Subpopulations of Normal Peripheral Blood and Bone Marrow Cells Express a Functional Multidrug Resistant Phenotype

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The multidrug-resistance gene, *MDR*1 is expressed in many normal tissues, but little is known about its expression in normal hematopoietic cells. Using the monoclonal antibody C219 and flow cytometric analysis, P-glycoprotein (P-gp) was found to be expressed in all peripheral blood (PB) subpopulations (CD4, CD8, CD14, CD19, CD56) except granulocytes. To specifically determine *MDR*1 gene expression, these PB subpopulations were isolated by fluorescence-activated cell sorting (FACS) and analyzed for *MDR*1 mRNA by polymerase chain reaction (PCR). All subsets were positive by PCR, but only minimal *MDR*1 mRNA was detected in monocytes and granulocytes. Significant efflux of Rhodamine-123 (Rh-123), a measure of P-gp function, was detected in $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, and $CD56^+$ cells but not in granulocytes. Next, PCR-analysis was performed on FACS-sorted bone marrow (BM) cells to assess *MDR*1 expression in different maturational stages. Precursors ($CD34^+$), early and late myeloid cells ($CD33^+/CD34^+$, $CD33^+/CD34^-$) as well as lymphocytes of the B-cell lineage ($CD19^+/CD10^+$, $CD19^+/CD10^-$) expressed the *MDR*1 gene. BM monocytic cells ($CD33^{++}/CD34^-$) were negative, and a very weak signal was detected in erythroid cells (glycophorin A^+). Significant Rh-123 efflux was found in $CD34^+$, $CD10^+$, $CD33^+$, and $CD33^{++}$ BM cells, but not in glycophorin A^+ cells. We conclude that PB and BM lymphocytes, PB monocytes, BM progenitors, and immature myeloid cells, but not late BM monocytes, erythroid cells, and PB granulocytes, express *MDR*1 mRNA and a functional P-gp. These results have to be taken into account when *MDR*1 expression is determined in tumor samples containing normal blood cells.

CELLULAR RESISTANCE to antineoplastic drugs represents a major problem in clinical oncology. One type of resistance, induced by structurally unrelated drugs, such as vinca alkaloids, anthracyclins, and epipodophyllotoxins, is termed multidrug resistance (*MDR*). *MDR* has been related to overexpression of the *MDR*1 gene and production of a 170 to 180 Kd membrane-associated P-glycoprotein (P-gp). P-gp is believed to function as an energy-dependent drug efflux pump for various lipophilic xenobiotics, resulting in reduced intracellular accumulation.

P-gp is not only expressed by malignant cells but has been also identified in normal tissues with excretory function, such as the proximal tubules of the kidney, and in liver biliary ducts.[1] P-gp was also detected in endothelial cells of the blood-brain barrier.[2] With regard to hematopoiesis, little is known about *MDR*1 gene expression in normal bone marrow (BM) and peripheral blood (PB) cells. Although low expression of *MDR*1 mRNA has been described in blood and BM samples,[3] P-gp expression could not be detected in these cells by immunohistochemical assays.[1,4] Expression and activity of P-gp has been shown in BM progenitor cells[5] and recently, *MDR*1 mRNA could be detected by in situ mRNA hybridization in a subset of cells with myeloid and lymphoid morphology, respectively.[6] Andreeff and Hegewisch detected expression of P-gp in peripheral blood cells with the monoclonal antibody (MoAb) HYB241 and a sensitive statistical technique (the Kolmogorov-Smirnov test) to analyze flow cytometric data (unpublished observation, March 1988). Therefore, we investigated the expression of *MDR*1 mRNA and of P-gp in normal blood and BM cells to define its distribution in different hematopoietic lineages and maturational stages and investigated the functional activity of P-gp by means of rhodamine-123 (Rh-123) efflux in these PB and BM subpopulations.

