Effect of Folic Acid Supplementation on the Folate Status of Buccal Mucosa and Lymphocytes

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Abstract

Folate deficiency may be associated with an increased risk of cancer at certain sites. There is a need to measure folate status and putative biomarkers of cancer risk in the same target tissue, or in surrogate tissues. A study was carried out to develop a method for the rapid measurement of folate in human buccal mucosa and lymphocytes and to evaluate the responsiveness of this measurement in both tissues to folic acid supplementation in healthy subjects, relative to conventional markers of folate status. Three hundred and twenty-three adults, ages between 20 and 60 years, were screened for RBC folate concentrations. Sixty-five subjects with red cell folate between 200 and 650 nmol/L participated in a randomized, double blind, placebo-controlled, folate (1.2 mg) intervention trial, lasting 12 weeks. As anticipated, a significant baseline correlation \( r = 0.36, P < 0.01 \) was observed between red cell folate and plasma 5-methyltetrahydrofolate (5-MeTHF). Lymphocyte total folate was significantly associated with plasma 5-MeTHF \( r = 0.28, P < 0.05 \) and plasma total homocysteine concentration \( r = -0.34, P < 0.05 \). Buccal mucosa total folate showed no correlation with either red cell folate or 5-MeTHF, but was significantly associated with lymphocyte total folate \( r = 0.35, P < 0.01 \). Supplementation elicited a significant increase in lymphocyte total folate \( P < 0.01 \), and this was strongly associated with the increase in RBC total folate \( P < 0.01 \) and plasma 5-MeTHF \( P < 0.01 \). Buccal mucosa total folate was not influenced by folate supplementation. Methods have been developed for the rapid measurement of lymphocyte and buccal mucosal total folate. Lymphocyte folate is sensitive to folate intake and is reflected by plasma 5-MeTHF.

Introduction

Epidemiological studies have consistently revealed an association between the consumption of fruit and vegetables and reduced incidence of cancers. A relative deficiency in folate has been associated with pre-cancerous lesions or cancer of the breast, large bowel, cervix, lung, esophagus, and pancreas (1-6). There is an increasing need to measure folate in these target tissues to understand the cellular effects of folate on site-specific cancers.

Conventional measures of folate have relied on RBC or plasma total folate, and more recently, plasma 5-methyltetrahydrofolate (5-MeTHF). Plasma folate is a dynamic marker of folate status, and reflects short-term changes in folate intake (7, 8). RBC total folate is an accurate marker of long-term folate status (8), because folate is incorporated into the developing erythroblast during erythropoiesis in the bone marrow (9). Red cell folate is also considered to reflect long-term liver folate concentrations (10-12). Folate, as a supplement or a food fortificant, usually exists as pteroylmonoglutamate, which is metabolized by the enterocyte into 5-MeTHF monoglutamate, the major plasma form of the vitamin (13, 14).

Lymphocytes are increasingly used as a cellular source to investigate the effect of folate status on putative markers of DNA damage, such as strand breakage, microsatellite instability, hypomethylation, and uracil misincorporation (15-21). To our knowledge, there are no reports of the direct measurement of folate in human lymphocytes and nothing is known of the folate responsiveness of this variable. A previous study (22) reported the activity of a folate-dependant enzyme system in lymphocytes. A lymphocyte deoxyuridine suppression test as an indirect measure of lymphocyte folate has also been reported (23).

Buccal cell mucosa has been successfully used to investigate the relationship between exposure to carcinogenic hydrocarbons of tobacco smoke, DNA damage, and site-specific folate deficiency (as reviewed in refs. 24, 25). However, whether buccal mucosal total folate is sensitive to folate intake is not known. Lymphocytes and buccal mucosa provide non-invasive, ethically sound, cost-effective alternatives to biopsy material for folate analysis, making them particularly suitable for population-based folate intervention studies.

