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Genotoxic effects induced by formaldehyde in human blood and implications for the interpretation of biomonitoring studies

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Formaldehyde (FA) was tested for its genotoxicity in human blood cultures. We treated blood samples at the start of the culture to follow FA-induced DNA damage (DNA–protein crosslinks, DPX), its repair and its genetic consequences in form of sister chromatid exchanges (SCE) and micronuclei (MN). Our results clearly indicate that DPX (determined by the comet assay) are induced at FA concentrations of ≥25 μM. DPX induced by FA concentrations up to 100 μM are completely removed before lymphocytes start to replicate. SCE are induced at concentrations >100 μM parallel to the induction of cytotoxicity (measured as reduction of the replication index). MN were not induced by FA concentrations up to 250 μM (the highest analyzable concentration) added at the start of the blood cultures in the cytokinesis-block micronucleus (CBMN) test. FA-induced cytotoxicity (measured as reduction of the nuclear division index) possibly prevented division of damaged cells. MN were only significantly induced in human blood when proliferating cells were exposed to FA during the last cell cycle before preparation. Several human biomonitoring studies reported increased frequencies of SCE and MN in lymphocytes of subjects exposed to FA. Our results characterize the genotoxic potential of FA in cultured lymphocytes and lead to the conclusion that cytogenetic effects of FA are very unlikely to occur in blood cultures of FA-exposed subjects.

Introduction

Formaldehyde (FA) is an important industrial compound but it is also a naturally occurring biological compound that is present in all cells, tissues and body fluids. Many studies have demonstrated a genotoxic potential of FA *in vitro* (1,2). FA-induced various genotoxic effects in proliferating cultured mammalian cell lines (3–6) and in human whole blood cultures (7–10). It is generally accepted that the primary DNA alterations induced by FA are DNA–protein crosslinks (DPX). DPX can be induced in proliferating and non-proliferating cells. They are removed by spontaneous hydrolysis and active DNA repair in different cell types with half-lives between 12 and 18 h (11). In proliferating cells, unrepaired DPX can arrest DNA replication and lead to the induction of other genotoxic effects such as sister chromatid exchanges (SCE) (3,7,8). Incomplete repair of DPX can lead to the formation of mutations (12). Chromosomal effects such as chromosome aberrations and micronuclei (MN) seem to be most efficiently induced (3,6).

Induction of DPX, SCE, chromosome aberrations and MN has also been reported in human biomonitoring studies (13–23). In cultured peripheral blood of human subjects exposed to FA increased genotoxic effects were measured in a few studies but not in others. These studies have been critically reviewed and the reported genotoxic effects were considered to be implausible because systemic distribution of inhaled FA is unlikely (24). A recent assessment of the genotoxicity of FA by the German Federal Institute for Risk Assessment (BfR) came to the conclusion that 'there is no sufficient evidence to reject the plausible assumption that FA does not induce systemic genotoxicity in man' (2). It was noted that none of the studies (positive or negative) was fully reliable because of shortcomings in the study design (e.g. insufficient definition of the exposure) and/or the survey and evaluation of the data.

FA-induced genotoxic effects in blood cultures of human subjects exposed to FA can only occur when the blood investigated carries increased FA concentrations or increased amounts of DPX at the time of blood sampling. The amount of DPX can be indirectly determined in blood samples by various methods such as the K-SDS method or the comet assay (10,13,15). DPX need to persist until replication of lymphocytes to induce SCE or cause DNA-strand breaks (e.g. because of incomplete repair) leading to the occurrence of chromosomal aberrations and MN in the course of the next mitosis.

Because SCE and MN (in binucleated cells following the CytB protocol) are formed during the cultivation of lymphocytes we systematically investigated the induction and removal of FA-induced DPX in human blood samples *ex vivo* and tried to define the conditions that are necessary for the induction of cytogenetic effects as a consequence of FA exposure. Our results obtained with this approach support the assumption that systemic genotoxic effects of FA measured with cytogentic methods in human biomonitoring lack plausibility.

Materials and methods

Blood samples

Heparinized blood samples were obtained by venepuncture from healthy young volunteers. Freshly collected blood was diluted in chromosome medium for the genotoxicity tests. Cell culture media and ingredients were obtained from Invitrogen (Karlsruhe, Germany). If not specifically indicated, the chemicals used in these experiments were purchased from Sigma (Munich, Germany). Agarose (MEEO) was supplied by Roth (Karlsruhe, Germany) and low melting agarose (LMA, SeaPlaque) was from Biozym (Hameln, Germany). Formaldehyde (FA; CAS No. 50-00-0, 16% ultrapure, methanol free) was bought from Polysciences, Inc., Warrington, PA, USA. FA was diluted in Hank's solution immediately before use.

