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Reversal of Resistance to Methotrexate by Hyperthermia in Chinese Hamster Ovary Cells[1]

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ABSTRACT

Our Chinese hamster ovary cells are extremely resistant to methotrexate (MTX) (100% survival after 500 μg/ml for 13 hr). However, exposure to 43° (but not 41° or 42°) for 1 hr sensitizes the cells to MTX so that a 50% cell kill in excess of that due to hyperthermia occurs. Treatment of cells at 43° increases net MTX uptake by about 30% at 30 min but causes a substantial reduction after 1 hr. This negative effect is greater in cells continually heated at 43° than in those exposed for only 1 hr. Treatment at 43° for 1 hr also markedly increases efflux of MTX out of cells over the first 2 hr. Dihydrofolate reductase activity was found to decrease to about 50% of control values by 4 to 5 hr after exposure to 43°. The biological half-life of dihydrofolate reductase in Chinese hamster ovary cells was determined to be about 4.5 hr, indicating that hyperthermia-induced cessation of protein synthesis may explain both the decrease in dihydrofolate reductase activity and the sensitization to MTX observed with heat exposure. In scheduling experiments, lethality due to exposure to 43° for 1 hr in conjunction with MTX was maximum when 1-hr drug exposure began just at the end of heat treatment.

INTRODUCTION

There is now considerable clinical interest in the treatment of cancer with a combination of chemotherapeutic drugs and hyperthermia (9, 13). In general, however, early clinical results have been less than outstanding when patient morbidity and the difficulties in delivering the hyperthermia (at least whole body) are considered (4, 16, 19). *In vitro* and *in vivo* studies conducted in this laboratory and others (9, 17) have suggested that the scheduling of chemotherapeutic drugs with heating may be critical to maximization of cellular lethality. Better clinical results might be anticipated if these factors were understood. Because other studies had shown that exposure to temperatures greater than 43° could transiently shut off protein synthesis, causing significant decreases in the levels of important intracellular enzymes (8, 10), we chose to investigate the interaction between exposure of cells to hyperthermia and MTX,[2] since MTX acts by inhibiting one such enzyme, DHFR (1). Our strain of CHO cells proved an excellent model because it is resistant to MTX due to high levels of this enzyme (11). We further attempted to define the best scheduling of this drug with heat to better plan for eventual clinical application.

MATERIALS AND METHODS

Method of Cell Culture. CHO cells, originally obtained in 1974 from Dr. Ronald M. Humphrey, University of Texas Cancer Center, M. D. Anderson Hospital, Houston, Texas, and RT9 cells, originally obtained in 1975 from Dr. Kenneth Wheeler, University of Rochester, Rochester, N. Y., were both grown in McCoys' Medium 5A supplemented with 20% (v/v) fetal calf serum, penicillin (11 units/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) (all from Grand Island Biological Co., Grand Island, N. Y.). Cells were maintained in logarithmic growth phase at 37° in a 5% CO_2-95% air atmosphere. Doubling time was 13 to 14 hr for the CHO cells and 15 to 16 hr for the RT9 cells. Cell numbers were determined with an electronic particle counter (Coulter Electronics, Hialeah, Fla.). Cells were replated approximately 12 hr before experimental manipulation.

Cell Viability Measurements. Cell viability was measured by the ability of single cells to form colonies *in vitro*. For this assay, known numbers of cells were plated in plastic Falcon No. 3002 Petri dishes (Falcon Plastics, Oxnard, Calif.) containing 5 ml of fresh medium. Three dilutions of the known cell number were made for each drug concentration, and each was plated in triplicate. After incubation for 7 to 8 days, colonies were fixed and stained with 0.5% crystal violet (Fisher Scientific Co., Fair Lawn, N. J.) in 95% ethanol. Viable cells were those that produced colonies of more than 50 cells upon examination under a dissecting microscope. The plating efficiencies of control cultures of CHO cells were between 80 and 90%. The results from colony formation experiments, described in Charts 1 and 6, represent 3 to 5 replicate experiments per data point.

