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Membrane glycoprotein changes associated with anthracycline resistance in HL-60 cells*

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Summary. The glycoproteins on the surface of HL-60/S wild-type, drug-sensitive human leukemia cells and HL-60/AR anthracycline-resistant cells which do not overexpress the P-glycoprotein, were characterized by labeling with [^{35}S]-methionine, NaB[$^{3}H_4$], phosphorus 32, or sodium iodide I 125. HL-60/S and HL-60/AR cell lysates and membrane fractions tagged with [^{35}S]-methionine or phosphorus 32 showed no significant differences in their protein patterns as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by autoradiography. HL-60/S cells labeled with NaB[$^{3}H_4$] yielded glycoproteins that were smeared predominantly in the molecular-weight range of 210,000 and 160,000 Da, with pI values ranging between pH 4 and pH 4.4. In contrast, NaB[$^{3}H_4$]-labeled HL-60/AR cells showed 7–8 discrete glycoproteins within a molecular-weight range of 170,000 and 140,000 Da, with pI values also ranging between pH 4 and pH 4.4. In addition, [^{3}H]-glucosamine incorporation into HL-60/S and HL-60/AR cells revealed that the latter showed lower uptake of [^{3}H]-glucosamine than did the former. Following treatment with tunicamycin, [^{3}H]-glucosamine uptake in HL-60/S cells decreased, whereas that in HL-60/AR cells remained unchanged. Surface-membrane radioiodination of HL-60/S and HL-60/AR cells showed two distinct protein electrophoretic patterns, with differences being observed in both the high- (220–95 kDa) and low-molecular-weight ranges (21 kDa). Flow cytometric analysis of HL-60/S and HL-60/AR cells using myeloid and lymphoid antigen-specific antibodies demonstrated no antigenic differences between HL-60/S and HL-60/AR cells. HL-60/S cells incubated in the presence of tunicamycin, an inhibitor of *N*-linked glycosylation, or the protein kinase C agonist phorbol 12-myristate 13-acetate (PMA) developed a glycoprotein pattern similar to that observed in HL-60/AR cells. In addition, tunicamycin treatment of HL-60/S cells decreased daunorubicin (DNR) retention and altered its intracellular distribution as compared with that in HL-60/AR cells. These data indicate that HL-60/AR cells do not possess either de novo or amplified high-molecular-weight surface-membrane proteins; instead, existing proteins are hypoglycosylated. These results also show that HL-60/AR cells exhibit the multidrug-resistant phenotype in association with altered membrane glycoproteins of both high (220–95 kDa) and low molecular weight (21 kDa), but without overexpression of the P-glycoprotein. Furthermore, in HL-60/S cells, the multidrug-resistant phenotype is partially inducible by inhibition of *N*-linked glycosylation of cell-surface proteins.

Introduction

In the study of experimental multidrug resistance (MDR), two forms have been described, classic and atypical MDR [5]. The classic form of MDR has been associated with cross-resistance between anthracyclines and vinca alkaloids, decreased drug accumulation due to active efflux of drug, circumvention of MDR with cationic amphophilic reagents such as verapamil or tamoxifen, and the overexpression of the mRNA and gene product for P-glycoprotein [1, 4, 20, 24, 28]. The atypical form of MDR has been characterized by cross-resistance between anthracyclines and epipodophyllotoxins but not vinca alkaloids [12]. Other characteristics of these cells include the absence of the P-glycoprotein, no change in drug accumulation, and the lack of circumvention of MDR following treatment with

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Abbreviations: HL-60/AR, anthracycline-resistant cells; HL-60/S, parental HL-60 cells; DNR, daunorubicin; RA, retinoic acid; PBS, phosphate-buffered saline (Hanks' Balanced Salt Solution); PMA, phorbol 12-myristate,13-acetate; DVFM, digitized video fluorescence microscopy; DFP, diisopropylfluorophosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MDR, multidrug resistance; 6GT, 6-thioguanine; NP-40, Nonidet-40 detergent

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verapamil [6]. The atypical MDR phenotype in these cells has also been correlated with changes in the nuclear enzyme topoisomerase II [27].

Our laboratory has previously reported an anthracycline-resistant human myeloid leukemic cell line (HL-60/AR) that possesses many of the features of the classic form of MDR described above [7]. This cell line was developed by exposure to increasing levels of anthracyclines. HL-60/AR cells are cross-resistant to vinca alkaloids. MDR in these cells is circumvented using verapamil. HL-60/AR cells have also been shown to accumulate less daunorubicin (DNR) due to increased drug efflux out of the cell, which can be blocked by verapamil. Additionally, after exposure to DNR, a different pattern of intracellular drug distribution has been found in HL-60/AR cells as compared with the sensitive HL-60/S cells [19]. The HL-60/AR line does not overexpress the P-glycoprotein [10] and may therefore be a prototype of a third category of MDR. The present study futher elucidates the mechanism(s) of drug resistance in HL-60/AR cells by examining high-molecular-weight glycoproteins before and after the inhibition of *N*-linked glycosylation with tunicamycin and then assessing their role in drug retention and intracellular drug distribution.

Materials and methods

Buffers and reagents. Hanks' phosphate-buffered saline (PBS) was buffered at pH 7.4; it and RPMI-1640 medium were purchased from Gibco Laboratories (Grand Island, N.Y.). The following were also purchased from Gibco: glutamine, pyruvate, nonessential amino acids, penicillin, streptomycin, and fetal calf serum (FCS). The following reagents were purchased from the sources indicated: [^{35}S]-methionine, sodium iodide I 125, phosphorus 32, NaB[^{3}H$_4$], [^{3}H]-glucosamine, and [^{14}C]-DNR, Amersham Corp. (Arlington Heights, Ill.); acrylamide, bisacrylamide, ammonium persulfate, *N,N,N′,N′*-tetramethyl ethylenediamine, sodium dodecyl sulfate (SDS), Coomassie blue dye, bromophenol blue, and molecular-weight standards, Bio-Rad Laboratories (Richmond, Calif.); Kodak XAR-5 X-ray film, Eastman Kodak Co. (Rochester, N.Y.); 14″ × 14″ X-ray cassette, Manostat Laboratories Instruments and Scientific Apparatus (New York, N.Y.); phorbol 12-myristate 13-acetate (PMA), retinoic acid (RA), Nonided detergent (NP-40), iodoacetamide, hydrogen peroxide, lactoperoxidase, diisopropylfluorophosphate (DFP), tunicamycin, and poly-L-lysine, Sigma Chemical Co. (St. Louis, Mo.); Scintillation fluid and vials, Fisher Scientific Co. (Springfield, N.J.); neuraminidase, Calbiochem (La Jolla, Calif.); DNl, Ives Laboratories (Menlo Park, Calif.). Monoclonal antibodies to cell-surface antigens were obtained from the following sources: anti-Ia, B1 (CD20), B4 (CD19), MY7 (CD13), and MY9 (CD33), Coulter (Hialeah, Fla.); Leu9 (CD7), Becton-Dickinson (Mountain, Calif.); OKM1 (CD11B) and OKM5, Ortho Pharmaceutical (Raritan, N.J.).

