Induction of Sister Chromatid Exchanges and Cell Division Delays in Human Lymphocytes by Microsomal Activation of Benzene

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ABSTRACT

Metabolic activation of benzene by rat liver microsomes and a reduced nicotinamide adenine dinucleotide phosphate-generating system (S-9 mix) induced sister chromatid exchanges (SCEs) and cell division delays in cultured human lymphocytes. There were optimal concentrations of S-9 mix for the conversion of benzene into the active metabolites that exerted these cytotoxic effects. Reduced glutathione prevented the induction of SCEs by benzene plus S-9 mix in a dose-dependent manner. Reduced glutathione (3 mM) also prevented the induction of SCEs by catechol or hydroquinone, active metabolites of benzene and potent inducers of SCEs, strongly suggesting that glutathione did not simply inhibit the activity of S-9 mix to activate benzene but actually prevented the production of DNA lesions by the active metabolites. Pulse treatment of cells with benzene plus S-9 mix produced the largest number of SCEs when administered at 40 hr of culture (fixed at 72 hr) but did not induce SCEs when administered immediately after the beginning of culture. This indicates that induced DNA lesions that could lead to formation of SCEs are removed in time.

INTRODUCTION

Because benzene, which has a direct association with human cancer, e.g., leukemia (9, 25), is contained in gasoline (8, 22), it is one of the most widely distributed environmental pollutants. Furthermore, the annual production of benzene for industrial use has been increasing worldwide; in the United States alone, an estimated 2 million workers are potentially exposed to it (8). Thus, there is an increased public health concern for the hematotoxicity and leukemogenicity of benzene, and many reviews have appeared on the subject (2, 4, 8, 9, 21, 25). However, the mechanisms by which benzene exerts its effects are not understood.

In the body, benzene is converted metabolically into phenolic compounds, the major metabolites being phenol, catechol, and hydroquinone (4, 23, 25). A previous study (18) showed that catechol and hydroquinone induce SCEs as well as cell cycle delays in human lymphocytes in vitro. Because benzene itself is negative in the Ames Salmonella test, even with microsomal activation (4), as well as in in vitro SCE test (4, 7, 18), it has been suggested (7, 18) that the metabolites of benzene, which also interact with rat DNA (16, 26), might be responsible for its biological effects.

The present study with human lymphocytes demonstrates the mutagenic potential of benzene by means of an in vitro metabolic activating system that can produce the phenolic metabolites of benzene (5, 10, 27, 32). After metabolic activation, benzene leads to the induction of both SCEs, which is one of the most sensitive methods for detecting the effects of mutagenic carcinogens (3, 13, 15, 22, 28), and cell cycle delays. The results also imply that induced DNA lesions that lead to SCE formation are removed in time.

MATERIALS AND METHODS

Heparinized peripheral blood samples were obtained from healthy adult men. Whole blood (0.2 ml) was added to 5 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 15% fetal bovine serum and 3% phytohemagglutinin M (Grand Island Biological Co., Grand Island, N.Y.). Bromodeoxyuridine (20 µM) was also present in medium for the entire culture period. The cultures were incubated at 37° for 72 hr in complete darkness. Three hr before fixation, Colcemid (final concentration, 2 x 10⁻⁷ M) was added. The cells were then collected by centrifugation, exposed to 0.075 M KCl hypotonic solution for 5 min to spread the chromosomes and hemolyze the RBC, and fixed 3 times in methanol:acetic acid (3:1). Air-dried chromosome preparations were made, and a modification of the fluorescence-plus-Giemsa method (11) was applied to obtain harlequin chromosomes as reported previously (18, 19). Cells dividing for the first (X1), second (X2), and third or more (X3+) time in culture can be determined in such preparations (18, 19, 30). X1 cells contain chromosomes with both sister chromatids stained uniformly darkly. X2 cells contain only harlequin chromosomes with one chromatid darkly stained and its sister chromatid lightly stained, whereas X3+ cells contain some harlequin chromosomes and other chromosomes with both sister chromatids stained uniformly lightly.

Benzene (Aldrich Chemical Co., Milwaukee, Wis.) was first dissolved in serum-free culture medium to give a 25 µM concentration. Aliquots of this freshly made solution were added to the cultures to give the appropriate final concentration.

