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Award Number: DAMD17-98-1-8345

TITLE: Effect of Folate on the Efficacy and Toxicity of Cancer Chemotherapy

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REPORT DATE: December 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2001	Final (1 Sep 98 - 30 Nov 01)

4. TITLE AND SUBTITLE
Effect of Folate on the Efficacy and Toxicity of Cancer Chemotherapy

5. FUNDING NUMBERS
DAMD17-98-1-8345

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this research project was to characterize the effect of dietary folate levels on the cellular pharmacology and toxicology of chemotherapeutic agents. The scope of the research involved *in vitro* studies with cell lines and *in vivo* assessments in rats of folate-chemotherapy interactions. Studies at a molecular level with human cells confirmed the model developed in rodent cells to explain the synergy between nutritional folate deficiency and alkylating agents. Cells expressing p53 activity exhibited a higher rate of mutation induction but were more sensitive to the toxic effects of alkylating agents than those lacking p53. Folate deficiency tended to reduce toxicity but increase mutation induction after alkylator treatment. Studies in rat liver confirmed that folate metabolism modulates glutathione levels. Studies in rats of the interaction of diet and cyclophosphamide indicated that deaths were predicted by dose and diet. The combination of high doses of folate and chemotherapy caused renal damage, indicating that there may be an optimal amount of dietary folate to modulate toxicity. Folate supplementation decreased the frequency of mitochondrial DNA deletions in liver from untreated rats and from animals treated with cyclophosphamide. Studies with 5-fluorouracil confirmed that dietary changes profoundly affect the outcome of cancer chemotherapy.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
142

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

A. Branda, R.F., Lafayette, A.R., O'Neill, J.P., Nicklas, J.A. The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary (CHO) cells treated with monofunctional alkylating agents. Mutation Res. 427:79-87 (1999).

B. Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status. Mutat. Res.473:51-71 (2001).

C. Branda, R.F., Chen, Z., Brooks, E.M., Naud, S.J., McCormack, J.J. Diet modulates the toxicity of cancer chemotherapy in rats. I. Cyclophosphamide. Submitted.

D. Branda, R.F., Chen, Z., Brooks, E.M., Naud, S.J., Trainer, T.D., McCormack, J.J. Diet modulates the toxicity of cancer chemotherapy in rats. II. 5-Fluorouracil. Submitted.

E. Branda, R.F., Brooks, E.M., Chen, Z., Naud, S.J., Nicklas, J.A. Dietary modulation of mitochondrial DNA deletions and copy number after chemotherapy in rats. Mutation Res, in press.

F. Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. P53 activity modulates the effect of folate deficiency on genetic damage caused by alkylating agents in human lymphoblastoid cells. Proc. AACR 41:69 (2000).

INTRODUCTION

The general subject of this research project is the effect of diet and nutrition on the efficacy and toxicity of chemotherapy in women with breast cancer. More specifically, the research focuses on the interactions of a micro-nutrient, folic acid, with chemotherapeutic drugs frequently used clinically in women with breast cancer in either the adjuvant or metastatic setting; namely, cyclophosphamide, 5-fluorouracil and doxorubicin. The hypothesis to be tested is whether dietary supplementation with a non-toxic nutrient, folic acid, may reduce the toxicity and increase the efficacy of chemotherapy in women with breast cancer. The purpose of the research is to better understand the effect of folate metabolism and varying dietary folate levels on the cellular pharmacology and clinical toxicity of chemotherapeutic agents and then utilize this knowledge to decrease the toxicity and increase the effectiveness of these drugs. The scope of the research involves *in vitro* studies with cell lines to assess folate-drug interactions and *in vivo* assessments in rats of folate-chemotherapeutic drug interactions.

BODY

Task 1. *In vitro* assessment of folate-drug interactions

- **Determine the impact of folate levels on the cytotoxicity of 5-FU, doxorubicin and hydroperoxycyclophosphamide**

The cell lines described in the grant application: MCF-7 (mammary adenocarcinoma); BT-474 (mammary ductal carcinoma); SK Br3 (mammary ductal carcinoma); MDA-MB-435 (mammary adenocarcinoma); and Hs578 Bst (human breast fibroblast cells) were located in tissue repositories and obtained. They were grown out in their original media and adapted to growth in media that can be formulated as folate-free. In the meantime, experiments were performed with TK6 human lymphoblastoid cells instead of the RPMI 1788 human lymphoblastoid cells described in the grant. We decided that the TK6 cells were preferable because we already had them adapted to growth in media that can be formulated folate-free, and a variant mutated at the p53 gene (WTK1) was available in our laboratory. The latter cells proved useful to investigate the role of p53 mutations on the interaction of folate metabolism and chemotherapeutic drugs.

As noted in the 1999 Annual Report, we encountered considerable difficulty identifying a source of hydroperoxycyclophosphamide. It is not commercially available, and most of the currently synthesized drug is committed to studies of bone marrow purging in preparation for bone marrow transplantation. We were able to obtain a small quantity from Dr. Carol Miller at the Johns Hopkins Medical School. Our plan was modified to perform the studies described in the grant with 5-fluorouracil (5-FU) and doxorubicin, and use ethyl methanesulfonate (EMS) as a surrogate for cyclophosphamide, since its mechanism of action is similar.

The TK6 and WTK1 cells were cultured for 3 days in folate-replete or -free RPMI medium, then treated overnight with EMS. Cells then were grown in complete medium for 7-8 days to go through phenotypic lag. Cells were seeded for cloning efficiency and for mutant selection in the presence of 10 uM 6-thioguanine into 96 well microtiter plates. At 10-14 days,

colony growth was scored and cloning efficiency calculated by use of the Poisson relationship. The mutant frequency is the ratio of the mean cloning efficiency in the presence and absence of 6-thioguanine.

After synthesis of cDNA, amplification was done in two rounds of nested PCR in a Perkin-Elmer 2400 Thermal Cycler. The final product was run on a 1% agarose gel, stained with ethidium bromide and observed under UV light. The product was excised, Gene Cleaned and sequenced with an ABI 373 sequencer. Some clones were studied further by multiplex PCR.

Treatment of the cell lines with increasing concentrations of EMS resulted in a progressive decline in cloning efficiency. Figure 1 shows the results of 5 separate experiments in which TK6 and WTK1 cells were incubated in folate-replete or deficient media and then treated with EMS in the concentration range of 0 to 50 ug/ml. WTK1 cells were more resistant to the cytotoxic effects of EMS than TK6 cells in both folate replete and deficient media. Folate

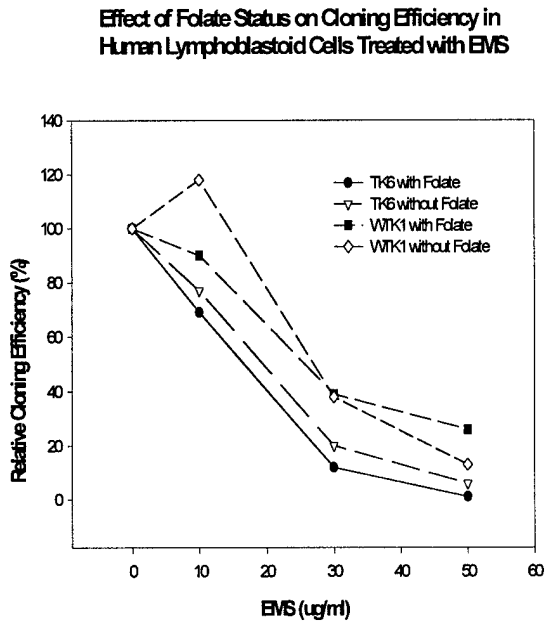


Figure 1

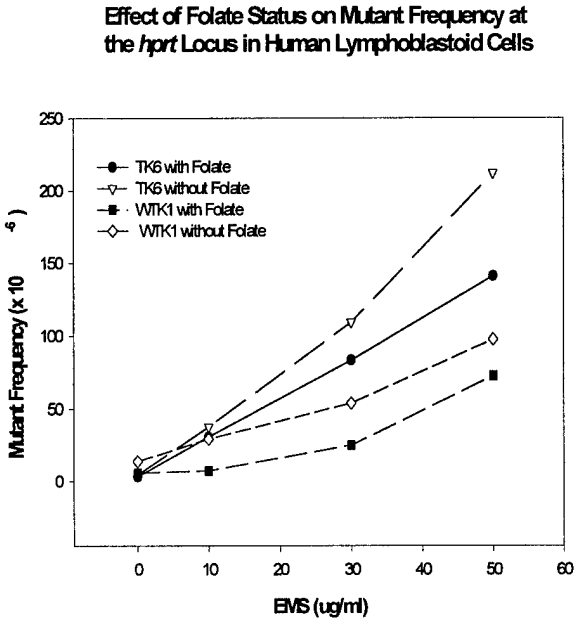


Figure 2

deficient TK6 were more resistant to the toxic effects of EMS than folate replete cells. However, there was considerable variability among the experiments, and statistical analyses indicated that the slopes for the cloning efficiencies were similar.

The mutant frequencies at the *HPRT* locus determined from these 5 experiments are shown in Figure 2. After treatment with EMS, the mutant frequencies were higher in TK6 than WTK1 cells in both folate replete and deficient medium. The mutant frequencies were higher in folate deficient than replete cells, but the effect was greater in TK6 cells. Statistical analysis of

the slopes indicated significantly different *HPRT* mutation dose-response relationships at the 0.01 level.

Molecular analyses of 152 6-thioguanine resistant mutant clones are shown in detail in Table 2 of the appended manuscript (1) (the full table could not be included in this application because of its length). A summary of this data is presented here as Table I. The predominant mutation (63%) in both cell types grown in the presence or absence of folic acid was a G>A transition on the non-transcribed strand. These transitions were mainly at non-CpG sites, particularly when these bases were flanked 3' by a purine or on both sides by G:C base pairs. The section of exon 3 that contains six guanines in a row was especially susceptible to mutation (19 of the 95 G>A mutations). A smaller number of G>A transitions occurred on the transcribed strand, reflected as C>T transitions (14%) and were more common in the folate-deficient WTK1 cells. The most striking difference between the folate-replete and deficient cells was an increased frequency of deletions in the cells of both types grown under low-folate conditions (17%) as compared to replete cells (4%). In addition, four mutations in low folate cells resulted in no cDNA and no obvious change in the nine *HPRT* exons and could represent translocation events, or deletions that interfered with RNA splicing. Therefore the total deletion frequency could be as high as 17 of 75 (23.7%) in the low folate mutations.

The predominant mutation in EMS-treated folate-replete WTK1 cells was a G>A transition. EMS is mutagenic by reaction with the O^6 and N^7 positions of guanine. O^6-ethylguanine is mutagenic by pairing with thymine during replication, while N^7-alkylation products lead to apurinic sites that are processed by base excision repair and may cause mutations by defective repair or by mis-incorporation (2-6). Under folate-replete conditions, most mutations (69%) in the WTKI cells were G>A transitions, suggesting that the deficiency of the AGT repair mechanism was a major contributing factor to persistent mutations. The smaller number of genomic deletions (8%) probably reflects error-prone base excision repair, because base excision repair defective cell lines exhibit increased percentages of deletion mutations after EMS treatment (7,8). The mutational spectrum in the p53-competent TK6 cell line was similar to WTK1 cells after EMS treatment in folate-containing medium, showing 76% G>A transitions but no deletions. This observation suggests that the p53 gene product does not have a major influence on the molecular spectrum after treatment with monofunctional alkylating agents.

Folate deficient TK6 and WTK1 cells, like folate replete cells, showed a predominance of G>A transitions. However they also exhibited an increased percentage of deletions compared to folate-replete cells. This finding supports and confirms our previous report that folate-deficient CHO cells had more intragenic deletions after EMS treatment than folate replete cells (9, manuscript appended). Folate deficient WTK1 cells were found to have a higher percentage of C>T transitions (21%) than either folate replete WTK1 cells (10%) or TK6 cells regardless of folate status (16% replete, 8% deficient). This higher percentage of C>T transitions may represent persistence of G>A transitions on the transcribed strand of these folate-deficient, p53 mutant cells. Alternatively, O^2-ethylcytidine may act as uracil and code for thymine (10) giving the pathway: O^2-ethylcytidine $\rightarrow$ U $\rightarrow$ T.

The distribution of mutation types, shown in Figure 3 differs significantly between the +folate and -folate cells ($p=0.024$), primarily due to the increase in deletion mutations.

Table I
Summary of mutation types in the HPRT gene in 6-thioguainine resistant human lymphoblastoid cells exposed to EMS

Mutation Type (No. of mutations)	NTS	Folate Replete		Low Folate	
		TK6 (38)	WTK1 (39)	TK6 (37)	WTK1 (38)
Transitions					
GC>AT	G>A	29 (14)[a]	27 (12)[a]	21 (10)[a]	18 (7)[a]
	C>T	6	4	3	8
AT>GC	A>G	0	1	0	0
	T>C	0	1	0	0
Transversions					
GC>TA	G>T	0	2	2	1
	C>A	1	0	0	0
GC>CG	G>C	0	0	2 (1)[a]	0
	C>G	0	0	0	0
AT>TA	A>T	0	0	1 (1)[a]	1 (1)[a]
	T>A	0	0	0	0
AT>CG	A>C	0	0	0	0
	T>G	0	0	1	0
Genomic deletion		0	3	3	10
"new exon"		1			
no change		1[b]	1[c]	4[d]	

NTS = nontranscribed strand
[a]mutations which affected splicing
[b]exon 8 exclusion in cDNA with no change in genomic found
[c]exon 4 exclusion in cDNA with no change in genomic found
[d]four mutations with all nine exons present in genomic DNA and no change in the sequence of exons 1, 7, 8 and 9.

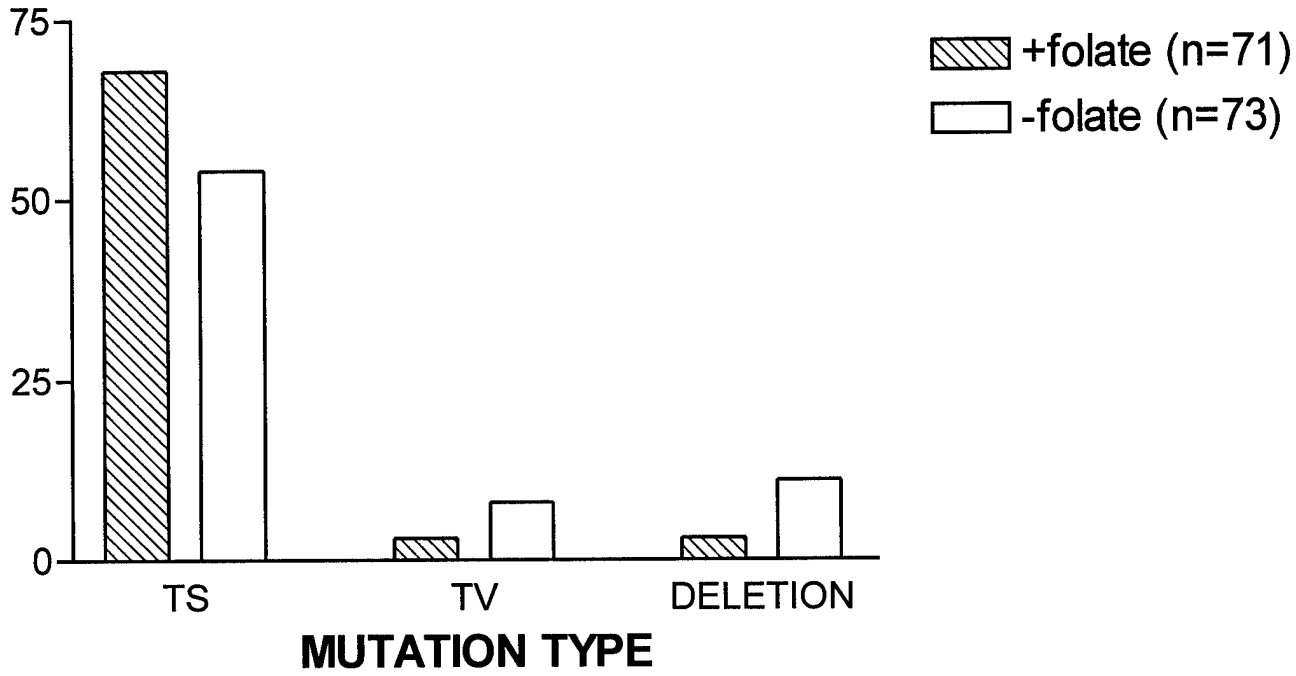


Figure 3: Summary of EMS mutation spectrum in human lymphoblast cells treated in the presence and absence of folate. Mutation types are grouped into transitions (TS), transversions (TV) and genomic deletions.

Calculations of the ID_{50} for the breast cancer cell lines by EMS are shown in Table II. The cells were cultured in either folate-deficient medium or in 2 concentrations of folate, 2 ug/ml (a concentration found in many standard culture media) and 50 ug/ml. The data in Table II suggest that higher folate levels tend to protect against the toxic effects of EMS in the MCF 7, MDA-MB-435 and MADB 106 breast cancer cell lines. This observation is consistent with our original animal studies, in which rats on high folate diets treated with cyclophosphamide had less toxicity than folate deficient rats. However, this data does not support the observation that breast cancers in folate deficient rats were less sensitive to alkylating agent.

Table II. The Effect of Culture Medium Folate Level on the Concentration of EMS that Inhibits Growth by 50% of Breast Cancer Cell Lines of Varying Estrogen Receptor and Erb B2 status.

				Folate Concentration (ug/ml)		
Cell Line	**Pathology**	**ER**	**Erb B2**	**0**	**2**	**50**
MCF 7	Adenocarcioma	+	+/-	0.56*	0.56	0.70
BT-474	Ductal carcinoma	+	++	0.38	0.44	0.40
SK Br 3	Ductal carcinoma	-	+++	0.21	0.19	0.22
MDA-MB-435	Adenocarcinoma	-	-	0.07	0.08	0.11
MADB 106	Rat adenocarcinoma			0.43	0.50	0.46
TK 6	Lymphoblast			44.6	40.5	35.9

* ID_{50} in mg/ml EMS

The ID_{50}'s for doxorubicin and 5-fluorouracil are shown in Tables III and IV. The doxorubicin and 5-FU data suggest that, in contrast to alkylating agents, folate levels have a more variable effect on toxicity associated with these drugs. Thus high folate levels protect against doxorubicin toxicity in the BT-474 and TK 6 cell lines, but enhance toxicity in the MCF 7, SK Br 3 and MADB 106 breast cancer cell lines. 5-FU toxicity was either enhanced by high folate levels, as seen in the MCF 7, BT-474, and SK Br 3 breast cancer cell lines, reduced (MDA-MB-435 and TK6), or unchanged (MADB 106).

Table III. The Effect of Culture Medium Folate Level on the Concentration of Doxorubicin that Inhibits Growth by 50% of Breast Cancer Cell Lines of Varying Estrogen Receptor and Erb B2 status

				Folate Concentration (ug/ml)		
Cell Line	Pathology	ER	Erb B2	**0**	**2**	**50**
MCF 7	Adenocarcinoma	+	+/-	0.27*	0.21	0.21
BT-474	Ductile carcinoma	+	++	0.33	0.39	0.54
SK Br 3^	Ductile carcinoma	-	+++	0.72	0.43	0.32
MDA-MB-435	Adenocarcinoma	-	-	0.25	0.18	0.22
MADB 106 ^	Rat adenocarcinoma			8.5	5.8	6.1
TK 6	B cell Lymphocyte			0.45	0.56	0.58

* ID_{50} in uM Doxorubicin
^ N=2

Table IV. The Effect of Culture Medium Folate Level on the Concentration of 5-FU that Inhibits Growth by 50% of Breast Cancer Cell Lines of Varying Estrogen Receptor and Erb B2 status

				Folate Concentration (ug/ml)		
Cell Line	Pathology	ER	Erb B2	**0**	**2**	**50**
MCF 7	Adenocarcinoma	+	+/-	.0107	.0075	.0057
BT-474^	Ductile carcinoma	+	++	.021	.014	.011
SK Br 3^	Ductile carcinoma	-	+++	.0207	.0139	.0111
MDA-MB-435	Adenocarcinoma	-	-	.0076	.0095	.0091
MADB 106^	Rat adenocarcinoma			.029	.028	.031
TK 6	B cell lymphoma			.0007	.0032	.0019

* ID_{50} in uM 5 FU
^ N=2

Thus, of the three drugs, the effect of folate status was most consistent with alkylating agents, with high folate levels reducing toxicity *in vitro*. Therefore our mechanistic studies, described below, focused on this class of chemotherapeutic agent.

- **Determine mechanism of folate-drug interactions**

Studies of the pharmacologic mechanisms that account for the amelioration of alkylating agent-induced toxicity by folate supplementation illustrated by the data in Table II have focused on the enzyme systems that are involved in alkylating agent metabolism and on mitochondrial DNA damage. With regard to the former, it appears that altered glutathione levels may be involved. It is well established that glutathione protects against the cytotoxicity of alkylating agents (reviewed in 11). The multiple steps at which glutathione modulates alkylating agent metabolism, as illustrated by cyclophosphamide, is shown in Figure 4. Since folate metabolism is involved in the synthesis of the three amino acids that constitute glutathione (glutamate, cysteine and glycine), illustrated in Figure 5, we measured glutathione levels in liver samples from rats on diets of varying folate content and treated with increasing doses of cyclophosphamide. As shown in Figure 6, glutathione levels increased in the order: folate deficient<folate replete<high folate, and this result was statistically significant ($p<0.0001$) using analysis of variance. These results suggest that folate supplementation promotes higher levels of glutathione production and protection against toxicity.

This result in rat liver was confirmed with tissue culture cells *in vitro.* As illustrated in Figure 7, flow cytometric analysis was used to quantitate total glutathione. The difference between Peaks A and B represents intra-cellular glutathione. In this experiment, glutathione increased progressively during the culture period. When cells were grown for four days in culture medium of varying folate content, we found the relative glutathione contents were: 0 μM = 0.2; 2 μM = 1.62; 50 μM = 2.94. Thus, folate supplementation increases glutathione levels both *in vitro* and *in vivo*, and this effect probably accounts, at least in part, for the reduced cytotoxicity of alkylating agents seen with the high folate state.

Other mechanistic studies were undertaken to characterize the folate-drug interaction. As illustrated in Figure 4, alkylating agent metabolism is influenced by the microsomal cytochrome P450 system and by aldehyde dehydrogenase levels in addition to glutathione. Therefore, we assayed these systems in livers from rats maintained on diets of varying folate content. The results of these experiments are described in detail in the appended manuscript and are illustrated in Figures 2 and 3 in the manuscript (12). Unfortunately we could not detect a diet-related difference in these enzyme systems to account for the observed differences in toxicity associated with alkylating agents.

Figure 4. Metabolism of Cyclophosphamide

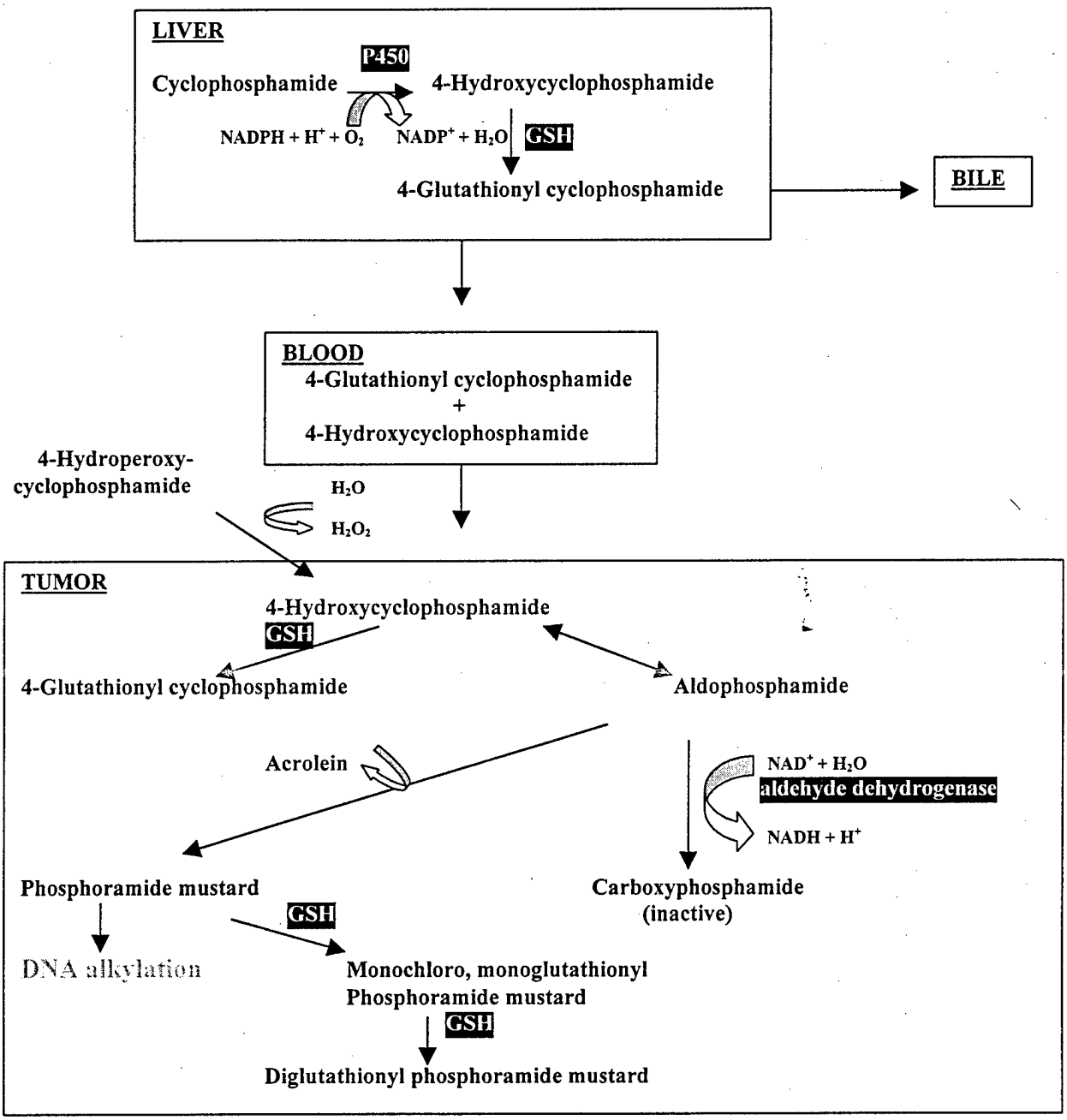
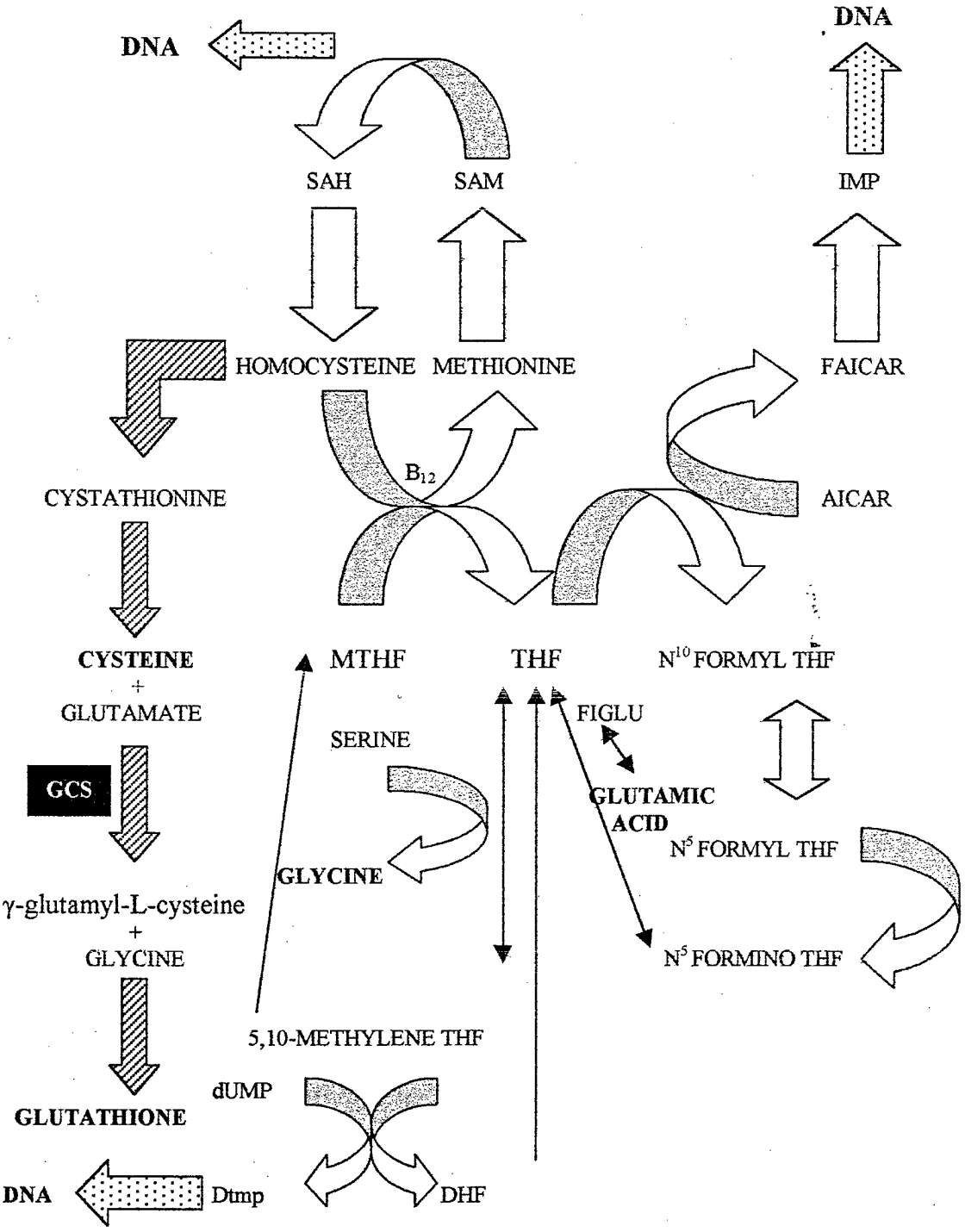


Figure 5. Relationship between folate metabolism and glutathione synthesis.



MTHF, 5-methyltetrahydrofolate; *B_{12}*, vitamin B_{12}; *SAM*, S-adenosyl-L-methionine; *SAH*, S-adenosyl-L-homocysteine; *THF*, tetrahydrofolate; *dUMP*, deoxyuridylic acid; *dTMP*, thymidylic acid; *DHF*, dihyrofolate; *FIGLU*, formino-glutamic acid, *AICAR*, 5-amino-4-imidazolecarboxamide; *FAICAR*, formyl-AICAR; *IMP*, inosinic acid, GCS, γ-glutamylcysteine synthetase

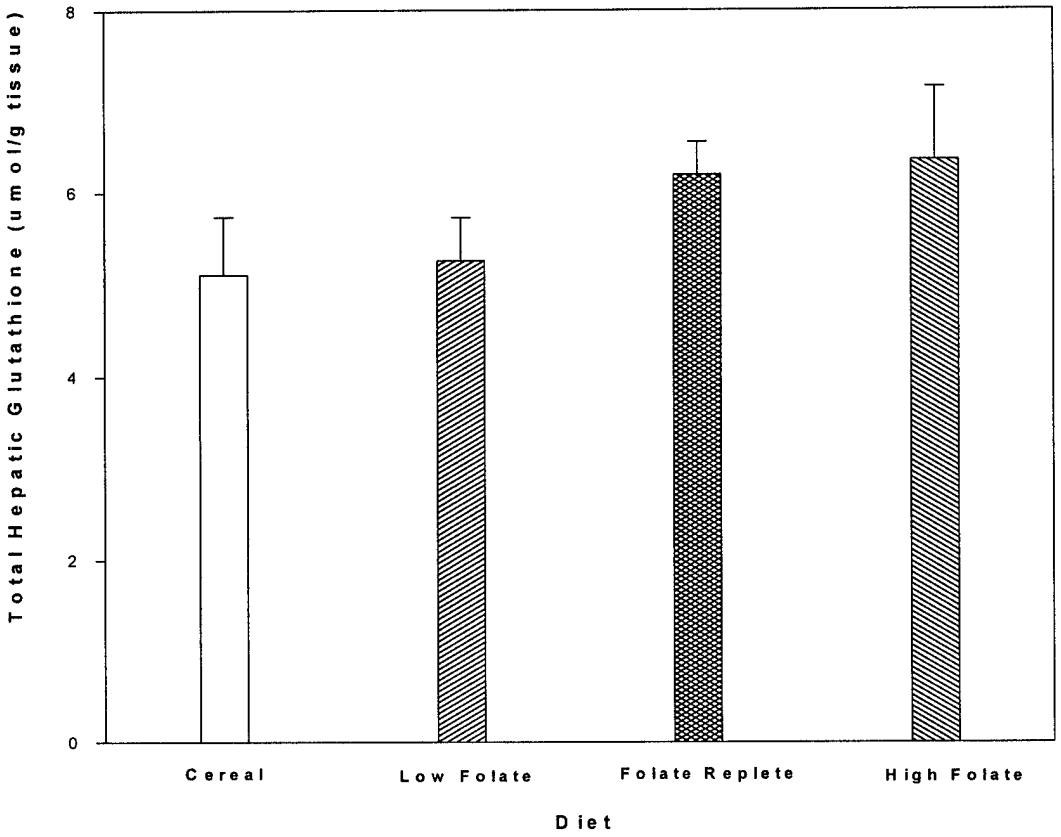


Figure 6. Total hepatic glutathione levels in rats fed either a cereal-based diet or a purified diet of varying folate content.

