

Involvement of P-Glycoprotein in the Transmembrane Transport of Interleukin-2 (IL-2), IL-4, and Interferon- γ in Normal Human T Lymphocytes

By Johannes Drach, Andrea Gsur, Gerhard Hamilton, Shourong Zhao, Jutta Angerler, Michael Fiegl, Niklas Zojer, Markus Raderer, Ines Haberl, Michael Andreeff, and Heinz Huber

The physiological role of the multidrug resistance P-glycoprotein (P-gp), which is expressed by normal human T lymphocytes, is still largely unknown. To investigate whether or not P-gp is involved in the transport of cytokines, peripheral blood lymphocytes were stimulated with phytohemagglutinin (PHA) in the absence or presence of P-gp inhibitors, and concentrations of cytokines (interleukin-2 [IL-2], IL-4, IL-6, interferon- γ [IFN- γ]) in the supernatants of these cultures were quantitated by enzyme-linked immunosorbent assay. P-gp inhibitors included verapamil (Ver), tamoxifen (Tmx), and the P-gp specific monoclonal antibody UIC2. Release of IL-2 was significantly suppressed by these inhibitors at concentrations that were also effective in blocking efflux of Rhodamine-123 from normal T lymphocytes. IL-2 mRNA expression in lymphocytes was not different between PHA control and the cultures with P-gp inhibitors. Ver and Tmx did not interfere with T-cell activation as determined by

CD25 and CD69 expression. In a nonhematological model, the P-gp expressing HCT-8 adenocarcinoma cell line, exogenously added IL-2 was shown to exert an inhibitory effect on P-gp mediated Rhodamine-123 efflux. In addition, trans-epithelial transport of IL-2 by electrophysiologically tight and polarized HCT-8 monolayers was examined. A time-dependent flux of IL-2 across dense monolayers, which was partially inhibited by Ver, was observed. We also investigated whether or not P-gp inhibitors suppressed release of other cytokines produced by activated T cells (IL-4, IL-6, IFN- γ). Release of IL-4 and IFN- γ was significantly inhibited by Ver, Tmx, and UIC2; however, release of IL-6 remained unaffected. These data show P-gp mediated transmembrane flux of IL-2 in T lymphocytes and HCT-8 cells. We conclude that P-gp participates in the transport of cytokines (IL-2, IL-4, and IFN- γ) in normal peripheral T lymphocytes.

© 1996 by The American Society of Hematology.

P-GLYCOPROTEIN (P-gp), the 170-kD product of the *MDR1* gene, is a member of the ABC (adenosine triphosphate [ATP]-binding cassette) superfamily of active transporters¹ and was first discovered in drug resistant tumor cells.² As an energy dependent transmembrane protein, P-gp uses ATP hydrolysis to pump cytotoxic drugs out of cells thus reducing the intracellular concentration of drug and, as a consequence, toxicity. P-gp was also found to be expressed in normal tissues, particularly on the luminal surfaces of transporting epithelia, such as proximal tubules of the kidney, epithelial cells of the small intestine and colon, and biliary hepatocytes of the liver.³⁻¹⁰ The pattern of tissue distribution suggested a physiological role of P-gp in secreting metabolites and natural xenobiotics. P-gp was also detected at high levels in the adrenal cortex, and subsequent studies provided evidence that cortisol and aldosterone are physiological substrates of P-gp.^{11,12}

Several groups, including ours, have reported that expression of P-gp is a feature of certain cell populations from normal human bone marrow and peripheral blood (PB).¹³⁻²² Besides hematopoietic progenitor cells, normal T lymphocytes express significant levels of a functional P-gp as determined by efflux of fluorescent dyes. The function of P-gp on normal lymphocytes is still largely unknown. It has been recently suggested that P-gp in CD8⁺ lymphocytes and natural killer (NK) cells may play a role in mediating cytotoxicity.^{19,23} However, P-gp is also abundantly expressed on lymphocytes not exerting cytotoxicity, which implies that additional functional roles of P-gp must exist.

Upon stimulation, lymphocytes exert their immunological functions in part by production of various cytokines. Because P-gp may be involved in the transmembrane transport of endogenous proteins,²⁴⁻²⁶ we hypothesized that P-gp could have a physiological function as a transporter of cytokines. In this report, we provide evidence that inhibition of P-gp by both small molecules and a monoclonal antibody (MoAb) blocking P-gp activity results in a significantly suppressed

release of interleukin-2 (IL-2), IL-4, and interferon- γ (IFN- γ) from phytohemagglutinin (PHA)-stimulated normal T lymphocytes. In addition, we have tested the effects of IL-2 on P-gp-mediated transport in a nonhematological in vitro system, namely the P-gp expressing human ileocecal adenocarcinoma cell line HCT-8. We also present data showing transepithelial translocation of IL-2 across polarized HCT-8 monolayers.

MATERIALS AND METHODS

Stimulation of lymphocytes. PB lymphocytes were obtained from 10 healthy individuals (median age 26 years, range 22 to 31) and separated by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After 2 washes with RPMI-1640 medium, cells were resuspended at a concentration of 10⁶ cells/mL in growth medium (RPMI-1640 supplemented with 10% fetal calf serum, penicillin [100 U/mL], and streptomycin [100 μ g/mL]). Stimulation was performed by adding PHA (Sigma, St Louis, MO) at 1 μ g/mL or 10 μ g/mL. After 24 and 48 hours, cells were procured

From the University of Vienna, First Department of Internal Medicine, the Division of Clinical Oncology; Ludwig Boltzmann Institute of Clinical Oncology, KH Lainz, Vienna, Austria; and the University of Texas M.D. Anderson Cancer Center, the Department of Hematology, Section of Experimental Hematology, Houston.

Submitted July 31, 1995; accepted April 30, 1996.

Supported in part by grants from the Austrian Science Foundation (P10893-MED to J.D.), the Austrian National Bank (No 4851 to G.H.), and the National Institutes of Health (CA 57639 and CA 55164 to M.A.).

Address reprint requests to Johannes Drach, MD, University of Vienna, First Department of Internal Medicine, Division of Clinical Oncology, Waehringer Guertel 18-20, A-1090 Vienna, Austria.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

0006-4971/96/8805-0032\$3.00/0

for mRNA analysis, and culture supernatants were obtained for determination of cytokine levels.