We aimed to develop procedures for the measurement of folate in human buccal mucosa and lymphocytes using an established folate-binding assay, to determine the relationship between conventional
measures of folate status with buccal mucosa and lymphocyte folate and to evaluate the responsiveness of cellular folate to folate supplementation in a healthy population.

**Subjects and Methods**

**Study Design.** A randomized, double blind, placebo-controlled, folic acid intervention trial was carried out. Men and women between 20 and 60 years of age, employed by the University of Sheffield, Doncaster and Bassetlaw Hospitals NHS Trust, and the Sheffield Teaching Hospitals NHS Trust, United Kingdom, were eligible for the study. Volunteers who were pregnant or planning a pregnancy; receiving medical care; taking methotrexate, antiepileptics drugs, or vitamin supplements; who were smokers; or who had a red cell folate concentration less than 200 nmol/L were excluded. Volunteers were screened for red cell folate concentration, providing an accurate marker of long-term folate status (26). Healthy subjects with red cell folate concentrations in the range of 200-650 nmol/L were recruited to take part in the intervention trial; this range represented the lowest 20% (n = 65) of the screened sample and satisfied sample size requirements while allowing for dropouts. The sample size was calculated from a reported effect of folate supplementation on lymphocyte DNA methylation (15). To detect a 30% improvement in DNA methylation in response to folate supplementation, with a power of 95% and a probability of 5%, required 28 subjects per group. Volunteers were randomized to receive either 1.2 mg folic acid or glucose placebo, daily for 12 weeks. The folate dose represents a significantly higher dose than the current United Kingdom and United States recommended nutrient intake of 200 μg, but is considerably lower than the pharmacologic doses used in many clinical trials (27-31).

Folic acid (1.2 mg) and placebo (glucose) capsules were manufactured by the Clinical Directorate of Pharmacy Services, Sheffield Teaching Hospitals NHS Trust, United Kingdom. The study was reviewed and approved by the North Sheffield Ethics Office of the Sheffield Teaching Hospitals NHS Trust, United Kingdom. Written informed consent was obtained from all subjects.

**Specimen Collection.** Subjects were initially screened for red cell folate concentration by donating a 500-μL sample of whole blood, obtained from the finger tip using a pro-lancet device (Roche, Welwyn Garden City, United Kingdom) into an EDTA-coated tube (Sarstedt, Leicester, United Kingdom). The mouth was then rinsed with 20 mL PBS and five 50-μL aliquots were added to 1 mL cell lysis buffer containing 2% ascorbic acid and 3 mol/L guanidine hydrochloride (Abbot Laboratories, Chicago, IL) at −80°C for the later measurement of red cell folate. Lymphocytes were prepared from fresh whole blood (detailed below) and plasma separated from the remaining sample was stored with ascorbic acid (2% w/v) at −80°C for the later measurement of 5-MeTHF and without preservative, for total homocysteine, a functional marker of folate status. Plasma cotinine was also measured, as an indicator of tobacco smoke exposure.

Buccal mucosa was collected (as detailed below), at week 0, 4, and 12, into 20 mL PBS for the later measurement of total folate. In addition, at week 4, a 500-μL sample of blood was obtained from the finger using a pro-lancet device drawn into an EDTA-coated tube for red cell folate measurement, as a marker of compliance.

**Lymphocyte Preparation.** Human lymphocytes were isolated, washed, and counted according to a method specifically developed to prepare the lymphocytes for measurements of DNA (32). Brief, whole blood was centrifuged at 24,000 × g at 4°C for 15 minutes The ‘buffy coat’ layer was removed, re-suspended in RPMI (Life Technologies, Inc., Paisley, United Kingdom), and layered onto an equal volume of lymphoprep gradient solution (Robbins Scientific, West Midlands, United Kingdom). Following centrifugation at 700 × g, 25°C for 30 min, the cells were washed 3 times using RPMI (25°C, 15 min, 700 × g), and subsequently counted using either a haemocytometer or automated cell counter (Coulter, Miami, FL). A portion of the cells were cryopreserved in 90% FCS/10% DMSO at a final cell density of 3 × 10^6/mL and stored at −80°C. The remaining cells were washed 3 times with PBS and incubated in lysis buffer containing 2% ascorbic acid and 3 mol/L guanidine hydrochloride, in the absence of light, for 4 hours at 25°C. To determine the precision of the isolation and folate extraction method, 50 mL of whole blood were collected and split into five identical replicates, which were then processed as above.

