High-Dose Methotrexate Does Not Affect the Pharmacodynamics of Phenobarbital Hypnotic Action but Decreases the Central Nervous System (CNS) Sensitivity to Pentylenetetrazol-Induced Maximal Seizures in Rats

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Chemotherapy with high-dose methotrexate (HD-MTX) is often associated with acute neurotoxicity. We determined whether the altered neuronal function after HD-MTX [such as the reduced regional cerebral metabolic glucose rate (rCMRGlc) and slow electroencephalographic pattern] affects the sensitivity of the CNS to centrally acting drugs: the depressant phenobarbital, which reduces rCMRGlc, and the analeptic agent pentylenetetrazol (PTZ), which elevates rCMRGlc. Adult male Sabra rats received an i.v. infusion of MTX, 0.51 mg/min, to induce neurotoxicity or saline solution for 24 hr. Subsequently, MTX-treated and control groups were infused in one experiment with phenobarbital until loss of the righting reflex and in the second experiment with PTZ until the onset of maximal seizures. HD-MTX did not affect the infused hypnotic dose or serum, brain, and cerebrospinal fluid concentrations of phenobarbital at the onset of anesthesia. The convulsive dose and PTZ concentrations in the serum and brain at the onset of maximal seizures were significantly higher in the HD-MTX-treated animals. These outcomes indicate that HD-MTX and the reduced rCMRGlc that follows this treatment do not contribute to the hypnotic action of phenobarbital. On the other hand, treatment with HD-MTX exhibited anticonvulsant properties as evidenced by the reduced CNS sensitivity to PTZ-induced seizures.

KEY WORDS: pharmacodynamics; high-dose methotrexate; neurotoxicity; pentylenetetrazol; phenobarbital; induced seizures; brain; brain glucose metabolism; drug interaction.

INTRODUCTION

Methotrexate (MTX) has an important role in the treatment of various types of neoplastic disease, such as trophoblastic neoplasms and leukemias. In certain treatment protocols, such as for osteogenic sarcoma and brain tumors, high-dose methotrexate (HD-MTX) (defined as a regimen over 5 g/m^2) is required. In addition to the common adverse (toxic) effects that follow cancer chemotherapy (e.g., bone marrow inhibition), HD-MTX treatment is associated with neurotoxicity, generally expressed by several types of encephalopathies (1). This clinical syndrome develops within 24 hr of treatment and is characterized by confusion, lethargy, and coma. In adolescent cancer patients treated with HD-MTX, there is a reduction in the regional cerebral metabolic rate of glucose (rCMRGlc) as measured by ^{18}F-fluorodeoxyglucose and positron emission tomography (2).

Because HD-MTX-induced encephalopathies are observed together with reduced rCMRGlc (2), it has been proposed that the neurotoxicity is a consequence of the reduction in rCMRGlc (3). Many other drugs such as phenobarbital and other depressants, are also known to reduce rCMRGlc proportionally to the administered dose (4); specifically, a high reduction in rCMRGlc was observed in rats during barbiturate induced coma (5). On the other hand, stimulant substances such as bicuculline and pentylenetetrazol (PTZ) greatly increase rCMRGlc at the onset of convulsions (6). Investigation of the interaction between concurrent treatments with HD-MTX and neuroactive agents can provide information about the required dosage adjustments of these drugs, as well as the contribution of rCMRGlc to their pharmacologic action.

In order to investigate the neurological dysfunctions induced by HD-MTX, an animal model of acute HD-MTX neurotoxicity was developed by Phillips *et al.* (7). This rat model is characterized by a dose-dependent depression of rCMRGlc, electroencephalographic slowing, abnormalities in plasma amino acids and behavior, and an absence of systemic toxicity. These symptoms are in agreement with clinical observations (2).

The purpose of this study was to investigate the effects of (patho-)physiological influences induced by acute HD-MTX on the pharmacodynamics (i.e., concentration–effect relationship) of two centrally acting drugs: a depressant, phenobarbital, and an analeptic agent, PTZ [a parameter that has been suggested to evaluate neurotoxicity (8)].

An animal model has been developed previously in order to evaluate the effect of (patho-)physiologic states on the pharmacodynamics of certain centrally acting medications, including phenobarbital and PTZ (9,10). It has been established that phenobarbital concentrations in the cerebrospinal fluid (CSF) (but not in the serum or the whole brain) at the onset of loss of the righting reflex (LRR) are independent of pharmacokinetic variables and reflect the drug concentrations at the site of action (biophase). On the other hand, PTZ distributes very rapidly from the blood to receptor sites in the brain. Consequently, PTZ concentrations in the serum, brain, or CSF are equally suitable to serve as sampling sites for pharmacodynamic evaluation (10).

