Extruded micronuclei induced by colchicine or acrylamide contain mostly lagging chromosomes identified in paintbrush smears by minor and major mouse DNA probes

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In the mouse bone marrow micronucleus assay, it was studied whether micronuclei (MN) could be expelled from polychromatic erythrocytes (PCE) in a similar way to the main nucleus. To avoid the disrupting centrifugation step of the conventional bone marrow preparation procedure, the paintbrush technique was used in the present experiments. With May-Grunwald-Giemsa staining of paintbrush slides, 5% of the colchicine (COL)-induced MN were found attached to the outside membranes of PCE and were regarded as extruded. Of the acrylamide (AA)-induced MN, 22% were extruded. After fluorescence in situ hybridization (FISH) of a total of 300 MN per chemical treatment with the mouse minor and major satellite DNA probes, 9.7% MN were extruded in the COL group and 8.3% MN were extruded in the AA group. FISH showed that 76% of the retained COL-induced MN were signal-positive, indicating that they contained entire chromosomes. With AA, 29% minor-positive and 28.3% major-positive retained MN were found, confirming its known clastogenicity. However, the observed frequency of signal-positive MN (1.7 MNPEPc 1000 PCE) in the AA group was about three times higher than in the control (0.5 MNPEPc 1000 PCE) which indicates that AA has aneugenetic potential. FISH analysis of the extruded MN showed 72-100% major as well as minor signals. It is concluded that expelled MN contain mostly entire chromosomes.

Introduction

During erythrocyte maturation, the main nucleus is expelled. It is possible that large micronuclei (MN) undergo expulsion by the same mechanism as the main nucleus. To verify the existence of extruded MN, the preparation method was changed from the common smear method to the paintbrush method described by Styles et al. (1983). This paintbrush technique avoids the centrifugation step and, therefore, may enable the observation of MN which are in the process of leaving the erythrocyte or have just left the erythrocyte and are still attached to the outer membrane. The question was 'do extruded MN exist and do they contribute significantly to the MN yield?' Parton et al. (1991) described extruded MN induced by demicolcine which were mostly large in size. The second question then was whether these large extruded micronuclei contained entire chromosomes or acentric fragments.

In the past decade, centromere detection methods have been used to distinguish MN formed by acentric fragments from those formed by whole chromosomes (Miller et al., 1991, 1992, Salassidis et al., 1992; Miller and Nüssle, 1993; Farooqi et al., 1993; Schriever-Schwemmer and Adler, 1994). For mice, the major DNA probe was used primarily; it hybridizes to the pericentric heterochromatic block. The major probe labels all chromosomes, except the Y chromosome, in a one dot pattern (Pietras et al., 1983; Miller et al., 1991; Weier et al., 1991). Another centromere probe is the minor DNA probe which labels all chromosomes except the Y at or near the centromere in the kinetochore region with two dots, similar to the labelling by CREST antibodies (Broccoli et al., 1990; Moens and Pearlman, 1990; Schriever-Schwemmer and Adler, 1993). The safest way to determine centromere-containing MN is to use double labelling with the major and the minor DNA probe (Eastmond et al., 1993; Chen et al., 1994; Schriever-Schwemmer and Adler, 1994).

Currently, colchicine (COL) is used as a typical aneugen and as a positive control (Miller and Adler, 1989; Adler et al., 1991; Schriever-Schwemmer and Adler, 1994). Acrylamide (AA) was tested for its possible aneugenicity. One of the best documented effects of AA is its clastogenicity in somatic and germinal cells. Chromosomal aberrations (chromatid gaps, breaks and exchanges) were observed in mammalian cell cultures (Moore et al., 1987; Tsuda et al., 1993), as well as in mouse bone marrow cells and in mouse spermatogonia (Shiraishi, 1978; Adler et al., 1988) after AA treatment. Furthermore, AA induced dominant lethal mutations, heritable translocations and specific locus mutations in male mouse germ cells (Shirai et al., 1987; Ehling and Neuhaus-Klaus, 1992; Gutierrez-Espeleta et al., 1992; Adler et al., 1994). Aneugenicity data of AA are scarce, however, some studies in mouse bone marrow and spermatocytes indicate that AA has aneugenetic potential (Gassner and Adler, 1995, 1996).

