Cytogenetic monitoring of hospital workers occupationally exposed to ionizing radiation using the micronucleus centromere assay

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A cytogenetic study was performed in lymphocytes of hospital workers occupationally exposed to X- and γ-rays using the micronucleus centromere assay. A comparison of the data for the exposed group and an age-matched group of non-exposed hospital workers showed a significant (P < 0.05) increase in centromere-positive micronuclei for the radiation workers, while no effect on centromere-negative micronuclei was present. The observed systematic increase in micronucleus frequency with age was mainly due to increased chromosome loss, reflected in the centromere-positivity of the micronuclei. The micronucleus frequencies were 40% higher in females than in males, which can again be attributed to higher chromosome loss. Two exposed individuals showed exceptionally high micronucleus yields, 90% of which were centromere-positive. In situ hybridization with a centromeric probe for chromosome X shows that X chromosome loss is responsible for these high micronucleus yields. In the studied population, smoking had no significant effect on the micronucleus yields. The results obtained indicate that in contrast to the predominantly clastogenic action of acute exposure to ionizing radiation, the aneugenic properties of radiation may be important after long-term chronic low dose exposure.

Introduction

There are indications from epidemiological studies that radiologists and other medical X-ray workers may show increased mortality due to cancer and leukemia (Smith and Doll, 1981; Wang et al., 1988; Aoyama, 1989). Cytogenetic studies of hospital workers occupationally exposed to low doses of ionizing radiation also revealed an enhanced baseline level of chromosomal aberrations compared with control populations (Bigatti et al., 1988; Jha and Sharma, 1991; Barquinero et al., 1993). Cytogenetic monitoring of this group of workers is of special value as dose estimates from personal dosimetry in hospitals are very crude and unreliable compared with the nuclear industry. Usually the film badge is worn underneath the lead apron, which introduces a very serious underestimation of the real dose.

Although cytogenetic analyses for dicentrics and translocations are the state of the art techniques for biological dosimetry, application of these techniques for biomonitoring or screening of relatively large groups of radiation workers is difficult as chromosome analyses are time consuming and require highly skilled personnel. The cytokinesis-block micronucleus assay of peripheral blood lymphocytes (Fenech and Morley, 1985) is a valuable and less laborious alternative for large scale studies (Thierens et al., 1999). Fluorescence in situ hybridization (FISH) with a pancentromeric probe on micronucleus preparations allows distinction between the clastogenic and aneugenic actions of radiation (Kirsch-Volders et al., 1997). Scoring of micronuclei with and without centromeres has increased the sensitivity of the technique substantially for monitoring of radiation workers (Vral et al., 1997). According to Streffer et al. (1998), the micronucleus centromere assay is able to detect the effects of chronic exposure in uranium miners a long time after exposure.

Using the micronucleus centromere assay we performed a large scale cytogenetic study of hospital workers occupationally exposed to X-rays or to radiation from radioactive sources. An age-matched population of doctors and nursing staff of departments such as pediatrics, where exposure to ionizing radiation is negligible, served as a control group. Apart from a direct comparison between the occupationally exposed and control groups, the effect of donor age, gender and smoking on micronucleus frequencies with and without centromeres was investigated. This study was performed within the framework of the Programme of Scientific Support to the Protection of Workers in the Area of Health of the Services of the Prime Minister Science Policy Office of the Belgian Government.

Materials and methods

Subjects

The population under study, exposed occupationally to ionizing radiation, comprised 71 individuals, 35 males and 36 females, working as doctors, nurses or technicians in the Departments of Radiology, Radiotherapy, Nuclear Medicine, Cardiology, Urology and Gastroenterology of the University Hospital Ghent. Concerning Cardiology and the other internal medicine departments staff members dealing with interventional radiology procedures especially were included. These medical workers are known to receive the highest doses of X-ray exposure. The control group, age-matched to the population under study, comprised 60 individuals, 23 males and 37 females, not exposed occupationally, which was confirmed by their film badge readings. This control group consisted of doctors and nurses working in the Department of Pediatrics and the Burns Unit of the hospital. Informed consent was obtained from all donors. Hospital workers preparing chemotherapeutic drugs were excluded from the study. Preceding blood collection on the occasion of a regular occupational medical examination, the volunteers were asked to fill in a questionnaire. Gender, date of birth, smoking habit, work-related exposure to hazardous agents, previous exposure to diagnostic X-rays as a patient, nuclear medical examination and use of therapeutic drugs were registered. None of the studied subjects received chemotherapeutic drugs. The blood samples were coded in the Occupational Medicine Service. Processing of the samples for the exposed and control groups and scoring were performed blind and concurrently in the laboratory. At the end of the study the data from the questionnaire and the radiation burden records were linked to the code number by the Occupational Medicine Service for data analysis.

