Jpn. J. Cancer Res. 86, 607–615, June 1995

New Flow Cytometric Method for Detection of Minimally Expressed Multidrug Resistance P-Glycoprotein on Normal and Acute Leukemia Cells Using Biotinylated MRK16 and Streptavidin-RED670 Conjugate

Akihiro Takeshita,[1] Kaori Shinjo, Kazunori Ohnishi and Ryuzo Ohno

The Division of Hematology, Department of Medicine III, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-31

To evaluate the expression of multidrug resistance (MDR) on normal and leukemia cells, we examined P-glycoprotein (P-gp) by a newly devised flow cytometric method, utilizing a biotinylated monoclonal antibody (mAb) against P-gp (MRK16), a streptavidin-RED670 conjugate (SA-RED670) and appropriate emission filters. The combination of biotinylated MRK16 (b-MRK16) and SA-RED670 resulted in higher sensitivity as compared with standard methods such as the use of streptavidin-phycoerythrin (SA-PE) conjugate. The sensitivity was examined in K562, K562/ADR, NOMO-1, NOMO-1/ADR and HL60 cells, and compared with the data obtained from reverse transcription polymerase chain reaction (RT-PCR) of mdr-1 gene. P-gp positivity on flow cytometry was 10.4%, 99.9%, 1.4%, 90.4% and 0%, respectively. Mdr-1 mRNA was well expressed in K562/ADR and NOMO-1/ADR cells, but not in NOMO-1 and HL60 cells. In K562 cells, mdr-1 was found after 40 cycles of PCR, but not 25 cycles. These data are well correlated with those from the flow cytometry. We then studied the P-gp expression on normal peripheral blood cells and acute leukemia cells. P-gp was little expressed on peripheral lymphocytes, monocytes and granulocytes. It was also little expressed on blast cells from 5 patients with acute promyelocytic leukemia (APL) at diagnosis, ranging from 0.2 to 10.6% (4.6±3.9%). Ten other acute myeloid leukemia (AML) and 5 acute lymphocytic leukemia (ALL) expressed P-gp at diagnosis, ranging from 8.5% to 34.5% (16.9± 11.8%) and from 2.3% to 45.6% (24.0±17.8%), respectively. All 9 relapsed or refractory cases expressed P-gp, ranging from 21.1% to 99.8% (52.2±29.9%). Significant differences were found in APL, CD34-positive and relapse and refractory cases ($P=0.0006$, 0.0007 and 0.0088, respectively). These results indicate that this flow cytometric analysis is useful for the evaluation of clinical MDR status and can identify a group of patients with resistant leukemia.

Key words: P-glycoprotein — Multi-drug resistance (MDR) — Leukemia — MRK16 — Flow cytometry

Resistance to multiple chemotherapeutic drugs is one of the main reasons for treatment failure in cancer chemotherapy.[1–3] The most extensively studied type of multidrug resistance (MDR) is associated with overproduction of a 170-kd to 180-kd membrane glycoprotein (P-gp), which is encoded by mdr-1 gene located on chromosome 7.[4] Overexpression of P-gp is related with lower intracellular drug accumulation, mediated through increased membrane efflux of the drugs. P-gp is associated with clinical resistance to chemotherapy, and has been detected on resistant or refractory tumor cells. MDR is induced by treatment with anthracyclines and vinca alkaloid in hematological malignancies.[5–8] In some studies, expression of P-gp was a negative prognostic factor for response,[9] and was associated with the presence of an immature acute myeloid leukemia (AML) phenotype.[10, 11]

In untreated *de novo* AML, the expression of P-gp has been detected in 27% to 71% of patients.[10–13] This variation seems to arise mainly from the detection methods of P-gp and from diverse phenotypic differences of leukemia blast cells. There have been 3 main methods of analysis; flow cytometry using an anti P-gp monoclonal antibody (mAb) such as MRK16, C219, C494, UIC2 and JSB1,[4, 14–18] analysis of mdr-1 mRNA by reverse transcription polymerase chain reaction (RT-PCR),[19] and functional staining with anthracyclines or mitochondrial fluorescent dyes such as rhodamine-123.[20, 21] Both the standard flow cytometry and the staining method have relatively low sensitivity and can not detect minimal expression of P-gp, while RT-PCR has higher sensitivity, but has an intrinsic disadvantage for quantification, especially in heterogeneous populations of cells.

Thus, a more specific and sensitive method is needed for the detection of P-gp in a specified population of hematopoietic cells. Moreover, we need to overcome nonspecific binding of mAb, when we analyze a small amount of P-gp on cell membranes. Some investigators have studied P-gp expression by improved methods using biotinylated mAb, Fab or indirect immunofluores-

[1] To whom correspondence should be addressed.

cence,[7, 22, 23] but satisfactory sensitivity and specificity for P-gp have not been obtained.

We have developed a new technique in flow cytometry, using a combination of biotinylated MRK16 and a streptavidin-RED670 (SA-RED670; Gibco BRL, Grand Island, NY) conjugate, based on our recently developed technique of non-isotopic quantitative flow cytometric analysis of receptors for granulocyte-colony stimulating factor using SA-RED670. We not only solved the above-mentioned problems, but also succeeded in reducing the non-specific binding of MRK16, the most sensitive antibody to P-gp.[18] Using this highly sensitive method, we analyzed P-gp expression on normal hematopoietic cells and leukemia cells.

MATERIALS AND METHODS

Cell samples Cells used in this study were human peripheral blood (PB) cells, human bone marrow (BM) cells, the human chronic myelogeneous leukemia cell line K562, Adriamycin-resistant K562 (K562/ADR) (kindly provided by Dr. T. Tsuruo, University of Tokyo, Tokyo),[24] the human promonocytic leukemia cell line NOMO-1, NOMO-1/ADR (kindly provided by Dr. M. Ogura, Aichi Cancer Institute, Nagoya, and Dr. M. Tanimoto, Nagoya University, Nagoya)[25] and human promyelocytic leukemia HL60 cells (kindly provided by Dr. M. Tanimoto, Nagoya University, Nagoya). K562/ADR and NOMO-1/ADR are MDR cell lines established from K562 and NOMO-1, respectively.[24, 25] ADR (4×10^{-7} *M*) was added every 2 weeks to the culture medium for MDR cell lines.

