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Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide

A. C. M. Kroes, J. Lindemans, M. Schoester, and J. Abels

Institute of Hematology, Erasmus University, Rotterdam, The Netherlands

Summary. Exposure to nitrous oxide interferes selectively with the coenzyme function of vitamin B12 and causes inactivation of methionine synthetase, with subsequent impairment of folate metabolism and reduction of cellular proliferation. In a rat leukemia model (BNML) we investigated the combined administration of nitrous oxide, inactivating vitamin B12, and methotrexate (MTX), a folate antagonist inhibiting the enzyme dihydrofolate reductase. Through different mechanisms, both agents decrease the availability of tetrahydrofolate, and subsequently of other reduced folates, with increased impairment of folate-dependent synthesis of thymidylate. Effects on leukemic growth and on hematological values in rats demonstrated enhancement of the therapeutic effect of MTX by exposure to nitrous oxide. With several treatment schedules, the results of combined treatment were seen to be better than additive when compared with the effects of single agents. In particular, pretreatment of leukemic rats with nitrous oxide for 3 days before administration of MTX appeared effective. With higher doses of MTX, concomitant exposure to nitrous oxide even resulted in toxic effects. These findings were in accordance with the results of some metabolic studies performed in leukemic rats. De novo synthesis of thymidylate in leukemic cells, when studied by means of the deoxyuridine suppression test, showed the most severe disturbance with combined treatment consisting in MTX (0.5 mg/kg) and nitrous oxide pretreatment for 3 days. Intracellular levels of folate and dTTP were lowest with 2 and 3 days' pretreatment before MTX, respectively. It is concluded that this interaction of nitrous oxide and MTX can result in enhanced metabolic and therapeutic effects of low doses of MTX. Inactivation of vitamin B12 appears to be a potentially useful addition in cancer chemotherapy.

Introduction

The similarity of hematological disturbances caused by deficiency of either folic acid or cobalamin (vitamin B12) is well established. Both vitamins are involved in pathways essential in the synthesis of nucleotides, and consequently of DNA. The important function of folic acid in cellular proliferation is also reflected in the striking cytostatic activity of folate antagonists of which methotrexate (MTX) is the best known example. This antimetabolite is widely used in cancer chemotherapy [14]. In contrast, until recently, the role of vitamin B12 in neoplastic growth has remained unclear, because no effective method was available to interfere with its coenzyme function. In 1978, however, it was recognized that selective inactivation of vitamin B12 could be achieved with exposure to the anesthetic gas nitrous oxide, or N_2O [1, 7]. Megaloblastic hematopoiesis after prolonged exposure to nitrous oxide had been observed much earlier [21], and a chemical interaction of nitrous oxide with complexes of cobalt was also known for some time [2]. It appeared that a specific oxidative action of nitrous oxide on the cobalt moiety of vitamin B12 caused a nearly complete inactivation of the methylcobalamin-requiring enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase (E. C. 2.1.1.13). Nitrous oxide effectively established a state of functional deficiency of vitamin B12, with severely disturbed folate metabolism [6] and toxic effects on hematopoiesis [38]. Methionine synthetase is essential in folate metabolism, because it provides the only pathway by which 5-methyltetrahydrofolate, the major extracellular folate, can be converted into tetrahydrofolate (THF). THF and other, subsequently formed, reduced folates can be converted into folylpolyglutamates, which are then retained in the cell and are important coenzymes in one-carbon transfer reactions. De novo synthesis of thymidylate requires such folate-dependent methylation. MTX also interferes with folate metabolism by limiting the generation of THF, but through a different mechanism. Its inhibition of dihydrofolate (DHF) reductase prevents the reconversion of DHF to THF.

