ABSTRACT

Erythrocytes containing micronuclei serve as an indicator of genotoxic exposure in splenectomized individuals. Micronucleated erythrocytes, derived from cytogenetically damaged RBC precursors, are not selectively removed from peripheral blood in individuals who lack splenic function. The relationship between micronucleated cell frequencies and demographic, environmental, and dietary factors was examined in 44 subjects with previous splenectomy due to trauma. Their micronucleated cell counts followed a log-normal distribution, with geometric means of 3.3 micronucleus-containing cells/1000 reticulocytes and 2.7/1000 normochromatic erythrocytes. A multiple regression analysis showed that drinking five cups of coffee or tea/day (relative to none) was associated with an approximately 2-fold higher frequency of micronucleated cells. Weaker statistical associations were also noted with micronucleus frequency and the consumption of calcium supplements (associated with a higher frequency) and vitamins A, C, or E (lower frequency). An apparent trend of micronucleus formation through intervention studies.

INTRODUCTION

With the current concern for the potential genotoxic and carcinogenic effects of environmental exposures (in air, water, diet, occupation, etc.), sensitive indicators of DNA damage are needed to determine the extent to which such exposures actually contribute to genotoxic damage in humans.

The measurement of micronuclei in human erythrocytes and lymphocytes is one such marker of the effect of genotoxic exposures (1). The measurement of micronuclei in bone marrow erythrocytes has become established as a routine assay for cytogenetic damage in experimental animals (2) and has been applied to humans with exposures to chemotherapeutic drugs or with occupational exposures (3–7). However, the invasive nature of bone marrow sampling severely limits the usefulness of this procedure as a routine assay for cytogenetic damage in humans. Interest in the peripheral blood lymphocyte assay (8) has been stimulated recently by the introduction of the cytokinesis-block method (9), which allows first-division nuclei to be accumulated in culture as binucleate cells which are easily recognized and scored. The ability to determine the cell division being scored is a critical requirement because the micronucleus frequency depends on the proportion of cells which have divided following the induction of DNA damage and the fate of micronuclei in cells which have divided more than once (9). Nevertheless, the assay is still limited by a lack of sensitivity to agents which do not produce long-lived lesions in nondividing cells, due to the test cell population not actively dividing in vivo.

This report suggests using micronucleated peripheral blood erythrocytes in splenectomized subjects as a marker for chromosomal damage. The end point measured is the prevalence of micronuclei (also known as Howell-Jolly bodies) among peripheral erythrocytes. Under normal conditions, the human spleen removes micronucleus-containing erythrocytes from the peripheral blood. But in asplenic individuals, micronucleated cells persist and accumulate with repeated genotoxic exposure (10). As a consequence, evidence of cytogenetic damage is uniquely visible in splenectomized persons as an increased frequency of micronucleus-containing RBCs. Although limited to splenectomized individuals, the advantages of the assay include simplicity and greater ease of scoring than other currently applied assays.

The assay can be conducted on two erythrocyte populations: the immature polychromatic erythrocytes which stain positively for residual RNA (hereafter referred to as reticulocytes) and the mature normochromatic erythrocytes which do not stain positively for RNA (hereafter simply erythrocytes). Newly formed reticulocytes mature into circulating erythrocytes after only 1–2 days (11); hence, micronucleated reticulocytes demonstrate only very recent genotoxic exposures. Erythrocytes have an average life span of 120 days (11); hence, micronucleated erythrocytes reflect genotoxic exposures within approximately 4 months prior to sampling (10).

The studies of micronuclei in circulating erythrocytes which have been reported to date have examined relatively small numbers of subjects splenectomized due to illness (idiopathic thrombocytopenia, Hodgkin’s disease, leukemia, etc.) or traumatic damage to the spleen. Chemotherapeutic agents known to damage DNA cause >10-fold increases in micronucleated cells, and the kinetics of the appearance and disappearance of micronucleated cells have been shown to be consistent with the kinetics of RBC formation and turnover (10). A marked effect of mild folate deficiency on the frequency of micronucleated cells has also been reported (12).

