Diet modulates the toxicity of cancer chemotherapy in rats

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The effects of diet and folate status on cyclophosphamide or 5-fluorouracil toxicity were studied in Fischer 344 rats maintained on either a cereal-based diet or a purified diet (AIN-93G). The rats fed the purified diet were divided into 3 groups: folate deficient (no dietary folic acid), folate replete (2 mg folic acid/kg diet), and high folate (2 mg folic acid/kg diet plus 50 mg/kg body weight folic acid intraperitoneally daily). The LD50 for cyclophosphamide was significantly higher for the cereal diet than for the purified diets, but there was no difference among the purified diets. Deaths were predicted by dose, diet, white blood cell count, and BUN on Day 4 after treatment. In the saline-treated rats fed the purified diet, hepatic total glutathione levels increased in the following order: folate deficient < folate replete < high folate. There was no significant difference in aldehyde dehydogenase activities or of microsomal P450 levels in livers from rats on the different diets. In the rats treated with 5-fluorouracil, the high folate rats developed more severe anemia, azotemia, and leukopenia than the other groups. Weight, white blood cell count, hematocrit, and BUN were important predictors of death. The kidneys from rats fed the cereal-based diet were histologically normal, but rats ingesting the purified diet had increasing renal pathology that correlated with folate intake. These results indicate that diet has an important influence on the toxicity of cyclophosphamide and 5-fluorouracil and that folate status modulates hepatic glutathione levels, which is a major cellular defense against oxidant and alkylating agent damage. (J Lab Clin Med 2002;140:358-68)

Abbreviations: 5-FU = 5-fluorouracil; FdUMP = 5-fluoro-2′-deoxyuridine-5′-monophosphate; IP = intraperitoneal; BUN = blood urea nitrogen; DNA = deoxyribonucleic acid; LDH = lactic dehydrogenase; SGPT = serum glutamic pyruvic transamidase; CPK = creatine phosphokinase; CYP = cytochrome P450; dTTP = deoxythymidine triphosphate; ANOVA = analysis of variance; ANCOVA = analysis of covariance

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Relatively little is known about the influence of diet on the efficacy and toxicity of cancer chemotherapy. Folic acid is a dietary constituent that may serve as a model for studies of these interactions. Folate nutritional status varies widely in cancer patients, and folate metabolism plays a central role in the one-carbon transfers that critically affect the activity of chemotherapeutic agents. Folate compounds are essential co-factors for the synthesis of pyrimidine and purine nucleotides, for DNA methylation reactions, and for amino acid (homocysteine/methionine, serine/glycine, and glutamic acid) metabolism.[1] Folic acid deficiency is associated with DNA strand breaks,[2-5] chro-

mosomal abnormalities,[6,7] increased uracil incorporation into DNA,[3,5] defective DNA repair,[2,5,8] increased somatic mutation rates,[2,9] and anomalous DNA methylation patterns.[4] Because most cancer chemotherapeutic agents damage DNA, it is not surprising that folate nutritional status can modulate this damage. Our laboratory previously reported that folic acid deficiency acts synergistically with alkylating agents to increase the number of DNA strand breaks and somatic mutations in Chinese hamster ovary cells in vitro[2] and in human lymphoblasts,[10] and in vivo in rat splenocytes.[9] We also have presented evidence that nutritional folate deficiency may increase genetic damage in peripheral blood lymphocytes from women treated with chemotherapy for breast cancer.[11] Our studies characterizing the mutational spectra in folate-deficient cells treated with alkylating agents, taken together with the work of others, support the following mechanism to explain the synergy between folic acid deficiency and alkylating agents: (1) folate deficiency causes extensive uracil incorporation into DNA, and (2) the greatly increased utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair.[10,12]

Folate metabolism also influences the efficacy and toxicity of 5-fluorouracil (5-FU), an anti-metabolite that is useful in the treatment of breast cancer and squamous carcinoma of the head and neck and is a mainstay of therapy for gastrointestinal adenocarcinoma. After conversion to the nucleotide level, the principal mechanism of action of 5-FU is the inhibition of thymidylate synthase and subsequent DNA synthesis by 5-fluoro-2′-deoxyuridine-5′ monophosphate (FdUMP).[13] Thymidylate synthase requires the folate compound 5,10-methylene tetrahydrofolate to act as the methyl donor.[14] FdUMP forms a strong covalent bond with thymidylate synthase in the presence of 5,10-methylene tetrahydrofolate.[13,14] Though enzymatic inhibition of thymidylate synthase by FdUMP occurs at intracellular folate concentrations that are adequate for cell growth, the stability of the ternary complex is directly related to intracellular folate concentration, and inhibition is enhanced by high folate levels.[14] Consequently, folate compounds have been used extensively in the clinic to modulate the cytotoxicity of 5-FU.[14] For the most part, leucovorin (5-formyltetrahydrofolate) has been used experimentally and clinically, but folic acid (pteroylglutamic acid) is also effective.[15] It appears that the schedule of leucovorin administration is more important than dose, and that a continuous infusion over 24 hours or repetitive dose scheduling is more effective than single intermittent bolus in raising tumor concentrations of 5,10-methylene tetrahydrofolate.[14] The dose-limiting toxicities with 5-FU are myelosuppression and gastrointestinal.[13] The addition of leucovorin appears to change the toxicity profile in that leukopenia is less frequent, while diarrhea is more common, and stomatitis more severe with the combination than with 5-FU alone.[16]

Previously we reported that mammary tumors in rats maintained on a folate-deficient diet were somewhat less responsive to 5-FU than tumors in either folate-replete or supplemented rats, but this difference was not statistically significant.[17] However, we found that folate-deficient animals were significantly more sensitive to the toxic effects of 5-FU than folate-replete rats, and that folate supplementation appeared to protect further against 5-FU toxicity.[17] In the same report we also noted that cyclophosphamide, an alkylating agent, was less effective in restricting the growth of implanted mammary carcinoma cells in folate-deficient animals than in folate-replete rats, and that increasing levels of dietary folate ameliorated the chemotherapy-induced toxicity.[17] This result was somewhat surprising, since it indicated that folate supplementation increased the toxicity of cyclophosphamide to tumor cells but protected normal tissues against drug toxicity. The present studies were performed to more comprehensively characterize the effects of diet, and particularly nutritional folate status, on the toxicity associated with cyclophosphamide and 5-FU. In addition, a cereal-based dietary group was included as a control for the purified diet.