During the past year we completed studies on the effect of nutritional folic acid levels on alkylator-induced mitochondrial DNA damage. This work was just accepted for publication in Mutation Research, and a copy of the manuscript is appended (13). Cancer chemotherapeutic drugs are known to damage mitochondria, and this damage may contribute to their efficacy and toxicity. Approximately half of intracellular folate compounds are compartmentalized in the mitochondria, and mitochondrial folate is responsible for the generation of one-carbon units for cytoplasmic anabolic reactions including the synthesis of DNA precursors. These studies were initiated to investigate the relationship between diet, particularly nutritional folate status, and mitochondrial DNA damage after cancer chemotherapy in rats. The animals were maintained on either a cereal-based diet or a purified diet of varying folate content (deficient, replete or supplemented) for 6 weeks. Then they were treated with a single intraperitoneal injection of increasing doses of either cyclophosphamide or 5-fluorouracil. Two weeks later, the amount of the "common deletion" (4.8kb, bases 8103-12937) in liver was measured by quantitative co-amplification of the mitochondrial D-loop and the mitochondrial deletion using a real-time quantitative polymerase chain reaction assay. The relative abundance of mitochondrial DNA was determined by co-amplifying mitochondrial D-loop versus rat β-actin gene. Overall, there was a significant effect of dose ($P=0.0001$) and of diet ($P=0.0001$) on mitochondrial deletions in rats treated with cyclophosphamide. Within the group of animals that received no cyclophosphamide, the high folate group had fewer deletions than either the low folate ($P=0.0001$) or replete ($P=0.006$) groups. After treatment with cyclophosphamide, rats maintained on the high folate diet had significantly fewer deletions than folate-deficient rats. The rats fed the folate-replete purified diet had fewer deletions than rats fed the cereal-based diet. There was no effect of diet on mitochondrial copy number after cyclophosphamide treatment. In rats treated with 5-fluorouracil, a significant dose by diet interaction was found for both deletions and relative abundance of mtDNA ($P<0.01$). The rats maintained on the folate-replete purified

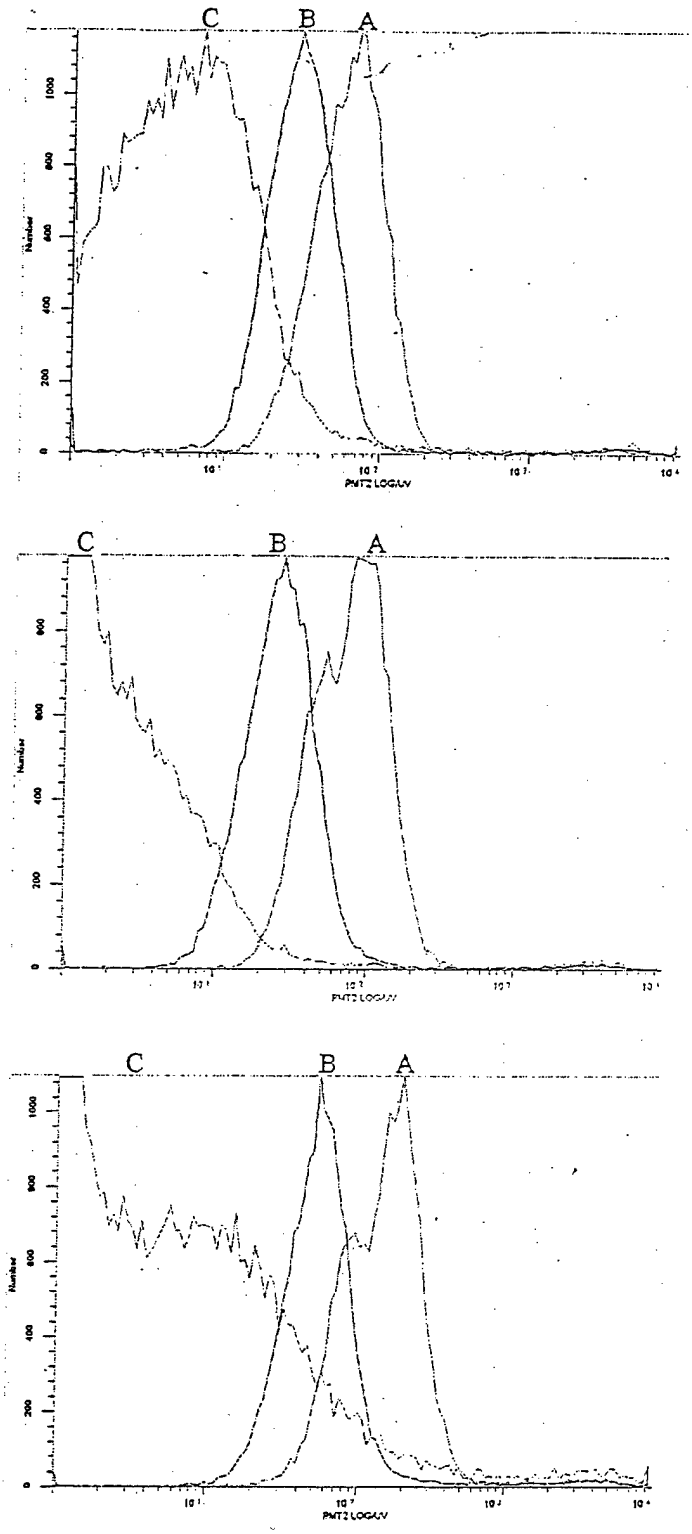


Figure 7. Flow cytometric analysis of total glutathione in MADB106 rat mammary carcinoma cells. The cells were cultured for 2 (top), 3 (middle) or 4 (bottom) days in complete DMEM medium. Peak A represents total intra-cellular thiols. Peak B represents depletion of glutathione by buthionine sulfoximine (BSO) treatment. Peak C represents depletion of all intra-cellular thiols. The difference between Peaks A and B represents intra-cellular gluthathione. In the experiment, intra-cellular glutathione increased progressively with duration of the culture period.

diet had fewer deletions than rats fed the cereal-based diet, but there was no consistent effect of nutritional folate status on deletions at the various 5-fluorouracil dosage levels. There was a trend for mtDNA relative abundance to be higher in the cereal-based and high folate groups. These results indicate that diet can modulate the extent of mitochondrial damage after cancer chemotherapy, and that folic acid supplementation may be protective against some types of mitochondrial DNA damage. Thus, protection against mitochondrial DNA damage is another mechanism by which folate status may modulate the toxicity associated with chemotherapy.

Task 2. *In vivo* assessment of folate drug interactions

- **Determine the effect of folate status on drug toxicity**

These experiments proceeded as described in the Statement of Work. We elected to start with the toxicity studies, since these experiments are the most time-consuming because of the numerous assays for toxicity. In addition, used the results for dose-finding for the efficacy studies. The results of the toxicity studies are presented in detail in the appended manuscripts (12,14).

Weaning female Fischer 344 rats were maintained on either standard rat chow (Teklad 7012) or a Purified Diet containing either no folic acid or 2 mg folic acid/kg of diet, as previously reported by our laboratory (15). Some rats on the folate-containing diet received additional folic acid, 50 mg/kg, intraperitoneally (IP) daily. After 6 weeks on these diets, the rats were injected with a single dose of either cyclophosphamide or 5-FU IP. Blood was obtained for laboratory determinations prior to the chemotherapy injection, and on days 4, 9 and 14 afterward. Surviving rats were sacrificed on day 14 and the livers collected and frozen.

Measurements of hepatic folate levels by the *Lactobacillus casei* method (15) gave the following results: standard rat chow, 34.0 μg/g; folate replete diet, 32.1 μg/g; folate deficient diet, 9.1 μg/g; high folate animals, 45.8μg/g. These results indicate that the dietary conditions caused important differences in tissue folate levels.

The number of deaths in each dietary group (6 animals/group) after treatment with increasing doses of cyclophosphamide, and the measurements of growth, bone marrow toxicity (HCT, WBC), renal toxicity (BUN), liver toxicity (LDH, SGPT) and cardiac toxicity (CPK) are presented in the appended manuscipt (12).

The following conclusions can be drawn from this experiment:

1. The median lethal dose (LD_{50}) are:

cereal control	232 mg/kg
folate deficient	154 mg/kg
folate replete	159 mg/kg
high folate	148 mg/kg

- The cereal control was significantly different from the other groups; there was no significant difference among the folate groups.

2. There were no significant differences in rat weights at 6 weeks on the various diets.

3. Rat weight at 6 weeks did not predict survival after cyclophosphamide (ctx) treatment.

- Diet group and ctx dose level influence survival.

4. Weight gain (growth) during the first 6 weeks was the same in the various dietary groups.

5. Weight at week 7 did not differ among the dietary groups at any of the ctx dose levels.

6. At week 8, the cereal controls were significantly heavier than the other groups at the 85 mg/kg dose, while the folate deficient rats lost significantly more weight than the other groups at the 110 mg/kg dose.

7. At week 6 (pre-treatment) the high folate group had a significantly higher BUN than the other dietary groups.

8. There was no difference in WBC or HCT pre-treatment among the dietary groups.

9. The cereal control group generally had higher HCT's during the treatment than the other groups.

10. On day 9, the WBC was higher in the high folate group at 50 mg/kg, and higher in the cereal group at 65 mg/kg.

11. The BUN generally was higher in the high folate group on days 9 and 14 than in the other dietary groups.

12. After logistic regression, diet and dose caused the main effects; pre-treatment BUN, WBC and HCT were not significant when added to diet and dose, or just dose.

13. After logistic regression, deaths were predicted by dose, diet, WBC and BUN on day 4.

14. Measurements of covariance indicated that:

- With regard to HCT, there were interactions of diet and time and dose and time, indicating that different diets and different doses act differently over time. Therefore all of the curves are different over time from each other.
- With regard to WBC, dose and diet have a differential effect over time; there is no diet interaction with time. Therefore dose rather than diet is more important for interaction over time for WBC. Diet has a more unique effect on dose over time on HCT than WBC.
- With regard to BUN, there is a dose-time but not diet-time interaction, but diet has an effect on BUN.

The above results suggested that the causes of death were bone marrow suppression, an expected effect of cyclophosphamide treatment, and renal failure, an unanticipated effect. The renal failure appeared to be most prominent in the high folate dietary group. Therefore histologic examination of kidneys from rats in the different dietary groups was performed by a pathologist at the University of Vermont. He found that the kidneys from rats on the 4 diets

alone were normal. Similarly, the kidneys from rats on the cereal control diet and treated with chemotherapy also were normal. However, kidneys from rats on the low folate and folate replete diets showed focal tubular regeneration, while the kidneys from rats on the high folate diet showed acute tubular necrosis, often marked (see Figures 3, 4 and 5 in the appended manuscript, 14). These results indicate that the combination of a purified diet and chemotherapy is nephrotoxic compared to a cereal-based diet and chemotherapy. The addition of high levels of folic acid to the purified diet and chemotherapy exacerbates the renal toxicity. There have been previous suggestions in the literature that high levels of folic acid may cause kidney damage. For example, Achon and colleagues recently reported that high dietary folate supplementation of rats was associated with impaired dietary protein metabolic utilization and higher urinary nitrogen elimination (16). Fetuses of folate supplemented dams had lower body weight and shorter vertex-coccyx length compared to unsupplemented dams (16). Klingler et al found that folic acid administered to rats in doses of 100, 200, 300 and 400 mg/kg of body weight resulted in changes in tubular morphologic features and renal function, and the severity of the changes were proportional to dose (17). Similarly, Schubert and colleagues reported that repeated injections of folic acid, 250 mg/kg, to rats caused severe chronic kidney damage (18). Renal toxicity in our animals occurred at much lower folate levels (50 mg/kg), suggesting a synergistic interaction between diet, chemotherapy and folic acid. To the extent that these studies are relevant to humans, they suggest that there may be an optimal dose of folic acid supplementation to modify the effects of cancer chemotherapy.

Statistical analyses confirmed the resistance to alkylating agent toxicity shown by the animals on the Teklad 7012 rat chow. This resistance is not mediated by dietary folate levels or hepatic glutathione levels. Other possible mechanisms were explored to explain the reduced toxicity including increased quenching of reactive oxygen species or the enhanced induction of the cytochromes P-450 drug/toxin metabolizing system and mixed function oxidases in animals fed cereal-based diets such as Tekad 7012 (19).

Toxicity studies were also performed with 5 FU and are reported in detail in the appended manuscript (14). These studies suffered a major set-back because 3 groups of animals (an untreated control group, 190 mg/kg and 250 mg/kg) were infected with a virus, sialodacryoadenitis, during the experiment. Therefore these animals are not evaluable. This loss caused a 3 month delay in the progress of these experiments and considerable expense for replacement animals and technician time.

The rats in the different dietary groups that later would be treated with 5-FU grew at approximately the same rate during the first six weeks of the experiment, although analysis by repeated measures ANOVA indicated that for nearly all weeks the high folate group was the lightest and the cereal diet group was usually the heaviest. The maximum difference was relatively small, 4.6g in Week 3, and by Week 6 there were no significant differences in weights among the diet groups.

The rats were injected IP with 5-FU after six weeks on the four diets. The weights in Week 7 did not differ among any of the diet groups after adjusting for baseline weight. At Week 8, the rats on the cereal-based diet were heavier than the other three groups, with a maximum difference of 15.1g, between cereal-based diet and high folate group (ANOVA, overall p=0.015).

Rats treated with the lowest 5-FU dose, 110 mg/kg, had relatively stable weights during the two weeks following treatment, while rats treated with the highest dose, 546 mg/kg, lost weight during this period.

The 5-FU related deaths in animals on the four diets are shown in Table V. Six or seven rats per group were treated 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. Inspection of the data suggests that at each 5-FU dose level, the high folate group had equal or greater mortality than the other dietary groups, while the rats on the cereal-based and low folate diets tended to have fewer deaths than the folate replete and high folate groups.

Table V. Deaths in rats maintained on either a cereal-based (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR; or high folate, HF) and treated with 5-FU in the dose indicated.

	Diet							
Dose	**CR**	**n**	**FD**	**n**	**FR**	**n**	**HF**	**n**
110 mg/kg	0	6	0	6	1	6	4	7
144 mg/kg	1	6	1	6	1	6	3	7
325 mg/kg	2	12	1	12	2	12	2	13
420 mg/kg	1	6	0	6	1	6	5	6
546 mg/kg	0	6	1	6	3	6	6	7

n = number of animals

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 overall $p<0.0005$). There were no significant differences among the dietary groups for white blood cell count at pretreatment.

Repeated measures ANOVA indicated that diet showed significant interactions with time for all three post-treatment blood analyses (hematocrit, white blood cell count and BUN; all models had $p<0.01$). Analyses showed that the BUN levels on Day 14 were significantly higher on Days 9 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 on Day 14 was significantly higher in the cereal diet group than in rats on the high and folate replete diets. The white blood cell count changes after chemotherapy were somewhat variable. On Day 4, the high folate and folate replete dietary groups had lower values than the folate deficient group. At Day 9, the white blood cell count in the high folate group remained depressed, and was significantly lower than in the other three dietary groups. 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. 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 $p<0.001$).

Logistic regression revealed that diet, but neither dose nor baseline weight, was related to death ($p<0.0001$). 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.

Survival analysis with post-treatment data included Days 0, 4, and 9 blood measurements as time-varying covariates. That is, baseline measurements were used to predict deaths up to Day 4, Day 4 measurements used to predict deaths to Day 9, and Day 9 measurements used to predict deaths to Day 14. Weights at Week 6 and 7 were included as another time-varying variable; dose was also included in this model. Model fitting yielded two models with similar fit indices. The better model was: weight, white blood cell count and hematocrit. The other model included: weight, white blood cell count and BUN. However, BUN was not significant when the hematocrit was in the model.

These results indicate that diet has an important influence on the toxicity of a bifunctional alkylating agent, cyclophosphamide, and of an anti-metabolite, 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, 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, and that rodents fed purified diets have higher tumor incidences compared to rodents fed natural ingredient diets.

- **Determine the effect of folate status on tumor growth rate**

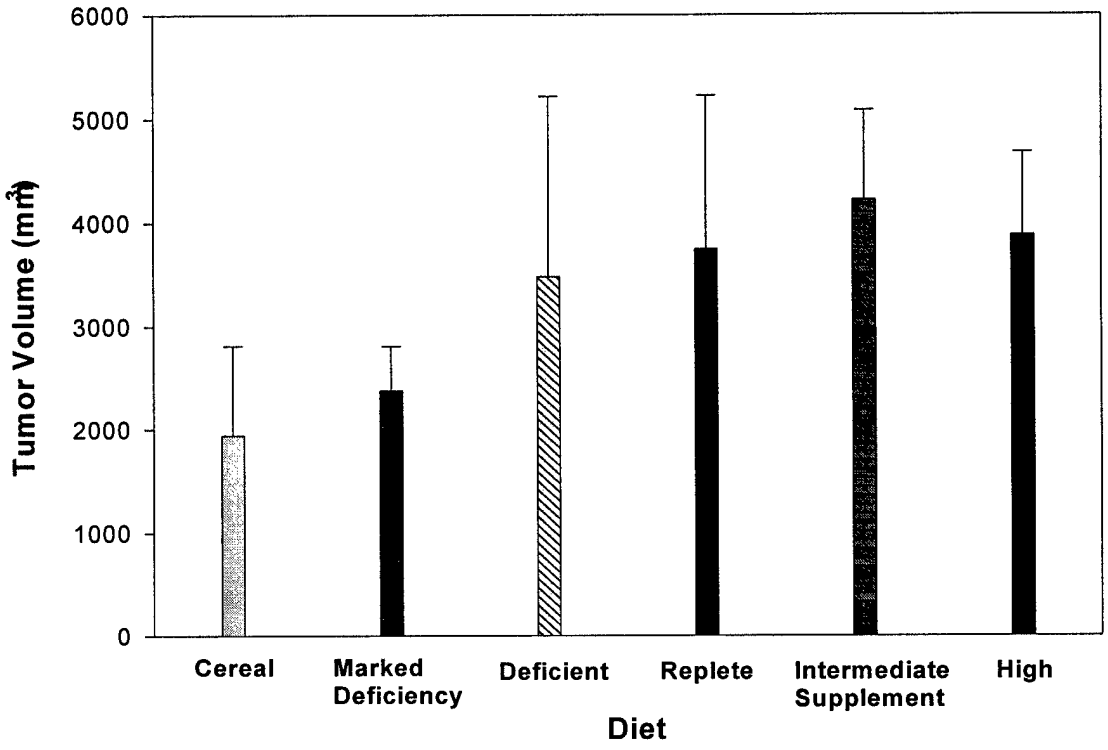


Figure 8. MADB 106 mammary tumor size in rats fed a cereal-based diet or a purified diet of varying folate content. The error bars represent the SEM.

These studies were performed exactly as described in the Statement of Work. Weanling Fischer 344 rats were divided into 5 dietary groups: marked folate deficiency, moderate folate deficiency, folate replete, intermediate folate supplementation and high folate supplementation. Rats in the marked folate deficiency group were fed AIN-93G diet with added 1.0% succinyl sulfathiazole (DYETS, Bethlehem, PA). A second group of animals was placed on the low folate diet (AIN-93G) to produce moderate folate deficiency. A third group received AIN-93G with 2 mg/kg of folic acid/g of chow (standard or folate-replete diet). A fourth group of animals received folic acid 10 mg/kg i.p. daily, and the fifth group received 50 mg/kg i.p. daily. Five weeks later, MADB 106 rat mammary tumor was injected orthotopically into the mammary fat pads. Tumor growth rate was measured and compared between the folate groups to determine if

folate status influences growth. Figure 8 shows the results of this experiment. It can be seen that the tumors were smaller in the rats fed a cereal-based diet that those fed a purified diet, and that tumor volume was proportional to the folate content of the diet within the purified diet.

- **Determine the effect of folate status on drug efficacy (combination of cyclophosphamide and doxorubicin)**

A second group of rats was maintained as in the experiment described above. When the tumor was barely palpable, chemotherapy treatment was initiated i.p. for each dietary group and repeated 96 hr later. The doses given were cyclophosphamide, 33 mg/kg, and doxorubicin, 3 mg/kg. Folic acid treatment was continued throughout the drug treatment phase of the study. Figure 9 shows the results of this experiment.

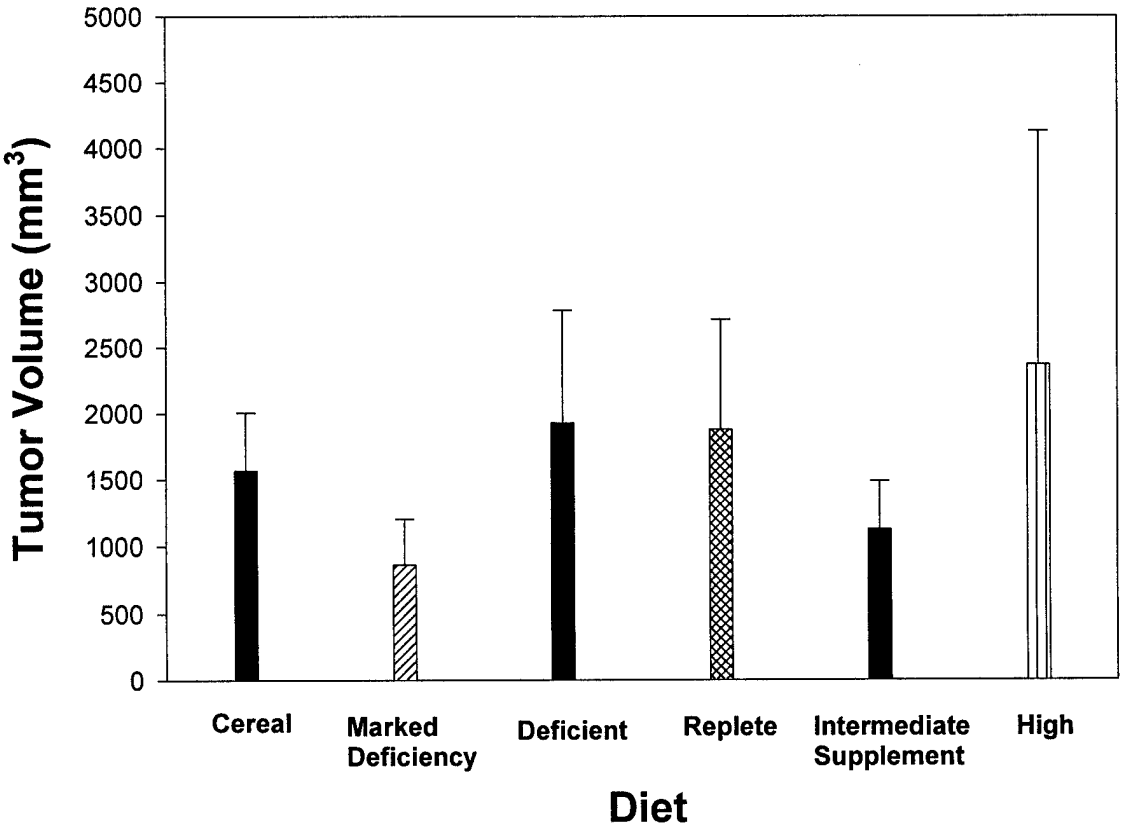


Figure 9. MADB 106 mammary tumor volume in rats fed a cereal-based diet or a purified diet of varying folate content and treated with cyclophosphamide and doxorubicin. Error bars represent the SEM.

The efficacy of therapy was determined by measuring the tumor growth inhibition (TGI), calculated as: $TGI = 100 - TV_{treatment}/TV_{control}$. Significance of differences was assessed using ANOVA. Significant differences between the treated and control TV values were found for rats in the intermediate folate supplementation diet ($p < 0.01$) and the marked folate deficient diet ($p = 0.01$). The differences for the other diet types were not significant. There were no significant differences among the survival times for rats on the different diet types.

Diet	TGI (%)	Mean Survival (days)
Cereal	46.0	14.0
Marked folate deficiency	63.5	11.8
Folate deficient	44.6	13.2
Folate replete	49.7	14.0
Intermediate folate supplementation	73.2	13.0
High folate supplementation	38.9	10.8

These data confirm that diet has an important influence on the efficacy of cancer chemotherapy in the rat mammary tumor model. Tumor growth inhibition was enhanced by the marked folate deficiency state because tumor growth was retarded, while chemotherapy inhibition was enhanced by added folate. However, consistent with our single agent toxicity studies, there appeared to be an optimal folate concentration. Very high levels of folate supplementation actually reduced efficacy.

- **Determine the effect of folate status on drug toxicity (combination of cyclophosphamide and doxorubicin)**

Weanling Fischer 344 rats were divided into the 6 dietary groups as described above. After 5 weeks, they were treated on Days 0, 4 and 8 with cyclophosphamide 33 mg/kg and doxorubicin 5 mg/kg. Animals were weighed daily and observed twice daily for signs of toxicity---loss of grooming behavior, lethargy and loss of righting reflex. After 14 days, surviving animals were euthenized.

Diet	Survival
Cereal	10.00
Marked folate deficiency	11.83
Folate deficient	10.17
Folate replete	8.33
Intermediate folate supplementation	8.67
High folate supplementation	9.17

Statistical analysis found significant differences among the survival times for rats on the different diet types. The following pairs of diet types differed significantly:

High	vs	Low
Cereal		Intermediate folate supplementation
Marked folate deficient		Folate replete
Marked folate deficient		Intermediate folate supplementation
Folate deficient		Folate replete

Thus folate deficiency tended to decrease the toxicity of the combination of cyclophosphamide and doxorubicin.

KEY RESEARCH ACCOMPLISHMENTS

- Studies of the interaction of folate metabolism and chemotherapeutic drugs at the molecular level in human lymphoblastoid cell lines supported our model to explain the synergy between nutritional folate deficiency and alkylating agents.

- The inhibitory concentrations (ID_{50}) of an alkylating agent (EMS), doxorubicin and 5-FU for 5 breast carcinoma cell lines and a human lymphoblast cell line grown in media of varying folate levels were identified. These results indicated that high folate levels tended to reduce the toxicity of alkylating agents but had a variable effect on the toxicity associated with doxorubicin and 5-FU.

- A previously unrecognized relationship between folate metabolism and cellular glutathione levels was confirmed in rat liver samples and by *in vitro* experiments.

- Folic acid supplementation of rats reduced the frequency of hepatic mitochondrial DNA deletions. Thus protection against mitochondrial DNA damage is another mechanism by which folate status may modulate the toxicity associated with chemotherapy.

- Statistical analyses of toxicity studies with cyclophosphamide in rats indicated that:
  - Rats on a cereal-based diet were significantly more resistant to the toxicity of cyclophosphamide than rats on a Purified Diet.
  - There was no significant difference among rats on the Purified Diet.
  - Diet and dose caused the main effects; pre-treatment BUN, WBC and HCT were not significant when added to diet and dose, or just dose.
  - Deaths were predicted by dose, diet, WBC and BUN on day 4.

- The combination of a purified diet and chemotherapy was nephrotoxic compared to a cereal-based diet and chemotherapy. The addition of high levels of folic acid to the purified diet and chemotherapy exacerbated the renal toxicity. Therefore there may be an optimal dose of folic acid supplementation to modify the effects of cancer chemotherapy.

- In toxicity studies with 5-FU, folate supplementation tended to increase mortality, while rats on the cereal-based and low folate diets tended to have fewer deaths. Model fitting indicated that mortality was predicted by weight, white blood cell count and BUN.

- Growth of the rat mammary tumor MADB 106 was retarded in rats fed a cereal-based diet or a purified diet that was markedly folate deficient.

- Treatment of tumor-bearing rats with the combination of cyclophosphamide and doxorubicin resulted in significant tumor growth inhibition in the intermediate folate supplementation group and the marked folate deficient group. These data confirm that nutritional folate status has an important influence on the efficacy of cancer chemotherapy in the rat mammary tumor model.

- In a toxicity study with the combination of cyclophophamide and doxorubicin, rats maintained on a cereal-based diet or folate deficient diets had significantly less toxicity than rats on diets with higher folate content.

REPORTABLE OUTCOMES

Manuscripts: Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status. Mutation Res. 473:51-71 (2001).

Branda, R.F., Lafayette, A.R., O'Neill, J.P., Nicklas, J.A. The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary (CHO) cells treated with monofunctional alkylating agents. Mutation Res. 427:79-87 (1999).

Branda, R.F., Chen, Z., Brooks, E.M., Naud, S.J., McCormack, J.J. Diet modulates the toxicity of cancer chemotherapy in rats. I. Cyclophosphamide. Submitted.

Branda, R.F., Chen, Z., Brooks, E.M., Naud, S.J., Trainer, T.D., McCormack, J.J. Diet modulates the toxicity of cancer chemotherapy in rats. II. 5-Fluorouracil. Submitted.

Branda, R.F., Brooks, E.M., Chen, Z., Naud, S.J., Nicklas, J.A. Dietary modulation of mitochondrial DNA deletions and copy number after chemotherapy in rats. Mutation Res, in press.

Abstract and Presentation: Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. P53 activity modulates the effect of folate deficiency on genetic damage caused by alkylating agents in human lymphoblastoid cells. Proc. AACR 41:69, 2000. Presented at the Annual Meeting of the AACR, April, 2000

Funding applied for based on work supported by this award:

- Effect of Folate on Chromosomes in Breast Cancer, NCI, R.F. Branda, P.I.
- Effects of St. John's Wort and Vitamin E on Breast Cancer Chemotherapeutic Agents, DOD, R.F. Branda, P.I.

CONCLUSIONS

The experiments described in this Final Report analyze the relationship between a micro-nutrient, folic acid, and the toxicity of chemotherapeutic drugs used to treat women with breast cancer. Our studies of the interaction of folate metabolism and alkylating agents at a molecular level confirm that intragenic deletions and G>A transitions are the predominant chromosomal changes, and indicate that human cells manifest the same mutational spectra as rodent cells. Since the findings reported here occurred in human cell lines, they may be clinically relevant. Our results suggest that cells expressing p53 activity exhibit a higher rate of mutation induction but are more sensitive to the toxic effects of alkylating agents than those lacking p53 activity. Folate deficiency tends to reduce toxicity but increase mutation induction after alkylating agent treatment. TK6 cells are deficient in alkyltransferase activity and resemble in this regard bone marrow myeloid precursors (20). The bone marrow is a frequent target organ for alkylating agent-induced transformation, and acute myelogenous leukemia has been reported following exposure to cyclophosphamide, melphalan and busulfan as single agents and to the combination of nitrogen mustard and procarbazine (21). Cytogenetic analyses indicate that transformation to myelodysplasia and acute leukemia is associated with deletions and translocations (21). Thus the combination of alkylating agent exposure and nutritional folate deficiency in a cell type deficient in alkyltransferase but expressing wild-type p53 creates an environment that increases the risk of developing carcinogenic genetic changes. The addition of a mutation at the p53 locus would alter further the biology of a developing tumor cell, in that loss of p53 activity reduces toxicity from monofunctional alkylating agents, and those cells lacking p53 activity and folate are most resistant. The corollary of these observations is that correction of nutritional folate deficiency may reduce the risk of developing carcinogenic genetic changes in normal cells after alkylating agent exposure and enhance the sensitivity to alkylating agents in p53-mutant malignant cells. The latter interpretation may explain, at least in part, our observation that mammary carcinoma was more resistant to cyclophosphamide chemotherapy in folate deficient rats than in folate replete animals (15).

Other mechanisms by which folate status might modulate alkylator-induced toxicity were elucidated by studies at a cellular level. Observations in rat liver cells from animals maintained on diets of varying folate content and treated with cyclophophamide indicate that folate metabolism can modulate GSH levels. These results were confirmed *in vitro* by studies with cell lines. Therefore, dietary supplementation with folic acid may represent a new and non-toxic approach to change the levels of an important determinant of alkylating agent toxicity. Other studies found that folate supplementation decreased the frequency of mitochondrial DNA deletions induced by alkylating agents in rat liver cells.

Finally, our animal studies indicate that there is an optimal amount of dietary folate to modify the toxicity of alkylating agents. Moreover, very high doses of folate added to chemotherapy can lead to kidney damage. Therefore future treatment protocols in patients will need to carefully consider the amount of supplemental folic acid administered. In addition, it may be necessary to alert patients receiving chemotherapy who are taking dietary supplements that high doses of folic acid should be avoided. An unexpected finding was that rats fed a cereal—based diet had more slowly growing tumors and less chemotherapy-associated toxicity

than rats fed a purified diet. The latter observation emphasizes the important but still poorly understood influence that diet has on the biology and treatment of cancer.

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The effect of folate deficiency on the *hprt* mutational spectrum in Chinese hamster ovary cells treated with monofunctional alkylating agents

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Received 7 December 1998; received in revised form 16 April 1999; accepted 20 April 1999

Abstract

Folic acid deficiency acts synergistically with alkylating agents to increase DNA strand breaks and mutant frequency at the *hprt* locus in Chinese hamster ovary (CHO) cells. To elucidate the mechanism of this synergy, molecular analyses of *hprt* mutants were performed. Recently, our laboratory showed that folate deficiency increased the percentage of clones with intragenic deletions after exposure to ethyl methanesulfonate (EMS) but not *N*-nitroso-*N*-ethylurea (ENU) compared to clones recovered from folate replete medium. This report describes molecular analyses of the 37 *hprt* mutant clones obtained that did not contain deletions. Folate deficient cells treated with EMS had a high frequency of G > A transitions at non-CpG sites on the non-transcribed strand, particularly when these bases were flanked on both sides by G:C base pairs. Thirty-three percent of these mutations were in the run of six G's in exon 3. EMS-treated folate replete cells had a slightly (but not significantly) lower percentage of G > A transitions, and the same sequence specificity. Treatment of folate deficient CHO cells with ENU resulted in predominantly T > A transversions and C > T transitions relative to the non-transcribed strand. These findings suggest a model to explain the synergy between folate deficiency and alkylating agents: (1) folate deficiency causes extensive uracil incorporation into DNA; (2) greatly increased utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair. In the case of EMS, this results in more intragenic deletions and G:C to A:T mutations due to impaired ligation of single-strand breaks generated during base excision repair and a decreased capacity to remove O^6-ethylguanine. In the case of ENU additional T > A transversions and C > T transitions are seen, perhaps due to mis-pairing of O^2-ethylpyrimidines. Correction of folate deficiency may reduce the frequency of these types of genetic damage during alkylator therapy.

Keywords: Folate; Alkylating agent; *hprt*; DNA repair

1. Introduction

The results of epidemiologic and interventional studies suggest that nutritional folic acid deficiency promotes carcinogenesis. Interest has focused particularly on colon cancer, where retrospective case-control studies indicating that folate supplementation lowered the risk of colon cancer were confirmed by prospective studies in both men and women [1–4]. Experimental studies in rats found that nutritional folate deficiency enhanced the development, while increasing dietary folate content progressively re-

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0027-5107/99/$ - see front matter
PII: S0027-5107(99)00095-0

duced the incidence of macroscopic colonic neoplasia after dimethylhydrazine treatment [5,6]. In addition, suggestive evidence has accumulated to implicate folate deficiency in the development of cancer at other sites in humans such as the uterine cervix, esophagus and brain (reviewed in Refs. [7,8]), and studies of dietary folate deficiency in mice described an increased incidence of leukemia after Friend Virus infection [9].

Although many mechanisms have been proposed to explain the promotion of carcinogenesis by dietary folate deficiency [7,8], most attention has concentrated on alterations of DNA synthesis and repair. Folic acid deficiency has several important effects on DNA metabolism. DNA synthesis is defective in megaloblastic anemia due to deficiency of either folic acid or vitamin B12, manifesting a reduced rate of replication fork movement [10,11]. Because the biosynthesis of DNA precursors is impaired in folate deficiency, there is inhibition of gap-filling and/or joining of Okazaki pieces, resulting in persistent single-stranded regions [10]. In addition, stalled replication forks are prone to double-strand breaks and induction of deletions [12,13]. Decreased synthesis of thymidylate due to a lack of the folate cofactor leads to extensive incorporation of uracil into DNA and strand breaks [14–16]. Double strand breaks and deletions may occur as a result of excision repair of opposing uracil residues by uracil–DNA glycosylase [17]. There is evidence that folate deficiency also impairs DNA repair. Thus, nucleotide pool imbalance, as is found in folate-deficient cells [18], inhibits the repair or enhances error-prone repair of spontaneous DNA damage [19,20], nutritional folate deficiency impairs the repair of γ-irradiation or ethyl methanesulfonate (EMS)-induced strand breaks [21], methotrexate inhibits DNA excision repair following exposure to ultraviolet irradiation or EMS [22], and folate depletion impairs DNA excision repair in rat colon [23]. Finally, diminished folate status alters the pattern of DNA methylation [24,25], rendering the hypomethylated regions more susceptible to nuclease attack and thereby resulting in DNA strand breaks [26,27].

Given the diverse and serious effects of folate deficiency on DNA metabolism, it is not surprising that numerous types of genetic damage have been reported in folate deficient cells. These include chromosomal gaps and breaks, fragments, triradial, quadriradial and ring forms, allocyclic chromosomes, micronucleus formation, increased rates of sister chromatid exchanges, and expression of fragile sites (reviewed in Refs. [28,29]). In addition, our laboratory has shown that deficiency of this vitamin acts synergistically with alkylating agents to increase DNA strand breaks and mutant frequencies at the *hprt* locus both in vitro and in vivo [21,30]. To elucidate the mechanism underlying this synergy, we performed molecular analyses of *hprt* mutant CHO cells that were treated with EMS or *N*-nitroso-*N*-ethylurea (ENU). An analysis by multiplex PCR amplification of *hprt* exons from cells treated with EMS showed intragenic deletions in 9/46 (19.6%) clones derived from folate-deficient cells, but in none in 16 mutants isolated from cells grown in folate-replete medium [31]. In these experiments, the mutant frequency increased from $618 \pm 61 \times 10^{-6}$ in folate replete medium to $1796 \pm 165 \times 10^{-6}$ in low folate medium, a 200% increase. Since the mutant frequency for the folate replete cells was 1/3 of the mutant frequency in low folate, 1/3 of the mutations can be considered 'background' and 2/3 (67%) as low folate induced. Therefore, the 20% frequency of deletions in the mutants in the low folate cells only explains about 30% of EMS-induced mutations which are due to the low folate treatment. The number of deletions in mutants generated by ENU was equally low in folate-deficient and folate-replete clones (4% vs. 3.8%) and thus cannot explain the large mutant frequency differences between the folate replete and low folate cultures (838×10^{-6} vs. 307×10^{-6}) [31]. Because deletions cannot explain all of the increase in mutant frequency after EMS and especially after ENU treatment of low folate cells, we now describe the mutational spectrum at the *hprt* locus in mutants that did not display intragenic deletions. Our results indicate that the combination of folate deficiency and EMS produces *hprt* mutants with a high frequency of G > A transitions at non-CpG sites on the non-transcribed strand, particularly when these bases are flanked on both sides by G:C base pairs. Folate deficient cells treated with ENU were found to have high proportions of C > T transitions and T > A transversions, but surprisingly few G > A transitions on the non-transcribed strand.

2. Materials and methods

2.1. Cell culture

Clones of the CHO-K1 cell line were obtained as described previously [21,31] and cryopreserved in liquid nitrogen. Experiments were carefully scrutinized so that only clones from independent cultures were included in the final analysis. Clones were quick thawed in a 37°C water bath and added to Ham's F-12 medium (JRH Biosciences, Lenexa, KS) containing 10% calf bovine serum (Hyclone Laboratories, Logan, UT) in a 75 cm tissue culture flask and placed in a 37°C, 5% CO_2 incubator. Cells were allowed to attach overnight, then covered with fresh medium, and grown for 3–4 days until confluent. The cells were then trypsinized, counted and aliquoted to eight 0.5 ml tubes, six at 1×10^4 and two at 5×10^4 cells, and stored at −70°C. The remaining cells were frozen in liquid nitrogen 1.2 ml cryotubes in 1 ml of 8% DMSO, 50% serum, 42% Ham's F-12 medium.