MATERIALS AND METHODS

Cells and cell lines. Normal PB and BM from healthy individuals and PB from two leukemia patients (acute myelogenous leukemia [AML] at diagnosis, common acute lymphoblastic leukemia [cALL] at relapse) were obtained after informed consent. Mononuclear cells and granulocytes were separated by density gradient centrifugation over Ficoll Hypaque (density 1.077 g/mL; Organon Teknika Corp, Durham, NC; and density 1.119 g/mL; Sigma, St Louis, MO, respectively), washed twice with phosphate-buffered saline (PBS) and resuspended in PBS at 1×10^7 cells/mL.

The multiple myeloma parent cell line 8226/S and its doxorubicin-resistant mutants 8226/DOX6 and 8226/DOX40 were kindly provided by Dr W. S. Dalton (University of Arizona, Tucson).[7] All cells were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% penicillin, 1% streptomycin, and 1% L-glutamine (all from GIBCO-BRL, Gaithersburg, MD). Cell cultures were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere. Using the MoAb HYB-241 it was recently shown that different amounts of P-gp were expressed in DOX-6 and DOX-40 cells, which were inversely related to the uptake of daunorubicin.[8]

Antibodies and immunofluorescence. The following MoAbs were used: Leu-4/CD3, Leu-3/CD4, Leu-M3/CD14, Leu-M1/CD15, Leu-12/CD19, Leu-M9/CD33, Leu-19/CD56, goat-anti mouse Ig (Becton Dickinson, San Jose, CA), J5/CD10, MY-4/CD14 (Coulter Immunology, Hialeah, FL), IOM-34/CD34, Glycophorin-A

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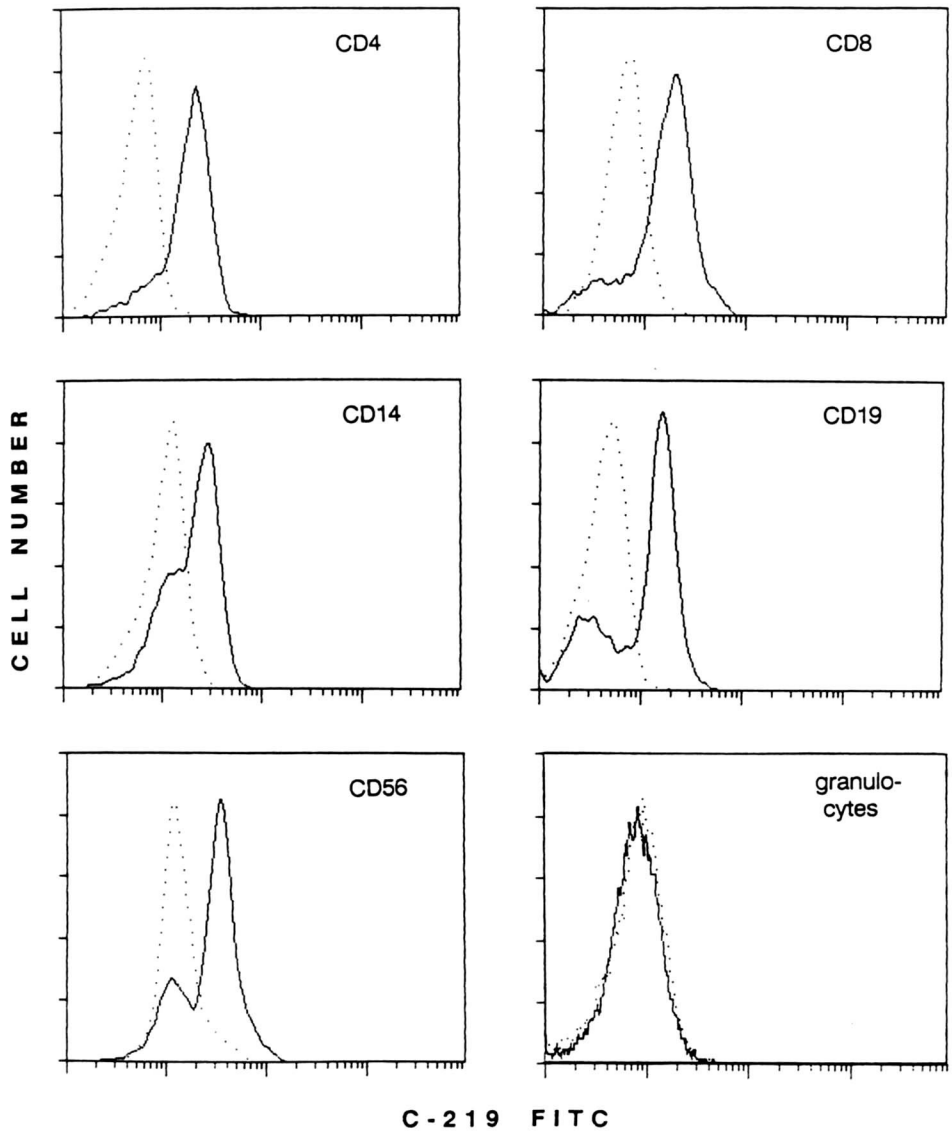

