Non-P-Glycoprotein-mediated Multidrug Resistance in Detransformed Rat Cells Selected for Resistance to Methylglyoxal Bis(guanylhydrazone)¹

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ABSTRACT

Three independent variants (G2, G4, G5), resistant to methylglyoxal bis(guanylhydrazone), an anticancer drug, have been isolated by single step selection from an adenovirus-transformed rat brain cell line (1). These variants display selective cross-resistance to several natural product drugs of dissimilar structure and action. Multidrug resistance has recently been shown to be caused by overexpression of the membrane-associated p-glycoprotein, most often caused by amplification of the mdr gene. Several types of experiments were conducted to determine whether the observed drug resistance in our cell lines could be due to changes at the mdr locus. The following results were obtained: (a) the mdr locus was not amplified; (b) transcription of the mdr gene and p-glycoprotein synthesis were not increased; (c) multidrug resistance cell lines, which carry an amplified mdr locus, were not cross-resistant to methylglyoxal bis(guanylhydrazone); (d) verapamil did not reverse the resistance of G cells or mdr cells to methylglyoxal bis(guanylhydrazone), nor that of G cells to vincristine; and (e) methylglyoxal bis(guanylhydrazone) resistance was recessive and depended on a block to drug uptake, as opposed to mdr cells which are dominant and express increased drug efflux. The results obtained suggest that the drug resistance in the G2, G4, and G5 cells was atypical and may be due to a mechanism distinct from that mediated by the mdr locus.

INTRODUCTION

Three independent variants (G2, G4, G5) resistant to MGBG, an anticancer drug, have been isolated by single step selection from an adenovirus-transformed rat brain cell line (1, 2). These variants display selective cross-resistance to several natural product drugs of dissimilar structure and action (3, 4). These agents include vincristine, doxorubicin, and colchicine. MGBG resistance was attributable to decreased drug uptake (2). Multidrug resistance has been shown to be caused by overexpression of the membrane-associated p-glycoprotein, most often occasioned by amplification of the mdr gene (5–8). The pharmacological basis for mdr appears to be a reduced steady-state accumulation of drugs which has been attributed to decreased retention (9–11).

Several types of experiments were conducted to determine whether the observed cross-resistance in our cell lines could be due to changes at the mdr locus. Our results showed no amplification of the mdr genes. Furthermore the MGBG-resistant cell lines did not show any increased expression of mdr RNA or p-glycoprotein. These conclusions were reinforced by pharmacological experiments diagnostic of classical multidrug resistance. Therefore these MGBG-resistant cell lines are atypical drug-resistant cells, the mechanism of resistance being unrelated to that found in classical multidrug-resistant cells. Therefore we conclude that these drug-resistant variants are atypical multidrug-resistant variants, possibly similar to such variants reported recently (12).

MATERIALS AND METHODS

Cells. F4 cells were derived from embryonic rat brain cells transformed by adenovirus (2 (13). F4 cells were recloned 3 times in success and mutagenized by different methods, and variants resistant to MGBG were isolated from these subclones. These isolates were named G2 (EMS mutagenized), G4 (mutagenized by DNA transfection), and G5 (mutagenized by infection with murine Moloney leukemia virus infection) and have been described before (1, 2, 14). Subclones derived from G2 and G4 were named G2a and G4F. Both of these cell lines maintained MGBG resistance and a detransformed phenotype. AuxBl is a auxotrophic mutant of the Chinese hamster ovary, and CHPC⁰ was isolated from AuxBl by selection with colchicine (15). CHPC⁰ is a classical multidrug-resistant cell line which contains an amplified mdr locus and expresses high levels of the p-glycoprotein (16). F4 and G cells were cultured in Petri dishes in Dulbecco's modified minimal essential medium and 10% fetal calf serum in air-CO₂ incubators at 37°C. AuxBl and CHPC⁰ were cultured in α-minimal essential medium (Gibco) supplemented with 10% fetal calf serum.

