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BIOCHEMICAL CHARACTERIZATION OF A MITOMYCIN C RESISTANT COLON CANCER CELL LINE VARIANT*

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Abstract—Resistance may limit the clinical usefulness of a variety of chemotherapeutic drugs including mitomycin C (MMC). The MMC-sensitive HT-29 colon cancer cell line and its MMC-resistant subline, HT-29R13, were studied *in vitro* under aerobic conditions to help characterize the mechanisms associated with MMC resistance. HT-29R13 cells exhibit approximately 2-fold resistance to MMC compared with HT-29 cells and lack the typical multidrug-resistance pattern; resistance is stable in the absence of drug exposure. Levels of glutathione (GSH) and total glutathione-*S*-transferase (GST) activity were not different between the two cell lines; however, levels of GSH reductase and GSH peroxidase were increased significantly in HT-29R13. Although total GST activity was unchanged, GST-π and GST-α isoenzyme expression as measured using western blot were increased significantly in HT-29R13 compared with HT-29. DT-diaphorase levels and topoisomerase II activity were decreased significantly in HT-29R13. Both cell lines had equal P-glycoprotein expression. Multiple drug resistance mechanisms are present in HT-29R13 including decreased drug activation (decreased DT-diaphorase), increased drug detoxification (increased GST-π and GST-α, GSH reductase, GSH peroxidase), and decreased accessibility of DNA targets (decreased topoisomerase II). Further work will be necessary to determine the degree to which each of these mechanisms contribute to MMC resistance in this model.

Key words: P-glycoprotein, glutathione-*S*-transferase, topoisomerase II, multidrug resistance

Mitomycin C (MMC)‡ is one of the few agents active against gastrointestinal malignancy, including colorectal cancer [1]. Limiting the usefulness of chemotherapeutic drugs including MMC is the initial presence or subsequent emergence of drug resistance. Various mechanisms have been described as being responsible for MMC resistance, including failure of MMC activation as a result of decreased ability to reduce the quinone structure to the active form [2], deficiency of DT-diaphorase (DTD) [3], cell surface protein alterations [4], and increased efflux mechanisms mediated by P-glycoprotein [5]. The development of cytosolic protein and phosphoprotein changes have been found to parallel the development of MMC resistance [6].

Much of the work examining MMC resistance mechanisms has been performed in the HCT116 human colon cancer cell line and several variants that were selected specifically for MMC resistance [7]. Other models that have been used include cell lines selected for Adriamycin® resistance, which exhibit the multidrug-resistant (MDR) phenotype and are cross-resistant to MMC [8].

To help further characterize MMC resistance, we recently developed an MMC-resistant subline of the HT-29 colon cancer cell line (HT-29R13). Compared with HT-29, this variant exhibits 2-fold higher resistance to MMC given for 1 hr at the 95% inhibitory concentration (IC_{95}) [9]. This low level of resistance does not increase with additional drug exposure, and resistance is stable for at least 14 months in the absence of drug exposure. HT-29R13 cells exhibit cross-resistance to melphalan and 5-fluorouracil, but not to Adriamycin, *cis*-platinum or etoposide, and thus these cells do not exhibit the typical MDR phenotype.

This study examined the biochemical parameters associated with MMC resistance in this model under aerobic conditions. We found that multiple drug-resistance mechanisms were present simultaneously and may potentially contribute to MMC resistance in HT-29R13. MMC resistance in this model did not appear to be due to increased expression of the MDR gene product P-glycoprotein, since P-glycoprotein expression was unchanged in the resistant variant.

MATERIALS AND METHODS

Human cancer cell lines. The HT-29 cell line was obtained from the American Type Culture Collection (Rockville, MD). The HT-29R13 subline was isolated by repeated exposures to 2 μM MMC for 1 hr, given weekly whenever possible, as previously described [9]. Cells were maintained in Ham's F-10 medium supplemented with 20% newborn calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL

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‡ Abbreviations: MMC, mitomycin C; MDR, multidrug resistance; GSH, glutathione; GST, glutathione-*S*-transferase; GR, glutathione reductase; GPX, glutathione peroxidase; DTD, DT-diaphorase; and IC_{95}, 95% inhibitory concentration.

streptomycin. The cells were incubated at 37° in a 95% air/5% CO_2 environment. Cells in logarithmic growth phase were used in all experiments.