Because the lymphocytes and buccal cells are washed before lysis, there will be an absence of endogenous plasma conjugases, which convert polyglutamates to monoglutamates. To confirm that the lack of plasma conjugases does not affect the total folate measurement, we compared washed erythrocytes with whole blood. Five milliliters of whole blood were collected as above, and five 50-μL aliquots were added to 1 mL cell lysis buffer. The remaining volume was centrifuged at 1000 × g at 4°C for 10 min, plasma was removed and retained. The remaining erythrocytes were washed in PBS, the red cell fraction was then re-suspended in PBS to the original volume, and five 50-μL aliquots were added to 1 mL cell lysis buffer. All samples were incubated according to the Abbott IMx (Abbott Laboratories) method and assayed immediately.

**Buccal Cell Preparation.** The buccal cell collection protocol was an adaptation of a method previously reported (33). Following a 16-hour fast, the subjects rinsed their mouths 3 times with distilled water to remove cell debris. The buccal mucosa was firmly scraped 5 times using a small paddle (Rosslab, Cheshire, United Kingdom). The mouth was then rinsed with 20 mL PBS and the cells collected into a centrifuge tube. The cells were protected from light and placed on ice during preparation. The cell suspension was centrifuged at 750 × g, 4°C for 5 minutes, and the wet weight of the pellet calculated by removing the supernatant and then subtracting the weight of the pre-weighed microfuge.
tube. The pellet was then re-suspended in 500 μL cell lysis buffer containing 2% ascorbic acid and 3 mol/L guanidine hydrochloride (Abbot Laboratories) and the cells stored at −80°C. Before measurement, the cell suspension was sonicated, on ice, protected from light, using a sonic probe (Jencons GE50, Bedfordshire, United Kingdom) for 40 seconds. Cells were investigated under light microscopy (Olympus CHA10, 10×) to confirm complete cell lysis.

To determine the effect of sonication and freezing on folate, five replicates of a known amount (14 ng/mL) of a standard solution of pteroylglutamic acid (folic acid; Abbot Laboratories) were processed using the above method. The reproducibility of the assay was determined using buccal mucosa collected on 2 separate days, split into either six or four equal parts, and processed individually. The automated ion capture assay requires the presence of ascorbic acid, which interferes with most common protein determination assays, and we, therefore, also evaluated the suitability of relating total folate concentration to either pellet wet weight or protein. Buccal mucosa samples were split into six equal parts, and sonicated separately using PBS instead of lysis buffer, obtained wet weight using the above method, or protein concentration using a modified bicinchoninic acid assay (ref. 34; Sigma Chemical Co., BCA-1, Surrey, United Kingdom).

**Labratory Methods.** A previously described HPLC procedure was used to measure 5-MeTHF in the plasma, buccal mucosa, and lymphocyte lysates (7). The automated Ion Capture Assay system (Abbot IMx; Abbot Laboratories) was used to determine the total folate concentration in RBCs, buccal mucosa, and lymphocyte cell lysates. Briefly, the universal bovine milk folate-binding protein (FBP) is used to bind folates from the sample, the polyanion FBP complexes are bound to a glass fiber matrix and washed. Alkaline phosphatase conjugate is added to bind the unoccupied FBP sites, the unbound conjugate is washed and then 4-methylumbelliferyl phosphate is added to matrix, and the fluorescent product is measured by the optical assembly. An automated Fluorescence Polarisation Immunoassay (Abbot IMx, FPIA homocysteine assay; Abbot Laboratories) was used to measure plasma total homocysteine. Plasma cotinine was determined using an anti-cotinine antibody enzyme immunoassay kit (Cozart Biosciences, Oxford, United Kingdom).