Samples from PB and BM were collected in heparinized tubes from normal volunteers and from patients with acute leukemia after informed consent had been obtained. Mononuclear cells (MNC) were isolated by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/ml; Pharmacia, Uppsala, Sweden). Cells were mixed with a hemolysing solution (0.826% NH_4Cl, 0.1% $KHCO_3$, 0.0037% EDTA-2Na, pH 7.3) for 10 min at 37°C. After hemolysis, cells were washed twice with phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS; Gibco BRL), and suspended in PBS containing 2% FCS and 0.1% NaN_3. Leukemia cell samples which contained more than 90% leukemia cells were used for surface marker analysis and P-gp evaluation.

Biotinylation of MRK-16 and a control mouse monoclonal antibody A half ml of 0.1 *M* $NaHCO_3$ was added to 1 ml of 2.5 mg/ml MRK16 stock solution (kindly provided by Dr. T. Tsuruo, University of Tokyo, Tokyo),[14, 26] and dialyzed for 24 h. Then NHS-LC-biotin (Pierce, Rockford, IL) was added, and the mixture was stirred at 37°C for 4 h. Then the solution was dialyzed in PBS containing 0.1% NaN_3 to remove biotin which had not reacted with MRK16. A mouse mAb (DAKO, Denmark) with the same immunoglobulin (Ig) subclass as MRK16 (IgG2a) was biotinylated in the same way, and used as a negative control (b-IgG2a). The Ig titer examination of biotin-labeled MRK16 (b-MRK16) and b-IgG2a was performed by means of enzyme-linked immunosorbent assay (ELISA). First, the wells of the ELISA plate (Becton Dickinson, Lincoln Park, NJ) were coated with 50 μl of rabbit anti-mouse IgG (50 μg/ml) (MBL, Nagoya) to block non-specific binding. Then 50 μl of biotinylated mAb was added. After washing 3 times, 50 μl of peroxidase-conjugated avidin (Gibco BRL) was added. Then 50 μl of 0.1% *o*-phenylenediamine (Sigma, St Louis, MO) solution contaning 0.02% H_2O_2 was added. The reaction was stopped by adding 50 μl of 2*M* H_2SO_4 and A_{490nm} was measured with a microplate photometer (Corona Electronic Corp., Tokyo). The assay revealed that these monoclonal antibodies were biotinylated similarly. The Ig titers of the two mAbs were examined similarly by ELISA using peroxidase-conjugated rabbit anti-mouse IgG, and were made equal for subsequent flow cytometric assay. Negative b-IgG2a was routinely used in our assay system because the Ig subclass IgG2a generally shows a higher tendency to bind non-specifically of Fc receptors on cell membranes.

Blocking of unsaturated Fc receptors by human immunoglobulin preparation to reduce non-specific binding We first tested the blocking effect of a 0.5% solution of a human immunoglobulin preparation, Polyglobin N (Miles, West Haven, NJ), on the non-specific binding of b-IgG2a and the SA-RED670 conjugate to K562/ADR cells, and compared the fluorescence intensity (FI) of the b-IgG2a-SA-RED670-reacted cells between samples with and without the blocking solution treatment.

Then we directly monitored unsaturated Fc receptors by using FITC-conjugated CD16 (Coulter, Hialeah, CA), CD32 (Pharmingen, San Diego, CA) and CD64 mAb (Pharmingen), all of which are specific to Fc receptor. Unsaturated Fc receptors on K562/ADR cells were blocked with the blocking solution. Then the cells were incubated with negative IgG2a or MRK16, and thereafter reacted with FITC-conjugated CD16, CD32 and CD64 mAb. FI was compared between the two groups.

Flow cytometric determination of P-gp expression Washed cells were incubated in a 0.5% solution of Polyglobin N at room temperature for 1 h in order to block non-specific binding. All supplements were heat-inactivated at 56°C for 30 min. Fifty μl of the cell suspension in the blocking solution (1×10^6 cells/ml) was incubated with 2.5 μl (1 μg) of b-MRK16 at 0°C for 30 min. A subclass-matched mAb (b-IgG2a) was routinely used as a negative control. After washing, the cell pellet was resuspended and incubated with 4 μl of SA-RED670 conju-

gate at 0°C for 30 min. Next, the cells were washed and resuspended in PBS containing 2% FCS for flow cytometric assay. Ten thousand events were counted using an Epics Elite flow cytometer (Coulter). An individual target cell population was gated using light-scattering parameters. The fluorochromes were excited by a 488 nm laser beam. The main filter to detect P-gp was a 630 nm long pass. Adoption of the biotin-avidin reaction, an energy transfer fluorochrome (i.e., SA-RED670) and a suitable emission filter in this assay system afforded high sensitivity. The filter used detects a broad range of red wavelength (over 630 nm), and covers most of the SA-RED670 emission curve. Data analysis was performed using Epics Cytometer software (Coulter). The viability of the cells after these treatments was more than 99.9% by the dye exclusion test with trypan blue.

In one experiment, we compared the FI of K562/ADR cells reacted with b-MRK16-SA-RED670 or with b-MRK16-SA-phycoerythrin (PE). The latter is a standard fluorochrome conjugate which reacts with biotin-labeled mAb.

Data analysis on flow cytometry Data were collected for 10,000 events gated on the basis of light-scattering properties. The data were analyzed on the Epics Elite. Cells were identified using the light-scattering display, and gated into lymphocytes, monocytes, granulocytes or leukemia blast cells. Then, the gated cells were analyzed for the expression of P-gp.

