Detection of 1,2,4-benzenetriol induced aneuploidy and microtubule disruption by fluorescence in situ hybridization and immunocytochemistry

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(Received 3 March 1993)
(Revision received 2 September 1993)
(Accepted 9 September 1993)

Keywords: 1,2,4-Benzenetriol; FISH; Aneuploidy; Microtubules; Leukemia

Summary

Fluorescence in situ hybridization (FISH) is becoming increasingly used to detect chromosomal changes in cancer cytogenetics. Here, we report its use in human HL60 cells to detect aneuploidy induced by the benzene metabolite, 1,2,4-benzenetriol (BT). Human centromeric probes specific for chromosomes 9 and 7 were used. Untreated HL60 cells were 0.72 ± 0.29% hyperdiploid for chromosome 9. Treatment with 5 μM BT increased this level 3-fold to 2.20 ± 0.87% and 50 μM increased it 4-fold to 2.96 ± 0.74%. Similar results were obtained with the chromosome 7 probe. The induction of aneuploidy by BT is therefore not chromosome-specific nor is it artifactual. Immunocytochemical staining with anti-tubulin antibodies also showed that BT disrupted microtubule organization at these concentrations. Thus, mitotic spindle disruption probably plays an important role in BT-induced aneuploidy. Trisomy and not tetrasomy accounted for the majority of the hyperdiploidy induced by BT in the two C-group chromosomes 7 and 9. Since trisomy of C-group chromosomes is commonly observed in leukemia, BT-induced aneuploidy may be involved in benzene-induced leukemia.

Benzene is an important industrial chemical and environmental pollutant that produces leukemia and other bone marrow disorders in humans (Aksoy, 1977; Yardley-Jones et al., 1991). It is metabolized primarily to phenol, which is subsequently metabolized to polyhydroxylated metabolites, namely hydroquinone (HQ), catechol (CAT) and 1,2,4-benzenetriol (BT) (Inoue et al., 1989a,b). All of these metabolites are capable of inducing various forms of genetic damage (Erexson et al., 1985; Glatt et al., 1989) including micronucleus formation (Yager et al., 1990; Robertson et al., 1991). Recently, we showed that BT, a minor metabolite of benzene with highly...
active chemical properties, significantly increased micronucleus formation in both human lymphocytes and HL60 cells (Zhang et al., 1993). Further, by using an anti-kinetochore antibody to delineate the mechanism of micronucleus formation, we observed that the majority of micronuclei formed were kinetochore-positive and therefore mainly resulted from chromosome lagging (Zhang et al., 1993). This suggested that BT could be a potent inducer of aneuploidy. In order to test this hypothesis, we have used a novel rapid method of detecting aneuploidy, namely fluorescence in situ hybridization (FISH) with chromosome-specific DNA probes (Pinkel et al., 1986; Gray and Pinkel, 1992). This method has been widely used in cancer cytogenetics (Cremer et al., 1988a,b; Hopman et al., 1991) and in cultured lymphocytes (Eastmond and Pinkel, 1990) to detect changes in both chromosome structure and chromosome number.

Aneuploidy is the loss or gain of whole chromosome(s) in the cell karyotype. Recent evidence suggests that it plays an important role in cancer progression and metastasis (Barrett et al., 1987; Fearon and Vogelstein, 1990) and many carcinogens are also aneuploidogens (Oshimura and Barrett, 1986). Aneuploidy is commonly detected in leukemia and involves changes in specific chromosomes. For example, trisomy of chromosome 8 is often found in acute myeloid leukemia (Le Beau, 1990) and trisomy of chromosome 12 is common in chronic lymphocytic leukemia (Han et al., 1983; Losada et al., 1991). Occupational exposure to benzene has been also shown to cause changes in specific chromosomes especially of the C-group (Erdogan and Aksoy, 1973; Sasiadek, 1992). Here, we have used FISH with centromeric probes specific to chromosomes 7 and 9 to determine whether BT can induce aneuploidy of these chromosomes in the human myeloid HL60 cell line. Further, in an effort to investigate the mechanism of aneuploidy induction, we have also used immunocytochemistry with anti-tubulin antibodies to determine if BT is capable of interfering with mitotic spindle assembly. In this study, we demonstrate that BT induces hyperdiploidy of chromosomes 7 and 9 and disrupts microtubules in HL60 cells. This suggests that BT-induced aneuploidy could play a role in benzene-induced leukemia.

