Effect of Methotrexate with 5-Methyltetrahydrofolate Rescue and Dietary Homocystine on Survival of Leukemic Mice and on Concentrations of Liver Adenosylamino Acids

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ABSTRACT

We have increased significantly the survival time of DBA/2 mice bearing methionine-dependent L1210 or L5178Y leukemia cells by i.p. administration of lethal doses of methotrexate (five daily doses of 25 mg/kg body weight) followed by rescue with 5-methyl tetrahydrofolate (five daily doses of 20 mg/kg body weight). The mice were maintained on a semipurified choline- and cyst(e)ine-free diet containing 0.32% L-methionine. We further increased significantly the survival time of the treated animals bearing L5178Y cells, but not those bearing L1210 cells, by substitution of 0.86% DL-homocysteine for the methionine in the diet.

We have examined the effects of both diets in mice treated with methotrexate and 5-methyl tetrahydrofolate, singly and in combination, on the concentrations of S-adenosylmethionine and S-adenosylhomocysteine in the liver, a tissue highly active in the metabolism of these amino acids. The substitution of homocysteine for methionine in the diet of untreated animals led to a significant increase in S-adenosylhomocysteine and decrease in S-adenosylmethionine in the liver, with a resultant profound decrease in the ratio of S-adenosylmethionine to S-adenosylhomocysteine which was not further altered significantly by administration of methotrexate.

INTRODUCTION

Observations that certain normal and malignant cells exhibit differences in the metabolism of methionine (17, 31, 40) have generated interest in the metabolic pathways involving methionine as possible sites of intervention in the chemotherapy of cancer (28). Chart 1 outlines the metabolism in mammalian cells (13) of methionine, which plays a key role in cell division through participation in the synthesis of polyamines and the initiation of protein synthesis. Methionine also figures prominently in the metabolism of THF, a cofactor which is essential for the biosynthesis of the purines and thymidylate required for the synthesis of DNA and RNA also required for cell division. The ability of antifolates, such as MTX, to inhibit the rapid division of neoplastic cells has long been recognized (11) and results from inhibition by this drug of dihydrofolate reductase with subsequent depletion of cellular THF. The chemotherapeutic efficacy of MTX against murine leukemia has been improved by administration of lethal doses of the drug which are tolerated by the host when followed by “rescue” with 5-formyl-THF (citrovorum factor) (16), a fully reduced form of folate which bypasses the need for the inhibited enzyme. As shown in Chart 1, 5-formyl-THF (citrovorum factor) can be converted to other forms of THF carrying d units in the CH oxidation state, one of which (10-formyl-THF) participates in purine biosynthesis and, after reduction to methylene-THF, in the biosynthesis of thymidylate, thus supporting cell division in the face of significant concentrations of MTX. Protocols using high-dose MTX: citrovorum factor rescue have been adapted successfully to the treatment of cancer in humans (29).

Examination of Chart 1 reveals the importance of Reaction A in normal cells in (a) the recycling of homocysteine to methionine and (b) converting the major circulating form of reduced folate, 5-methyl-THF (20), to THF which can then participate in the biosynthesis of the purines and thymidylate essential for cell division. In the presence of MTX, there is an accumulation of dihydrofolate, generated by the synthesis of TMP, with a resultant decrease in the concentration of 5-methyl-THF (33), impeding Reaction A.

In the work reported, we have sought to manipulate the relationships shown in Chart 1 to produce a selective attack on the growth of the neoplastic cells, L1210 and L5178Y, injected i.p. into mice. These cells do not grow in vitro when homocysteine is substituted for methionine in the culture medium and thus are said to be methionine dependent (7, 17). We fed mice a semipurified diet in which DL-homocystine was substituted for L-methionine, injected the tumor cells, and later administered lethal doses of MTX in conjunction with 5-methyl THF, which others (2, 37) have found to provide effective “rescue” from the antifolate. Both the homocystine diet and the form of rescue were designed to provide adequate nutrition for host cells, which are capable of meeting the need for methionine through Reaction A, while selectively impairing the growth of tumor cells, which are not.

