Assessment of Cytogenetic Damage by Quantitation of Micronuclei in Human Peripheral Blood Erythrocytes

Robert Schlegel, James T. MacGregor, and Richard B. Everson

Department of Biomedical and Environmental Health Sciences, University of California, Berkeley, California 94720 [R. S., J. T. M.]; Department of Cell Growth and Regulation, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 [R. S.;] Western Regional Research Center, United States Department of Agriculture, Berkeley, California 94710 [J. T. M.]; Epidemiology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709 [R. B. E.]; and Division of Hematology-Oncology, Department of Medicine, Duke University, Durham, North Carolina 27710 [R. B. E.]

ABSTRACT

The incidence of micronuclei (Howell-Jolly bodies) in peripheral blood erythrocytes of splenectomized and nonsplenectomized humans was evaluated as an index of genotoxic exposure. Subjects with intact spleens had very low frequencies of micronucleated cells among circulating erythrocytes, even when these individuals were exposed to known clastogenic agents used in cancer therapy (no micronuclei were seen in 100,000 cells). After splenectomy, the frequency of micronuclei among erythrocytes of untreated subjects rose slowly and after 4 mo established a steady-state level of approximately 2.0/1000, a value similar to that reported for human bone marrow (Goetz et al. Relationship between experimental results in mammals and man: cytogenetic analysis of bone marrow injury induced by a single dose of cyclophosphamide. Mutat. Res., 31: 247-254, 1985; and Hogstedt et al. Micronuclei and chromosome aberrations in bone marrow cells and lymphocytes of humans exposed mainly to petroleum vapors. Hereditas, 94: 179-187, 1981. Chemotherapy increased these levels, with individual samples from patients on daily treatment often having values greater than 5 times higher than control levels. The frequency of micronucleated erythrocytes rose as the duration of clastogenic exposure increased and returned to near base-line levels approximately 4 mo after treatment was discontinued. These findings suggest that it will be possible to use analyses of circulating erythrocytes to assess genotoxic exposures among splenectomized human populations. The ease of sample preparation and scoring should make it possible to monitor individuals with greater statistical power than is feasible with conventional cytogenetic techniques.

INTRODUCTION

Animal studies have established associations between the incidence of micronuclei (Howell-Jolly bodies) and the standard cytogenetic indices of chromosomal breakage (1, 2). Micronuclei arise when replicating cell populations are subjected to chromosomal breakage by clastogens or to chromosome loss by mitotic spindle dysfunction. The measurement of micronuclei in bone marrow cells of experimental animals has become established as a routine screening test for in vivo cytogenetic damage (see Ref. 2 for review). This technique has also been applied to bone marrow specimens of chemotherapeutically and occupationally exposed humans (3-7), but its use is limited by the invasive procedures required for bone marrow sampling. Although micronucleated erythrocytes are not selectively removed from the peripheral blood of mice and therefore accumulate during repeated exposures (8), the spleen of most animals (9), including humans (10), removes micronucleated erythrocytes from the circulation. The splenectomized human, however, should not remove micronucleated erythrocytes and should provide a model for monitoring cytogenetic damage without invasive techniques or complex sample preparation. Investigations of this model could provide useful comparisons with the animal literature and a new approach to monitoring clastogenic damage in humans. This paper evaluates the use of micronucleus frequencies in peripheral blood erythrocytes of splenectomized and nonsplenectomized humans as a method for assessing cytogenetic damage by analyzing blood smears from control subjects and from patients with known exposures to genotoxic agents used for cancer therapy. Whenever possible, micronucleus frequencies were determined from blood samples taken before, during, and after therapy.