### Materials and methods

#### Cell culture

HL60 cells, a human myeloid cell line originally derived from the peripheral blood leukocytes of a patient with acute myelocytic leukemia (Gallagher et al., 1979; Dalton et al., 1988), were obtained from American Type Culture Collection (Rockville, MD). HL60 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 50 mg/ml gentamycin sulfate (UCSF, Cell Culture Facility, San Francisco, CA) at 37 °C in a 5% CO₂ moist atmosphere and passaged twice weekly to maintain a density between 10⁵ and 10⁶ cells/ml. The cells in the plateau phase of growth were then seeded in 15-ml sterile centrifuge tubes and diluted with fresh complete medium to a density of 0.5 × 10⁶ cells/ml (2 ml per each culture). At this starting concentration of cells, the doubling time of HL60 cells was approximately 36–40 h.

#### Chemical treatment

1,2,4-Benzentriol (99%) (Aldrich, Milwaukee, WI) and colchicine (Sigma, St. Louis, MO) were dissolved in phosphate-buffered saline (PBS, Ca²⁺ and Mg²⁺ free, pH 7.4) immediately prior to treatment. At 24 h after culture initiation, HL60 cells were treated with BT in complete medium for 24 h or 1 h and in PBS for 1 h (FISH assay). Colchicine, as a positive control, was added to HL60 cells for 24 h in the media. All treatments were performed in duplicate for each dose. The treated cells were then washed in PBS and harvested following 48 h of culture initiation. The treated cells were then washed in PBS and harvested following 48 h of culture initiation. The washed cells were incubated with hypotonic solution (0.075 M KCl) for 15 min at 37 °C and fixed 3 times with Carnoy’s solution (methanol: glacial acetic acid = 3:1). The fixed cells were dropped onto glass slides, allowed to air dry, and stored at −20°C under a nitrogen atmosphere.

#### In situ hybridization

Biotinylated human centromeric probe specific for chromosome 9 (classical-satellite) and centromeric cocktail probe for chromosome 7 (α-satellite) were purchased from Oncor Inc. (Gaithersburg, MD). Detailed procedures for performing fluorescent in situ hybridization with
repetitive DNA probes have been described previously (Pinkel et al., 1986; Eastmond and Pinkel, 1990). Briefly, the best spots on each slide were located and marked. Slides were immersed in 70% formamide (Fluka, Buchs, Switzerland) in 2× SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0) at 72°C for 2 min to denature the cell DNA, and then quickly removed to ice-cold 70%, 85% and 100% ethanol series to dehydrate and air dried. The chromosome probes (0.1 ng/µl) was mixed with sonicated salmon sperm carrier DNA (50 ng/µl) in Master Mix 2.1 solution (55% formamide/1× SSC/10% dextran sulfate). The probe mixture was heated at 70°C for 5 min to denature the probe DNA. After rapid removal to ice, the denatured probe was applied to prewarmed slides (10–15 µl/spot) at 37°C on a slide warmer. The slides were coverslipped, air bubbles carefully removed, and incubated overnight at 37°C in a humidified chamber.

Detection and amplification

The first post-wash was performed 3 times in 50% formamide/2× SSC and once in 2× SSC at 45°C for 5 min each followed by a second wash in 2× SSC at room temperature for 5 min. The biotinylated probe was detected using FITC conjugated avidin (Vector, Burlingame, CA) 5 µg/ml in pre-block solution (4× SSC, 0.1% Triton X-100, 0.02% sodium azide, and 5% Carnation non-fat dry milk). After 3 washes in 0.1 M phosphate buffer (pH 8.0) of 3 min each with intermittent agitation at room temperature, the hybridization signal was amplified using a biotinylated goat anti-avidin antibody (Vector, Burlingame, CA) 5 µg/ml in pre-block solution followed by a second layer of FITC–avidin. The red fluorescent dye propidium iodide (0.5 µg/ml) in a mounting medium (Vector, Burlingame, CA) was used as counterstain DNA.

Scoring procedures and criteria

The stained slides were randomized and coded prior to scoring. 1000 cells per dose from each treatment were then scored by two observers for the presence of fluorescent probe spots in each nucleus. A Nikon microscope equipped with epifluorescent illumination, a 100× oil immersion lens and a filter for fluorescein (excitation at 450–490 nm, dichroic at 510 nm, emission at 520 nm) was used. The nuclei appeared red with bright green-yellow spots indicating the hybridization regions. Both the number of interphase nuclei with 0, 1, 2, 3, 4 and ≥5 spots, and the total number of scored cells were recorded. Each observer scored exactly half of the cells (500 nuclei per dose) from each replicate. Both were trained in the same manner according to the standard scoring criteria described briefly as follows: (1) Scorable interphase nuclei were intact and separated from each other; (2) hybridization regions appeared bright and had the same homogeneous fluorescence intensity; (3) two regions were scored as two spots only if they were clearly separated, otherwise two overlapping spots or split spots were scored as one if they could not be separated by changing focus; (4) if more than 2 spots presented in a single nucleus, they appeared the same in spot size and staining intensity even with a change of focus; and (5) if a nucleus contained no hybridization regions, it was scored as a zero only when its neighboring cells were clearly stained with fluorescent spots. When cells did not follow these 5 criteria, they were recorded as unscorable. However, very few, less than 1 per 1000 nuclei in our HL60 cell preparations were unscorable. In general, over 99.8% of the interphase nuclei of HL60 cells showed 1, 2, 3 or more in situ hybridization regions in all preparations.

