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Acute leukemias

Heterogeneity of isolated mononuclear cells from patients with acute myeloid leukemia affects cellular accumulation and efflux of daunorubicin

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ABSTRACT

Background and Objectives. **Pharmacologic studies on blasts from patients with leukemia are generally performed on density gradient isolated blood or bone marrow cells. Thereby, cellular drug accumulation and efflux are determined as mean values of the entire cell population. The objective of the present study was to characterize the heterogeneity in the accumulation and efflux of daunorubicin in various subpopulations of mononuclear cells isolated from patients with acute myeloid leukemia (AML).**

Design and Methods. **Mononuclear cells from 33 patients with AML were isolated from peripheral blood by density gradient centrifugation on Lymphoprep (1.077 g / mL). Cellular accumulation of fluorescent daunorubicin was determined by flow cytometry after incubation of the cells at +37°C for 1 hour. Thereafter, the cells were washed and reincubated in drug-free medium. Kinetics of drug efflux were determined by frequent determination of cellular fluorescence during 30 min. Daunorubicin accumulation and efflux were compared in the total isolated mononuclear cell population and in the various blast cell populations gated on FSC / SSC according to the results of immunophenotyping.**

Results. **In 8 of these 33 (24%) patient samples, two distinct blast cell populations could be identified. In 7 out of 8 these cases the more immature blasts had a lower drug accumulation and in 6 out of the 8 cases also a higher efflux rate than the differentiating cell population. Cyclosporin A increased daunorubicin accumulation and reduced efflux in the immature blast population. In the differentiating cell population cyclosporin A increased both the accumulation and the efflux. In patients with a single blast cell population, the gated blast cells had a significantly lower drug accumulation but also a lower drug efflux rate than the total cell population.**

Interpretation and Conclusions. **The results imply that drug transport studies on cells isolated from patients with AML give somewhat different results depending on the cell population studied. Some, but not all, of these differences in daunorubicin accumulation and efflux as well as in the effect of cyclosporin A can be explained by a heterogenous expression of the *mdr1*-gene. The observed heterogeneity may be of special relevance with regard to drug resistance. The presence of even a small resistant cell clone may jeopardize the effect of the chemotherapy due to expansion resulting in relapse of disease.**

Key words: acute myeloblastic leukemia, drug resistance multiple, flow cytometry, immunophenotyping, daunorubicin

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One of the most important challenges in leukemia research is how to prevent, detect and overcome resistance to chemotherapy. Patients with acute myeloid leukemia (AML) often suffer from treatment failure even after an initially good response and only one third can be cured by chemotherapy.[1]

The most studied mechanism of resistance is multidrug resistance (MDR) mediated by the P-glycoprotein (Pgp), a transmembrane protein with a pump function encoded by the *mdr1* gene.[2] MDR is characterized by cross resistance to a wide range of chemotherapeutics of natural origin e.g. anthracyclines, vinca alkaloids and taxanes.[3] MDR is well defined in cultured cells, in which it can easily be induced by incubation with increasing drug concentrations for a number of cell generations. Resistant cells have a reduced intracellular drug concentration caused by an increased drug efflux. Addition of resistance modifiers such as cyclosporin A can reverse drug resistance in cultured cells by inhibition of drug efflux thereby restoring the intracellular drug concentration.[4]

Increased levels of Pgp have been found in leukemic cells in more than 50% of patients with AML at diagnosis and at a still higher frequency at relapse or in secondary leukemia.[5,6] In several studies, the *mdr1* gene expression was associated with a poor initial response to chemotherapy and shorter duration of complete remission.[7-11] In other studies, the correlation between Pgp expression by leukemic samples and clinical outcome was weak.[12] Accurate determination of the expression of Pgp in clinical samples can be difficult due to much lower levels than in resistant cell lines.[13] Additional proteins, multidrug resistance associated protein (mrp)[14] and lung resistance related pro-

Table 1a. Patient characteristics and immunophenotype* of the blast cell populations.
Patients with two blast cell populations.

Pt	Sex/age	FAB	WBC x 10^9/L	% cells in blast gate	Immature blasts		Maturing blasts	
					%	phenotype	%	phenotype
1	M/33	M4	142	71	48°	19,45,10,DR+,22	25#	34,33,15,65,DR+,13
2	M/44	M4	206	76	35	34,DR+,13	60	15,13,33,4,11b,14
3	M/39	M2	58	77	51	33,117, DR-,13	32	33,15,65,DR-,11b,13
4	M/79	M4	30	50	42	33,117, DR+,4	53	33,15,65,DR+,14,11b
5	F/29	M5	33	80	15	34,117,DR+,7	61	117,33,13,14,DR+
6	F/40	M2	37	56	28	34,DR+,7,56,2	40	13,33,15,2,56,DR-
7	F/80	M2	59	66	15	34,117,DR+	70	15,65,33,13,11b,DR-
8	F/43	M4	136	64	66	34,117	36	117,13,15,14

