Lack of elevated drug efflux in adriamycin-resistant immunoblastic B lymphoma cells with *mdr*1 overexpression

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Abstract **A multidrug-resistant (MDR) subline of the immunoblastic B lymphoma cell line was established by sequentially selecting in increasing concentrations of adriamycin. The adriamycin-resistant cell line (HOB1/ADR) demonstrated resistance to a wide spectrum of chemotherapeutic agents including MDR drugs (Vinca alkaloids and anthracycline), antimicrotubule drug (colchicine), and DNA-damaging agents (cisplatin and mitomycin C). The expression of human *mdr*1 gene, as analyzed by RT-PCR and Western blotting, revealed a 13–15-fold increase in resistant cells. Unexpectedly, HOB1/ADR cells demonstrated a lack of reduced accumulation and of enhanced efflux of adriamycin. More than 60% adriamycin was effluxed at the same rate in both cell lines within 10 min. In contrast, the initial rate of vincristine accumulation was reduced by 3 fold in this resistant cell line. The maximal level of vincristine accumulation was 50% lower in the resistant cells than the parental cells. The maximal efflux rate was enhanced by 5 fold in the resistant cells. Inhibition of vincristine resistance by verapamil associated with restoration of drug accumulation, suggesting that acquired resistance in these cells is due to P-glycoprotein. These studies demonstrated that immunoblastic B lymphoma cells selected for adriamycin resistance preferentially developed P-glycoprotein-mediated vincristine efflux which plays an important role in vincristine resistance. In contrast, the resistant cells did not elevate adriamycin efflux, suggesting an additional mechanism responsible for adriamycin resistance.**

Key words: Drug efflux; Immunoblastic lymphoma; MDR; P-Glycoprotein

1. Introduction

Resistant cells occasionally arise from residual tumors during chemotherapy. Different treatment strategy in chemotherapy may affect the type of resistant tumor cells. Multidrug resistance (MDR) appears to be a major obstacle to chemotherapeutic success. Selection of numerous cell lines in vitro to study the MDR phenomenon by a single anticancer drug, such as an anthracycline, a Vinca alkaloid, or a taxane often results in a coordinate resistance to other structurally and functionally unrelated drugs [1–7]. In many instances, MDR-cells overexpress P-glycoprotein, a 150,000–180,000 Da integral membrane protein, by the *mdr*1 gene which is believed to confer resistance by acting as a drug efflux pump [8–10]. Resistant sublines show increased efflux of cytotoxic agents and reduced intracellular drug accumulation [8 for review; 11]. However, a number of cell lines have been described, such as the human small cell lung cancer cell line H69AR [7,12,13] and the human non-small cell lung cancer cell line PC-9 [14], which display multidrug resistance but do not overexpress P-glycoprotein. Recently, cDNA clones corresponding to an mRNA encoding a 190,000 Da integral membrane protein (designated multidrug resistance-associated protein (MRP)) markedly overexpressed in H59AR cells were isolated and characterized [15]. MRP belongs to the same superfamily of ATP-binding cassette transporter protein as P-glycoprotein [16,17]. Whereas, cells resistant to genotoxic agents such as cisplatin are due to either one or a combination of the several mechanisms, including reduced drug accumulation and enhanced DNA repair independent of P-glycoprotein [18 for review;19]. Multidrug resistance in the clinical setting appears to be multifactorial and may involve mechanisms besides P-glycoprotein overexpression [20]. A clinical case in our hands showed lack of response of an extranodal immunoblastic lymphoma to chemotherapy in a Taiwanese male patient [21]. The patient failed to respond to a combination of CHOP (cyclophosphamide, adriamycin, vincristine and prednisone) and MOPP (nitrogen mustard, vincristine, procarbazine and prednisone) chemotherapy regimens, and died three months following treatment. A lymphoma cell line (termed HOB1), which overexpresses c-H-*ras* and c-*myc* oncogenes, was established from the patient [21]. In this study, an adriamycin-resistant cell line was established from HOB1 cells, which showed MDR phenotype including P-glycoprotein-mediated drug resistance. However, the resistant lymphoma cells did not demonstrate an enhanced efflux and reduced accumulation of the selecting agent.

