Micronuclei are induced in rat spermatids in vitro by 1,2,3,4-diepoxoybutane but not by 1,2-epoxy-3-butene and 1,2-dihydroxy-3,4-epoxybutane

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The genotoxic effects of three 1,3-butadiene metabolites, 1,2-epoxy-3-butene (monepoxide, EB), 1,2,3,4-diepoxo- butane (diepoxide, DEB) and 1,2-dihydroxy-3,4-epoxybutane (dielepoxide, DiolEB), on male rat germ cells were studied by the meiotic micronucleus method in vitro. Seminiferous tubular segments from stages XII to XIII containing late pachytene-diakinetic spermatocytes were cultivated in the presence of the test chemical for 4 days. During the culture, spermatocytes passed through meiotic divisions and developed into early spermatids in which micronuclei could be scored. DEB was found to be a very potent micronuclear inducer in rat meiosis. All concentrations tested (5–20 μM) were able to cause a statistically significantly higher frequency of micronuclei (P < 0.05) compared with controls and a linear dose-dependent trend for micronuclear induction was seen (P < 0.01). However, EB and DiolEB caused no increase in micronuclear frequencies in spermatids at the concentrations tested (100–1000 μM for EB and 10–100 μM for DiolEB) and at higher concentrations cytotoxic effects were seen upon dividing cells causing a significant reduction in the number of spermatids. According to these results DEB is the most genotoxic butadiene metabolite in rat germ cells during meiosis.

Introduction

1,3-butadiene (BD) is an industrial chemical which is mainly used in the production of polymers in rubber industry but is also found in cigarette smoke and in the combustion products of fossil fuels. Inhaled BD is metabolized in the body to epoxide intermediates, which are responsible for its mutagenic and carcinogenic effects (de Meester 1988). 1,2-epoxy-3-butene (monepoxide, EB) is the first epoxide intermediate of BD, produced through oxidation by cytochrome P450. The EB can be further oxidized to 1,2,3,4-diepoxoybutane (diepoxide, DEB) by cytochrome P450. 1,2-dihydroxy-3,4-epoxybutane (dielepoxide, DiolEB) is a third metabolite of BD which may also have some mutagenic or carcinogenic potential. It can be produced by two different routes; either from DEB via hydroxylation by epoxide hydrolase or from EB via hydroxylation and oxidation by epoxide hydrolase and cytochrome P450 respectively (Malvoisin and Roberfroid, 1982).

The genotoxicity of BD has been tested in somatic cells both in mice and in rats. In mouse bone marrow cells, a clear induction of sister chromatid exchange and chromosomal aberrations and an increase in micronuclei in poly- or normochromatid erythrocytes have been observed after BD inhalation (Cunningham et al., 1986; Irons et al., 1987; Tice et al., 1987; Adler et al., 1994; Autio et al., 1994). However, the rat seems to be less sensitive to the clastogenic action of inhaled BD than the mouse, which is mainly explained by differences in BD metabolism between these two species (Cunningham et al., 1986; Autio et al., 1994). Analysis of mutations at the HPRT locus in splenic T-cells of B6C3F1 mice (Cochrane and Skopek, 1994) and evaluation of the lacZ-mutation frequency of transgenic mice carrying the lacZ transgene (Recio et al., 1992) demonstrated that inhaled BD is a somatic cell mutagen. Studies of the genotoxicity of different butadiene metabolites showed that both EB and DEB can cause micronuclei and sister chromatid exchanges in somatic cells of different species (Conner et al., 1983; Sharief et al., 1986; Sasiadek et al., 1991a,b; Xiao and Tates, 1995) but DEB is a stronger mutagen than EB (Xiao and Tates, 1995). EB, DEB and DiolEB are all able to induce mutations at the TK and HPRT loci of human TK cells and mice splenic T-cells in vitro but DEB exhibits its activity at a 100-fold lower concentrations than EB and DiolEB (Cochrane and Skopek 1993; Cochrane and Skopek, 1994).

In germ cells, BD inhalation is able to cause sperm head abnormalities (Morrissey et al., 1990), heritable translocations (Adler et al., 1995) and dominant lethal mutations in late spermatids and spermatozoa of mice (Hackett et al., 1988; Morrissey et al., 1990; Anderson et al., 1993; Adler et al., 1994). In addition, in the mouse spermatid micronucleus assay in vivo clastogenic effects of BD inhalation in preleptotene spermatocytes have been observed (Xiao and Tates, 1995). BD metabolites, EB and DEB were also able to cause meiotic micronuclei both in mice and in rats (Xiao and Tates et al., 1995) in vivo and the damage could be induced both in early and late meiotic stages of spermatocytes.

