Dietary Nucleotides: Effects on Cell Proliferation Following Partial Hepatectomy in Rats Fed NIH-31, AIN-76A, or Folate/Methyl-Deficient Diets

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ABSTRACT The requirement of a number of tissues for dietary nucleotides could explain some of the differences observed in animals fed natural ingredient diets vs. those fed purified diets lacking a source of dietary nucleotides. Lack of dietary nucleotides is exacerbated in animals fed folate- or methyl-deficient semipurified diets, in which both salvage and folate-dependent de novo synthetic pathways are diminished. We examined hepatocyte proliferation following partial hepatectomy in weanling male Fischer-344 rats fed natural ingredient NIH-31 diet, nucleotide-free purified AIN-76A diet or a basal diet similar to AIN-76A but deficient in the methyl donors folate, choline and methionine. Additional groups were fed AIN-76A or folate/methyl-deficient diets supplemented with 0.25% yeast RNA. Compared with NIH-31, AIN-76A increased dUMP/dTTP ratios, reduced the mitotic index (MI) and increased the ratio of proliferating cell index (PCI) to mitotic cells, an indication that hepatocytes were delayed in S-phase. Addition of yeast RNA to AIN-76A reversed (by approximately 50%) the effects of AIN-76A on dUMP/dTTP and cell proliferation. A folate/methyl-deficient diet also produced an increased dUMP/dTTP ratio and markedly reduced the MI, increasing the PCI/MI, which suggested even further delay of cells in S-phase. Addition of yeast RNA to the folate/methyl-deficient diet was effective in significantly reversing the effects of folate/methyl deficiency.

KEY WORDS: • dietary nucleotides • cell proliferation • purified diets • F344 rats

Many investigators have observed that animals maintained on purified or semipurified diets exhibit different toxicological responses compared with similar animals fed natural ingredient diets and that animals fed a crude diet were at lower risk of cancer than those fed a purified diet. Ip (1987) found that rats fed purified AIN-76A formulation were more sensitive to 9,10-dimethyl-1,2-benzanthracene—induced mammary tumorigenesis than those fed NIH-07 natural ingredient diet. Also, B6C3F1 mice administered AIN-76A diet had a higher incidence of spontaneous liver tumors and a higher incidence of both liver and bladder tumors induced by 2-acetylaminofluorene than those fed NIH-07, a natural ingredient diet (Fullerton et al. 1991). In addition, 2-acetylaminofluorene induced hepatoblastomas, a malignant tumor of primitive liver cells, in animals fed AIN-76A diet but not in those fed NIH-07 diet. The number of atypical pancreatic acinar cell nodules induced by azaserine was greater in animals fed a semipurified diet than in those fed a standard laboratory diet (Appel et al. 1990). [3H]Thymidine labeling and mitotic indices in the colorectum, jejunum and duodenum were reduced in mice fed AIN-76A compared with mice fed NIH-07 (Lok et al. 1988). In contrast, the AIN-76A diet induced basal cell hyperplasia of the forestomach of Fischer rats, whereas Purina, Purina and NIH-07 diets, all natural ingredient diets, did not (Masui et al. 1990). Requirements for dietary nucleotides by a number of tissues were reviewed recently by Carver and Walker (1995). A possible increased requirement for a dietary source of nucleotides during rapid cell proliferation was discussed by Seegmiller et al. (1977).

Dietary nucleotides and liver. Novak et al. (1994) found liver weights and glycogen levels were lower in mice fed a nucleotide-free diet rather than Purina Rat Chow, a natural ingredient diet, or diet supplemented with nucleotides or adenosine 5′-monophosphate. Parenterally administered nucleotides promote recovery of liver injury due to dietary D-galactosamine and improve liver function and nitrogen balance after liver injury or partial hepatectomy (Ogoshi et al. 1988). Further evidence that liver may be conditionally dependent on dietary nucleotides comes from interaction of nucleotide-free diets and inhibitors of purine and pyrimidine synthesis. Methotrexate, a folic acid antagonist that blocks de novo purine and pyrimidine biosynthesis, was lethal to rats maintained on a liquid, chemically defined diet but produced no clinical signs in rats fed regular chow diet (Harvey et al. 1987). Similarly, azaserine, an inhibitor of purine biosynthesis, induced a higher incidence of pancreatic prene-
plastic foci, nodules and carcinomas in rats maintained on AIN-76A diet compared with rats fed a natural ingredient diet (Longnecker et al. 1987).