METHODS

Animals. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Female Fischer 344 rats, 30 days old and weighing approximately 60 gm, were obtained from Charles River Canada (St.-Constant, Quebec). The rats were maintained in groups of 3 or 4 for 10 days and fed a cereal-based rat chow (Harlan Teklad LM-485; Harlan Teklad, Madison, WI). This diet consists of 19.92% protein, 5.67% fat, 4.37% fiber, and 4.05 Kcal/g gross energy. Its principal ingredients include ground corn, soybean meal, ground oats, wheat middlings, and alfalfa meal. It contains no animal protein. The folic acid content is 8.21 mg/kg, and the vitamin B12 content is 30.00 μg/kg. Then the rats were housed individually in stainless steel wire-bottomed cages. One group of 6 rats continued on the cereal-based diet, while the others were maintained on the AIN-93G diet (Dyets, Inc, Bethlehem, PA).[18] This purified diet is based upon vitamin-free casein and cornstarch and consists of 19.3% protein, 16.7% fat, 5% fiber and 3.8 Kcal/kg of diet. Folate-replete diets consisted of AIN-93G with a vitamin supplement that provided 2mg folic acid/kg and 25 μg of vitamin B12/kg of diet. The folate-deficient diet consisted of

AIN-93G with vitamin mix lacking folic acid. The rats receiving high folate were fed the AIN-93G with vitamin supplement and were injected intraperitoneally (IP) daily with folic acid, 50 mg/kg dissolved in 8.4% sodium bicarbonate solution. There were 6 rats in the folate-replete and -deficient groups, and 7 rats in the high folate group. Blood samples were obtained from tail veins prior to the injection of chemotherapy, and 4, 9, and 14 days after the chemotherapy treatment. Upon completion of the study, the rats were anesthetized with pentobarbital sodium (60 mg/kg IP) and exsanguinated by cardiac puncture. Gross necropsies were performed, and the livers were collected for subsequent analyses.

Folic acid analyses. The liver was weighed and 1 g homogenized in 3 vol 140 mmol KCl/L, as previously described.[17] The homogenate was diluted 1:9 with 50 mmol potassium phosphate/L, pH 4.8, containing 1% ascorbic acid, and incubated for 24 hours at 37° C to allow endogenous conjugase to convert folate polyglutamates to monoglutamates. Then the homogenates were autoclaved, cooled on ice, and centrifuged at 2,000*g* for 10 minutes. The supernatants were frozen at −70°. The growth turbidity of *Lactobacillus casei*, which grows in proportion to the amount of folic acid present, was measured in a 96-well plate.[17,19,20] A standard curve ranging from 0.0625 to 2 ng/well was made using folinic acid, since it is more stable than folic acid.[19] The plates were read at a wavelength of 595 nm at 24 hours and sample concentrations were determined by linear regression.

Measurements of blood counts and chemistries. White blood cell counts were measured by using a Coulter Counter (Model ZBI) according to the manufacturer's instructions. Analyses of blood urea nitrogen (BUN), lactic dehydogenase (LDH), serum glutamic pyruvic transamidase (SGPT), and creatine phosphokinase (CPK) were performed using Sigma Diagnostics Procedures No. 66-UV, 500, 505 and 661, respectively (St. Louis, MO). The BUN is a measure of renal function, LDH and SGPT release into serum of liver damage, and CPK release of cardiac muscle damage.

Cytochrome P450 (CYP). Liver microsomes were prepared by differential ultracentrifugation as described by Shapiro and colleagues.[21] The final microsomal pellet was suspended in 50 mM Tris buffer (pH 7.4) containing 0.25 M sucrose. Aliquots were stored at −80 °C until use. Total CYP content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum, using a millimolar extinction coefficient of 100 mM-1 × cm-1.[22] Microsomal protein concentration was determined using a Coomassie Protein Assay Reagent kit (PIERCE, Rockford, Ill).

Aldehyde dehydrogenase. Aldehyde dehydrogenase activity in liver was quantified by the method of Kohn et al.[23] Protein concentration was determined using a Coomassie Protein Assay Reagent kit.

Total glutathione. Total glutathione levels were determined by enzymatic recycling as previously reported by Gallagher et al.[24]Briefly, frozen rat liver was homogenized in 9 volumes of ice-cold 5% 5-sulfosalicylic acid, centrifuged, and stored on ice until assayed. Seven hundred μL of 125 mM potassium phosphate containing 6.3 mM disodium EDTA, 100 μL of 6 mM 5,5′-dithiobis-2-nitrobenzoic acid, 150 μL water, 20 μL of 20 mM β-nicotinamide adenine dinucleotide, and 20 μL of sample was added to a cuvette, and equilibrated to room temperature. Then 10 μL of 50 U/mL glutathione reductase was added to the cuvette and mixed. The formation of 2-nitro-5-thiobenzoic acid was monitored spectrophotometrically at 412 nm. Total glutathione levels were determined by comparing the rates observed to standard curve rates of known glutathione concentration.

