Use of the Fluorescent Micronucleus Assay to Detect the Genotoxic Effects of Radiation and Arsenic Exposure in Exfoliated Human Epithelial Cells

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The exfoliated cell micronucleus (MN) assay using fluorescent in situ hybridization (FISH) with a centromeric probe is a rapid method for determining the mechanism of MN formation in epithelial tissues exposed to carcinogenic agents. Here, we describe the use of this assay to detect the presence or absence of centromeric DNA in MN induced in vivo by radiation therapy and chronic arsicken (As) ingestion. We examined the buccal cells of an individual receiving 6,500 rad of photon radiation to the head and neck. Exfoliated cells were collected before, during, and after treatment. After radiation exposure a 16.6-fold increase in buccal cell MN frequency was seen. All induced MN were centromere-negative (MN-) resulting from chromosome breakage. This finding is consistent with the clastogenic action of radiation and confirmed the reliability of the method. Three weeks post-therapy, MN frequencies returned to baseline. We also applied the assay to exfoliated bladder cells of 18 people chronically exposed to high levels of inorganic arsenic (In-As) in drinking water (average level, 1.312 μg As/L) and 18 matched controls (average level, 1.4 μg As/L). The combined increase in MN frequency was 1.8-fold (P = 0.001, Fisher's exact test). Frequencies of micronuclei containing acentric fragments (MN-) and those containing whole chromosomes (MN+) both increased (1.65-fold, P = 0.07, and 1.37-fold, P = 0.15, respectively), suggesting that arsenic may have both clastogenic and weak aneuploidogenic properties in vivo. After strontiation on sex, the effect was stronger in male than in female bladder cells. In males the MN- frequency increased 2.05-fold (P = 0.07) while the frequency of MN+ increased 1.84-fold (P = 0.08). In addition, the frequencies of MN- and MN+ were positively associated with urinary arsenic and its metabolites. However, the association was stronger for micronuclei containing acentric fragments. By using FISH with centromeric probes, the mechanism of chemically induced genotoxicity can now be determined in epithelial tissues. © 1996 Wiley-Liss, Inc.

Key words: aneuploidy, chromosome damage, genotoxicity, micronuclei, radiation, arsenic, biomarkers, exfoliated cells, human

INTRODUCTION

Exfoliated cells from the intermediate and superficial layers of the epithelium have been widely used in cytology to detect abnormal morphology, premalignant changes, and cancer [Brawn, 1984; Bryan and Cohen, 1983]. In epidemiological biomarker studies, genetic changes in these cells are of particular interest because the epithelial tissues from which they arise are frequently the target of chemical agents. Exfoliated cells from some of these tissues are ideal for use in field studies since a large number of cells can be rapidly and non-invasively collected from study participants. In the laboratory, the resulting sample can be fixed on microscope slides and stored for future use months or years from the date of collection.

Exfoliated cells must be studied in interphase because the majority are non-viable. For this reason it is not possible to use traditional cytogenetic techniques requiring cell culture and metaphase spreads to study genetic damage and chromosomal changes in these cells. Presently only two assays are available which measure genetic damage in interphase cells, the micronucleus (MN) assay and interphase cytogenetics with DNA probes. The MN assay measures the frequency with which chromosomes and chromosomal fragments are lost to the nucleus during cell division. This assay was first used in exfoliated cells by Stich and Rosin [1983]. They used a non-fluorescent Feulgen-fast green staining to dye the nuclei a bright pink and the cytoplasm a light green. The assay has been used on

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a wide variety of cell types to measure the effects of environmental and occupational exposures such as smoking, pesticides, and parasitic infection in tissues where the highest cancer risks exist [Fontham et al., 1986; San et al., 1989; Rosin and Anwar, 1992]. The second method, in situ hybridization with labeled chromosome probes, can be used to measure gains and losses of particular chromosomes or genes in interphase cells collected from target tissues of interest. The application of this method is relatively new and has only recently been applied to exfoliated epithelial cells [Moore et al., 1993a,b].

The recently developed fluorescent MN assay is similar to the standard MN assay with regard to identifying and scoring MN and abnormal nuclear events, but uses fluorescent in situ hybridization (FISH) to identify the presence or absence of centromeres within each MN by employing a probe specific for all human centromeres [Titenko-Holland et al., 1994]. MN are formed due to whole chromosomes or chromosomal fragments being left outside of the main nucleus at telophase. By identifying the presence (MN+) or absence (MN−) of the centromeric probe within an MN, one can determine whether the MN was formed by an aneuploidigenic or clastogenic mechanism, respectively. In earlier studies, this assay was used to determine baseline MN frequencies in exfoliated buccal cells from healthy non-smoking female volunteers [Titenko-Holland et al., 1994]. To further validate the assay as an indicator of genetic damage in humans we sought to test it in exfoliated cells from individuals who had undergone in vivo exposure to established genotoxic agents.