Chemicals. Colchicine, doxorubicin, vincristine, podophyllotoxin, actinomycin D, and puromycin were from Sigma Chemical Co. (St. Louis, MO), and MGBG was purchased from Aldrich (Milwaukee, WI).

Assay of Drug Resistance. Dose-response curves of MGBG-resistant variants were determined from various drugs by plating 10⁶ cells, in duplicate, in 100-mm dishes. After incubation of 37°C for 10 to 14 days the dishes were stained with Coomassie blue. D₀ values were calculated from survival curves of colony formation and represent the concentration of drug which reduces the plating efficiency to 10%.

DNA Extraction and Hybridization. When cells reached confluence, they were scraped off the Petri dishes, and DNA was extracted as described earlier (1). DNA was restricted to completion with BamH1 restriction endonuclease, electrophoresed, transferred to nylon membrane, and hybridized with a DNA probe representing the most conserved 3' exon in the λ-DRII complementary DNA clone (kindly supplied by P. Gros) of the mouse mdr sequences (6). The quantity of DNA on the filter was determined by rehybridization with the β-actin probe.

RNA Hybridization. RNA was probed according to the in situ hybridization method of Paeratakul et al. (17). Briefly, cells were washed, scraped off the Petri dishes, and adjusted to 10⁶ cells per ml in 0.8% NaCl-0.115% Na₂HPO₄-0.02% KH₂PO₄-2H₂O-0.02% KCl, pH 7.4. Serial dilutions in 100 μl were applied onto a nitrocellulose filter using a 96-well manifold filtering apparatus (Biological Research Laboratories) and fixed with phosphate-buffered 1% glutaraldehyde containing 3% NaCl. The filters were washed in proteolytic buffer (50 mM EDTA-0.1 M Tris-hydrochloride, pH 8), digested in the same buffer with 20 μg/ml of proteinase K (Boehringer-Mannheim), dried, and hybridized with the mdr and β-actin probes according to the method of Maniatis et al. (18).

Photoaffinity Labeling of p-Glycoprotein. Purified plasma membranes were prepared as described before (19). Protein concentration was determined using the Biorad protein assay. Photoaffinity labeling of membrane samples with ³²I-NAS-verapamil and detection by electrophoresis were kindly performed by R. L. Fiselst (National Cancer Institute, Bethesda, MD) as described before (20).

Cellular Uptake and Retention of [³H]Colchicine. Methods described previously were followed (21). Twenty-four h after seeding, the cells...
were washed by centrifugation and resuspended in 0.14 M NaCl and 0.01 M K2HOP4. For uptake studies a final suspension was made in transport buffer (0.001 μg/ml of colchicine, 107 mM NaCl, 10 mM Tris, 26.2 mM NaHCO3, 5.3 mM KCl, 1.9 mM CaCl2, 1 mM MgCl2, and 7 mM D-glucose, pH 7.4). Each experimental point contained 10^6 cells and 0.5 to 1 μCi of [3H]colchicine (specific activity, 39.4 Ci/mol) in 300 μl of uptake buffer. Uptake was measured in at least triplicates at 37°C. Drug efflux was measured under similar conditions after loading the cells with [3H]colchicine for 60 min followed by rapid washing and resuspension in 300 μl of 37°C transport buffer. After incubation for different time periods, the cells were washed rapidly, and the pellet was lysed in 600 μl of 4 N NaOH at 70°C for 45 min. Cell-associated radioactivity was counted in 5 ml of Ready Safe (Beckman).

RESULTS

Cross-Resistance of MGBG-resistant Cell Lines to Other Drugs. Although MGBG has not been part of the list of drugs normally associated with multidrug resistance, and because the mechanism of resistance to this drug remains obscure, it appeared imperative to ask if these cells had acquired a cross-resistance to other drugs. Table 1 shows that indeed the three MGBG-resistant cell lines tested showed significant cross-resistance to doxorubicin and colchicine (7- to 40-fold) but particularly to vincristine (up to 200-fold). By contrast, little or no cross-resistance manifested itself to podophyllotoxin, actinomycin D, or puromycin. These results raise the question of whether or not the observed cross-resistance was due to an amplification or increased expression of the mdr locus.