2.2. cDNA Synthesis, PCR amplification and DNA sequencing

A master mix was made for cDNA synthesis prior to obtaining dry cell pellets from the −70°C freezer.

Table 1
Oligonucleotide primers used for PCR analysis of CHO DNA

RT-PCR primers

Name	Sequence	Base numbers[a]
215 +	ACCTCACCGCTTTCTCGTGC	Ex1 − 88 to Ex1 − 68
216 −	AAGCAGATGGCTGCAGAACT	IVS9 + 122 to IVS + 141
ZEE-1 +	GGCTTCCTCCTCACACGCT	Ex1 − 50 to Ex1 − 31
VRL16 −	GCAGATTCAACTTGAATTCTCATC	IVS9 + 58 to IVS9 + 82

Multiplex primers

Name	Sequence	Base numbers[a]
Exon 1 +	TCTGCAGGCTTCCTCCTCACACCG	Ex1 − 56 to Ex1 − 33
Exon 1 −	ACATGTCAAGGCAACGCCATTTCCA	IVS1 + 301 to IVS1 + 326
Exon 2 +	AGCTTATGCTCTGATTTGAAATCAGCTG	IVS1 − 42 to IVS1 − 15
Exon 2 −	ATTAAGATCTTACTTACctgtccaraatc	Base 123 (exon 2) to IVS2 + 29
Exon 3 +	CCGTGATTTTATTTTTGTAGgactgaaag	IVS2 − 28 to base 143 (exon 3)
Exon 3 −	TACATACAAAACTAGGATTGCCATATT	IVS3 + 30 to IVS3 + 57
Exon 4 +	TGTGTGTATTCAAGAATATGCATG	IVS3 − 48 to IVS3 − 24
Exon 4 −	CCAAGTGAGTGATTGAAAGCACAG	IVS4 + 57 to IVS4 + 81
Exon 5 +	AACATATGGGTCAAATATTCTTTCTAATAG	IVS4 − 161 to IVS4 − 132
Exon 5 −	GCTGAGAAAATTTAACAGTATTTTAG	IVS5 + 19 to IVS5 + 46
Exon 6 +	ACTTACCACTTACCATTAAATACC	IVS5 − 41 to IVS5 − 17
Exon 6 −	AAGCAATTGCTTATTGCTCCCAATG	IVS6 + 36 to IVS6 + 61
Exon 7,8 +	GTTCTATTGTCTTTCCCATATGTC	IVS6 − 49 to IVS6 − 25
Exon 7,8 −	TCAGTCTGGTCAAATGACGAGGTGC	IVS8 + 88 to IVS8 + 113
Exon 9 +	CAATTCTCTAATGTTGCTCTTACCTCTC	IVS8 − 69 to IVS8 − 42
Exon 9 −	GACAATCTATCGAAGGCTCATAGTGC	Ex9 + 180 to Ex9 + 206

Long PCR primers

Name	Sequence	Base numbers
S2 +	TGCTCTGATTTGAAATCAGC	IVS1 − 33 to IVS1 − 17
S3 −	CATACAAAACTAGGATTGCC	IVS3 + 34 to IVS3 + 53

[a]The bases listed as Ex1 − X to −Y are 5′ (upstream) to the 'A' of the ATG start codon, i.e., Ex1 − 88 to −68 is the sequence from 88 bases 5′ to the 'A' of the ATG start codon to 68 bases 5′ of that 'A'. Base 123 and base 143 in the exon 2 antisense and exon 3 sense primers, respectively, are cDNA base numbers. The base listed as IVSX − Y is Y bases 5′ to the 3′ end of intron X while the base listed as IVSX + Y is Y bases 3′ of the start of intron X. The bases listed as Ex9 + N are N bases 3′ to the A of the TGA stop codon.

Cells were kept on dry ice until the addition of a mix containing 0.5 ul NP-40 (Sigma, St. Louis, MO), 4 ul 25 mM $MgCl_2$, 2 ul PCR II Buffer (Perkin-Elmer/Cetus, Norwalk, CT), 8 ul 2.5 mM dNTP's (Perkin-Elmer), 1 ul Reverse Transcriptase (Murine Moloney Leukemia Virus, Perkin Elmer), 1 ul RNAse inhibitor (Perkin-Elmer), 1 ul Oligo (dT) (Perkin-Elmer) and 2.5 ul HPLC water (Sigma). Cells were thawed in the reaction mix and cDNA was generated in a Perkin-Elmer 2400 Thermal Cycler with a profile of 42°C for 15 min and 99°C for 5 min. Amplification of the cDNA was done in two rounds of nested PCR. A 5 ul aliquot of cDNA was transferred into a first round mix of 1.9 ul 25 mM $MgCl_2$, 2.45 ul PCR II Buffer (Perkin-Elmer), 2 ul 2.5 mM dNTP's (Perkin-Elmer), 17.25 ul HPLC water (Sigma), 0.125 ul each 30 pmole forward (215 +) and reverse (216 −) primers (Gibco BRL Life Technologies, Grand Island, NY) and 0.15 ul Amplitaq polymerase (Perkin-Elmer). The second round amplification consisted of 3 ul 25 mM $MgCl_2$, 5 ul PCR II Buffer (Perkin-Elmer), 4 ul 2.5 mM dNTP's (Perkin-Elmer), 36.25 ul HPLC water (Sigma), 0.25 ul each 30 pmole forward (ZEE1 +) and reverse primers (VRL16 −) (Gibco BRL, Life Technologies), 0.25 ul Amplitaq polymerase (Perkin-Elmer) and 1 ul of the first round product. A PCR profile of 94°C–1′, 60°C–1′, 72°C–1′30″ was used for both rounds. The final product was run on a 1.5% Agarose (Gibco BRL, Life Technologies) gel, stained with ethidium bromide and observed under UV light. If multiple products were obtained (an exon 2 + 3 deletion product can often be seen even in wildtype cells), the largest product was excised, Gene Cleaned (Bio 101, Vista, CA) and sequenced with an ABI 373 sequencer.

Genomic PCR was performed as described previously for multiplex PCR [31] except using only the primer pair for the exon of interest (primers are listed in Table 1).

The Long PCR method consisted of combining 45 ul HPLC water, 3 ul 10 mM dNTPs, 1 ul 50 pM forward (S2 +) and 1 ul 50 pM reverse (S3 −) primer and a single wax bead (Ampliwax, Perkin-Elmer) (Table 1). The mixture was heated to 80°C–5′ then 25°C–5′ followed by addition of 10 ul Long PCR Buffer 2 (Perkin-Elmer), 0.75 ul Enzyme (Platinum Taq, Perkin-Elmer), 1 ul DNA template and 38.25 ul HPLC water. An initial cycle of 95°C–1′30″, 58°C–30″, 68°C–8′ followed by 40 cycles of 94°C–15″, 58°C–30″, 68°C–8′ with a 10′ extension at 68°C was used on the Perkin-Elmer 2400 Thermal Cycler.

3. Results and discussion

DNA sequence analysis was performed on 13 *hprt* mutant clones collected after EMS treatment of folate deficient CHO cells, nine clones produced by EMS treatment of folate replete cells, nine clones from ENU-treated folate deficient cells, and six clones obtained after ENU treatment of folate replete cells. None of these clones had previously demonstrated intragenic deletions after multiplex PCR amplification of *hprt* exons. Initially, RT-PCR and cDNA sequencing was performed. Fourteen mutants lacked a whole exon(s) by RT-PCR. Two EMS folate replete mutants were missing exon 2 and two were missing exons 2 + 3. One EMS folate deficient mutant was missing exons 2 + 3 and one was missing exons 2 to 6. Three ENU folate replete mutants were missing exons 2 + 3, one was missing exon 3 and one was missing exon 5. One ENU folate deficient mutant was missing exon 8 and two were missing exons 2 + 3. We then used genomic PCR of the relevant exon(s) utilizing the CHO multiplex primers (Table 1, Ref. [32]) followed by automated sequencing to attempt to define the mutation causing the splice alteration in these mutants. This was a successful approach for three mutants. However, for the exon 2 or 3 loss mutants, while we could sequence the splice acceptor sequence for exon 2 and the splice donor sequence for exon 3, the primers overlapped the other splice site. Because there is limited intronic sequence information available for the CHO *hprt* gene, we were unable to synthesize the more appropriate primers. We did utilize long PCR using new exon 2 sense (S2 +) and exon 3 antisense (S3 −) primers. However, we were still only able to successfully PCR three of the mutants and of these three only one mutation (in the exon 3 acceptor) was determined despite repeated attempts.

The results of the sequencing studies are shown in Table 2. With the exception of a single splice site mutation in exon 6, all of the characterized mutations were in either exon 3 or exon 8. Exon 3 is the largest

Table 2
Base pair changes in the *hprt* gene of 6-thioguanine resistant CHO cells exposed to EMS or ENU when the cells were folate replete or deficient

EMS + folate			EMS − folate		
Mutation	Target sequence	Codon and amino acid change	Mutation	Target sequence	Codon and amino acid change
209G > A (exon 3)	AAG GGG GGC	70Gly > Glu	197G > A (exon 3)	CTC TGT GTG	66Cys > Tyr
389T > A (exon 5)	AAT GTC TTG	130Val > Asp	200T > G (exon 3)	TGT GTG CTG	67Val > Gly
551C > T (exon 8)	ATT CCA GAC	184Pro > Leu	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
574G > A (exon 8)	TAT GCC CTT	192Ala > Thr	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
574G > A (exon 8)	TAT GCC CTT	192Ala > Thr	208G > A (exon 3)	AAG GGG GGC	70Gly > Arg
			209G > A (exon 3)	AAG GGG GGC	70Gly > Glu
			485G > A (exon 6)	CCA GAC TTT	177Asp > Glu (splice 6)
			551C > T (exon 8)	ATT CCA GAC	184Pro > Leu
			568G > A (exon 8)	GTT GGA TAT	190Gly > Arg
			569G > A (exon 8) [exclusion of exons 2–6]	GTT GGA TAT	190Gly > Glu
			575C > A (exon 8)	TAT GCC CTT	192Ala > Asp
			575C > A (exon 8)	TAT GCC CTT	192Ala > Asp

ENU + folate			ENU − folate		
Mutation	Target sequence	Codon and amino acid change	Mutation	Target sequence	Codon and amino acid change
74C > T (exon 2)	ATT CCT CAT	38Pro > Leu	389T > A (exon 5)	AAT GTC TTG	130Val > Asp
IVS4-1G > A	ttctagAAT	Intron (splice exon 5)	389T > A (exon 5)	AAT GTC TTG	130Val > Asp
			416C > T (exon 6)	GAC ACT GGT	139Thr > Ile
			464C > T (exon 6)	AAG CCC CTC	155Pro > Leu
			464C > T (exon 6)	AAG CCC CTC	155Pro > Leu
			Δ534–536 (exon 8)	TTT [GTT] GGA	Δ165Val
			IVS3-9T > G[a]	ttt att ttt	Intron (splice exon 2,3) (new ag splice site)

[a]Splice junction sequenced using the Long PCR method (see Section 2).

exon and exons 3 and 8 the most frequently mutated exons in the human *HPRT* gene [33]. Particularly susceptible to mutation were guanine bases in exon 3 where six guanines are found in a row (cDNA bases 207–212).

EMS follows a mixed S_N1/S_N2 type reaction and is believed to be mutagenic by reaction with the O^6 position, and, to a lesser extent, to the N^7 position of guanine [34,35]. The *N*-alkylation products yield apurinic sites that are processed further by base excision repair and may cause mutations by mis-incorporation or error-prone repair processes [35–38]. The O^6-ethylguanine is mutagenic through direct miscoding by pairing with thymine during replication [35,39]. The spectrum of EMS is dominated by G > A transitions, and EMS has a relatively low efficiency for deletions in exponentially growing cells [35].

In the EMS treated cells, both the folate replete and folate deficient grown cells showed a high percentage of GC:AT mutations (80% and 75%, respectively). The major mutagenic lesion for EMS is O^6-ethylguanine which is directly mutagenic and is not removed in CHO cells because they do not contain O^6-alkylguanine–DNA alkyltransferase (AGT) [40,41]. Assuming that the G is the mutagenic target, then three of the four (75%) GC:AT mutations in the folate replete cells and eight of the nine

(89%) GC:AT mutations in the low folate cells occurred on the non-transcribed strand. Op het Veld et al. [42] also studied EMS treated CHO9 cells (folate replete) and found that 78% (14/18) of point mutations were at GC:AT basepairs with 64% on the non-transcribed strand (seven mutations were not determined in genomic DNA). When they studied a DNA repair defective cell line, EM-C11, they obtained 21/22 (95%) GC:AT mutations among the point mutations with 16/21 (76%) on the non-transcribed strand (nine mutants were deletions, two were frameshifts and seven mutants were undefined). The large proportion of deletions were ascribed to the DNA repair defect in the EM-C11 cell line similar to our observations of a larger percentage of deletions in EMS-treated folate deficient cells [39]. Our result of 89% G > A transitions in the folate deficient cells therefore suggests that low folate may also increase the proportion of mutations occurring on the non-transcribed strand.

In the studies here, of the eight G > A transitions on the non-transcribed strand in the low folate cells, six were flanked 3′ by a purine and four were flanked on both sides by G:C base pairs. One of the three G > A transitions in the EMS folate replete cells was flanked by a 3′ purine (G) (at 209 in the run of six G's). Op het Veld et al. [42] also found a high proportion of 3′ purines (8/9-CHO9 and 13/16-EM-C11) in their experiments.

The sequence specificity of G > A transitions may reflect the fact that excision-repair enzymes are less efficient at removing O^6-ethylguanine if this adduct is flanked by G:C base pairs [34]. However, excision repair is known to be less effective in rodent than human cells to remove O^6-ethylguanine lesions and therefore may have relatively little to do with the sequence specificity in CHO cells [42,43]. On the other hand, nucleotide excision repair may be active in folate deficient cells, since nucleotide excision repair is involved in removal of *N*-ethylpurines from the transcribed strand, and folate deficient cells showed the expected bias to mutations in the non-transcribed strand [43–45]. This strand bias does not appear to be due to a highly disproportionate number of mutable bases on the non-transcribed strand, since there were 120 mutable guanines (that is, guanines that if mutated would result in an amino acid change) on the non-transcribed strand (59%) and 83 mutable guanines on the transcribed strand (41%). Studies in cells that have O^6-alkylguanine–DNA alkyltransferase have lower levels of EMS induced mutations and do not show a strand bias in GC:AT mutations [46].

The molecular analyses of EMS-treated folate deficient CHO cells resemble the findings in the base excision repair defective cell line EM-C11 in that both have G > A transitions as the largest class of *hprt* mutations and both have high fractions of deletion mutations [44,47]. These similarities suggest that folate deficiency may impair base excision repair and are consistent with reports that nutritional folate depletion impairs DNA excision repair in rat colon [23], and that methotrexate inhibits DNA excision repair after exposure to EMS or ultraviolet irradiation [22].

A possible mechanism for impaired DNA excision repair in folate-deficient cells is mispairing and overloading of the repair system by excessive incorporation of uracil into DNA. Folate deficiency limits the methylation of dUMP to dTMP, resulting in extensive incorporation of uracil into DNA by DNA polymerase [14–16]. Repair of uracil residues utilizes excision repair [17,48]. Thus, a model to explain the synergistic interaction of EMS and nutritional folate deficiency, and the resulting mutational spectra, can be proposed: folate deficiency increases uracil incorporation into DNA, thereby increasing utilization of base excision repair and in turn promoting defective DNA repair in EMS-treated cells. This results in a higher number of intragenic deletions and G > A mutations due to impaired ligation of single-strand breaks generated during base excision repair and a reduced capacity to remove O^6-ethylguanine.

ENU reacts by an S_N1 mechanism and produces significant levels of alkylation of oxygens, such as the O^6-position of guanine and the O^2 and O^4 positions of thymine, resulting in miscoding or misrepair [34,35]. The mutational spectrum in ENU-treated mammalian cells is predominantly G > A and to a lesser extent A > G transitions and T > A transversions [35,49,50]. The latter type of mutation may reflect thymidine incorporation opposite O^2-ethylthymine [50].

The interpretation of the molecular analyses in ENU-treated CHO cells is limited by the large proportion of clones that had splice site mutations that

could not be fully characterized (the actual base change could only be determined in two mutants, which made statistical comparisons between the folate replete and deficient groups impossible). These mutations must be in the exon 2 donor or exon 3 acceptor sites that could not be sequenced for technical reasons. It is unclear why five of the six (83%) ENU folate replete mutations would be in splice sites unless a hotspot(s) exists in these regions. Four of the nine (44%) EMS folate replete mutations are also splice site mutations; however, splice site mutations make up only 2/13 (15%) and 3/9 (33%) of the EMS folate deficient and ENU folate deficient mutations, respectively. We previously reported that intragenic deletions were relatively uncommon in both folate deficient and replete cells after ENU exposure [31]. The spectrum of the remaining mutants was mainly C > T transitions and T > A transversions relative to the non-transcribed strand (Table 2).

In cells with O^6-alkylguanine–DNA alkyltransferase, ENU-induced mutations are predominantly TA:AT (38–45%), with a very strong bias for T > A transversions on the non-transcribed strand [46,50–53]. T > C and T > G mutations each make up ~ 15% of the mutations and G > A and C > T mutations each about 7%. In CHO cells, Op het Veld et al. [42] found 22% T > A (13/59), 8% T > C (5/59), 10% T > G(5/59) and 32% G > A (19/59) and 10% C > T (6/59). Our spectrum in folate deficiency, though very small, found 33% (2/6) T > A, 0% T > C, 17% (1/6) T > G, 0% G > A and 50% (3/6) C > T. This is a very high percentage of C > T which is not seen in AGT competent or incompetent cells. The ratio of G > A:C > T is also much different with a 3:1 ratio seen in the previous study of folate replete CHO cells and an approximately 1:1 study in AGT competent cells.

Consistent with prior observations by others, T > A transversions in our clones occurred preferentially at a thymine with an adjacent purine at its 5′ site and on the non-transcribed strand [50]. C > T transitions are distinctly unusual in ENU-treated mammalian cells [35,49,50]. One mechanism proposed for C > T transitions is deamination of cytosine to uracil under conditions of limited availability of *S*-adenosylmethionine [54], as occurs in folate deficiency. Uracil then codes for thymine during DNA replication [54]. In addition, decreased levels of *S*-adenosylmethionine enhance binding of DNA methyltransferase, thereby inhibiting repair of uracil [54,55]. Although an attractive hypothesis, this mechanism may not apply to our experimental results since the cytosines involved were not at CpG sites, and hence may not have been methylated. Alternatively, O^2-ethylcytidine may act as uracil and code for thymine [56] giving the pathway O^2-ethylcytidine → U → T. This mechanism also would promote saturation of base excision repair by utilizing uracil–DNA glycosylase.

The studies reported here will require confirmation in a larger number of clones. Study of approximately 35–50 mutants per group would be required to detect a 30–40% difference in frequency of one mutation type at 80% power, while it would require over 500 mutants per group to reach significance for the 7% difference in frequency in G > A mutations seen here between the EMS + folate and EMS − folate groups. Approximately 120 mutations per group would be needed to verify the high proportion (89%) of mutations occurring on the non-transcribed strand. These preliminary results suggest, however, that correction of folate deficiency may protect against certain types of genetic damage during alkylator therapy. In addition, similar studies in other cell types would be of interest. The mutational spectrum after alkyator treatment in CHO cells probably is not representative of many other mammalian cell types because CHO cells lack alkyl transferase [40,41]. Therefore, studies of the effect of folate deficiency on the mutational spectrum in lymphocytes from rats treated with alkylating agents [30] and in human lymphocytes are planned. Studies in human cells may be useful both to provide additional insights into the synergy between folate deficiency and mutation induction and for molecular epidemiologic approaches to elucidate the relationship between dietary components such as folic acid and environmental mutagens or carcinogens.

Acknowledgements

Supported by grants from the National Cancer Institute, Bethesda, MD (CA 41843) and the Department of the Army (DAMD17-98-1-8345). The content of the information does not necessarily reflect

the position or policy of the U.S. government, and no official endorsement should be inferred. Automated sequencing was performed in the Vermont Cancer Center DNA Analysis Facility which is supported by NCI P30 CA 22435.

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The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status

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Received 11 May 2000; received in revised form 21 September 2000; accepted 26 September 2000

Abstract

Folic acid deficiency acts synergistically with alkylating agents to increase genetic damage at the *HPRT* locus in Chinese hamster ovary cells in vitro and in rat splenocytes in vivo. The present studies extend these observations to human cells and, in addition, investigate the role of p53 activity on mutation induction. The human lymphoblastoid cell lines TK6 and WTK1 are derived from the same parental cell line (WI-L2), but WTK1 expresses mutant *p53*. Treatment of folate-replete or deficient WTK1 and TK6 cells with increasing concentrations (0–50 μg/ml) of ethyl methanesulfonate (EMS) resulted in significantly different *HPRT* mutation dose-response relationships ($P < 0.01$), indicating that folate deficiency increased the EMS-induced mutant frequency in both cell lines, but with a greater effect in TK6 cells. Molecular analyses of 152 mutations showed that the predominant mutation (65%) in both cell types grown in the presence or absence of folic acid was a $G > A$ transition on the non-transcribed strand. These transitions were mainly at non-CpG sites, particularly when these bases were flanked 3′ by a purine or on both sides by G:C base pairs. A smaller number of $G > A$ transitions occurred on the transcribed strand ($C > T = 14\%$), resulting in 79% total G:C > A:T transitions. There were more genomic deletions in folate-deficient (15%) as compared to replete cells (4%) of both cell types. Mutations that altered RNA splicing were common in both cell types and under both folate conditions, representing 33% of the total mutations. These studies indicate that cells expressing p53 activity exhibit a higher rate of mutation induction but are more sensitive to the toxic effects of alkylating agents than those lacking p53 activity. Folate deficiency tends to reduce toxicity but increase mutation induction after EMS treatment. The *p53* gene product did not have a major influence on the molecular spectrum after treatment with EMS, while folate deficiency increased the frequency of deletions in both cell types.

Keywords: Folate; Alkylating agent; *HPRT* mutation spectrum; Human lymphoblast cells; p53

1. Introduction

Folate compounds are essential cofactors for purine and pyrimidine synthesis and for DNA methylation [1]. Consequently a deficiency of the vitamin, folic acid, is associated with a variety of genetic abnormalities. Chromosomal gaps and breaks, fragments, triradial and quadriradial forms, allocyclic chromosomes, micronucleus formation, increased rates of sister chromatid exchanges, expression of fragile sites and gene amplification have been described after nutritional deprivation of folic acid both in vitro and in

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0027-5107/01/$ – see front matter
PII: S0027-5107(00)00138-X

vivo (reviewed in [2])[3,4]. Measurements of mutant frequencies at the *aprt* and *HPRT* locus in Chinese hamster ovary (CHO) cells [5,6], the *HPRT* locus in rat splenocytes [7], and the human *HPRT* locus in peripheral blood lymphocytes indicate that nutritional folate deficiency is slightly mutagenic [8,9]. However, treatment of folate-deficient CHO cells in culture or nutritionally depleted rats in vivo with alkylating agents results in a synergistic increase of mutant frequencies at the *HPRT* locus [5,7].

The mechanism for this synergy is not completely understood. Several studies have demonstrated that folic acid deficiency alone results in decreased thymidylate synthesis, extensive incorporation of uracil into DNA, and strand breaks [4,6,10–15]. Repair of uracil residues and *N*-alkylation products utilize base excision repair [16,17]. However, defective DNA excision repair has been described in folate-deficient CHO cells and rat colon [18,19]. Molecular studies in CHO cells from our laboratory found that treatment of folate-deficient CHO cells with ethyl methanesulfonate (EMS), which causes a mixed S_N1/S_N2 type reaction, resulted in a large number of intragenic deletions and $G > A$ mutations at non-CpG sites, particularly when these bases were flanked on both sides by G:C base pairs [20,21]. We postulated that these genetic changes were due to impaired ligation of single-strand breaks generated during base excision repair and a decreased capacity to remove O^6-ethylguanine. Taken together, these investigations by our laboratory and by others support the following model: (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 [21].

The current studies were performed to test this model in human cells. The human lymphoblast cell line, TK6 (*HPRT*$^+$, thymidine kinase heterozygous), was employed because it is near diploid with a stable karyotype (47, X, Y 13+), and it has been used extensively for studies of mutations at the *HPRT* locus [22,23]. Like CHO cells, TK6 cells are deficient in O^6-alkylguanine-DNA-alkyltransferase (AGT) [24–27]. Parallel studies were performed with the WTK1 lymphoblast cell line that was derived from the same donor as TK6 [22]. It has the same karyotype, identical growth and morphologic characteristics, is also *HPRT*$^+$ and thymidine kinase heterozygous and has a similar population doubling time, but differs from TK6 in that it has a homozygous mutation in codon 237 of exon 7 in the *p53* gene, resulting in expression of mutant p53 protein and no wild-type p53 protein [22,28,29]. The gene product of *p53* plays a role in excision repair [30–32] and since CHO cells are mutant at the *p53* locus [33–35], it was of interest to compare the interactive effects of folate deficiency and alkylating agents on human cells that have wild-type and mutant p53 protein and contrast the results with our prior findings in CHO cells.

2. Materials and methods

2.1. Cell culture

The TK6 and WTK1 cell lines were obtained from Dr. Howard Liber [22]. Cells were grown in folate-free RPMI Medium 1640 (Gibco cat. #2716-021) or complete RPMI supplemented with 10% calf serum (Hyclone cat. #A-2151-1). For folate-free conditions, the cells were grown for 3 days in folate-free medium, and treated in folate-free medium before returning to complete medium. For EMS treatment and mutant isolation, the cells were spun down and resuspended at 1×10^6 cells/ml in six identical 5 ml cultures. Five of these cultures were treated with 30 mg/ml EMS overnight. The cells were then spun down and washed twice. Survival plates were seeded from each of the original five cultures and the control. Each culture was then divided into four subcultures (giving 20 independent treated cultures) and grown in complete medium for 7–8 days to go through phenotypic lag. Cells from each of the 20 cultures were then plated in the presence and absence of 10 mM 6-thioguanine (TG) for mutant frequency determination. Two mutants were isolated from each TG selection plate and expanded. Samples of 1×10^4 cells from each of these mutant cultures were then snap frozen for mutational analysis and also stored in liquid nitrogen for subsequent growth if necessary.

The lymphoblast cloning assay was performed as previously described [36]. For determination of non-selected and TG selected CE, cells were seeded into 96-well microtiter plates (round bottom; Nunc) at 1–10 and $1–2 \times 10^4$ cells per well, respectively. At

10–14 days, colony growth was determined using an inverted phase contrast microscope and the CE calculated by use of the Poisson relationship, $P_0 = e^{-x}$, where P_0 is the fraction of wells without cell growth. The CE(X) equals $-\ln P_0/N$, where N = the average number of cells/well. The mutant frequency is the ratio of the mean CE in the presence (selected) and absence (non-selected) of TG.

2.2. cDNA synthesis, PCR amplification, multiplex PCR, long PCR, and DNA sequencing

Cell lysis and synthesis of cDNA was performed as described previously by Yang and colleagues [37]. Amplification of the cDNA was done in two rounds of nested PCR in a Perkin-Elmer 2400 Thermal Cycler. A 5 μl aliquot of cDNA was transferred into a first round mix of 1 μl 25 mM $MgCl_2$, 2 μl 10 × PE Buffer II (Perkin-Elmer), 0.5 μl each 2.5 mM dNTPs (Perkin-Elmer), 14.625 μl HPLC water (Sigma), 0.125 μl each 10 pM/μl forward (ssj) and reverse (rsj) primers (Table 1) (Gibco BRL Life Technologies, Grand Island, NY) and 0.125 μl Amplitaq polymerase (Perkin-Elmer) and amplified with a PCR profile of 94°C: 5 min, 30 cycles of 94°C: 1 min, 65°C: 1 min, 72°C: 2 min and a final extension of 72°C for 7 min. The second round amplification consisted of 3 μl 25 mM $MgCl_2$, 5 μl 10 × PE Buffer II, 1 μl each 2.5 mM dNTPs, 36.25 μl HPLC water, 0.25 μl each 10 pM/μl forward (B) and reverse (4b) primers (Table 1), 0.25 μl Amplitaq, and 1 μl of the first round product and amplified with a PCR profile of 30 cycles of 94°C: 1 min, 55°C: 1 min, 72°C: 2 min with a final extension of 72°C for 7 min. The final product was run on a 1% agarose (Gibco BRL Life Technologies, Grand Island, NY) gel, stained with ethidium bromide and observed under UV light. The product was excised, Gene Cleaned (Bio 101, Vista, CA) and sequenced with an ABI 373 sequencer (primers in Table 1).

Lysis for genomic PCR was carried out in a Thermolyne Amplitron II thermal cycler with a cycle profile of 56°C for 1 h followed by 96°C for 10 min. A 10 μl aliquot consisting of 40 μl Tris-EDTA (TE), 2.5 μl 10% Tween in TE, 2.5 μl 10% NP-40 in TE and 5 μl 1mg/ml proteinase K was added to the cell pellets and mixed well. Multiplex PCR was performed as described previously by Gibbs and colleagues [38] with the following exceptions: a 5 μl aliquot of lysed genomic DNA was added to the PCR mix and PCR was carried out with a cycle profile of 94°C for 5 min, 35 cycles of 94°C: 1min, 59°C: 1 min, 68°C: 2 min, with a final extension of 68°C for 5 min in a Perkin-Elmer 2400 Thermal Cycler. The PCR product was run on a 2% agarose gel, stained with ethidium bromide and observed under UV light. The bands of interest were excised, Gene Cleaned and sequenced with an ABI 373 sequencer.

DNA for Long PCR was purified using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Long PCR was performed in a Perkin-Elmer 2400 Thermal Cycler using the Expand Long Template PCR System (Boehringer Mannheim) following the protocol except with the following PCR profile; 94°C: 1 min, 20 cycles of 94°C: 30 s, 65°C: 12 min, 17 cycles of 94°C: 30 s, 6°C: 12 min with a cycle elongation of an additional 15 s per cycle, and a final extension of 72°C for 10 min. The multiplex primers were utilized to perform long PCR (2s–3a, 3s–4a, etc.) across the *HPRT* gene [38]. The PCR product was run on a 0.6% agarose gel stained with ethidium bromide and observed under UV light.

Table 1
Oligonucleotide primers used for RT-PCR and sequence analysis of *HPRT* mutations[a]

Name	Sequence	Base Numbers
PCR primers		
ssj (sense)	cctctgctccgccaccg	1614–1630
rsj (antisense)	cgcccaaagggaactgatagtctataggc	41719–41691
B (sense)	cctgagcagtcagcccgcgc	1641–1660
4b (antisense)	gcaaaaagctctactaagcagatggccacag	41608–41578
Sequence primers		
B (sense)	cctgagcagtcagcccgcgc	1641–1660
A (antisense)	tcaataggactccagatgttt	41546–41526

[a] Base numbers listed are 5′ to 3′ for the *HPRT* genomic sequence [71].

The bands of interest were excised, Gene Cleaned (Bio 101, Vista, CA) and sequenced on an ABI 373 sequencer.

2.3. Deoxyuridine suppression tests

Deoxyuridine suppression tests were performed as previously described [39].

2.4. Statistical analysis

Each group in the experiments relative CE versus EMS and mutant frequency versus EMS was entered into the general linear model as indicator variables while the EMS was entered along with interactions of EMS with the group indicator variables. This model was compared to the model that only included the indicator variables and EMS using an F-statistic. That is, we modeled the data using different slopes and modeled the data with equal slopes, and then compared the fit of the two models. A difference in the fit of the two models provided evidence that the slopes are different.

3. Results

TK6 cells displayed a slightly more rapid population doubling time (mean of 0.63 days, range 0.3–0.86 days, $n = 8$) than WTK1 cells (mean of 0.8 days, range 0.64–1.1 days, $n = 6$) in both folate-replete and deficient media (Fig. 1). The TK6 cells began showing evidence of slowed growth after about 3 days in low folate medium, while the WTK1 cells exhibited slowed growth beginning a day later, on Day 4. Cell number remained stable in the TK6 cells in low-folate medium but trended downward in the WTK1 cell cultures. After the addition of folic acid, the TK6 cells began increasing in number the next day, but recovery did not occur in the WTK1 cell cultures until after 48 h. The addition of folic acid to the culture medium led to a resumption of cell proliferation, indicating that the cells remained viable in the folate-free medium, as illustrated in Fig. 1. However, metabolic folate deficiency, as measured by the deoxyuridine suppression test, was detectable by Day 3 and became more severe on Day 5 in both cell lines after incubation in folate-free medium (data not shown).

Treatment of the cell lines with increasing concentrations of EMS resulted in a progressive decline in CE. The range of 0–50 μg/ml was most informative. Fig. 2 shows the results of five separate experiments in which TK6 and WTK1 cells were incubated for 3 days in folate-replete or deficient media and then treated with EMS in this concentration range. WTK1 cells were more resistant to the cytotoxic effects of EMS than TK6 cells in both folate-replete and deficient media. Folate-deficient TK6 and WTK1 cells were slightly more resistant to the toxic effects of EMS than folate-replete cells. However, there was considerable variability among the experiments, and statistical analyses indicated that the slopes for the survival curves were similar.

The mutant frequencies at the *HPRT* locus determined from these four treatment conditions are shown in Fig. 3. The background mutant frequency was slightly higher in folate-replete WTK1 cells than in TK6 (5.9 ± 4.6 versus $3.3 \pm 2.8 \times 10^{-6}$, respectively (mean ± S.D.)), and rose somewhat higher in folate-deficient medium ($13.9 \pm 11.6 \times 10^{-6}$ for WTK1; $4.5 \pm 3.1 \times 10^{-6}$ for TK6). None of these differences was significant by the unpaired t test. After treatment with EMS, the mutant frequencies were higher in TK6 than WTK1 cells in both folate-replete and deficient medium. The mutant frequencies were higher in folate-deficient than replete cells, but the effect was greater in TK6 cells. Statistical analysis of the slopes indicated significantly different *HPRT* mutation dose-response relationships ($P < 0.01$).

