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Micronuclei in lymphocytes and exfoliated buccal cells of postmenopausal women with dietary changes in folate

Nina Titenko-Holland [a,*], Robert A. Jacob [b], Nong Shang [a], Anita Balaraman [a], Martyn T. Smith [a]

[a] *Division of Environmental Health Sciences, 217 Warren Hall, School of Public Health, University of California, Berkeley, CA 94720-7360, USA*

[b] *USDA, Western Human Nutrition Research Center, POB 29997, Presidio of San Francisco, San Francisco, CA 94129, USA*

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Abstract

Folate deficiency is associated with anemia, birth defects, cancer and neuropsychiatric disorders. The purpose of this study was to determine if a moderate folate deficiency during controlled changes in folate intake would affect chromosomal damage in lymphocytes and buccal cells. A study of nine healthy postmenopausal women volunteers (age 49–63 years) was carried out in a metabolic unit (baseline week with folate intake of 195 μg/day, five-week depletion at 56 μg/day, and gradual repletion including four weeks at 111 μg/day, 11 days at 286 μg/day and 9 days at 516 μg/day). Plasma folate, vitamin B-12, and homocysteine were measured weekly. Cytogenetic damage was assessed by scoring micronucleus (MN) frequency in lymphocytes and buccal cells three times: (1) at the beginning of the study, (2) at the end of depletion, and (3) after repletion. The MN frequency increased in binucleated lymphocytes, as well as in all lymphocytes, after depletion ($p = 0.037$), and later decreased following repletion ($p = 0.028$). Both kinetochore-positive and kinetochore-negative MN were increased after depletion ($p = 0.015$ and 0.028), but after repletion only the change in kinetochore-positive MN was statistically significant ($p = 0.048$). The main variables affecting MN were: (1) vitamin B-12 level, (2) plasma folate level, and (3) baseline frequency of MN. The MN frequency in exfoliated buccal cells was decreased after dietary supplementation of 516 μg/day folate ($p = 0.010$). Thus, low folate, without clinical symptoms of anemia, results in higher levels of cytogenetic damage in both the blood and oral cavity of postmenopausal women.

Keywords: Dietary folate; Micronucleus frequency; Postmenopausal woman; Lymphocyte; Buccal cell

1. Introduction

Folate deficiency has been associated with cancers of epithelial origin [1,19,21,25], as well as neural tube defects and other birth abnormalities [22,35,48]. Folate deficiency may also contribute to myocardial infarction and neuropsychiatric disorders, especially in the elderly [6,7]. Folate requirements in different health groups have been the subject of extensive discussion [3,26]. More than 10% of the general population, an estimated 50% percent of

* Corresponding author. Tel.: +1-510-642-8781; Fax: +1-510-642-0427; E-mail: ninah@uclink4.berkeley.edu

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some low-income minority populations, and at least 25% of elderly people have a folic acid deficiency [3].

Biochemical studies have suggested that disturbances in the nucleotide pool, DNA synthesis, and cell growth are responsible for the adverse health effects associated with folate deficiency [18,47]. A recent study [4] showed that in folate deficiency, synthesis of thymidylic acid from dUMP, its precursor, was inhibited, causing dUMP to accumulate in the cell. This condition increases the chance of misincorporation of uracil into DNA in place of thymine, and when the DNA repair system removes the uracil it creates breaks in both DNA and chromosomes. In the group of folate deficient volunteers, massive incorporation of uracil into DNA and an increased frequency of micronucleated erythrocytes, presumably resulting from chromosome breaks, were observed [4].

A possible link between chromosome damage and folate deficiency was suggested in early experimental studies with hereditary fragile sites in human chromosomes induced as a result of cultivation under low-folate conditions [41]. Increased micronucleus (MN) frequency and chromosome breakage in human lymphocytes, erythrocytes and bone marrow cells were observed in individuals low in folate (reviewed in Ref. [23]). One large population study in Australia showed plasma folate and vitamin B-12 levels in young individuals were more important than vitamin C and E levels in minimizing chromosome damage in binucleated lymphocytes [12,13]. The highest levels of MN erythrocytes were observed in splenectomized individuals with exceptionally low values of plasma folate, red cell folate, or plasma B-12 [24]. All these data suggest that low folate status is associated with increased cytogenetic damage. The frequency of chromosome aberrations and MN also increases with age [46], although there is limited data available on the relationship between low folate and cytogenetic damage in older people. Neither of these studies of folate deficiency in human populations used antikinetochore-antibody staining to analyze the mechanism of MN formation. Very few data are available on effect of folate status and MN frequency in exfoliated cells [33].

Metabolism of folic acid is intimately connected with vitamin B-12 and homocysteine. Several studies have shown a significant association between low plasma folate and B-12 levels, on one hand, and increased MN frequency on the other [9,13,24]. Moderate folate deficiency has been linked to increased plasma homocysteine in both men and women [17,32]. A unique opportunity to study whether folate deficiency caused by controlled dietary folate depletion will result in cytogenetic changes, and whether vitamin B-12 and homocysteine were associated with these changes, was presented in a project carried out at the metabolic unit of the Western Human Nutrition Research Center, San Francisco [16]. While a group of women volunteers lived at the Center for 13 weeks, their folate intake was controlled to create a mild folate depletion (56 μg/day) followed by gradual folate repletion reaching 3 times the RDA-1998 of 180 μg/day (~ 516 μg/day). Postmenopausal women were chosen for this study to avoid the potential reproductive risk associated with low folate status. Multiple parameters of diet and energy consumption, as well as folate, vitamin B-12, and homocysteine status were monitored weekly. Results of this study showing that moderate folate depletion increased plasma homocysteine and decreased lymphocyte DNA methylation have been described elsewhere [16]. Here, we report our findings with the MN assay in this group, where MN levels in both lymphocytes and buccal epithelial cells were determined.