mdr Gene Analysis. The possible amplification of the mdr gene was determined by Southern blot analysis of DNA from the drug-resistant G cells and the parental drug-sensitive F4 cells (Fig. 1). A classical multidrug-resistant cell line (CHP3) and its sensitive parent (AuxBl) served as positive control. The CHP3 cell line contains about 40 copies of the mdr gene, because the 20-fold-diluted sample (Lane b) shows a 2-fold increase compared to the signal of AuxBl (Lane c). As shown by hybridization to the β-actin probe, the filter contained approximately the same amount of DNA from each cell line except Lane b (Fig. 1). Hybridization with the mdr probe gave the same amount of signal for G-cells as for F4. We conclude that the drug resistance phenotype of the G-cells is not caused by mdr gene amplification.

mdr RNA Expression. Lack of DNA amplification does not exclude a role for mdr in drug resistance, for there are instances where drug-resistant cells do not contain an amplified mdr locus but express a higher level of RNA (22). To explore this possibility, we compared the level of mdr-specific RNA in these cell lines. Again, we failed to observe any increase in mdr expression in the drug-resistant G-cells (Fig. 2).

p-Glycoprotein. Membrane preparations from resistant and sensitive cells were examined for the presence of p-glycoprotein by photoaffinity labeling with 125I-NAS-verapamil in the presence or absence of excess unlabeled verapamil to identify specifically labeled proteins. As shown in Fig. 3 the M, 170,000 p-glycoprotein could only be detected in CHP3 cells. Similarly, Western blots probed by the anti-p-glycoprotein antibody C219 failed to detect any signal in other than CHP3 cells. It may be concluded that the G-cells do not express significantly increased levels of p-glycoprotein.

Relative Resistance of mdr Cell Lines to MGBG. If the observed cross-resistance of the G-cells were mediated by the mdr gene, then one might expect classical multidrug-resistant cells to be resistant to MGBG. To test this hypothesis, the effect of MGBG was measured on three mdr cell lines and their

Table 1 Cross-resistance of MGBG-resistant cell lines to other drugs (determined by colony formation after 10 days)

<table>
<thead>
<tr>
<th>Drug</th>
<th>D0 of F4 cells (μg/ml)</th>
<th>Relative resistance^a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G2a</td>
<td>G4F</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.005</td>
<td>10</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.001</td>
<td>7</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>0.0008</td>
<td>102</td>
</tr>
<tr>
<td>Podophyllotoxin</td>
<td>0.001</td>
<td>5</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.020</td>
<td>4</td>
</tr>
<tr>
<td>Puromycin</td>
<td>0.5</td>
<td>1.6</td>
</tr>
<tr>
<td>MGBG</td>
<td>0.55</td>
<td>30</td>
</tr>
</tbody>
</table>

^a Values are means of 4 to 6 experiments.

^b Ratio of D0 of test cell over D0 of F4 cell.
Calcium channel blocker which specifically reverses the resistance that Tween-80 increases the toxicity of MGBG in drug-sensitive corresponding drug-sensitive parent cell lines (Table 2). One mdr cell line (LTA 42.2), which carried a 50-fold-amplified locus and was 70-fold more resistant to colchicine, showed no evidence of gene amplification*15-8150140 resistance to MGBG. Because two of the three cell lines showed a low level cross-resistance to MGBG, we suggest, though cannot prove, that the mdr locus is unlikely to be a determining factor in the mechanism of resistance to MGBG in the presence of Tween-80. This result suggests that MGBG resistance, like multidrug resistance, is at the level of drug transport across the membrane.

Verapamil failed to increase MGBG toxicity. Therefore MGBG is not transported by the p-glycoprotein pump. Verapamil also failed to increase the toxicity of vincristine in the G-cells, while an 1800-fold increase in toxicity was recorded for CH*C5 cells. This result confirms our conclusion that drug resistance in the G-cells is unlikely to be mediated by p-glycoprotein.

