Does formaldehyde induce aneuploidy?

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Formaldehyde (FA) was tested for a potential aneugenic activity in mammalian cells. We employed tests to discriminate between aneugenic and clastogenic effects in accordance with international guidelines for genotoxicity testing. The cytokinesis-block micronucleus test (CBMNT) in combination with fluorescence in situ hybridisation (FISH) with a pan-centromeric probe was performed with cultured human lymphocytes and the human A549 lung cell line. FA induced micronuclei (MN) in binuclear cells of both cell types under standard in vitro test conditions following the OECD guideline 487. FISH analysis revealed that the vast majority of induced MN were centromere negative, thus indicating a clastogenic effect. A similar result was obtained for MN induced by γ-irradiation, whereas the typical aneugens colcemid (COL) and vincristine (VCR) predominantly induced centromere-positive MN. Furthermore, COL and VCR clearly enhanced the MN frequency in mononuclear lymphocytes in the CBMNT, whereas such an effect was not observed for γ-irradiation and FA. In experiments with the Chinese hamster V79 cell line, the aneugens COL and VCR clearly increased the frequency of tetraploid second division metaphases, whereas FA did not cause such an effect. Altogether, our results confirm the clastogenicity of FA in cultured mammalian cells but exclude a significant aneugenic activity.

Introduction

Many studies have shown that formaldehyde (FA) is genotoxic and mutagenic in cultured mammalian cells in vitro. FA induces various DNA adducts and it is generally accepted that the most important DNA alterations induced by FA are DNA–protein cross-links (DPX). In proliferating cells, unpaired DPX can arrest DNA replication and lead to the induction of other genotoxic effects such as sister chromatid exchanges (SCE) (1,2). All mammalian cells investigated so far are capable to efficiently repair DPX but incomplete repair of DPX can lead to the formation of mutations. Chromosomal effects such as chromosome aberrations and micronuclei (MN) seem to be most efficiently induced, whereas FA is a poor inducer of true gene mutations (3,4). Not many data are available on a potential aneugenic activity of FA and published results are conflicting (1,5–7). A recently published human study suggested that FA might induce aneuploidy in myeloid progenitor cells and in this way cause leukaemia (8). Although this study suffered from severe shortcomings and the results lacked plausibility (9), this study was considered by the International Agency for Research on Cancer (IARC) as a potential mechanistic explanation for the induction of leukaemia by FA (10).

The micronucleus test (MNT) is the standard genotoxicity test for the detection of clastogenic and aneugenic activity of chemicals. An OECD guideline (No. 487) for the in vitro mammalian cell micronucleus test has recently been published (11). MN may originate from chromosome breaks leading to acentric chromosome fragments (i.e. lacking a centromere) or from whole chromosomes that did not reach the spindle poles during cell division. Chromosome fragments or whole chromosomes that are not included in the main daughter nuclei during nuclear division lead to MN, which can be analysed in the next interphase. Thus, MN represent a measure of both chromosome breakage (clastogenic effect) and chromosome malsegregation (aneugenic effect). Aneuploidy is mainly the consequence of non-disjunction or chromosome loss. During mitosis, chromosome non-disjunction occurs when chromatids do not separate normally but stick together and move to one pole during anaphase. This results in two daughter cells containing 2n + 1 and 2n − 1 chromosomes at the next mitosis (i.e. trisomy and monosomy of the respective chromosome). Chromosome loss may be the result of either non-attachment of the chromosome kinetochore to the spindle microtubules or chromosome lagging at anaphase.

The MNT detects the activity of clastogenic and aneugenic chemicals in cells that have undergone cell division during or after exposure to the test substance. The addition of the actin polymerisation inhibitor cytochalasin B (CytB) prior to the targeted mitosis allows for the identification and selective analysis of the MN frequency in cells that have completed one mitosis because such cells are binucleate (11). This modification of the MNT, the cytokinesis-block micronucleus test (CBMNT), was established by Fenech and Morley in 1985 (12) and is the preferred method for the detection of clastogenic and aneugenic chemicals in genetic toxicology (13). For the differentiation of chemicals inducing aneuploidy from those inducing clastogenicity, fluorescence in situ hybridisation (FISH) with centromeric probes can be applied. MN that do not reveal a centromeric FISH signal (cen−) are interpreted as harbouring acentric chromosomal fragments (clastogenic effect). The presence of centromere-specific DNA analysed by FISH (cen+) is indicative of MN containing whole chromosomes (aneugenic effect).