Comet assay

Aliquots of 5 μl heparinized freshly collected whole blood were mixed with 120 μl low melting agarose (0.5% in phosphate-buffered saline; PBS) and added to microscope slides (with frosted ends), which had been covered with a bottom layer of 1.5% agarose. Slides were lysed (pH 10; 4°C) and processed as described previously (25) using a time of alkali denaturation of 25 min and electrophoresis (0.86 V/cm) of 25 min at a pH >13 (4°C). Slides were coded

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and stained with ethidium bromide. Images of 50 randomly selected cells were measured by image analysis (Comet Assay II, Perceptive Instruments). For all experiments, we evaluated three image analysis parameters: tail migration, tail intensity and tail moment. In none of the experiments there was a significant difference among these parameters. Therefore, in accordance with our previous studies, we chose one parameter (tail moment) for the presentation of the results. Tail moment (TM) is calculated according to the formula: TM = (tail intensity/total comet intensity) × (tail centre of gravity − peak position).

For the detection of DPX, FA-treated blood cultures and controls were exposed to 2 Gy with Cs-137 γ-rays (Gammacell 2000, Nuclear Data, Germany) at 4 Gy/min. In the presence of DPX, γ-ray-induced DNA migration is reduced. The reduction of γ-ray-induced DNA migration is used as an indirect measure of DPX. Removal of DPX is determined by the cancellation of the inhibition of γ-ray-induced DNA migration when cells are irradiated at different time points after treatment with FA (3).

SCE test

SCE tests were performed according to Trenz *et al*. (26). Cultures were initiated by mixing 0.3 ml of heparinized blood with 3 ml chromosome medium 1A supplemented with 2% PHA-L (final concentration) and 10 μg/ml 5-bromodeoxyuridine (BrdUrd). Two parallel cultures were set up for each experiment and the total culture time was 72 h. Blood was treated with FA at the beginning of the culture. Colcemid (5×10^{-6} M) was added for the final 2 h. Chromosome preparation was done following standard procedures. Cells were centrifuged, resuspended in 0.4% KCl for 20 min and fixed three times in methanol:glacial acetic acid (3:1). For sister chromatid differentiation, air-dried slides were covered with Sörensen buffer (pH 6.8) and irradiated with an 8-W UV lamp (254 nm) at a distance of 15 cm for 30 min. Subsequently, slides were incubated in 2× SSC (standard saline concentration) for 30 min at 60°C and then stained with 7% Giemsa in Sörensen buffer. SCE were scored in 50 cells per sample (25 in each of the two parallel cultures) from coded slides. Toxicity was determined by scoring first division mitoses (M1), second division mitoses (M2) and third division mitoses (M3) among 100 metaphases and calculating the proliferation index (PI) according to the formula: PI = [M1 + (2 × M2) + (3 × M3)]/100.

Micronucleus test (MNT)

The MNT was performed as described previously (26). 0.3 ml blood was added to 3 ml of chromosome medium A, supplemented with 2% PHA-L and incubated at 37°C. Two parallel cultures were set up for each experiment. Cytochalasin B (CytB) was added to the cultures at a final concentration of 6 μg/ml 44 h after PHA stimulation. Cultures were harvested 24 h later, giving a total culture time of 68 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. Air-dried slides were stained with acridine orange (125 μg/ml in phosphate buffer). The frequency of MN was determined by analysing 2000 binucleated cells from coded slides (1000 from each of the parallel cultures). Toxicity was measured using the nuclear division index (NDI) which was calculated from 500 cells according to the formula: NDI = (M1 + 2M2 + 3M3 + 4M4)/*N*, where M1 − M4 indicates the number of cells with 1–4 nuclei and *N* the total number of cells scored. For the induction of MN, blood samples were treated with FA according to three different protocols.

- Protocol I: FA-treatment at the start of the cultures.
- Protocol II: FA-treatment 24 h after the start of the cultures.
- Protocol III: FA-treatment 44 h after the start of the cultures (together with the CytB).

