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Peroxidase-Dependent Metabolism of Benzene's Phenolic Metabolites and Its Potential Role in Benzene Toxicity and Carcinogenicity

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The metabolism of two of benzene's phenolic metabolites, phenol and hydroquinone, by peroxidase enzymes has been studied in detail. Studies employing horseradish peroxidase and human myeloperoxidase have shown that in the presence of hydrogen peroxide phenol is converted to 4,4′-diphenoquinone and other covalent binding metabolites, whereas hydroquinone is converted solely to 1,4-benzoquinone. Surprisingly, phenol stimulates the latter conversion rather than inhibiting it, an effect that may play a role in the *in vivo* myelotoxicity of benzene. Indeed, repeated coadministration of phenol and hydroquinone to B6C3F$_1$ mice results in a dramatic and significant decrease in bone marrow cellularity similar to that observed following benzene exposure. A mechanism of benzene-induced myelotoxicity is therefore proposed in which the accumulation and interaction of phenol and hydroquinone in the bone marrow and the peroxidase-dependent formation of 1,4-benzoquinone are important components. This mechanism may also be responsible, at least in part, for benzene's genotoxic effects, as 1,4-benzoquinone has been shown to damage DNA and is shown here to induce multiple micronuclei in human lymphocytes. Secondary activation of benzene's phenol metabolites in the bone marrow may therefore play an important role in benzene's myelotoxic and carcinogenic effects.

Introduction

Understanding the mechanism by which benzene exerts its toxic and carcinogenic effects on the hematopoietic system may be essential to determine whether low-level chronic exposure to benzene poses a significant health threat. Previous research has shown that benzene itself is probably not the actual toxicant, but is converted by hepatic metabolism to a metabolite(s) that travels to the bone marrow and exerts its toxic effects (*1,2*). There is, however, also the potential for benzene to be directly activated within the bone marrow itself (*3*). The primary metabolite of benzene *in vivo* is phenol with hydroquinone, catechol, and *trans,trans*-muconic acid occurring as significant secondary metabolites (*4-6*). Following benzene exposure, the three principal phenolic metabolites of benzene (Fig. 1) rise in concentration within the bone marrow (*7,8*). The rise in phenol is transient, but hydroquinone and catechol accumulate to relatively high concentrations (*8*). This selective accumulation

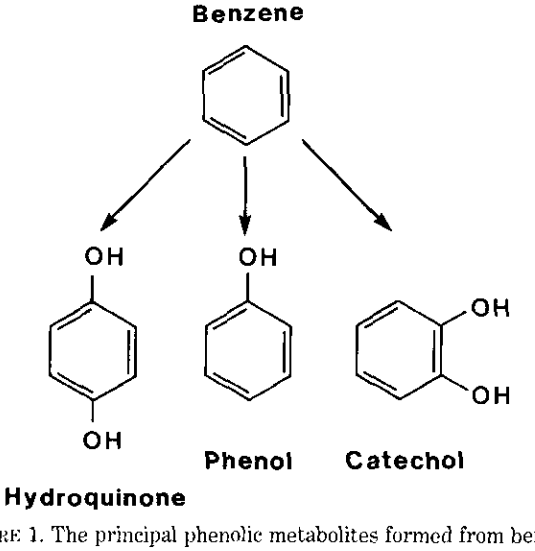

FIGURE 1. The principal phenolic metabolites formed from benzene.

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raises the possibility that a secondary bioactivation of one or more of these phenolic metabolites may occur within the bone marrow and generate the actual toxic species.

The bone marrow is an organ roughly equivalent in size with that of the liver in which 90% of the body's granulocytic leukocytes are located (*3*). These leukocytes are capable of undergoing a unique type of oxidative metabolism known as the "oxidative burst" in which numerous lysosomal and peroxidative enzymes, as well as various oxidants including H_2O_2, are released into phagosomes and the extracellular space (*9,10*). One of these enzymes, myeloperoxidase (MPO), accounts for 5% of the dry weight of mature peripheral neutrophils and is a significant cellular component of bone marrow granulocytic cells (*11*). The immature granulocytes located within the bone marrow have, in fact, been shown to contain more MPO than their mature circulating counterparts (*12*). All three principal phenolic metabolites of benzene are potential substrates for MPO (*13*). We therefore decided to investigate the role of this enzyme and its release, together with H_2O_2, in the activation of benzene's phenolic metabolites during the oxidative burst and its possible role in benzene hematotoxic effects.

