Lack of induction of micronuclei in human peripheral blood lymphocytes treated with hydroquinone

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Hydroquinone (HQ) has been reported to produce chromosomal effects in some in vivo and in vitro animal models. Its potential for inducing similar effects in human lymphocytes is less clear. The purpose of this study was to examine human lymphocytes treated with HQ for the presence of chromosomal anomalies, using an accepted assay for micronuclei. In addition, the stability of HQ in culture medium was determined to verify exposures. Lymphocyte cultures were obtained from eight donors so that variable responses amongst individuals could be assessed. The micronucleus assays utilized were a common 72 h assay with no wash, as well as two assay variations to maximize cell division. Assay variations consisted of either cell washing at 44 h or allowing unwashed cultures an extra 24 h recovery period before harvest. In all assays treatment was at 24 h post-mitogenic stimulation and cytochalasin B was added to stop dividing cells from undergoing cytokinesis. Thus, cells that were scored had undergone one division in the presence of the chemical. Stability results showed that while HQ was detectable in cultures at least for 15 h, it was considerably more stable at 25 than at 100 or 250 μM treatment levels. Results generated using any of the three micronucleus assay variations showed no significant increase in micronuclei in cultures treated with 12.5–200 μM HQ. Colchicine, the positive control and a known spindle disrupter, produced elevated levels of micronuclei. At certain HQ concentrations, a block in cell division was observed, as evidenced by a decrease in percent binucleated cells and replicative index end-points. By varying the assay conditions, cell cultures overcame this block in division and divided at HQ concentrations up to 200 μM, depending on the donor. The reversible block in cell division observed may be a protective response, allowing cells to recover without gross chromosomal damage. This study has substantially expanded the database with regard to the effects of HQ treatment on lymphocytes.

Introduction

Hydroquinone (HQ) occurs naturally in bacteria, plants and some animals and is also manufactured for commercial use. HQ is utilized in cosmetics as a skin lightening agent, in photography as a black and white developer, in the production of antioxidants for rubber and as a polymerization inhibitor for vinyl acetate and acrylic monomers (International Programme on Chemical Safety, 1994). HQ is also present in cigarette tar as a result of the combustion of tobacco leaf pigments and is a hydroxy metabolite of benzene (Li et al., 1996). The most common and most frequent exposures to HQ occur through the diet, as HQ is a naturally occurring antioxidant in many foods (Deisinger et al., 1996).

As one of several benzene metabolites, HQ has been extensively studied for its genotoxic potential and possible role in benzene-induced leukemia. Reviewing the published genotoxicity assays that have been performed with HQ, Whysner et al. (1995) reported that mutagenicity assays typically resulted in a lack of positive response, while rodent in vivo micronucleus assays were characteristically positive with HQ. Micronuclei (MN) serve as markers of gross chromosome anomalies as they are generated through clastogenic and/or aneugenic insult (Countryman and Heddle, 1976). A micronucleus is formed when an acentric fragment (clastogenic) or whole chromosome (aneugenic) fails to incorporate into the main nucleus at the time of cell division. Consistent chromosomal aberrations are often associated with human leukemias and metabolites of benzene that induce chromosomal anomalies have been implicated as contributing factors in benzene-induced leukemia (Morimoto and Wolff, 1980; Yager et al., 1990; Robertson et al., 1991).

The typical in vitro assay for MN induction, referred to here as the 72 h assay, entails chemical treatment of human peripheral blood lymphocytes (PBL) at 24 h post-mitogenic stimulation and the addition of cytochalasin B at 44 h to block cytokinesis. This timing ensures that binucleated cells will represent cells that have undergone one division in the presence of the chemical treatment. In addition, the mechanism of micronucleus formation can be investigated through the combination of the micronucleus assay with an anti-kinetochore antibody probing or fluorescence in situ hybridization techniques (Eastmond and Tucker, 1989; Eastmond and Pinkel, 1990).

Studies of MN induction by HQ in human PBLs in vitro (Table I) have yielded mixed results (Yager et al., 1990; Migliore and Nieri, 1991; Robertson et al., 1991; Van Hummelen and Kirsch-Volders, 1992; Ferguson et al., 1993; Vian et al., 1995). Assay variations that may in part explain the varied findings include use of isolated PBLs versus whole blood, 72 versus 96 h assays and the addition of a cell wash step in some studies. Human PBLs from whole blood cultures treated with HQ demonstrated either a weakly positive response (2- to 3-fold increase in MN over spontaneous levels) or no micronucleus induction at all (Migliore and Nieri, 1991; Van Hummelen and Kirsch-Volders, 1992; Ferguson et al., 1993). In those studies reporting MN induction, the effective concentrations varied among individuals (9–273 μM HQ). Positive responses were not statistically significant in some studies and no evidence for a concentration-related response was reported. When isolated PBLs were used rather than whole blood an 11-fold increase in MN and evidence of a dose–response effect were reported using cells from a single donor (Yager et al., 1990).