*Note: *only positive markers are given, with exception of HLA-DR. In case #1 two populations with °lymphoid and #myeloid characteristics were found. Double expression of °lymphoid and #myeloid markers was also found in 15% of cells.*

tein (lrp)[15] involved in drug transport and intracellular distribution have also been identified. Mrp expression has not been found to be an adverse factor[16-18] but studies on lrp expression in AML are more contradictory. Some studies report an adverse effect on treatment outcome[19-22] but others have not found this correlation.[17,23]

Functional assays determining cellular response to chemotherapy, drug accumulation, retention and efflux to chemotherapy are important in measuring drug resistance since drugs must be able to enter the cell and to stay in it long enough to exert cytotoxicity.

Since it has been shown that relapses often occur in the most immature cell population[24] we aimed our study at the elucidation of the functional heterogeneity in daunorubicin accumulation and efflux in various subpopulations of mononuclear cells isolated from patients with AML.

Design and Methods

Patients

The study was performed on leukemic blasts from 33 patients with AML with a WBC count > 10×10^9 cells/L. Patient characteristics are given in Tables 1a and 1b. The diagnosis was based on morphology and immunophenotyping[25] and classification was made according to FAB criteria.[26] The study was approved by the local ethics committee.

Processing of blood samples

Mononuclear cells from peripheral blood were isolated by density gradient centrifugation (1.077 g/mL, Lymphoprep®, Nycomed Pharma AS, Oslo, Norway), at 500g for 20 min at room temperature and washed twice in phosphate-buffered saline. Cell samples were analyzed immediately or frozen in a programmed freezer to –160°C and stored in liquid nitrogen. Before analyzis, frozen cells were rapidly thawed in a water bath at 37°C, diluted in 10 mL newborn calf serum (Gibco BRL, Life Technologies Ltd, Paisley, Scotland) centrifuged and washed in medium RPMI 1640 with 20 mM HEPES supplemented with 10% newborn calf serum and 2 mM L-glutamine (all from Gibco BRL, Life Technologies Ltd, Paisley, Scotland). Cell viability after thawing was determined using the trypan blue exclusion test and was always above 85%. Previous studies and our own experience have shown that functional assays can be equally well performed on fresh or vitally frozen and thawed cells.[8,27]

Table 1b. Patient characteristics and immunophenotype* of the blast cell populations.
Patients with one blast cell population.

Pt No	Sex/age	FAB	WBC x 10^9/L	% cells in blast gate	Blast phenotype
9	M/68	M2	20	80	13, 33, 15, DR
10	F/43	M2	25	60	13, 33, DR, 4
11	F/43	M5	146	85	13,33,15,65,11b,4,14
12	M/65	M2	92	62	13,33,15,4
13	M/61	M5	11	84	34,13,11b,DR,19,2
14	F/83	M0	110	71	34,117, 7,DR
15	M/60	M2	35	95	34,13,33,7
16	F/67	M1	91	85	13,33,4
17	M/70	M4	31	65	34,13,33
18	M/72	M2	89	50	34,117,33
19	M/74	M0	98	95	34
20	M/87	M5	22	84	117,13,33,DR,19
21	M/73	M2	17	60	13,33,7,4
22	M/73	M5	47	80	117,33,11b,DR,7,5
23	M/66	M5	13	62	13,33,15,DR,11b,4,14,56
24	F/74	M5	30	50	13,33,15,4,14
25	F/82	M5	12	84	33,56
26	F/74	M1	46	64	34,117,33,56
27	M/53	M5	125	87	33,15,65,DR,56,4
28	M/73	M0	100	80	33,56
29	M/45	M5	35	89	$34,33^{dim},65,15,56$
30	F/62	M4	14	56	34,13,33,15,DR,11b,4,14
31	F/30	M1	100	89	34,13,15,DR
32	M/66	M2	22	42	34,13,33,15,DR,4
33	M/67	M2	62	76	33,15,56

