Pergamon

Biochemical Pharmacology, Vol. 49, No. 6, pp. 755–762, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(94)00511-7

CHARACTERIZATION OF AN ANTHRACYCLINE-RESISTANT HUMAN PROMYELOCYTE LEUKEMIA (HL-60) CELL LINE WITH AN ELEVATED MDR-1 GENE EXPRESSION

KERSTIN JÖNSSON,* NITA DAHLBERG,* ULF TIDEFELT,‡ CHRISTER PAUL* and GÖRAN ANDERSSON†

*Division of Clinical Hematology and Oncology, Department of Medicine, †Division of Pathology, Department of Immunology, Microbiology, Pathology and Infectious Diseases, Huddinge Hospital, Karolinska Institute, S-141 86 Huddinge; ‡Department of Medicine, Örebro Medical Centre Hospital, S-701 85 Örebro, Sweden

(*Received* 22 *April* 1994; *accepted* 5 *November* 1994)

Abstract—Multidrug resistance to a variety of cytotoxic drugs is due to decreased drug accumulation at the intracellular site of drug action. When due to increased energy-dependent drug efflux, this transport change is often associated with increased expression of an efflux pump for various lipophilic compounds, for example the P-glycoprotein which is the product of the MDR-1 gene. However, previously described HL-60 human promyelocytic leukemia cell lines resistant to the cytotoxic effect of anthracyclines have been reported *not* to express P-glycoprotein. We have isolated, by drug selection, an anthracycline-resistant HL-60 cell line that, in comparison to parental drug sensitive cells, exhibits a multidrug resistant phenotype including diminished intracellular drug retention, cross-resistance to multiple cytotoxic drugs, increased expression of a monoclonal antibody C219-reactive 180 kDa P-glycoprotein detected by Western blot analysis as well as increased expression of MDR-1 mRNA as determined by Northern blot and solution hybridization/RNAse protection analyses. Evidence is presented that the anthracycline-resistant HL-60 cells have amplified the MDR-1 gene.

Key words: HL-60; leukemia; MDR-1; multidrug resistance; solution hybridization; doxorubicin

Tumor cell resistance to cytotoxic agents is considered one of the major obstacles to successful chemotherapy. Multidrug resistance (MDR§), i.e. resistance to the cytotoxic effects of several different, structurally unrelated compounds (i.e. vinca alcaloids, anthracycline antibiotics and epipodophyllotoxin derivatives), can either be present at the time of initial diagnosis or be acquired during chemotherapy. Progress in elucidation of mechanisms of multiple drug resistance has been favored by the availability of cultured cell lines resistant to cytotoxic drugs with different modes of action. One of the most consistent changes in multidrug resistant cell lines is the over-expression of a 170–180 kDa P-glycoprotein encoded by the multidrug resistance (MDR)-1 gene [1]. This P-glycoprotein is a transmembrane protein which functions as an ATP-dependent efflux pump for lipophilic compounds [2, 3]. The P-glycoprotein belongs to the ATP-binding cassette (ABC) transporter superfamily, which also includes certain bacterial transport proteins, the cystic fibrosis chloride conductance regulator and the major histocompatibility complex class II-linked peptide transporters [4].

MDR genes and the MDR phenotype are often expressed in hematological malignancies [5–7]. P-glycoprotein is expressed in hematopoietic progenitor cells [8], and malignancies derived from hematopoetic cells at an early stage of differentiation exhibit an intrinsic type of drug resistance [9, 10]. Furthermore, after chemotherapy, an increased proportion of leukemic cells expressing the P-glycoprotein can sometimes be observed [11].

Previous studies have shown that HL-60 promyelocytic leukemia cells selected to tolerate the cytotoxic effect of the anthracycline doxorubicin exhibit increased energy-dependent drug efflux [12, 13] without over-expressing P-glycoprotein [14] or MDR-1 mRNA [15]. In this report, we have characterized doxorubicin-resistant HL-60 cells that express an MDR phenotype, i.e. functional multidrug resistance, over-express P-glycoprotein and the corresponding mRNA transcript and exhibit amplification of the MDR-1 gene.

MATERIALS AND METHODS

Materials. The chemotherapeutic drugs used were obtained from the following sources: doxorubicin (Adriamycin®), epirubicin (Farmorubicin®) and idarubicin (Idarubicin®) were purchased from Farmitalia Carlo Erba, Italy, and daunorubicin (Cerubidin®) from Rhône-Poulenc Rorer, France.

† Corresponding author: Department of Pathology, Huddinge Hospital, S-141 86 Huddinge, Sweden. Tel. +46-8-746-5646; FAX +46-8-779 5520.

§ Abbreviations: Mab, monoclonal antibody; MDR, multidrug resistance; ara-C, 1-β-D-arabinofuranosyl cytosine; CdA, chlorodeoxyadenosine; IC_{50}, drug concentration that reduced cell growth by 50%; cDNA, complementary DNA; AB complex, avidin–biotin complex; HRP, horseradish peroxidase.

Cytosine arabinoside (ara-C, Cytosar®) came from Upjohn, U.S.A.; chlorodeoxyadenosine (CdA) from Dr Zygmunt Kazimierczuk, Foundation for the Development of Diagnostics and Therapy, Warsaw, Poland; amsacrine (Amekrin®) from Parke-Davis, U.S.A.; etoposide (Vepesid®) from Bristol-Myers Squibb, U.S.A.; melphalane (Alkeran®) from Wellcome, U.K.; and vincristin (Vincristine Lederle®) from David Bull/Cyanamid, U.S.A. Verapamil (Isoptin®) was obtained from Knoll, Germany. All other reagents were of analytical grade or higher.