Fig 1. P-glycoprotein expression in normal peripheral blood subpopulations. Lymphocytes and monocytes were stained with MoAbs after Ficoll-Hypaque separation, granulocytes were isolated by density gradient centrifugation. All cell populations were subsequently stained with MoAb C-219 as described in Materials and Methods. C-219 fluorescence is shown for each cell population gated on the respective surface marker. (····) isotypic control; (—) C-219.

(AMAC, Westbrook, ME), and OKT-8/CD8 (Ortho Diagnostic System, Raritan, NJ). All MoAbs were used at saturation concentrations following the manufacturers' recommendations. Incubation was performed for 30 minutes on ice, followed by two washes with PBS. Isotypic control antibodies (Becton Dickinson) were used in all experiments.

C-219 MoAb against P-gp was obtained from Centocor Diagnostics (Malvern, PA). A previously published staining protocol was used[9] and slightly modified for double-staining of surface markers and P-gp. Briefly, after staining of the surface antigen, cells were fixed in paraformaldehyde (0.8% in PBS) at 4°C for 2 minutes, permeabilized with methanol 70% at −20°C for 2 minutes, and incubated with C-219 (2.5 μg/mL) for 60 minutes.

Rhodamine efflux studies. Efflux of Rh-123 (Sigma) was studied as previously described[5] with minor modifications. Briefly, for double labeling with surface markers and Rh-123, cells were stained with MoAb against the surface antigen as described above and incubated with 200 ng/mL Rh-123 in 1 mL of media for 15

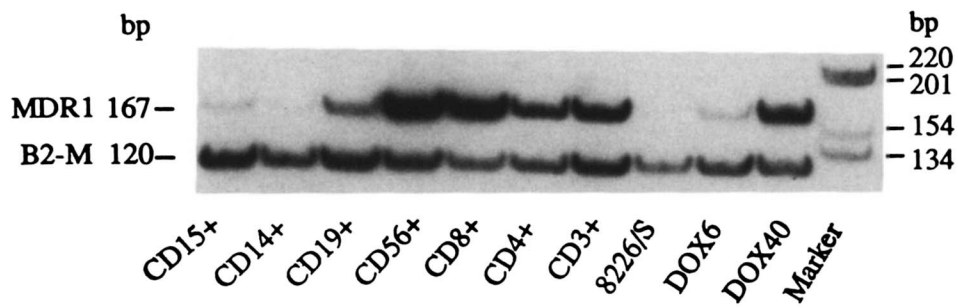

Fig 2. Analysis of *MDR*1 in normal peripheral blood cells. FACS-sorted lineage-specific subpopulations of cells were analyzed for *MDR*1 expression using reverse transcription PCR. Sensitive (8226/S) and MDR cell lines (8226/DOX-6, 8226/DOX-40) were used as controls.

