A comparison of folic acid and 5-methyltetrahydrofolate for prevention of DNA damage and cell death in human lymphocytes in vitro

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Folic acid (FA), the most oxidized and stable form of folate, is commonly used as a dietary supplement and in culture media. FA must be reduced and methylated to become the metabolically active form found in blood and utilized by tissues, i.e. 5-methyltetrahydrofolate (5-MeTHF). 5-MeTHF is the methyl group donor required for the conversion of homocysteine to methionine catalyzed by vitamin B12-dependent methionine synthase. It is hypothesized that 5-MeTHF may be more effective than FA in reducing spontaneous DNA damage and improving cell proliferation because, unlike FA, it can donate a methyl group for methionine synthesis, which is required for cell division via polyamine production and for maintenance methylation of DNA after its conversion to S-adenosylmethionine. We aimed to determine whether FA and 5-MeTHF differed in their capacity to prevent genetic damage and cell proliferation of human lymphocytes in vitro. Lymphocytes from eight female volunteers (40–48 years) were cultured in RPMI 1640 medium containing 12–120 nM FA or 5-MeTHF for 9 days. Mitogenesis was stimulated with phytohemagglutinin and the medium changed on days 3 and 6. Cytokinesis was inhibited by adding cytochalasin B on day 8 and cells were harvested and transferred to microscope slides on day 9. Chromosome damage, cell death and cytostasis was measured using the cytokinesis-block micronucleus assay in its comprehensive mode. The results showed that the frequency of micronucleated binucleate cells was significantly lower at 120 nM FA compared with 120 nM 5-MeTHF (P < 0.05), however, at 12 nM concentration both forms of folate were associated with increased frequency of micronuclei and nuclear buds relative to 120 nM (P < 0.05). Apoptosis tended to be significantly higher in 5-MeTHF cultures compared with FA cultures, however, necrosis and nuclear division were similar between cultures. We conclude that 5-MeTHF is not more efficient than FA in preventing human lymphocyte genomic instability in this in vitro system. Further research is needed to clarify the role of choline and methionine concentration and the importance of the reduced folate carrier and the folate receptor in determining the relative bioavailability of 5-MeTHF and FA with regard to genome stability.

Introduction
Folate plays a critical role in the prevention of chromosome breakage and hypomethylation of DNA. It is required for the synthesis of dTMP from dUMP. Under conditions of folate deficiency dUMP accumulates and, as a result, uracil is incorporated into DNA instead of thymine (Eto and Krumdieck, 1986; Wagner, 1995; Fenech, 2001). There is good evidence suggesting that excessive misincorporation of uracil in DNA not only leads to point mutations but also results in single- and double-strand DNA breaks, chromosome breakage and micronucleus (MN) formation (Blount and Ames, 1995; Blount et al., 1997). Folate and vitamin B12 are also required for the synthesis of methionine and S-adenosylmethionine. The latter is the common methyl donor required for the maintenance of methylation patterns in DNA that determine gene expression and DNA conformation (Zingg and Jones, 1997). Folate deficiency is an important risk factor for cancer, cardiovascular diseases and neurological abnormalities (Panchurinuti et al., 1994; Blount and Ames, 1995; Blount et al., 1997; Lindahl and Wood, 1999). We have performed a series of studies to investigate the effect of folate concentration on minimizing genomic instability in human lymphocytes. In a study in 64 older healthy men we showed that MN frequency was significantly negatively correlated with folate and vitamin B12 concentration in plasma and positively correlated with homocysteine, even though these men had apparently ‘normal’ range plasma values for these micronutrients (Fenech et al., 1997). We also found that a dietary supplement intake of 700 µg folic acid (FA) and 7 µg vitamin B12 minimized micronucleated (MNed) cell frequency and homocysteine in young adults in a randomized double-blind placebo-controlled dietary intervention study (Fenech et al., 1998). Other studies have shown that global DNA methylation in lymphocytes or colonic tissues is increased when FA intake is increased (Cravo et al., 1994; Fowler et al., 1998; Jacob et al., 1998). FA modulates DNA repair, DNA strand breakage and uracil misincorporation in immortalized human colonocytes and FA deficiency substantially increases DNA instability in these cells (Duthie et al., 2000). FA is not a significant natural form of folate. However, FA is the form used in tablets and fortified foods because it is the most stable form of this vitamin. FA must be reduced and methylated before it can function as a methyl donor, either for the conversion of dUMP to dTMP or to donate a methyl group to homocysteine (Figure 1). Unlike FA, 5-methyltetrahydrofolate (5-MeTHF) can immediately enter the methionine synthesis cycle and provide a methyl group for maintenance methylation of DNA and lowering of homocysteine, which may be genotoxic (Crott and Fenech, 2001).