Microtubule staining

The organization of microtubules was assessed in HL60 cells by a new immunocytochemical method (Leung et al., 1992). HL60 cells were treated with BT or colchicine in media for 1 h, and washed twice with PBS (Ca²⁺/Mg²⁺ free, pH 7.4). They were then resuspended in a microtubule stabilizing buffer (MTSB) consisting of 0.1 M piperazine-N,N'-bis-2-ethane-sulfonic acid (PIPES), 1 mM MgSO₄, 2 mM ethylene glycolbis(β-amino-ethyl ether) (EGTA) and 2 M glyc erol, pH 6.9 (all from Sigma, St. Louis, MO). Washed cells were collected directly onto glass slides using a Cytospin-II cytocentrifuge (Shandon, Sewickley, PA) for 10 min at 600 rpm. Slides were fixed in freshly prepared 3.7% para-form-
aldehyde (Sigma, St. Louis, MO) in MTSB (pH 6.9) for 30 min at room temperature. Fixed cells were incubated with PBS and 0.1 M glycine (Sigma, St. Louis, MO) in PBS for 5 min each, then extracted with 0.3% Nonidet P-40 (Sigma, St. Louis, MO) in PBS for 10 min, and washed in PBS twice. Cells were then stained with a mouse monoclonal anti-β tubulin antibody (Sigma, St. Louis, MO) diluted 1:200 with PBS followed by a 30 min incubation at 37°C in a humidified incubator. Following 2 rinses in PBS, slides were then incubated with a 1:64 dilution with PBS of fluoresceinated goat anti-mouse IgG (Sigma, St. Louis, MO) under the same conditions. The stained slides were washed twice in PBS, mounted using a fresh antifade solution and then stored at −5°C in the dark. Microtubule integrity was qualitatively assessed by immunofluorescence microscopy using the following criteria: (1) presence or absence of microtubule staining; (2) fluorescence intensity; and (3) abnormal mitotic figures e.g. lagging chromosomes, micronuclei, monopolar or multipolar mitoses etc..

Photography
Photography was conducted using a Nikon 35-mm camera attachment with either a 5 X or 2.5 X projection lens coupled with a 100 x oil immersion lens. Since the fluorescein label rapidly fades upon long exposure of the slide to light, high ASA rating films were, therefore, used such as Kodak Ektachrome color slide film (ASA 400) or Fujicolor (ASA 1600) color print film. The black and white prints displayed in Figs. 2 and 3 were made by laser-imaging from the color slides.

Statistical analysis
To test for consistency of BT treatment replicates, one-way ANOVA (analysis of variance) was performed on transformed data. In order to stabilize the variance, the arcsine of the square root of the proportion of hyperdiploid cells was used as the transformation. To examine for trend of elevated frequency of hyperdiploidy with increasing concentrations of BT, the Chi-square Test for Trend (Binomial Trend Test) was performed on the raw data. The potential difference in frequency of hyperdiploidy at various doses versus the control was analyzed by using Fisher’s Exact Test with the allowance for Type I error adjusted appropriately. Critical values were determined using a 0.05 probability of Type I error unless otherwise stated. The “Stata” program for statistical analyses was used on a MacIntosh IIx computer.

Results

Fluorescence in situ hybridization in HL60 cells
Scoring cells stained with chromosome-specific fluorescent probes enumerates the number of cell nuclei with 0, 1, 2, 3, 4 and ≥ 5 hybridization regions (fluorescent spots) corresponding to numbers of a specific chromosome present (Eastmond and Pinkel, 1990). To determine the background frequencies of hybridization regions observed in

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total cells scored</th>
<th>Number of spots/1000 cells</th>
<th>Total hyperdiploidy per 1000 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Controls</td>
<td>10,000</td>
<td>0.2</td>
<td>99.2</td>
</tr>
<tr>
<td></td>
<td>(S.D.) a</td>
<td>(0.3)</td>
<td>(14.0)</td>
</tr>
<tr>
<td>Colchicine</td>
<td>5,000</td>
<td>0.8</td>
<td>101.8</td>
</tr>
<tr>
<td>(0.1 μM)</td>
<td>(S.D.) a</td>
<td>(1.3)</td>
<td>(13.3)</td>
</tr>
</tbody>
</table>

a Standard deviation (S.D.) of 5 separate experiments is shown in parentheses. 2000 cells each for controls and 1000 cells each for colchicine treatments were scored.