*Note: *only positive markers are given.*

Immunodiagnosis

The phenotypic analysis was performed on whole bone marrow, or on peripheral blood (patients #4 and #31), by the three-color direct immunofluorescence technique using combinations of fluorescein isothiocyanate (FITC), phycoerythrin (PE) and peridinine chlorophyll protein (PerCP) or phycoerythrincyanine 5 (PE/Cy5) conjugated monoclonal antibodies (Mabs) at pretitrated optimal conditions. Mabs were against the following antigens: CD2, CD3, CD4, CD8, CD11b, CD13, CD20, CD22, CD33, CD34, CD38, CD45, CD56, HLA-DR from Becton Dickinson, (B&D, San José, CA, USA), CD5, CD7, CD10, CD15, CD19, lambda, kappa, myeloperoxidase (MPO) from Dako, (Glostrup, Denmark), CD13, CD14, CD19, CD33 from Caltag, (Burlingame, CA, USA), CD34, CD117, GPA from Immunotech, (Marseille, France), CD65 from Scandic GmbH, (Vienna, Austria) and terminal deoxynucleotidyl transferase (TdT) from Supertechs (Bethesda, MD, USA). For membrane staining, cell suspensions were incubated for 15 minutes at room temperature with Mabs and FACS-lysing solution (B&D) was applied subsequently to lyse erythrocytes. Detection of cytoplasmic MPO and nuclear TdT was performed after permeabilization and fixation with Permeafix (Ortho, Raritan, NY, USA) used according to manufacturers' recommendations.[24] Samples stained with irrelevant antibody were used as negative controls. Analysis was performed on a FACScan with Paint-a-gate and Lysys-II softwares (B&D). The percentage of positivity for various Mabs was determined after gating blast cell populations by their light scatter features. Samples were considered positive for the given marker if there were more than 20% positive cells in the gate. Blast cells were defined as immature when positive for Mabs against CD34 and/or CD117. In two cases both the immature and differentiating blast cell populations were positive for CD117 and those expressing CD34 were defined as immature. Blast cells were defined as differentiating when they expressed granulocyte or monocyte differentiation markers CD15 or CD14 but were negative for CD34 and/or CD117. Using so-called *back gating* analyses it was defined that immature and differentiating blast populations had different positions on forward scatter (FSC) and side scatter (SSC) plots (Figure 1a).

Flow cytometric determination of daunorubicin accumulation and efflux

Isolated cells were resuspended at a concentration of 5×10^5 cells/mL medium and incubated with 1 µM daunorubicin (Rhône-Poulenc Rorer, Birkeröd, Denmark) at 37°C with regular shaking for 60 min whereafter daunorubicin accumulation was measured. FSC and SSC-signals were collected using linear scales and fluorescence (Fl-2) using a logarithmic scale. The intracellular daunorubicin content was expressed by the mean intensity (MFI) of fluorescence 2 (Fl-2) using a FACScan flow cytometer with an argon laser (excitation at 488 nm) and Lysys-II software. The flow cytometer settings were standardized by fluorescent microspheres (Coulter Standard-Brite, Coulter Corp, Hialeah, FL, USA). Gating on FSC was done to exclude dead cells and debris. Leukemic blast cells