MATERIALS AND METHODS

Animals and Operations. Male Sabra rats weighing 190 to 245 g were acquired from the Animal Breeding Unit of the Hebrew University–Hadassah Medical School, Israel. They had an indwelling cannula implanted in the right jugular vein under light ether anesthesia (11), 1 day before the pharmacodynamic experiment. The cannulas were filled with saline solution (no heparin). During the experimental period, all animals were housed in individual plastic cages in a light-controlled room and had free access to food and water.

Drugs and Chemicals. Methotrexate solution (100mg/ml; Emthexate PF) was purchased from Pharmachemie B.V. (Haarlem, The Netherlands). Phenobarbital sodium (Phamir

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Ltd., Israel) was dissolved with distilled water to produce a 40 mg/ml solution. PTZ, purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI), was dissolved in normal saline to make a 18.54 mg/ml solution. Normal saline was used in the control rats.

MTX Infusion Technique. MTX was administered as a continuous intravenous infusion for 24 hr by a syringe infusion pump at a rate of 0.0051 ml/min (total dose, 730 mg). The infusion tubing was protected from rodent bites by a flexible metal spring. The pharmacodynamic experiments were performed immediately following the MTX infusion.

Pharmacodynamic Experiments. To evaluate the effect of HD-MTX on the pharmacodynamics of phenobarbital, sodium phenobarbital solution was administered at a constant rate of 1.2 ml/hr (48 mg/hr) until the onset of the pharmacologic end point (LRR) (9). Samples were immediately collected in the following order: cerebrospinal fluid from the cisterna magna, blood (for serum) from abdominal aorta, and brain (which was stripped of its external vasculature and meninges). Similarly, to evaluate the effect of HD-MTX on the pharmacodynamics of PTZ-induced seizures, the rats were placed individually in a plastic basin, and PTZ solution was administered intravenously at a continuous rate of 2 ml/hr (37.08 mg/hr), until the onset of maximal seizures, which were evidenced by tonic flexion of the forelimbs and, usually, tonic extension of the hindlimbs (10). At that moment, the rats were lightly anesthetized with ether, and samples of blood and brain were taken. The biological samples were frozen pending analysis. Rectal temperature was monitored before the pharmacodynamic studies began. The dose of each of these two drugs, required to induce the pharmacologic end point, was calculated as (infusion time) × (infusion rate), normalized by body weight.

Analytical Methods. Phenobarbital concentrations in serum, brain, and CSF were assayed by a high-performance liquid chromatography (HPLC) method, according to Danhof and Levy (9), with certain modifications. The standard curve was linear in the range 50–300 mg/L. PTZ concentrations in serum and brain were assayed using an HPLC method described by Ramzan (12), with certain modifications. The standard curve was linear in the range 50–250 mg/L. Serum analysis of BUN was performed using a commercially available kit (No. 535, Sigma Chemical Co., St. Louis, MO). Because little serum was left, the determination of free concentrations of citrulline and phenylalanine in the serum was performed on pooled serum of each of the HD-MTX-treated groups and the corresponding control groups. Amino acid determination was made by Aminolab (Kiryat Weizmann Science Part, Rehovot, Israel) using an amino acid analyzer (Biotronik LC 5000) according to Moore and Stein (13).

Statistical Analysis. The data were analyzed for statistical significance using Student's unpaired t test. Statistically significant difference was taken as $P < 0.05$.

RESULTS

Pertinent characteristics of the animals used to determine the effect of HD-MTX on the pharmacodynamics of phenobarbital and the concentrations of phenobarbital at onset of LRR are summarized in Table I. Serum urea nitrogen and body temperature were not altered following HA-MTX treatment. The citrulline concentration in the serum of the phenobarbital-anesthetized rats was lower after HD-MTX treatment, while the phenylalanine concentration was elevated. The phenobarbital infusion time to onset of LRR and the total relative dose of phenobarbital required to reach this pharmacodynamic end point were not affected by HD-MTX. Phenobarbital concentrations in the serum, brain, and CSF at the onset of LRR in HD-MTX-treated rats and controls were not significantly different.

