Toxicity of Methotrexate in Rats Preexposed to Nitrous Oxide

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ABSTRACT

Several chemotherapeutic protocols for the treatment of malignancies include administration of methotrexate (MTX) during or shortly after total anesthesia. Clinical observations in patients treated for breast carcinoma or childhood cancer have shown unexpected myelosuppression and mucosal damage. This phenomenon may be attributed to the synergistic effects of nitrous oxide, which inactivates the cobalamin coenzyme of methionine synthase, and MTX, which inhibits dihydrofolate reductase, on folate metabolism. However, no quantitative data on dose-effect relationships are available regarding the combined toxicity of MTX and N2O. We investigated the effect of exposure to N2O on the toxicity of MTX. Groups of male Wistar rats were exposed to either 50% N2O/50% O2 or air for 12–48 h. Subsequently, a single i.p. injection of 10, 20, 40, or 80 mg MTX/kg body weight was given. Gastrointestinal toxicity resulted in diarrhea and weight loss in all groups for 5 days after MTX administration. Concomitantly, bone marrow depression with leukocytopenia and thrombocytopenia occurred. Exposure to N2O did not alter the plasma clearance of MTX. No substantial liver or kidney toxicity could be detected, but the 50% lethal dose for MTX was reduced from 60/100 mg/kg to 10 mg/kg if rats had been exposed to N2O for 48 h; the main causes of death were dehydration and bleeding. The administration of 5-formyltetrahydrofolate (4 x 10 mg i.p.) but not 5-methyltetrahydrofolate protected completely against the lethal effect of the drug combination. Altogether, cytotoxic effects of MTX on proliferating cells are potentiated by N2O. Therefore, the use of this anesthetic shortly before or during MTX administration should be avoided.

INTRODUCTION

The folate antagonist MTX1 has become a widely used antimetabolite in cancer chemotherapy (1, 2). The drug is a potent inhibitor of dihydrofolate reductase. It is subject to intracellular polyglutamation which results in increased cellular retention and higher affinity for other folate-dependent enzymes (3). Subsequent severe disturbance of folate metabolism, accompanied by a decrease of cellular reduced folate compounds which are required for dTMP and purine biosynthesis, is considered to be the primary effect of MTX interference with DNA replication.

Not surprisingly, common side effects of MTX treatment are myelosuppression and gastrointestinal toxicity. Renal and liver damage may occur as well, in particular after high dose and/or chronic administration of MTX (1, 2). Combination of MTX with several other drugs has proven to increase the therapeutic efficiency and/or the toxicity in vitro and in vivo (1, 2, 4). The clinical significance of many of these interactions remains to be determined (4).

Observations in patients treated for metastatic breast carcinoma (5, 6) or childhood leukemia (7) have shown that ordinary MTX administration during or shortly after N2O anesthesia may provoke severe symptoms, the most pronounced of which are bone marrow depression and gastrointestinal toxicity. This may be explained by the fact that the anesthetic gas N2O inactivates the cobalamin coenzyme of methyltetrahydrofolate:homocysteine methyltransferase or methionine synthase (EC 2.1.1.13). Since the discovery of this effect of N2O on cobalamin, many studies have been performed to elucidate the consequences of prolonged exposure to N2O on cellular folate metabolism. These data were recently reviewed (8, 9). Methionine synthase appears to be essential for the cellular retention of reduced folates because methyltetrahydrofolate (5-methyl-THF), the major extracellular folate, must be demethylated to THF before it can be polyglutamated and converted to the other active coenzyme forms. Prolonged exposure to N2O therefore leads to depletion of cellular folates by loss of 5-methyl-THF and severe disturbance of folate-dependent methylation reactions such as the de novo synthesis of dTMP. Regarding the specific biochemical lesions caused by MTX and N2O it is not surprising that both agents, through inhibition of different metabolic pathways of folate, mutually potentiate their effects on the DNA synthesis of proliferating cells. In vitro studies on human normal bone marrow have proved that MTX and N2O have a synergistic effect on nucleotide synthesis (10). Both in lymphoblast cell lines (11) and in a rat model for myeloid leukemia (12), exposure to N2O increased the sensitivity of proliferating leukemic cells for MTX.

