Comparison between micronucleated lymphocyte rates observed in healthy subjects and cancer patients

F. Duffaud1, T. Oursière2, P. Villani2, A. L. Pelissier3, F. Volot1, R. Favre1 and A. Botta2

1 Service d'Oncologie Médicale, CHU Timone Adultes, Boulevard Jean Moulin, 13385 Marseille cédex 5, 2 Laboratoire de Biogénotoxicologie (EA 1784), Service de Médecine du travail, Faculté de Médecine, 27 Boulevard Jean Moulin, 13385 Marseille cédex 5, 3 Service de Médecine Légale, Faculté de Médecine de Marseille and 4 Service d'Informatique Médicale, CHU Timone Adultes, Marseille, France

5 To whom correspondence should be addressed

Micronucleated cell rates were assessed in cytokinesis-blocked lymphocytes of 198 male and female healthy subjects (HS) not occupationally exposed to genotoxic risks and of 70 male and female cancer patients (CP) prior to any anticancer treatment. In the HS group, spontaneous micronucleated cell rates (MN cell rates) were 9.7 ± 2.8 per 1000 binucleated lymphocytes and 9.8 ± 3.1 for males and females respectively. In the CP group, spontaneous MN cell rates were 21.1 ± 15.3 per 1000 binucleated lymphocytes and 19.1 ± 11.2 for males and females respectively. Moreover, they were shown to have a large inter-individual variability in the two groups. The study of inter-individual variation factors showed that only tobacco could affect MN cell rate in HS whereas age and sex apparently had no significant effect. In the CP group, only age significantly affected MN cell rate, whereas sex, tobacco, alcohol, imaging techniques and tumour stage had no significant effect. There was no significant difference in the distribution of gender between HS and CP, whereas there was a significant difference in the distribution of age and tobacco between the two groups. The comparison of MN cell rates in 54 HS and 54 CP matched for age and sex showed a statistically significant difference. Spontaneous MN cell rates of these two populations reflect environmental exposure. Moreover, for CP it most probably refers to various cellular lesions and genetic damage.

Introduction

The survey of human genetic integrity has become of special interest since the development of industrial activities and consequent exposure to chemical and physical genotoxins. Moreover, other factors such as lifestyle, various medical therapies and climatic changes may promote genetic damage. Human genetic damage can be assessed by using different in vitro and in vivo assays (Ishidate, 1988).

The validity of the cytokinesis-blocked micronucleus assay (CBMN assay) as a biomarker of chromosome damage in dividing mammalian cells is well established (O'Donovan, 1990). This assay offers an easier and less tedious alternative to metaphase chromosome analysis, with the advantage that exposure to both clastogens and aneugens may be detected (Arlett et al., 1989; Fenech, 1990). This method has led to increased interest by some cytogeneticists in the application of the CBMN assay for population monitoring (Di Georgio et al., 1994; Duffaud et al., 1996; Gutierrez et al., 1995; Klemans et al., 1995; Norppa et al., 1993; Villani et al., 1995), although some authors do not agree on this point (Van Hummel et al., 1994). The occurrence of spontaneous micronuclei (MN) in healthy subjects (HS) is well established (Fenech, 1993). Most authors report spontaneous micronucleated (MN) cell rates in HS with mean value of ~10 MN per 1000 binucleated lymphocytes (range 4–15) (Hogstedt, 1984; Fenech, 1993).

In order to determine baseline data in healthy subjects the CBMN assay was applied to the lymphocytes of 198 male and female subjects. The effects of age, sex and tobacco on spontaneous MN cell rates were studied. Then, the baseline MN cell rate in 70 cancer patients (CP) was determined by application of the same assay, and the effects of sex, age, tobacco and alcohol consumption, imaging techniques and tumour stage on MN cell rates were studied. Moreover, the variation factors distribution in the two groups was studied in order to compare spontaneous MN cell rates in HS with those in CP.

Materials and methods

Subjects

Two groups of subjects were investigated. The first group of 198 HS, consisted of 120 males and 78 females (19–72 years old) of mixed social class, not occupationally exposed to known mutagens or aneugens. The detailed age structure of male and female HS is detailed in Table I. Prior to the beginning of the experimentation, subjects informed consent was obtained and the absence of additional exposures which could influence cytogenetic parameters was verified: absence of anticancer therapy, absence of radiography or vaccination for at least 3 months, absence of any medical treatment for at least 1 month. Prior to sampling, information concerning their individual lifestyle was given by anonymous questionnaire (sex, age, occupation, tobacco consumption, chronic medical therapy). HS characteristics are reported in Table I. The second group was composed of 70 CP with 50 males and 20 females (17–76 years old). Patients with previous cancer treated with chemotherapy and/or radiotherapy were excluded from this experimentation. Prior to sampling, information concerning individual lifestyle was obtained. Tobacco consumption was easily and precisely evaluated (smokers yes/no; g/day) whereas alcohol consumption was quantitatively difficult to estimate (significant alcohol daily consumption, yes/no). Histological diagnosis was confirmed by biopsy or surgical resection. Cancer clinical staging required chest X-ray (1 or 2 weeks before treatment) and computer tomography of the involved region (2–3 weeks before treatment) for all of the patients, and bone scintigraphy with 99Tc (2–3 weeks before treatment) for some of them. The influence of radiation exposure on MN cell rates is considered in the discussion. For some patients, folate status and vitamin B12 status were measured. Distribution of CP characteristics (sex, age, tumour site, tumour stage, tobacco and alcohol consumption, imaging techniques) are reported in Table II.