Drugs and biochemicals. MMC was provided by Daniel Elliot of the Bristol-Myers Co. (Evansville, IN). NADPH, glutathione reductase (GR), and oxidized glutathione (GSSG) were obtained from Boehringer–Mannheim Biochemicals (Indianapolis, IN). Glutathione (GSH) and other chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO).

GSH assay. GSH was extracted by adding 0.6% 5-sulfosalicylic acid to culture dishes containing plated cells and then incubating at 4° for 20 min [10]. Supernatants were removed from the dishes, and measurements for total GSH were performed using a modified Tietze assay [11]. Absorbance was monitored at 412 nm using a Hitachi U-2000 spectrophotometer (Hitachi Instruments, Inc., Danbury, CT). GSH levels were normalized to protein content as measured using the Bradford method [12].

Enzyme assays. Cells were trypsinized, washed, and resuspended in 5 mM diethylenetriamine pentaacetic acid. Cells were disrupted using a Branson sonicator, and total protein of the homogenates was measured using the Bradford method [12]. The homogenates were spun down at 16,000 *g* for 10 min, and the supernatants were stored at −155° until assay. Glutathione-*S*-transferase (GST) was measured, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate, by the method of Habig *et al.* [13]. GR was measured using the method of Massey and Williams [14]. Glutathione peroxidase (GPX) was measured at 37°, using hydrogen peroxide as a substrate, by a modification of the method Paglia and Valentine [15], after preincubation of the samples at 42° for 10 min. DTD was measured, using menadione as the electron acceptor, by the method of Ernester [16]. The dicumarol-sensitive part of the activity was taken as a measure of DTD.

Immunoblotting to detect P-glycoprotein expression. Cells were harvested with 0.05% trypsin/0.02% EDTA and homogenized by gentle sonication on ice in 2× lysis buffer (0.125 Tris–HCl, pH 6.8, 25% glycerol, 25% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol). Plasma membrane preparations from the cells were purified according to the method of Riordan and Ling [17], and protein was determined by the Bradford method [12]. One hundred micrograms of membrane protein was separated using SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (6.5% resolving gel with 4.5 M urea) as described by Laemmli [18]. The protein was then transferred to a 0.45-μm nitrocellulose sheet (Bio-Rad, Richmond, CA) by electro-blotting (16 hr at 100 mA constant current) using a Bio-Rad Mini Transblot Cell and accompanying instructions. The nitrocellulose sheet was blocked with blocking buffer [1% bovine serum albumin (BSA)/0.05% Tween 20 in 10 mM Tris–HCl buffer, pH 7.4] and incubated with 2.5 μg/mL of mouse C219 monoclonal antibody (Centocor, Malvern, PA) in fresh blocking buffer at room temperature for 4 hr. This antibody is not species-specific and recognizes a highly conserved internal epitope in P-glycoprotein [19]. The nitrocellulose sheet was washed with Tris–saline buffer (150 mM NaCl, 0.05% Tween 20, 10 mM Tris–HCl, pH 8.0), and then incubated with biotinylated goat anti-mouse IgG_{2a} (Vector Laboratories, Burlingame, CA) in blocking buffer at room temperature. The nitrocellulose sheet was then rewashed with Tris–saline buffer, and developed using 0.02% H_2O_2 and 0.1% diaminobenzidine (DAB) tetrahydrochloride (in 100 mM Tris–HCl buffer, pH 7.2). A P-glycoprotein-negative Chinese hamster ovary (CHO) cell line, AuX B1, and its MDR variant, ChR C5 [20], were used as negative and positive controls of P-glycoprotein expression. All immunoblots were quantified using laser densitometry.

