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Multidrug resistance in acute leukemia: a comparison of different diagnostic methods

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Accurate measurement of P-glycoprotein (P-170) expression in clinical samples still remains a controversial issue. In this study tumor cell P-170 expression was assessed in 29 patients suffering from acute leukemia (17 acute myeloid leukemia (AML) and 12 acute lymphoblastic leukemia (ALL)) using three different techniques: flow cytometry measuring rhodamine 123 (Rh123) efflux (functional level), immunocytochemistry (protein level) and RT-PCR (mRNA level). Rh123 efflux was detectable in 10/29 (34%) of all cases, in 9/17 (53%) of AML and in 1/12 (8%) of ALL samples. In AML patients a significant association of CD34 expression and P-170 activity was observed ($P < 0.02$). All AML patients with the FAB subtype M5 were Rh123 negative ($P < 0.007$). Cytospin preparations were analyzed for staining with monoclonal antibodies JSB1 and MM4.17. Eight of 16 (50%) AML and 0/9 (0%) ALL cases expressed the multidrug resistance (MDR) protein assessed by JSB1. With MM4.17 87% of AML and 50% of ALL patients were scored positive. Agreement between both antibodies was found in only 13/23 (57%) samples. Extracted RNA from 12 patients was analyzed by RT-PCR to evaluate the expression of MDR1 and multidrug resistance-associated protein (MRP) mRNA. An increased level of MDR1 mRNA was detectable in 4/7 AML and 0/5 ALL cases. MRP expression was found in 3/7 AML and 0/5 ALL patients. Comparison of Rh123 assay and immunocytochemistry revealed a very good correlation when using MoAb JSB1 ($P < 0.004$) but not with MM4.17 (not significant (NS)). JSB1 also showed a much better association with the PCR results ($P < 0.05$) than MM4.17 (NS). Finally, we compared the results of the functional Rh123 assay and RT-PCR and observed a high correlation for Rh123/MDR1 ($r = 0.819$, $P < 0.001$) but low for Rh123/MRP ($r = 0.562$, NS). We conclude that measurement of Rh123 efflux and immunocytochemical staining of cytospin preparations with JSB1 allows the accurate monitoring of P-170 expression in acute leukemia. The simplicity of these two MDR assays suggests their use for routine MDR screening.

Keywords: multidrug resistance; acute leukemia; MDR assays

Introduction

Resistance to cytotoxic drugs is one of the major obstacles in the treatment of hematological malignancies. Different types of drug resistance have been identified. One of these is the so-called multidrug resistance (MDR), characterized by the overexpression of P-glycoprotein (P-170),[1,2] encoded by the MDR1 gene, and/or the overexpression of multidrug resistance-associated protein (MRP).[3] As we know today, both of them are transmembrane proteins that function as energy-dependent pumps, decreasing the intracellular drug concentrations.[3,4] This process is not specific for one single substance at all, but tumor cells showing the MDR phenotype are resistant against a wide range of apparently, in structure and mechanism of toxicity, unrelated anticancer drugs. Meanwhile there also exists clear data about the clinical significance of MDR,[5–7] and a number of MDR modulator trials have been initiated to estimate the value of adding them to the regular treatment protocols for the reversal of clinical resistance.[8,9]

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Detection methods and diagnosis of the MDR phenotype in clinical tumor samples have not been standardized so far. To date a number of different methods have been used:[10] reverse transcriptase polymerase chain reaction (RT-PCR), Northern blot, slot blot, *in situ* hybridization, Western blot, immunocytochemistry and flow cytometry. Quite often these studies showed differing results, even when identical blood samples were analyzed in different laboratories. This fact was discussed again at an international workshop upon this issue, asking for standardization of detection methods.[11]

What are the properties we need to demand? Of course, first of all the method has to meet both criteria of high specificity and sensitivity, especially as P-170 levels occurring *in vivo* are considered to be much lower than in cell lines. Furthermore, MDR screening techniques should discriminate between tumor cells and normal cells in clinical samples, avoiding false positive results caused by contamination of normal P-170 expressing cells (ie lymphocytes in peripheral blood).[12] As we also know, P-170 can be phosphorylated,[13] which might change its level of activation. Finally, any method used should not be too time consuming and expensive.

Therefore the aim of our study was to compare three different methods in the MDR diagnosis of acute leukemia, each of them detecting MDR expressing cells at different levels: immunocytochemistry, using the monoclonal antibodies JSB1[14] and MM 4.17[15] directed against P-glycoprotein (protein level), flow cytometry, using the fluorescent dye rhodamine 123 (Rh123) (functional level)[16] and RT-PCR, measuring the amounts of MDR1-specific mRNA and MRP-mRNA (genetic level).