Activation of Phenol by Peroxidases and Stimulated Human Leukocytes

Phenol has been shown to be a good reducing cofactor in the reduction of H_2O_2 by peroxidases. Early *in vitro* studies (*14,15*) demonstrated that phenol is metabolized by horseradish peroxidase (HRP) to 2,2′-biphenol,4,4′-biphenol and an oxidation product with an UV absorbance maximum at 400 nm, presumably 4,4′-diphenoquinone. The HRP-dependent conversion of phenol to DNA- and protein-binding metabolites has also been reported by several investigators (*16–18*). More recently, studies in our laboratory (*19*) have shown that phenol is converted to covalently binding metabolites during the oxidative burst of stimulated human leukocytes (Table 1). This covalent binding is blocked by either catalase and azide, suggesting roles for both H_2O_2 and peroxidases in the binding process (Table 1).

Table 1. Activation of phenol to covalent binding metabolites during the phorbol myristate acetate-induced oxidative burst of human leukocytes.

Treatment	Covalent binding,[a] nmole phenol equiv./mg protein
Complete[b]	2.7 ± 1.0[c]
+ Catalase[d]	0.5 ± 0.1
+ Azide[d]	0.4 ± 0.2
− PMA	0.1 ± 0.05

[a]Convalent binding to protein was determined after a 20-min incubation as described in Eastmond et al. (19).

[b]Complete incubations contained freshly isolated human leukocytes (10^7 cells/mL), [^{14}C] phenol (30 μM), and PMA (1 μg/mL) in Dulbecco's phosphate buffer, pH 7.1, at 37°C.

[c]Data from Eastmond et al. (*19*). Mean ± SD of three experiments are shown.

[d]Catalase was added at 13,000 U/mL and sodium azide at 10 mM.

We have also shown that extensive phenol binding to protein occurs very rapidly in the presence of human MPO and H_2O_2, as well as with HRP (*20*). This binding is inhibited by the presence of glutathione or ascorbate, most likely because of their ability to act as antioxidants and reduce the phenoxy radical back to phenol (Fig. 2) (*20*). In our studies, 4,4′-biphenol and 4,4′-diphenoquinone were the principal identifiable products, but it is clear that 2,2′-biphenol is also formed (Fig. 2). In fact, a polymerization product of 2,2′-biphenol is likely to be the major DNA-binding species formed from phenol (*17,18*), with 4,4′-diphenoquinone being responsible for a portion of the protein binding observed (*20*). Both the biphenols and 4,4′-diphenoquinone produce a variety of cytotoxic and genotoxic effects on human lymphocytes (*21*) and could potentially be involved in benzene's *in vivo* bone marrow toxicity.

Peroxidase-Dependent Metabolism of Hydroquinone and the Stimulating Effect of Phenol

During studies of the peroxidase-dependent metabolism of phenol (*20*), it was noted that phenol appeared to stimulate the metabolism of 4,4′-biphenol to 4,4′-diphenoquinone. The similarity in structure between 4,4′-biphenol and hydroquinone suggested that phenol may have a similar effect on the peroxidase-dependent metabolism of hydroquinone. Hydroquinone is converted by a two-electron oxidation to 1,4-benzoquinone in a stoichimetric fashion by a process dependent on the presence of HRP or MPO and H_2O_2 (*22*). The removal of hydroquinone during this process is stimulated by the presence of phenol (Fig. 3). The concentration of phenol must be higher than that of hydroquinone, but significant stimulation of HRP-dependent hydroquinone metabolism occurs when the phenol to hydroquinone ratio is as low as 1.33 (Fig. 4*A*). Much more significant effects are observed however when this ratio rises above 10 (Fig. 4*A*).