However, no experimental data exist on the toxicological consequences of the N2O-MTX interaction. In the present study we investigated the potentiation of MTX by N2O and the impact of this combination on the tissues which are most vulnerable for MTX toxicity.

MATERIALS AND METHODS

Animals. Male Wistar rats were used at the age of 12–16 weeks (200–250 g). Food and water were supplied ad libitum during all experiments.

Materials. Both sodium methotrexate and calcium 5-formyl-THF were obtained from Lederle (Etten-Leur, The Netherlands). N2O was obtained from Hoekloos (Schiedam, The Netherlands). 5-Methyl-THF was obtained from Sigma (St. Louis, MO).

Exposure of Rats to N2O. Rats were placed in a 40-liter flow chamber through which a gas mixture of 50% N2O/50% oxygen was blown at a rate of 800 ml/min. Moreover, excess carbon dioxide, water, and contaminating volatile compounds were eliminated in a cleaning circuit (13). Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). In one experiment various amounts of N2O (0–50%), replenished by nitrogen (50–0%) and 50% oxygen, were used to study the effect of the inhaled N2O concentration on MTX toxicity. Rats not exposed to N2O were kept in air but otherwise treated identically.

LD50 Study. Groups of 25 rats were exposed to N2O for 12, 24, or 48 h or to air. Directly thereafter each group was divided in 5 clusters of 5 rats which received an i.p. injection of 1 ml of MTX solution in 0.9% NaCl solution, resulting in a dose of 0 (controls), 10, 20, 40, or 80 mg MTX/kg of body weight. Subsequently, they were kept in normal housing conditions for 3 weeks and body weight and number of deaths were checked daily.

Persistence of N2O Induced Effects on MTX Toxicity. For this experiment 25 rats were exposed to N2O for 48 h. Subsequently, groups of 5 rats received 40 mg MTX/kg 0, 6, 12, 24, and 48 h after termination of N2O exposure. Rats were observed for 3 weeks.

Clearance of MTX. The plasma MTX clearance following i.p. injection of 20 mg MTX/kg body weight was monitored in 2 groups of 5 rats.
rats, one of which had been exposed to N\textsubscript{2}O for 48 h. Rats were anesthetized mildly by ether inhalation when blood (0.8 ml) was sampled by orbita plexus puncture to determine plasma concentrations of MTX and 7-hydroxymethotrexate (7-OH-MTX) as described before (14).

Evaluation of Gastrointestinal Toxicity. Gastrointestinal tract toxicity was assessed on the basis of body weight and the occurrence of diarrhea in rats used for determination of LD\textsubscript{50} values. The gastrointestinal tract of rats sacrificed for evaluation of hematological, liver, and kidney toxicity was also inspected macroscopically.

Evaluation of Hematological Toxicity. In the first experiment 2 groups of 5 rats, one of which had been exposed to N\textsubscript{2}O for 48 h, were given i.p. injections of 10 mg of MTX/kg of body weight. At days 1, 4, 7, 13, and 21 after MTX, 0.8 ml blood was collected by orbita plexus puncture. Leukocyte count and hemoglobin concentration were determined with a Sysmex Cellcounter cc-120. Thrombocytes were counted with a Platelet Analyser 800 (Baker). Bone marrow toxicity was investigated in a second experiment. Rats were exsanguinated directly or 48 or 96 h after (N\textsubscript{2}O-)MTX administration (10 mg/kg body weight). The femurs were flushed with 10 ml α-medium containing 0.1% fetal calf serum. The collected bone marrow cells were washed, and 10⁵ cells were plated in triplicate in methylcellulose cultures for assessment of CFU-F (15) on a coagulometer (Amelung), of plasma total protein by the biuret assay and of creatinine and urea on a R. A. 1000 (Technicon).

Evaluation of Hepatotoxicity and Renal Toxicity. EDTA plasma, already sampled for other purposes, was also used for determination of alanine aminotransferase and aspartate aminotransferase on an ACP (17). on a coagulometer (Amelung), of plasma total protein by the biuret assay and of creatinine and urea on a R. A. 1000 (Technicon).