The present experiments were carried out to verify the existence of extruded MN, to analyse whether these extruded MN contain lagging chromosomes and to analyse the aneugenicity of AA in the mouse micronucleus assay in vivo. To answer these questions, both chemicals were used at only one dose which previously had been shown to induce a high frequency of MN (Adler et al., 1988, 1991).

Materials and methods

Chemicals and animal treatment

Male (102EI×C3H/Ei)F1 mice from the GSF animal colony were used at the age of 10-14 weeks weighing 25-29 g. Mice were injected i.p. at a volume of 0.1 ml/10 g body weight with 25 mg/kg of COL and 125 mg/kg of AA. COL and AA were purchased from Sigma (Deisenhofen, Germany). Both compounds were dissolved in bidistilled water. Control animals were injected i.p. with equal volumes of bidistilled water.

Slide preparation

The mice were killed 24 h after treatment and the femurs were extracted. The femur was opened at the knee end, so that a paintbrush of size 0 or 00 could be inserted. The bone marrow was sampled by gently turning the paintbrush, which had been wetted with fetal calf serum (FCS), in the opening of the bone. The bone marrow was then brushed onto a silane-coated slide which had been wetted before with a drop of FCS. Five or six slides were made for
erythrocytes (PCE). 

Debris and possible nuclear fragments of ruptured 
magnification for the presence of MN inside of or attached to polychromatic 

The conventional micronucleus scoring and the signal analysis after double-
Microscopic and statistical analysis 

per chemical treatment and two animals per .solvent control.

Altlußheim. Germany).
The micron mouse satellite DNA probe, pMKB6 (Wong and Rattner, 1988), a 273 bp fragment that represents approximately two tandem repeats, in plasmid pPTZ191L, was a gift from B.Vig (Reno, USA). It was propagated in 

for the possibility of different detection systems for the haptens.
biostrap and digoxygenin as haptens allowed the use of two DNA probes at the 

and colour of PCE as well as MN. At least 1000 PCE were scored per animal.

apoptotic nucleated cells were clearly distinguished from true MN by shape and colour of PCE as well as MN. At least 1000 PCE were scored per animal. 

and the MNCE ratio was also evaluated. For signal detection 100 MN per animal were scored at x1250 magnification. The number of signals per MN were counted.
The x² test was used to determine significant differences in the MN data.

Results 

Paintbrush technique and conventionally analysed micronuclei 

Instead of the widely used bone marrow smear technique the paintbrush technique described by Styles et al. (1983) was used to preserve cells undergoing enucleation and especially the expulsion of the MN from the erythrocytes. Figure 1 illustrates the expulsion process of micronuclei at the cell with MN still attached to the plasmalemma (extruded MN; c-e) and totally extruded MN (f).

The frequencies of retained and extruded MN, the PCE/ NCE ratios and the MNPEp/1000 PCE obtained in the present experiments are shown in Table I. In the two solvent-control animals, the average of 1.0 MN/1000 PCE is in the normal range for solvent controls in our laboratory and no extruded MN were found. At 24 h after treatment with 1 mg/kg COL, 7.3 retained MN/1000 PCE and 0.4 extruded MN/1000 PCE were found. Thus, 4.4% of all COL-induced MN were extruded. At 24 h after AA treatment, 4.7 retained MN/ 1000PCE and 1.3 extruded MN/1000PCE were observed with a dose of 125 mg/kg. Thus, 22.2% of all AA-induced MN were extruded. The MN frequencies in the treated groups (retained or total) were significantly higher than the control. No significant difference could be detected comparing the frequency of total (retained + extruded) MN to the retained MN frequency in either group.

To correlate the FISH data with the conventional MN data we calculated a MNPEp/1000 PCE frequency. For example, after treatment with COL, 7.7 MN/1000 PCE were found in the conventional MNT and 76% MN were centromere-positive, thus 5.9 MN/1000 PCE were calculated to be centromere-positive. 

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The MNCE frequencies were between 0.25 and 0.5/1000 NCE in all groups (data not shown). Thus, no discrimination between MN induced in PCE and NCE was required for the FISH analysis since the NCE only contributed minimally to the total number of MN. No significant reduction of the PCE/ NCE ratio was found in either of the two treatment groups compared with the solvent control.

