Differential Inhibition of DNA Synthesis in Human T Cells by the Cigarette Tar Components Hydroquinone and Catechol

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Hydroquinone (HQ), catechol, and phenol exist in microgram quantities in cigarette tar and represent the predominant form of human exposure to benzene. Exposure of human T lymphoblasts (HTL) in vitro to 50 μM HQ or 50 μM catechol decreased IL-2-dependent DNA synthesis and cell proliferation by >90% with no effect on cell viability. Phenol had no effect on HTL proliferation at concentrations up to 1 mM. The addition of HQ or catechol to proliferating HTL blocked 3H-TdR uptake by >90% within 2 hr without significantly affecting 3H-UR uptake, suggesting that both compounds inhibit a rate-limiting step in DNA synthesis. However, the effects of HQ and catechol appear to involve different mechanisms. Ferric chloride (FeCl3) reversed the inhibitory effect of catechol, but not HQ, corresponding with the known ability of catechol to chelate iron. HQ, but not catechol, caused a decrease in transferrin receptor (TIR, CD71) expression, comparable to the level observed in IL-2-starved cells. HQ also inhibited DNA synthesis in cultures of transformed Jurkat T lymphocytes, primary and transformed fibroblasts, and mink lung epithelial cells, indicating that its antiproliferative effect was not restricted to IL-2 mediated proliferation. However, DNA synthesis by primary lymphocytes was more sensitive to HQ (IC50 = 6 μM) than that of the transformed Jurkat T cell line (IC50 = 37 μM) or primary human fibroblasts (IC50 = 45 μM), suggesting that normal lymphocytes may be particularly sensitive to HQ. The effects of HQ and catechol on DNA synthesis could be partially reversed by a combination of adenosine deoxyriboside and guanosine deoxyriboside, suggesting that both compounds may inhibit ribonucleotide reductase. © 1997 Society of Toxicology.

Although an association between smoking and lung cancer has been evident since the 1930s, only recently have the immunosuppressive effects of smoking been recognized. Haynes et al. (1966) first reported that smoking increased the incidence of respiratory tract infections in adolescents, and Daniele et al. (1977) demonstrated that the proliferative response of T cells from the lungs of cigarette smokers was reduced by >85% compared to nonsmokers. The fact that peripheral T cells from cigarette smokers exhibit normal responses suggests that cigarette smoke contains substances that induce a localized state of immune suppression.

The immunosuppressive effect of cigarette smoke is directly related to the level of tar present (Holt et al., 1976). Tar contains several compounds with demonstrated immunosuppressive activity, including nicotine (Geng et al., 1995) and benzo[a]pyrene (Mudzinski, 1993). In addition, the pyrolysis of tobacco leaf pigments yields up to 100 μg of hydroquinone (HQ), 280 μg of catechol, and 70 μg of phenol per cigarette (Waltz et al., 1965; Hecht et al., 1981; Hoffmann et al., 1983), and cigarette smoking represents the major source of human exposure to benzene (Wallace, 1989). These phenolic derivatives of benzene differ widely in immunosuppressive activity. HQ and catechol inhibit the mitogenic response of rat spleen cells to phytohemagglutinin (PHA) at concentrations below 10 μM, while phenol has no effect even at millimolar concentrations (Pfeifer and Irons, 1981, 1982). It is widely believed that HQ and catechol must be further oxidized to quinones before they are capable of altering most lymphoid responses (Greenlee et al., 1981; Irons, 1985; Bodell et al., 1993). However, we recently observed that HQ blocks IL-2-dependent proliferation of human T lymphoblasts (HTL) without affecting the ability of these cells to produce IL-2 upon mitogenic stimulation (Li et al., 1996). In contrast, p-BQ preferentially inhibits IL-2 production (Li et al., 1996; Post et al., 1985; Freed and Geiselhart, 1997; Geiselhart et al., 1997). Furthermore, HQ did not diminish intracellular glutathione levels and its effect

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Abbreviations used: AdR, CdR, GdR, deoxyribonucleosides; AR, GR, UR, ribonucleosides; p-BQ, para-benzoquinone; HQ, hydroquinone; HTL, human T lymphoblasts; MFI, mean fluorescence intensity; MLE, mink lung epithelium; TIR, transferrin receptor; PE, phycoerythrin.
on DNA synthesis could be reversed by washing, suggesting that its effects are not mediated by the thiol-reactive p-BQ. These observations suggest that HQ may interfere with either an IL-2-mediated event or a rate-limiting step in DNA synthesis.