2. Materials and methods

2.1. Sample collection

Blood and exfoliated buccal cells were collected from a group of ten postmenopausal women. Health prestudy screening, study protocol and parameters of diet during folate depletion and repletion have been described elsewhere [16]. Briefly, healthy volunteer nonsmoking women, ages 49–63 years (57.9 ± 5.5 years, Table 1), were admitted to the metabolic unit of the USDA, ARS Western Human Nutrition Research Center (WHNRC) after medical and physiological screening. Tests for plasma folate and vitamin B-12, alcohol, tobacco, and drug use were also performed. Signed informed consent was obtained

Table 1
Demographics and vitamin levels in postmenopausal women during dietary study with controlled intake of folate

	Group description		
No. of participants	9		
Age (years)	57.9 ± 5.5[a]		
Weight (kg)	70.6 ± 11.9		
Smoking	No		
Alcohol	No		
Hormonal supplements	2 Yes/7 No		
	Sample collection		
	d1-5 Baseline	d6-41 Depletion	d42-91 Repletion
Folate intake (μg/day)	196	56	228
Folate in plasma (ng/ml)	9.6 ± 5.8	4.4 ± 2.3[c]	7.4 ± 1.9[d]
B-12 in plasma (pg/ml)	462 ± 184	412 ± 151[b]	391 ± 128
Plasma homocysteine (μmol/l)	10.0 ± 1.2	12.0 ± 2.7[c]	13.2 ± 4.3

[a]Mean ± standard deviation for all data in the table.
[b]$p < 0.05$, [c]$p < 0.01$, [d]$p < 0.001$ statistically significant vs. previous collection (by-pair comparison, Student's t-test).

from each volunteer. For the duration of the 91 day study, the subjects lived and ate all meals in the WHNRC metabolic unit. Subjects consumed the same experimental low folate diet in a four day menu rotation for the entire period. The diet provided 56 μg/day of folate and was supplemented with varying amounts of synthetic folic acid to provide depletion (56 μg/day, no folate supplement) and repletion (up to 516 μg/day) of folate. Baseline screening (day 1–5) with folate intake of 195 μg/day was followed by the five-week depletion period with 56 μg/day, and repletion period consisting of 4 weeks of 111 μg/day, 11 days of 286 μg/day and 9 days of 516 μg/day of dietary folate. The five week folate depletion period (day 6–41) was designed to reduce body folate stores and produce moderate but not severe folate deficiency, analogous to that of free-living women with chronically low folate intake and body status but without macrocytosis or anemia. The folic acid supplement solutions were mixed into applesauce served at each breakfast and dinner during the repletion period (day 42–91, an average folate intake of 228 μg/day). The diet was supplemented daily with three different vitamin/mineral tablets to provide at least 80% of the RDA-1989 [28] for each essential micronutrient except folate (the diet plus supplements provided 124% of the RDA for vitamin B-12). Individual energy intake and body weights were estimated and adjustments were made to energy intake if the subjects deviated beyond ±5% of the baseline weight (70.6 ± 11.9 kg, Table 1).

Specimens of blood and exfoliated buccal cells were collected from each individual three times: (1) at the beginning of the study (background), (2) at the end of depletion (day 41), and (3) at the end of repletion (days 89, 90 and 92). Blood for serum and red cell folate, plasma B-12 and homocysteine determinations was collected by venipuncture using EDTA (7.5%) as anticoagulant and processed immediately. Methods for determining folate and B-12 levels, using a competitive protein binding radioassay kit (Quantaphase II B-12/Folate Radioassay, BioRad, Hercules, CA), were described previously [16]. Total plasma homocysteine was analyzed by HPLC fluorescence detection after derivatization with a fluorescent sulfonic acid reagent [2]. One of the individuals with an abnormally high level of homocysteine (more than three standard deviations (sd) from the mean) was excluded from further analysis.

2.2. Isolated human lymphocyte culture

Fasting blood was collected in heparin-vacutainers and lymphocytes were isolated using Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradients

and cultured as described previously [45]. Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS) and layered onto Ficoll-Paque with a ratio of blood + PBS: Ficoll maintained at 4:3. The blood was centrifuged at 2000 rpm for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 1200 rpm for 10 min each, and then washed with RPMI 1640 media. Cell density was counted with a hemocytometer. Typically, each culture consisted of an initial density of 1×10^6 cells in 2 ml of culture medium. Culture medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin, 100 ug/ml streptomycin (Gibco, Grand Island, NY) and 1.5% phytohemagglutinin (PHA, HA15, Burroughs-Wellcome, Greenville, NC). The lymphocyte cultures were grown in a humidified incubator with 5% CO_2 at 37°C in 15 ml conical polystyrene centrifuge tubes. Cytochalasin B (Sigma, St. Louis, MO) (5 μg/ml) was added to the cultures at 44 h post-initiation as described in Ref. [11]. Cytochalasin B prevents the cells from completing cytokinesis resulting in the formation of multinucleated cells. At 72 h, lymphocyte cultures were spun directly (48 g, 10 min) onto glass slides using a cytocentrifuge (Shandon, Sewickley, PA). Slides were allowed to air dry at room temperature for 15 min before methanol fixation. Slides were stored at −20°C in a sealed box, desiccated under a N_2 atmosphere.

2.3. Immunofluorescent staining

Detailed procedures for performing the antikinetochore antibody staining method have been described elsewhere [8]. Briefly, methanol fixed slides were incubated for 5 min in PBS containing 0.1% Tween 20. Excess fluid was drained from the slide and 40–50 μl of the antikinetochore antibody (Chemicon, Temecula, CA) diluted 1:1 with PBS containing 0.2% Tween 20 is applied. The slide was then coverslipped and placed in a humidified chamber at 37°C for 1 h. Following two washes in PBS containing 0.1% Tween 20 for 5 min each, excess fluid was again drained and the slides are covered with a 1:50 dilution of fluorescent goat anti-human IgG (Chemicon, Temecula, CA) and incubated again for 1 h. Because the fluorescent-labeled antibodies fade upon exposure to light, this and all subsequent steps were conducted under yellow light. The slides were rinsed twice in buffer plus 0.1% Tween 20 and counterstained with DNA-dye 4′6-diamidino-2-phenylindole (DAPI) (2 μg/ml) in an antifade solution [20]. Slides were stored refrigerated for up to a week prior to analysis by microscopy.