The degree of dissociation between the two populations was calculated with the Immuno-4 routine of the Epics Cytometer software.[27] Statistical analysis of P-gp expression among populations was performed by using Student's paired t statistics.

RNA PCR Total cellular RNA was isolated according to the acid-guanidium-phenol-chloroform technique. cDNA was synthesized with a cDNA synthesis kit (Gibco BRL) following the manufacturer's instructions. PCR was performed using cDNA synthesized from 150 ng of RNA, 10 pmol of mdr-1 specific primer, 1.5 unit of Taq-polymerase, 5 μl of 10×PCR buffer (Roche Diagnostic Systems, Branchburg, NJ) and 0.5 μl of 25 mmol dNTP in a final volume of 50 μl. β2-Microglobulin

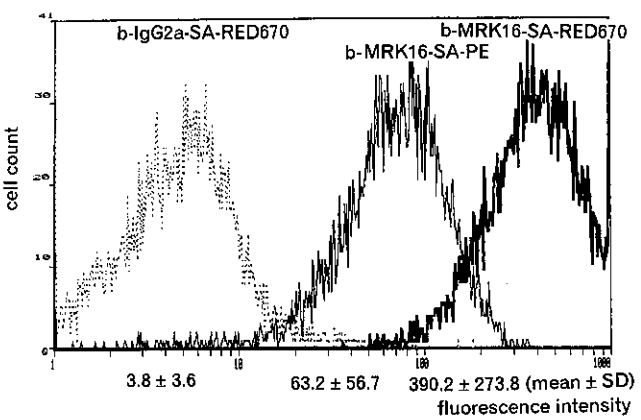

Fig. 1. Comparison of sensitivity to detect P-gp on K562/ADR cells stained with b-MRK16 and SA-RED670 or with b-MRK16 and SA-PE. K562/ADR cells (5×10^6/ml, 50 μl) were incubated with 5 μl of b-MRK16, then stained with 1 μg of SA-RED670 or SA-PE, and analyzed with the flow cytometer. Dark and light solid lines show the results from b-MRK16-SA-RED670-reacted cells and b-MRK16-SA-PE-reacted cells, respectively. Dotted lines show the result from a sample incubated with the b-IgG2a and SA-RED670 conjugate. The mean fluorescence intensity (FI) is represented at the bottom of the figure. Although not shown in the figure, FI for the b-IgG2a-SA-PE-reacted cells was 4.3±4.3.

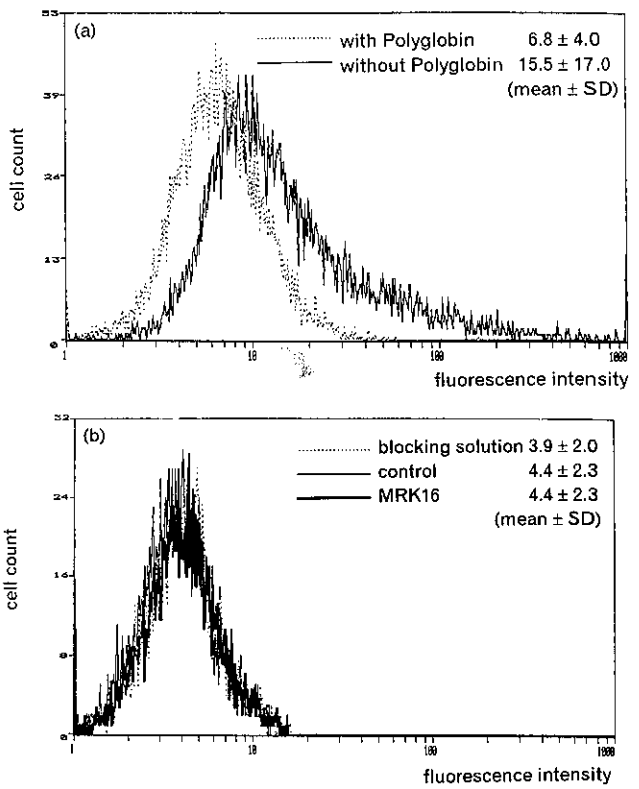

Fig. 2. (a) The difference of fluorescence intensity (FI) of samples preincubated with (dotted lines) or without (solid lines) the Polyglobin N blocking solution in K562/ADR cells stained with the b-IgG2a and SA-RED670 conjugate. (b) FI of FITC-conjugated CD16 on K562/ADR cells after pretreatment with the different antibodies. Light and dark solid lines indicate FI of cells treated with control IgG2a and MRK16, respectively. Dotted lines indicate FI of cells preincubated only with the blocking solution. No significant difference was observed among the three curves.

was used as a control. Each cycle contained a denaturation step at 94°C for 1 min, an annealing step at 63°C for 1 min, and an elongation step at 72°C for 2 min. A total of 40 cycles was performed, using an automatic PCR processor (Perkin Elmer Cetus, Norwalk, CT). Ten μl aliquots of the PCR products were separated electrophoretically through a 3% agarose mixture, consisting of 1% Agarose S (Nippon Gene, Tokyo) and 2% NaSieve GTG agarose (FMC Bioproducts, Rockland, ME). All experiments were performed in duplicate.