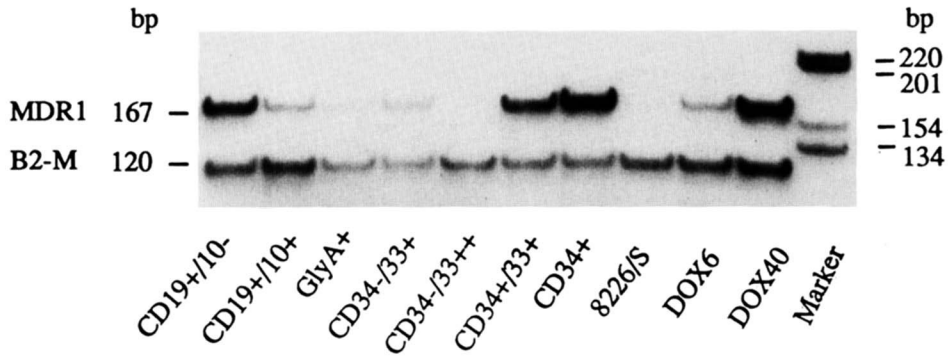

Fig 3. Analysis of *MDR*1 in normal bone marrow cells. FACS-sorted lineage- and stage-specific subpopulations of cells were analyzed for *MDR*1 expression using PCR. Sensitive (8226/S) and multidrug-resistant cell lines (8226/DOX-6, 8226/DOX-40) were used as controls.

minutes at 37°C, washed twice in ice cold RPMI-1640 medium, and then transfered into dye-free medium with or without 10 μg/mL verapamil and incubated at 37°C for 90 minutes.

Statistical analysis. Rh-123 efflux data were statistically analyzed using Student's *t*-test for independent samples. Within each subpopulation, the percent decrease of mean fluorescence with and without verapamil was compared. Flow cytometric measurements were quantitated using the Kolmogorov-Smirnov analysis.[10]

Flow cytometry. Flow cytometric analysis and cell sorting was conducted with a FACStar PLUS flow cytometer (Becton Dickinson), equipped with an argon ion laser (Spectra Physics, Mountain View, CA) operated at 488 nm and 300 mW. Green (FITC, Rh-123) and red (phycoerythrin) fluorescence was detected using 530/30 nm and 585/40 nm bandpass filters, respectively. Spectral overlap between green (FITC, Rh-123) and red (PE) signals was electronically compensated. Data acquisition and analysis was performed with the FACStar PLUS Research software (Becton Dickinson). Forward and side scatter signals were collected using linear scales, and fluorescence signals were collected on logarithmic scales. Cells displaying more fluorescence than their controls were considered positive. Cell sorting was performed using the Normal R sorting mode. All cells were kept on ice during the sorting procedure. An aliquot of sorted cells was reanalyzed for purity.

RNA preparation and polymerase chain reaction (PCR). Total cellular RNA was isolated according to the acid-guanidinium-phenol-chloroform technique.[11] cDNA was synthesized with a cDNA synthesis kit obtained from Boehringer Mannheim Corp (Indianapolis, IN) following the manufacturer's instructions. PCR was performed using cDNA synthesized aliquots from 50 ng of RNA, 150 ng of each *MDR*1-specific primer, 1.5 U of Taq-Polymerase, and 5 μL of 10 × PCR buffer (both from Boehringer Mannheim Corp), 0.5 μL of 25 mmol dNTP and 3 μCi of ^{32}P-dCTP (Du Pont, Wilmington, DE) in a final volume of 25 μL. *MDR*1-specific primers were kindly provided by Dr I.B. Roninson and used as previously described.[5] β_2-Microglobulin was used as control as previously described.[5] Each cycle contained a denaturation step at 94°C for 1 minute, an annealing step at 63°C for 1 minute, and an elongation step at 72°C for 2 minutes. A total of 32 cycles was performed followed by a final elongation step at 72°C for 10 minutes, using an automatic PCR processor (Perkin Elmer Cetus, Norwalk, CT).

Ten-microliter aliquots of the PCR products were separated electrophoretically through a 6% acrylamide gel (BIO-RAD, Richmond, CA) for 2 hours and subsequently exposed overnight to a Kodak X-Omat film (Eastman Kodak, Rochester, NY) at −70°C without intensified screen. ^{32}P incorporation was quantitated by radioactivity measurements using Betascope 603 (Betagen, Waltham, MA).

All experiments were performed in duplicate from 3 to 7 PB and BM samples.