**Data Analysis.** Statistical analyses were done with SPSS software, version 10.0 (SPSS Inc., Chicago, IL). Simple descriptive statistics (mean, median, interquartile range, skewness, and distribution) were used to describe the characteristics of the subjects and baseline measurements. Correlation between variables was evaluated with the use of Spearman’s rank-order coefficient correlation. Kruskall-Wallis non-parametric independent samples test was used to evaluate the effect of intervention, comparing differences between baseline and follow-up in the folate group with those in the placebo group, for each variable of interest. The effect of intervention over time is represented using a clustered box plot with summaries of separate variables against intervention group. The effect of plasma cotinine as confounder was evaluated by Kruskall-Wallis non-parametric independent samples test, with the folate concentrations as test variable against smoker or nonsmoker as grouping variable. All tests were two-tailed and were considered significant when $P < 0.05$.

**Results**

**Reproducibility of Measurements.** The coefficient of variation for the measurement of buccal mucosa total folate relative to either protein or wet weight was 7% and 2%, respectively. The use of wet pellet weight offers an accurate, rapid method of relating buccal mucosa folate to cell number without the additional problems of ascorbate interference often associated with protein measurements. The sonication processing of the buccal mucosa, while essential for extraction of cellular folate, had no significant effect on folate concentration. A whole blood sample, which was split into 5 equal volumes, from which the lymphocytes were separately isolated and folate extracted, produced a coefficient of variation of 2.8%. The intra- and inter-batch coefficients of variation were: red cell folate 6%, 7%; plasma 5-MeTHF 3%, 8%; and homocysteine 2%, 2%, respectively. The IMx was also used to measure red cell folate in a sample (M05302103) obtained from the United Kingdom National External Quality Assessment Service (UKNEQAS). The mean for this sample, as determined by IMx in 21 external laboratories, was 350.7 ng/mL (28/10/02); we reported a mean of 311 ng/mL.

There was no significant difference in the calculated value for whole blood folate measured using the IMx folate binding immunofluorescent assay on whole blood (541 nmol/L ± 24) or in washed RBCs (499 nmol/L ± 19) corrected for plasma folate (34 nmol/L ± 1). As anticipated, the monoglutamate form of 5-MeTHF was not detectable within the cell lysates of either lymphocytes or buccal mucosa.

**Response to Intervention.** Sixty-five subjects were recruited to the study, to allow for a 15% dropout rate. Sixty-one subjects (folate $n = 28$, placebo $n = 33$) completed the intervention. There were no significant differences in the distribution of age or sex between the two groups: folate: $M = 5$, $F = 23$, 42 years ($±9$ years) and placebo: $M = 3$, $F = 30$, 40 years ($±8$ years). As shown in Table 1, the baseline values are within expected ranges.

**Table 1. Baseline concentrations of total folate in red cells, buccal mucosa, and lymphocytes, and related plasma variables**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Folate</td>
</tr>
<tr>
<td>Buccal mucosa folate (pg/mg)</td>
<td>284 (193-467)</td>
</tr>
<tr>
<td>Lymphocyte folate (ng/10⁶ cells)</td>
<td>0.21 (0.17-0.32)</td>
</tr>
<tr>
<td>Red cell folate (nmol/L)</td>
<td>552 (469-655)</td>
</tr>
<tr>
<td>Plasma 5-MeTHF (nmol/L)</td>
<td>24 (16-32)</td>
</tr>
<tr>
<td>Plasma homocysteine (μmol/L)</td>
<td>11.2 (10-15)</td>
</tr>
<tr>
<td>Plasma cotinine (ng/mL)</td>
<td>0.93 (0.0-5.82)</td>
</tr>
</tbody>
</table>

**NOTE:** Median (interquartile range).
for a healthy western population. Because subjects were recruited from the lowest 20% of those screened (red cell folate = 200-650 nmol/L), these values fall into the lower part of the reference range. There were no significant differences observed between groups for any of the baseline measurements. Plasma cotinine concentrations suggested that 10 subjects had been exposed to sufficient tobacco smoke to elevate plasma cotinine, despite claiming to be nonsmokers. These subjects had a lower buccal mucosa total folate concentration than the rest of the sample [mean 277 (±205) pg/mg pellet wet weight, mean 362 (±239) pg/mg pellet wet weight, respectively], although this did not reach significance.