Inhibitors of P-gp. Small molecule inhibitors and P-gp specific MoAbs were used in stimulation experiments. Small molecule inhibitors included verapamil (Ver) (used at final concentrations of 1 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$), tamoxifen (Tmx) (1 $\mu\text{mol}/\text{L}$, 10 $\mu\text{mol}/\text{L}$, and 50 $\mu\text{mol}/\text{L}$), and cyclosporin-A (CsA) (0.1 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, and 10 $\mu\text{g}/\text{mL}$). All these drugs were shown to bind to P-gp and block its function by competitive inhibition of other substrates.^{27,33} Experiments with antibodies were performed with UIC2 (generously provided by Dr I.B. Roninson, University of Illinois at Chicago, to M.A.), which is a P-gp specific MoAb also blocking its function,³⁴ and MRK-16 (Immunotech, Marseille, France). MRK-16 reacts with a different external epitope of P-gp³⁵ and was shown to be P-gp inhibitory only for certain substrates.^{34,35} An isotypic IgG2a antibody (Becton Dickinson, San Jose, CA) was used as control. All antibodies were used at a concentration of 10 $\mu\text{g}/\text{mL}$, which was shown to be a saturating concentration for these P-gp antibodies.^{15,34}

Determination of cytokine production. Levels of IL-2, IL-4, IL-6, and IFN- γ in the culture supernatants were quantified by enzyme-linked immunosorbent assay (ELISA) according to instructions of the manufacturer (Quantikine ELISA kits for IL-2, IL-4, IL-6, IFN- γ ; R&D Systems, Minneapolis, MN).

RNA preparation and reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated according to the acid-guanidinium-phenol-chloroform method.³⁶ cDNA sequences were synthesized from 2 μg of total cellular RNA in the presence of 5 \times cDNA buffer (Promega, Southampton, UK), 3 μg of DNase/RNase-free bovine serum albumin, 10 mmol/L of deoxytriphosphate nucleotides, 100 pmol/L of pd(N)6 as primer (Boehringer Mannheim, Mannheim, Germany), 40 U of RNasin (Promega), and 150 units of M-MLV (Promega) in a final reaction volume of 25 μL . After preparation of this mixture on ice, incubation was performed at 37°C for 60 minutes, then at 95°C for 5 minutes, followed by a quick chill to 4°C.

Five microliters of the large scale cDNA preparation (equivalent to 200 ng of starting RNA template) was used for amplification of specific DNA sequences. PCR was performed in a final volume of 50 μL containing 100 pmol/L of the specific primer set, 1.25 mmol/L of deoxytriphosphate nucleotides, 1 \times PCR buffer, and 2 U Taq DNA polymerase (all reagents were obtained from Promega). The IL-2 specific primer set was obtained from Clontech (Palo Alto, CA) and is expected to generate a cDNA fragment of 305 base pairs. β -Actin was used as internal control (primers were also purchased from Clontech). Amplifications were performed for 30 cycles. Each cycle contained a denaturation step at 94°C for 45 seconds, an annealing step at 60°C for 45 seconds, and an elongation step at 72°C for 2 minutes. After the last cycle, a final elongation step was performed at 72°C for 7 minutes. Twenty microliter aliquots of the PCR products were separated electrophoretically on 2% agarose gels containing ethidium bromide. The products were then visualized on an UV transilluminator and photographed. HL-60 cells were included as IL-2⁻ control (data not shown).

Efflux of Rhodamine-123 (Rh-123). Flow cytometric determination of P-gp function using efflux of Rh-123 was performed as previously described.¹⁶ To study the effect of P-gp inhibitors on efflux of Rh-123 from lymphocytes, cells were incubated with 200 ng/mL Rh-123 in RPMI-1640 for 15 minutes at 37°C, washed twice with ice-cold medium, and then transferred into dye-free medium with or without P-gp inhibitors. After incubation at 37°C for 90 minutes, cells were analyzed on a FACScan flow cytometer (Becton Dickinson) using the LYSIS II Research software provided by the manufacturer (Becton Dickinson).

Immunofluorescence and flow cytometry. For the determination of antigen expression on T lymphocytes, the following MoAbs were used: CD3/Leu-4 (conjugated with fluorescein isothiocyanate

[FITC]), CD25/anti-IL-2R (conjugated with phycoerythrin [PE]), CD69/Leu-23 (conjugated with PE), all obtained from Becton Dickinson. Experiments were performed as two-color immunofluorescence studies combining CD3-FITC with either CD25-PE or CD69-PE. Following the manufacturer's recommendations, 10⁶ cells were incubated with the antibodies at saturation concentrations for 30 minutes at 4°C and washed twice with phosphate-buffered saline (PBS) afterwards. Samples with appropriate isotype-matched control antibodies were processed in parallel. Analysis was performed on a FACScan flow cytometer, and events from 10,000 cells were acquired using the LYSIS II Research software.

HCT-8 cells. Human ileocecal adenocarcinoma HCT-8 cells were obtained from ATCC (American Tissue Culture Collection, Rockville, MD). Cells were maintained in bicarbonate-buffered 10% fetal bovine serum/RPMI 1640 medium (Seromed, Berlin, Germany) supplemented with 4 mmol/L L-glutamine and 75 $\mu\text{g}/\text{mL}$ gentamycin at 37°C in a humidified atmosphere containing 5% CO₂. Confluent monolayers were subcultured every 3 to 4 days by treatment with 0.05% trypsin and 0.02% EDTA in Ca²⁺- and Mg²⁺-free PBS.

For experiments in which efflux of Rh-123 was examined, HCT-8 cells were procured, washed with medium, and loaded with Rh-123 as previously described. The effects of Ver (1 $\mu\text{mol}/\text{L}$), CsA (1 $\mu\text{g}/\text{mL}$), and IL-2 (300 ng/mL) on Rh-123 efflux were quantitated by comparing the slopes of linear regression lines for the mean fluorescence intensities of aliquots sampled at 0, 10, and 20 minutes of incubation.

Directed IL-2 flux across HCT-8 epithelial monolayers. Preparation of HCT-8 epithelial monolayers and directed transepithelial transport were performed as previously described.^{37,38} In brief, 1 \times 10⁶ HCT-8 cells in RPMI-1640 medium were seeded into porous filter cups (25 mm diameter, low density and 0.45 μm pore size; Falcon, Becton Dickinson) and cultured until polarized monolayers of transepithelial resistance exceeding 800 Ohm cm² were obtained. IL-2 (3 $\mu\text{g}/\text{mL}$) was added to the basolateral compartment, medium without or with the P-gp inhibitor Ver (1 $\mu\text{mol}/\text{L}$) to the apical compartment, and the transport rates were calculated from linear fits to IL-2 ELISA determinations using samples taken each hour for a total of 5 hours from the apical side of the cultures.

RESULTS

Release of IL-2 from normal lymphocytes in the presence of small molecule inhibitors of P-gp. Normal PB lymphocytes were stimulated with PHA (final concentrations 1 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$) in the presence or absence of P-gp inhibitors (Ver or Tmx). IL-2 present in the supernatant of these cultures was measured by ELISA. Data from the literature suggested that a significant inhibition of P-gp in *MDR1* expressing cell lines can be achieved with 10 $\mu\text{g}/\text{mL}$ of Ver as well as with 10 $\mu\text{mol}/\text{L}$ and 50 $\mu\text{mol}/\text{L}$ of Tmx.^{29,30} These concentrations of small molecule inhibitors of P-gp were also effective in blocking P-gp pumping activity in normal lymphocytes as shown in efflux experiments with Rh-123 as P-gp substrate (Fig 1).