ESULTS

Difference between two fluorochromes, SA-RED670 and SA-PE, for the detection of P-gp Firstly, we compared the sensitivity of our newly developed method using

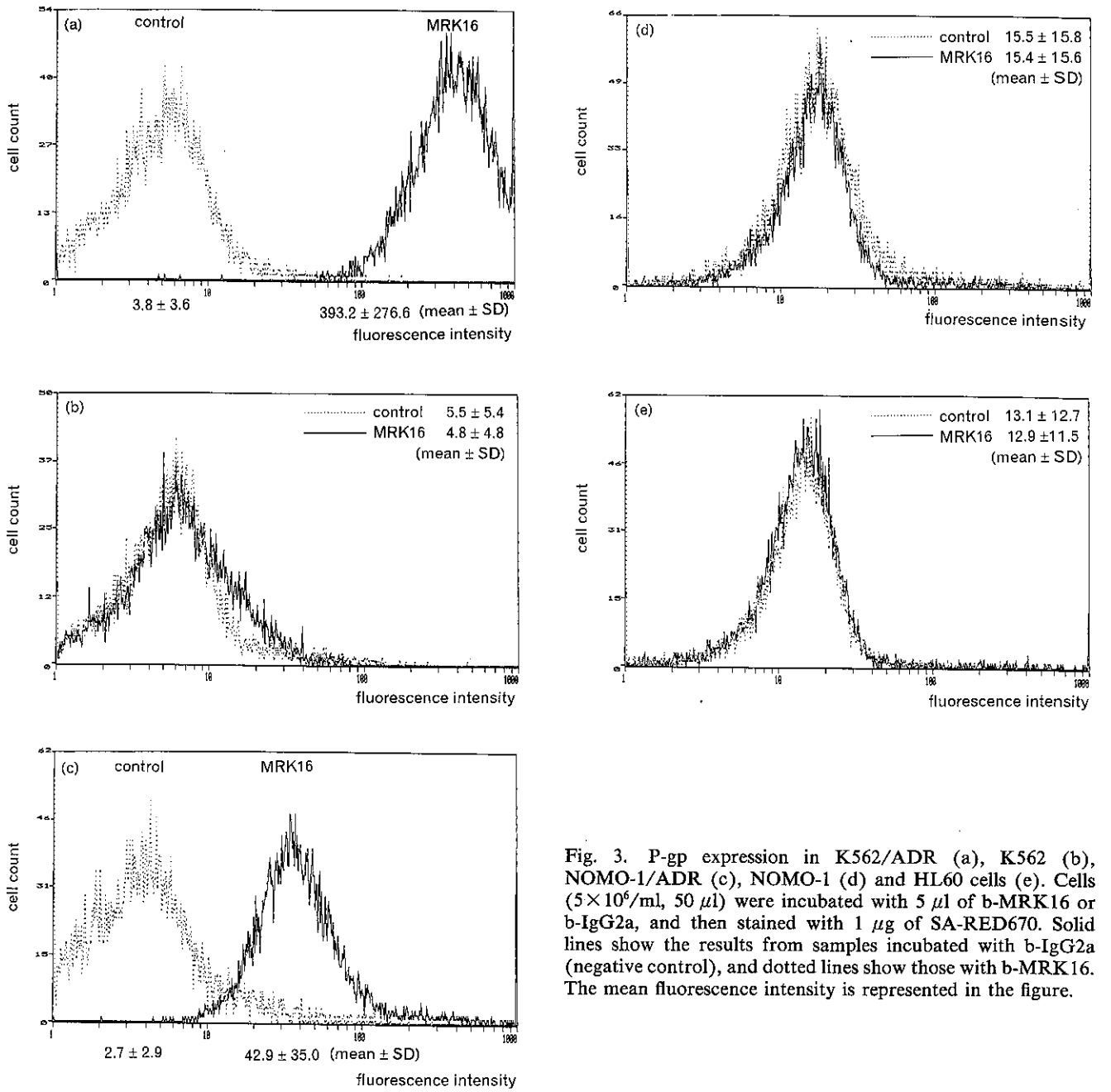

Fig. 3. P-gp expression in K562/ADR (a), K562 (b), NOMO-1/ADR (c), NOMO-1 (d) and HL60 cells (e). Cells (5×10^6/ml, 50 μl) were incubated with 5 μl of b-MRK16 or b-IgG2a, and then stained with 1 μg of SA-RED670. Solid lines show the results from samples incubated with b-IgG2a (negative control), and dotted lines show those with b-MRK16. The mean fluorescence intensity is represented in the figure.

b-MRK16 and the SA-RED670 conjugate with that of the conventional method using b-MRK16 and the SA-PE conjugate for the detection of P-gp on K562/ADR cells (Fig. 1). The degree of P-gp expression was represented by FI. Values of FI for the b-MRK16-SA-RED670-reacted cells, the b-MRK16-SA-PE-reacted cells and the b-IgG2a-SA-RED670-reacted cells were 390.2±273.8 (mean±SD), 63.2±56.7 and 3.8±3.6, respectively. Although not shown in Fig. 1, FI for the b-IgG2a-SA-PE-reacted cells was 4.3±4.3. The greatest ($P<0.001$) shift of the fluorescence profile was seen in the SA-RED670 assay. We concluded that the sensitivity of our method using the SA-RED670 conjugate is higher than that of the conventional method using the SA-PE conjugate.

Blocking of unsaturated Fc receptors by human immunoglobulin preparation to reduce non-specific binding Next, we tested the blocking effect of a 0.5% solution of Polyglobin N on the non-specific binding of b-IgG2a and the SA-RED670 conjugate to K562/ADR cells. As shown in Fig. 2a, K562/ADR cells which were not incubated with this blocking solution showed considerable FI (15.5±17.0), while the cells which were incubated with the blocking solution revealed minimal FI (6.8±4.0) ($P<0.001$). The result showed that the blocking procedure was necessary, and that the b-IgG2a-SA-RED670-reacted cells gave minimal FI after the blocking procedure. Therefore, all further experiments were performed after this blocking procedure, and with the b-IgG2a and SA-RED670 conjugate as a negative control.