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Isolated PBLs, apparently more sensitive than whole blood cultures, were subsequently used by Robertson et al. (1991) and Vian et al. (1995) with mixed results. Two of the isolated PBL studies were from the same laboratory (Yager et al., 1990; Robertson et al., 1991) and the isolated PBLs were taken from the same donor (personal communication). Vian et al. (1995) used a single donor in a 72 h no wash assay and found a delay in division, but no statistically significant increase in MN. They also utilized shorter treatment times without a wash, which allowed the testing of extremely high concentrations of HQ. Here they found an increase in MN in one donor at the highest levels of HQ, after which they observed cytotoxicity.

The purpose of the present study was to investigate further the ability of HQ to induce MN in cells from various healthy donors, as well as to examine the mechanism by which HQ-induced MN are generated. The procedure followed was essentially that described in the Commission of the European Communities validation study of *in vitro* micronucleus induction in human lymphocytes (Van Hummelen and Kirsch-Volders, 1992), except that isolated PBLs rather than whole blood cultures were used. The conditions used here reproduced those used by Yager *et al.* (1990) and were chosen based on the expectation that significant induction of MN in lymphocytes from a single subject could be reproduced in lymphocytes obtained from other donors. The stability of HQ in cell culture medium both in the presence and absence of human PBLs was also determined so that the actual concentration of HQ and its rate of degradation under assay conditions would be known. These studies were performed according to OECD Principles of Good Laboratory Practice (as revised in 1997) [C(97)186/Final].

### Materials and methods

#### Chemicals

HQ was obtained from Eastman Kodak (Rochester, NY) and dissolved in saline: Hank’s balanced salt solution without calcium chloride, magnesium chloride, magnesium sulfate and sodium bicarbonate (HBSS) (Gibco, Grand Island, NY); Dulbecco’s phosphate-buffered saline solution (DPBS) (Gibco). Cytoschalin B (Cyt B) (Sigma, St Louis, MO) was dissolved in dimethylsulfoxide (Eastman Chemical Co., Kingsport, TN) and kept as a stock solution of 1.5 mg/ml under refrigeration. Colchicine (Colch) (Sigma) was initially dissolved in ethyl alcohol (Fisher, Fairlawn, NJ) and then diluted in HBSS before being delivered to the cultures.

#### Stability studies

The stability of HQ in culture medium with and without human PBLs was studied at 25, 100 and 250 μM concentrations. Conditions of a typical 72 h

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**Table I. Comparison of literature findings from studies in which human whole blood or isolated PBLs have been exposed to hydroquinone**

<table>
<thead>
<tr>
<th>Reference</th>
<th>Culture method/ donor</th>
<th>HQ conc.</th>
<th>Basic assay</th>
<th>Time of HQ admin./ Cyt B addition</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Migliore and Nieri (1991)</td>
<td>Whole blood</td>
<td>10–200 μM</td>
<td>72W</td>
<td>24/44 h</td>
<td>50 μM, 2X increase in MN; 200 μM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10–200 μM</td>
<td>96W</td>
<td>24/44 h</td>
<td>10–200 μM, no increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Van Hummelen and Kirsch-Volders (1992)</td>
<td>Whole blood</td>
<td>1 and 100 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>1 μM, 4X increase in MN; 100 μM, no increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1 and 100 μM) +S9</td>
<td>72W</td>
<td>24/44 h</td>
<td>1 μM, no increase in MN; 100 μM, 2X increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200 and 300 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>200 μM, no increase in MN; 300 μM, 4X increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 and 150 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>50 or 150 μM, no increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(50 and 150 μM) +S9</td>
<td>72W</td>
<td>42/44 h</td>
<td>50 or 150 μM, no increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 and 150 μM</td>
<td>72W</td>
<td>42/44 h</td>
<td>50 or 150 μM, no increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ferguson <em>et al.</em> (1993)</td>
<td>Whole blood</td>
<td>100, 300 and 1000 μM</td>
<td>96W</td>
<td>24/48 h</td>
<td>100 μM, no increase in MN; &gt;300 μM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100, 200 and 400 μM</td>
<td>96W</td>
<td>24/48 h</td>
<td>100 μM, no increase in MN; 200 μM, 3X increase in MN&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50–300 μM</td>
<td>96W</td>
<td>24/48 h</td>
<td>50, 100, 150 μM, no increase in MN; 200 μM, 2X increase in MN; 300 μM, 2X increase in MN&lt;sup&gt;b&lt;/sup&gt;; 400 μM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yager <em>et al.</em> (1990)</td>
<td>Isolated PBLs</td>
<td>2–150 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>75 μM, 3X increase in MN</td>
</tr>
<tr>
<td>Robertson <em>et al.</em> (1991)</td>
<td>Isolated PBLs</td>
<td>75 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>3 μM, no increase in MN</td>
</tr>
<tr>
<td></td>
<td>Donor (same individual as in Yager <em>et al.</em> 1990)</td>
<td>75 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>3 μM, no increase in MN</td>
</tr>
<tr>
<td>Vian <em>et al.</em> (1995)</td>
<td>Isolated PBLs</td>
<td>5–50 μM</td>
<td>72NW</td>
<td>24/44 h</td>
<td>5 μM, no increase in MN; 10 and 20 μM, 2X increase in MN&lt;sup&gt;b&lt;/sup&gt;; 50 μM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(250 μM–2 mM) +S9</td>
<td>72W 1.5 h</td>
<td>24/44 h</td>
<td>250, 500, 750 μM, 2X increase in MN&lt;sup&gt;b&lt;/sup&gt;; 1.0 and 1.5 m, 4–5X increase in MN; 2 mM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(250 μM–2 mM) +S9</td>
<td>72W 1.5 h</td>
<td>42/44 h</td>
<td>250 μM, no increase in MN; 500 μM, 4X increase in MN; 750 μM, no increase in MN; &gt;1 mM, cytotoxic&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MN, micronuclei; W, wash; NW, no wash.