TABLE 1
BASELINE AND EFFECT OF COLCHICINE ON NUCLEAR SPOT FREQUENCIES IN HL60 CELLS USING A CENTROMERIC PROBE SPECIFIC FOR CHROMOSOME 9
untreated HL60 cells, a total of 10 000 interphase nuclei were examined after hybridization with a centromeric probe specific for chromosome 9. The frequencies of nuclei containing 0, 1, 2, 3, 4 and ≥ 5 hybridization regions for this chromosome were 0.02, 9.92, 89.34, 0.63, 0.09 and 0%, respectively (Table 1). A normal diploid cell should contain 2 copies of chromosome 9, indicated as 2 bright yellow-green hybridization spots in the cell nucleus. The frequency of apparent hypodiploidy (9.94%) was considerably higher than the hyperdiploid frequency (0.72%). The same phenomenon has been found in untreated human lymphocytes, in which the frequencies of hypodiploidy and hyperdiploidy have been reported to be 9.04% and 0.39%, respectively (Eastmond and Pinkel, 1990). This is most likely due to overlap and visual fusion of the hybridization regions from both copies of the chromosome, which leads to an artifactually-high rate of monosomy. The baseline level of hyperdiploidy in HL60 cells was significantly higher than that of lymphocytes (p < 0.01). The frequency of hypodiploidy observed in both untreated HL60 cells and lymphocytes was also statistically different. These differences are probably due to gene instability in the long-term transformed cell line.

**Colchicine as a positive control for aneuploidy induction**

Colchicine is a well-known inducer of aneuploidy. It interacts with microtubules by binding to tubulin proteins and inhibits microtubule assembly leading to mitotic arrest (Wallin et al., 1988). Colchicine was chosen as a positive control to determine whether FISH could be used as a sensitive assay in the HL60 cell line. HL60 cells were treated with 0.1 μM colchicine for 24 h in media. A total of 5000 interphase nuclei of treated HL60 cells were examined with 1000 cells from each treatment being scored. The number of hybridization regions per 1000 cells is presented as mean and standard deviation (S.D. in parentheses) in Table 1. The hyperdiploid frequency (3.04 ± 1.26%) was significantly elevated above control values (p < 0.0001), whereas the frequency of hypodiploid cells (10.26 ± 1.46%) was approximately the same as in controls (Table 1). Similar results for colchicine-induced aneuploidy, hypodiploidy (12.4%) and hyperdiploidy (5.0%), were obtained in lymphocytes by Eastmond and Pinkel (1990). Thus, colchicine induces similar levels of aneuploidy in both HL60 cells and lymphocytes. The human myeloid HL60 cell line, therefore, is a suitable model system for using FISH to detect aneuploidy-inducing agents.

**BT-induced hyperdiploidy of chromosome 9**

HL60 cells were treated with BT for 24 h in media and hybridized with DNA probe specific for chromosome 9. Identical experiments with BT treatment were repeated 5 times for statistical precision, and no significant difference in these replicates were detected by ANOVA. 5000 inter-

### TABLE 2

<table>
<thead>
<tr>
<th>[BT] μM</th>
<th>Number of domains/1000 nuclei</th>
<th>Total number of scored cells</th>
<th>Total hyperdiploidy per 1000 nuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0, 1, 2, 3, 4, ≥ 5 hyperdiploidy</td>
<td></td>
<td></td>
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<tr>
<td>0</td>
<td>5000</td>
<td>0</td>
<td>93.2 ± 18.3</td>
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<tr>
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<td>0.2 ± 0.4</td>
<td>84.4 ± 21.1</td>
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<tr>
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<td>5000</td>
<td>0</td>
<td>89.2 ± 12.4</td>
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<tr>
<td>20</td>
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<td>88.4 ± 3.4</td>
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<td>50</td>
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<td>0</td>
<td>75.4 ± 9.7</td>
</tr>
<tr>
<td>80</td>
<td>2000</td>
<td>0.5 ± 0.7</td>
<td>87.5 ± 9.2</td>
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<tr>
<td>100</td>
<td>1000</td>
<td>0</td>
<td>89</td>
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*The data presented here are the mean ± S.D. (standard deviation).*
phase cells were scored (1000 for each experiment) at each non-toxic dose of BT (0, 5, 10, 20 and 50 μM). Notable cytotoxicity as indicated by trypan blue dye exclusion was observed only after treatment with 80 and 100 μM BT in HL60 cells, when the cell viability was decreased to 32% and 15%, respectively. Only one to two thousand cells were scorable at these toxic doses. The number of hybridization domains per 1000 nuclei are shown in Table 2. BT treatment at all concentrations ranging from 5 to 100 μM resulted in a significant increase in the frequency (2.20–3.40%) of hyperdiploid cells over the control (p < 0.001).