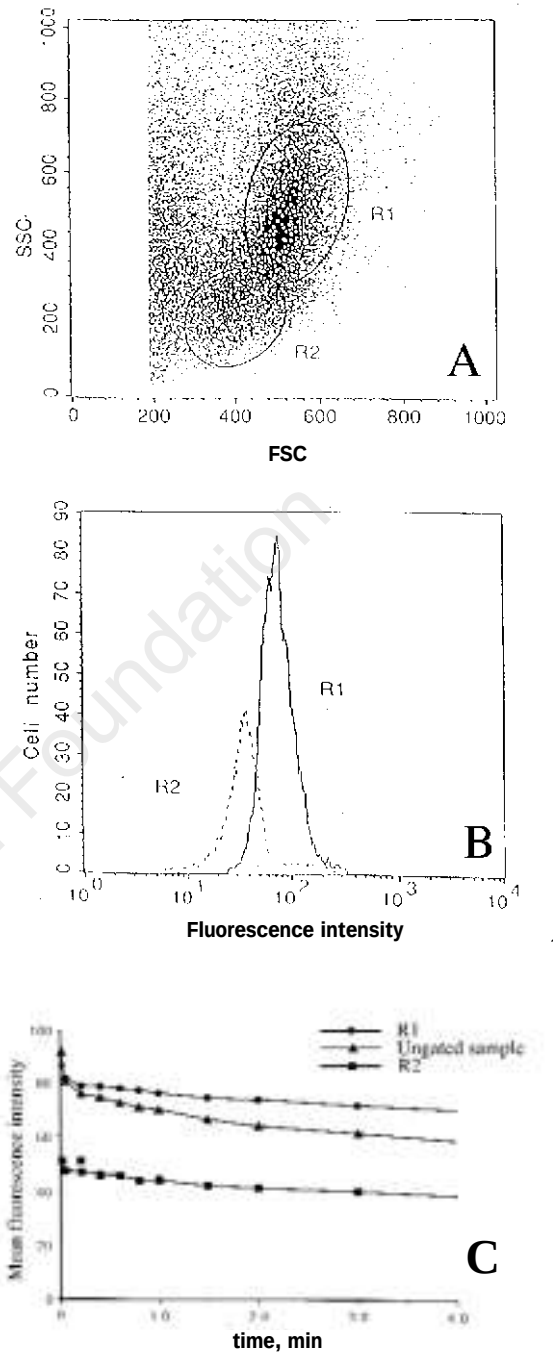

Figure 1.
(A) Dot plot picture (patient sample #6) showing gates for differentiating blasts (R1) and immature blasts (R2). Gating was based on light scatter properties according to results from immunophenotyping with monoclonal antibodies.
(B) Histograms with cellular fluorescence intensities (Fl-2) for the gated differentiating blasts (R1) and immature blasts (R2). After 1 hr incubation of leukemic cells with 1 µM daunorubicin the differentiating population (R1) shows higher drug accumulation.
(C) Efflux curves with rate k (from $C_t = C_0e^{-kt}$) = 3.9 differentiating blasts (R1), 6.0 immature blasts (R2) and 8.9 total ungated population.

were further gated based on light scatter properties according to results from immunophenotyping with Mabs. For determination of drug efflux, cells were centrifuged at 4°C and resuspended in prewarmed drug-free medium whereafter incubation continued at 37°C. Cellular drug concentration was quantified by assessment of MFI at frequent intervals (at 0, 2, 4, 6, 8, 10, 15, 20 and 30 min). For the samples with two blast cell populations, the effect of 3 µM cyclosporin A (Sandimmun, Novartis, Sweden) on daunorubicin accumulation and efflux is also presented. In these studies cyclosporin A was present during both the loading and efflux periods. Efflux curves were plotted from the MFI values assuming a one-compartment pharmacokinetic model (following a negative exponential curve $C_t = C_0e^{-kt}$).

mdr1-mRNA quantification with competitive RT-PCR

Total cellular RNA was extracted by using the ULTRASPEC™ - II RNA (Biotecx Lab., Houston, TX, USA). RNA yield and purity were determined spectrophotometrically at 260/280 nm (Perkin Elmer, Norwalk, CT, USA) and the integrity of RNA verified by electrophoretic size separation in 1% ethidium bromide stained agarose gels. cDNA was synthesized using random priming (N6, Pharmacia, Uppsala, Sweden) and MMLV reverse transcriptase, as described earlier.[28]

*Mdr*1 and β_2-microglobulin (β_2-M) specific primers and PCR conditions have been described elsewhere.[28, 29] The single competitive template which contains both *mdr1* and β_2-M primer sequences was constructed by using mimic PCR and cloned into TA vectors (Invitrogen, San Diego, CA, USA). The wild type *mdr1* and β_2-M fragments were 239 bp and 266 bp, which were 44 bp and 69 bp longer than their competitive PCR products, respectively. cDNA corresponding to 50 ng of RNA was co-amplified with appropriate competitive molecules (3×10^3 competitors for *mdr1* and 5×10^5 competitors for β_2-M) in 25 µL of reaction mixture containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM of each primer and 0.625 U Taq DNA polymerase (Perkin Elmer). PCR was performed using 30 cycles for *mdr1* and 25 cycles for β_2-M.

The same amount of cDNA derived from the mixture of 98% *mdr1*-negative human myloid leukemia K562 cells and 2% vincristine-resistant *mdr1* expressing cells K562/Vcr150 (about 200 *mdr1* mRNA molecules per K562/Vcr150 cell)[30] was tested in parallel as a reference. That mixed cell population resulted in approximately 4 *mdr1* mRNA molecules per cell, which made the comparison between reference cells and patients' samples easier.

PCR products were resolved in 2% agarose gels stained with ethidium bromide, visualized in UV light and photographed. Volumetric integration of signal intensities was performed by using NIH Image software (version 1.58). The relative levels of *mdr1* mRNA expression were arbitrarily given by the ratio of the target and competitor signal density and normalized to the ratio of β_2-M and its competitors. The final value of the *mdr1* mRNA level in patients' samples was expressed as the percentage of that in the cell mixture of 98% K562 and 2% K562/Vcr150.

With the PCR conditions described above, the linearity of the assay with less than 20% coefficient of variation was obtained over a 70 fold difference in *mdr1* gene expression. The signal ratio of *mdr1* and co-amplified competitor PCR products corresponded well to the dilution factor of cDNA from the mixed cells of 98% K562 and 2% K562/Vcr150 and a two-time difference in cDNA input could be clearly distinguished. To avoid possible variation due to different RNA loading or reverse transcription efficiency, or the presence of PCR inhibitors, β_2-M and its competitor molecules were co-amplified as internal controls in parallel with the *mdr1* sequences. Five of the 33 samples could not be evaluated because of RNA degradation and/or presence of PCR inhibitors.