Cell culture and incubations. Human promyelocyte leukemia cells, HL-60, obtained from American type culture collection (ATCC, Rockville, U.S.A.), were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, NY, U.S.A.) and 1 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO) and incubated in a humified atmosphere with 5% CO_2 at 37°.

Isolation of doxorubicin-resistant cells. HL-60 cells were selected for doxorubicin resistance by continuous stepwise increments of doxorubicin concentration from 0.04 μM to 10 μM. 2.5×10^5 cells/mL were suspended in culture medium in the presence of doxorubicin. Medium was changed every fourth day, and the concentration of doxorubicin was increased when cell numbers increased at a similar rate as the parental HL-60-S cells. Resistant sublines tolerating 0.5 μM, 5 μM or 10 μM doxorubicin, i.e. HL60-$R_{0.5}$, HL-60-R_5 and HL-60-R_{10}, respectively, were collected and stored in liquid nitrogen with 10% DMSO (Merck, Darmstadt, Germany) as the cryoprotective agent. The isolates exhibited a 14-fold (HL60-$R_{0.5}$), 142-fold (HL-60-R_5) and 328-fold (HL-60-R_{10}) increased tolerance to doxorubicin. Karyotyping of both HL-60-S and HL-60-R_{10} showed that the number of normal chromosomes in HL-60-R_{10} compared to HL-60-S was reduced. The two marker chromosomes, del 9p13 and del 10p11-12, were observed in both sublines.

Drug uptake assay. Three pairs of cell suspensions (2 mL, 10^6 cells/mL) were incubated with 1 μM doxorubicin for 3 hr. After sonication and TCA extraction, the intracellular uptake and retention of doxorubicin was determined by spectrophotofluorometry [16]. All incubations were performed in duplicate. To study the effect of verapamil on intracellular drug retention in HL-60-S and HL-60-R_{10} cells, incubations were performed in the presence or absence of 5 μg/mL verapamil; 2 pairs of incubations were continued for 2 and 18 hr, respectively after a change to doxorubicin-free medium.

In vitro *drug sensitivity assay.* For determination of IC_{50} values for HL-60-S and HL-60-R sublines, 2 mL cell suspension (10^5 cells/mL) was incubated with increasing concentrations of cytostatic drugs. The cells were incubated with ara-C and CdA continuously, with doxorubicin for 3 hr and with daunorubicin, idarubicin, epirubicin, mitoxantrone, etoposide, amsacrine, melphalane, and vincristine for 1 hr. After the 1 or 3 hr incubations, the cells were resuspended in fresh medium and cultured for 4 days. The incubation times for the different drugs were those that have been shown to best mimic *in vivo* concentrations in tumor cells in patients during therapy [17–19]. Drug cytotoxicity was determined at the end of the culture period by an automated bioluminiscent assay for cellular ATP [20, 21] and expressed as the ratio of the ATP content in drug-exposed to unexposed cells.

Western blot analysis. Plasma membranes and endoplasmic reticulum membranes from resistant HL-60-R_{10} cells and sensitive HL-60-S cells were isolated using a minor modification of the method described in [22]. Cells were hypotonically lysed in 1 mM Tris (pH 7.2) and the nuclei were separated from the membranes by forcibly pipetting the lysed cells 15 times through a Falcon 10 mL serological pipet. The nuclei were removed by centrifugation for 10 min at 3,000 *g* and the supernatants were centrifuged at 48,500 *g* for 1 hr. The membrane pellets were resuspended in 1 mM Tris (pH 7.2). Protein concentrations were determined according to Lowry [23]. Electrophoresis of 25 μg protein aliquots was carried out in a 6% SDS-polyacrylamide mini-gel at 100 V for 2 hr and proteins were transferred to a nitrocellulose filter (Hybond-C extra, Amersham Corp.). The P-glycoprotein was detected by staining with Mab C219 (Centocor Diagnostics, U.S.A.) at 10 μg/mL for 2 hr according to the instructions of the manufacturer. A biotinylated rabbit anti-mouse IgG1 secondary antibody diluted 1:200 together with AB complex HRP kit 1:500 dilution (Dako A/S, Denmark) was used for visualization.

MDR-1 gene fragment. A 1098 bp *Eco*R1 restriction fragment of the human MDR-1 cDNA, corresponding to nucleotides 3074–4172 of the full-length cDNA sequence [24, 25] and inserted in a pUC 13 plasmid, was kindly provided by Dr Takashi Tsurou, Institute of Applied Microbiology, University of Tokyo, Japan [26]. The entire cDNA fragment was subcloned into the *Eco*R1 site of the plasmid pT7T3 19U (Pharmacia LKB Biotechnology Inc.). The orientation of the insert was confirmed by dideoxy-sequencing.