One of the earliest events induced by IL-2 is the expression of transferrin receptors (TfR, CD71), which facilitate the uptake of extracellular iron. Since TfR expression is necessary for initiation of S phase of the cell cycle (Neckers and Cossman, 1983; Seiser et al., 1993), inhibition of TfR expression could explain the antiproliferative effects of HQ. The purpose of these experiments was to determine if HQ and catechol suppress T cell proliferation by inhibiting TfR expression or by a direct effect on DNA synthesis.

**MATERIALS AND METHODS**

**Chemicals.** HQ, catechol, phenol, hydroxyurea, deoxyribonucleosides, and the ribonucleosides were purchased from Sigma Chemical Co. (St. Louis, MO) and dissolved in sterile distilled water immediately prior to use. Concentrations of HQ, catechol, and phenol were adjusted based on their extinction coefficients at 288, 275, and 271 nm, respectively. Further dilutions were made in PBS. Desferrioxamine and ferric chloride (FeCl₃) (Sigma) were dissolved in distilled water and then diluted into the culture medium.

**Cells.** IL-2-dependent HTL were derived from PHA-stimulated peripheral blood mononuclear cells as previously described (Li et al., 1996). The cells were cultured in complete medium, consisting of RPMI 1640 (BioWhitaker, Walkersville, MD) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 0.075% sodium bicarbonate, 25 μg/ml gentamicin, and 10% fetal bovine serum (FBS). Primary human fibroblasts were obtained from cadaveric pancreas organ donors. Pancreata were stored in ice-cold EuroCollins solution and cannu-lated with a 16-gauge stainless-steel pipetting needle via the main pancreatic duct. The pancreas was dissociated by agitating in a shaking water bath at 110 rpm for 10 mm at 37°C. The released cells were washed four times with balanced salt solution (HBSS). After the organ was distended with the initial 40-60 ml, the remaining volume was delivered with a peristaltic pump (Cole-Palmer, Niles, IL) at a rate of 7 ml/min at 37°C for 60 min. The pancreas was dissociated by agitating in a shaking water bath at 110 rpm for 10 min at 37°C. The released cells were washed four times with ice-cold HBSS at 4°C and cultured in DMEM supplemented as described for 3T3 fibroblasts. Primary fibroblasts were used up to 10 passages.

**Measurement of viability.** Cell viability was determined by a modification of the method of Duke et al. (1994). The cells were stained with 10 μg/ml each of acridine orange (Sigma) and ethidium bromide (Fisher Biotech) for 5 min and enumerated using a fluorescent microscope.

**IL-2 production.** HTL were cultured in complete medium at 10⁵ cells/ml and stimulated with 3 nm phorbol-12-myristate-13-acetate (PMA, Sigma) and 1 μM A23187 (Sigma) for 24 hr. Supernatants were then collected and the IL-2 levels were determined in a bioassay using the IL-2-dependent murine HT-2 cell line as previously described (Li et al., 1996).