The present investigation sought to examine the frequency of micronucleated cells in a group of splenectomized (but otherwise healthy) subjects and to identify common demographic, environmental, and dietary factors associated with micronucleated erythrocytes in splenectomized subjects.
environmental, and dietary factors that may be correlated with this frequency.

MATERIALS AND METHODS

Enrollment of Study Subjects. Potential study subjects were identified from the records of the Northern California Kaiser Permanente Medical Care Program. Computer-stored discharge records for the years 1971–1984 were searched to obtain a list of individuals who were hospitalized with trauma/injury (International Classification of Diseases, Rev. 9, 800–999 and E codes) and whose medical records had splenectomy (surgical procedure code 45.1) as the primary discharge diagnosis. This produced a list of 303 individuals with a trauma-related splenectomy for whom computer-stored information, including date of birth, date of surgical procedure, and address, was available.

To each person was mailed a recruitment packet that contained a letter describing the study and requesting their participation, a human subjects consent form that followed Kaiser Permanente guidelines, and a self-administered questionnaire. The questionnaire included basic demographic information, several dietary questions, and questions about exposures to potential genotoxic agents, including X-rays, chemotherapy, and potential occupational exposures. Because of the limited life span of erythrocytes and the previous demonstration that micronucleated cell frequencies return to their baseline values within 4 months after cessation of genotoxic exposures (10), the questionnaire asked only about exposures within the last 4 months. Subjects who agreed to participate were directed to their local health plan facility for blood drawing.

Laboratory Procedures. A blood sample was drawn from each subject into a heparinized Vacutainer tube by Kaiser Permanente laboratory staff. Two drops of blood were transferred to a vial containing 0.5 ml of 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The sample was gently mixed and held at room temperature at least 24 h. Six or more standard blood smears were prepared using the freshly drawn heparinized blood. Smears were air dried, immersed in absolute methanol for 2–5 min, and air dried again. All samples were delivered to the United States Department of Agriculture laboratory for analysis. Upon receipt at the United States Department of Agriculture laboratory, the glutaraldehyde-fixed preparations were refrigerated until scored.

The methanol-fixed slides were immersed in a solution of acridine orange (0.02 mg/ml in 0.01 M sodium phosphate buffer, pH 7.4) for 4 min and were then rinsed in phosphate buffer for 8 min. Prior to scoring, a coverslip was placed over the wet smear area, and the slide was blotted to remove excess buffer. The stained slides were scored as wet mounts.

Blood samples were scored for the frequency of micronucleus-containing cells in newly formed RNA-positive reticulocytes and mature RNA-negative erythrocytes. Slides were scored as wet mounts at 6.30 by epifluorescence microscopy using an oil immersion objective and a Zeiss fluorescein isothiocyanate filter. The excitation wavelength was 450–490 nm, the dichromatic beam splitter was set at 510 nm, and the barrier filter was 520 nm. Under these conditions of staining and excitation, RNA-containing bodies fluoresce red-orange, and DNA-containing bodies (nuclei and micronuclei) fluoresce bright yellow. RNA-negative erythrocytes are a dull green color and are easily distinguished from the red-orange RNA-positive cells. Micronuclei were defined as intracellular bodies with the characteristic yellow fluorescence of DNA in addition to the other characteristics of micronuclei described in the literature (13). The frequencies of micronucleus-containing erythrocytes in each sample were determined using a semiautomated procedure in which the count of total cells scored was based upon specified subfields of known area.

The numbers of micronucleus-containing cells among 2,000 RNA-positive erythrocytes and among at least 10,000 RNA-negative erythrocytes were determined for each sample. The total number of RNA-positive cells scored was estimated by counting the number of RNA-positive cells in every fifth field scored and was based upon a count of at least 400 cells. The total number of RNA-negative cells scored was estimated by counting the number of RNA-negative cells in an area that was approximately 0.05 times the area of every fourth field and is based upon at least 100 cells.