To determine if there was a trend in frequency of hyperdiploidy as the dose of BT was increased, we categorized scored cells as either hyperdiploid or non-hyperdiploid and assessed the trend using the Binomial Trend Test. A significant trend (p < 0.001) was observed indicating that elevated hyperdiploid frequency is associated with increasing doses of BT (Fig. 1A). However, the frequency of hypodiploidy in BT treated cells was not significantly different from that found in untreated HL60 cells.

To avoid potential confounding effects of thiol-containing compounds and antioxidants, such as glutathione, ascorbate and serum, present in complete medium, HL60 cells were also treated with BT at 5–50 μM for only 1 h in PBS and harvested at 48 h after culture initiation. Comparable increases in hyperdiploid frequency were observed (Table 3). The increase (at 50 μM of BT) is statistically significant in comparison with the PBS control (p < 0.001). Surprisingly, exposure of HL60 cells to PBS, instead of media, significantly increased the background frequency of hyperdiploidy to 1.70%, which is twice that of cells in media. Thus, PBS may act as an inducer of hyperdiploidy in HL60 cells. However, treatment with BT for 1 h in medium produced only a minimal increase in hyperdiploidy, and a plateau phase was observed at increasing doses (Fig. 1B). This shows the protective effect of complete

**TABLE 3**

<table>
<thead>
<tr>
<th>[BT] μM</th>
<th>Total number of scored cells</th>
<th>Total number</th>
<th>Number of hybridization domains</th>
<th>[BT] μM</th>
<th>Total number of scored cells</th>
<th>Total number</th>
<th>Number of hybridization domains</th>
<th>Total number of scored cells</th>
<th>Total number</th>
<th>Number of hybridization domains</th>
<th>Total number of scored cells</th>
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Fig. 1. Dose–response for 1,2,4-benzenetriol-induced hyperdiploidy in HL60 cells following treatment with 5–50 μM 1,2,4-benzenetriol in complete medium for 24 h (A) and in the medium or PBS for 1 h (B). Doses are expressed as concentration×time (C × T) so that 24- and 1-h exposures can be directly compared. Fig. 1A also shows the level of hyperdiploidy from 5 separate experiments. Means (solid dots) ± standard deviation (error bars) are shown. Details of the procedures are described in Material and methods.
medium. In addition, the hyperdiploid frequency observed at 50 μM × 1 h in medium (Fig. 1B) is in agreement with the observation at this same dose (C × T) in Fig. 1A. This indicates that 24-h exposure in medium delivered a much higher dose than 1-h exposure even though the exposure level was the same. Overall, BT induced a dose-related increase in hyperdiploidy of chromosome 9 in HL60 cells independent of whether the treatment was performed in media or in PBS.

**BT-induced hyperdiploidy of chromosome 7**

In addition to the chromosome 9 classical satellite probe, the smaller sized α-satellite probe (Cocktail) specific for chromosome 7 was used. Table 4 shows the background frequencies of nuclei containing 0, 1, 2, 3 and 4 hybridization regions in untreated HL60 cells stained with the chromosome 7 probe. These were 0, 6.40, 92.90, 0.50 and 0.20%, respectively. Similar background levels (0.30, 6.60, 92.70, 0.40 and 0%) have been found in human lymphocytes using the same probe (Eastmond and Pinkel, 1990). However, the frequency of monosomy (6.40%) of chromosome 7 in control cells was significantly lower (p < 0.01) than the frequency (9.32%) of chromosome 9 (Tables 2 and 4). Similar results were also found in treated cells. This suggests that the use of the smaller sized hybridization probe decreases overlapping events which artificially increase the frequency of monosomy. Nuclear spot frequencies in HL60 cells treated with BT at non-toxic doses (5–50 μM) for 24 h in media were obtained using the α-satellite probe of chromosome 7 (Table 4). BT, again, significantly increased hyperdiploid frequencies of chromosome 7 at levels similar to that found with chromosome 9. Moreover, the increase in hyperdiploidy was mostly dependent on the increase in the frequency of 3 hybridization domains per nucleus (Table 4).

