ABSTRACT

Three established human colon carcinoma cell lines (LoVo, SW620, and SW403) with different degrees of phenotype differentiation were investigated for their sensitivity to the cytotoxic effects of cyclophosphamide (CP) and to its active metabolite, 4-hydroxy-cyclophosphamide (4-OH-CP), and for their mixed function oxidation (MFO) activities. None of the cell lines showed sensitivity to CP as determined by the inhibition of colony formation assay, even after continuous drug treatment at high concentrations (200 µg/ml) for up to 72 h. CP also had no effect on the cellular doubling time or on the incorporation of [3H]-thymidine. Pretreatment with phenobarbital (PB) plus hydrocortisone (HC) was unable to induce CP cytotoxicity. In contrast, 4-OH-CP, the major metabolite formed from CP by MFO, was highly toxic to the cells. About 90% cell kill was obtained at drug concentrations of 17.5 µg/ml (LoVo), 15 µg/ml (SW620), and 55 µg/ml (SW403) after 1-h incubation at 37°C. MFO activities were determined by measuring p-nitroanisole demethylase (PNAD) and aryl hydrocarbon hydroxylase (AHH) in microsomes prepared from noninduced cells or from cells treated with benzo[a]pyrene or PB plus HC. Intrinsic AHH activities were below the level of detection for all cell lines. Treatment with benzanthracene resulted in AHH activities of 12 to 15 pmol of 3-OH-BP per min per mg of protein, but treatment with PB plus HC failed to induce significant AHH activities. PNAD activities in noninduced cells as well as in cells treated with benzo[a]pyrene were 0.05 to 0.08 nmol of p-nitrophenol formed per min per mg of protein; treatment with PB plus HC increased PNAD activities by only 1.5-fold. Thus, in contrast to reports for rat colon and for a single human colon cancer cell line, CP is inactive when applied directly to several other human colon carcinoma cell lines. Because these cells have minimally detectable intrinsic and induced MFO activities, we conclude that CP cannot be successfully metabolized into 4-OH-CP to induce a significant degree of cell kill.

INTRODUCTION

CP, a cytotoxic immunosuppressive and chemotherapeutic agent, has been used to treat a gamut of human tumors. Native CP exerts minimal alkylating activity (1-4) and is ineffective when applied directly to both normal and neoplastic cells in vitro (1, 2, 5, 6). Several investigators have demonstrated that CP must first be hydroxylated to 4-OH-CP in order to exert its cytotoxic effect. CP activation occurs via the cytochrome P-450-dependent MFO enzyme system present principally in the microsomal fraction of the liver (2, 7-9), but also in many extrahepatic tissues (10-12). Fang and Strobel reported the existence of this drug metabolizing system in the microsomes isolated from the mucosal layer of rat colon and demonstrated the inducibility of this system after pretreatment of experimental animals with PB plus HC, β-naphthoflavone, BA, and 3-methylcholanthrene (13, 14). These investigators further demonstrated the presence and inducibility of the cytochrome P-450-dependent activating system in the human colon cancer cell line LS174T (15). This cell line was shown sensitive to CP alone, as well as to various combinations of CP plus MFO inducers, as judged by the decrease in the number of exponentially growing cells in short-term culture, and by inhibition of [3H]dThd incorporation (15).

Our laboratory has categorized several established human colon carcinoma cells into three major phenotypic groups based on their morphology, degree of differentiation, CEA production, growth kinetics, and capacity for heterotransplantation into nude animals (16). These cell lines can be used to evaluate the lethal efficacy of antitumor agents using the colony formation technique (16, 17). Thus, we were interested in investigating the correlation between the intrinsic induced activities of the cytochrome P-450-dependent MFO system present in these cell lines and their in vitro sensitivity to CP and its commital metabolite, 4-OH-CP, which exists in equilibrium with its open-ringed tautomer, aldoxorubicin (18, 19), in order to extend and generalize Fang and Strobel’s findings to other colon cancer lines. However, our results, obtained using several in vitro cytotoxicity assays, have shown that CP is ineffective in three such cell lines by virtue of their minimal intrinsic and inducible MFO activities. Therefore, we conclude that local activation-induction of CP by colon carcinoma would not prove to be a valid chemotherapeutic venture.