In cultures of normal lymphocytes without P-gp inhibitors, levels of released IL-2 were 53.8 ng/mL (median, range 48.8 to 156.2) after 24 hours of stimulation with 1 $\mu\text{g}/\text{mL}$ of PHA, 620.7 ng/mL (median, range 300.5 to 1,905) after 24 hours of stimulation with 10 $\mu\text{g}/\text{mL}$ of PHA, and 783.4 ng/mL (median, range 315.3 to 2408.2) after 48 hours of stimulation with 10 $\mu\text{g}/\text{mL}$ of PHA. However, PHA-stimulation of lymphocytes in the presence of Ver (10 $\mu\text{g}/\text{mL}$) or

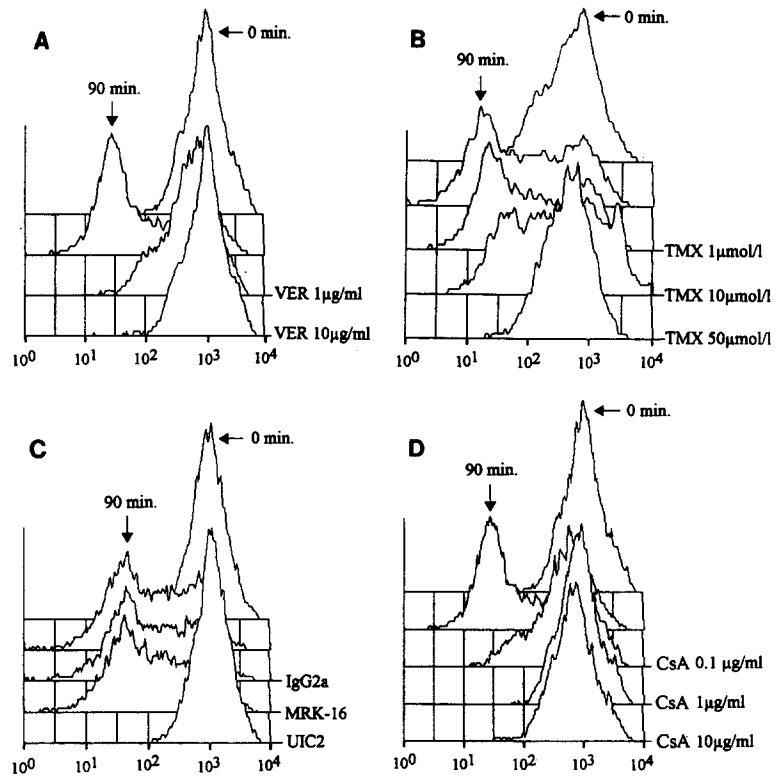


Fig 1. Effect of small molecule inhibitors and MoAbs on Rh-123 efflux from normal lymphocytes. Lymphocytes were loaded with Rh-123 (200 ng/mL) and efflux was measured after incubation in dye-free medium for 90 minutes. Fluorescence intensity as determined by flow cytometry is shown on the abscissa (logarithmic scale, arbitrary units), and the ordinate gives the relative cell number. Zero minutes, Rh-123 fluorescence of lymphocytes after loading; 90 minutes, Rh-123 fluorescence of lymphocytes after 90 minutes of efflux in the absence of P-gp inhibitors. All other histograms show Rh-123 fluorescence of lymphocytes when efflux was performed in the presence of verapamil (VER, A), tamoxifen (TMX, B), (MoAbs, C), and cyclosporin A (CsA, D), respectively.

Tmx (10 $\mu\text{mol/L}$ and 50 $\mu\text{mol/L}$) resulted in a significant reduction of IL-2 levels in the supernatant ($P < .05$, Fig 2). Tmx was almost as effective as CsA (Fig 2C), which is an inhibitor of P-gp function (Fig 1D), but it also has an immunosuppressive effect by inhibiting transcription of the IL-2 gene.³⁹

To exclude the possibility that low levels of IL-2 in cultures with Ver and Tmx were a consequence of altered IL-2 expression, we examined IL-2 mRNA expression in lymphocytes from these cultures. Using RT-PCR and IL-2 specific primers, we did not observe downregulation of IL-2 mRNA expression in response to Ver and Tmx, which was in contrast to cultures treated with CsA at concentrations between 0.1 and 10 $\mu\text{g/mL}$ (Fig 3).

We also tested whether or not stimulation of lymphocytes in the presence of Ver or Tmx had any effect on expression of CD25 and CD69, which are surface markers known to be expressed by activated T cells. Lymphocytes from 3 healthy individuals were stimulated with PHA (10 $\mu\text{g/mL}$), and as expected expression of CD25 and CD69 was upregulated as determined by flow cytometry at 24 and 48 hours (Table 1). Addition of Ver (10 $\mu\text{g/mL}$) or Tmx (10 $\mu\text{mol/L}$) resulted in a similar pattern of antigen expression on CD3⁺ cells, and neither the percentage of antigen expressing cells nor fluorescence intensity of positive cells (Fig 4) was different compared to control (PHA only). In contrast, in the presence of CsA (1 $\mu\text{g/mL}$) we observed not only a lower frequency of CD25 expressing T cells ($P = .02$), but also reduced fluorescence intensity of CD25 expression (Fig 4).

Inhibition of P-gp function by MoAbs. Next, we used the P-gp specific MoAb UIC2 which is a potent inhibitor of

P-gp function in both *MDR1* expressing cell lines³⁴ and normal lymphocytes.¹⁷ At a saturating concentration of 10 $\mu\text{g/mL}$, UIC2 efficiently blocked Rh-123 efflux from normal lymphocytes (Fig 1C). In contrast, MRK-16, which is also P-gp specific, as well as an IgG2a isotypic control antibody did not have any blocking effect on Rh-123 efflux (Fig 1C). These antibodies (final concentrations 10 $\mu\text{g/mL}$) were then used in experiments in which normal lymphocytes from 5 individuals were stimulated with 10 $\mu\text{g/mL}$ of PHA for 24 hours. In cultures with the IgG2a antibody, we did not observe suppressed levels for IL-2 compared to PHA controls. Significantly suppressed levels of IL-2 were detected in cultures with the UIC2 MoAb (16.6% \pm 5.8% of IgG2a control, mean \pm SD; $P = .0001$), but only a minor effect could be seen with the MRK-16 antibody (84.9% \pm 1.8%) (Fig 5). As shown in Fig 6, strong bands for IL-2 mRNA were detected in lymphocytes obtained from all cultures confirming that IL-2 gene expression was not influenced by the addition of any of these antibodies.