Cyclophosphamide. A 20 mg/ml solution was used for IP injections. Lyophilized CYTOXAN (Mead Johnson/Bristol Myers Squib Co, Princeton, NJ) was reconstituted with sterile water for injection, USP (Abbott Laboratories, North Chicago, Ill). Single doses of 50, 65, 85, 110, 144, 190, and 250 mg/kg were given.

5-Fluorouracil. A 50 mg/ml solution was used for IP injections. 5-FU was obtained from American Pharaceutical Partners, Inc. (Los Angeles, Calif). Single doses of 110, 144, 325, 420, and 546 mg/kg were given.

Statistical analyses. Analysis of variance was used to test the significance of differences in hematocrit, white blood cell counts, assays of renal and liver function, cardiac toxicity, folate levels, cytochrome P450, aldehyde dehydogenase and glutathione levels. Repeated measures ANOVA were used to examine gains in rat weights. If a significant F value was found, Fisher exact test was used to compare means. Probit analysis was used to determine median lethal dose (LD50). Logistic regression and survival analysis (SAS procedures LIFETEST & PHREG) were used to model predictors of mortality. In the experiments with 5-FU, two variables (BUN and white blood cell count) were transformed using the natural logarithm. Analysis of covariance (ANCOVA) was used to perform a glutathione trend analysis.

RESULTS

Cyclophosphamide. There were no significant differences in weight gain or in rat weight at 6 or 8 weeks on the various diets in any of the treatment groups (data not shown).

After 8 weeks on the various diets, the hepatic folate levels were (mean ± SD): cereal diet, 30.24 ± 7.30 μg/g liver; folate-deficient diet, 8.92 ± 0.99 μg/g liver;

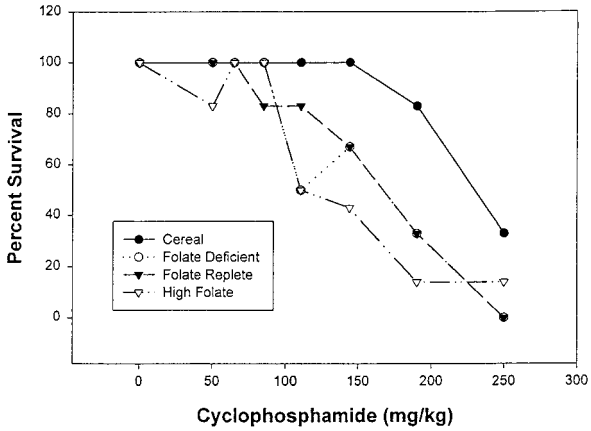

Fig 1. Effect of diet on survival in rats treated with increasing doses of cyclophosphamide. The rats were maintained on a cereal-based diet (*closed circles*) or a purified diet that was folate deficient (*open circles*), folate replete (*closed triangles*) or folate supplemented (*open triangles*). The LD50 for the cereal diet was significantly higher than for the purified diets ($P = .001$). There was no significant difference among the purified diets

folate-replete diet, 28.61 ± 8.84 μg/g liver; and high folate diet, 43.01 ± 7.22 μg/g liver. The mean folate levels of rats on the high folate diet was significantly higher than the other 3 groups, while the mean folate levels of rats on the low folate diet was significantly lower than the other 3 groups by ANOVA. The folate levels of the animals ingesting the cereal diet and the folate replete diet were not significantly different.

The calculated median lethal doses (LD50) for the cyclophosphamide-related deaths were: cereal diet, 232 mg/kg; folate-deficient diet, 154 mg/kg; folate-replete diet, 159 mg/kg; high folate diet, 148 mg/kg (Figure 1). The LD50 for the cereal diet was significantly higher than for the purified diets by probit analysis. There was no significant difference among the purified diets.

Blood samples were obtained to monitor bone marrow, renal and liver function, and evidence of cardiac damage following cyclophosphamide treatment. Hematocrit, white blood cell count, and BUN determinations were obtained prior to chemotherapy administration after Week 6 and on Days 4, 9, and 14 following chemotherapy. At Week 6 (pre-treatment), the high folate group had a significantly higher BUN ($P = .01$) than the other two purified diet groups (data not shown). There was no difference in hematocrit or white blood cell count pre-treatment among the dietary groups (data not shown).

The effects of diet and cyclophosphamide dose on BUN on Day 14 are illustrated in Table I. The BUN was significantly higher in the high folate group than in the other dietary groups on Days 9 (data not shown) and 14 ($P < .03$) at cyclophosphamide doses of 65, 144, and 190 mg/kg. Significant differences for hematocrits among the diet groups were found only for specific combinations of dose levels and times (Table I). Generally the cereal diet group tended to have higher hematocrits than the other three groups.

Pretreatment measures of hematocrit, white blood cell count, or BUN were not found to be significant predictors of survival when added to diet and dose. However, death was best predicted by dose, diet, and Day 4 measures of BUN and white blood cell count.

Determinations of serum LDH, CPK, and SGPT were made from samples collected on Day 14 in surviving rats; therefore, the numbers are small in the higher cyclophosphamide dose groups. For LDH, diet was a highly significant factor ($P < .0006$). A comparison of the mean LDH values in the different dietary groups found that the replete diet was significantly lower than the cereal and the low folate diets (Table I). The high folate diet was not significantly different from any of the other diets. Dose was also found to be highly significant ($P < .0001$). The general trend was that LDH decreased as the dose increased, although not all dose levels had LDH means that were significantly different from the adjacent levels. For CPK, the diet by dose (categorical variable) interaction was significant ($P = 0.028$). Diet means were found to be significantly different for two dose levels, 65 mg/kg and 190 mg/kg (data not shown). For SGPT, the overall model was significant ($P < .001$) and the dose by diet interaction was significant ($P = 0.035$). The diet comparisons at each dose level found that the cereal group had a higher SGPT than the other 3 groups (all $P < .003$) at 5 of the dose levels (data not shown).