Thirdly, we directly tested the existence of Fc receptors on K562/ADR cells with FITC-conjugated CD16, CD32 and CD64 mAb. With and without the blocking procedure, the FI values of FITC-CD16, CD32, and CD64 mAb-reacted cells were 4.2±2.3 vs. 6.0±4.1, 37.1±25.9 vs. 75.3±53.8 and 5.7±2.9 vs. 5.9±3.1, respectively. We directly monitored remaining unsaturated Fc receptors in K562/ADR cells, which had been incubated with control IgG2a or MRK16 by using FITC-conjugated CD16, CD32, and CD64 mAb. The FI values were 4.4±2.3 vs. 4.4±2.3 (Fig. 2b), 39.2±27.9 vs. 37.1±25.9 and 5.8±3.1 vs. 5.7±2.9, respectively. The result indicated that the non-specific binding, mainly induced by Fc receptors, was blocked equally well. These data suggested that unsaturated Fc receptors would hardly influence our assay system.

Detection of P-gp on K562, K562/ADR, NOMO-, NOMO-1/ADR and HL60 cells Fig. 3a shows FI profiles of P-gp expression on K562/ADR cells. The FI value of the b-MRK16-SA-RED670-reacted cells was 393.2±276.6, while that of the b-IgG2a-SA-RED670-reacted cells was 3.8±3.6. From these results, the P-gp positivity of K562/ADR cells was calculated to be 99.9% by the Immuno-4 program. Fig. 3b shows the FI profiles of K562 cells. The FI value of the b-MRK16-SA-RED670-reacted cells was 5.5±5.4, while that of the b-IgG2a-SA-RED670-reacted cells was 4.8±4.8, and the calculated P-gp positivity was 10.4%.

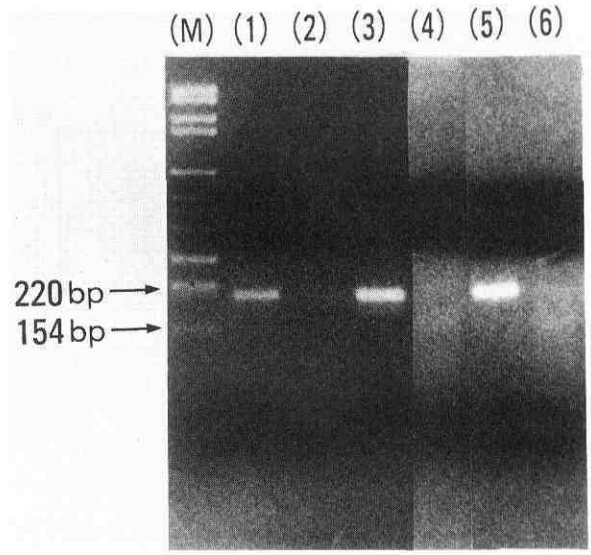

Fig. 4. Mdr-1 gene expression by RT-PCR in K562, K562/ADR, NOMO-1, NOMO-1/ADR and HL60. The results from a size marker (M), K562 (40 cycles of PCR) (1), K562 (25 cycles of PCR) (2), K562/ADR (3), NOMO-1 (4), NOMO-1/ADR (5) and HL60 (6) are shown. Mdr-1 gene was expressed in K562/ADR, and NOMO-1/ADR, and minimally in K562 (expressed at 40 cycles of PCR, but not at 25 cycles), but not in NOMO-1 and HL60 cells.

Fig. 3c shows the FI profiles on NOMO-1/ADR cells. The FI value of the b-MRK16-SA-RED670-reacted cells was 42.9±35.0, while that of the b-IgG2a-SA-RED670-reacted cells was 2.7±2.9, and the calculated P-gp positivity was 90.4%. Fig. 3d shows the FI profiles on NOMO-1 cells. The FI value of the b-MRK16-SA-RED-670-reacted cells was 15.4±15.6, while that of the b-IgG-2a-SA-RED670-reacted cells was 15.5±15.8, and the calculated P-gp positivity was 1.4%.

Fig. 3e shows the FI profiles on HL60 cells. The FI value of the b-MRK16-SA-RED670-reacted cells and that of the b-IgG2a-SA-RED670-reacted cells were 12.9±11.5 and 13.1±12.7, respectively, and the calculated P-gp positivity was 0%.

RT-PCR detection of mdr-1 in K562, K562/ADR, NOMO-1, NOMO-1/ADR and HL60 On RT-PCR, mdr-1 mRNA was overexpressed in K562/ADR and NOMO-1/ADR cells. In K562 cells, mdr-1 was minimally expressed at a total of 40 cycles of PCR, and was not expressed at 25 cycles. In NOMO-1 and HL60 cells, it was not expressed (Fig. 4). The data are well correlated with the data from flow cytometry using the b-MRK16 and RED-670 conjugate.

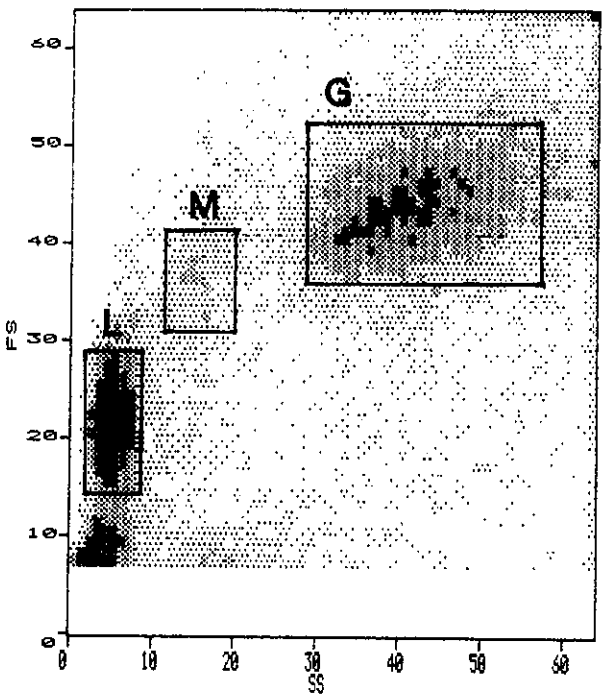

Fig. 5. Cells were identified using light-scattering parameters, and gated into lymphocytes (L), monocytes (M) and granulocytes (G). The gated cells were analyzed for the expression of P-gp as shown in Table I.