Tissue culture. HL-60/S and HL-60/AR cells were cultured at 5×10^5 cells/ml in RPMI-1640 medium containing the following: 2 mM glutamine, 1 mM pyruvate, 0.1 mM nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. HL-60/AR cells were grown in the absence of 0.1 μM DNR at 2 weeks prior to all experiments. Culture flasks were incubated at 37° C in a humidified atmosphere containing 5% CO_2. Cells were routinely tested for mycoplasma contamination.

Radiolabeling of HL-60/S and HL-60/AR proteins and surface glycoproteins. Both HL-60/S and HL-60/AR cells were grown in media supplemented with 1 mM [^{35}S]-methionine (45.4 TBg/mmol) for 24 h. These cells were harvested, washed three times with PBS, and sonicated in the presence of the protease inhibitors DFP (1 mM) and iodoacetamide (1 mM). Cell lysates were centrifuged at 8,000 *g* for 15 min. In addition, membrane fractions were isolated from cell lysates by ultracentrifugation at 100,000 *g* for 1 h [13]. Both the lysate supernatants and membrane fractions were then analyzed by electrophoresis under reducing conditions in 7.5% polyacrylamide slab gels containing SDS. SDS-PAGE was run using a constant current of 30 mA. Gels were stained with Coomassie blue dye, heat-dried under vacuum, then exposed to Kodak XAR-5 X-ray film and developed by an X-omat automated developer.

In other experiments, HL-60/S and HL-60/AR cells were analyzed for their surface glycoproteins using a previously described method [7]. Briefly, HL-60/S and HL-60/AR cells (10 million) were treated with *Vibrio cholerae* neuraminidase (0.1 units/ml) for 15 min at 37° C, washed and incubated with 5 units/ml galactose oxidase for 30 min at 37° C, then washed and resuspended in PBS containing 2 mCi NaB[^{3}H$_4$] for 20 min at 0° C. The cells were then washed and sonicated as described above. Cell lysates were analyzed in the first dimension by isoelectric focusing using a pH gradient of 4.5–7 and in the second dimension by SDS-PAGE (7.5% polyacrylamide). The gels were processed as described above except that they were silver-stained and autoradiographed.

To determine the amount of [^{3}H]-glucosamine incorporated into membrane compounds, HL-60/S and HL-60/AR cells (3×10^5 cells/ml) were grown in the presence and absence of tunicamycin for 24 h, then exposed to 1.7 mCi [^{3}H]-glucosamine for an additional 24 h. Cells were lysed and the membranes were isolated by ultracentrifugation at 100,000 *g* for 1 h. The total amount of [^{3}H]-glucosamine was determined by counting the membrane pellets in a scintillation counter.

Immunofluorescent cytofluorometric analysis of HL-60/S and HL-60/AR cells. The immunophenotypic profiles of HL-60/S and HL-60/AR cells were determined by indirect immunofluorescence using a fluorescent-activated cell sorter as previously described [22].

Immunochemical staining for P-glycoprotein. The Chinese hamster ovary cell lines AuxB$_1$ and CH^{r}C5 were kindly provided by Dr. V. Ling of the Ontario Cancer Institute (Toronto, Canada). The anti-P-glycoprotein monoclonal antibodies MRK16, 265/F4, and C219 were used in these studies. The MRK16 antibody [18] was a gift from Dr. T. Tsuruo of the Japanese Foundation for Cancer Research (Tokyo, Japan). The 265/F4 antibody [23] was generously supplied by Dr. W. L. McGuire and co-workers at the University of Texas, San Antonio. The C219 antibody [21] was a gift from Dr. Ling and was also obtained from Centocor, Inc. (Malvern, Pa.).

AuxB$_1$ and CH^{r}C5 cells were grown directly on glass microscope slides using 8-well microculture chambers (Lab-Tek, Inc.). HL-60 cells were placed on glass microscope slides coated with poly-L-lysine. Cells on glass slides either left were unfixed (for MRK16 studies) or were fixed with 1 : 1 v/v ether: ethanol for 10 min (for 265/F4 or C219 studies). Reaction with the anti-P-glycoprotein monoclonal antibody was accomplished at room temperature by overlaying the cells with 35 μl TBS (TRIS-buffered saline: 0.05 M TRIS, 0.15 M NaCl, pH 7.6) containing the appropriate amount of monoclonal antibody (10 μg/ml for MRK16, 12.5 μg/ml for 265/F4, or 5 μg/ml for C219) for 30 min in a humidified chamber. Antibody binding to the cells was detected by an alkaline phosphatase-anti-alkaline phosphatase method (APAAP) [11]. A positive result was indicated by the appearance of red color associated with the cell membrane as viewed by light microscopy; results were graded from 0 to +4 on the basis of color intensity by an observer blinded to the cell type used.

Analysis of phosphoproteins by in vivo labeling of HL-60/S and HL-60/AR cells. A total of 10 million cells were washed and resuspended in 20 ml medium containing 0.05 M TRIS-HCl (pH 7.6), 0.15 M NaCl, 5 mM KCl, 0.5 M $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM D-glucose, 1 × minimal essential medium, amino acids, and vitamins. The cells were incubated with phosphorus 32 (10 μCi/ml) for 2 h at 37° C, washed three times in the same buffer, then resuspended in ice-cold lysis buffer, sonicated, and centrifuged at 8,000 *g* for 15 min in the cold room. In addition, membrane fractions were isolated from cell lysates by ultracentrifugation at 100,000 *g* for 1 h. Both the cell lysates and the membrane fractions were then analyzed by SDS-PAGE and autoradiography.

Analysis of HL-60/S and HL-60/AR cells following tunicamycin, PMA or RA treatment. HL-60/S and HL-60/AR cells were cultured either for 1 or 24 h in the presence of 20 nM PMA or 2 μg/ml RA or for 48 h with 2 μg/ml tunicamycin. These concentrations of PMA or RA were not toxic to the cells as assessed by trypan blue exclusion. Tunicamycin at this concentration was also not toxic to the cells as assessed by the two-color fluorochromatic cytotoxicity test [29]. At the end of the incubation period, cells were harvested and their extracts were analyzed by electrophoresis. In addition, DNR accumulation, retention and intracellular distribution was also assessed using radioactive drug and digitized video fluorescence microscopy (DVFM) [19] following treatment with Tunicamycin of intact cells.