Statistical analysis

Correlations between drug accumulation and efflux were estimated using the Spearman rank correlation test. Differences in drug accumulation and efflux rates between the different cell populations were analyzed with a paired t-test and Wilcoxon's signed-rank test.

Results

Daunorubicin accumulation and efflux in the total cell population

There was a pronounced variability in daunorubicin accumulation in the total isolated cell populations with MFI varying from 24 to 165 (Tables 2a and b). Pronounced variability was also seen in the drug efflux rate (range 5-45% of the accumulated drug during 30 min) (Tables 3a and b). A correlation ($r = 0.492$, $p = 0.005$) was found between high drug accumulation and high efflux rate (Figure 2). The total cell populations were analyzed for *mdr1* expression. The *mdr1* mRNA levels in the 28 studied patients are summarized in Tables 2a and b. There was a weak correlation between *mdr1* gene expression and daunorubicin accumulation ($r = -0.419$, $p = 0.029$) but none between *mdr1* and efflux rate ($r = 0.125$, $p = 0.51$).

Patients with two blast cell populations

In eight of the 33 patients, two distinct blast cell populations could be identified by gating based on light scatter properties (FSC/SSC) and the results of immunophenotyping (Tables 1a and b). The blasts could be separated into a population showing signs of maturation and an immature blast population. The immature population had lower FSC/SSC characteristics than the differentiating one. The leukemic origin of the populations was ascertained by the fact that they were both found in the blast region of the light scatter plot and also by morphologic control of the cell population (> 80% blast cells). The accumulation and efflux of daunorubicin could be calculated separately for the total cell population and for the two blast cell populations described above (Figure 1). In comparison to the differentiating blast cell population, the immature population had a lower daunorubicin accumulation (7 of 8 samples, $p = 0.018$, Table 2a) and a higher efflux rate (6 of 8 samples, $p = 0.21$, Table 3a). Cyclosporin A increased the daunorubicin

Table 2a. Daunorubicin accumulation and *mdr1* mRNA expression.
Patients with two blast cell populations.

Pt No	Dnr accumulation, MFI						mdr1 mRNA
	immature blasts		maturing blasts		total		
	-CyA	+CyA	-CyA	+CyA	-CyA	+CyA	level*
1	56	60	63	60	59	58	43
2	66	77	103	117	84	97	5
3	48	51	56	57	52	55	14
4	53	59	103	116	97	113	0
5	62	60	86	84	103	102	5
6	51	57	87	99	91	100	19
7	62	61	62	71	66	69	21
8	48	50	72	75	55	56	26
mean	56	59	79	85	76	81	
sd	6.9	8.3	18.5	23.6	20.2	24.1	

**expressed in relative terms, see Design and Methods.*

Table 2b. Daunorubicin accumulation and mdr1 mRNA expression.
Patients with one blast cell population.

Pts no.	Daunorubucin accumulation, MFI		mdr1 mRNA
	Blast cells	Total population	Level*
9	43	56	15
10	74	108	10
11	110	146	1
12	59	86	48
13	56	63	5
14	45	60	73
15	50	62	80
16	70	87	7
17	25	24	na
18	48	48	89
19	43	43	3
20	62	67	43
21	69	74	66
22	56	59	na
23	214	161	na
24	64	95	24
25	47	86	6
26	40	64	78
27	64	72	0
28	82	92	32
29	90	92	na
30	58	62	na
31	37	44	117
32	129	165	37
33	53	85	0
mean	68	80	
sd	38.1	34.9	

**Expressed in relative terms, see Design and Methods; na, not analyzed.*

accumulation (p = 0.044, Table 2a) and reduced the efflux rate (p = 0.044, Table 3a) in the immature cell population. In contrast, cyclosporin A slightly increased both the accumulation of daunorubicin (p = 0.048) and the efflux rate in the differentiating population. The latter effect was, however, not significant (p = 0.33).

Patients with a single blast cell population

In 25 of the 33 patient samples, only one distinct blast cell population could be identified. In comparison with the total Lymphoprep-isolated cell population, the gated blast cells had a lower daunorubicin accumulation (p = 0.0005, Table 2b) and also a lower efflux rate (p = 0.0002, Table 3b). There was hardly any correlation (r = 0.286, p = 0.16) between accumulation and efflux rate in the blast cell population while in the total isolated cell population the correlation was noticeable (r = 0.513, p = 0.012) (Figure 3). In ten cases we separately analyzed the non-blast cell population. This population had a higher accumulation than the total cell population and also a higher efflux rate (data not shown).

Within these 25 cases we identified patient samples #14, 18, 19, and 26 as having the most immature phenotype (i.e. the stem cell marker CD34 and c-kit CD117 and lacking the differentiation markers CD15, CD14 and CD11b). In contrast, patient samples #9, 11, 23, 27, and 30 had the most differentiated immunophenotypes. The cases having the most differentiating phenotype had a higher drug accumulation (MFI 98 compared to 44) and a lower drug efflux rate (mean k-value 5.6 vs 10.4) than the group with the most immature phenotype.