Table I. P-gp Positivity in Lymphocytes, Monocytes and Granulocytes from 5 Normal Volunteers

Volunteer	MRK16 positivity (%) in		
	Lymphocytes	Monocytes	Granulocytes
1	0.2	0.3	0.5
2	0.5	0.7	0.3
3	1.5	2.1	1.1
4	3.1	2.8	0.6
5	4.3	2.5	2.3
Total (mean±SD)	1.9±1.6	1.7±1.1	1.0±0.8

P-gp positivity was calculated with the Immuno-4 program on the Epics Cytometer software. No significant difference was observed among the positivity of these three populations.

Detection of P-gp on peripheral blood cells from normal volunteers To characterize P-gp expression on peripheral blood cells from 5 normal volunteers, we studied the FI of lymphocytes, monocytes and granulocytes which were reacted with the b-MRK16 and SA-RED670 conjugate. Lymphocytes, monocytes and granulocytes were identified and gated with suitable light-scattering parameters (Fig. 5). P-gp was little expressed on these cells (Table I).

Detection of P-gp on blast cells of patients with acute leukemia To characterize P-gp expression on blast cells from patients with acute leukemia, we studied P-gp positivities of blast cells from 29 patients which were reacted with the b-MRK16 and SA-RED670 conjugate (Table II). The results of the statistical analysis are summarized in Table III. P-gp was little expressed on blast cells from 5 patients with acute promyelocytic leukemia (APL) at diagnosis, ranging from 0.2 to 10.6% (4.6±3.9%). Ten other AML and 5 acute lymphocytic leukemia (ALL) expressed P-gp at diagnosis, ranging from 8.5% to 34.5% (16.9±11.8%) and from 2.3% to 45.6% (24.0±17.8%), respectively. All 9 relapsed or refractory cases expressed P-gp, ranging from 21.1% to 99.8% (52.2±29.9%). Significance was found in APL, CD34-positive phenotype, and relapse and refractory cases ($P=0.0006$, 0.0007 and 0.0088, respectively).

DISCUSSION

Many investigators have attempted to detect P-gp with several mAbs such as MRK16, C219, C494, UIC2 and JSB1.[14-18] MRK16 is one of the most specific mAbs for P-gp, and has the advantage of being able to be used without fixation.[4,28] However, non-specific binding of MRK16 to Fc receptors hampers the analysis of the minimally expressed P-gp. To overcome this problem, several techniques including biotinylation or fractionation of the mAb have been tried.[10,29] However, the results have not necessarily accorded with those obtained by other methods, such as RT-PCR.[13] In this study, we succeeded in reducing non-specific binding. Non-specific binding was not only reduced by blocking Fc receptor with Polyglobin, but also could be compensated by using a non-MRK16 mAb of IgG2a subclass as a negative control. The successful reduction of non-specific binding was confirmed by the data on the P-gp expression on normal peripheral monocytes, which generally possess abundant Fc receptors. Monocytes expressed little P-gp in the present analysis. In our assay, whole immunoglobulins were biotinylated because fractionated immunoglobulins, Fab for example, were not biotinylated sufficiently. An indirect immuno-fluorescence method was not adopted because it generally has too high nonspecific binding to allow analysis of the minimally expressed P-gp.

The b-MRK16 and SA-RED670 conjugate resulted in higher sensitivity and enabled multicolor analysis. SA-RED670 is an energy-transfer fluorochrome consisting of the dye Cy5 which is covalently conjugated to R-phycoerythrin (R-PE). Light energy absorbed at 488 nm by R-PE is transferred to the Cy5 dye and emitted at 670 nm. The loss of R-PE emission energy at 575 nm during the transfer is very small, and the emission ratio of 575

Table II. P-gp Positivity in Leukemia Blast Cells of Acute Leukemia

Case	FAB classification	Stage of disease	CD expression	MRK16 positivity (%)
1	M1	at diagnosis	CD13, CD33, CD34	31.5
2	M1	at diagnosis	CD13, CD33	20.6
3	M2	at diagnosis	CD13, CD33	15.6
4	M2	at diagnosis	CD13, CD33	13.2
5	M2	at diagnosis	CD33, CD34	25.7
6	M2	at diagnosis	CD13, CD33	16.7
7	M2	at diagnosis	CD33, CD34	29.0
8	M3	at diagnosis	CD13, CD33	4.2
9	M3	at diagnosis	CD13, CD33	2.5
10	M3	at diagnosis	CD13, CD33	5.8
11	M3	at diagnosis	CD13, CD33	0.2
12	M3	at diagnosis	CD13, CD33	10.6
13	M4	at diagnosis	CD13, CD14, CD33	8.5
14	M6	at diagnosis	CD13, CD33, CD34	34.7
15	M6	at diagnosis	CD13, CD13, CD34	13.8
16	L1	at diagnosis	CD5, CD7	36.9
17	L1	at diagnosis	CD10, CD19, HLA-DR	23.9
18	L2	at diagnosis	CD19, CD20, HLA-DR	11.2
19	L2	at diagnosis	CD10, CD19, HLA-DR	2.3
20	L2	at diagnosis	CD10, HLA-DR	45.6
21	M1	relapse	CD13, CD33, CD34	21.1
22	M2	relapse	CD13, CD33, CD34	76.5
23	M2	relapse	CD33, CD34	23.6
24	M2	relapse	CD13, CD33	46.9
25	M3	relapse	CD13, CD33	24.2
26	L2	relapse	CD5, CD7, HLA-DR	53.9
27	L2	relapse	CD10, CD34, HLA-DR	34.5
28	L3	relapse	CD5, CD7	89.9
29	L2	refractory	CD5, CD7, CD34, HLA-DR	99.8

P-gp positivity was calculated with the Immuno-4 program on the Epics Cytometer software. Leukemia blast cells were collected at diagnosis in patients No. 1–20, at relapse in No. 21–28 and at the refractory stage in No. 29.