It has been shown that the effect of nitrous oxide on vitamin B12 can be utilized to reduce growth of leukemia in vitro [18] and in vivo, in rats [20]. This effect is associated with a disturbance of folate-dependent de novo synthesis of thymidylate. Therefore, it appears that nitrous oxide and MTX, through inhibition of different pathways, ultimately may have similar effects on folate metabolism. These observations suggest that the inactivation of vitamin B12 can modify, and possibly enhance, the efficacy of MTX. In vitro studies on human bone marrow have demonstrated a synergistic effect of MTX and nitrous oxide

Offprint requests to: A. C. M. Kroes, Institute of Hematology, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

Abbreviations used in this paper: MTX, methotrexate; THF, tetrahydrofolate; DHF, dihydrofolate; dTTP, deoxythymidine triphosphate.

with regard to impairment of nucleotide synthesis [17]. The purpose of the present study is to investigate the influence of nitrous oxide on the effects of MTX in vivo, using a rat leukemia model: the Brown Norway myeloid leukemia (BNML). This transplantable acute promyelocytic leukemia has been described in detail elsewhere [12] and is considered to be a suitable model for chemotherapeutic studies [40]. In addition to experiments intended to assess effects on leukemic growth, a number of metabolic studies were performed in leukemic rats, to investigate some effects of treatment on folate metabolism. These studies included deoxyuridine suppression tests, and determinations of intracellular folate and deoxythymidine triphosphate (dTTP) levels.

Materials and methods

Animals. Male rats of the Brown Norway inbred strain were used at the age of 12–16 weeks (body weight 200–275 g). Food and water were supplied ad libitum during the experiments.

Brown Norway myeloid leukemia (BNML). Cryopreserved leukemic cells were kindly provided by Dr. A. Hagenbeek from the Radiobiological Institute (TNO), Rijswijk, The Netherlands, where this transplantable rat leukemia model was developed. Origin, classification and proliferation kinetics were described elsewhere [12]. For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. A standard dose of 10^7 cells suspended in Hanks' balanced salt solution was injected IV, which leads to progressive leukemic infiltration of bone marrow, spleen and liver, with death after 20–24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of tumor load and, along with hematological determinations, can be used effectively to assess effects of chemotherapy [12]. To avoid a gradual change in growth properties, serial transplantations were limited to only two passages, after which spleen cells were used from rats freshly inoculated with cells from a cryopreserved stock.

Treatment with nitrous oxide and MTX. Leukemic rats were treated according to different schedules in groups of at least four. Exposure to nitrous oxide was carried out in a 40-l flow chamber into which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water, and contaminating volatile compounds were eliminated in a cleaning circuit, essentially as described by Rupreht and Dzoljic [37]. Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. Sodium methotrexate (Ledertrexate SP, from Lederle) was injected IP. Rats not receiving MTX received injections of 0.15 *M* NaCl IP instead.

Evaluation of leukemic growth. Experiments intended to assess effects on leukemic growth were all evaluated by the same procedure. To allow a simultaneous investigation of several aspects of leukemia these experiments were terminated after a fixed period of 18 days (in some instances: 19 days) of leukemia, just before death from leukemia was to be expected. Rats were killed by exsanguination, after recording of body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically, and in some experiments differential blood cell counts were done. Plasma levels of vitamin B12 were measured in a competitive radioisotope binding assay using purified intrinsic factor [22]. Normal values for organ weights, leukocyte counts and plasma vitamin B12 were derived from at least 12 comparable nonleukemic Brown Norway rats.

Metabolic studies. In separate experiments, rats with advanced leukemia were treated for short periods, after which leukemic cells were used in metabolic studies. Three similar experiments were carried out separately. In each, eight leukemic rats inoculated at day 0 were divided in four pairs. These pairs were treated with nitrous oxide for 3, 2, 1, or 0 days. Immediately afterwards one rat in each pair received MTX, 0.5 mg/kg IP, the other rat receiving saline only. At 18 h after administration of MTX, on day 15 of leukemia, leukemic cells were obtained from the spleens of all rats, washed, and resuspended in Hanks' balanced salt solution. These cell suspensions were counted electronically and used in deoxyuridine suppression tests and determinations of intracellular folate and dTTP.