Radiation was chosen for this purpose for a number of reasons. First, the MN assay has been widely used to study the clastogenic effects of radiation in many cell types including exfoliated cells [Abraham et al., 1993; Miller et al., 1993; Stich and Rosin, 1983]. Further, it has been determined that radiation-induced MN in blood cell cultures are primarily clastogenic in origin. Generally, dose-dependent increases are seen except in cases where cell death [Stich and Rosin, 1983], cell proliferation [Wutke et al., 1993], or cell cycle delay [McFee et al., 1994] was shown to influence the MN frequencies found. Human epithelial cells exposed to radiation during the course of therapy are also relatively easy to obtain. Cells can be collected before, during, and after treatment, making each subject his or her own control. During therapy, patients receive known doses of ionizing radiation to particular tissues. By taking samples at different intervals of treatment, the response of epithelial cells to increasing cumulative dose can be examined.

We also applied the new assay to a population environmentally exposed to inorganic arsenic (In-As) through their drinking water. In-As is known to be a cause of lung cancer by inhalation and skin cancer via ingestion [IARC, 1980]. Increasing epidemiological evidence suggests that ingestion of In-As can also cause lung, bladder, kidney, and liver cancer [Bates et al., 1992]. A number of biomarker studies of human populations environmentally exposed to As have been conducted [Vig et al., 1984; Ostrosky-Wegman et al., 1991; Nilsson et al., 1993; Lerdal, 1994]. The findings to date do not correlate well with findings in vitro and have revealed little about the mechanism of As-induced genotoxicity in humans. Arsenic has been shown to have both clastogenic and aneuploidigenic properties in lymphocytes in vitro using both the MN assay and chromosome specific probes [Eastmond and Tucker, 1989; Eastmond and Pinkel, 1990; Eastmond et al., 1993]. Vega et al. [1995] also evaluated the aneuploidigenic potential of As in vitro and found a dose-related increase in hyperploid lymphocytes. To further test the new fluorescent MN assay we decided to apply it to slides from a study of As-exposed and -unexposed urothelial cells [Warner et al., 1994] to determine the mechanism of As-induced genotoxicity in vivo. In both radiation- and As-exposed cells the method was capable of distinguishing MN induced by chromosome breakage or lagging.

**MATERIALS AND METHODS**

**Radiation-Exposed Individual**

A 29-year-old male received radiation therapy to the right side of the jaw in the course of treatment for a salivary gland tumor. A cumulative dose of 6,400 rads of photon radiation was given 5 days/week for a period of 9 weeks. Before, during, and after treatment, cells from the oral mucosa were collected by rubbing the inside of the mouth with a premoistened tongue depressor as described elsewhere [Moore et al., 1993a].

**Arsenic-Exposed Population**

The study population included residents of a county in Nevada with private water supply wells. Exposed subjects were defined as individuals with wellwater As levels >500 μg A/VL, more than ten times the U.S. maximum contaminant level (MCL). Unexposed subjects were defined as individuals with wellwater As levels <10 μg A/VL. To ensure chronic exposure, the study was restricted to individuals who had resided in their home for at least 1 year and used no additional water source such as filtered or bottled water. To reduce confounding, each exposed subject was individually matched on age (±4 years), sex, and smoking status to a similarly identified unexposed subject. As an additional measure of As exposure, urinary In-As and its metabolites were measured as previously described [Kalman, 1988]. Other details of the study population and epidemiological methods are described elsewhere [Warner et al., 1994].

**Cell Collection and Staining**

Buccal cells were dropped, fixed, and stored and FISH was performed as previously described [Moore et al., 1993a; Titenko-Holland et al., 1994]. To obtain bladder cell samples from the As-exposed and -unexposed individuals, each subject was asked to provide a urine sample from the second and third voids of the day. The first void of the day was not collected because exfoliated bladder cell degradation occurs when the cells have been in contact with urine overnight. Because
females generally provide more cells per void than males, a total of two urine samples were obtained from females and four from males. Participants were supplied with precoded polypropylene bottles and instructions for urine collection. Bladder cells were collected from the urine specimens within 2 hr via centrifugation and the cell pellet was washed with 0.9% NaCl. After centrifugation, cells were dropped on a coded slide, air-dried, fixed in 80% methanol at 0°C, and stored in a nitrogen atmosphere at ~20°C until use for the micronucleus assay.

**Micronucleus Assay**

A new version of the MN assay that employs the fluorescent dye propidium iodide and FISH with a biotin-labeled probe for all human centromeres was used. This procedure appears to be easier and more reliable than the previous method of Feulgen-fast green staining and also allows for the mechanism of MN formation to be determined [Moore et al., 1993; Titenko-Holland et al., 1994].

Briefly, the slides were preheated for 30 min on a slide warmer at 63°C to fully adhere cells to the slide. They were then treated with 300 µg/ml pepsin for 30 min at 37°C to permeabilize the cells [Moore et al., 1993b]. The slides were subsequently rinsed twice in phosphate-buffered saline and fixed in buffered 4% parafomaldehyde for 20 min at 0°C. After washing, the slides were baked for 20 min at 63°C. They were then hybridized with a biotin-labeled alpha-satellite probe specific for all human centromeres (Oncor) as described by Titenko-Holland et al. [1994]. The fluorescent dye, propidium iodide at 1 µg/ml in antifade solution, was used to counterstain the DNA.