Table III. Summary of the Statistical Analysis of P-gp Positivity in Leukemia Blast Cells

	P-gp positivity (mean±SD)	Statistical significance
APL vs. other type of leukemia	4.6±3.9 vs. 21.9±11.9	0.0006[a)]
CD34 positive vs. negative	26.9±8.1 vs. 9.8±6.7	0.0007[a)]
AML vs. ALL	16.9±11.8 vs. 24.0±17.8	0.3120[a)]
at diagnosis vs. relapse and refractory	17.6±12.9 vs. 52.2±29.9	0.0088

a) Calculated at diagnosis.

nm/670 nm is less than 10%. Because the peak of emission of RED670 is more than 100 nm from those of FITC, PE and the excitation beam, the background of scattered light could be reduced. Therefore, the background fluorescence level could be reduced. Moreover, using a 630 nm long-pass filter, we could detect almost all the emission spectrum of SA-RED670. With the combination of the fluorochrome and the 630 nm long-pass filter, the conjugate enables us to detect a minimal difference between the fluorescence profiles of a sample and its negative control.

By the present improved flow-cytometry method, minimally expressed P-gp could be measured clearly, directly and easily. Further, by adequate gating according to light-scattering parameters, a highly purified population of blast cells was easily obtained in our assay. The finding that contamination of mdr-1 cells at a rate as low as 1% results in mdr-1 positivity in the PCR technique is

noteworthy.[29] In our assay, we can avoid overdiagnosis arising from contamination with other cell populations.

Our reliable determination of P-gp expression on normal peripheral blood cells has important implications for MDR studies. In leukemia patients' clinical samples, which are generally mixtures of normal and leukemia cells, positive results may possibly be attributable to contaminating normal cells. In fact, since many published studies were not performed on pure leukemia cells, some authors have warned that the results have to be interpreted with caution.[29] The possibility of error would be further reduced by the combined use of the b-MRK16 and SA-RED670 conjugate and multi-color analysis using other mAbs which recognize a certain cell phenotype or maturation stage of normal and leukemia cells.

In AML, P-gp overexpression was reported in patients with refractory or relapsed disease, as well as in some patients at diagnosis and in some high-risk patients in complete remission.[8, 9, 14] P-gp was little expressed on blast cells from all 5 patients with APL in our study, ranging from 0.2% to 10.6%. It was reported that APL was frequently negative for P-gp,[10] and our data are compatible with that report. This is probably because APL cells express little CD34 antigen. We further showed that CD34 had an intimate association with P-gp ($P=0.0007$). Campos *et al.* reported that P-gp overexpression in AML was restricted to the $CD34^+$ cell population and was related to drug resistance.[10] They further reported that both P-gp and CD34 expression were unfavorable prognostic factors in AML patients. te Boekhorst *et al.* found that mdr-1 expression was associated with reduced intracellular daunorubicin retention in AML with $CD34^+$ phenotype, but not in AML with $CD34^-$ phenotype.[11] Our data clarify these findings. Ten other AML expressed P-gp ranging from 8.5% to 34.7%, and 5 ALL expressed it ranging from 2.3% to 56.9% at diagnosis. No significant difference was observed in P-gp expression of blast cells between AML and ALL. The P-gp expression was clearly increased in 9 relapsed or refractory cases tested ($P=0.0088$).

Our results demonstrate that this flow cytometric analysis of P-gp is reliable for evaluating clinical MDR status, and identifies a group of patients with resistant leukemia. Phenotypic analysis has generally been performed at diagnosis of leukemia, but P-gp expression should also be adopted as a routine diagnostic procedure for leukemia in the future. We believe that our rapid and reproducible flow cytometric method will be suitable for this purpose.

ACKNOWLEDGMENTS

We thank Dr. Takashi Tsuruo, Institute of Applied Microbiology, University of Tokyo for providing MRK16, Hiroko Hasegawa for her laboratory assistance and Kiyoshi Shibata, Equipment Center, Hamamatsu University School of Medicine for his cooperation.

(Received December 19, 1994/Accepted March 9, 1995)

REFERENCES

1) Holmes, J., Jacobs, A., Carter, C., Wieczorek, A. J. and Padua, R. A. Multidrug resistance in haematopoietic cell lines, myelodysplastic syndrome and acute myeloblastic leukemia. *Br. J. Haematol.*, **72**, 40–44 (1989).
2) Musto, P., Melillo, L., Lombardi, G., Matera, R., Giorogio, C. and Carotenuto, M. High risk of early resistant relapse for leukemic patients with presence of multidrug resistance associated P-glycoprotein positive cells in complete remission. *Br. J. Haematol.*, **77**, 50–53 (1991).
3) List, A. F. and Spier, C. M. Multidrug resistance in acute leukemia: a conserved physiologic function. *Leuk. Lymphoma*, **8**, 9–14 (1992).
4) Hamada, H. and Tsuruo, T. Functional role for the 170- to 180-kDa glycoprotein specific to drug-resistant tumor cells as revealed by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA*, **83**, 7785–7789 (1986).
5) Grogan, T., Dalton, W., Rybski, J., Spier, C., Meltzer, P., Richter, L., Gleason, M., Pindur, J., Cline, A., Scheper, R., Tsuruo, T. and Salmon, T. Optimization of immunocytochemical P-glycoprotein assessment in multidrug-resistant plasma cell myeloma using three antibodies. *Lab. Invest.*, **63**, 815–824 (1990).
6) Michieli, M., Raspadoli, D., Damiani, D., Geromin, A., Gallizia, C., Michelutti, A., Fanin, R., Fasola, G., Russo, D., Tazzari, P., Pileri, S., Mallardi, F. and Baccarani, M. The expression of the multidrug resistance-associated glycoprotein in B-cell chronic lymphocytic leukemia. *Br. J. Haematol.*, **77**, 460–465 (1991).
7) List, A. F., Spier, C. M., Cline, A., Doll, D. C., Carewal, H., Morgan, R. and Sandberg, A. A. Expression of the multidrug resistance gene product (P-glycoprotein) in myelodysplasia is associated with a stem cell phenotype. *Br. J. Haematol.*, **78**, 28–34 (1991).
8) Pilarski, L. M. and Belch, A. R. Circulating monoclonal B cells expressing P glycoprotein may be a reservoir of multidrug-resistant disease in multiple myeloma. *Blood*, **83**, 724–736 (1994).
9) Michieli, M., Damiani, D., Geromin, A., Michelutti, A., Fanin, R., Raspadori, D., Russo, D., Visani, G., Dinota, A., Pileri, S., Tsuruo, T., Grandi, M., Baccarani, M. and Tura, S. Overexpression of multidrug resistance-associated p170-glycoprotein in acute non-lymphocytic leukemia. *Eur. J. Haematol.*, **48**, 87–92 (1992).