Fluorescence in situ hybridization (FISH) analysis of MN

The origin of MN was assessed by FISH using biotin-labeled pan-centromeric chromosome paint specific for all human centromeres (Cambio, Cambridge, UK). The hybridization was carried out as previously described (26). Frozen slides were brought to room temperature and rehydrated in 2× SSC (0.3M sodium chloride, 0.03 M sodium citrate, pH 7.0) for 5 min prior to treatment with 0.1% Triton X-100 in 2× SSC for 3 min. After washing the slides in 2× SSC for 2 min the slides were fixed in 4% formaldehyde for 10 min at room temperature and washed in 2× SSC for 5 min. The slides were denaturated for 10 min in 70 % formamide, 2× SSC, pH 7.0 at 72–74°C and dehydrated in an ice-cold 70, 90 and 100% ethanol series. After air drying the slides were brought to 37°C on a warming plate. The hybridization mix was denatured for 10 min at 85°C and chilled on ice. Aliquots of 25 μl of the hybridization mix were added to the slides, the slides were covered with a 24 × 50 mm coverslip and sealed with rubber cement. Hybridization was carried out overnight at 37°C. After removing the coverslips, the slides were washed in 50% formamide/2× SSC (pH 7; 45°C) three times for 10 min, then rinsed three times in 2× SSC for 5 min at 45°C, two times for 5 min in 0.2× SSC at 60°C. The slides were collected in 4× SSC/0.1% Tween-20 before blocking in bovine serum albumin (BSA)-solution (5% BSA in 4× SSC/0.1% Tween-20) for 0.5 h. The biotin-labeled probe was detected with 150 μl FITC-Avidin (1:200) in 1% BSA for 40 min at 37°C. Then the slides were washed three times for 5 min in 4× SSC/0.1% Tween-20. The signals were enhanced by biotinylated anti-avidin (1:100) and FITC-Avidin. After each detection step the slides were washed three times in 4× SSC/0.1% Tween-20. The cells were counterstained with DAPI (4,6-Diamidino-2-phenylindol)/propidium iodide and embedded in antifade solution. Slides were kept in dark and analyzed using a fluorescence microscope (Zeiss Axioplan, 100 W HBO lamp) with a triple dye filter for the detection of DAPI, propidium iodide and FITC. Depending on whether a fluorescent spot was observed inside the MN, the MN was classified as centromere-negative or centromere-positive.

Flow cytometry

For flow cytometry, 0.3 ml blood was cultured at 37°C in 3 ml chromosome medium 1A, supplemented with 2% PHA-L. At the time points indicated, cells were harvested by centrifugation and resuspended in 300 μl chromosome medium 1A. 10 μl of the cell suspension were added to 1.6 ml DAPI-staining solution (Partec CyStain), mixed thoroughly and incubated at room temperature for 1 min. This solution was analyzed with a Partec CCA flow cytometer (Partec GmbH, Münster, Germany) using the following settings: L–L 25; U–L 999; speed 3.0; gain 400 to achieve a cell counting rate of 50–80 cells/s. Each sample was analyzed twice and all experiments were performed five times with blood from five different donors.

Statistical analysis

Pre-experiments were performed with the comet assay, the SCE test and the MNT to define the FA concentrations for the study. The main experiments (shown in Figures 1, 2 and 4–7) were then performed three times under the same conditions with blood from different donors. Differences between mean values of the three experiments were tested for significance using Student's *t*-test. A statistically significant difference was set at $P < 0.05$.

Results

Figure 1 summarizes the induction of DPX by FA in human blood samples (mean of three experiments with blood from different donors). FA-induced a concentration-related reduction in gamma radiation induced DNA migration. A significant difference was obtained with FA concentrations of 25 μM and higher. DPX induced in blood samples at the start of the culture are removed in time (Figure 2) and 8 h after the start of the culture, DPX induced by 100 μM FA are completely removed while a portion of DPX induced by higher FA concentrations (200 and 300 μM) still persists after 24 h.

Figure 3 shows the results of the flow cytometric analysis of five blood cultures from different donors. It can be seen that stimulated lymphocytes start to enter S-phase later than 24 h after the start of the blood cultures. The percentage of cells in S-phase then increases steadily.

SCE were significantly induced by FA in human blood cultures treated at the start of the culture at a FA concentration of 200 μM (Figure 4A). However, this treatment strongly reduced the proliferation index of the blood cultures indicating a clear cytotoxic effect (Figure 4B). Figure 4B also depicts that cytotoxicity (reduction in the PI) is already seen at 100 μM FA. Thus, it can be concluded from these experiments that FA-induced cytotoxic effects occur in parallel or even precede the genotoxic effect in the SCE test under these experimental conditions.