**Nucleotide pool imbalances.** Not only does the absence of dietary nucleotides place an increased demand on de novo synthesis of purines and pyrimidines for DNA and RNA synthesis, this deficiency also produces imbalances in intracellular nucleotide pools, affecting the fidelity of DNA polymerase in DNA replication and repair (Das et al. 1985). Numerous studies, primarily utilizing folate deficiency, have shown that conditions that increase the ratio of dUTP to dTTP can be extremely detrimental to DNA replication by promoting the misincorporation of uracil into DNA as a substitute for thymidine. WIL-2 cells, a human lymphoid cell line, incorporate uracil into DNA when treated with exogenous deoxyuridine and metoprine, an antifolate, and the misincorporation of uracil is accompanied by fragmentation of newly synthesized DNA (Sedwick et al. 1981).

More recently, uracil incorporation into DNA of folate-deficient HL-60 cells and lymphocytes was reported (Wickramasinghe and Fida 1993). Increased dUTP/dTTP ratios delayed progression of the DNA replication fork (Wickramasinghe and Hoffbrand 1980) and passage of cells through the cell cycle (James et al. 1994) and promoted genomic instability (Foghrby et al. 1995). In vitro studies demonstrated that increased dUTP/dTTP ratios promoted folate fragile site expression (Reid 1987), DNA strand breaks (Li and Kaminskas 1984), error-prone DNA repair (Hollday 1985) and mutagenesis (Mattano et al. 1990). The role of folate deficiency in DNA damage was reviewed recently (Blout and Ames 1995).

Further evidence that an imbalance in nucleotide pools, specifically an increase in dUTP, is tumorigenic comes from studies indicating that an excess of dietary orotic acid, a precursor for uracil, promotes carcinogenesis in liver (Rao et al. 1983), mammary gland (Elliot and Visek 1989) and duodenum (Rao et al. 1986). Interestingly, liver tumorigenesis due to orotic acid is potentiated by partial hepatectomy (Laconi et al. 1993). Tumor promotion also occurs from increased endogenous orotic acid due to genetic disorders of the urea cycle (Vasudevan et al. 1995), arginine deficiency or high dietary levels of the orotic acid precursor carbamylaspartate (Vasudevan et al. 1994). Orotic acid–induced hepatic foci, which are resistant to the toxicity of orotic acid, have increased ability to degrade dUMP (Backway et al. 1995), which presumably decreases incorporation of uracil into DNA.

To determine whether the lack of dietary nucleotides in purified diets has an adverse effect on nucleotide pools and cell proliferation, we compared the effects of various diets with and without an added source of dietary nucleotides (yeast RNA) on soluble nucleotide pools and cell proliferation following partial hepatectomy in rats.

### MATERIALS AND METHODS

Weanling male F344 rats (weighing approximately 100 g) were obtained from the National Center for Toxicological Research (NCTR) breeding colony. Five groups of rats (24 per group) were fed NIH-31 meal (Purina Mills, Richmond, IN) for 1 wk and then fed one of the following test diets: 1) NIH-31; 2) AIN-76A (Teklad, Madison, WI); 3) AIN-76A + RNA (yeast RNA, 2.5 g/kg diet, Sigma Chemical, St. Louis, MO); 4) folate/methyl-deficient diet (Kang-Lee et al. 1983); or 5) folate/methyl-deficient diet + RNA (yeast RNA, 2.5 g/kg diet). Thus, the NIH-31 diet is representative of a natural ingredient diet containing precursors for both salvage and de novo purine and pyrimidine synthesis, whereas the AIN-76A diet contains de novo but not salvage pathway precursors. The folate/methyl-deficient diet was administered as an example of a diet deficient in both salvage and de novo pathway precursors. Purine and pyrimidine content of the RNA used to supplement diets was equivalent to that found in chow diet (Pizzini et al. 1990). Food and water were available ad libitum. After 3 wk of consumption of the test diets, the rats were anesthetized with ether and partially hepatectomized by the method of Higgins and Anderson (1931). Six rats of each group were anesthetized with ether and their livers removed at zero time, 48 h, 7 d and 14 d following partial hepatectomy. Deoxyribonucleotide triphosphates (dNTP) were extracted from liver samples obtained by freeze-clamp

### TABLE 1

Deoxyribonucleotide pools in weanling male F344 rats fed the experimental diets for 3 wk