Immunohistochemical staining to detect P-glycoprotein. To detect P-glycoprotein on individual cells, the murine monoclonal antibody C219 was used as the primary antibody using the biotin-avidin conjugated immunoperoxidase method of Warnke *et al.* [21] with some modifications. Tumor cells were suspended in F-10 medium with 10% BSA (Gamma Biological, Houston, TX) at approximately 0.8 to 1.0×10^6 cells/mL and one drop was placed onto each slide. The slide preparations were fixed in ice-cold acetone, air-dried, and stored at −70°. C219 antibody was used at 5 μg/mL in 2% BSA in phosphate-buffered saline (PBS). The second stage employed biotin-conjugated goat anti-mouse IgG_{2a} (Vector Laboratories) diluted to 1/200 in 2% BSA in PBS. The third stage employed avidin-D conjugated with horse-radish peroxidase (Vector Laboratories). The color reaction was produced by using DAB (Sigma) and hydrogen peroxide (0.02%) followed by 5 min in 0.5% copper sulfate in 0.85% NaCl to intensify the color. A negative control slide repeated all these steps using an irrelevant, isotype-matched mouse IgG_{2a} as the primary antibody. As standards, Chinese hamster ovary AuX B1 cells and their drug-resistant variant (ChR C5) cells were routinely stained simultaneously in each experiment.

Immunoanalysis of GST isoenzymes. The procedure for isolation of cytosolic protein from the cells was essentially as described above. Cytosolic protein (50 μg per lane) was resolved by 12% SDS–PAGE followed by electrophoretic transfer to 0.45 μm nitrocellulose according to the manufacturer's directions (Bio-Rad). The sheet was blocked with 5% goat serum for 1 hr and then incubated overnight with 1 : 200 anti-GST-π isoenzyme antibody (Oncor, Gaithersburg, MD) or with 1 : 200 polyclonal anti-GST-α or anti-GST-μ isoenzyme antibody (originally from Dr. J. D. Hayes, Department of Biochemistry, University of Edinburgh). Biotinylated goat-anti-rabbit IgG was used as the secondary antibody (Vector Laboratories) for GST detection. The nitrocellulose sheet was then rewashed and developed as described above. All immunoblots were quantified using laser densitometry.

DNA topoisomerase II activity. To determine topoisomerase II activity, first a nuclear extract was obtained. Exponentially growing cells were collected and washed with ice-cold nuclear buffer (pH 6.5) containing 2 mM dipotassium phosphate, 5 mM $MgCl_2$, 1 mM NaCl, and 0.1 M dithiothreitol. The washed cells were resuspended and mixed with nuclear buffer supplemented with 0.35% Triton

Table 1. Glutathione levels and enzyme activity in HT-29 and HT-29R13 cells

	GSH (nmol/mg protein)	GR (nmol/min/ mg/protein)	GST (nmol/min/ mg/protein)	GPX (nmol/min/ mg/protein)	DTD* (nmol/min/ mg/protein)
HT-29	68 ± 6	18 ± 1	172 ± 10	23 ± 2	963 ± 41
HT-29R13	63 ± 5	34 ± 3†	167 ± 12	34 ± 2†	507 ± 33†

Measurements of glutathione levels and enzyme activity were performed as described in Materials and Methods. Values are means ± SEM; N = 5 independent experiments. Abbreviations: GSH, glutathione; GR, glutathione reductase; GST, glutathione-*S*-transferase; GPX, glutathione peroxidase; and DTD, DT-diaphorase.

* Represents the dicumarol-sensitive part of total diaphorase activity.

† $P < 0.05$ compared with HT-29.