Rescue Study. Three groups of rats were exposed to N\textsubscript{2}O for 48 h and given injections of 40 mg MTX/kg. One group received no further treatment, a second group received 4×10 mg 5-methyl-THF/kg every 12 h starting 24 h after MTX, and a third group received 4×10 mg 5-formyl-THF in a similar schedule. Toxic deaths were recorded for 3 weeks thereafter.

Statistical Analysis. The Mann-Whitney U test was used to evaluate significance of observed differences among treatment groups.

RESULTS

LD\textsubscript{50} Study. In order to quantify the interaction of N\textsubscript{2}O with MTX, a matrix of combinations of both agents was administered to groups of 5 rats. Table 1 shows that N\textsubscript{2}O exposure for 12 h induced lethal toxicity when combined with 40 mg MTX/kg body weight. N\textsubscript{2}O preexposure for 24 or 48 h resulted in an increased lethality of all MTX dosages. Animals usually died between 6 and 10 days after MTX administration. Interpolation of combined data resulted in a LD\textsubscript{50} for i.p. MTX of 60 mg/kg body weight. In combination with prior exposure to N\textsubscript{2}O for 48 h the LD\textsubscript{50} of MTX became approximately 10 mg/kg body weight.

Effect of Various N\textsubscript{2}O Concentrations on MTX Toxicity. Toxicity of 20 mg MTX/kg in combination with 48 h exposure to several N\textsubscript{2}O concentrations was studied. Table 2 shows that already 10% N\textsubscript{2}O increases the lethal toxicity of MTX. Table 2 also reveals that 50% oxygen by itself (in combination with 50% nitrogen) does not enhance MTX toxicity.

Exposure to N\textsubscript{2}O after MTX Administration. Table 3 demonstrates that MTX toxicity is substantially less when N\textsubscript{2}O exposure follows when it precedes MTX (see also Table 1).

Persistence of N\textsubscript{2}O Induced Effect on MTX Toxicity. Table 4 demonstrates that exposure to N\textsubscript{2}O for 48 h prior to 40 mg MTX/kg i.p., leading to 100% morbidity when MTX is given directly after N\textsubscript{2}O exposure, becomes less toxic when the interval between N\textsubscript{2}O and MTX is prolonged to more than 6 h.

MTX Clearance. The plasma clearance of MTX (20 mg/kg i.p.) in control rats (O) and in rats exposed to N\textsubscript{2}O for 48 h (○). Values are the mean ± SD (bars) of 5 rats.

METHOTREXATE TOXICITY AFTER NITROUS OXIDE EXPOSURE

Table 1 Toxicity of i.p. MTX after N\textsubscript{2}O exposure in rats

Clusters of 5 rats were exposed to N\textsubscript{2}O for various periods prior to i.p. MTX (0-80 mg/kg). The number of toxic deaths in each cluster is shown.

<table>
<thead>
<tr>
<th>MTX (mg/kg)</th>
<th>N\textsubscript{2}O preexposure (h)</th>
<th>Toxic deaths</th>
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<tr>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>10</td>
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<tr>
<td>20</td>
<td>0</td>
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<tr>
<td>80</td>
<td>0</td>
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<thead>
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<th>MTX (mg/kg)</th>
<th>N\textsubscript{2}O preexposure (h)</th>
<th>Toxic deaths</th>
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<td>0</td>
<td>12</td>
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<tr>
<td>80</td>
<td>12</td>
<td>1</td>
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Table 2 Effect of various N\textsubscript{2}O concentrations on MTX toxicity

Rats were exposed to gas mixtures containing 0-50% N\textsubscript{2}O for 48 h. Subsequently they received i.p. 20 MTX/kg. The number of toxic deaths is shown.

<table>
<thead>
<tr>
<th>Composition of inhaled gas mixture (% by volume)</th>
<th>Toxic deaths</th>
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<tr>
<td>N\textsubscript{2}O</td>
<td>O₂</td>
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<tr>
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</tr>
<tr>
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<td>50</td>
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<td>10</td>
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<td>25</td>
<td>50</td>
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<td>50</td>
<td>50</td>
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</table>

Table 3 Toxicity of i.p. MTX followed by N\textsubscript{2}O exposure for 48 h

Table 4 Toxicity of i.p. MTX (40 mg/kg) administered directly or 6, 12, 24, or 48 h after N\textsubscript{2}O exposure for 48 h

Table 5 Toxicity of i.p. MTX (40 mg/kg) administered directly or 6, 12, 24, or 48 h after N\textsubscript{2}O exposure for 48 h

Fig. 1. Plasma clearance of MTX (20 mg/kg i.p.) in control rats (O) and in rats exposed to N\textsubscript{2}O for 48 h (○). Values are the mean ± SD (bars) of 5 rats.