Cell culture

One whole blood sample from each subject was used for the micronucleus assay. For CP this 'pretreatment sample' was collected: (i) before adjuvant treatment (radiotherapy and/or chemotherapy) but after surgery for CP with localized/resectable disease; (ii) prior to any anticancer treatment (chemotherapy and/or radiotherapy) for CP with advanced/unresectable disease.

Whole blood samples were collected in 5 ml heparinized plastic tubes and kept at 15–20°C. The average storage time for all blood samples was 6 h. For each culture, 0.7 ml of whole heparinized blood, 0.1 ml of phytohaemagglutinin (Becton-Dickinson, Paris, France), 2.5 ml of fetal calf serum (Biochrom-Seromed, and 0.1 ml of penicillin-streptomycin 10 000 IU/ml
The study of the variation factors distribution in the two groups with \( \chi^2 \) test is shown in Table III. There was no statistically significant difference in the distribution of gender between HS and CP (\( P = 0.23 \)), whereas there was a statistically significant difference in the distribution of age and tobacco between the two groups (\( P < 0.0001 \) and \( P = 0.0002 \)).

Summary statistics concerning micronucleated cells rates in the HS group and in the CP group are shown in Table IV. In the HS group, spontaneous micronucleated cell rates (MN cell rates) were \( 9.7 \pm 2.8 \) per 1000 binucleated lymphocytes and \( 9.8 \pm 3.1 \) for males and females respectively. In the CP group, spontaneous MN cell rates were \( 21.1 \pm 15.3 \) per 1000 binucleated lymphocytes and \( 19.1 \pm 11.2 \) for males and females respectively. Mean values of folate status and vitamin B12 status measured for 17 CP were \( 1019 \pm 450 \) pg/ml (normal range 225–1000) and \( 10.2 \pm 8 \) ng/ml (normal range 2–19.7) respectively. Statistical analysis of inter-individual variation factors (age, sex, tobacco for the CP group) is shown in Table V for the HS group and in Table VI for the CP group. In the HS group, only tobacco consumption induced a statistically significant increase of micronucleated cell rate (\( P < 0.001 \)), whereas age and sex apparently had no statistically significant effect (\( P = 0.857 \) and \( P = 0.484 \)). In the CP group, only age induced a statistically significant increase of micronucleated cell rate (\( P = 0.01 \)), whereas sex, tobacco, alcohol, bone scintigraphy and tumour stage had no statistically significant effect on the micronucleated cell rate (\( P = 0.48 \), \( P = 0.25 \), \( P = 0.42 \), \( P = 0.7 \) and \( P = 0.10 \)).

Therefore, in order to compare the spontaneous micronucleated cell rates in HS and in CP, we matched for age and sex.
Table VI. Variation factors analysis by the Kruskal–Wallis test concerning CP (effect of sex, age, alcohol and tobacco consumption, tumour stage, bone scintigraphy on micronucleated cell rate)

<table>
<thead>
<tr>
<th>Variation factors</th>
<th>Defined groups</th>
<th>Sample size</th>
<th>Micronucleated cell rate (mean)</th>
<th>Significance level (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>males</td>
<td>48</td>
<td>21.1</td>
<td>0.48 (NS)</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td>22</td>
<td>19.1</td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td>yes</td>
<td>41</td>
<td>21.1</td>
<td>0.25 (NS)</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>29</td>
<td>20.1</td>
<td></td>
</tr>
<tr>
<td>Alcohol</td>
<td>yes</td>
<td>35</td>
<td>20.6</td>
<td>0.42 (NS)</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>30</td>
<td>19.9</td>
<td></td>
</tr>
<tr>
<td>Tumoral stage</td>
<td>localized</td>
<td>19</td>
<td>14.9</td>
<td>0.10 (NS)</td>
</tr>
<tr>
<td></td>
<td>advanced</td>
<td>51</td>
<td>22.6</td>
<td></td>
</tr>
<tr>
<td>Bone scintigraphy</td>
<td>yes</td>
<td>27</td>
<td>19.3</td>
<td>0.7 (NS)</td>
</tr>
<tr>
<td></td>
<td>no</td>
<td>43</td>
<td>21.3</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>males and</td>
<td>70</td>
<td>20.1</td>
<td>0.01 (S)</td>
</tr>
<tr>
<td></td>
<td>females</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NS, not statistically significant; S, statistically significant.