Deoxyuridine suppression test. This test demonstrates impaired de novo synthesis of thymidylate. ^{3}H-Thymidine incorporation into DNA is measured with and without addition of deoxyuridine. Deoxyuridine will suppress incorporation of ^{3}H-thymidine in DNA if it can be converted to thymidylate through folate-dependent methylation. This suppression is reduced by vitamin B12 or folate deficiency [42], inactivation of vitamin B12 by nitrous oxide [28], and treatment with other agents interfering with de novo synthesis of thymidylate [5].

Leukemic spleen cells (approx. 5×10^6 per test) were used from rats of various groups, as described above. The test was carried out essentially according to Metz [30], with some modification as described elsewhere [20]. Deoxyuridine (Sigma, St. Louis, USA) was used in a concentration of 0.1 mmol/l. All incubations were performed in triplicate. Incorporation of ^{3}H-thymidine (0.3 μCi per test, specific activity 25 Ci/mmol, from Amersham, UK) is expressed as a percentage of the maximal incorporation, measured in each case in incubations without addition of deoxyuridine.

Intracellular folate. In suspensions of leukemic spleen cells, intracellular folate content was determined. After centrifugation a pellet of approx. 10^8 cells was resuspended in a total volume of 1 ml 10% (≈1.3 mol/l) mercaptoethanol, heated in a water bath of 100 °C for 5 min, and cooled. Hog kidney polyglutamate hydrolase, prepared as described by McMartin et al. [29], was added and allowed to incubate at room temperature for 2 h. The samples were frozen at −20 °C until assayed. After thawing, the extracts were centrifugated (1500 *g* for 10 min at 4 °C), and aliquots of the supernatants were used in a folate radioisotope dilution assay, essentially as described by Dunn and Foster [11], with ^{125}I-folic acid (Becton Dickinson, Orangeburg, N.Y., USA) as a tracer, and β-lactoglobulin (Sigma, St. Louis, USA) as a folate binder. 5-Methyl-THF was used as a standard, and results are expressed as picomoles of folate per 10^6 cells.

Intracellular dTTP. In leukemic cells obtained from rats as described above, dTTP was determined using the DNA polymerase assay system originally developed by Solter and Handschumacher [39], with the modifications and corrections published by Hunting and Henderson [13]. A different extraction method was used, however. After being washed once in Hanks' balanced salt solution, cell suspensions were centrifuged and the supernatant was removed. To the pellet of about 10^8 cells, 1.2 μg cyano [^{57}Co]cobalamin, or 10^5 dpm, (Amersham, UK) was added, as an internal standard for cell quantities in the assay. The pellet was then extracted with 5 ml ice-cold 60% methanol and stored at −20 °C until assayed. After centrifugation the supernatant was dried using a rotary evaporator at 25 °C, dissolved in 50 m*M* potassium cacodylate and subsequently used in the DNA polymerase catalyzed assay. DNA polymerase I from *E. coli*, dATP, dTTP, and poly(d(A,T)) were all obtained from Boehringer Mannheim (FRG) and ^{3}H-dATP and ^{3}H-dTTP were from Amersham (UK). Procedures, and calculated corrections for dilution of specific activities by the endogenous nucleotides were carried out as described elsewhere [13]. Concentrations of dTTP are expressed as picomoles per 10^6 cells, based upon counts of the internal standard and cell concentration of the initial suspension.