Northern blot analysis. RNA was extracted with guanidine thiocyanate (Fluka, Buchs, Switzerland) followed by centrifugation in CsCl with modifications according to [27]. RNA (20 μg) was electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde. The intactness of RNA and the location of 18 and 28S rRNA was determined by ethidium bromide staining. Following transfer of RNA to a nylon filter (Hybond-N, Amersham Corp.), the filter was prehybridized for 3 hr and then hybridized with MDR-1 cDNA (1×10^6 cpm/mL) labeled with [^{32}P]-dCTP by random primer extension according to [28, 29]. Hybridization was performed at 55° for 16 hr in 50% formamide, 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 25 mM KPO_4, 5 × Denhardt's solution (1 × Denhardt's = 0.02% BSA, 0.02% Ficoll and 0.02% polyvinylpyrrilidone), 50 μg denatured calf thymus DNA and 10% dextran sulfate. Filters were washed twice at 65° in 2 × SSC/0.1% SDS, then 1 × SSC/0.1% SDS and finally in 0.5 × SSC/0.1% SDS. Filters were exposed to X-ray film (Hyperfilm, Amersham Corp.) at −70° for 6 days with enhancing screens.

Solution hybridization assay. Total nucleic acids (TNA) from 20×10^6 cells were isolated according to [30]. In short, cells were lysed and homogenized in an SDS-containing buffer and digested by proteinase K (0.2 mg/mL) (Boehringer Mannheim, Germany) followed by phenol/chloroform extraction of nucleic acids. After linearization with BamHI, a [^{35}S]CTP-labeled cRNA probe was synthesized *in vitro* using the T3 promotor in the pT7T3 vector and 10 U T3 RNA polymerase with reagents from Promega Biotech as described in [27]. [^{35}S]-labeled MDR-1 cRNA (50,000 cpm/incubation) was hybridized to TNA according to [31] with slight modifications. Hybridization was carried out at 70° for 16 hr in a total volume of 40 μL containing 0.6 M NaCl, 20 mM Tris-HCl (pH 7.5), 4 mM EDTA, 0.1% SDS, 7.5 mM dithiothreitol and 25% formamide. Following digestion by RNase A (40 μg) and RNase T1 (2 ng) (Boehringer Mannheim), hybrids were collected by filtration on GF/C glass microfibre filters (Whatman). Results are expressed as cpm/μg total nucleic acids.

Southern blot analysis. Genomic DNA from the parental HL-60-S and the resistant HL-60-R sublines were digested with *Eco*R1 (Boehringer Mannheim) and separated by electrophoresis in 0.8% agarose. Denatured DNA was transferred to a nylon filter (Hybond-N, Amersham Corp.) and hybridized with MDR-1 cDNA (1×10^6 cpm/mL) labeled with [^{32}P]-dCTP by random primer extension according to [28,29]. Hybridization was performed at 42° for 16 hr in 45 mM Tris-HCl (pH 7.5), 0.91 M NaCl, 45.5% formamide, 9.1% dextran sulfate, 9.1 × Denhardt's solution, 0.91% SDS, 0.08% sodium pyrophosphate and 0.91 mg salmon sperm DNA/mL. Filters were washed twice at room temperature in 2 × SSC/0.5% SDS, at 42° in 0.1 × SSC/0.1% SDS, and once at 65° in 0.1 × SSC/0.1% SDS and then exposed to X-ray film (Hyperfilm, Amersham Corp.) at −70° for 1 day with enhancing screens.

RESULTS

Isolation of anthracycline-resistant HL-60 cells

Parental, sensitive human promyelocyte leukemia cells, HL-60-S, were selected for tolerance to the cytotoxic effect of doxorubicin by increasing the concentration of the drug stepwise from 0.04 to 10 μM. During the first year of selection, it required approximately 200 days for the cells to tolerate twice the initial concentration, i.e. 75 instead of 40 nM doxorubicin. Increasing the tolerance from 75 to 150 nM took considerably less time (80 days) whereas from 150 nM the concentration of doxorubicin could be doubled every 30 days. A separate flask of HL-60-R_{10} cells was maintained in doxorubicin-free media for 6 months without spontaneous reversion to the drug sensitive phenotype. During the selection protocol, isolates tolerating 0.5 μM, 5 μM and 10 μM (HL-60-$R_{0.5,5,10}$) were frozen with cryoprotective agents.

Drug transport and resistance profile

After a 3 hr incubation with doxorubicin at a concentration of 1 μM, the intracellular uptake of the drug in the HL-60-R_{10} subline was 56% of that in the parental HL-60-S cells (Table 1). Following an additional 2 hr incubation in the absence of drug, 61% of the initial drug content was retained in the sensitive HL-60-S cells compared to 11% in the resistant HL-60-R_{10} cells. After 18 hr in drug-free medium, 44% of the initial drug content was retained in the HL-60-S cells compared to 5% in the HL-60-R_{10} cells. In the presence of verapamil, the intracellular concentration of doxorubicin immediately after the incubation in HL-60-R_{10} cells was 1.7 times higher than without verapamil (Table 1). This difference increased to reach a 5-fold higher intracellular drug concentration after 18 hr of incubation in drug-free medium. Interestingly, a small but significant effect on retention of doxorubicin was also noted in the parental cell line, which may be due to a basal expression of MDR-1 mRNA in the parental HL-60-S cells (see below).

The IC_{50} concentration for various antileukemic drugs to HL-60-S cells and the cross-resistance profile for the three doxorubicin-resistant sublines are shown in Table 2. There was a pronounced cross-resistance to epirubicin, daunorubicin, idarubicin, mitoxantrone and vincristine, which paralleled the resistance to doxorubicin in the sublines. Etoposide and amsacrine showed a level of resistance similar to that of the anthracyclines and mitoxantrone in the HL-60-$R_{0.5}$ subline, but a lower degree of resistance in the HL-60-R_5 and HL-60-R_{10} sublines. Even ara-C showed a moderate, 6-fold resistance in the HL-60-$R_{0.5}$ subline, which diminished however with an increasing degree of doxorubicin resistance.