<sup>a</sup>Cytotoxic, defined as a 50% decrease in the mitotic index or BN cell frequency.

<sup>b</sup>No cytotoxicity.

<sup>c</sup>Less than 500 cells scored per experiment.

<sup>d</sup>Not statistically significant.
MN assay (see Yager et al., 1990) were used, with HQ additions at 24 h following the addition of mitogen (phytohemagglutinin, PHA). Samples were removed from cultures at varying times (up to 27 h post-treatment), analyzed and compared with initial measurements taken at the time of treatment (∼t). Samples containing PBLs were centrifuged, and the supernatant was removed and transferred to amber vials. Samples were analyzed using high performance liquid chromatography (HPLC) (1090 series II; Hewlett-Packard, Palo Alto, CA) with a reverse phase Supelcosil LC-18 column (Supelco, Bellefonte, PA) and electrochemical detection [LC-4C Amperometric Detector (BAS, West Lafayette, IN); CoulArray Coulometric Electrode Array Detector (ESA, Chelmsford, MA)]. The mobile phase was an isocratic mixture of 98% water, 2% acetonitrile (J.T. Baker, Phillipsburg, NJ). Peak areas were identified and compared with calibration data generated by analyzing HQ standards on each study day. HQ standards were prepared in DPBS with the addition of 0.01% ascorbate (L-ascorbic acid; Eastman Kodak). Standards were run at intervals during the course of the day’s study when appropriate. The detector response to HQ standards was found to be linear over the concentration range analyzed. The percent reduction in HQ concentration over time in the presence of PBLs from each donor (or in medium alone) was modeled using a generalized exponential function:

\[
\text{reduction} = \left(1 - \exp\left[-\beta_2 \times (\text{time})^{\beta_1}\right]\right) \times 100
\]

A statistical software package, JMP v.3.1 (SAS Institute, Cary, NC), was used to fit the non-linear model, resulting in unique estimates of the parameters \(\beta_1\), \(\beta_2\) and \(\beta_3\) for each donor at each HQ concentration. The model was used to estimate the percent reduction occurring at 2 h incubation. Alternatively, to estimate the time until 50% reduction in HQ occurred, the model was solved for time as a function of the parameters and 50% reduction.

**Cultures**

Human peripheral blood samples were obtained from a total of eight healthy male and female volunteers. The eight blood donors were non-smokers and ranged in age from 17 to 45 years with a mean age of 32.3 years at the start of the study. Blood was drawn by venipuncture into sodium heparin Vacutainers (Becton-Dickinson, Franklin Lakes, NJ). Lympocytes (PBLs) were isolated using density centrifugation (Histopaque-1077; Sigma) and cultured at 37°C for 72 or 96 h in an incubator with a 5% CO₂ atmosphere. Cultures were set up in 15 ml clear polypropylene culture tubes (Corning, Corning, NY) with for each donor and assay. For a particular donor, the number of tests ranged from 5 to 8 per donor and assay. For a particular donor, the number of tests ranged from one to five with each test treated as a replicate for the given dose/donor/assay combination. The counts were standardized to proportions and then transformed using the arcsine square root transformation (Snedecor and Cochran, 1989). Because each proportion was based on a variable number of cells counted, ranging from 500 to 1800, the number of cells was used as a weighting factor in the ANOVA. For each assay and donor, the relevant control was tested against each HQ treatment concentration. A P value of ≤0.05 was used to determine statistical significance.

**Results**

Before investigating the effects of HQ on micronucleus induction, the concentration of HQ in the cultures following treatment was examined. Stability studies were run in culture medium with and without human PBLs. These studies enabled the verification of treatment concentrations by demonstrating the presence of HQ in the cultures following treatment. Rates of HQ loss under typical culture conditions were also determined.