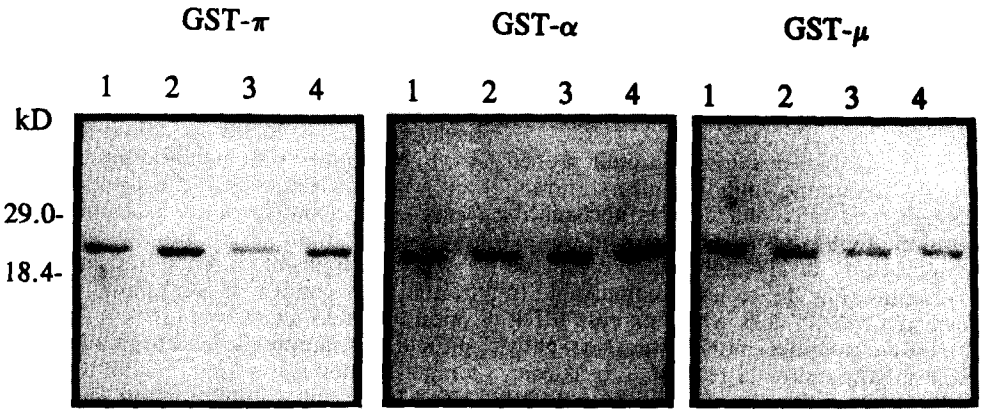

Fig. 1. GST isoenzyme expression in HT-29 and HT-29R13 cells. Each lane contained 50 μg of cytosolic protein, as described in Materials and Methods. Lane 1: AuX B1 as control (non-MDR); lane 2: ChR C5 as control (MDR); lane 3: HT-29, passage 27; and lane 4: HT-29R13, passage 27. GST-π and GST-α expression were increased in resistant HT-29R13 cells compared to parent HT-29 cells. GST-μ expression was unchanged.

X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). The mixture was incubated with shaking at 4° for 10 min. The lysed cells were centrifuged at 1000 *g* for 10 min, and the nuclear pellet was washed three times with nuclear buffer. The protein was extracted from the nuclear pellet by incubating for 60 min at 4° in nuclear buffer containing 0.35 M NaCl and 1 mM PMSF. DNA and nuclear debris were pelleted by centrifugation at 17,000 *g* for 25 min, and the supernatant was collected. Glycerol was added immediately to the supernatant to achieve 30% concentration. Protein content for the nuclear extract was determined by the Bradford method [12].

Topoisomerase II activity was then assayed by the ability of nuclear extracts to relax supercoiled pBR322 DNA. In this assay, only topoisomerase II activity is capable of converting highly supercoiled pBR322 DNA into the relaxed DNA form [22]. The reaction mixture (50 μL) contained 50 mM Tris–HCl (pH 7.5), 85 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM EDTA, 30 μg/mL BSA, 1 mM ATP, 0.4 μg of pBR322 DNA (GIBCO, Gaithersburg, MD), and various dilutions of the nuclear extract. The reaction was carried out at 37° for 30 min and stopped by the addition of 10 μL of a solution containing 2% SDS, 0.05% bromophenol blue, and 30% Ficoll. The samples were analyzed by electrophoresis on 1.0% agarose gels in 89 mM Tris–borate buffer (pH 8.0). Gels were stained with 2 μg/mL ethidium bromide, washed, and photographed under a UV light source. One unit of topoisomerase II activity was defined as the complete conversion of 1 μg of pBR322 supercoiled DNA to the relaxed form in 10 min at 37°.

RESULTS

The levels of intracellular GSH and the activities of GST, GR, GPX, and DTD in HT-29 parent and HT-29R13 MMC-resistant cells are shown in Table 1. GSH levels in HT-29 and HT-29R13 were not significantly different. There was no significant difference in total GST activity (toward CDNB) between the two cell lines. GR was increased 1.9 times and GPX was increased 1.5 times in HT-29R13 cells compared with HT-29 cells ($P < 0.05$). Differences in the levels of GR and GPX were also

Table 2. GST isoenzyme expression measured by laser densitometry

	GST-π	GST-α	GST-μ
HT-29	44 ± 6.0	38 ± 4.0	30 ± 4.0
HT-29R13	95 ± 5.0*	56 ± 7.0†	25 ± 3.0
AuX B1	46 ± 4.0	41 ± 5.0	33 ± 2.0
ChR C5	94 ± 5.0	40 ± 6.0	36 ± 4.0

Measurements were performed on western blots such as shown in Fig. 1. Values expressed in arbitrary units/mg cytosolic protein are means ± SEM; N = 3 independent experiments.

* $P < 0.01$ compared with HT-29.

† $P < 0.05$ compared with HT-29 and ChR C5.

evident when the results were normalized to relative cell volume as measured using flow cytometry rather than cell protein content (data not shown). DTD activity was 1.9 times higher in HT-29 cells compared with HT-29R13 cells ($P < 0.05$).