Cultures to be treated were first centrifuged, and the supernatant was removed, leaving 0.5 ml of medium which contained the pelleted cells. The cells were then resuspended in serum-free culture medium containing benzene and the metabolic activation system [Ames, S-9 mix, a rat liver microsomal suspension supplemented with an NADPH-generating system (1)]. The S-9 mix consisted of 10% (v/v) S-9 rat liver extract (Lilton Bionetics, Inc., Kensington, Md.), prepared according to the procedure described by Ames et al. (1); 8 x 10⁻³ M MgCl₂; 3.3 x 10⁻² M KCl; 5 x 10⁻³ M glucose 6-phosphate; 4 x 10⁻³ M NADP; and 1 x 10⁻⁷ M Na₂HPO₄, NaH₂PO₄ (pH 7.4). The proportion of S-9 mix in the medium is expressed as a percentage of undiluted S-9 mix; e.g., 30% S-9 mix consists of 30% undiluted S-9 mix and 70% Roswell Park Memorial Institute Tissue Culture Medium 1640 with supplements. Except as otherwise described, the cells were incubated at 37° with benzene and S-9 mix for 2 hr (40 hr after the beginning of culture) in tightly capped glass tubes in a shaking water bath. This agitation ensured even distribution of active metabolites among the cells (34). After treatment, the medium containing benzene and S-9 mix was removed by centrifugation, and the cells were washed 3 times with prewarmed complete culture medium. The cells were then resuspended in the same culture medium for further incubation.
SCEs were analyzed in 35 consecutive second-division cells for each point. Two hundred metaphase cells were scored to determine the percentage of cells in X1, X2, and X3+ divisions. The S.E. for the percentages of X1, X2, and X3+ cells was determined by the use of polynomial variances.

RESULTS

When cells from cultures exposed to benzene in the presence of metabolic activation were examined, it was found that 10% S-9 mix caused the largest increase in the number of SCEs (Table 1; Chart 1). Cells treated with benzene and 1% or even 90% S-9 mix, or in the absence of activation, showed no increase of SCEs (Chart 1). Treatment with S-9 mix alone also gave an increase in the frequency of SCEs when added at concentrations of more than 10% (Table 1). The data indicate that 10% S-9 mix is the optimal concentration for converting benzene into the active metabolites that might be responsible for the induction of SCEs. Benzene induced SCEs in a clearly dose-dependent manner only after the appropriate activation. It was further shown that treatment with 5 mM benzene plus 10% S-9 mix leads to highly significant increases in the frequency of SCEs in lymphocytes from 4 different individuals (Table 2).

Only when 10 to 30% S-9 mix was used was benzene converted into active form(s) that were cytotoxic in that they delayed cell turnover times (Chart 2). It has been shown before (18) that a chemically induced delay in cell division is clearly manifested as a change in the relative proportion of X1, X2, and X3+ cells. In this study, the ratio of X3+ cells to X2 cells was calculated as another index of cell division delay (Chart 2), which is given in untreated cultures at 67 hr (Chart control cultures, cell division delays can be estimated (18). For X3+ cells or the ratios of X3+ to X2 cells from treated and agreement with those found previously for untreated cells (18, 19, 30). By comparing the relative frequencies in X1, X2, and X3+ cells or the ratios of X3+ to X2 cells from treated and control cultures, cell division delays can be estimated (18). For instance, treatment with 5 mM benzene plus 10% S-9 mix gave a distribution of 11% X1, 39% X2, and 50% X3 cells at 72 hr (Chart 2), which is given in untreated cultures at 67 hr (Chart 3). It thus appears that this treatment leads to a division delay of 5 hr. The delay in cell division produced by benzene after activation is evident in both the frequency curves of X1, X2, and X3+ cells (Chart 2) and the ratios of X3+ to X2 cells in treated cultures (Table 1). Benzene exerted a greater cytotoxic effect in the presence of 30% S-9 mix than in the presence of 10% S-9 mix. However, 5 mM benzene plus 30% S-9 mix induced only half as many SCEs as did 5 mM benzene plus 10% S-9 mix (Chart 1). There was no increase in the SCE frequency in

### Table 1

<table>
<thead>
<tr>
<th>Concentration of benzene (mM)</th>
<th>% of S-9 mix</th>
<th>Control</th>
<th>2.0 × 10⁻⁴</th>
<th>1.0 × 10⁻³</th>
<th>5.0 × 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SCEs/cell</td>
<td>X3+:X2</td>
<td>SCEs/cell</td>
<td>X3+:X2</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>7.69 ± 0.48D</td>
<td>2.44</td>
<td>7.46 ± 0.46</td>
<td>2.13</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>7.46 ± 0.46</td>
<td>2.33</td>
<td>8.14 ± 0.45</td>
<td>2.10</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>8.10 ± 0.45</td>
<td>2.58</td>
<td>8.49 ± 0.51</td>
<td>2.30</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>7.54 ± 0.43</td>
<td>1.71</td>
<td>9.37 ± 0.51D</td>
<td>1.83</td>
</tr>
<tr>
<td>30</td>
<td></td>
<td>9.69 ± 0.53D</td>
<td>1.51</td>
<td>9.60 ± 0.53D</td>
<td>1.49</td>
</tr>
<tr>
<td>90</td>
<td></td>
<td>10.46 ± 0.57D</td>
<td>0.94</td>
<td>10.97 ± 0.59D</td>
<td>1.08</td>
</tr>
</tbody>
</table>