Micronucleus centromere assay

Heparinized blood samples were drawn by venipuncture from medical workers. The same day as blood sampling, whole blood cultures containing 0.3 ml blood in RPMI 1640 medium with l-glutamine and 25 mM HEPES buffer (Gibco Laboratories, Gent, Belgium) supplemented with 10% fetal calf serum

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observed difference in MNCM\(^+\) and MNCM\(^−\) in the studied populations, the MN data were corrected for the effect of age and 60% of both populations were non-smokers. The exposed out using the Wilcoxon test. To evaluate the effect of radiation exposure on The same holds for a possible in

Table I. Direct comparison between the exposed and control populations of the average number of micronuclei (MN), number of MN containing a centromere (MNCM\(^+\)) and MN without a centromere (MNCM\(^−\))

<table>
<thead>
<tr>
<th></th>
<th>MN (MNCM(^+))</th>
<th>MN (MNCM(^−))</th>
<th>Age</th>
<th>H (mSv)</th>
<th>M</th>
<th>F</th>
<th>Smoker</th>
<th>Non-smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed</td>
<td>21.88 (13.46)</td>
<td>14.74 (11.71)</td>
<td>67%</td>
<td>41.6 (8.3)</td>
<td>35</td>
<td>36</td>
<td>30</td>
<td>41</td>
</tr>
<tr>
<td>Control</td>
<td>18.63 (7.53)</td>
<td>11.22 (6.98)</td>
<td>60%</td>
<td>11.25 (18.02)</td>
<td>23</td>
<td>37</td>
<td>24</td>
<td>36</td>
</tr>
</tbody>
</table>

The mean age and radiation burden over the last 10 years (H) for both groups are also given together with the number of male and female workers and the number of smokers and non-smokers in the populations. The figures within parentheses refer to the standard deviation. The percentages of centromere-positive and centromere-negative MN in the total number of MN are also given. The \(P\) values of the Wilcoxon analysis, indicating the probability for equality between two populations, are also included.

(Gibco) were initiated. Purified phytohaemagglutinin (PHA, P, 30 µg/ml; Sigma Chemical Co., St Louis, MO) was added asmitogen. Cytochalasin B (6 µg/ml, stock solution 2 mg/ml in dimethylsulphoxide; Sigma Chemical Co.) was added 42 h after culture initiation to block cytokinesis. After an incubation period of 70 h the cells were collected, treated with a hypotonic solution of 0.075 M KCl and fixed with a mixture of methanol/glacial acetic acid as described (Vral et al., 1994). After fixation the cells were dropped onto clean slides, allowed to dry and stored at −20°C before in situ hybridization with the centromeric DNA probe.

In situ hybridization was performed as described by Nederlof et al. (1990) with some modifications. A centromeric DNA probe, p82H (provided by A.R. Mitchell), which is a cloned alphoid sequence present in the centromeric region of all human chromosomes, was used (Mitchell et al., 1985). The probe was biotinylated (0.4 mM biotin-16-dUTP; Boehringer Mannheim) by standard nick translation. The slides, stored at −20°C, were pretreated with 4 µg/100 µl RNase (30 min at 37°C) and 7.5 µg/ml pepsin (5 min at 37°C; both Sigma), followed by post-fixation in 4% paraformaldehyde (UCB, Vel, Leuven, Belgium). A probe concentration of 4 ng/µl (in 50% deionized formamide/SSC; UCB) was added to the slides. Probe and target DNA were denatured simultaneously (5 min at 80°C). Following overnight hybridization, immunofluorescence detection of the probe was performed by means of the tyramide signal amplification method (NEN Life Science). The tyramide signal amplification technology uses streptavidin/horseradish peroxidase to catalyze the deposition of biotin-labeled tyramides at the hybridization sites. These labeled tyramides were then visualized by means of streptavidin/ fluorescent isothiocyanate (FITC) with significant enhancement of the signal. The slides were counterstained with propidium iodide. As validation of the centromere detection method, FISH treatment of metaphase spreads showed that the p82H probe hybridizes to the centromeres of all chromosomes. On slides of two individuals with high micronucleus yields FISH with a commercial centromeric probe for chromosome X (Vysis CEP X) was performed.

