Isolation and Characterization of an Anthracycline-resistant Human Leukemic Cell Line

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ABSTRACT

An anthracycline-resistant subline of HL-60 promyelocytic leukemia cells (HL-60/AR) has been isolated in vitro by subculturing in progressively higher concentrations of Adriamycin. The resistant cells are capable of sustaining continuous growth in 10^{-6} M Adriamycin which is more than 50 times the 50% inhibitory dose for the parent line. HL-60/AR expressed variable degrees of cross-resistance to daunorubicin, dihydrooxanthracenedione, vincristine, vinblastine, and actinomycin D, but it remained sensitive to methotrexate and 1-β-o-arabinofuranosylcytosine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of glycoproteins of HL-60/AR revealed two prominent glycoproteins with molecular weights of 160,000 ± 10,000 and 110,000 ± 10,000 which were not detected in the sensitive cells. Cellular uptake and retention of daunorubicin was studied in the resistant and sensitive cells utilizing digitized video fluorescence microscopy. The sensitive cells accumulated more drug and showed at least 2-fold greater levels of brightness than the resistant cells. Studies of total intracellular accumulation, utilizing 10^{-8} M [³¹C]-daunorubicin as a marker, showed a 1-h accumulation of 98 ± 20 pmoles/10^6 cells in HL-60/AR versus 255 ± 25 pmoles/10^6 cells in HL-60. Exposure to nontoxic concentrations of the calcium channel blocker Verapamil (10^{-5} M) led to enhanced accumulation (175 ± 8 pmoles/10^6 cells) and retention of the drug in HL-60/AR, resulting in increased cytotoxicity in HL-60/AR. These anthracycline-resistant leukemic cells may serve as a valuable experimental model in studying the phenomenon of multiple drug resistance as well as strategies to circumvent it in human myeloid leukemia.

INTRODUCTION

The anthracycline antibiotics DNR and Adriamycin have significant activity against acute nonlymphocytic leukemia (1, 2). However, resistance to anthracyclines has been commonly observed in patients who relapse after achieving a complete remission with anthracycline-containing regimens (3, 4). Subclinical persistence and eventual emergence of drug-resistant cells are recognized as underlying mechanisms of relapse (5). To study the phenomenon of anthracycline resistance in greater detail, several investigators have developed in vitro or in vivo mammalian cell lines exhibiting varying degrees of resistance to Adriamycin or DNR (6–9). These cells also exhibit cross-resistance to structurally unrelated compounds with different mechanisms of action, i.e., a multiple drug resistance phenotype (8, 10–12). In this initial paper, we report the isolation and characterization of an anthracycline-resistant human promyelocytic leukemia cell line (HL-60/AR), derived from the line initially described by Collins et al. (13). HL-60/AR cells are capable of sustaining continuous growth in 1 μM Adriamycin, more than 50 times the 50% inhibitory drug concentration for the parent cells. Amplified membrane glycoproteins have been identified in several mammalian cell lines exhibiting multiple drug resistance phenotype (14–16). In this study, we have found a similar expression of these glycoproteins in HL-60/AR. Earlier studies involving anthracycline-resistant murine P388, Erhlich ascites, and CHO lines have suggested that resistance may be related to reduced cellular accumulation and retention of the drug (17–19). Moreover, calcium channel blockers (e.g., Verapamil) and calmodulin inhibitors (e.g., trifluoperazine) have been shown to increase the cellular accumulation and retention of anthracyclines, resulting in enhanced cytotoxicity in the drug-resistant cells (20–22). The aim of the present study was to compare the cellular accumulation and retention of DNR in HL-60/AR and HL-60 through labeled [³¹C]DNR studies, as well as the recently described technique of DVFM (23). The effect of Verapamil in intracellular accumulation and retention of DNR was also examined by the 2 methods. A further aim was to determine whether Verapamil could overcome anthracycline resistance in these human myeloid leukemia cells.