MATERIALS AND METHODS

Cell Lines. Studies were performed on three permanently established human colorectal cancer cell lines representing three biological groups with distinct phenotypic expressions (16): Group I (well-differentiated and moderate production of CEA) consisted of line LoVo; Group II (intermediate differentiation and low production of CEA) was represented by line SW620; and Group III (undifferentiated and high CEA production) was represented by line SW403. LoVo cells, established at the University of Texas, M. D. Anderson Hospital in 1972, are propagated as monolayer cultures at 37°C in a 5% CO₂ atmosphere in Ham’s F-10 medium supplemented by 20% fetal bovine serum, vitamins, glutamine, and antibiotics (20). Both of the SW lines were obtained from the Scott and White Clinic, Temple, TX, and they were maintained in a 1% CO₂
atmosphere in L-15 medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics (21).

Cell Survival. Stock cultures of each cell line were harvested by procedures reported previously (16) and counted with the aid of an electronic particle counter (Model ZBI Coulter Counter; Coulter Electronics, Inc., Hialeah, FL). Cell suspension aliquots were seeded into 60-mm Petri dishes (5 x 10^6 cells/dish). The cells were incubated for 48 to 72 h to achieve exponential growth. The medium was discarded, and the cells were exposed to increasing drug concentrations at 37°C for 1 h (and for 24 to 72 h for CP only). The drug was decanted, and the cells were washed twice in Hanks' balanced salt solution, harvested as a monodispersed suspension, and counted. Known aliquots of each cell suspension were dispensed into 60-mm Petri dishes so that 50 to 100 colonies would appear after 21 days of incubation in a humidified incubator at 37°C. The colonies were stained with 2% crystal violet in 5% ethanol and scored under a stereomicroscope. Viability was defined as the ability of single cells to give rise to a colony ≥ 50 cells. In each experiment, the plating efficiency of at least six control cultures was assessed simultaneously. Control cultures consisted of cells treated in exactly the same manner as the test cells but without receiving the drug. The survival fractions for the different drug concentrations were normalized with respect to the individual controls for each experiment. All experiments were repeated at least twice with triplicate samples for each drug concentration.

In other experiments, CP cytotoxicity was tested on MFO-induced cells. Induction of MFO was performed by treating with a fresh mixture of 3 ml of PB (75 μg/ml) and HC (50 μg/ml) every 24 h for a total of 72 h as detailed below. Controls were cells treated with fresh medium alone and with induction mixture alone but no subsequent drug treatment.

Growth Kinetics. To test the effect of CP and 4-OH-CP on population growth kinetics, exponentially growing LoVo, SW620, and SW403 cells were treated either for only 1 h followed by reincubation with fresh medium, or continuously by replenishing every 24 h with medium containing freshly dissolved drug. Drug concentrations were selected for each cell line based on the degree of cell kill (25%, 50%, and 90%) as determined by inhibition of colony formation. Two cell counts per duplicate plate per time point were made 1 h following addition of drug, and every 24 h thereafter for a total of 96 to 120 h. Cell counts were then compared with those of duplicate control plates (medium only) run simultaneously at each time point.

Incorporation of [3H]dThd. Cells were allowed to enter exponential growth and used without further manipulation or induced with PB and HC as described below. The medium was decanted, and the cells were treated with either CP or 4-OH-CP for 1 h at 37°C. Concentrations were selected as described in the preceding paragraph. The drug solution was decanted, and the cells were washed with 0.9% NaCl solution and reincubated with fresh medium containing [3H]dThd (1 μCi/ml; specific activity, 60 μCi/mmol). Duplicate dishes were harvested after 1-, 5-, 24-, and 48-h incubation. The resulting cell suspensions were washed twice with 0.9% NaCl solution, recentrifuged, and treated with 5% cold TCA for 10 min at 4°C. The acid-insoluble precipitate was centrifuged, washed twice with cold 5% TCA, and dissolved in 1 ml of 0.1 M NaOH. Incorporated [3H]dThd was determined with a Beckman Model LKB800 liquid scintillation counter (Beckman Instruments, Inc., Irvine, CA). Results were expressed as the ratio of [3H]dThd incorporated by the drug-treated cells to that of control cells.