For the scoring of micronuclei (MN) and analysis for the presence of centromeres in the MN the preparations were examined under a Leitz fluorescence microscope with 400\(×\) magnification. Scoring was performed with FITC/Texas Red (Chroma) filter set was used. The MN frequencies were scored according to the criteria proposed by Fenech (1993): the diameter of an MN is less than one third of the diameter of the macronucleus, it is non-refractile and is not linked to the macronucleus by a nucleoplasmic bridge. MN partly overlapping with the nucleus or with each other were also taken into account. MN were scored as centromere-positive when the brightness of the pancentromere probe signal in the micronucleus was comparable with the spots in the nucleus. One thousand binucleated (BN) cells were scored on two slides per individual. The results are expressed as the total number of MN per 1000 BN cells, the number of MN containing a centromere (MNCM\(^+\)) per 1000 BN cells and the number of MN without a centromere (MNCM\(^−\)) per 1000 BN cells.

### Results

A direct comparison of the average values of MN, MNCM\(^+\) and MNCM\(^−\) yields and other variables of the study (age and radiation burden over the last 10 years) between the exposed and the control populations is given in Table I. The higher MN frequency in the exposed population compared with the controls is clearly due to an elevated number of MNCM\(^+\).

The difference in MNCM\(^+\) is statistically significant at the 95% confidence level applying the Wilcoxon test. As the populations are age-matched an effect of age can be ruled out. The same holds for a possible influence of smoking: about 60% of both populations were non-smokers. The exposed group comprised relatively more males. Taking into account the age–gender effect, discussed further below, increases the observed difference in MNCM\(^+\) between the exposed and the control group of workers: 14.40 versus 10.24 for a half-male/half-female population 41.65 years old.

In an overview of the individual data, the number of MN, MNCM\(^+\) and MNCM\(^−\) per 1000 BN cells versus donor age for the control and exposed populations is presented in Figure 1. The slopes of the linear regression lines for the relationship...
of MN, MNCM+ and MNCM− to age of the total population of 131 individuals were 0.52 (r = 0.42), 0.44 (r = 0.34) and 0.08 MN/year (r = 0.20), respectively. The results of this linear regression are also shown in Figure 1. The centromere-positive MN account for ~80% of the increase in the total number of MN with age. Two female radiological workers showed exceptionally high numbers of MN, more than 90% of which were centromere-positive. It is of interest to note that for these individuals a number of binucleated cells showed two or more micronuclei, resulting in a slight overdispersion compared with the Poisson distribution (σ²/µ = 1.18 and 1.53) usually observed in MN frequency distributions (Thierens et al., 1995). A second analysis of blood samples drawn 6 months later confirmed these two high values: instead of 72 and 59 (first analysis), MN frequencies of 64 and 68 were found. FISH analysis with a centromeric probe for chromosome X of these two individuals showed 67 and 41 centromere-positive MN/1000 BN cells, respectively.

Sorting out the data according to donor gender shows MN and MNCM+ frequencies which are significantly (P < 0.01) higher for females than for males (Table II). The difference between MNCM− frequencies is not significant. These conclusions were not altered when the two females with an exceptionally high MN yield were excluded from the statistical data analysis. The MN, MNCM+ and MNCM− yields for females were 1.43, 1.62 and 1.16 times greater, respectively, than for males. As the male and female populations were also age matched, the observed gender effect is not influenced by the age dependence of MN frequencies. An analysis of the MN− and MNCM+ frequencies versus age shows a stronger dependence for the female population than for the male population (MN, 0.58 versus 0.44 MN/year; MNCM+, 0.54 versus 0.33 MN/year).