The percentage of RNA-positive cells in each sample was determined by counting the number of RNA-positive cells in each fourth field and counting the number of RNA-negative cells in a small sector of that field. For a typical proportion of RNA-positives to RNA-negatives, this percentage was based upon a total count of approximately 30 RNA-positive cells and approximately 100 RNA-negative cells.

Absence of splenic function was verified by determining the frequency of “pitted” erythrocytes (14) in the glutaraldehyde-fixed blood samples. Pitted cells accumulate in the erythrocyte population of subjects who lack a functioning spleen but occur at a very low frequency (<2%) in individuals with normal spleen function. A sample of fixed blood in 1–2 drops of fixative solution was placed on a glass slide with coverslip. The specimen was examined under Normarski optics at ×1000 magnification. Pitted cells were defined by the presence of an apparent cell surface depression with a continuous, smooth, and defined edge and evident shadowing. The size of this apparent depression may range from 0.05–0.2 times the cell diameter. Cells with one or more surface depressions meeting these criteria were classified as pitted. At least 500 cells were scored/specimen. Subjects with a frequency of pitted erythrocytes of ≥12% were considered to lack splenic function (14).

Statistical Analysis. Laboratory and questionnaire data were merged and analyzed using the statistical software packages BMDP (15) and SAS (16, 17). Questionnaire responses were used to define various demographic, dietary, or life-style factors that might be associated with the frequency of micronucleated cells. All of the codings and groupings of these factors were made without knowledge of any individual's micronucleated frequency.

Coffee and tea consumption were combined to create a variable representing daily total caffeine intake from these two sources. The daily consumption of each was weighted proportionately to their approximate caffeine content (18) and summed as follows: 1 × (cups of caffeinated coffee/day) + 0.4 × (cups of tea/day) = cups of coffee equivalents/day.

We also asked subjects whether they took regular doses of vitamin or mineral supplements. Because relatively few subjects reported taking each specific vitamin or mineral, the responses were combined for those taking B vitamins and those taking vitamins C, E, or A. Grouping of vitamins C, E, and A was based on evidence that their antioxidant properties may defend against genetic and carcinogenic damage (19).

Occupational title, job duties, and reported exposures to solvents, metal fumes, pesticides, or other chemicals were used to group subjects into those with a high potential for genotoxic exposure, those with relatively less potential, and those with little or no potential.

To examine possible effects of X-ray exposures, we considered only X-rays to major portions of the body (such as trunk, pelvis, limbs) and excluded dental X-rays or X-rays of distal extremities.

The frequencies of micronucleus-containing cells in the two cell populations (reticulocytes and erythrocytes) were analyzed separately. To investigate the association between various demographic or life-style factors and the frequency of micronucleus-containing cells, we compared the frequencies between subgroups of categorical variables (such as gender) or examined scatter plots of micronucleus-containing cell counts against continuous variables, such as age and coffee consumption.

The frequency distribution of micronucleated cells/1000 cells was skewed to the right and appeared to follow a log-normal distribution. Therefore, we derived a log transformation of the frequency of micronucleus-containing cells as In (number of micronucleus-containing cells/number of cells scored). Hence, the means we report for micronucleated cell counts/1000 cells are geometric means.

To assess the independent statistical relationships of several variables simultaneously, we used a multiple linear regression analysis (20). Model goodness of fit was assessed by R2, which is interpreted as the proportion of the variance in the log micronuclei frequency accounted for by the independent variables in the model (20). The dependent variable in each model was the natural log of the number of micronucleus-containing cells/1000 cells, as described above. The regressions used as independent variables the demographic and life-style factors

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that were associated with micronucleus counts in the unadjusted analysis or that we thought may be associated a priori. For ease of presentation and interpretation, the independent variables were kept untransformed. More complicated representations (e.g., logarithmic, quadratic) did not significantly improve the fit. Hence, the regression coefficient for each independent variable indicates the change in log frequency of micronucleated cells associated with a one unit change in the independent variable. Alternatively, the antilog of the regression coefficient can be interpreted as the multiplier of the micronucleated cell frequency associated with a unit change in the independent variable.