These results indicated that animals ingesting the cereal-based diet had markedly less toxicity from cyclophosphamide than animals on the purified diet and that folate status was not an important determinant of toxicity. We next investigated whether this variation in toxicity was due to a dietary alteration of the metabolism of cyclophosphamide. At 3 different cyclophosphamide doses, 50, 110, and 190 mg/kg, there was no significant difference in hepatic microsomal cytochrome P450 levels in rats on the different diets (data not shown). Similarly, aldehyde dehydrogenase activities in both the soluble fractions and solubilized particulate fractions of liver from rats treated with cyclophosphamide, 50 mg/kg, were not significantly different (data not shown). In contrast, total glutathione levels were significantly influenced by the folate content of the diet in the saline-treated ($P = 0.01$) but not in the cyclophosphamide-treated animals. As shown in Figure 2, in the group treated with saline, the levels were highest in the high folate dietary group, intermediate in the folate replete group, and lowest in the low folate

Table I. Fischer 344 rat hematocrit, serum BUN, and serum LDH measured 8 weeks after maintenance on a cereal-based diet (CR) or a purified diet of varying folate content

Dose	Diet	Hct	BUN	LDH	*n*
0 mg/kg	CR	42.2 ± 2.7	15.7 ± 1.0	799.8 ± 118.0	6
	FD	42.9 ± 1.6	15.5 ± 1.8	679.2 ± 197.1	6
	FR	40.2 ± 1.3	15.4 ± 2.2	697.9 ± 149.4	6
	HF	37.6 ± 7.4	26.6 ± 15.4†	514.5 ± 192.9	6
50 mg/kg	CR	38.9 ± 3.6	20.8 ± 1.8	942.9 ± 67.4	6
	FD	41.3 ± 0.8	18.5 ± 1.9	916.0 ± 60.2	6
	FR	40.3 ± 4.5	20.4 ± 2.2	834.5 ± 106.3	6
	HF	42.7 ± 2.2	20.9 ± 2.8	862.8 ± 79.1	5
65 mg/kg	CR	42.5 ± 1.1	19.4 ± 1.9	964.8 ± 21.0	6
	FD	41.4 ± 1.4	19.3 ± 2.1	932.2 ± 35.6	6
	FR	39.3 ± 6.4	16.6 ± 2.9	883.5 ± 68.7	6
	HF	41.8 ± 1.2	24.8 ± 4.7†	860.8 ± 91.4	7
85 mg/kg	CR	44.7 ± 2.3*	23.1 ± 2.7	821.2 ± 138.6	6
	FD	41.3 ± 1.5	16.7 ± 3.2	815.8 ± 91.1	6
	FR	41.3 ± 0.9	18.4 ± 2.1	702.8 ± 32.8	5
	HF	40.9 ± 2.1	23.0 ± 5.1	783.6 ± 124.2	7
110 mg/kg	CR	38.6 ± 1.7	21.2 ± 3.7	819.2 ± 110.5	6
	FD	32.8 ± 4.3	36.3 ± 23.8	766.0 ± 21.8	3
	FR	38.9 ± 2.6	16.1 ± 2.8	579.0 ± 156.3	5
	HF	42.7 ± 2.5	24.9 ± 0.5	682.4 ± 244.8	3
144 mg/kg	CR	39.6 ± 1.4	14.9 ± 2.4	695.9 ± 269.3	6
	FD	36.6 ± 4.9	13.9 ± 2.3	505.0 ± 131.3	4
	FR	39.6 ± 2.2	14.8 ± 2.1	579.9 ± 210.5	4
	HF	31.0 ± 9.4	35.9 ± 23.2†	562.2 ± 249.8	3
190 mg/kg	CR	41.1 ± 1.0	16.7 ± 3.1	647.8 ± 105.2	5
	FD	38.5 ± 1.4	17.3 ± 5.7	601.4 ± 147.6	2
	FR	40.0 ± 0.0	13.3 ± 1.3	354.2 ± 97.7	2
	HF	41.0 ± 0.0	35.5 ± 0.0†	905.8 ± 0.0	1
250 mg/kg	CR	22.8 ± 3.2	15.8 ± 3.5	252.9 ± 81.8	2
	FD	—	—	—	0
	FR	—	—	—	0
	HF	41.0 ± 0.0	15.5 ± 0.0	477.6 ± 0.0	1

*Significantly higher ($P < .05$) than other dietary groups.
†Significantly higher ($P < .03$) than other dietary groups.
Cyclophosphamide was injected after week 6 in the doses indicated.
FD, folate deficient; *FR,* folate replete; *HF,* high folate.

group. Although this pattern was also seen at the 50 mg, 85 mg, and 190 mg cyclophosphamide dose levels, these differences were not significant by ANCOVA. Figure 2 also shows that glutathione levels were higher in the saline-treated animals on the folate replete diet than in rats maintained on the cereal diet ($P = .0048$).

5-fluorouracil. At Week 6, when the rats were injected IP with 5-FU, there were no significant differences in weights among the diet groups (data not shown). At Week 8, the rats on the cereal-based diet were heavier than the other three groups, with a maximum difference of 15.1 g, between cereal-based diet and high folate group (ANOVA, overall $P = .015$; data not shown).