Heat Treatments. Exponentially growing cells were grown in T_{25} flasks (Falcon Plastics). Flasks were then immersed in a specially designed plexiglass water tank with continuous flow of heated water for a Haake Model FK water circulator (temperature variability, ±0.1°) (Haake, Inc., Saddle Brook, N. J.). Five ml of fresh media or media plus drug were added directly prior to heating. Thermometry was accomplished with mercury and glass thermometers calibrated by the method of Cetas and Conner (6).

Drug Treatments. Exponentialiy growing cells were exposed to varying concentrations of MTX (Lederle Laboratories, Pearl River, N. Y.) in 60-mm Petri dishes for 1 hr at 37°, or in T_{25} flasks as above at 41°, 42°, or 43°. Drug solutions were always prepared immediately prior to use by dissolving the drug in sterile water. Appropriate dilutions of drugs were added to fresh medium, with the volume of drug solution never exceeding 20% (1 ml) of the total volume of 5 ml. After treatment, the medium was removed, and the cultures were washed twice with warmed Puck's Saline A (Grand Island Biological Co.) and trypsinized (0.05% for 2 to 3 min). Following this, known numbers of cells were plated into plastic Petri dishes for colony growth as described above.

Measurement of DHFR. The activity of DHFR was measured by the method of Mathews and Huennekens (18). Twenty million cells were used for each determination. CHO and RT9 cells were removed from monolayer cultures by scraping with a rubber policeman. These cells were suspended (1.0×10^7 cells/ml) in 0.1 M Tris HCl buffer, pH 7.4, and were disrupted by sonication (E/MC Corp. ultrasonic cell disruptor; 4.5-in probe, 20-sec pulse). The resulting cell homogenate was centrifuged at 12,000 × *g* for 10 min. The pellet was discarded, and the supernatant extract was placed on ice. The reaction mixture included 40 mM potassium phosphate (pH 7.4), 8 mM 2-mercaptoethanol, 0.08 mM dihydrofolate, 0.08 mM NADPH, and 0.20 ml of sample supernatant in a total volume of 1.00 ml. DHFR activity was then determined at 37° by measuring the decrease in absorbance at 340 nm of the solution as

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[2] The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; CHO, Chinese hamster ovary.

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NADPH was oxidized and dihydrofolate was reduced to tetrahydrofolate. Absorbance was measured with a temperature-controlled Beckman Model DU spectrometer. The results obtained, as described in Chart 4, represent the mean of triplicate determinations and are confirmed by 3 replicate experiments.

Determination of DHFR Half-Life. Asynchronous, exponentially growing cultures of CHO cells were exposed to a 20-μg/ml dose of the protein synthesis-inhibiting drug cycloheximide (20). At 15, 30, 60, 120, and 270 min following the beginning of exposure, the remaining DHFR activity was determined as above. The biological half-life is defined as the time required after cycloheximide addition for one-half of the measurable DHFR activity to be lost.

Drug Uptake Studies. MTX uptake was measured using [^{3}H]MTX (150 to 250 mCi/mmol; Amersham/Searle, Arlington Heights, Ill.). CHO cells growing exponentially in 60-mm plastic Petri dishes (10^6 cells/dish) were exposed to 2 μCi of [^{3}H]MTX (56 μg/ml) for varying times at 37° or 43°. In other experiments, cells were exposed to labeled MTX for varying times after experiencing a heat shock of 43° for 1 hr. After treatment, cells were placed on ice, washed twice with warmed Puck's Saline A, and scraped off the dishes. Cells were lysed in 0.1 ml of 1 M NaOH and solubilized in a toluene fluor containing PPO, 0.4 mg/ml, and POPOP, 5 μg/ml, and then adjusted to 25% Triton X-100 (v/v). Samples were counted in a Searle Mark II liquid scintillation counter (65% efficiency for tritium).