2. Materials and methods

2.1. Chemicals, drugs, and sera

Chemotherapeutic drugs and cytotoxic agents (Adriamycin, colchicine, mitomycin C, cisplatin, puromycin, and vinblastine) were purchased from Sigma, St. Louis, MO. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin and streptomycin were ordered from Gibco, Gaithersburg, MD. RPMI-1640 medium was purchased from Hyclone (Logan, Utah).

2.2. Cells and drug sensitivity

Human lymphoma HOB1 cell line was established from a gingival biopsy of a male Taiwanese patient having immunoblastic lymphoma [21]. The cells were maintained in RPMI-1640 medium supplemented with 10% FBS, and 100 units/ml penicillin and 100 μg/ml streptomycin. The adriamycin-resistant HOB1 cell line (HOB1/ADR) was established from an initial exposure of HOB1 cells to a low concentration of adriamycin (0.001 μg/ml), followed by a 10-fold increment of the drug concentration up to 0.1 μg/ml. HOB1/ADR cells were cultured in this drug concentration to maintain resistant phenotype, and grown in

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Abbreviations: DMEM, Delbecco's modified Eagle's medium; EBV, Epstein-Barrvirus; MDR, multidrug resistance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction.

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drug-free medium for three population doublings before experiments. Parental and resistant pairs of colon adenocarcinoma cells (SW620, SW620/MDR) and non small-cell lung cancer cells (PC9 and PC9/VCR) were cultured as previously described [14,22]. Drug sensitivity was determined by drug-induced growth inhibition of cells using MTT dye (3-(4,5-dimethylthiazol)-2,5-diphenyltetrazilium bromide) (Sigma) method as described by Mosmann [23]. The drug resistance of the cells is defined as the ratio of IC_{50}, the drug concentration causing 50% cell growth inhibition, of resistant cells divided by that of parental cells.

2.3. RNA detection by RT-PCR

DNA and RNA, and the related enzymatic reactions for reverse transcription and polymerase-chain reaction (RT-PCR) were prepared and conducted by accepted methods [24]. PCR was performed with cDNA transcribed from 50 ng of total RNA, 1 unit of Taq DNA Polymerase (Stratagene) in a microprocessor-driven thermal cycler (Perkin-Elmmer/Cetus) in a final volume of 25 μl reaction buffer. Each cycle of PCR included denaturation at 95°C for 30 s, primer annealing at 55°C for 1 min, and extension/synthesis at 72°C for 2 min. PCR primers were synthesized in an Applied Biosystems DNA synthesizer (model 380B); the primer yield and quality were tested by UV spectroscopy and gel electrophoresis. Each primer was included at 37.5 pmol per reaction. *MDR1*-specific sequences were amplified by using the sense-strand primer 5′-GAGGTGAAGAAGGGCCAGACG-3′ (nucleotides 3175–3195 relative to the first nucleotide of translation initiation codon) and the antisense-strand primer 5′-TTCTGGATGGTGGACAGGCGGTGA-3′ (nucleotides 3716–3693) [25]; PCR using these primers yields a 542-bp product. PCR primers used for amplification of β_2-microglobulin-specific sequences were 5′-ACCCCCACTGAAAAAGATGA-3′ (nucleotides 1544–1563; sense strand) and 5′-ATCTTCAAACCTCCATGATG-3′ (nucleotide 2253–2262 and 3507–3516; antisense strand) [26]; PCR using these primers yields a 114-bp product. For quantitation, 2 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) was added to each reaction mixture. PCR products were separated on 12% polyacrylamide gels, dried and exposed to X-ray films (X-AR5, Kodak) in cassettes with an intensifying screen at −70°C. Films and intensifying screens were calibrated by different time exposure of the standard [α-^{32}P]dCTP. Autoradiographs were determined by 10 scans of one-dimensional densitometry (Hoefer GS300).

2.4. Protein detection by Western blotting

A polyclonal antibody to P-glycoprotein, mdr (Ab-1), was purchased from Oncogene Science, Inc., Uniodale, New York. For Western blotting, cellular proteins were separated on 10% SDS-PAGE in duplicates, and visualized by silver staining according to Laemmli [27] or processed for immunostaining with the antiserum for 3 h, treated by the avidin–biotin–peroxidase complex method, and stained with hematoxylin and photographed [28].