Aneugens are chemicals that affect cell division and the mitotic spindle apparatus resulting in the loss or gain of whole chromosomes, thereby inducing aneuploidy. They usually act on
non-DNA targets such as the inhibition of spindle function with no direct interaction with DNA. It is generally assumed that a critical number of target sites must be occupied before the biological effect occurs. For these mechanistic reasons and based on the available data, a threshold concept for the induction of aneuploidy is well accepted by the scientific community (14). A threshold mode of action (MOA) is characterized by a non-linear dose–response relationship and a level of exposure below which the genotoxic effect is not observed. Aneugens induce cen+ MN in the CBMNT with FISH analysis and spindle poisons such as colchicine/coleoside (COL) or vinblastine/vincristine (VCR) are recommended as positive controls (11). It has also been shown that aneugens exert a characteristic effect on the frequency of MN in mononuclear cells in the CBMNT with human lymphocytes and it was therefore recommended to include the analysis of mononuclear cells to distinguish aneugens from clastogens and increase the sensitivity of the test (15,16). Furthermore, aneugens also may induce polyploidy and induction of polyploid cells has been used as an indicator of the substance’s potential to inhibit mitotic processes and to induce numerical chromosome aberrations in standard in vitro mammalian chromosome aberration tests according to the OECD guideline 473 (17).

To identify and characterise a potential aneugenic activity of FA, we now performed a comprehensive study using three different cell systems (human blood cultures, A549 cells and V79 cells) and considering various genetic endpoints (MN frequencies in binuclear cells in the CBMNT, MN frequencies in mononuclear cells, FISH analysis of MN and induction of polyploidy). Results obtained for FA were compared to effects of typical aneugens (COL and VCR) and to γ-irradiation as a typical clastogen.

Materials and methods

Materials

Cell culture media and ingredients were obtained from Biochrom (Berlin, Germany). RPMI medium and phytohaemagglutinin (PHA) were purchased from Invitrogen (Darmstadt, Germany). If not specifically indicated, the chemicals used in these experiments were purchased from Sigma-Aldrich (Munich, Germany). FA (CAS No. 50-00-0, 16% ultrapure, methanol free) was bought from Polysciences, Inc., Warrington, PA, USA. FA was diluted in distilled water immediately before use. COL was purchased from Biochrom and VCR from Sigma-Aldrich. Both substances were diluted in distilled water immediately before use. CytB was added to the cultures at a final concentration of 3 g/ml 45 h after PHA stimulation. Cells were harvested 27 h later, giving a total culture time of 72 h. Cells were harvested by centrifugation, treated with a hypotonic solution (0.56% KCl) and fixed once with methanol/glacial acetic acid (5 + 1) mixed with an equal amount of 0.9% NaCl and then fixed three times with methanol/glacial acetic acid (5 + 1). Air-dried slides were stained with acridine orange (60 µg/ml in phosphate buffer). The frequency of MN and micronucleated cells was determined by analysing 1000 binucleated and 100 micronucleated cells from coded cultures. Toxicity was measured using the cytokinesis-block proliferation index (CBPI), which was calculated from 500 cells according to the formula: \( CBPI = \left( \frac{M1 + 2M2 + 3M3 + 4M4}{N} \right) \), where M1–M4 indicates the number of cells with one to four nuclei and N the total number of cells scored. For the induction of MN, blood cultures were irradiated or treated with FA, COL or VCR 44 h after PHA stimulation. A549 cells were cultivated for 24 h and then exposed to FA for 2 h or irradiated with γ-rays and further cultivated in the presence of CytB for 48 h. Alternatively, cells were treated with FA or VCR for 30 h in the presence of CytB. For preparation and staining, the same protocols were applied as described for blood cultures. The frequency of micronucleated cells was measured in 1000 binucleated cells from coded slides.