We also examined the effects, singly and in combination, of the homocystine diet and of MTX and the rescue protocol on the metabolism of the sulfur amino acids as revealed by measurements of the molar ratio of AdoMet to AdoHcy in the liver, which participates actively in the metabolism of these amino acids. By this means, we hoped to determine whether or not MTX and the homocystine diet might produce additive effects on methionine metabolism, as monitored in the liver.

MATERIALS AND METHODS

Animals and Diets. Male DBA/2 mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. Animals, weighing 25 ± 3 g at the start of each experiment, were caged individually in wire-bottomed cages.
MTX, Reduced Folate, Homocystine, and Tumors

in which 20% methanol in 15 mM H2PO4 elutes 5-methyl-THF at 11 min and 5-formyl-THF at 16 min. Ammonium sulfate, special enzyme grade, was obtained from Schwarz/Mann, Inc., division of Mediscience, Spring Valley, N. Y. All other chemicals were reagent grade and were obtained from Fisher Scientific Co., Pittsburgh, Pa. Solutions of MTX for injection were prepared by dissolving MTX in 0.15 M NaCl by gradually adjusting the pH to 7 with NaOH. Solutions of 5-methyl-THF were prepared in 0.15 M NaCl containing 10 mM sodium ascorbate, pH 6.5 to 7, and bubbled with nitrogen to protect against oxidation of the reduced folate. Each solution used for i.p. injection was filtered through an Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, Mich.; pore size, 0.45 μm), dispensed in equal volumes into 5 sterile tubes, and kept frozen at -20° until the day of use. Solutions were injected in volumes equivalent to 0.01 ml per g body weight.

Determination of S-Adenosyl Amino Acids. Mice were anesthetized with ether, the abdominal cavity was opened with minimal blood loss, and a 0.4- to 0.8-g portion of the liver was quickly removed and dropped into a preweighed centrifuge tube containing 2.5 ml 5% sulfosalicylic acid. Homogenization, using a Polytron homogenizer (Kinematica GmbH, Lucerne, Switzerland) at a setting of “5,” was completed within 5 sec of the time the blood supply to the liver was severed. Homogenates were weighed to determine the weight of the liver sample, and the tubes were centrifuged in the cold at 5500 × g for 10 min. Supernatants were frozen at -20° for later analysis by HPLC.

HPLC analyses were made at ambient temperature on a 10-μm Partisil SCX column, 250×4.6-mm internal diameter (Whatman, Inc., Clifton, N. J.), fitted with a guard column (42-× 3.2-mm internal diameter), and liver metabolites were determined by a modification of the method of Hoffman (25). Equipment consisted of a Beckman Model 332 liquid chromatograph, equipped with an Altex Model 210 injection valve, a Hitachi Model 110-10 spectrophotometer, a Beckman Model 155 variable-wavelength detector equipped with a 20-μl flow cell with a 10-mm light path, and a Beckman Model B-5000 Omni-Scribe 28-cm strip-chart recorder, all obtained from Beckman Instruments, Inc., Fullerton, Calif. Elution, at 2 ml/min, utilized Solvent A, 10 mM ammonium formate, adjusted to pH 3.7 with 88% formic acid, and Solvent B, 0.2 mM ammonium sulfate in Solvent A, pH 3.7. Liver supernatant (20 μl) was injected in running Solvent A at 0 time. At 5 to 7.5 min, the flow of Solvent B was increased linearly from 0 to 12.5%. From 10 to 12.5 min, the flow of Solvent B was increased linearly from 12.5 to 100%. At 17 min, the flow of Solvent B was decreased linearly to 0% over a period of 0.1 min. The column was then reequilibrated with a flow of 30 μl of Solvent A before addition of the next sample. Under these conditions, AdoHcy and AdoMet, detected by absorbance at 254 nm, 0.01 A units full scale, are eluted at 11.2 and 13.6 min, respectively. Quantitation was achieved by comparison of peak heights with those of standard adenosylamino acids obtained from Sigma Chemical Co. Solvents were prepared in glass-redistilled water and were filtered through a Millipore type HA 0.45-μm pore size, before analysis.