**Scoring Procedure and Criteria**

All slides were first examined with low-power magnification to observe the quality of the slide and screen for the presence of polymorphonuclear leukocytes, bacteria, and fungi since heavy infections may interfere with scoring. Slides were then scored using a Nikon microscope equipped with epifluorescent illumination, a 100× oil immersion lens and a filter for fluorescein and propidium iodide (excitation at 450-490 nm, dichroic at 510 nm, and emission at 520 nm).

The radiation-exposed buccal cell slides were coded and stained simultaneously with the goal of counting at least 1,000 normal cells/slide. The As-exposed and -unexposed slides were coded, stained, and scored in groups so that each matched pair was scored simultaneously with the scorer blind as to which slide was an exposed subject and which slide was the matched control. Because of interindividual variations in number of normal cells per urine void, between 500 and 2,900 normal cells were scored for each subject. Only cells that were not smeared, clumped, or overlapped and that contained intact nuclei were included in the analysis. Cells undergoing abnormal cell division and degenerative processes such as karyorrhexis, karyolysis, nuclear fragmentation, and pyknosis were recorded separately [Tolbert, 1992]. The frequency of micronucleated cells was estimated based on the number of normal exfoliated cells scored.

**Statistical Analysis**

**Radiation Exposure**

The data were analyzed using STATA [STATA Computing Resource Center, 1992] software. Statistical analyses to compare micronucleus frequencies before, during, and after radiation exposure were performed using the Fisher exact test. The analysis focused on the effect of radiation exposure on the frequency of cells with micronuclei. All counts were converted to frequency of micronucleated cells (MNC) per 1,000 normal exfoliated cells. It was hypothesized a priori that radiation exposure would be associated with an increase in the frequency of micronucleated cells so one-tailed tests were used.

**Arsenic Exposure**

The data were analyzed using STATA [STATA Computing Resource Center, 1992] and PC SAS software [SAS Institute Inc., 1988]. The analysis focused on the effect of As exposure on the frequency of cells with micronuclei. Selection of study subjects and methods for determining the mean frequency of exposed and unexposed groups were performed as previously described [Warner et al., 1994]. The 90% confidence limits were determined, statistics for significance of the effect measure (frequency ratio) was assessed by the Wilcoxon sign-rank test. The apparent discrepancy between the P value and the confidence intervals excluding 1 is due to the test of significance being nonparametric whereas confidence limits were derived with parametric statistics [Warner et al., 1994]. A Student's t-test was used to evaluate the differences in percent normal cells for each group. In the analysis of centromere-specific probe frequencies the Fisher exact test was used to compare the frequency of MN/+/ for the exposed and unexposed groups. The frequencies compared were calculated by dividing the total number of MN/+/ by the total number of normal cells scored in each group.

The dose-response relationship between urinary arsenic measures and centromere-specific frequency was assessed by regression analysis. Two measures of urinary As were examined, In-As and the sum of In-As and its metabolites, monomethylarsonic acid (MMA) and dimethylarsonic acid (DMA). The associations between MN+/ and MN− frequencies and As exposure were assessed with the Spearman non-parametric correlation coefficient.

**RESULTS**

**Effect of Radiation Therapy on Buccal Mucosal Cells**

**Percentage of Abnormal Cells**

The percentage of abnormal and normal cells at each therapy time point is shown in Table I. The percentage of abnormal cells was calculated by dividing the number of intact nuclei undergoing the degenerative processes of karyorrhexis and karyolysis by the total number of cells scored. The percentage of abnormal cells prior to therapy and 3 weeks after therapy commenced was 24.0% and 21.0% respectively. Six weeks and 12 weeks (3 weeks after cessation of therapy) after commencement of exposure, the percent abnormal cells was significantly elevated to 41.3% and 39.6% respectively ($P < 0.001$).

**MN Frequencies**

The numbers of micronucleated cells (MNC) in buccal mucosa before, during, and after radiation therapy are also presented in Table I. Approximately 2,000 exfoliated buccal cells were analyzed for each time point. Pre-therapy MN frequencies were similar to baseline frequencies of healthy volunteers using both the standard Feulgen fast-green and the new fluorescent methods of staining [Titenko-Holland et al., 1994]. A 16.6- and 12.6-fold increase in MN frequency was observed 3 and 6 weeks after initiation of radiation exposure, respectively ($P < 0.001$). Three weeks post-therapy, MN frequencies returned close to baseline. MN frequencies were not significantly different pre- and post-radiotherapy ($P =$
TABLE 1. Micronucleus Frequencies in Exfoliated Buccal Cells of an Individual Receiving Radiation Therapy to the Neck Before, During, and After Treatment