Defining the optimal composition of culture media for genomic stability is important for two reasons: (i) genotoxicity test results may be dependent on the micronutrient composition of the medium; and (ii) the results may be relevant for defining optimal micronutrient concentration in vitro. In a series of in vitro studies with long-term cultured human lymphocytes we previously showed that genomic instability increases in a...
dose-dependent manner as FA concentration is decreased from 120 to 12 nM using a wide range of genotoxicity biomarkers integrated into the cytokinesis block micronucleus (CBMN) assay, namely micronuclei (MNs), micronucleated binucleated cells (MnBNCs), nucleoplasmic bridges (NPBs), nuclear buds (NBuds), apoptosis and necrosis (Crott et al., 2001a,b). Because MN frequency was minimized at concentrations of FA (≥60 nM) above the normal range observed for folate in plasma (20–40 nM), we reasoned that the accepted ‘normal’ range values are inadequate for genomic stability. However, this conclusion is uncertain given that the circulating form of folate, 5-MeTHF, may be more effective than FA in minimizing DNA damage. In this study we aimed to determine, using the same system, whether there are differences between FA and 5-MeTHF with regard to genomic stability of human lymphocytes in vitro.

Materials and methods

Approval for this study was obtained from CSIRO Health Sciences and Nutrition Human Ethics Committee. Eight healthy women aged 40–48 years and not receiving anti-folate therapy or cancer treatment were recruited for the study. We focused this study on women to avoid the possible confounding effect of gender given that in vivo studies suggest significant differences between males and females with respect to folate, vitamin B12 and homocysteine status in blood (Fenech et al., 1998; Poirier et al., 2001).

Volunteers donated a fasted blood sample (40 ml in lithium-heparinized vacuette). Lymphocytes were isolated using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Lymphocyte cultures were prepared at a concentration of 0.5 × 10^6 cells/ml in 5 ml of RPMI 1640 medium containing either 120 or 12 nM FA (F7876; Sigma) or 5-MeTHF (M0132; Sigma), 5% dialyzed fetal bovine serum (Trace Biosciences, Victoria, Australia), 10 U/ml interleukin-2 (Roche Diagnostics, Basel, Switzerland), 2 mM L-glutamine (Sigma), 100 U/ml penicillin G (Sigma) and 100 µg/ml streptomycin (Sigma). The concentrations of folate chosen were intended to investigate effects within the deficiency and sufficiency concentration range for which we had already shown clear differences in genomic stability (Crott et al., 2001a,b). Mitogenesis was stimulated by the addition of phytohemagglutinin (PHA) (45 µg/ml) (Murex Biotech, Kent, UK) and cultures were incubated at 37°C and 5% CO2 in a humidified incubator. After 3 days, cell number and viability were determined using a Coulter counter and Trypan blue exclusion, respectively. The cultures were continued in 4.7 ml of fresh medium and 0.3 ml of ‘conditioned’ medium from the previous 3 day culture with 0.5×10^6 viable cells/ml. The components of the medium were the same as above but without PHA. This process of counting and re-culturing cells was repeated 6 days post-PHA treatment and a final viable cell count was measured on day 9.

At 8 days post-PHA treatment, two 750 µl aliquots of each culture were transferred to 6 ml culture tubes for the CBMN assay to measure chromosome damage, cytostasis and cell death. Cytocentrifuging (Shandon Southern Products, Cheshire, UK). Slides were then air dried, fixed and stained with Diff-Quik (LabAids, New South Wales, Australia). Coded slides were scored for the frequency of MnBNCs, MNed mono (M Ned mono) cells, NBuds, NPBs, apoptotic cells and necrotic cells. The nuclear division index (NDI) was determined as previously described (Fenech, 2001). Samples of 1000–1500 BNCs were scored per culture to determine frequency of MNed BNCs, NPBs and NBuds. Samples of 500 cells were scored per culture to determine NDI and frequency of necrotic and apoptotic cells. MNed mono cell frequency was determined by scoring 500 mono nuclelated cells. The scoring criteria used were those described by Fenech (2000).

