Toxicity of Methotrexate in Rats Preexposed to Nitrous Oxide

Anton A. M. Ermens, Martijn Schoeter, Lidwien J. M. Spijkers, Jan Lindemans, and Johan Abels

Institute of Hematology Er2202, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

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 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 MTX 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 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 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.

Received 2/17/89; revised 7/5/89; accepted 8/11/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The abbreviations used are: MTX, methotrexate; 7-OH-MTX, 7-hydroxy-methotrexate; THF, tetrahydrofolate; CFU-GM, colony forming unit-granulocyte/macrophage; LD₅₀, dose lethal to 50% of animals.
rats, one of which had been exposed to N₂O 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 LD₅₀ 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 N₂O 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 (N₂O-)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) and CFU-GM (16).

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 5040 (Eppendorf), of fibrinogen, according to the method of Clauss (17) on a coagulometer (Amelung), of plasma total protein by the biuret assay and of creatinine and uric acid on a R. A. 1000 (Techiron).

Rescue Study. Three groups of rats were exposed to N₂O for 48 h and given injections of 40 mg MTX/kg. One group received no further treatment, a second group received 4 x 10 mg 5-methyl-THF/kg every 12 h starting 24 h after MTX, and a third group received 4 x 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

LD₅₀ Study. In order to quantify the interaction of N₂O with MTX, a matrix of combinations of both agents was administered to groups of 5 rats. Table 1 shows that N₂O exposure for 12 h induced lethal toxicity when combined with 40 mg MTX/kg body weight. N₂O 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 LD₅₀ for i.p. MTX of 60 mg/kg body weight. In combination with prior exposure to N₂O for 48 h the LD₅₀ of MTX became approximately 10 mg/kg body weight.

Effect of Various N₂O Concentrations on MTX Toxicity. Toxicity of 20 mg MTX/kg in combination with 48 h exposure to several N₂O concentrations was studied. Table 2 shows that already 10% N₂O increases the lethality of MTX. Table 2 also reveals that 50% oxygen by itself (in combination with 50% nitrogen) does not enhance MTX toxicity.

Exposure to N₂O after MTX Administration. Table 3 demonstrates that MTX toxicity is substantially less when N₂O exposure follows than when it precedes MTX (see also Table 1).

Persistence of N₂O Induced Effect on MTX Toxicity. Table 4 demonstrates that exposure to N₂O for 48 h prior to 40 mg MTX/kg i.p., leading to 100% morbidity when MTX is given directly after N₂O exposure, becomes less toxic when the interval between N₂O and MTX is prolonged to more than 6 h.

MTX Clearance. The plasma clearance of MTX was studied after exposure to N₂O for 48 h. Fig. 1 reveals that elimination of the drug from the systemic circulation does not change after exposure to N₂O. Only trace amounts of 7-OH-MTX, the major metabolite of MTX, were detected in plasma of both treatment groups.
Gastrointestinal Toxicity. The physical condition of all rats used for the LD₅₀ 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 ulcerations throughout the gastrointestinal tract of rats suffering from diarrhea.

N₂O 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 N₂O (48 h) on the number of peripheral leukocytes and platelets are presented. Both numbers were suppressed when MTX was preceded by N₂O 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 N₂O-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 N₂O-MTX on myelopoiesis. Stromal integrity was measured by culturing CFU-F. Fig. 4B reveals that both MTX and N₂O-MTX cause a reduction of CFU-GM in bone marrow. However, recovery after MTX alone is much faster than after preexposition to N₂O 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 N₂O-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 N₂O 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. Inclusion of N2O also in the test gas mixture, may result in enhanced polyglutamation of the drug (20).

Moreover, increased activity of folylpolyglutamate synthase (9, 20) as a consequence of the induced folate depletion (8, 9). Prolonging N2O exposure to 48 h results in massive depletion of rat tissue folates within 48 h of N2O exposure alone on the mucosa although inactivation 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 damage, loss of body weight by diarrhea, induced dehydration and reduced food intake by the sick animals. The effects of N2O-MTX on hematopoiesis also show synergistic features. Bone marrow depression and pancytopenia 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 synthesis 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...
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).

N2O 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 efficiency in case of N2O-MTX toxicity may be expected.

Recently it has been suggested that N2O reduces the antineoplastic effects of MTX as a consequence of induced toxicity (7). However, Kros et al. (12) have already demonstrated the chemotherapeutic benefit of MTX when combined with N2O 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 N2O (35) implicating a possible antineoplastic synergism of both drugs as has been suggested before (10).

Conclusion. Exposure to N2O 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 N2O 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 N2O and MTX on dividing cells merits further investigation of its possible applicability in the treatment of neoplastic diseases.

REFERENCES

Toxicity of Methotrexate in Rats Preexposed to Nitrous Oxide


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/22/6337

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.