With MPO-dependent hydroquinone metabolism, the picture is slightly more complex. When 0.55 U/mL MPO is incubated with 75 μM hydroquinone, concentrations of phenol below 5 mM have an inhibitory effect on hydroquinone removal (Fig. 4*B*). A significant stimulatory effect is observed only at phenol concentrations above 10 mM (Fig. 4*B*). If, however, the MPO and hydroquinone concentrations are lowered to 0.3 U/mL and 10 μM, respectively a significant stimulatory effect is observed with phenol concentrations as low as 100 μM (K. L. Steinmetz, D. A. Eastmond, and M. T. Smith, manuscript in preparation). A significant stimulatory effect of phenol on MPO-dependent hydroquinone oxidation can therefore be observed at concentrations that are at least achievable within the bone marrow *in vivo* (*8*).

Similar results to those described, where phenol stimulates the metabolism of hydroquinone, are also observed in rodent bone marrow cell cultures and bone marrow homogenates (*23*) and in lysates from elicited murine peritoneal macrophages (*24*), which contain a peroxidase

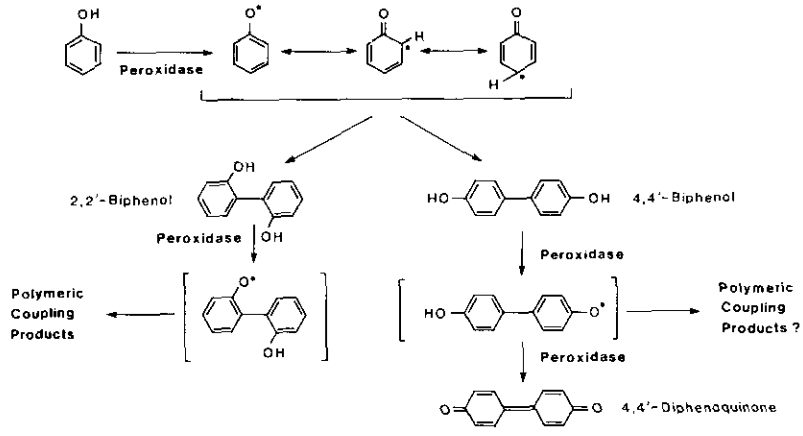

FIGURE 2. Peroxidase-mediated metabolism of phenol to biphenols and 4,4'-diphenoquinone.

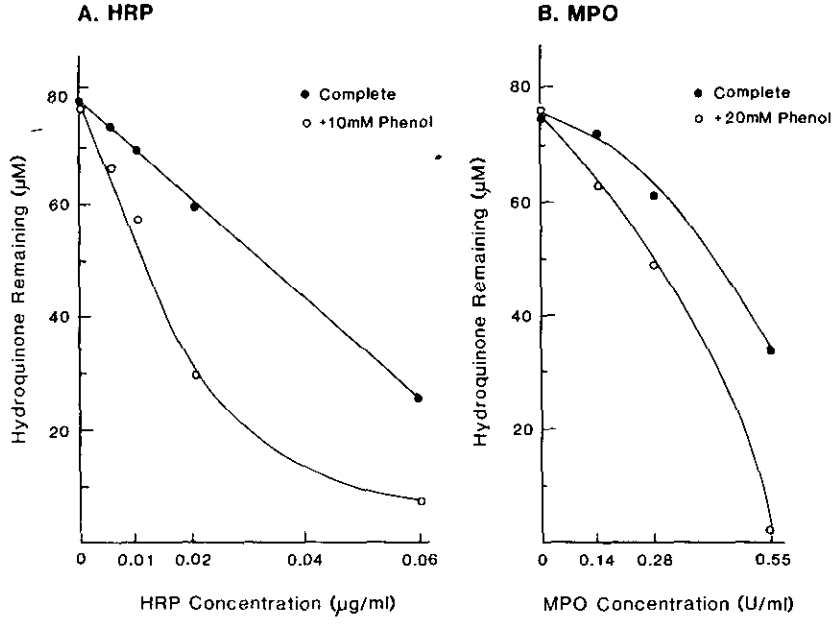

FIGURE 3. Phenol-induced stimulation of hydroquinone metabolism by horseradish peroxidase (*A*) and human myeloperoxidase (*B*). Complete incubations consisted of 75 μM hydroquinone, 1 mM H_2O_2, and variable concentrations of HRP and MPO in 0.1 M phosphate buffer, pH 7.4, at 37°C. The reaction was terminated at 2 min by the addition of 5% trichloroacetic acid. Hydroquinone concentration was measured by HPLC with electrochemical detection as described in Eastmond et al. (*22*).