To determine the molecular spectrum of the *HPRT* mutations, cDNA sequencing was initially performed followed by genomic multiplex PCR and/or sequencing as necessary to define the mutation. 159 mutant clones were analyzed. Seven pairs of mutants with identical mutations and arising in the same culture were found (mutations #9, 14, 63, 90, 107, 116 and 120). These pairs are believed to be non-independent and are considered as one mutation in the following analyses. Of the 152 mutations thus analyzed, 15 did not yield a cDNA after attempts on three different cell pellets; 11 of these 15 showed genomic deletions. Of the 137 cDNAs sequenced, 37 showed exclusion of one or more complete exons, nine showed partial exon exclusion and eight showed

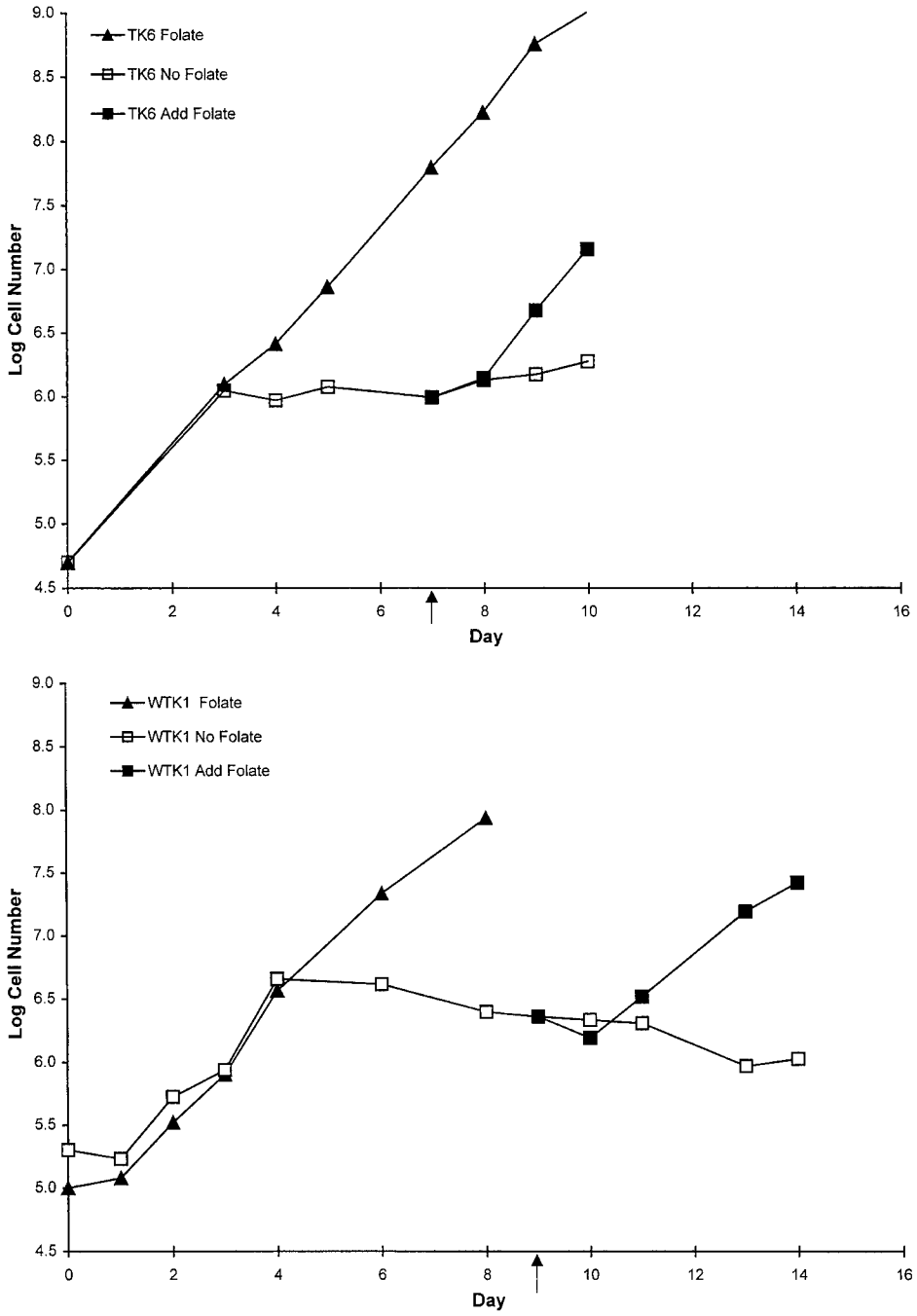

Fig. 1. Proliferation of human lymphoblastoid cells in folate-replete and deficient culture media. Top: TK6 cells in folate-replete medium proliferated with a doubling time of about 0.63 days (closed triangles). Cells in folate-deficient medium proliferated at the same rate until Day 3, when cell growth rate slowed and cell number stabilized. The addition of folic acid on Day 7 (arrow) was followed a day later by a resumption of cellular proliferation. Bottom: WTK1 cells in folate-replete medium proliferated with a doubling time of about 0.8 days (closed triangles). Cells in folate-deficient medium proliferated at approximately the same rate until Day 4, when they entered a quiescent phase with a downward trend in cell numbers (open squares). Addition of folic acid to the culture medium of these cells on Day 9 (arrow) led to a resumption of proliferation at the prior rate (closed squares) after 48 h, while cells remaining in folate-deficient medium did not increase in number.

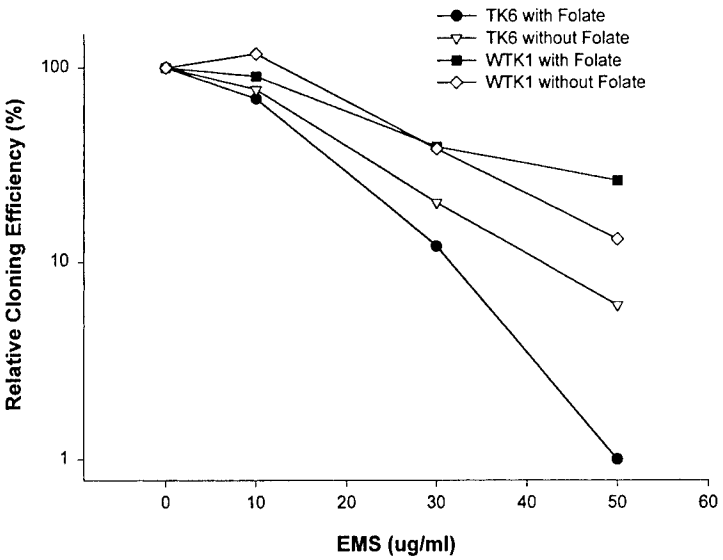

Fig. 2. Effect of folate status and EMS treatment on relative cloning efficiency of human lymphoblastoid cells. Cells were cultured in media containing or lacking folic acid. They were treated with EMS in the indicated concentrations, and cloning efficiency was determined relative to untreated cells. Each point represents the mean of five separate experiments.

inclusion of intron sequences. Of the 37 complete simple exon exclusion(s) mutants, 32 showed single base substitution mutations in splice sequences, three mutants showed no change in the genomic region containing the exon and only two mutants were genomic deletions. Of the other 17 splice alteration mutations, 15 were single base substitutions, one contained a deletion, and one did not show a genomic change. Of the 15 mutants that did not yield a cDNA, 11 showed deletion of *HPRT* exons in genomic DNA, two had

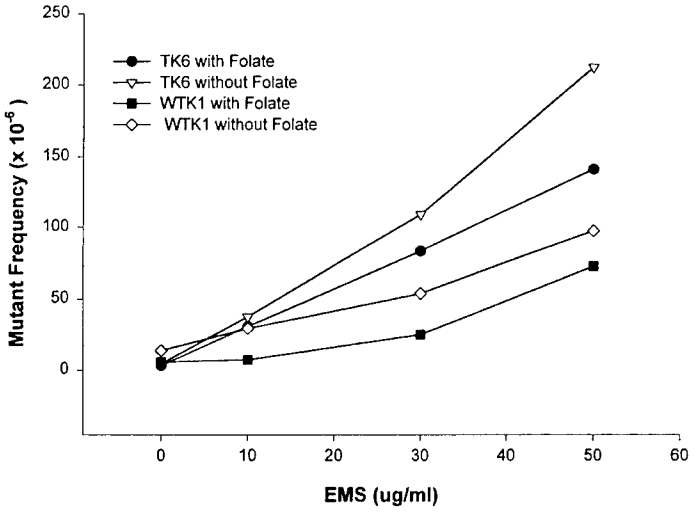

Fig. 3. Mutant frequency at the *HPRT* locus in human lymphoblastoid cells after treatment with EMS. Each point represents the mean of five separate experiments.

Table 2
Base pair changes in the *HPRT* gene of 6-thioguanine resistant human lymphoblastoid cells exposed to EMS

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino acid change
A. Folate-replete TK6 p53^{+}					
1	LT2-5D1	Intron inclusion 9192–9230[b]			Insertion of 13 aa between exon 1 and exon 2
2	LT2-3D2	Exclusion 28–32 (exon 2)	IVS1-1G>A	ttt cag ATT	TGA STOP at new codon 10
3	LT2-5D2	148G > A (exon 3)		CTT GCT CGA	50ala>thr
4	LT2-1B2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
5	LT2-1D2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
6	LT2-4B1	208G > A (exon 3)		AAG GGG GGC	70gly > arg
7	LT2-1C1	209G > A (exon 3)		AAG GGG GGC	70gly > glu
8	LT2-4D2	209G > A (exon 3)		AAG GGG GGC	70gly > glu
9	LT2-5A1 & 5A2	209G > A (exon 3)		AAG GGG GGC	70gly > glu
10	LT2-5B1	209G > A (exon 3)		AAG GGG GGC	70gly > glu
11	LT2-1C2	Exon 4 exclusion	IVS3−1G > A	aac tag AAT	Inframe loss of exon 4 (aa107–128)
12	LT2-2A1	Exon 4 exclusion	IVS3−1G > A	aac tag AAT	Inframe loss of exon 4 (aa107–128)
13	LT2-1B1	Exon 4 exclusion	IVS4+1G > A	AAG gta tgt	Inframe loss of exon 4 (aa107–128)
14	LT2-3A1 & 3A2	Exon 5 exclusion	IVS4−1G > A	ttc tag AAT	Inframe loss of exon 5 (aa129–146)
15	LT2-2B1	400G > A (exon 5)		GTG GAA gta	134glu > lys
16	LT2-2D1	416C > T (exon 6)		GAC ACT GGC	139thr > ile
17	LT2-2D2	454C > T (exon 6)		AGG CAG TAT	152gln > STOP
18	LT2-4D1	454C > T (exon 6)		AGG CAG TAT	152gln > STOP
19	LT2-4A1	Mix exclusion 403–452 and exons 2/3 exclusion with exclusion 403	IVS5−1G > A	tga aag GAT	TAA STOP at new codon 137 OR TGA STOP at new codon 48
20	LT2-1D1	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 166
21	LT2-2C2	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 166
22	LT2-3D1	Mix exon 8 exclusion and exclusion 533–553	IVS7−1G > A	tta gTT GTT	TGA STOP at new codon 183 or inframe loss of 1st 8 aa of exon 8 and 178phe > tyr
23	LT2-1A1	539G > A (exon 8)		GTT GGA TTT	180gly > glu
24	LT2-5B2	539G > A (exon 8)		GTT GGA TTT	180gly > glu
25	LT2-5C2	539G > A (exon 8)		GTT GGA TTT	180gly > glu
26	LT2-4B2	Exon 8 exclusion	551C > T	ATT CCA GAC	TAG at new codon 183
27	LT2-4C1	569G > A (exon 8)		GTA GGA TAT	190gly > glu
28	LT2-3C2	575C > A (exon 8)		TAT GCC CTT	192ala > asp
29	LT2-3B2	577C > T (exon 8)		GCC CTT GAC	193leu > phe
30	LT2-5C1	577C > T (exon 8)		GCC CTT GAC	193leu > phe

Table 2 (*Continued*)

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino acid change
31	LT2-2C1	580G > A (exon 8)		CTT GAC TAT	194asp > asn
32	LT2-4C2	Exon 8 exclusion	IVS8+5G > A	Tgta agt aat	TGA STOP at new codon 183
33	LT2-3B1	Exon 8 exclusion	No change		TGA STOP at new codon 183
34	LT2-2B2	Inclusion IVS8−14 to −1	IVS8−16G > A	ttt ctg gat	TAG STOP at new codon 257
35	LT2-3C1	Inclusion IVS8−14 to −1	IVS8−16G > A	ttt ctg gat	TAG STOP at new codon 257
36	LT2-4A2	Exclusion 610–626 (exon 9)	IVS8−1G > A	tta tag CAT	Immediate TGA STOP at new codon 204
37	LT2-1A2	617G > A (exon 9)		GTT TGT GTC	206cys > tyr
38	LT2-2A2	617G > A (exon 9)		GTT TGT GTC	206cys > tyr
B. Folate-replete WTK1 p53$^-$					
39	LT3-4C2	Exclusion IVS0−22[c] to IVS1+27 and inclusion IVS1 +28 to +49	Del 1655–1730		Loss of ATG START
40	LT3-4A2	IVS1+5G > T with inclusion IVS1+1 to +49		G gtg agc agc	TAG STOP at new codon 27
41	LT3-5D2	118G > A (exon 2)		CAT GGA CTA	40gly > arg
42	LT3-2D2	134G > A (exon 2)		GAC AGg gta	45arg > lys
43	LT3-3B2	Exclusion 28–32 (exon 2)	IVS1−1G > A	ttt cag ATT	Immediate TGA STOP at new codon 10
44	LT3-2A2	Exon 2 exclusion	IVS2+1G > A	GAC AGg taa	TGA STOP at new codon 11
45	LT3-5B2	Exon 2 exclusion	IVS2+1G > A	GAC AGg taa	TGA STOP at new codon 11
46	LT3-3D2	Mix exons 2 & 3 exclusion and exon 3 exclusion	IVS2−1G > A	tgt agG ACT	Inframe loss of exons 2 & 3 (aa10–106) OR TAA STOP at new codon 53
47	LT3-4C1	Exons 2 & 3 exclusion	IVS2−1G > A	tgt agG ACT	Inframe loss of exons 2 & 3
48	LT3-4A1	145C > T (exon 3)		CGT CTT GCT	49leu > phe
49	LT3-1C2	148G > A (exon 3)		CTT GCT CGA	50ala > thr
50	LT3-3B1	151C > T (exon 3)		GCT CGA GAT	51arg > STOP
51	LT3-2C2	196T > C (exon 3)		CTC TGT GTG	66cys > arg
52	LT3-5B1S	197G > A (exon 3)		CTC TGT GTG	66cys > thr
53	LT3-2C1	208G > A (exon 3)		AAG GGG GGC	70gly > arg
54	LT3-3D1	208G > A (exon 3)		AAG GGG GGC	70gly > arg
55	LT3-4D2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
56	LT3-5C2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
57	LT3-1A1	209G > A (exon 3)		AAG GGG GGC	70gly > glu
58	LT3-3C2	209G > A (exon 3)		AAG GGG GGC	70gly > glu
59	LT3-5C1	209G > T and +/− exclusion 208–318 (exon 3)		AAG GGG GGC	70gly > val ± deletion of 37 aa
60	LT3-1A2	217A > G (exon 3)		TAT AAA TTC	73lys > glu

Table 2 (*Continued*)

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino acid change
61	LT3-1B1	Exon 4 exclusion	IVS4+1G > A	AAG gta tgt	Inframe loss of exon 4 (aa107–128)
62	LT3-2B1	Exon 4 exclusion	No change		Inframe loss of exon 4 (aa107–128)
63	LT3-3A1 & 3A2	400G > A (exon 5)		GTG GAA gta	134glu > lys
64	LT3-4D1	416C > T (exon 6)		GAC ACT GGC	139thr > ile
65	LT3-1C1	508C > T (exon 7)		CCA CGA AGT	170arg > STOP
66	LT3-1B2	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 166
67	LT3-4B1	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 166
68	LT3-5A1	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 166
69	LT3-5A2	Exclusion 533–553 (exon 8)	IVS7−1G> A	tta gTT GTT	Inframe loss of 1st 7 aa of exon 8 and 178phe > tyr
70	LT3-2A1	539G > A (exon 8)		GTT GGA TTT	180gly > glu
71	LT3-2B2	539G > A (exon 8)		GTT GGA TTT	180gly > glu
72	LT3-2D1	539G > A (exon 8)		GTT GGA TTT	180gly > glu
73	LT3-3C1	539G > A (exon 8)		GTT GGA TTT	180gly > glu
74	LT3-1D1	601G > A (exon 8)		AGG GAT TTG	201asp > asn
75	LT3-5D1	635G > A (Exon 9)		ACT GGA AAA	212gly > glu
76	LT3-4B2	No cDNA	Del exon 1 thru 9		No protein made
77	LT3-1D2	No cDNA	Del exon 4 thru 9		No protein made
C. Low-folate TK6 p53$^+$					
78	LT1-4D1	2T > G (exon 1)		gtt ATG GCG	1met > arg
79	LT1-4C2	27G > A and inclusion IVS1+1 to+49		GTC GTG gtg	TAG STOP at new codon 27
80	LT1-4A2	IVS1+1G > A and inclusion IVS1+1 to +49		GTG gtg agc	TAG STOP at new codon 27
81	LT1-5C1	Mix exon 2 exclusion and exclusion 28–32	IVS1−1G > A	ttt cag ATT	Immediate TGA STOP at new codon 10 OR TGA STOP at new codon 11
82	LT1-1A1	119G > A (exon 2)		CAT GGA CTA	40gly > glu
83	LT1-1D2	134G > A (exon 2)		GAC AGg taa	45arg > lys
84	LT1-4C1	134G > A (exon 2)		GAC AGg taa	45arg > lys
85	LT1-1C2	Exon 2 exclusion	IVS2+1G > C	GAC AGg taa	TGA STOP at new codon 11
86	LT1-2A1	Exons 2 & 3 exclusion	IVS3+1G > A	TGT gtg agt	Inframe loss of exons 2 & 3 (aa10–106)
87	LT1-1B1	152G > C (exon 3)		GCT CGA GAT	51arg > pro
88	LT1-4B2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
89	LT1-2C1	209G > A (exon 3)		AAG GGG GGC	70gly > glu
90	LT1-3B1 & 3B2	209G > A (exon 3)		AAG GGG GGC	70gly > glu

Table 2 (*Continued*)

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino acid change
91	LT1-4A1	211G > T (exon 3)		GGG GGC TAT	71gly > cys
92	LT1-5B2	(1) Exclusion 317–318 with inclusion 26894–26934[b] (2) exon 3 exclusion with inclusion 26894–26934 (3) exclusion exons 2 & 3	IVS3+1G > A	TGT gtg agt	Immediate TAA STOP at codon 106 OR inframe loss of exons 2 & 3 (aa10–106) OR TAA STOP at new codon 47
93	LT1-2C2	Exon 4 exclusion	IVS3−1G > A	aac tag AAT	Inframe loss of exon 4 (aa107–128)
94	LT1-4B1	No cDNA	IVS3−1G > A	aac tag AAT	Inframe loss of exon 4 (aa107–128)
95	LT1-5B1	355G > A (exon 4)		GGT GGA GAT	119gly > arg
96	LT1-1A2	IVS5+1G > A and inclusion IVS5+1 to +67		GAA gta agt	TAA STOP at new codon 136
97	LT1-2B1	+/− Exon 6 exclusion	IVS5−2A > T	tga aag GAT	Normal or TAA STOP at new codon 147
98	LT1-2A2	425C > T (exon 6)		AAA ACA ATG	142thr > ile
99	LT1-5D1	Ambiguous	430C > T	ATG CAG ACT	144gln > STOP
100	LT1-1B2	+/− Exon 8 exclusion	538G > A	GTT GGA TTT	180gly > arg
101	LT1-3A2	+/− Exon 8 exclusion	577C > T	GCC CTT GAC	193leu > phe
102	LT1-3A1	+/− Exon 8 exclusion	589G > T	AAT GAA TAC	197glu > STOP
103	LT1-1D1	568G > A (exon 8)		GTA GGA TAT	190gly > arg
104	LT1-5A2	568G > A (exon 8)		GTA GGA TAT	190gly > arg
105	LT1-1C1	599G > A (exon 8)		TTC AGG GAT	200arg > lys
106	LT1-4D2	599G > A (exon 8)		TTC AGG GAT	200arg > lys
107	LT1-3C1 & 3C2	601G > A (exon 8)		AGG GAT TTG	201asp > asn
108	LT1-3D1	626G > A (exon 9)		ATT AGT GAA	209ser > asn
109	LT1-2B2	No cDNA	Del exon 1		No ATG START codon
110	LT1-5D2	No cDNA	Del exon 1		No ATG START codon
111	LT1-2D2	No cDNA	Del exon 6, 7/8, 9		No protein
112	LT1-5A1	No cDNA	Del exon 1 thru 9		No protein
113	LT1-2D1	No cDNA	No change		
114	LT1-5C2	No cDNA	No change		
D. Low-folate WTK1 p53					
115	LT4-2D2	130G > T (exon 2)		ATG GAC Agg	44asp > tyr
116	LT4-2B1 & 2B2	Exon 2 exclusion	IVS2+1G > A	GAC AGg taa	TGA STOP at new codon 11
117	LT4-5B1	Exon 2 exclusion	IVS2+1G > A	GAC AGg taa	TGA STOP at new codon 11
118	LT4-5D1	Exon 2 exclusion	IVS2+1G > A	GAC AGg taa	TGA STOP at new codon 11
119	LT4-4B2	145C > T (exon 3)		CGT CTT GCT	49leu > phe
120	LT4-4D1 & 4D2	151C > T (exon 3)		GCT CGA GAT	51arg-stop
121	LT4-1B2	208G > A (exon 3)		AAG GGG GGC	70gly > arg
122	LT4-1A1	209G > A (exon 3)		AAG GGG GGC	70gly > glu

Table 2 (*Continued*)

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino acid change
123	LT4-4C2	209G > A (exon 3)		AAG GGG GGC	70gly > glu
124	LT4-3B1	No cDNA	IVS3+1G > A	TGT gtg agt	Inframe loss of exons 2 & 3 (aa10–106)
125	LT4-4B1	Exons 2 & 3 exclusion	IVS3+1G > A	TGT gtg agt	Inframe loss of exons 2 & 3 (aa10–106)
126	LT4-5A1	325C > T (exon 4) ± exon 2 & 3 exclusion		GAC CAG TCA	109gln > stop
127	LT4-3D2	355G > A (exon 4)		GGT GGA GAT	119gly > arg
128	LT4-1C1	Exon 5 exclusion	IVS4−1G > A	ttc tag AAT	In frame loss of exon 5 (aa129–146)
129	LT4-2A2	400G > A (exon 5)		GTG GAA gta	134glu > lys
130	LT4-5A2	400G > A (exon 5)		GTG GAA gta	134glu > lys
131	LT4-3A1	416C > T (exon 6)		GAC ACT GGC	139thr > ile
132	LT4-3A2	419G > A (exon 6)		ACT GGC AAA	140gly > asp
133	LT4-1C2	430C > T (exon 6) ± exons 2 & 3 exclusion		ATG CAG ACT	144gln-STOP
134	LT4-3B2	464C > T (exon 6)		AAT CCA AAG	155pro > leu
135	LT4-5D2	464C > T (exon 6)		ATT CCA AAG	155pro > leu
136	LT4-3D1	508C > T (exon 7)		CCA CGA AGT	170arg > stop
137	LT4-2A1	Exon 7 exclusion	IVS7+1G > A	GAC Tgt aag	TGA STOP at new codon 165
138	LT4-1D1	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 165
139	LT4-2C2	Exon 7 exclusion	IVS7+5G > A	Tgt aag tga	TGA STOP at new codon 165
140	LT4-2C1	538G > A (exon 8)		GTT GGA TTT	180gly > arg
141	LT4-4A1	+/− Exon 8 exclusion	538G > A	GTT GGA TTT	180gly > arg
142	LT4-4C1	569G > A (exon 8)		GTA GGA TAT	190gly > glu
143	LT4-5B2	599G > A (exon 8)		TTC AGG GAT	200arg > lys
144	LT4-1A2	Exon 8 exclusion	IVS8+4A > T	Tgta agt aat	TAG STOP at new codon 187
145	LT4-3C1	617G > A (exon 9)		GTT TGT GTC	206cys > tyr
146	LT4-1B1	No cDNA	Del exon 1		No ATG START codon
147	LT4-1D2	No cDNA	Del exon 1		No ATG START codon
148	LT4-4A2	Exon 4 exclusion	Del exon 4 (del 22729–28260; insert ca)		In frame loss of exon 4 (aa107–128)
149	LT4-5C1	Exon 4 exclusion	Del exon 4 (del 24438–30995)		In frame loss of exon 4 (aa107–128)
150	LT4-2D1	No cDNA	Del exon 1 thru 9		No protein
151	LT4-3C2	No cDNA	Del exon 2 thru 9		No protein
152	LT4-5C2	No cDNA	Del exon 4 thru 9		No protein

[a] Mutations # 9, 14, 63, 90, 107, 116, and 120 were found in replicate isolates from the same independent culture and are treated as a single event.
[b] Genomic base numbers (Genbank acession # M26434).
[c] This is the 22nd base upstream (5') of the ATG START codon.

Table 3
Summary of mutation types in the *HPRT* gene in 6-thioguanine resistant human lymphoblastoid cells exposed to EMS

Mutation type (no. of mutations)	NTS[a]	Folate-replete		Low-folate	
		TK6 (38)	WTK1 (39)	TK6 (37)	WTK1 (38)
Transitions					
GC > AT	G > A	29 (13)[b]	27 (10)[b]	22 (9)[b]	21 (10)[b]
	C > T	6 (1)	4	3 (1)	8
AT > GC	A > G	0	1	0	0
	T > C	0	1	0	0
Transversions					
GC > TA	G > T	0	2 (2)	2 (1)	1
	C > A	1	0	0	0
GC > CG	G > C	0	0	2 (1)[b]	0
	C > G	0	0	0	0
AT > TA	A > T	0	0	1 (1)[b]	1 (1)[b]
	T > A	0	0	0	0
AT > CG	A > C	0	0	0	0
	T > G	0	0	1	0
Genomic deletion		0	3	4	7
'new exon'		1	0	0	0
No change		1[c]	1[d]	2[e]	0

[a] NTS = non-transcribed strand.
[b] Mutations which affected splicing.
[c] Exon 8 exclusion in cDNA with no change in genomic found.
[d] Exon 4 exclusion in cDNA with no change in genomic found.
[e] Two mutations with all nine exons present in genomic DNA and no change in the sequence of any exons. Long PCR analysis of the entire *HPRT* gene showed no large alteration in either mutation.

single base substitutions and two showed no genomic change. These molecular analyses of the *HPRT* mutants are presented in detail in Table 2; the analyses are summarized in Table 3. The predominant mutation (99 of 152 = 65%) in both cell types grown in the presence or absence of folic acid was a $G > A$ transition on the non-transcribed strand. A smaller number (21 of 152, 14%) of $G > A$ transitions occurred on the transcribed strand. (These transitions on the transcribed strand appeared to be more frequent in the folate-deficient WTK1 cells). The $G > A$ transitions were mainly (116 of 120) at non-CpG sites, and occurred primarily at Gs flanked 5′ by a purine or on both sides by purine bases. The section of exon 3 that contains six consecutive guanines (207–212) was especially susceptible to mutation (19 of the 99 G > A mutations on the non-transcribed strand, 19%).

Splice site mutations were common in both cell types and under both folate conditions, representing approximately 31% of mutations. Of the 52 mutations that affected splicing, 35 caused simple exon exclusions in the cDNA. The other 17 are presented in detail in Table 4. There were nine partial exon exclusions (Table 4) (mutations #2, 19, 22, 36, 43, 59, 69, 81 and 92) and eight intron inclusions (mutation #1, 34, 35, 39, 40, 79, 80 and 96). There were two unusual intron inclusion splice effects. One mutant (#1, Table 4) showed the inclusion of 38 bases between exons 1 and 2 in cDNA. Inspection of the genomic sequence flanking these 38 bases revealed a reasonable splice acceptor and donor sequences, consistent with this inclusion being a cryptic exon. The reason for the use of these splice sequences is not yet known. We hypothesize that it is recognized as the result of a deletion or insertion in intron 1. Mutant #92 (Table 4) showed multiple cDNA products. The actual mutation is a G > A transition in the first base of the splice donor sequence (IVS3 + 1G > A). One cDNA results from the use of the new splice donor sequence GT_{318}atga resulting in the exclusion of the last two bases of exon 3 (317–318) and, in addition, the inclusion of 41 bases from the middle of intron. Inspection of these latter sequences flanking

Table 4
HPRT splice alterations in 6-thioguanine resistant human lymphoblastoid cells exposed to EMS[a]

Mutation #	Mutant code	Alteration	Sequence context	Comments
A. Insertion of cryptic exons				
1	LT2-5D1	Intron inclusion 9192–9230	ttttttttttttttag|A_{9192}C(A)GGAGTCTCGCTCT-GTCACTCAGGCTGGAGTGTAGTG$_{9230}$|gtatga	Analysis of the genomic region around the inserted bases indicates that there is an apparent acceptor just 5′ and an apparent donor 3′. Thus, these bases form a reasonable although somewhat small exon (38bp). The area around exon 1 (b1651–1841) and the inserted bases (b9014–9546) was sequenced and no alterations were found. We speculate a large deletion or insertion elsewhere in intron 1 alters the splicing pattern and causes utilization of this new 'exon.' NOTE: There appears to be an error or polymorphism in the GeneBank sequence as A_{9194} was not present in our wild type sequence.
92	LT1-5B2	IVS3+1G > A	gctttatttttttacatttag|A_{26894}TTTTAATCCGTCT-GGAATTTATTTTTGTGTATGCTGTGAG$_{26934}$|gtaggg	Multiple cDNA products were found: (1) exclusion of b317–318 with the inclusion of b26894–26934 (2) exclusion exon 3 with the inclusion b26894–26934 and (3) exclusion exons 2&3. Exclusion 317–318 GT was due to utilization of a new created splice sequence TT_{316}|GT_{318}atga. The sequences 5′ and 3′ of the included 26894–26934 appears to be an acceptor and donor, respectively. Because of the mutation of the exon 3 donor this 26894–26934 is processed as an alternative or additional 'exon'.
B. Creation of new splice sites				
59	LT3-5C1	209G > T	AG_{207}|$G\underline{T}_{209}$GGGC...TATTGT$_{318}$|gtgagt	Two cDNA products were found: (1) Loss of bases 208–318 in exon 3 due to creation of a new splice donor site at b208 and (2) and normal length cDNA with 209G > T (gly > val).
19	LT2-4A1	IVS5−1G > A	aaaa$\underline{a}_{IVS5-1}G_{403}$|ATA...$AAG_{485}$ |aaaa$\underline{a}_{IVS5-1}G_{403}$ATA...$CAG_{452}$|GCA...$AAG_{485}$|	Splice acceptor site mutation that causes the exclusion of b 403 (the first base of exon 6) utilizing a new acceptor created one base shifted, or the exclusion of b403–452 utilizing a cryptic acceptor at 452.
34	LT2-2B2	IVS8−16G > A	ct$\underline{ag}$|$a_{IVS8-14}$ttttttttatag$_{IVS8-1}$CATGTT	Inclusion of intron 8, b1 to b14 due to use of a new splice site (tag$_{IVS-15}$|a). This results in a chain terminating codon TAG at new codon 251 creating a larger protein product of 255 amino acids since the normal TAA at codon 251 is out of frame.

Table 4 (*Continued*)

Mutation #	Mutant code	Alteration	Sequence context	Comments
35	LT2-3C1	IVS8−16G > A		
C. Use of cryptic splice sites				
79	LT1-4C2	27G > A	GT<u>A</u>g$_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	Splice donor site mutations that cause the use of a cryptic splice site at IVS1+49. This 49bp inclusion is out of frame and yields a chain termination codon TAG at new codon number 27.
80	LT1-4A2	IVS1+1G > A	GTG<u>a</u>$_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	
40	LT3-4A2	IVS1+5G > T	GTG<u>t</u>$_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	
39	LT3-4C2	Del 1655–1730	gc[c$_{IVS0-22}$cg...gg$_{IVS1-27}$]cc...ag$_{IVS1+49}$|gtggcg	Genomic deletion of 22 bases 5′ to exon 1, exon 1, and 27 bases of intron 1 including the exon 1 splice site which causes the use of the cryptic splice site at IVS1 + 49 (gtggcg) to splice to exon 2.
2	LT2-3D2	IVS1−1G > A	cttttt<u>c</u>aa$_{IVS1-1}$A$_{28}$TTAG$_{32}$|GTG	Splice acceptor site mutation that results in 2 cDNAs: exclusion of exon 2 or the exclusion of bases 28–32 utilizing a cryptic acceptor at b32. This results in an immediate chain terminating TGA at new codon 10.
43	LT3-3B2			
81	LT1-5C1			
96	LT1-1A2	IVS5+1G > A	GAA<u>a</u>$_{IVS5+1}$taagt...aaag$_{IVS5+67}$|gtaagc	Splice donor site mutation that causes the use of a cryptic splice site at IVS5 + 67 which splices in 67 bases of intron 5. This inclusion results in a chain termination codon, TAA, at new codon 136.
22	LT2-3D1	IVS7−1G > A	ttaa$_{IVS7-1}$T$_{533}$TGTT...CCAG$_{553}$|ACAAG	Splice acceptor site mutation that creates mixed cDNA: (1) exon 8 exclusion and (2) b533–553 exclusion because of the use of a cryptic acceptor site at b553. The latter yields an inframe mRNA which predicts a protein lacking 7 amino acids.
69	LT3-5A2			
36	LT2-4A2	IVS8−1G > A	ataa$_{IVS8-1}$C$_{610}$ATG...TTAG$_{626}$|TGAA	Splice acceptor site mutation that causes the exclusion of b610–626 in exon 9 due to the use of cryptic splice site TAG$_{626}$|T. This results in an immediate chain terminating TGA at new codon 204.

[a] Does not include 33 simple exon exclusions listed in Table 4.

the inclusion reveals reasonable splice acceptor and donor sequences, defining it as another cryptic exon.

New splice sites were created in three mutations and cryptic splice sites were utilized because of mutations in the normal splice sequences in eight mutations. Most of these mutations have been reported previously [40]. Nearly all splice site mutations involved a G > A transition, often at a GT sequence (Table 4).

The most striking difference between the folate-replete and deficient cells was an increased frequency of deletions in the cells of both types grown under low-folate conditions. In folate-replete cells, three of 77 mutations (3.9%) were genomic deletions compared to 11 of 75 (14.7%) in folate-deficient cells. In addition, two mutations in low-folate cells resulted in no cDNA and no change in the nine *HPRT* exons and could represent small intron deletions that interfered with RNA splicing. Long PCR analysis of the entire *HPRT* gene showed no large alteration in either mutation. Two of the genomic deletion mutations in WTK1 cells treated in low folate (mutants 148 and 149, both deletions of exon 4) have been characterized for the deletion breakpoints. The sequence junctions are shown in Fig. 4.

Mutation # 149 shows a deletion of 6558 bases, with a four base sequence identity at the intron 3 and 4 breakpoints of TTTC. Thus, the actual breakpoints are ambiguous because of the four base sequence identity, and the deletion is designated as if it came from the 5′ donor sequence ($TTTC_{24437}$). Mutation # 148 shows a deletion of 5532 bases, with no sequence identity at the breakpoints, but has a two base pair insertion (CA) at the junction. This insertion creates a CA repeat. Both deletions have TGT at the 5′ donor sequence in intron 3, but no other clear similarities.

4. Discussion

These studies indicate that the combination of nutritional folic acid deficiency and a monofunctional alkylating agent (EMS) causes similar genetic damage in human lymphoblast cell lines and CHO cells. Both the WTK1 lymphoblastoid cell line and CHO cells are derived from mammalian cells, and both are deficient

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Mutation #149       cDNA: exclusion of exon 4
WTK1, Low Folate    Genomic: deletion 24438 (intron 3) to 30995 (intron 4)

                    Intron 3: AATGTTGAGA GAATGACTGT TTTC24437 cgataaaaaa aaaaagtcca
                              |||||||||| |||||||||| ||||           |          |
                    SEQUENCE: AATGTTGAGA GAATGACTGT TTTC     TCGATTTTGA GGGGTAATTA
                              |   |    | ||   |     ||||     |||||||||| ||||||||||
                    Intron 4: agcataagaa cagataggac TTTC30995 TCGATTTTGA GGGGTAATTA


Mutation #148       cDNA: exclusion of exon 4
WTK1, Low Folate    Genomic: deletion 22729 (intron 3) to 28260 (intron 4);
                             insertion CA

                    Intron 3: TTTCTTAAAG TGTCTCATGT22728   aagagggcag tagctttcag
                              |||||||||| ||||||||||        | |     | |   | | |
                    SEQUENCE: TTTCTTAAAG TGTCTCATGT  CA  CAAACCTATG TGAGTCTATG
                               ||   |||        |         |||||||||| ||||||||||
                    Intron 4: ctttaaaaaa aaaaaaaaaa28260  CAAACCTATG TGAGTCTATG
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Fig. 4. Breakpoints of two *HPRT* intragenic deletions induced by EMS in WTK1 cell in low-folate conditions. For both mutations, the line labeled SEQUENCE designates the sequence found in the mutated DNA. The sequence above and below is that of the appropriate region of the introns from which they derive. The base numbers are according to Edwards et al. [71]. Capital letters designate the intron origin of the bases, small letters designate the flanking bases lost through the deletion event and | designates identical bases flanking the junction. The bold underlined bases designate identical sequences at the breakpoints (**TTTC** in mutation #149) and the bold italicized bases designate inserted bases at the junction (**CA** in mutation # 148).

in AGT [24–27] and in p53 protein [28,29,33–35]. Therefore, it is not surprising that the WTK1 and CHO cell lines react similarly to these DNA damaging agents. The present studies extend and confirm our prior results in CHO cells and support the notion that they are relevant to human cells.

Treatment of WTK1 and TK6 cells with EMS resulted in dose-dependent cytotoxicity. However, inhibition of colony formation occurred at much lower concentrations than was seen previously with CHO cells. For example, the relative survival of CHO cells was about 40% with concentrations of EMS of 800 μg/ml, while the CE dropped to below that range with as little as 50 μg/ml of EMS in the lymphoblastoid cells treated under the same conditions [5]. Other laboratories have published similar relative sensitivities of these cell lines to EMS [23,41]. In standard culture medium containing folic acid, WTK1 cells were more resistant to the cytotoxic effects of EMS than TK6 cells, confirming the previous report of Honma and colleagues [23]. The WTK1 cell line also has been shown to be more resistant than TK6 to X-ray induced cell killing [22,42,43]. Since p53 protein mediates apoptosis after DNA damage, a delay or partial abrogation of apoptosis in the p53-deficient WTK1 cell line may account for its superior survival after alkylating agent treatment. For example, apoptotic death is delayed in WTK1 compared to TK6 after X-ray treatment [28,42,43]. In our experiments, growth arrest in folate-deficient medium was delayed about a day in the WTK1 cells compared to TK6 cells. Incubation of the lymphoblastoid cell lines in folate-deficient medium tended to increase their resistance to EMS cytotoxicity. Studies in other laboratories have shown that in vitro folate deficiency induces apoptosis in CHO cells, accumulation of human hepatoma HepG2 cells in S-phase and coincident apoptosis, and apoptosis of late-stage mouse erythroblasts [6,44,45]. It is therefore surprising that folate deficiency did not increase the sensitivity of the human lymphoblastoid cells to alkylating agent treatment. A possible explanation with regard to TK6 cells may lie in the timing of the EMS treatment on Day 4 of culture. At that point the TK6 cells in folate-deficient medium are already in growth arrest (Fig. 1) and thereby perhaps less susceptible to the cytotoxic effects of alkylating agents.