Statistical Analysis. Data were analyzed for significant differences by analysis of variance, with post hoc analysis by Duncan's studentized range test [10]. Further statistical treatments are noted in legends to Charts 2 and 3.

RESULTS

The basal diet, which contained no choline or cyst(e)ine, was designed to provide adequate nutrition but also to place a high demand on added methionine, both as the sole dietary source of preformed methyl groups and of cysteine sulfur (Chart 1). The dry diet contained 5 mg of pyridoxine-HCI per 100 g, or 10 times the usual supplement, so that any complex formation between homocystine and pyridoxal phosphate would not produce a deficiency of vitamin B6 (38).
Chart 2. Mean survival time of leukemic animals as a function of dietary sulfur amino acid and of treatment with MTX and 5-methyl-THF. In Experiments A to D, animals received 0.1 ml 0.15 M NaCl containing leukemia cells at 5 to 7 p.m. on Day 0 as follows: Experiment A, 4 x 10^3 L1210 cells; Experiment B, 4 x 10^3 L1210 cells; Experiment C, 10^3 L1210 cells; and Experiment D, 10^3 L5178Y cells. Treated animals were given injections i.p. of MTX (25 mg/kg body weight) on 5 successive days at 4 to 5 p.m., beginning on Day 1, in a volume of 0.01 ml/g body weight; they were similarly given injections of 5-methyl-THF (20 mg/kg body weight) at 11 a.m. to 12 noon on 5 successive days beginning on Day 1 (Experiment A) or Day 2 (Experiments B to D). Untreated animals were given injections of equivalent volumes of 0.15 M NaCl when treated animals received MTX and with 0.15 M NaCl containing 10 mM ascorbate, pH 6.5 to 7.0, when treated animals received 5-methyl-THF. The basal diet was supplemented with either 0.32% L-methionine (Met) or 0.86% DL-homocystine (Hcy). Number at the bottom of each column, number of animals tested. Columns with asterisks, survival times of treated animals within a single experiment which differ significantly (p < 0.001), when compared by t test for groups of unequal size. Bars, S.E.

We showed earlier that the basal diet, supplemented with 0.32% L-methionine, provided for adequate growth with no microscopic evidence of fatty liver in animals maintained on the diet for 52 days (22). We then tested diets in which the methionine was replaced by levels of DL-homocystine representing 1.5 and 3 times the sulfur content of the methionine diet. In animals initially weighing 25 to 26 g, diets containing either level of DL-homocystine in place of methionine, fed for 28 days, produced...
mean gains in body weight of 11.5 ± 7.1% (S.E.) and 11.9 ± 4.9%, respectively, as compared to 21.5 ± 4.7% for the diet containing methionine. Older animals, initially weighing 32 to 36 g, were shown on the average to maintain these weights when diets containing either level of homocystine were fed for 90 days; when animals in this weight range were fed the methionine diet for 3 months, body weights increased an average of 29.8 ± 8.2%. Thus, although the choline-free diet containing homocystine did not support continued increases in body weight of older animals as did the methionine diet, both levels of homocystine tested did permit long-term maintenance of body weight, with no toxicity evident in 3-month feeding trials.

In experiments testing the survival of animals hosting ascitic leukemia cells, the animals were fed the methionine diet for 4 to 5 days to permit adjustment to the agar gel. When all animals had accepted the gel diet, designated animals were fed the homocystine diet for 1 to 2 days before leukemia cells were injected. We have shown previously that, within 2 days, dietary homocystine induces near-maximal changes in metabolism of S-adenosylamino acids in the liver (23).