2.5. Drug accumulation study

Drug uptake and efflux was determined by a modification of Lemontt et al. [29]. Exactly 10^6 cells were mixed with 0.5 ml of serum-free medium containing 0.5 μM of [^{14}C]adriamycin (55 mCi/mmol; Amersham) or [^{14}C]vincristine (6.5 Ci/mmol; Amersham). For drug uptake analysis, labeled cells were layered onto 0.2 ml of a silicon oil:mineral oil mixture (5:1, v/v) and centrifuged for 10 s at 12,000 × *g*. The cell pellets were washed 3–4 times with the medium. Radioactivity was counted in 2-ml of scintillation cocktail T (BDH Laboratory Supplies, England) in a Beckman LS5000TD liquid scintillation counter. For drug efflux analysis, cells were depleted of ATP with 20 ng/ml retenone (Sigma) before being loaded with 0.5 μM of [^{14}C]adriamycin (25°C, 1 h). The cells were centrifuged, and drug efflux was initiated by resuspending the cell pellets in the medium and incubating at 25°C. At specific times 0.8 ml of the cell suspension (10^6 cells) were extracted. The aqueous medium (0.8 ml) remaining over the oil was removed for radioactivity counting as described above.

3. Results

3.1. Viability and cell growth in response to drugs

HOB1/ADR, an adriamycin-resistant human immunoblastic lymphoma cell line, was established from HOB1 [21]. Cytotoxicity, determined by MTT (Fig. 1, upper panel) exhibited a 240-fold resistance to the selecting drug. The dose-response in the resistant cells demonstrated a 'shoulder' at low concentrations of ADR (within 1 μg/ml)). In contrast, the parental cells lacked a tolerance response. The IC_{50} was 0.05 and 12 for parental and resistant cells, respectively. Cell growth curve was also measured (Fig. 1, lower panel). Both cell lines exhibited a similar population doubling time, 22–24 h. Resistance of HOB1/ADR cells to other cytotoxic agents was also estimated. Calculated IC_{50} values are listed in Table 1. HOB1/ADR cells also demonstrated high resistance to MDR-type drugs: colchicine (133 fold), vinblastine (500 fold), vincristine (200 fold); and to DNA-damaging agents: cisplatin (65 fold), mitomycin C (25 fold). HOB1/ADR cells appeared to be resistant to a wide spectrum of anticancer drugs and cytotoxic agents.

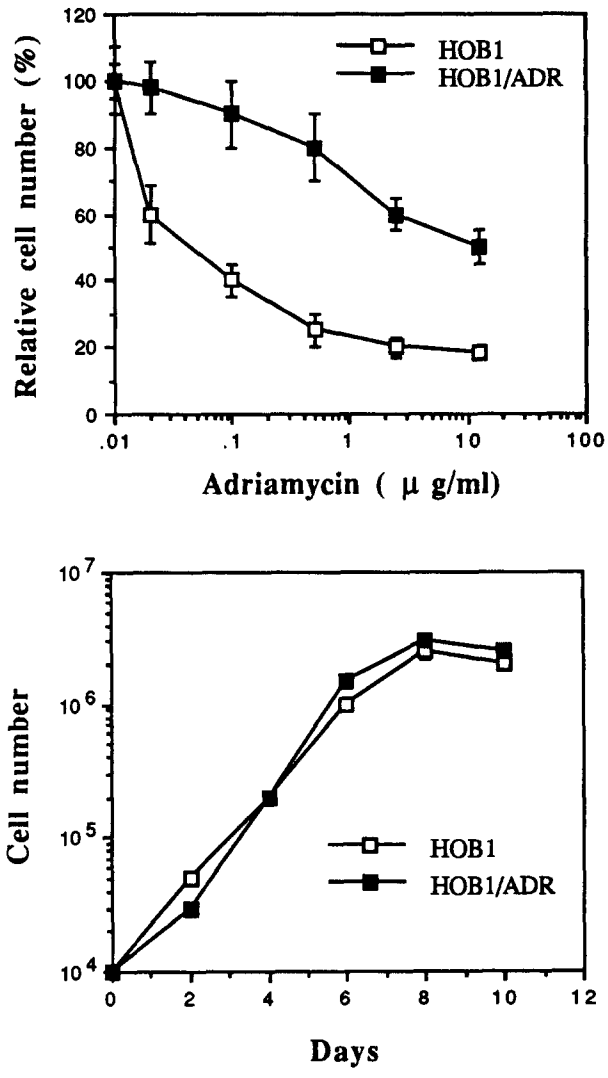

Fig. 1. Drug sensitivity and cell growth pattern in HOB1 and HOB1/ADR cells. Upper Panel: % cell number relative to mock-treated cells was determined by MTT assay. Points, means; bars, ± S.D.; $n = 5$. Lower panel: cell growth pattern was estimated for 10 days (means ± S.D.; $n = 5$).