Materials and methods

Patients

Twenty-nine consecutive patients with newly diagnosed acute leukemia were studied, 17 of them suffering from acute myeloid leukemia (AML) and 12 from acute lymphoblastic leukemia (ALL). The diagnosis was based on cytological and cytochemical examination of bone marrow smears according to the French–American–British (FAB) criteria.[17] Surface markers were analyzed with a panel of T, B and myeloid lineage-associated monoclonal antibodies (MoAbs). The AML patients' age ranged from 26 to 80 years, with a median of 54 years and the ALL patient's age ranged from 1 to 64 years with a median of 23 years. FAB classification of AML revealed: four M1, five M2, two M4, six M5. The immunophenotypes of ALL were: nine common-ALL, three T-ALL. Patients' characteristics and results are summarized in Table 1.

Samples from bone marrow or peripheral blood were collected in 10 ml tubes containing heparin as anticoagulant. Mononuclear cells were separated by density gradient centrifugation (Lymphoprep; Nycomed, Oslo, Norway) and washed twice in PBS. All samples were processed within 10 h. Cells for RT-PCR were frozen immediately in liquid nitrogen.

Table 1 Clinical characteristics of all patients, together with assay results

Patient No.	*Classification*	*Age*	*% Rh123-effluxing cells*	*JSB1*	*AB %*	*MM4.17*	*AB %*	*PCR/MDR1 (mdr1/β_2 -mg)*	*PCR/MRP (mrp/β_2 -mg)*
1	T-ALL	23	0	–	0	+	>75	ND	ND
2	c-ALL	64	15	–	0	+	50–75	ND	ND
3	c-ALL	42	0	–	0	–	0	ND	ND
4	c-ALL	4	0	ND		ND		ND	ND
5	T-ALL	1	0	–	0	ND		–	–
6	c-ALL	40	0	ND		ND		–	–
7	c-ALL	5	0	–	0	+	>75	ND	ND
8	c-ALL	25	0	–	0	–	0	ND	ND
9	c-ALL	4	0	–	0	–	0	–	–
10	c-ALL	52	0	–	0	+	0–25	–	–
11	c-ALL	5	0	–	0	–	0	ND	ND
12	T-ALL	7	0	ND		ND		–	–
13	AML-M5	33	0	–	0	–	0	–	–
14	AML-M2	57	67	–	0	+	50–75	ND	ND
15	AML-M1	59	70	+	50–75	+	25–50	+ (1.36)	+ (1.70)
16	AML-M5	41	0	–	0	+	>75	ND	ND
17	AML-M5	74	0	–	0	+	50–75	ND	ND
18	AML-M5	72	0	+	>75	+	>75	+ (0.24)	+ (0.69)
19	AML-M4	67	0	–	0	+	25–50	ND	ND
20	AML-M5	50	0	ND		ND		ND	ND
21	AML-M2	50	0	–	0	+	25–50	ND	ND
22	AML-M4	80	44	+	50–75	+	50–75	ND	ND
23	AML-M1	50	16	+	50–75	+	50–75	+ (0.36)	–
24	AML-M2	57	95	+	>75	+	25–50	ND	ND
25	AML-M2	62	16	+	50–75	+	>75	ND	ND
26	AML-M1	72	50	+	>75	+	>75	–	–
27	AML-M2	26	60	+	50–75	+	50–75	+ (0.99)	+ (0.15)
28	AML-M5	33	0	–	0	–	0	–	–
29	AML-M1	34	16	–	0	+	50–75	ND	ND

AB, antibody; ND, not done; +, positive; –, negative.

Rh123 efflux assay and flow cytometric analysis

The assay and analysis were performed as described before.[16,18] Aliquots of 500 μl cell suspension (1×10^6 cells) were incubated for 45 min at room temperature simultaneously with 150 ng/ml Rh123 and with phycoerythrin (PE)-labeled MoAbs (CD33/CD13/CD15/CD14 for AML; CD1/CD3/CD7/CD10/CD19 for ALL and CD34 for both entities). After two washes in ice-cold medium, cells were resuspended in Rh123-free medium containing either an MDR inhibitor (10 μmol/l verapamil) or no MDR inhibitor. The Rh123 efflux was selectively determined for the PE-labeled cells after 1 h and 2 h. Between measurements cells were kept at 37°C.