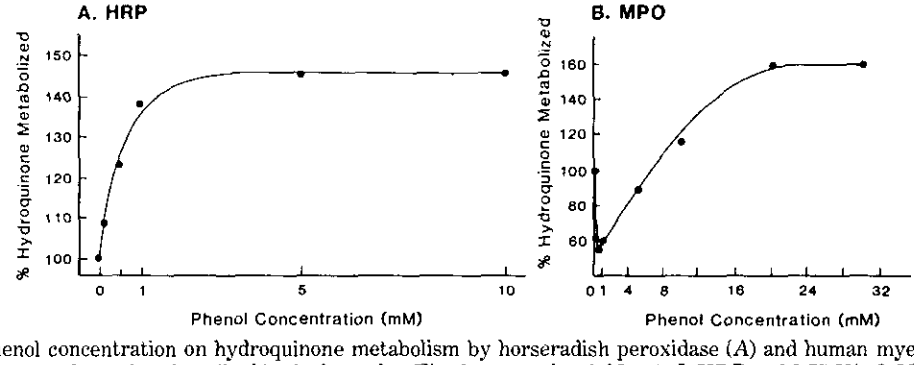

FIGURE 4. Effect of phenol concentration on hydroquinone metabolism by horseradish peroxidase (*A*) and human myeloperoxidase (*B*). Incubations and analyses were performed as described in the legend to Fig. 3 except that 0.06 μg/mL HRP and 0.55 U/mL MPO were used and phenol was added at different concentrations between 0.1 and 32 mM.

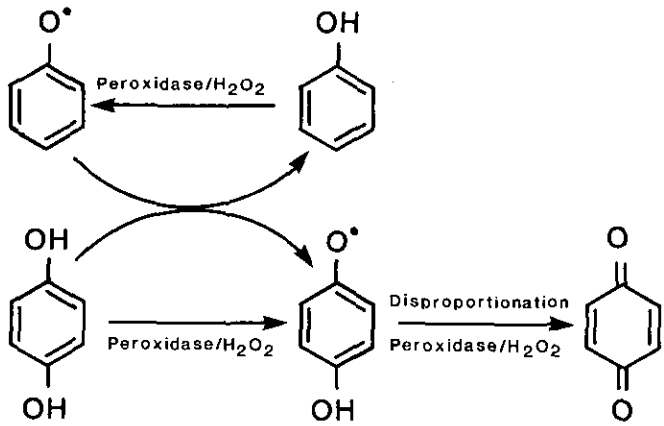

FIGURE 5. Proposed scheme for the phenol-induced stimulation of hydroquinone metabolism.

component. The precise mechanism by which phenol stimulates the oxidation of hydroquinone is unclear. The most likely mechanism, however, would involve the enzymatic oxidation of phenol to its phenoxy radical that would then directly oxidize the hydroquinone to its semiquinone radical (Fig. 5). The latter is the rate-limiting step in the overall oxidation of hydroquinone to 1,4-benzoquinone with the quinone species then being derived from a disproportionation of the semiquinone radicals (Fig. 5). This mechanism is consistent with the observation that phenol is only minimally consumed during the process (*22*).

The stimulatory effects of phenol on hydroquinone oxidation suggested that a similar phenomenon may occur following benzene exposure *in vivo*. In order to test this hypothesis, R. Irons of the Chemical Industry Institute of Toxicology administered combinations of benzene's phenolic metabolites to mice and measured their effect on bone marrow cellularity. The concomitant administration of phenol (75 mg/kg) and hydroquinone (25–75 mg/kg) to $B6C3F_1$ mice twice daily produced a dramatic and significant decrease in bone marrow cellularity. Other combinations of the phenolic metabolites of benzene did not produce similar effects, nor did administration of the metabolites alone (*22*).