**DNA and RNA synthesis.** HTL were cultured in 96-well plates at 2 × 10⁵ cells/well in 200 μl of complete medium with or without 20 units/ml of rIL-2. HQ, catechol, or phenol were added at the initiation of the cultures. The cells were then pulsed at the times shown in the figures with 0.6 μCi/ml of [3H]Tdr or 1.2 μCi/ml of [3H]UR for 2 hr, frozen and thawed, treated with 0.4% NP-40, and then harvested onto glass fiber filters using a cell harvester (Skatron Inc., Sterling, VA). Radioactivity was quantitated by liquid scintillation spectrophotometry. Mouse 3T3 fibroblasts, human pancreatic fibroblasts, and mink lung epithelial cells were detached by 0.5% trypsin-EDTA (Sigma) and washed with medium plus 10% FBS before plating into 96-well plates. Mouse 3T3 fibroblasts and human pancreatic fibroblasts were cultured at 2 × 10⁴ cells/well in 200 μl of complete medium with 10% FBS. Mink lung epithelial (MLE) cells were cultured at 10⁴ cells/well. The cells were cultured for 24 hr prior to the addition of phenol, catechol, or HQ in order to allow the cells to attach and initiate growth. Phenol, catechol, or HQ was added to the cultures, which were then pulsed for 2 hr with 0.6 μCi/ml of [3H]Tdr or 1.2 μCi/ml of [3H]UR, harvested, and quantitated as described for HTL.

**Measurement of transferrin receptor.** HTL were stained with anti-Cd71 antibody (Becton-Dickinson, San Jose, CA) followed by phycoerythrin (PE)-conjugated rat anti-mouse IgG (Becton-Dickinson, San Jose, CA) followed by phycoerythrin (PE)-conjugated rat anti-mouse IgG (Becton-Dickinson, San Jose, CA). The cells were analyzed by flow cytometry using CellQuest software.

**Statistical analysis.** All values for statistical significance were derived by the Student's t test unless otherwise indicated.

**RESULTS**

**Effects of HQ, catechol, and phenol on IL-2-dependent lymphoblast proliferation.** Both HQ and catechol inhibited IL-2-dependent HTL proliferation in a dose-dependent manner (Fig. 1). At 50 μM, catechol and HQ blocked [3H]-Tdr...
uptake by 80–90% (p < 0.001) without affecting IL-2 production or cell viability. The inhibition of 3H-TdR uptake correlated with >90% inhibition of cell proliferation, as determined by counting the cells with a Coulter counter (data not shown). In contrast, 50 μM phenol did not inhibit HTL proliferation, IL-2 production, or viability and had no effect on these T cell functions at concentrations up to 1 mM (data not shown).

The antiproliferative effect of catechol is reversible by iron. Catechol is a known iron chelator (Avdeef et al., 1978) that might be expected to inhibit lymphocyte proliferation by removing iron. In order to test this hypothesis, we attempted to block the effect of 50 μM catechol by treating HTL in the presence of 100 μM FeCl3. As a positive control, HTL were treated with an equal concentration of another known iron chelator, desferrioxamine. As can be seen in Fig. 2, FeCl3 reversed the antiproliferative effects of both desferrioxamine and catechol. However, FeCl3 did not block the effect of HQ.

The inhibition of DNA synthesis by catechol was more rapid than that of desferrioxamine. As can be seen in Fig. 3, 50 μM catechol caused an immediate cessation of 3H-TdR uptake, while desferrioxamine required >2 hr to produce the same level of inhibition. The difference in kinetics is presumably due to the fact that, unlike catechol, desferrioxamine is membrane-impermeant and therefore limited to chelating extracellular iron (Richardson et al., 1994). It was interesting to note that despite differences in the mode of action of catechol and HQ, the kinetics of the catechol effect were identical to those previously observed with HQ (Li et al., 1996).

HQ preferentially inhibits DNA synthesis. Although HQ blocked 3H-TdR uptake by >90% within 2 hr after its addition to the cultures, suppression of 3H-UR uptake was not statistically significant at this time point (Fig. 4). This experiment indicates that the effect of HQ on 3H-TdR uptake was not an artifact of decreased nucleoside transport and suggests HQ preferentially inhibits DNA, rather than RNA, synthesis. The inhibition of 3H-UR uptake observed after 24 hr was probably secondary to the overall suppression of HTL proliferation.

