Isolation and Characterization of Adriamycin-resistant HL-60 Cells Which Are Not Defective in the Initial Intracellular Accumulation of Drug

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ABSTRACT

Two human leukemia cell lines (Molt-4 and HL-60) have been used for establishing cells which exhibit a low level resistance to Adriamycin. Analysis of drug uptake patterns shows that the Molt-4 resistant cells are defective in the initial intracellular accumulation of drug. In contrast to Molt-4 the levels of drug which accumulate in the sensitive and resistant HL-60 cells during a 60-min incubation period are essentially the same. However, when incubations are continued there is a major reduction in intracellular drug levels in the resistant cell. Further studies show that resistant cells incubated in the presence of drug for extended time periods efflux drug at a rate considerably greater than that exhibited by the sensitive parental line. Similar efflux patterns are obtained with nuclei isolated from drug-sensitive and -resistant cells. Additional studies using an in vitro phosphorylation system demonstrate distinct protein changes in membranes of Molt-4 and HL-60 resistant cells. Thus, we have found that a membrane fraction from the Molt-4 resistant line contains a M, 170,000 protein which is not detected in a similar fraction from cells sensitive to drug. HL-60 resistant membranes contain two proteins with molecular weights of 150,000 and 120,000 which are not found in membranes from drug-sensitive cells. The results of this study suggest that drug resistance in HL-60 cells is related to an efflux mechanism which is triggered only after cells are exposed to drug for prolonged periods.

INTRODUCTION

During the past several years a number of cell lines have been isolated which exhibit various levels of resistance to the antitumor agent Adriamycin (1-11). Analysis of these cells has shown that resistance is related, in all cases, to a reduced intracellular accumulation of drug (3-10). It has also been observed that, in most cell lines examined, reduced drug levels occur as a result of a defect in the initial cellular uptake of drug (3-10, 12-14). The exact basis of this phenotype is not known but may be due to decreased drug binding (15) or transport (12) or to an enhanced efflux mechanism which extrudes drug from the cell (3, 7-9, 11, 16).

In the present study we describe the isolation of an Adriamycin-resistant human leukemia cell line (HL-60) which is not defective in the initial cellular accumulation of drug. Analysis of these cells shows, however, that resistance can be explained by the presence of an efflux mechanism which is activated when cells are exposed to drug for prolonged periods.

MATERIALS AND METHODS

Materials. [14C]Adriamycin (50 mCi/mmole) and [3H]Daunomycin (4.2 Ci/mmol) and [γ-32P]ATP (2900 Ci/mmol) were from New England Nuclear. [32P]Pi was from ICN. Unlabeled Adriamycin and daunomycin were provided by the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute.

Isolation of Cells Resistant to Adriamycin. Drug-resistant cells were prepared from the two human leukemia cell lines, Molt-4 (T-lymphoblast) (17) and HL-60 (promyelocyte) (18). Resistant lines were obtained by continuous growth in increasing levels of Adriamycin. Cells were grown in RPMI medium containing 10% fetal calf serum. Dose-response curves for Adriamycin-resistant lines were determined by growing cells in increasing levels of drug and counting cells capable of excluding trypan blue in a hemacytometer. Values of the concentration of drug resulting in 50% loss of cell viability were determined from the dose-response curves. Under these conditions the HL-60 and Molt-4 isolates are 10- and 15-fold, respectively, more resistant to Adriamycin than the sensitive parental cell lines. These cells also exhibit similar levels of resistance to daunomycin. Before use the resistant cells were grown in drug-free medium for several generations. The drug-resistant phenotype has remained stable during growth in the absence of drug for 1.5 years.