The lethality of doses of 5 to 25 mg of MTX per kg of body weight was tested on animals fed 0.32% L-methionine or 0.43% or 0.86% D(-)-homocystine diets. All animals receiving 5 daily i.p. injections of 7.5 mg of MTX or more per kg of body weight died after mean survival times ranging from 6.3 to 8.2 days. Mean survival time was similar among animals on methionine and homocystine diets and was little influenced by the dose of the drug within the range tested. The average loss of body weight from the day of the first injection of drug to the time of death was 26%, again with little variation induced by the diet or by the dose of the drug. All control animals receiving injections of equivalent volumes of 0.15 M NaCl survived.

Rescue of 10 mice from the lethality of 5 daily injections of 25 mg of MTX per kg of body weight was effected by 5 daily injections of 20 mg of dl-5-methyl-THF per kg of body weight; one-half of this dosage of the reduced folate rescued only 9 of 10 animals tested. Rescue by the higher dose of 5-methyl-THF was achieved whether the reduced folate was given 5 hr before (2), or delayed until 19 hr after, each injection of MTX; the average maximum weight losses before recovery with these 2 modes of rescue were 13 and 17%, respectively. White et al. (39) have shown that both the natural (l) and unnatural (d) isomers of 5-methyl-THF are substrates for the high-affinity THF cofactor membrane transport carrier in Ehrlich ascites tumor cells and that both isomers stimulate a net efflux of MTX from these cells when they have been preloaded with the antifolate.

In preliminary experiments, we established that the mean survival time of mice given injections i.p. of L1210 cells did not change significantly when the number of cells injected varied from 10⁶ to 2 x 10⁷ cells in 0.1 ml of 0.15 M NaCl, or when standard laboratory chow was fed in place of the semipurified diets. The effects of diets supplemented with methionine or homocystine, and of treatment with MTX and 5-methyl-THF, on the survival of mice bearing the ascitic form of L1210 or L5178Y cells are shown in Chart 2. In every experiment, where each animal received the same inoculum of cells, the mean survival time was prolonged significantly by treatment with 5 daily i.p. injections of MTX and of 5-methyl-THF, as compared with the survival of tumor-bearing animals receiving 0.15 M NaCl injections. We first followed the procedure of Blair and Searle (2), who had shown that 5-methyl-THF could protect non-tumor-bearing F₁ hybrid mice from the toxicity of 5 daily injections of MTX when the reduced folate was administered 5 hr before each injection of MTX. This procedure was followed in Chart 2, Experiment A. We then found that the mean survival time of tumor-bearing animals could be further increased significantly (p < 0.01) by delaying the rescue with reduced folate until 19 hr after each injection of MTX, as can be seen by comparing Experiments A and B of Chart 2. The greatest increase in survival time is seen in Experiment C where treated, homocystine-fed animals lived 108% longer than did their untreated counterparts. Examination of Experiments A through C of Chart 2 reveals that the substitution of dietary homocystine for methionine leads to no significant differences in the mean survival time of animals hosting L1210 cells, when the effects of the 2 diets are compared in either treated or untreated groups of animals. In Chart 2, Experiment D, in which animals were given injections of L5178Y cells, the methionine and homocystine diets are seen to have no differential effect on the mean survival time of untreated animals; however, among treated animals, the homocystine diet produced an 80% increase in mean survival time which was significantly greater (p < 0.001) than the mean survival time of treated animals receiving the methionine diet.

We wished to explore whether or not the above combination of chemotherapy and the homocystine diet could be shown to affect the metabolism of sulfur amino acids and chose to measure the concentrations of AdoMet and AdoHcy in the liver, which is the organ containing the highest combined specific activity of enzymes catalyzing the formation of the former and hydrolysis of the latter amino acid. Accordingly, we treated groups of animals, fed methionine or homocystine diets, for 5 days with MTX and 5-methyl-THF, as outlined in Experiment A of Chart 2, and sacrificed the animals beginning at 10 a.m. on the sixth day. The livers were removed and analyzed as described in "Materials and Methods." The results of these experiments, along with appropriate controls, are depicted in Chart 3. Liver AdoHcy and AdoMet concentrations and the molar ratios of AdoMet to AdoHcy are compared between groups of animals on the methionine and homocystine diets receiving the same treatments, with significant differences indicated in Chart 3.