The concentration of HQ remained at or above 50% of the original concentration over at least the first 2 h following treatment (Figure 1). Concentrations then declined at varying rates depending on initial HQ concentration and presence and/or source of cells. A comparison of percent HQ remaining versus time for the 25 and 250 μM concentrations is shown in Figure 2. The rate of HQ loss was greater in cultures containing only cell culture medium than in most cultures containing human PBLs, especially at the low (25 μM) HQ concentration (see also Figure 3). There also appears to be variability among cultures containing PBLs, indicating that the source of the cells (i.e. donor) is a source of variability (Figures 1 and 3). Further, the lowest concentration (25 μM) of HQ appears to be more stable than either of the higher concentrations (100 or 250 μM) regardless of the presence of PBLs (Figures 2 and 3). Average rates of loss of HQ based
deemed unscorable (U). Cultures were classified as U if at least 500 BN cells/replicate experiment could not be counted for the end-point of MN. Table III, which contains the binucleated frequencies (%BN) and RI, provides some explanation for why 500 binucleated cells were not available for scoring in cultures designated U. BN frequency is a reflection of the percent of mononucleated, BN and multinucleated cells in a culture \([(1\times\text{mononucleated}) + (2\times\text{BN}) + (3\times\text{multinucleated})]/100 \text{ cells}]\]. In instances where the BN frequency was seen to be low (<14%), a high percentage of mononucleated cells was observed. Such cultures are designated U/L in Table II, indicating that the cultures were unscorable for MN because of a low binucleated frequency (high mononucleated frequency). Such an increase in the frequency of mononucleated cell is considered evidence of a block in cell division or slowing of cell cycling. The majority of donors exhibited this apparent block at 75 \(\mu\text{M}\) HQ, resulting in an inability to score MN in binucleated cells. Mononucleated cells were also scored for MN in case the cells had escaped cell block; no evidence of an increase in MN was manifest (data not shown). In addition to the block of cell division, some of the preparations contained mainly cellular and nuclear debris, and they were referred to as U/D for being unscorable caused by evidence of cellular damage (i.e. nuclear debris, lack of intact cells). In the 72 h no wash assay the majority of the donors displayed evidence of cellular damage as well as a low binucleated frequency following treatment with 75 \(\mu\text{M}\) HQ.

Statistically significant increases in MN over control levels were seen in cultures from each donor when Colch, the positive control, was administered. These results indicate that the assay was indeed working. Colch is a known spindle disrupter and the micronucleus assay must be slightly modified when it is utilized (Parry and Sors, 1993). Thus, a similar 72 h standard assay is run except that Colch is washed from the cultures 20 h post-treatment, prior to the addition of Cyt B. This allows the cells to recover from the induced mitotic arrest. It was decided to implement the wash method utilized for Colch with HQ cultures, because HQ appeared to cause a similar block in cell division. Reversing the block would enable cells to undergo subsequent divisions as required for micronucleus induction. Thus, the 72 h no wash assay was run again except the cells were washed at 44 h of culture, before Cyt B treatment. This method was designated 72 h wash and was run using cells from four of the original eight donors.

Results from the 72 h wash assay are displayed in Tables II and III. Cells from donor 03 that had previously been sensitive to HQ-induced block now divided readily, with the %BN cell frequency increasing >10-fold at 75 \(\mu\text{M}\) HQ (from 4 to 45%). Similar large increases in this end-point were observed in the three other donors examined. These results suggest that the previously seen cells designated U/L were attributable to a reversible block in cell division. Although this assay improved cell replication such that higher concentrations of HQ resulted in cell preparations that were on linear regression analysis were 1.8 and 3.8% per h (cell cultures and medium alone, respectively) at the 25 \(\mu\text{M}\) treatment concentration. At the 250 \(\mu\text{M}\) treatment concentration, HQ loss rates averaged 14 and 17% per h, respectively, in cell cultures and medium alone. HQ was below the detection limit at 15 and 18 h at the high treatment concentrations, whereas it was still present at 24 h at the 25 \(\mu\text{M}\) treatment level. Despite the variation observed, it was concluded that HQ was available to interact with the cells at all concentrations and its effect on PBLs could be evaluated under these cell culture conditions.

In conducting the micronucleus assay, a 72 h assay as described by Yager et al. (1990) was initially followed, with delivery of the chemical at 24 h post-mitogenic stimulation and Cyt B addition at 44 h of culture. The assay was designated 72 h no wash because the chemical was not washed from the system, and the treatment time was 48 h. No effect of HQ on micronucleus frequencies in cells from any of the eight donors (Table II) was observed. Treatment at some of the higher concentrations of HQ resulted in cell preparations that were

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**Fig. 2.** HQ stability in the presence of PBLs (solid symbols) or medium alone (open symbols), as analyzed by HPLC with electrochemical detection. Initial HQ concentrations of 25 \(\mu\text{M}\) (■, □) and 250 \(\mu\text{M}\) (▲, △). Trend lines were created by applying a linear regression model.