MATERIALS AND METHODS

Cells. HL-60 cells were obtained from the original line initially described by Collins et al. (13). They are maintained in suspension culture in RPMI medium (Grand Island Biological Co., Grand Island, NY), supplemented with 1% nonessential amino acids and 10% heat-inactivated FCS. Sterile 75-cm^2 tissue culture flasks containing the cells are kept in a 37°C, 5% CO₂, controlled atmosphere incubator (NAPCO, Portland, OR). Cells are passed twice weekly and routinely examined for Mycoplasma contamination. Logarithmically growing cells are utilized for all experiments. Anthracycline-resistant cells were obtained utilizing the method of Bodner et al. (24) to isolate 6-thioguanine-resistant HL-60 cells. Briefly, 100 ml of cells at a density of 10^6 cells/ml were placed in a 150-ml tissue culture flask. Adriamycin was added to achieve a final concentration of 2 × 10^{-8} M. The flask was placed in an incubator, and at the end of 4 days, the cells were centrifuged at 1200 x g for 6 min, and the cell pellet was resuspended in 200 ml of fresh RPMI medium containing 2 × 10^{-8} M Adriamycin. After an additional 4 days of incubation, the cell suspension was gently layered over 5 ml of lymphocyte separation medium (specific gravity, 1.077; Bionetics, Kensington, MD) in sterile 50-ml centrifuge tubes and spun at 400 × g for 35 min. At the end of this period, the interface layer containing viable cells was transferred by pipet into sterile
15-ml centrifuge tubes, and the cells were washed 3 times with fresh RPMI medium containing 10% FCS and 5 x 10⁻⁴ M Adriaycin and maintained in the same manner as parent HL-60 cells. At weekly intervals, the concentration of Adriaycin was increased by 100 nM increments until a concentration of 10⁻⁴ M Adriaycin was reached. Cells have been maintained in RPMI medium containing 10⁻⁴ M Adriaycin for 6 months and have not exhibited any changes in growth characteristics over this period. A separate flask of Adriaycin-resistant cells has been maintained in Adriaycin-free medium for 3 months without spontaneous reversion to the parent phenotype.

Drugs and Chemicals. SDS, Coomassie Brilliant Blue R-250, and high-molecular-weight standards were purchased from Bio-Rad (Richmond, CA); acrylamide, EDTA, and bisacrylamide were purchased from Eastman (Rochester, NY); Adriaycin was obtained from Adria Laboratories (Wilmington, DE). Trizma base, Tris-HCl, mercaptoethanol, NBT, galactose oxidase, DNR, vincristine, vinblastine, and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO). Mitomycin C was obtained from Bristol Laboratories (Syracuse, NY). DHAD and [¹⁴C]DNR (30.9 mCi/mmol) were supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Verapamil was purchased from Knoll Pharmaceutical Co. (Whippany, NJ); [³H]thymidine (30 Ci/mmol) and sodium [³H]borohydride (500 mCi/mmol) were purchased from Amersham Radiochemicals (Arlington Heights, IL). All other chemicals were of reagent grade and were purchased from Fisher Scientific Co. (Springfield, NJ). Drugs were stored as dry powders at -20°C and reconstituted in sterile medium prior to use.

Radioactively Labeled Drug Accumulation and Retention Studies. The intracellular accumulation of [¹⁴C]DNR in parent and anthracycline-resistant cells was determined by a previously described rapid centrifugation technique (25). Cells were exposed to 10⁻⁴ M [¹⁴C]DNR with and without 10⁻⁶ M verapamil for 60 min, and the total intracellular accumulation was expressed as pmol of DNR per 10⁶ cells. To study the retention of [¹⁴C]DNR, HL-60/AR and HL-60 cells were incubated for 60 min with 1 µM [¹⁴C]DNR, washed, and then resuspended in drug-free PBS for 30 min, with and without 10⁻⁶ M verapamil. At the end of this period, the amount of radioactive drug remaining in the cells was determined and expressed as a percentage of the initial 1-h drug accumulation.

Uptake and Efflux of Daunomycin by DVFM. Uptake and efflux of DNR were quantitated by measuring the fluorescence intensity emitted by the drug associated with the cells, using the DVFM technique previously described in detail (23).