Western blot analysis of cytosolic protein probed with anti-GST isoenzyme antibodies clearly showed that although total GST activity was unchanged, the expression of GST-π isoenzyme protein was increased significantly in HT-29R13 cells compared with HT-29 cells (Fig. 1), to levels comparable to those seen in ChR C5 cells which express MDR (Table 2). GST-α isoenzyme protein was also slightly but significantly increased in HT-29R13 cells compared with HT-29 cells. This differed from MDR ChR C5 cells, which showed no increase in GST-α expression compared with the parental AuX B1 cells (Fig. 1, Table 2). No significant changes in GST-μ expression were noted (Fig. 1).

Shown in Fig. 2 are the results of the western blot analysis for P-glycoprotein expression in which membrane proteins from HT-29 and HT-29R13 cells were reacted with the P-glycoprotein specific C219 monoclonal antibody. Densitometric analysis of the P-170 bands demonstrated that both cell lines expressed P-glycoprotein, and the levels of expression were identical (HT-29: 31.5 ± 3.5 arbitrary units/μg membrane protein; HT-29R13: 29 ± 1.0 arbitrary units/μg membrane protein). Similar results were obtained when P-glycoprotein expression was measured using immunohistochemical staining (results not shown). The percentage of cells staining positive for P-glycoprotein was 42.4 ± 12.1% for HT-29 cells, and 39.2 ± 9.7% for HT-29R13 cells. P-Glycoprotein expression measured using either technique did not change with increasing cell passage number.

The activity of topoisomerase II in 0.35 M NaCl extracts of nuclei isolated from parental HT-29 and acquired resistant HT-29R13 cells was compared by using the topoisomerase II specific DNA relaxation reaction. In Fig. 3 are shown the results obtained with serial dilutions of nuclear extracts from HT-29 and HT-29R13 cells. The level of topoisomerase II present in salt extracts of HT-29 nuclei was significantly higher (86 ± 21 U/μg nuclear protein) than the level present in HT-29R13 nuclei (40 ± 16 U/μg nuclear protein) ($P < 0.05$).

DISCUSSION

Multiple drug-resistance mechanisms are present simultaneously in HT-29R13 cells, which may alone or together contribute to MMC resistance. Elevated levels of GR, GPX, GST-π, and GST-α were present, suggesting that HT-29R13 cells are capable of increased drug or free radical detoxification. Levels of DTD were decreased, suggesting that the resistant variant is less able to reduce MMC to its active form. Also, decreased levels of topoisomerase II were present in the resistant variant, making the DNA targets in the resistant line less accessible to MMC, and therefore possibly contributing to resistance. Although no significant difference in P-glycoprotein expression was noted between the sensitive parent HT-29 line and the resistant HT-29R13 variant, suggesting that P-glycoprotein expression does not modulate resistance in this model, a difference in P-glycoprotein activity between the two variants has not been excluded. In addition, it is possible that with further clonal isolation of HT-29R13 cells, subpopulations of cells may be identified with greater degrees of resistance and with different individual drug-resistance mechanisms not apparent when examining the whole population.

The development of MMC resistance has been related to a number of mechanisms including cell surface protein alterations [4] as well as cytosolic protein and phosphoprotein changes [6]. Decreased drug activation may account for MMC resistance in HCT116 variants [2], particularly since a synthetic MMC analog whose quinone structure is more easily reduced to its active form successfully reverses resistance both *in vitro* [23] and *in vivo* [24]. Mitomycin C resistance is also part of the classic MDR phenotype modulated by P-glycoprotein [5, 8].

The precise mechanism whereby MMC is activated remains controversial. Evidence suggests the importance of DTD in two-electron reduction of MMC to its active form under aerobic conditions [3, 25], whereas other mechanisms, including one-electron activation by NADPH cytochrome P450 oxidoreductase, appear important under hypoxic conditions [26]. Expression of the DTD gene (NQO1) in a panel of colorectal cell lines correlates with sensitivity to MMC [27]. The results in our study of HT-29R13 further support the role of DTD in modulating MMC resistance, but other resistance mechanisms were found in the model as well.