**Fig. 3.** Time at which 50% loss of HQ occurs as analyzed by HPLC with electrochemical detection. Two to five replicate cultures were run per treatment in the presence of PBLs from one of five individuals (light gray columns) or in medium alone (dark gray). Scale reflects 20 h treatment time.
designated 96 h. The 96 h assay was conducted with cells from all of the eight original donors.

Higher rates of cell division were observed in most of the donors using the 96 h assay when compared with the 72 h no wash studies. Data indicate that the cells, given additional time, were able to recover from the insult and divide at concentrations up to 150 \( \mu \text{M} \). Despite the change in assay conditions, no evidence for any effect of HQ on the induction of MN was observed. Interestingly, donor 03 demonstrated a significantly lower \((P < 0.05)\) number of MN at the 75 \( \mu \text{M} \) HQ treatment level as compared with controls.

### Discussion

The original intent of this study was to demonstrate induction of MN and to investigate the mechanism of micronucleus formation by HQ in human PBLs \textit{in vitro}. The expectation that HQ might induce MN was based primarily on the results of the 1990 study reported by Yager et al. (72 h no wash assay). However, the results of the Yager et al. report were not reproduced by this laboratory, as no evidence was found of HQ interaction with genomic material or the cellular machinery, manifested as elevated MN. Thus, an investigation of mechanism was not feasible. Exposure of human PBLs to HQ did, however, appear to suppress mitogenesis. Although an HQ-induced mitotic delay is not a novel finding, this report is unique in that assay conditions were optimized to investigate micronucleus induction following removal of the block to cell division. Furthermore, the investigation of HQ stability in the presence of PBLs \textit{in vitro} presented here provides a possible explanation for the observed delay in mitosis. A review of the literature is also presented, which indicates that HQ micronucleus induction \textit{in vitro} in human PBLs is inconsistent. The results of this study, especially when discussed in the context of the existing literature, suggest that much caution must be utilized when considering the current belief that HQ is a critical metabolite in benzene leukemogenicity.

In the Yager et al. (1990) study cells from the one individual tested produced 2- to 11-fold increases in MN when treated with HQ \((25-125 \mu \text{M})\). Cytotoxicity was noted at 125 \( \mu \text{M} \) HQ and no block in cell division was reported, indicating that scoring was not limited (Yager et al., 1990). In the present study where the Yager protocol was reproduced \((72 \text{ h no wash})\), none of the cells from the eight donors tested demonstrated significant increases in MN at any of the concentrations utilized \((12.5-200 \mu \text{M})\). Interestingly, scoring was limited at concentrations of 25, 50 or 75 \( \mu \text{M} \) and above, depending on the donor. This lack of scorability was attributable to the decrease in cell division defined by dose-dependent decreases in percentage of binucleated cells. Morimoto and Wolff (1980) reported similar findings in human PBLs and there are a number of published reports which refer to HQ inhibition of cell division \textit{in vitro} in various other cell types (Irons, 1985; Carbonelle et al., 1995; Taysse et al., 1995; Hader et al., 1996; Li et al., 1996).

In an effort to overcome the block in cell division observed with HQ and allow expression of MN, assay conditions were varied. The addition of a wash 24 h after chemical treatment \((72 \text{ h wash})\) alleviated the effect of HQ on mitogenesis,
However, no significant increases in MN over control values were observed in PBLs of any of the four donors. This finding indicated reversibility of the mitotic arrest that is in agreement with the findings of Li et al. (1996), who also demonstrated that a washing step enabled human T lymphoblasts to recover from HQ-induced mitotic delay.

The third assay (96 h) provided for an additional 24 h in culture to allow cells to recover from the inhibition of division. Investigating chromosomal damage 48 h post-chemical treatment has been recommended as a means of optimizing first division cells in the event a test chemical may delay or block division (Henderson et al., 1997). With the 96 h assay the cells from the majority of donors were able to recover from HQ treatment and divide. When PBLs from donors that were able to replicate under these conditions were examined for micronucleus induction, again, no increases were observed. Thus, it was found that cells possess the intrinsic ability to overcome the delay in cell division resulting from HQ treatment, without the manifestation of gross molecular damage.

Colch (a known aneugen and clastogen) was consistently seen to induce MN. It should be noted that treatment with colch required a wash step (identical to that utilized in the 72 h wash assay with HQ) because colch causes mitotic delay. The implementation of a wash allowed for removal of the cell block and manifestation of chromosomal damage as MN. Thus, we can be confident that the variations in assay conditions utilized should have maximized the ability of MN to be detected if HQ were capable of generating MN in these donors.

The stability of HQ was examined to ensure that the compound was, in fact, present and bioavailable (not immediately spontaneously oxidized) under the typical cell culture conditions utilized in the micronucleus assay. The present study demonstrated that under the conditions used in the isolated PBL micronucleus assay, HQ was detectable in culture medium and its rate of disappearance from the cultures was donor and treatment dependent. This is the first published study that has documented the conditions under which HQ is stable in culture medium. Analytical verification of HQ concentration, as presented here, is necessary if conflicting results from in vitro studies with HQ are to be reconciled.