Figures 5–7 summarize the results of the MNT according to Protocols I, II and III, respectively. Treatment of blood samples at the start of the culture (Protocol I) did not lead to increased MN frequencies in binucleated cells (Figure 5A). FA concentrations up to 250 μM could be tested which already caused strong cytotoxic effects measured as a reduction in the NDI (Figure 5B). In one out of the three experiments only 1349 cells (419 and 930 cells from each culture) were scored. Higher

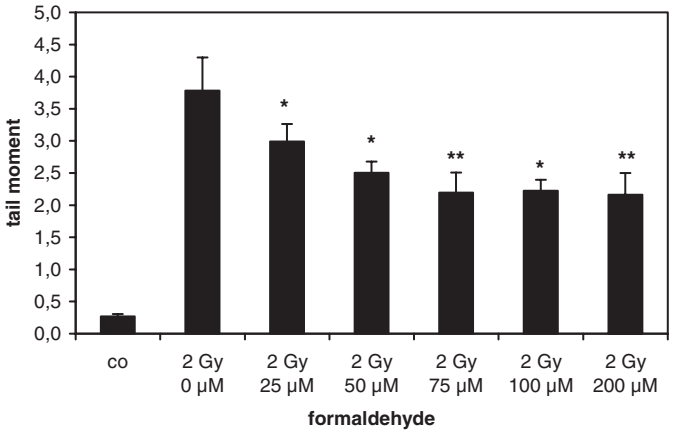

Fig. 1. Induction of DPX by formaldehyde (FA) in human whole blood cultures. Reduction of gamma ray (2 Gy) induced DNA migration (TM) by increasing FA concentrations. Mean ± SEM of three independent tests with blood from different donors.*$P < 0.05$; **$P < 0.01$.

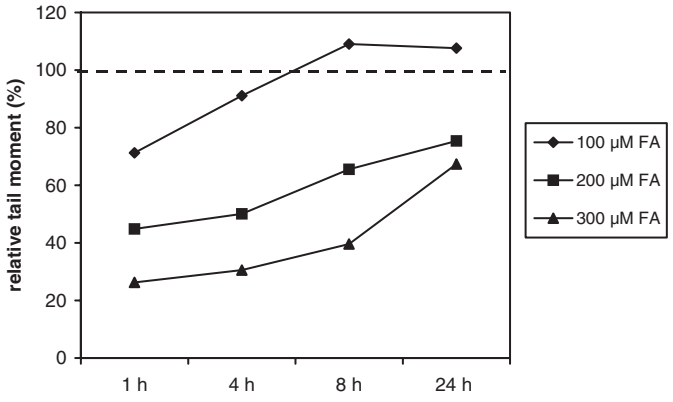

Fig. 2. Removal of FA—induced DPX in human blood cultures in time. Cancellation of the reduction of gamma-ray-induced DNA migration (relative TM; effect of gamma radiation alone corresponds to 100 %). Mean of three independent tests with blood from different donors.

concentrations totally inhibited proliferation of lymphocytes. When blood cultures were treated with FA 24 h after the start of the cultures (Protocol II) FA concentrations up to 400 µM could be tested but no significant induction of MN was measured (Figure 6A). In the experiments with 400 µM <1000 could be scored in the four cultures of two experiments and the figure is based on a total of 3812 cells. Figure 6B clearly indicates that the NDI is strongly reduced under these experimental conditions. The use of 500 µM FA led to a complete failure of two out of the three tests. The results of the experiments according to Protocol II suggest that FA induces a strong cytotoxic effect under these experimental conditions but no significant induction of MN. When blood cultures were treated with FA according to Protocol III, i.e. 44 h after the start of the cultures, a clear and concentration-related induction of MN is observed (Figure 7A). A statistically significant induction of MN is measured at concentrations of 300 µM and higher. In the experiments with 400 µM FA <1000 binucleated cells (between 516 and 963) were scored in five out of the six cultures (a total of 4482 cells). Reduction in the NDI as an indicator of cytotoxicity parallels the induction of MN (Figure 7B).