3.2. Overexpression of mdr*1 gene*

The mature RNA of *mdr*1 gene in HOB1 and HOB1/ADR

cells was measured by RT-PCR (Fig. 2). Quantitation of *mdr*1 amplification (HOB1/ADR vs. HOB1) was shown (Fig. 2A). PCR was conducted in 30 cycles using cDNA transcribed from 1- or 10-fold diluted total RNA. The quantitated data indicated 8–10-fold amplification of *mdr*1 gene; or 13–15-fold amplification of *mdr*1 if normalized to β_2-microblobulin. RT-PCR for the detection of *mdr*1 yielded a single PCR product of the expected size of 543-bp (indicated with an arrowhead in Fig. 2B). The specificity of the amplified *mdr*1 cDNA was confirmed by partial sequence, and by the same size of PCR product amplified from 50 ng of pGEM3Zf(−)-*mdr*1, as plasmid DNA containing full-length *mdr*1 cDNA (a kind gift from Professor P. et Borst, The Netherlands Cancer Institute). PCR product of

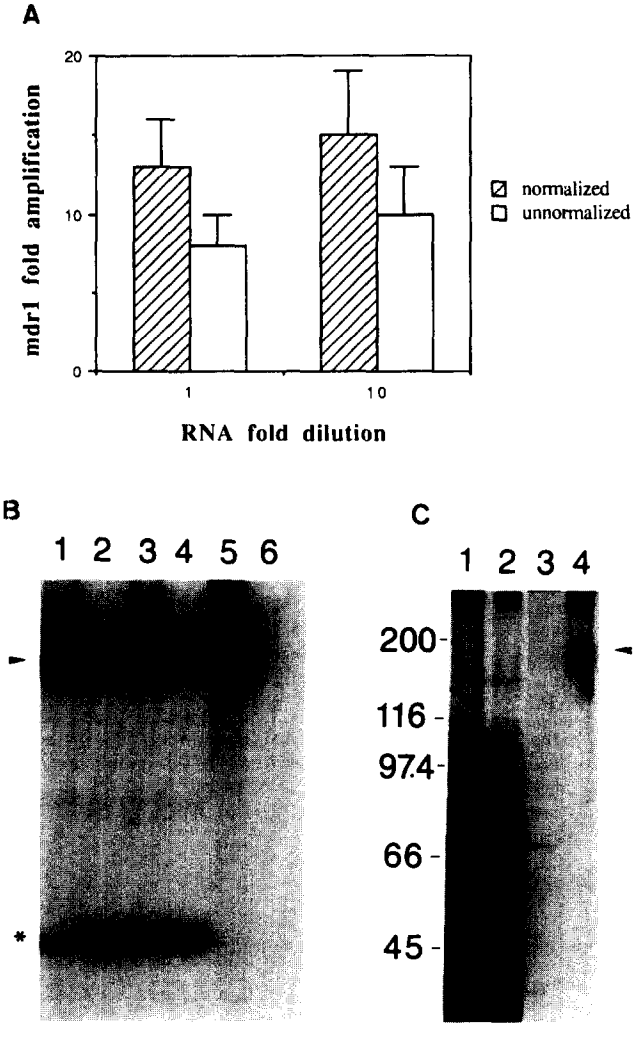

Fig. 2. (A) The fold amplification of *mdr* gene detected by RT-PCR. The mRNA was 1- or 10-fold diluted for RT-PCR. *mdr*1 was compared without normalization or by normalizing to β_2-microtubuline. Points, means; bars, ± S.D.; $n = 3$. A representative example of RT-PCR was shown (B). Reverse transcribed cDNA from HOB1/ADR (lanes 1 and 3) or HOB1 (lanes 2 and 4) mRNA was amplified in PCR programmed in 30 reaction cycles. pGEM3Zf(−)-*mdr*1 (lane 5) and buffer (lane 6) were used as controls. PCR products for *mdr*1 and β_2-microglubulin are indicated with arrowhead and star, respectively. (C) Immunodetection of P-glycoprotein. Fifty μg of cell lysates was separated on 10% SDS-PAGE, and silver stained (lanes 1 and 2) or Western blotting using mdr1 antibody (lanes 3 and 4). Lanes: 1 and 3, HOB1; lanes 2 and 4, HOB1/ADR. Molecular weight size markers (in kDa) are indicated.