Results

Effects of growth of leukemia in rats

In Table 1, results of six experiments with several different treatment schedules are summarized. In all these experiments, one group of leukemic rats treated with MTX only is compared with one or more groups of rats treated with a combination of MTX and exposure to nitrous oxide. Most experiments included a group receiving nitrous oxide continuously throughout the treatment period (day 7 to day 18 or 19 of leukemia). In other groups, nitrous oxide treatment was limited to either 3 days before administration of MTX (pretreatment) or 3 days after administration of MTX (post-treatment). This is illustrated in Fig. 1, which shows the treatment schedules as applied in experiment 3 of Table 1. In addition, all experiments included rats receiving no treatment or nitrous oxide only (days 7–19), without MTX. Cumulative results in these rats are also shown in Table 1. From the data in Table 1, it follows that low doses of MTX alone do not have substantial effects on leukemic growth. In all experiments the addition of nitrous oxide enhanced the therapeutic effects of MTX. The differences between rats treated with MTX only and rats treated with both MTX and nitrous oxide (continuously) are statistically significant according to Wilcoxon's nonparametric rank sum test. For all three parameters of leukemia, results did not overlap, yielding p-values of 0.05 or less, in any of the experiments 1–4. In addition, the results of combined treatment appeared to be better than additive in these experiments, with the possible exception of experiment 1, in which the lowest dose of MTX was administered. In other cases, the reduction of leukemic growth obtained with combined treatment is often greater than the added effects of the two single agents, which is indicative of a synergistic interaction. From experiments 3 and 5 it can be derived that by far the major part of the added effect is contributed by the period of exposure to nitrous oxide before administration of MTX (pretreatment schedules). In experiment 6 the highest dose of MTX was used (4 mg/kg). In this experiment, rats treated with both MTX and nitrous oxide (continuously) died prematurely at 14–16 days after inoculation, without any evidence of leukemia. Spleen and liver weights at autopsy were subnor-

Table 1. Effects of treatment on growth of leukemia

Expt no	Treatment	No. of. rats	Spleen weight[a] (g) mean ± SEM	Liver weight[b] (g) mean ± SEM	Leukocytes[c] (10^9) mean ± SEM
	None (untreated controls)	22	3.90 ± 0.09	17.39 ± 0.45	24.8 ± 2.1
	N_2O, continuous (days 7–18/19)	14	2.89 ± 0.09	14.33 ± 0.44	11.1 ± 1.0
1	MTX, 1 × 0.5 mg/kg (day 11)	5	3.64 ± 0.10	15.60 ± 0.69	18.1 ± 1.2
	+ N_2O, continuous (days 7–19)	5	2.34 ± 0.10	12.50 ± 0.55	7.7 ± 0.7
2	MTX, 2 × 0.5 mg/kg (days 10 and 14)	4	3.29 ± 0.09	12.95 ± 0.52	16.6 ± 1.3
	+ N_2O, continuous (days 7–18)	4	1.43 ± 0.14	9.76 ± 0.47	3.8 ± 0.5
3	MTX, 2 × 0.5 mg/kg (days 10 and 16)	4	3.38 ± 0.18	16.04 ± 0.78	20.2 ± 1.4
	+ N_2O, 2 × 3 days after MTX (days 10–13/16–19)	4[d]	3.32	17.05	20.0
	+ N_2O, 2 × 3 days before MTX (days 7–10/13–16)	4	2.37 ± 0.11	13.08 ± 0.44	8.1 ± 0.6
	+ N_2O, continuous (days 7–19)	4	1.97 ± 0.17	11.68 ± 0.81	7.0 ± 1.1
4	MTX, 1 × 2 mg/kg (day 10)	4	3.44 ± 0.14	14.00 ± 0.46	20.8 ± 2.9
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	2.58 ± 0.24	12.59 ± 0.94	7.7 ± 1.0
	+ N_2O, continuous (days 7–19)	4	1.95 ± 0.08	11.47 ± 0.17	6.1 ± 0.6
5	MTX, 2 × 1 mg/kg (days 6 and 14)	4	3.19 ± 0.22	12.37 ± 0.59	15.9 ± 1.8
	+ N_2O, 2 × 3 days after MTX (days 6–9/14–17)	4	3.37 ± 0.10	14.70 ± 0.80	14.0 ± 0.7
	+ N_2O, 2 × 3 days before MTX (days 3–6/11–14)	4	2.31 ± 0.14	10.64 ± 0.51	10.8 ± 1.2
6	MTX, 1 × 4 mg/kg (day 10)	4	2.85 ± 0.32	13.30 ± 0.74	14.9 ± 1.1
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	1.47 ± 0.43	10.12 ± 0.49	4.2 ± 1.4
	+ N_2O, continuous (from day 7)	4	all rats died (without evidence of leukemia)		