FISH double labelling with the minor and major DNA probes 

Examples of retained and extruded MN with FISH using major and minor DNA probes are shown in Figure 2.

The results of the total of 300 scored MN after double labelling with the minor and major probes are shown in Table II. In the control, 80 (53.3%) of the 150 MN analysed showed no signal and 70 MN (46.7%) were double-labelled with the minor and the major probe. After treatment with 1 mg/kg of COL, 72 out of 300 (24%) MN showed no signal and 228 MN (76%) were major- as well as minor-positive. With 125 mg/kg AA, 215 MN (71.7%) of 300 MN analysed had no signal, two MN (0.7%) were minor-positive only and 85 MN (28.3%) were double-labelled with the minor and major DNA probe.
The distribution of the numbers of signals in the minor- or major-positive MN is shown in Table III. In the control, 10 MN (14.3%) out of 70 minor-positive MN had one signal, 57 MN (81.4%) showed two signals, two MN (2.9%) showed three signals and one MN (1.4%) showed four signals. After treatment with 1 mg/kg of COL, 65 (28.5%) of 228 minor-positive MN had one signal, 135 MN (59.2%) showed two signals, nine MN (4%) had three signals and 21 MN (9.2%) had four signals. With 125 mg/kg of AA, 23 MN (26.4%) of 87 minor-positive MN had one signal, 49 MN (56.3%) showed two signals, eight MN (9.2%) had three signals and six MN (7%) had more than three signals. With the major DNA probe
Table I. Frequencies of retained and extruded MN in polychromatic erythrocytes of bone marrow after i.p. treatment of male mice with COL and AA

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (mg/kg)</th>
<th>No of animals</th>
<th>Individual animal scores per 1000 PCE</th>
<th>Mean MNPCE (% ± SD)</th>
<th>PCE/NCE (± SD)</th>
<th>MNPE_{total} per 1000 PCE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total MN</td>
<td>extruded MN</td>
<td>total MN</td>
<td>retained MN</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>2</td>
<td>2, 0</td>
<td>0, 0</td>
<td>1.0 ± 1.0</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td>COL</td>
<td>1</td>
<td>3</td>
<td>6, 8, 9</td>
<td>0, 0, 1</td>
<td>7.7 ± 1.5*</td>
<td>7.3 ± 2.1*</td>
</tr>
<tr>
<td>AA</td>
<td>125</td>
<td>3</td>
<td>4, 7, 7</td>
<td>0, 2, 2</td>
<td>6.0 ± 1.2b</td>
<td>4.7 ± 0.6b</td>
</tr>
</tbody>
</table>

*P < 0.01; bP < 0.05 (χ² test)

MNPCE, micronucleated polychromatic erythrocytes; NCE, normochromatic erythrocytes; PCE, polychromatic erythrocytes.

Fig. 2. Examples of mouse erythrocytes with MN labelled by FISH with minor (CY3-red) and major (FITC-yellow) satellite DNA probes. The MN are counterstained with DAPI (blue). Erythrocytes show autofluorescence in green. The pictures were digitized using the ISIS program (MetaSystems, Altlussheim, Germany). (a) Minor- and major-negative retained MN; (b) minor- and major-negative MN that is just about to be expelled; (c) minor- (red) and major- (yellow) positive retained MN; (d) minor- and major-positive extruded MN still attached to the membrane of the erythrocyte. The overlap of red, yellow and blue colour signals results in a white spot, however, the individual colours were always seen with the respective individual filters.

The following frequencies were found: in the control 62 MN (88.6%) out of 70 major positive MN had one signal and 8 MN (11.4%) showed two signals. After treatment with 1 mg/kg of COL, 191 MN (83.8%) of 228 major-positive MN had one signal and 37 MN (16.2%) showed two signals. With 125 mg/kg of AA, 63 MN (74.1%) of 85 major-positive MN had one signal. 19 MN (22.4%) showed two signals and three MN (3.5%) had three signals.