<table>
<thead>
<tr>
<th>Time</th>
<th>Cells scored</th>
<th>Normal cells (%)</th>
<th>Abnormal* cells (%)</th>
<th>Cells w/MN</th>
<th>MN−/+−/+*</th>
<th>% MN cells</th>
<th>%MN−</th>
<th>%MN+</th>
<th>MN−/MN+</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 weeks (pre-therapy)</td>
<td>2.050</td>
<td>76.0 ± 0.5</td>
<td>24.0 ± 0.5</td>
<td>2</td>
<td>1/20</td>
<td>0.098 ± 0.003</td>
<td>0.048 ± 0.141</td>
<td>0.100 ± 0.141</td>
<td>33.3%/66.7%</td>
</tr>
<tr>
<td>3 weeks</td>
<td>2.029</td>
<td>79.0 ± 6.5</td>
<td>21.0 ± 6.5</td>
<td>33</td>
<td>24/210</td>
<td>1.627 ± 0.073</td>
<td>1.185 ± 0.163</td>
<td>0.097 ± 0.137</td>
<td>92.3%/7.7%</td>
</tr>
<tr>
<td>6 weeks</td>
<td>2.029</td>
<td>58.7 ± 12.4</td>
<td>41.4 ± 12.4</td>
<td>25</td>
<td>17/210</td>
<td>1.230 ± 0.131</td>
<td>0.836 ± 0.192</td>
<td>0.097 ± 0.137</td>
<td>89.5%/10.5%</td>
</tr>
<tr>
<td>12 weeks (3 weeks after</td>
<td>2.000</td>
<td>60.4 ± 17.1</td>
<td>39.6 ± 17.1</td>
<td>3</td>
<td>0/0/3</td>
<td>0.150 ± 0.050</td>
<td>N.C.</td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>cessation of therapy)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Cells undergoing the degenerative processes of karyorrhexis and karyolysis.

**Cells for which the mechanism of MN formation could not be determined or did not fit all criteria, unscorable.

N.C. = not calculable.

0.488. In contrast, the percentage of normal cells had not returned to baseline 3 weeks post-exposure.

**Centromere-Specific MN Frequencies**

In Table 1 frequencies of MN characterized for the presence or absence of centromeric probe are presented. To calculate the frequency of centromere-positive and -negative MN, the total number of MN of each sub-type was divided by the total number of normal cells scored. The increase in MN− and MN+ was calculated by dividing the frequency of MN− and MN+ by the pre-treatment frequency. After 3 weeks of radiotherapy a 24.4-fold increase in MN− was seen. There was no increase in the frequency of MN+. Similarly, after 6 weeks of radiotherapy a 17.4-fold increase in only MN− was seen. The effect of radiation on the induction of MN with different mechanisms of formation was also determined by calculating the change in the proportion of MN+ and MN−. The MN−/MN+ ratio was calculated by dividing the number of MN+ and MN− by the total number of MN scored for that time point (Table 1). Prior to radiation exposure, 33% of all characterizable MN were MN+. After 3 and 6 weeks exposure, the fraction was raised to 92.3% and 89.5% respectively. The change in proportion correlates with the finding of a significant rise in MN− buccal cells. Post-radiation exposure (3 weeks after cessation of therapy), frequencies of MN+ and MN− could not be determined because the buccal cells did not hybridize well. Additional protein digestion and staining were performed, but staining remained suboptimal. Cells were covered by a thick cell membrane and nuclei appeared shrunken but they were not considered pycnotic.

**Cells Containing Multiple Micronuclei**

Frequently, severe genotoxic insults may result in multiple MN being formed when spindle disturbances or multiple clastogenic events take place within a cell. Multiple MN are rarely seen in unexposed or control cells; therefore, they can be a good indicator of genetic damage. Before treatment, only one multiple MNC was seen containing two MN positive (2MN+) for the centromeric probe. After 3 weeks exposure, three cells containing 2MN− were found. At 6 weeks exposure two cells with 2MN− and one cell with 3MN− were found, suggesting the induced micronuclei were clastogenic in origin and contained acentric fragments of DNA.

**Effect of Inorganic Arsenic Exposure on Urothelial Cells**

**Percentage of Abnormal Cells**

When the percentage of abnormal cells was compared in the exposed and unexposed group, evidence of an association between the frequency of cells undergoing abnormal cell division and degenerative processes and As exposure was not found. The percentage of abnormal bladder cells was 22 ± 20 and 20 ± 18 respectively. However, after adjusting for sex, the percentage of abnormal cells was significantly elevated in exposed versus unexposed females but not males. The percentage of abnormal cells in exposed and unexposed females was 15 ± 12 and 9 ± 4 (P < 0.01), respectively. The percentage of abnormal cells in exposed and unexposed males was 32 ± 24 and 34 ± 19, respectively. We also found the percentage of abnormal cells to be consistently higher in male bladder cells compared to female bladder cells. It is noteworthy that a large variation exists in the percentage abnormal cells between individuals in a group [Titenko-Holland et al., 1994].