HQ concentration was determined immediately following its addition to culture medium and at intervals for up to 24 h, in both the presence and absence of cells. The presence of cells in the medium appeared to enhance the stability of HQ (particularly at 25 μM); when cells were absent, HQ loss occurred at a slightly faster rate. In general, HQ concentrations remained at, or above, 50% of the initial concentration over at least the first 2 h of incubation, after which they declined at varying rates depending upon initial concentration and cell source. Interestingly, the two higher initial concentrations showed higher rates of HQ loss. It is important to note that ‘loss’ as defined here refers to declining concentration of HQ as analyzed by HPLC with electrochemical detection. Loss to volatilization can be ruled out due to the very low vapor pressure of HQ. Rather, it is suspected that the observed loss of HQ may be accounted for by oxidation resulting in the presence of a breakdown product(s) in the cultures.

HQ can undergo auto-oxidation to form p-benzoquinone (BQ) in vitro at physiological pH (Greenlee et al., 1981; Irons et al., 1982). The most common oxidation mechanism in

Table III. Effects of HQ on cell division in human PBLs

<table>
<thead>
<tr>
<th>Assay</th>
<th>Treatment</th>
<th>Donor</th>
<th>%BN</th>
<th>RI</th>
<th>%BN</th>
<th>RI</th>
<th>%BN</th>
<th>RI</th>
<th>%BN</th>
<th>RI</th>
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<td>Untreated</td>
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<td>20</td>
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</tr>
<tr>
<td></td>
<td>12.5 μM HQ</td>
<td>03</td>
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HQ, hydroquinone; Colch, colchicine; HBSS, Hank’s balanced salt solution; U, unscorable cultures; L, low binucleated frequency; D, cellular destruction (see text); ND, no data available; %BN, binucleated cell frequency; RI, replication index.

* A minimum of 600 PBLs per treatment were counted for each end-point.

*75 nM Colch.
aqueous medium for HQ is sequential one-electron oxidations via the semiquinone to BQ. The semiquinone radical anion may then react with another HQ molecule in a self-condensation reaction forming a dimer (Monks et al., 1992). A high concentration of HQ in the medium would be expected to drive this reaction towards polymerization. This may explain the higher rate of HQ loss in incubations containing the higher added HQ concentrations. The extent of BQ and polymer formation is unknown because these putative products were not identifiable by the analytical method used here, but the formation of polymeric products was suggested by coloration of the HQ-treated culture medium that increased in intensity over time.

Applying what is known about the potential for HQ to oxidize in vitro provides a number of possible explanations for the HQ stability findings in this study. The relatively slow loss of HQ, which occurred at 25 µM treatment concentrations, may be due to the presence of trace levels of glutathione and ascorbate in the cell medium that could contribute to maintenance of reduced HQ. Additionally, the presence of cells may act to lower the rate of HQ disappearance by reducing BQ back to HQ enzymatically [e.g. by NAD(P)H:quinone oxidoreductase] or by maintaining HQ in the reduced state through reversible binding to cell surface proteins. Other cell types, including rat kidney proximal tubule cells and human red blood cells, as well as plasma, have been found to exhibit similar stabilizing effects on HQ (R.Boatman and L.Perry, unpublished data). If HQ is stabilized enzymatically or through reversible protein binding, it follows that saturation could occur at higher added concentrations of HQ and the cell’s reducing or stabilizing capacity overwhelmed. The resultant auto-oxidation of HQ could create a pro-oxidant environment through the generation of excess reactive oxygen species. Such conditions may contribute to the sustained rapid loss of HQ that was observed when cultures were treated with high initial HQ concentrations. Under conditions where HQ was stable (25 µM), mitotic delay was rarely observed. Thus, the mitotic delay observed in this study may have been caused by substances other than HQ, including semiquinone, BQ, polymeric products or excess reactive oxygen species. Similarly, induction of mitotic delay or MN reported elsewhere may also be attributable to one or more of these degradation products, where the same or similar methodology was used.