**FISH analysis of MN**

The origin of MN was assessed by FISH using biotin-labelled pan-centromeric chromosome paint specific for all human centromeres (Cambio, Cambridge, UK). Frozen slides were brought to room temperature on a heating plate. RNase was diluted in 2× standard saline concentration (SSC) (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0) to a final concentration of 100 µg/ml and added to the slides for 1 h at 37°C in a humidified box. Thereafter, the slides were washed three times in 2× SSC for 5 min and immersed in a pepsin solution (30 µg/ml) for 8 min at 37°C. After washing the slides in 1× phosphate-buffered saline (PBS) for 5 min and for 5 min in PBS with MgCl2 (50 mM), the slides were fixed in 1% FA for 15 min at room temperature. After another washing step in 1× PBS for 5 min, the slides were dehydrated in 70, 80, 90 and 99% ethanol at room temperature. The slides were denaturated for 1 min in 70% formamide, 2× SSC, pH 7.0, at 70°C and dehydrated in an ice-cold 70, 80, 90 and 99% ethanol. Thereafter, the slides were air-dried for 30 min. The pan-centromeric probe was denatured for 10 min at 80°C. Aliquots of 15 µl of the probe were added to the slides. The slides were covered with a 24 × 50 mm coverslip and sealed with rubber cement. Hybridisation was carried out overnight at 37°C. After removing the coverslips, the slides were washed in 2× SSC and fixed in 50% formamide/2× SSC (pH 7; 45°C) for 15 min and then rinsed in 2× SSC for 5 min at 45°C. The slides were collected in 4× SSC/0.1% Tween20 before blocking in bovine serum albumin (BSA) solution (5% BSA in 4× SSC/0.1% Tween20) for half an hour at 37°C. Then the slides were washed in 4× SSC/0.1% Tween20. The biotin-labelled probe was detected with 150 µl fluorescein isothiocyanate (FITC–avidin (1:200) in 1% BSA for 30 min at 37°C in a humidified box. Afterwards, the slides were washed three times for 5 min in 2× SSC/0.1% Tween20 at 45°C. The slides were washed two times by biotinylated anti-avidin (1:100) and FITC–avidin. After each detection step, the slides were washed three times in 4× SSC/0.1% Tween20 at 45°C. The cells were counterstained with 4,6-diamidino-2-phenylindol (DAP) and embedded in antifade solution. Slides were kept in dark and analysed using a fluorescence microscope (Zeiss Axiosplan, 100 W HBO lamp) with a triple dye filter for the detection of DAPI, propidium iodide and FITC. Deposition of whether a fluorescent spot was observed inside the MN, the MN was classified as centromere negative (cen−) or centromere positive (cen+). Cells with more than one MN, each MN was classified as cen− or cen+. The percentage of induced cen− and cen+ MN in treated cultures was calculated after subtracting the background frequencies of cen− and cen+ MN from the parallel negative control culture.

**Induction of polyploidy in V79 cells**

V79 cells were cultivated for 24 h, then exposed to FA, COL or VCR and further cultivated in the presence of 10 µg/ml BrdUrd (5-bromodeoxyuridine) for 24 or 28 h. COL (5 × 10−7 M) was added for the final 2 h. Chromosome preparation was done following standard procedures. Cells were centrifuged, resuspended in 0.56% KCl (37°C) for 20 min and fixed three times in methanol/glacial acetic acid (3 + 1). For sister chromatid differentiation, air-dried slides were covered with Sorenson buffer (pH 6.8) and irradiated with an 8-W UV lamp (254 nm) at a distance of 15 cm for 20 min. Subsequently, slides were incubated in 2× SSC for 20 min at 60°C and then stained with 7% Giemsa for 20 min in Sörensen buffer. The frequency of tetraploid metaphases was determined among 100 second division metaphases (i.e. chromosomes with complete sister chromatid differentiation). The frequency of SCE was measured in 30 diploid second division metaphases per culture. Toxicity was determined by scoring first division metaphases (M1), second division metaphases (M2), and third division metaphases (M3) among 100 metaphases and calculating the proliferation index (PI) according to the formula: PI = (M1 + 2M2 + 3M3)/100.
Pre-experiments were performed to define the appropriate concentrations for the different tests. The main experiments (shown in the figures and tables) were then performed with concentrations, which led to a clear induction of MN and independently repeated three times. Differences between mean values of the independently repeated experiments were tested for significance using Student’s t-test. A statistically significant difference was set at $P < 0.05$.