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Gastrointestinal Toxicity. The physical condition of all rats used for the LD<sub>50</sub> determinations was checked daily. Usually the first sign of toxicity was loss of body weight followed by diarrhea and bleeding from the nose. If the weight loss had not been regained within 5 days after MTX administration, death usually followed within a few days. In Fig. 2 the average body weights per group on day 5 after MTX are expressed as the percentage of the body weight on the day of MTX administration. Because the rats were only 12 weeks old, absence of toxicity resulted in increased body weight (approximately 15% in 5 days). Macroscopic dissection of rats after death revealed extensive bleeding from ulcers throughout the gastrointestinal tract of rats suffering from diarrhea.

N<sub>2</sub>O alone had no noticeable effect on body weight (data not shown).

Hematological Toxicity. In Fig. 3 the effects of 10 mg MTX/kg with or without exposure to N<sub>2</sub>O (48 h) on the number of peripheral leukocytes and platelets are presented. Both numbers were suppressed when MTX was preceded by N<sub>2</sub>O and reached a nadir approximately 7 days after MTX administration. MTX alone also induced a decrease in the leukocyte count, but 7 days later. The hemoglobin concentration or peripheral blood in the combination treatment group had increased 15% on day 4 after MTX but returned to 10% below normal thereafter (data not shown). In the N<sub>2</sub>O-MTX group 2 rats died on days 8 and 9, respectively; therefore, data from days 14 and 21 are the average of 3 rats.

Because granulocytes and monocytes make up only 10% of peripheral leukocytes in rats, clonogenic assays were performed to investigate the impact of N<sub>2</sub>O-MTX on myelopoiesis. Stromal integrity was measured by culturing CFU-F. Fig. 4B reveals that both MTX and N<sub>2</sub>O-MTX cause a reduction of CFU-GM in bone marrow. However, recovery after MTX alone is much faster than after preexposure to N<sub>2</sub>O as is clear from the total marrow cellularity (Fig. 4A) and the amount of CFU-GM present in the bone marrow on day 4. The cytotoxic effect of N<sub>2</sub>O-MTX on CFU-F (Fig. 4C) is maximal after 4 days, but by this time bone marrow stroma in MTX treated rats has already recovered.

Exposure to N<sub>2</sub>O for 48 h alone causes no changes in the studied parameters afterwards (data not shown).

Hepatotoxicity and Renal Toxicity. Table 5 presents a panel of plasma factors determined to assess hepatic and renal tox-
coenzyme functions in thymidylate and purine synthesis, also exposure (thereby increasing the sensitivity of proliferating cells further increase of lethality from MTX. These observations activity is completely suppressed and the conversion of 5-
ring during cobalamin inactivation. N2O exposure for 12 h
in literature (18, 19) data.

already 10% N2O in the inhaled gas mixture is sufficient to
increase the lethal toxicity of MTX. This is in agreement with Kondo et al. (22) who showed that inhibition of methionine synthase already occurs during exposure to 2% N2O.

Although complete recovery of methionine synthase activity from N2O exposure and subsequent repletion of tissue folates require 3–5 days (22, 23), the mortality from 48 h of exposure to N2O plus 40 mg MTX/kg decreases rapidly if MTX administration is postponed. This suggests that partial restoration of methionine synthase activity is sufficient enough to prevent the synergistic interaction between N2O and MTX.

Specific Organ Toxicity. The effect of N2O-MTX consists of cytotoxic action towards the rapidly dividing cells of mucosa and bone marrow. Little is known about the effect of N2O exposure alone on the mucosa although inactivation of methionine synthase and reduced uptake of dietary folates (without diarrhea) have been demonstrated (24). Combination of nonlethal dosages of N2O and MTX causes liver weight loss of body weight by diarrhea induced dehydration and reduced food intake by the sick animals. The effects of N2O-MTX on hemopoiesis also show synergistic features. Bone marrow depression and pancyclopenia in N2O pretreated rats is more pronounced and the recovery of myelopoiesis is delayed. The effects of N2O-MTX on stromal elements, which are considered to be essential for hematopoiesis, increase up to 4 days, suggesting cytotoxic activity towards pre-CFU-F cells, and may contribute to the sustained bone marrow depression. N2O alone induces an increase of the myeloid progenitors. This agrees with earlier observations in rats in which N2O induced accumulation of the myeloid precursors in the bone marrow (25).