Six or 7 rats per group were treated with 5-FU at each dose level. However, because of the difficulty in obtaining linear dose-response relationships, the 325 mg/kg dose level was repeated for a total of 12 rats/dietary group. Nevertheless, the median lethal doses could not be determined by Probit analyses. Too few deaths occurred in three of the groups to make a reliable estimate while death did not vary linearly in relationship to dose in the high folate group.

Blood samples were obtained to measure bone marrow, renal and liver function, and to detect evidence of cardiac damage following 5-FU therapy. Prior to chemotherapy, the high folate dietary group had significantly higher BUN values and lower hematocrit values than the other three dietary groups (ANOVA, both

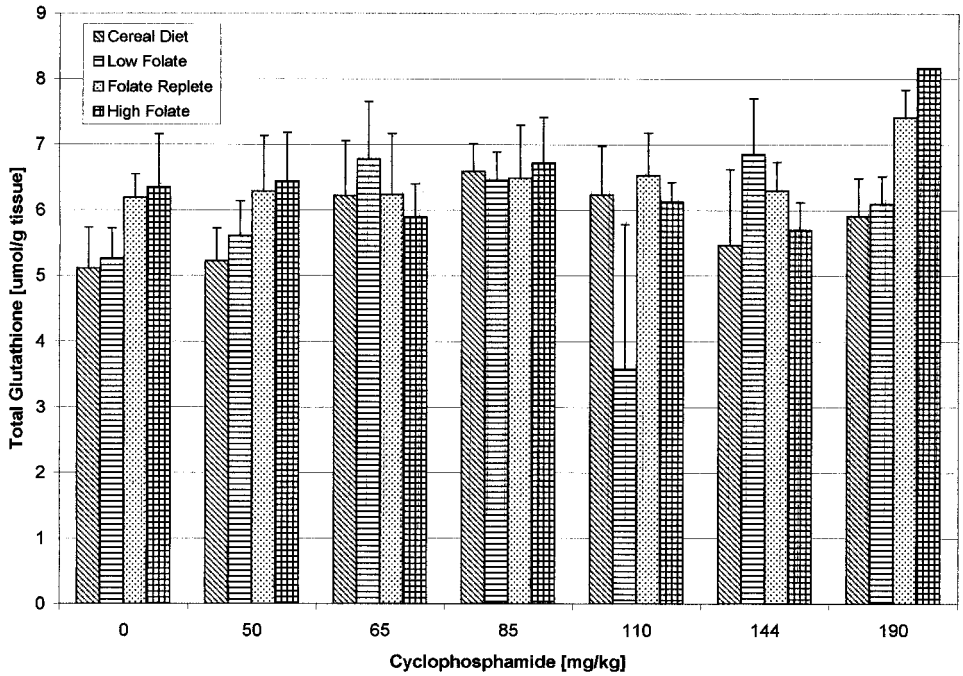

Fig 2. Hepatic total glutathione levels in rats fed a cereal-based diet or a purified diet of differing folate content (low folate, folate replete, or high folate) for 8 weeks. After 6 weeks on the diets, rats were treated either with saline or with cyclophosphamide in the indicated doses. The error bars represent the standard deviation. There were 6 saline-treated rats per dietary group except for high folate, where there were 7 animals. The number of rats per group surviving to 8 weeks after cyclophosphamide treatment is provided in Table I. In the saline-treated group, glutathione levels were significantly ($P = 0.01$) influenced by the folate content of the diet. A glutathione trend analysis (ANCOVA) found no dose diet interaction in the cyclophosphamide-treated rats

overall $P < .0005$; data not shown). There were no significant differences among the dietary groups for white blood cell count at pretreatment (data not shown).

Repeated measures ANOVA indicated that diet showed significant interactions with time for three post-treatment blood analyses (hematocrit, white blood cell count and BUN; all models had $P < .01$). The BUN levels on Day 14 are shown in Table II; analyses showed that the BUN was significantly higher on Days 9 (data not shown) and 14 in the high folate group compared to the other dietary groups. The hematocrit was significantly lower in the high folate group on Days 4 and 9. By Day 9, the maximum difference in hematocrit between the cereal and high folate dietary groups, 9.9%, was substantial, and this maximum difference persisted through Day 14 at 9.1%. The hematocrit levels on Day 14 are shown in Table II. The hematocrit on Day 14 was significantly higher in the cereal diet group than in rats on the high and folate replete diets. The changes in white blood cell count after chemotherapy were somewhat variable. However, by Day 14, the folate-deficient rats as a group had significantly lower white blood cell counts than the cereal and folate replete groups (data not shown). On Day 14, the CPK and LDH levels were significantly higher in the cereal group than in the other 3 dietary groups, and the SGPT was higher in the cereal group than in the folate-deficient and -replete groups (all overall $P < .001$). The data for LDH are shown in Table II.

Logistic regression revealed that diet, but neither dose nor baseline weight, was related to death ($P < .0001$). Pretreatment white blood cell count in Week 6 improved the logistic model predicting death. Survival analysis confirmed that white blood cell count was the only pretreatment blood analysis that was associated with survival. The white blood cell count was a more significant predictor of deaths that occurred early, soon after the administration of chemotherapy. Its significance for predicting later deaths was borderline. When Day 4 blood analyses were added to the logistic model, pretreatment white blood cell count was dropped as a significant predictor, but Day 4 white blood cell and BUN were found to be significant.