In addition to the concentration-related differences in rate of HQ loss, there appeared to be variability in the rate of loss of HQ among donors, suggesting a difference in stabilization capacity among individuals. Biological variability, in the form of differences in enzyme or other protein expression, may account for the observed differences in rate of HQ loss among individuals. Despite these differences, study findings demonstrate that cells were successfully exposed to HQ in all treated cultures. At the same time, stability results indicate that as the ‘dose’ of HQ added to cultures increases, there is a disproportionate increase in the formation of uncharacterized degradation products of HQ. These findings underscore the importance of determining the test substance concentration during in vitro studies designed to test the genotoxic potential of HQ and like compounds. Studies employing typical cell culture conditions, and high added concentrations of HQ in particular, are likely to test the effect not of HQ itself, but of a mixture of substances. Although the in vitro degradation products were not characterized, we speculate that they differ significantly both qualitatively and quantitatively from metabolites of HQ formed in vivo. Concentrations of free HQ and oxidation products in vivo remain relatively low, as shown in experiments where HQ was administered to rats (DiVincenzo et al., 1984; Hill et al., 1993; Deisinger and English, 1999). HQ is rapidly and extensively metabolized to glucuronide and sulfate ester conjugates (reviewed in DeCaprio, 1999). In a study where rats were given [14C]HQ by oral administration, first-pass elimination exceeded 99% based on measurements of free HQ and total 14C in plasma (English et al., 1988). The estimated in vivo half-life of HQ in arterial blood is 1.2–3.6 min (Deisinger and English, 1999) and the potential for concentration of HQ in tissues is modest (Greenlee et al., 1981). The concentration of free HQ in the human also appears to be tightly controlled. The peak blood concentration in a human volunteer after ingesting 275 mg HQ was <0.9 µM (Corley et al., 2000). Free HQ is stabilized to oxidation by plasma and a variety of cell types and the extent of oxidation in vivo is known to be ~5% of the administered dose. This estimate of oxidation represents the combined total thioether conjugates and protein, respectively. Thioether conjugates and protein S-adducts (Boatman et al., 1994) that have been measured in vivo. These are the stable products of reaction between BQ and glutathione or protein, respectively. Thioether conjugates and protein S-adducts are indirect indicators of BQ formation due to the preferential reactivity of BQ with protein and non-protein sulfhydryl groups (Hanzlik et al., 1994). The standard in vitro conditions used in the present investigation resulted in an apparent self-catalyzed degradation of HQ at 100 and 250 µM treatment levels with the probable formation of polymeric products, discussed above. Such a process is unlikely to occur in vivo, given the body of knowledge concerning HQ disposition in vivo. Indeed, HQ is stable in medium (with or without added protein) when the O2 content of the atmosphere is reduced to 5%, approximating physiological conditions (Colinas et al., 1994).

The results reported here, showing a lack of MN induction in human PBLs by HQ, are consistent with a body of literature. A closer look at the existing literature indicates that previous evidence (but lack of emphasis) of cytotoxicity exists. Additionally, interpretation of the stability work presented here suggests some reasoning for the differences in micronucleus induction and mitotic delay found between various studies in the literature. Six previous reports have been identified (Table 1) in which the effects of HQ on micronucleus induction in human lymphocytes in vitro were described. Migliore and Nieri (1991), Van Hummelen and Kirsch-Volders (1992) and Ferguson et al. (1993) used whole blood, whereas Yager et al. (1990), Robertson et al. (1991) and Vian et al., (1995), as well as the present study, used isolated PBLs. Reviewing the literature for reports of PBLs in whole blood that were treated with HQ reveals that cells from a total of eight donors had been tested in this manner and under various conditions (e.g. 72 wash, 72 no wash or 96 wash; see Table I for details). Studies performed in whole blood cultures according to the standard 72 h assay, and variations thereof, provide no reproducible evidence of MN induction by HQ. Timing of HQ addition was also examined by Van Hummelen and Kirsch-Volders (1992), who found that whether HQ was administered in G1 or G2 was immaterial to the outcome and the addition of S9 appeared to be inconsequential. Although not reproduced, significant increases in MN (2- to 4-fold) were reported at 1, 9, 50, 90, 100, 200 and 300 µM HQ in cultures of cells from certain donors under various conditions (72 wash, 72 no wash or 96
wash). Interestingly, these same concentrations of HQ produced no MN in cultures of cells from other donors, even though identical conditions were employed.

In contrast to the present studies using isolated PBLs, high concentrations of HQ (150, 200 and 300 \(\mu M\)) did not appear to suppress cell division in whole blood cultures. Thus, utilizing the whole blood method allows testing with high concentrations of HQ (100–400 \(\mu M\)) without the limitations imposed by cytotoxicity. These results are consistent with the proposed theory that oxidation of HQ is critical for the mitotic delay. In whole blood the presence of additional proteins and cell types would stabilize HQ, decrease the formation of oxidative products and provide additional binding sites for reactive BQ. The concentrations of oxidative degradation products available to interact with the mitotic apparatus to induce cell cycle delay would thus be greatly reduced. Oxidative products of HQ have been reported to interfere with proteins such as DNA polymerase (Lee et al., 1989) and spindle tubulin (Irons and Neptun, 1980), which further supports a role of oxidative degradation of HQ in the observed inhibition of mitosis.

Unlike the random micronucleus induction (discussed above) which was reported by researchers using whole blood, Yager et al. (1990) saw a clear dose–response effect in HQ induction of MN in PBLs from one individual. The most puzzling part of this finding is that a 72 h no wash assay was utilized. Unlike the cells from the eight donors used here, no cytoxicity was observed in cells from this individual at HQ concentrations <200 \(\mu M\). Because Robertson et al. (1991) utilized the isolated PBL method with cells from the same donor used by Yager et al. (1990), there is published evidence that this finding is reproducible by laboratories, at least at the 75 \(\mu M\) concentrations.