MATERIALS AND METHODS

Wright's stained peripheral blood smears prepared as part of standard medical care were obtained from files maintained by the Clinical Hematology Laboratory at Duke University. Blood smears were assembled from groups of patients which had received similar treatments, emphasizing instances where smears were available before, during, and after treatment. In addition to these existing materials, unstained smears of blood specimens were obtained from several splenectomized and spleen-intact subjects. Specimens from individuals undergoing treatment with cytotoxic drugs were obtained from patients attending clinics of the Departments of Medicine or Surgery at Duke University Medical Center, while specimens from individuals splenectomized because of traumatic rupture of the spleen were enrolled by newspaper advertisements. These blood smears were air dried, fixed for 5 min in absolute methanol, and stained with either Wright's stain or with acridine orange. Wright's stained slides were used for studies of total erythrocytes, while acridine orange-stained slides were used for studies of reticulocytes. Acridine orange staining was performed by immersion of slides for 5 min in pH 7.4 sodium phosphate buffer (1% by weight) containing 0.02 mg of acridine orange per ml, followed by a 10-min rinse in pH 7.4 phosphate buffer. Slides were then wet mounted and examined by fluorescent microscopy using a Zeiss fluorescein isothiocyanate filter. Under these conditions, RNA (including that in reticulocytes) fluoresces red, and DNA (including that in micronuclei), green-yellow. For all studies, the frequency of micronuclei was manually scored at \( \times 1000 \) under oil. The number of cells scored from each sample was 2000 total erythrocytes or 4000 reticulocytes. The criteria used for the identification of micronuclei are described by Schmid (1). All slides were coded using random numbers to prevent the reader's knowledge of the exposure and splenectomy status of the study subjects. The binomial comparison described by Kastenbaum and Bowman (11) was used for statistical analysis.

Since it has been reported that splenosis (functioning ectopic spleen tissue) is a frequent occurrence after splenectomy for trauma, all trauma controls were evaluated for spleen function by determining the percentage of "pitted" erythrocytes (12). Eusplenic normal subjects generally have frequencies of pitted erythrocytes below 1%, while asplenic individuals exhibit frequencies between 12 and 35%. Individuals splenectomized for trauma, but not those splenectomized for hematological or oncological indications, often have intermediate values (between 1 and 8%) due to splenosis (12). Only those trauma control subjects with greater than 12% pitted erythrocytes were assumed to have no spleen function and were included in the study. All patients with cancer or...
chemotherapy patients with intact spleens. Because all but a few of these specimens were obtained from existing slide files, percentages of pitted erythrocytes could not be measured. Since elevated micronuclei frequencies have been reported in acute nonlymphocytic leukemia (13) and megaloblastic or hemolytic anemias (14), subjects whose clinical records suggested these conditions were excluded from this study. One control subject who was splenectomized for trauma had micronucleus values that were of magnitude higher than the mean values of the other controls. This case will be discussed in a later report, and these values were excluded from the data presented here.

RESULTS

Twelve subjects with intact spleens who received neither chemotherapy nor radiotherapy prior to sampling had no micronuclei among 56,000 erythrocytes from 28 Wright's stained blood smears (Table 1). Likewise, 16 nonsplenectomized patients who received at least 1 mo of chemotherapy during the 4 mo prior to sampling had no micronucleated cells among 100,000 erythrocytes from 50 Wright's stained blood smears (Table 2). Micronucleated erythrocytes therefore do not accumulate in the peripheral blood of nonsplenectomized humans, even though their presence has been demonstrated in the bone marrow of both normal and chemically exposed subjects (3, 4).

Among individuals splenectomized for ITP who received either no therapy or steroid treatment only, micronucleated erythrocytes began to appear in the peripheral blood shortly after splenectomy. The incidence of these cells slowly increased for about 4 mo, after which a steady-state frequency was established at approximately 4/2000 (Table 1). Similar control values have been reported for human bone marrow samples (3, 4). No differences were seen between subjects splenectomized for ITP and those for trauma who did not have any return of spleen function (Table 1), as determined by the percentage of pitted erythrocytes (see "Materials and Methods").