<table>
<thead>
<tr>
<th>Deoxyribonucleotide (nmol/mg protein)</th>
<th>NIH-31 diet</th>
<th>AIN-76A diet</th>
<th>AIN-76A diet + RNA</th>
<th>Folate/ methyl-deficient diet</th>
<th>Folate/ methyl-deficient diet + RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>dCTP</td>
<td>0.92 ± 0.18</td>
<td>0.80 ± 0.16</td>
<td>1.31 ± 0.24</td>
<td>0.95 ± 0.18</td>
<td>0.85 ± 0.13</td>
</tr>
<tr>
<td>dUMP</td>
<td>1.56 ± 0.41</td>
<td>1.11 ± 0.17</td>
<td>1.42 ± 0.45</td>
<td>3.30 ± 0.62</td>
<td>1.91 ± 0.40</td>
</tr>
<tr>
<td>dTTP</td>
<td>1.55 ± 0.20</td>
<td>0.66 ± 0.14*</td>
<td>1.21 ± 0.27</td>
<td>1.51 ± 0.21</td>
<td>1.34 ± 0.27</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.47 ± 0.18</td>
<td>0.48 ± 0.42</td>
<td>0.48b</td>
<td>0.62 ± 0.24</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>dATP</td>
<td>0.99 ± 0.21</td>
<td>0.79 ± 0.11</td>
<td>1.14 ± 0.23</td>
<td>1.24 ± 0.23</td>
<td>1.31 ± 0.19</td>
</tr>
<tr>
<td>dUMP/dTTP ratio</td>
<td>1.0</td>
<td>1.68</td>
<td>1.17</td>
<td>2.01</td>
<td>1.43</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM (4–6 rats/group). * P < 0.05 relative to NIH-31 group.

2 Only one sample.

### TABLE 2

Effect of dietary nucleotides on cell proliferation

<table>
<thead>
<tr>
<th>Dietary group</th>
<th>Mitotic index</th>
<th>Proliferating cell index</th>
<th>PCI/MI ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH-31</td>
<td>25.42 ± 1.93a</td>
<td>762.0 ± 12.26a</td>
<td>30.82 ± 3.35a</td>
</tr>
<tr>
<td>AIN-76A</td>
<td>11.87 ± 0.62b</td>
<td>781.6 ± 9.27a</td>
<td>66.98 ± 4.48a</td>
</tr>
<tr>
<td>AIN-76A + RNA</td>
<td>13.67 ± 0.66c</td>
<td>782.8 ± 7.47a</td>
<td>50.37 ± 2.00a</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Each diet group contained six rats fed the respective diet for 3 wk before and following partial hepatectomy. AIN-76A + RNA diet contained 0.25% yeast RNA. Mitotic index = mitotic cells per 1000 hepatocytes. Proliferating cell index = PCNA positive cells per 1000 hepatocytes. PCI/MI ratio = the ratio of proliferating cell index to mitotic index. Within a column, values with different superscripts are significantly different (P < 0.05).
in situ and determined as described previously (Cross et al. 1993). Liver tissue specimens were processed and scored for mitotic figures and proliferating cells as described previously (Jackson et al. 1995). Because of the large number of hepatocytes in some stage of replication at 48 h after partial hepatectomy, cells in S-phase could not be distinguished from those in G1 and G2 with sufficient precision. Consequently, all PCNA-positive cells in G1, G2 and S were combined as total proliferating cells. Statistical differences were determined by one-way ANOVA. When significant differences (P < 0.05) between treatment groups were found, pairwise comparisons of all groups were performed using the Student-Newman-Keuls method. Data for proliferating cell index/mitotic index (PCI/MI) ratios did not pass the normality test for a parametric analysis, and Kruskal-Wallis one-way ANOVA on ranks was used. Statistical analysis was performed with the SigmaStat™ software (Jandel Scientific, San Rafael, CA). Our study was conducted under Good Laboratory Practice guidelines with prior approval of the NGCP Animal Care and Use Committee.