The numbers of extruded MN labelled with the minor and major DNA probe are shown in Table IV. In the control, six MN (4%) out of 150 MN were extruded and all six (100%) had minor and major signals. After treatment with 1 mg/kg COL, 29 MN (9.7%) of 300 MN were extruded and only four (13.8%) of the extruded MN had no signals while 25 (86.2%) of the extruded MN had minor and major signals. With 125 mg/kg of AA, 25 MN (8.3%) of 300 MN were extruded and seven (28%) of the extruded MN had no signal whereas 18 MN (72%) showed minor and major signals.

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Lagging chromosomes in extruded micronuclei induced by colchicine or acrylamide

Table II. Double labelling of MN with minor and major DNA probes by FISH

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (mg/kg)</th>
<th>No. of MN scored</th>
<th>MN without any signal</th>
<th>MN without minor signal</th>
<th>MN with major signals</th>
<th>MN with minor signals</th>
<th>MN with major and minor signals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>%</td>
<td>total</td>
<td>%</td>
<td>total</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>150</td>
<td>80</td>
<td>53.3</td>
<td>70</td>
<td>46.7</td>
<td>70</td>
</tr>
<tr>
<td>COL</td>
<td>1</td>
<td>360</td>
<td>72</td>
<td>24.0</td>
<td>228</td>
<td>76.0</td>
<td>228</td>
</tr>
<tr>
<td>AA</td>
<td>125</td>
<td>360</td>
<td>215</td>
<td>71.7</td>
<td>85</td>
<td>28.3</td>
<td>85</td>
</tr>
</tbody>
</table>

Table III. Distribution of the signal frequencies among MN after double labelling with minor and major DNA probes by FISH

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (mg/kg)</th>
<th>MN with minor signals</th>
<th>One minor signal (%)</th>
<th>Two minor signals (%)</th>
<th>Three minor signals (%)</th>
<th>Four minor signals (%)</th>
<th>More than five minor signals (%)</th>
<th>MN with major signals</th>
<th>One major signal (%)</th>
<th>Two major signals (%)</th>
<th>Three major signals (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>70</td>
<td>10</td>
<td>14.3</td>
<td>57</td>
<td>(81.4)</td>
<td>(2.9)</td>
<td>0</td>
<td>70</td>
<td>62</td>
<td>(88.6)</td>
</tr>
<tr>
<td>COL</td>
<td>1</td>
<td>228</td>
<td>65</td>
<td>(28.5)</td>
<td>135</td>
<td>(59.2)</td>
<td>(4.0)</td>
<td>21</td>
<td>0</td>
<td>228</td>
<td>(83.8)</td>
</tr>
<tr>
<td>AA</td>
<td>125</td>
<td>87</td>
<td>23</td>
<td>(26.4)</td>
<td>49</td>
<td>(56.3)</td>
<td>(9.2)</td>
<td>5</td>
<td>1</td>
<td>63</td>
<td>(74.1)</td>
</tr>
</tbody>
</table>

Table IV. Number of extruded MN labelled with minor and major DNA probes by FISH

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Dose (mg/kg)</th>
<th>No. of MN scored</th>
<th>No of MN extruded</th>
<th>Extruded MN without any signals</th>
<th>Extruded MN with major and minor signals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>%</td>
<td>total</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>150</td>
<td>6</td>
<td>4.0</td>
<td>0</td>
</tr>
<tr>
<td>COL</td>
<td>1</td>
<td>300</td>
<td>29</td>
<td>9.7</td>
<td>4</td>
</tr>
<tr>
<td>AA</td>
<td>125</td>
<td>300</td>
<td>25</td>
<td>8.3</td>
<td>7</td>
</tr>
</tbody>
</table>

Discussion

The purpose of the present study was to verify whether: (i) extruded MN could be detected; (ii) extruded MN contained lagging chromosomes; and (iii) AA had an aneugenic effect.

The present results using the paintbrush method for the conventional micronucleus staining yielded MN frequencies for all groups which were a little lower than the published data (Adler et al., 1988, 1991; Schriever-Schwemmer and Adler, 1994). For the control, the MN frequency of 1.0/1000 PCE agreed well with the published data. For COL, Schriever-Schwemmer and Adler (1994) found 10.5 MN/1000 PCE and Adler et al. (1991) found 12.3 MN/1000 PCE compared with these in the present study. These lower MN frequencies could be due to a better preservation of all erythrocytes so that no loss of erythrocytes occurred as it may when a centrifugation step is involved or to inter-animal variation and the small number of animals in the present study.