Additionally, it is possible to compare the effects of the 4 treatments shown in Chart 3 on the concentrations of liver adenosylamino acids in animals on either diet. In Chart 3A, among methionine-fed animals, the liver AdoMet concentration in the group treated with MTX is significantly lower than that of animals treated with both MTX and 5-methyl THF (p < 0.01) or of 0.15 M NaCl-treated controls (p < 0.05). The concentration of liver AdoMet in methionine-fed animals receiving both MTX and reduced folate was significantly higher (p < 0.01) than that of all groups on the homocystine diet. The concentration of liver AdoMet in the methionine-fed 0.15 M NaCl control group was significantly higher (p < 0.01) than that of all animals on the homocystine diet except those treated with MTX only, where the differences were less significant (p < 0.05). The concentration of liver AdoMet in homocystine-fed animals was not significantly different when comparisons were made among the 4 treatment groups. Treatment of homocystine-fed animals with both MTX and 5-methyl THF led to liver AdoMet concentrations which were not different from those of methionine-fed animals treated with MTX alone but were significantly lower than those
of methionine-fed reduced folate-treated control animals \( (p < 0.05) \) or of methionine-fed 0.15 \( \mu \) NaCl-treated control animals or those treated with both MTX and reduced folate \( (p < 0.01) \).

In Chart 3B, there are no significant differences in liver concentrations of AdoHcy in homocystine-fed animals when treated groups are compared with 0.15 \( \mu \) NaCl controls. However, the concentration of AdoHcy in the livers of homocystine-fed animals receiving both MTX and 5-methyl-THF was significantly lower \( (p < 0.01) \) than that in animals receiving either MTX or reduced folate alone. In animals on the methionine diet, the AdoHcy concentration in the livers of groups treated with MTX was significantly higher \( (p < 0.05) \) than that of either the 0.15 \( \mu \) NaCl- or reduced folate-treated control groups; treatment of methionine-fed animals with both MTX and reduced folate did not lower the liver AdoHcy concentration significantly from that of those treated with MTX only. Additionally, Chart 3B reveals that the homocysteine diet fed to the 0.15 \( \mu \) NaCl control group, or treatment of methionine-fed animals with MTX, elevates liver AdoHcy to about the same extent (differences not significant).

Treatment of homocystine-fed animals with MTX elevates liver AdoHcy still further so that, although it is not increased significantly over that of homocystine-fed 0.15 \( \mu \) NaCl- or reduced folate-treated control animals, it is significantly higher \( (p < 0.01) \) than that of all methionine-fed animals, including those treated with MTX. Reduced folate-treated animals on the homocystine diet have liver AdoHcy concentrations which are significantly higher \( (p < 0.01) \) than those of homocystine-fed animals treated with both MTX and reduced folate, as well as those of all groups on the methionine diet.

In Chart 3C, the liver AdoMet:AdoHcy ratio is significantly higher in methionine-fed animals receiving 0.15 \( \mu \) NaCl, reduced folate, or both MTX and reduced folate than in all other control groups. These differences are all highly significant \( (p < 0.01) \) except for that between homocysteine- and methionine-fed animals receiving both MTX and the reduced folate, where the \( p \) value is < 0.05, as indicated in Chart 3. Treatment of methionine-fed animals with MTX alters the liver AdoMet:AdoHcy ratio to significantly \( (p < 0.01) \) lower than that of methionine-fed animals treated with 0.15 \( \mu \) NaCl or reduced folate and above that of reduced folate-treated animals on the homocystine diet \( (p < 0.01) \). Treatment of methionine-fed animals with both MTX and reduced folate leaves the liver AdoMet:AdoHcy ratio still significantly below \( (p < 0.05) \) that of 0.15 \( \mu \) NaCl controls, although it is significantly higher \( (p < 0.01) \) than that of methionine-fed animals treated with MTX only. In homocysteine-fed animals, treatment with both MTX and 5-methyl-THF leads to an increase in the liver AdoMet:AdoHcy ratio over that seen in groups treated with reduced folate or MTX alone \( (p < 0.01) \).