Stained cells were analyzed on a FACStar flow cytometer (Becton Dickinson, Mountain View, CA, USA) equipped with a 5 W argon-ion laser emitting at 488 nm with 250 mW power. Rh123 fluorescence was collected through a 530/30 nm and PE fluorescence through a 585/40 nm bandpass filter. Dual fluorescence studies were performed as described before. To determine the fraction of cells showing Rh123 efflux a marker was set to the left of the fluorescence peak of cells, which were coincubated with MDR inhibitors. Samples were scored positive when, without MDR modulator, a clear subpopulation of cells left of the marker was detectable after a 60 or 120 min incubation. Earlier studies using cell lines showed that the percentage of the P-170 negative KB-3-1 cells showing Rh123 efflux was always below 10% (range 0–8%) whereas the percentage of P-170 expressing KB-8-5 cells was always clearly above 10%. Interpretation of results by mean channel fluorescence intensities instead of percentage positive cells gave comparable results. Data were processed with a Consort 30 software (Becton Dickinson). All flow cytometric studies were carried out in duplicate.

Cytospin preparations and immunocytochemistry

Cells were suspended in PBS and centrifuged on object-slides, air dried for at least 2 h (maximum 24 h), then fixed in acetone for 5 min and afterwards frozen at –80°C. For immunocytochemical staining the slides were incubated with 50 μl of diluted MoAbs JSB1, directed against an internal epitope, and MM4.17, directed against an external epitope of P-170, or isotype-matched controls for 18 h at 4°C. After three washes in Tris buffer, peroxidase-conjugated anti-mouse (rabbit) and swine anti-rabbit Ig were added for 30 min at 20°C (dilution 1:10) and after washing, the slides were incubated with diaminobenzidine tetrahydrochloride (DAB) for 10 min at 20°C and washed three times. Cells were counter-stained with Mayer's hemalum solution. The MDR cell line KB-8-5 was used as a positive control, the cell line KB-3-1 as a negative control (both provided by Dr M Gottesmann, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA). Stained slides were analyzed for percentage of positive cells (0–25%, 26–50%, 51–75%, 76–100%) and for staining intensity (+, ++. +++) by two independent investigators.

Detection of MDR1 and MRP-mRNA levels by RT-PCR

Total RNA was isolated using RNAzol (Biotexs Laboratories, Houston, TX, USA) as described by the manufacturer. cDNA was synthesized with 500 ng of total cellular RNA and 5 pmol/μl hexanucleotide random primers (Boehringer Mannheim, Germany) at 37°C in a volume of 50 μl, containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 100 μg/ml bovine serum albumin, 20 mM β-mercaptoethanol, 1 mM each of deoxynucleotide, 1.25 units RNasin (Promega, Madison, WI, USA) and 120 units MMLV reverse transcriptase (Promega). Amplification of MDR1 and MRP was carried out by polymerase chain reaction using 2 μl cDNA (corresponding to 5 ng of total RNA) in a volume of 50 μl, containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM deoxynucleotides each and 3 units Taq-polymerase (Promega). β_2-microglobulin was used as a control for employing similar amounts of RNA in the experiment. The following primers were used (Microsynth, Balgach, Switzerland): MDR1 (product length 157 base pairs): 5′ CCC ATC ATT GCA ATA GCA GG 3′ (sense) and 5′ GTT CAA ACT TCT GCT CCT GA 3′ (antisense);[19] MRP (product length 296 base pairs): 5′ GGA AAC CAT CCA CGA CCC TAA TCC CT 3′ (sense) and 5′ CCA CCT CCT CAT TCG CAT CCA CCT TG 3′ (antisense); B_2-mg (product length 120 base pairs): 5′ACC CCC ACT GAA AAA GAT GA 3′ (sense) and 5′ ATC TTC AAA CCT CCA TGA TG 3′ (antisense).[19] Thirty cycles were performed, with a denaturation temperature of 94°C for 25 s, an annealing temperature of 57°C for 30 s and an extension temperature of 73°C for 1 min. Following cycle 16 the time for synthesis at 73°C was extended for 5 s per cycle. The reaction products were separated on a 10% polyacrylamide gel (BioRad, Hercules, CA, USA) and stained with ethidium bromide. Intensity of the DNA bands was determined by densitometry (Vilber-Lourmat, Marne La Vallée, France). mRNA levels were quantified as ratio MDR1/β_2-mg and MRP/β_2-mg, respectively.[20] The following cell lines served as controls: parental KB-3-1 and resistant KB-8-5 for MDR1 mRNA expression and parental HL-60 and resistant HL-60/AR for MRP mRNA expression (provided by Dr Gekeler, Byh Gulder, Konstanz, Germany).