Table 2 summarizes results from Spearman’s bivariate one-tailed correlation test that evaluated baseline associations. The correlation between plasma 5-MeTHF and red cell folate was highly significant. A significant baseline association was also observed between intracellular lymphocyte folate and plasma 5-MeTHF and with the functional folate marker plasma total homocysteine. A significant association was also observed between buccal mucosa and lymphocyte folate. No significant associations were found at week 0 between buccal mucosa and plasma 5-MeTHF, red cell folate, or plasma homocysteine.

The 12 weeks of supplementation elicited a significant increase in plasma and red cell folate (Figs. 1 and 2) in contrast with the values in the placebo group, which were unchanged from baseline. Lymphocyte total folate was shown, for the first time, to be highly responsive to folate intake (Fig. 3), increasing significantly in the supplement group, with no change in the placebo group. This increase was significantly associated with the increase in red cell folate and plasma 5-MeTHF. Conversely, buccal mucosa total folate was not responsive to folate supplementation (Fig. 4). Plasma cotinine concentration had no effect as a confounder on the sensitivity of the mucosal cells to folate intervention.

Table 2. Summary of baseline correlations between variables

<table>
<thead>
<tr>
<th>Tested variables</th>
<th>Spearman’s rho (r)</th>
<th>95% Confidence Interval</th>
<th>P value for correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte folate vs:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma 5-MeTHF</td>
<td>0.28</td>
<td>0.08 to 0.58</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RBC folate</td>
<td>0.17</td>
<td>0.21 to 0.50</td>
<td>0.80</td>
</tr>
<tr>
<td>Buccal cell folate</td>
<td>0.35</td>
<td>0.02 to 0.62</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Plasma homocysteine</td>
<td>-0.34</td>
<td>-0.03 to -0.62</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Buccal cell folate</td>
<td>-0.13</td>
<td>-0.25 to -0.46</td>
<td>0.36</td>
</tr>
<tr>
<td>Plasma 5-MeTHF</td>
<td>0.36</td>
<td>0.01 to 0.63</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Figure 1. The effect of intervention on plasma 5-MeTHF concentration. The change seen in the folate-treated group at 12 weeks was significantly different from that seen in the placebo group (P < 0.01).

Figure 2. The effect of intervention on red cell folate concentration. The change seen in the folate-treated group at 4 and 12 weeks was significantly different from that seen in the placebo group (P < 0.01).

Figure 3. The effect of intervention on lymphocyte folate concentration. The change seen in the folate-treated group at 12 weeks was significantly different from that seen in the placebo group (P < 0.01).
Discussion

In this study, we have developed relatively high-throughput methods to measure total folate concentrations in buccal mucosa and lymphocytes, both of which are increasingly used to study biomarkers of cancer risk. In intervention trials in healthy people, it is unethical and inappropriate to obtain tissue from target cells via biopsy, but it is important to investigate the levels of folate and the various folate metabolites in a surrogate, easily accessible cell type, such as lymphocytes or epithelial cells. Current interest in the link between folate status and DNA instability has focused on measuring DNA damage in lymphocytes and buccal cells while largely relying on RBCs or plasma for measures of folate status. It would be preferable to make both sets of measurements in the same material. Using buccal cells or lymphocytes allows this.