In each of the 8 groups of animals receiving the 2 diets and the 4 treatments, one animal was not sacrificed but was continued on the appropriate diet to monitor the response to the 4 treatments. All animals survived except those treated with MTX only; these died on the seventh day after the first injection of the drug with losses in body weight of 29 and 32.5% for mice on the homocystine and methionine diets, respectively. Animals treated with both MTX and 5-methyl-THF survived after a maximal loss in body weight of 11.0% on Day 5 and 11.5% on Day 6 for homocysteine- and methionine-fed animals, respectively. Reduced folate- and 0.15 \( \mu \) NaCl treated control animals on either diet showed only minor fluctuations in body weight during the course of the daily injections.

**DISCUSSION**

Malignant and transformed cells characteristically show repression \( (4) \) or inadequate activity \( (26) \) of endogenous metabolic pathways leading to the biosynthesis of nutrients which can be supplied by the host. Examples of various approaches to manipulation of these metabolic changes were discussed in a series of reports on amino acid imbalance in the treatment of cancer, summarized by Muggia et al. \( (35) \). Simply removing an amino acid from the diet of the host animal is generally ineffective in reducing the supply reaching malignant cells \( \text{in vivo} \) if the normal tissues of the host are capable of synthesizing the amino acid. The complement of enzymes present in the liver endows this organ with the ability to control plasma concentrations of most of the dietary amino acids including the sulfur amino acids \( (12) \).

The work reported here describes a combined nutritional and chemotherapeutic approach to depriving methionine-dependent tumor cells of methionine. Substitution of homocysteine for all dietary methionine leaves the normal animal dependent upon 2 known routes of methionine regeneration from homocysteine. One of these involves the transfer of a methyl group from betaine to homocysteine (Chart 1B), catalyzed by betaine:homocysteine methyltransferase \( (EC 2.1.1.5) \), which has been shown to have significant activity in liver only among 11 rat tissues tested \( (12) \). We have attempted in these studies to minimize the contribution of this enzyme to the synthesis of methionine from homocysteine by eliminating choline, the metabolic precursor of betaine, from the semipurified diet used.

A second route for the conversion of homocysteine to methionine \( (Chart 1A) \) involves the vitamin \text{B}_{12}-dependent enzyme \( [5\text{-methyl-THF}:L\text{-homocysteine methyltransferase (EC 2.1.1.13)}] \), which permits endogenous conversion of homocysteine to methionine. This enzyme is widely distributed in mammalian tissues \( (12) \), with the small intestine the only tissue found to lack activity of this enzyme. The 2 murine tumor cell lines we have used are incapable of growing in culture medium in which \( \text{L} \)-homocysteine replaces \( \text{L} \)-methionine \( (7, 31) \). This suggests deficient utilization of both homocysteine in the biosynthesis of methionine and of 5-methyl-THF in meeting cellular needs for THF. The use of MTX in conjunction with a diet in which homocysteine is substituted for methionine should block the formation in most tissues of both methionine and THF. Administration of supplements of 5-methyl-THF should rescue only those cells capable of converting this compound to THF as methionine is formed from homocysteine.