RESULTS AND DISCUSSION

dNTP pools. Effects of diet on acid-soluble dNTP pools at the time of partial hepatectomy are summarized in Table 1. There was a significant decrease in dTTP in rats fed the AIN-76A diet compared with those fed the NIH-31 diet. Although dUMP was also lower in AIN-76A–fed rats, a greater decrease in dTTP resulted in a marked increase in dUMP/dTTP ratios. Supplementation of the AIN-76A diet with 0.25% yeast RNA lowered the dUMP/dTTP ratio to near that of NIH-31–fed rats. Table 1 shows that dUMP levels were highest in the folate/methyl-deficient diet group, resulting in the highest dUMP/dTTP ratio of all the diet groups tested. Addition of yeast RNA to the folate/methyl-deficient diet decreased both dUMP and dTTP but resulted in a lower dUMP/dTTP ratio.

Cell proliferation. Effects of diet on the mitotic index and number of PCNA-positive cells are summarized in Table 2 and Table 3. There were no significant differences among the various diet groups at any time other than at the peak of cell proliferation 48 h after partial hepatectomy; therefore only data obtained at 48 h are presented. Approximately 80% of hepatocytes were positive for PCNA in all diet groups, and there were no significant differences in the proliferating cell index among different diet groups. There was a significant and marked decrease in the hepatic mitotic index of AIN-76A–fed animals compared with those fed NIH-31 diet (Table 2). This resulted in a twofold increase in the PCI/MI ratio, suggesting that hepatocytes of rats fed the AIN-76A diet were delayed in the cell cycle prior to mitosis. Supplementation of the AIN-76A diet with RNA significantly increased the mitotic index and lowered the PCI/MI ratio, indicating the hepatocytes were progressing through S phase into mitosis at a more normal rate.

The mitotic index in folate/methyl-deficient rats was reduced even further when compared with any other diet group, resulting in a greater PCI/MI ratio (Table 3). The delay of cell cycle progression due to lack of salvage pathway precursors was further exacerbated by a deficiency of methyl donors for de novo pathway synthesis of purines and pyrimidines. Deficiency in both salvage and de novo synthetic pathways produced a marked delay of hepatocytes in the cell cycle prior to mitosis. Supplementation of the folate/methyl-deficient diet with RNA significantly increased the mitotic index and decreased the PCI/MI ratio.

In summary, our results indicate that consumption of the purified AIN-76A diet, which lacks a source of dietary nucleotides, results in increased dUMP/dTTP ratios and a delay of hepatocytes through the cell cycle following partial hepatectomy when compared with consumption of the natural ingredient NIH-31 diet. Supplementation of the AIN-76A diet with yeast RNA partially, but not completely, reversed the effect of AIN-76A on dNTP pools and cell proliferation. Folate/methyl deficiency in the absence of dietary nucleotides resulted in a further increase in dUMP/dTTP ratios and a greater delay of hepatocytes through the cell cycle. Supplementation of the folate/methyl-deficient diet with yeast RNA increased the mitotic index, indicating a partial reversal of the effect of folate/methyl deficiency on cell proliferation. Failure of 0.25% yeast RNA to completely reverse the deficiencies of the AIN-76A diet to support cell proliferation, compared with NIH-31 diet, indicates that either the optimum level or type of supplemental nucleic acid was not used or that there are other necessary components in the NIH-31 diet missing from the AIN-76A diet. In either case, our results suggest that increased toxicity and tumor promotion observed in animals fed AIN-76A and other purified diets may in part be due to resulting imbalances in dNTP pools and interference with DNA replication. Further studies are needed to define the optimum level and source of nucleic acids for reversing the deficiency of the AIN-76A diet and to determine whether increased dUMP/dTTP ratios lead to increased incorporation of uracil into DNA under these conditions.

LITERATURE CITED


<table>
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<tr>
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<th>Proliferating cell index</th>
<th>PCI/MI ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate/methyl deficient</td>
<td>9.16 ± 0.71a</td>
<td>803.0 ± 13.2a</td>
<td>90.50 ± 7.45a</td>
</tr>
<tr>
<td>Folate/methyl deficient + RNA</td>
<td>12.88 ± 0.74b</td>
<td>802.3 ± 11.0a</td>
<td>63.23 ± 3.42b</td>
</tr>
</tbody>
</table>

1 Experimental conditions same as Table 1. Folate/methyl-deficient diet was deficient in folate, choline and methionine. Folate/methyl-deficient + RNA diet contained 0.25% yeast RNA. Mitotic index = mitotic cells per 1000 hepatocytes. Proliferating cell index = PCNA-positive cells per 1000 hepatocytes. PCI/MI ratio = ratio of proliferation cell index to mitotic index. Within a column, values with different superscripts are significantly different (P < 0.05).