**Results**

**Induction of MN**

Figure 1 summarises the results of the CBMNT with human blood cultures exposed to FA 45 h after the start of the cultures. FA treatment for the last 27 h in the presence of CytB led to a clear and concentration-related increase in binuclear cells with MN. The increase in MN in comparison with the negative control was statistically significant after treatment with 300 μM FA. Only a marginal increase in the frequency of mononuclear cells with MN was measured (from 9.25% in controls to 12.25% at 200 μM FA and 13.75% at 300 μM FA). The concentration-related reduction of the CBPI (from 1.8 to 1.3) indicates the cytotoxicity of FA under these experimental conditions.

Figure 2 summarises the results of the CBMNT with blood cultures exposed to 2 Gy γ-rays, 0.02 μg/ml COL or 0.01 μg/ml VCR. In blood cultures irradiated with 2 Gy 45 h after the start and then cultured in the presence of CytB for another 27 h, a strong induction of MN was measured in binuclear cells (377% cells with MN compared to controls with 34%). The frequency of mononuclear cells with MN increased from 5.7 to 32.2% and the CBPI decreased from 2.1 to 1.7. Treatment of blood cultures for the last 27 h with COL slightly increased the frequency of binuclear cells with MN but clearly induced MN in mononuclear cells. VCR clearly induced MN in binuclear and mononuclear lymphocytes. There was a 5-fold increase in binuclear cells with MN but a 11-fold increase in mononuclear cells with MN. Higher concentrations of COL and VCR (0.03 μg/ml and 0.02 μg/ml, respectively) increased the frequency of mononuclear cells with MN but strongly reduced the amount of binuclear cells, thus preventing an appropriate evaluation (data not shown).

Table I summarises the results for the MNTs performed with A549 cells. Cells were treated with FA for 2 h and then cultivated for 48 h in the presence of CytB or exposed to FA for 30 h in the presence of CytB. A549 cells were also irradiated with 2 Gy γ-rays and then cultivated in the presence of CytB for 48 h or exposed to VCR for 30 h in the presence of CytB. A549 cells treated with VCR for 2 h and then cultivated in the presence of CytB did not produce increased MN frequencies. Therefore, FA was tested under both conditions, i.e. treatment for 2 h and post-cultivation with CytB (48 h) and permanent treatment (30 h) in the presence of CytB. The frequency of binuclear cells with MN slightly increased in FA-treated cultures (200 and 300 μM for 2 h) in a concentration-related manner (from 22.3 to 37.7 and 47.7%). The increase was statistically significant ($P = 0.033$) in comparison with the
negative control after exposure to 300 μM FA. After permanent (30 h) treatment, evaluation of MN was possible for the FA concentration of 150 μM. Under these experimental conditions, the frequency of cells with MN increased from 20 to 52% \( \pm \) (\( P < 0.01 \)). Higher concentrations were cytotoxic and inhibited proliferation of A549 cells. Ionising radiation (98.3% \( \pm \)) and VCR (66.3% \( \pm \)) had a stronger MN-inducing effect than FA under the respective treatment conditions.

**FISH analysis of MN**

The results of the FISH analysis in binuclear lymphocytes with MN (from the experiments shown in Figure 1) are summarised in Table II. The total number of MN analysed per treatment, the number and percentage of MN without centromere signal (cen−) and the number of MN with centromere signal (cen+) are specified. The percentage of induced cen− and cen+ MN in treated cultures was calculated after subtracting the background frequencies of cen− and cen+ MN of the parallel negative control culture. It can be seen that FA mainly induced cen− MN (73% at 200 μM and 92% at 300 μM), indicative of a clastogenic effect. The effect of FA is similar to the effect of ionising radiation (93% cen− MN) but quite different from the effects of the aneugens COL and VCR (95% and 81% cen+ signals, respectively). Altogether, 3538 MN from 13 untreated control cultures were analysed. Among these, 956 were cen− and 2582 were cen+.

FISH analysis of MN induced in A549 cells (from the experiments shown in Table I) basically confirmed the results obtained with lymphocytes. Table III shows the number of analysed MN, the number and percentage of MN without centromere signal (cen−) and with centromere signal (cen+) as well as the calculated percentage of induced MN without and with centromere signal. Seventy-seven percent of MN induced by 150 μM FA (30 h treatment) were centromere negative and 81% of MN induced by 300 μM FA (2 h treatment) were centromere negative. Ninety-six percent of γ-ray-induced MN were centromere negative, whereas only 22% of VCR-induced MN were centromere negative. A total of 1841 MN were analysed from untreated control cultures after cultivation in the presence of CytB for 30 h; 733 had cen− and 1108 had cen+. A total of 1841 MN were analysed from untreated control cultures after cultivation in the presence of CytB for 30 h; 733 had cen− and 1108 had cen+.