The buccal mucosa method represents a novel alternative to current labor-intensive methods for tissue folate determination. The lymphocyte method is particularly appropriate for lymphocyte isolation procedures currently used for measures of DNA stability in lymphocytes (32). Using the FBP assay automated for the IMx analyzer, we have shown that washed red cells, when corrected for plasma, report the same total folate concentration as whole blood. Evidently, the FBP assay does not require a separate conjugase treatment. Indeed, the FBP, which is the basis of this assay, has already been shown to bind folate polyglutamates in washed red cells, liver, and brain tissue (35-42). Cellular folate can include 5-methyl-THF, formyl-THF, methenyl-THF, methylene-THF, and un-substituted tetrahydrofolates. Bagley and Selhub (35-42) have elegantly shown that a bovine milk FBP can bind to all these forms of cellular folate. FBP is the basis for many commercial folate assays, including radioimmunoassay, although there is still some uncertainty with respect to the binding affinities for different forms of folate. However, the bovine milk FBP, assumed to be the FBP in all commercial assays, is evidently able to detect differences in the distribution of intracellular folates in people homozygous for the MTHFR (C677T) genotype, compared with wild-type or heterozygotes (35-42). In our study, we observed significant association at baseline between red cell and plasma folate, as anticipated from previous work (43-45). Total lymphocyte folate correlated strongly at baseline with plasma 5-MeTHF, and homocysteine levels, but not with red cell folate, suggesting that lymphocyte folate may be a more dynamic marker of folate status than red cell folate, which reflects folate incorporated during erythropoiesis. Buccal mucosa folate was not associated with red cell folate or plasma folate concentrations, and this is consistent with previous studies (33, 46). However, we found that buccal mucosa folate did correlate strongly with lymphocyte folate, suggesting that they both reflect some aspect of the cellular folate pool.

Following a supplement of folic acid for 12 weeks, red cell folate and plasma 5-MeTHF increased significantly and to an extent comparable with that reported in other studies (43-45, 47). In addition, we have shown, for the first time, a significant response of intracellular lymphocyte total folate to folic acid intervention. The percentage change in lymphocyte folate was remarkably similar to that of red cell folate and plasma 5-MeTHF during intervention. This suggests that both red cell folate and plasma 5-MeTHF are good predictors of lymphocyte folate status and may be suitable surrogates for this cellular material. However, because the methods described here allows for a rapid, efficient, and accurate measurement of lymphocyte folate, this should be considered the preferred source of material for determining folate status when interactions between folate and DNA stability is a focus of interest. A human intervention study to determine effects of folate from measures of DNA stability and folate in the same cells is now underway.

Despite 12 weeks of folate supplementation, at a dose significantly higher than the United Kingdom recommended nutrient intake, no increase in buccal mucosa folate was observed. There has been some interest in the use of buccal mucosa as a cellular sample for the measurement of biomarkers of DNA damage and folate status (25, 33, 46). The baseline measurements we obtained for buccal mucosa total folate are almost identical to the baseline values previously obtained using the microbiological assay (33). As buccal mucosa total folate does not respond to folate supplementation and shows no association with other measures of folate status following intervention, its value as a general biomarker of tissue responsiveness to folate intake is limited. This lack of response may reflect the turnover time of mucosal cells although we are unaware of any published information about this. In addition, the buccal mucosa may have specific apical membrane folate uptake properties, which make this cell type less useful as a marker of longer-term folate status. Finally, differences in the precise technique of exfoliating the cells may influence the maturity of the cells collected and thereby the folate concentration measured.

The measurement of lymphocyte folate we have developed is rapid and cost effective and more importantly can be used alongside established protocols for the isolation of lymphocytes to evaluate DNA damage. This allows the reporting of DNA damage and folate
concentration from the same source, which is also sensitive to changes in folate status. The measurement itself is strongly associated with conventional measures of folate status and is extremely sensitive to changes in folate intake.

References

42. Stern LL, Bagley PJ, Rosenberg IH, Selhub J. Conversion of 5-formyltetrahydrofolic acid to 5-methyltetrahydrofolic acid is unimpaired in folate-adapted persons homozygous for the C677T mutation in the methylenetetrahydrofolate reductase gene. J Nutr 2000;130(9):2238-42.
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