2.4. Cell division kinetics and cell viability

Cell viabilities were determined at 72 h using Trypan blue (0.16%). Replicative index (RI), a measure of cell division kinetics, was calculated by scoring at least 400 cells per dose or sample, by counting the percent of cells containing 1, 2, 3 or more nuclei per individual. RI was calculated as follows:

$$\mathrm{RI} = [(1 \times \%\ \text{mononuclear cells}) + (2 \times \%\ \text{binuclear cells}) + (3 \times \%\ \text{tri}) + (4 \times \%\ \text{tetra})]/100.$$

2.5. Exfoliated cell collection

Buccal cells were collected and analyzed according to a procedure described earlier [43]. Oral mucosa was swabbed with a moistened wooden tongue depressor. We prepared slides both by direct smearing of buccal cells or by dropping the washed cell suspension with determined density onto the slides. Washes were performed twice in a buffer solution containing 0.01 M Tris–HCl, 0.1 M EDTA and 0.02 M NaCl at pH 7.0, and cells (50–100 μl of 1.5×10^6–2×10^6/ml suspension) were dropped onto prewarmed slides (37°C). Washes with this buffer helped to inactivate endogenous DNases present in the oral cavity and to remove bacteria and cell debris that would complicate scoring. Dropping was preferable to smearing because it allowed for the even distribution of at least 3000–5000 cells per slide at an optimal density without overlap and eliminated debris and bacteria. After dropping, the cells were allowed to air-dry and fixed in methanol (80% v/v) at 0°C for 20 min. After all specimens were collected, they were mixed, coded and stained with propidium iodide (1 μg/ml, Sigma) in antifade solution [20].

2.6. Scoring procedure and criteria

Lymphocytes were analyzed under a total magnification of ×1000 using a Nikon microscope with a filter for fluorescein (excitation at 470 nm, dichroic at 510 nm, emission at 520–560 nm) and quinacrine (excitation at 400–440 nm, dichroic at 450 nm, barrier at 470 nm). At least 1000 binucleated lymphocytes (those that had undergone one mitotic division) were scored for the number of MN. All mono-, tri- and tetranucleated cells available at the same slide area where these binucleated cells were analyzed were also scored for the presence of MN. The number of these cells was dependent on proliferation (replicative index) and was defined by default (~ 850, 20 and 50 per individual, respectively). When a MN was located using the quinacrine filter, the presence or absence of kinetochore staining was determined by switching to the fluorescent filter.

Exfoliated buccal cells were analyzed under a total magnification of ×1000 using a Nikon microscope with a filter for propidium iodide (excitation at 470 nm, dichroic at 510 nm, emission at 520–560 nm). Two thousand cells were scored for each individual and the presence of MN was recorded separately for each thousand cells. Only cells that were not smeared, clumped or overlapped and that contained intact nuclei were included in the analysis. Cells undergoing degenerative processes such as karyorhexis, karyolysis, and/or fragmentation of the nucleus [43] were recorded separately and were not included in MN analysis. The frequency of MN was estimated based on the number of normal exfoliated cells.

The following criteria for MN analysis were used in lymphocytes and exfoliated cells. MN must have: (a) been less than 1/3 diameter of the main nucleus, (b) been on the same plane of focus, (c) had the same color, texture and refraction as the main nucleus, (d) had smooth oval or round shape; and (e) been clearly separated from the main nucleus. Questionable MN were disregarded.

2.7. Statistical analysis

Statistical analysis of data was defined by the design of the study with a relatively small number of subjects who had a high initial variability in both MN and plasma folate level. Longitudinal design allowed by-pair comparison of the data from each individual who served as her own control. This approach eliminated a significant part of the variability by excluding (1) inter-individual variability, and (2) a part of intra-individual variability unrelated to dietary folate changes. A controlled environment of the metabolic unit with standardized diet and lifestyle allowed the changes introduced by folate to become a major variable. Differences in plasma folate, vitamin B-12, and homocysteine between baseline, depletion and repletion were assessed by paired *t*-tests of values at the end of each period. Non-parametric Wilcoxon's sign-rank test was used to assess the significance of the change in the frequency of MN and other parameters due to folate depletion or repletion. All data were expressed as an average ±sd throughout the text and in the tables and graphs since sd is an index of the inter-individual differences. The change in the MN frequency may depend on the baseline and change in plasma folate, homocysteine and vitamin B-12 levels. Age, body weight, and hormonal supplements were also considered as possible covariates. These associations were measured by non-parametric Spearman rank correlation coefficients. Linear correlation coefficients were also assessed. *p*-Values were calculated based on the Spearman rank test. In all cases differences were considered significant where p was ≤ 0.05.