Suspensions (50 x 10⁶/ml) of HL-60 and HL-60/AR cells were allowed to settle onto the surface of a coverslip that had been rinsed in polylysine (25). Five ml of logarithmically growing cells (cell density, 5 x 10⁴ cells/ml) were seeded into 35-mm 6-well tissue culture plates. Various drugs, including Adriaycin, DNR, DHAD, actinomycin D, mitomycin C, vincristine, and vinblastine were added to the plates at several concentrations, and the cells were then placed in the 37°C, 5% CO₂, fully humidified incubator. At 24-h intervals, 1-ml aliquots of cell suspension were obtained for cell density determination utilizing a Model ZBI Coulter Counter (Hialeah, FL). The IC₅₀ for a particular agent is defined as that drug concentration which resulted in 50% reduction in cell number at 72 h relative to untreated control. Values were obtained by linear regression analysis of dose-response curves as previously described (28). The effect of 10⁻⁷ M verapamil on the IC₅₀ of Adriaycin and DNR for HL-60 and HL-60/AR was determined by incubating the cells in the simultaneous presence of verapamil and either of the anthracyclines.

Drug Studies: Soft Agar Colony Growth. HL-60 cells spontaneously form colonies in soft agar with a cloning efficiency of approximately 4 to 10% (27). The effect of continuous exposure to various concentrations of Adriaycin and DNR on the growth of anthracycline-sensitive and -resistant HL-60 cells in soft agar was determined utilizing a previously reported technique (28). The IC₅₀ for a particular drug was defined as that concentration of the drug which reduced colony formation to 50% of the values for untreated control cells. Experiments were performed in duplicate and repeated at least 3 times.

Thymidine Suicide Index Studies. Logarithmically growing HL-60/AR and HL-60 cells were exposed to high specific activity [³H]thymidine (60 Ci/ml) for 20 min in a 37°C incubator. At the end of this period, the cells were washed twice with RPMI medium containing 4 x 10⁻⁶ M unlabeled thymidine. Cells were then plated in soft agar as described above, and the colonies were scored at Day 10. The ratio of the percentage of cells in S phase during the period of [³H]thymidine exposure was determined by comparing colony formation in control and drug-treated cells (29).

Studies of HL-60/AR Cell Morphology and Differentiation. HL-60 cells are capable of undergoing morphological and functional terminal differentiation under the influence of compounds such as DMSO, TPA, and retinoic acid (27). The ability of HL-60/AR and HL-60 to respond to various concentrations of DMSO was determined by assessing morphological criteria of maturation, as well as the functional capacity of the cells to reduce NBT dye. Five ml of cells (10⁵ cells/ml) were seeded into 35-mm 6-well tissue culture plates in the presence of DMSO (1.1 to 1.5%). After 5 days, the cells were centrifuged, and cytospin preparations were made from the cell pellet utilizing a Shandon cytocentrifuge (Shandon Scientific Co., Ltd., London, England). The slides were air dried and stained with Wright-Giemsa. Differential counts were performed using light microscope examination of a minimum of 200 cells per condition. The percentage of mature cells (myelocytes, metamyelocytes, bands, and neutrophils) was determined for each condition. The mitotic index was also ascertained by determining the percentage of cells in mitosis counting a total of 200 cells.

For NBT reduction studies, 1 ml of cells was suspended at 2 x 10⁶ cells/ml in RPMI medium supplemented with 20% FCS. The cells were incubated for 20 min with an equal volume of 0.2% NBT in the presence of 200 ng of TPA. The percentage of cells containing intracellular reduced blue formazan deposits was then determined by counterstaining the slide with safranin solution.

Analysis of Cell Surface Glycoproteins of HL-60 and HL-60/AR Cells. HL-60 and HL-60/AR cells were grown for 5 days without doxorubicin, then washed twice with PBS, resuspended to a concentration of 2.0 x 10⁶ cells/ml, and treated with Vibrio cholerae neuraminidase (0.1 units/ml) for 20 min at 37°C. At the end of this period, the cells were washed twice and further incubated in the presence of galactose oxidase (5 units/ml) for 30 min. The cells were centrifuged, and cytospin preparations were made from the cell pellet utilizing a Shandon cytocentrifuge (Shandon Scientific Co., Ltd., London, England). The slides were air dried and stained with Wright-Giemsa. Differential counts were performed using light microscope examination of a minimum of 200 cells per condition. The percentage of mature cells (myelocytes, metamyelocytes, bands, and neutrophils) was determined for each condition. The mitotic index was also ascertained by determining the percentage of cells in mitosis counting a total of 200 cells.

