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Cytogenetic damage induced by folate deficiency in mice is enhanced by caffeine

(chromosomal damage/micronucleus)

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ABSTRACT **Folate deficiency in Swiss mice increased the incidence of micronuclei in peripheral blood erythrocytes, indicating increased chromosomal damage in nucleated erythrocyte precursors. Caffeine enhanced the incidence of micronuclei in blood and bone marrow by up to 5-fold in folate-deficient mice at doses that did not significantly alter the micronucleus frequency in the presence of adequate dietary folate. The lower dose of caffeine used in this study (75 mg/kg) approaches doses received by humans who consume large amounts of caffeinated beverages. Since folate deficiency and caffeine consumption are highly prevalent in the human population, the potential for a similar interaction in man should be evaluated.**

The occurrence of micronuclei in peripheral blood and bone marrow erythrocytes has been used as an indicator of cytogenetic damage in laboratory animals and man (1–3). Two recent studies indicate that folate deficiency and caffeinated-beverage consumption may elevate the frequency of micronucleated erythrocytes in man. Caffeinated-coffee consumption was associated with an elevated frequency of micronucleated erythrocytes in a cross-sectional study of genotoxic risk factors (4), and folate supplementation lowered micronucleus frequency to normal in an individual with a marginal folate deficiency and an abnormally high micronucleus frequency (5). These studies suggest that folate status and caffeinated-beverage consumption may be risk factors for cytogenetic damage in man.

Mild-to-moderate folate deficiency (6–9) and caffeinated-beverage consumption (10) are both highly prevalent in the general population. Folate deficiency affects up to 60% of low-income and elderly persons (8, 11) and is also common in subpopulations, such as alcoholics (12) and those with gastrointestinal disorders (13) or receiving anticonvulsant therapy (14). Severe folate deficiency in man leads to megaloblastic anemia, with an accompanying increase in chromosomal aberrations (15, 16). *In vivo* and *in vitro* studies have reported that low-folate conditions increase chromosomal breakage, micronucleus formation, defects in chromosome condensation (15, 17, 18), metastatic potential of cultured murine melanoma cells (19), and expression of chromosomal fragile sites (20, 21). Evidence indicates that certain *in vivo* human cell populations may be folate-deficient even when blood folate values are normal and that cellular dysplasia associated with low-folate status in cervix and lung can be reversed by folate administration (22–24).

Folate deficiency in pregnant women has been reported to range from 2% to 50%, depending on the population studied (25). Folate deficiency during pregnancy is associated with an elevated incidence of birth defects (26).

Severe folate deficiency induced by antifolate drugs has been shown to increase the frequency of micronuclei in mouse bone marrow erythrocytes (27, 28), but we are not aware of previous reports of increased micronucleus frequencies induced by dietary folate deficiency in laboratory animals.

Fragile-site expression induced *in vitro* by conditions of limiting folate is known to be strongly enhanced by caffeine, leading to micronucleus formation and increased frequencies of chromosome breaks (18, 29). Under these conditions, caffeine increases folate-related breakage events, often by more than an order of magnitude. There appear to be no data indicating whether a similar interaction occurs in mammals *in vivo*. Although earlier studies of caffeine effects in animals and man have failed to demonstrate significant genotoxic effects *in vivo* at doses near those that would result from the consumption of caffeinated beverages (30–33), the recent report by Chen *et al.* (34) that chromosomal fragility in human lymphocytes cultured under low-folate conditions is linearly related to the level of coffee consumption of the donor supports the possibility that caffeine could exert significant effects in man under low-folate conditions.

Previous studies of caffeine have shown little or no effect on micronucleus frequencies in mouse bone marrow at doses up to 200 mg/kg (31–33). However, these studies were carried out in mice maintained on commercial laboratory feeds, which contained adequate folate.

We report here that dietary folate deficiency increases the frequency of micronucleated erythrocytes in Swiss mice and that caffeine dramatically increases the frequency of micronucleated cells under conditions of folate deficiency.