Efflux of MTX. In these experiments, cells were exposed to 2 μCi [^{3}H]MTX (56 μg/ml) for 1 hr at 37° or at 43°. After these treatments, cells were washed twice with warmed Puck's Saline A and placed in standard medium at 37°. At various times thereafter, cells were removed from the monolayer cultures by scraping and solubilized, and the remaining [^{3}H]MTX was determined as described above.

RESULTS

The survival kinetics of CHO cells exposed to increasing concentrations of MTX for 1 hr at 37°, 41°, 42°, or 43° are shown in Chart 1. When cell killing due to heat treatments at 42° and 43° was accounted for, increased cell kill due to MTX was seen only at 43°. At 43°, a 50% cell kill above that due to heat was seen, which was independent of the concentration of MTX above 5 μg/ml. Approximately 50% of CHO cells are in S phase during exponential growth (data not shown). As we have previously reported, our CHO cells are extremely resistant to MTX-induced lethality and are not killed by up to 500 μg of MTX per ml applied for up to 13 hr at 37° (11). Thus, exposure to 43° sensitized these resistant cells to MTX, allowing additive lethality to be achieved.

In order to understand how heating at 43° overcame MTX resistance, we first examined the uptake of drug into heated and unheated cells. These results are shown in Chart 2. At 30 min, MTX uptake into heated cells was about 30% greater than into unheated cells (0.42 μmol/10^9 cells *versus* 0.32 μmol/10^9 cells). Thereafter, however, while net uptake of MTX reached a plateau at 1 hr in unheated cells, an increasing net loss was observed in heated cells which was larger in cells continually exposed to 43° than in cells heated at this temperature for only 1 hr. Thus, after 5 hr, the levels of MTX in cells heated for 1 hr at 43° or continually at 43° was about 25 and 50% less, respectively (0.38 μmol/10^9 cells and .24 μmol/10^9 cells), than in unheated cells (0.53 μmol/10^9 cells).

The reason for these results became clear when efflux of MTX out of unheated cells and cells heated at 43° for 1 hr was examined. As shown in Chart 3, loss of MTX from cells occurs much more rapidly over the first 2 hr in CHO cells heated for 1

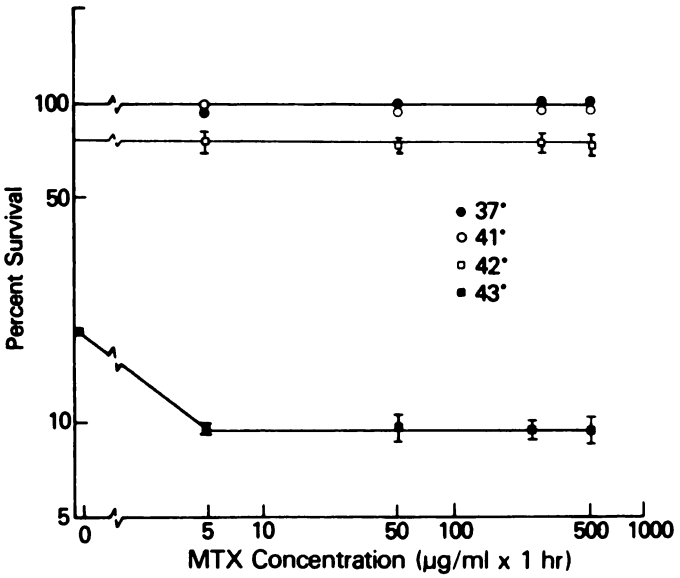

Chart 1. Survival of asynchronous CHO cells after concurrent 1-hr exposure to MTX and hyperthermia. CHO cells in asynchronous, exponential growth were exposed to increasing concentrations of MTX for 1 hr at 37°, 41°, 42°, or 43°, and survival was determined by colony formation. *Points*, mean of 3 to 5 replicate experiments; *bars*, S.E.