**BT-induced hyperdiploidy is primarily trisomy**

BT increased the frequency of hyperdiploidy of chromosomes 7 and 9 in HL60 cells treated with BT both in media (Tables 2 and 4) and in PBS (Table 3). The proportion of nuclei with 3 spots (trisomy) in total hyperdiploidy was consistently 2–4-fold higher than the proportion of cells with 4 hybridization regions (tetrasomy) in both control and treated cells (Table 5). Treatment with BT in PBS tended to increase the proportion of hyperdiploidy in chromosome 9 caused by trisomy. A similar pattern was also seen for treatment with BT in media, which was observed for chromosome 7 but not for chromosome 9 (Table 5). Thus, trisomy accounts for majority of the background and BT-induced hyperdiploidy observed in HL60 cells.

**BT-induced microtubule disruption**

Disruption of the mitotic spindle leads to improper chromosome segregation during mitosis, resulting in chromosome lag and eventually leading to aneuploidy. To investigate mechanisms of BT-induced aneuploidy, the effect of BT on microtubule assembly was therefore tested in HL60 cells using an immunofluorescence staining assay with anti-tubulin antibody. The normal patterns of microtubule distribution in interphase and

<table>
<thead>
<tr>
<th>[BT] μM</th>
<th>Total number of scored cells</th>
<th>Number of domains/1000 nuclei</th>
<th>Total number of hyperdiploidy per 1000 cells</th>
</tr>
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<tbody>
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<td>0</td>
<td>1000</td>
<td>0-1 2 3 4</td>
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<td>50</td>
<td>1000</td>
<td>0 43 931 24 2</td>
<td>19</td>
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TABLE 5
PROPORTION OF TRISOMY AND TETRASOMY IN TOTAL HYPERDIPLOIDY INDUCED BY 1,2,4-BENZENETRIOL IN HL60 CELLS

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<thead>
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<th>Chromosome 7</th>
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<tr>
<td></td>
<td>BT (24 h in media)</td>
<td>BT (1 h in PBS)</td>
<td>BT (24 h in media)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trisomy (%)</td>
<td>Tetrasomy (%)</td>
<td>Trisomy (%)</td>
<td>Tetrasomy (%)</td>
</tr>
<tr>
<td>0</td>
<td>73.4 ± 8.2</td>
<td>22.1 ± 8.4</td>
<td>64.7</td>
<td>35.3</td>
</tr>
<tr>
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<td>81.6 ± 18.8</td>
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<td>21.4</td>
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<td>74.8 ± 8.2</td>
<td>22.1 ± 7.7</td>
<td>95.0</td>
<td>5.0</td>
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</table>

* The data presented here are the mean ± S.D. (standard deviation).

metaphase of untreated cells are shown in Fig. 2. The full complement of cytoplasmic microtubules in the interphase cell (Fig. 2A) and spindle microtubules in the mitotic cell (Fig. 2B) was observed. Experiments on microtubule disruption by BT were performed for 1 h in complete medium. Treatment of HL60 cells with BT and colchicine resulted in decreased microtubule integrity as indicated by decreased fluorescence intensity as compared with controls (Fig. 3). At the lowest dose of BT tested (20 μM), the microtubules were intact and the fluorescence intensity

![A](image1.png)

![B](image2.png)

Fig. 2. Immunocytochemical anti-tubulin antibody staining of control HL60 cells. Microtubules are clearly visible in the interphase cell (A) and the cell undergoing mitosis (B).
slightly increased, but abnormal tripolar configurations were observed (Fig. 3A). An intermediate concentration of BT (50 μM) dramatically decreased the fluorescence staining intensity and disrupted most microtubules (Fig. 3B). At the maximal dose tested (100 μM BT), microtubules were essentially absent due to complete disruption, and only microtubule organizing centers were visible (Fig. 3C). Colchicine (1 μM), used as a positive control (Fig. 3D), produced complete

Fig. 3. Effects of 1,2,4-benzenetriol and colchicine on microtubule integrity and cytoskeletal organization in HL60 cells. The fluorescence intensity of treated HL60 cells stained with anti-tubulin antibodies was decreased as the concentration of 1,2,4-benzenetriol was increased. HL60 cells treated with 1,2,4-benzenetriol at 20 μM (A), 50 μM (B) and 100 μM (C), and treated with 1 μM colchicine as a positive control (D) are shown.
microtubule disruption in both mitotic and interphase cells. Thus, both BT and colchicine disrupt microtubule assembly in HL60 cells.