METHODS

Male weanling Swiss mice (10–12 g; Simonsen Laboratories, Gilroy, CA) were placed on either (*i*) a folate-free diet containing vitamin-free casein and 1% succinylsulfathiazole (catalogue number 117758, batch 8123; Dyets, Bethlehem, PA) or (*ii*) the same diet supplemented with folic acid (5 mg/kg), for 7 weeks. Each dietary group initially contained 24 mice. After 2, 4, and 6 weeks on the diets, ≈10 μl of blood was removed from the ventral tail vessel of each animal, 2–3 μl was spread on each of three precleaned microscope slides, air-dried, and fixed for 5 min in absolute methanol. During the last week on the diet, groups of 8 mice received caffeine (lot 36F-0495; Sigma) at 150 mg/kg, caffeine at 75 mg/kg, or the solvent vehicle (isotonic saline at 5 ml/kg) each day for 4 successive days by intraperitoneal injection. On the fifth day the animals were anesthetized with diethyl ether, and ≈1 ml of blood was withdrawn from an axillary cut into a syringe containing 0.2 ml of EDTA at 10 mg/ml in isotonic saline.

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Samples were withdrawn for the preparation of slides as described above and for determination of hematological parameters. Plasma was separated from the remaining sample by centrifugation and frozen for subsequent folate analysis. One femur was removed from each animal and bone marrow smears were prepared for micronucleus analysis, as described by Schmid (1), using a reduced volume (0.5 ml) of fetal bovine serum. Smears were stained with Hoechst 33258/pyronin Y in M/15 Sorensen's buffer (35) or acridine orange (36), and 1000 RNA-positive erythrocytes (reticulocytes) and 10,000 RNA-negative erythrocytes were scored for micronuclei, as described (37). Plasma samples were analyzed for folate content using a commercial competitive radiobinding assay (Bio-Rad Quantaphase). Reticulocyte frequency was determined by counting RNA-positive cells among 10,000 erythrocytes by using Hoechst 33258/pyronin Y staining (37). Erythrocyte and leukocyte counts were obtained by counting blood samples diluted with Isoton or Isoton plus Zapoglobin (Coulter Electronics) on a model ZBI Coulter counter. Hemoglobin content was determined by absorbance of Zapoglobin-treated cells at 540 nm.

RESULTS

Mice maintained on a low-folate diet exhibited an elevated background frequency of micronucleated erythrocytes (Fig. 1*A*). This effect was significant in the RNA-positive (youngest) erythrocyte population after 2 weeks and remained approximately at steady state in this cell population between 4 and 7 weeks on the diet. The background frequency was also significantly elevated in the longer-lived RNA-negative cell population. With more prolonged periods of folate deficiency, larger increases in the background micronucleus frequency were observed (data not shown).

Under the above conditions of folate deficiency, which approximately doubled the normal background frequency of micronucleated erythrocytes, caffeine induced high frequencies of micronucleated erythrocytes at doses that have little