RESULTS

Expression of P-gp is detectable by MoAbs when mononuclear PB cells are analyzed. To determine which normal cells express the protein, double staining with lineage-specific surface antibodies and C-219 (an MoAb that recognizes a cytoplasmic epitope of P-gp[12]) was performed (Fig 1). Kolmogorov-Smirnov analysis showed D-values (mean ± SD) of 0.48 ± 0.30 for CD4$^+$ cells, 0.62 ± 0.13 for CD8$^+$ cells, 0.64 ± 0.30 for monocytes, 0.00 ± 0.00 for

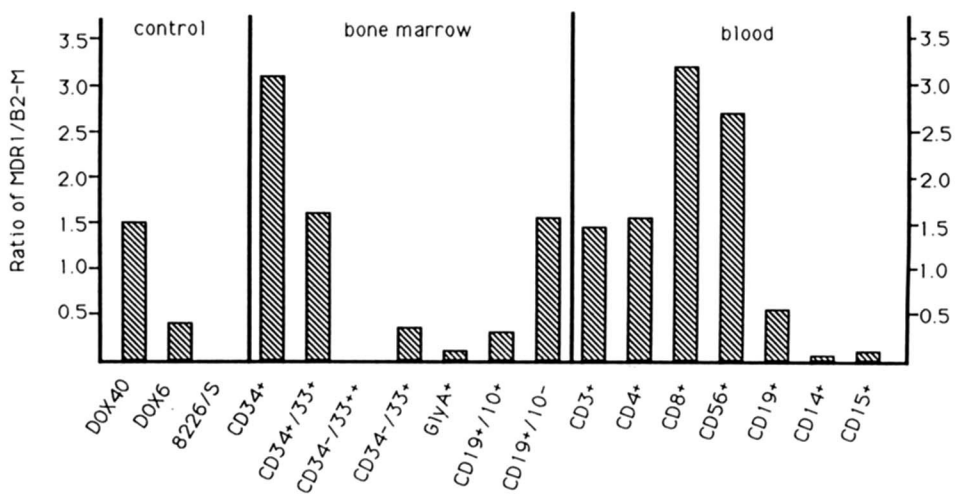

Fig 4. Ratio of *MDR*1 to beta$_2$-microglobulin (B2-M) expression in normal PB and BM cells. ^{32}P was incorporated during PCR of FACS-sorted lineage- and stage-specific subpopulations and quantified using Betascope-603. Sensitive (8226/S) and multidrug-resistant cell lines (8226/DOX-6, 8226/DOX-40) were used as controls.

granulocytes, 0.48 ± 0.07 for B cells, and 0.48 ± 0.10 for natural killer (NK) cells. Because C-219 is known to detect not only the *MDR*1 but also the *MDR*3 gene product,[13] we subsequently analyzed *MDR*1 mRNA.

Analysis of normal unseparated PB and BM cells for *MDR*1 mRNA was positive (data not shown). PB cells from three healthy volunteers were stained with lineage-specific MoAbs and subsequently sorted by FACS. Staining was performed with antibodies for pan–T-cells (CD3), major T-cell subsets (CD4, CD8), NK cells (CD56), B cells (CD19), monocytes (CD14) and granulocytes (CD15). Subpopulations ranged from 3.8% to 55%, and the purity after sorting was 98.3% ± 0.98% (median ± SD). Immediately after sorting, RNA was extracted and analyzed for *MDR*1 mRNA by PCR amplification using *MDR*1-specific primers.[5] A sensitive (8226/S) and two multidrug-resistant (8226/DOX-6 and 8226/DOX-40) cell lines were included as controls for the PCR technique. *MDR*1 expression was undetectable in 8226/S cells and elevated in 8226/DOX-6 and 8226/DOX-40 cell lines. Results of the PCR study are shown in Fig 2 for PB and in Fig 3 for BM cells. Strong bands for *MDR*1 mRNA were detected in NK cells, B and T cells. T-cell subsets were studied in seven normal individuals;in all cases, $CD8^+$ cells appeared to have higher levels of *MDR*1 expression than $CD4^+$ cells. This was confirmed by quantitative ^{32}P measurements of *MDR*1 and β_2-microglobulin using the Betascope: For $CD8^+$ cells, the ratio was significantly higher than for $CD4^+$ cells (Fig 4). Monocytes and granulocytes expressed *MDR*1 mRNA at very low levels.