3. Results

3.1. Micronuclei and replicative index in lymphocytes

Controlled folate intake during the depletion phase of the study resulted in a significant decrease of plasma folate (9.6 ± 5.8 ng/ml baseline level vs. 4.4 ± 2.3 ng/ml at day 42, $p < 0.01$, Table 1) followed by an increase after repletion (7.4 ± 1.9, $p < 0.001$). Initially, the nine study subjects differed appreciably in their plasma folate (2.9–19.5 ng/ml) and B-12 (251–831 pg/ml), but this variability decreased at the end of the study due to the controlled diet and environment (4.6–8.7 ng/ml and 314–698 pg/ml for folate and B-12, respectively) [16]. Vitamin B-12 was decreased from the baseline level of 462 ± 184 pg/ml to 412 ± 151 pg/ml at the end of

depletion period ($p < 0.05$). A slightly lower level was observed after the repletion period (391 ± 128 pg/ml) but this difference was not significant compared to the day 42 level. At the end of the folate depletion period, five of the nine subjects had plasma folate concentrations below or near the lower limit of normal (3–20 ng/ml, [42]), while all vitamin B-12 concentrations remained in the normal range of 200–835 pg/ml [42]. Individual changes in the MN frequency during folate depletion and repletion are shown in Table 2. Though the average levels of total MN, micronucleated cells, and kinetochore-positive and kinetochore-negative MN reflect the changes in the study group, more precise statistical analysis was performed using by-pair individual comparison of these parameters after depletion and repletion. The decrease in folate intake was accompanied by a significant increase in total MN frequency in binucleated cells ($p = 0.038$, Table 3). Less than 20% of binucleated cells with MN had more than one MN, and there was no significant difference in the frequency of multiple MN between different cell collections. No significant change in the replicative index was observed due to folate depletion and repletion (Table 2).

To assess whether MN changes occur in subpopulations of lymphocytes other than binucleated cells, we also analyzed mononucleated, trinucleated and tetranucleated cells. This approach allowed us to obtain more information regarding the effect of folate depletion on cytogenetic damage in human lymphocytes in vivo. The same pattern of increased MN frequency after folate depletion followed by decreased frequency after repletion, described above for binucleated lymphocytes, was also observed for mononucleated cells (Fig. 1). The frequency of MN

Table 2
Cytogenetic effects of dietary folate changes on micronuclei in binucleated human lymphocytes

	Micronuclei/1000 cells		
	d1-5 Baseline	d6-41 Depletion	d42-91 Repletion
Individuals (ID no.)			
1	5.9[a]	6.6	3.9
2	22.7	19.0	24.7
3	6.9	8.7	12.7
4	25.1	23.0	16.7
5	11.6	33.5	9.6
7[b]	17.0	20.5	26.4
8	8.2	14.9	8.9
9	22.0	23.7	14.8
10	14.0	25.2	8.9
Group total			
MN cells/1000 cells	14.8 ± 7.3[c]	19.5 ± 8.4[d]	14.1 ± 7.5[e]
MN(total)/1000 cells	17.2 ± 9.2	23.3 ± 11.1[f]	16.2 ± 9.0
Kinetochore-positive MN + per 1000 cells	9.4 ± 4.8	12.5 ± 6.4[g]	8.7 ± 4.6
Kinetochore-negative MN − per 1000 cells	7.4 ± 5.5	10.8 ± 7.6	7.4 ± 5.0
Replicative index[h]	1.6 ± 0.2	1.7 ± 0.1	1.7 ± 0.2

[a] Micronucleated cells per 1000 binucleate lymphocytes (between 980 and 1055 cells were scored from two parallel cultures for each individual).
[b] Subject 6 was excluded as an outlier based on an unusually high level of plasma homocysteine.
[c] Mean ± sd.
[d] Difference between the levels of MN at the first and second collection was calculated using by-pair comparison, Wilcoxon's sign rank test, $p = 0.066$.
[e] Difference between the levels of MN at the second (depletion) and third (repletion) collections, by Wilcoxon's one-tail test, $p = 0.082$.
[f] $p = 0.038$.
[g] $p = 0.029$.
[h] Replicative index = [(% mononuclear cells) + (2 × % binuclear cells) + (3 × % trinuclear cells) + (4 × %tetra or > tetranuclear cells)]/100.

Table 3
Micronucleus frequency in subpopulations of lymphocytes with different nuclear number

Lymphocytes	Mononucleated	Binucleated	Trinucleated	Tetranucleated	Total nuclei
Cell number	823.6 ± 419.2[a]	1014.6 ± 36.4	17.9 ± 15.1	51.5 ± 33.0	3112.2 ± 355.9[b]
MN per 1000 cells					
1st collection	3.4 ± 3.1	17.2 ± 9.2	32.2 ± 43.6	238.5 ± 301.2	8.9 ± 3.3[c]
2nd collection	5.1 ± 4.7	23.3 ± 11.1	128.9 ± 116.1	195.1 ± 111.4	13.0 ± 5.7
3rd collection	3.4 ± 2.1	16.2 ± 9.0	167.7 ± 119.5	192.2 ± 174.8	9.7 ± 5.2
p-Values for changes between 1st and 2nd collection					
MN cells	0.103[d]	0.066	0.038	0.367	
Total MN	0.103	0.038	0.038	0.455	0.037
MN +	0.066	0.029	0.038	0.103	0.037
MN −	0.285	0.126	0.455	0.455	0.082
p-Values for changes between 2nd and 3rd collection					
MN cells	0.150	0.082	0.066	0.249	
Total MN	0.125	0.082	0.212	0.249	0.028
MN +	0.049	0.180	0.325	0.064	0.020
MN −	0.410	0.213	0.455	0.367	0.248

[a]Average number of cells (mean ± sd) scored for each of three cell collections for each of nine study subjects.
[b]To compare the MN frequency for lymphocytes with different nuclear number, total nucleus number for all scored cells was determined as a sum of mononucleated cells + number of binucleated cell multiplying by two, +trinucleated cells × 3, and +tetranucleated cells × 4.
[c]Weighted MN frequency per 1000 nuclei of mono-, bi-, tri- and tetranucleated cells normalized for the nucleus number (mean ± sd).
[d]*p*-Values for the MN changes by Wilcoxon's one-tail rank test.

in mononucleated cells varied between 3.4 MN (1st and 3rd collections) and 5.1 MN (2nd collection) per 1000 cells (Table 3). The frequency was significantly higher for binucleated cells (16.2–23.3 MN per 1000 cells, $p < 0.01$). An estimation for tri- and tetranucleated cells was less stable since comparatively small numbers of these cells were available for analysis (~ 20 and 50 for each individual in each of the three cell collections). However, they had the highest frequency of MN per cell (Table 3). The MN frequency for trinucleated cells (32.2–167.7 MN per 1000 cells) was statistically different from that in the tetranucleated cells (192.2 and 238.5 MN per 1000 cells, $p < 0.05$). After MN frequencies for all cells were normalized per nucleus number to account for the difference in mono-, bi-, tri- and tetranucleated cells, the significance of the differences in the MN frequency per 1000 nuclei between these subpopulations was sustained ($p < 0.05$, Fig. 2). The effect of both depletion and repletion on total MN frequency adjusted for nucleus number was significant ($p =$ 0.037 and 0.028, respectively, Table 3).