The importance of bone marrow suppression, as measured by decreases in the hematocrit and white blood cell count (as a predictor of death after 5-FU), was expected. The prominence of an elevated BUN as a predictor was more surprising, both with 5-FU and with

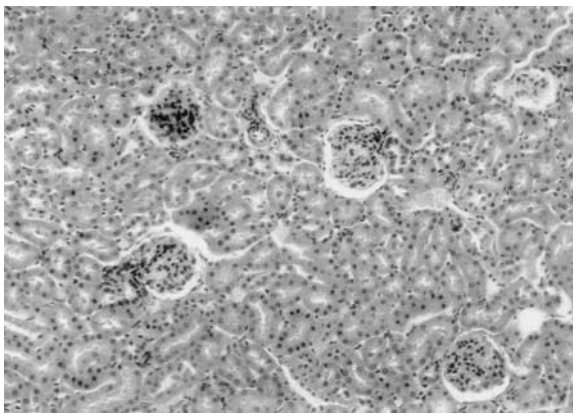

Fig 3. Kidney microscopic anatomy in Week 8 from a rat maintained on the cereal-based diet and treated with 5-FU, 110 mg/kg, in Week 6. The histology was normal. Magnification x 500.

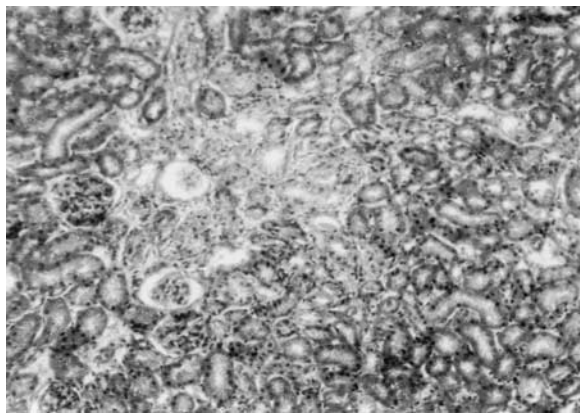

Fig 4. Kidney microscopic anatomy in Week 8 from a rat maintained on the folate-replete purified diet and treated with 5-FU, 110 mg/kg, in Week 6. There is focal tubular degeneration with regeneration. Magnification × 500.

Table II. Fischer 344 rat hematocrit, serum BUN, and serum LDH measured 8 weeks after maintenance on a cereal-based diet (CR) or a purified diet of varying folate content

Dose	Diet	Hct	BUN	LDH	*n*
0 mg/kg	CR	42.2 ± 2.7†	15.7 ± 1.0	799.8 ± 118.0^	6
	FD	42.9 ± 1.6	15.5 ± 1.8	679.2 ± 197.1	6
	FR	40.2 ± 1.3	15.4 ± 2.2	697.9 ± 149.4	6
	HF	37.6 ± 7.4	26.6 ± 15.4*	514.5 ± 192.9	6
110 mg/kg	CR	38.2 ± 4.3†	19.1 ± 2.6	785.0 ± 210.7^	6
	FD	40.8 ± 6.9	19.2 ± 1.9	763.3 ± 147.6	6
	FR	32.1 ± 4.9	22.9 ± 11.4	701.3 ± 303.8	5
	HF	33.7 ± 0.6	25.8 ± 13.8*	695.3 ± 350.6	3
144 mg/kg	CR	35.7 ± 8.3†	17.0 ± 2.2	674.6 ± 100.8^	5
	FD	28.9 ± 2.6	17.0 ± 4.6	492.8 ± 145.8	5
	FR	26.1 ± 5.5	18.7 ± 0.9	626.6 ± 207.4	5
	HF	29.3 ± 3.8	20.7 ± 4.8*	486.4 ± 343.9	4
325 mg/kg	CR	29.0 ± 9.4†	14.5 ± 2.0	640.9 ± 128.4^	10
	FD	19.0 ± 5.8	16.4 ± 4.9	394.8 ± 133.1	11
	FR	26.1 ± 8.6	16.0 ± 1.8	445.9 ± 191.3	10
	HF	16.3 ± 6.7	29.2 ± 12.8*	340.5 ± 86.0	11
420 mg/kg	CR	26.7 ± 9.5†	14.8 ± 2.4	719.6 ± 193.0^	6
	FD	23.7 ± 10.6	15.8 ± 2.0	341.8 ± 131.0	6
	FR	16.7 ± 9.0	18.7 ± 6.4	409.1 ± 157.9	5
	HF	12.0 ± 0.0	32.6 ± 0.0*	136.4 ± 0.0	1
546 mg/kg	CR	21.0 ± 10.0†	14.9 ± 2.6	463.3 ± 165.8^	6
	FD	22.8 ± 11.5	15.0 ± 2.6	272.0 ± 146.6	5
	FR	16.7 ± 5.0	15.2 ± 2.1	368.9 ± 114.1	3
	HF	11.0 ± 0.0	39.9 ± 0.0*	223.3 ± 0.0	1

5-fluorouracil was injected after week 6 in the doses indicated.
†Significantly higher than other dietary groups ($P < .05$ by ANOVA)
*Higher than other dietary groups ($P = .08$ by ANOVA)
^Significantly higher than other dietary groups ($P = < .05$ by ANOVA)
FD, folate deficient; *FR,* folate replete; *HF,* high folate.

cyclophosphamide, since renal toxicity is not a common feature of the toxicity profile for either drug.[25,26] Therefore, kidneys from rats in the four dietary groups that received saline alone and rats that were treated with the lowest dose of 5-FU, 110 mg/kg, were examined histologically by an expert in renal pathology. None of