Table VII. Comparison of micronucleated cell rates in age and sex-matched HS and CP. Result of Mann–Whitney rank sum test

<table>
<thead>
<tr>
<th></th>
<th>HS</th>
<th>CP</th>
<th>Significance level</th>
</tr>
</thead>
<tbody>
<tr>
<td>MN cell rate:</td>
<td>9.8 ± 4.4</td>
<td>18.7 ± 16.8</td>
<td>P &lt; 0.0001 (S)</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

S, statistically significant.

Discussion

The baseline level of 198 HS observed in this study averaged 9.7 ± 2.8 micronucleated cells per 1000 binucleated lymphocytes and 9.8 ± 3.1 for males and females respectively. Previous studies reported spontaneous micronucleated lymphocytes mean value ~10 MNi per 1000 binucleated lymphocytes (Hogstedt, 1984; Fenech and Morley, 1985; Arlett et al., 1989; Heddle, 1990; Yager, 1990; Norppa et al., 1990). The difference between lower and upper levels of MN cell rates in HS (2 and 22 per thousand respectively) presented evidence that a large inter-individual variability could be observed for MN cell rates in human lymphocytes. Among the variation factors considered in this study (sex, age and tobacco consumption), only tobacco was shown to be statistically significant and induced an increase of ~25% of the micronucleated cell rates. Tobacco has been shown to induce chromosome aberrations (Ashby and Richardson, 1985; Littlefield and Joiner, 1986; Au et al., 1991) and micronuclei (Arlett et al., 1989; Fenech et al., 1990; Heddle et al., 1990; Norppa et al., 1990; Yager, 1990; Tomanin et al., 1991; Fenech, 1993; Da Cruz et al., 1994; Holmen et al., 1995), and plays a role in head and neck, lung and bladder cancer development. In our population, mainly aged 40–59 years, age had no statistically significant effect on micronucleated cell rate. This result agrees with some previous reports (Arlett et al., 1989), although several authors have shown an increase in micronucleated cells correlated with age (Fenech and Morley, 1986; Heddle, 1990; Yager, 1990; Barregard et al., 1991; Migliore et al., 1991; Fenech, 1993; Da Cruz et al., 1994; Holmen et al., 1995). When present, this effect could be explained by either accumulated genetic damage in lymphocytes (Evans and O’Riordan, 1975; Natarajan and Obe, 1980; Fenech and Morley, 1989; Eastmond and Tucker, 1989; Fenech, 1993) or true ageing process such as altered cell metabolism and/or decreased DNA repair capability (Fenech and Morley, 1986; Franceschi, 1989). In our HS population, which has an excess of males (60.1%), sex had apparently no statistically significant effect on micronucleated cell rate: this is in accordance with some previous reports concerning the micronucleus assay (Arlett et al., 1989; Heddle, 1990), whereas Fenech reported the influence of sex on spontaneous micronucleated cell rate (Fenech, 1993; Fenech et al., 1994). The rest of inter-individual variations observed in our study may be explained by lifestyle, which includes various environmental factors, or individual susceptibility. Vegetarian diet and alcohol drinking, for example, may increase micronuclei induced by smoking (Arlett et al., 1989; Au et al., 1991; Xue et al., 1992). Individual susceptibility due to variation in metabolic activation of mutagens and in DNA repair efficiency (Huber et al., 1989; Remmer, 1987; Wiencke et al., 1991) has been shown to induce inter-individual differences in responses of micronucleated lymphocytes in vitro exposures (Thiérens et al., 1991).

The spontaneous micronucleated cell rates in CP averaged 21.1 ± 15.3 per 1000 binucleated lymphocytes and 19.1 ± 11.2 for males and females respectively. Among the variation factors considered in this CP group, tobacco and alcohol did not significantly affect the base line level of micronucleated cells, whereas (i) smokers and alcohol consumers represent 60 and 50% respectively of patients in this group, and (ii) smokers are heavy tobacco consumers (average consumption between 30 and 40 g/day). However, tobacco is considered to be an important variation factor which influences micronuclei rates in HS (Fenech, 1993; Holmen et al., 1995; Arlett et al., 1989; Fenech, 1990; Heddle, 1990; Norppa et al., 1990; Yager, 1990). Sex did not significantly affect micronucleated cell rates in CP or HS groups. The apparent absence of an age effect could be partly explained by the excess of males (71.4%) in the CP group. Moreover, tumour stage was inclined to show an increase in micronucleated cell rates when tumours are advanced and/or metastatic, but this effect was not statistically significant, probably because of the small number of patients.