Induction of Cytochrome P-450-dependent MFO. Cells were allowed to enter exponential growth, and sets of twenty 100-mm Petri dishes were used to test each inducer. The medium was removed, and each dish received 5 ml of fresh medium containing either HC (50 μg/ml) plus PB (75 μg/ml), or BA (10 μM). The medium was replenished every 24 h for a total of 3 to 5 days with freshly dissolved inducer. Twenty control dishes exposed to fresh medium only were processed concurrently. After the induction period, cells were removed from dishes by scraping with a rubber policeman and transferred into cold Tris-HCl buffer (10 mM Tris-HCl, 0.14 M KCl-10 mM EDTA-1 mM dithiothreitol, pH 7.4). Cells were sedimented by centrifugation and resuspended in Tris-HCl buffer. Phenylmethylsulfonyl fluoride was added for a final concentration of 0.25 mM before disrupting the cells by sonication (Model W-225R, Heat Systems-Ultrasonics, Inc., Plainview, NY) using a microtip at 80 W for 1 to 1.5 min at 4°C. The homogenate was centrifuged at 9,000 x g for 10 min in a Sorval centrifuge equipped with an SM-24 rotor (Sorval RC-5B; Du Pont Instruments, Wilmington, DE), and the microsomal fraction was isolated from the resulting supernatant by ultracentrifugation at 105,000 x g for 45 min (Model 15-65; Beckman Instruments, Inc., Irvine, CA). The pellet was resuspended in 0.15 M KCl-10 mM EDTA, pH 7.4, by homogenization and resedimented in 0.25 M sucrose-1.0 mM EDTA, pH 7.8. Protein content was determined according to the method of Lowry et al. (22) using bovine serum albumin as standard. MFO (AHH and PNAD) activities were determined at 37°C in a mixture that contained microsomal protein and an NADPH-generating system consisting of 50 mM Tris-HCl, pH 7.8, 5 mM MgCl2, 5 mM isocitrate, 1 mM NADP+, and sufficient isocitrate dehydrogenase to reduce 0.64 μmol of NADP+ per min per ml of mixture. AHH activity was determined by the method of Dehne et al. (23). In addition to the NADPH-generating system, the incubation mixture contained 60 μM benzo[a]pyrene and 0.1, 0.3, or 0.5 μg of microsomal protein per ml. After exactly 5 min, the formation of fluorescent phenolic products was quantified in a Perkin-Elmer MFP-44A fluorescence spectrophotometer (Norwalk, CT) using authentic 3-hydroxybenzo[a]pyrene as a standard. The O-demethylation of p-nitroanisole was determined essentially as described by Moldeus et al. (24). Mixtures contained microsomal protein (1 mg/ml), the NADPH-generating system, and 3.5 mM p-nitroanisole. Reactions were stopped after 15 and 30 min by the addition of 50% TCA. The precipitate was pelleted by centrifugation, and 1 ml of the clear supernatant was transferred to tubes containing 150 μl of 3 M Tris-HCl, pH 9.0. Absorbance was measured in a Beckman DU-8 UV-visible spectrophotometer (Beckman Instruments, Inc., Irvine, CA) at 400 nm and quantified using p-nitrophenol as a standard.

Drugs. CP (Cytoxan) was obtained from Mead Johnson and Co. (Evansville, IN), and PB-sodium was from Wyeth Laboratories, Inc. (Philadelphia, PA). HC-sodium was supplied by Abbott Laboratories (North Chicago, IL), and PNAD was from Aldrich Chemical Co., Inc. (Milwaukee, WI). 4-OH-CP was supplied by Asta-Werke (Degussa Pharma Gruppe, Biefield, West Germany). Authentic 3-hydroxybenzo[a]pyrene was obtained from the National Cancer Institute, Bethesda, MD. The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): p-nitrophenol; 1,2-BA benzo[a]pyrene; N-isocitric acid; isocitrate dehydrogenase (type IV from porcine heart, 4.2 units/mg of protein); and NADP (yeast-β-NAD).