Discussion

In spite of the evidence implicating the involvement of aneuploidy and aneuploidy-inducing agents in the carcinogenic process, rapid methods for detecting aneuploidy in systems relevant to humans have not been well established (Oshimura and Barrett, 1986). Standard cytogenetic techniques are time consuming and labor intensive, require highly trained personnel, and are prone to technical artifacts. In addition, karyotypic analyses are restricted to cells and tissues from which good metaphase spreads can be obtained. In recent years, a new method, named fluorescence in situ hybridization (FISH) for detecting numerical and structural chromosomal changes in both interphase nuclei and metaphase spreads has become available (Gray and Pinkel, 1992). This methodology utilizes in situ hybridization with DNA probes specific to blocks of repetitive DNA sequences on defined regions of specific chromosomes (Willard and Waye, 1987; Pinkel et al., 1986, 1988). The visualization of chemically-modified probes involves the use of non-radioactive fluorescent antibodies. The determination of aneuploidy is performed by simply counting the number of label regions representing a particular chromosome of interest within the isolated nucleus. This method has been widely used for the molecular cytogenetic analysis of cancer cells (Cremer et al., 1988a,b; Hopman et al., 1991), but has also been used in cultured human lymphocytes (Eastmond and Pinkel, 1990) and bone marrow (Poddighe et al., 1991; Jenkins et al., 1992). Recently, our laboratory has also adapted this procedure so that it can be used in exfoliated human cells (Moore et al., 1993).

In this study, we have used FISH to determine if the benzene metabolite, 1,2,4-benzenetriol (BT), can induce aneuploidy in a model cell system, HL60 cells. These cells contain high levels of the enzyme myeloperoxidase, which activates BT (Subrahmanyam et al., 1992) and are representative of immature myeloid cells. Although the background level of aneusomy was slightly higher in HL60 cells than that in human lymphocytes, use of a positive control (colchicine) showed that it increased the frequency of hyperdiploidy in HL60 cells in a manner similar to that found in lymphocytes. Thus, HL60 cells are a suitable model for studying the aneuploidyogenic effects of BT. Further, our data show that BT induces a dose-related increase in aneuploidy of chromosomes 7 and 9 in HL60 cells.

Virtually all of the BT-induced aneuploidy in HL60 cells detected by FISH was in the form of hyperdiploidy. No BT-induced change in hypodiploidy was detected. However, no significant change in the level of hypodiploidy was observed after treatment with the known aneuploidy-inducing agent, colchicine. This is because the background level of hypodiploidy (monosomy) detected by FISH is artificially high due to probe overlap or close juxtaposition of signals. Indeed, with the larger sized classical-satellite probe of chromosome 9, the amount of overlap would be expected to be greater than that seen with the smaller α-satellite centromeric probe for chromosome 7. This most likely explains the higher frequency of monosomy detected by FISH for chromosome 9 as compared with chromosome 7 in this study. Eastmond and Pinkel (1990) have calculated that as much as 85% of the apparent monosomy detected by FISH is due to probe overlap. FISH is, therefore, unsuitable for the detection of monosomy in interphase nuclei. It is, however, a sensitive method for the detection of hyperdiploidy, and both colchicine and BT increased the level of hyperdiploidy significantly in HL60 cells.

Although an increase in hyperdiploidy of chromosomes 9 was observed after BT treatment, this could have been due to chromosomal breakage occurring within the region stained by the DNA probe. For example, the target region 9q12 for the classical-satellite chromosome 9 probe used in this study, has been reported to be susceptible to non-random chromosomal breakage by γ-rays (Dubos et al., 1978). To determine whether the effects of BT on hyperdiploidy of chromosome 9 were caused by true aneuploid events or non-random chromosome breakage, we used a smaller α-satellite probe specific to another chromosome, chromosome 7. A similar increase in hyper-
diploidy of chromosome 7 was observed in HL60 cells (Table 4) as that detected using the chromosome 9 probe (Table 2). We, therefore, conclude that the BT-induced hyperdiploidy is real and not artificial. Moreover, our results also show the effects of BT on inducing hyperdiploidy are not chromosome specific, since they are observed in both chromosome 7 and 9 probes. Chromosome 7 and 9 are, however, both C-group chromosomes which benzene has been shown to specifically affect (Erdogan and Aksoy, 1973; Sasiadek, 1992). Thus, further work is needed to determine if benzene's metabolites produce specific effects on the C-group chromosomes as compared to chromosomes from other groups.

Trisomy of group C chromosomes has also been observed in myeloid metaplasia (Sandberg et al., 1964), myeloproliferative disorders (Winkelstein et al., 1966), and in benzene-induced leukemia (Erdogan and Aksoy, 1973). Although possible mechanisms of trisomy induction are obscure, it is clear that trisomy of a chromosome with a dominant-acting gene will result in an increased expression of the gene product. Here, we have shown the majority of BT-induced hyperdiploidy of chromosome 7 and 9 in HL60 cells is in the form of trisomy. The ratio of trisomy to tetrasomy induced by BT was around 3:1 (Table 5).