**MN Frequencies**

A comparison of the weighted mean frequencies of MN in bladder cells between exposed and unexposed groups has previously been presented in Warner et al. [1994]. In this study, a 1.8-fold increase in the weighted mean frequency of MNC of the bladder was seen. The weighted mean frequency in the exposed group was 2.79 per 1,000 cells as compared with 1.57 per 1,000 cells in the unexposed groups (90% CI 1.06–2.99; P = 0.09). After stratification on sex the weighted mean frequency of MNC in bladder cells was clearly elevated among exposed males compared to unexposed males (FR); (FR =
TABLE II. Micronuclei in Urothelial Cells of Arsenic-Exposed and -Unexposed Individuals

<table>
<thead>
<tr>
<th>Pair</th>
<th>Normal cells</th>
<th>MN cells</th>
<th>MN+/-fu</th>
<th>%MN cells</th>
<th>Normal cells</th>
<th>MN cells</th>
<th>MN+/-fu</th>
<th>%MN cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.139</td>
<td>0/0/0</td>
<td>0.00</td>
<td>1.842</td>
<td>4</td>
<td>0/0/0</td>
<td>0/0/0</td>
<td>0.00</td>
</tr>
<tr>
<td>2</td>
<td>1.558</td>
<td>0/1/1</td>
<td>0.13</td>
<td>2.286</td>
<td>3</td>
<td>0/0/0</td>
<td>0/0/0</td>
<td>0.00</td>
</tr>
<tr>
<td>3</td>
<td>462</td>
<td>0/1/1</td>
<td>0.43</td>
<td>1.856</td>
<td>1</td>
<td>0/0/0</td>
<td>0/0/0</td>
<td>0.00</td>
</tr>
<tr>
<td>4</td>
<td>1.609</td>
<td>0/2/6</td>
<td>0.62</td>
<td>2.690</td>
<td>4</td>
<td>3/1/0</td>
<td>0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>5</td>
<td>2.827</td>
<td>0/1/0</td>
<td>0.04</td>
<td>1.288</td>
<td>5</td>
<td>5/0/0</td>
<td>0.22</td>
<td>0.00</td>
</tr>
<tr>
<td>6</td>
<td>1.159</td>
<td>3/7/1</td>
<td>0.35</td>
<td>763</td>
<td>2</td>
<td>1/0/1</td>
<td>0.26</td>
<td>0.00</td>
</tr>
<tr>
<td>7</td>
<td>2.286</td>
<td>4/1/1</td>
<td>0.21</td>
<td>1.449</td>
<td>0</td>
<td>0/0/0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>578</td>
<td>2/0/5</td>
<td>0.69</td>
<td>731</td>
<td>3</td>
<td>2/2/0</td>
<td>0.17</td>
<td>0.00</td>
</tr>
<tr>
<td>9</td>
<td>1.118</td>
<td>3/2/8</td>
<td>0.98</td>
<td>1.748</td>
<td>3</td>
<td>2/2/0</td>
<td>0.17</td>
<td>0.00</td>
</tr>
<tr>
<td>10</td>
<td>1.848</td>
<td>3/1/0</td>
<td>0.22</td>
<td>1.875</td>
<td>1</td>
<td>0/0/0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>11</td>
<td>1.824</td>
<td>0/0/0</td>
<td>0.00</td>
<td>1.847</td>
<td>1</td>
<td>0/0/0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>12</td>
<td>1.069</td>
<td>8/4/8</td>
<td>1.31</td>
<td>2.231</td>
<td>6</td>
<td>2/2/0</td>
<td>0.27</td>
<td>0.00</td>
</tr>
<tr>
<td>13</td>
<td>430</td>
<td>0/0/1</td>
<td>0.23</td>
<td>1.170</td>
<td>0</td>
<td>0/0/0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>14</td>
<td>2.061</td>
<td>0/0/1</td>
<td>0.05</td>
<td>0.655</td>
<td>2</td>
<td>0/0/0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>15</td>
<td>1.408</td>
<td>1/1/0</td>
<td>0.14</td>
<td>1.654</td>
<td>3</td>
<td>1/0/2</td>
<td>0.18</td>
<td>0.00</td>
</tr>
<tr>
<td>16</td>
<td>402</td>
<td>0/0/1</td>
<td>0.25</td>
<td>1.712</td>
<td>4</td>
<td>3/1/0</td>
<td>0.23</td>
<td>0.00</td>
</tr>
<tr>
<td>17</td>
<td>2.875</td>
<td>4/3/2</td>
<td>0.31</td>
<td>1.667</td>
<td>5</td>
<td>3/1/3</td>
<td>0.30</td>
<td>0.00</td>
</tr>
<tr>
<td>Total</td>
<td>27.561</td>
<td>31/25/35</td>
<td>0.28</td>
<td>29.287</td>
<td>46</td>
<td>24/16/11</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

TABLE III. Characterization of Micronuclei With a Centromeric Probe in Exfoliated Urothelial Cells of Arsenic-Exposed and -Unexposed Individuals