- Mean ± S.E.
- Significant by Student's t test (one tailed) (p < 0.01).
- Significant by Student's t test (two tailed) (p < 0.05).
- Significant by Student's t test (two tailed) (p < 0.001).
cells exposed to 1 mM benzene plus 30% S-9 mix, which induced about the same amount of delay as did 5 mM benzene plus 10% S-9 mix (Chart 2), whereas 1 mM benzene plus 10% S-9 mix still caused the production of SCEs (Chart 1). Thus, we have excluded the possibility that a longer division delay induced by exposure to benzene plus 30% S-9 mix would have caused an apparently smaller induction of SCEs. These data therefore strongly suggest that the metabolites predominantly responsible for cell division delay might be different from those that induce SCEs.

When benzene is incubated with S-9 mix, metabolites are formed that bind irreversibly to biomacromolecules, and the addition of reduced GSH to the incubation mixture prevents this binding in a dose-dependent manner (31). In the present study, the addition of GSH to the culture caused a dose-dependent decrease in SCEs in cells exposed to benzene and S-9 mix (Chart 4). The induction of SCEs was almost completely prevented by 3 mM GSH. In the repeated experiment (Table 3), it was further shown that 3 mM GSH treatment also completely prevented the induction of SCEs by catechol or hydroquinone, major phenolic metabolites of benzene and potent inducers of SCEs (18), as well as that by benzene plus S-9 mix. This strongly suggests that GSH treatment did not merely prevent activation of benzene by inhibiting the S-9 mix activity but really prevented the induction of SCE-leading lesions by the metabolites of benzene. These data are in excellent agreement with the finding of Tunek et al. (31) that 2 mM GSH prevented 90 to 95% of the irreversible binding of benzene metabolites to biomacromolecules.

When cells from cultures exposed to $5 \times 10^{-3}$ M benzene plus 10% S-9 mix at various times were examined for SCEs, it was found that different numbers of SCEs were induced at different times of treatment (Chart 5). After treatment at 0 hr, no SCEs were induced. After treatment at subsequent times, however, the frequency of SCEs increased, peaking at 40 hr, and then decreased at later treatment times. The data thus indicate that 40 hr after the beginning of culture is the most effective treatment time for the induction of SCEs by benzene plus S-9 mix.

In earlier experiments, cells in culture were exposed to

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**Chart 2.** Percentage of X1, X2, and X3+ cells in cultures exposed to benzene and S-9 mix. The frequency curves in control cultures (a) are shown by broken lines in c and d for comparison. Bars, S.E.

**Chart 3.** Frequency of SCEs/cell (a); the percentage of X1, X2, and X3+ cells (b); and the ratio of X3+ to X2 cells (c) in untreated cultures fixed at various times. Bars, S.E.

**Chart 4.** Effects of GSH on the induction of SCEs by benzene plus S-9 mix. Cells were treated with various concentrations of GSH along with benzene ($5 \times 10^{-3}$ M) and 10% S-9 mix from 40 to 42 hr after initiation. Bars, S.E.
Table 3
Preventive effects of GSH on the induction of SCEs by catechol, hydroquinone, or benzene plus S-9 mix
Cells were treated in the same protocol as in Chart 4. Each value was based on 35 second-division cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor</th>
<th>GSH (3 mM) treatment</th>
<th>None</th>
<th>Benzene (5 mM) + S-9 mix (10%)</th>
<th>Catechol (0.3 mM)</th>
<th>Hydroquinone (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A -</td>
<td></td>
<td>7.17 ± 0.45a</td>
<td>13.23 ± 0.61</td>
<td>13.26 ± 0.79</td>
<td>11.37 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>7.66 ± 0.43</td>
<td>8.26 ± 0.55</td>
<td>7.54 ± 0.53</td>
<td>8.63 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>t test</td>
<td></td>
<td>NSb</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>B -</td>
<td></td>
<td>7.71 ± 0.45</td>
<td>12.91 ± 0.61</td>
<td>12.83 ± 0.61</td>
<td>12.69 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td></td>
<td>7.11 ± 0.49</td>
<td>6.91 ± 0.53</td>
<td>7.60 ± 0.50</td>
<td>7.54 ± 0.50</td>
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</tr>
<tr>
<td>t test</td>
<td></td>
<td>NSb</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± S.E.
b NS, not significant (p = 0.05).