Results

Rh123 assay (flow cytometry) = functional level

All 29 acute leukemias were examined using this assay, 10 of them showing significant Rh123 efflux (34.4%). There were evident differences between AML, with nine positive cases out of 17 (52.9%), and ALL, with one out of 12 (8.3%). The percentage of positive cells ranged from 16 to 95% (mean 48%) for AML, and was 15% in the single positive ALL. These data are in good accordance with earlier studies.[21] Examples for positive and negative assays are shown in Figure 1.

In AML we found a statistically significant correlation between Rh123 efflux and the blasts expressing CD34 ($P < 0.03$). On the other hand, analysis revealed an inverse correlation between a positive functional Rh123 assay and the expression of the CD15 surface marker by the AML blasts ($P < 0.008$). All the other markers examined in AML (CD33, CD13, CD14, CD7), did not show significant correlation.

As far as FAB classification was concerned, none of the six monocytoid differentiated AML cases (M5) showed Rh123 pumping activity ($P < 0.007$ when compared with the remaining FAB subtypes). For both groups (AML + ALL) there was no association between Rh123 efflux and leukocyte or thrombocyte count, hemoglobin level, blast infiltration of the bone marrow or lactate dehydrogenase level.

Immunocytochemistry = protein level

Twenty-five cytospin preparations were examined for staining with the monoclonal antibody JSB1, specific for a cytoplasmatic domain of P-170. Among these 25 cases there were 16 AML and nine ALL. Eight of 25 revealed positive results, representing 32%. In AML eight of 16 cases showed positive staining (50%), while in ALL none of the nine cases was scored positive. In all the positive AML cases staining was weak, with a cytoplasmatic pattern (characteristic for transmembrane proteins on cytospin preparations) and the percentage of positive cells exceeded at least 50%.

The second monoclonal antibody used in our study, MM4.17, which is directed against an external epitope of P-170, was applied to the tumor specimens of 24 patients (16 AML and eight ALL). Eighteen of 24 were scored positive for this antibody (75%), with 14 AML (87%) and four ALL (50%). Although the staining was quite strong, the pattern was not that clear cytoplasmatically, and in some cases an apparently nonspecific staining of the nuclei occurred. The percentage of MM4.17-positive cells thereby always exceeded at least 25%.

An agreement between the two monoclonal antibodies was observed in only 13/23 (57%) patients where both tests were performed.

RT-PCR = genetic level

The leukemic blasts of 12 patients have also been examined by RT-PCR, searching for the respective levels of MDR1- and MRP-mRNA. An increased level of MDR1 was found in 4/12 cases (33.3%) and for MRP in 3/12 cases (25%). For MDR1 in AML four of seven cases were positive, the levels of mRNA being 1.36/0.99/0.24/0.36 (ratio MDR1/β_2-mg). The resistant cell line KB-8-5 was used as a positive control with a MDR1/β_2-mg ratio of 1.08. All five ALL cases were negative. The three cases with elevated MRP-mRNA levels also belonged to the group of AML exclusively. Their levels were also correlated to β_2-mg (1.7/0.15/0.69). The corresponding value of the positive control HL-60/AR was 5.89.

All three MRP-positive AML cases also expressed MDR1 mRNA. Results of the PCR analysis are shown in Figure 2.

Correlation between different diagnostic methods

Comparing the flow cytometric Rh123 assay with immunocytochemistry we observed a very good correlation between MoAb JSB1 ($P < 0.004$), but not MM4.17 (NS). This trend also continued when looking at the PCR results, measuring MDR1-mRNA. The correlation to JSB1 was quite good ($P < 0.05$), compared with MM4.17 (NS). Neither antibody showed a significant correlation with MRP-mRNA expression.

Association of the results of the functional assay and PCR analysis was high for Rh123/MDR1 ($r = 0.819$, $P < 0.001$; Figure 3a) and low for Rh123/MRP ($r = 0.562$, NS; Figure 3b).