[a] Normal spleen weight in comparable nonleukemic BN rats: 0.45 ± 0.07 g
[b] Normal liver weight in comparable nonleukemic BN rats: 8.25 ± 0.99 g
[c] Normal value of leukocyte count in nonleukemic BN rats: 3.9 ± 0.4 × 10^9/l
[d] 2 rats dying prematurely of leukemia are not included

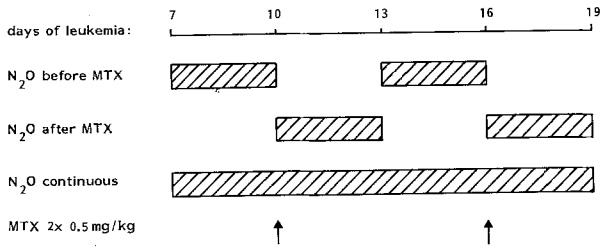

Fig. 1. Treatment of leukemic rats with nitrous oxide (N_2O) and methotrexate (MTX), as given in experiment 3 of Table 1. Periods of exposure to nitrous oxide are *shaded*, and *arrows* indicate time of administration of MTX. Rats receiving MTX or nitrous oxide only and untreated rats were also included

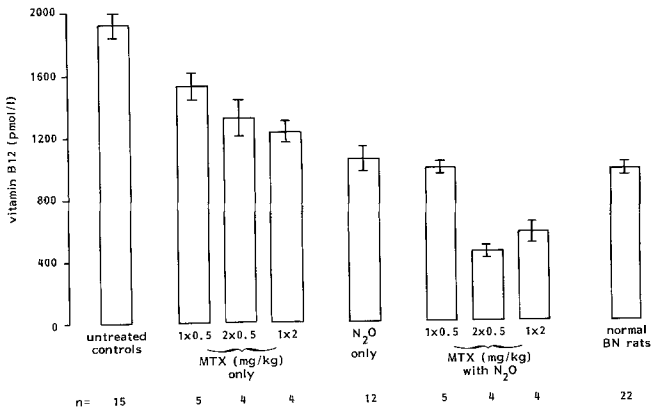

Fig. 2. Plasma levels of vitamin B12 in leukemic rats treated in several experiments with different doses of MTX. Groups with combined treatment received nitrous oxide continuously (days (7–18/19 of leukemia). Values in normal (nonleukemic) BN rats are also shown. *Bars* indicate SEM