The mechanism of MN formation was examined using antikinetochore-antibody staining to distinguish between kinetochore-positive (MN +) and kinetochore-negative (MN −) micronuclei. A slightly larger fraction (54–56%) of lymphocyte MN at all collection times were MN + (Table 3), reflecting aneugenic mechanism of cytogenetic damage. Both MN + and MN − were similarly affected by dietary changes in folate intake, but only changes in the MN + frequency were significant after depletion ($p = 0.029$) and repletion ($p = 0.027$) for all analyzed lymphocytes (Table 3). An increase in MN − after depletion approached statistical significance (p $= 0.066$). Similar results were obtained when only mononucleated and binucleated cells were included in the analysis (Fig. 3). Total MN were increased after depletion ($p = 0.014$) and decreased after repletion ($p = 0.066$) as were MN + ($p = 0.015$ and $p = 0.048$ for depletion and repletion, respectively). MN − were elevated after depletion ($p = 0.028$), while a small decrease following repletion was not statistically significant ($p = 0.41$). These data suggest that both chromosome breakage and aneuploidy were induced by folate depletion.

3.2. Micronuclei in exfoliated buccal cells

Between 0 and 6 MN per thousand buccal cells were observed during dietary folate study of post-

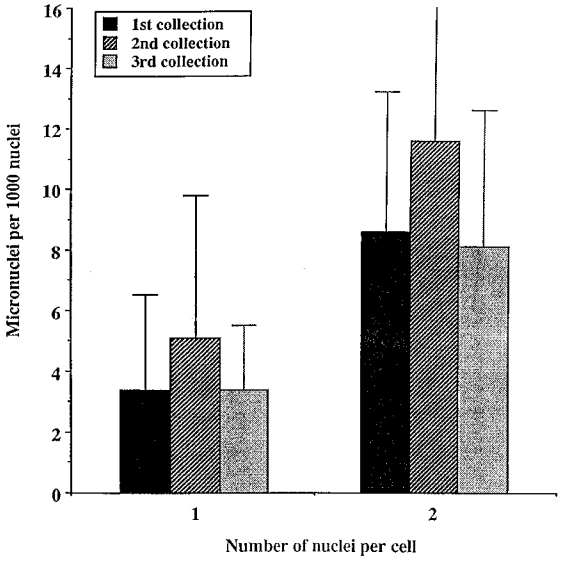

Fig. 1. Micronuclei in mono and binucleated lymphocytes during dietary folate changes. The average number of mononucleated cells scored for each of the three cell collections was 824 ± 419, and the number of binucleated was 1015 ± 36. The MN frequency was normalized for the number of nuclei per cell. The difference in the MN frequency of three cell subpopulations was statistically significant ($p < 0.01$).

menopausal women (Table 4). An average baseline frequency of 2.56 ± 1.78 MN cells was not appreciably affected by folate depletion but was significantly decreased after repletion (1.06 ± 0.73, $p = 0.11$, Table 4). Very few cells had more than one MN; thus, the frequency of MN cells and MN per 1000 cells were almost identical. Degenerated cells resulting from karyorhesis, karyolysis, and fragmentation were decreased by the end of the depletion period (2.9 ± 1.3 vs. 5.4 ± 3.6 baseline, $p = 0.014$), though there was no further change after repletion.

3.3. Factors affecting MN variability

Dietary folate intake changes were accompanied by changes in levels of vitamin B-12 and homocysteine, which are involved in folate metabolism. Since we observed a high individual variability in the MN frequency in both lymphocytes and exfoliated buccal cells, we performed a correlation analysis to reveal the most significant covariates. Associations with the following variables were analyzed by both Spearman rank correlation and linear regression: (1) age, (2) body weight, (3) hormonal supplements, (4) individual folate level and change in plasma folate, (5) vitamin B-12 baseline and change in plasma level, (6) homocysteine level, and (7) MN, MN +, MN − baseline and changes after depletion and repletion. Variables that were significant or approaching significance are shown in Table 5. Data shown were based on the analysis of all nuclei of mono-, bi, tri- and tetranucleated lymphocytes. We also performed a similar analysis for binucleated lymphocytes only and for binucleated and mononucleated cells together. Since these results generally supported our findings based on the analysis of all lymphocytes, we present the latter as more comprehensive. In lymphocytes during the depletion phase of the study, the most noticeable negative association was observed between MN frequency and plasma vitamin B-12 level ($p = 0.02$ for total MN, and