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RESULTS

Growth and Differentiation Studies. Anthracycline-resistant HL-60 cells shared a variety of biological characteristics with the parent cell line as illustrated in Table 1. Approximately 90 to 95% of cells of both cell lines is comprised of promyelocytes, and the remainder of the cells displays spontaneous differentiation to more mature forms. HL-60/AR cells retained the capacity of the parent cells to respond to inducers of differentiation, such as DMSO. The majority of cells exposed to 1.25% DMSO for 5 days differentiated into mature neutrophils on examination of Wright-stained cytospin preparations. These cells were also capable of reducing NBT dye. No maturation was observed in HL-60/AR and HL-60 cells exposed to Adriamycin concentrations ranging from $10^{-10}$ to $10^{-6}$ M.

The growth characteristics of anthracycline-sensitive and -resistant cells were also virtually identical. Both cell types are capable of forming colonies in soft agar in the absence of exogenous colony-stimulating activity with a plating efficiency of approximately 4.5%. Addition of a source of colony-stimulating activity increased the cloning efficiency by a factor of about 2.5 for both cell types. The percentage of clonogenic cells in S phase, reflected by thymidine suicide index, was $80 \pm 5.0$ for HL-60/AR and $76 \pm 6$ for HL-60. The mitotic indices of the sensitive and resistant cells were also identical (i.e., $2.0 \pm 1.0$).

Suspension Culture and Soft Agar Colony Growth Studies. In contrast, significant differences between the IC_{50} of Adriamycin were noted in HL-60/AR when compared to the parent cells (Table 2). The Adriamycin concentrations resulting in 50% inhibition of suspension culture growth at 72 h were $1.8 \pm 0.5 \times 10^{-5}$ M and $2.0 \pm 10^{-6}$ M for Adriamycin-sensitive and -resistant cells, respectively. Similar values were observed for DNR (e.g., $5 \times 10^{-6}$ M and $2.5 \times 10^{-6}$ M). The corresponding Adriamycin concentrations resulting in a 50% reduction in soft agar colony formation were $1.0 \pm 0.5 \times 10^{-5}$ M and $1.5 \pm 0.5 \times 10^{-6}$ M. The calcium channel blocker verapamil, at a nontoxic concentration of 10 \mu M, enhanced the cytotoxicity of Adriamycin and daunorubicin for HL-60/AR but not for HL-60. The IC_{50} of Adriamycin for HL-60/AR growing in suspension culture decreased from 2 \mu M to 0.3 \mu M when these cells were coincubated with verapamil.

Table 1

<table>
<thead>
<tr>
<th>Biological characteristics</th>
<th>HL-60/AR</th>
<th>HL-60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphology (%)</td>
<td>90 ± 2</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Plate efficiency (%)</td>
<td>4.0 ± 1.5</td>
<td>4.5 ± 1.0</td>
</tr>
<tr>
<td>Mitotic index (%)</td>
<td>3.0 ± 1.0</td>
<td>2.0 ± 1.5</td>
</tr>
<tr>
<td>Response to colony-stimulating activity (%)</td>
<td>2.5 ± 0.3</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Thymidine suicide index (%)</td>
<td>80 ± 5</td>
<td>76 ± 7</td>
</tr>
<tr>
<td>Response to 1.25% DMSO-d_6, % of mature myeloid cells, NBT reduction (%)</td>
<td>88 ± 5</td>
<td>90 ± 5</td>
</tr>
</tbody>
</table>

* Mean ± SD of at least 3 separate experiments.

Cross-Resistance Studies. As shown in Table 2, HL-60/AR exhibited cross-resistance to the closely related analogue DNR comparable to that displayed by Adriamycin. A decreasing magnitude of cross-resistance to DHAD, vincristine, vinblastine, and actinomycin D was observed. The resistant and the sensitive HL-60 cells were equally sensitive to the alkylating agent mitomycin C and the antimetabolites 1-\beta-d-arabinofuranosylcytosine and methotrexate.