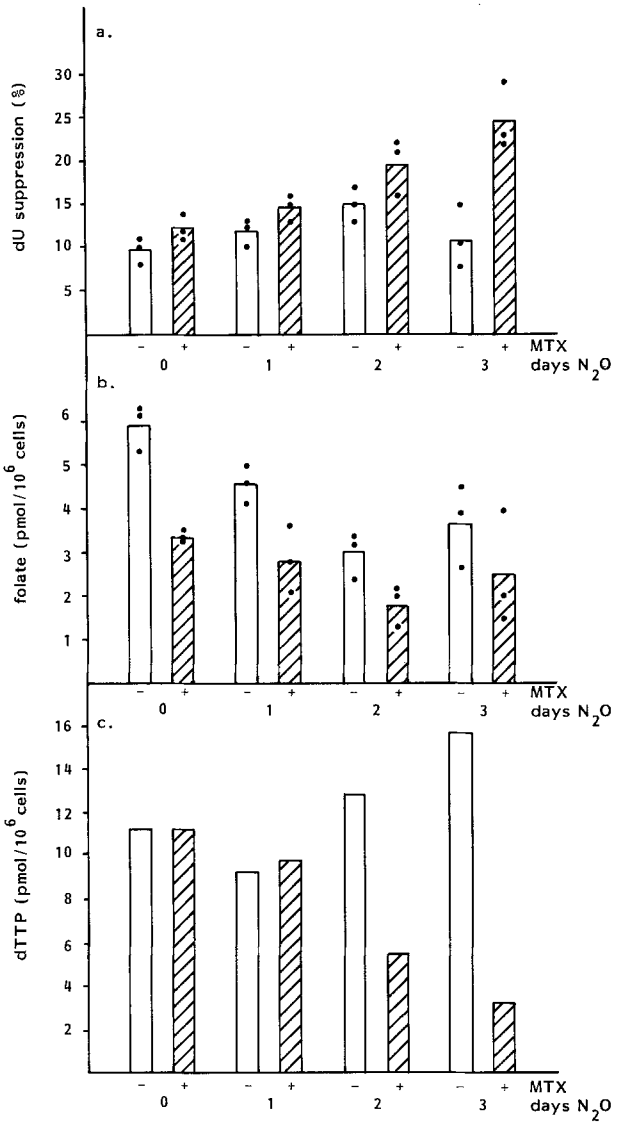

Fig. 3a–c. Results of metabolic experiments with leukemic spleen cells after in vivo treatment of rats with 0–3 days' exposure to nitrous oxide, followed or not by administration of MTX (0.5 mg/kg): **a** deoxyuridine suppression values, expressed as percentages of maximal incorporation of ^{3}H-thymidine, in incubations without deoxyuridine; **b** intracellular levels of folate (pmol/10^6 cells); **c** intracellular levels of dTTP (pmol/10^6 cells). *Shaded columns* indicate MTX treatment. The experiments were performed 18 h after administration of MTX and/or exposure to nitrous oxide

mal, and body weight was seriously reduced (77% of weight before treatment). This was considered to be a toxic effect of treatment. In other experiments, however, no toxicity was observed and nitrous oxide treatment was well tolerated by rats without evident effects on consciousness. Even with combined treatment, loss of body weight was always less than 10%.

In some experiments differential blood cell counts were made, which demonstrated that the observed reductions in peripheral leukocyte counts were also accompanied by a striking relative decrease of leukemic cells (promyelocytes), as shown in Table 2.

Plasma levels of vitamin B12, as determined in these experiments, are presented in Fig. 2. Compared with normal BN rats, vitamin B12 levels in untreated leukemic controls are very high. With treatment these levels are reduced, as is shown for three doses of MTX. Exposure to nitrous oxide alone has marked effects on the vitamin B12 level in plasma, but much lower and even subnormal levels are found after combined treatment.

Metabolic effects

In separate experiments, pairs of leukemic rats were treated with nitrous oxide for 0, 1, 2, or 3 days. One rat of each pair subsequently received MTX (0.5 mg/kg), and 18 h afterwards leukemic cells of all rats were used for deoxyuridine suppression tests and determinations of intracellular folate and dTTP. This experimental procedure was repeated twice, and results of all rats used in this study are presented together (Fig. 3).

Deoxyuridine suppression tests (Fig. 3a) show increased disturbance with longer duration of nitrous oxide exposure before the administration of MTX. Higher values in this test indicate a decreased ability of deoxyuridine to suppress the uptake of ^{3}H-thymidine, demonstrating impaired de novo synthesis of thymidylate. MTX without nitrous oxide treatment is clearly less effective: mean values are 12% without nitrous oxide and 25% with 3 days of pretreatment. Figure 3b shows the results of intracellular folate determinations. Both nitrous oxide and MTX de-

Table 2. Effect of treatment on differential blood cell counts

Treatment	No. of. rats	Promyelocytes[a] (leukemic cells)	Lymphocytes[a]	Neutrophils[a]
None (untreated controls)	8	17.4±1.9	74.4±2.0	8.3±1.6
N_2O, continuous (days 7–18/19)	6	13.5±3.4	83.2±2.7	3.3±0.8
MTX, 2×0.5 mg/kg (days 10 and 14)	4	13.5±2.2	74.5±1.8	12.0±1.0
+ N_2O, continuous (days 7–18)	4	0.8±0.2	98.3±0.6	1.0±0.4
MTX, 1×2 mg/kg (day 10)	3	30.6±1.5	65.0±1.5	4.3±1.2
+ N_2O, 1×3 days before MTX (days 7–10)	3	8.0±6.0	90.3±6.2	1.7±0.3
+ N_2O, continuous (days 7–19)	4	3.3±0.9	95.5±1.0	1.3±0.2
MTX, 1×4 mg/kg (day 10)	4	13.3±3.9	81.5±5.2	5.3±1.6
+ N_2O, 1×3 days before MTX (days 7–10)	4	2.0±0.7	97.5±0.9	0.5±0.3
Normal BN rats (nonleukemic)	5	0	91.4±1.4	6.0±1.5[b]