Proposed Mechanism of Benzene-Induced Myelotoxicity

The findings described are consistent with the proposed mechanism for benzene-induced myelotoxicity outlined in Figure 6. Briefly, the following events seem to be important in benzene-induced myelotoxicity: a) the hepatic conversion of benzene to phenol and hydroquinone; b) the selective accumulation of these metabolites in the bone marrow; c) the localized phenol-dependent stimulation of hydroquinone oxidation in the bone marrow; and d) the formation of 1,4-benzoquinone in the bone marrow. Although the phenol-induced stimulation of hydroquinone metabolism in the bone marrow can occur *in vitro* and may be important *in vivo*, one cannot also rule out a toxicokinetic interaction between these two compounds in benzene-induced myelotoxicity. An alternative or further contributing factor in the *in vivo* toxicity of coadministered phenol and hydroquinone may be that they compete for conjugating enzymes and co-factors, thereby raising their free concentration in the plasma, and presumably the bone marrow, following coadministration. Studies are presently underway to test the importance, if any, of this potential toxicokinetic interaction.

The proposed mechanism of benzene-induced myelotoxicity outlined in Figure 6 shows MPO to be the activating enzyme and 1,4-benzoquinone as the ultimate toxic metabolite. Two questions are raised immediately by this scheme: Any peroxidase enzyme is likely to be able to catalyze the conversion of hydroquinone to 1,4-benzoquinone, so why is MPO shown as the sole activating enzyme? What role, if any, does another putative toxic metabolite of benzene, i.e., *trans,trans*-muconaldehyde (*25,26*), play since it is omitted from this scheme? Clearly some caveats must be placed on the proposed scheme.

The rodent bone marrow contains at least two distinct peroxidases, myeloperoxidase and eosinophil peroxidase, with different properties (*27*). Either of these two enzymes could potentially activate hydroquinone, as could the hydroperoxidase component of prostaglandin synthase (PGS), which may also be present in the bone marrow. Recent studies demonstrating that benzene-induced myelotoxicity can be ameliorated by the simultaneous administration of indomethacin (*28,29*) are suggestive of a

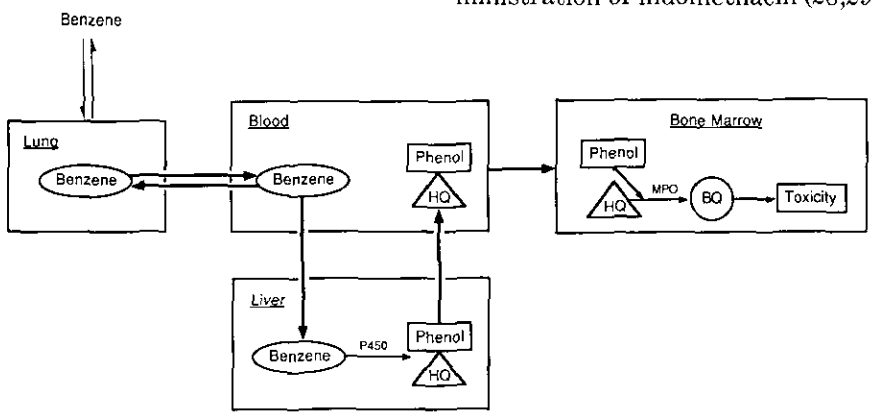

FIGURE 6. Proposed mechanism for benzene-induced myelotoxicity. HQ, hydroquinone; BQ, 1,4-benzoquinone; P450, cytochrome P-450; and MPO, myeloperoxidase.