The background mutant frequencies at the *HPRT* locus in the experiments reported here were slightly higher in WTK1 cells than in TK6. Amundson and colleagues found that the formation of spontaneous *HPRT*$^-$ mutants was the same in WTK1 and TK6 [22], while Honma et al. reported an 8.3-fold higher spontaneous mutant frequency at the *HPRT* locus in WTK1 cells than in TK6 cells [23]. Our findings were intermediate between these two reports. Background mutant frequency increased modestly in both cell lines after incubation in folate-deficient medium, consistent with prior experiments in CHO cells wherein folate-deficient cells had slightly higher background mutant frequencies than folate-replete cells at the *HPRT* [5] and *aprt* loci [6].

After treatment with EMS, TK6 cells had a higher mutant frequency than WTK1 at all drug concentrations tested. Amundson et al. found that *HPRT* mutant frequencies were slightly but not significantly higher in TK6 than in WTK1 after EMS treatment [22]. In contrast, X-ray induced mutant frequencies at the *HPRT* locus were higher in WTK1 than in TK6 [22], and mutant frequencies at another locus, thymidine kinase, also were higher in WTK1 than in TK6 after EMS or X-ray treatment [22,23,46]. The greater mutability of the autosomal thymidine kinase locus may be due to more frequent inter- and intramolecular recombination events [47]. Folate deficiency enhanced the mutagenesis of EMS in both TK6 and WTK1, consistent with our previous findings in CHO cells [5], but the effect was greater in the p53 positive TK6 cells.

The *p53* gene product has been implicated in several DNA repair pathways. For example, p53 is involved in the induction of O^6-methylguanine-DNA methyltransferase through promoter activation after DNA damage [48]. The *hMSH2* gene is a *p53*-regulated target gene, suggesting that p53 is also involved in DNA mismatch correction [49]. Wild-type *p53* is required for global genomic nucleotide excision repair but not transcription-coupled repair [30,50], for base excision repair [32], and for ionizing radiation-induced modulation of excision repair [31]. DNA strand breaks associated with excision repair are potent inducers of p53 [51], and p53 binds to ends and single-stranded gaps in DNA [52]. In addition, Lee and coworkers found that p53 recognized insertion/deletion mismatches [52]. Therefore, increased p53 activity has been associated with the types of DNA lesions previously described in folate-deficient cells treated with alkylating agents; namely, strand breaks, intragenic dele-

tions, and single base mismatches. In the experiments reported here, we did not find that enhanced DNA repair mediated by p53 activity ameliorated either the cytotoxic or mutagenic activities of monofunctional alkylating agents in folate-deficient cells. Nutritional folate deficiency augmented genetic damage by EMS in the *p53* mutant WTK1 cells, and, if anything, the augmentation was greater in folate-deficient TK6 cells that expressed wild-type *p53*.

As in CHO cells, the predominant mutation in EMS-treated folate-replete WTK1 cells was a G > A transition. EMS is mutagenic by reaction with the O^6 and N^7 positions of guanine. O^6-ethylguanine is mutagenic by pairing with thymine during replication, while N^7-alkylation products lead to apurinic sites that are processed by base excision repair and may cause mutations by defective repair or by mis-incorporation [53–57]. Under folate-replete conditions, most mutations (79%) in the WTKI cells were G:C > A:T transitions, suggesting that the deficiency of the AGT repair mechanism was a major contributing factor to persistent mutations. The smaller number of genomic deletions (8%) probably reflects error-prone base excision repair, because base excision repair defective cell lines exhibit increased percentages of deletion mutations after EMS treatment [58,59]. The mutational spectrum in the p53-competent TK6 cell line was similar to both CHO cells and WTK1 cells after EMS treatment in folate-containing medium, showing 92% G:C > A:T transitions, but no deletions. This observation suggests that the *p53* gene product does not have a major influence on the molecular spectrum after treatment with monofunctional alkylating agents.

Folate-deficient TK6 and WTK1 cells, like folate-replete cells, showed a predominance of G:C > A:T, 68 and 76%, respectively, transitions. However they also exhibited an increased percentage of deletions compared to folate-replete cells, 11 and 18%, respectively. This finding supports and confirms our previous report that folate-deficient CHO cells had more intragenic deletions after EMS treatment than folate-replete cells [20].

Folate-deficient WTK1 cells were found to have a higher percentage of C > T transitions (21%) than either folate-replete WTK1 cells (10%) or TK6 cells regardless of folate status (16% replete, 8% deficient). This higher percentage of C > T transitions may represent persistence of G > A transitions on the transcribed strand of these folate-deficient, *p53* mutant cells. Alternatively, O^2-ethylcytidine may act as uracil and code for thymine [60] giving the pathway: O^2-ethylcytidine → U → T.

Treating all G/C → A/T transition mutations, except the four that occur at CpG dinucleotides (two each of 151C → T and 508C → T), as the result of adducts on the G base, there are 116 G → A mutations. Considering the flanking bases, 54% (63 of 116) occurred at Gs flanked by purines (21 at GGG, 17 at GGA, 14 at AGG and 11 at AGA), 22% (26 of 116) at Gs with a 5′ purine (15 at AGT, 7 at GGT, 3 at AGC and 1 at GGC) and 12% (14 of 116) at Gs with a 3′ purine (9 at TGG and 5 at TGA). Only 11% (13 of 116) occurred at Gs flanked by pyrimidines (9 at TGT and 4 at TGC). Forty of these 116 G → A mutations occurred in splice donor or acceptor sequences (34%). Thirty-one of these 40 (78%) occurred at ag(a/g) (12/40) or (a/g)gt (19/40) sequences, reflecting the known importance of the acceptor ag dinucleotide and the donor gt dinucleotide [40]. Of the total 25 AG(A/G) mutations, 12 occurred in splice sequences (12 of 40, 30%) compared to 13 in coding sequences (13 of 76, 17%). Of the total 22 (A/G)GT mutations, 19 occurred in splice sequences (19 of 40, 48%) compared to three mutations in coding sequences (3 of 76, 4%). The most frequent mutation in splice sequences was at AGT (11 of 40, 28%); mutations at AGT were found only rarely in the coding sequence (4 of 76, 5%).

The above result demonstrates the importance of defining a complete spectrum. Table 5 presents a summary of the cDNA phenotype and the actual mutation type. The 37 complete simple exon exclusion cDNAs consisted of 32 single base substitution mutations and only two genomic deletions (three were uncharacterized). Eleven of the 14 genomic deletion mutations did not yield a cDNA. These results demonstrate the importance of not simply characterizing mutations only through cDNA sequencing. It was only through genomic analysis that the increase in genomic deletions resulting from treatment of cells in low-folate medium was demonstrated, i.e. 4% (3 of 77) in folate-replete versus 15% (11 of 75) in low-folate conditions.

While these studies were performed with human cell lines, these lines differ in important characteristics from other human tissues. Namely they are most likely Epstein–Barr virus transformed [61], lack alkyltransferase activity [24,25], and are thymidine kinase

Table 5
Summary of cDNA phenotype mutation type

	Folate-replete		Low-folate		Total
	TK6	WTK1	TK6	WTK1	
No. of Mutants	38	39	37	38	152
cDNA positive	38	37	30	32	137
'Normal' length	22	23	18	20	83
Complete simple exon exclusion	9	9	7	12	37
Single base substitution	9	8	7	8	32
Genomic deletion	0	0	0	2	2
No change	0	1	0	2	3
Partial exon exclusion	4	3	2	0	9
Single base substitution	4	3	2	0	9
Intron inclusion	3	2	3	0	8
Single base substitution	2	1	3	0	6
Genomic deletion	0	1	0	0	1
Uncharacterized	1	0	0	0	1
cDNA negative	0	2	7	6	15
Single base substitution	0	0	1	1	2
Genomic deletion	0	2	4	5	11
Uncharacterized	0	0	2	0	2

deficient heterozygotes [62]. These differences may influence the relevance of our studies to some other human tissues. For example, thymidine kinase is a salvage enzyme that helps maintain deoxyribonucleoside triphosphate pools. Thymidine kinase-deficient cell lines showed enhanced sensitivity to killing and increased mutagenesis by ultaviolet and gamma irradiation, and by EMS [63–65]. Moreover, mutational loss of thymidine kinase was associated with increased methotrexate-induced DNA strand breaks and cytotoxicity [66]. These effects appeared to be dependent upon a concomitant decrease of the TTP pool and severe enzyme deficiency (0–1.4%) [63–65]. TK6 cells, with thymidine kinase levels of 25–50% [62], did not have increased aberration induction after treatment with methotrexate [67], but the combination of methotrexate and an alkylating agent was not tested. It is possible that the partial thymidine kinase deficiency in TK6 cells magnified the nucleotide pool disruption caused by folate deficiency and enhanced the chromosome damage caused by EMS reported here. Thus, these studies may be most relevant to differentiating bone marrow cells, such as occurs during erythropoiesis and granulopoiesis, that also have a decreased capacity to salvage thymidine [68]. There appears to be a negative correlation between the extent to which cells depend upon salvaged thymidine for DNA synthesis and their degree of differentiation [68].

TK6 lymphoblasts also resemble bone marrow myeloid precursors cells in that they are deficient in alkyltransferase activity [24,25,69]. The bone marrow is a frequent target organ for alkylating agent-induced transformation, and acute myelogenous leukemia has been reported following exposure to cyclophosphamide, melphalan and busulfan as single agents and to the combination of nitrogen mustard and procarbazine [70]. Cytogenetic analyses indicate that transformation to myelodysplasia and acute leukemia is associated with deletions and translocations [70]. Thus, the combination of alkylating agent exposure and nutritional folate deficiency in a cell type deficient in alkyltransferase but expressing wild-type *p53* creates an environment that increases the risk of developing carcinogenic genetic changes. The addition of a mutation at the *p53* locus would alter further the biology of a developing tumor cell, in that loss of p53 activity reduces toxicity from monofunctional alkylating agents, and those cells lacking p53 activity and folate are most resistant. The corollary of these observations is that correction of nutritional

folate deficiency may reduce the risk of developing carcinogenic genetic changes in normal cells after alkylating agent exposure and enhance the sensitivity to alkylating agents in *p53*-mutant malignant cells.

Acknowledgements

Supported by grants from the National Cancer Institute, Bethesda, MD (CA 41843) and the Department of the Army (DAMD17-98-1-8345). The content of the information does not necessarily reflect the position or policy of the US government, and no official endorsement should be inferred. Automated sequencing was performed in the Vermont Cancer Center DNA Analysis Facility and was supported in part by NCI P30 CA 22435. We thank Dr. Takamaru Ashikaga, Medical Statistics Department, University of Vermont, for his valuable assistance with the statistical analyses.

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amplification for either *PIK3CA* or *PIK3CB*. In contrast, none of the 61 adenocarcinomas showed a copy number ratio greater than 2 neither for *PIK3CA* nor for *PIK3CB*. Only 4/62 tumors had a ratio greater than 2. This data suggests that the amplification of genes in the PI3-Kinase pathway may be involved in the development of squamous cell carcinoma but not adenocarcinoma of the lung. Further investigations on PI3-Kinase pathway genes are under way to determine the functional significance of copy number increase of these genes in lung cancer development and progression. (Supported in part by a Parker B. Francis Fellowship).

#440 SPECIFIC CHROMOSOMAL CHANGES IN MOUSE LUNG ADENOCARCINOMA CELL LINES DETECTED BY SPECTRAL KARYOTYPING. Linda M Sargent, J R Senft, D T Lowry, A M Jefferson, F Tyson, and S H Reynolds, *National Institute for Occupational Safety and Health, Morgantown, WV, and National Institute of Environmental Health Sci, Research Triangle Park, NC*

Adenocarcinoma is rapidly becoming the most common form of lung cancer in the United States. The difficulty in obtaining lung cancer families and the problems in obtaining samples for various stages of human lung adenocarcinoma have lead to the use of primary lung tumors in mice as an experimentally manipulable model for human lung adenocarcinoma. Chromosomal changes in mouse lung tumors have been reported using standard chromosome banding analysis. Due to the difficulty in identification of marker chromosomes based on banding alone, we have analyzed 12 early passage mouse lung adenocarinoma cell lines by Spectral Karyotyping (Applied Spectral Imaging, Carlsbad California). An entire copy of chromosomes 6 (78%) and 15 (78%) were gained while chromosome 7 (67%) and 14 (33%) were lost. There was a gain of a portion of chromosomes 1 (67%) and 2 (56%) and a loss of a portion of chromosomes 4 (67%) and 8 (44%). The minimal region of alteration is 1G1,2F2, 4C6 and 8B3. The minimal regions of alteration of chromosomes 1, 2, 4 and 8 contains putative susceptibility genes for mouse lung adenocarinoma. In addition, the deleted regions contain linkage groups that are altered in human adenocarcinoma. Due to the conservation of linkage groups between mouse and human, the identification of susceptibility genes in the mouse may aid in the identification of similar genes in the human population.

#441 RELATIONSHIPS BETWEEN DNA INCORPORATION, MUTANT FREQUENCY, AND LOSS OF HETEROZYGOSITY AT THE *TK* LOCUS IN HUMAN LYMPHOBLASTOID CELLS EXPOSED TO AZT. Quanxin Meng, T. Su, O. A Olivero, M. C Poirier, X. Shi, X. Ding, and V. E Walker, *National Cancer Inst, Bethesda, MD, and Wadsworth Ctr, NYSDOH, Albany, NY*

The purpose of this study was to investigate the potential mechanisms of AZT mutagenicity and carcinogenicity by determining the AZT-DNA incorporation, AZT-induced thymidine kinase (*TK*) mutant frequencies (M*f*s), and the percentage of loss of heterozygosity (LOH) in AZT-induced *TK* mutants in the human lymphoblastoid cells. Cells were exposed to 300 μM AZT for 0, 1, 3, or 6 days, or to 0, 33, 100, 300, or 900 μM AZT for 3 days (n = 5 flasks/group). An AZT-RIA and a cell cloning assay were used to measure AZT-DNA incorporation and *TK* M*f*s, respectively. AZT was incorporated into DNA in a dose-related manner at concentrations up to 300 μM, above which no further increase was observed. *TK* M*f* increased with the extended duration and with incremental concentrations of AZT exposure. There was a positive correlation (P = 0.036, coefficient = 0.903) between AZT-DNA incorporation and AZT-induced *TK* M*f*s, suggesting that AZT incorporation into cellular DNA has a direct role in the genotoxicity of AZT. Southern blot analyses indicated that 84 % of AZT-induced mutants were attributable to LOH, consistent with the known mechanism of AZT as a DNA chain terminator.

#442 ARSENITE INDUCED P53 ACCUMULATION THROUGH THE ATM-DEPENDENT PATHWAY. Ling-Huei Yih, and Te-Chang Lee, *Institute of Biomed Sci, Acad Sinica, Taipei, Taiwan ROC*

Accumulated evidence has shown that arsenite-induced cytogenetic alterations are associated with its carcinogenicity. In the present study, arsenite-induced DNA strand breaks were demonstrated by alkaline single cell gel electrophoresis (Comet assay) in human fibroblasts (HFW). As accompanied to the appearance of DNA strand breaks in arsenite-treated HFW cells, we observed p53 accumulation by immunoblotting and immunofluorescence techniques. In addition, p53 down-stream proteins, such as p21 and MDM-2, were significantly induced by arsenite treatment. Furthermore, the kinetic of arsenite-induced p53 accumulation was similar to that induced by X-ray irradiation, but different from that induced by UV irradiation. Wortmannin, an inhibitor of ATM-kinase and/or PI-3 kinase, inhibited arsenite- or X-ray irradiation-induced p53 accumulations, but did not alter UV irradiation-induced p53 accumulation. These results suggest the involvement of ATM in arsenite-induced p53 accumulation. To confirm this point, we also demonstrated that arsenite treatment, similar to X-ray irradiation, did not induce p53 accumulation in ATM defective cells, GM3395. In contrast, UV irradiation caused p53 accumulation in GM3395 cells. Therefore, our present study suggested that arsenite induced DNA strand breaks which may lead to p53 accumulation through an ATM-dependent pathway in HFW cells.

#443 FRAGILE SITE (FRA) UPREGULATION DEFINES A DISTINCT PATHWAY OF HEAD AND NECK (HN) TUMORIGENESIS. Julie G Izzo, Maranke I Koster, Vali A Papadimitrakopoulou, Adel K El-Naggar, Waun K Hong, and Walter N Hittelman, *U T M D Anderson Cancer Ctr, Houston, TX*

FRA expression is involved in chromosomal rearrangements (deletions, amplifications, exchanges) and is inducible by environmental factors and carcinogens. Previously we demonstrated that FRA expression at the 11q13 region is detectable *in vivo* as split signals of a region-specific probe during HN tumorigenesis and that it precedes gene amplification. To study the relationship between FRA expression and specific events, 15 HN tumor specimens (8 with, 7 without 11q13 amplification) with adjacent premalignant lesions (PL) and normal mucosa (AN) were examined for loss of heterozygosity (LOH) at 9p21 and 3p14 (loci of known FRA), 11q13 FRA upregulation and cyclin D1 (CCND1) expression. FRA expression was higher in PL adjacent to amplified tumors (mean±SD 4.7%±. 4 *vs* .78±. 03, p=. 0006), in these epithelia FRA frequency increased sharply (3 fold) in regions with CCND1 dysregulation. LOH at 9p21 was observed in 7/8 (88%) PL of amplified cases, always with concomitant dysregulated CCND1, while in only 1/7 (14%) PL of non amplified cases. In contrast 3p14 LOH was mainly found in PL of non amplified cases (6/7 [86%] *vs* /8 [25%]) without CCND1 dysregulation. Remarkably, ANL of amplified cases (without CCND1 or 9p21 abnormalities) harbored higher FRA expression than ANL of non amplified cases (mean±SD: 3.6%±. 3 *vs*.3±.0, p=. 0002), suggesting an inherent defect. Although no difference in tobacco exposure was evident between the two groups, the FRA expressing group demonstrated clinical evidence of field cancerization [multiple asynchronous primaries (3/8)/premalignant lesions (3/8)]. These data suggest the possible existence of genetically predetermined and distinct pathways to tumor development in the form of FRA with tobacco acting as a promoter. (NIH CA-52051)

#444 P53 ACTIVITY MODULATES THE EFFECT OF FOLATE DEFICIENCY ON GENETIC DAMAGE CAUSED BY ALKYLATING AGENTS IN HUMAN LYMPHOBLASTOID CELLS. Richard F Branda, J Patrick O'Neill, Elise M Brooks, Lucy M Trombley, and Janice A Nicklas, *Univ of Vermont, Burlington, VT*

Relatively little is known about the effects of diet on the toxicity of chemotherapy. Our laboratory previously has shown that deficiency of folic acid acts synergistically with alkylating agents to increase genetic damage at the *hprt* locus in Chinese hamster ovary cells *in vitro* and in rat splenocytes *in vivo*. The present studies extend these observations to human cells and investigate the role of p53 activity. The human lymphoblastoid cell lines TK6 and WTK1 are derived from the same parental cell line (WI-L2), but WTK1 expresses mutant p53. Treatment of folate replete or deficient cells with increasing concentrations (0-50 μg/ml) of ethyl methanesulfonate (EMS) resulted in significantly different *hprt* mutation dose-response relationships ($P<0.01$), indicating that folate deficiency increased the EMS-induced mutant frequency in both cell lines, but with a greater effect in TK6 cells. Molecular analyses of 153 mutant clones showed more intragenic deletions, complex chromosomal changes and G>T transitions in folate deficient as compared to replete TK6 cells, and a striking increase of C>T transitions in folate deficient WTK1 cells as compared to the other 3 groups. These results indicate that folate deficiency augments genetic damage in human cells, particularly those that express p53, and changes the mutational spectra caused by alkylating agents.

#445 EXPRESSION OF BASE EXCISION REPAIR ENZYMES IN RAT AND MOUSE LIVER IS INDUCED BY PEROXISOME PROLIFERATORS AND IS DEPENDENT UPON CARCINOGENIC POTENCY. Ivan Rusyn, Ronald G Thurman, Michael L Cunningham, and James A Swenberg, *NIEHS, RTP, NC, and Univ of North Carolina at Chapel Hill, Chapel Hill, NC*

Sustained elevation of cell replication and inhibition of apoptosis are considered main mechanisms of tumor promotion for peroxisome proliferators (PPs). A potential role of oxidative stress and DNA damage has also been proposed. In view of the possible formation of DNA adducts by PPs, DNA repair may be an important factor to consider in the mechanism of PPs. Here, the ability of PPs to induce expression of base excision repair enzymes was examined in livers of rats or mice fed PP-containing diets using an RNAse protection assay and quantitative RT-PCR. In mice, WY-14,643 (WY, 500 ppm, 1 wk), a potent carcinogen, caused a 3-fold increase in mRNA for 8-OH-dG glycosylase (OGG1), thymine DNA glycosylase (TDG), AP endonuclease (APE), N-methylpurine DNA glycosylase (MPG) and endonuclease III (Nth1). In contrast, administration of diisononyl phthalate (8000 ppm), a less potent carcinogen, had little effect. In rats, when WY (1000 ppm) and diethylhexyl phthalate (12000 ppm), a weak carcinogen, were given for 7 days, induction of OGG1, APE, and Nth1 was observed only in WY-treated animals. Since these two compounds cause a similar initial increase in cell proliferation, this effect could not be attributed solely to a rapid growth of liver mass. Moreover, WY (0-500 ppm; 34 and 90 d) induced both time- and dose-dependent increases in expression of OGG1, APE, Nth1 and MPG. Similar effects were observed with gemfibrosil (0-16000 ppm, 90 d), a rodent carcinogen, but not with dibutyl phthalate (0-10000 ppm, 34 and 90 d). Collectively, these data suggest that DNA base excision repair may be an important factor in PP-induced carcinogenesis and could provide further evidence supporting a role of oxidative DNA damage by PPs. (ES 9785)

DIET MODULATES THE TOXICITY OF CANCER CHEMOTHERAPY IN RATS. I. CYCLOPHOSPHAMIDE[1]

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[1]Supported by grants from the Department of Defense (DAMD17-98-1-8345) and the National Cancer Institute, Bethesda, MD (CA 41843 and P30-CA 22435). The content of the information does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

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Running Title: Diet and Cyclophosphamide Toxicity

ABSTRACT

The effects of diet and nutritional folate status on toxicity caused by cyclophosphamide were studied in Fischer 344 rats maintained on either a cereal-based diet (Harlan Teklad LM-485) or a purified diet (AIN-93G). The rats fed the purified diet were randomly 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 folic acid intraperitoneally/kg body weight). Rats on all 4 diets grew at the same rate and had similar hematocrits, white blood cell counts and BUN levels after 6 weeks. Then the rats were treated with increasing doses of cyclophosphamide (0, 50, 65, 85, 110, 144, 190 and 250 mg/kg). The median lethal doses (LD50) were: cereal diet, 232 mg/kg; folate deficient diet, 154 mg/kg; folate replete diet, 159 mg/kg; high folate diet, 148 mg/kg. The LD50 for the cereal diet was significantly higher 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. Diet had no effect on hepatic microsomal P450 levels or aldehyde dehydrogenase activities. Hepatic total glutathione levels were significantly higher in folate replete rats compared to rats fed the cereal-based diet, and increased in the order: folate deficient<folate replete<high folate in rats fed the purified diet. These results indicate that diet has an important influence on the toxicity of cyclophosphamide. They also show that folate status modulates hepatic glutathione levels, a major cellular defense against oxidant and alkylating agent damage.

INTRODUCTION

Patients receiving cancer chemotherapy frequently ask whether a change in their diet would be beneficial. Unfortunately the answer is usually unsatisfactory to both the patient and physician. There is relatively little scientific information on which to base a recommendation because, while the question is important, the investigational issues are complex. Patients vary widely in their nutritional status, dietary components are numerous, and chemotherapy regimens often employ several drugs in complicated combinations and schedules. Folic acid is a dietary constituent that may serve as a model for studies of the interactions of diet and cancer chemotherapy. Folate status varies widely in the general population as well as in patients with cancer. Dietary folic acid lack has been said to be the most common vitamin deficiency in the United States and the rest of the world (1). Populations at particular risk are pregnant women, alcoholics, people in lower socio-economic groups, and patients with proliferative diseases such as hemolytic anemia and psoriasis (1,2). Folic acid deficiency was reported to be particularly prevalent in patients with cancer because of their poor dietary intake and increased utilization of the vitamin (3,4). More recently, dietary fortification with folic acid has raised the blood levels of the vitamin in the general population (5). However, this fortification potentially may lead to supra-normal levels of folic acid in individuals whose diet already is rich in folic acid or who take supplemental vitamins. Thus the interactions of both unusually low and high levels of folic acid with cancer chemotherapy need to be considered.

In addition to the fact that folate nutritional status varies widely in cancer patients, the possible relevance of this vitamin to chemotherapy outcome derives from the effects of folate on cellular metabolism. Folate compounds play a central role in one-carbon transfers. Therefore they 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 (6-9), chromosomal abnormalities (10-12), increased uracil incorporation into DNA (7,9,13-15), defective DNA repair (6,9,16,17), increased somatic mutation rates (6,18,19), and anomalous DNA methylation patterns (8). Since 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 vitro,* in Chinese hamster ovary cells (6) and in human lymphoblasts (20), and *in vivo* in rat splenocytes (19). 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 (21). 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: (i) folate deficiency causes extensive uracil incorporation into DNA and, (ii) the greatly increased utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair (20,22).

Alkylating agents as a class are used extensively in the clinic to treat various types of cancer. Perhaps the most widely employed alkylating agent is cyclophosphamide (23). Used either as a single agent or in combination with other drugs, cyclophosphamide is a standard treatment for breast cancer, lymphomas, and lymphatic leukemias, and is used in high doses in preparation for bone marrow transplantation (24). Cyclophosphamide is a bifunctional alkylating agent that requires metabolic activation, usually in the liver by P450 enzymes (23). Subsequently its metabolites either produce DNA alkylation that results in cell death or are

inactivated by glutathione or aldehyde dehydrogenase (25). Previously we reported that nutritional folate status appeared to modulate the efficacy and toxicity of cyclophosphamide in rats (26). We found that cyclophosphamide 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 (26). 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.

MATERIALS AND METHODS

Animals. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Female Fischer 344 rats, 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. The rats were housed individually in stainless steel wire-bottomed cages. One group continued on the cereal-based diet, while the others were maintained on the AIN-93G diet (Dyets, Inc., Bethlehem, PA) (27). This purified diet is based upon vitamin-free casein and cornstarch. Folate-replete diets consisted of AIN-93G with a vitamin supplement that provided 2 mg 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. Blood samples were obtained from tail veins prior to the injection of chemotherapy, and 4, 9 and 14 days after the chemotherapy treatment. At the 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 liver 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 (26). The homogenate was diluted 1:9 with 50 mmol potassium phosphate/L, pH 4.8, containing 1% ascorbic acid, and incubated for 24 hr at 37° C to allow endogenous conjugase to convert folate polyglutamates to monoglutamates. Then the homogenates were autoclaved, cooled on ice, and centrifuged at 2,000g for 10 min. 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 (26,28,29). A standard curve ranging from 0.0625 to 2 ng/well was made using folinic acid, since it is more stable than folic acid (28). The plates were read at a wavelength of 595 nm at 24 hrs 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 BUN, LDH, SGPT and CPK were performed using Sigma Diagnostics (St. Louis, MO) Procedures No. 66-UV, 500, 505 and 661, respectively.

Cytochrome P450 (CYP). Liver microsomes were prepared by differential ultracentrifugation as described by Shapiro and colleagues (30). 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 $mM^{-1} \cdot cm^{-1}$ (31). Microsomal protein concentration was determined using a Coomassie® Protein Assay Reagent kit (PIERCE, Rockford, IL).

Aldehyde dehydrogenase. Aldehyde dehydrogenase activity in liver was quantified by the method of Kohn *et al.* (32). Protein concentration was determined using a Coomassie® Protein Assay Reagent kit (PIERCE, Rockford, IL).

Total glutathione. Total glutathione levels were determined by enzymatic recycling as previously reported by Gallagher *et al.* (33). Briefly, frozen rat liver was homogenized in 9 volumes of ice cold 5% 5-sulfosalicylic acid, centrifuged and stored on ice until assayed. 700 µL of 125 mM potassium phosphate containing 6.3 mM disodium EDTA, 100 µL of 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), 150 µL water, 20 µL of 20 mM β-nicotinamide adenine dinucleotide (NADPH), and 20 µL of sample was added to a cuvette, and equilibrated to room temperature. Then 10 µL of 50 U/mL GSSG 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, IL).

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, folate levels, cytochorme

P450, aldehyde dehydogenase and glutatione levels. Repeated measures ANOVA were used to examine gains in rat weights. If a significant F value was found, Fisher's least significant difference test was used to compare means. Probit analysis was used to determine median lethal dose (LD50). Logistic regression was used to model predictors of mortality.

RESULTS

The growth of Fischer 344 rats on the four diets over an eight week period is shown in **Figure 1**. These animals received only saline injections rather than chemotherapy in week 6. Similar weight measurements were made for rats subsequently treated with cyclophosphamide in Week 6 (data not shown). There were no significant differences in weight gain or in rat weight at 6 weeks on the various diets in any of the treatment groups. Rat weight at 6 weeks did not predict survival after cyclophosphamide treatment. The rat weights in Weeks 6, 7 and 8 for animals on the four diets treated with increasing doses of cyclophosphamide in Week 6 are shown in **Table 1**. Weight at Week 7 did not differ among the dietary groups at any of the cyclophosphamide levels. At Week 8, the cereal diet animals were significantly heavier than the other groups at the 85 mg/kg dose ($P = 0.006$), while the folate deficient rats lost significantly more weight than the other groups at the 110 mg/kg dose ($P = 0.005$).

After 8 weeks on the various diets, the hepatic folate levels were (mean $\pm$ SD) : cereal diet, 30.24 $\pm$ 7.30 μg/g liver; folate deficient diet, 8.92 $\pm$ 0.99 μg/g liver; folate replete diet, 28.61 $\pm$ 8.84 μg/g liver; and high folate diet, 43.01 $\pm$ 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 cyclophosphamide-related deaths in rats on the different diets is shown in **Table 2.** The calculated median lethal doses (LD50) were: cereal diet, 232 mg/kg; folate deficient diet, 154 mg/kg; folate replete diet, 159 mg/kg; high folate diet, 148 mg/kg. 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 obtained prior to chemotherapy administration in Week 6 and on Days 4, 9 and 14 following chemotherapy are shown in **Tables 3, 4 and 5.** At Week 6 (pre-treatment), the high folate group had a significantly higher BUN ($P = 0.01$) than the other two purified diet groups. The BUN levels in animals on the cereal-based diet were not significantly different from any of the purified diets. There was no difference in hematocrit or white blood cell count pre-treatment among the dietary groups.

A repeated measures analysis for unbalanced designs was run with the post-treatment data. A significant three-way interaction was found for hematocrits. Significant differences among the diet groups were found only for specific combinations of dose levels and times. At the lower doses, the cereal diet group tended to have significantly higher hematocrits than the other three groups ($p<0.05$ for 50 mg/kg, Day 4, and for 85 mg/kg on Days 9 and 14). At the highest dose level the cereal diet group was significantly higher only in comparison to the high folate group ($p = 0.01$, Day 4 for 250 mg/kg). The overall analysis with white blood cell count as the outcome found a significant interaction between dose and time. Diet differences,

however, were not significant. The BUN was significantly higher in the high folate group than in the other dietary groups on Days 9 and 14 ($p<0.03$) at all cyclophosphamide doses except 85 mg/kg. At that dose on Day 14, the BUN of the cereal group was higher than the other groups ($p = 0.007$).

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 are shown in **Table 6.** These samples were collected on Day 14 in surviving rats. Therefore in the higher cyclophosphamide dose groups, the numbers are small. For LDH, diet was a highly significant factor ($p<0.0006$). 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. The high folate diet was not significantly different from any of the other diets. Dose was also found to be highly significant ($p<0.0001$). 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. Specifically, the 50 mg/kg and 65 mg/kg groups were not significantly different from each other, nor 85 mg/kg and 110 mg/kg groups or 144 mg/kg and 190 mg/kg groups. The group that did not receive cyclophosphamide had LDH levels similar to the groups treated with drug dosages in the middle range. The dose control group had significantly lower LDH levels than the 50, 65 and 85 mg/kg dose groups, while its mean LDH was significantly higher than the 250 mg/kg group. The model that included a dose by diet interaction did not find the latter to be significant ($p = 0.062$).

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. At 65 mg/kg, the cereal group had a mean CPK level that was significantly higher than the 3 other groups ($p<0.004$). At 190 mg/kg, the high folate group had a higher CPK level than the other 3 groups ($p<0.009$).

For SGPT, the overall model was significant ($p<0.001$) 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 $p<0.003$) at 5 of the dose levels. Exceptions were at the 50 mg/kg dose level, where the cereal group was significantly higher than the low and high folate groups only. No significant differences were found between the group means at dose levels 144 mg/kg (where there was an extreme outlier) and 250 mg/kg (where there was high attrition).

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 were interested in investigating whether this variation in toxicity was due to a dietary alteration of the metabolism of cyclophosphamide. **Figure 2** shows that 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. 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 (**Figure 3**). 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 4**, the levels were highest in the high folate dietary group, intermediate in the folate replete group, and lowest in the low folate group.

Although this pattern was also seen at the 50 mg, 85 mg, and 190 mg dose levels, these differences were not significant. **Figure 5** shows that glutathione levels tended to be higher in the animals on the folate replete diet than in rats maintained on the cereal diet, and these differences were significant in the saline-treated ($P = 0.0048$) and 50 mg/kg cyclophosphamide-treated rats ($P = 0.02$). Since the hepatic folate levels of rats on these 2 diets are similar, this difference is probably due to other factors. In neither the rats on the purified diet nor the cereal diet was there a dose-diet interaction.

DISCUSSION

These results indicate that diet has an important influence on the toxicity of a bifunctional alkylating agent, cyclophosphamide. Rats on a cereal-based diet were much 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 cyclophosphamide. The cereal diet-fed animals tended to maintain their weight better and had higher hematocrits and white blood cell counts after chemotherapy. Since the principal adverse effect of cyclophosphamide is bone marrow suppression with leukopenia (24), it was not surprising that the white blood cell count 4 days after chemotherapy was predictive of 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 (27), and that rodents fed purified diets have higher tumor incidences compared to rodents fed natural ingredient diets (34,35).

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 (23, 36,37). 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 (38). Ingestion of a casein-based purified diet (Normal Protein Test Diet) by rats was associated with an almost total loss of hydroxylation of aromatic polycyclic hydrocarbons by the microsomal mixed-function oxidase system, while alfalfa was identified as an inducer of this system in cereal-based diets (38). Dietary alfalfa has been effective in ameliorating the toxic effects of drugs and chemicals in rodents (39). The antioxidant phenolic compound, butylated hydroxyanisole (BHA), causes changes in the spectral characteristics of the cytochrome P450 system (38). The related compound, tertiary-butylhydroquinone, is a component of the AIN-93 diet but not of the cereal-based diet used in these experiments (27). While high protein diets have increased the activity of hepatic microsomal enzymes (40), the protein content of the cereal-based and AIN-93 diets were similar (19.92% and 17%, respectively). Rats on folate-deficient diets were reported to have adequate basal levels of hepatic microsomal cytochrome P450 synthesis but were unable to respond with enzyme induction by drugs (41). 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 (Figure 2).

We next measured hepatic aldehyde dehydrogenase levels in animals on the different diets, since this enzyme can modify cyclophosphamide toxicity by inactivating aldophosphamide (25,42-44). An elevation of aldehyde dehydrogenase levels is thought to account for the

decreased toxicity of cyclophosphamide in mice fed menhaden oil (45). 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 (Figure 3).