The BT-induced increases in hyperdiploidy of chromosomes 7 and 9 in HL60 cells were dose-dependent and independent of whether the treatment was performed in media or in PBS (Fig. 1). The presence of complete medium was protective, however, and incubations with BT had to be much longer in media than in PBS for an effect to be observed (Fig. 1A). Interestingly, incubation of HL60 cells in PBS for 1 h elevated the baseline of hyperdiploid frequency by 2-fold, but BT still markedly increased this level. The enhanced background may be explained by our unexpected finding that microtubular structures were unstable when HL60 cells were suspended in PBS for 1 h (data not shown). Thus, PBS may act as a possible aneuploidy-inducer under these circumstances.

Since the mitotic apparatus consists predominantly of microtubules, any compound that affects microtubules might be a potential aneuploidy-inducer. We, therefore, hypothesized that the mechanism by which BT induces hyperdiploidy in HL60 cells most likely involves microtubule disruption and dysfunction. Microtubules are composed primarily of tubulin polymers which are rich in nucleophilic sulfhydryl groups important for microtubule assembly. Their integrity is required for spindle formation and proper segregation of chromosomes into the daughter nuclei during cell division. It has been shown that the polyhydroxy benzene metabolites, BT and HQ, inhibit tubulin polymerization in vitro, with BT being approximately twice as potent as HQ (Irons et al., 1981). These effects are oxygen-dependent and significantly inhibited under anaerobic conditions, showing that the oxidation of BT and HQ to their semiquinone intermediates and/or quinone metabolites must be responsible for their effects on microtubules (Irons, 1985; Pfeiffer and Irons, 1983). BT can be spontaneously (Zhang and Davison, 1990) or enzymatically (Subrahmanyam et al., 1992) oxidized to electrophilic quinone and semiquinone intermediates which may interact with nucleophilic sulfhydryls of tubulin and cause mitotic abnormalities resulting in aneuploidy. This may also explain our finding of a dose-related increase in hyperdiploidy of chromosome 9 was found in HL60 cells treated with BT 1 h in PBS, whereas a plateau phase was observed at increasing doses of BT treated 1 h in medium (Fig. 1B). The difference may be due to the presence of serum or other sulfhydryl-rich proteins and glutathione (GSH) in the medium which act as additional targets for BT's quinone metabolites.

A new immunocytochemical assay (Leung et al., 1992) was used to allow us to visualize the microtubular structure within HL60 cells and showed that BT causes disruption of the intercellular microtubular apparatus. Although BT almost completely disrupted the microtubular structure at high aneuploidogenic concentrations, it unexpectedly produced a small increase in anti-tubulin fluorescent intensity and abnormal mitotic figures, such as tripolar spindle formation, at relatively low concentrations (Fig. 3A). Similar results were obtained in another study, in which human lymphocytes were treated with other benzene metabolites, HQ and catechol alone and in
combination. These treatments decreased microtubule integrity at higher concentrations but increased fluorescent intensity at lower doses (Robertson, 1992). A previous study on BT-induced genotoxic damage also showed that BT increased micronucleus formation predominantly in the form of kinetochore-positive micronuclei, indicating whole chromosomes, in both lymphocytes and HL60 cells (Zhang et al., 1993). Since microtubule disruption is observed in HL60 cells treated with BT at the same concentrations as those which produce aneuploidy and micronuclei, we conclude that the effect of BT on microtubule disruption is most likely involved in its production of hyperdiploidy and kinetochore-positive micronuclei.

In summary, we have shown that the benzene metabolite BT is able to induce aneuploidy and microtubular disruption in human myeloid HL60 cells. The disruption of microtubules may be involved in the production of BT-induced aneuploidy. The observed aneuploidy was mainly in the form of hyperdiploidy. Specifically, BT induced hyperdiploidy of the C-group chromosomes 7 and 9. Moreover, the majority of the hyperdiploidy induced was due to trisomy. Since trisomy of C-group chromosomes is commonly found in the leukemias, we suggest that BT-induced aneuploidy could play a role in benzene-induced leukemia.

Acknowledgements

The authors are indebted to Dr. David Eastmond for his helpful comments and discussions, and to Malia D. Beaulieu for her assistance in the statistical analysis. L. Zhang is an awardee of The William and Ada Isabell Steel Memorial Graduate Scholarship from Simon Fraser University, B.C. Canada and is also a trainee of the Health Effects Component of the University of California Toxic Substances Program. This work was financially supported by NIH grants P42-ES04705 and P30-ES01896.

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