The present paper confirms the utility of 5-methyl THF in the rescue of mice from the toxicity of MTX, while producing highly significant increases in the mean survival times of animals receiving i.p. injections of either leukemic cell line over those receiving no chemotherapy. This finding extends the pioneering work of Goldin et al. \( (15) \) in the use of 5-formyl-THF \( (\text{citrovorum factor}) \) in overcoming drug toxicity and in extending the survival of mice inoculated with L1210 cells and treated with the antifolate, aminopterin. Mead et al. \( (34) \) used 5-methyl-THF to reduce the toxicity of MTX given to leukemic \( (L1210) \) hybrid \( F_1 \) mice; these workers reported a 27% increase in the median survival time of leukemic animals treated with this form of rescue. Smith et al. \( (37) \) found 5-methyl-THF superior to 5-formyl-THF \( (\text{citrovorum factor}) \) in overcoming the toxicity of high doses of MTX administered \( 7 \) times, with Cytoxan administered \( 6 \) times. Both
drugs were given at 3- to 10-day intervals to C57BL × DBA/2 F1 mice given injections s.c. of L1210 cells. These workers reported that rescue with 5-methyl-THF, administered twice as frequently as MTX, produced a 328% increase in mean survival time over that of untreated tumor-bearing animals. Deaths in the group receiving chemotherapy were attributed to drug toxicity as well as to tumors.

Borsa et al. (3) have shown that the persistence in mouse serum of MTX injected i.p. is biphasic, with a half-life of 31 min over the first 4 hr and of 12 hr thereafter. Since 5-methyl-THF is transported into L1210 cells by an active transport system which also transports MTX (19), we have separated by 5 hr the i.p. injections of these compounds on a single day so as to limit the activity of the reduced folate in competitively inhibiting the penetration of MTX into tumor cells or stimulating efflux of MTX from preloaded cells.

The higher (0.86%) of the 2 concentrations of dietary DL-homocysteine treated has been used in the experiments outlined in Chart 2 in an attempt to exploit the reported effect of homocysteine in retarding the growth of L5178Y cells in culture (7). The higher concentration of dietary homocysteine should also interfere more effectively with the transport of methionine into tumor cells. Methionine, which normally occurs in low concentrations in the plasma of mice (1), is an excellent substrate for both the A and L systems for transport of neutral amino acids (9). Homocysteine also interacts with both of these systems and is more effective than is L-methionine in inhibiting the transport of L-histidine by both the A and L systems of S37 ascites tumor cells (32). Homocysteine, with its sulfhydryl group, should also be efficiently transported by the ASC system, for which methionine is not a substrate (9).

It is apparent that, in animals hosting L1210 cells, the homocysteine diet in conjunction with chemotherapy with MTX and 5-methyl-THF does not increase the mean survival time over that of methionine-fed animals receiving the same chemotherapy. In untreated animals inoculated with 107 L5178Y cells, the mean survival time of animals on either diet is longer than that in untreated animals inoculated with 107 L1210 cells (Chart 2). Although Halpern et al. (17) have shown L5178Y cells grown in culture to be more sensitive to methionine deprivation than are L1210 cells, we see no change in mean survival time of animals bearing L5178Y cells when homocysteine is substituted for methionine in the diet; presumably, the host tissues provide adequate methionine. However, the combination of the homocysteine diet and chemotherapy does produce a significant increase in mean survival time of these animals over those on the methionine diet. This finding provides an interesting puzzle in view of the observations that L5178Y cells grown in vitro are capable of utilizing 5-methyl-THF, when it is supplied in the culture medium (7), and that certain methionine-dependent rat cells are capable of converting DL-homocysteine thiolactone to methionine and grow well in cultures containing homocysteine when low levels of methionine are also present (26). Chelko and Bertino (8) have reported that preincubation of L5178Y cells in methionine-free medium interferes with MTX cell kill in vitro.

We have observed that treated animals hosting either L1210 or L5178Y cells characteristically eat little for 3 to 4 days before they die. Thus, any effect of the diet on survival must occur before that time. We have found previously that the livers of animals on the choline-free homocysteine diet accumulate large amounts of lipid, compared to the normal amount of lipid found in the livers of animals fed the basal diet containing methionine (23). The data in Chart 2 indicate that these stores of lipid in the liver play no significant role in extending the survival of anorexic animals bearing either L1210 or L5178Y cells, as the homocysteine diet produced no increase in survival of untreated tumor-bearing animals over those on the methionine diet.