[a] Expressed as percentages of the total number of nucleated cells, counting 200 cells, with indication of SEM
[b] With additional 0.4% eosinophils and 2.2% monocytes

creased folate levels, and the lowest levels are found with combined treatment after 2 days of nitrous oxide exposure. With 3 days of nitrous oxide exposure it appears that folate contents recover to some extent. Figure 3c, presenting levels of intracellular dTTP, is based upon the last metabolic experiment only, involving eight rats, in contrast to the other results. From this experiment it can be concluded that dTTP levels in leukemic cells are lowest with combined treatment consisting in MTX and 2 or 3 days' pretreatment with nitrous oxide. Nitrous oxide treatment alone appears to cause increased levels of dTTP, but it should be emphasized that these and other values were obtained 18 h after exposure and some recovery may have occurred.

Discussion

Nitrous oxide interferes specifically with the coenzyme function of vitamin B12 and thereby inactivates methionine synthetase [8]. This severely affects folate metabolism, because methionine synthetase is required in the conversion of 5-methyl-THF, the predominant extracellular folate, into THF. This conversion is essential for folate coenzyme functions and also for the cellular retention of folates. In contrast to other reduced folates, 5-methyl-THF is not a substrate for synthesis of folylpolyglutamates, as is evident from metabolic studies [23, 27] and from properties of the purified enzyme [31]. The synthesis of folylpolyglutamates is decreased by nitrous oxide [27, 34, 35], which explains the serious cellular depletion of folates occurring on exposure [25]. The decreased availability of reduced folates impairs folate-dependent synthesis of thymidylate, and subsequently reduces DNA synthesis and cellular proliferation. The inhibition of leukemic growth by nitrous oxide in vitro has been demonstrated [18], and in a previous study we described in vivo antileukemic effects of nitrous oxide in rats [20]. We also showed that these effects were enhanced in combined treatment with cycloleucine, which inhibits the conversion of methionine into S-adenosylmethionine and indirectly interferes with folate metaboilism [19]. A number of studies have investigated the combination of nitrous oxide, as a vitamin B12-inactivating agent, and MTX, as a typical folate antagonist. Kano et al. [17] have demonstrated synergistic effects with regard to inhibition of thymidylate synthesis in normal human bone marrow. Black and Tephly [4] compared metabolic effects of both agents in rat liver cells. The inhibition of methionine synthetase by nitrous oxide considerably decreased the availability of THF while the inhibition of DHF reductase by MTX was much less effective. This difference probably can be explained by the low activity of thymidylate synthetase in liver cells. Dudman et al. [10] found increased sensitivity of leukemic cell lines to MTX with nitrous oxide-induced inhibition of methionine synthetase, which was further exploited by the use of 5-methyl-THF instead of 5-formyl-THF as a rescue agent.