Table I. Description of Male Sabra Rats Used in this Investigation and the Effect of HD-MTX on Concentrations of Phenobarbital at the Onset of Loss of Righting Reflex[a,b]

Parameter	Control	MTX treated
No. of animals	10	11
Weight (g)	244 ± 35	243 ± 36
Rectal temp. (°C)	37.3 ± 0.4	36.9 ± 0.4
BUN (mg/dl)	21.1 ± 4.1	25.9 ± 3.0
Serum amino acids (nmol/ml)[c]		
Citrulline	32.8	22.5
Phenylalanine	92	735
Time to end point (min)	45.8 ± 6.5	40.3 ± 8.6
Dose (mg/kg)	152 ± 26	132 ± 22
Serum conc. (mg/L)	233 ± 23	236 ± 22
Brain conc. (mg/kg)	121 ± 14	126 ± 15
CSF conc. (mg/L)[d]	91.6 ± 9.6	87.3 ± 14.2

[a] HD-MTX was achieved by a continuous i.v. infusion of MTX at a rate of 0.51 mg/min during 24 hr, just before the experiment.
[b] The animals received an i.v. infusion of the drug at a rate of 48 mg/hr. Results are reported as mean ± SD.
[c] Results of pooled serum measured after phenobarbital administration.
[d] Number of animals = 8.

The effect of HD-MTX on the pharmacodynamics of PTZ-induced seizures is described in Table II. The citrulline concentration in the serum of HD-MTX-treated rats, after induction of maximal seizures, was lower in comparison to

Table II. Description of Male Sabra Rats Used in this Investigation and the Effect of HD-MTX on Concentrations of PTZ at the Onset of Maximal Seizure[a,b]

Parameter	Control	MTX treated
No. of animals	12	12
Weight (g)	192 ± 20	213 ± 28
Rectal temp. (°C)	37.3 ± 0.6	37.0 ± 0.5
Serum amino acids (nmol/ml)[c]		
Citrulline	177	60.5
Phenylalanine	459	1288
Infusion time (min)	30 ± 10	42 ± 12
Dose (mg/kg)	92 ± 24	121 ± 28*
Serum conc. (mg/L)	114 ± 27	134 ± 17*
Brain conc. (mg/kg)	110 ± 27	144 ± 22*

[a] HD-MTX was achieved by a continuous i.v. infusion of MTX at a rate of 0.51 mg/min during 24 hr, just before the experiment.
[b] The animals received an i.v. infusion of the drug at a rate of 37.08 mg/hr. Results are reported as mean ± SD.
[c] Results of pooled serum measured after PTZ administration.
* Significantly different from control value, $P < 0.04$.

that in saline-treated controls, while the phenylalanine concentration was higher. The concentrations of both citrulline and phenylalanine in the saline-treated controls of phenobarbital-induced anesthesia (Table I) were markedly lower than the corresponding concentrations in the serum obtained following PTZ-induced convulsions (Table II). HD-MTX-treated rats required a larger dose of PTZ than saline-treated control rats to produce onset of maximal seizures. Consequently, their drug infusion time was considerably longer than that of the control group. The concentration of PTZ at the pharmacologic end point was significantly higher in the serum and brain of MTX-treated animals than in controls. In both experiments, the HD-MTX-treated rats exhibited lethargic behavior evidenced by reduced spontaneous activity.

DISCUSSION

The phenomenon of HD-MTX-induced acute encephalopathy is being recognized with increasing frequency (14), although the mechanisms responsible are unclear (15). A number of theories have been suggested including the accumulation of toxic oxidized folates and inhibition of tetrahydrobiopterin production [which is a cofactor required for the hydroxylation of endogenous amines (16)] and direct injury which selectively affects astrocytes (17). Since various changes in neuronal function such as rCMRGlc, behavior, and electroencephalographic pattern are evidenced following HD-MTX treatment, we hypothesized that HD-MTX may modify the CNS sensitivity to the depressant and stimulant effects of centrally acting drugs.

Phillips *et al.* developed an HD-MTX rat model that resembles very closely the occurrences in humans following HD-MTX treatment (2). This animal model enables us to investigate various aspects of HD-MTX neurotoxicity and was utilized previously to evaluate the effect of high-dose leucovorin on HD-MTX neurotoxicity (18). In the present work this model was utilized to study the effect of HD-MTX on the pharmacodynamics of phenobarbital and PTZ.