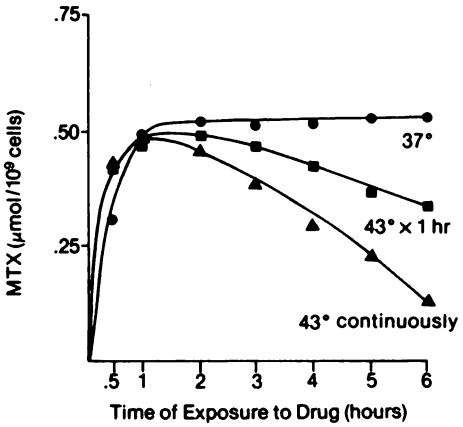

Chart 2. MTX uptake into CHO cells. Exponentially growing, asynchronous CHO cells were exposed to 2 Ci of [^{3}H]MTX (56 μg/ml) for 0.5, 1, 2, 3, 4, 5, or 6 hr at 37°, at 43°, or at 37° following a 1-hr exposure to 43°. After being washed, cells were solubilized, and remaining radioactivity was determined. *Points*, mol/10^9 cells, representing the mean of 4 or 5 replicate experiments.

hr at 43° than in unheated cells. After 1 to 2 hr, the rate of MTX loss from cells was similar for both heated and unheated cells.

Since we did not think that we could explain the cytotoxicity due to MTX in heated cells by an increase in drug uptake, and because previous studies indicated that heating decreased the levels of certain enzymes by shutting off protein synthesis (8, 10), we examined DHFR activity levels at times after heating. As can be seen in Chart 4, in both CHO cells and RT9 cells, DHFR activity dropped significantly 3 hr following a 1-hr exposure to 43°, reaching a low point at 4 to 5 hr after heating was started. This decrease in enzyme activity probably was not the result of an altered enzyme since heating does not change the ability of MTX to inhibit DHFR *in vitro* (data not shown). It is also interesting that the level of DHFR activity showed recovery to near base-line values at 7 hr in CHO cells (the

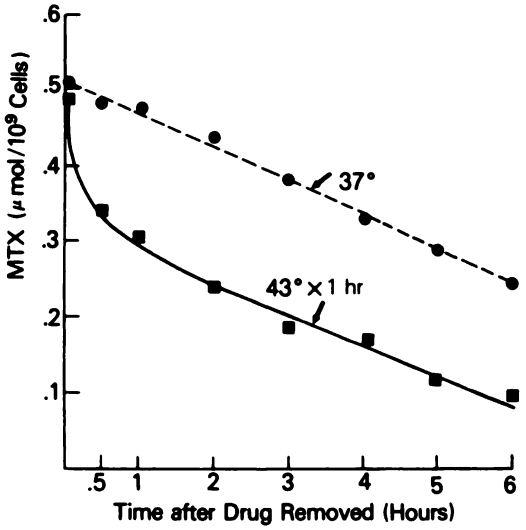

Chart 3. MTX efflux from heated or nonheated CHO cells. Exponentially growing, asynchronous CHO cells were exposed to 2 Ci of [^{3}H]MTX (56 μg/ml) for 1 hr at 37 or 43°. Cells were then washed and placed in fresh drug-free media, and the remaining radioactivity determined after 0.5, 1, 2, 3, 4, 5, or 6 hr. *Points*, mol/10^9 cells, representing the mean of 3 to 5 experiments.

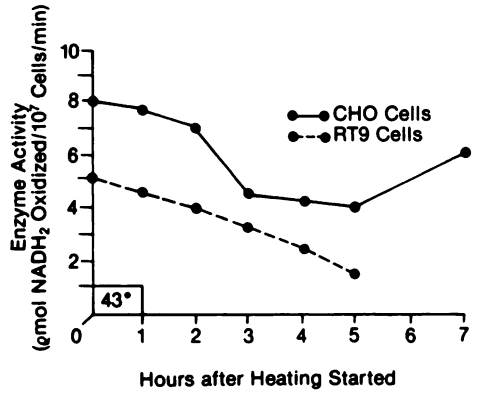

Chart 4. Effect of exposure to 43° for 1 hr on DHFR activity in CHO cells and RT9 cells. Asynchronous, exponentially growing CHO or RT9 cells were heated at 43° for 1 hr, and DHFR activity was determined by the method of Mathews and Huennekens (12) at 1, 2, 3, 4, 5, or 7 hr following the beginning of heat shock. *Points*, enzyme activity expressed as pmol $NADH_2$ oxidized per 10^7 cells per min, representing the mean of 3 experiments.

levels of DHFR in RT9 cells were not examined after 5 hr), suggesting a recovery in protein synthesis.