To investigate *MDR*1 expression during hematopoietic differentiation, the following BM subsets were studied in three normal individuals: $CD34^+$ (progenitor cells), $CD33^+/CD34^+$ (myeloid progenitors), $CD33^+/CD34^-$ (myeloid cells), $CD33^{++}/CD34^-$ (monocytic cells), $CD10^+/CD19^+$ (early B cells), $CD10^-/CD19^+$ (mature B cells) and glycophorin A^+ cells (erythroid precursors). BM subpopulations comprising 2.8% to 39.2% of unsorted BM were enriched to 96.1% ± 3.1% (median ± SD) by the sorting procedure. As shown in Figs 3 and 4, $CD34^+$ cells were positive for *MDR*1; within this population, myeloid-committed progenitors ($CD33^+/CD34^+$) expressed *MDR*1, whereas only a faint band was detected in CD34-negative myeloid cells. Monocytic cells at a more differentiated stage did not show any *MDR*1 expression. In B cells, *MDR*1 was detectable in both early and late maturational stages. Erythroid cells expressed *MDR*1 only at extremely low levels. This could be attributable to the 4.4% contamination with remaining glycophorin A negative cells after the sorting procedure. A dilution experiment with 8226/S and 8226/DOX-40 cells, which have an *MDR*1/β_2-microglobulin ratio comparable to T cells (Fig 4), was performed to test whether a low percentage of *MDR*1-expressing cells could cause positivity in this PCR-assay. Even 1% 8226/DOX-40 cells admixed to 8226/S cells resulted in detectable *MDR*1 expression using the same conditions as in the analysis of the PB and BM cells (data not shown).

Finally it is of importance to show that P-gp is not only present but also functionally active in normal cells. Efflux of Rh-123 was applied as a functional test using 8226/S and 8226/DOX-6 cell lines as negative and positive controls (Fig 5A). PB and BM cells were double stained with surface MoAb and Rh-123 and efflux was measured in the presence and absence of verapamil. In PB, significant efflux was seen in all lymphocyte subpopulations, which was most prominent in NK cells. In monocytes and granulocytes, a slight decrease of Rh-123 mean fluorescence was found after 90 minutes, but compared with the controls without verapamil, this decrease was significant only in $CD14^+$ cells at a level of $P < .05$. In BM, $CD34^+$ cells effluxed the dye as did $CD10^+$, $CD33^+$, and $CD33^{++}$, but to a lesser extent. In glycophorin A^+ cells, the efflux was not significantly different from its control ($P > .05$).