One way ANOVA analysis was used to determine the significance of differences in the parameters measured in relation to the type of folate and concentration in the culture medium. Pair-wise comparison of significance was determined using Tukey’s test. Cross-correlation analysis was performed using Spearman’s method. ANOVA analysis was performed using GraphPad Prism (GraphPad Inc., San Diego, CA) and the cross-correlation analysis was performed using CSS Statistica (Statsoft, Tulsa, OK). Significance was accepted at P < 0.05.

Results

The results are summarized and illustrated in Figures 2–4.

With regard to the DNA damage end-points it was evident that FA was significantly more effective in minimizing MN formation than 5-MeTHF at the 120 nM concentration and that MN formation was significantly increased at 12 nM compared with 120 nM for both folate types (Figure 2A). The formation of NBuds was lowest for 120 nM compared with 12 nM FA (P < 0.05), but the difference from the 5-MeTHF cultures did not achieve significance (Figure 2B). NPB and MNed mono cells tended to be decreased at 120 nM folate concentrations, however, differences between the four folate treatments did not achieve statistical significance (Figure 2C and D).

Fig. 1. The main metabolic pathways by which folate, vitamin B12, choline and methionine impact on DNA methylation, synthesis and repair. BHMT, betaine:homocysteine methyltransferase; DHF, dihydrofolate; DMG, dimethylglycine; 5-MeTHF, 5-methyltetrahydrofolate; 5,10-MeTHF, 5,10-methylenetetrahydrofolate; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; THF, tetrahydrofolate; TS, thymidylate synthase; VIT B12, vitamin B12.

Fig. 2. The relationship between folate type and concentration and the various DNA damage biomarkers measured in the comprehensive CBMN assay. Treatments not sharing the same letter are significantly different from each other. Results represent means ± SEM, n = 8 female subjects.

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The estimated total viable cell count on days 3, 6 and 9. ANOVA, \( P = 0.0045 \), with the higher apoptosis rates recorded for the 5-MeTHF cultures relative to the FA cultures and with statistical significance achieved for the pair-wise comparison between 12 nM 5-MeTHF and 120 nM FA (Figure 3D). The highest necrosis rate was observed for 12 nM 5-MeTHF, although differences between cultures were only marginally significant (ANOVA, \( P = 0.055 \)) (Figure 3C). The viable cell count tended to show an inverse trend to that observed for necrosis and apoptosis, with the highest viable cell counts obtained in the FA cultures relative to the 5-MeTHF cultures (ANOVA, \( P = 0.0372 \)) and statistical significance achieved for the pair-wise comparison between 120 nM FA and 12 nM 5-MeTHF (Figure 3B). In contrast, there were no differences in NDI of the viable cells in the different cultures (Figure 3A). We also estimated the total viable cell count by extrapolating the total number of cells using the viable cell count and dilution factor at days 3 and 6. The results for total viable cell count show an increasing trend of total viable cell number with days of culture for all treatments, however, for each day there was a clearly significantly larger number of viable cells in the 120 nM FA culture relative to the other cultures, which were not significantly different from each other (Figure 4).

Cross-correlation analysis of the combined data for FA and 5-MeTHF for all the cultures (Table I) revealed significant positive correlations between MNed BNC frequency and NBuds (\( r = 0.604, P = 0.000 \)), MNed monos (\( r = 0.506, P = 0.003 \)), NDI (\( r = 0.473, P = 0.006 \)) and apoptosis (\( r = 0.367, P = 0.039 \)). NDI was also positively correlated with two other DNA damage biomarkers, namely NBuds (\( r = 0.387, P = 0.029 \)) and MNed monos (\( r = 0.485, P = 0.005 \)). As expected, necrosis was negatively correlated with NDI (\( r = -0.339, P = 0.057 \)) and viable cell count on day 9 (\( r = -0.464, P = 0.007 \)).

**Discussion**

Folate plays a critical role in the prevention of chromosome breakage and hypomethylation of DNA (Fenech, 2001). Because it is required for the synthesis of dTMP from dUMP, folate deficiency will lead to dUMP accumulation and uracil incorporation into DNA. As a result, point mutation, single- and double-strand DNA breaks, chromosome breakage and MNi are induced (Blount and Ames, 1995; Blount et al., 1997).

To become biochemically active FA must be reduced by dihydrofolate reductase and methylated to become 5-methyltetrahydrofolate which then participates in the synthesis of dTMP by donating a methylene group to dUMP. In contrast, 5-MeTHF is the natural circulating form of folate in the blood and can participate directly in the methionine cycle by donating a methyl group to homocysteine, following which it is converted to tetrahydrofolate (THF) which is the point at which FA enters the thymidine synthesis cycle after it is reduced.