Table 1. Intracellular uptake and retention of doxorubicin in HL-60-S and HL-60-R_{10} cells with and without verapamil

Time after incubation (hr)	HL-60-S		HL-60-R_{10}	
	− verapamil (nmol/mg protein)	+ verapamil (nmol/mg protein)	− verapamil (nmol/mg protein)	+ verapamil (nmol/mg protein)
0	1.33 ± 0.06	1.32 ± 0.09	0.74 ± 0.21	1.29 ± 0.38
2	0.81 ± 0.05	0.96 ± 0.12	0.15 ± 0.06	0.53 ± 0.03
18	0.59 ± 0.04	0.84 ± 0.04	0.06 ± 0.02	0.29 ± 0.04

Cells were incubated with 1 μM doxorubicin for 3 hr and then in the absence of drug for 2 and 18 hr with and without 5 μg/mL verapamil as described in Materials and Methods. Doxorubicin was extracted with 27% TCA and the concentration determined by spectrophotofluorometry. The table consists of mean values and range from two experiments in duplicate.

Table 2. Cross-resistance profile to various antileukemic drugs for different HL-60 sublines selected for resistance to doxorubicin

Drug	Concentrations tested (μM)	IC_{50} HL-60-S (μM)	Degree of resistance HL-60-$R_{0.5}$	HL-60-R_5	HL-60-R_{10}
Doxorubicin	0.02–344	0.07	14	142	328
Epirubicin	0.01–100	0.16	23	85	72
Daunorubicin	0.01–1000	0.12	80	858	1367
Idarubicin	0.002–50	0.005	12	220	280
Mitoxantrone	0.002–200	0.03	40	167	3700
Vincristine	0.0002–100	0.017	4100	>5900*	>5900*
Etoposide	2–3400	16.8	12	19	19
Amsacrine	0.05–200	1.8	1.7	4.2	8.5
Malphalane	0.1–200	11.3	0.54	1.4	1.4
ara-C	0.05–2	0.082	6.5	2.1	3.2
CdA	0.002–0.1	0.005	0.8	0.8	1

Cells were incubated and cellular ATP was determined as described in Materials and Methods. The degree of resistance was calculated by dividing the IC_{50} concentrations of the resistant cells lines by those of the parental HL-60-S line.

* IC_{50} could not be reached.

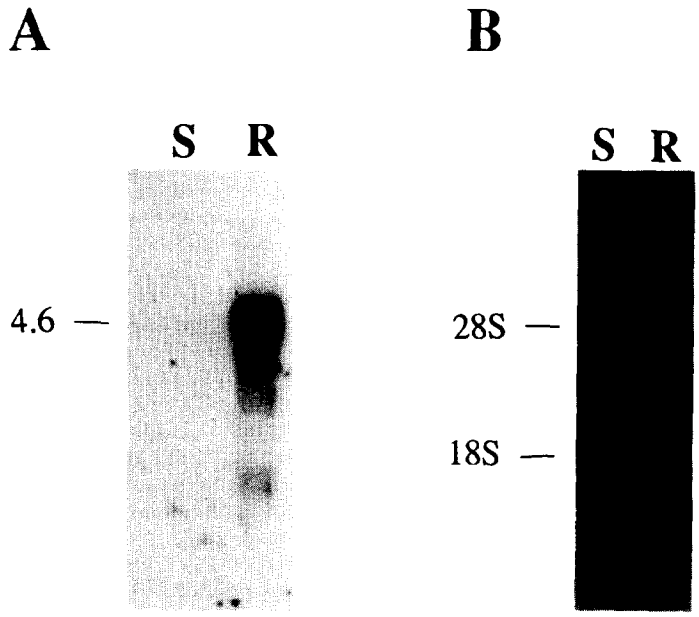

Fig. 1. Analysis of MDR-1 mRNA expression from HL-60-S and HL-60-R_{10} cells by Northern gel analysis. RNA was isolated as described in Materials and Methods and separated by agarose-formaldehyde gel electrophoresis. RNA was transferred to nylon filter and hybridized with a [^{32}P]-labeled MDR-1 cDNA probe. Lane S: 20 μg RNA from HL-60-S cells; lane R: 20 μg RNA from HL-60-R_{10} cells. (A) A 4.6 kb mRNA was detected in total cellular RNA from the HL-60-R_{10} cells. 18S and 28S rRNA were used as size markers. (B) Ethidium bromide stain of the gel prior to transfer.

For CdA and melphalane, no cross-resistance was observed.

Expression of MDR-1 mRNA

Northern blot analysis (Fig. 1) was performed to characterize the size and abundance of MDR-1 mRNA transcripts in total cellular RNA, extracted with guanidine thiocyanate followed by centrifugation in CsCl solution, from HL-60-S and HL-60-R_{10} cells. The [^{32}P]dCTP MDR-1 cDNA probe labeled by random primer extension hybridized to a single 4.6 kb mRNA in RNA from the HL-60-R_{10} cells. No hybridizing transcript could be detected in the HL-60-S cells by this method.