In five patients we noted two fluorescence peaks in the total isolated cell population after incubation with daunorubicin for 60 min. All of them had single blast cell populations according to immunophenotyping (#11, 16, 24, 25, and 33). The peak representing cells with the higher daunorubicin accumulation gradually disappeared during the efflux period (Figure 4). When the cell populations corresponding to the two peaks were visualized by *back gating* on the light scatter plots, the disappearing high accumulating population corresponded to the plot area where apoptotic cells are usually found.

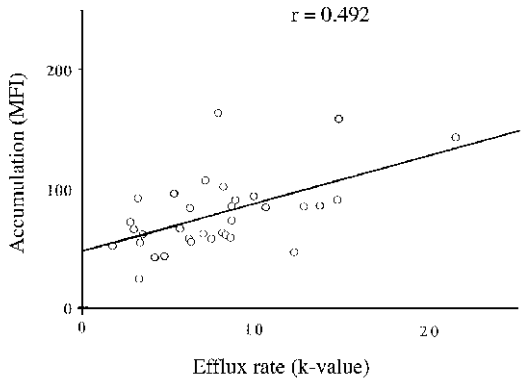

Figure 2. Correlation between accumulation and efflux rate in the total isolated cell population from 33 patient samples. Accumulation was measured after 1 hr incubation with 1 µM daunorubicin. Efflux rate was calculated after 30 min efflux in drug-free medium. Spearman's correlation coefficient (r) for the two parameters was equal to 0.492 (p=0.0054).

Table 3a. Daunorubicin efflux from the total and the gated blast cell populations.
Patients with two blast cell populations.

Pt no.	Efflux rate, k-value, x 10^{-3}						% efflux during 30 min					
	Immature blasts		Maturing blasts		Total		Immature blasts		Maturing blasts		Total	
	-CyA	+CyA	-CyA	+CyA	-CyA	+CyA	-CyA	+CyA	-CyA	+CyA	-CyA	+CyA
1	5.4	3.4	2.3	0.6	7.5	3.7	14.7	9.2	6.3	1.3	20.4	10.2
2	5.7	2.5	5.2	5.4	6.2	4.3	16.5	7.3	15.2	15.5	17.1	13.4
3	2.0	2.0	1.6	1.9	1.7	2.3	5.9	6.2	4.4	6.4	5.0	6.9
4	1.8	2.7	3.2	6.6	5.3	3.6	6.5	7.6	10.1	17.4	14.1	6.4
5	5.5	4.5	5.2	4.8	8.2	5.9	15.1	12.7	15.4	13.8	21.8	15.8
6	6.0	2.0	3.9	3.6	8.9	8.6	15.3	12.6	11.7	10.9	23.6	23.7
7	5.1	-1.5	-1.6	2.6	2.9	2.0	14.5	-3.4	-4.1	7.2	9.3	6.5
8	3.1	2.2	3.8	4.0	3.3	3.2	7.6	7.0	11.3	13.2	8.0	9.2
mean	4.3	2.2	3.0	3.7	5.5	4.2	12.0	7.4	8.8	10.7	14.9	11.5
sd	1.7	1.7	2.2	2.0	2.6	2.1	4.5	5.0	6.5	5.4	6.9	6.0

Table 3b. Daunorubicin efflux from the total and the gated blast cell populations.
Patients with one blast cell population.

Pt no.	Efflux rate, k-value, x10^{-3}		% efflux during 30 min	
	blasts	total	blasts	total
9	2.1	6.3	6.5	15.7
10	4.6	7.1	14.3	19.7
11	10.8	21.5	27.7	45.3
12	9.9	12.9	25.5	32.2
13	5.1	7.0	13.3	18.3
14	9.1	8.6	23.5	22.2
15	5.3	8.3	14.0	20.9
16	9.0	13.8	23.4	33.3
17	2.4	3.2	7.9	10.5
18	11.9	12.3	30.1	29.7
19	3.1	4.1	10.6	12.6
20	5.0	5.6	16.1	17.3
21	7.8	8.7	20.0	23.5
22	4.6	6.1	15.8	17.8
23	11.6	14.9	29.4	34.7
24	7.8	10.0	21.1	25.3
25	1.0	8.7	3.1	23.0
26	17.3	8.1	40.0	21.0
27	1.0	2.7	3.1	8.2
28	12.3	14.8	28.1	32.9
29	2.4	3.1	7.3	8.5
30	2.3	3.4	6.8	10.0
31	4.1	4.7	11.2	12.5
32	6.4	7.9	19.6	22.9
33	6.0	10.7	15.2	26.8
mean	6.5	8.6	17.3	21.8
sd	4.2	4.5	9.5	9.3