Parton et al. (1991) analysed the amount of extruded MN after treatment of mice with demicoline and cyclophosphamide using the paintbrush method to preserve cells undergoing enucleation. Treatment with demicoline, a classical aneugen, caused relatively high frequencies of extruded MN (15–40% of all MN), however, no extruded MN were observed after treatment with cyclophosphamide, a classical clastogen.

In this study, using May–Grünwald–Giemsa staining, no extruded MN were found in the control, however, after treatment with COL, 4.4% of all MN were extruded and after treatment with AA, 22.2% of all MN were extruded (Table I). Using FISH labelling, 4.0% MN were extruded in the control, 9.7% in the COL group and 8.3% in the AA group (Table IV). The results indicate that scoring only retained MN may underestimate the true level of MN induction, however, the difference between total and retained MN in our experiments was not significant. Extrusion of MN is not only a property of demicoline, as argued by Parton et al. (1991), but the extrusion of MN can be observed with other chemicals, as in the present study with COL and AA. Thus, the extrusion of MN may be a common phenomenon.

To verify whether the extruded MN contain lagging chromosomes or acentric fragments, FISH analysis with the minor and major DNA probes was carried out. This technique makes it possible to discriminate between MN formed by clastogens and by aneugenics (Miller et al., 1991; Chen et al., 1994; Schriever-Schwemmer and Adler, 1994). The discrimination is particularly important for chemical clastogens which may also have aneugenic potential. Such chemicals may intersect with the targets for chromosomal missegregation on the chromosomal level, i.e. with kinetochore proteins, centromeric DNA or telomeres.

In control animals, the yield of MN with minor and major probes by FISH was 46.7% of the 150 MN analysed (Table II). This result was in the same range as for controls of other in vivo bone marrow MN studies (Vanderkerken et al., 1989; Miller et al., 1991; Schriever-Schwemmer and Adler, 1994). After treatment with the classical spindle poison COL, the...
FISH labelling with the minor and major probe gave comparable results (Table II), i.e. 76% of the MN contained entire chromosomes as evidence by concurrent minor and major signals. This result was slightly higher than that of previous studies when between 67% major-positive MN and 75% minor-positive MN were found (Miller et al., 1991; Schriefer-Schwemmer and Adler, 1994).

AA is a well-known clastogen (Shelby et al., 1987; Adler et al., 1988, 1994; Gutierrez-Espeleta et al., 1992) and a suspect aneugen (Adler et al., 1993; Gassner and Adler, 1996). In the present study, 29% MN were minor-positive and 28.3% MN were major-positive. Thus, AA-induced MN containing acenric fragments were 2.5 times more frequent than AA-induced MN containing lagging chromosomes (Table II). However, the frequency of positive MN was more than three times higher after AA treatment than in the control, i.e. 1.7 MNPEpos/1000 PCE versus 0.5 MNPEpos/1000 PCE (Table II). Therefore, AA has to be regarded as having an aneugenic potential in addition to its clastogenic activity.

The distribution of the signal frequencies among the double-labelled MN show 82–95% of the double-labelled MN have one or two minor signals and 96–99% have one or two major signals, indicating that these MN contained whole chromatids or chromosomes (Table III). These distribution results are very similar to earlier observations in experiments with COL and mitomycin C in our laboratory (Schriefer-Schwemmer and Adler, 1994).

The MN ratios found using the FISH technique also included extruded MN, which were still attached to the erythrocyte membranes. In the control, 4% of the analysed MN were extruded. After COL treatment, 9.7% MN were extruded, and after AA treatment, 8.3% were extruded (Table IV). In the controls, 100% of these extruded MN showed major as well as minor signals. In the COL group, 86% and in the AA group, 72% of the extruded MN showed major as well as minor signals. Thus, only 0–28% of the extruded MN were without any signals. The signal negative extruded MN frequency was higher in the AA group which may be due to the stronger clastogenicity of AA.

We conclude that: (i) extruded MN do exist; (ii) mostly MN formed by lagging chromosomes stand a risk of expulsion, however, also MN containing acenric fragments can be expelled from the erythrocytes; and (iii) AA has an aneugenic potential.

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