The results of an investigation into the influence of smoking on MN, MNCM+ and MNCM− frequencies in the studied population are summarized in Table III. An increase in the MN and MNCM− frequencies is observed for the smokers but this increase is not statistically significant. Correction of the micronucleus data for age and gender using correction factors deduced from Table II and the linear regression represented in Figure 1 does not alter this conclusion.

Discussion

Detection of centromeres in the MN with a pancentromeric probe and by in situ hybridization allows distinction between the clastogenic and aneugenic actions of radiation (Kirsch-Volders et al., 1997). A direct comparison of the control population of medical workers with the exposed group shows an increase in MN and MNCM+ frequencies in the exposed population, the latter being statistically significant (P < 0.05). On the other hand, no increase in MNCM− frequencies was observed. The increase in MNCM+ cannot be attributed to

### Table II. Comparison of the average number of micronuclei (MN), number of MN containing a centromere (MNCM+) and number of MN without a centromere (MNCM−) of the males and females of the whole population studied

<table>
<thead>
<tr>
<th>Gender</th>
<th>MN (X±SD)</th>
<th>MNCM+ (X±SD)</th>
<th>MNCM− (X±SD)</th>
<th>Age</th>
<th>Exposed</th>
<th>Control</th>
<th>Smoker</th>
<th>Non-smoker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>16.43 (8.63)</td>
<td>9.75 (6.54) 59%</td>
<td>6.68 (3.27) 41%</td>
<td>41.4 (8.7)</td>
<td>35</td>
<td>23</td>
<td>27</td>
<td>31</td>
</tr>
<tr>
<td>Females</td>
<td>23.54 (12.07)</td>
<td>15.80 (11.34) 67%</td>
<td>7.74 (4.07) 33%</td>
<td>41.8 (7.1)</td>
<td>36</td>
<td>37</td>
<td>27</td>
<td>46</td>
</tr>
</tbody>
</table>

The mean age for both sexes is also given together with the number of smokers and non-smokers and the number of control and exposed individuals. The figures within parentheses refer to the standard deviation. The percentages of the centromere-positive and centromere-negative MN in the total number of MN are also given. The P values of the Wilcoxon analysis, indicating the probability of equality between two populations, are also included.

### Table III. Results of the micronucleus centromere analysis of subpopulations of the medical workers classified in the smoker and non-smoker groups

<table>
<thead>
<tr>
<th>Gender</th>
<th>MN (X±SD)</th>
<th>MNCM+ (X±SD)</th>
<th>MNCM− (X±SD)</th>
<th>Age</th>
<th>Male</th>
<th>Female</th>
<th>Exposed</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-smokers</td>
<td>19.87 (11.28)</td>
<td>12.36 (7.99) 62%</td>
<td>7.51 (4.33) 38%</td>
<td>42.7 (7.7)</td>
<td>31</td>
<td>46</td>
<td>41</td>
<td>36</td>
</tr>
<tr>
<td>Smokers</td>
<td>21.13 (11.30)</td>
<td>14.20 (10.18) 67%</td>
<td>6.93 (2.76) 33%</td>
<td>40.1 (7.6)</td>
<td>27</td>
<td>27</td>
<td>30</td>
<td>24</td>
</tr>
</tbody>
</table>

For meaning of the symbols we refer to the caption to Table II. The P values are the results of the Wilcoxon analysis of the comparison between the smoking and non-smoking populations.
the age distribution of males and females in the control group and the exposed population. Adjusting the MN data to a half-male/half-female population with a mean age of 41.65 years using the age dependence found for males and females slightly increased the observed difference in MNCM+. Other cytogenetic studies of hospital worker populations (Bigatti et al., 1988; Jha and Sharma, 1991; Barquinero et al., 1993) also showed a significant increase in structural chromosome type aberrations and numerical abnormalities in the exposed population compared with the controls. However, no correlation between the frequency of chromosomal aberrations and the dose received could be derived (Barquinero et al., 1993). An analysis of our MN data for the exposed group in two subpopulations of hospital workers according to a radiation burden over the last 10 years of more or less than 5 mSv also showed no dose-related effect on the observed increase in MN and MNCM+ (Figure 2).