In order to compare the strengths of the associations of various study variables, we used the fitted models to calculate the micronucleus counts predicted by varying each independent variable, holding the others constant (21).

RESULTS

Enrollment of Study Subjects. Of the 303 patients identified with a discharge diagnosis of splenectomy, 174 had either terminated their membership in the health plan, had moved without providing the health plan with a forwarding address, or were deceased. Of the remaining 129 persons, 48 (37%) declined to participate, or failed to provide both a blood specimen and a completed questionnaire. This left 81 subjects (63% of those who could be contacted) for whom we received both laboratory and questionnaire data.

In order to eliminate subjects with evidence of splenosis or regenerated splenic function, we excluded 37 subjects who had pitting in <12% of the erythrocytes examined. The statistical analysis below is based on the remaining 44 confirmed asplenic subjects.

The asplenic subjects contained more males than females (26 and 18, respectively), and the predominant ethnic group was white, non-Hispanic. The median age of the group was 42 years, with the youngest member being 5 years old and the eldest 80 years old. Nearly half (46%) of the group reported a total household income of ≥$30,000/year. None of these subjects reported receiving chemotherapy within the previous 4 months, although eight persons reported receiving major X-ray exposure.

The questions about dietary and personal habits asked respondents to report their average experience during the previous 4 months. Sixteen (36%) reported smoking cigarettes, with a median consumption in the category of 0.5–1 pack/day. Twenty-nine (66%) drank coffee (median consumption, 1–2 cups/day), 10 (23%) drank decaffeinated coffee (median, <1 cup/day), and 12 (27%) drank tea (median, <1 cup/day).

Ten (23%) subjects reported taking daily doses of at least one of the vitamins A, C, or E. Four (9%) took regular doses of B vitamins, 10 (23%) took multiple vitamin preparations, and six subjects (14%), all of whom were females, reported taking calcium supplements.

The four subjects classified as having a job with a high potential for genotoxic exposure included three workers (a radiation worker, a pipefitter, and a shipfitter) whose reported exposures included halogenated organic solvents or welding materials and a hairstylist who worked with hair dyes and permanent wave solutions. Subjects in the medium potential group included a wallpaper hanger and a rancher who used herbicides.

Results of the Micronucleus Assay. The numbers of micronucleus-containing cells/1000 reticulocytes in the 44 asplenic subjects ranged from 0.5–15.9/1000, with a geometric mean of 3.3/1000 (Fig. 1). The number of micronucleus-containing cells/1000 erythrocytes was 0.3–8.6/1000, with a geometric mean of 2.7/1000. These values are similar to values reported for bone marrow erythrocytes (7). In the unadjusted analysis, micronucleated cell frequencies appeared directly associated with age and consumption of coffee equivalents, while there was no apparent trend with cigarette consumption (Fig. 2).

For the multivariate analysis, Table 1 lists the independent variables included in the regression, the units for each variable, its regression coefficient and P value, and the predicted micronucleus frequency multiplier (the exponentiation of the regression coefficient) with its 95% confidence interval. These coefficients are "adjusted" for all other factors included in the model, in the sense that they are calculated in the presence of the other factors.

For reticulocytes, the regression model has an $R^2$ of 0.41, indicating that 41% of the variance in log micronucleus frequency was accounted for by the 11 variables in the regression. The variables in this model that were statistically significantly associated with higher micronucleus frequencies were the consumption of coffee equivalents and taking calcium supplements. Taking vitamin A, C, or E supplements was significantly associated with lower frequencies.

The equivalent analysis for erythrocytes had an $R^2$ of 0.43 (Table 1). Male gender and coffee equivalents consumption were significantly associated with increased micronuclei. The coefficients for taking vitamins A, C, or E and calcium supplements, while approximately similar in magnitude to those in the reticulocyte analysis, did not reach statistical significance ($0.1 \leq P \leq 0.2$).