Effect of IL-2 on P-gp mediated transport of HCT-8 suspension cells and polarized monolayers. To further investigate the association between IL-2 and P-gp mediated transport, we used the human ileocecal adenocarcinoma cell line HCT-8 as a nonhematological *MDR1* expressing model. HCT-8 cells exhibit significant P-gp expression and high rates of Rh-123 efflux, which is sensitive to P-gp inhibitors like Ver, CsA, and others.³⁸ The effect of IL-2 on Rh-123 efflux of HCT-8 cells in suspension was compared to media controls, 1 $\mu\text{mol/L}$ Ver and 1 $\mu\text{g/mL}$ CsA, respectively (Fig 7A). Exogenously added IL-2 could be shown to exert an inhibitory effect on P-gp mediated Rh-123 efflux, reaching

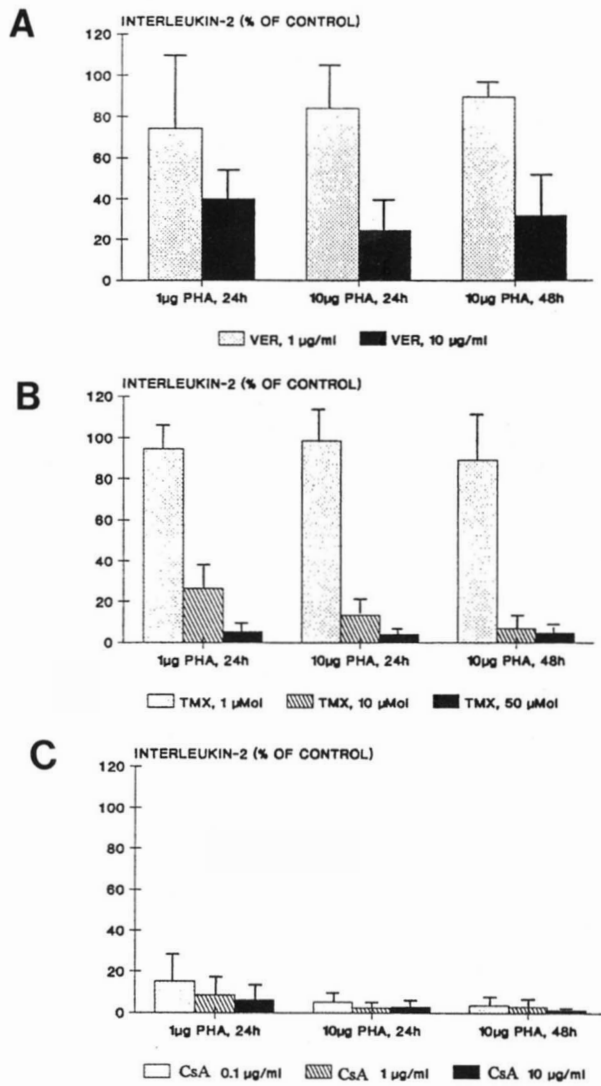


Fig 2. IL-2 present in supernatants of cultures of PHA-stimulated normal lymphocytes in the presence of P-gp inhibitors. Concentrations of IL-2 were measured by ELISA, and results are expressed as percent of control (no inhibitor). Control is designated as 100%. (A) verapamil (VER), (B) tamoxifen (TMX), (C) cyclosporin A (CsA). Results are derived from duplicate experiments with PB lymphocytes from 6 to 10 healthy individuals.

25% and 33% of the effects found in response to Ver and CsA, respectively.

Trans epithelial transport of IL-2 by HCT-8 monolayers was studied as described for the P-gp substrate vinblastine in a previous report.³⁸ Briefly, IL-2 was added to the basolateral compartment of electrophysiologically tight monolayers of polarized HCT-8 cells on porous filter cups (resistance > 800 Ohm cm²) and aliquots of the apical compartment were collected for up to 5 hours and tested for their IL-2 content. Because P-gp mediated transport is linear in the HCT-8 model, the measured IL-2 concentrations were approximated by a linear fit and the slopes of the regression lines for media controls and for the addition of 1 µmol/L Ver at the apical

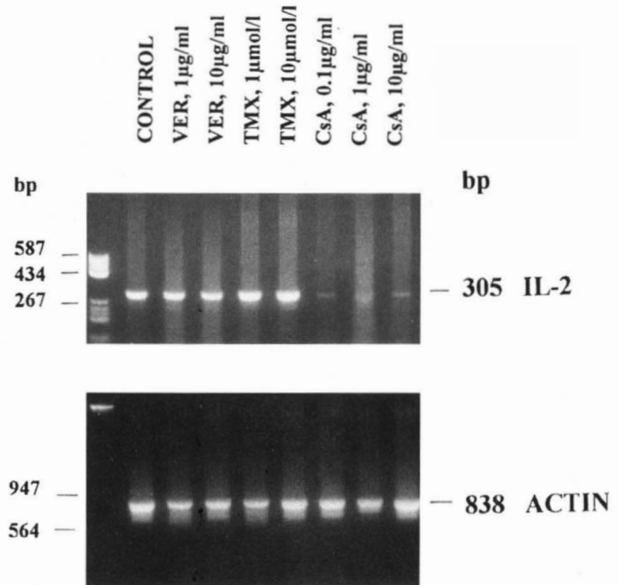


Fig 3. Expression of IL-2 mRNA in PHA-stimulated normal PB lymphocytes (10 µg/mL of PHA for 24 hours) as detected by RT-PCR (for details see Materials and Methods). IL-2 gene expression is inhibited by cyclosporin A (CsA), but not by verapamil (VER) and tamoxifen (TMX). β -Actin was used as internal standard.

side used for further calculations. These results show a time-dependent flux of IL-2 across dense monolayers (apical concentration of IL-2 [pg/mL] = $-11.5 + 135.2 \times \text{time [hours]}$), which could be partially inhibited by Ver ($24.3 \pm 3.6\%$ inhibition, Fig 7B). A low flux of IL-2 was observed in the apical to basolateral direction and this transport was stimulated by Ver, indicating passive and paracellular flux processes with opening of the gap junctions by apical Ver (data not shown).