A more sensitive method, solution hybridization/RNAse protection, was compared with the Northern analysis and employed to achieve a semi-quantitative estimate of MDR-1 mRNA expression (Fig. 2).

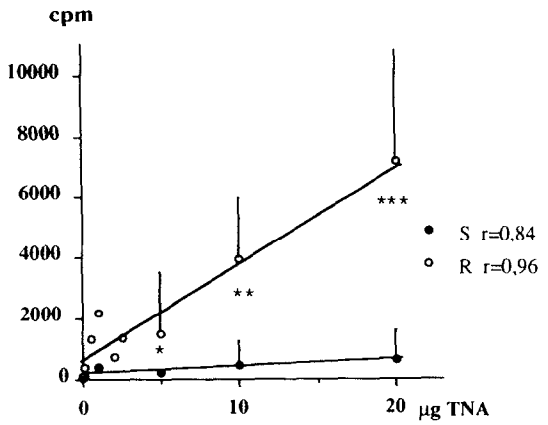

Fig. 2. Expression of MDR-1 mRNA in HL-60-S and HL-60-R_{10} cells as determined by solution hybridization. Total nucleic acids (TNA) were hybridized with [^{35}S]CTP-labeled MDR-1 cRNA probe as described in Materials and Methods. *$P < 0.06$, $N = 6$; **$P < 0.001$, $N = 15$; ***$P < 0.02$, $N = 10$, compared to the corresponding value in HL-60-S cells.

Total nucleic acids prepared from HL-60-S and HL-60-R_{10} cells were hybridized to a [^{35}S]CTP-labeled cRNA probe. The number of mRNA transcripts in the HL-60-R_{10} cells was calculated to be 14 times higher compared to the HL-60-S cells.

Correlation between doxorubicin resistance, expression of P-glycoprotein, MDR-1 mRNA expression and MDR-1 gene copy number

Cell membranes derived from plasma membrane and endoplasmic reticulum were isolated from HL-60-S and three HL-60-R subline cells. Following polyacrylamide gel electrophoresis under denaturing conditions, transfer to nitrocellulose and immunodetection by monoclonal antibody C219, a 180 kDa protein, co-migrating with purified P-glycoprotein standard as positive control, was detected in all resistant cell lines. However, the intensity of staining of the 180 kDa protein was clearly higher with membranes from the HL-60-R_5 and R_{10} cells (Fig. 3A). The protein could not be detected in the sensitive HL-60-S cells.