**Induction of polyploidy**

Table IV summarises the results of the experiments performed with V79 cells to investigate the induction of polyploidy by FA. The permanent V79 cell line used in our experiments is heterocentric with 21 chromosomes in more than 80% of the cells (The Chinese hamster has a normal karyotype of \( 2n = 22 \)). Metaphases with 19–22 chromosomes are called ‘diploid’ here; cells with a doubled chromosome number are called ‘tetraploid’. The background frequency of tetraploid metaphases was between 0 and 3% of all metaphases under our cell culture conditions. The frequency of tetraploid metaphases was not increased in metaphases with sister chromatid differentiation (i.e. in cells which had passed through two cell cycles in the presence of BrdUrd and FA) after treatment with FA (50 and 100 μM) for 24 or 28 h. In contrast, a clear induction of polyploidy was measured in cultures treated with COL (53% tetraploid metaphases at 0.02 μg/ml) or VCR (95% tetraploid metaphases at 0.02 μg/ml). An increase in tetraploid metaphases (from 1 to 16%) was observed among homogenously stained metaphases (i.e. in cells which had passed one G2-phase and/or mitosis and one complete cell cycle in the presence of FA) after treatment with 100 μM FA for 28 h in one experiment. In the two other experiments, the frequency of 

### Table I. Induction of MN by FA, γ-rays and VCR in A549 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Binuclear cells with MN</th>
<th>( P )-value (( t )-test)</th>
<th>CBPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (48 h CytB)</td>
<td>22.3 ± 12.10</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>FA (200 μM; 2 h)</td>
<td>37.7 ± 17.21</td>
<td>0.141</td>
<td>2.24</td>
</tr>
<tr>
<td>FA (300 μM; 2 h)</td>
<td>47.7 ± 12.50</td>
<td>0.033</td>
<td>2.03</td>
</tr>
<tr>
<td>γ-rays (2 Gy)</td>
<td>98.3 ± 40.65</td>
<td>0.037</td>
<td>1.96</td>
</tr>
<tr>
<td>Control (30 h CytB)</td>
<td>20.0 ± 7.00</td>
<td></td>
<td>2.33</td>
</tr>
<tr>
<td>FA (150 μM; 30 h)</td>
<td>51.7 ± 10.69</td>
<td>0.009</td>
<td>1.80</td>
</tr>
<tr>
<td>VCR (0.01 μg/ml; 30 h)</td>
<td>66.3 ± 25.74</td>
<td>0.040</td>
<td>1.87</td>
</tr>
</tbody>
</table>

\( ^* \)Mean ± SD of three experiments.

### Table II. FISH analysis of induced MN in binuclear human lymphocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN analysed</th>
<th>Cen− (%)</th>
<th>Cen+ (%)</th>
<th>Induced MN (%) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA (200 μM)</td>
<td>833</td>
<td>435 (52.2)</td>
<td>398 (47.8)</td>
<td>73.0</td>
</tr>
<tr>
<td>FA (300 μM)</td>
<td>850</td>
<td>590 (69.4)</td>
<td>260 (30.6)</td>
<td>92.0</td>
</tr>
<tr>
<td>γ-Rays (2 Gy)</td>
<td>1165</td>
<td>1027 (88.2)</td>
<td>138 (11.8)</td>
<td>93.2</td>
</tr>
<tr>
<td>COL (0.02 μg/ml)</td>
<td>914</td>
<td>130 (14.2)</td>
<td>784 (85.8)</td>
<td>5.1</td>
</tr>
<tr>
<td>VCR (0.01 μg/ml)</td>
<td>998</td>
<td>207 (20.7)</td>
<td>791 (79.3)</td>
<td>19.2</td>
</tr>
</tbody>
</table>

\(^a\)After subtracting the frequencies of cen− and cen+ of the parallel control.