Effect of P-gp inhibitors on release of IL-4, IFN- γ , and IL-6. Using lymphocytes from healthy individuals and PHA stimulation as described earlier, we investigated whether or not P-gp inhibitors also suppressed release of other cytokines (IL-4, IFN- γ , and IL-6). After 24 hours of stimulation with PHA at 10 µg/mL, the following concentrations of cytokines (mean \pm SD from 4 to 6 experiments)

Table 1. Expression of CD25 and CD69 on CD3⁺ T Lymphocytes

Culture Condition	% CD25 ⁺ (mean \pm SD)	% CD69 ⁺ (mean \pm SD)
Unstimulated	19.3 \pm 6.5	6.8 \pm 2.2
24 hours:		
PHA	89.4 \pm 4.6	96.8 \pm 0.9
PHA + VER	87.7 \pm 5.2	96.6 \pm 1.2
PHA + TMX	87.1 \pm 5.2	96.6 \pm 1.1
PHA + CsA	78.5 \pm 2.8	93.6 \pm 2.7
48 hours:		
PHA	97.1 \pm 1.8	95.5 \pm 0.9
PHA + VER	97.0 \pm 1.8	92.5 \pm 1.5
PHA + TMX	96.5 \pm 1.5	93.4 \pm 1.0
PHA + CsA	87.1 \pm 0.8	90.2 \pm 4.1

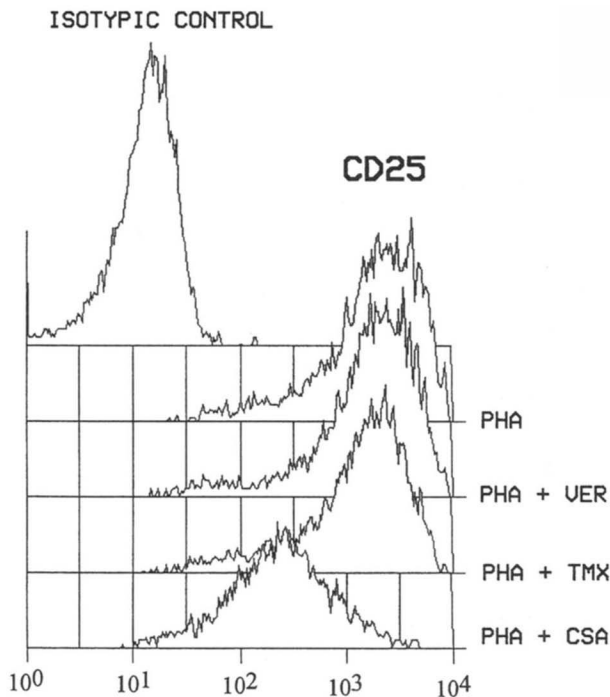


Fig 4. Expression of CD25 on CD3⁺ lymphocytes after 24 hours of stimulation with 10 μ g/mL of PHA. Lymphocytes treated with PHA without inhibitor and in the presence of verapamil (VER, 10 μ g/mL) or tamoxifen (TMX, 10 μ mol/L) expressed CD25 to a similar extent, whereas treatment of lymphocytes with cyclosporin A (CsA, 1 μ g/mL) resulted in a significantly lower expression of CD25.

were determined in the supernatants: 52.2 \pm 31.2 pg/mL for IL-4, 1.2 \pm 0.86 ng/mL for IFN- γ , and 290.5 \pm 18.9 ng/mL for IL-6. Both small molecule inhibitors of P-gp (Fig 8) and the UIC2 MoAb (Fig 5) significantly inhibited release

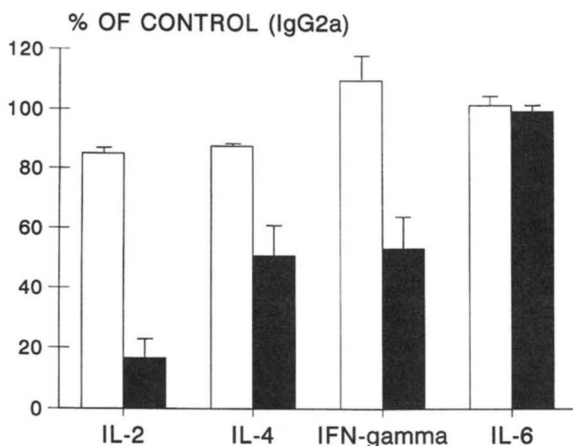


Fig 5. Cytokine levels determined in supernatants of PHA-stimulated normal lymphocytes (10 μ g/mL of PHA for 24 hours) in the presence of P-gp specific MoAbs. Results are expressed as percent of control (PHA-stimulated lymphocytes in the presence of an isotypic IgG2a antibody). All antibodies were used at 10 μ g/mL. Release of IL-2, IL-4, and IFN- γ was significantly inhibited by the UIC2 MoAb; release of IL-6, however, remained unaffected. (□), MRK-16; (■), UIC2.

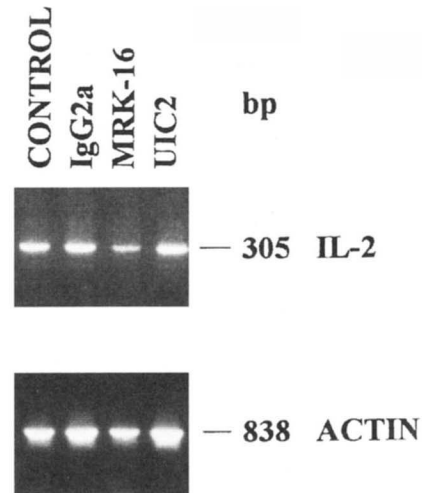


Fig 6. IL-2 gene expression in normal lymphocytes after 24 hours of PHA-stimulation (10 μ g/mL) in the absence (control) or presence of MoAbs (IgG2a isotypic control, MRK-16, UIC2). Bands for IL-2 mRNA were similar under each culture condition.

of IL-4 and IFN- γ , but there was no evidence for reduced concentrations of IL-6 in the presence of these P-gp inhibitors.

DISCUSSION

Several recent studies have reported on P-gp expression by normal human and murine peripheral T lymphocytes.^{13,14,16-21} Differential expression of P-gp by subpopulations of T cells has been noted, but its role in T-cell activation and function has been unclear. In this report, we show that release of IL-2, IL-4, and IFN- γ from stimulated normal lymphocytes is significantly suppressed in the presence of P-gp inhibitors. Several lines of evidence suggest that decreased levels of cytokines are a consequence of P-gp inhibition rather than a more generalized effect on T-cell function or interference with T-cell activation.