In order to determine whether the kinetics of the decline in DHFR activity was consistent with simple cessation of synthesis, we determined the half-life of DHFR activity after treatment of cells with the protein synthesis inhibitor cycloheximide (20). As can be seen in Chart 5, the half-life of DHFR activity was 4.5 hr. Assuming shut-off of protein synthesis over the first 30 min of exposure to 43°, we believe that it is reasonable to interpret the drop to one-half the base-line level of DHFR activity by 5 hr (observed in Chart 4) as compatible with decay of enzyme function due to cessation of synthesis. In addition, recovery of DHFR levels 7 hr after exposure to 43° is similar to that which we observed previously in CHO cells in the case of ornithine decarboxylase (8).

In an attempt to define the most cytotoxic scheduling of MTX with a 1-hr heat shock at 43°, we exposed CHO cells to MTX (5 μg/ml) for 1 hr, either concurrently with a temperature of 43° for 1 hr or at various times after this heat exposure. As shown in Chart 6, after the data are normalized for killing due to hyperthermia, additional cytotoxicity was observed only when MTX was administered either concurrently with the heat treatment or directly following heating. At times further removed from heating, no additive cytotoxicity was observed. In repeated experiments, exposure to MTX at the end of 1-hr heat treatments produced the most cell kill [about 55% above that due to heat alone (about 80%)].

DISCUSSION

MTX is a useful drug in the treatment of several neoplasms such as head and neck cancer (15), cervical cancer (23), and breast cancer (5), each of which are often amenable to treatment with local hyperthermia where a temperature of 43° can

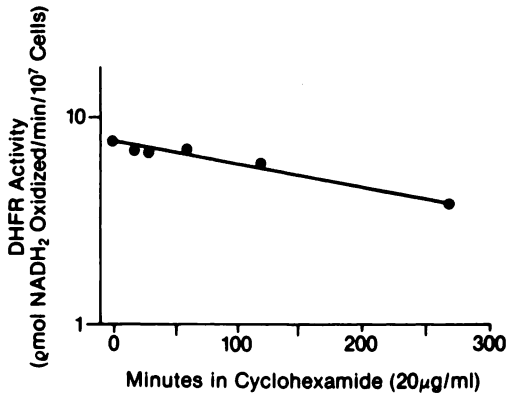

Chart 5. Determination of the half-life of DHFR activity in CHO cells. After CHO cells were exposed to a 20-μg/ml dose of the protein synthesis inhibitor cycloheximide (13), the remaining DHFR activity was determined at various times by the method of Mathews and Huennekens (12). *Points*, enzyme activity expressed as pmol $NADH_2$ oxidized per 10^7 cells per min, representing the mean of 3 experiments.