RESULTS

Cell Survival. Survival of LoVo, SW620, and SW403 cells as a function of increasing concentrations of CP or 4-OH-CP administered to the cells for 1 h is presented in Chart 1. The survival curves of all cell lines treated with 4-OH-CP exhibit a type C
MFO ACTIVITIES OF COLON CARCINOMA IN ACTIVATION OF CP

(Threshold exponential) pattern typical of radiomimetic agents (25). The curves corresponding to the well- and moderately well-differentiated cell lines show similar numerical characteristics (LoVo: D0 3 μg/ml, and D6 7 μg/ml; SW620: D0 3.5 μg/ml, and D6 6 μg/ml), while the poorly differentiated line SW403 is considerably more resistant (D0 24 μg/ml, and D6 14 μg/ml). Thus, at a concentration of 25 μg/ml, only 1% and 0.75% survival values are noted, respectively, for LoVo and SW620 cells, while for SW403, survival is decreased by less than 1 log. In contrast to the lethal activity exhibited by its commital metabolite, CP failed to induce any degree of cell kill. All cell lines demonstrated fully 100% survival, even when treated with concentrations as high as 200 μg/ml and for incubation intervals as long as 72 h. Induction of MFO activity failed to activate CP. All cell lines showed complete survival following the subsequent treatment with CP concentrations ranging from 25 to 200 μg/ml. Induction treatment alone did not affect cell survival (data not shown).

Growth Kinetics. Cell counts of LoVo and SW620 cells (CP alone, CP plus inducer, and 4-OH-CP) performed 1 h after drug treatment and daily for the 5 subsequent days showed no significant changes from untreated controls (data not shown). The maximal difference (14%) was observed between control counts and those of SW620 cells treated with 4-OH-CP [15 μg/ml (90% cell kill)] on Day 5. SW403 cells treated with 4-OH-CP [32 μg/ml (50% cell kill) and 55 μg/ml (90% cell kill)] showed a difference of 55% and 65% from control, respectively. Both CP-treated and CP plus inducer-treated SW403 cells exhibited a 26% decrease from control counts.

Incorporation of [3H]dThd. The effect of CP (200 μg/ml) and that of increasing concentrations of 4-OH-CP on the incorporation of [3H]dThd by LoVo, SW620, and SW403 cells is shown in Chart 2. The selected concentrations of 4-OH-CP for each cell used were based on their yield of 25%, 50%, and 90% cell kill as determined by colony formation. CP was used at the highest concentration utilized in cell survival analysis.

After 1-h treatment, all 3 concentrations of 4-OH-CP induced a similar decrease in [3H]dThd incorporation (60 to 65% of control) by LoVo cells. Removal of drug and reincubation with fresh medium were accompanied by prompt restitution to control values. LoVo cells treated with CP displayed a similar incorporation pattern to that of cells treated with 4-OH-CP, and pretreatment with MFO-inducing agents did not change results obtained with CP treatment alone.

For both SW620 and SW403 cells, treatment with CP of either induced or noninduced cells failed to change the pattern of [3H]dThd incorporation with respect to that of control cells. Treatment of SW620 cells with 4-OH-CP induced a dose-dependent decrease in incorporation. Treatment of SW403 cells with the same drug also yielded an apparent dose-dependent trend in decreasing [3H]dThd incorporation, but differences were not statistically significant.

Analysis of Cytochrome P-450-dependent MFO. AH and O-demethylation of PNAD activities of induced and noninduced LoVo, SW620, and SW403 cells are shown in Table 1. Intrinsic AH activity values were below detectable levels for all three cell lines (less than 1.0 pmol of 3-hydroxybenzo(a)pyrene formed per min per mg of protein) and did not increase following PB plus HC induction. BA-treated cells presented AH activity values from 12 to 15 pmol of 3-OH-BP per min per mg of protein. PNAD activities in both noninduced and BA-treated cells were 0.050 to 0.087 nmol of p-nitrophenol formed per min per mg of protein. These values were increased 50 to 70% following treatment with PB plus HC in LoVo and SW403 cells, but not in the SW620 cell line.