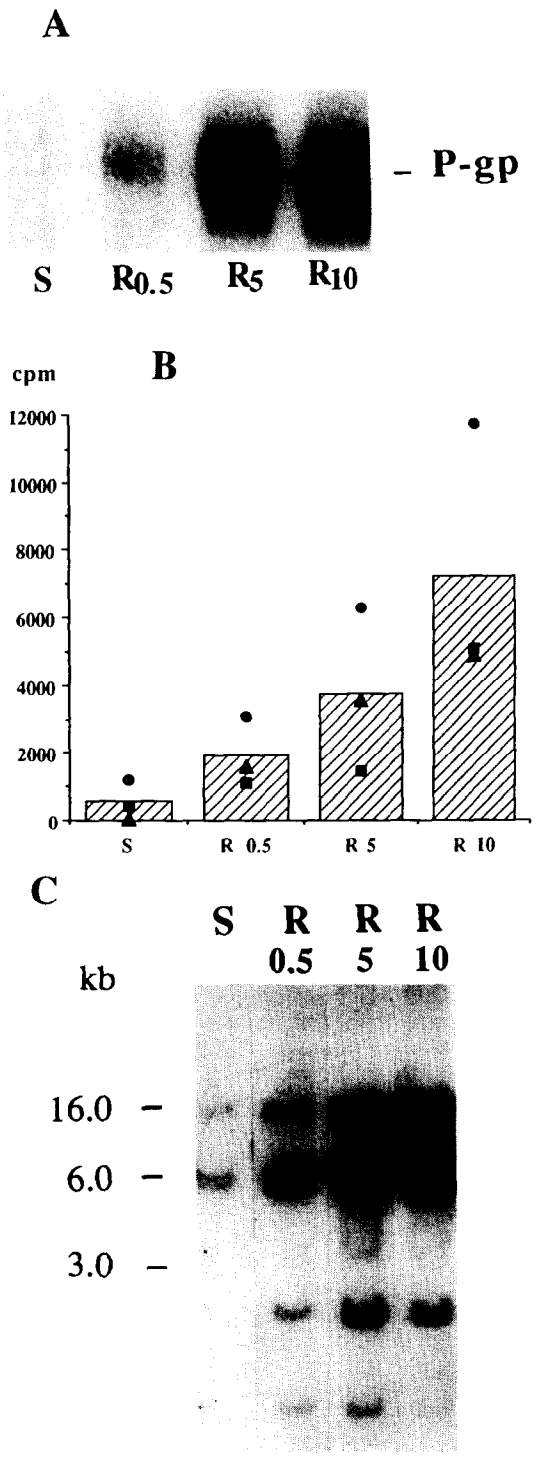

Fig. 3. Correlation between P-glycoprotein expression, MDR-1 mRNA expression and MDR-1 gene copy number in parental and drug-resistant HL-60 sublines. (A) Detection of P-glycoprotein by monoclonal antibody C219 in isolated membranes from HL-60-S and HL-60-R sublines. Cell membranes containing endoplasmic reticulum and plasma membranes were prepared as described in Materials and Methods and subjected to SDS–PAGE. The immunoreactive P-glycoprotein is indicated in the right margin. Kaleidoscope prestained standards (Bio-Rad) were used as size standards. Lane S: HL-60-S cells; lane $R_{0.5}$: HL-60-$R_{0.5}$ cells; lane R_5: HL-60-R_5 cells; lane R_{10}: HL-60-R_{10} cells. (B) Solution hybridization. Total nucleic acids (10 µg) from HL-60-S, HL-60-$R_{0.5}$, HL-60-R_5 and HL-60-R_{10} were hybridized with [^{35}S]CTP-labeled MDR-1 cRNA. The legends represent the actual values from three different experiments and the bars show the mean value. (C) Southern hybridization analysis of MDR-1 gene copy number. DNA was isolated from HL-60-S, HL-60-$R_{0.5}$, HL-60-R_5, HL-60-R_{10} cells and digested with *Eco*R1. DNA fragments were separated by agarose gel electrophoresis and hybridized with a [^{32}P]dCTP random primer-labeled MDR-1 cDNA probe. A DNA molecular marker II ladder (Boehringer Mannheim) was used as a size standard.

In order to investigate if the level of MDR-1 mRNA could be correlated to the degree of tolerance to the cytotoxic effect of doxorubicin (Table 2), total nucleic acids from HL-60-S and the resistant sublines were hybridized with [^{35}S]CTP-labeled MDR-1 cRNA (Fig. 3B). With an increased degree of doxorubicin tolerance, the level of MDR-1 mRNA transcripts also increased, indicating a positive correlation between MDR-1 gene transcription and enhanced tolerance to the cytotoxic effect of doxorubicin.

In order to elucidate whether the mechanism behind the elevated level of MDR-1 mRNA in the resistant HL-60-R sublines was correlated to amplification of the MDR-1 gene, the same amount of DNA from HL-60-S, HL-60-$R_{0.5}$, HL-60-R_5 and HL-60-R_{10} cells was digested with *Eco*R1 and the fragments separated by agarose gel electrophoresis, blotted and hybridized to a [^{32}P]-labeled MDR-1 probe (Fig. 3C). The MDR-1 probe hybridized to predominant 16 kb and 6 kb *Eco*R1 fragments. The intensity of these fragments was increased in the HL-60-R sublines in relation to the degree of resistance to the cytotoxic effect of doxorubicin.

DISCUSSION

The definition of a multidrug resistant phenotype usually includes cross-resistance to multiple chemotherapeutic agents, defective intracellular retention of drug, over-expression of the 170–180 kDa P-glycoprotein (usually due to gene amplification) and reversal of drug-resistance by ion-channel blockers, such as verapamil [1]. The analysis of drug-sensitive HL-60-S and drug-resistant HL-60-R cell lines, the latter developed by stepwise selection in increasing concentrations of doxorubicin *in vitro*, revealed an over-expression of MDR-1 mRNA in the resistant cells. Furthermore, in HL-60 cells manifesting various degrees of doxorubicin resistance, a positive correlation with respect to doxorubicin resistance, levels of P-glycoprotein detected by Mab C219, MDR-1 mRNA levels and amplification of the MDR-1 gene was apparent. There was a complete cross-resistance between doxorubicin and the other anthracyclines as well as mitoxantrone and vincristine. Etoposide and amsacrine constituted an intermediate group while the doxorubicin-resistant sublines showed unchanged sensitivity against melphalane and the purine analogue CdA. Thus the profile was that of classical MDR [1].

The intracellular retention of doxorubicin was poor but partly reversed by verapamil in the resistant sublines. A small effect on retention was also seen in the parental cell line, which is consistent with a low P-glycoprotein expression. Collectively, over-expression of P-glycoprotein as the result of amplification of the MDR-1 gene provides an explanation for the pattern of cross-resistance to cytotoxic drugs, as well as decreased drug retention in the HL-60-R cell populations. In a recent review [1], information on various drug-resistant cell lines now available, with particular respect to the various parameters of the MDR phenotype, was compiled. In this survey, approximately 75% of the various cell lines made resistant by exposure to anthracyclines exhibited a complete MDR phenotype including expression of P-glycoprotein. However, a significant number of multidrug resistant cells were found not to over-express the P-glycoprotein. This is also the case for the hitherto available anthracycline-resistant HL-60 cell lines, which accordingly have generated an interest in chemotherapy resistance mechanisms not involving expression of the MDR-1 gene [12, 13].

In the drug uptake and efflux experiment, the 2 hr time point was chosen to show the rapid efflux in the resistant subline while the 18 hr time point was selected to demonstrate the retention of drug in the sensitive, parental cell line. We have previously shown that doxorubicin is retained intracellularly for a long period of time [32]. After 18 hr, there was still approximately 50% of the drug remaining in the sensitive HL-60 cells.