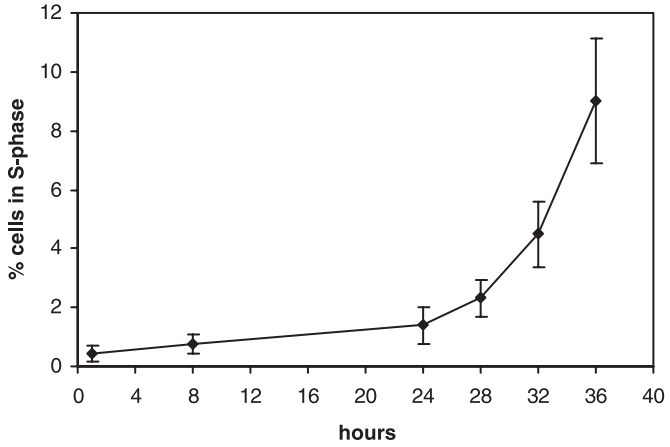

Fig. 3. Flow cytometry of whole blood at different time points after PHA stimulation. Percentage of lymphocytes entering S-phase. Mean ± SEM of five independent tests with blood from different donors.

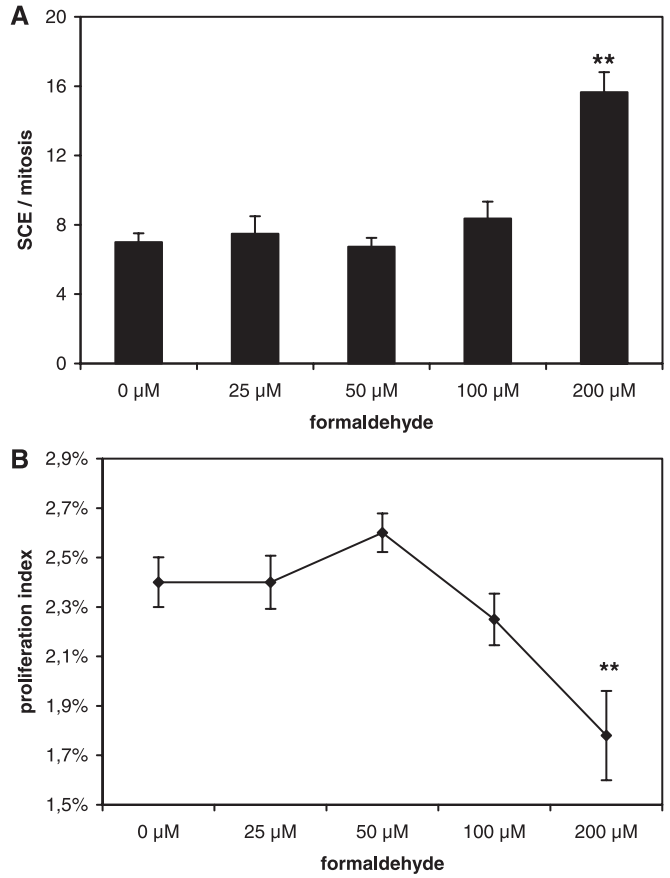

Fig. 4. Induction of SCE (**A**) and reduction of the PI (**B**) in human whole blood cultures treated with FA at the start of the cultures. Mean ± SEM of three independent tests with blood from different donors.**$P < 0.01$.

Two further experiments were performed according to Protocol III and cultures were treated with 350 µM FA. Slides were analysed by FISH to differentiate between a clastogenic effect (centromere-negative MN) and a aneugenic effect (centromere-positive MN) in the induction of MN. 81% of the analyzed MN in binuceated cells (119 out of 147) were centromere-negative and 19% were centromere-positive. In untreated cultures (negative control) the portion of