HQ inhibits DNA synthesis in primary and transformed cells. The antiproliferative effect of HQ was not specific to IL-2-dependent T cells. As can be seen in Fig. 5, HQ also inhibited 3H-TdR uptake by the transformed Jurkat T cell line, transformed 3T3 mouse fibroblasts (3T3), MLE cells and, to a lesser extent, primary human fibroblasts without

![FIG. 2. Iron salts reverse the inhibitory effects of catechol on HTL proliferation. HTL were cultured with 50 μM desferrioxamine (Def), 50 μM catechol (Cat), or 50 μM HQ in the presence or absence of 100 μM FeCl3 for 24 hr. Proliferation of the cells was compared to the control (untreated). The data represent the mean ± SD of four experiments.](image1)

![FIG. 3. Kinetics of the inhibitory effects of catechol and desferrioxamine on DNA synthesis. HTL were treated with 50 μM catechol (O) or desferrioxamine (●). At the different time points indicated, cells were pulsed with 3H-TdR for 2 hr before harvesting. The data represent the mean ± SD of three separate experiments.](image2)

![FIG. 4. HQ has an immediate inhibitory effect on DNA synthesis but not RNA synthesis. HTL were treated with 50 μM HQ (■) and immediately pulsed with 3H-TdR or 3H-UR for 2 hr and then harvested as described under Materials and Methods. Cells were also cultured with 50 μM HQ for 24 hr before pulsing and harvesting. Negative and positive controls consisted of HTL cultured without rIL-2 (□) or with rIL-2 (■), respectively. The results represent the mean ± SD of six samples performed in two separate experiments. At 2 hr, the inhibition of DNA synthesis by HQ was significant (p = 0.02), whereas RNA synthesis was not (p = 0.27). After 24 hr incubation, the inhibition on both DNA and RNA synthesis were significant (p = 0.003 and p = 0.02 for DNA and RNA synthesis, respectively) by the paired Student's t test.](image3)
TfR expression from 126 ± 27 MFI to 51 ± 5 MFI (p <

FIG. 5. Effect of HQ on the proliferative response of normal and transformed cells. HTL (○), Jurkat T cells (△), 3T3 fibroblasts (■), mink lung epithelial cells (□), and primary human pancreatic fibroblasts (●) were treated with HQ and then pulsed with 3H-TdR for 2 hr. The cells were then harvested onto glass fiber filters and counted. Data are presented as the mean percentage of control of the untreated HTL (9665 ± 2428 cpm), Jurkat (33,580 ± 1804 cpm), 3T3 (17,230 ± 6518 cpm), MLE (4232 ± 1531 cpm), or human pancreatic fibroblasts (1771 ± 124 cpm). The data represent the mean ± SD of three experiments.

Inhibition of DNA synthesis by HQ and catechol is partially reversed by deoxyribonucleosides. The observation that HQ preferentially inhibits DNA synthesis suggested that it might interfere with ribonucleotide reductase, a rate-limiting iron-dependent enzyme that catalyzes the reduction of ribonucleotides to deoxyribonucleotides. Treatment of proliferating cells with hydroxyurea has been shown to cause a depletion of dATP and dGTP without decreasing dTTP and dCTP levels (Snyder, 1984). We therefore attempted to block the inhibitory effect of HQ and catechol by treating the cells with adenosine deoxyribose (AdR) and guanosine deoxyribose (GdR). The corresponding ribonucleosides (AR and GR) were used as controls. As can be seen in Table 1, the combination of 0.1 mM AdR and 0.1 mM GdR reduced the inhibitory effects of HQ and hydroxyurea to comparable levels (35–37% of the control; p < 0.005), while the corresponding ribonucleotides had no effect. The addition of 1 μM CdR to the mixture of AdR and GdR did not further reverse the inhibitory effect of HQ (data not shown). AdR and GdR also partially reversed the effects of catechol, which is consistent with the role of iron in ribonucleotide reductase activity.