For electrophoretic analysis, HL-60/S and HL-60/AR cell-surface proteins were labeled with sodium iodide I 125 using the lactoperoxidase method [17]. These cells were washed twice in PBS containing 1 mM potassium iodide, rewashed twice in PBS, and resuspended in PBS containing 1% NP-40, DFP, and iodoacetamide. The cell lysates were centrifuged at 8,000 *g* for 15 min. The supernatants were then transferred to SDS sample buffer, boiled for 90 s, and analyzed by SDS-PAGE. For DVFM studies, treated HL-60/S and HL-60/AR cells (as described above) were centrifuged and resuspended in PBS (pH 7.4) at 1×10^6 cells/ml and incubated with 2.5 μM DNR for 1 h at 37° C in an atmosphere containing 5% CO_2. The cells were then centrifuged, resuspended in 15 μl ice-cold PBS, and examined by DVFM using a 530- to 560-nm excitation filter and a 580-nm barrier filter such as that used for rhodamine fluorescence tests. The data was recorded on 0.75-in. videotape. For DNR accumulation and retention, 2 μM [^{14}C]-DNR was incubated with untreated or tunicamycin-treated HL-60/S or HL-60/AR cells in RPMI-1640 (3.5 ml) for 1 h at 37° C in an atmosphere containing 5% CO_2. Two equal aliquots were removed: one was immediately centrifuged through mineral oil; the other was centrifuged, washed once in ice-cold RPMI-1640, and resuspended in 0.5 ml RPMI-1640 for an additional 30 min at 37° C in an atmosphere containing 5% CO_2 before being centrifuged through mineral oil. Both accumulation and retention pellets were cut and placed in 10 ml scintillation fluid, and [^{14}C]-DNR counts were determined in a scintillation counter.

Results

Radiolabeling of HL-60/S and HL-60/AR cellular proteins and surface glycoproteins

To investigate the difference between proteins in HL-60/S and HL-60/AR cells, we radiolabeled cells from each of these lines with [^{35}S]-methionine. Subsequently, the cells were sonicated and the lysates and membranes were analyzed by SDS-PAGE and autoradiography. As shown in Fig. 1 (lanes 1, 2) no significant differences were observed in the protein patterns exhibited by HL-60/S vs HL-60/AR cells, suggesting that there are no differences in protein expression between these two cell lines. As shown in Fig. 1 B (lane 2), HL-60/AR cells displayed all of the high-molecular-weight proteins observed in HL-60/S cells when the gel was exposed for longer periods, indicating that these proteins are expressed in both cell lines. In vivo labeling of HL-60/S and HL-60/AR cells with phosphorus 32 revealed no differences in their phosphoprotein patterns of lysates or membrane fractions following SDS-PAGE and autoradiographic analysis (Fig. 2, lanes 1, 2). These data do not rule out the possibility that the resistant HL-60/AR cells were hyperphosphorylated.

Alterations in HL-60/S and HL-60/AR surface-membrane glycoproteins were determined by external surface-carbohydrate labeling with NaB[^{3}H$_4$] using neuraminidase-treated cells. These cells were solubilized and ana-

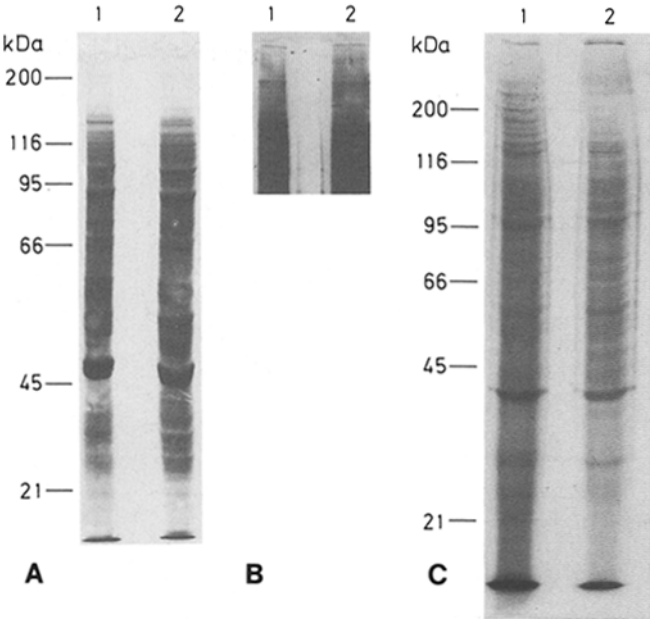

Fig. 1 A – C. Autoradiograph of SDS-PAGE analysis of biosynthetically labeled HL-60/S and HL-60/AR cells with [^{35}S]-methionine. HL-60/S and HL-60/AR cells were grown in the presence of [^{35}S]-methionine for 24 h. These cells were treated with lysis buffer, sonicated, and centrifuged, and the lysates or membrane fractions were analyzed by SDS-PAGE under reducing conditions (2-mercaptoethanol). An equal amount of protein was loaded for each lane. **A** HL-60/S cell lysates (*lane 1*) and HL-60/AR cell lysates (*lane 2*). **B** Exposure of the same gel for 5 days, showing only high-molecular-weight material. **C** HL-60/S cell-membrane fraction (*lane 1*) and HL-60/AR cell-membrane fraction (*lane 2*)