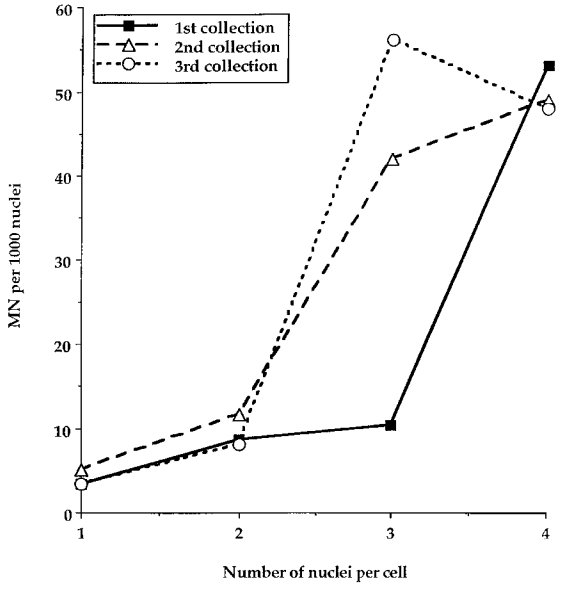

Fig. 2. Micronuclei in the lymphocytes with different nucleus number. For each cell collection ~1000 binucleated cells were scored, and the number of mono (~850), tri (~20) and tetranucleated (~50) cells was defined by the replicative index. Total of >3000 nuclei for each individual was analyzed for each of the three cell collections during folate depletion and repletion. The difference between the MN frequencies in mononucleated, binucleated, tri and tetranucleated lymphocytes was statistically significant ($p < 0.05$ by Wilcoxon's one-tail rank test).

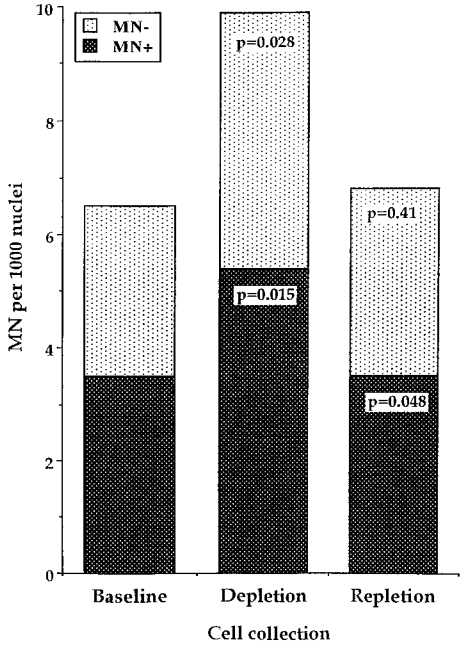

Fig. 3. Kinetochore-positive and kinetochore-negative micronuclei in human mono- and binucleated lymphocytes during dietary folate changes. Total number of nuclei analyzed per individual for each of the three cell collections was 2857.8 ± 420.7. Kinetochore-positive and kinetochore-negative micronuclei were differentiated by the presence of bright yellow-green signal after antikinetochore-antibody staining. Cells were counterstained with DAPI. Total MN were increased after depletion ($p = 0.014$) and decreased after repletion ($p = 0.066$) as were MN+ ($p = 0.015$ and $p = 0.048$ for depletion and repletion, respectively). MN− were elevated after depletion ($p = 0.028$), while a small decrease following repletion was not statistically significant ($p = 0.41$).

$p = 0.009$ for MN +). A weaker negative association was also observed for the baseline plasma folate level (−0.55, $p = 0.06$). However, Spearman correlation coefficients between MN and folate changes were positive (0.53–0.57) with borderline statistical significance ($p = 0.06$–0.07). Association with age was observed only for MN + ($p = 0.03$). Repletion phase was mostly characterized by positive association with vitamin B-12 level (0.62–0.67, $p = 0.03$–0.04) while the changes in MN + and B-12 were negatively correlated ($p = 0.038$). At this stage no direct association was observed between MN frequency and folate level or change in plasma folate. Nevertheless, such a link was found between the increase in MN due to depletion and the decrease in the MN frequency after repletion (from −0.73 to −0.55, $p = 0.01$–0.06).

Buccal cell MN were characterized by association with changes of vitamin B-12 in both the depletion and repletion phase (0.77–0.52, $p = 0.009$–0.074). A positive correlation with age ($p = 0.02$) and a negative correlation with MN level at the second collection ($p = 0.02$) were also observed.

In summary, based on the p-values estimated by Spearman rank coefficients, the main factors affecting MN frequency in lymphocytes were: (1) plasma vitamin B-12 and change in B-12 level, (2) MN and MN + baseline and change due to folate intake, and (3) plasma folate level and change in plasma folate due to depletion or repletion. Similar associations were observed for the buccal cell MN, though there

Table 4
Cytogenetic effects of dietary folate changes on micronuclei in human exfoliated buccal mucosa cells

	Micronuclei/1000 cells		
	Baseline	Depletion	Repletion
Individuals (ID no.)			
1	1.5[a]	2.5	3.5
2	1.5	2.5	1.5
3	2.0	2.0	0.5
4	1.0	1.0	0
5	1.5	1.0	1.5
7[b]	1.5	2.5	0.5
8	5.8	3.0	1.5
9	3.0	4.0	1.0
10	5.0	4.5	1.0
Group total			
MN cells/1000 cells[c]	2.56 ± 1.78	2.44 ± 1.26	1.06 ± 0.73[d]
MN per 1000 cells	2.56 ± 1.78	2.50 ± 1.27	1.22 ± 1.00[e]
Degenerated cells (%)	5.4 ± 3.6	2.9 ± 1.3[f]	3.6 ± 1.4

[a] Micronucleated cells per 1000 buccal cells (2000 cells were scored from two slides for each individual except no. 8 with 2028 cells).
[b] Subject 6 was excluded as an outlayer based on an unusually high level of plasma homocysteine.
[c] Mean ± sd.
[d] Difference between the levels of MN in exfoliated buccal cells at the second (depletion) and third (repletion) collections by Wilcoxon's one-tail test, $p = 0.010$.
[e] Difference between the levels of MN in exfoliated buccal cells at the second (depletion) and third (repletion) collections by Wilcoxon's one-tail test, $p = 0.020$.
[f] First vs. second collection by Wilcoxon's one-tail test, $p = 0.014$.