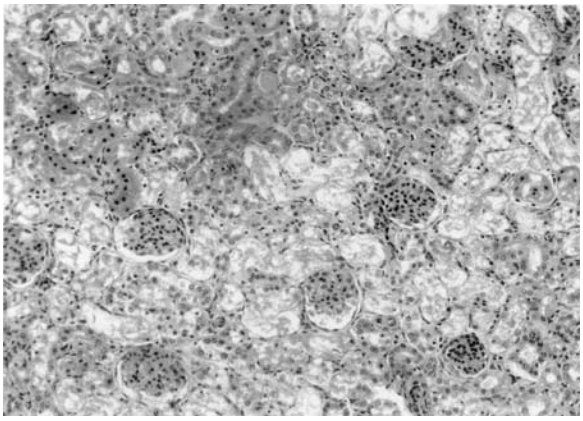

Fig 5. Kidney microscopic anatomy in week 8 from a rat maintained on the high folate purified diet and treated with 5-FU, 110 mg/kg, in Week 6. There is marked acute tubular necrosis. Magnification × 500.

the animals in these treatment groups died during the 14 day period after the administration of saline or chemotherapy. The kidneys were collected at post-mortem examination when the animals were euthanized on Day 14. Histologic examination showed that all rats in the saline-treated dietary groups had no evidence of renal pathology. Similarly, all 6 rats in the cereal-based dietary group that received 5-FU had normal renal morphology, as illustrated in Figure 3. In contrast, 3 of 6 rats in the folate-deficient group, and 5 of 6 rats in the folate-replete group had focal tubular degeneration and regeneration, and one animal in each group also had focal acute tubular necrosis (Figure 4). The rats in the high folate group had extensive renal damage. All 7 animals showed acute tubular necrosis, which was marked in 2 cases (Figure 5). Two rats also had focal tubular degeneration with regeneration, and one showed perirenal inflammation with old hemorrhage and organized perirenal thrombosis.

Purified diets have been reported to produce markedly acidic urine, with pH consistently below 6.0.[27] Since folic acid comes out of solution at acid pH, we tested the urine pH in rats in the dietary groups. After 6 weeks on the different diets, the mean urinary pH of each group were: cereal-based: 6.45 ± 0.8; low folate: 5.83 ± 0.5; folate replete: 5.50 ± 0.2; high folate: 5.75 ± 0.2 (mean ± SD). The urinary pH of the rats maintained on the cereal-based diet was significantly higher than that of the rats fed the purified diets ($P = .02$). There was no significant difference of urinary pH among the rats on purified diets of varying folate content.

DISCUSSION

The results reported here indicate that diet has an important influence on the toxicity of a bifunctional alkylating agent, cyclophosphamide, and of an antimetabolite, 5-FU. In the case of both drugs, rats on a cereal-based diet were more resistant to toxicity than animals on a purified diet. Although rats on both types of diets grew and gained weight at the same rate and had similar hematocrits and white blood cell counts prior to chemotherapy treatment, the rats on the cereal-based diet had less evidence of toxicity after either cyclophosphamide or 5-FU. The cereal diet fed animals tended to maintain their weight better and had higher hematocrits and white blood cell counts after chemotherapy. Survival analysis indicated that weight, white blood cell count, hematocrit and BUN were important predictors of death. Since a principal adverse effect of both cyclophosphamide and 5-FU is bone marrow suppression.[25,26] It was not surprising that the hematocrit and white blood cell count 4 days after chemotherapy were predictive of death. After drug treatment the anemia and azotemia became more pronounced, and the high folate animals had more severe neutropenia during the first week post therapy than the other groups. Logistic regression analysis confirmed that diet was related to death. Prior investigations have noted that animals fed purified diets and subjected to stress, toxins or carcinogens have more adverse effects than those fed cereal-based diets,[18] and that rodents fed purified diets have higher tumor incidences compared to rodents fed natural ingredient diets.[27,28] The observations reported here confirm the findings of Bounous et al that rats ingesting a natural ingredient diet are protected against 5-FU toxicity compared to rats fed a defined-formula diet.[29] They noted that maintenance on a defined-formula diet was associated with shorter survival time and more severe leukopenia than animals eating a conventional Purina rat chow after 5-FU injection.[29] Postulating that the defined diet lacked a crucial factor, they tested the roles of free amino acids, nitrogen level, vegetable fibers and arachidonic acid, but found no evidence to support any of these as the protective agent.[29,30]

We considered several possible explanations for the superiority of the cereal-based diet over the purified diet in protecting against cyclophosphamide toxicity. The hepatic microsomal cytochrome P450 system is important for the metabolic activation of cyclophosphamide.[31] The prodrug cyclophosphamide requires the action of hepatic microsomal mixed fuction oxidase for hydroxylation to 4-hydroxycyclophosphamide.[31] Activation is primarily catalyzed by cytochrome P-4502B and P-4502C enzymes, but CYP3A4/5 may play a role

at high cyclophosphamide concentrations.[32,33] 4-hydroxycyclophosphamide serves as a transport species that is converted intracellularly to aldophosphamide and then is further metabolized to acrolein and phosphoramide mustard.[31] The latter compound possesses significant DNA-alkylating activity.[31] A number of dietary components have been reported to modulate the cytochrome P450 system. For example, it has been suggested that naturally occurring organic compounds in cereal-based diets can cause induction of the cytochrome P450 system and thereby lessen the effects of drugs.[34] However, we could detect no significant differences in hepatic microsomal cytochrome P450 levels in rats on the different diets at any of the cyclophosphamide dosage levels tested.

We next measured hepatic aldehyde dehydrogenase levels in animals on the different diets, since this enzyme can modify cyclophosphamide toxicity by inactivating aldophosphamide.[23] However, there was no important effect of the different diets on this enzyme in either the soluble fractions or solubilized particulate fractions of rats treated with cyclophosphamide.