The results of all three methods used did not correlate with

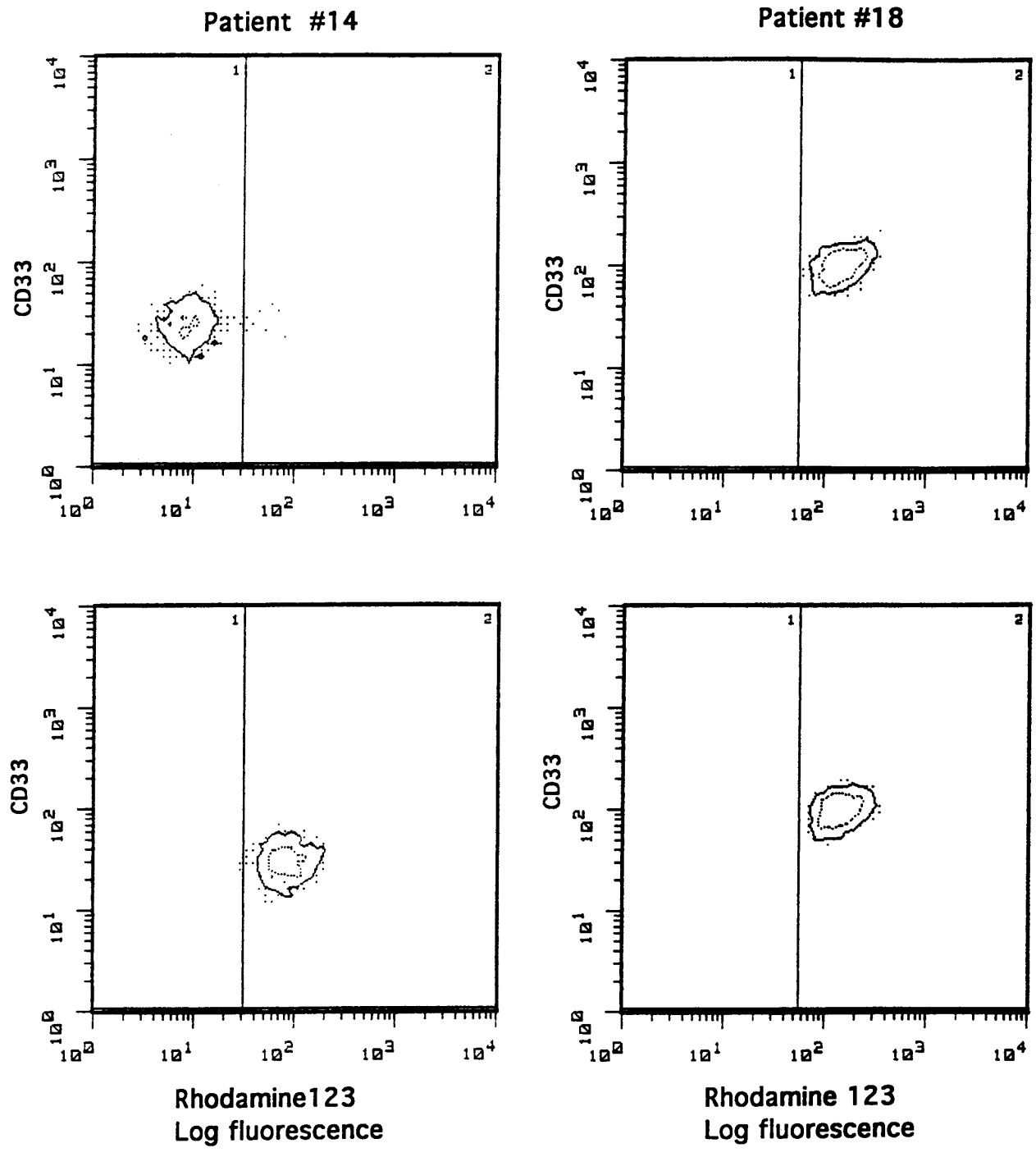

Figure 1 Two parameter contour plots of leukemia cells in a multidrug resistance-positive AML patient (patient No. 14) and a multidrug resistance-negative AML patient (patient No. 18). Cells were stained with Rh123 and CD33. Patient No. 14: loss of Rh123 is clearly evident after 60 min in Rh123-free medium (upper diagram). The cells left of the cursor show Rh123 efflux. Efflux is completely abolished in the presence of verapamil (lower diagram). Patient No. 18: no Rh123 efflux is evident after 60 min.

the following parameters: white blood cell count, thrombocytes, hemoglobin, LDH, percentage of blast cells.

One patient (No. 26) showed a rapid pumping activity of 50% of the leukemic blasts, but lacked MDR1 and MRP mRNA expression. Another interesting observation was made with patient No. 18 whose blast cells showed a high level of MRP mRNA but no pumping activity in the functional assay.