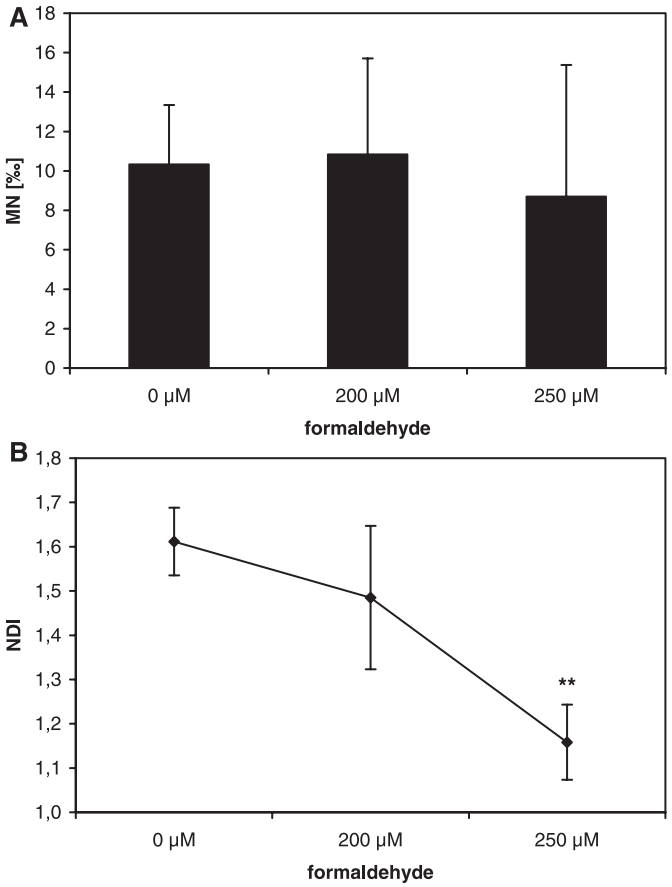

Fig. 5. Induction of MN (**A**) and reduction of the NDI (**B**) in human whole blood cultures treated with FA at the start of the cultures. Mean ± SEM of three independent tests with blood from different donors. $^{**}P < 0.01$.

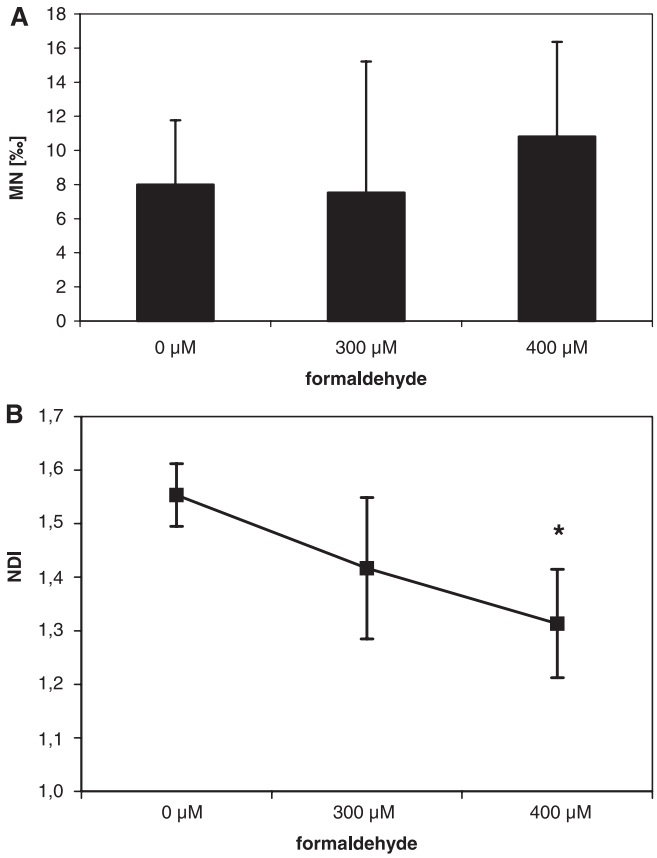

Fig. 6. Induction of MN (**A**) and reduction of the NDI (**B**) in human whole blood cultures treated with FA 24 h after the start of the cultures. Mean ± SEM of three independent tests with blood from different donors. $^{*}P < 0.05$.

centromere-negative MN was 55% (36 out of 65) and in blood cultures irradiated with 2 Gy gamma radiation (positive control for a clastogenic effect) the portion of centromere-negative MN was 92% (146 out of 159).

Discussion

Our results clearly indicate that FA induces DPX in human blood cells and that FA-induced DPX can lead to the formation of SCE and MN under certain conditions. DPX are detected by the comet assay after treatment with 25 µM FA. A similar sensitivity of the comet assay for the detection of FA-induced DPX has been reported for other mammalian cell lines (3,6,27). It should be mentioned that the actual FA concentration in the cell nucleus is much lower due to the volatility of the compound and its high reactivity with components of the medium, the cell membranes and the cytoplasm. Using the potassium-sodium dodecyl sulfate (K-SDS) assay (i.e. measuring DPX induction as the extent of DNA associated with protein after the protein is precipitated using K-SDS), Shaham *et al*. (28) reported a detection limit for FA-induced DPX of <1 µM and measured increased amounts of DPX in blood of humans occupationally exposed to FA (13,15). However, comparative investigations indicated a similar sensitivity of the comet assay and the K-SDS assay in the range of 25–50 µM (3,27) and the reliability of the results published by Shaham *et al*. (13,15,28) has been questioned (2,24,29).