When comparing the P-glycoprotein-negative, doxorubicin-resistant HL-60 cell lines of [12, 13] with the P-glycoprotein-positive HL-60 isolates of the present study, a number of differences can be observed that may be of relevance to explain their distinctive phenotypes. Firstly, the former cell lines have a lower degree of doxorubicin resistance, with IC_{50} doses 10–20-fold higher than the parental cell lines. In contrast, our HL-60 R_{10} subline tolerates more than a 300-fold higher toxic concentration of doxorubicin than the parental cells. Compared to one previously described anthracycline-resistant HL-60 cell line, our cell line has a much more pronounced resistance to vincristine and it also expresses an intermediate resistance to etoposide. It has been observed that a multidrug resistance mechanism not involving the P-glycoprotein can precede a resistance mechanism involving increased expression of P-glycoprotein [33, 34]. Thus, it remains to be tested whether the anthracycline-resistant HL-60 cell lines of [12, 13] would transcriptionally activate the MDR-1 gene and/or amplify it [35] by selecting for a higher degree of tolerance to doxorubicin.

Another more trivial reason for the absence of P-glycoprotein/MDR-1 mRNA expression may be due to methodological differences. Although MDR-1 mRNA expression was clearly detected in our 14-fold resistant HL-60 $R_{0.5}$ subline by solution hybridization, no signal could be detected by Northern blot analysis (data not shown). We have consistently observed that solution hybridization is between 20–40-fold more sensitive than Northern blot analysis for the detection of MDR-1 mRNA transcripts (K. Jönsson, U. Tidefelt, C. Paul, G. Andersson, unpublished data). Thus, interpreting the absence of signal as the equivalent of absence of cellular expression may be hazardous given the use of less sensitive methods.

Another possible explanation for anthracycline resistance in the absence of P-glycoprotein expression would obviously be a complementary expression of a drug transporter with a similar drug specificity but structurally non-identical with P-glycoprotein. It has been pointed out that P-glycoprotein-negative anthracycline-resistant HL-60 cells over-express a 190 kDa ATP-binding phosphorylated surface membrane protein that has a limited homology with P-glycoprotein [36]. Furthermore, a novel member of the ABC transporter superfamily, denoted MRP,

was recently identified by cDNA cloning from a doxorubicin-resistant small cell lung cancer cell line negative for the P-glycoprotein 170 [37]. Interestingly, this gene has recently been associated with the non-P-glycoprotein multidrug resistance of doxorubicin-resistant HL-60 cells [38]. The availability of anthracycline-resistant HL-60 cell lines with absent and variable expression of P-glycoprotein, the product of the MDR-1 gene, clearly provides a fine opportunity to analyse different mechanistic aspects of resistance to cytotoxic drugs used in cancer chemotherapy.

Acknowledgements—This work was supported by grants from the Swedish Cancer Society, the Swedish Medical Research Council and Örebro Council Research Committee. The authors would like to thank Ms Malin Ehnfors for her skillful technical assistance.