It is important to know whether FA and 5-MeTHF differ in their capacity to maintain genomic stability. In previous studies we had shown that MNed BNCs, NPBs and NBuds are minimized at 120 nM FA, which is much higher than the folate concentrations observed in the plasma of healthy individuals (Crott et al., 2001a,b). We have therefore questioned whether ‘normal’ values for folate in blood are adequate to minimize DNA damage. However, it is plausible that much less 5-MeTHF than FA is needed to maintain genomic stability.
because unlike FA it can donate a methyl group for methionine synthesis and maintenance methylation of DNA. In so doing, 5-MeTHF may be more effective than FA in (i) reducing the homocysteine concentration, which is potentially genotoxic (Crott and Fenech, 2001), and (ii) preventing hypomethylation of centromeric DNA, which could lead to undercondensation of the heterochromatin regions of chromosomes 1, 9 and 16 and their loss via micronucleation (Fenech, 2001).

We employed the CBMN assay to investigate differences between FA and 5-MeTHF in minimizing genomic instability in human lymphocytes cultured for 9 days because we had previously shown that this system is sensitive to relatively small changes in FA concentration (Crott et al., 2001b). The results of the present study show that frequencies of MNed BNCs were increased at 12 nM folate concentration relative to 120 nM, however, at this concentration FA reduced MNed cell frequency to a significantly greater extent than 5-MeTHF. These results not only provide further evidence that FA deficiency causes DNA damage but also indicate that 5-MeTHF may be less effective than FA in reducing genomic instability in this in vitro system. The frequency of apoptosis tended to be higher in 5-MeTHF cultures, which is probably the result of increased DNA damage relative to the FA cultures rather than a more efficient apoptotic process, because MNed BNC frequency was higher in the 5-MeTHF cultures and apoptosis was positively correlated with MNed cell frequency. The results of this experiment do not support the hypothesis that 5-MeTHF may be more effective than FA in reducing spontaneous DNA damage.

One of the possible explanations for this surprising result is that lymphocytes in culture take up FA more efficiently than 5-MeTHF. Cells can acquire folate by two separate mechanisms (Kamen and Capdevila, 1986; Antony, 1992; Doucette and Stevens, 2001). The first involves the reduced folate carrier, a transmembrane transporter with a high affinity for 5-MeTHF (Kd = 0.3–4.0 µmol/l) but a much lower affinity for FA (Kd = 100–200 µmol/l). The second involves the folate receptor, a glycoporphosphatidylinositol-anchored protein that transports folates into the cells via endocytosis and has a much higher affinity for FA and 5-MeTHF than the reduced folate carrier (~100 00-fold higher for FA and 100-fold higher for 5-MeTHF). This suggests that the folate receptor may play an important role in transporting folates when the concentration is in the nanomolar range, which is the physiological range and the one used in our studies. Consequently, it is plausible that there may have been different rates of uptake between FA and 5-MeTHF in our experiments which could partly explain the differences between these two forms of folate with respect to cell proliferation, cytotoxicity and genome damage.

Another possible explanation is that 5-MeTHF was not bioavailable because it had to compete with an excess of choline in the RPMI 1640 medium to enter the methionine cycle. Normal RPMI 1640 medium contains 21.5 µM choline chloride and 2.26 µM FA. However, the folate concentration in our RPMI 1640 medium was 12 or 120 nM, which is close to the physiological range (20–30 nM), but much lower than normal RPMI 1640 medium (2260 nM). According to a study by Jacob et al. (1999), human plasma choline concentration is between 6 and 7 µM, which is considerably lower than that in RPMI 1640 medium (21.5 µM). Therefore, the ratio of choline to folate in our in vitro system may not reflect the situation in vivo. The molar ratio of choline to folate in our experiments was 1791 (at 12 nM folate) or 179 (at 120 nM folate), while in vivo it may range between ~700 (at 7 µM choline, 10 nM folate) and 233 (at 7 µM choline, 30 nM folate) and in standard RPMI 1640 medium it is only 9.5, which is not physiological. As shown in Figure 1, choline can be oxidized to form a metabolite called betaine, which can donate three methyl groups for methylation reactions. The methyl groups of betaine may be used to convert homocysteine to methionine. Betaine may compete with 5-MeTHF to convert homocysteine to methionine. A higher concentration of choline may lead to a higher level of betaine and a decline in the homocysteine concentration in medium. Subsequently, efficient conversion of 5-MeTHF to THF and 5,10-methylenetetrahydrofolate could be inhibited by betaine to some extent and this may indirectly block the involvement of 5-MeTHF in thymidine synthesis and result in an elevated intracellular dUTP/dTTP ratio and, consequently, DNA damage. In contrast, FA can enter the thymidine synthesis pathway unhindered by betaine, after it is reduced to THF, so it may be more effective than 5-MeTHF in preventing uracil incorporation into DNA. However the possibility that betaine competes with 5-MeTHF may seem improbable given that the enzyme betaine:homocysteine methyltransferase may not be efficiently expressed in cultured human lymphocytes (Wang et al., 1991). It is also possible that the supra-physiological concentration of methionine (100 µM) in RPMI 1640 medium could minimize the effectiveness of 5-MeTHF in maintaining genome stability given that the role of 5-MeTHF is to donate a methyl group for the synthesis of methionine from homocysteine (Figure 1; Crott et al., 2001a).