However, various groups reported the induction of cytogenetic effects (SCE, chromosome aberrations and MN) in blood cultures from human subjects exposed to FA by inhalation (13,14,16–23). Although conflicting results exist and a critical assessment of these studies (positives and negatives) is partly hampered by the incomplete information on exposure and missing details of the study design and the methodology, the positive findings pose a matter of concern for the evaluation of the genotoxicity of FA.

After exposure of human subjects to FA *in vivo* SCE and MN in binucleated cells (CytB method) only develop during the cultivation period of the blood samples as a consequence of FA-induced DPX. We therefore tried in an *ex vivo* approach to get information on the amount of DPX present at the start of the culture (i. e. the time of blood sampling in biomonitoring studies) that is necessary to induce cytogenetic effects in cultured blood samples. We can show that DPX are removed from blood cells during the culture period in accordance with previously published data (11). SCE are induced when DPX persist until S-phase of stimulated lymphocytes. Actually, at a concentration of 200 µM, a significant amount of DPX persisted and SCE were induced. Induction of SCE was accompanied by a strong cytotoxic effect which actually seems to occur at lower concentrations than the genotoxic effect. Although it can be assumed that DPX are also a cause for the cytotoxic effect, FA-induced cytotoxicity may also be based on additional mechanisms (e.g. reactions with cellular proteins). Taken together, these experiments suggest that the induction of SCE by FA in human blood cultures requires high and

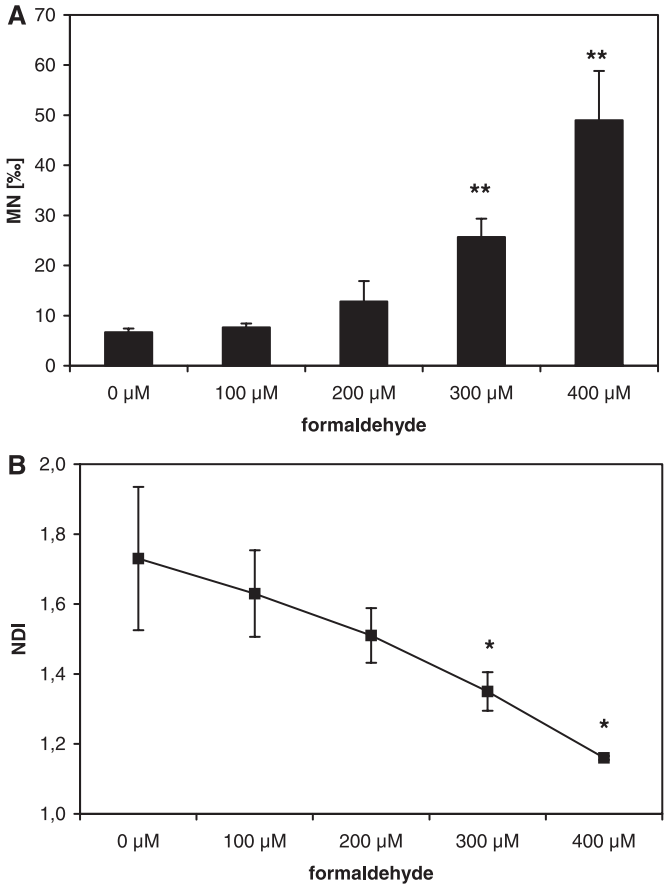

Fig. 7. Induction of MN (**A**) and reduction of the NDI (**B**) in human whole blood cultures treated with FA 44 h after the start of the cultures. Mean ± SEM of three independent tests with blood from different donors. *$P < 0.05$; **$P < 0.01$.

cytotoxic FA concentrations (200 µM) that produce enough DPX to persist until replication. Such a high amount of DPX cannot be expected in blood of humans occupationally exposed to FA. Therefore, the reported effects in the SCE test (13,14,16–18) are most likely not related to the FA exposure. In experimental animals (rats and Rhesus monkeys) no increased blood concentrations of FA were measured after exposure to high concentrations of FA. Furthermore, DPX were not increased in the bone marrow of normal and GSH-depleted rats (24). One might argue that in humans occupationally exposed to FA over several years, DPX accumulate in time in peripheral blood cells and high levels of DPX can be reached without accompanying cytotoxic effects. However, half-life of peripheral lymphocytes is limited (~3–6 months) and DPX are constantly removed by hydrolysis and DNA repair (11). Experiments with rats indicated that DPX did not accumulate in the nasal respiratory mucosa of rats exposed for 12 weeks to FA at airborne concentrations of 6 or 10 p.p.m. (30). At present, there is no evidence for accumulation of DPX in the *in vivo* situation in peripheral lymphocytes. Even if FA could cause DNA damage in some long-lived lymphocytes or in stem cells *in vivo* such effects in individual cells would not lead to a positive result in a standard cytogenetic test.