10) Campos, L., Guyotat, D., Archimbaud, E., Oriol, P. C., 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).
11) te Boekhorst, P. A. W., de Leeuw, K., Schoester, M., Wittebol, S., Nooter, K., Hagemeijer, A., Löenberg, B. and Sonneveld, P. Predominance of functional multidrug resistance (MDR-1) phenotype in CD 34^+ acute myeloid leukemia cells. *Blood,* **82,** 3157–3162 (1993).
12) Pirker, R., Wallner, J., Geissler, K., Linkesch, J., Haas, O. A., Bettelheim, P., Hopfner, M., Scherrer, R., Valent, P., Havelec, L., Ludwig, H. and Lechner, K. MDR1 gene expression and treatment outcome in acute myeloid leukemia. *J. Natl. Cancer Inst.,* **83,** 708–712 (1991).
13) 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).
14) Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S. and Mori, S. Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK16. *Cancer Res.,* **48,** 1926–1929 (1988).
15) Kuwazuru, Y., Yoshimura, A., Hanada, S., Utsunomia, A., Makino, T., Ishibashi, K., Kodama, M., Iwashashi, M., Arima, T. and Akiyama, S. Expression of the multidrug transporter, P-glycoprotein, in acute leukemia cells and correlation to clinical drug resistance. *Cancer,* **66,** 868–873 (1990).
16) Georges, E., Bradrey, G., Gariepy, J. and Ling, V. Detection of P-glycoprotein isoforms by gene-specific monoclonal antibodies. *Proc. Natl. Acad. Sci. USA,* **87,** 152–156 (1990).
17) Hart, S. M., Ganeshaguru, K., Lyttelton, M. P. A., Prentice, H. G., Hoffbrand, A. V. and Mehta, A. B. Flow cytometric assessment of multidrug resistance (MDR) phenotype in acute myeloid leukemia. *Leuk. Lymphoma,* **11** (Suppl. 1), 239–248 (1993).
18) van Acker, K. L., van Hove, L. M. and Boogaerts, M. A. Evaluation of flow cytometry for multidrug resistance detection in low resistance K562 cells using daunorubicin and monoclonal antibodies. *Cytometry,* **14,** 736–746 (1993).
19) Marie, J. P., Zittoun, R. and Sikic, B. I. Multidrug resistance (mdr1) gene expression in adult acute leukemias: correlation with treatment outcome and *in vitro* drug sensitivity. *Blood,* **78,** 586–592 (1991).
20) Marie, J. P., Faussat-Suberville, A. M., Zhou, D. and Zittoun, R. Daunorubicin uptake by leukemic cells: correlation with treatment outcome and mdr1 expression. *Leukemia,* **7,** 825–831 (1993).
21) Ludescher, C., Thaler, J., Drach, D., Spitaler, M., Gatteringer, C., Huber, H. and Hofmann, J. Detection of activity of P-gycoprotein in human tumor samples using rhodamin 123. *Br. J. Haematol.,* **82,** 161–168 (1992).
22) Chaudhary, P. M., Mechetner, E. B. and Robinson I. B. Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood,* **80,** 2735–2739 (1992).
23) Klimecki, W. T., Futscher, B. W., Grogan, T. M. and Dalton, W. S. P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood,* **83,** 2451–2458 (1994).
24) Tsuruo, T., Saito, H., Kawabata, H., Ohhara, T., Hamada, H. and Utakoji, T. Characteristics of resistance to Adriamycin in human myelogeneous leukemia K562 resistant to Adriamycin and in isolated clones. *Jpn. J. Cancer Res.,* **77,** 682–692 (1986).
25) Hamaguchi, M., Morishita, Y., Takahashi, I., Mitinori, O., Takamatsu, J. and Saito, H. FDP D-dimer induces the secretion of interleukin-1, urokinase-type plasminogen activator, and plasminogen activator inhibitor-2 in human promonocytic leukemia cell line. *Blood,* **77,** 94–100 (1991).
26) Ishida, Y., Ohtsu, T., Hamada, H., Sugimoto, Y., Tobinai, K., Minato, K., Tsuruo, T. and Shimoyama, M. Multidrug resistance in cultured human leukemia and lymphoma cell lines detected by a monoclonal antibody, MRK16. *Jpn. J. Cancer Res.,* **80,** 1006–1013 (1989).
27) Overton, W. R. Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry,* **9,** 619–626 (1988).
28) Georges, E., Tsuruo, T. and Ling, V. Topology of P-glycoprotein as determined by epitope mapping of MRK-16 monoclonal antibody. *J. Biol. Chem.,* **268,** 1792–1798 (1993).
29) Drach, D., Zhao, S., Drach, J., Mahadevia, R., Gattringer, C., Huber, H. and Andreeff, M. Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood,* **80,** 2729–2734 (1992).