The results of the cross-correlation analysis show significant positive correlations between MNed frequency in BN cells and NBuds and MNed frequency in mononucleated cells, which is in agreement with our previous observations in a different group of subjects (Crott et al., 2001a,b). We have observed a significant positive correlation between MNed cell frequency and apoptosis, which suggests that the apoptosis rate was simply a reflection of the extent of DNA damage experienced in viable cells, some of which proceeded to apoptosis. If the change in MNed cell frequency was subject

| Table I. Cross-correlation matrix with correlation coefficients and significant P values |
|---------------------------------|-----------------|---|---|----------------|-------|-----------------|-------|-----------------|
|                               | NBud MNed mono NPB NDI | Apoptosis Necrosis Viable cell count day 9 |
| MNed BNCs | 0.604 P = 0.000 | 0.506 P = 0.003 | 0.128 | 0.423 P = 0.006 | 0.367 P = 0.039 | 0.013 | −0.163 |
| NBud     | 0.230 | 0.191 | 0.387 P = 0.029 | 0.143 | 0.010 | −0.087 |
| MNed mono | 0.141 | 0.485 P = 0.005 | 0.212 | −0.104 | 0.261 | −0.167 |
| NPB      | 0.038 | 0.073 | −0.339 P = 0.057 | 0.207 | 0.183 | −0.464 P = 0.007 |
| NDI      | - | - | - | - | - | - |
| Apoptosis | - | - | - | - | - | - |
| Necrosis | - | - | - | - | - | - |
| Viable cell count day 9 | - | - | - | - | - | - | - |

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to altered capacity of DNA damaged cells to undergo apoptosis we would have expected a negative correlation with apoptosis because an increased propensity to apoptosis would have eliminated cells with DNA damage from the MN score, which is performed in viable cells. It is interesting to note the positive correlation of NDI with MNed BN, NBuds and M Ned monos because this suggests that cells with folate deficiency-induced genomic instability may achieve a proliferative advantage if they survive as viable cells, however, this result is opposite to what we have observed previously (Crott et al., 2001a) and may reflect differences in the subjects studied in terms of their proliferative/DNA damage response to folate deficiency conditions.

FA deficiency is known to cause gene amplification and chromosome damage (Melnyk et al., 1999; Crott et al., 2001a,b; Fenech and Crott, 2002). Shimizu et al. (1998, 2000) showed that amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via nuclear budding to form MNi during S phase of mitosis. Miele et al. (1989) showed that amplified dihydrofolate reductase genes in methotrexate-resistant V79 cells were accumulated in NBuds and MNi. In our previous studies we showed that MNed cells, NBPs and NBuds increased in a dose-related manner with lower FA concentration in the 12–120 nM concentration range (Crott et al., 2001a,b; Fenech and Crott, 2002). We concluded that these biomarkers were evidence of the breakage–fusion–bridge cycle that leads to gene amplification. In this study we were able to replicate the increase in MNed cells and NBuds with FA deficiency, however, the effect on NBPs was not significant. We can only attribute this difference to the possibility that susceptibility to NBP formation may vary between groups of individuals donating lymphocytes for these studies.

From the results of our study we conclude that 5-MeTHF is not more efficient than FA in preventing mammalian genomic instability and cytotoxicity in this in vitro system. However, other aspects of this comparison need further research, which we are currently pursuing. These include the influence of the various forms of folate on rates of DNA methylation and malsegregation of chromosomes. In addition, further research is needed to clarify the role of choline and methionine concentration and the importance of the reduced folate carrier and the folate receptor in determining the relative bioavailability of 5-MeTHF and FA with regard to genome stability.

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