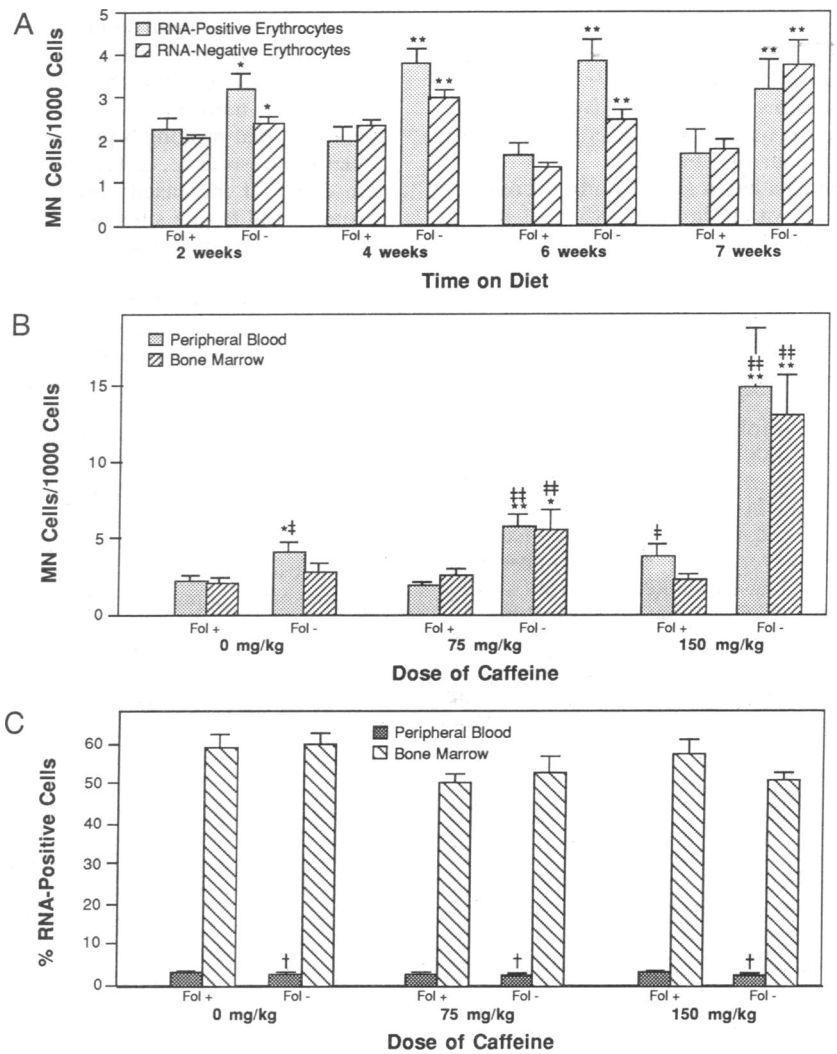

FIG. 1. Effect of folate status and caffeine on the frequency of micronucleated (MN) erythrocytes in Swiss mice. (*A*) Frequency of micronucleated erythrocytes in peripheral blood as a function of time on folate-deficient diet. Acridine orange staining was according to Hayashi *et al.* (36). Fol+, folate-sufficient diet; Fol−, folate-deficient diet. Bars represent mean values ± SEM for groups of 24 mice at weeks 2–6 and 8 mice at week 7. The frequencies of RNA-positive cells as a percentage of total erythrocytes at each sampling time were as follows: 5.57 ± 0.30, 4.62 ± 0.35; 3.48 ± 0.24, 3.01 ± 0.13; 3.91 ± 0.22, 2.98 ± 0.24; 3.59 ± 0.20, 3.55 ± 0.41 in the folate-sufficient and folate-deficient groups at 2, 4, 6, and 7 weeks, respectively. *, Significantly elevated value relative to folate-sufficient diet, $P < 0.05$; **, $P \leq 0.0001$. (*B*) Frequency of micronucleated RNA-positive erythrocytes in peripheral blood and bone marrow as a function of caffeine treatment and folate status. Hoechst 33258/pyronin Y staining was according to MacGregor *et al.* (35). *, Significantly elevated value relative to same dose of caffeine in folate-sufficient dietary group, $P < 0.05$; **, $P \leq 0.0001$; ‡, significantly elevated relative to the folate-sufficient no-caffeine control group, $P < 0.05$; ‡‡, $P \leq 0.0001$. (*C*) Frequency of RNA-positive erythrocytes among total erythrocytes. Data for same groups as in *B*. †, Significantly depressed relative to folate-sufficient no-caffeine control group, $P < 0.05$.

Table 1. Hematological parameters

Diet	Caffeine, mg/kg	Plasma folate, ng/ml	WBCs, no. $\times 10^{-3}/mm^3$	RBCs, no. $\times 10^{-6}/mm^3$	Hemoglobin, g/dl	PCV, %	Reticulocytes, % of RBCs
Low folate	0	0.69 ± 0.06*	2.5 ± 0.6†	6.1 ± 0.4†	11.4 ± 0.8	32 ± 1.2§	2.83 ± 0.10
	75	0.84 ± 0.08	1.7 ± 0.2†	7.1 ± 0.4†	11.4 ± 0.8	34 ± 0.5	2.33 ± 0.25
	150	0.79 ± 0.07	4.4 ± 0.9	7.3 ± 0.4	12.0 ± 0.4	36 ± 0.3‡	2.40 ± 0.15
Normal folate	0	74.5 ± 7.8	5.0 ± 0.9†	7.4 ± 0.5†	12.8 ± 1.0	36 ± 0.8	3.23 ± 0.13
	75	59.8 ± 6.7	3.8 ± 0.7†	8.0 ± 0.3†	12.4 ± 0.6	36 ± 1.0§	2.89 ± 0.23
	150	50.8 ± 3.7	9.0 ± 1.3‡	8.2 ± 0.3‡	14.2 ± 0.7	37 ± 1.0	3.04 ± 0.08

RBC, erythrocyte; WBC, leukocyte; PCV, packed cell volume.
*Numbers represent group mean ± SEM of eight mice (n), except as noted: †$n = 4$; ‡$n = 6$; §$n = 7$.

or no effect in folate-sufficient mice (Fig. 1*B*). Four daily doses of caffeine (150 mg/kg) increased the frequency of micronucleated reticulocytes by 4- to 5-fold in blood and bone marrow. Elevations of this magnitude are comparable to those observed after exposure to potent clastogenic agents, such as cyclophosphamide or mitomycin C [e.g., MacGregor *et al.* (38) and Salamone *et al.* (39)]. In mice maintained on a normal level of folate, these doses of caffeine induced little or no genotoxicity. A significant, but less pronounced, effect was also seen with caffeine at 75 mg/kg.