Analysis of Drug Uptake and Efflux. Sensitive and resistant HL-60 and Molt-4 cells were seeded at a density of 3 x 10^6/ml in 3.0 ml of RPMI-10% fetal calf serum. [14C]Adriamycin or [3H]daunomycin was added to the cell cultures to a final concentration of 2 μg/ml. The cells were incubated at 37°C in a CO_2 incubator and after various time periods were centrifuged. The cell pellets were suspended in 0.1 ml of N NaOH and incubated for 2 h at 55°C. An aliquot was taken for radioactivity and protein (19) determination.

For drug efflux studies sensitive or resistant cells at a density of 3 x 10^6/ml in complete RPMI medium were loaded with [3H]daunomycin for 30 min time periods. For each experiment 6 x 10^6 cells were used with daunomycin added to a final concentration of 1 μg/ml. After the drug-loading period the cells were centrifuged and suspended in fresh RPMI medium. The cells were incubated at 37°C, and at various time periods, an aliquot was taken and centrifuged. The cell pellet was suspended in 0.2 ml of N NaOH and incubated for 2 h at 55°C. An aliquot was taken for radioactivity determination. Additional studies have been carried out in which drug efflux was measured from nuclei isolated (20) from drug-sensitive and -resistant HL-60 cells. In these experiments cells were loaded with drug as described above and thereafter centrifuged. The cells were suspended in 1.5 ml of 10 mm Tris-HCl (pH 7.6)-2 mM MgCl_2 and allowed to swell for 5 min on ice. The cells were then disrupted with a glass homogenizer. Cell breakage as monitored by phase contrast microscopy was always greater than 95% (usually 25 strokes of the pestle). The nuclei isolated after centrifugation at 450 x g for 5 min were suspended in 2.0 ml of fresh RPMI medium and thereafter incubated at 37°C. After incubation for various time periods a 0.35-ml aliquot was taken and centrifuged at 8000 x g for 5 min. The nuclear pellet was solubilized in N NaOH, and an aliquot was taken for radioactivity determination.

Adriamycin Inhibition of DNA and RNA Synthesis. HL-60 sensitive and resistant cells were seeded at a density of 3 x 10^6 cells/ml in 60-mm tissue culture dishes. Adriamycin was added at various concentrations, and the cells were incubated for 3.5 h at 37°C. Control cells did not receive Adriamycin. At the end of the incubation period the [3H]thymidine added to a final concentration of 2 or 1 μCi/ml, respectively. The cells were incubated an additional 10 min at 37°C, centrifuged for 5 min at 2000 x g, and thereafter incubated in 0.5 ml of 0.1 m Tris-HCl (pH 7.6). To the cell suspension 0.5 ml of 10% trichloroacetic acid were added, and the cells were incubated on ice for 10 min. The cell suspension was poured over a glass fiber filter and washed with 10 ml of 0.01 n HCl. The filters were dried, and the radioactivity was determined.

In Vitro Phosphorylation of Proteins in Isolated Membranes. Sensitive and resistant cells were grown in RPMI medium for 48 h, after which time the cells were collected, and a membrane fraction containing both plasma membranes and endoplasmic reticulum was prepared as described previously (21). The membranes were suspended in 0.01 M
Tris-HCl (pH 7.6)-0.125 M sucrose and stored on ice. For in vitro phosphorylation isolated membranes (25 μg of protein) were incubated in a 25-μl reaction mixture containing 0.05 M Tris-HCl (pH 7.6), 2 mM β-mercaptoethanol, either 5 mM Mn²⁺ or Mg²⁺, and 3 μCi of [γ-32P] ATP. Incubations were carried out for various time periods at room temperature, and the reaction was stopped by the addition of 10 mM EDTA. Samples were thereafter electrophoresed in a 7% polyacrylamide gel (22), and the phosphorylated proteins were detected by autoradiography.