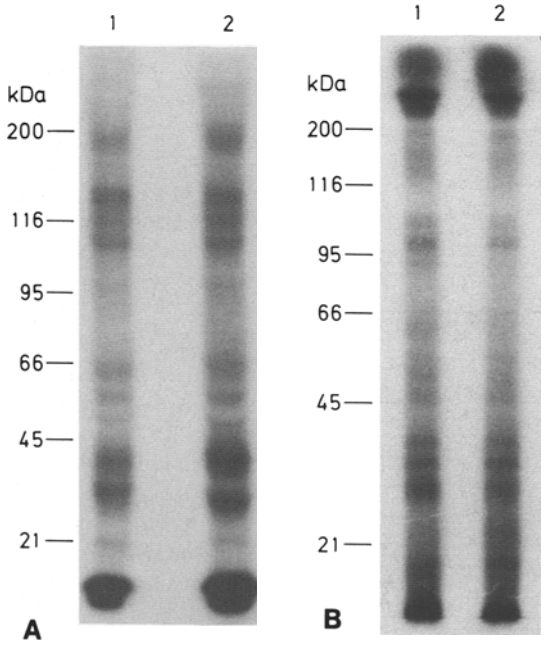

Fig. 2 A, B. Analysis of phosphoproteins by in vivo labeling of HL-60/S and HL-60/AR cells. In all, 10 million HL-60/S and HL-60/AR cells were washed and resuspended in 20 ml cell buffer. Cells were then incubated with phosphorus 32 (10 μCi/ml) for 2 h at 37° C. The cells were then washed three times in the same buffer, resuspended in lysis buffer, sonicated, and centrifuged at 8,000 *g* for 15 min or at 100,000 *g* for 1 h. The cell lysate and membrane fractions were then analyzed by SDS-PAGE and autoradiography. An equal amount of protein was loaded for each lane. **A** Lysates, *lane 1* (HL-60/S) and *lane 2* (HL-60/AR). **B** Membrane fractions, *lane 1* (HL-60/S) and *lane 2* (HL-60/AR)

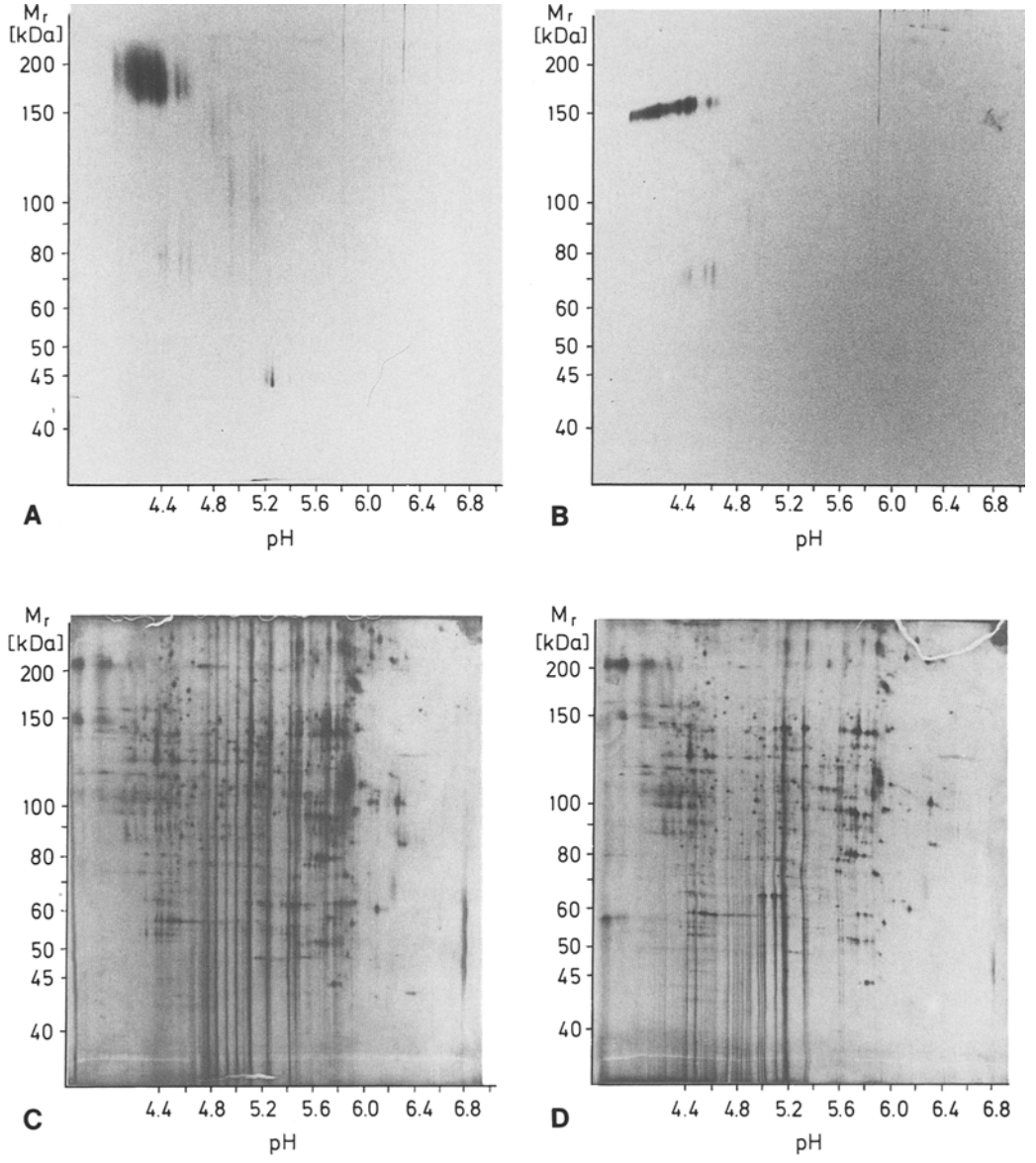

Fig. 3 A–D. Two-dimensional electrophoretic analysis of the cell-surface glycoproteins in HL-60/S and HL-60/AR cells. In all, 10 million HL-60/S and HL-60/AR cells were treated with 0.1 units/ml neuraminidase for 15 min at 37° C and then exposed to 5 units/ml galactose oxidase for 30 min at 37° C, followed by incubation with 2 mCi NaB[$^3H_4$] for 20 min at 0° C. These cells were sonicated in lysate buffer and centrifuged at 8,000 *g* for 20 min. The supernatants were analyzed by two-dimensional electrophoresis, the first dimension involving iso-electric focusing and the second, SDS-PAGE. The gels were then exposed to X-ray film for 15 days at –70° C. An equal amount of protein was loaded for each lane. The vertical values represent the molecular-weight standards and the horizontal numbers indicate the pH values. **A** HL-60/S. **B** HL-60/AR. Silver-stained gels: **C** HL-60/S, **D** HL-60/AR

lyzed by isoelectrofocusing and SDS-PAGE. The autoradiographs of these gels are shown in Fig. 3. The HL-60/S cells demonstrated a smeared pattern between molecular weights of 210,000 and 160,000 Da in the SDS-PAGE dimension (Fig. 3A). The isoelectrofocusing dimension showed denser streaks at pH 4 and 4.4. In contrast, HL-60/AR cells exhibited distinct glycoproteins within the molecular-weight range of 170,000–140,000 Da (Fig. 3B). We also observed 7–8 distinct bands between pH 4 and pH 4.4 following isoelectrofocusing. Figures 3C and 3D demonstrate the silver-stain patterns of the gels shown in Figs. 3A and 3B. The HL-60/S cell extracts were smeared (Fig. 3C) as compared with the HL-60/AR cells (Fig. 3D). Figures 3C and 3D appear to be very similar except that there seem to be some additional protein bands in the molecular-weight range of 60–70 kDa (pI 5.4–6) in HL-60/S cells that were not found in HL-60/AR cells. Whether these bands represent distinct proteins or altered forms of the same protein (via different glycosylation) is unknown. These data show that there are distinct differences between glycoproteins in HL-60/S vs HL-60/AR cells as analyzed by NaB[$^3H_4$] radiolabeling.