It has been suggested that the hepatotoxicity of chronic MTX administration may be related to a reduction of folate dependent methionine synthesis because this causes hepatic depletion of choline, the necessary precursor for the alternative, betaine dependent methionine synthesis (26, 27). However, N2O-induced inhibition of methionine synthase does not provoke acute liver toxicity from MTX treatment as is clear from the data presented in Table 5. This is probably related to the minor importance of the thymidylate synthase/dihydrofolate reductase pathway for the restoration of THF pools in the nondividing cells of the liver (28).

Clinical Implications and Perspectives. For this study rats were chosen as test animal inasmuch as substantial knowledge is available on both N2O and MTX effects on folate metabolism in rats. Moreover the biochemical events of N2O exposure in the rat closely resemble those in humans (9) although the latter is known to be more vulnerable to the effects of N2O on cell proliferation (29). Comparison of the presented data and reported clinical observations in breast cancer patients (5, 6) reveals some striking similarities. The incidence of unpredictable side effects after postoperative chemotherapy (including MTX) was inversely correlated with the period between N2O

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Toxic deaths</th>
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<tbody>
<tr>
<td>Aspartate aminotransferase (units/liter)</td>
<td>Alanine aminotransferase (units/liter)</td>
</tr>
<tr>
<td>Controls</td>
<td>5</td>
</tr>
<tr>
<td>N2O</td>
<td>5</td>
</tr>
<tr>
<td>10 mg MTX/kg</td>
<td>4</td>
</tr>
<tr>
<td>N2O + 10 mg MTX/kg</td>
<td>5</td>
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</tbody>
</table>

* P < 0.05.
** P < 0.01.
anesthesia and MTX administration. Toxicity could be alleviated with 5-formyl-THF, although the effect of this rescue therapy on the efficiency of the chemotherapeutic protocol used is not yet known.

In our study, 5-formyl-THF was also efficient as rescue agent. However, the exact mechanism by which this drug restores the intracellular reduced folate pool is still the subject of dispute. In vivo this rescue agent is rapidly converted to 5-methyl-THF (30) and several studies have revealed that the latter may also play an important role in the reversal of MTX toxicity (31, 32). In N\textsubscript{2}O treated subjects, however, only unmetabolized 5-formyl-THF is capable of entering the cellular reduced folate pools (11, 33). Because conversion of 5-formyl-THF to 5-methyl-THF differs considerably among the various routes of administration (34), differences in rescue efficacy in case of N\textsubscript{2}O-MTX toxicity may be expected.

Recently it has been suggested that N\textsubscript{2}O reduces the antineoplastic effects of MTX as a consequence of induced toxicity (7). However, Kroes et al. (12) have already demonstrated the chemotherapeutic benefit of MTX when combined with N\textsubscript{2}O exposure in a rat model for myeloid leukemia. Moreover, it is shown that the deleterious effects of MTX on the folate metabolism of fresh human leukemic cells could be significantly enhanced by N\textsubscript{2}O (35) implicating a possible antineoplastic synergism of both drugs as has been suggested before (10).

Conclusion. Exposure to N\textsubscript{2}O prior to MTX administration results in a dose dependent synergistic cytotoxicity towards the proliferating cells of mucosa and bone marrow. The biochemical mechanism through which the synergism between N\textsubscript{2}O and MTX occurs is not yet fully understood but is related to the inhibition of 2 important enzymes in the formation of reduced folates, necessary for both purine and dTMP synthesis. Effective alleviation from resulting toxicity can be achieved by i.v. 5-formyl-THF administration. However, the demonstrated synergistic action of the anesthetic gas N\textsubscript{2}O and MTX on dividing cells merits further investigation of its possible applicability in the treatment of neoplastic diseases.

REFERENCES

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