### Table III. FISH analysis of induced MN in binuclear A549 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MN analysed</th>
<th>Cen− (%)</th>
<th>Cen+ (%)</th>
<th>Induced MN (%) (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA (300 μM) (^b)</td>
<td>1042</td>
<td>672 (64.5)</td>
<td>370 (35.5)</td>
<td>80.7</td>
</tr>
<tr>
<td>FA (150 μM) (^b)</td>
<td>970</td>
<td>476 (49.1)</td>
<td>494 (50.9)</td>
<td>77.4</td>
</tr>
<tr>
<td>γ-Rays (2 Gy)</td>
<td>559</td>
<td>430 (76.9)</td>
<td>129 (23.1)</td>
<td>95.8</td>
</tr>
<tr>
<td>VCR (0.01 μg/ml) (^b)</td>
<td>920</td>
<td>245 (26.6)</td>
<td>675 (73.4)</td>
<td>22.0</td>
</tr>
</tbody>
</table>

\(^a\)After subtracting the frequencies of cen− and cen+ of the parallel control.

### Table IV. Induction of polyploidy by COL, VCR and FA in V79 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration (h)</th>
<th>Diploid metaphases (%)</th>
<th>Tetraploid metaphases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24</td>
<td>98.7 ± 0.58</td>
<td>1.3 ± 0.58</td>
</tr>
<tr>
<td>COL (0.01 μg/ml)</td>
<td>24</td>
<td>94.0 ± 1.73</td>
<td>6.0 ± 1.73</td>
</tr>
<tr>
<td>COL (0.02 μg/ml)</td>
<td>24</td>
<td>44.3 ± 12.50</td>
<td>53.0 ± 8.89</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>97.3 ± 0.58</td>
<td>2.7 ± 0.58</td>
</tr>
<tr>
<td>VCR (0.01 μg/ml)</td>
<td>24</td>
<td>8.7 ± 6.03</td>
<td>90.3 ± 5.51</td>
</tr>
<tr>
<td>VCR (0.02 μg/ml)</td>
<td>24</td>
<td>3.0 ± 2.65</td>
<td>94.7 ± 2.08</td>
</tr>
<tr>
<td>Control</td>
<td>24</td>
<td>98.3 ± 1.53</td>
<td>1.7 ± 1.53</td>
</tr>
<tr>
<td>FA (50 μM)</td>
<td>24</td>
<td>99.3 ± 0.58</td>
<td>0.7 ± 0.58</td>
</tr>
<tr>
<td>Control</td>
<td>28</td>
<td>99.0 ± 1.00</td>
<td>1.0 ± 1.00</td>
</tr>
<tr>
<td>FA (50 μM)</td>
<td>28</td>
<td>98.7 ± 0.58</td>
<td>1.3 ± 0.58</td>
</tr>
<tr>
<td>Control</td>
<td>28</td>
<td>98.7 ± 2.31</td>
<td>1.3 ± 2.31</td>
</tr>
</tbody>
</table>

Mean ± SD of three experiments.
homogenously stained tetraploid metaphases was 1 and 4%, respectively. After treatment with 50 μM FA, no homogenously stained tetraploid metaphases were found at all.

Interestingly, in second division diploid metaphases, the frequency of SCE was increased in a concentration-related manner after exposure to FA for 24 or 28 h (Figure 3). The increase in SCE frequency was statistically significant after treatment with 50 μM FA and cultivation for 24 h ($P < 0.01$) and after treatment with 100 μM FA and cultivation for 24 h ($P < 0.001$) or 28 h ($P < 0.01$). This result indicates that FA exerts a DNA-damaging effect on V79 cells under the condition of these experiments. A clear inhibition of cell proliferation (reduction in PI from ~2.0 to 1.8) was measured after exposure to 100 μM FA at both preparation times. Slight inhibition of proliferation was also measured after exposure to 50 μM FA and cultivation for 28 h. These results indicate that the DNA-damaging effect of FA is accompanied by a cytotoxic effect.