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cells scored</th>
<th>Frequency MN- (%)</th>
<th>Frequency MN+ (%)</th>
<th>MN-/MN+ (P values)</th>
<th>Ratio MN-/MN+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total exposed</td>
<td>N.A.</td>
<td>1.65/1.37</td>
<td>0.07/0.15</td>
<td>40%/60%</td>
<td></td>
</tr>
<tr>
<td>Total stranded</td>
<td>N.A.</td>
<td>1.65/1.37</td>
<td>0.07/0.15</td>
<td>45%/55%</td>
<td></td>
</tr>
<tr>
<td>Males exposed</td>
<td>9,833</td>
<td>0.081</td>
<td>0.102</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Males unexposed</td>
<td>8,406</td>
<td>0.167</td>
<td>0.190</td>
<td>2.06/1.86</td>
<td></td>
</tr>
<tr>
<td>Females unexposed</td>
<td>19,454</td>
<td>0.041</td>
<td>0.072</td>
<td>N.A.</td>
<td></td>
</tr>
<tr>
<td>Females exposed</td>
<td>19,155</td>
<td>0.057</td>
<td>0.078</td>
<td>1.39/0.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(0.31/0.48)</td>
<td></td>
</tr>
</tbody>
</table>

N.A. = not applicable.

2.34; 90% CI 1.27–4.29; P = 0.07). However, there was little difference between exposed females and unexposed females (FR = 1.42; 90% CI 0.76–2.65; P = 0.38).

Raw data from the As study are presented in Table II. The frequencies of MN+ ranged from 0 to 0.75% for exposed individuals and 0 to 0.24% for unexposed individuals. The frequencies of MN− ranged from 0 to 0.37% for exposed individuals and 0 to 0.30% for unexposed individuals. Table III presents the urothelial cell frequencies of both MN+ and MN−. For the total group and after stratification on sex the frequencies of both MN+ and MN− show small increases. These data show that the frequency of MN without centromeres (MN−) was 1.65-fold (P = 0.07) while the increase in MN+ was 1.37-fold (P = 0.15). When the frequency of centromere-specific MN is compared after stratification on sex a larger increase in both MN− and MN+ is seen in males than in females. In males the increase in frequency of MN− was 2.06-fold (P = 0.07), and the increase in MN+ was 1.86-fold (P = 0.08) while in females the increase in frequency of MN− was 1.39-fold (P = 0.31), and the increase in MN+ was 1.08-fold (P = 0.48).

We also examined the relative change in the proportion of MN+ and MN− with respect to arsenic exposure. In both exposed and unexposed urothelial cells MN− were less common than MN+; however, a small increase in the proportion of MN− was seen in the As-exposed group both before and after stratification on sex. In the total
TABLE IV. Summary of Spearman Correlation Coefficients for Urinary Arsenic and Frequency of Centromere-Specific MN in Bladder Cells Before and After Stratification on Sex

<table>
<thead>
<tr>
<th>Urinary arsenic exposure</th>
<th>Frequency MN−</th>
<th>Frequency MN+</th>
<th>Frequency MN−</th>
<th>Frequency MN+</th>
<th>Frequency MN−</th>
<th>Frequency MN+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum As</td>
<td>0.195</td>
<td>0.074</td>
<td>0.24</td>
<td>0.040</td>
<td>0.308</td>
<td>0.188</td>
</tr>
<tr>
<td>(P = 0.07)</td>
<td>(P = 0.07)</td>
<td>(P = 0.13)</td>
<td>(P = 0.10)</td>
<td>(P = 0.73)</td>
<td>(P = 0.88)</td>
<td></td>
</tr>
<tr>
<td>In-As</td>
<td>0.250</td>
<td>0.060</td>
<td>0.297</td>
<td>0.025</td>
<td>0.331</td>
<td>0.062</td>
</tr>
<tr>
<td>(P = 0.03)</td>
<td>(P = 0.06)</td>
<td>(P = 0.07)</td>
<td>(P = 0.03)</td>
<td>(P = 0.74)</td>
<td>(P = 0.86)</td>
<td></td>
</tr>
</tbody>
</table>

In-As = inorganic arsenic; Sum As = In-As + methylarsonic acid + dimethylarsinic acid.

The proportion of centromere-negative MN increased from 40 to 45%. In the exposed males the proportion of centromere-negative MN increased from 44 to 47% while in the exposed females the proportion of centromere-negative MN increased from 36 to 42%. This finding correlates with the consistently larger fold-increase in MN− in the exposed population both before and after stratification on sex (Table III). These results suggest that As induces both centromere-negative and centromere-positive MN, but the largest increases were seen in centromere-negative MN.

Association of Arsenic Exposure and Micronucleus Formation

In Warner et al. [1994], it was shown that As exposure was positively associated with micronucleus frequency in exfoliated bladder cells. The effect appeared to be stronger in male bladder cells than in female bladder cells. In Table IV we present associations between total urinary As, In-As, and the centromere-specific micronucleus frequencies with Spearman correlation coefficients and one-way P values before and after stratification on sex. Overall the correlation coefficients suggest that As exposure may be positively associated with the change in the frequency of MN− albeit non-significantly (Table IV). After adjusting for sex, a similar non-significant correlation between micronuclei containing acentric fragments is seen in male and female cells.