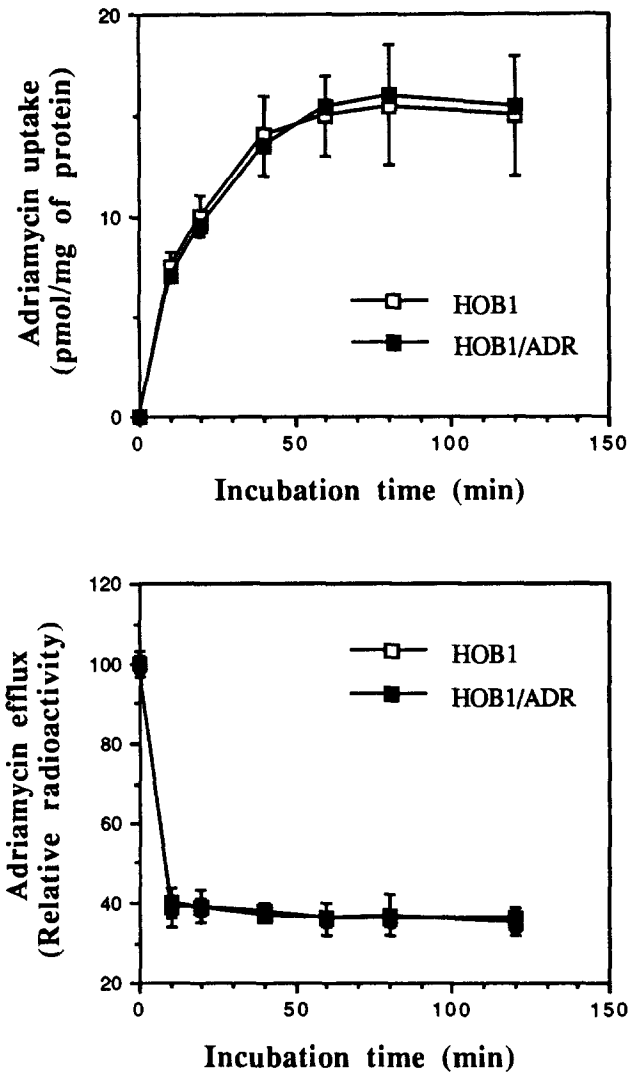

Fig. 3. Time course of the uptake and efflux of adriamycin in HOB1 and HOB1/ADR cells. Upper panel: Cells were incubated in medium containing [^{14}C]adriamycin for 120 min. Lower panel: Cells were loaded with [^{14}C]adriamycin for 60 min before assay for efflux. Points, means; bars, ± S.D.; $n = 3$.

the expected size of 120 bp for β_2-microblobulin was indicated with a star.

Since HOB1/ADR cells developed cross-resistance to classical MDR drugs and overexpressed *mdr*1 mRNA, it is reasonable to speculate that the resistance cells may overexpress P-glycoprotein. To test this hypothesis, 100 μg of cell membrane proteins was separated in acrylamide gel in duplicates, and either silver stained or processed for immunostaining with a commercial antibody against P-glycoprotein (Fig. 2C). Immunostaining indicates that HOB1/ADR cells (lane 4) showed a protein band with size similar to P-glycoprotein (indicated with arrowheads). In contrast, HOB1 showed barely detectable, if any, P-glycoprotein.