**Discussion**

The genotoxicity and mutagenicity of FA in mammalian cells *in vitro* have been investigated and characterised in various studies. It is generally assumed that the most important primary DNA alterations induced by FA are DPX (2,3) but other types of DNA adducts have also been measured (18). Unrepaired DNA lesions may lead to the formation of mutations in replicating cells and it has been shown that chromosomal effects (clastogenicity) are predominant, whereas true gene mutations are induced to a lesser extent (3,4). On the basis of the existing broad database, one would classify FA as a typical clastogen. However, FA also induces protein–protein adducts and thus might interact with the mitotic apparatus and disturb chromosome segregation. Until recently, there was no concern with regard to a potential of FA to induce aneuploidy and no need to consider such effects in risk assessment. It is generally accepted that the induction of aneuploidy is a so-called indirect mechanism of genotoxicity due to an interaction with redundant targets (e.g. tubulin) and not with DNA (19). In contrast with clastogenic effects, the induction of aneuploidy is expected to show threshold concentration–effect curves (14). Considering the strong clastogenic activity of FA in directly exposed cells, FA-induced aneuploidy in the absence of a clear clastogenic effect seems to be highly unlikely.

Up to now, a potential of FA for inducing aneuploidy has not been studied in detail. However, molecular characterisation of FA-induced MN by FISH in cultured human lymphocytes and V79 cells as well as MN measured in buccal and nasal cells of FA-exposed subjects indicated that MN occurred as a consequence of chromosome breakage and not of aneuploidy (1,5,6). An association between FA exposure and aneuploidy was reported in a biomonitoring study with subjects exposed to FA at the workplace (7). The frequency of monocentromeric MN was significantly higher in lymphocytes of exposed subjects than in controls while that of the acentromeric MN was similar in exposed subjects and controls. However, this finding is rather implausible and not fully reliable because the aneugenic effect occurred in the absence of clastogenicity. It can furthermore be assumed that MN measured in the CBMNT mainly arises *ex vivo* during the cultivation of lymphocytes (20). Enhanced formation of aneuploid cells would require that the cells sampled and investigated have high levels of FA-induced damage to the mitotic apparatus leading to malsegregation of chromosomes during mitosis.

A recently published study suggested that occupational exposure to FA caused leukaemia-specific chromosome aberrations (monosomy 7 and trisomy 8) in cultured myeloid blood progenitor cells (8). However, this study suffered from severe shortcomings in the evaluation and interpretation of these effects and the origin of these aneuploidies and their biological significance remained unclear (9). Besides the lack of evidence for a systemic availability of FA, induction of aneuploidy was an unproven assumption for FA’s genotoxic MOA. Nevertheless, the results of this study were considered by IARC as a potential mechanistic explanation for the induction of leukaemia by FA and supported the classification of FA as a leukaemogen (10).

Our results presented here clearly exclude any biologically significant potential of FA to induce aneuploidy. We used the CBMNT in combination with FISH as the standard genotoxicity tests for the determination of aneugenic effects that is generally accepted by the scientific community and part of international guidelines for genotoxicity testing (21). Exposure of human lymphocyte cultures to FA for 27 h in the presence of CytB led to a clear induction of MN in binuclear cells. The mean background frequency of micronucleated cells in untreated controls was 37 per 1000 in these experiments with a range between 21 and 59. This is somewhat higher than the usually expected range of 0–30 per 1000 binucleated cells (22). This seems to be related to the cell culture conditions used but the clear induction of MN by FA in accordance with earlier results (1) clearly demonstrates the sensitivity of the test. Under the same experimental conditions, known aneugens (COL and VCR) also induced MN in binuclear cells. However, whereas the
vast majority of FA-induced MN (up to 90%) were centromere negative (i.e., indicated a clastogenic effect), the vast majority of MN induced by COL and VCR (95 and 81%) were centromere positive (i.e., indicated an aneugenic effect). This finding clearly demonstrates that FA does not react like an aneugen but clearly demonstrates clastogenic activity. The distribution of induced MN with and without centromeric signals is rather similar to that measured for ionising radiation, which is an accepted positive control for a clastogen. These results were further confirmed by the analysis of MN frequencies in mononuclear lymphocytes. Previous investigations have shown that aneugens, but not clastogens, clearly induced an increase in MN in mononuclear lymphocytes. This effect was interpreted as being the result of mitotic slippage. It was suggested to include the analysis of MN in mononuclear cells for the reliable detection of aneugens in the CBMNT (15, 23). This finding was confirmed by another study (24) using a similar experimental protocol as employed in our study, i.e. treatment of lymphocyte cultures for the last 24 h in the presence of CytB. An international collaborative study on in vitro MNT (16) also demonstrated the induction of MN in mononuclear cells of human lymphocyte cultures and various mammalian cell lines for different aneugens. The authors also pointed out that for a direct comparison of the frequencies of mononuclear and binuclear cells with MN, one has to divide the incidence of micronucleated binuclear cells by two. This is necessary because one cell division leads to two mononuclear cells but only to one binuclear cell (16). Taking these considerations into account, our results even more clearly show that the aneugens COL and VCR lead to a higher frequency of mononuclear cells with MN and FA and γ-rays mainly enhance the frequency of binuclear cells with MN and only have a minor effect on the frequency of mononuclear cells with MN. In summary, our experiments with human lymphocyte cultures clearly demonstrate the clastogenic activity of FA but do not give a hint towards a significant induction of aneuploidy in lymphocytes exposed to FA under appropriate test conditions.