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 (25,46,47). Cyclophosphamide initially depletes glutathione levels (48) but also provides a drug selection pressure that leads later to higher glutathione levels in resistant tumor cells (25,46,47). 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. Since 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. The major determinants of glutathione synthesis are the availability of cysteine and the activity of the rate-limiting enzyme, γ-glutamylcysteine synthetase (49). The hepatic glutathione level is closely related to the cysteine content of the diet (49) and to methionine excess that leads to catabolism of homocysteine to cysteine via the cytathionine synthase pathway (50,51). Approximately half of the intracellular glutathione pool in human liver cells is derived from homocysteine (50), and glutathione synthesis is stimulated by a methionine load (51). Since folate deficiency is associated with elevated homocysteine levels, one might predict that glutathione levels would be

high. However the relationship is complex. Homocysteine decreases the expression of gene pathways involved in the production of antioxidant enzymes (glutathione peroxidase, NKEF-B PAG, superoxide dismutase, cluserin) thereby reducing the cellular antioxidant potential (52,53). This reduction promotes oxidative stress in rat livers (52). Hepatic glutathione levels have been reported to be lower in rats fed a methyl group deficient diet but not altered further by additional folate lack (54). 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 (55). The increased formation of free radicals possibly caused by reduced levels of hepatic glutathione may contribute to the development of hepatocellular carcinoma in methyl-deficient rats (54,56). 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 (57). 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 (57). The nucleotide pool abnormality was associated with a delay of hepatocytes through the cell cycle following partial hepatectomy (57). 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 (58), but further experimentation will be necessary to test this possibility more directly.

Also unexplained is the discrepancy between the results of the current experiments and our prior observations (26). The investigations reported here do not support our previous conclusion that high levels of folate protect against cyclophosphamide toxicity. A major difference between the two experiments is the schedule of drug administration. In the earlier experiments, the cyclophosphamide was 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, as for example an increase in glutathione levels, were obscured by renal toxicity (59) and premature death. This postulate is explored further in our report describing studies of the effects of diet on 5-fluorouracil toxicity (60).

LEGENDS

Figure 1. Growth of Fischer 344 rats fed a cereal-based diet (CR), or a purified diet of differing folate content: folate deficient (FD), folate replete (FR), or high folate (HF). There were no significant differences in either the rate of growth or of weights at 6, 7 and 8 weeks for rats fed the different diets.

Figure 2. Liver microsome cytochrome P450 levels (CYP) in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content: folate deficient (FD), folate replete (FR), or high folate (HF). The rats were treated with either 50, 110 or 190 mg/kg

cyclophosphamide after 6 weeks on the diets. The error bars represent standard deviations. There was no significant difference among the groups by ANOVA.

Figure 3. Hepatic aldehyde dehydrogenase activities in rats fed a cereal-based diet (CR), or a purified diet of differing folate content: folate deficient (FD), folate replete (FR), or high folate (HF). The error bars represent standard deviations. There was no significant difference among the groups by ANOVA.

Figure 4. Hepatic total glutathione levels in rats fed a purified diet of differing folate content (low folate, folate replete, or high folate) for 6 weeks and then treated with a single injection of cyclophosphamide in the doses indicated. Glutathione levels were measured 2 weeks later in surviving animals. The differences were significant in only the saline-treated group, and there was no dose-diet interaction.

Figure 5. Hepatic total glutathione levels in rats fed a cereal-based diet or a purified diet with adequate folate content (folate replete). After 6 weeks, the rats were treated with a single injection of cyclophosphamide in the doses indicated. Glutathione levels were measured 2 weeks later in surviving animals. The differences were significant in the saline-treated and 50 mg/kg cyclophosphamide-treated groups. There was no dose-diet interaction.

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Table 1: Fischer 344 rat weights measured 6, 7 and 8 weeks after maintenance on a cereal-based diet (CR) or a purifed diet of varying folate content (folate deficient, FD; folate replete, FR; or high folate, HF). Cyclophosphamide was injected in week 6 in the doses indicated.

Dose	Diet	Week 6	n	Week 7	n	Week 8	n
0 mg/kg	CR	133.91 ± 5.91	6	138.95 ± 5.23	6	141.33 ± 6.88	6
	FD	139.07 ± 7.60	6	141.19 ± 9.51	6	144.04 ± 10.07	6
	FR	143.38 ± 6.68	6	146.88 ± 5.86	6	147.08 ± 7.11	6
	HF	140.10 ± 7.61	7	146.70 ± 5.58	6	148.44 ± 7.76	6
50 mg/kg	CR	138.49 ± 6.12	6	140.15 ± 4.91	6	144.43 ± 7.52	6
	FD	152.10 ± 11.49	6	147.67 ± 12.00	6	152.99 ± 11.00	6
	FR	140.68 ± 9.20	6	143.63 ± 10.26	6	142.77 ± 10.66	6
	HF	133.95 ± 8.74	6	132.17 ± 8.36	6	136.83 ± 11.74	5
65 mg/kg	CR	143.69 ± 7.51	6	141.28 ± 3.50	6	150.89 ± 6.96	6
	FD	136.19 ± 7.09	6	135.73 ± 8.08	6	141.17 ± 6.06	6
	FR	138.75 ± 8.17	6	133.75 ± 6.43	6	140.80 ± 6.64	6
	HF	137.70 ± 6.78	7	131.56 ± 7.68	7	138.40 ± 11.65	7
85 mg/kg	CR	141.14 ± 7.93	6	133.36 ± 9.30	6	149.80 ± 7.63	6
	FD	138.49 ± 10.97	6	125.06 ± 11.56	6	137.97 ± 11.15	6
	FR	136.24 ± 8.72	6	128.74 ± 11.15	6	138.17 ± 6.77	5
	HF	138.11 ± 8.69	7	126.51 ± 10.82	7	141.39 ± 9.22	7
110 mg/kg	CR	146.04 ± 3.78	6	126.49 ± 8.38	6	144.50 ± 5.65	6
	FD	146.14 ± 7.13	6	124.49 ± 6.63	6	100.06 ± 29.36	3
	FR	151.09 ± 10.90	6	132.01 ± 7.76	6	143.14 ± 10.89	5
	HF	147.15 ± 6.77	6	137.13 ± 10.19	5	147.50 ± 6.45	3
144 mg/kg	CR	144.93 ± 7.77	6	123.93 ± 8.04	6	141.32 ± 14.11	6
	FD	147.23 ± 8.73	6	132.13 ± 8.61	5	138.34 ± 15.29	4
	FR	147.93 ± 6.86	6	130.90 ± 1.72	5	140.67 ± 5.94	4
	HF	147.60 ± 5.94	7	128.48 ± 5.32	6	134.16 ± 12.72	3
190 mg/kg	CR	140.64 ± 2.98	6	125.52 ± 10.37	6	141.37 ± 5.46	5
	FD	140.80 ± 8.94	6	125.38 ± 5.52	5	128.50 ± 8.35	2
	FR	138.14 ± 6.11	6	119.93 ± 12.95	6	131.64 ± 1.585	2
	HF	139.00 ± 7.65	7	121.24 ± 6.37	6	136.31 ± 0.0	1
250 mg/kg	CR	135.11 ± 12.38	6	114.01 ± 8.76	5	113.26 ± 18.02	2
	FD	141.00 ± 9.74	6	119.63 ± 9.40	5	-	0
	FR	139.23 ± 9.09	6	116.83 ± 8.79	5	-	0
	HF	142.70 ± 7.36	7	119.50 ± 14.26	4	147.71 ± 0.0	1

Table 2: Deaths in rats maintained on either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR; or high folate, HF) and treated with cyclophosphamide in the dose indicated.

	Diet							
Dose	**CR**	n[1]	**FD**	n[1]	**FR**	n[1]	**HF**	n[1]
50 mg/kg	0	6	0	6	0	6	1	6
65 mg/kg	0	6	0	6	0	6	0	7
85 mg/kg	0	6	0	6	1	6	0	7
110 mg/kg	0	6	3	6	1	6	3	6
144 mg/kg	0	6	2	6	2	6	4	7
190 mg/kg	1	6	4	6	4	6	6	7
250 mg/kg	4	6	6	6	6	6	6	7

[1]: n = number of animals

Table 3: Hematocrits measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with cyclophosphamide in the indicated doses. Meaurements were made before injection and 4, 9 and 14 days later.

Dose	Diet	Initial	n	Day 4	n	Day 9	n	Day 14	n
0 mg/kg	CR	48.9 ± 3.2	6	46.8 ± 1.2	6	43.6 ± 2.2	6	42.2 ± 2.7	6
	FD	52.0 ± 2.6	6	49.0 ± 3.4	6	44.3 ± 2.9	6	42.9 ± 1.6	6
	FR	52.4 ± 2.0	6	50.0 ± 2.0	6	43.4 ± 2.0	6	40.2 ± 1.3	6
	HF	49.3 ± 4.7	7	50.2 ± 1.2	6	42.6 ± 2.6	6	37.6 ± 7.4	6
50 mg/kg	CR	na[1]	6	52.3 ± 4.3	6	39.8 ± 1.3	6	38.9 ± 3.6	6
	FD	na[1]	6	46.4 ± 5.6	6	41.7 ± 2.4	6	41.3 ± 0.8	6
	FR	na[1]	6	46.4 ± 1.7	6	39.1 ± 3.1	6	40.3 ± 4.5	6
	HF	na[1]	6	45.6 ± 1.3	5	40.2 ± 2.3	5	42.7 ± 2.2	5
65 mg/kg	CR	49.5 ± 1.8	6	43.9 ± 0.7	6	34.8 ± 3.1	6	42.5 ± 1.1	6
	FD	50.1 ± 1.6	6	42.3 ± 2.0	6	37.0 ± 1.7	6	41.4 ± 1.4	6
	FR	50.8 ± 1.9	6	44.2 ± 3.1	6	36.4 ± 3.2	6	39.3 ± 6.4	6
	HF	50.5 ± 1.2	7	40.8 ± 2.2	7	34.6 ± 3.2	7	41.8 ± 1.2	7
85 mg/kg	CR	50.3 ± 1.3	6	43.8 ± 3.5	6	34.1 ± 3.2	6	44.7 ± 2.3	6
	FD	49.5 ± 1.6	6	41.6 ± 3.2	6	30.8 ± 2.2	6	41.3 ± 1.5	6
	FR	49.4 ± 1.1	6	41.7 ± 2.7	6	27.9 ± 4.8	5	41.3 ± 0.9	5
	HF	49.7 ± 1.2	7	41.1 ± 2.9	7	27.5 ± 5.3	7	40.9 ± 2.1	7
110 mg/kg	CR	51.3 ± 1.8	6	41.2 ± 1.7	6	31.8 ± 2.9	6	38.6 ± 1.7	6
	FD	51.8 ± 1.5	6	42.1 ± 1.9	6	32.1 ± 1.9	4	32.8 ± 4.3	3
	FR	50.5 ± 2.3	6	42.4 ± 2.7	6	27.9 ± 4.6	5	38.9 ± 2.6	5
	HF	48.6 ± 1.1	6	39.5 ± 2.9	6	33.3 ± 5.1	3	42.7 ± 2.5	3
144 mg/kg	CR	49.8 ± 1.5	6	38.8 ± 3.9	6	26.3 ± 2.4	6	39.6 ± 1.4	6
	FD	49.3 ± 1.5	6	40.1 ± 1.1	6	24.9 ± 4.3	4	36.6 ± 4.9	4
	FR	51.5 ± 2.4	6	39.8 ± 0.9	6	21.9 ± 5.9	5	39.6 ± 2.2	4
	HF	49.1 ± 2.0	7	41.1 ± 3.3	7	18.8 ± 10.8	4	31.0 ± 9.4	3
190 mg/kg	CR	48.5 ± 0.5	6	38.7 ± 1.9	6	31.7 ± 2.7	5	41.1 ± 1.0	5
	FD	50.3 ± 1.5	6	37.2 ± 1.2	6	27.2 ± 6.0	3	38.5 ± 1.4	2
	FR	49.8 ± 1.3	6	37.8 ± 5.9	6	28.7 ± 4.2	3	40.0 ± 0.0	2
	HF	48.4 ± 1.6	7	38.1 ± 5.9	7	29.0 ± 0.0	2	41.0 ± 0.0	1
250 mg/kg	CR	51.1 ± 1.2	6	40.4 ± 0.9	6	21.8 ± 7.5	3	22.8 ± 3.2	2
	FD	51.1 ± 1.6	6	41.8 ± 2.7	6	8.0 ± 0.0	1	-	0
	FR	51.1 ± 2.4	6	40.5 ± 3.2	6	-	0	-	0
	HF	50.2 ± 2.3	7	36.6 ± 3.1	7	32.0 ± 0.0	1	41.0 ± 0.0	1

[1]: na = not available

Table 4: White blood cell counts measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with cyclophosphamide in the indicated doses. Meaurements were made before injection and 4, 9 and 14 days later.

Dose	Diet	Initial	n	Day 4	n	Day 9	n	Day 14	n
0 mg/kg	CR	13232 ± 1615	6	10881 ± 2041	6	12707 ± 3417	6	3267 ± 1422	6
	FD	11232 ± 2376	6	8673 ± 3047	6	11133 ± 3112	6	2061 ± 394	6
	FR	10383 ± 1033	6	10792 ± 1858	6	10224 ± 2125	6	3281 ± 1838	6
	HF	13576 ± 9363	7	10424 ± 2049	6	17420 ± 12755	6	2599 ± 1046	6
50 mg/kg	CR	na[1]	6	2882 ± 525	6	7954 ± 1125	6	4486 ± 1512	6
	FD	na[1]	6	3185 ± 1195	6	11820 ± 4218	6	4326 ± 3578	6
	FR	na[1]	6	2736 ± 319	6	10240 ± 3248	6	2948 ± 1317	6
	HF	na[1]	6	3237 ± 798	5	18311 ± 10366	5	4165 ± 3269	5
65 mg/kg	CR	9020 ± 1462	6	2811 ± 1884	6	34844 ± 18957	6	4605 ± 4754	6
	FD	8035 ± 1908	6	1865 ± 308	6	13366 ± 1486	6	3099 ± 1791	6
	FR	8110 ± 977	6	1996 ± 348	6	17867 ± 3897	6	2762 ± 1115	6
	HF	8417 ± 798	7	1866 ± 518	7	25357 ± 17287	7	5933 ± 3421	7
85 mg/kg	CR	10335 ± 1191	6	1348 ± 215	6	17728 ± 5792	6	6524 ± 5315	6
	FD	11092 ± 2088	6	1757 ± 606	6	24452 ± 9240	6	31893 ± 32815	6
	FR	9739 ± 2113	6	1436 ± 383	6	23340 ± 8708	5	11519 ± 6583	5
	HF	9578 ± 1627	7	1631 ± 275	7	35695 ± 17714	7	23369 ± 23649	7
110 mg/kg	CR	10795 ± 2207	6	1971 ± 803	6	27868 ± 14851	6	28152 ± 24739	6
	FD	10417 ± 1269	6	1360 ± 256	6	20603 ± 9919	4	52699 ± 13250	3
	FR	11389 ± 1120	6	1913 ± 622	6	25713 ± 4983	5	37842 ± 48270	5
	HF	10402 ± 1265	6	1645 ± 538	6	17148 ± 6958	3	16905 ± 22012	3
144 mg/kg	CR	12361 ± 2426	6	1521 ± 474	6	21814 ± 7933	6	55703 ± 30651	6
	FD	10416 ± 1898	6	1405 ± 641	6	11388 ± 6560	4	86574 ± 61079	4
	FR	10109 ± 709	6	1117 ± 724	6	17835 ± 5345	5	62601 ± 44893	4
	HF	10702 ± 1514	7	969 ± 221	7	14342 ± 12852	4	84468 ± 38990	3
190 mg/kg	CR	11060 ± 3083	6	1410 ± 600	6	14208 ± 7608	5	24396 ± 27453	5
	FD	11176 ± 2177	6	1368 ± 194	6	9365 ± 2224	3	15336 ± 9912	2
	FR	11283 ± 1605	6	1349 ± 294	6	19233 ± 6601	3	42417 ± 48280	2
	HF	11927 ± 1067	7	1166 ± 242	7	21655 ± 13993	2	9658 ± 0.0	1
250 mg/kg	CR	11588 ± 2000	6	948 ± 357	6	2661 ± 579	3	88114 ± 49022	2
	FD	9825 ± 1774	6	931 ± 431	6	1790 ± 0.0	1	-	0
	FR	9228 ± 1168	6	943 ± 217	6	-	0	-	0
	HF	9452 ± 1713	7	866 ± 473	7	32079 ± 0.0	1	7853 ± 0.0	1

[1]: na = not available

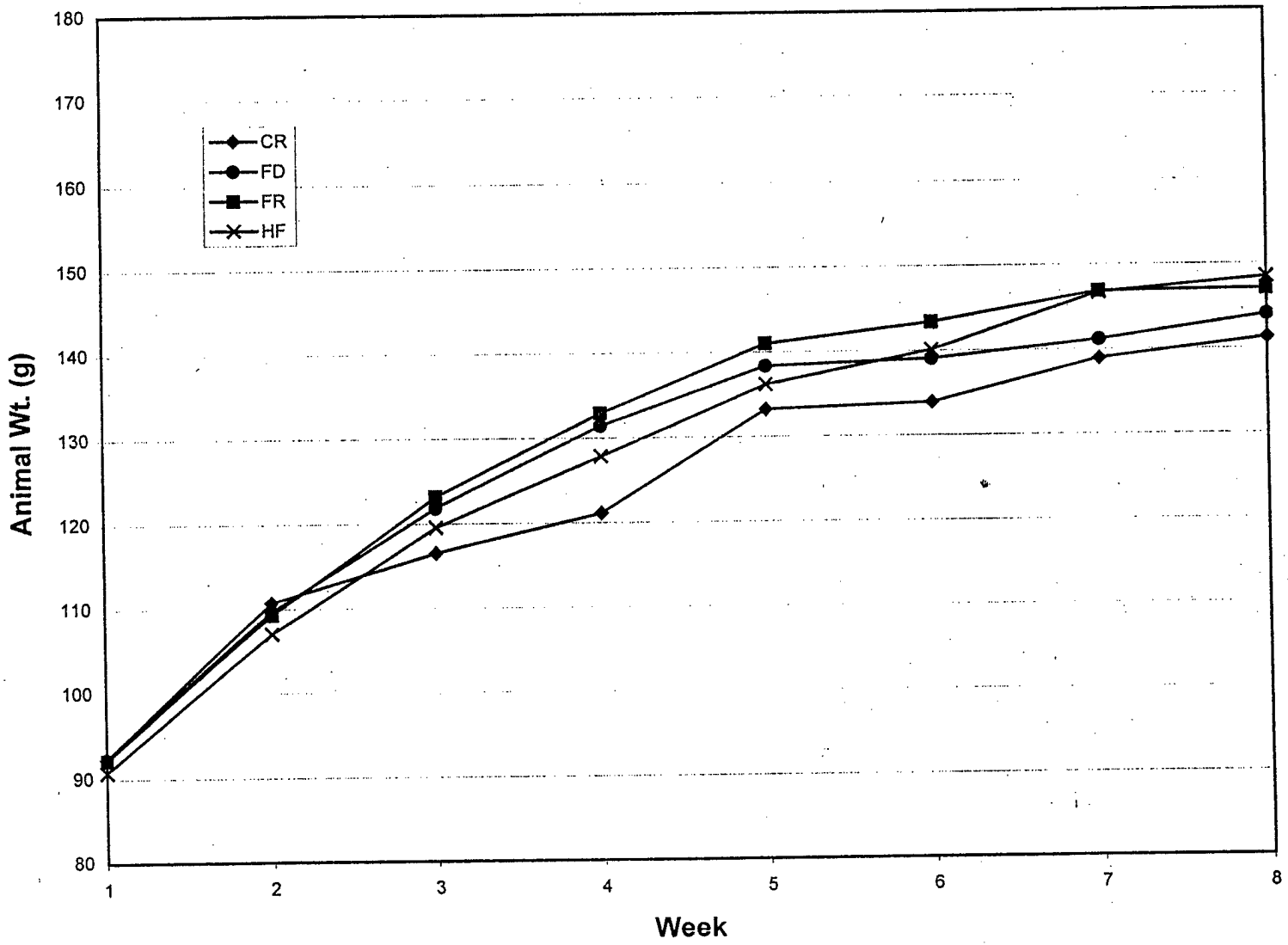
Table 5: BUN measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with cyclophosphamide in the indicated doses. Meaurements were made before injection and 4, 9 and 14 days later.

Dose	Diet	Initial	n	Day 4	n	Day 9	n	Day 14	n
0 mg/kg	CR	16.4 ± 2.6	6	13.6 ± 0.8	6	14.9 ± 3.1	6	15.7 ± 1.0	6
	FD	14.6 ± 3.6	6	14.2 ± 3.0	6	16.4 ± 5.0	6	15.5 ± 1.8	6
	FR	14.0 ± 2.6	6	15.1 ± 6.3	6	14.5 ± 2.9	6	15.4 ± 2.2	6
	HF	16.4 ± 3.4	7	15.0 ± 1.8	6	16.3 ± 4.1	6	26.6 ± 15.4	6
50 mg/kg	CR	na[1]	6	12.9 ± 1.1	6	14.0 ± 2.7	6	20.8 ± 1.8	6
	FD	na[1]	6	13.5 ± 1.3	6	11.3 ± 1.7	6	18.5 ± 1.9	6
	FR	na[1]	6	13.4 ± 1.2	6	13.0 ± 1.9	6	20.4 ± 2.2	6
	HF	na[1]	6	13.6 ± 3.9	5	13.2 ± 0.7	5	20.9 ± 2.8	5
65 mg/kg	CR	13.5 ± 1.5	6	11.5 ± 1.5	6	12.4 ± 1.8	6	19.4 ± 1.9	6
	FD	14.1 ± 1.7	6	11.3 ± 1.0	6	12.6 ± 2.3	6	19.3 ± 2.1	6
	FR	13.3 ± 3.8	6	12.3 ± 1.9	6	11.9 ± 2.4	6	16.6 ± 2.9	6
	HF	13.4 ± 1.6	7	12.7 ± 2.6	7	15.6 ± 2.9	7	24.8 ± 4.7	7
85 mg/kg	CR	26.3 ± 17.6	6	15.9 ± 3.6	6	14.3 ± 2.8	6	23.1 ± 2.7	6
	FD	15.0 ± 2.9	6	15.8 ± 9.1	6	9.3 ± 3.1	6	16.7 ± 3.2	6
	FR	14.0 ± 3.0	6	12.0 ± 3.0	6	12.7 ± 3.5	5	18.4 ± 2.1	5
	HF	20.3 ± 5.7	7	12.3 ± 1.6	7	20.6 ± 12.9	7	23.0 ± 5.1	7
110 mg/kg	CR	15.8 ± 4.1	6	20.7 ± 6.5	6	11.8 ± 1.4	6	21.2 ± 3.7	6
	FD	15.4 ± 3.5	6	18.0 ± 11.4	6	26.5 ± 23.6	4	36.3 ± 23.8	3
	FR	14.3 ± 1.3	6	15.3 ± 1.6	6	9.9 ± 4.1	5	16.1 ± 2.8	5
	HF	20.8 ± 6.3	6	22.3 ± 4.8	6	17.0 ± 1.3	3	24.9 ± 0.5	3
144 mg/kg	CR	15.0 ± 3.1	6	20.5 ± 19.9	6	19.2 ± 20.6	6	14.9 ± 2.4	6
	FD	16.5 ± 3.4	6	18.5 ± 19.7	6	22.2 ± 23.6	4	13.9 ± 2.3	4
	FR	15.6 ± 3.2	6	16.2 ± 15.2	6	13.1 ± 7.4	5	14.8 ± 2.1	4
	HF	15.8 ± 2.9	7	22.7 ± 10.1	7	31.2 ± 20.8	4	35.9 ± 23.2	3
190 mg/kg	CR	22.0 ± 8.2	6	20.2 ± 10.3	6	22.4 ± 12.4	5	16.7 ± 3.1	5
	FD	16.2 ± 5.5	6	21.0 ± 18.5	6	16.4 ± 13.4	3	17.3 ± 5.7	2
	FR	27.5 ± 17.0	6	27.0 ± 23.6	6	20.8 ± 17.1	3	13.3 ± 1.3	2
	HF	34.4 ± 13.0	7	16.1 ± 4.3	7	20.6 ± 5.7	2	35.5 ± 0.0	1
250 mg/kg	CR	16.4 ± 4.9	6	39.0 ± 20.9	6	33.9 ± 19.9	3	15.8 ± 3.5	2
	FD	16.0 ± 1.9	6	31.6 ± 23.6	6	38.7 ± 0.0	1	-	0
	FR	16.5 ± 5.0	6	16.3 ± 8.6	6	-	0	-	0
	HF	25.4 ± 10.1	7	27.5 ± 18.5	7	15.0 ± 0.0	1	15.5 ± 0.0	1

[1]: na = not available

Table 6: LDH, SGPT & CPK measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with cyclophosphamide in the indicated doses.

Dose	Diet	LDH	n	SGPT	n	CPK	n
0 mg/kg	CR	799.8 ± 118.0	6	19.4 ± 1.7	6	33.5 ± 10.3	6
	FD	679.2 ± 197.1	6	13.2 ± 1.5	6	29.9 ± 12.8	6
	FR	697.9 ± 149.4	6	12.9 ± 1.2	6	31.4 ± 9.1	6
	HF	514.5 ± 192.9	6	12.6 ± 1.3	6	22.2 ± 21.0	6
50 mg/kg	CR	942.9 ± 67.4	6	19.0 ± 7.2	6	44.4 ± 8.6	6
	FD	916.0 ± 60.2	6	13.2 ± 4.1	6	38.3 ± 7.5	6
	FR	834.5 ± 106.3	6	14.2 ± 3.5	6	35.1 ± 6.1	6
	HF	862.8 ± 79.1	5	11.5 ± 0.9	5	37.9 ± 3.9	5
65 mg/kg	CR	964.8 ± 21.0	6	26.4 ± 7.7	6	63.9 ± 12.7	6
	FD	932.2 ± 35.6	6	15.3 ± 4.7	6	48.6 ± 7.3	6
	FR	883.5 ± 68.7	6	18.3 ± 5.8	6	37.6 ± 8.5	6
	HF	860.8 ± 91.4	7	13.2 ± 2.2	7	38.8 ± 8.0	7
85 mg/kg	CR	821.2 ± 138.6	6	19.8 ± 4.2	6	55.8 ± 16.2	6
	FD	815.8 ± 91.1	6	12.3 ± 0.6	6	49.0 ± 14.4	6
	FR	702.8 ± 32.8	5	13.8 ± 3.0	5	37.1 ± 2.7	5
	HF	783.6 ± 124.2	7	12.6 ± 1.1	7	41.6 ± 11.2	7
110 mg/kg	CR	819.2 ± 110.5	6	14.7 ± 1.0	6	44.8 ± 11.3	6
	FD	766.0 ± 21.8	3	11.3 ± 0.4	3	45.0 ± 4.2	3
	FR	579.0 ± 156.3	5	10.3 ± 1.6	5	37.8 ± 7.3	5
	HF	682.4 ± 244.8	3	12.3 ± 1.2	3	42.7 ± 19.0	3
144 mg/kg	CR	695.9 ± 269.3	6	13.7 ± 1.8	6	37.1 ± 20.7	6
	FD	505.0 ± 131.3	4	11.0 ± 0.1	4	24.9 ± 3.7	4
	FR	579.9 ± 210.5	4	10.9 ± 2.1	4	30.0 ± 8.3	4
	HF	562.2 ± 249.8	3	32.1 ± 38.6	3	30.0 ± 13.5	3
190 mg/kg	CR	647.8 ± 105.2	5	20.0 ± 2.2	5	35.8 ± 9.2	5
	FD	601.4 ± 147.6	2	14.6 ± 2.0	2	32.0 ± 0.0	2
	FR	354.2 ± 97.7	2	12.8 ± 1.0	2	21.5 ± 3.5	2
	HF	905.8 ± 0.0	1	9.6 ± 0.0	1	83.0 ± 0.0	1
250 mg/kg	CR	252.9 ± 81.8	2	13.0 ± 0.3	2	4.5 ± 3.5	2
	FD	-	0	-	0	-	0
	FR	-	0	-	0	-	0
	HF	477.6 ± 0.0	1	12.1 ± 0.0	1	20.0 ± 0.0	1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170
160
150
140
130
120
110
100
90
80
Animal Wt. (g)
CR
FD
FR
HF
1
2
3
4
5
6
7
8
Week

Figure 1

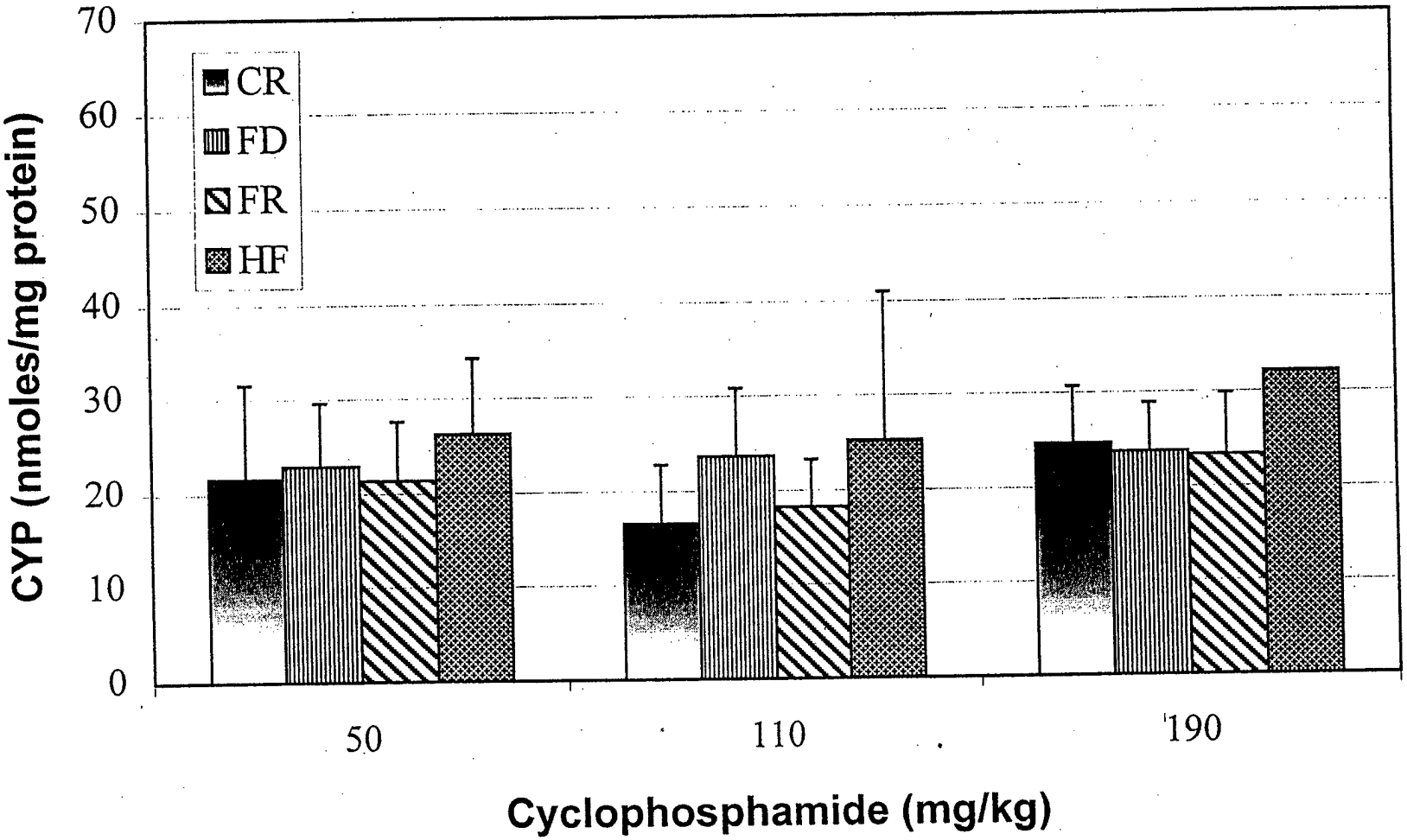
70
60
50
40
30
20
10
0
CR
FD
FR
HF
CYP (nmoles/mg protein)
50
110
190
Cyclophosphamide (mg/kg)

Figure 2

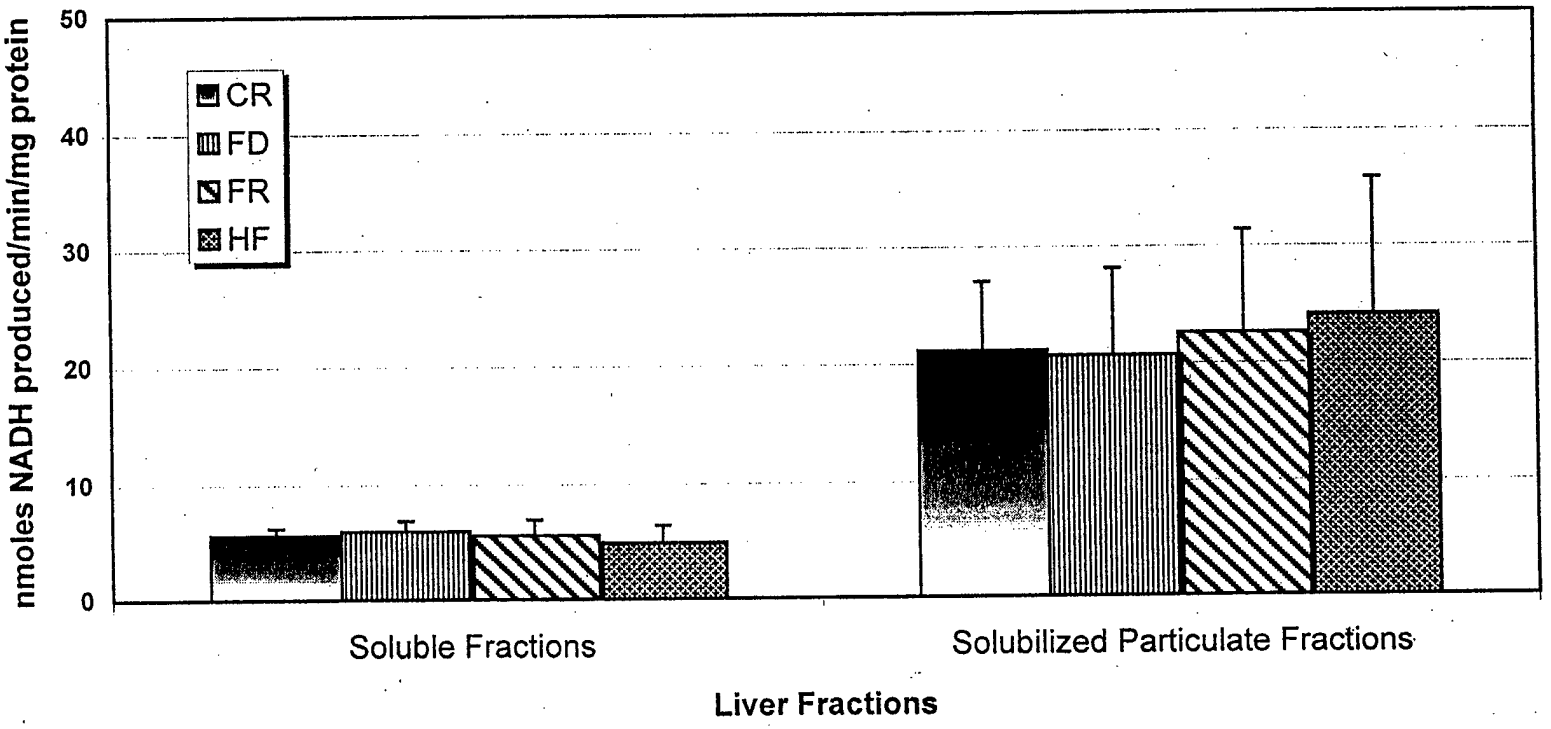
nmoles NADH produced/min/mg protein
50
40
30
20
10
0
CR
FD
FR
HF
Soluble Fractions
Solubilized Particulate Fractions
Liver Fractions