Drug Uptake and Efflux. We have previously shown that resistance to MGBG was due to a reduced uptake of the drug (2). In order to characterize the nature of cross-resistance of G-cells to other drugs, we measured the pattern of [%H]colchicine uptake and efflux in the parental F4 cells and the resistant G2 cells. In both cases, the results were comparable to those obtained with the classical mdr cell line CH*C5 and its drug-sensitive parent AuxB1. Colchicine uptake was 8-fold lower in the resistant G2a cells than in the parental F4 cells (Fig. 4A). By comparison, the highly drug-resistant CH*C5 cell line accumulated 170-fold less drug than its parent (Fig. 3B). To determine if drug retention could account for these marked differences in uptake, efflux was measured in these cell lines after a 60-min incubation with [%H]colchicine. Data are expressed as the percentage of [%H]colchicine remaining in the cells at various times after the 60-min pretreatment. The kinetics of efflux appeared to be similar for all cell lines (Fig. 5). The rate of efflux was 10% greater from G2a cells than F4 cells during the first 10 min, followed by convergence such that both cells retained the same drug concentration after 60 min. By contrast, the rate of efflux was 25 to 30% greater from the CH*C5 cells than from the parental AuxB1 cells, and it still remained 8% greater at 60 min. Considering the much higher level of drug resistance of CH*C5 cells, this might be expected.

In summary, these results demonstrate that the drug resistance mechanism of the G2 cells is distinct from that of classical mdr cells in that the reduced intracellular accumulation of colchicine (for example) is probably due to failure of intracellular binding, rather than due to an active efflux mechanism.

MGBG Resistance Is Recessive. The multidrug resistance phenotype is dominant because it is expressed through the p-glycoprotein drug-efflux pump (23, 24). Based on studies of MGBG influx and efflux, we and others have reported that MGBG resistance resulted from decreased drug uptake (2, 25, 26). Drug efflux, however, has also been reported (27). Because of the intrinsic difficulty of distinguishing between influx and efflux by means of drug-uptake studies, we reexamined this question using a biological assay. We reasoned that MGBG, being small enough to traverse gap junctions between cells, would kill resistant cells in a mixed culture of resistant and sensitive cells. In both cases, the results were compared to those obtained with the classical mdr cell line CH*C5 and its drug-sensitive parent AuxB1. Colchicine uptake was 8-fold lower in the resistant G2a cells than in the parental F4 cells (Fig. 4A). By comparison, the highly drug-resistant CH*C5 cell line accumulated 170-fold less drug than its parent (Fig. 3B). To determine if drug retention could account for these marked differences in uptake, efflux was measured in these cell lines after a 60-min incubation with [%H]colchicine. Data are expressed as the percentage of [%H]colchicine remaining in the cells at various times after the 60-min pretreatment. The kinetics of efflux appeared to be similar for all cell lines (Fig. 5). The rate of efflux was 10% greater from G2a cells than F4 cells during the first 10 min, followed by convergence such that both cells retained the same drug concentration after 60 min. By contrast, the rate of efflux was 25 to 30% greater from the CH*C5 cells than from the parental AuxB1 cells, and it still remained 8% greater at 60 min. Considering the much higher level of drug resistance of CH*C5 cells, this might be expected.

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Table 3 Effect of verapamil and Tween-80 on the cytotoxicity (D_{10}, the concentration of drug which reduces plating efficiency to 10%) of MGBG and vincristine in resistant and sensitive cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGBG +100 µg/ml of Tween-80</th>
<th>MGBG +10 µM verapamil</th>
<th>Vincristine +10 µM verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGBG (µM)</td>
<td>Fold decrease</td>
<td>MGBG (µM)</td>
</tr>
<tr>
<td>F4</td>
<td>2</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>G2a</td>
<td>60</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>G4F</td>
<td>25</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>G5</td>
<td>30</td>
<td>16</td>
<td>9</td>
</tr>
<tr>
<td>AuxB1</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>CH^RC5</td>
<td>10</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

Fig. 4. Time course for total accumulation of [3H]colchicine. [3H]Colchicine was added at 0 min, and cell-associated radioactivity was determined at the indicated times thereafter. A shows the parental drug-sensitive F4 cells and the drug-resistant G2 derivative. B shows the parental drug-sensitive AuxB1 and the drug-resistant mdr derivative CH^RC5.