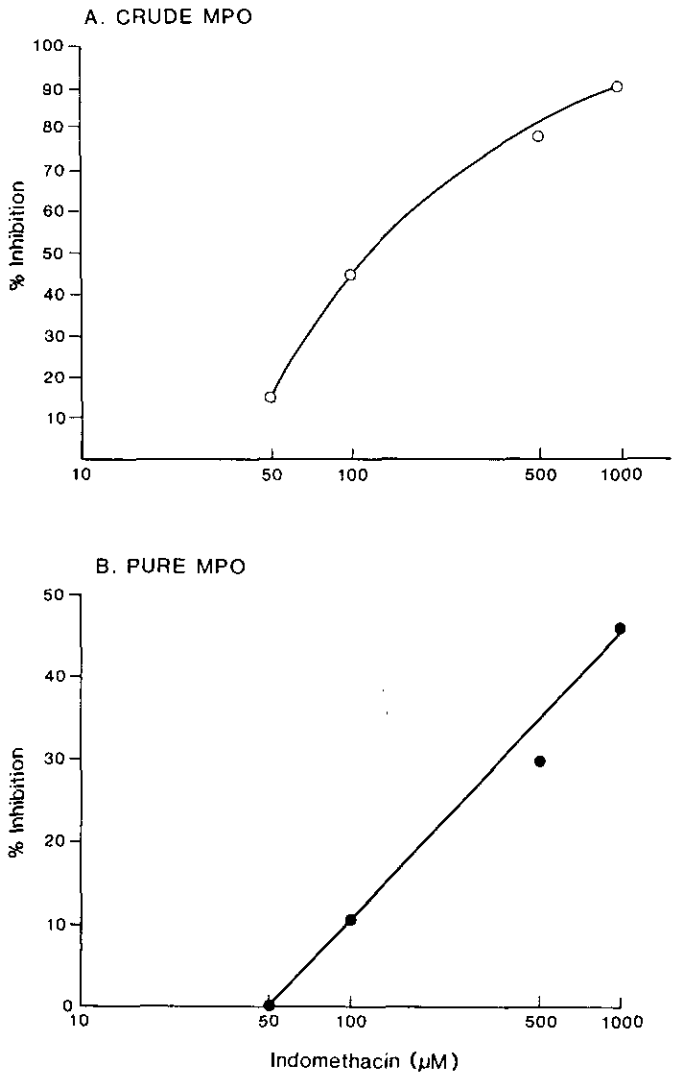

FIGURE 7. Effect of indomethacin on hydroquinone metabolism by crude (*A*) and pure (*B*) human myeloperoxidase. Incubations contained 10 μM hydroquinone, 1 mM H_2O_2, and either 0.2 (crude) or 0.3 (pure) U/mL of myeloperoxidase in 0.1 phosphate buffer (pH 7.4). All incubations were performed at 37°C for 5 min and terminated by addition of trichloroacetic acid. Hydroquinone concentration was measured as described in Eastmond et al. (*22*).

role for PGS. This drug is a known inhibitor of the cyclooxygenase component of PGS but has no effect on the hydroperoxidase component of this enzyme (*30*). Not surprisingly, however, these drugs also have numerous nonspecific effects, including inhibitory effects on the oxidative burst of leukocytes (*31*) and even the MPO-catalyzed oxidation of hydroquinone, where both indomethacin (Fig. 7) and aspirin (data not shown) produce significant inhibitory effects. Caution must therefore be exercised in reaching conclusions derived from data employing these relatively nonspecific agents. The elucidation of the precise roles of MPO, PGS, and other peroxidases in the myelotoxicity of benzene will require further work and is the subject of continuing research in our laboratory. The reportedly high levels of MPO in the bone marrow (*11,12,32*) in contrast to the low levels of bone marrow PGS (*33*) would lend support, however, to a more important role for MPO. In addition, recent experiments have indicated that addition of arachidonic acid, the physiological substrate for PGS, produces only a minimal increase in hydroquinone binding in human and rodent bone marrow *in vitro*, whereas H_2O_2 produces highly significant increases in the amount of hydroquinone equivalents bound (D. Ross, personal communication).

1,4-Benzoquinone and *trans,trans*-Muconaldehyde as Toxic Metabolites of Benzene

The peroxidase-dependent generation of 1,4-benzoquinone in the bone marrow clearly seems to be important for the myelotoxicity observed following phenol and hydroquinone coadministration to mice. It may also be important for benzene's myelotoxic effects, but does not rule out a role for another putative toxic metabolite of benzene, i.e., *trans,trans*-muconaldehyde (*25,26*). Both 1,4-benzoquinone and *trans,trans*-muconaldehyde are α,β-unsaturated diketones and as such may have similar effects on cells. Some of the known effects of 1,4-benzoquinone are listed in Table 2. Clearly, a similar list could be generated for *trans,trans*-muconaldehyde and one cannot rule out an additive role for these two toxic species in benzene-induced toxicity and carcinogenicity.