Figure 3

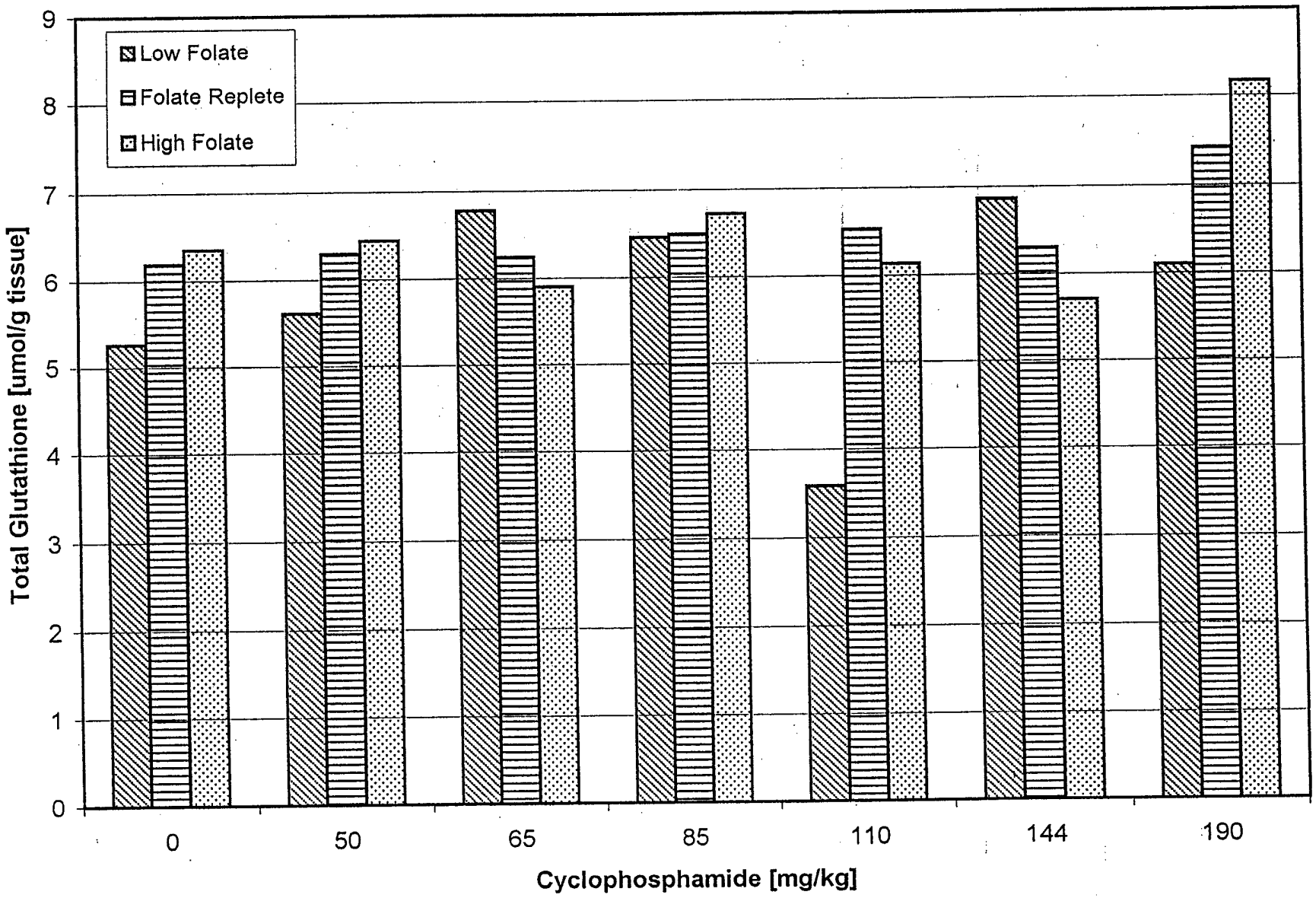
Low Folate
Folate Replete
High Folate
Total Glutathione [umol/g tissue]
0
1
2
3
4
5
6
7
8
9
0
50
65
85
110
144
190
Cyclophosphamide [mg/kg]

Figure 4

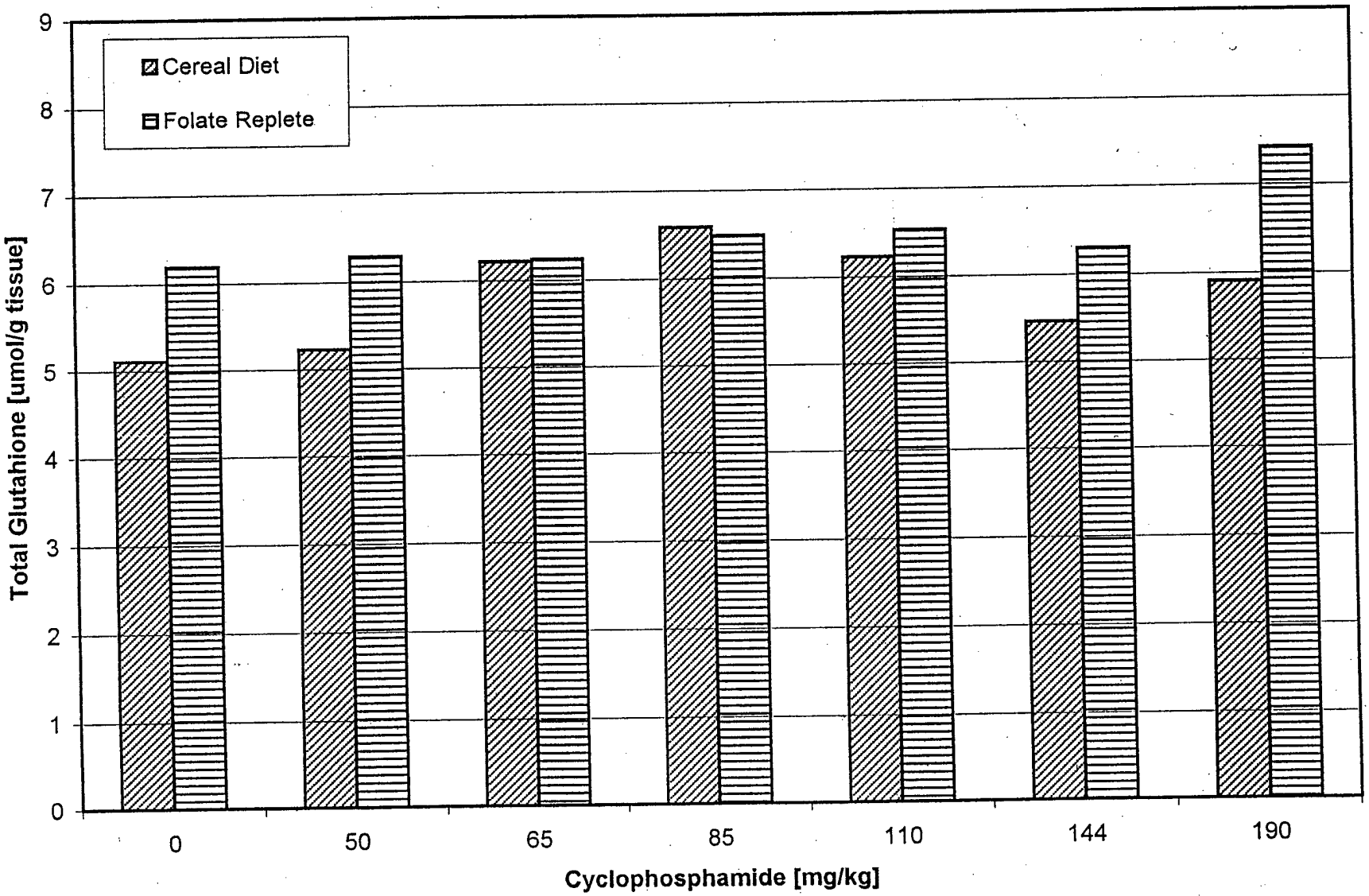
Cereal Diet
Folate Replete
Total Glutahione [umol/g tissue]
0
1
2
3
4
5
6
7
8
9
0
50
65
85
110
144
190
Cyclophosphamide [mg/kg]

Figure 5

DIET MODULATES THE TOXICITY OF CANCER CHEMOTHERAPY IN RATS. II. 5-FLUOROURACIL[1]

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[1]Supported by grants from the Department of Defense (DAMD17-98-1-8345) and the National Cancer Institute, Bethesda, MD (CA 41843 and P30-CA 22435). The content of the information does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred.

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Running Title: Diet and 5-Fluorouracil Toxicity

ABSTRACT

The effects of diet and nutritional folate status on 5-fluorouracil (5-FU) toxicity was studied in Fischer 344 rats maintained on either a cereal-based diet (Harlan Teklad LM-485) or a purified diet (AIN-93G). The rats fed the purified diet were randomly 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 folic acid intraperitoneally/kg body weight). After 6 weeks the weights among the diet groups were similar. However, the high folate group had higher BUN and lower hematocrit levels than the other 3 dietary groups prior to treatment with chemotherapy. In Week 6, 5-FU was injected intraperitoneally. By Week 8, the rats on the cereal-based diet had maintained their weights and hematocrits at higher levels, while the high folate rats had developed more severe anemia, azotemia and leukopenia, than the other groups. Survival analysis indicated that weight, white blood cell count, hematocrit and BUN were important predictors of death after 5-FU. Logistic regression analysis indicated that diet was related to death. The microscopic anatomy of the kidneys from saline-treated animals was normal in all dietary groups. After 5-FU treatment, the kidneys from rats fed the cereal-based diet also were histologically normal, but rats ingesting the purified diet had increasing renal pathology that correlated with folate intake. The rats in the high folate group had extensive acute tubular necrosis. These results indicate toxicity is modulated by an interaction of folate and 5-FU in rats. Folate supplementation appears to increase the myelotoxicity caused by 5-FU, while 5-FU exacerbates the renal damage associated with high levels of folic acid.

INTRODUCTION

5-Fluorouracil (5-FU) is an antimetabolite 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'deoxy-5' monophosphate (5dUMP) (1,2). Thymidylate synthase requires the folate compound 5,10-methylene tetrahydrofolate to act as the methyl donor (3). FdUMP forms a tight-binding covalent bond with thymidylate synthase in the presence of 5,10-methylene tetrahydrofolate (1-3). Although 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 (3). Consequently folate compounds have been used extensively in the clinic to modulate the cytotoxicity of 5-FU (3). For the most part leucovorin (5-formyltetrahydrofolate) has been used experimentally and clinically, but folic acid (pteroylglutamic acid) is also effective (4). Studies of the combination of 5-FU and leucovorin have shown a significant benefit over 5-FU alone for tumor response but no consistent survival advantage in patients with colorectal cancer (5). 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 (3,5,6). The dose-limiting toxicities with 5-FU are myelosuppression and gastrointestinal (1). 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 (7). Quality of life appears to be better with the combination, in that patients

treated with 5-FU and leucovorin had better performance status, greater weight gain, and were more likely to note an improvement of symptoms than patients receiving 5-FU alone (7).

While there is strong biochemical, pharmacologic and clinical evidence to support the idea that folate supplementation increases the cytotoxicity of 5-FU, the effects of folate nutritional status on its efficacy and toxicity are less well understood. Hoshiya and colleagues reported that dietary methionine depletion enhanced the antitumor activity of 5-FU by about 2-fold in mice (8). They postulated that methionine deprivation increased levels of methionine synthase and methionine synthesis within the tumor cells, resulting in a release of 5-methyltetrahydrofolate for production of 5,10-methylene tetrahydrofolate (8). Mice maintained on a folic acid-deplete diet had decreased hepatic 5,10-methylene tetrahydrofolate levels and were less responsive to 5-FU alone compared to folate replete animals (9). However, tumors in the dietary folate-deficient mice had a greater response to the combination of 5-FU and leucovorin than tumors in folate replete mice (9). These authors also noted that tumor growth stimulation occurred if 5-FU was given at times when folates were not elevated sufficiently (9). In a study of 95 patients with head and neck squamous cell carcinoma, response to 5-FU-based chemotherapy was correlated with the reduced-folate status of the tumors; thus, the distribution of 5,10-methylene tetrahydrofolate was significantly higher for complete responders compared to patients with a partial or no response (10). In contrast, Backus et al. found that folate depletion increased the sensitivity of solid tumor cell lines to 5-FU (11). Colon cancer and squamous cell carcinoma cell lines cultured in low folate medium had decreased levels of thymidylate synthase and reduced folate carrier, rendering them more sensitive to 5-FU (11). Our laboratory previously 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 (12). 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 (12). The current studies were performed to investigate further the relationship between diet, and particularly folate nutritional status, on the toxicity associated with 5-FU.

MATERIALS AND METHODS

Animals. The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Female Fischer 344 rats, 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 continued on the cereal-based diet, while the others were maintained on the AIN-93G diet (Dyets, Inc., Bethlehem, PA) (13). This purified diet is based upon vitamin-free casein and cornstarch. Folate-replete diets consisted of AIN-93G with a vitamin supplement that provided 2 mg 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, 10 mg/ml dissolved in 8.4% sodium bicarbonate solution. Blood samples were obtained from tail veins prior to the injection of

chemotherapy, and 4, 9 and 14 days after the chemotherapy treatment. At the 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 liver and kidney collected for subsequent analyses.

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 BUN, LDH, SGPT and CPK were performed using Sigma Diagnostics (St. Louis, MO) Procedures No. 66-UV, 500, 505 and 661, respectively.

5-Fluorouracil. A 50 mg/ml solution was used for IP injections. 5-FU was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA).

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 and weight at Week 6. Repeated measures ANOVA were used to examine gains in rat weights. When a significant F value was found, Fisher's least significant difference test was used to compare means. Logistic regression and survival analysis (SAS Proe LIFETEST & PHREG) were used to model predictors of mortality. Two variables, BUN and white blood cell count, were transformed using the natural logarithm.

RESULTS

The weight gain in rats on the four diets is illustrated in **Figures 1 and 2.** The animals grew at approximately the same rate during the first six weeks of the experiment, although analysis by repeated measures ANOVA indicated that for nearly all weeks the high folate group was the lightest and the cereal diet group was usually the heaviest. Among the rats that went on

to treatment with 5-FU, the maximum difference was relatively small, 4.6g in Week 3, and by Week 6 there were no significant differences in weights among the diet groups. However, there were significant differences among the dose groups ($p<0.0001$). The 110 mg/kg group weighed less on average than all of the other groups. The 325 mg/kg group weighed less than the other three groups (144, 420, and 526 mg/kg). The maximum weight difference was 13.5g (ANOVA).

The rats were injected IP with 5-FU after six weeks on the four diets. Their weights in Weeks 6, 7 and 8 are shown in **Table 1**. The weights in Week 7 did not differ among any of the diet groups after adjusting for baseline weight. At Week 8, the rats on the cereal-based diet were heavier than the other three groups, with a maximum difference of 15.1g, between cereal-based diet and high folate group (ANOVA, overall $p=0.015$). Figure 1 shows that rats treated with the lowest 5-FU dose, 110 mg/kg, had relatively stable weights during the two weeks following treatment, while rats treated with the highest dose, 546 mg/kg, lost weight during this period (Figure 2).

Measurements of hepatic folate levels showed that the mean folate levels of rats on the high folate diet were significantly higher than the other three groups, while the mean folate levels of rats on the low folate diet were significantly lower than the other three groups (14).

The 5-FU related deaths in animals on the four diets are shown in **Table 2.** Six or seven rats per group were treated 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. Inspection of the data suggests that at each 5-FU dose level, the high folate group had equal or greater mortality than

the other dietary groups, while the rats on the cereal-based and low folate diets tended to have fewer deaths than the folate replete and high folate groups.

Blood samples were obtained to measure bone marrow, renal and liver function and to detect evidence of cardiac damage following 5-FU therapy. Hematocrit, white blood cell count and BUN determinations obtained prior to 5-FU treatment in Week 6 and on Days 4, 9 and 14 following chemotherapy are shown in **Tables 3, 4 and 5.** 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 overall $p<0.0005$). There were no significant differences among the dietary groups for white blood cell count at pretreatment.

Repeated measures ANOVA indicated that diet showed significant interactions with time for all three post-treatment blood analyses (hematocrit, white blood cell count and BUN; all models had $p<0.01$). Analyses showed that the BUN was significantly higher on Days 9 and 14 in the high folate group compared to the other dietary groups, and that 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 on Day 14 was significantly higher in the cereal diet group than in rats on the high and folate replete diets. The white blood cell count changes after chemotherapy were somewhat variable. On Day 4, the high folate and folate replete dietary groups had lower values than the folate deficient group. At Day 9, the white blood cell count in the high folate group remained depressed, and was significantly lower than in the other three dietary groups. However, by Day 14, the folate deficient rats had significantly lower white blood cell counts than the cereal and folate replete groups. 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 $p<0.001$) **(Table 6)**.

Logistic regression revealed that diet, but neither dose nor baseline weight, was related to death ($p<0.0001$). 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.

Survival analysis with post-treatment data included Days 0, 4, and 9 blood measurements as time-varying covariates. That is, baseline measurements were used to predict deaths up to Day 4, Day 4 measurements used to predict deaths to Day 9, and Day 9 measurements used to predict deaths to Day 14. Weights at Week 6 and 7 were included as another time-varying variable; dose was also included in this model. Model fitting yielded two models with similar fit indices. The better model was: weight, white blood cell count and hematocrit. The other model included: weight, white blood cell count and BUN. However, BUN was not significant when the hematocrit was in the model.

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 in this study with 5-FU and in our prior study with cyclophosphamide (14), since renal toxicity is not a common feature of the toxicity profile of either drug (15,16). 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 (T.D.T.). None of 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 six 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. These changes are shown in **Figure 4.** The rats in the high folate group had extensive renal damage. All 7 animals showed acute tubular necrosis, which was marked in two cases **(Figure 5)**. Two rats also had focal tubular degeneration with regeneration, and one showed peri-renal inflammation with old hemorrhage and organized peri-renal thrombosis.

Purified diets have been reported to produce markedly acidic urine, with pH consistently below 6.0 (17). 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's (± SD) 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. The urinary pH of the rats maintained on the cereal-based diet was significantly higher than that of the rats fed the purified diets ($p=0.02$). There was no significant difference of urinary pH among the rats on purified diets of varying folate content. In a simple titration of a folic acid solution, 10 mg/ml in sodium bicarbonate (pH 8.4), the folic acid precipitated at pH 6.33. Therefore it appears possible that a higher urinary pH is at least a partial explanation for the protective effect of the cereal-based diet against renal damage.

DISCUSSION

These studies suggest that dietary differences have an important effect on the toxicity of 5-FU. Rats fed a cereal-based diet maintained their weights and hematocrits after chemotherapy better than rats ingesting a purified diet. Rats fed a purified diet supplemented with folic acid had lower hematocrit values and higher serum BUN levels prior to chemotherapy than animals in the other dietary groups. After drug treatment the anemia and azotemia became more pronounced, and the high folate animals had white blood cell counts lower during the first week post therapy than the other groups. Survival analysis indicated that weight, white blood cell count, hematocrit and BUN were important predictors of death after 5-FU. Not surprisingly, then, logistic regression analysis indicated that diet was related to death.

The observations reported here confirm the findings of Bounous and colleagues that rats ingesting a natural ingredient diet are protected against 5-FU toxicity compared to rats fed a defined-formula diet (18). 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 (18). 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 these as the protective agent (18,19). An alternative explanation may be provided by the work of Jackson et al., who found a marked decrease in dTTP pools in rats fed the purified diet, AIN-76A, compared to those fed NIH-31, a natural ingredient diet (20). An important part of the mechanism of action of 5-FU is inhibition of thymidylate synthase with resulting depletion of dTTP (5). Since there does not appear to be a role for salvage pathway involvement in resistance to 5-FU (21), 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 (3).

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 (16). 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 (22-24). 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 (25). High doses of folic acid have been associated with nephropathy in rats (26,27). 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 (27). 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 (27). The most prominent changes are related to tubular function, particularly concentrating ability, resulting in polyuria (27). Perhaps of greatest relevance to the present work, Klingler and colleagues 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 (27). Taken together, these

observations suggest the following sequence of events to explain the renal damage observed in our study caused by the combination of 5-FU and a relatively low dose of folic acid (50 mg/kg): 1) supplemental folic acid becomes less soluble at the acid pH associated with ingestion of the purified diet; 2) renal tubular damage occurs; 3) the combination of 5-FU and folic acid inhibit thymidine synthesis and thereby limit regenerative processes. Although speculative, this mechanism is plausible based upon the observations and is subject to further testing. Clearly the higher urinary pH is not the only explanation for the protective effect of the cereal-based diet compared to the purified diet, because some renal damage was also seen in the folate deficient rats.

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 (3,5,6). The possible relevance of these observations to patients with cancer is unclear. Certainly many patients ingest multiple nutritional supplements, often in large quantities. However, it seems unlikely that chronic use of folic acid supplementation by patients might lead to severe renal toxicity. Rats typically have serum folate levels in the range of 85 ng/ml, and this is approximately 8 to 10 times the level in humans (28). A patient would need to ingest about 5 mg/kg to be exposed to comparable levels of folic acid. Most vitamins contain 0.4 to 0.8 mg of folic acid, and the medicinal dose is 1 mg. Nevertheless, since some renal damage was seen with the dietary levels of folate usually found in standard rat chow, it would seem prudent for patients receiving

chemotherapy to avoid large chronic supplemental amounts of folic acid until further studies clarify this relationship.

While the studies reported here showing that folic acid supplementation enhanced the toxicity of 5-FU are consistent with other published reports (4), they do not support our previous observation that folic acid supplementation protected against 5-FU toxicity (12). The reason for this inconsistency is uncertain. In part, it may be due to a difference in drug schedule, in that the prior set of experiments was performed with multiple doses of 5-FU while the current experiments used single dose 5-FU. In addition, renal toxicity was more prominent in the current experiments than in the previous ones, and this may have obscured any possible beneficial effect from folate supplementation. Additional experimentation will be necessary to fully define the apparently complex relationship between folic acid dietary supplementation and chemotherapy toxicity.

LEGENDS

Figure 1. Growth of Fischer 344 rats fed a cereal-based diet (CR), or a purified diet of differing folate content: folate deficient (FD), folate replete (FR) or high folate (HF). The rats were treated with 5-FU, 110 mg/kg, intraperitoneally, in Week 6.

Figure 2. Growth of Fischer 344 rats fed a cereal-based diet (CR), or a purified diet of differing folate content: folate deficient (FD), folate replete (FR) or high folate (HF). The rats were treated with 5-FU, 546 mg/kg, intraperitoneally, in Week 6.

Figure 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.

Figure 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 x 500.

Figure 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 x 500.

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Table 1: Fischer 344 rat weights measured 6, 7 and 8 weeks after maintenance on a cereal-based diet (CR) or a purifed diet of varying folate content (folate deficient, FD; folate replete, FR; or high folate, HF). 5-FU was injected in week 6 in the doses indicated.

Dose	Diet	Week 6	n	Week 7	n	Week 8	n
0 mg/kg	CR	133.91 ± 5.91	6	138.95 ± 5.23	6	141.33 ± 6.88	6
	FD	139.07 ± 7.60	6	141.19 ± 9.51	6	144.04 ± 10.07	6
	FR	143.38 ± 6.68	6	146.88 ± 5.86	6	147.08 ± 7.11	6
	HF	140.10 ± 7.61	7	146.70 ± 5.58	6	148.44 ± 7.76	6
110 mg/kg	CR	134.69 ± 6.45	6	135.43 ± 6.44	6	141.59 ± 8.14	6
	FD	134.35 ± 3.37	6	136.99 ± 4.17	6	142.16 ± 5.51	6
	FR	134.88 ± 6.81	6	136.06 ± 6.96	6	129.15 ± 26.58	5
	HF	126.30 ± 9.83	7	130.35 ± 11.28	6	142.88 ± 8.16	3
144 mg/kg	CR	149.11 ± 9.82	6	148.03 ± 12.52	6	148.69 ± 25.63	5
	FD	143.28 ± 7.01	6	136.44 ± 4.57	6	138.50 ± 6.68	5
	FR	145.81 ± 5.72	6	139.71 ± 6.28	6	140.09 ± 9.06	5
	HF	141.39 ± 5.86	7	136.80 ± 8.41	6	142.37 ± 7.23	4
325 mg/kg	CR	139.21 ± 5.41	12	133.79 ± 8.00	12	146.47 ± 3.22	10
	FD	133.93 ± 13.93	12	130.24 ± 8.13	12	129.66 ± 17.71	11
	FR	140.49 ± 7.69	12	134.48 ± 5.76	12	137.59 ± 13.28	10
	HF	137.45 ± 6.52	13	132.62 ± 7.11	13	123.38 ± 17.40	11
420 mg/kg	CR	145.75 ± 7.62	6	133.98 ± 13.00	6	140.69 ± 13.98	6
	FD	142.97 ± 8.30	6	130.24 ± 10.17	6	138.16 ± 12.65	6
	FR	142.73 ± 10.27	6	127.75 ± 13.03	6	127.73 ± 21.59	5
	HF	145.71 ± 3.10	6	133.90 ± 8.28	6	123.90 ± 0.00	1
546 mg/kg	CR	144.67 ± 4.10	6	132.70 ± 7.29	6	144.66 ± 11.70	6
	FD	146.75 ± 6.16	6	131.90 ± 9.04	6	138.07 ± 10.56	5
	FR	146.24 ± 9.27	6	128.31 ± 11.53	5	138.81 ± 13.96	3
	HF	145.69 ± 8.24	7	132.30 ± 7.03	7	110.31 ± 0.00	1

Table 2: Deaths in rats maintained on either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR; or high folate, HF) and treated with 5-FU in the dose indicated.

	Diet							
Dose	**CR**	n[1]	**FD**	n[1]	**FR**	n[1]	**HF**	n[1]
110 mg/kg	0	6	0	6	1	6	4	7
144 mg/kg	1	6	1	6	1	6	3	7
325 mg/kg	2	12	1	12	2	12	2	13
420 mg/kg	1	6	0	6	1	6	5	6
546 mg/kg	0	6	1	6	3	6	6	7

[1]: n = number of animals

Table 4: White blood cell counts measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with 5-FU in the indicated doses. Meaurements were made before injection and 4, 9 and 14 days later.

Dose	Diet	Initial	n	Day 4	n	Day 9	n	Day 14	n
0 mg/kg	CR	13232 ± 1615	6	10881 ± 2041	6	12707 ± 3417	6	3267 ± 1422	6
	FD	11232 ± 2376	6	8673 ± 3047	6	11133 ± 3112	6	2061 ± 394	6
	FR	10383 ± 1033	6	10792 ± 1858	6	10224 ± 2125	6	3281 ± 1838	6
	HF	13576 ± 9363	7	10424 ± 2049	6	17420 ± 12755	6	2599 ± 1046	6
110 mg/kg	CR	12420 ± 1558	6	6805 ± 1707	6	10213 ± 5236	6	43355 ± 21611	6
	FD	9457 ± 1868	6	12678 ± 12514	6	9216 ± 3258	6	15123 ± 17022	6
	FR	9342 ± 1977	6	5085 ± 1870	6	5227 ± 2053	5	56603 ± 35423	5
	HF	12464 ± 3904	7	4947 ± 1054	7	7196 ± 3801	4	72690 ± 24891	3
144 mg/kg	CR	11426 ± 2447	6	8000 ± 3570	6	8573 ± 4650	6	38617 ± 37444	5
	FD	10018 ± 2418	6	5625 ± 1527	6	5156 ± 1015	6	56625 ± 15954	5
	FR	11947 ± 3798	6	5276 ± 391	6	4963 ± 1612	6	91840 ± 14154	5
	HF	12581 ± 2535	7	5605 ± 1178	7	6453 ± 2541	6	78157 ± 25619	4
325 mg/kg	CR	10550 ± 2458	12	6471 ± 2632	12	5104 ± 2919	11	26853 ± 11900	10
	FD	12411 ± 9844	12	7940 ± 3307	12	3343 ± 1891	11	19969 ± 8061	11
	FR	9861 ± 2825	12	6733 ± 1535	11	5813 ± 5737	11	25814 ± 15726	10
	HF	11034 ± 6373	13	5381 ± 1271	13	2402 ± 1138	13	30039 ± 27704	11
420 mg/kg	CR	11408 ± 865	6	5432 ± 2578	6	5133 ± 3802	6	39208 ± 12358	6
	FD	11726 ± 1373	6	7436 ± 1548	6	5936 ± 6351	6	12270 ± 6258	6
	FR	10613 ± 747	6	5196 ± 1719	6	4168 ± 2331	5	22852 ± 24237	5
	HF	10085 ± 2605	6	4543 ± 1103	6	2949 ± 1314	6	12885 ± 0	1
546 mg/kg	CR	9713 ± 1819	6	5662 ± 1936	6	3419 ± 2141	6	29598 ± 21896	6
	FD	7747 ± 1535	6	5369 ± 1975	6	2490 ± 2630	6	20339 ± 13703	5
	FR	7526 ± 991	6	4099 ± 883	5	1633 ± 854	4	19832 ± 7037	3
	HF	16012 ± 17367	7	3678 ± 469	7	874 ± 266	5	7311 ± 0	1

Table 5: BUN measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with 5-FU in the indicated doses. Meaurements were made before injection and 4, 9 and 14 days later.

Dose	Diet	Initial	n	Day 4	n	Day 9	n	Day 14	n
0 mg/kg	CR	16.4 ± 2.6	6	13.6 ± 0.8	6	14.9 ± 3.1	6	15.7 ± 1.0	6
	FD	14.6 ± 3.6	6	14.2 ± 3.0	6	16.4 ± 5.0	6	15.5 ± 1.8	6
	FR	14.0 ± 2.6	6	15.1 ± 6.3	6	14.5 ± 2.9	6	15.4 ± 2.2	6
	HF	16.4 ± 3.4	7	15.0 ± 1.8	6	16.3 ± 4.1	6	26.6 ± 15.4	6
110 mg/kg	CR	17.4 ± 9.4	6	12.7 ± 3.8	6	14.7 ± 2.7	6	19.1 ± 2.6	6
	FD	15.4 ± 1.9	6	12.1 ± 2.7	6	13.0 ± 2.5	6	19.2 ± 1.9	6
	FR	12.3 ± 4.7	6	21.6 ± 16.4	6	17.2 ± 9.6	5	22.9 ± 11.4	5
	HF	16.9 ± 2.9	7	16.8 ± 5.4	7	21.7 ± 6.4	4	25.8 ± 13.8	3
144 mg/kg	CR	18.3 ± 2.1	6	23.0 ± 12.1	6	17.0 ± 3.2	6	17.0 ± 2.2	5
	FD	16.2 ± 4.0	6	30.6 ± 20.7	6	18.3 ± 13.6	6	17.0 ± 4.6	5
	FR	14.9 ± 3.4	6	30.4 ± 17.8	6	21.8 ± 18.2	6	18.7 ± 0.9	5
	HF	25.3 ± 8.5	7	20.0 ± 5.5	7	24.6 ± 11.0	6	20.7 ± 4.8	4
325 mg/kg	CR	12.7 ± 3.2	12	25.7 ± 12.4	12	14.2 ± 1.8	11	14.5 ± 2.0	10
	FD	13.0 ± 1.7	12	26.3 ± 15.8	12	12.5 ± 2.8	11	16.4 ± 4.9	11
	FR	12.8 ± 2.5	12	19.5 ± 11.0	11	12.9 ± 3.4	11	16.0 ± 1.8	10
	HF	14.9 ± 2.9	13	18.7 ± 12.9	13	21.2 ± 11.6	13	29.2 ± 12.8	11
420 mg/kg	CR	18.1 ± 6.2	6	38.7 ± 17.2	6	19.9 ± 16.4	6	14.8 ± 2.4	6
	FD	15.0 ± 3.0	6	28.7 ± 18.1	6	14.5 ± 2.5	6	15.8 ± 2.0	6
	FR	17.1 ± 4.8	6	35.8 ± 18.4	6	13.9 ± 3.2	5	18.7 ± 6.4	5
	HF	21.5 ± 9.4	6	37.0 ± 20.0	6	31.9 ± 17.2	6	32.6 ± 0.0	1
546 mg/kg	CR	15.2 ± 2.4	6	32.6 ± 17.9	6	10.0 ± 1.1	6	14.9 ± 2.6	6
	FD	14.1 ± 1.8	6	20.1 ± 16.5	6	16.2 ± 18.4	6	15.0 ± 2.6	5
	FR	13.6 ± 0.9	6	30.0 ± 17.7	5	10.2 ± 1.5	4	15.2 ± 2.1	3
	HF	16.9 ± 2.4	7	15.3 ± 9.2	7	12.0 ± 1.2	5	39.9 ± 0.0	1

Table 6: LDH, SGPT & CPK measured in rats fed either a cereal-based diet (CR) or a purified diet of varying folate content (folate deficient, FD; folate replete, FR, or high folate HF) for 6 weeks and then treated with 5-FU in the indicated doses.