Effect of HQ and catechol on transferrin receptor expression. As shown in Fig. 6, removal of IL-2 from the culture medium for 24 hr resulted in a decrease in cell-surface TfR expression from 126 ± 27 MFI to 51 ± 5 MFI (p <

DISCUSSION

Although the immunosuppressive effects of catechol and HQ have been known for more than a decade, it had been widely assumed that these benzene derivatives had to be further oxidized to quinones in order to exert an immunosuppressive effect (Greenlee et al., 1981; Post et al., 1985; Irons and Neptun, 1980; Irons et al., 1981; Pellack-Walker et al., 1985; Kalf et al., 1987; Luster et al., 1987). While the quinones are more toxic to lymphocytes than the corresponding hydroxylated benzenes, we have clearly demonstrated that HQ and catechol have potent antiproliferative effects on human T cells at concentrations that do not cause cell death. Unlike previous studies that measured proliferation in mitogen-stimulated cultures, we analyzed the early (IL-2 production) and late (DNA synthesis) events separately. Neither HQ nor catechol significantly inhibited IL-2 production, yet both were capable of inducing an immediate cessation of DNA synthesis. In contrast, p-BQ preferentially inhibits IL-2 production and does not affect DNA synthesis at nonlethal concentrations (Li et al., 1996).

The extent to which HQ is oxidized in lymphocytes may be significantly different from that in myeloid cells. Myeloid cells contain high levels of enzymes (myeloperoxidase and prostaglandin H synthetase) that are capable of catalyzing the oxidation of HQ to p-BQ (Smith et al., 1989; Schlosser et al., 1989; Kettle and Winterbourn, 1992). Many of the effects of HQ on these cells, including inhibition of IL-1...
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TABLE 1
Reversal of the Inhibitory Effect of HQ and Catechol by Deoxyribonucleosides

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*HTL were incubated for 2 hr with 50 μM HQ or 1.5 mM hydroxyurea in the presence or absence of the various nucleosides (0.1 mM) and 0.6 μCi of 3H-TdR. The cells were then harvested and counted by liquid scintillation. The proliferative response for the controls shown above was 6,110–10,205 cpm. The data are presented as the mean ± SD of six experiments.

production, suppression of phagocytosis, and the formation of protein and DNA adducts, have been attributed to p-BQ (Lewis et al., 1988; Renz and Kalf, 1991; Subrahmanyam et al., 1991). In contrast, lymphocytes have very low levels of peroxidases and may therefore be less able to oxidize HQ to p-BQ. In support of this hypothesis, we noted that HQ did not diminish GSH levels in lymphocytes and its antiproliferative effect could be reversed by washing (Li et al., 1996). Thus, the primary effect of HQ on lymphocyte proliferation appears to be due to the hydroxylated benzene rather than the quinone.

Although catechol and HQ exhibited nearly identical effects on HTL, the two compounds appear to have different mechanisms of action. FeCl₃ completely reversed the inhibitory effects of catechol, but it had no effect on the antiproliferative properties of HQ (Fig. 4). The ortho position of the hydroxyl groups in catechol allows it to chelate iron with an equilibrium constant of $10^{13}$ (Avdeef et al., 1978), and catechol has been shown to inhibit ribonucleotide reductase activity by mobilizing iron from the iron-binding R2 subunit (Atta et al., 1993; Swarts et al., 1995). Our data support this observation, since the inhibitory effect of catechol could be partially reversed by the addition of deoxyribonucleosides. In contrast, the para position of the HQ hydroxyl groups in HQ does not permit iron binding, and the antiproliferative effect of HQ consequently could not be reversed with FeCl₃. Nevertheless, HQ also appears to inhibit ribonucleotide reductase, as evidenced by the fact that its effect on DNA synthesis was partially reversed by deoxyribonucleosides but not by the corresponding ribonucleosides (Table 1). Although the reversal did not exceed 40%, it was nevertheless equivalent to that observed in cells treated with 1.5 mM hydroxyurea, a known inhibitor of ribonucleotide reductase. In addition, HQ caused an immediate cessation in 3H-TdR.
incorporation rather than suppressing \(^{3}\text{H}-\text{UR}\) uptake, further suggesting that it preferentially blocks a rate-limiting step in DNA synthesis.