Fig. 5. Efflux of [3H]colchicine from drug-sensitive and -resistant cells. The cells were loaded with [3H]colchicine for 1 h at 37°C and washed, and the percentage of retained radioactivity was measured at the indicated times thereafter. Points, mean; bars, SE

Fig. 6. Cytotoxicity of MGBG on mixed cultures of cells. MGBG-resistant G4F and MGBG-sensitive F4 cells were mixed in a ratio (1:1) and cultured in the presence of 25 µM MGBG. Colonies were counted at Day 10. All surviving colonies showed the morphology of G4F cells. There were no survivors in the plates where 10^6 cells were seeded.

DISCUSSION

We have previously reported the isolation and characterization of MGBG-resistant variants (G-cells) that had concomitantly lost their virus-transformed phenotype (1, 14). Here we examined the nature of this drug resistance in comparison with classical multidrug-resistant cells. The G-cells expressed a pattern of cross-resistance to several natural product drugs, similar to that expressed by multidrug-resistant mdr cells. The phenotypes of G-cells and mdr cells, however, were found to be significantly different in several respects: (a) unlike mdr cells, G-cells did not have amplified mdr genes nor increased expression; (b) mdr cell lines were not resistant to MGBG; (c) verapamil did not abrogate the resistance of G-cells or mdr cells to MGBG, nor that of G-cells to vincristine; and (d) MGBG resistance was recessive and depended on a membrane block to drug uptake, as opposed to mdr cells which have a dominant phenotype and express increased drug efflux. Therefore we conclude that the G-cells express a different type of multidrug resistance which is distinct from that of classical mdr cell lines.

Beck et al. (12) have reported studies on an unusual mdr cell line which had been selected for resistance to epipodophyllotoxin. This line had selective cross-resistance, failed to respond to verapamil, and showed no increase in p-glycoprotein expression. Other non-p-glycoprotein-mediated, drug-resistant cell lines include human leukemia HL-60 cells (28, 29), breast cancer cell line MCF-7 (30), and a human fibrosarcoma cell line (21). Furthermore, drug resistance due to altered DNA topoisomerase II activity was demonstrated in the K562 cells (31). A comparative discussion of the properties of several of these cells can be found in Beck et al. (12). Another mechanism of drug resistance in cells moderately resistant to vinblastine was caused by a change in lipid composition of the plasma membrane (32).

Our studies add to this list of unusual mdr cell lines, the G-cells, and the new dimension of a relationship to MGBG resistance. MGBG is an antileukemic agent that acts as a polyamine antagonist by inhibiting 5-adenosylmethionine decarboxylase (33). It also causes damage to mitochondria (27). It is thought that MGBG enters the cell via several pathways, one of them being the energy-dependent polyamine transport system (26, 33). Unfortunately, the molecular biology of this transport system is unknown. The decreased uptake of MGBG in the G-cells appears to extend to the polyamines (2) as has
been reported for other MGBG-resistant mutants (34). This could be a contributing factor to the detransformed phenotype of the G-cells, particularly as elevated polyamine levels play an important role in the metabolism of cancer cells (33). Our experiment demonstrated that the MGBG resistance phenotype behaves as a recessive character. This conclusion was confirmed by means of cell fusion experiments. These results also agree with a recent report on MGBG-resistant Chinese hamster ovary cells (35).

Studies on atypical mdr cells may have clinical implications because of the spectrum of drugs against which these cells are cross-resistant. It is possible that cells displaying the phenotype of atypical mdr cells will be found in clinical specimens, just as classical mdr cells have been found (36, 37). Indeed the heterogeneity of cell types in clinical specimens of drug resistance may be a reflection of the presence of atypical as well as classical multidrug-resistant cell types.

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