Although GST may be involved in MMC resistance in HCT116R44 [2], another study examining a variety of cell lines failed to show a relationship between MMC resistance and GST-π expression [28]. Although we did not find elevated levels of total GST activity in HT-29R13, we did find elevated expression of GST-π and GST-α protein, but GST-μ expression was unchanged. There are several possible reasons for the increase in expression of two GST isoenzyme proteins without a corresponding change in total GST activity (against CDNB). Although CDNB is a relatively good GST substrate at high concentrations, the K_m for CDNB ranges from 70 to 2000 mM for various GSTs [29]. Thus,

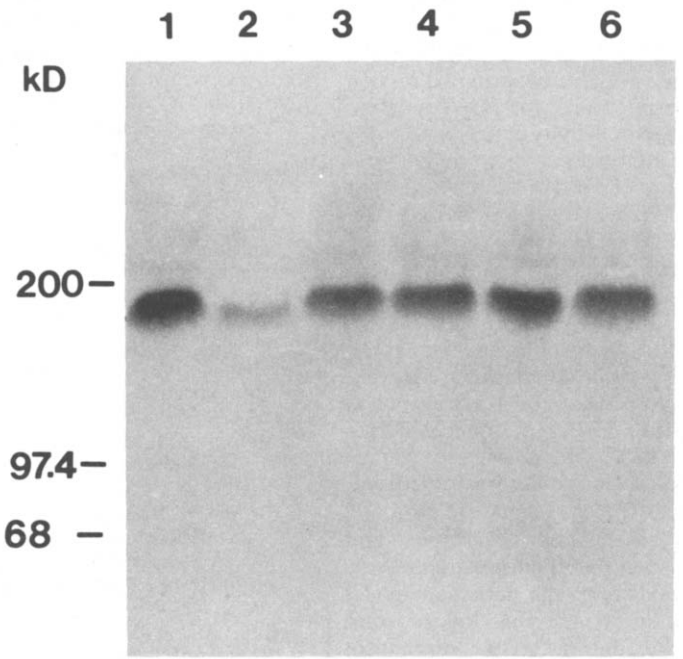

Fig. 2. P-Glycoprotein expression in HT-29R13 and HT-29 cells. Each lane contained 100 μg of membrane protein, as described in Materials and Methods. Lane 1: positive control (ChR C5); lane 2: negative control (AuX B1); lane 3: HT-29R13, passage 12; lane 4: HT-29R13, passage 27; lane 5: HT-29, passage 12; and land 6: HT-29, passage 27. No significant difference in P-glycoprotein expression was noted between HT-29 and the resistant variant HT-29R13.