3.3. Reduced accumulation of vincristine, but not adriamycin

To analyze adriamycin accumulation, radioactive ^{14}C-labelled adriamycin was used to study drug uptake and efflux in parental and resistant HOB1 cells (Fig. 3). Unexpectedly, both

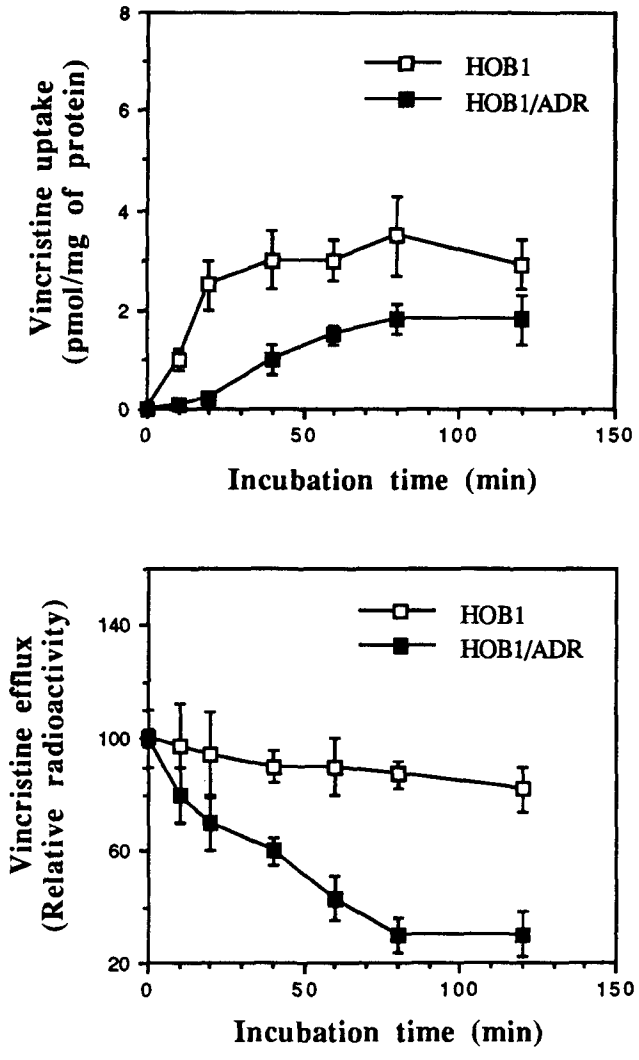

Fig. 4. Time course of the uptake and efflux of vincristine in HOB1 and HOB1/ADR cells. Upper panel: Cells were incubated in medium containing [^{14}C]vincristine for 120 min. Lower panel: Cells were loaded with [^{14}C]vincristine for 60 min before assay for efflux. Points, means; bars, ± S.D.; $n = 3$.

cells showed a similar accumulation rate (upper panel) and efflux rate (lower panel). There was a rapid uptake rate of adriamycin with 0.3 pmol/mg protein/min within 40 min, followed by an uptake plateau. For drug efflux, more than 60% of adriamycin was excluded in 10 min in both cell lines. Similar analysis was conducted for vincristine accumulation (Fig. 4). For drug uptake (upper panel), parental cells showed a nearly linear pattern of drug accumulation following 20 min incubation. There was a uptake plateau after 60 min incubation in HOB1/ADR cells. For this condition, the drug uptake rate was about 0.075 and 0.025 pmol/mg protein/min in HOB1 and HOB1/ADR cells, respectively. The results indicate that the drug-uptake rate in HOB1/ADR cells is 3 fold lower than parental cells. Furthermore, the resistant cells accumulated less vincristine than parental cells following prolonged incubation. Vincristine efflux was also compared in HOB1/ADR and parental cells (Fig. 4, lower panel). Cells were incubated with vincristine for 60 min before the level of drug remaining intracellularly was measured. As shown, there was a rapid decrease of vincristine in HOB1/ADR cells. The rate of vincristine efflux was 10%/min and 0.2%/min for HOB1 and HOB1/ADR cells, respectively. The drug efflux rate in HOB1/ADR cells was 5-fold increased. Only ~50% of the drug remaining following 50 min incubation of the resistant cells. In contrast, 90% or more was retained in the parental cells. Taken together, HOB1/ADR cells exhibited a reduced accumulation of vincristine. However, adriamycin accumulation was not lowered in the resistant cells.