Cells Containing Multiple Micronuclei

As seen in the radiation-exposed cells, severe genotoxic insult frequently results in multiple micronuclei being formed within a cell. Thus, the presence of multiple MN is a good indicator of genetic damage. In this study a total of 12 multiple micronucleated cells were found. Of these 12 multiple micronucleated cells, seven were found in exposed males, three in unexposed males, and three in unexposed females. Although many of the multiple micronucleated cells were unscarable, it is interesting to note that the majority of micronuclei contained in these cells from exposed males contained acentric fragments (7−, 1+, 10u). Only one MN found was positive for the centromeric probe. The frequency of centromere-positive - and -negative micronuclei remained equal in unexposed males (1+, 1−, 4u) and females (1+, 1−, 2u).

DISCUSSION

We have demonstrated that the fluorescent MN assay can be used to determine the mechanism involved in the elevation of MN frequency observed in exfoliated epithelial cells following in vivo radiation exposure. The majority of MN observed contained acentic fragments of DNA (MN−), consistent with the known clastogenic action of radiation. Further, in radiation-exposed epithelial cells the ratio of MN− to MN+ was similar to that found by other investigators in vitro [Fenech and Morley, 1989; Eastmond and Tucker, 1989; Salissidis et al., 1992]. Earlier methods used MN size [Kormos and Koteles, 1987] and DNA content [Pincu et al., 1985] to assess the mechanism of MN formation. Recently, antikinetochore antibodies and centromeric DNA probes have been used in lymphocytes and cultured cells to assess the mechanism of MN formation. Now using the fluorescent MN assay with FISH, one can study mechanisms of genetic damage in epithelial tissues, the site of the majority of human cancers.

To our knowledge only two other laboratories have used the exfoliated cell MN assay to measure the effects of radiotherapy on epithelial tissues. Both found radiation exposure to be a strong inducer of MN formation, but there was considerable variation in the MNC frequencies reported. For example, Tolbert et al. [1992] found a sixfold increase in the prevalence of medium and high certainty MNC of the radiation-exposed group (10/1,000 cells) compared to the unexposed group (1.6/1,000 cells). The mean prevalence of high certainty MNC for the radiation-exposed group was 5.2/1,000 cells, a 17-fold increase over control levels. Similarly, we found the MNC frequency was 16.3/1,000 cells at 3 weeks exposure, a 16.6-fold increase over pre-treatment levels, and 12.3/1,000 cells at 6 weeks exposure, a 12.5-fold increase. In an earlier study, Stich and Rosin [1983] found that up to 30% of the buccal cells from a patient receiving 3,400
rads to the left and right cheek contained micronuclei after 3 weeks exposure. Although interindividual variability can be substantial, some variability may be due to differences in scoring criteria of each laboratory and the presence of degenerating cell nuclei in the cell sample.

Using structured scoring criteria [Tolbert et al., 1992] helped to correctly identify normal cells in which to score MN, and to exclude degrading cells which could be confused with MNC. During oral radiation therapy a condition known as mucositis is commonly seen in patients exposed to high levels of radiation [Silverman, 1990]. This condition is secondary to radiation-induced mitotic death of the basal cells in the oral mucosa and results in ulceration and necrosis of epithelial tissues. Mucositis was observed in this patient at the end of therapy. Slides obtained at 9 weeks, just prior to cessation of treatment, were not considered scorables and could not be used for this study. The fluorescent MN assay can make scoring easier and more precise by correctly identifying degenerate nuclei and accurately selecting only normal cells in which to score micronuclei. By using strict criteria when scoring, cytotoxic or degenerative cell processes will be less likely to be confused with normal cells containing MN in the sample.

The mechanism of As-induced genotoxicity in this field study was also consistent with in vitro data. Arsenic appeared to have both clastogenic and weak aneuploidyogenic properties in vivo. The frequencies of MN+ and MN− in the exposed group compared to the unexposed group increased but non-significantly. Additionally, after stratification on sex the frequencies of both MN+ and MN+ in bladder cells are elevated among exposed males compared to unexposed males while there is little difference between exposed females and unexposed females. The apparent lack of consistency may be due to the fact that males exfoliate primarily transitional epithelial cells in the urine, those that line the bladder wall. Females, on the other hand, exfoliate mainly squamous cells that originate from other regions of the genitourinary tract and only a small percentage of transitional cell carcinoma of the bladder. Therefore, squamous cell contamination of the female urothelial cell samples without the ability to differentiate them from transitional cells could mask any association between chronic ingestion of As and bladder cell micronuclei observed. The occurrence of bladder cell misclassification among females but not males could be expected to bias the results among females towards no effect.