Discussion

One of the major investigational issues concerning chemoresistance is to establish the best combination of methods for the detection of the MDR phenotype in clinical samples. In the study presented here, we tried to compare three different MDR screening techniques in 29 patients suffering from acute

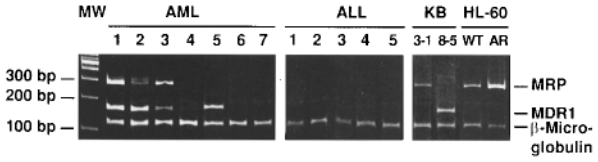

Figure 2 Analysis of MDR1, MRP and β_2-microglobulin mRNA expression in seven AML and five ALL patients determined by RT-PCR. Corresponding amplification products of the control cell lines KB-3-1, KB-8-5, HL-60 and HL-60/AR are shown.

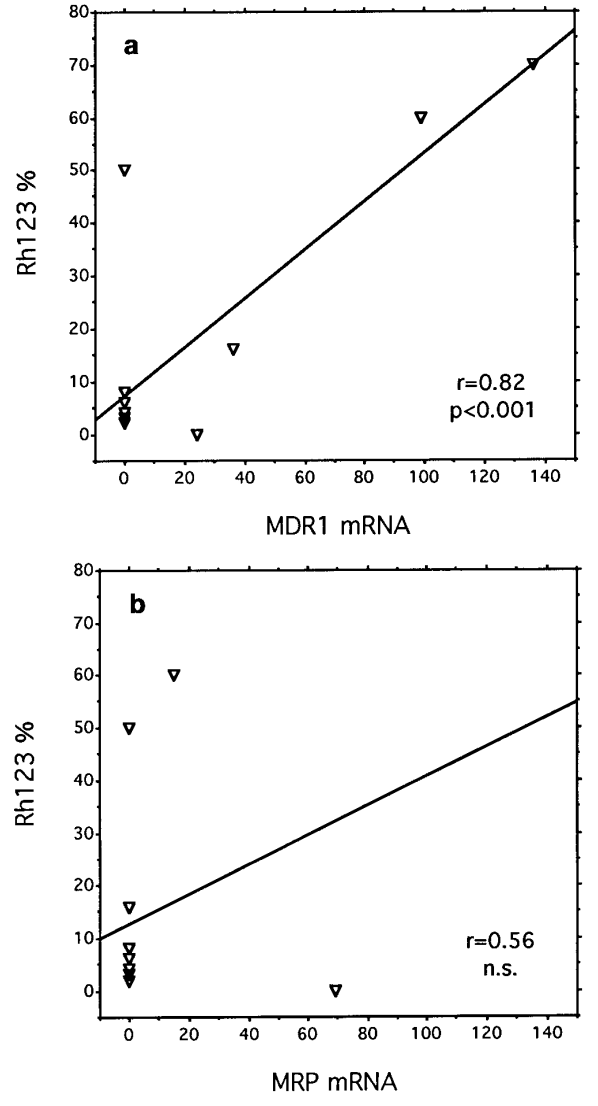

Figure 3 Statistical significant correlation is seen between MDR1 mRNA levels and the percentage of cells showing Rh123 efflux in 12 patients with acute leukemia (a). Lack of correlation is evident between MRP mRNA levels and the percentage of cells showing Rh123 efflux (b).

leukemia, and examined how far they meet the criteria demanded above (see Introduction).

Using flow cytometry, we performed a functional assay, with the fluorescent dye Rh123 acting as a substrate of P-170. We observed Rh123 efflux in 9/17 (53%) AML cases. In ALL the frequency of P-170 function was much lower with 1/12 (8%) positive cases. These results are in good accordance with currently published studies.[21,22] Co-staining of Rh123 with specific surface markers enables the discrimination of blast cells from normal blood cells. Thus, this assay meets the criterium of specificity. Additionally, even low levels of drug resistance are detectable by measuring Rh123 efflux including cells with only 1.7-fold increase in resistance.[23] Furthermore, this method offers the possibility of checking the state of activity of P-170 and not only its presence or absence. A very similar method, using the same fluorescent dye, also proved reliable for monitoring the MDR1 phenotype in leukemias, in a recently published study.[24]

An important issue of our study was to gain information as to whether the Rh123 assay is also able to recognize the function of MRP, as inconsistent data exist, whether Rh123 is one of its substrates or not.[25] Interestingly, we found that the pumping out of Rh123 did not always take the same length of time. In four of our samples we found slow pumping activity that lasted continuously for 2 h and more, while usually after an interval of 1 h the fluorescence patterns did not change further. We presumed that transport mechanisms different from P-170, like MRP, might be responsible for this phenomenon. However, our study did not reveal any significant correlation between the results of Rh123 assay and PCR for MRP-mRNA ($P < 0.48$).