The aim of this study was to evaluate the genotoxicity of three BD metabolites EB, DEB and DiolEB during the male rat meiosis in vitro. The usefulness of this method for testing of potential germ cell mutagens is also discussed.

Materials and methods

Animals

Adult, male Sprague-Dawley rats aged 2–3 months were used. They were killed by CO2 and cervical dislocation. One rat was used per one set of cultures consisting of 12–15 seminiferous tubular segments treated with different concentrations of the chemical studied. Altogether five rats per chemical were used.

Separation of defined seminiferous tubule segments

Seminiferous tubule segments were separated as described earlier (Sjöblom et al., 1994). Briefly, testes were removed and segments of seminiferous tubules were isolated by transillumination-assisted microdissection in testis isolation medium (TIM). Using phase contrast microscopy of live cell squashes, stages XII and XIII of the epithelial cycle containing late pachyneme and diakinetic spermatocytes just going to start meiotic divisions were identified. Segments of 2 mm in length were separated from these stages under sterile conditions for culture.

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Tissue culture

Segments of seminiferous tubules were cultured for 4 days in 100 µl of Ham's F12/Dulbecco's minimal essential medium (DMEM) 1:1 (Flow Laboratories, Irvine, UK) in 96-well culture plates (Nunclon, A/S Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO₂ in air at 34°C. One set of cultures contained altogether 12 tubule segments including tubules treated with different concentrations of test chemical (three tubules per treatment) and three untreated tubules. DEB (CAS No. 938-19-0, purity 97%; Aldrich-Chemie, Steinheim, Germany) was dissolved in the culture medium at concentrations of 5, 10, and 20 µM before the initiation of the culture. EB (CAS No. 930-22-3, purity 98%; Aldrich-Chemie) was first dissolved in ethanol and then in the culture medium at concentrations of 100, 500 and 1000 µM. Each of these different dilutions and the control contained 0.1% ethanol. DioleEB (purity >92%) was diluted in the culture medium at the concentrations of 10, 50, and 100 µM. DioleEB was synthesized by Drs Kimmo Peltonen and Constantin Neagu at the Finnish Institute of Occupational Health and at the Department of Chemistry, University of Helsinki, Finland. Etoposide (Delta West Pty Ltd, Bentley, Australia) 1 µM was used as a positive control and was diluted directly in culture medium. The medium with and without added chemicals was not changed during the 4 days of culture.

Scoring of micronuclei

During the culture, late pachytene and diakinesis spermatocytes passed through meiotic divisions and developed into round spermatids. After the culture, squash preparations were made from the tubule segments by squeezing them carefully between a glass slide and a coverslip, freezing them in liquid nitrogen and fixing them in acetic acid-ethanol (1:3) after the coverslips were removed. The slides were stained with Hoechst 33258 fluorescent dye and mounted with Fluoromount (Onola, Espoo, Finland). The slides were checked for technical quality and 10 slides per chemical concentration were selected for micronuclei scoring. Slides were coded and usually scored SPTs = spermatids.

Statistical analysis

Statistical calculations were based on Tukey studentized range method performed by program 7D in BMDP statistical program package (BMDP Statistical Software, Los Angeles, USA). Linearity of the decrease at frequency of micronuclei after DEB treatment was studied by polynomial regression analysis by the 5R program of BMDP. A square root transformation of the frequencies of micronuclei was made before testing to equalize the variances between different treatment groups.

Results

The effects of DEB, EB and DioleEB on the frequency of micronuclei in newly formed spermatids are shown in Table I. DEB caused a significant increase in the frequency of micronuclei at all the concentrations tested. The control level of micronuclei was 2.8 per 1000 cells and micronuclei increased in a dose-dependent manner from 6.4 with 5 µM through 12.4 (10 µM DEB) to 19.0 with 20 µM concentration of DEB. Over this concentration range, a statistically significant linear trend for micronucleus induction was seen (P < 0.01). Higher concentrations of DEB (40, 80 and 160 µM) were also tested in pilot experiments but they showed arrest and killing of dividing cells, observed by brightly phase-contrasting spermatocytes and by a significantly reduced number of spermatids in phase contrast microscopic evaluation after culture.

Germ cell mutagenicity of EB was studied at concentrations of 100, 500 and 1000 µmol/l which did not cause any increase in micronucleus formation in early spermatids. The control level in these experiments with 0.1% ethanol was 2.2 micronuclei per 1000 spermatids and EB treated seminiferous tubule segments showed 2.7 (100 µM), 1.8 (500 µM) and 2.6 (1000 µM) micronuclei per 1000 spermatids. The highest concentration tested (1000 µM) was slightly cytotoxic and still higher concentrations (e.g. 5000 µM) of EB showed very severe cytotoxicity.