Dose	Diet	LDH	n	SGPT	n	CPK	n
0 mg/kg	CR	799.8 ± 118.0	6	19.4 ± 1.7	6	33.5 ± 10.3	6
	FD	679.2 ± 197.1	6	13.2 ± 1.5	6	29.9 ± 12.8	6
	FR	697.9 ± 149.4	6	12.9 ± 1.2	6	31.4 ± 9.1	6
	HF	514.5 ± 192.9	6	12.6 ± 1.3	6	22.2 ± 21.0	6
110 mg/kg	CR	785.0 ± 210.7	6	22.5 ± 5.8	6	44.6 ± 8.3	6
	FD	763.3 ± 147.6	6	14.9 ± 2.9	6	36.6 ± 5.0	6
	FR	701.3 ± 303.8	5	14.1 ± 1.5	5	30.6 ± 21.3	5
	HF	695.3 ± 350.6	3	16.2 ± 4.6	3	24.0 ± 7.1	3
144 mg/kg	CR	674.6 ± 100.8	5	19.1 ± 3.6	5	31.3 ± 2.3	5
	FD	492.8 ± 145.8	5	12.0 ± 1.0	5	24.7 ± 5.9	5
	FR	626.6 ± 207.4	5	11.7 ± 0.4	5	29.5 ± 8.1	5
	HF	486.4 ± 343.9	4	12.0 ± 1.0	4	24.5 ± 17.1	4
325 mg/kg	CR	640.9 ± 128.4	10	20.8 ± 3.7	10	39.4 ± 36.9	10
	FD	394.8 ± 133.1	11	16.2 ± 4.0	11	27.7 ± 22.4	11
	FR	445.9 ± 191.3	10	14.3 ± 2.5	10	26.2 ± 22.5	10
	HF	340.5 ± 86.0	11	25.3 ± 23.1	11	13.9 ± 6.8	11
420 mg/kg	CR	719.6 ± 193.0	6	23.8 ± 2.9	6	36.4 ± 10.4	6
	FD	341.8 ± 131.0	6	14.5 ± 1.1	6	15.2 ± 6.0	6
	FR	409.1 ± 157.9	5	19.0 ± 6.9	5	22.1 ± 10.0	5
	HF	136.4 ± 0.0	1	16.6 ± 0.0	1	7.0 ± 0.0	1
546 mg/kg	CR	463.3 ± 165.8	6	19.5 ± 1.2	6	36.5 ± 22.8	6
	FD	272.0 ± 146.6	5	12.2 ± 0.9	5	20.4 ± 7.3	5
	FR	368.9 ± 114.1	3	13.8 ± 0.4	3	16.5 ± 7.1	3
	HF	223.3 ± 0.0	1	22.7 ± 0.0	1	11.0 ± 0.0	1

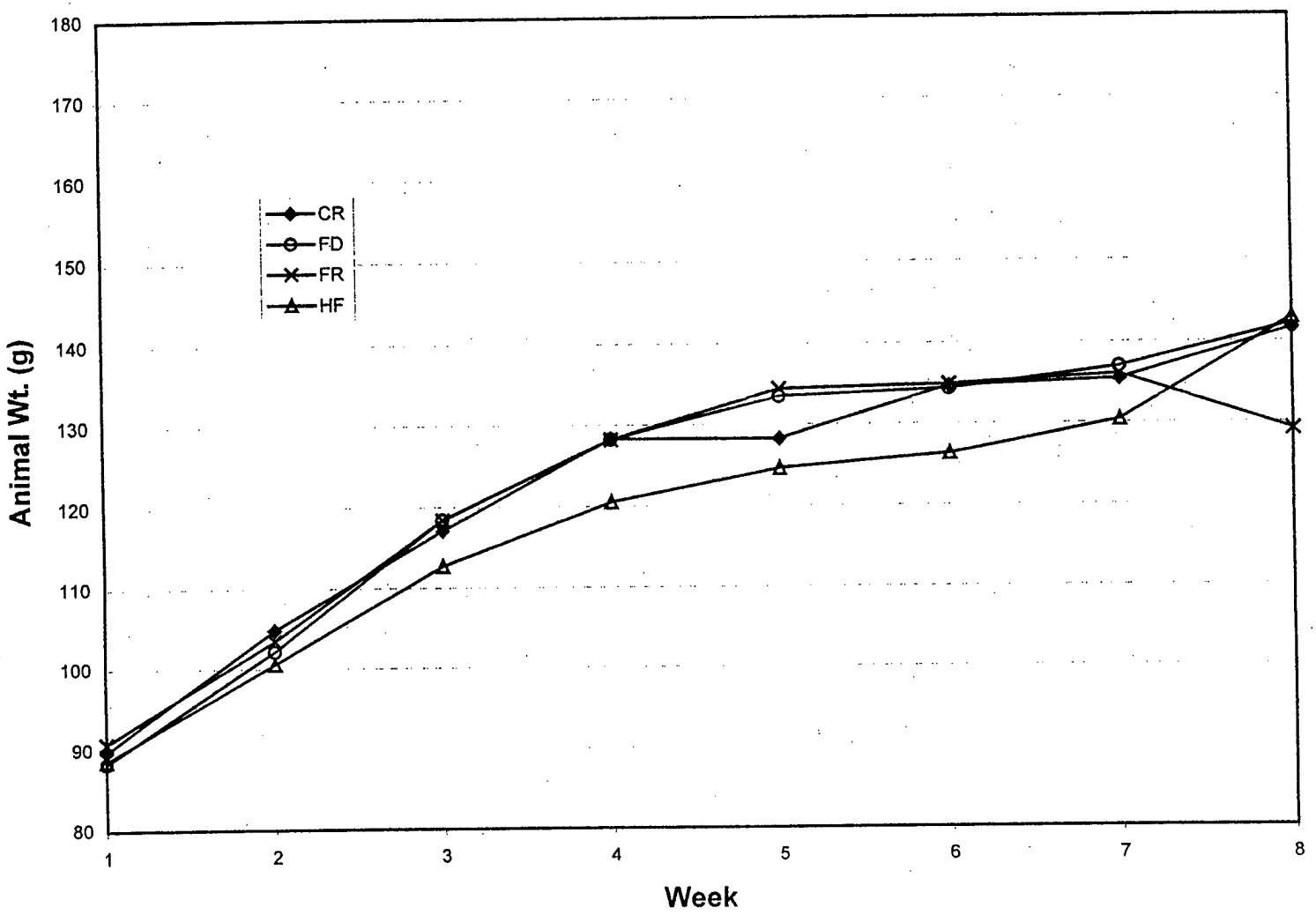
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Figure 1

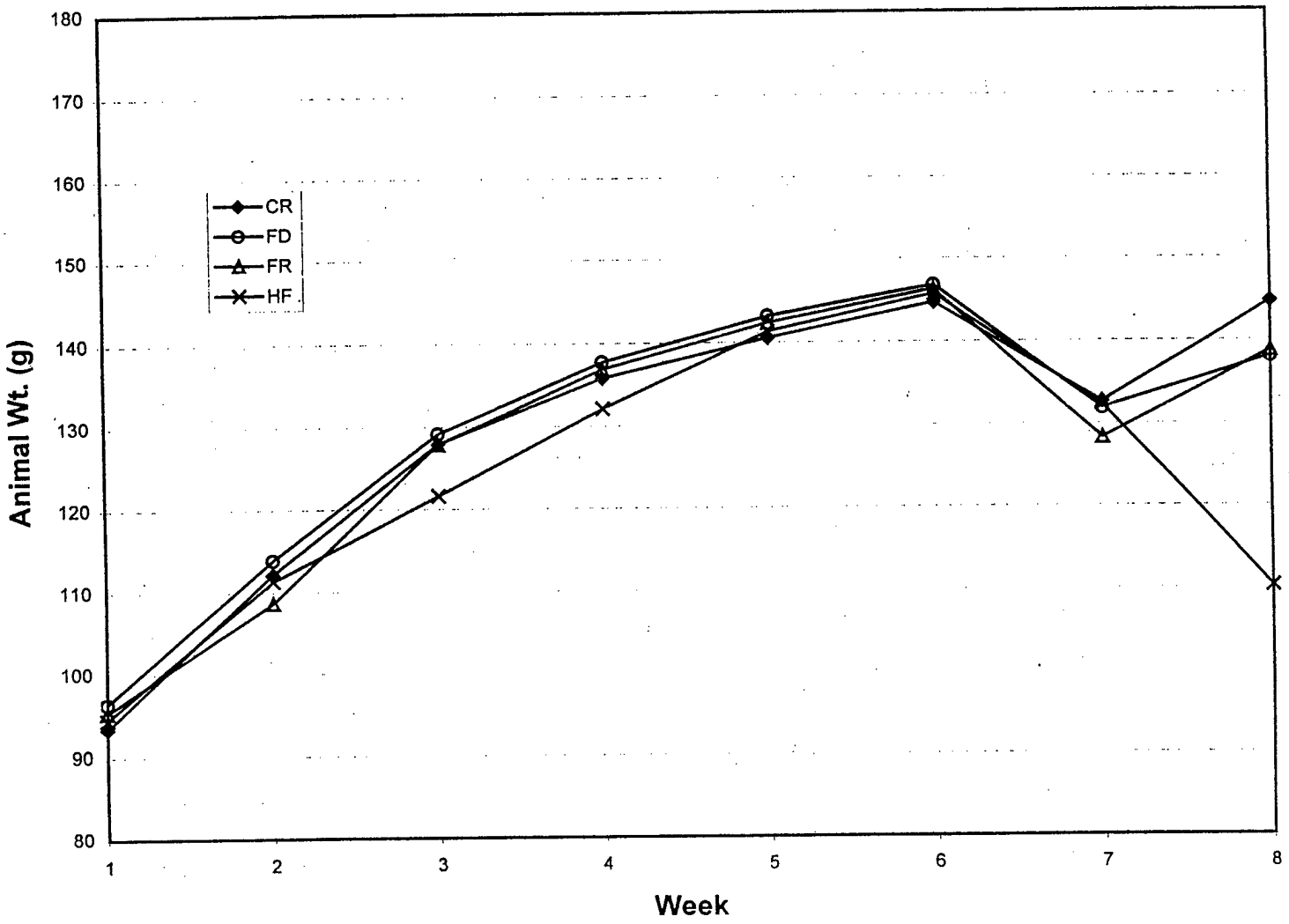
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Figure 2

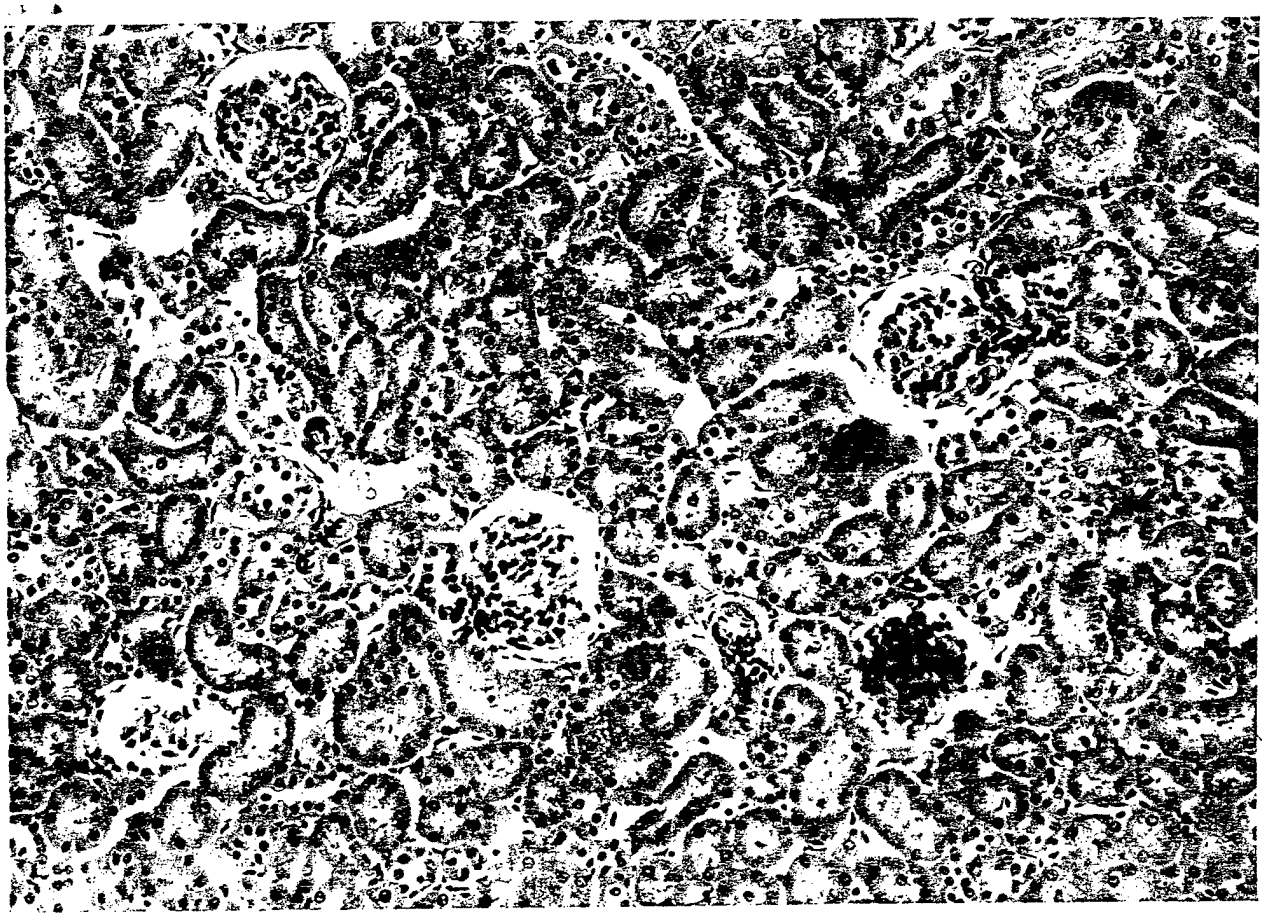

Figure 3

Figure 4

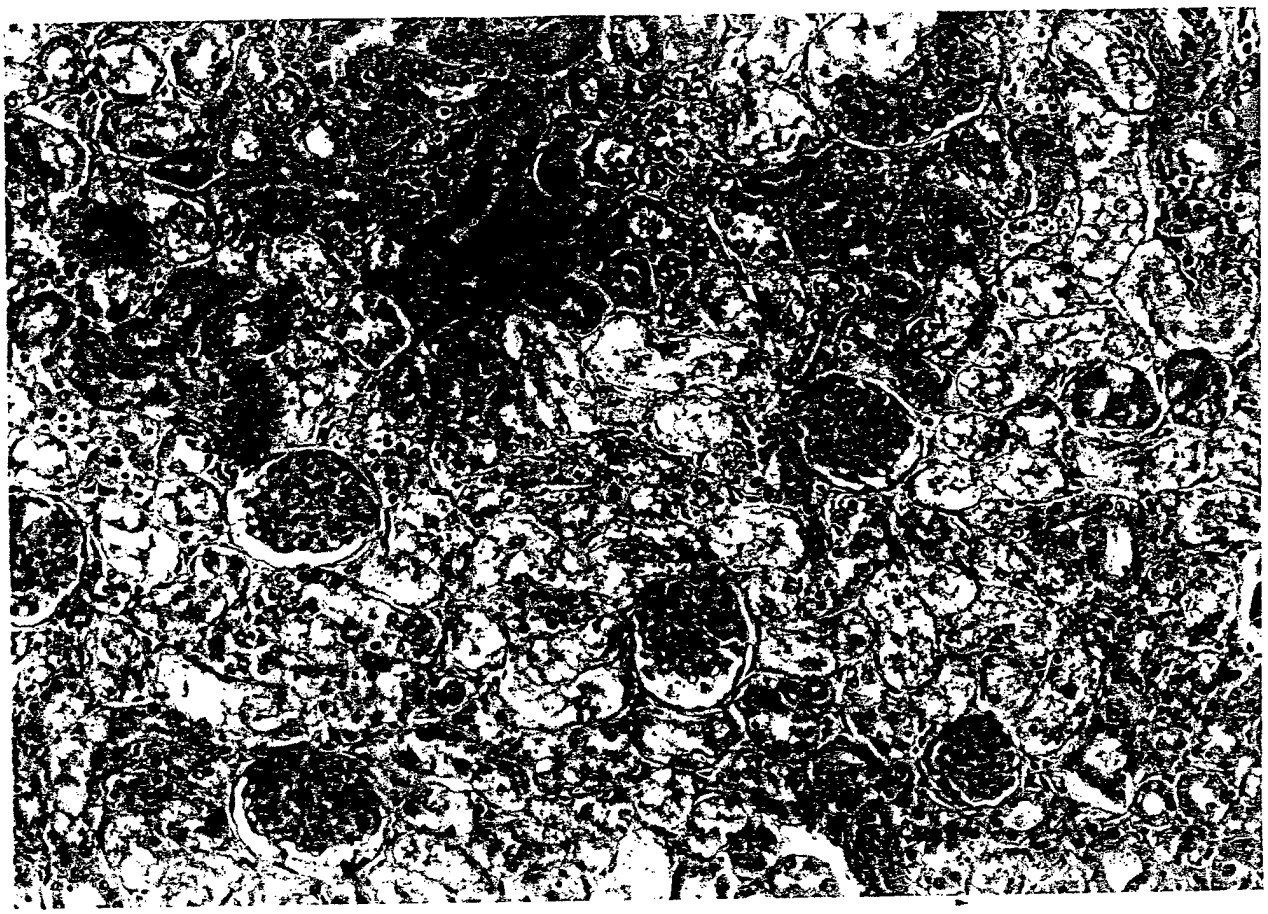

Figure 5

FINAL

DIETARY MODULATION OF MITOCHONDRIAL DNA DELETIONS AND COPY NUMBER AFTER CHEMOTHERAPY IN RATS

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Abstract

Mitochondrial DNA is particularly susceptible to mutation by alkylating agents, and mitochondrial damage may contribute to the efficacy and toxicity of these agents. We found that folate supplementation decreased the frequency of the "common deletion" (4.8-kb, bases 8103-12936) in liver from untreated rats and from animals treated with cyclophosphamide but not 5-fluorouracil. The relative abundance of mitochondrial DNA was greater after chemotherapy but there was no effect of diet. Rats fed a purified diet had fewer mitochondrial deletions than those maintained on a cereal-based diet after chemotherapy. These results indicate that diet can modulate the extent of mitochondrial damage after cancer chemotherapy, and that folic acid supplementation may be protective against mitochondrial DNA deletions.

Key Words: Mitochondrial DNA, folate, cyclophosphamide, 5-fluorouracil

1. Introduction

The late complications of cancer chemotherapy are of increasing concern. For example, more patients with Hodgkin's Disease now die of treatment-related causes than of Hodgkin's Disease itself [1]. Of these late complications, deaths from second malignancies are the most common [1]. The risk of developing myelodysplasia or acute leukemia increases with the age of the patient and is linearly related to the total dose of alkylating agents [1]. Karyotypic analyses of therapy-related myelodysplasia or acute myelogenous leukemia demonstrate frequent deletions of chromosomes 5 and 7, and less often of chromosome segments 17p, 12p and 20q [2]. Our laboratory has reported that folic acid deficiency increases the percentage of intragenic deletions caused by alkylating agents in Chinese hamster ovary cells and in human lymphoblastoid cells [3,4]. These observations support the idea that folate supplementation of

deficient patients may decrease the frequency of intragenic deletions caused by alkylating agents and thereby reduce the risk of developing secondary malignancies. Mitochondrial DNA (mtDNA) deletions also have been associated with several types of malignancy, including breast, gastric, colon and renal carcinomas [5]. Studies with alkylating agents indicate that mtDNA is especially susceptible to mutation and that mitochondrial damage may contribute to the cytotoxic activity of these agents [reviewed in 6,7]. Because of the reports of the possible involvement of mitochondrial alterations in carcinogenesis [5,8], we investigated the relationship of diet, and particularly nutritional folate status, to mtDNA deletions after cancer chemotherapy in rats.

2. Materials and Methods

2.1 Animals and diets.

The research protocol was approved by the Institutional Animal Care and Use Committee of the University of Vermont. Female Fischer 344 rats 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 B_{12} content is 30.00 μg/kg. Then the rats were housed individually in stainless steel wire-bottomed cages. One group continued on the cereal-based diet, while the others were maintained on the AIN-93G Purified Rodent Diet (Dyets, Inc., Bethlehem, PA) of varying folate content (deficient, replete or supplemented) for 6 weeks. This purified diet is based upon vitamin-free casein and cornstarch. Folate-replete diets consisted of AIN-93G with a vitamin supplement that provided 2 mg folic acid/kg and 25 μg of vitamin B_{12}/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 i.p. daily with folic acid, 50 mg/kg dissolved in 8.4% sodium bicarbonate solution. Then they were treated with a single intraperitoneal injection of increasing doses of either cyclophosphamide or 5-fluorouracil (5-FU). Lyophilized CYTOXAN® (Mead Johnson/Bristol Myers Squibb Co., Princeton, NJ) was reconstituted with sterile water for injection, USP (Abbott Laboratories, North Chicago, IL). A 20 mg/ml solution of cyclophosphamide was used for i.p. injections. 5-FU was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA). A 50 mg/ml solution of 5-FU was used for i.p. injections.

2.2 Measurement of mtDNA deletions and copy number.

Two weeks after chemotherapy administration, liver was collected for subsequent analyses after cardiac puncture and exsanguination of the rats. The liver tissue was stored at -80°C until processing. Total hepatic DNA was isolated using the Qiagen DNeasy Tissue kit (Valencia, CA). The amount of the "common deletion" (4.8-kb, bases 8103-12936) in liver was measured by quantitative co-amplification of the mitochondrial D-loop and the mitochondrial deletion using a real-time quantitative polymerase chain reaction assay. The relative abundance of mitochondrial DNA was determined by co-amplifying mitochondrial D-loop versus rat β-actin gene.

2.3 Oligonucleotide Primers and TaqMan Probe Design.

Primers and probes for the rat D-loop, rat mitochondrial deletion and rat β-actin were designed using Primer Express software (PE, Foster City, CA) with rat mitochondrial genome from GenBank (accession X14848). Primers and probes were synthesized and HPLC purified by the PE Oligo Factory (Foster City, CA) and primer limiting experiments were performed to determine the proper primer concentrations. Sequence for the primers and probes can be found in Table 1.

2.4 Real-time PCR for Mitochondrial Deletion.

Mitochondrial deletion expression was quantified with a 5'VIC reporter and a 3'TAMRA quencher dye and D-loop expression with a 5'6-FAM reporter and a 3'TAMRA labeled quencher dye. PCR amplification was carried out in a 50μL reaction consisting of 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 200nM each mitochondrial deletion forward and reverse primers, 100nM each D-loop forward and reverse primers, and 100nM each mitochondrial deletion and D-loop probe. The cycling condition included an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each sample was assayed in duplicate and fluorescence spectra were continuously monitored by the 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA) with sequence detection software version 1.6.3.

2.5 Real-time PCR for Mitochondrial D-loop.

D-loop expression was quantified with the probe detailed above and β-actin expression was quantified with a 5'VIC reporter and a 3'TAMRA labeled quencher dye. PCR amplification was carried out in a 50μL reaction consisting of 1X TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems, Foster City, CA), 200nM each β-actin forward and reverse primers, 50nM each D-loop forward and reverse primers, and 100nM each β-actin and D-loop probe. The cycling condition included an initial phase of 2 min at 50°C, followed by 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C.

2.6 Statistical Analyses.

Analysis of variance was used to test the significance of differences in mtDNA deletions and copy number.

3. Results and Discussion

These studies utilized livers from rats that were maintained on a cereal-based diet or on a purified diet of varying folate content. The amount of the "common deletion" (4.8-kb, bases 8103-12936) was measured by quantitative co-amplification of the mitochondrial D-loop and the mitochondrial deletion using a real-time reverse transcriptase polymerase chain reaction assay with the specific primers described in Table 1. The amount of mitochondrial DNA (abundance) relative to genomic DNA was determined by co-amplifying mitochondrial D-loop versus rat β-actin gene. This methodology allows not only the determination of changes in the amount of mitochondrial deletion relative to total mitochondrial DNA after chemotherapy drug treatment in rats on different diets but also the measurement of changes in total mitochondrial DNA relative to genomic DNA. In this system, a smaller ΔC_T Deletion (mitochondrial deletion [C_{Tdel}] - mitochondrial D-loop [$C_{T\ D\text{-}loop}$]) indicates more deletions, while a smaller ΔC_T Copy Number (mitochondrial D-loop [$C_{T\ D\text{-}loop}$] - β-actin [$C_{T\ \beta\text{-}actin}$]) indicates less total mitochondrial DNA.

3.1 Mitochondrial deletions after cyclophosphamide treatment.

The ΔC_T values and Relative Expression (which indicates the fold difference in deletions or copy number compared to the folate replete purified diet) for mitochondrial deletions and copy number in livers from rats treated with cyclophosphamide are shown in Table 2. Overall, there was a significant effect of dose ($P \leq 0.0001$) and of diet ($P \leq 0.0001$) on mitochondrial deletions in rats treated with cyclophosphamide (Table 2). For any particular dose, the diet effects were similar on mitochondrial deletions ($P = 0.44$).

Within the group that received no cyclophosphamide, the high folate group had significantly fewer deletions than either the low folate ($P \leq 0.0001$) or replete ($P = 0.006$) groups. There was no significant difference between the low folate and replete groups ($P = 0.22$). These findings indicate that the folate content of the diet influenced the number of spontaneous or

background "common deletions" in rat liver cells. Mitochondrial defects, particularly deletions, accumulate in senescent tissues and have been implicated in chronic progressive external ophthalmoplegia, Kearns-Sayre Syndrome and its associated condition, Pearson syndrome, in ischemic heart disease, and in cirrhotic liver [reviewed in 9,10]. A "common deletion" that is the result of recombination between repeats occurs in both humans and rats. In humans, a 4977bp deletion occurs by recombination between copies of a 13-bp repeat in the ATP8 (repeat at 8470-8482) and NAD5 (repeat at 13447-13459) genes while in rat, a 4834bp deletion occurs by recombination between copies of a 16-bp repeat in the ATP6 (repeat at 8103-8118) and ND5 (repeat at 12937-12952) genes [11,12]. The phenomenon could be caused by a homologous recombinational event in the region bordered by the repeats, via slipped mis-pairing. In humans, this common deletion is found with increasing frequency in aging tissues and in approximately 30% of patients with chronic progressive external ophthalmoplegia [9]. The "common deletion" is often used as a specific indicator of general damage [12]. Experiments in adult and senescent rats confirm that the "common deletion" accumulates during aging [12,13]. At the same time senescent rats have a higher mtDNA content in the liver, heart and brain than the adult counterparts [13]. The data presented here suggest that folate deficiency may enhance, and supplemental folate reduce the frequency of these deletions.

Compared to untreated controls, rats that received 110 or 144 mg/kg cyclophosphamide had significantly more deletions in all dietary groups. For rats receiving 50 or 110 mg/kg cyclophosphamide, there were significantly fewer deletions in the high folate group compared to low folate ($P = 0.01$). For rats that received 50 or 144 mg/kg cyclophosphamide, there were significantly more deletions in rats on the cereal-based diet than on the folate replete diet ($P<0.01$).

These data confirm previous studies indicating that mitochondrial damage is increased after treatment with alkylating agents [6, 14-16]. Our findings also provide evidence that diet can modulate the deleterious effects of alkylating agents on mtDNA. After treatment with cyclophosphamide, rats maintained on a purified diet and supplemented with folic acid had significantly fewer mtDNA deletions than rats fed the same diet but lacking folic acid. The composition of the diet also appeared to be important, in that rats fed the purified diet had fewer mtDNA deletions than rats fed a cereal-based diet. Since the folate-replete purified diet and the cereal-based diet contained approximately the same amounts of folic acid, there are other, as yet unidentified, dietary constituents that can protect against alkylating agent-induced mtDNA damage.

3.2 Effect of cyclophosphamide treatment on mitochondrial copy number

Overall, there was a significant effect of dose ($P \leq 0.0001$) and of diet ($P = 0.014$) on mitochondrial DNA relative abundance in rats treated with cyclophosphamide (Table 2). For any particular dose, the diet effects were similar on mitochondrial relative copy number ($P = 0.16$) for interaction. Mitochondrial relative abundance increased in cyclophosphamide-treated rats. Compared to untreated animals, differences were significant ($P < 0.02$) in all dietary groups except for low folate and replete diets at the 110 mg/kg dose level and high folate at the 144 mg/kg dose level. Rats on the low folate diet had significantly fewer copies of mtDNA than animals on the high folate diet at the 110 mg/kg level ($P = 0.04$) and significantly higher relative copies than rats maintained on the high folate diet at the 144 mg/kg level ($P = 0.04$). Otherwise there was no significant effect of diet on mtDNA relative abundance in untreated or cyclophosphamide-treated animals. There was no difference in relative copy number in rats on the cereal-based vs folate replete diets in untreated or cyclophosphamide-treated rats. Therefore, mtDNA relative abundance increased in cyclophosphamide-treated animals, but there was no

consistent effect of diet on mitochondrial relative copy number in untreated or cyclophosphamide-treated rats.

Treatment of patients with alkylating agents such as cyclophosphamide has been associated with an increased risk of secondary malignancies, particularly acute myelogenous leukemia and its precursor condition, myelodysplasia [17]. Gattermann has described mtDNA mutations in sideroblastic anemia and postulated that age-dependent accumulation of mtDNA mutations in stem cells contributes to the development of myelodysplasia [18]. Boultwood and colleagues reported that mtDNA is invariably amplified in acute myelogenous leukemia patients, with levels 2- to 50-fold higher than levels found in blood or bone marrow samples from normal individuals [1]). Since cyclophosphamide treatment increased both mtDNA damage and relative copy number, these mtDNA changes may contribute to the role of alkylating agents in carcinogenesis [8].

3.3 Mitochondrial deletions and copy number after 5-FU treatment

The ΔC_T values for mitochondrial deletions and mtDNA relative abundance in livers from rats treated with 5-FU are shown in Table 3. In the rats treated with 5-FU, a significant dose by diet interaction was found for both deletions and relative copy number ($P<0.01$) in an analysis of rats fed the purified diet of varying folate content. Significant differences were found with regard to deletion number at 3 of the 5 dosages in rats on the purified diet (Table 3). At 110 mg/kg, the high folate group was lower than the low folate group; at the 325 mg/kg dose level, the low folate group was lower than the high folate or replete groups; at the 546 mg/kg dose level, the folate replete group was lower than the low folate group. With regard to mtDNA relative abundance, significant differences were found at only 2 dose levels: at the 110 and 144 mg/kg dose levels, the high folate group was higher than the low folate or folate replete groups (Table 3).

In a comparison of the mtDNA analyses of rats maintained on the cereal-based diet and the folate-replete purified diet and treated with 5-FU, there was a significant dose by diet interaction for both deletions and relative copy number ($P<0.01$). The folate-replete group had significantly fewer deletions than the cereal group at nearly all dosage levels ($P \leq 0.02$) except at the 325 mg/kg dose level, where the cereal group had fewer deletions ($P=0.001$). In the rats that received no drug, these two dietary groups were not significantly different. With regard to mtDNA relative abundance, the two dietary groups were significantly different at 2 dose levels: 325 mg/kg and 420 mg/kg. In both cases the cereal group had the higher relative copy number ($P \leq 0.001$).

Thus, in contrast to the findings with cyclophosphamide, 5-FU treatment and diet had a more variable effect on mtDNA. A significant dose by diet interaction was found for both deletions and relative abundance. The rats maintained on the folate-replete purified diet had fewer deletions than rats fed the cereal-based diet, but there was no consistent effect of nutritional folate status on deletions at the various 5-FU dosage levels. Relative mtDNA copy number tended to be higher in the cereal-based and high folate groups.

Currently there is a limited amount of information available regarding the effect of diet on mtDNA damage. Kang and colleagues showed that dietary restriction to 60% of *ad libitum* intake in rats prevented the age-related increase in mtDNA deletions in the liver but not the brain [20]. More recently Reddy et al. confirmed this result using a 50% dietary restriction and a real-time quantitative PCR, "TaqMan", assay similar to the one used in present study [21]. Dietary restriction is believed to protect both nuclear and mtDNA from oxidative damage [22]. Our studies suggest that a specific dietary component, folic acid, also may be protective against accumulation of the "common deletion".

The mechanism(s) by which folate might protect against mtDNA damage is unclear at present. Mitochondria contain a substantial proportion of the cell's folate content [23-26]. Mitochondrial folate metabolism is a primary source of one-carbon units for cytoplasmic anabolic processes such as purine and pyrimidine synthesis [24,27]. Therefore one could speculate that mitochondrial folate deficiency may impair the synthesis and repair of both nuclear and mitochondrial DNA. Another possible mechanism is that folic acid deficiency, by causing elevated homocysteine levels, decreases cellular antioxidant potential (glutathione peroxidase, NKEF-β PAG, superoxide dismutase, clusterin) and promotes oxidative damage of mtDNA [28]. Regardless of mechanism, these studies suggest that dietary supplementation with a non-toxic nutrient, folic acid, may decrease the frequency of background mtDNA deletions and of the mtDNA alterations associated with cancer chemotherapy.

Acknowledgements

We thank Timothy Hunter for his assistance in designing the primers and probes used in this study. This work was supported by grants from the Department of Defense (DAMD17-98-1-8345) and the National Cancer Institute, Bethesda, MD (CA 41843). The content of the information does not necessarily reflect the position or policy of the U.S. government, and no official endorsement should be inferred. Automated sequencing was performed in the Vermont Cancer Center DNA Analysis Facility and was supported in part by NCI P30 CA 22435.

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Table 1. Sequence of PCR Primers and Probes

Primers	
Rat D-loop FWD	GGTTCTTACTTCAGGGCCATCA
Rat D-loop REV	GATTAGACCCGTTACCATCGAGAT
Rat Actin FWD	GGGATGTTTGCTCCAACCAA
Rat Actin REV	GCGCTTTTGACTCAAGGATTTAA
Rat Mito Del FWD	AAGGACGAACCTGAGCCCTAATA
Rat Mito Del REV	CGAAGTAGATGATCCGTATGCTGTA
Probes	
Rat Mito Del	VIC-TCACTTTAATCGCCACATCCATAACTGCTGT-TAMRA
Rat Actin	VIC-CGGTCGCCTTCACCGTTCCAGTT-TAMRA
Rat D-loop	6FAM-TTGGTTCATCGTCCATACGTTCCCCTTA-TAMRA

Table 2. Mitochondrial DNA deletions and copy number in rat liver were measured two weeks after injection of cyclophosphamide (CTX) in the indicated doses. The rats were maintained on either a cereal-based diet or a purified diet of varying folate content. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean ± SEM. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C}{}_T$ where $\Delta\Delta C_T$ equals $\Delta C_{TDeletion} - \Delta C_{TCalibrator}$ where the calibrator is the ΔC_T of 0 mg/kg CTX in the folate replete purified diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β-actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean ± SEM. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C}{}_T$ where $\Delta\Delta C_T$ equals $\Delta C_{TCopyNumber} - \Delta C_{TCalibrator}$ where the calibrator is the ΔC_T of 0 mg/kg CTX in the folate replete purified diet. Relative Expression indicates the fold difference in deletions or copy number compared to the folate replete purified diet.

A. Deletions

	Cereal Based Diet		Purified Diet					
			Low Folate		Folate Replete		High Folate	
CTX [mg/kg]	Δ C_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp	Δ C_T (n^a)	Rel Exp	Δ C_T (n^a)	Rel Exp
0	4.71±0.53(6)	1.17	4.67±0.23(6)	1.21	4.94±0.43(6)	1.00	5.54±0.64(6)	0.66
50	4.11±0.27(6)	1.78	4.47±0.27(6)	1.39	4.82±0.14(6)	1.09	5.04±0.18(5)	0.93
65	4.37±0.35(6)	1.48	4.56±0.73(6)	1.30	4.68±0.28(6)	1.20	4.76±0.54(7)	1.13
110	4.09±0.12(6)	1.80	4.09±0.30(3)	1.80	4.38±0.17(4)	1.47	4.85±0.15(3)	1.06
144	3.78±0.21(6)	2.23	4.07±0.18(4)	1.83	4.42±0.22(4)	1.43	4.28±0.17(3)	1.58
190	4.73±0.51(5)	1.16	4.37±1.00(2)	1.48	4.32±0.78(2)	1.54	3.62(1)	2.50

B. Copy Number

	Cereal Based Diet		Purified Diet					
			Low Folate		Folate Replete		High Folate	
CTX [mg/kg]	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp
0	-6.73±0.27(6)	0.89	-6.78±0.13(6)	0.92	-6.90±0.29(6)	1.00	-6.83±0.25(6)	0.95
50	-8.01±0.19(6)	2.16	-7.86±0.34(6)	1.95	-7.82±0.11(6)	1.89	-7.64±0.11(5)	1.67
65	-8.22±0.45(6)	2.50	-7.78±0.35(6)	1.84	-7.95±0.25(6)	2.07	-7.85±0.34(7)	1.93
110	-7.32±0.32(6)	1.34	-6.86±0.43(3)	0.97	-7.13±0.57(4)	1.17	-7.38±0.26(3)	1.39
144	-7.60±0.35(6)	1.62	-7.44±0.18(4)	1.45	-7.37±0.20(4)	1.39	-6.94±0.09(3)	1.03
190	-7.41±0.11(5)	1.42	-7.18±0.35(2)	1.21	-7.11±0.33(2)	1.16	-7.45(1)	1.46

[a]n=number of animals

Table 3. Mitochondrial DNA deletions and copy number in rat liver measured two weeks after injection of 5-fluorouracil (5-FU) in the indicated doses. The rats were maintained on either a cereal-based diet or a purified diet of varying folate content. The number of PCR cycles required to exceed a threshold (C_T) just above background was calculated for test and reference reactions. C_T values were determined in duplicate and averaged for the mitochondrial deletion and D-loop and then subtracted to obtain the ΔC_T Deletion, expressed as the mean ± SEM. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C}{}_T$ where $\Delta\Delta C_T$ equals $\Delta C_{TDeletion}$ - $\Delta C_{TCalibrator}$ where the calibrator is the ΔC_T of 0 mg/kg 5-FU in the folate replete purified diet. C_T values were determined in duplicate and averaged for the mitochondrial D-loop and β-actin and subtracted to obtain the ΔC_T Copy Number, expressed as the mean ± SEM. Relative Expression (Rel Exp) was calculated using the equation $2^{-\Delta\Delta C}{}_T$ where $\Delta\Delta C_T$ equals $\Delta C_{TCopyNumber}$ - $\Delta C_{TCalibrator}$ where the calibrator is the ΔC_T of 0 mg/kg 5-FU in the folate replete purified diet. Relative Expression indicates the fold difference in deletions or copy number compared to the folate replete purified diet.

A. Deletions

	Cereal Based Diet		Purified Diet					
			Low Folate		Folate Replete		High Folate	
5-FU [mg/kg]	Δ C_T (n^a)	Rel Exp	Δ C_T (n^a)	Rel Exp	Δ C_T (n^a)	Rel Exp	Δ C_T (n^a)	Rel Exp
0	4.71±0.53(6)	1.17	4.67±0.23(6)	1.21	4.94±0.43(6)	1.00	5.54±0.54(6)	0.66
110	4.99±0.26(6)	0.97	5.55±0.33(6)	0.66	5.97±0.65(5)	0.49	7.02±1.13(3)	0.24
144	3.50±0.33(6)	2.71	4.22±0.12(5)	1.65	4.71±0.43(5)	1.17	4.55±0.29(4)	1.31
325	6.46±0.87(10)	0.35	6.60±1.09(11)	0.32	5.18±0.86(10)	0.85	5.04±0.88(11)	0.93
420	4.07±0.15(6)	1.83	4.61±0.66(6)	1.26	4.55±0.32(5)	1.31	4.49(1)	1.37
546	4.82±0.40(6)	1.09	4.97±0.18(5)	0.98	5.80±0.61(3)	0.55	5.93(1)	0.50

B. Copy Number

	Cereal Based Diet		Purified Diet					
			Low Folate		Folate Replete		High Folate	
5-FU [mg/kg]	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp	ΔC_T (n^a)	Rel Exp
0	-6.73±0.27(6)	0.89	-6.78±0.13(6)	0.92	-6.90±0.29(6)	1.00	-6.83±0.25(6)	0.95
110	-7.11±0.27(6)	1.16	-7.02±0.31(6)	1.09	-7.00±0.21(5)	1.07	-7.87±0.70(3)	1.96
144	-6.03±0.36(6)	0.55	-6.08±0.26(5)	0.57	-5.95±0.31(5)	0.52	-6.96±0.23(4)	1.04
325	-8.92±0.51(10)	4.06	-8.02±0.56(11)	2.17	-8.02±0.57(10)	2.17	-7.96±0.56(11)	2.08
420	-6.95±0.22(6)	1.04	-6.51±0.49(6)	0.76	-5.92±0.44(5)	0.51	-5.62(1)	0.43
546	-9.28±0.60(6)	5.21	-9.18±0.60(5)	4.86	-8.93±0.33(3)	4.08	-8.72(1)	3.53

[a]n=number of animals