First, decreased levels of cytokines were observed not only in the presence of small molecule inhibitors of P-gp (Ver, Tmx), which are drugs with known pleiotropic effects, but also in cultures with the MoAb UIC2, a specific inhibitor of P-gp function. In contrast to UIC2, almost no inhibitory effect on cytokine release was found with another P-gp specific MoAb, MRK-16, which recognizes a different external epitope of P-gp. In efflux experiments with Rh-123 as P-gp substrate, inhibition of P-gp function was observed with UIC2, but not with MRK-16. Both antibodies gave the expected positive results when *MDR1* expressing cell lines were stained and analyzed by flow cytometry (data not shown), which rules out the possibility of antibody degradation. We believe that our present data are in agreement with previous observations that MRK-16 is inhibitory only for some P-gp substrates,^{34,35} and that inhibition of P-gp function by means of MoAbs may be epitope specific.³⁴

Second, stimulation of normal lymphocytes in the presence of P-gp inhibitors (Ver, Tmx, and UIC2) did not result

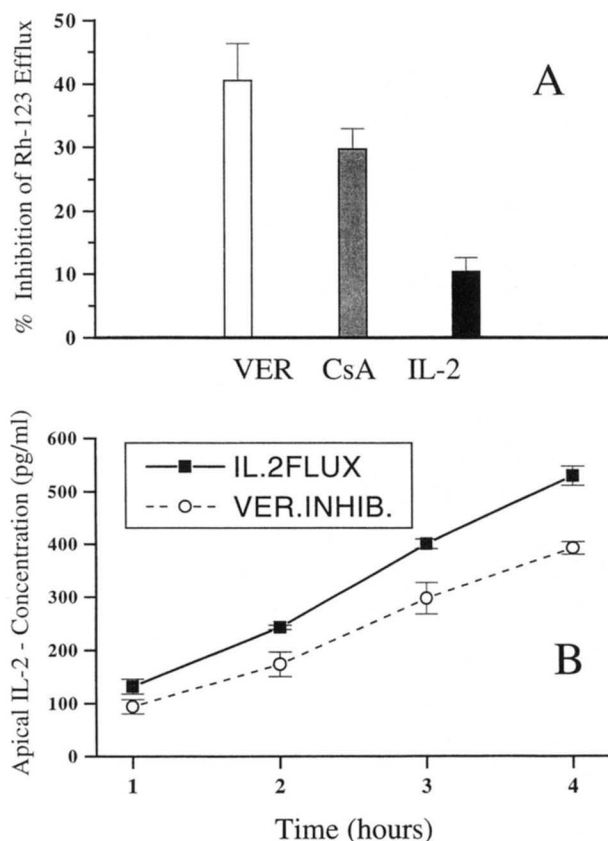


Fig 7. (A) Effects of 1 $\mu\text{mol/L}$ Ver, 1 $\mu\text{g/mL}$ CsA, and 300 ng/mL IL-2 on Rh-123 efflux from HCT-8 cells (mean \pm SEM; 5 independent experiments). The slope of the regression line for efflux tests (20 minutes) using medium alone was taken as 100%. (B) Transepithelial flux of IL-2 across electrophysiologically tight polarized HCT-8 monolayers following addition of 3 $\mu\text{g/mL}$ IL-2 to the basolateral compartment (mean \pm SEM; 5 independent experiments). The increase in apical IL-2 concentration is shown in the absence (solid line) and presence (broken line) of 1 $\mu\text{mol/L}$ Ver. Values of these two series are significantly different at all time points ($P < .02$; paired t -test). Inhibition was calculated from the slopes of linear regression lines fitted to each data set.

in a significant downregulation of IL-2 mRNA expression, although small differences in gene expression may be difficult to detect by a semiquantitative RT-PCR assay. However, a significantly lower expression of IL-2 mRNA was found in the presence of CsA, which is known to inhibit IL-2 gene transcription.³⁹ P-gp inhibitors at blocking concentrations suppressed IL-2 release almost as effectively as CsA. Even if these inhibitors caused a slight change in IL-2 mRNA expression that was undetectable by RT-PCR, this effect could not be the primary explanation for the marked reduction of released IL-2.

Third, we have also examined if Ver and Tmx had any effect on other events of early T-cell activation. There was no difference in CD69 and CD25 expression compared to control with PHA only as determined by flow cytometry at 24 and 48 hours of stimulation. This was again in contrast to T cells obtained from cultures with the immunosuppressive agent CsA, which exhibited significantly lower CD25

expression. In addition, if P-gp inhibitors exerted a more generalized effect on T cells with interference with activation events, one would expect decreased levels for all cytokines produced by activated T lymphocytes. Similar to IL-2, concentrations of IL-4 and IFN- γ were lower in cultures with P-gp inhibitors compared to controls. However, when IL-6 was examined, neither small molecule inhibitors nor MoAb UIC2 had any effect on released IL-6.

To further exclude any possibility that decreased levels of IL-2 were caused by alterations of lymphocyte biology under these culture conditions, experiments were performed with P-gp expressing HCT-8 adenocarcinoma cells. HCT-8 cells show formation of electrophysiologically tight and polarized monolayers upon cultivation of cells on porous filter supports. These layers are almost impermeable to small molecules transported by the paracellular route like mannitol, but exhibit directed transepithelial flux of P-gp substrates like doxorubicin and vinca alkaloids by passive basolateral uptake and active efflux by apically located P-gp.³⁷ Endocytosis, which is a well-characterized mechanism of protein uptake by intestine derived cell lines,⁴⁰ is the most likely pathway of IL-2 uptake through the basolateral barrier in HCT-8 cells. In our experiments with HCT-8 cups of proven resistance, IL-2 passed from the basolateral to apical compartment with near linear kinetics as previously measured for the P-gp substrate vinblastine.³⁸ Because transport of IL-

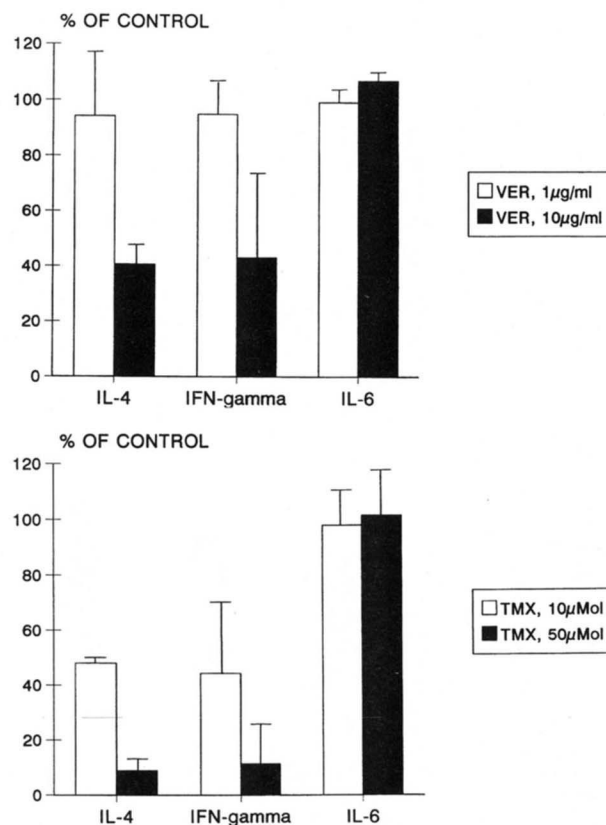


Fig 8. Cytokine release from PHA-stimulated normal lymphocytes in the presence of (top) verapamil or (bottom) tamoxifen: Small molecule inhibitors suppress release of IL-4 and IFN- γ , but not of IL-6.