The groups of splenectomized patients that received chemotherapy during the 4 mo preceding sampling showed significant increases in the frequency of micronucleated erythrocytes when compared to splenectomized controls (Table 2). When chemotherapy patients are classified into subgroups according to the length of treatment during the 4 mo before sampling, a direct relationship is seen between treatment length and micronucleus frequency. Hodgkin's disease patients were treated every fourth wk with similar doses of BVCP. This relatively homogeneous group illustrates, as do the chemotherapy patients in general, the direct relationship between the extent of exposure during the 4 mo prior to sampling and the micronuclear frequency among erythrocytes in the peripheral circulation. Treatment during the 4 mo preceding sampling is important because erythrocytes persist in the circulation of splenectomized humans for approximately 120 days (10). Micronucleus frequencies therefore increase as preexisting erythrocytes are replaced by cells formed during treatment and decrease when treatment is stopped and micronucleated erythrocytes are removed from the circulation. As expected, 26 samples taken from 12 patients 4 mo or longer after therapy was discontinued displayed micronuclear frequencies which were no longer elevated above those from ITP control patients who had been splenectomized.

Table 1 Frequency of micronucleated cells among 2000 erythrocytes from Wright's stained blood smears of splenectomized subjects receiving no therapy or steroid therapy only.

<table>
<thead>
<tr>
<th>Subjects splenectomized for ITP</th>
<th>Mean ± SE</th>
<th>No. of patients</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presplenectomy</td>
<td>0.00 ± 0.00</td>
<td>12</td>
<td>28</td>
</tr>
<tr>
<td>&gt;0 and ≤ 2 mo</td>
<td>1.31 ± 0.24</td>
<td>6</td>
<td>15</td>
</tr>
<tr>
<td>&gt;2 and ≤ 4 mo</td>
<td>3.12 ± 0.52</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>&gt;4 and ≤ 12 mo</td>
<td>4.12 ± 0.59</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>&gt;12 mo</td>
<td>4.00</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Subjects splenectomized for trauma</td>
<td>4.78 ± 1.32</td>
<td>9</td>
<td>9</td>
</tr>
</tbody>
</table>

A single value from each patient is used for the determination of the mean. Multiple samples from the same patient are averaged.

The abbreviations used are: ITP, idiopathic thrombocytopenic purpura; BVCP, 1,3-bis(2-chloroethyl)nitrosourea-vinblastine sulfate-cyclophosphamide-procarbazine-prednisone.

Table 2 Summary table of patient diagnosis, therapy, and mean frequency of micronuclei per 2000 erythrocytes

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>n</th>
<th>Presplenectomy</th>
<th>Pre-Rx*</th>
<th>0–4 mo on Rx</th>
<th>&gt;4 mo on Rx</th>
<th>&lt;4 mo off Rx</th>
<th>&gt;4 mo off Rx</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vinblastine</td>
<td>4</td>
<td>0 (4/8)</td>
<td>2.8 (2/7)</td>
<td></td>
<td></td>
<td></td>
<td>5.0 (4/6)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>2</td>
<td>0 (2/5)</td>
<td>4.0 (1/3)</td>
<td>18.7 (1/3)</td>
<td>48.5 (1/2)</td>
<td></td>
<td>15.0 (2/4)</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BVCP</td>
<td>7</td>
<td>0 (4/4)</td>
<td>0.7 (3/3)</td>
<td>6.1 (6/18)</td>
<td>11.1 (6/14)</td>
<td>10.7 (3/3)</td>
<td>2.0 (3/3)</td>
</tr>
<tr>
<td>Other chemotherapy</td>
<td>5</td>
<td>0 (4/6)</td>
<td>0 (1/1)</td>
<td>11.6 (4/16)</td>
<td>15.2 (5/19)</td>
<td>6.1 (3/5)</td>
<td>6.4 (4/11)</td>
</tr>
<tr>
<td>Lymphoma or chronic lymphocytic leukemia</td>
<td>5</td>
<td>0 (4/6)</td>
<td>2.0 (3/4)</td>
<td>8.1 (5/11)</td>
<td>10.5 (3/10)</td>
<td>12.0 (2/2)</td>
<td>8.5 (2/7)</td>
</tr>
<tr>
<td>Chlorambucil or hydroxyurea p.o.</td>
<td>5</td>
<td>0 (4/6)</td>
<td>1.0 (1/1)</td>
<td>7.2 (2/6)</td>
<td>32.8 (2/6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miscellaneous combination chemotherapy</td>
<td>4</td>
<td>0 (4/24)</td>
<td>1.0 (1/1)</td>
<td></td>
<td>7.2 (2/6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy patients with intact spleens</td>
<td>17</td>
<td>NA</td>
<td>0 (4/4)</td>
<td>0 (14/33)</td>
<td>0 (9/22)</td>
<td>0 (3/4)</td>
<td></td>
</tr>
<tr>
<td>All splenectomized chemotherapy patients</td>
<td>27</td>
<td>0 (22/53)</td>
<td>1.7 ± 0.6 (11/19)</td>
<td>8.7 ± 1.4 (18/54)</td>
<td>16.9 ± 3.9 (17/51)</td>
<td>8.9 ± 1.9 (14/20)</td>
<td>5.0 ± 1.1 (12/26)</td>
</tr>
</tbody>
</table>