Among variation factors of the spontaneous micronucleated cell rate in CP, a role of imaging technique [chest radiograph, local computed tomography (scanner) and/or bone scintigraphy] used for cancer staging could be supposed in the increase of spontaneous MNi cell rate. Nevertheless, the X-ray dose delivered by chest radiograph or by scanner does not seem high enough to explain this result. It is very difficult to measure the X-ray doses delivered by these imaging techniques exactly. The dose received by the bone marrow during chest radiograph averages 0.019-0.038 mGy (Archer et al., 1991). The dose received by the bone marrow during cerebral scanning 4.7 and 0.7 cGy. Moreover, the radiation dose absorbed by bone marrow during bone scintigraphy using 99mTc is also insignificant (0.68 cGy) (Gambini and Granier, 1988), and the
The comparison of spontaneous micronucleated cell rate of CP with or without bone scintigraphy did not find a statistically significant difference between the two groups, \( P = 0.7 \). The radiation dose delivered by imaging techniques, therefore, seems to have no influence on MN cell rate in the CP group. Moreover, folate status and Vitamin B12 status measured for some CP (25%) did not show any deficiency. Only age showed a statistically significant influence on base line levels of micronucleated cells in CP, with patients between 19 and 79 years old and a median age of 56 years.

Despite the significantly different distribution of age and tobacco consumers in HS and in CP, the comparison of micronucleated cell rates in the two groups was possible after CP and HS were matched for age and sex.

The comparison of baseline levels of micronucleated cells in lymphocytes from age and sex-matched CP and HS showed a statistically significant increase of micronucleated cell rate in the CP group.

Moreover, tobacco does not seem to play a role in the significant elevation of micronucleated cell rate in our CP; since MN cell rates of smokers and non-smokers are not significantly different. Therefore, the greater number of smokers in the CP group does not enable us to explain the significant difference of micronucleated cell rates between HS and CP.

One major explanation of this significant difference could be linked to the various cellular lesions and genetic damage associated with cancer status. The role of inflammation in cancer development and the association between inflammation and DNA damage are of importance in this explanation. The observation that inflammatory cells can induce genetic damage in co-cultures has been well supported by in vitro studies (Kaplan, 1987). Moreover, an association between inflammation induced by chronic trauma and the development of tumours in humans has been noted repeatedly since the mid-1800s (Kaplan, 1987; Rosin, 1991; Frenkel, 1992; Correa, 1992). It has been suggested that during prolonged inflammation the release of oxygen-derived free radicals (reactive oxygen species) generated by activated inflammatory cells or chronic irritation cause incessant damage to normal cells in adjacent tissue (Weitzman and Gordon, 1990; Frenkel, 1992; Simmonds et al., 1992). This would lead to an elevation in genetic instability in the target tissue and plays a role in the development of cancer. The exact mechanism by which inflammatory reactions induce DNA damage is therefore unclear. These reactions could increase DNA damage indirectly by stimulating cell proliferation during tissue regeneration (Rosin, 1991). Preston-Martin et al. (1990) recently have summarized evidence for an association between exposure to agents that increase cell division (physical and chemical agents, hormones, drugs) and the development of human cancers. The common factor in these mitogenic agent exposures was their ability to invoke elevated proliferation and, in many cases, inflammation. The pathways by which proliferation is stimulated by these agents are not clearly understood. Elevated epithelial proliferation is postulated to increase carcinogenesis in two main ways. First it increases genetic damage: (i) by facilitating the interaction of a carcinogen with the DNA, since replicating DNA has an altered structural configuration; (ii) by decreasing the probability of DNA damage being repaired; and (iii) by allowing the occurrence of genetic changes which require cell division (chromosome loss, duplication, translocation, nondisjunction) (Preston Martin et al., 1990; Ames et al., 1990). Secondly, elevated cell proliferation allows selective clonal expansion to occur more rapidly (Boone et al., 1992).

The significance of the more elevated spontaneous micronucleated cell rate in CP is very likely related to their pathological status or inherited genetic instability. The detection of a tumour stage tendency to increase the spontaneous MN cell rate in CP with advanced and/or metastatic tumour in our study, are consistent with this hypothesis. The accumulation of numerous chromosomal or genetic mutations in tumour cells is well established. The frequency and the rate of progression of malignant tumours depend on the mutation frequency (Alberts et al., 1992). The mutation rates can be more elevated because of environmental mutagenic agents or intracellular defects in DNA replication, recombination or repair mechanisms. The alteration of DNA repair mechanisms (mismatch repair, excision-resynthesis process, 'bulky lesion repair') could therefore be involved in the cancer development, and also partly explain the elevated micronucleated cell rate in CP.

The previous exposition of genotoxic or mutagenic environmental agents for CP may also play a role in their spontaneous micronucleated cell rate.

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