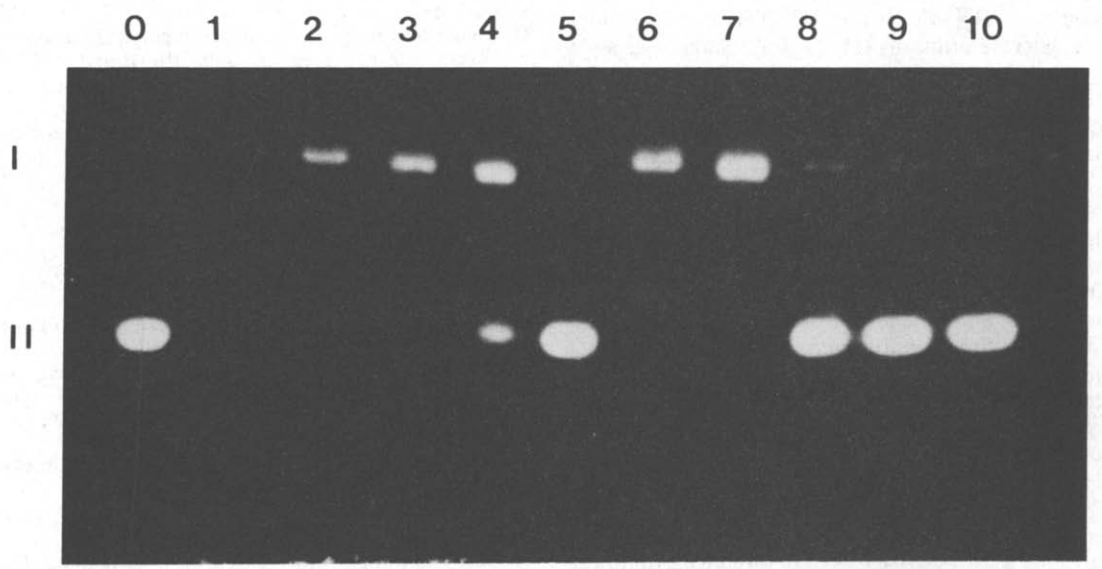

Fig. 3. Topoisomerase II activity in HT-29 and HT-29R13 cells. Topoisomerase II activity was assessed by the ability of nuclear extracts from the cell lines to relax supercoiled pBR322 DNA, as described in Materials and Methods. The untreated control was loaded in lane 0. Nuclear extracts from HT-29 were loaded in lanes 1–5 with 0.25, 0.125, 0.0625, 0.0313, and 0.0157 μg of nuclear protein per well. HT-29R13 was loaded in lanes 6–10 with 0.25, 0.125, 0.0625, 0.0313, and 0.0157 μg of nuclear protein per well. Ordinate I corresponds to the "relaxed" form of pBR322 DNA, and II corresponds to the "supercoiled" form. Note the decrease in topoisomerase activity in HT-29R13 cells, with the transition between the supercoiled and relaxed forms occurring in the third dilution (lane 8), as opposed to between the fourth and fifth dilution in HT-29 cells (lanes 4 and 5).

total GST activity (toward CDNB) may not be sensitive enough to reflect the increased GST-π and GST-α expression noted in HT-29R13 cells. Alternately, an increase in GST isoenzyme expression may result in down-regulation of total GST function [30]. Also, the high specific activity of GST-μ towards CDNB [31] suggests that even a slight decrease in this isoenzyme (Table 2) might compensate for overexpression of GST-π and GST-α, resulting in no change in total GST activity.

Changes in topoisomerase II expression have been associated with resistance to several chemotherapeutic agents, including etoposide [32, 33], Adriamycin [32, 33], and mitoxantrone [32]. Recently, decreased levels of topoisomerase II were noted in Cho-Adr^r cells resistant to Adriamycin and cross-resistant to MMC [34]. In our HT-29R13 model, selected specifically for MMC resistance, decreased topoisomerase II activity was noted, but cross-resistance to Adriamycin was not seen.

Which resistance mechanisms develop, and whether multiple resistance mechanisms are present, appear to be dependent upon the particular model as well as the method of selecting the resistant variant as shown by the different Adriamycin resistance mechanisms in MCF-7 cells [30] compared with Friend erythroleukemia cells [35]. Although most studies of MMC resistance have examined individual resistance mechanisms, one recent study investigated multiple mechanisms. Cho-Adr^r cells selected for Adriamycin resistance exhibit the classic MDR phenotype, with increased P-glycoprotein expression [34]. Verapamil completely reverses Adriamycin resistance, but only partially reverses MMC resistance. These cells also possess increased levels of GST-α and decreased levels of topoisomerase II [34]. In our HT-29R13 model which was selected primarily for MMC resistance, we show increased levels of GST-π and GST-α and decreased topoisomerase II activity as well as the presence of other mechanisms, but P-glycoprotein expression was not increased.

Although multiple drug-resistance mechanisms were present in HT-29R13, it is difficult to ascertain the degree to which each of the mechanisms contributes to the low level MMC resistance present in this model. It certainly is possible that multiple mechanisms may be responsible for MMC resistance, similar to the situation with Adriamycin resistance [30, 36]. It is not known whether the drug-resistance mechanisms in our model develop simultaneously or in a particular sequence. It will be interesting to determine the effect of a more easily reducible MMC analog, and the effects of certain enzyme inhibitors (i.e. ethacrynic acid to inhibit GST), on MMC resistance in HT-29R13. MMC in some models appears to be more active under hypoxic conditions [25], so it will be especially important to determine the effects of hypoxia on MMC-resistance mechanisms. These areas are currently being investigated by our laboratory.

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