* Rx, therapy; NA, not applicable.
* Numbers in parentheses, number of subjects/number of samples.
* P < 0.01 when compared to either ITP or trauma controls >4 mo after splenectomy.
* P < 0.05.
* Mean ± SE.
for at least 4 mo (mean ± SE of 2000 erythrocytes, 4.99 ± 1.12 versus 4.10 ± 0.46, respectively).

Fig. 1 illustrates the changes in micronucleus frequencies during the course of medical treatment for four splenectomized patients who had a relatively complete series of blood smears available. These patients were chosen to illustrate that, in the same subject, the micronucleus frequency increases with greater treatment duration and that the incidence of micronucleated erythrocytes returns to control values within approximately 4 mo after therapy is discontinued. Two of these subjects (Fig. 1, A and B) are ITP patients who received cyclophosphamide therapy. These treated subjects are therefore closely matched with the untreated ITP controls.

Although micronucleated erythrocytes were selectively removed from the circulation of nonsplenectomized humans, the possibility existed that micronucleated cells could be detected among the newly formed reticulocytes if the selective removal was not too rapid. Since the interval between treatment and peak incidence of micronucleated reticulocytes in the peripheral blood is not known precisely, patients receiving daily treatment were examined. Four nonsplenectomized prostate cancer patients who were receiving daily p.o. therapy with either L-phenylalanine mustard or cyclophosphamide had a significantly increased ($P < 0.01$) frequency of micronucleated reticulocytes compared with 5 untreated controls (mean ± SE of 4000 reticulocytes, 3.75 ± 1.38 versus 0.40 ± 0.40, respectively). The frequencies of micronucleated reticulocytes in the 2 patients receiving 2 mg per day of L-phenylalanine mustard were 1 and 2 per 4000, while significantly higher frequencies ($P < 0.05$) of 5 and 7 per 4000 were seen in the 2 patients receiving daily