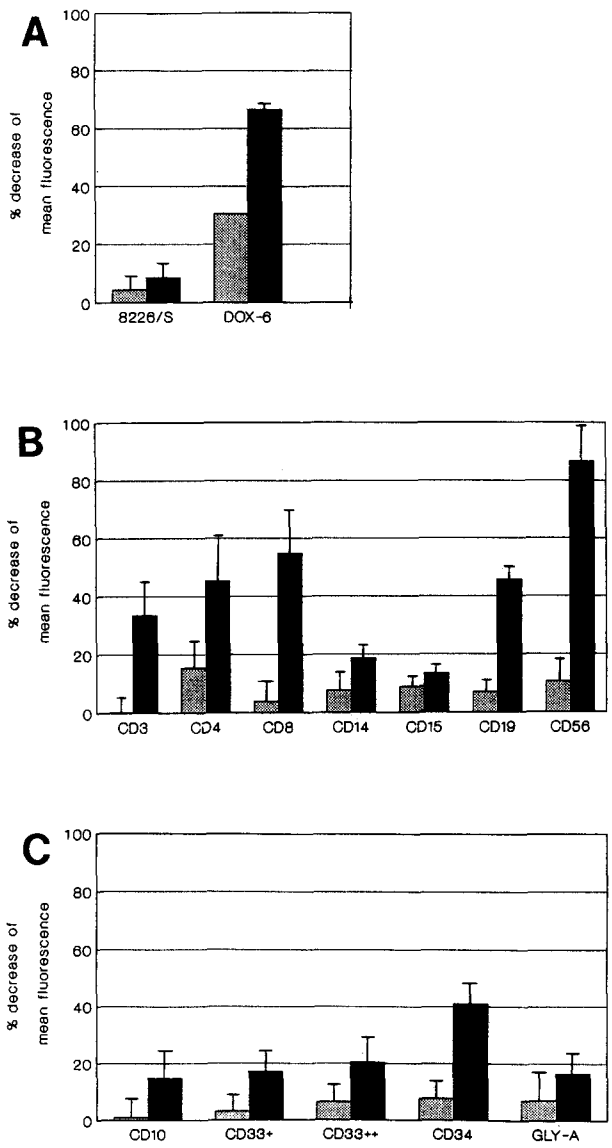

Fig 5. (A) Rh-123 efflux in control cell lines and (B) normal peripheral blood and (C) bone marrow cells. Cells were double stained with surface markers and fluorescence intensity for Rhodamine was measured on gated subpopulations at timepoint 0 and after 90 minutes. Results are given as percent decrease of mean fluorescence in the presence (open bars) or absence (black bars) of verapamil.

To test whether residual normal cells could influence the assessment of *MDR*1 expression in patient samples, we analyzed sorted blast cells and normal T cells from two leukemic patients for *MDR*1 expression (newly diagnosed AML, relapsed cALL). In both samples, not only blast cells but also T cells were shown to be positive (Fig 6).

DISCUSSION

This study investigates the expression of P-gp and *MDR*1 mRNA in blood and marrow cells from normal individuals. P-gp was found to be expressed and functionally active in all PB subpopulations except in granulocytes. Following reverse transcription PCR, a band for *MDR*1 mRNA was detected in all PB-subpopulations. Both expression and function appeared to be highest in NK cells and $CD8^+$ T cells, as determined by ^{32}P-incorporation during PCR and by Rh-123 efflux measurements.

Previous studies showed that normal lymphocytes have the ability to eliminate Rh-123[14,15] and to bind the antibody HYB241 (unpublished observation, March 1988), suggesting an *MDR*1-like activity in these cells. However, these studies did not investigate the expression of P-gp or *MDR*1 mRNA in specific cell types. The data reported here provide evidence that subpopulations of normal PB cells express *MDR*1 mRNA at different levels. This pattern is consistent with the results of rhodamine-efflux studies,[14,15] and we can now attribute those findings specifically to *MDR*1 expression.

Antibody studies have shown that P-gp is expressed in $CD34^+$ progenitor cells.[5] In a recent publication, in situ mRNA hybridization showed positivity for *MDR*1 in a subset of BM cells that could be assigned to the myeloid and lymphoid lineage.[6] Therefore, it was of interest to analyze *MDR*1 expression in marrow cells of different hematopoietic lineages and maturational stages. In a purified (97.7%) population of normal $CD34^+$ progenitor cells, *MDR*1 mRNA was found at high levels. *MDR*1 was also detected in myeloid-committed progenitors ($CD33^+$/$CD34^+$), CD34-negative myeloid cells, and in different subsets of B-cell maturation, but not in CD34-negative monocytic cells. In contrast, the Rh-123 efflux study showed pumping activity in a $CD33^{++}$ monocytic BM population, which suggests P-gp expression at least at an early stage of monocytic differentiation. The Rh-123 study showed a decrease in the mean fluorescence for Rh-123 in all BM subpopulations. This efflux could be blocked by verapamil, which implies that the efflux is caused by P-gp. Glycophorin A^+ BM cells showed no significant efflux of Rh-123 and ^{32}P-incorporation during PCR was very low. Because the glycophorin-A–positive population was only 95.6% pure, we cannot definitely conclude that erythroid cells express *MDR*1. We have found that contamination of *MDR*1-negative cells with as few as 1% *MDR*1-positive cells results in *MDR*1 positivity using the PCR technique.