HD-MTX treatments do not cause systemic toxicity, as evidenced by normal values of body temperature, mean arterial pressure, arterial blood pH, blood gases, plasma glucose, serum electrolytes, calcium, blood urea nitrogen, creatinine, aspartate aminotransferase, and bilirubin (7). Lack of systemic toxicity following 24-hr MTX infusion was confirmed in the present work by normal values of body temperature and serum urea nitrogen concentrations. The normal systemic physiological values indicate that MTX toxicity develops predominantly in the brain. The characteristic changes in serum amino acid profile following HD-MTX treatment (7) (i.e., higher phenylalanine and lower citrulline concentration) were also ascertained in this work. The values of both amino acids in the serum obtained after PTZ-induced maximal seizures were markedly high in comparison with the corresponding values in serum obtained from phenobarbital-anesthetized rats. The elevated amino acid concentration was probably due to convulsions, as cerebral concentrations of several amino acids are known to be significantly altered after PTZ-induced seizures (6).

It has been suggested that HD-MTX modifies the blood–brain barrier (BBB) function and increases the BBB permeability of low molecular weight hydrophilic compounds (2). The results of the present work, in both phenobarbital and PTZ studies, do not support such an alteration in BBB permeability. Enhanced BBB permeability is expected to require lower doses and lower serum concentrations in order to produce the certain phenobarbital concentration in the biophase needed to induce LRR. Such differences were not detected (Table I). Similarly, the ratio between the total PTZ dose required to induce maximal seizures in the HD-MTX-treated animals and that in the corresponding control group equals the ratio of PTZ brain concentrations at that pharmacologic end point between these groups (Table II), unlike anticipated in the case of increased BBB permeability.

HD-MTX had no effect on phenobarbital concentrations in the CSF at onset of LRR. This outcome indicates that chemotherapy does not alter CNS sensitivity to the hypnotic effect of the barbiturate. In view of the general depressive state of the HD-MTX-treated rats, manifested by lethargy, reduced spontaneous activity, and diminished startle response to loud noise or vibrissal stimulation [observed previously (7), as well as in the present experiments]; this finding was unanticipated. One would expect that a lower barbiturate concentration would be required to produce general anesthesia in these lethargic rats. The result implies that the mechanism(s) by which the general depressive state was produced does not coincide with the mechanism of the depressant action of phenobarbital. In addition, the fact that the pharmacodynamics of phenobarbital were not affected by HD-MTX clearly demonstrates that a reduced rCMRGlc does not have a role in the depressant action of barbiturates. In a case where reduced rCMRGlc would play such a role, the concentration–effect relationship of the hypnotic effect of the barbiturate should have been modified.

Unlike the effect of HD-MTX on the pharmacodynamics of phenobarbital, the pharmacodynamics of PTZ-induced maximal seizures were altered following HD-MTX treatment. This pharmacodynamic effect was evidenced by the higher PTZ concentrations in the serum and brain of the HD-MTX-treated group at the onset of maximal seizures. That is, elevated PTZ concentrations were required in order to produce the same pharmacologic response.

PTZ is used clinically as an analeptic (19), but its major use has been as an experimental tool for identifying and testing anticonvulsant agents (20). Previously, PTZ-induced seizure thresholds were suggested as a parameter to evaluate the neurotoxic activity of certain medications and to quantify their convulsive liability (8). While HD-MTX produces electroencephalographic slowing, representing anticonvulsant characteristics, seizures were reported in some cases following HD-MTX treatment (21), suggesting the possible proconvulsant potency of this treatment. The outcome of the present experiment clearly demonstrates that HD-MTX in fact produces anticonvulsant properties.

The convulsant effect of PTZ occurs via its effect on the picrotoxin-sensitive site of the benzodiazepine–γ-aminobutyric acid (GABA) receptor–ionophore complex (22). Therefore, it is possible that the effect of MTX on the PTZ-induced seizure thresholds may be caused by interference with GABA transmission. Such interference could be precipitated directly by MTX or indirectly by its metabolites, an endogenous compound (whose concentration in the CNS was altered secondarily to HD-MTX treatment), or any

other (patho-)physiological changes secondary to HD-MTX (e.g., changes that produce slowing of the electroencephalographic activity). Another possible explanation for the reduced CNS sensitivity to PTZ-induced convulsions is associated with the antifolate activity of MTX. Folic acid is known to produce seizures after i.v. injection in mice. These seizures resemble the effect of stimulants that act on GABA receptors (23). Folic acid metabolites, such as folinic acid, exhibit the same property. Folic acid can reverse the antiepileptic effect of phenytoin and phenobarbital. In addition, seizures can be aroused in patients treated with folic acid (24). According to this evidence, antifolate activity seems to be engaged in anticonvulsant activity. At least one antiepileptic drug, lamotrigine, was developed on the basis of this hypothesis (25).

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