To determine the relative degree of glycosylation of membrane-associated glycoproteins in HL-60/S and HL-60/AR cells, we incubated these cells in the presence or absence of tunicamycin prior to [^{3}H]-glucosamine exposure. As shown in Table 1, HL-60/S cells demonstrated increased [^{3}H]-glucosamine incorporation as compared with HL-60/AR cells. This label decreased in HL-60/S

Table 1. Incorporation of [^{3}H]-glucosamine in HL-60/S and HL-60/AR cells following tunicamycin treatment

	[^{3}H]-Glucosamine	
	Media	Media Plus tunicamycin
HL-60/S	4,130 ± 648	1,538 ± 232
HL-60/AR	2,476 ± 430	2,525 ± 441

Data are expressed as cpm of precipitable counts (± SD)

Table 2. Antigenic determination of HL-60 and HL-60/AR cells

	Surface antigens[a]							
	Ia	CD20	CD19	CD11	OKM5	CD13	CD33	CD7
HL-60	–	–	–	2+	2+	3+	3+	–
HL-60/AR	–	–	–	1+	1+	3+	3+	–

[a] Surface antigens were determined by indirect immunofluorescence of intact cells as described in Materials and methods
Values represent the relative fluorescence intensity of positive cells stained with each monoclonal antibody tested. +, positive; –, negative

Table 3. Immunochemical staining for P-glycoprotein

Cell line	MRK16	265/F4	C219
Human leukemia:			
HL-60/S	+1 (6)	+0 (5)	+1 (6)
HL-60/AR	+1 (6)	+0 (5)	+1 (6)
Chinese hamster ovary:			
$AuxB_1$ (sensitive)	+1 (6)	+0 (3)	+1 (3)
CH^rC5 (MDR)	+2 (6)	+4 (3)	+3 (3)

Values represent a scale of positivity ranging from 0 (negative result) to +4 (intense positivity; see Materials and methods). Each value represents the median of values obtained in separate individual experiments done on different days. For each individual experiment, each treatment was performed in duplicate. Numbers in parentheses represent the number of individual experiments carried out

cells following tunicamycin treatment, whereas the HL-60/AR line showed no decrease in [^{3}H]-glucosamine labeling following drug treatment. These data support the hypothesis that HL-60/AR cells are hypoglycosylated as compared with HL-60/S cells.

Antigenic determination of HL-60/S and HL-60/AR cells

HL-60/S and HL-60/AR cells were analyzed for the expression of common myeloid and lymphoid surface-membrane antigens using a panel of monoclonal antibodies. As shown in Table 2, both HL-60/S and HL-60/AR cells have similar immunophenotypic profiles, these being Ia, CD20, CD19, and CD7 (negative) and CD11B, OKM5, CD13, and CD33 (positive). The results indicate that HL-60/AR cells do not show altered expression of common myeloid or lymphoid antigens as compared with the HL-60/S line.

Immunochemical staining for P-glycoprotein

HL-60/S and HL-60/AR cells were studied for the presence of P-glycoprotein using the immunochemical APAAP method (see Materials and methods) in conjunction with three monoclonal antibodies known to react with P-glycoprotein: MRK16 [18], 265/F4 [23], and C219 [21]. These antibodies have been shown to detect P-glycoprotein overexpression in multidrug-resistant murine leukemia cells (P388/ADR), human myeloma cells (RPMI 8226/dox6 or RPMI 8226/dox40), Chinese hamster lung cells (DC3F/AD-X), or Chinese hamster ovary cells (CH^rC5) as compared with the respective drug-sensitive parental cell lines [30]. Both HL-60/S and HL-60/AR cells showed borderline reactions (+1), if any, to APAAP staining using these three antibodies (Table 3). By comparison, the known multidrug-resistant Chinese hamster cell line CH^rC5 displayed specifically higher immunoreactivity with all three antibodies as compared with the drug-sensitive parental Chinese hamster ovary cell linie $AuxB_1$ (Table 3).

Analysis of HL-60/S and HL-60/AR cells following tunicamycin, PMA and RA treatment

Radioiodination of surface-membrane proteins found in HL-60/S and HL-60/AR cells was performed not only to analyze differences in proteins but also to assess their accessibility to iodine 125. HL-60/S and HL-60/AR cells were grown in the presence of 2 μg/ml tunicamycin (48 h), PMA and RA (1 h or 24 h) and were then radioiodinated and analyzed by SDS-PAGE and autoradiography.

As shown in Fig. 4, lanes 1 and 3, and Fig. 5, lane 1, HL-60/S cells differ in their electrophoretic pattern following surface radioiodination as compared with HL-60/AR cells. HL-60/S cells show a smeared electrophoretic pattern between the molecular-weight range of 200,000 and 95,000 Da as compared with HL-60/AR cells (Fig. 4, lanes 1, 3); when treated with tunicamycin (Fig. 4, lane 2), the former lost this smeared electrophoretic pattern. In addition, the amount of radioiodinated glycoprotein was reduced in HL-60/S cells treated with tunicamycin as compared with untreated HL-60/S cells. A distinct glycoprotein weighing approx. 130,000–150,000 Da was the only one labeled with iodine 125 within the range of 200,000–95,000 Da in tunicamycin-treated HL-60/S cells. We also observed an increase in the labeling of low-molecular-weight material, in particular a 21,000 Da protein in HL-60/S cells treated with tunicamycin and the loss of a smeared band at approx. 30,000 Da. In contrast, following tunicamycin treatment HL-60/AR cells retained the high-molecular-weight glycoproteins observed in untreated HL-60/AR cells, including the 130,000- to 150,000-Da glycoprotein (lane 4) and the 21,000-Da band. The only differences observed between untreated and tunicamycin-treated HL-60/AR cells involved changes in the mobility of the 95,000-Da protein and increases in radioiodinated material at approx. 30,000 Da (Fig. 4, lanes 3, 4). Unlike HL-60/S cells treated with tunicamycin, the HL-60/AR cells showed an increase in radioiodinated proteins with electrophoretic patterns that were not smeared but instead displayed well-resolved protein bands.

