the normal albumin concentration in humans (normal range, 3.6 to 6 g/dl) to make a meaningful extrapolation to an in vivo system. The present study suggests that the dose of LV required to protect or rescue DCM-induced toxicity in humans should be at least equal to that of the high-dose MTX and LV rescue regimen. However, it may be necessary to increase the duration of LV administration over that of the high-dose MTX and LV regimen, in consideration of the high binding of DCM to HA which may serve as a circulating reservoir for free DCM.

An in vitro screening system has been used routinely to detect biologically active new anticancer agents in conjunction with in vivo screening. Advances in cell culture techniques have contributed to the use of human tumor cells in an in vitro system. Changes in biological activities of DCM and MTX in the presence of HA in the culture medium warrant the further modification of the extracellular environment in an in vitro screening system. Such an improvement should bring the in vitro system closer to a more realistic in vivo system.

REFERENCES

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