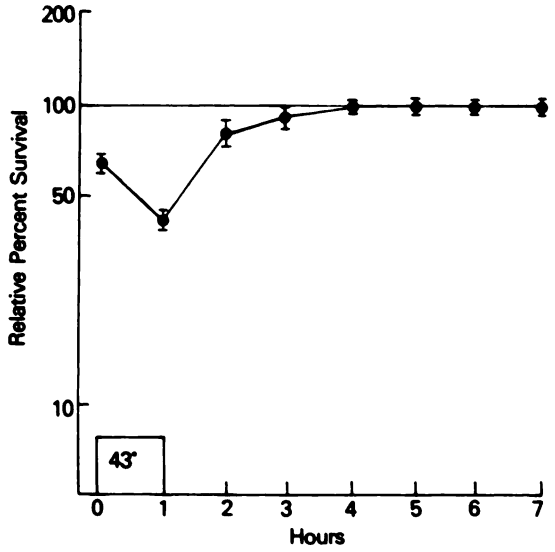

Chart 6. Survival of asynchronous CHO cells after 1-hr exposures to 5 μg/ml of MTX either concurrently with or at times following heat shock at 43°. Asynchronous, exponentially growing CHO cells were exposed to MTX (5 μg/ml) for 1 hr either concurrently with or at 1, 2, 3, 4, 5, 6, or 7 hr following the beginning of a 1-hr heat shock at 43°. Survival was determined by colony formation. *Points*, mean of 3 to 5 replicate experiments; *bars*, S.E. Survival data are normalized for killing due to exposure to 43° for 1 hr.

be achieved (21). As with essentially all other chemotherapeutic agents, however, advanced stages of these tumors are not curable with MTX and eventual drug resistance emerges. We have shown that the high degree of MTX resistance exhibited by our CHO cells can be overcome by exposure to 43° for 1 hr so that lethality due to hyperthermia and to MTX become additive. Indeed, when a 1-hr exposure to MTX is given at the end of a 1-hr heat exposure at 43°, fully 55% of the cells which survive heating are killed, exceeding the predicted additive lethality. Since S-phase cells are more sensitive to hyperthermic cytotoxicity than G_1 cells (3), one would expect fewer cells surviving heat treatment to be in S phase where MTX is active (12) compared to the approximately 50% of CHO cells normally in S phase during unperturbed exponential growth (data not shown).

Our previous studies (11) showed that our CHO cells were resistant to MTX-induced lethality on the basis of high levels of DHFR activity, a situation which has been previously noted in human tumors (2) as well as in other experimental cell lines (7). Our data indicate that, although net MTX uptake is stimulated by exposure to 43° at 30 min, net uptake at later times is actually decreased. The probable mechanism by which exposure to 43° sensitizes these CHO cells to MTX is by shutting off protein synthesis, causing the activity of the enzyme DHFR to fall as a function of its half-life. Both the initial fall of DHFR and the rebound of activity at about 7 hr is very like that previously reported in the case of ornithine decarboxylase also after exposure of CHO cells to 43° (8). Our observation of MTX uptake is very like that described by Kowal *et al.* (14), who have also found early stimulation and later inhibition. Resistance to other chemotherapeutic drugs may also be due to high levels of target enzymes. Since the temporary cessation of protein synthesis induced by temperatures above 43° may be a general phenomenon, other antimetabolite drugs with actions that require inhibition of critical enzymes should be tested in systems with hyperthermia.

We were surprised that optimum scheduling required close approximation of exposure to both hyperthermia and drug, although this is probably the case for radiation and heat (22) as well as for some other chemotherapeutic drugs and heat (9). Before performing the scheduling experiments, we had predicted that lethality would be maximum when MTX followed heating by 3 to 5 hr, a time when DHFR activity is lowest (Chart 4). However, net uptake of MTX is substantially impaired 3 to 4 hr after exposure to 43° for 1 hr (Chart 2), and poor uptake of drug may explain the failure of MTX to add to hyperthermic cell kill at this time. Furthermore, the rebound of DHFR activity by 7 hr following heat shock may be sufficient to restore folate pools before irreversible damage occurs to cells. In addition, the previously reported (3) cell cycle blocks due to heat, which cause cells to accumulate in the MTX-insensitive G_2 and M phases, may also provide a partial explanation.

Our experiments demonstrate that an extremely high degree of resistance to a cytotoxic drug can be overcome by hyperthermia. Most patients who are now candidates for clinical trials with hyperthermia have been heavily pretreated and have become resistant to chemotherapeutic drugs. This study suggests that hyperthermia may resensitize tumors to some chemotherapeutic drugs, although sensitization of normal tissues to these agents may also occur.

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