FA did not induce MN in human blood cultures treated at the start of the culture. It is likely that low concentrations did not induce enough DPX to induce MN and high concentrations prevented damaged cells to pass through a mitosis under these experimental conditions. Because FA is known to be a potent inducer of MN in other cell systems, we further investigated its MN-inducing potential in blood cultures. The draft OECD guideline (487) for the *in vitro* MNT suggests to add the test substance 24 h after the start of the blood cultures (to add CytB 20 h later and to prepare the slides 72 h after the start of the cultures). Using this approach (Protocol II) we were able to test higher FA concentrations (up to 400 µM) but did not measure a significant increase in the MN frequency. Again, considerable cytotoxicity was obvious under these test conditions. The OECD draft guideline for the *in vitro* MNT recommends to test substances with an equivocal or negative response with a modified protocol and to add the test compound (together with the CytB) 48 h after the start of the blood culture. This protocol is expected to be most sensitive because proliferating lymphocytes are treated during the last cell cycle before preparation. Applying this protocol (Protocol III), we could demonstrate that FA induces MN in human whole blood cultures when added at this late time point (i. e. treating the proliferating cells during their last cell cycle before preparation). Nevertheless, cytotoxic effects were obvious under these experimental conditions as well. Taken together, these experiments clearly indicate that FA is a weak inducer of MN in human blood cultures and that the induction of MN in human blood cultures requires specific conditions. Positive effects are only obtained when high amounts of DPX are directly induced in proliferating cells and MN are analysed after one mitosis. This is in agreement with MNT results from proliferating permanent cell lines (3,5). Blood cells with increased amounts of DPX at the start of the cell culture are either able to remove/repair induced DPX or are too heavily damaged to be detected as binucleated cells with induced MN in the standard CBMN. Consequently, increased MN frequencies measured in blood cultures from FA-exposed subjects (20,23) are most likely not related to the FA exposure. This conclusion is supported by an experimental cytogenetic study with lymphocytes from rat following FA inhalation. Exposure to FA concentrations up to 15 p.p.m. for 6 h/day for 5 days did not cause an increase in either SCE frequency in cultured lymphocytes or in the number of metaphases displaying chromosome aberrations (31).

Because a recent human biomonitoring study reported increased frequencies of centromere-positive MN (i. e. induction of aneuploidy) in workers exposed to FA (20) we also characterized MN induced by FA in blood cultures by FISH. Our results show that the majority of FA-induced MN are centromere-negative indicating a clastogenic mode of action. The portion of centromere-negative MN is similar to the effect induced by γ-rays and the results obtained for γ-rays (positive control) and for untreated blood cultures (negative control) are in accordance with our previously published data (26). A clastogenic effect of FA on human blood cultures is in agreement with the known clastogenic activity of FA in other *in vitro* studies (9,32). Aneugenic effects are usually thresholded and require high substance concentrations in the target cells. The reported aneugenic effect in the MNT in a biomonitoring study (20) is even less likely than a clastogenic effect and is most likely not related to the assumed FA exposure.

A further conclusion with regard to the assessment of biomonitoring studies after FA exposure can be drawn from our results. FA exposure of blood cells and increased DPX levels at the start of the blood culture do more efficiently lead

to an induction of SCE than an induction of MN. Therefore, increased MN frequencies in the absence of increased SCE frequencies in blood cultures from FA-exposed subjects (19) suggest that the effects are not related to FA exposure. The same may hold true for increased frequencies of chromosome aberrations without increased SCE frequencies in the same blood samples (21,22).

In summary, our systematic investigations on the induction and removal of DPX in FA-treated blood and their relationship to the formation of SCE and MN demonstrate that cytogenetic effects can be induced after exposure to FA under specific conditions. However, cytogenetic effects in blood cultures of humans exposed to FA are unlikely to occur because these conditions are not met.

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