Intracellular Accumulation and Retention Studies. Results of total intracellular accumulation of [\textsuperscript{14}C]DNR are presented in Chart 1. The 1-h accumulation of $10^{-4}$ M [\textsuperscript{14}C]DNR was 98 ± 20 pmol/10^6 cells in HL-60/AR versus 255 ± 25 pmol/10^6 cells in HL-60/AR + Verapamil 10^{-5} M/L.
the sensitive HL-60 cells. Simultaneous exposure to 10^{-5} M verapamil enhanced the intracellular accumulation of [14C]DNR to 175 ± 9 pmol/10^6 cells in the HL-60/AR without significantly altering the accumulation in parent cells. The accumulated [14C] DNR retained in the sensitive and resistant HL-60, after incubation for 30 min in the drug-free PBS, was 75 ± 5 and 35 ± 4%, respectively (Chart 2). Coincubation with verapamil (10^{-5} M) increased the drug retention to 78 ± 5% of the accumulated drug in HL-60/AR without augmenting retention in HL-60.

**DVFM.** Serial observations of HL-60 cells in microscopy chambers that were perfused with 10^{-5} M DNR showed rapid cellular uptake of the drug (Chart 3) by both sensitive and resistant cells. In resistant cells, the rate of uptake of drug began to decline within 4 min after the start of drug perfusion. Sensitive cells accumulated more drug and showed at least 2-fold greater levels of brightness at 30 min than the resistant cells. Their brightness declined after washout with PBS. Resistant cells showed a marked decrease in brightness even before drug washout. Sensitive and resistant cells perfused with 10^{-6} M daunorubicin also showed detectable uptake of the drug, greater in sensitive cells (data not shown). Throughout the perfusion, the nucleus was conspicuously less fluorescent than the cytoplasm in all viable cells studied.

**Analysis of Cell Surface Glycoproteins.** SDS-polyacrylamide gel electrophoresis of glycoproteins from cells treated with neuraminidase and labeled with tritiated sodium borohydride after oxidation with galactose oxidase showed that HL-60/AR cells contain 2 prominent tritiable glycoproteins (gp110 and gp160) (Fig. 1). These glycoproteins are either absent or present in minimal quantities in HL-60 parent cells. Coomassie blue staining of the proteins demonstrated easily identifiable protein bands in both HL-60 and HL-60/AR cells but failed to show a corresponding increase in any of the glycoproteins detected by fluorography.

**DISCUSSION**

Although the exact mechanism by which leukemic cells develop resistance to the anthracyclines DNR and Adriamycin remains obscure, the present studies suggest that these cells share a number of characteristics with their murine counterparts. As previously described for Adriamycin-resistant murine P388 and CHO cells and velban-resistant human lymphoblastic cells CEM/VLB100 (7, 8, 10, 33), HL-60/AR also expressed a variable degree of cross-resistance with respect to DHAD, vincristine, velban, and actinomycin D, but not to the alkylating agent mitomycin C or antimetabolites such as 1-β-D-arabinofuranosylcytosine and methotrexate. The cross-resistance of these cells to DHAD, Vinca alkaloids, and actinomycin D is significantly less.

**MOLECULAR WEIGHT**

![Molecular Weight Chart]

Fig. 1. Analysis of cell glycoprotein of HL-60 and HL-60/AR cells by SDS-polyacrylamide gel electrophoresis. Cell surface glycoproteins were tritiated and separated by SDS-polyacrylamide gel electrophoresis as described in text. Lane 1 represents molecular weight standards. Lanes 2 and 3 represent a Coomassie blue protein stain of solubilized glycoproteins from HL-60/AR and HL-60 cells, respectively. Lanes 2a and 3a represent fluorographs of Lanes 2 and 3, respectively.
than the primary resistance to Adriamycin. In this respect, HL-60/AR differs from the previously reported, daunorubicin-resistant CHO cells. However, in vein-resistant human leukemia lymphoblasts CEM/VLB10o, Beck et al. (33) have documented that the cross-resistance to other classes of natural product drugs is not as great as the primary resistance to the selective drug. It is possible that the variable and complex patterns of cross-resistance in multiple drug-resistant phenotypes may result from the differences in membrane alterations, which have been reported, and the diverse selection pressures, which were applied to obtain these phenotypes.