Vian et al. (1995) noted their concern with variance between individuals and utilized isolated PBLs from only one donor per type of treatment in their experiments. When they utilized an assay similar to our 72 h no wash assay, the results agreed with the findings presented here, i.e. cells from their donors demonstrated treatment-induced delay of cell division, which hindered scoring at the same concentration (50 \(\mu M\)), as did cells from four of the eight donors in the present study. Assays employing a shorter treatment time (1.5 h) did result in statistically significant increases in MN (Vian et al., 1995). Following treatment in G\(_1\), 4- to 5-fold increases at 909 and 1364 \(\mu M\) in one donor were reported. Treatment in G\(_2\) resulted in a statistically significant increase (4-fold) in MN only at 455 \(\mu M\). The concentrations utilized by these investigators for these shorter treatment assays were extremely high compared with those used in other studies. Cell division at such high concentrations of HQ was surprising in the light of the cytotoxic effects which have been reported in the literature for cells treated with >200 \(\mu M\) HQ. It would appear that positive increases in MN were only manifest in cells that were not affected by a cytotoxic delay of division. The fact that Yager et al. (1990) did not observe a cell block and reported a clear concentration–response effect in micronucleus induction is consistent with the idea that the cell block is a protective mechanism. It is expected that cells expressing the block have sufficient repair time resulting in no MN induction. In contrast, cells failing to exhibit the block may sustain unrepaired damage, which could become manifest as MN. Measured concentrations of HQ were not reported in these previous studies, so it is not known if the lack of cell block was related to disposition of HQ in cultures. It is possible that minor differences in culture conditions may influence the generation of reactive products. For example, trace levels of reduced transition metals can generate the highly reactive hydroxyl radical from hydrogen peroxide, an oxidation by-product of HQ. Alternatively, variable effects of HQ on human lymphocytes in vitro may be attributable to varying expression of biotransformation enzymes in different donors. The presence of CYP1A1, CYP2E1 and peroxidases has been reported in lymphocytes (Schmidt, 1985; Vanden-Heuvel et al., 1993; Wilson et al., 1995). These enzymes may be capable of bioactivating HQ and P450 isoforms, in particular, are known to be expressed variably in individuals due to genetic and environmental factors. An example of bioactivation by human lymphocytes via ring oxidation was recently reported by Johnson et al. (1998) for benz[a]anthracene.

Recently, Andreoli et al. (1999) reported evidence of single-strand DNA breaks after in vitro treatment of resting human PBLs with HQ, but no excess MN after cell division, consistent with the present investigation. Damage was ameliorated by the addition of catalase, suggesting that damage occurs from hydrogen peroxide formation. Damage was not observed in whole blood cells, in contrast to the results in isolated PBLs, even when tested at cytotoxic millimolar concentrations. These findings suggest that the type of damage caused to DNA by the in vitro addition of HQ is amenable to repair prior to cell division and is less likely to occur at all under more physiologically relevant conditions.

Although a large database exists for HQ cytogenetic test results, the current stability study suggests that in vitro exposures in previous studies have been inadequately characterized. This, together with inconsistent findings, leads to uncertainty as to whether HQ is clastogenic, aneugenic or even consistently induces MN. The belief that HQ is a critical metabolite in benzene leukemogenicity relies heavily on its potency relative to other benzene metabolites in a variety of in vitro tests. In the light of the present study and the inconsistencies within the literature, one must use caution before agreeing that such a conclusion is supported. Problems in reproducing a dose-related response may arise both from non-proportional treatment-related changes in the culture concentration of HQ and the in vitro mix of degradation and oxidation products present, as well as differences in PBL characteristics among individual donors. It is understood that variations in diet and, in particular, folate levels, age, sex, general health and underlying genetic types would stabilize HQ, decrease the formation of oxidative products. For example, trace levels of reduced transition metals can generate the highly reactive hydroxyl radical from hydrogen peroxide, an oxidation by-product of HQ. Alternatively, variable effects of HQ on human lymphocytes in vitro may be attributable to varying expression of biotransformation enzymes in different donors. The presence of CYP1A1, CYP2E1 and peroxidases has been reported in lymphocytes (Schmidt, 1985; Vanden-Heuvel et al., 1993; Wilson et al., 1995). These enzymes may be capable of bioactivating HQ and P450 isoforms, in particular, are known to be expressed variably in individuals due to genetic and environmental factors. An example of bioactivation by human lymphocytes via ring oxidation was recently reported by Johnson et al. (1998) for benz[a]anthracene.

In conclusion, HQ does not appear to induce MN in isolated human lymphocytes in the preponderance of individuals tested thus far. Further studies are necessary to confirm and understand individual differences. Examination of a larger population may be required to identify individuals who are not responsive to HQ-induced cell cycle delay and are thus more sensitive to MN formation. Finally, HQ is considerably more stable when added at 25 \(\mu M\) to cell cultures or culture medium alone than at \(\geq 100 \mu M\), a factor to be considered in the design and interpretation of future studies.

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