3.4. Enhancement of drug accumulation by verapamil

To test whether drug accumulation is mediated by P-glycoprotein, inhibition by verapamil was used. Adriamycin accumulation was compared in HOB1 and HOB1/ADR cells (Fig. 5 upper panel). As shown, there is no significant enhancing effect by verapamil on adriamycin accumulation in HOB1 and HOB1/ADR cells. Reduced adriamycin in SW620/MDR cells was reversed by verapamil to a level comparable to that in SW620 cells. Vincristine accumulation was also compared in the parental and resistant cells (lower panel). Cells were incubated with labelled vincristine for 60 min, and the intracellular level of vincristine was measured. There was 3.5 pmol/mg of protein in HOB1 cells, and no significant verapamil effect was detected. In contrast, in the presence of verapamil the vincristine level of HOB1/ADR cells increased from 2.5 to 4.2 pmol/mg of protein, a level comparable to that in the HOB1 cells. For comparison, SW620/MDR and SW620 cells were also

Table 1
Sensitivity to anticancer and cytotoxic agents of HOB1 and HOB1/ADR cells

Agents	IC_{50} (μg/ml)[a]		
	HOB1	HOB1/ADR	Fold resistance[b]
Adriamycin	0.05 ± 0.003	12 ± 0.5	240
Colchicine	0.009 ± 0.001	1.2 ± 0.2	133
Vinblastine	0.001 ± 0.0003	0.5 ± 0.1	500
Vincristine	0.05 ± 0.004	10 ± 1.5	200
Cisplatin	0.1 ± 0.01	6 ± 2	65
Mitomycin C	0.04 ± 0.005	1 ± 0.08	25

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by MTT assay after 4 days of continuous exposure to the drug. Mean ± S.D. ($n = 5$).
[b] Fold resistance was determined by the ratio of IC_{50} of HOB1/ADR to the IC_{50} of HOB1 cells.

Table 2
Effect of verapamil on the IC_{50} values of adriamycin

Cell lines	IC_{50} (μg/ml)[a]		
	− verapamil	+ verapamil	Fold reduction[b]
HOB1	0.05 ± 0.003	0.04 ± 0.004	1.25
HOB1/ADR	11.5 ± 0.5 (230)[c]	0.23 ± 0.01	50
SW620	0.53 ± 0.04	0.48 ± 0.05	1.1
SW620/MDR	48 ± 2.5 (90.6)[c]	0.45 ± 0.02	106
PC9	0.04 ± 0.002	0.038 ± 0.001	1.05
PC9/VCR	0.039 ± 0.007 (0.98)[c]	0.037 ± 0.005	1.05

[a] IC_{50} is the drug concentration effective in inhibiting 50% of the cell growth measured by MTT assay after 4 days of continuous exposure to the drug; results are means ± S.D. ($n = 5$).
[b] Fold reduction was determined by the ratio of IC_{50} without verapamil divided by IC_{50} with verapamil (1 μg/ml).
[c] Fold resistance relative to the parental cells.

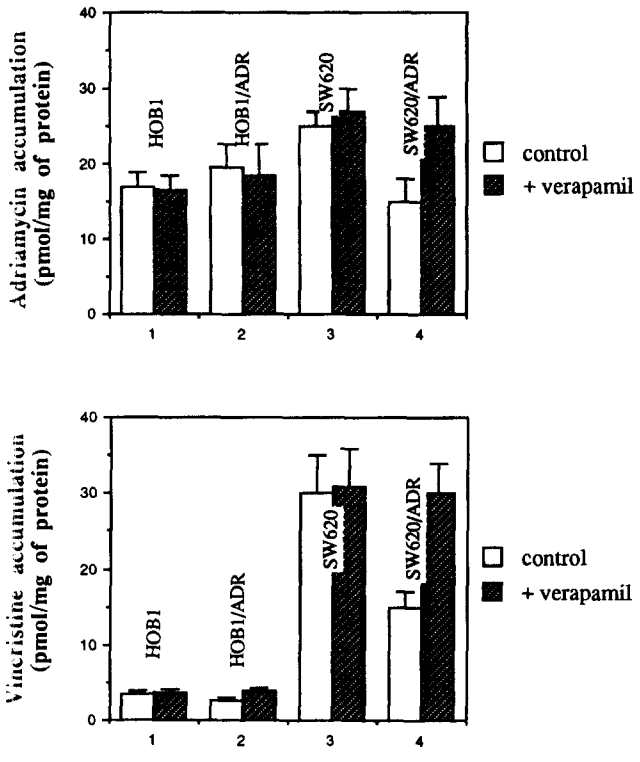

Fig. 5. Verapamil effect on the accumulation of adriamycin (upper panel) or vincristine (lower panel) in parental or resistant cells. Cells were incubated in medium containing [^{14}C]adriamycin or [^{14}C]vincristine for 60 min in the presence or absence of verapamil (10 μM). Points, means; bars, ± S.D.; n = 3.

analyzed in parallel. Verapamil restored vincristine accumulation in SW620/MDR cells to ~30 pmol/mg of protein which is similar to the level in parental cells; whereas with little verapamil effect on SW620 cells. The results indicate that verapamil enhances the accumulation of vincristine, but not adriamycin, in HOB1/ADR cells.