Labeling the Cell Surface of Drug-sensitive and -resistant Cells. Drug-sensitive or -resistant cells growing in RPMI medium were centrifuged and washed once in PBS. The cells were suspended in 2 ml of PBS-5 mM glucose at a density of 1 × 10⁷/ml. Cell surface labeling with ¹²⁵I was carried out as described by Hynes (23). Incubations with laccoperoxidase and glucose oxidase were carried out for 15 min at room temperature. At the end of the incubation period the cells were washed 3 times with PBS-0.1% NaCl. The final pellet was suspended in 0.2 ml of 0.01 M Tris-HCl (pH 7.6)-1.5% Triton X-100-0.2% sodium dodecyl sulfate. The cells were incubated 2 h on ice and thereafter centrifuged at 10,000 × g for 15 min. Samples were taken for acid precipitation, and equal amounts of radioactivity were applied to a 7% polyacrylamide gel. Labeled proteins were detected by autoradiography.

In Vivo Labeling and Analysis of Phosphoproteins in HL-60 Sensitive and Resistant Cells. Sensitive or resistant cells were grown in 40 ml of complete RPMI medium for 48 h. The cells were thereafter centrifuged and suspended in 20 ml of medium containing 0.05 M Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM KCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 5.5 mM d-glucose, 1× minimal essential medium, amino acids, and vitamins. 32Pi was added to a concentration of 10 μCi/ml, and the cells were incubated for 60 min at 37°C. After the incubation period the cells were centrifuged, and membranes containing both plasma membranes and endoplasmic reticulum were prepared as previously described (21). The membranes were suspended in 0.01 M Tris-HCl (pH 7.6), and an aliquot was taken for radioactivity determination. Equal amounts of radioactively labeled protein were electrophoresed in a 7% polyacrylamide gel.

RESULTS

Analysis of Cellular Drug Accumulation. In these experiments sensitive and resistant cells were incubated with radioactively labeled Adriamycin or daunomycin, and the time course of cellular drug uptake was determined. Molt-4 resistant cells were found to be defective in the initial uptake of drug and thus to accumulate less drug than the sensitive cell through the entire incubation period (Fig. 1). This pattern of drug uptake is closely similar to that found for several other cell lines which exhibit resistance to Adriamycin (3, 4, 6–9). We have also noticed that, during the early period of drug accumulation, there is a transient increase in the uptake of Adriamycin or daunomycin (Fig. 1). The basis of this drug uptake pattern is not known. In contrast to the Molt-4 cells the levels of drug uptake in the sensitive and resistant HL-60 cells are essentially the same through a 60-min incubation period (Fig. 2). However, after this time period the levels of drug in the resistant cell begin to decline, whereas in the sensitive cell drug levels continue to increase or reach a plateau (Fig. 2). In different experiments of this type this same pattern of cellular drug accumulation has been obtained with Adriamycin and daunomycin concentrations ranging from 0.3–2 μg/ml. In a number of experiments we have found some variation in the exact time period when drug levels begin to decline in the resistant cell. Thus in some experiments loss of drug from the cell begins after a 40- to 60-min incubation period. The reason for the variation is not known, but it does not seem to be related to the use of different concentrations of drug in the uptake experiments. The HL-60 sensitive and resistant cells were also found to exhibit an early period of drug uptake where Adriamycin or daunomycin levels increase and then rapidly decline (Fig. 2). Like Molt-4 this occurs at about 10 min after the addition of drug to the cells.

Drug Efflux in HL-60 Cells. Sensitive or resistant HL-60 cells were loaded with [³H]daunomycin, and the cells were thereafter collected and suspended in fresh medium. Drug efflux was determined over a 60-min incubation period. During this time there is about a 15% loss of drug from the sensitive cell (Fig. 3A). In contrast, resistant cells efflux about 40% of the

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3 The abbreviations used are: PBS, phosphate-buffered saline containing 0.01 M phosphate (pH 7.6) and 0.15 M NaCl; p150, M, 150,000 protein (p170 and p120 defined similarly).
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Fig. 3. Efflux of daunomycin from HL-60 cells and isolated nuclei. Drug-sensitive and resistant cells were incubated with [3H]daunomycin for 70 min at 37°C. The cells were collected, and drug efflux was measured from either whole cells (A) or isolated nuclei (B) as described in "Materials and Methods." x, sensitive cells or nuclei; o, resistant cells or nuclei. [3H]Daunomycin was used in these studies, since it is available at a specific activity considerably greater than that for [14C]Adriamycin.