2 is partially inhibited by Ver added to the apical compartment, it is concluded that P-gp participates in the translocation of this cytokine across HCT-8 monolayers. The high concentrations of IL-2 in the basolateral compartments were a precondition to obtain significant transepithelial fluxes in our filter inserts with low density of pores ($1.6 \times 10^6/4.3 \text{ cm}^2$) where only a small fraction of the basolateral cell surface is accessible. We have also shown that addition of IL-2 to Rh-123 preloaded HCT-8 cells resulted in a significant inhibition of P-gp mediated dye efflux. In agreement with the transepithelial flux of IL-2 observed with HCT-8 monolayers, this finding points to an inhibitory modulation of P-gp function by IL-2 in this nonhematological in vitro model.

Results from previous studies suggested that the function of P-gp in mammalian cells may include the transmembrane transport of endogenous peptides or proteins. In yeast, the product of the STE6 gene has been identified as a homologue of the mammalian *MDR1/P-gp*.²⁴ Its function includes export of hydrophobic peptides, which is different from the classical signal sequence-recognizing secretory pathway.^{24,25} Based on this observation, IL-1 would be a candidate for secretion by P-gp because IL-1 precursors lack recognizable signal sequences⁴¹ and are absent from organelles of the classical secretory pathway.⁴² A subsequent report indeed showed an association between *MDR1* expression and transport of IL-1 β : When COS cells were cotransfected with vectors encoding for IL-1 β and *MDR1*, secretion of IL-1 β was detected; in contrast, COS cells transfected with IL-1 β only or with IL-1 β and 3 failed to secrete IL-1 β .⁴³ Our present report as well as the findings by Raghunath et al⁴⁴ provide evidence for the involvement of P-gp in the transport of IL-2 and other cytokines from stimulated normal human lymphocytes.

Human and murine T cells can be subdivided into several populations that differ in their functions. In the mouse system, P-gp expression was found to identify a novel subset of activated CD4⁺ T lymphocytes that could not be distinguished by staining for other surface markers.^{21,45} Using the ability of these cells to extrude Rh-123, it was shown that expression of mRNA for IL-4 and IFN- γ in addition to IL-2 mRNA occurred only in the subsets with Rh-123 efflux, which also correlated with production of these cytokines upon polyclonal stimulation.⁴⁵ Based on our findings we may postulate that the described association between expression of P-gp and cytokine mRNA by T-cell populations is not coincidental but rather is a reflection of a physiological function of P-gp. However, it is worth mentioning that P-gp expressing T-cell subpopulations may still be heterogeneous in their function because in mice Rh-123 extruding, CD4⁺ T cells were reported to show diminished IL-2-driven IL-4 production.²¹

There is now increasing evidence for an important role of P-gp in metabolic and secretory functions of normal human cells, independent of their tissue distribution. Expression of P-gp has been shown to be associated with a volume-regulated chloride channel,⁴⁶ and P-gp has also been found to be a channel for the export of ATP.⁴⁷ In addition, cellular products like steroid hormones^{11,12} and metabolites (eg, bilirubin)⁴⁸ are transported by P-gp. Our results extend these observations and show that P-gp is involved in the

transmembrane transport of certain cytokines. P-gp can be important for the pharmacology of drugs other than anticancer agents, such as dexamethasone, digoxin, and morphine.⁴⁹ Functions of P-gp in normal cells such as hematopoietic progenitors^{15,16,22} and epithelial cells at the blood-brain barrier^{7,8} still need to be elucidated. Future work should also determine the function of P-gp in immune cells other than T lymphocytes, because also NK cells and B lymphocytes express significant levels of P-gp.^{16,17,20,23} Knowledge of physiological functions of P-gp may be important for our understanding of both beneficial and unwanted effects of cancer therapies combining multidrug resistant-reversing agents with chemotherapy.

REFERENCES

- Hyde SC, Emsley P, Hartshorn M, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard R, Higgins CF: Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346:362, 1990
- Roninson IB: *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*. New York, NY, Plenum, 1991
- Fojo AT, Ueda K, Slamon DJ, Poplack DG, Gottesmann MM, Pastan I: Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci USA* 84:265, 1987
- Thiebaut F, Tsuruo T, Hamada H, Gottesmann MM, Pastan I, Willingham MC: Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc Natl Acad Sci USA* 84:7734, 1987
- Arceci RJ, Croop JM, Horwitz SB, Housman D: The gene encoding multidrug resistance is induced and expressed at high levels during pregnancy in the secretory epithelium of the uterus. *Proc Natl Acad Sci USA* 85:4350, 1988
- Sugawara I, Kataoka I, Morishita Y, Hamada H, Tsuruo T, Itoyama S, Mori S: Tissue distribution of P-glycoprotein encoded by a multidrug-resistant gene as revealed by a monoclonal antibody, MRK 16. *Cancer Res* 48:1926, 1988
- Cordon-Cardo C, O'Brien JP, Casals D, Rittman GL, Biedler JL, Melamed MR, Bertino JR: Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci USA* 86:695, 1989
- Thiebaut F, Tsuruo T, Hamada H, Gottesmann MM, Pastan I, Willingham MC: Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: Evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. *J Histochem Cytochem* 37:159, 1989
- Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR: Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. *J Histochem Cytochem* 38:1277, 1990
- van der Valk P, van Kalken CK, Ketelaars H, Broxterman HJ, Scheffer G, Kuiper CM, Tsuruo T, Lankelma J, Meijer CJ, Pinedo H, Scheper R: Distribution of multidrug resistance associated P-glycoprotein in normal and neoplastic human tissues. *Ann Oncol* 1:56, 1990
- Ueda K, Okamura N, Hirai M, Tanigawara Y, Saeki T, Kioka N, Komano T, Hori R: Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J Biol Chem* 34:24248, 1992
- van Kalken CK, Broxterman HJ, Pinedo HM, Feller N, Dekker H, Lankelma J, Giaccone G: Cortisol is transported by the multidrug resistance gene product P-glycoprotein. *Br J Cancer* 67:284, 1993