---

Fig. 1. Incidence of micronucleated cells among 2000 erythrocytes from Wright's stained blood smears of four splenectomized patients during the course of medical treatment. The durations of chemotherapy and radiotherapy (RT) are represented by the bars at the bottom of each graph. The dashed vertical line represents the date of splenectomy. Patient diagnoses and treatments were: A and B, idiopathic thrombocytopenic purpura. A, treatment with 60–150 mg of p.o. cyclophosphamide per day ($\Delta$) and 10–150 mg of p.o. prednisone per day. B, treatment with a single i.v. dose of 2 mg of vincristine (\(\Delta\)) followed by p.o. cyclophosphamide (V) at a dose of 100 mg/day for Months 12 to 22, 50 mg/day for Months 23 to 31, and 30 mg every other day for Months 32 and 33 and p.o. prednisone at doses of 5–120 mg/day. C, Hodgkin's disease. An i.v. BCOP (\(\Delta\)) regimen was repeated every 4 wk consisting of: BCNU (200 mg); vinblastine sulfate (12 mg); cyclophosphamide (1500 mg); procarbazine (50 mg (Day 1), 100 mg (Day 2), 250 mg (Days 3–10)); and p.o. prednisone [150 mg/day (Days 1–10)]. D, melanoma/lymphoma. A CAOP i.v. regimen repeated every 3 wk consisted of: cyclophosphamide (1000 mg); Adriamycin (70 mg); vincristine sulfate (2 mg); and p.o. prednisone [50 mg/day (Days 1–5)], followed by a B C O P i.v. regimen repeated every 4 wk that consisted of: 1,3-bis(2-chloroethyl)-1-nitrosourea (200 mg); cyclophosphamide (1200 mg); vincristine sulfate (1 mg); and p.o. prednisone [100 mg/day (Days 1–5)].
MICRONUCLEI AS A MEASURE OF CYTOGENETIC DAMAGE

cyclophosphamide doses of 50 mg or 100 mg, respectively. Thus, increases in the frequency of micronucleated reticulocytes are detectable in nonsplenectomized patients receiving chemotherapy, even though there is a rapid removal of micronucleated erythrocytes from the circulation.

DISCUSSION

The rapid removal of micronucleated erythrocytes from the peripheral circulation of nonsplenectomized individuals produces very low frequencies of micronucleated cells among total erythrocytes in both exposed and unexposed subjects. Significant increases were seen, however, in the frequency of micronucleated reticulocytes in the circulation of nonsplenectomized patients receiving daily chemotherapy. Because the observed frequencies in peripheral blood are low relative to the frequency in the newly formed erythrocyte population entering the circulation, it is likely that changes in the efficiency of splenic removal would alter the observed spontaneous frequency in the blood of normal individuals. While there appear to be no published examples of chemicals which impair the splenic removal of micronucleated erythrocytes from the circulation, it is known that splenic infarction is capable of increasing the frequency of these micronucleated cells in the blood (14). It is therefore possible that the frequency of micronucleated reticulocytes will not provide a reliable index of genotoxic damage in eu-splenic individuals unless changes in spleen function can be carefully monitored.

In splenectomized individuals, micronucleated erythrocytes persist in the peripheral blood of both patients who are exposed and those who are unexposed to cytogenetically active agents. The observed spontaneous frequency of approximately 2/1000 in individuals splenectomized for at least 4 mo agrees well with previously reported values among reticulocytes in human bone marrow (3, 4). Among chemotherapy patients who received 2 or more days of treatment, 16 of 22 individuals had at least one sample which was elevated above the highest value recorded in 28 samples from 7 splenectomized ITP controls. Even though the measurement of micronuclei among total erythrocytes is not optimal for detecting infrequent exposures (see following paragraph) and we could not test for residual spleen function in these study subjects, these results compare favorably with results from lymphocyte chromosome analysis performed by Schinzel and Schmid (15) on 67 patients treated with chemotherapy or combinations of chemotherapy and radiotherapy. They reported values which were higher than 10 samples from 10 control individuals for chromatid breaks or chromosome aberrations in 13 of 67 and 29 of 67 patients, respectively. Although the differences in chemotherapeutic agents used and cell types examined do not permit a reliable comparison of the sensitivity of the methods used in this study with those used by Schinzel and Schmid (15), several other human studies have directly compared bone marrow micronucleus and classical cytogenetic analysis for detecting chromosomal damage and have reported increased sensitivity with the micronucleus test (4–6, 16).