The finding that *MDR*1 is expressed in normal blood cells has important implications for MDR studies of patient samples. In a leukemia sample with a mixture of normal and leukemic cells, positive results may be attributable to residual normal cells. Likewise, *MDR*1 expression in normal cells has implications for the interpretation of results obtained from lymph nodes and other tissues with high numbers of reactive lymphocytes. Because none of the published studies[16-22] were performed on "pure" leukemic cells, the results have to be interpreted with caution. Depending on the sensitivity of the assay used, it is possible that low levels of residual normal cells do not influence the results of MDR studies. However, if a sensitive technique like PCR is applied, it is necessary to study the MDR phenotype exclusively within the malignant cell population. Also, determination of the level of gene expression may be influenced by the presence of normal cells, which could lead to overestimates or underestimates of the actual *MDR*1 level in malignant cells.

Our study also raises the question of the physiologic role of *MDR*1 in normal leukocytes. As P-gp is known to function as an efflux pump, it may facilitate the extrusion of

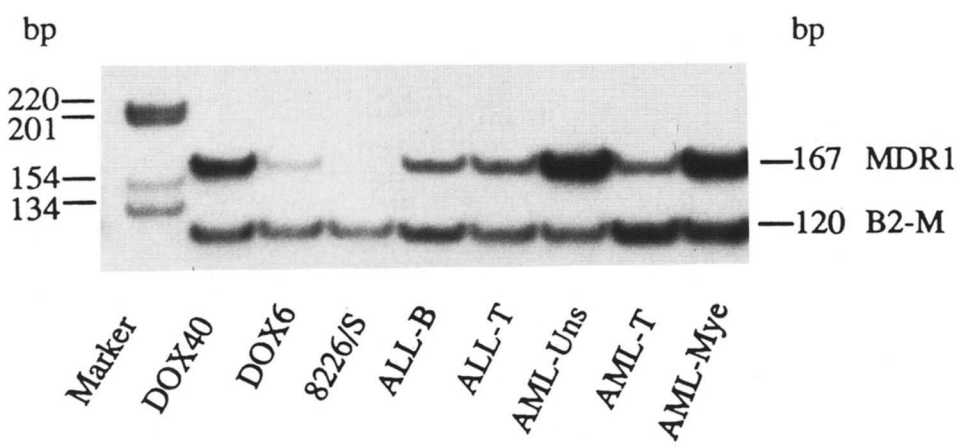

Fig 6. *MDR*1 analysis using PCR of FACS-sorted leukemic blasts and of T lymphocytes from two leukemia patients. Blasts were labeled with CD10 (cALL-specific) and CD33 (AML-specific) antibodies and CD3 was used as T cell marker that was not expressed by the leukemic cells. 8226/S, DOX-6, and DOX-40 cells are shown as controls. ALL-B, $CD10^+$ lymphoblasts; ALL-T, $CD3^+$ T cells from the cALL patient; AML-Uns, unsorted AML; AML-T, $CD3^+$ T cells from the AML patient; AML-Mye, $CD33^+$ myeloblasts. T cells represent 8.3% (cALL) and 15.7% (AML) of the unsorted mononuclear cells and are positive for *MDR*1 in both samples.

toxic or perhaps even regulatory substances in these cells. Do lymphocytes use P-gp as a pump for enzymes and other factors exerting immunologic effects? What level of *MDR*1 expression is of clinical significance in malignant cells? Furthermore, expression of *MDR*1 in hematopoietic progenitor and functional, differentiated cells may render them more vulnerable to chemotherapy if a P-gp inhibitor is administered at the same time. Increased myelosuppression was observed in one clinical trial using cyclosporine A and etoposide in combination,[23] but the physiologic and pathophysiologic roles of P-gp have to be better defined to fully understand the implications for chemotherapy/P-gp antagonist combination therapies.

Our data provide evidence that normal PB and BM cells express *MDR*1 and that its gene product P-gp is functionally active in these cells. These results have to be considered in the analysis of *MDR*1 expression in any tumor sample that may contain normal blood cells.

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