The regression models given in Table 1 were used to show the changes in the mean micronucleus-containing cell counts (and 95% confidence intervals) that would be predicted from varying each independent variable (Fig. 3). These predictions were calculated against the predicted micronucleated cell counts
average of 5 cups/day and taking regular doses of calcium supplements are approximately comparable (an increase of about 2/1000). An increase of about 1/1000 reticulocytes is predicted/30-year increase in age, but this association was not observed for erythrocytes. Regular daily doses of vitamin A, C, or E would be predicted to lower micronucleus-containing cell counts to approximately one-half the baseline value.

The results in Fig. 3 are intended to illustrate the relative strengths of the statistical associations predicted by the factors investigated in this cross-sectional sample. These are, of course, hypothetical predictions derived from a statistical model. In practice, changing the value of one variable (e.g., reducing a person's coffee consumption) might well result in the changing of another as well (e.g., increasing tea or decaffeinated coffee consumption).

**DISCUSSION**

This study has demonstrated the feasibility of recruiting splenectomized subjects for an epidemiological study and has shown statistically significant associations between the micronucleus frequency in circulating reticulocytes or erythrocytes and three dietary factors: consumption of caffeinated coffee and/or tea, vitamin A, C, or E, and calcium supplements.

Caffeine has long been a suspected carcinogen, primarily for cancers of the pancreas, bladder, kidney, and ovary (22). Caffeine is known to have adverse effects on DNA synthesis and mitosis as well as DNA repair processes (23), but, in general, human epidemiological studies have not revealed any strong or consistent relationship to any cancer (22). Pancreatic cancer has been most studied because of an initially positive association with coffee consumption (24), but subsequent studies have not borne out this relationship (25). Nevertheless, on the basis of the long-term suspicion of caffeine as a carcinogen, the findings of the current study are of special interest and deserve further study. We also note that reported intake of decaffeinated coffee was not associated with a similar increase in micronuclei.

Consumption of coffee has recently been reported to be associated with increased chromosomal fragility (26) and sister chromatid exchange frequency (27) in peripheral blood lymphocytes.

The possible protective role of vitamins A, C, and E in the etiology of cancer has received an increasing amount of support from animal as well as human observational and intervention studies (28). Vitamin A is important for cell differentiation and proliferation, a deficiency of which is an important factor in

**Table 1 Multiple regression analysis of micronucleated cell frequency among reticulocytes and erythrocytes in 44 splenectomized subjects**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
<th>Reticulocytes</th>
<th>Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male vs. female</td>
<td>0.292 (0.22)</td>
<td>0.604 (0.02)</td>
</tr>
<tr>
<td>Age</td>
<td>10 y</td>
<td>0.109 (0.10)</td>
<td>0.025 (0.72)</td>
</tr>
<tr>
<td>Coffee equivalents</td>
<td>1 cup/day</td>
<td>0.135 (0.05)</td>
<td>0.154 (0.03)</td>
</tr>
<tr>
<td>Decaffeinated coffee</td>
<td>1 cup/day</td>
<td>-0.018 (0.87)</td>
<td>0.085 (0.87)</td>
</tr>
<tr>
<td>Vitamin A, C, or E</td>
<td>Daily dose vs. no</td>
<td>-0.648 (0.02)</td>
<td>-0.359 (0.19)</td>
</tr>
<tr>
<td>Calcium supplements</td>
<td>Daily dose vs. no</td>
<td>0.737 (0.05)</td>
<td>0.587 (0.13)</td>
</tr>
<tr>
<td>B vitamins</td>
<td>Daily dose vs. no</td>
<td>0.378 (0.36)</td>
<td>-0.641 (0.14)</td>
</tr>
<tr>
<td>Multiple vitamins</td>
<td>Daily dose vs. no</td>
<td>0.018 (0.94)</td>
<td>0.020 (0.94)</td>
</tr>
<tr>
<td>Genotoxic exposures</td>
<td>High vs. low potential</td>
<td>-0.103 (0.78)</td>
<td>-0.196 (0.62)</td>
</tr>
<tr>
<td>X-ray</td>
<td>Yes vs. no</td>
<td>0.021 (0.94)</td>
<td>0.529 (0.10)</td>
</tr>
<tr>
<td>Cigarette smoking</td>
<td>1 pack/day</td>
<td>-0.159 (0.47)</td>
<td>0.040 (0.86)</td>
</tr>
</tbody>
</table>