DioleEB did not cause any increase in the number of micronuclei at the concentrations tested. The control level in this case was 2.0 per 1000 cells and micronucleus frequencies of 2.2, 1.5 and 1.6 per 1000 spermatids were obtained with 10, 50 and 100 µM concentrations of DioleEB. No or very mild cytotoxicity was seen at these concentrations but at higher (e.g. 500 µM) concentrations, in preliminary studies, severe killing of spermatocytes during the divisions was seen. Etoposide (1 µM) which was used as a positive control, induced a micronuclear frequency of 4.7% which was statistically significantly higher than in the control (P < 0.01) and consistent with our earlier observations (4.12%, Sjöblom et al., 1994).

Discussion

Our results indicate that DEB is able to cause a dose-dependent increase in the frequency of micronuclei in late spermatocytes during meiosis. This is in agreement with earlier observations of Xiao and Tates (1995) who showed that in rats DEB is a potent inducer of micronuclei both in early spermatocyte stages (in preleptotene and zygotene) and in late spermatocyte stages.

<table>
<thead>
<tr>
<th>Dose (µmol/l)</th>
<th>No. tubular segments</th>
<th>No. SPTs scored</th>
<th>No. MN scored</th>
<th>Frequency of MN/1000 SPTs</th>
<th>SEM</th>
</tr>
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<tbody>
<tr>
<td>EB 0</td>
<td>10</td>
<td>5000</td>
<td>11</td>
<td>2.20</td>
<td>0.36</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>5070</td>
<td>14</td>
<td>2.73</td>
<td>0.88</td>
</tr>
<tr>
<td>500</td>
<td>10</td>
<td>5000</td>
<td>9</td>
<td>1.80</td>
<td>0.63</td>
</tr>
<tr>
<td>1000</td>
<td>10</td>
<td>5000</td>
<td>13</td>
<td>2.60</td>
<td>0.52</td>
</tr>
<tr>
<td>DEB 0</td>
<td>10</td>
<td>5000</td>
<td>14</td>
<td>2.80</td>
<td>0.90</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>5000</td>
<td>32</td>
<td>6.40*</td>
<td>0.83</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>5000</td>
<td>62</td>
<td>12.40**</td>
<td>2.15</td>
</tr>
<tr>
<td>20</td>
<td>10</td>
<td>5000</td>
<td>95</td>
<td>19.00**</td>
<td>2.28</td>
</tr>
<tr>
<td>DioleEB 0</td>
<td>10</td>
<td>5000</td>
<td>10</td>
<td>2.00</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
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<td>5000</td>
<td>11</td>
<td>2.20</td>
<td>0.63</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>4830</td>
<td>7</td>
<td>1.50</td>
<td>0.34</td>
</tr>
<tr>
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<td>10</td>
<td>5000</td>
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</tr>
<tr>
<td>Etoposide</td>
<td>1</td>
<td>9393</td>
<td>233</td>
<td>47.00**</td>
<td>4.29</td>
</tr>
</tbody>
</table>

SPTs = spermatids
MN = micronuclei
*P < 0.05
**P < 0.01

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(in diplotene and diakinesis) in vivo. Similar results have been obtained in our laboratory in rats in vivo (J.Lähdetie, unpublished). Also in mice DEB was found to be able to cause chromosomal damage in late spermatocyte stages although the effect was not as prominent as in rats (Xiao and Tates, 1995).

In splenocytes of rats and mice it has been shown by fluorescence in situ hybridization (FISH) that the majority of micronuclei induced by DEB and EB lack a centromeric DNA region suggesting that the main mechanism of micronucleus formation is chromosome breakage (Xiao et al., 1996). On the other hand, also the frequencies of micronuclei containing the centromeric signal were found to be significantly higher than in controls, suggesting that whole chromosome lagging may be induced (Xiao et al., 1996). However, there are major differences between the cell cycle of splenocytes and spermatocytes which will make it difficult to judge whether the mechanisms of micronucleus formation are similar in the two cell types.

DEB is known to produce DNA interstrand crosslinks. Other bifunctional agents with cross-linking ability such as mitomycin C or cyclophosphamide do not produce micronuclei when late meiotic G2 phase is exposed (Tates, 1992; Lähdetie, 1983a). Thus DEB must have alternative effects in meiosis. In cultured somatic cells topoisomerase II inhibitors cause chromosomal breakage in G2 phase of the cell cycle (Darroudi and Natarajan, 1986). We have observed that topoisomerase II inhibitors such as adriamycin and etosopside are extremely potent inducers of micronuclei during meiotic G2 phase both in vivo and in vitro (Lähdetie, 1983b; Toppari et al., 1986; Lähdetie et al., 1994; Sjöblom et al., 1994). Thus we can speculate that chemicals producing spermidial micronuclei after treatment of late stages of meiotic prophase, such as DEB, ethynitrosourea (Tates, 1992) or trophosphamide (West et al., 1995) might exert their effect through interaction with the topoisomerase II enzyme.