Table 5
Significant variables associated with micronucleus frequency in lymphocytes and exfoliated buccal cells

Endpoint	Variable	L-association[a]	S-association[b]	*p*-Value
Lymphocytes[c]				
Depletion				
MN^{d}_{1-2}	F^{e}_{1}	−0.65	−0.53	0.067
MN_{1-2}	B^{f}_{1}	−0.68	−0.68	0.021
MN_{1-2}	F_{1-2}	0.70	0.57	0.059
$MN+_{1-2}$	F_1	−0.56	−0.55	0.060
$MN+_{1-2}$	B_1	−0.74	−0.77	0.009
$MN+_{1-2}$	F_{1-2}	0.60	0.53	0.073
$MN+_{1-2}$	Age	−0.73	−0.64	0.033
$MN-_{1-2}$	F_{1-2}	0.58	0.57	0.059
Repletion				
MN_{2-3}	MN_{1-2}	−0.63	−0.57	0.054
MN_{2-3}	B_1	0.37	0.62	0.040
MN_{2-3}	B_2	0.35	0.55	0.064
$MN+_{2-3}$	$MN+_{1-2}$	−0.58	−0.55	0.060
$MN+_{2-3}$	B_2	0.40	0.67	0.028
$MN+_{2-3}$	B_{2-3}	−0.34	−0.62	0.038
$MN-_{2-3}$	$MN-_{1-2}$	−0.82	−0.73	0.013
Buccal cells				
Depletion				
MN_{1-2}	B_{1-2}	0.39	0.77	0.009
Repletion				
MN_{2-3}	MN_2	−0.76	−0.69	0.021
MN_{2-3}	Age	0.58	0.68	0.024
MN_{2-3}	B_{2-3}	−0.27	−0.52	0.074

[a]Linear regression coefficient.
[b]Spearman rank correlation coefficient.
[c]All lymphocytes (mono-, bi-, tri- and tetranucleated) were included into analysis, the frequency of MN was normalized by the number of nuclei per cell, e.g., the number of MN per nucleus was used.
[d]MN_{1-2} was used to describe the change in MN between first and second collection (2–3 between second and third).
[e]F_1 is plasma folate level at the first collection, while F_{1-2} stands for the change of folate level between first and second collection (2–3 are used respectively for the change between second and third collections).
[f]B_1 is plasma vitamin B-12 level at the first collection.

was no significant correlation with folate. In two cases, one for lymphocytes and one for buccal cells, MN were associated with age. Thus, MN frequency in lymphocytes and exfoliated cells appeared to be affected by multiple factors both reflecting controlled dietary intake and also individual genetic differences.

4. Discussion

The MN assay in binucleated human lymphocytes and cultured cell lines has been shown to be an effective tool in measuring cytogenetic damage of agents with different mechanisms of genotoxicity in vitro and in vivo [8,11,29]. The MN assay has been used to analyze the effect of low folate status in several cohorts using binucleated lymphocytes [12,13] and erythrocytes in splenectomized patients [4,24]. A principal conclusion was that low folate is generally associated with increased cytogenetic damage.

We reached the same conclusion after monitoring a group of postmenopausal women living in a highly controlled environment in the metabolic unit. Though initially these women had quite different MN, folate, B-12 and homocysteine levels, the difference in micronutrients was noticeably decreased during the study, while MN variability did not change appreciably. Moreover, although we observed a statistically significant increase in lymphocyte MN after folate depletion and decrease as a result of repletion, there was no correlation between individual folate measurements and MN frequencies at any collection time. Our findings corroborate those in Ref. [13], where in a large study of 126 vegetarians and 138 non-vegetarians of different ages (including a small group of individuals older than 60 years), where such a correlation, except in young females, was not seen either. The lack of association in older individuals may be explained by insufficient statistical power and high inter-individual variability in epidemiological studies. It is also possible that such an association could be disguised by the effect of other nutrients closely related to folate. In fact, Fenech and Rinaldi [13] observed a negative correlation between MN frequency and the level of B-12 both in young males and females, and in males vitamin C was also significant in defining the level of MN.

A study of 122 splenectomized healthy individuals [24] also found no correlation between individual levels of folate and MN. However, seven individuals with the highest observed frequencies of micronucleated erythrocytes from this group together with 64 individuals studied by Everson et al. [9] and Smith et al. [39] all had exceptionally low values of plasma folate, red cell folate, and plasma vitamin B-12.

Moreover, such an association was observed in an individual with Crohn's disease, a condition which results in the inhibition of dietary folate absorption and transport. These data suggest that folate is one of the major determinants of the types of damage that lead to spontaneous MN formation in erythrocytic cells. Another important observation made in Refs. [13,24], and our study is that vitamin B-12 status also appears to have a major influence on the frequency of micronucleated cells. Plasma B-12 values in our group of postmenopausal women indicated no apparent vitamin B-12 deficiency throughout the study. Nevertheless, it is possible that a negative B-12 balance may have occurred since plasma B-12 values declined from baseline. According to Ref. [15], a negative vitamin B-12 balance begins when vitamin B-12 absorption falls low enough to deplete the amount of vitamin B-12 on its primary delivery protein, TCII, resulting in a low holo-TCII level, even though total vitamin B-12 levels remain within normal limits. Fenech [10] found the same connection between MN and vitamin B-12 in a group of 50- to 70-year-old men whose diet was supplemented with folate above RDA either through tablets or cereal. A significant improvement in folate status was not accompanied by a change in MN frequency in binucleated lymphocytes, while an inverse correlation observed between plasma B-12 and MN frequency was statistically significant. Both folate and B-12 are intimately involved in metabolic pathways responsible for maintenance of the DNA precursor pool.