Tunicamycin treatment of HL-60/S and HL-60/AR cells did not result in any gross diminution in protein per million cells, nor did it induce nonspecific esterase activity, NBT reduction, or the common myeloid-differentiation markers (data not shown). As shown in Fig. 5A, HL-60/S cells treated with PMA for 24 h (lane 3) gained the 130,000- to 150,000-Da glycoprotein. HL-60/S cells treated with PMA for 1 h (lane 2) or with RA for 1 and 24 h (lanes 4 and 5, respectively) remained unchanged as compared with the untreated cells (lane 1). HL-60/AR cells

were unchanged in terms of electrophoretic pattern following all treatments (Fig. 5B, lanes 1–5). Both PMA and RA differentiated HL-60/S cells but did not affect HL-60/AR cells as assessed by nonspecific esterase activity, NBT reduction, and common myeloid-differentiation markers (data not shown). These data indicate that the differences in glycoproteins observed between HL-60/S and HL-60/AR cells were due in part to hypoglycosylation of surface proteins possessing *N*-linked sugars in the latter cells. The hypoglycosylated status observed in HL-60/AR cells is inducible in HL-60/S cells following treatment with PMA for 24 h or with tunicamycin for 48 h. In addition, perturbation of the normal *N*-linked glycosylation pathway enabled the low-molecular-weight (21 kDa) protein to become accessible to membrane radioiodination.

Both HL-60/S and HL-60/AR cells treated with tunicamycin were also analyzed for retention and intracellular distribution of DNR using DVFM. As shown in Fig. 6, HL-60/S cells treated with 2 μg/ml tunicamycin for 48 h retained less drug than did untreated HL-60/S cells. In contrast, tunicamycin-treated HL-60/AR cells retained amounts of drug similar to those measured in untreated cells. DVFM analysis demonstrated that HL-60/S cells distribute DNR in the cytoplasmic and nuclear compartments (Fig. 7A), whereas HL-60/AR cells localize DNR into vesicular organelles (Fig. 7C). When HL-60/S cells were treated with tunicamycin (Fig. 7B), DNR was distributed into vesicular organelles in a fashion similar to that observed in untreated and tunicamycin-treated HL-60/AR cells (Fig. 7C, D). These data indicate that following tunicamycin treatment, HL-60/S cells are partially converted into the HL-60/AR phenotype in terms of DNR retention and distribution.

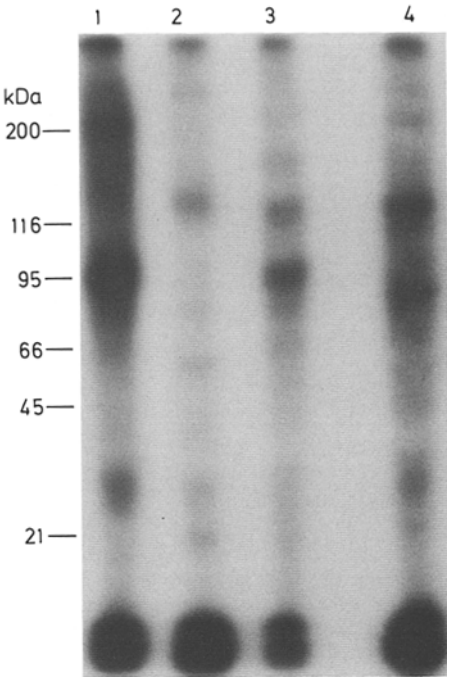

Fig. 4. SDS-PAGE analysis of the surface glycoproteins on HL-60/S and HL-60/AR cells following treatment with 2 μg/ml tunicamycin for 48 h. Cell-surface glycoproteins were iodinated and separated by SDS-PAGE and autoradiography. The molecular-weight standards are illustrated on the *far left*. An equal amount of protein was loaded for each lane. This gel was exposed to X-ray film for 24 h. *Lane 1*, HL-60/S cells; *lane 2*, HL-60/S cells treated with tunicamycin, *lane 3*, HL-60/AR cells, *lane 4*, HL-60/AR cells treated with tunicamycin

Discussion

The present study demonstrates that the phenotypic changes associated with anthracycline resistance in HL-60/AR cells are in part due to hypoglycosylation of the surface-membrane glycoproteins. Following [^{35}S]-methionine treatment, HL-60/S and HL-60/AR cells showed no significant differences in their electrophoretic patterns as assessed by SDS-PAGE and autoradiography (Fig. 1). HL-60/S and HL-60/AR cells labeled with phosphorus 32 also displayed no difference in their electrophoretic pattern (Fig. 2), demonstrating that there is no increase in specific phosphoproteins in HL-60/AR cells.

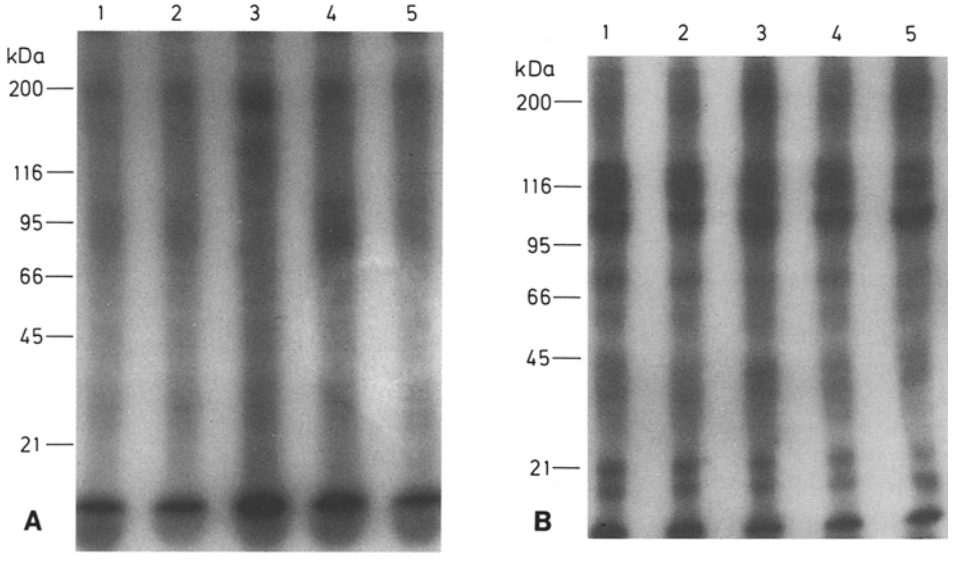

Fig. 5A, B. SDS-PAGE analysis of the iodinated surface glycoproteins on HL-60/S and HL-60/AR cells following PMA and RA treatment. HL-60/S and HL-60/AR cells were treated with either PMA or RA for 1 h and 24 h. The intact cells were iodinated and the cell-surface proteins were analyzed by SDS-PAGE and autoradiography. An equal amount of protein was loaded for each lane. The HL-60/S gel was exposed to X-ray film for 2 days and the HL-60/AR gel was exposed for 4 days. Increased exposure time only made the patterns darker and did not reveal additional bands. **A** HL-60/S. **B** HL-60/AR. *Lane 1*, untreated cells; *lane 2*, 1-h PMA; *lane 3*, 24-h PMA; *lane 4*, 1-h RA; *lane 5*, 24-h RA