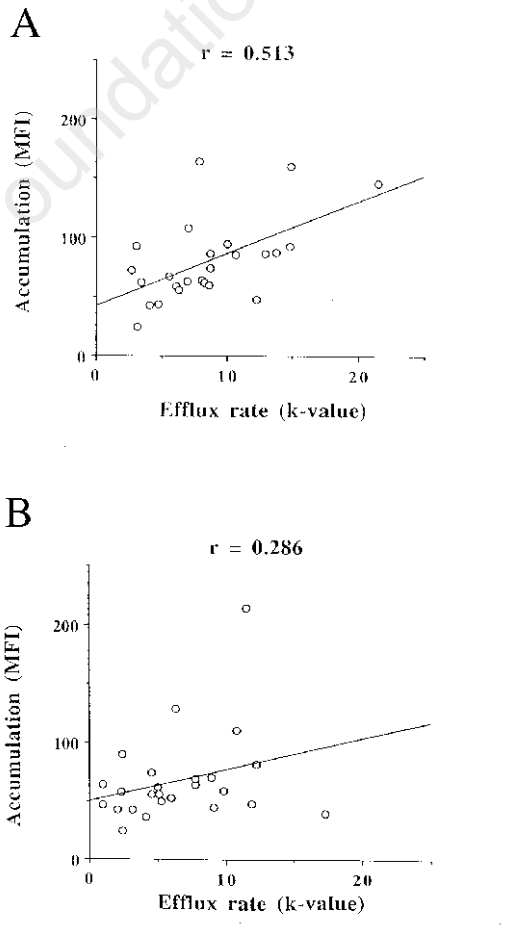

Figure 3. Correlation between accumulation and efflux rate in 25 patient samples with one blast cell population. Accumulation after 1 hr incubation with 1 µM daunorubicin and efflux rate was measured after 30 min efflux in drug-free medium. A) shows the results in the total isolated cell populations with correlation coefficient 0.513 (*p*=0.012); B) shows the blast cell populations with correlation coefficient 0.286 (*p*=0.16).

Discussion

Prior to the study of resistance factors on peripheral blood samples from patients with AML, mononuclear cells are usually isolated by density gradient centrifugation. The obtained cell population does not only contain leukemic blast cells but also, to a varying extent, normal monocytes and lymphocytes.

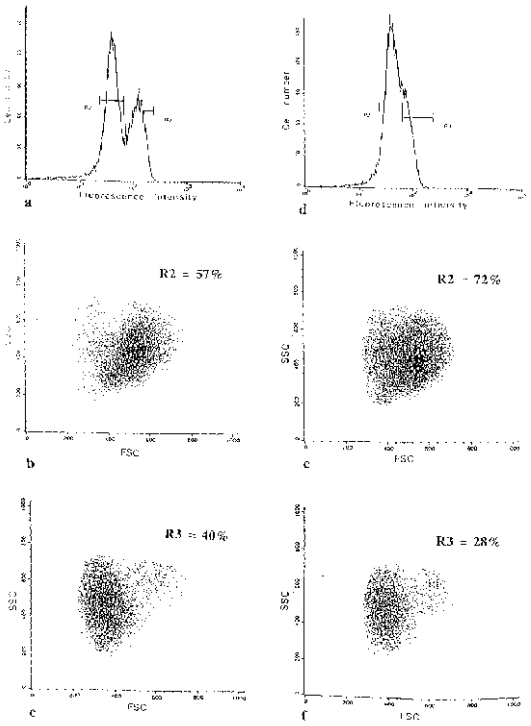

Figure 4. Heterogeneity of daunorubicin acumulation in a case with one blast population (patient sample #25). (a) Histogram from leukemic cells, incubated with 1 µM daunorubicin, with double fluorescence peaks after 1 hr accumulation but a single peak (d) at the end of the efflux period. FSC/SSC (b) and (c) corresponding to the two peaks R3 and R2 respectively in histogram (a), FSC/SSC (e) and (f) corresponding to R3 and R2 in histogram (d) show that the population with higher accumulation is located in the *apoptotic cell* area and disappears during the observation time.

Virtually all normal hematopoietic blood cells express Pgp although some cell types only at a low level.[31]

Our study demonstrates differences in daunorubicin accumulation and efflux between the total isolated cell population and the leukemic blast cells. Daunorubicin was selected for the transport studies as it is more clinically relevant than rhodamine 123. We could identify two distinct blast cell populations in 25% of the patients. The FAB categories in 7 of 8 cases were AML M2 or M4, which by definition show signs of maturation and thus can consist of blast cell populations at various levels of differentiation. In other studies as many as 74% of AML samples were shown to contain more than one blast cell population in the bone marrow.[32] The lower fraction of these cases in our material may have been due to the selection of patients with a white blood cell count exceeding 10×10^9 cells/L and because the study was made on peripheral blood.