Additional experiments were performed with A549 cells because this cell line has previously been used for characterising the genotoxic potential of FA (25, 26). The results obtained clearly confirmed the results of the CBMNT with lymphocytes. FA-induced MN were predominantly the result of a clastogenic event (cen− MN). This finding corresponds to the result obtained for γ-irradiation but is quite different from the result obtained for the aneugen VCR (78% cen+ MN). Two experimental protocols were used for the induction of MN by FA in A549 cells: short-term treatment (2 h) followed by cultivation in the presence of CytB for 48 h (according to the protocol used for γ-rays) or permanent treatment for 30 h in the presence of CytB (according to the protocol used for VCR). MN induced by FA under both conditions did not reveal any significant aneugenic effect.

We already characterised the genotoxic and mutagenic potential in V79 cells, a cell line frequently used in genotoxicity testing (2, 3). On the basis of these published results, one would classify FA as a typical clastogen. FISH analysis performed with FA-induced MN in V79 cells confirmed that FA induced MN in V79 cells mainly via a clastogenic MOA and did not show aneugenic activity (5). We now used V79 cells to study the induction of polyploidy by FA, a phenomenon associated with the induction of aneuploidy. V79 cells were used for these experiments because (in contrast to A549 cells) this cell line has a relatively stable karyotype and a low degree of heteroploidy. Polyploid metaphases have to be evaluated in the second mitosis after exposure because disturbance of mitosis occurs in the first mitosis and the resulting polyploid karyotype is seen in the second metaphase. BrdUrd-labelling unambiguously allowed the identification of second division metaphases after FA exposure and a clear result was obtained. In contrast to COL and VCR, which strongly induced polyploidy, FA did not show such an effect under the same experimental conditions. To be sure that an effect was not missed because of a cell cycle delay in exposed cultures, a second preparation time point (4 h later) was investigated. However, there was no induction of polyploidy by FA in metaphases after (potential) proliferative delay. Interestingly, some tetraploid metaphases were observed on these slides. These tetraploid metaphases did not show sister chromatid differentiation but were homogenously stained. These cells might have been in the G2-phase or early mitosis of the first cell cycle when they were exposed to FA (and BrdUrd). This finding might indicate that V79, which are exposed to high FA concentrations shortly before the mitotic apparatus is built up, may become polyploid. This potential MOA needs further investigations before its biological relevance can be assessed. Altogether, the experiments with V79 clearly show that FA does not induce polyploidy under experimental conditions, which lead to clearly enhanced frequencies of tetraploid cells after exposure to reference aneugens. The experiments also confirmed the DNA-damaging and cytotoxic action of FA under these test conditions (2). SCE were induced in a concentration-related manner and cell proliferation was inhibited. We conclude that an effect of FA on the mitotic apparatus in the absence of a DNA-damaging or clastogenic effects and cytotoxicity is highly unlikely.

In summary, our study did not find any indication for a potential of FA to induce aneuploidy. Although it cannot completely be excluded that the genotoxic action of FA may in rare cases also lead to chromosome malsegregation, this MOA cannot explain the findings reported for cultured myeloid progenitor cells from humans occupationally exposed to FA (8). The increased frequency of aneuploid cells is obviously not causally related to FA exposure and cannot be used as a plausible mechanism for the induction of leukaemia by FA. The reliability and plausibility of the results presented by Zhang et al. (8) were already critically commented (9) and the results presented here seem to exclude a significant aneugenic activity of FA.

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