* a Dependent variable expressed as ln(micronucleated cells/1000 cells).

b P value for two-tailed test of hypothesis that regression coefficient is 0.
Carcinogenesis. Also, carotene is known to have antioxidant effects which protect against damage to DNA (29). In epidemiological studies, an association with reduced lung cancer risk has been most strongly supported (28, 30) and enough evidence exists to justify randomized double-blind controlled trials of the effect of β-carotene supplements on cancer outcomes (31). Vitamins C and E are also antioxidants which reduce DNA damage and mutations and could thereby protect against carcinogenesis (28). Little evidence currently exists from human studies for a protective effect of vitamin C, but a recent study of serum vitamin E in a large cohort of Finnish men suggests a reduced risk of all cancers among those with high levels compared to those with low levels (32).

Calcium has, until recently, not been considered of etiological importance in cancer. However, recent studies from several sources have suggested that dietary calcium may be associated with a reduced risk of colon cancer (33, 34). Calcium may bind with fats in the intestine which make these fats less accessible to metabolism by bile salts and the subsequent formation of carcinogenic breakdown products (35). Our findings are not consistent with the epidemiological literature in that persons with high calcium intake in our study had higher levels of micronuclei. It may be that taking calcium supplements was a marker for something else not measured in our study, such as low estrogen levels in women or contaminants in over-the-counter calcium supplements.

Several studies have reported a strong association of micronucleus frequency with age in human peripheral blood lymphocytes (9, 36–38). Simple scatter plots of our erythrocyte data against age (Fig. 2) showed a trend similar to that reported for lymphocytes. However, the multiple regression analysis suggested that the age effect apparent in our unadjusted analysis was partly due to confounding by other factors (such as coffee consumption) that were correlated with age. An analysis of our data relating micronucleated cell counts to age only, as, for example, was done by Fenech and Morley (9), would have predicted a 17% increase in micronuclei among reticulocytes, and a 12% increase among erythrocytes, per 10-year increase in age. In contrast, the multivariate analysis predicted an increase in micronuclei per 10-year increase in age of only 11% for reticulocytes and 3% for erythrocytes.

Although smoking has been reported to be associated with an increased micronucleus frequency in both peripheral blood lymphocytes (37, 39) and in erythropoietic bone marrow cells (39), we observed no evidence of an association between smoking and increased micronucleus frequency in the present study. This may have been due in part to the relatively low exposures in this study group. No subject reported smoking as many as 2 packs of cigarettes/day, and only three smoked >1 pack/day.

Serum folate deficiency has recently been shown to increase the frequency of micronuclei in erythrocytes (12) but could not be evaluated in the present study. Recent observations in laboratory animals demonstrated a strong enhancement of caffeine-induced micronucleus formation by folate deficiency (40). These findings suggest an interaction between folate status and caffeinated beverage consumption. It will be important to monitor folate status in future studies and to assess its possible interaction with other factors.

The main limitation of this study was its small sample size. Our group of 44 confirmed asplenic subjects was too small to adequately assess the effects of factors with low prevalence, such as specific occupational exposures. Even though 81 subjects had completed questionnaire and laboratory work, we were required to eliminate 37 subjects (46%) because they showed evidence of residual or regenerated splenic function. The proportion of individuals with such regenerated splenic function was not known in this study.
function is believed to be highest among those splenectomized because of trauma (14). In spite of this, we chose to restrict the study to those with traumatic splenectomies in order to assemble a group that had no underlying illness.

Although a multiple regression analysis was used in this cross-sectional study to examine the associations of several factors simultaneously, confirmation of some of these associations must await controlled intervention studies. Because the cell population studied is rapidly dividing in vivo, short-term experimental studies in which suspected factors are added or withheld for several days should permit this confirmation.

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Micronucleated Erythrocytes as an Index of Cytogenetic Damage in Humans: Demographic and Dietary Factors Associated with Micronucleated Erythrocytes in Splenectomized Subjects


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