The fluorescent MN assay appears to make the identification of MN easier and more precise, and to allow the mechanism of MN formation to be studied. However, to analyze exfoliated cell MN data from an exposed population, one must be aware that there are a number of factors that can seriously influence MN frequency, and the frequency of MN+ and MN− found in a sample. In control populations we have shown how number of cells scored per person, timing of sample collection, and scoring criteria can influence MN frequencies found [Moore et al., 1993b; Titenko-Holland et al., 1994]. In exposed populations, some additional factors should be considered when analyzing MN data and determining the significance of results.

First, exfoliated cell MN frequencies are influenced by chemical-induced cytotoxicity. In vitro, MN frequencies generally decline as concentrations of genotoxic chemicals reach cytotoxic levels. We have attempted to use methods of Tolbert et al. [1992] to measure abnormal nuclear events such as karyolysis, karyorrhexis, chromosome fragmentation, and nuclear pyknosis as measures of cytotoxicity; however, it is still possible to underestimate the frequency of cell death in a sample. Finding high levels of cytotoxicity in an exposed tissue is a possible explanation for not finding an exposure-dependent increase in MN frequency. Second, cell proliferation rates can be influenced by chemical exposure. Decreased cell proliferation can negatively influence MN frequencies since 1) proliferation is needed to produce MN after genotoxic insult has taken place and 2) decreased cell proliferation could conceivably give cells more time to repair themselves via cellular repair mechanisms. It is possible that decreased cell proliferation could result in fewer cells per slide or fewer cells per urine void; however, sampling time of day, age of subject, length of time samples remained in urine before fixation, previous physical activities of subject, and female hormonal cycle can also influence these factors. If a decrease in cell proliferation has occurred, it is possible that a negative dose-dependent response would result. Third, it appears as if hybridization efficiency varies between individuals in a group and between cell types.

Hybridization efficiency decreased with increasing exposure to both As and radiation. In earlier studies of buccal cells from young, healthy, non-smoking volunteers, generally over 90% of cells hybridized with centromeric and chromosome specific probes [Titenko-Holland et al., 1994; Moore et al., 1993b] and it was possible to evaluate all MN for the presence or absence of centromeric probe. Variability in hybridization efficiency began to appear when urothelial cells from five female and five male volunteers were examined [Moore et al., 1993b]. In females only 2–10% of exfoliated urothelial cells did not hybridize, whereas in males 9–24% of cells did not hybridize. This difference is probably caused by the difference in exfoliated cell types in male and female urine. It is possible that male cells are exposed to the osmotic forces of urine and therefore vary with respect to their external environment more so than female cells. In exposed epithelial cells of any type, variation may also occur.
due to a thickening of the cell membrane as a protective response to genotoxic or cytotoxic exposure. In unexposed pre-radiation therapy buccal cells it was possible to determine in all MN whether or not they contained centromeric probe. After radiation exposure, almost 30% of the cells were unscorable. This trend continued until 3 weeks post-exposure. At this time point, cells appeared covered by a thick cell membrane and nuclei appeared shrunken but were not considered pyknotic. Additional protein digestion and staining were performed; however, MN remained unscorable.

The same phenomenon was seen in urothelial cells of the As-exposed group when compared to the unexposed group. In the unexposed group 17% of micronucleated cells and 21.5% of micronuclei were unscorable due to variations in protein digestibility within the group. In the As-exposed group 33% of MNC and 28% of MN were considered unscorable, suggesting that As exposure somehow caused a thickening of the cell membrane and impeded probe penetration to the nucleus.

To confirm this finding, we examined the percentage of unscorable MN and MNC in the four individuals most highly exposed and sensitive to As. Their samples alone contained 27/35 (77%) of the unscorable MN and 18/26 (69%) of the unscorable MNC. This provides additional evidence that in vivo exposure to a genotoxic or cytotoxic agent can cause variability in the hybridization efficiency and centromere detection within MN. To overcome these problems we suggest beginning with a lower concentration of pepsin to avoid over-digestion of cells and, subsequently, increasing proteinase concentrations on the same or a subsequent sample if hybridization efficiency is suboptimal. Similar methods are employed when interphase cytogenetics are used to evaluate aneuploidy frequencies in tumor samples since they also vary with respect to their protein digestibility.

In conclusion, we have shown that the fluorescent MN assay is a useful method for determining the mechanism of MN formation in epithelial tissues. A centromeric probe was used with this assay to determine the mechanism of radiation-induced micronucleus formation. A distinct increase in centromere-negative MN was found in radiation-exposed buccal cells, confirming the clastogenic action of radiation. The mechanism of As-induced genetic damage to the human bladder epithelium was also examined. Small, yet non-significant, increases in both centromere-negative and centromere-positive MN were found in exfoliated urothelial cells from 33-exposed people, suggesting that As may have clastogenic and weak aneuploidogenic properties in vivo. However, due to 1) the small sample size of the present study and 2) the potential for bladder cell misclassification that occurs among females, a larger study including only males should be conducted to confirm these findings. Such a study is currently underway on an As-exposed population in Chile.

REFERENCES


Accepted by—
J. Yager