Previous work (Vral et al., 1997) on micronucleus induction after in vitro low dose γ-irradiation showed that radiation-induced MN are for the most part centromere-negative, reflecting the clastogenic action of radiation. After in vitro irradiation only a very small increase in centromere-positive MN with dose (5 MNCM+ /1000 BN cells/Gy) was observed. Surprisingly, in the present study no difference in MNCM+ between the exposed and control populations was noticed, while a significant increase in MNCM+ was observed. Our results indicate that, in contrast to the predominantly clastogenic action of acute exposure to ionizing radiation, the aneugenic properties of radiation may be important after long-term chronic low dose exposure. In a study of thyroid cancer patients by interphase FISH, Ramirez et al. (1997) reported that radioiodine treatment also had an aneugenic effect apart from the clastogenic effect. Furthermore, young and elderly patients showed the same increase in MNCM+ frequency on radioiodine treatment, while the increase in MNCM+ was 2.3-fold higher in elderly patients. Thus, our observations are supported by data from thyroid cancer patients treated with radioactive iodine. An aneugenic activity of ionizing radiation after in vivo exposure was also reported by the group of Natarajan for mouse (Boei and Natarajan, 1995; Hande et al., 1996a,b) and victims of the Goiania accident (Natarajan et al., 1991).

The results of the present work show that the systematic increase in MN frequency with donor age is for the great part due to an increase in MNCM+, reflecting increased chromosome loss with age. Previous studies showed that the X-chromosome is almost completely responsible for spontaneously occurring chromosome loss (Hande et al., 1994; Richard et al., 1994; Surralles et al., 1996; Catalan et al., 1998; Carere et al., 1999). The slopes of 0.58 and 0.44 MN/year, obtained from a linear regression analysis of the total number of MN for the female and the male populations, respectively, are close to the values of 0.52 and 0.31 MN/year reported for Fenech (1993). The observed overall increase of 0.52 MN/year is higher than the value of 0.19 MN/year reported by Ban et al. (1993) from a study of ~1000 atomic bomb survivors with ages between 42 and 84 years.

According to our data the MN frequencies are 1.4 times greater in females than in males. This observation is in agreement with the findings of Fenech (1998) in his large scale study of variables influencing baseline micronucleus frequency: MN frequencies are systematically larger in females with a factor increasing from 1.2 to 1.6 with age. The centromere analysis performed in the present work shows that this gender effect can be attributed almost completely to a greater number of chromosome loss events in females as the number of centromere-positive MN is 60% higher in females than in males. The observed age and gender effects can be explained by preferential loss of the X-chromosome during aging, as demonstrated in previous studies (Hande et al., 1994; Richard et al., 1994; Carere et al., 1999). Furthermore, Zijno et al. (1996a,b) also reported an age-related increase in X-chromosome non-disjunction. Following Surralles et al. (1996), the higher susceptibility of chromosome X to malsegregation in comparison with autosomes is due to a constitutive defect in the machinery involved in mitotic segregation of this chromosome. According to our data this effect is also responsible for the very high MN yields found in two subjects of our study.

Although smokers show a slight increase in MN and MNCM+ frequencies, the observed differences between smokers and non-smokers are not statistically significant. This also holds after correction of the data for differences in age and gender. Concerning the effect of tobacco on micronucleus frequency, the data in the literature are contradictory. According to some authors (Au et al., 1991; Tomanin et al., 1991; Da Cruz et al., 1994; Holmen et al., 1995) micronucleus frequency is elevated in smokers. On the other hand, no significant effect of smoking habit on micronucleus frequency was observed in the large-scale studies of Migliore et al. (1991) and Ban et al. (1993). Less unambiguous are the conclusions of Fenech (1993) and Duffaud et al. (1997). In his review paper Fenech (1993) stated that MN frequency is influenced by the number of cigarettes per day but not dependent on the number of years smoking. According to Duffaud et al. (1997) tobacco consumption affects the micronucleated cell rate in healthy subjects but not in cancer patients.

In conclusion, our data show a significantly elevated number of centromere-positive MN in medical workers exposed to ionizing radiation. This observation points to the possible importance of aneugenic effects after chronic exposure to radiation. The high susceptibility of chromosome X to malsegregation is responsible for the very high MN yields found in two females. No significant influence of smoking on MN, MNCM+ and MNCM– frequencies was observed.

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