After EB treatment of cultured seminiferous tubules we were not able to see any change in the frequency of micronuclei in early spermatids. In vivo, Xiao and Tates (1995) have noticed that 40 mg EB/kg caused chromosomal damage only in early spermatocyte stages but a larger dose of 80 mg/kg affected also late spermatocyte stages (diplotene and diakinesis) of rats. In mice they saw an effect only on early stages. Results from our laboratory (J.Lähdetie, unpublished) of EB in vivo are similar to those of Xiao and Tates (1995) showing positive micronucleus induction in late spermatocyte stages of the rat. The lack of effect of EB on micronucleus formation in vitro could indicate that EB is not able to induce chromosomal damage in germ cells by itself but it rather needs oxidation to DEB by cytochrome P450 for its action. This oxidation cannot occur in microsomes of testicular cells (Sharer et al., 1992).

Also DioIEB had no effect on the micronuclear frequency in early spermatids in vitro. This result is in agreement with almost negative results of J.Grawé and J.Lähdetie (unpublished) in bone marrow cells of rat. However, in the Ames test DioIEB increased the number of revertants with and without added metabolic activation (S9-mixture, Nylund et al., unpublished) and it also induced mutations at the TK and HPRT loci in TK6 human lymphoblastoid cells (Cochrane and Skopek, 1994). In addition, slight induction of spermatid micronuclei in vivo was observed in our laboratory (J.Lähdetie, unpublished). Therefore further studies are needed to conclude whether DioIEB is a significant mutagenic metabolite of BD.

In our study DioIEB was assayed at the concentrations of 10–100 μM while EB was tested at the concentrations of 100–1000 μM due to the limitations by toxicity at higher doses. In contrast, the study of Cochrane and Skopek (1994) EB caused toxic effects at lower concentrations than DioIEB. This higher toxic activity of DioIEB in our study could be due to the higher water solubility of DioIEB or to the volatility of EB. There may also be differences in the detoxification of these BD metabolites by glutathione-S-transferase and by epoxide hydrolase between rat testis and human somatic cells.

Our results are in agreement with earlier studies with somatic and germ cells of different mammals showing differences in the mutagenicity of these three BD metabolites with DEB as the most mutagenic BD metabolite (Sasiadek et al., 1991a,b; Cochrane and Skopek, 1994; Xiao and Tates, 1995). Also in bacterial mutagenicity assays DEB has been found to be more mutagenic than EB (de Meester, 1988) which is due to the double epoxide structure of DEB. According to these studies DEB may account for most of the genotoxic effects of BD and thus the amount of BD metabolized to DEB mainly determines the degree of mutagenic effect after BD exposure. Several studies have shown that although BD metabolism is qualitatively similar between different species there are large quantitative differences in the formation of BD oxides and detoxification of BD (Bond et al., 1986; Csanády et al., 1992; Sharer et al., 1992; Johanson and Filser, 1993; Kohn and Melnick, 1993; Bechtold et al., 1994; Thornton-Manning et al., 1995). Differences between BD metabolism in rat, mouse and human must be further clarified before it is possible to do reliable estimations of human genetic risks.

In conclusion, these results show that DEB is a strong inducer of micronuclei during meiotic divisions and can cause genetic damage in germ cells of the rat. Two other BD metabolites, EB and DioIEB did not induce any micronuclei in early spermatids in this assay. However, there are still very few results of the genotoxicity of the various BD metabolites in germ cells of different laboratory animals.

The meiotic micronucleus method in vitro seems to be a sensitive system for testing of male germ cell mutagenicity. However, its disadvantage is that it does not give information about the mutagens that act during earlier phases of meiosis (for example during S-phase) because at present it is not possible to cultivate rat seminiferous tubules from the onset of meiosis to its completion. In addition, metabolic differences between in vivo and in vitro conditions can also affect the final result as evidenced by present results on EB.

Acknowledgements

Dr Kimmo Peltonen is greatly acknowledged by providing the BD metabolites studied. This study was performed as a part of European Union Project called 'Multi-endpoint analysis of genetic damage induced by 1,3-butadiene and its major metabolic derivatives in somatic and germinal cells of mice, rats and man. Genetic risk estimation by the parallelogram method' and was financially supported by CEC Contract EV5-V-CT94-0543, and the Finnish Ministry of Environment.

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Received on March 15, 1996; accepted on June 4, 1996.