intracellular drug through a 60-min incubation period. Additional studies have been carried out in which drug efflux was measured with nuclei isolated from cells previously loaded with [3H]daunomycin. The results of these studies also show a greater efflux of drug from resistant nuclei as compared to nuclei isolated from cells sensitive to drug. Thus after a 60-min incubation period 10% of the drug has been removed from sensitive nuclei, whereas 40% of the [3H]daunomycin has been effluxed from nuclei isolated from resistant cells (Fig. 3B). At the present time the exact nuclear location of the drug which is being effluxed is not known.

Adriamycin Inhibition of DNA and RNA Synthesis in Sensitive and Resistant HL-60 Cells. Sensitive and resistant HL-60 cells were incubated in the absence or presence of Adriamycin, and the synthesis of DNA and RNA was determined after labeling with [3H]thymidine (Fig. 4A) or [3H]uridine (Fig. 4B). The results of these studies show that, in order to inhibit DNA and RNA synthesis by 50%, resistant cells require 2-3 times as much drug as the sensitive cell. These results thus suggest that the levels of drug removed from the resistant cell after prolonged incubation periods are significant and could contribute to a major reduction in the cytotoxic activity of Adriamycin.

Identification of Protein Changes in Membranes of Drug-resistant Cells. In these studies a membrane fraction containing both plasma membranes and endoplasmic reticulum was isolated from Molt-4 and HL-60 cells and thereafter incubated in an in vitro protein phosphorylation system containing [γ-32P]-ATP. The proteins phosphorylated were analyzed after polyacrylamide gel electrophoresis. Incubation of the Molt-4 resistant cells. In contrast to Molt-4, the HL-60 membranes in the in vitro system in the presence of Mg2+ ATP. The proteins phosphorylated were analyzed after polyacrylamide gel electrophoresis. Incubation of the Molt-4 resistant cells. In contrast to Molt-4, the HL-60

DISCUSSION

In the present study we have isolated two human leukemic cell lines (HL-60 and Molt-4) which exhibit resistance to Adriamycin. The cells were isolated for low level resistance in an effort to avoid the possibility that prolonged exposure to high levels of drug induces multiple cellular changes which obscure the identification of a primary lesion which contributes to drug resistance. Cells with low level resistance may also be more closely related to the clinical setting. The results of this study show that Molt-4 resistant cells are defective in the initial cellular accumulation of drug and thus exhibit a drug uptake pattern similar to a number of other Adriamycin-resistant cell lines which have been previously described (3, 4, 6-9). It has also been observed that resistant cells exhibiting this phenotype contain a M, 150,000 protein which is absent in membranes from drug-sensitive cells (Fig. 7, Lanes C and D). In studies carried out thus far we have not been able to identify p120 with in vivo 32P labeling or by 125I labeling in the presence of lactoperoxidase.