Cellular cytotoxicity was low, as evidenced by the lack of a marked change in the percentages of RNA-positive erythrocytes in blood and bone marrow (Fig. 1*C*).

The folate-deficient animals had a significantly decreased leukocyte count, but there was little or no effect of folate status on erythrocyte count, reticulocyte count, packed-cell volume, or g % hemoglobin (Table 1). Plasma folate levels of the control vs. folate-deficient mice reflected the dietary folate levels.

DISCUSSION

Our results demonstrate that folate deficiency causes cytogenetic damage in mice and that caffeine can act synergistically with folate deficiencies to induce cytogenetic damage *in vivo*. It is difficult to relate this effect of caffeine in mice directly to the risk of genetic damage in humans consuming caffeinated beverages. However, since caffeine is absorbed rapidly and completely (40) and eliminated relatively slowly [$t_{1/2} = \approx 3.5$ hr (41)], the dose that might reasonably be consumed in a 1-hr period may be roughly comparable to the acute administration used in the present experiments. A 50-kg human subject consuming three cups of high-caffeine coffee [up to 150 mg per cup (10, 41)] would receive a caffeine dose of ≈9 mg/kg. On a body-weight basis, this is less than an order of magnitude lower than the dose of 75 mg/kg that induced micronuclei in folate-deficient mice. On a surface-area basis, a dose of 75 mg/kg to a mouse would extrapolate to a dose of 7.0 mg/kg in man (42, 43). Although such extrapolations are limited by the wide variation in biological response among species, the conditions under which these effects were observed in the mouse are close enough to those that occur in man to dictate the need for a direct determination in man of the cytogenetic consequences of folate deficiency combined with consumption of caffeineated beverages.

The mechanism by which caffeine potentiates cytogenetic damage in folate-deficient mice is unknown. Folate is required for the synthesis of dTMP and purines, and folate deficiency can induce DNA damage by modifying cellular nucleotide pools (44). Caffeine has been reported to have many biochemical and genetic effects, including inhibition of purine and pyrimidine synthesis, inhibition of DNA repair, and enhancement of chromosomal damage induced by other genotoxins (45–47). Alterations in nucleotide pools may be an important factor in fragile-site expression (44), and the fact that caffeine and folate deficiency are known to affect nucleotide synthesis suggests modification of nucleotide pools as a possible mechanism for the observed interaction. However, relatively high concentrations of caffeine are required for inhibition of nucleotide synthesis (47, 48). An alternative hypothesis is that the increased expression of these breakage sites may be related to the ability of caffeine to disturb the cell cycle and to induce premature chromosome condensation. Such an effect has been demonstrated to occur *in vitro*, even among cells that have not completed DNA replication (49). An overlap between the completion of DNA replication and the onset of mitosis, a situation that can be promoted by conditions that prolong S phase (e.g., folate deficiency) and advance mitosis (e.g., caffeine exposure), has been proposed as a cause of increased chromosomal breakage at human chromosomal fragile sites (50).

It is possible that the observed enhancement of folate-induced micronucleus formation by caffeine *in vivo* occurs through the same mechanism responsible for enhancement of fragile-site expression by caffeine *in vitro* (18, 29). Fragile sites occur at chromosomal locations associated with oncogenes and with breakpoints characteristically seen in certain malignancies, especially leukemias and lymphomas (51–53). Chromosomal rearrangements involving fragile sites occur in these cancer cells at frequencies greater than expected by chance (18). The role of fragile-site expression in initiating malignancy is a subject of debate (51, 53, 54), with evidence both for and against a causative role (53).

The observed statistical associations between caffeine and chromosomal damage in man (4, 34) and the known susceptibility of human bone marrow to DNA damage induced by folate deficiency (9) suggest the possibility of a quantitatively important role of these factors in modulating the spontaneous rate of chromosomal damage in man. The present observations in mice demonstrate that these factors interact synergistically *in vivo*. In view of the high prevalance of folate deficiency and caffeine consumption in man, it is important to determine if the synergistic induction of chromosomal damage observed in mice occurs in folate-deficient humans who consume high levels of caffeine. It will be of interest to determine if the observed anticarcinogenic effects of green vegetables in numerous epidemiological studies (55) may in part be explained by a protective effect of folic acid.

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