Immunocytochemistry also offers the possibility of single cell analysis, and meanwhile there exists a great panel of monoclonal antibodies specific for P-170. As previously demanded by different investigators,[11,24] we used two of these antibodies directed against different epitopes, JSB1 and MM4.17, in our study. The cytospin preparations stained with JSB1 were easy to interpret, although the staining in the positive cases was quite weak (+). However, we observed a good correlation of this method with the functional test ($P < 0.004$).

As far as MM4.17 is concerned, the situation was much more difficult. The staining was considerably stronger, but it did not always seem to be limited to the plasma membrane and unspecific staining of the nuclei was hard to exclude. This fact also led to considerable disagreement in the interpretation of results by two independent investigators. Furthermore, there was no correlation between MM4.17 staining and Rh123 efflux. From this point of view we do not consider this antibody to be of value for the detection of P-170 on cytospin preparations, although our group has experienced better results applying this antibody to cryostat sections.[21] The reason for this discrepancy is not clear at present.

Finally, we also performed a RT-PCR as a third method. One major problem concerning this technique was to prevent mRNA from immediate degradation, as our clinical samples were also processed for routine diagnosis. Thus, mRNA was extractable only from 12 patients' samples. This issue may limit the use of RT-PCR for routine assessment of MDR in clinical samples. However, comparing RT-PCR with the other methods we found good correlation between the results obtained by flow cytometry and immunocytochemistry using JSB1, but not MM4.17.

Taking all these facts together, we suggest the combined use of a functional flow cytometric assay with Rh123 and a immunocytochemical staining of acetone-fixed cytospin preparations with the monoclonal antibody JSB1, as performed in our study, as a reliable and practical approach to determine P-170 expression in clinical samples. The combination of both methods not only fulfills the criteria mentioned above, but also sufficiently corresponds with the recommendations demanded by a recent workshop on this issue:[11] use of standardized MDR cell lines as positive controls to calibrate the

method, sample fixation with acetone, multiparameter analysis, immunostaining data reported as staining intensity and percentage of positive cells.