One effect consistently observed following benzene exposure is the induction of micronuclei (*34–36*). The possibility arises that 1,4-benzoquinone could be responsible for this effect. To test this hypothesis, cultured human lymphocytes were exposed to various concentrations of 1,4-benzoquinone for different time periods. In these cultures, we consistently observed a cytotoxic effect on one subpopulation of cells and no adverse effect on another subpopulation, which went on to replicate normally (Table 3). In another small and highly selective subpopulation, a broad spectrum of genetic damage was observed with the occurrence of multiple micronuclei (Fig. 8). A cell with 4 micronuclei is shown in Figure 8, but as many as 11 micronuclei in a single cell can be observed. In the modified procedure of Fenech and Morley (*37*) a similar pattern of multiple micronuclei formation is classically observed with microtubule inhibitors, such as colchicine and vincristine. As 1,4-benzoquinone has also been shown to

Table 2. Biological effects of 1,4-benzoquinone.

Binds to DNA and protein
Interferes with microtubule assembly
Inhibits DNA and RNA sythesis
Causes single-stranded DNA breaks
Causes SCEs in humans lymphocytes *in vitro*
Causes chromosomal fragmentation during cell division
Inhibits cell division resulting in pyknosis of the chromosomes during metaphase
Interferes with the growth of bone marrow stromal cells
Interferes with lymphocyte blastogenesis and agglutination

Table 3. Effect of 1,4-benzoquinone on human lymphocyte viability and replicative index.[a]

Treatment	Time of addition, hr	% Viable		Replicative index, female
		Male	Female	
Control	24	87	82	—
BQ, 2.5 μM		57	42	—
BQ, 15 μM		31	36	—
Control	38	—	74	1.47
BQ, 2.5 μM		—	56	1.42
BQ, 15 μM		—	37	1.42
Control	41	—	84	1.67
BQ, 2.5 μM		—	49	1.65
BQ, 15 μM		—	35	1.86
Control	43	82	—	1.75
BQ, 2.5 μM		51	—	1.71
BQ, 15 μM		30	—	1.65

[a]Human lymphocytes were grown in RPMI culture media with supplements and harvested onto slides at 72 hr for the measurement of replicative index. Viability was determined as the exclusion of trypan blue. 1,4-Benzoquinone (BQ) was added in DMSO at two different concentrations at the time points shown. Control incubations received DMSO alone.

block the thiol-sensitive GTP binding site on microtubules with remarkable potency (*38*), it is perhaps not surprising to find such an effect. 1,4-Benzoquinone can also form DNA adducts and cause strand breaks in DNA (*39*). It is also mutagenic, at least in V79 cells (*40*). One may therefore conclude that 1,4-benzoquinone could cause a broad spectrum of DNA damage in cells, including mutations, chromosomal breaks, and potentially aneuploidy, and may be responsible for the genotoxic and carcinogenic effects of benzene both in animals and humans.

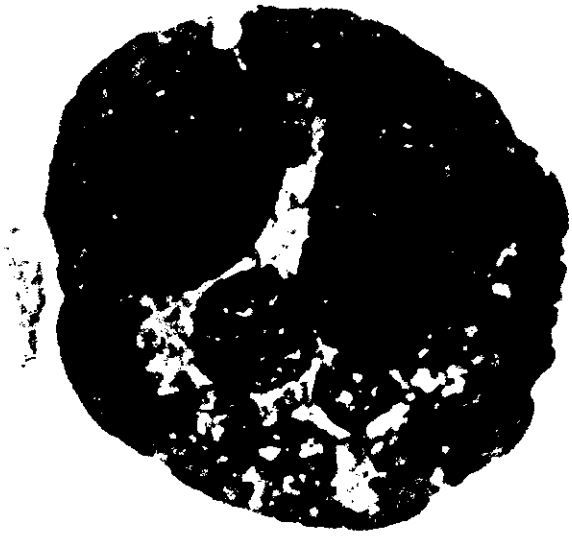

FIGURE 8. Human binucleated lymphocyte showing four micronuclei after treatment with 2.5 μM 1,4-benzoquinone (magnification, ×500). Cells were grown in RPMI culture media with supplements and harvested onto slides at 72 hr. Slides were fixed in methanol and stained with May-Grunewald Giemsa for enumeration of micronuclei in 500 binucleated cells per duplicate culture.

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