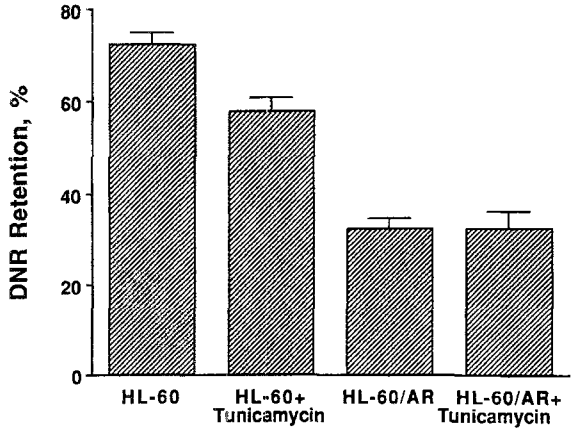

Fig. 6. DNR intracellular-retention in HL-60/S and HL-60/AR cells following treatment with tunicamycin. HL-60/S and HL-60/AR cells were grown in the presence of 2 μg/ml tunicamycin for 48 h. These cells were then incubated for 60 min with [^{14}C]-DNR, washed, resuspended in drug-free PBS, and incubated at 37° C for 30 min. At the end of this period, the [^{14}C]-DNR retained in the cell pellet was determined. Columns, represent the percentage of the labeled drug retained at 30 min relative to the values obtained at time zero; bars indicate the SD. Experiments were performed in triplicate on at least 2 separate occasions. The difference between HL-60/S and HL-60/S treated with tunicamycin is significantly (P <0.01)

This makes HL-60/AR cells distinct from the other P-glycoprotein-negative, multidrug-resistant (MDR) HL-60 cell line described by Marsh et al. [26]. Our results indicate that no proteins were overexpressed or preferentially phosphorylated in HL-60/AR cells as compared with HL-60/S cells. Following carbohydrate labeling with NaB[^{3}H$_4$], HL-60/S and HL-60/AR cells exhibited different electrophoretic patterns (Fig. 3). HL-60/S cells predominantly possess high-molecular-weight glycoproteins (range, 210,000–160,000 Da; pI values, pH 4–4.4); this was evident by smearing of the material labeled between the above molecular-weight and pI values, characteristic of an increased and heterogeneous glycosylation of proteins. The silver-stained gels seemed to demonstrate some protein differences at the molecular weight of 60–70 kDa (pI 5.4–6) in HL-60/S as compared with HL-60/AR cells. The relevance of these proteins in MDR is not known. In contrast, HL-60/AR cells exhibited 7–8 discrete glycoprotein bands, predominantly in the molecular-weight range of 170,000–140,000 Da, with pI values ranging between pH 4 and pH 4.4. In addition, Table 1 demonstrates that HL-60/AR cells were hypoglycosylated as compared with HL-60/S cells. These results indicate that the high-molecular-weight surface glycoproteins found in HL-60/S and HL-60/AR cells differ in their sugar residues and that those in HL-60/AR cells are more homogeneous

These data are compatible with our previously published work showing that HL-60/AR cells express different glycoproteins at an approx. molecular weight of 160,000–120,000 Da as compared with HL-60/S cells. The above data show that there are various species of glycoproteins with different pI ranges, further illustrating glycosylation differences between HL-60/S and HL-60/AR cells [7].

HL-60/S and HL-60/AR cells were previously analyzed for the presence of the MDR gene product using a human 5′mdr cDNA probe in dot-blot studies and the monoclonal antibody C219 in Western blot analysis [10]. These analyses showed no increased expression of the MDR mRNA or of the surface-membrane P-glycoprotein in HL-60/S or HL-60/AR cells. In the present study, the anti P-glycoprotein monoclonal antibodies MRK16, 265/F4, and C219 failed to detect any specific increase in immunochemical staining (APAAP method) in HL-60/AR cells as compared with HL-60/S cells. The results of these APAAP studies could be interpreted as indicating either the absence of overexpression of P-glycoprotein in HL-60/AR cells or

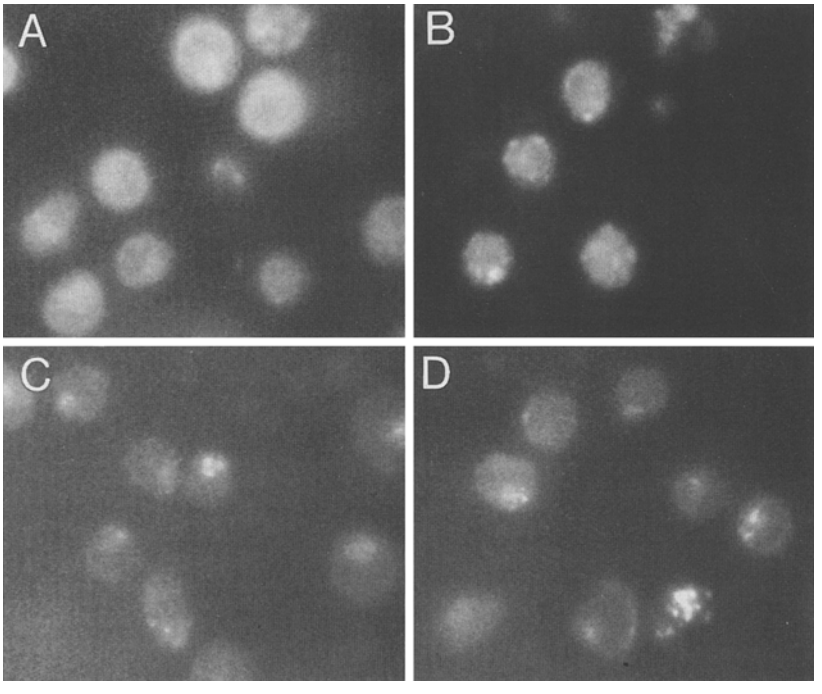

Fig. 7 A – D. Intracellular distribution of DNR in HL-60/S and HL-60/AR cells following treatment with tunicamycin as assessed by DVFM. HL-60/S and HL-60/AR cells were grown in the presence of tunicamycin for 48 h. These cells were then incubated with DNR for 1 h and analyzed by DVFM. Each photograph represents DVFM images following the averaging of 15 frames. **A** Untreated HL-60/S. **B** HL-60/S treated with tunicamycin. **C** Untreated HL-60/AR. **D** HL-60/AR treated with tunicamycin