We hypothesize that HQ hydroxyl groups might be capable of quenching the tyrosyl radical in the M2 subunit of ribonucleotide reductase in a manner similar to that proposed for hydroxyurea and other hydroxy compounds (Petersson et al., 1980; Swarts et al., 1995). The tyrosyl radical promotes the abstraction of an electron from the ribose sugar (Reichard and Ehrenberg, 1983) and is critical for enzyme activity. The inability of phenol to inhibit HTL proliferation could be due to the fact that phenol has a sevenfold lower reduction potential than HQ (Pellack-Walker et al., 1980). Similarly, the fully oxidized p-BQ is incapable of donating an electron to quench the tyrosyl radical, and this may explain why p-BQ does not inhibit HTL proliferation. Experiments are now in progress to measure the ability of HQ to quench the tyrosyl radical.

The effect of HQ on TfR expression appears to be separate and distinct from its effects on DNA synthesis. HQ blocks \(^{3}\text{H}-\text{TdR}\) incorporation in proliferating HTL prior to a decrease in cell-surface expression of the TfR, suggesting that it has a direct effect on DNA synthesis. However, when HTL were starved of IL-2 for 48 hr, HQ blocked the de novo expression of these TfR after the readdition of IL-2.

FIG. 7. HQ blocks de novo induction of TfR. HTL were starved of IL-2 for 2 days (starting on Day 0) to allow cell-surface expression of TfR to drop to baseline levels. The cells were then reincubated on Day 2 in fresh medium with 20 units/ml of IL-2 (□), fresh medium without IL-2 (□), or fresh medium with IL-2 plus 50 \(\mu\)M HQ (loit). In the top panel the cells were pulsed with \(^{3}\text{H}-\text{TdR}\) at the times shown and harvested 24 hr later. The bottom panel shows the level of TfR (CD71) expression on each day, coinciding with the start of the \(^{3}\text{H}-\text{TdR}\) pulse. The data represent the mean ± SD of six samples performed in two separate experiments.

Since TfR expression preceded DNA synthesis, this experiment suggests that HQ also inhibits an IL-2-mediated event regulating cell-surface expression of TfR. Thus, the overall immunosuppressive effect of HQ may be due to separate effects on DNA synthesis and TfR expression.

The inhibition of DNA synthesis by HQ was not limited to lymphocytes, although they appear to be considerably more sensitive than fibroblasts and transformed lymphocytes. While it is possible that the increased sensitivity is merely an in vitro artifact, we could not attribute it to different rates of \(^{3}\text{H}-\text{TdR}\) incorporation by the various cell lines. The simplest explanation is that the level of ribonucleotide reductase varies between these cell lines, and Tagger and Wright (1984) have reported a 3.5-fold increase in transformed fibroblasts compared to the normal strains from which they were derived. However, further experimentation is required to determine if this is true in lymphocytes as well.

The inhibitory effect of catechol on DNA synthesis appears to be entirely a function of chelating iron, since the addition of FeCl\(_3\) completely reversed the effect. Iron is an important cofactor in ribonucleotide reductase activity, and it was therefore not surprising that the effect of catechol was at least partially reversed by the addition of deoxyribonucleosides. However, iron depletion has been reported to block the appearance of cyclin A and p34cdc2 proteins in human T cells (Terada et al., 1993; Lucas et al., 1995), suggesting that catechol may also affect earlier events that control cell cycle progression from G\(_0\)/G\(_1\) to S phase.

The immunosuppressive potential of HQ and catechol in cigarette tar appears to be substantial. A single cigarette deposits up to 100 \(\mu\)g of HQ and 280 \(\mu\)g of catechol in the lungs (Hoffmann et al., 1983; Waltz et al., 1965). Since the volume of fluid in the lung is relatively small, the concentration of HQ and catechol in the lungs of cigarette smokers probably exceeds the 50 \(\mu\)M solutions used in our experiments. Experiments are now in progress to determine the effects of HQ on ribonucleotide reductase activity and TfR gene transcription/translation.

ACKNOWLEDGMENT

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REFERENCES