References

1 Rothenberg M, Ling V. Multidrug resistance: molecular biology and clinical relevance. *J Natl Cancer Inst* 1989; **81**: 907–910.
2 Pastan I, Gottesman M. Multiple-drug resistance in human cancer. *New Engl J Med* 1987; **316**: 1388–1393.
3 Cole S, Bhardwaj G, Gerlach J, Mackie J, Grant C, Almquist K, Stewart A, Kurz E, Duncan A, Deeley R. Overexpression of a transporter gene in a multidrug resistant human lung cancer cell line. *Science* 1992; **258**: 1650–1654.
4 Weinstein RS, Kuszak JR, Kluskens LF, Coon JS. P-glycoprotein in pathology: the multidrug resistance gene family in humans. *Hum Pathol* 1990; **21**: 34–48.
5 Pirker R, Wallner J, Geissler K, Linkesch W, Haas OA, Bettelheim P, Hopfner M, Scherrer R, Valent P, Havelec L, Ludwig H, Lechner K. MDR1 gene expression and treatment outcome in acute myeloid leukemia. *J Natl Cancer Inst* 1991; **83**: 708–712.
6 Marie JP, Faussat-Suberville AM, Zhou D, Zittoun R. Daunorubicin uptake by leukemic cells: correlation with treatment outcome and mdr1 expression. *Leukemia* 1993; **7**: 825–831.
7 Goasguen JE, Dossot JM, Fardel O, Le Mee F, Le Gall, E, Leblay R, LePrise PY, Chaperon J, Fauchet R. Expression of the multidrug resistance-associated P-glycoprotein (P-170) in 59 cases of *de novo* acute lymphoblastic leukemia: prognostic implications. *Blood* 1993; **81**: 2394–2398.
8 Sonneveld P, Durie BGM, Lokhorst HM, Marie J, Solbu G, Suciu S, Zittoun R, Löwenberg B, Nooter K. Modulation of multidrug-resistant multiple myeloma by cyclosporin. *Lancet* 1992; **340**: 255–259.
9 Miller TP, Grogan TM, Dalton WS, Spier CM, Scheper RJ, Salmon SE. P-glycoprotein expression in malignant lymphoma and reversal of clinical drug resistance with chemotherapy plus high-dose verapamil. *J Clin Oncol* 1991; **9**: 17–24.
10 Zhou DC, Marie JP, Suberville AM, Zittoun R. Relevance of mdr1 gene expression in acute myeloid leukemia and comparison of different diagnostic methods. *Leukemia* 1992; **6**: 879–886.
11 Beck WT, Grogan TM, Willmann CL, Cordon-Cardo C, Parham DM, Kuttesch JF, Andreeff M, Bates SE, Berard CW, Boyett JM, Brophy NA, Broxterman HJ, Chan HSL, Dalton WS, Dietel M, Fojo AT, Gascoyne RD, Head D, Houghton PJ, Srivastava DK, Lehnert M, Leith CP, Paietta E, Pavelic ZP, Rimza L, Roninson IB, Sikic BI, Twentyman PR, Warnke R, Weinstein R. Methods to detect P-glycoprotein-associated multidrug resistance in patients' tumors: consensus recommendations. *Cancer Res* 1996; **56**: 3010–3020.
12 Chaudhary PM, Mechetner EB, Roninson IB. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood* 1992; **80**: 2735–2739.
13 Hait WN, Aftab DT. Rational design and pre-clinical pharmacology of drugs for reversing multidrug resistance. *Biochem Pharmacol* 1992; **43**: 103–107.
14 Scheper RJ, Bulten JWM, Brakkee JGP, Quak JJ, Van der Schoot E, Balm AJM, Meijer CJL, Broxterman HJ, Kuiper CM, Lankelma J, Pinedo HM. Monoclonal antibody JSB-1 detects a highly conserved epitope on the P-glycoprotein associated with multi-drug resistance. *Int J Cancer* 1988; **42**: 389–394.
15 Cianfriglia M, Willingham MC, Tombesi M, Scagliotti GV, Frasca G, Chersi A. P-glycoprotein epitope mapping. Identification of a linear human-specific epitope in the fourth loop of the P-glycoprotein extracellular domain by MM4.17 murine monoclonal antibody to human multi-drug-resistant cells. *Int J Cancer* 1994; **56**: 153–160.
16 Ludescher C, Thaler J, Drach D, Drach J, Spitaler M, Gattringer C, Huber H, Hofmann J. Detection of activity of P-glycoprotein in human tumour samples using rhodamine 123. *Br J Haematol* 1992; **82**: 161–168.
17 Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposed revised criteria for the classification of acute myeloid leukemia. *Ann Intern Med* 1985; **103**: 620–626.
18 Ludescher C, Eisterer W, Hilbe W, Hofmann J, Thaler J. Decreased potency of MDR-modulators under serum conditions determined by a functional assay. *Br J Haematol* 1995; **91**: 652–657.
19 Noonan KE, Beck C, Holzmayer TA, Chin JE, Wunder JS, Andrulis IL, Gazdar AF, Willman CL, Griffith B, Von Hoff DD, Roninson IB. Quantitative analysis of MDR1 (multidrug resistance) gene expression in human tumors by polymerase chain reaction. *Proc Natl Acad Sci USA* 1990; **87**: 7160–7164.
20 Ludescher C, Hilbe W, Eisterer W, Preuss E, Huber C, Gotwald M, Hofmann J, Thaler J. Activity of P-glycoprotein in B-cell chronic lymphocytic leukemia determined by a flow cytometric assay. *J Natl Cancer Inst* 1993; **85**: 1751–1758.
21 Ludescher C, Eisterer W, Hilbe W, Gotwald M, Hofmann J, Zabernigg A, Cianfriglia M, Thaler J. Low frequency of activity of P-glycoprotein (P-170) in acute lymphoblastic leukemia compared to acute myeloid leukemia. *Leukemia* 1995; **9**: 350–356.
22 Ivy SP, Olshefski RS, Taylor BJ, Patel KM, Reaman GH. Correlation of P-glycoprotein expression and function in childhood acute leukemia: a children's cancer group study. *Blood* 1996; **88**: 309–318.
23 Webb M, Raphael CL, Asbahr H, Erber WN, Meyer BF. The detection of rhodamine 123 efflux at low levels of drug resistance. *Br J Haematol* 1996; **93**: 650–655.
24 Broxterman HJ, Sonneveld P, Feller N, Ossenkoppele GJ, Währer DCR, Eekman CA, Schoester M, Lankelma J, Pinedo HM, Löwenberg B, Schuurhuis GJ. Quality control of multidrug resistance assays in adult acute leukemia: correlation between assays for P-glycoprotein expression and activity. *Blood* 1996; **87**: 4809–4816.
25 Feller N, Kuiper CM, Lankelma J, Ruhdal JK, Scheper RJ, Pinedo HM, Broxterman HJ. Functional detection of MDR1/P170 and MRP/190-mediated multidrug resistance in tumour cells by flow cytometry. *Br J Cancer* 1995; **72**: 543–549.