13. Neyfakh AA, Serpinskaya AS, Chervonsky AV, Apasov SG, Kazarov AR: Multidrug-resistance phenotype of a subpopulation of T-lymphocytes without drug selection. *Exp Cell Res* 185:496, 1989
14. Coon JS, Wang Y, Bines SD, Markham PN, Chong ASF, Gebel HM: Multidrug resistance activity in human lymphocytes. *Hum Immunol* 32:134, 1991
15. Chaudhary PM, Roninson IB: Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. *Cell* 66:85, 1991
16. Drach D, Zhao S, Drach J, Mahadevia R, Gatringer C, Huber H, Andreeff M: Subpopulations of normal peripheral blood and bone marrow cells express a functional multidrug resistant phenotype. *Blood* 80:2729, 1992
17. Chaudhary PM, Mechetner EB, Roninson IB: Expression and activity of the multidrug resistance P-glycoprotein in human peripheral blood lymphocytes. *Blood* 80:2735, 1992
18. Marie JP, Brophy NA, Ehsan MN, Aihara Y, Mohamed NA, Cornbleet J, Chao NJ, Sikic BI: Expression of multidrug resistance gene *mdr1* mRNA in a subset of normal bone marrow cells. *Br J Haematol* 81:145, 1992
19. Gupta S, Kim CH, Tsuruo T, Gollapudi S: Preferential expression of multidrug resistance gene 1 product (P-glycoprotein), a functionally active efflux pump, in human CD8+ T cells: A role in cytotoxic effector function. *J Clin Immunol* 12:451, 1992
20. Klimecki WT, Futscher BW, Grogan TM, Dalton WS: P-glycoprotein expression and function in circulating blood cells from normal volunteers. *Blood* 83:2451, 1994
21. Witkowski JM, Li SP, Gargas G, Miller RA: Extrusion of the P-glycoprotein substrate rhodamine-123 distinguishes CD4 memory T cell subsets that differ in IL-2-driven IL-4 production. *J Immunol* 153:658, 1994
22. Drach J, Zhao S, Drach D, Körbling M, Engel H, Andreeff M: Expression of 1 by normal bone marrow cells and its implication for leukemic hematopoiesis. *Leuk Lymphoma* 16:419, 1995
23. Chong AS-F, Markham PN, Gebel HM, Bines SD, Coon JS: Diverse multidrug-resistance modification agents inhibit cytolytic activity of natural killer cells. *Cancer Immunol Immunother* 36:133, 1993
24. McGrath T, Varshavsky A: The yeast *STE6* gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. *Nature* 340:400, 1989
25. Raymond M, Gros P, Whiteway M, Thomas DY: Functional complementation of yeast *ste6* by a mammalian multidrug-resistance *mdr* gene. *Science* 256:232, 1992
26. Scharma RC, Inoue S, Roitman J, Schimke RT, Simoni RD: Peptide transport by the multidrug resistance pump. *J Biol Chem* 267:5731, 1992
27. Cornwell MM, Safa A, Felsted RL, Gottesmann MM, Pastan I: Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150- to 170-kDa protein detected by photoaffinity labeling. *Proc Natl Acad Sci USA* 83:3847, 1986
28. Safa AR: Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. *Proc Natl Acad Sci USA* 85:7187, 1988
29. Yusa K, Tsuruo T: Reversal mechanism of multidrug resistance by verapamil: Direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. *Cancer Res* 49:5002, 1989
30. Berman E, Adams M, Duiyou-Osterndorf R, Godfrey L, Clarkson B, Andreeff M: Effect of tamoxifen on cell lines displaying the multidrug-resistant phenotype. *Blood* 77:818, 1991
31. Foxwell BMJ, Mackie A, Ling V, Ryffel B: Identification of the multidrug related P-glycoprotein as a cyclosporin binding protein. *Mol Pharmacol* 36:543, 1989
32. Tamai I, Safa A: Competitive interaction of cyclosporins with vinca alkaloid-binding site of P-glycoprotein in multidrug-resistant cells. *J Biol Chem* 266:16509, 1990
33. Saeki T, Ueda K, Tanigawara Y, Hori R, Komano T: Human P-glycoprotein transports cyclosporin A and FK506. *J Biol Chem* 268:6077, 1993
34. Mechetner EB, Roninson IB: Efficient inhibition of P-glycoprotein-mediated multidrug resistance with a monoclonal antibody. *Proc Natl Acad Sci USA* 89:5824, 1992
35. Hamada H, Tsuruo T: Functional role for the 170-180 kDa glycoprotein specific to drug resistant tumor cells as treated by monoclonal antibodies. *Proc Natl Acad Sci USA* 83:7785, 1986
36. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156, 1987
37. Hunter J, Hirst BH, Simmons NL: Epithelial secretion of vinblastine by human intestinal adenocarcinoma cell (HCT-8 and T84) layers expressing P-glycoprotein. *Br J Cancer* 64:437, 1991
38. Zacherl J, Hamilton G, Thalhammer T, Riegler M, Cosentini EP, Ellinger A, Bischof G, Schweitzer M, Teleky B, Koperna T, Wenzl E: Inhibition of P-glycoprotein mediated vinblastine transport across HCT-8 intestinal carcinoma monolayers by verapamil, cyclosporine A and SDZ PSC 833 in dependence of extracellular pH. *Cancer Chemother Pharmacol* 34:125, 1994
39. Elliott JF, Lin Y, Mizel SB, Bleackly RC, Harnish DG, Paetkau V: Induction of interleukin-2 messenger RNA inhibited by cyclosporin A. *Science* 226:1439, 1984
40. Hughson EJ, Hopkins CR: Endocytic pathways in polarized Caco-2 cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces. *J Cell Biol* 110:337, 1990
41. March CJ, Mosley B, Larsen A, Cerretti DP, Braedt G, Price V, Gillis S, Henney CS, Kronheim SR, Grabstein K, Conlon PJ, Hopp TP, Cosman D: Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs. *Nature* 315:641, 1985
42. Singer II, Scott S, Hall GI, Limjoco G, Chin J, Schmidt JA: Interleukin-1 beta is localized in the cytoplasmic ground substance but is largely absent from the golgi apparatus and plasma membranes of stimulated human monocytes. *J Exp Med* 167:389, 1988
43. Young PR, Krasney PA: Stimulation of interleukin-1 beta secretion in monkey kidney cells by coexpression of the mammalian P-glycoprotein 1, in Ghezzi P, Mantovani A (eds): *Pathophysiology and Pharmacology of Cytokines*. Biomedical, 1992, p 21
44. Raghu G, Park SW, Roninson IB, Mechetner EB: P-glycoprotein transports interleukin-2 in human T-lymphocytes. (submitted for publication)
45. Bommhardt U, Cerottini J-C, MacDonald HR: Heterogeneity in P-glycoprotein (multidrug resistance) activity among murine peripheral T cells: correlation with surface phenotype and effector function. *Eur J Immunol* 24:2974, 1994
46. Hardy SP, Goodfellow HR, Valverde MA, Gill DR, Sepulveda V, Higgins CF: Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume-activated chloride channels. *EMBO J* 14:68, 1995
47. Abraham EH, Prat AG, Gerweck L, Seneviratne T, Arceci RJ, Kramer R, Guidotti G, Cantiello HF: The multidrug resistance (*mdr1*) gene product functions as an ATP channel. *Proc Natl Acad Sci USA* 90:312, 1993
48. Gosland MP, Brophy NA, Duran GE, Yahanda AM, Adler KM, Hardy RI, Halsey J, Sikic BI: Bilirubin—A physiologic substrate for the multidrug transporter. *Proc Am Assoc Cancer Res* 32:426a, 1991
49. Schinkel AH, Wagenaar E, van Deemter L, Mol CA, Borst P: Absence of the *mdr1a* P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* 96:1698, 1995