Blount et al. [4] made a further step in establishing the mechanism by which folate deficiency may cause chromosome damage. They supplemented the diet of 19 splenectomized individuals with folic acid (5 mg pteroylglutamic acid per day) and measured plasma and erythrocyte folate, DNA uracil levels, and MN frequency. The study concluded that DNA uracil levels are closely associated with frequencies of MN, and that both are minimized by supplementation with folic acid. Uracil misincorporation is associated with an increased frequency of abasic sites and subsequent strand incisions [4], suggesting that folate deficiency can result in chromosome breakage. Also supporting this hypothesis is data indicating a high frequency of fragile sites in low folate cultures [41]. However, no analysis of the mechanism of MN formation was performed in any previous MN study of lymphocytes or erythrocytes.

In the present study, we applied antikinetochore-antibody staining, which allows differentiation of MN formed due to either chromosome lagging or chromosome breakage. Both types of MN were increased due to folate depletion ($p = 0.015$ and $p = 0.028$ for MN+ and MN−, respectively). These results provide the first evidence that not only chromosome breakage, but the integrity of chromosome segregation and the cell division mechanism as well, could be affected by folate deficiency.

Advances in the MN assay of human lymphocytes (cytokinesis block, antikinetochore antibody staining or FISH with centromeric probe) have led to its increased use and its recommendation as a 'routine' index of cytogenetic damage [14]. Binucleated lymphocytes have been used for MN analysis in the majority of studies. Although reports of the MN assay as a biomarker of environmental exposures continue to accumulate, many questions exist regarding its limitations and interpretation of the data from population studies. These important issues include intra- and inter-individual variability [34], the effect of cytochalasin B [30], differential sex effect favoring a greater MN frequency in women [5,34], age, smoking, and other sources of variability [46]. The modification of the MN assay introduced in Ref. [11] allows differentiation of subpopulations of lymphocytes which have undergone a different number of divisions in culture by arrest of the cytoplasm division (using cytochalasin B) while nuclei divide normally.

In our study, we initially performed analysis of binucleated lymphocytes separately to obtain data comparable to other published results. A mild response to dietary changes in folate was observed, but only the increase in total MN and MN+ due to depletion was significant ($p = 0.038$ and $p = 0.029$, respectively). We also collected data on the MN frequency in mono-, tri- and tetranucleated cells. Since the number of mononucleated and binucleated cells was similar, as was their response to folate changes, we combined MN data for all nuclei. This approach provided more statistical power by increasing the total cell number, and incorporating all cells possibly affected by folate deficiency in vivo. As a result, a significant change in MN frequency due to

depletion and repletion ($p = 0.037$ and 0.028, respectively) was revealed.

These findings highlight the importance of proper selection of cell and target tissue for folate deficiency analysis. In all previous publications, only binucleated lymphocytes and erythrocytes were used for MN analysis. Since folate deficiency was associated with cancers of epithelial origin [19], and a significant reduction in oral leukoplakias accompanied by a lower MN frequency in buccal cells was observed in tobacco/betel quid chewers treated with vitamins [36,40], it was of interest to determine if cytogenetic damage in buccal cells would be associated with controlled changes in folate intake. The MN assay in exfoliated cells proved to be a sensitive biomarker of environmental exposure to smoking/tobacco chewing [36], aerial formaldehyde [44], and a high level of arsenic in drinking water [27]. However, in the study of smokers performed in Ref. [33] no association between folate status and MN frequency was found.

The folate repletion protocol in the present study included the gradual increase in folate intake from 111 μg/day (day 42–69) to 286 μg/day (day 70–80), and finally 516 μg/day (day 81–91), with an average level during the repletion period of 228 μg/day. It is possible that the cytogenetic effect of the increased folate level was accumulating throughout the entire repletion period. This scenario is even more plausible for lymphocytes, since they circulate in the blood stream for several weeks and even months [46]. Therefore, the most adequate measure of their exposure will be defined by an average level of folate intake (228 μg/day). On the other hand, the period in which the effect of folate repletion in exfoliated cells could be registered (by MN decrease) is the last 7–14 days before cell collection. Based on the dynamics of cells with MN induced by radiotherapy and chemotherapy, this is the typical lag period between an occurrence of MN in the basal layer of epithelium and the time cells migrate through the cellular strata and are sloughed off [37,38]. Thus, the MN that were observed in these exfoliated cells would reflect damage caused by factors in the oral cavity present 7–14 days earlier. In our study, the intake of 516 μg/day was maintained for the last 9 days before cell collection. This more than tripling of the RDA-1989 for folate resulted in a significant decrease in MN frequency in buccal cells. Lymphocytes appear to be more sensitive to dietary folate changes than exfoliated cells, since a statistically significant MN change in the former was observed after only a two-fold decrease/increase in folate intake, while in the latter a three-fold increase was necessary. Further studies are warranted to compare dose response of cytogenetic damage to dietary folate changes in different cell types.

We speculate that the cytogenetic response in both exfoliated cells and lymphocytes would be even stronger were we to study a group of individuals with clinical folate deficiency. Our data add strength to the recent suggestions that a higher RDA for elderly people, premenopausal and pregnant women, and children may better protect their health [31]. Further, our study also indicates that MN in exfoliated cells are a sensitive biomarker of cytogenetic damage.

In conclusion, cytogenetic monitoring of postmenopausal women showed that MN frequency in lymphocytes increased after controlled dietary folate depletion, and later decreased following repletion. MN analysis in all lymphocytes was more statistically powerful than analysis of binucleated cells to assess cytogenetic damage in vivo. Both kinetochore-positive and kinetochore-negative MN demonstrated similar changes, suggesting that anomalies in cell division and chromosome segregation, in addition to chromosome breakage, are associated with folate deficiency. The main covariables affecting MN changes during dietary depletion and repletion were plasma vitamin B-12, folate, and the individual baseline frequency of MN. MN frequency in buccal exfoliated cells did not respond to depletion, but was significantly decreased after dietary folate repletion up to 516 μg/day folate. Further study is needed to establish whether higher folate intake will lower cytogenetic damage in different epithelial tissues and thus decrease the risk of cancer, especially in older people.

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