Hryniuk (27) has studied the mechanism of action of MTX in cultured L5178Y cells and has concluded that interference with purine synthesis contributes initially to cell kill but that, ultimately, the cells die predominantly a thymineless death. As a third action, MTX should interfere with the generation of methyl groups de novo via the THF-mediated C1 pool, blocking the recycling of homocysteine to methionine (Chart 1). Because of the unique role of methionine in its activated form (AdoMet) as the primary biological methylating agent, we sought to examine the effect of MTX on the metabolism of this amino acid in the liver, which is highly active in methyltransferase reactions. Cantoni (5) has postulated that the intracellular ratio of AdoMet to AdoHcy plays a critical role in regulating biological methylation reactions, and he and his coworkers (6) have studied the relative sensitivity of a number of methyltransferases to changes in this ratio. Since methyltransferases catalyze the methylation of proteins, nucleic acids, lipids, and a variety of small molecules such as neurotransmitters, a disturbance in the ratio of AdoMet to AdoHcy induced by MTX might be expected to affect many aspects of cellular function. The data in Chart 3 reveal that administration of MTX does lower the ratio of hepatic AdoMet to AdoHcy significantly in methionine-fed animals. However, 0.15 M NaCl-treated animals on a diet containing 0.43% DL-homocysteine experience the same decrease in this ratio, and administration of MTX to these animals has no further effect on the concentrations of liver adenosylaminoc acids. Since mice tolerate the diet containing homocysteine at 0.86% quite well for as long as 3 months, and since the homocysteine diet alone has no effect on the mean survival time of leukemic mice, we suggest that the lowering of the hepatic ratio of AdoMet to AdoHcy produced by MTX is not physiologically significant. These findings also demonstrate that the effects of MTX and of dietary homocysteine on the ratio of AdoMet to AdoHcy in the liver are not additive.

The effects of MTX and dietary homocysteine on concentrations of S-adenosylmethionine acids in other tissues and in tumor cells in vivo remain to be examined. Isolation of tumor cells from the ascitic fluid will demand special care if the in vivo state is to be revealed. Hoffman et al. (24) have shown that levels of AdoHcy in the liver increase markedly within sec of the death of the animal, possibly due to rapid accumulation of adenosine.

The significant increase in the AdoMet:AdoHcy ratio in the livers of homocysteine-fed animals treated with both MTX and 5-methyl-THF over those receiving either the drug or reduced folate alone results from a significant decrease in liver AdoHcy in the former group of animals. The equilibrium of the reaction for the hydrolysis of AdoHcy favors condensation of homocysteine and adenosine; thus, the hepatic concentrations of both of these compounds should affect the amount of AdoHcy in the liver. MTX, by inhibiting purine synthesis, would be expected to decrease liver adenosine; the administration of 5-methyl-THF should favor the conversion of homocysteine to methionine via Reaction A (Chart 1). The combination of MTX and the reduced folate could reduce the hepatic concentrations of both adenosine and homocysteine with a resultant decrease in AdoHcy.

The present work extends our understanding of the effects of MTX, Reduced Folate, Homocysteine, and Tumors.

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MTX on hepatic methionine metabolism in vivo and demonstrates the reversal of MTX-induced changes in the concentrations of S-adenosylamino acids by "rescue" with 5-methyl-THF. We have also demonstrated the utility of combined nutritional and chemotherapeutic regimens, both affecting a metabolic pathway which functions differently in normal and in malignant cells, in increasing the survival of mice bearing L5178Y cells. Nutritional manipulation may be more successful in larger animals, including humans, where continuous infusion of amino acid mixtures is feasible. This nutritional and chemotherapeutic approach to selective starvation of cancer cells may benefit from augmentation with the catabolic enzyme, methionase (EC 4.4.1.11), which has been used by Kreis (30) to inhibit the growth of certain malignant cells in culture. Also, the use of vinristine and probenecid may be helpful in prolonging the retention by tumor cells of MTX in polyglutamate forms (14).

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Effect of Methotrexate with 5-Methyltetrahydrofolate Rescue and Dietary Homocystine on Survival of Leukemic Mice and on Concentrations of Liver Adenosylaminolibc Acids

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