A number of in vitro pharmacological studies of anthracyclines in various resistant murine and human sublines have demonstrated reduced intracellular drug accumulation (34). This phenomenon has been ascribed to a decreased capacity of the drug-resistant cells to retain the accumulated drug (19). Some investigators have suggested that diminished retention is due to an energy-dependent enhanced drug efflux (35). The evidence for this is provided by studies demonstrating that metabolic inhibitors enhance drug retention in resistant cells (17, 35). Other investigators have invoked reduced drug binding and a greater fraction of "releasable" drug in the drug-resistant mutants (33).

The present study utilizing DVFM technique confirms a marked decrease in intracellular accumulation of DNR in the anthracycline-resistant human leukemic cells. Furthermore, a reduced slowly exchangeable drug fraction, which may represent bound intracytoplasmic drug, was observed in HL-60/AR. This could account for the reduced anthracycline accumulation and retention in the resistant sublines. In contrast to a previous study of anthracycline uptake into P388 cells (36), cellular uptake appeared grossly homogeneous. In our study, intranuclear DNR was not well visualized by DVFM, possibly because of fluorescence quenching by DNA. Utilizing radioactively labeled DNR as a marker of anthracycline accumulation, similar differences in drug accumulation and retention were observed between the sensitive and resistant cells.

An overproduction of a high-molecular-weight membrane glycoprotein (gp160) is the most commonly described biochemical marker in anthracycline-resistant cells displaying multiple drug phenotype (14–16). Using sodium [3H]borohydride and galactose oxidase to label the cell surface glycoproteins, 2 prominent glycoproteins, gp160 and gp110, were detected in HL-60/AR but not in HL-60. This is the first description of an enhanced expression of 2 separate membrane glycoproteins in a human myeloid leukemia cell line, selected for resistance to Adriamycin, and exhibiting multiple drug-resistant phenotype. It is also of interest that these cells retain the capacity to undergo terminal differentiation on exposure to inducers of differentiation such as DMSO. At the present time, we have not attempted to correlate the degree of drug resistance with the amount of expression of gp160 as other investigators have reported (37).

Associated genotypic changes, which have been demonstrated in these cells, include double minute chromosomes and homogeneous staining regions (38, 39). Recently, Roninson et al. (40) have provided evidence for gene amplification in multiple drug-resistant Chinese hamster lines. However, gene products of these amplified genes have yet to be identified. Studies are currently under way in our laboratory to assess genotypic changes in HL-60/AR in an effort to elucidate the mechanism of resistance in these cells.

Reversal of anthracycline resistance by calcium channel blockers (e.g., verapamil), calmodulin inhibitors (e.g., trifluoperazine), and other agents such as reserpine has been well documented (20–22, 41). The present study confirms previous findings that a nontoxic concentration of verapamil increases intracellular accumulation and retention of DNR in HL-60/AR but not in HL-60. This resulted in a significant, but partial, reduction of the Adriamycin and DNR IC50 in HL-60/AR. Our data indicate that, although the fraction of retained drug is completely normalized in HL-60/AR treated with verapamil, the total intracellular drug accumulation in HL-60/AR remained lower compared to HL-60. This may explain why verapamil produced only a partial reversal of anthracycline resistance in HL-60/AR.

The biochemical basis of reversal of resistance by calcium channel blockers is incompletely understood. Whether these agents affect drug resistance by modulating intracellular calcium environment and drug efflux across cell membranes is currently under investigation (42). The potentiating effect of verapamil upon anthracycline cytotoxicity is being explored in clinical trials in cancer patients known to be refractory to anthracyclines (43). The availability of HL-60/AR may serve as a valuable experimental model in elucidating the mechanism of multiple drug resistance as well as strategies to circumvent it in human myeloid leukemia.

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