Total glutathione levels in livers from rats on the different diets then were determined. Glutathione participates at two separate locations in the cyclophosphamide metabolic pathway and thereby plays an important role in determining the toxicity and efficacy of the drug.[35] Cyclophosphamide initially depletes glutathione levels[36] but also provides a drug selection pressure that leads later to higher glutathione levels in resistant tumor cells.[35] We found that hepatic glutathione levels were significantly higher in the animals on the folate replete purified diet compared to rats fed the cereal-based diet. Because these two dietary groups have nearly the same hepatic folate levels, this glutathione effect is not due to the somewhat different levels of dietary folate content. The diet-associated difference in glutathione levels probably does not account for the decreased toxicity of cyclophosphamide in cereal-fed animals because these animals had lower glutathione levels than rats ingesting the purified diet.

Folate status did influence glutathione levels in rats fed the purified diet. The levels increased in the order: folate deficient $<$ folate replete $<$ high folate. Hepatic glutathione levels have been reported to be lower in rats fed a methyl group deficient diet but not altered further by additional lack of folate.[37] Others found that folate-deficient rats had elevated plasma total glutathione levels and attributed this change to a stimulated oxidized glutathione efflux from cells due to an oxidative stress.[38] Our studies suggest that high levels of folate might raise hepatic glutathione levels further and provide additional protection against carcinogenic compounds and oxidative stress.

Taken together, our studies do not provide an explanation for the decreased cyclophosphamide toxicity enjoyed by rats on the cereal diet. Others have reported imbalances in intracellular nucleotide pools in rats fed purified diets compared to natural ingredient diets.[39] Rats ingesting the AIN-76A diet had a marked increase in dUMP/dTTP ratios, and this abnormality was exaggerated in folate/methyl-deficient diet groups.[39] It is possible that the increased toxicity seen in the purified diet groups after cyclophosphamide treatment was due to restricted DNA replication and repair caused by nucleotide imbalances. Observations in rats on the purified diet AIN-93M noting lower lymphocyte cloning efficiencies and less reduction in mutant frequencies at the *hprt* locus with calorie restriction compared to rats on the natural-formula NIH-31 diet are consistent with this postulate.[40] The marked decrease in dTTP pools in rats fed the purified diet, AIN-76A, compared to those fed NIH-31, a natural ingredient diet, reported by Jackson et al[39] may also be relevant to our observations with 5-FU. An important part of the mechanism of action of 5-FU is inhibition of thymidylate synthase with resulting depletion of dTTP. Since there does not appear to be a role for salvage pathway involvement in resistance to 5-FU,[41] the purified diet and 5-FU may work additively or synergistically to reduce dTTP availability for DNA synthesis and thereby increase toxicity. Folate supplementation in turn would further enhance toxicity by promoting inactivation of thymidylate synthase.[14]

Thus changes in nucleotide pools, with resulting abnormalities of DNA synthesis and repair, may explain the increased bone marrow toxicity we observed in rats fed a purified diet and supplemented with folic acid, as well as the protective effect of the cereal-based diet. These changes may also contribute to the renal failure seen in the 5-FU treated rats. 5-FU rarely if ever causes nephrotoxicity.[26] We found that the cereal-based diet was protective against renal damage, and that damage was proportional to dietary folate levels in the rats fed a purified diet, but only in 5-FU treated animals. Thus the renal toxicity appears to involve an interaction of folate and 5-FU in the setting of the purified diet. Rat kidney is particularly enriched in a high affinity folate binding protein that together with the reduced folate carrier promotes the renal reabsorption of folates.[42] Since folate is loosely bound to albumin and is filtered by the glomerulus, renal reabsorption in the proximal tubule is necessary to avoid rapid depletion of folate stores. High doses of folic acid have been associated with nephropathy in rats.[43,44] Beginning at a dose of 100 mg/kg of folic acid, relatively mild changes in tubular cell morphology and impairment of renal tubular and glomerular function are found.[44] As the folic

acid dose is increased in the range of 100 to 400 mg/kg, the magnitude of the renal injury is dose-related, and severe functional and structural abnormalities are seen at the higher doses.[44] The most prominent changes are related to tubular function, particularly concentrating ability, resulting in polyuria.[44] Perhaps of greatest relevance to the present work, Klingler et al found that there was a high degree of tritiated thymidine uptake, high labeling index, and increased kidney tissue DNA concentration after injection of high doses of folic acid, indicating a regenerative process in response to tubular injury.[44] In planning these experiments, we specifically chose a supplemental folic acid dose that was well below published levels known to cause nephrotoxicity. Since this dose was given daily for 6 weeks prior to the injection of 5-FU, the enhanced nephrotoxicity in our experiments is consistent with the observation that repetitive dose scheduling of folate compounds increases the toxicity of 5-FU more than single intermittent bolus administration.[14]

The investigations reported here do not support our previous conclusion that high levels of folate protect against cyclophosphamide and 5-FU toxicity. A major difference between the two experiments is the schedule of drug administration. In the earlier experiments, the cyclophosphamide and 5-FU were given in divided doses, whereas in the current experiments a single dose was administered. A second difference is that we noted more renal toxicity in the current experiments, and statistical analysis indicated that deaths were predicted by dose, diet, white blood cell count and BUN on Day 4. It is possible that any beneficial effects from the folate supplementation on cylophosphamide toxicity, as in an increase in glutathione levels, were obscured by renal toxicity and premature death. Additional experimentation will be necessary to fully define the apparently complex relationship between folic acid dietary supplementation and chemotherapy toxicity. These experiments might include utilizing a hybrid diet that is both cereal-based and supplemented to high folate levels. This approach could lead to a better understanding of the relationship between a cereal-based diet and folate levels with regard to cyclophosphamide toxicity.

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