3.5. *Inhibition of drug resistance by verapamil*

The acquired adriamycin resistance of HOB1/ADR cells was tested by a sublethal concentration of verapamil (1 μg/ml) (Table 2). Resistance phenotypes of P-glycoprotein-dependent (SW620/MDR) and independent (PC-9/VCR) cells were used as controls. IC_{50} of these resistant cell lines with or without verapamil is indicated in comparison with their parental counterpart. Adriamycin resistance in HOB1/ADR cells (230 fold) was partially reversed by verapamil. There was 50-fold reduction. The adriamycin resistance of SW620 and SW620/MDR cells was reduced by verapamil by 1.25 and 50 fold, respectively. In contrast, vincristine resistance in PC-9 and PC-9/VCR cells was not affected by verapamil. The results indicate that verapamil affects vincristine toxicity in P-glycoprotein-overexpressing SW620/MDR cells [22], but not in P-glycoprotein-independent PC-9/VCR cells [14]. Thus, adriamycin resistance in HOB1/ADR cells is at least partly due to P-glycoprotein.

4. Discussion

In this report, an MDR lymphoma cell line was selected by adriamycin. The resistant cells demonstrated the overexpression of *mdr*1 gene, and were phenotypically sensitive to verapamil, an effective reversing agent of P-glycoprotein-mediated MDR [30,31]. The HOB1/ADR cells selectively pumped vincristine, but not adriamycin, out of cells, associated with cross-resistance to vincristine. Treatment of the HOB1/ADR cells with verapamil partially reversed acquired resistance, suggesting that P-glycoprotein is partly responsible for adriamycin resistance. A simple explanation is that a verapamil-sensitive mechanism(s) other than adriamycin efflux is responsible for the resistant phenotype in the immunoblastic lymphoma cells. The above observation is not due to analytical artifacts because verapamil clearly reverses resistance in P-glycoprotein-dependent SW620/MDR [22] but ineffective in a P-glycoprotein-independent resistant PC9/VCR [14]. It has previously been demonstrated that mutations in the P-glycoprotein gene, *mdr*1, in some cases, but not in others, may explain drug specificity [10,32]. The co-enhancement in efflux of adriamycin and vincristine, associated with co-resistance to both drugs, was detected in SW620/MDR cells [this study;22]. Perhaps, the sublethal pressure by vincristine-based chemotherapy of the patient [21] pre-selects for cells with potential in P-glycoprotein overexpression and preferential efflux of vincristine. Further treatment of the cultured HOB1 cells with adriamycin or perhaps other agents establishes cells with *mdr*1 overexpression and selected drug efflux. It should be interesting to have more detailed analysis in view of drug efflux specificity in resistant immunoblastic lymphoma cells. Western and Northern blotting analyses indicated that MRP and GST are not involved in the non-efflux resistance of HOB1/ADR cells (Chao, C.C.-K., unpublished data). Alternatively or additionally, *ras* overexpression may play a role in the drug resistance of HOB1/ADR cells. Conflicting results have been reported that *ras* overexpression is associated with resistant phenotype in some cells [33–35] but not in others [36–38]. Therefore, events associated with the presence of an acitvated *ras* oncogene, such as protein kinase C [39,40], glutathione S-transferase [41,42], and transcriptional factor AP-1 [43] activities, may be related to the mechanisms involved in the response of HOB1/ADR cells to adriamycin and other drugs. We and others have previously demonstrated that adriamycin-selected MDR cells are associated with overexpression of anionic glutathione S-transferase and cytochrome P450-related enzymes, potentially able to inactivate anthracyclines and possibly other MDR drugs [22,44,45]. Perhaps, endogeneously activated *ras* expression in parental HOB1 cells [20] in cooperation with other mechanism(s) elicited by the selecting agent results in the resistant phenotype, which is not necessary to be associated with drug accumulation. This is the first evidence, to my knowledge, showing adriamycin-resistant cells which display enhanced efflux of the other but not the selecting drug.

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