If an exposure under evaluation is not given continuously, a disadvantage of measuring the incidence of micronucleated cells among total erythrocytes is that many circulating erythrocytes would be derived from preexisting erythroblasts which were not subjected to the agent under study. Sensitivity for studying episodic exposures would be greatly enhanced by measuring instead the micronucleus frequency among reticulocytes which are derived only from the exposed erythroblast population. The interval between the formation of micronucleated reticulocytes in the bone marrow and their appearance in the peripheral blood following chemotherapy or radiotherapy is not precisely known, and it is likely that the timing varies with the type of therapy and the extent of exposure. These variables have been well documented in the mouse (17–20), and it is reasonable to assume that similar conditions prevail in the human. Reticulocytes which are derived from exposed erythroblasts probably begin entering the peripheral circulation about 3 to 4 days after exposure is initiated (21). The return to control values should occur between 4 and 10 days after exposure is stopped, since the incidence of micronucleated reticulocytes remains elevated in the blood of mice for only about 2 to 3 days (19), and the rate of erythropoiesis and the life span of reticulocytes apparently do not differ by more than a factor of 2 between mice and humans (22, 23). Despite lowered sensitivity for detecting effects from intermittent exposures, measuring the micronucleus frequency in total erythrocytes rather than reticulocytes greatly reduces the danger of missing transiently elevated micronucleus frequencies by reducing the chance of sampling at inappropriate times. Since each of the two cell populations offers different advantages, it is recommended that micronuclei be examined in both reticulocytes and total erythrocytes whenever possible.

The kinetic considerations described above may explain why larger increases in frequencies of micronucleated red cells were obtained in our study than were reported in two studies by Goetz et al. (3, 24). Their findings showed only 2- to 3-fold increases in the frequency of micronucleated reticulocytes from the bone marrow of patients who had received 40 mg of cyclophosphamide per kg i.v. Unfortunately, marrow samples were taken 24 h after exposure, a length of time hardly sufficient for nuclear extrusion among nondividing normoblasts (22) and certainly inadequate for micronucleus formation during the previous cell cycle.

Potential clinical and epidemiological applications of the peripheral blood micronucleus test can now be explored. Further studies of splenectomized patients receiving cytotoxic drugs should be considered. Materials from previous or ongoing clinical trials containing such subjects could be used to investigate the frequency of different agents. Collection of materials from ongoing studies would allow refinements in the timing of samples available in hematology laboratory files. Analyses of pitted erythrocytes for assessment of residual spleen function, uniform sampling times, serum levels of folate or other nutrients that might affect levels of breakage, as well as comparisons with other assays for genotoxic damage would therefore be possible. In epidemiological studies, chemical exposures or other factors potentially associated with clastogenic damage could be evaluated by relating them to the frequency of micronuclei among circulating erythrocytes of splenectomized subjects in cross-sectional studies. Since the incidence of micronucleated reticulocytes should return to base-line levels within approximately 1 to 2 wk after exposure is discontinued, factors causing cytogenetic damage could perhaps best be identified by analyzing serial blood specimens obtained from splenectomized volunteers before and after controlled interventions such as the removal of specific exposures. This protocol would have the advantage of each subject serving as his or her own control. Comparable studies using lymphocyte chromosome analysis would be appreciably more complicated and labor intensive, because the persistence of chromosomal aberrations can vary from a few hours (25) to longer than 1 yr (26), and the scoring of chromosomal aberrations takes approximately 15
times as long as the scoring of micronuclei to achieve equivalent statistical power (27).

ACKNOWLEDGMENTS

The authors thank Dr. John Laszlo of the Department of Medicine, Duke University, for advice in the planning of this project and for facilitating our access to hematological specimens. We also thank Mary Jo Fyfe of Survey Research Associates, Inc., for assistance in collecting blood specimens and clinical data. Special thanks go to Dr. Howard A. Pearson and Edward Sullivan of the Department of Pediatrics, Yale University, for scoring pitted erythrocytes.

REFERENCES

Assessment of Cytogenetic Damage by Quantitation of Micronuclei in Human Peripheral Blood Erythrocytes

Robert Schlegel, James T. MacGregor and Richard B. Everson


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/46/7/3717

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.