The immature blast cell population displayed a lower drug accumulation and a higher rate of efflux than the blast cells showing signs of differentiation towards granulocytic or monocytic lineage. We also found a similar correlation between differentiation and drug accumulation and efflux rate in blasts from patients with only one blast cell population. The correlation between immaturity and low accumulation and high efflux is in agreement with the strong association between the stem cell marker CD34 and the expression of Pgp which are concomitantly downregulated with progressive hematopoetic lineage commitment.[33,34] A significant association between drug efflux and CD34 expression has also been found in other studies, in which decreased daunorubicin accumulation was noted in leukemic blast subsets expressing $CD34^+$ as compared with subsets that were $CD34^-$.[7,35]

With currently applied chemotherapy regimens most patients with AML achieve complete remission[36] but less than one third of them is cured.[1] The high risk of relapse may imply a presence of a minor resistant cell clone already at diagnosis. It has been demonstrated that relapses usually derive from the most immature cell population.[24] This is in accordance with our observation that the most immature blast cells both in the samples with two cell populations and in the samples with one blast cell population show lower drug accumulation and higher efflux, which probably leads to less killing and to the possible expansion of surviving blasts. This may be further confirmed by another type of heterogeneity demonstrated by the presence of cell populations with higher daunorubicin uptake. These populations showed time scatter properties which suggested apoptosis and after 45 min of efflux study disappeared. This strengthens the possibility of the presence of drug sensitive and resistant cells even within a leukemic clone that does not show various subpopulations by immunophenotyping.

Cyclosporin A exerted the expected effects of reducing the efflux rate of daunorubicin and increasing drug accumulation in the immature blasts. Accumulation also increased in the differentiating population but the efflux was not reduced; indeed it increased. This may imply that cyclosporin A, in addition to inhibiting Pgp, also increases drug influx and/or intracellular storage capacity. Comparing a panel of AML cell lines, Bailly *et al*.[37] found that in immature cell lines, efflux of rhodamine 123 could be blocked by resistance modifiers. In contrast, more mature cell lines showed no rhodamine 123 efflux despite high expression of the *mdr1* gene (analyzed by RT-PCR). There are differences in cellular pharmacology between rhodamine 123 and daunorubicin. Only daunorubicin is stored intracellulary linked to DNA while rhodamine 123 is a mitochondrial dye with much lower cytotoxicity.[38]

The results of daunorubicin accumulation and efflux for total isolated samples were compared with gated single blast populations and lower accumulation and efflux were noted in the latter. Daunorubicin is highly concentrated intracellularily and the concentration gradient favors a leakage from the cells into the medium. Therefore a higher drug accumulation leads to a higher leakage component which in certain populations may dominate over the active efflux mechanism. That may be one of the reasons for lack of correlation between *mdr1* expression in the total cell population and accumulation and efflux in

our material. Leith *et al*.[7] studied the correlation between functional drug efflux and *mdr1* expression in samples from 60 previously untreated AML patients. Functional efflux, correlated to *mdr 1* expression, was identified in 39 cases but several discrepant cases were also found: 10 cases with efflux but no *mdr1* expression and 6 cases with *mdr1* expression but no efflux.

In summary, we have demonstrated various aspects of heterogeneity in daunorubicin accumulation and efflux in leukemic cell populations. The clinical relevance of this heterogeneity should be further investigated in samples from more patients, and preferably using sorted, homogenously immature blast cell populations. Our results suggest that when resistance factors are examined in patient samples, the possibility of variable expression of these factors within the cell sample should be considered and various blast populations, if present, should be studied separately.

Contributions and Acknowledgments

All authors participated in designing the study and writing the paper, EK carried out the cytometric studies and handled the data, APMD was responsible for the immunophenotyping, DX carried out the RT-PCR mRNA quantification, and AG provided the patient samples and clinical data. All authors critically revised the intellectual content of the study.

The criteria for the order in which the authors' names appear are based on the amount of work expended in the study, except for the last name, Professor Peterson, who is the head of the department in which the study was performed.

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Disclosure

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Potential implications for clinical practice

- The final outcome of leukemia chemotherapy will depend on the effect on the least responsive cell population.
- In some cases of AML, immature blast populations that are resistant to cytostatics may be present already at diagnosis. This may have a great impact on the response to treatment and treatment outcome of the patients.
- Studies on drug efflux and effect of resistance modifers in leukemic cell populations may provide a tool to identify patients that benefit from the addition of resistance modifiers to chemotherapy.
- Resistance reversing agents will be most effective if added to the front-line therapy when the number of resistant cells is small.

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