Additional studies show that membranes isolated from resistant cells labeled in vivo with 32P also contain p150 (Fig. 7, Lane B). Under identical labeling conditions this protein is essentially absent in membranes from drug-sensitive cells (Fig. 7, Lane A). Cell surface labeling also shows that resistant cells contain a M, 150,000 protein which is absent in cells sensitive to drug (Fig. 7, Lanes C and D). In studies carried out thus far we have not been able to identify p120 with in vivo 32P labeling or by 125I labeling in the presence of lactoperoxidase.
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the same. If, however, the cells are exposed to drug for prolonged periods a major efflux mechanism is activated which removes drug from the resistant but not the sensitive cell. Of interest is the finding that nuclei isolated from resistant cells also exhibit enhanced levels of drug efflux. HL-60 resistant cells may thus contain two types of efflux mechanisms, one which removes drug from the cytoplasm and a second in which drug is effluxed from the nucleus to the cytoplasm with subsequent removal from the cell. It is also of interest that, although the Molt-4 and HL-60 cells were isolated during parallel treatments with Adriamycin, the drug uptake patterns are distinct. Whether the pattern found in the HL-60 cells is in some way related to their myeloid origin remains to be determined. Recently Bhalla et al. (11) have isolated HL-60 cells which exhibit a 50-fold increase in resistance to Adriamycin. It was observed that sensitive and resistant cells also accumulate equal levels of drug but only during the first 4 min of the incubation period. After this time period there is a rapid loss of drug from the resistant cell. It thus may be indicated that, as cells reach higher levels of resistance, the efflux mechanism exists in an active state and thus removes drug soon after it enters the cell. Such seems to be the case for P388 leukemic cells which exhibit a 100-fold increase in resistance to Adriamycin (3). The results of the present study also suggest that drug efflux (3, 12) and not drug binding (15) or transport (12) is the basis of resistance in the HL-60 isolates.

In the present study we have found that membranes of resistant HL-60 cells contain 2 proteins (p150 and p120) which are essentially absent in membranes of cells sensitive to drug. The identification of these 2 proteins has been greatly facilitated by the use of an in vitro protein phosphorylation system in the presence of high-specific-activity [gamma-32P]ATP. Previously we have also used a similar in vitro system to identify multiple protein changes in membranes of Chinese hamster lung cells resistant to Adriamycin (26). The results obtained thus far suggest that in vitro phosphorylation may provide a highly sensitive system for detecting protein changes in a variety of cell lines which exhibit Adriamycin resistance. It is also of interest that p150 is the major membrane protein which is phosphorylated in the in vitro system. Since this protein is only a very minor membrane component it must contain many sites for phosphorylation and be located in the membrane such that it is highly accessible for phosphorylation by a protein kinase. Drug-resistant related membrane phosphoproteins have also been identified in other cell lines (10, 21). The finding that p150 is labeled in vivo with 32P suggests the possibility that phosphorylation may play a role in regulating the biological activity of this protein. It is also of interest that previous studies

![Fig. 5. In vitro phosphorylation in membranes isolated from sensitive and resistant Molt-4 cells. Isolated membranes were incubated in the standard reaction mixture containing either 5 mM Mg2+ (Lanes A and B) or 5 mM Mn2+ (Lanes C and D). After a 10-min incubation period the phosphorylated proteins were analyzed after polyacrylamide gel electrophoresis. Lanes A and C, sensitive membranes; Lanes B and D, resistant membranes.](image1)

![Fig. 6. In vitro phosphorylation in membranes isolated from sensitive and resistant HL-60 cells. Isolated membranes were incubated in the standard reaction mixture containing 5 mM Mn2+. After an incubation period of 5 min (Lanes A and B), 10 min (Lanes C and D), or 20 min (Lanes E and F), the phosphorylated proteins were analyzed after polyacrylamide gel electrophoresis. Lanes A, C, and E, sensitive membranes; Lanes B, D, and F, resistant membranes.](image2)
have provided evidence that phosphorylation may regulate the function of 2 drug-resistant related proteins in Chinese hamster lung cells (27–29). Bhalla et al. (11) have also found by using cell surface labeling techniques that 50-fold Adriamycin-resistant HL-60 cells contain proteins with molecular weights of 160,000 and 110,000 which are greatly reduced in cells sensitive to drug. It is also indicated from the present work that, in low level resistant cells, p150 and p120 are not active in restricting the initial cellular accumulation of drug. Thus if these proteins are involved in drug efflux they must be activated after prolonged exposure to drug. The activation process may involve some topological change of the protein in the membrane and/or some structural modification, such as phosphorylation.

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