DISCUSSION

Chemotherapy of colon carcinoma is a particularly frustrating experience because very few drugs show any significant clinical activity (26). Paradoxically, because of the high rate of primary unresectability and recurrence after resection, most patients afflicted with this disease cannot be treated but with a systemic chemotherapy regimen. Zubrod (27) has suggested that increments in the efficacy of chemotherapeutic activity against large bowel cancers could reside in the efficient exploitation of presently available antitumor agents matched to the biological characteristics of the target colon cancer cell. Theoretically, the possibility of tumor selectivity, specificity, and regional utilization of antitumor agents may be applied to localized cellular activation of CP via the colon mucosa cytochrome P-450-dependent MFO microsomal enzyme system. In 1962, Wattenburg et al. (28) were first to report the presence of benzo(a)pyrene hydroxylase activ-

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<table>
<thead>
<tr>
<th>Cell line</th>
<th>Inducing agent</th>
<th>PNAD (nmol p-nitrophenol/min/mg protein)</th>
<th>AHH (nmol 3-hydroxybenzaldehyde/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LoVo</td>
<td>None</td>
<td>0.071 ± 0.003</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PB + HC</td>
<td>0.126 ± 0.010</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>0.083 ± 0.009</td>
<td>12.0 ± 0.5</td>
</tr>
<tr>
<td>SW620</td>
<td>None</td>
<td>0.078 ± 0.007</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PB + HC</td>
<td>0.073 ± 0.019</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>0.087 ± 0.019</td>
<td>ND</td>
</tr>
<tr>
<td>SW403</td>
<td>None</td>
<td>0.057</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>PB + HC</td>
<td>0.080</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>BA</td>
<td>0.050</td>
<td>15.2 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SD of at least two independent experiments.

ND, not detectable.

Cells failed to respond to CP, it was of interest to determine whether increased cytotoxicity was possible following induction of these cell lines. Results of colony formation inhibition for all of these cell lines treated with CP after inducer pretreatment showed no deviation from control, although [3H]dThd incorporation at 1 h resulted in a 37.5% reduction from control for LoVo cells and a 5.5% and 17.3% reduction for SW620 and SW403 cells, respectively. Mild CP activity, as measured by the decreased number of cells per plate, was only noted for both noninduced and induced SW403 cells (26%). Increased PNAD activities were anticipated for the PB-HC-induced cells; however, the method we used to measure PNAD activity was only moderately sensitive, and the levels of MFO activity, both intrinsic and induced, were either not detectable or only moderately elevated by this method. Thus, although it is possible that established cell lines may have lost cytochrome P-450-associated enzymes that are normally present in fresh tissues, and that because of the multiplicity of P-450-dependent MFO enzymes, activities of both PNAD and AHH may not be predictive of CP metabolism, our data would indicate that MFO activities in colon cancer lines are too low to be significant in the activation of CP. The lack of significant activation was clearly reflected in the "bioassay" constituted by the colony formation technique which demonstrated lack of CP cytotoxic activity (the desired end point) regardless of minor, erratic, and transient changes in [3H]dThd incorporation or short-term inhibition of cell proliferation (cell cycle transit delay?). The fact that some minor changes were actually seen could suggest that the low intrinsic MFO activities might metabolize some CP, thus causing these transient changes, but are insufficient to elicit adequate intracellular concentrations of active metabolites to ultimately abrogate cellular capacity for unlimited proliferation.

In view of these data and the fact that Sladek (31) considered that the therapeutic efficacy of CP cannot be altered by pretreating animals with agents that merely increase the rate of CP activation, we conclude that speculative, localized, induced metabolism of CP by in vivo colon cancer cells would not prove a valid chemotherapeutic venture.

ACKNOWLEDGMENTS

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MFO ACTIVITIES OF COLON CARCINOMA IN ACTIVATION OF CP

Mixed Function Oxidase Activities of Established Human Colon Carcinoma Cell Lines in the Activation of Cyclophosphamide

P. S. Moskwa, H. Vadi and B. Drewinko


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