that the three different epitopes recognized by MRK16, 265/F4, and C219 [18, 21, 23] are not conserved in the "putative P-glycoprotein" expressed in HL-60/AR cells. However, in view of the above-mentioned dot-blot analyses [10], we favor the former interpretation of the APAAP results. Thus, HL-60/AR cells appear to represent a third category of MDR, and we suggest that the P-glycoprotein is not overexpressed in these cells and, hence, is not a necessary component of the local intracellular events determining drug resistance in cells expressing the MDR phenotype.

To determine whether the observed differences between glycoproteins in HL-60/S and HL-60/AR cells were attributable to a change in known cell-surface antigens, the expression of common myeloid and lymphoid antigens was analyzed in the two cell types (Table 2). Both HL-60/S and HL-60/AR cells showed identical myeloid and lymphoid cell-surface antigens. These results are consistent with the [^{35}S]-methionine labeling study (Fig. 1), which also demonstrated no differences between proteins in HL-60/S and HL-60/AR cells.

The relationship of altered carbohydrate moieties of cell-surface glycoproteins to MDR is not known. Several studies of differences between carbohydrates in drug-sensitive vs -resistant cells have shown changes in glycosylation patterns [2, 8, 25]. CEM/VLB cells (a human leukemic lymphoblastic cell line resistant to vinblastine) showed a prominently increased expression of glycosylated proteins at molecular weights between 190,000 and 170,000 Da as compared with parent CEM cells [3]. In the same study, a series of 90,000 Da glycoproteins found on the drug-sensitive cells were noted to be diminished in the drug-resistant cells. Treatment of CEM/VLB cells with tunicamycin deleted the resistance-associated glycoproteins. The deletion of hyperglycosylated proteins by tunicamycin did not alter the accumulation or retention of [^{3}H]-VLB in CEM and CEM/VLB cells. In a distinct doxorubicin-resistant HL-60 cell line developed by Marsh et al. [26], it was demonstrated that both the resistant and the parent sensitive subline possess a 150,000-Da protein that is predominantly glycosylated. Gallagher et al. [16] showed that HL-60 cells resistant to the differentiation inducers retinoic acid, dimethylsulfoxide (DMSO), or 6-thioguanine (6TG) demonstrate hyposialylation of surface-membrane glycoproteins as compared with wild-type HL-60 cells. In the latter cells, following treatment with neuraminidase, surface-membrane glycoproteins tagged with iodine 125 resembled the two-dimensional electrophoretic pattern shown by glycoproteins observed in RA- and 6TG-resistant HL-60 cells [14].

In the present study, SDS-PAGE analysis of drug-sensitive HL-60/S cells grown in the presence of tunicamycin for 48 h revealed radiolabeling of surface-membrane glycoproteins similar to that observed in untreated HL-60/AR cells (Fig. 4). In addition, untreated HL-60/S cells tagged with iodine 125 displayed a smeared pattern as compared with tunicamycin-treated HL-60/S cells (Fig. 4, lanes 1, 2). HL-60/S cells grown in tunicamycin also acquired a drug-resistant phenotype with respect to DNR retention and intracellular distribution (Fig. 6, 7). In contrast, tunicamycin treatment of HL-60/AR cells had no effect on the radiolabelling of membrane glycoproteins or on DNR distribution or retention. Moreover, HL-60/AR cells exhibited distinct and well-resolved glycoprotein patterns as compared with HL-60/S cells following SDS-PAGE and autoradiography (Fig. 4, 5A, lane 1; Fig. 5B) These results indicate that the carbohydrate moieties on these glycoproteins may partially mediate drug accumulation and retention.

Another protein alteration observed in HL-60/AR cells that was not seen in HL-60/S cells was the surface expression of a 21,000-Da protein, which was not expressed following PMA treatment but was noted following tunicamycin treatment (Fig. 4; 5A, B). It has been shown that phosphorylation of a 21,000-Da protein in human breast-cancer cell lines and small-cell lung cancer lines is associated with MDR [15]. The present study showed no differences in the phosphorylation status of cellular proteins between HL-60/AR and HL-60/S cells. Thus, neither the increased expression of the P-glycoprotein nor the phosphorylation of distinct proteins appear to be required for decreased net drug accumulation or increased drug efflux in the HL-60/AR cell line.

In our study, parent HL-60/S cells acquired a 130,000- to 150,000-Da surface glycoprotein, as was observed in the drug-resistant HL-60/AR cells following treatment with PMA for 24 h. HL-60/S surface glycoproteins labeled with iodine 125 remained unchanged following a 1-h incubation with PMA or any RA treatment (Fig. 5A). HL-60/AR cells also did not change their surface-glycoprotein profile following treatment with either PMA or RA (Fig. 5B). Both PMA and RA had no effect on surface markers (as shown in Table 2) in HL-60/AR cells following 1 or 24 h incubation. In contrast, following PMA or RA incubation, HL-60/S cells showed an increased expression of Table 2 markers as assessed by immunofluorescent cytofluorometry (data not shown). These data suggest that HL-60/AR cells are resistant to the effect of both PMA and RA as compared with HL-60/S cells.

DNR is known to bind to model membranes in two ways: by ionic attractions between the amino group of daunosamine and charged membrane groups and by hydrophobic interactions of the DNR aglycone moiety with the membrane hydrocarbon interior [9]. It has been speculated in other MDR cell lines that increases in cellular phosphorylation and/or increased expression of P-glycoprotein may increase the binding of anthracyclines to membranes, causing the drug to be more rapidly exchangeable with the extracellular milieu via membrane trafficking [2, 9]. It is possible that after the removal of *N*-linked sugars, DNR is more effectively bound to membranes because of their lower net charge. DNR would then colocalize intracellularly with these membrane glycoproteins during normal metabolic turnover; in turn, the drug would partition with these membranes, enabling drug to efflux more readily with the exterior of the cell.

In summary, the present study indicates that the P-glycoprotein is not required for the classic MDR phenotype and that changes in the glycosylation of preexisting surface proteins may play a role in resistance in HL-60/AR cells. These cells should prove to be useful models for the study of alternative mechanisms of MDR.

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