REFERENCES

1. Nielsen D and Skovsgaard T, P-glycoprotein as multidrug transporter: a critical review of current multidrug resistant cell lines. *Biochim Biophys Acta* **1139**: 169–183, 1992.
2. Juranka PF, Zastawny RL and Ling V, P-glycoprotein: multidrug-resistance and a superfamily of membrane-associated transport proteins. *FASEB J* **3**: 2583–2592, 1989.
3. Roninson IB, The role of the MDR1 (P-glycoprotein) gene in multidrug resistance *in vitro* and *in vivo*. *Biochem Pharmacol* **43**: 95–102, 1992.
4. Higgins C, ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* **8**: 67–113, 1992.
5. Marie J-P, Zittoun R and Sikic B. Multidrug resistance (MDR1) gene expression in adult acute leukemias: correlations with treatment outcome and *in vitro* drug sensitivity. *Blood* **78**: 586–592, 1991.
6. Campos L, Guyotat D, Archimbaud E, Calmard-Oriol P, Tsuruo T, Troncy J, Treille D and Fiere D, Clinical significance of multidrug resistance P-glycoprotein expression on acute nonlymphoblastic leukemia cells at diagnosis. *Blood* **79**: 473–476, 1992.
7. Zhou D-C, Marie J-P, Suberville A-M and Zittoun R, Relevance of mdr1 gene expression in acute myeloid leukemia and comparison of different diagnostic methods. *Leukemia* **6**: 879–885, 1992.
8. Preet MC, Chaudhary PM and Roninson I, Expression and activity of P-glycoprotein, a multidrug efflux pump in human hematopoietic stem cells. *Cell* **66**: 85–94, 1991.
9. List AF, Spier CM, Cline A, Doll DC, Garewal H, Morgan R and Sandberg AA, Expression of the multidrug resistance gene product (P-glycoprotein) in myelodysplasia is associated with stem cell phenotype. *Br J Haematol* **78**: 28–34, 1991.
10. Campos L, Guyotat D, Jaffar C, Solary E, Archimbaud E and Treille D, Correlation of MDR/1P-170 expression with daunorubicin uptake and sensitivity of leukemic progenitors in acute myeloid leukemia. *Eur J Haematology* **48**: 254–258, 1992.
11. Tiirikainen M and Krusius T, Multidrug resistance. *Ann Med* **23**: 509–520, 1991.
12. Bhalla K, Hindenburg A, Taub R and Grant S, Isolation and characterization of an anthracycline-resistant human leukemic cell line. *Cancer Res* **45**: 3657–3662, 1985.
13. Marsh W, Sicheri D and Center MS, Isolation and characterization of Adriamycin-resistant HL-60 cells which are not defective in the initial intracellular accumulation of drug. *Cancer Res* **46**: 4053–4057, 1986.
14. McGrath T and Center MS, Adriamycin resistance in HL60 cells in absence of detectable P-glycoprotein. *Biochem Biophys Res Com* **145**: 1171–1176, 1987.
15. McGrath, T, Latoud C, Arnold ST, Safa AR, Felsted RL and Center MS, Analysis of resistance associated membrane proteins and levels of mdr gene expression. *Biochem Pharmacol* **38**: 3611–3619, 1989.
16. Paul C, Peterson C, Gahrton G and Lockner D, Uptake of free and DNA-bound daunorubicin and doxorubicin in human leukemic cells. *Cancer Chemother Pharmacol* **2**: 49–52, 1979.
17. Sundman-Engberg B, Tidefelt U, Liliemark J and Paul C, Intracellular concentrations of anti-cancer drugs in leukemic cells *in vitro* vs *in vivo*. *Cancer Chemother Pharmacol* **25**: 252–256, 1990.
18. Tidefelt U, Sundman-Engberg B and Paul C, Comparison of the intracellular pharmokinetics of doxorubicin and 4'-epi-doxorubicin in patients with acute leukemia. *Cancer Chemother Pharmacol* **24**: 225–229, 1989.
19. Sundman-Engberg B, Tidefelt U, Gruber A and Paul C, Intracellular concentrations of mitoxantrone in leukemic cells *in vitro* vs *in vivo*. *Leukemia Res* **4**: 347–352, 1993.
20. Lundin A, Extraction and automatic luminometric assay of ATP, ADP and AMP. In: *Analytical Applications of Bioluminescence and Chemoluminescence, Proceedings of the Third International Symposium on Analytical Applications of Bioluminescence and Chemiluminescence, Birmingham, U.K.*, 17–19 *April* 1984 (Eds. Kricka LJ, Thorpe C, Whitehead W), pp. 491–501. London, Academic, 1984.
21. Rhedin AS, Tidefelt U, Jönsson K and Paul C, Comparison of a bioluminescence assay with differential staining cytotoxicity for cytostatic drug testing *in vitro* in human leukemic cells. *Leukemia Res* **17**: 271–276, 1993.
22. Esko JD, Gilmore JR and Glaser M, Use of a fluorescent probe to determine the viscosity of LM cell membranes with altered phospholipid compositions. *Biochemistry* **16**: 1881–1890, 1977.
23. Lowry OM, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
24. Roninson IB, Chin J, Choi K, Gros P, Housman DE, Fojo A, Shen D-W, Gottesman MM and Pastan I, Isolation of human mdr DNA sequences amplified in multidrug-resistant KB carcinoma cells. *Proc Natl Acad Sci USA* **83**: 4538–4542, 1986.
25. Ueda K, Clark D, Chen C-J, Roninson IB, Gottesman MM and Pastan I, The human multidrug resistance (Mdr-1) gene: cDNA cloning and transcription initiation. *J Biol Chem* **262**: 505–508, 1987.
26. Sugimoto Y, Asami N and Tsurou T, Expression of P-glycoprotein mRNA in human gastric tumors. *Jpn J Cancer Res* **80**: 993–999, 1989.
27. Sambrook J, Fritsch E and Maniatis T, *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York, 1989.
28. Feinberg A and Vogelstein B, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13, 1983.
29. Feinberg A and Vogelstein B, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **137**: 266–267, 1984.
30. Durnam D and Palmiter R, A practical approach for quantitating specific mRNA by solution hybridization. *Anal Biochem* **131**: 385–393, 1983.
31. Mathews L, Norstedt G and Palmiter R, Regulation of insulin-like growth factor I gene expression by growth hormone. *Proc Natl Acad Sci USA* **83**: 9343–9347, 1986.

32. Paul C, Liliemark J, Tidefelt U, Gahrton G and Peterson C, Pharmacokinetics of daunorubicin and doxorubicin in plasma and leukemic cells from patients with acute nonlymphoblastic leukemia. *Ther Drug Monit* **11**: 141–148, 1989.
33. Baas F, Jongsma APM, Broxterman HJ, Arceci RJ, Housman D, Scheffer GL, Riethorst A, van Groeningen M, Nieuwint AWM and Joenje H, Non-P-glycoprotein mediated mechanism for multidrug resistance precedes P-glycoprotein expression during *in vitro* selection for doxorubicin resistance in a human lung cancer cell line. *Cancer Res* **50**: 5392–5398, 1990.
34. Slapak CA, Lecerf J-M, Daniel JC and Levy SB, Energy-dependent accumulation of daunorubicin into subcellular compartments of human leukemia cells and cytoplasts. *J Biol Chem* **267**: 10638–10644, 1992.
35. Shen D-W, Fojo A, Chin JE, Roninson IB, Richert N, Pastan I and Gottesman MM, Human multidrug-resistant cell lines: increased mdr1 expression can precede gene amplification. *Science* **232**: 643–645, 1986.
36. Marquardt D, McCrone S and Center MS, Mechanisms of multidrug resistance in HL60 cells: detection of resistance-associated proteins with antibodies against synthetic peptides that correspond to the deduced sequence of P-glycoprotein. *Cancer Res* **50**: 1426–1430, 1990.
37. Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AMV and Deeley RG, Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**: 1650–1654, 1992.
38. Krishnamachary N and Center MS, The MRP gene associated with a non-P-glycoprotein multidrug resistance encodes a 190 kDa membrane bound glycoprotein. *Cancer Res* **53**: 3658–3661, 1993.