The present study shows effects of combined therapy with nitrous oxide and MTX on in vivo growth and metabolism of rat leukemia. The exposure of rats to nitrous oxide enhanced inhibition of leukemic growth by low doses of MTX. A period of exposure before the administration of MTX (pretreatment) appeared essential for this effect. The effects on leukemic infiltration in spleen and liver correlated well with hematologic results (total and differential leukocyte counts) and with determinations of vitamin B12 in plasma. An interesting feature of this leukemia is a continuous rise of vitamin B12 levels in the course of leukemic growth [20], which is also observed in human acute promyelocytic leukemia [36]. Plasma vitamin B12 can be used as a kind of tumor marker, and treatment leads to reduced levels. The particularly striking decrease caused by nitrous oxide, however, is explained by its specific effect on cobalamin. Analogues of cobalamin are formed after oxidation and are excreted rapidly [33]. In addition to effects on leukemic growth, some metabolic aspects of this interaction were studied in leukemic cells after in vivo treatment of rats. On the basis of the results discussed before, pretreatment with nitrous oxide was administered for periods up to 3 days. Deoxyuridine suppression tests demonstrated increased disturbance of de novo synthesis of thymidylate with longer duration of nitrous oxide pretreatment before MTX. Treatment with a single agent was clearly less effective. Simultaneous determination of intracellular folates, to confirm the presumed cellular folate depletion, indeed showed a decrease in folate content, particularly with combined treatment. Some comments on these folate measurements are warranted, however. In the radioisotope competitive binding assay the

β-lactoglobulin folate binder is used, and probably not all relevant folate derivatives show the same affinity for this binder. Our own observations (not included in this study) indicated that 5-methyl-THF has slightly lower affinity than THF. As these folates are intracellularly predominant [4, 29], the observed decrease in total folate content could also be explained, at least partially, by a shift of folates from THF toward 5-methyl-THF. This, however, is functionally about equivalent, because the conversion of 5-methyl-THF is blocked on nitrous oxide exposure, as discussed before. Moreover, the results of this folate radioassay in the measurement of tissue folates were recently found to be comparable to microbiological assays [26]. Changes in cellular folate on nitrous oxide exposure in our study are similar to earlier observations [25, 35], including an indication of recovery at more than 2 days of exposure, an adaptive mechanism which is not yet understood. Finally, decreased dTTP levels show that the impairment of folate-dependent synthesis of thymidylate has noticeable effects on this direct precursor of DNA. Considered together, the findings in metabolic experiments are in accordance with the results of studies directed at inhibition of growth, showing maximum effects after about 3 days of pretreatment with nitrous oxide before MTX.

Several potential mechanisms could explain the results of this interaction. First, a reduction of intracellular folates by nitrous oxide pretreatment can obviously induce greater susceptibility to folate antagonists [16]. A second mechanism is closely related and concerns the reduced synthesis of folylpolyglutamates on nitrous oxide exposure. Polyglutamation of MTX, leading to increased activity and cellular retention [15], occurs in competition with normal folate substrates [3, 16]. In cells pretreated with nitrous oxide a larger proportion of MTX may be converted into polyglutamate forms. A third potential mechanism to explain the results relates to the observed marked increase in activity of thymidylate synthetase, after nitrous oxide exposure of up to 3 days [9]. It is well established that thymidylate synthetase has a central role in mediating the cytotoxic effects of MTX [32, 41], because this enzyme actually causes THF depletion by its conversion of reduced folates into DHF. Increased activity of this enzyme, as a possible adaptation to nitrous oxide treatment, thus may result in enhanced effects of MTX. All these mechanisms can explain the observed importance of pretreatment with nitrous oxide before MTX.

The results presented in this study demonstrate that in vivo metabolic manipulation of leukemic cells with nitrous oxide can enhance metabolic and therapeutic effects of MTX. The clinical relevance of this interaction is illustrated by the recent observation of increased toxicity of adjuvant chemotherapy involving MTX started directly postoperatively [24], which the authors attributed to inactivation of vitamin B12 by nitrous oxide during anesthesia. Our findings lend support to this suggestion. In experimental chemotherapy, the inactivation of vitamin B12 represents a new method to enhance activity of MTX. The relative contributions of increased polyglutamation of MTX, and increased activity of thymidylate synthetase, should be subjects of further research. It also remains to be demonstrated that the effects described are applicable to human leukemia, but it is known that man is more susceptible to vitamin B12 deficiency than any animal [6]. Finally, these results indicate the significance of vitamin B12 in leukemic proliferation and the value of vitamin B12-related metabolism as an additional target in cancer chemotherapy.

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