Table 4
Effects of treatment conditions on the induction of SCEs by benzene plus S-9 mix
Cells were treated with 5 × 10^-3 M benzene plus 10% S-9 mix from 40 to 42 hr after the beginning of culture.

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Type of cap</th>
<th>Agitation</th>
<th>SCEs/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silicone</td>
<td>+</td>
<td>8.66 ± 0.49b</td>
</tr>
<tr>
<td></td>
<td>Silicone</td>
<td>+</td>
<td>12.46 ± 0.60b</td>
</tr>
<tr>
<td></td>
<td>Silicone</td>
<td>+</td>
<td>9.43 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>Cork</td>
<td>+</td>
<td>9.26 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>Cork</td>
<td>+</td>
<td>9.14 ± 0.52</td>
</tr>
</tbody>
</table>

a Incubated without benzene plus S-9 treatment.
b Mean ± S.E.
c Significant at p < 0.001 (Student’s t test).
d Incubated without a stopper.

DISCUSSION
The present study demonstrates that, after appropriate in vitro metabolic activation, benzene causes both an increase in SCEs and a delay in cell division in human lymphocyte cultures. Previous experiments (18) showed that the phenolic metabolites, rather than benzene itself, are the cytotoxic substances that induce SCEs and cell cycle delays. Because it has been shown (24, 31, 32) that benzene is converted into the phenolic metabolites in vitro when incubated with rat liver microsomes in the presence of an NADPH-generating system (S-9 mix), the present results indicate that the formation of phenolic metabolites might be the cause of benzene toxicity. This is consistent with the findings of Tunek et al. (31), that incubation of cultures with GSH hardly affected the formation of phenol but did decrease the irreversible binding of benzene metabolites to biomacromolecules. With the present finding that GSH led to a dose-dependent decrease in SCEs induced by catechol and hydroquinone as well as benzene plus S-9 mix, this strongly suggests that further metabolites of the phenolic metabolites, possibly benzo(semi)quinones (32), might be the ultimate mutagenic toxicants that induce irreversible binding, SCEs, and cell cycle delays and thus might be leukemogenic.

There are optimal concentrations of S-9 mix for the conversion of benzene into reactive forms that induce SCEs and cell cycle delays, because only after appropriate activation does benzene exert these effects in a clearly dose-dependent manner. Similar results with known promutagens such as benzo(a)pyrene (1, 14, 29), aflatoxin B1 (14, 17, 29), furylfuramide (20), and dimethyl nitrosamine (6, 14, 17) indicate the critical importance of S-9 concentrations in mutagenesis assays. The existence of optima for concentrations of S-9 mix to metabolize benzene may result from the balance between activation of benzene into reactive forms and their subsequent reactions (detoxication) (12). At lower than optimal concentrations of S-9 mix, benzene cannot be converted into the phenolic metabolites at sufficient concentrations. At higher concentrations, both the conversion of benzene into phenolic compounds and their subsequent reactions would be so fast that the metabolites could not be accumulated sufficiently. But at optimal concentrations of S-9 mix, benzene can be metabolized to produce sufficient amounts of the compounds that enter cells and cause damage in DNA and other macromolecules, resulting in a marked increase in SCEs and cell cycle delays.

It has been reported recently (33) that there was no increase in SCEs in cultured lymphocytes obtained from benzene-exposed workers, although there was a significant increase in...
chromosome aberrations. One should be careful in interpreting such results, because the present study suggests that the DNA lesions leading to SCE formation that are induced by benzene metabolites are repairable. It is necessary for cells to pass through the S phase before DNA damage in the cells can result in the formation of SCEs (35). If induced DNA damage is repaired completely before cells enter S phase, then those cells show no increase in SCE frequency. It has been noted (19) that second-division metaphase cells in 72-hr cultures enter their first S phase at about 48 hr after the beginning of culture. In the present experiments, benzene plus 10% S-9 mix, which induced a 5-hr delay in cell division, gave the largest increase in SCEs when added for 2 hr immediately before cells entered their first S phase, i.e., at 40 hr of culture. The same treatment at 0 hr of culture resulted in no increase in SCEs because there was enough time for repair of the induced DNA damage that would have led to SCE formation. This suggests the possibility that, even when mutagenic carcinogens such as benzene produce DNA damage in the circulating blood (G0) lymphocytes, the damage can be repaired completely before the cells enter S, resulting in no increase of SCEs in cultured lymphocytes.

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REFERENCES

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