

A NEW PROCEDURE FOR THE QUANTITATIVE ASSESSMENT OF P-GLYCOPROTEIN EFFLUX PUMP ASSOCIATED WITH HUMAN T LYMPHOCYTES

Introduction: P-glycoprotein (P-gp), a multi-drug transporter located in plasma membranes, reduces intracellular availability of some drugs. Upregulation of P-gp has been observed in some clinical situations, including chronic inflammatory disease and viral infection. However, P-gp is expressed in only a small subset of peripheral blood mononuclear cells (PBMC) and at much lower quantities than it is on P-gp-positive cell lines used in other studies.

Methods: P-gp expression was assessed by flow cytometry by using a commercially available, anti-P-gp, allophycocyanin-conjugated monoclonal antibody. Flow cytometry was also used to determine the efflux activity associated with P-gp; with this process, re-fluxed fluorescent P-gp substrate, rhodamine 123 (Rho123), was determined by the subsequently identified P-gp-positive PBMC subset. Use of verapamil during the dye-loading procedure maximized the amount of dye retained by the cells.

Results: The use of allophycocyanin-conjugated monoclonal antibody allowed for the identification of P-gp-positive PBMC subsets, even when the cells were fully loaded with Rho123. We used a logical gating strategy to identify a P-gp-positive PBMC subset, after which P-gp efflux activity of the PBMC subset could be quantitatively assessed. This new procedure enabled us to assess the P-gp efflux function of T lymphocytes in some clinical situations, which induced P-gp upregulation *in vivo*.

Conclusion: This new procedure enables us to quantitatively assess the P-gp efflux activity associated with PBMC. (*Ethn Dis.* 2008;18[Suppl 2]:S2-75-S2-80)

Key Words: New Procedure, P-gp Efflux, Quantitative Assessment, Peripheral Blood Mononuclear Cells

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INTRODUCTION

P-glycoprotein (P-gp) is a plasma membrane-bound multidrug transporter that actively pumps out a wide variety of chemicals from the interior of the cell, critically determining the intracellular bioavailability of numerous drugs. It is found in different cells, including T lymphocytes and B lymphocytes, which determine the function of host immune responses. P-gp is expressed in only a small subset of peripheral blood mononuclear cells (PBMC) and at much lower quantities than it is on P-gp-positive cell lines used in other studies.^{1,2} Previous studies have shown that P-gp is upregulated under some stress conditions and also in inflammatory diseases such as lupus.³ Several studies have demonstrated that P-gp is upregulated on CD4-positive T cells infected by human HIV-1; such upregulation could severely minimize the intracellular bioavailability of HIV-1 protease inhibitors, which are excellent P-gp substrates.^{4,5}

Both the quantity of P-gp expressed in certain cells and its efflux pump activity are usually assessed by flow cytometry, after loading P-gp-positive cell lines with the green-fluorescent P-gp substrate, rhodamine 123 (Rho123).⁶⁻⁸ However, P-gp is detectable only at a much lower density and only in a small subpopulation of PBMCs. Currently available procedures could not, because of a number of technical problems, be used to assess the efflux pump activity of the P-gp-expressing PBMC subset.

The present study described the modification of the procedures that enabled us to quantitatively assess the efflux pump activity associated with P-gp-expressing lymphocytes from

normal individuals or from patients with chronic inflammatory diseases.

METHODS

Participants

Venous blood samples were aseptically collected by using BD Vacutainer Blood Collection Tubes containing sodium heparin (Becton, Dickinson and Co., Franklin Lakes, NJ). EDTA-containing Vacutainer tubes were avoided because of their potential for compromising P-gp pump activity. The present study protocol and the informed consent form were fully reviewed and approved by the Ponce School of Medicine Institutional Review Board on the Protection of Human Subjects (FWA00000345).

Preparation of PBMC

Approximately 10 mL blood was loaded on top of a Ficoll-Hypaque gradient in an ACCUSPIN System-HISTOPAQUE tube (Sigma-Aldrich, Inc., Saint Louis, Mo) and centrifuged at room temperature at 2500 rpm for 30 minutes. The plasma layer on the top was gently aspirated off, and the mononuclear cells banding at the interphase were collected by aspiration with Pasteur pipettes. Mononuclear cells were collected by centrifugation and washed twice in sterile RPMI-1640 medium (Sigma-Aldrich, Inc.) supplemented with 10% fetal bovine serum. The cell concentration was adjusted, whenever feasible, to $\approx 1 \times 10^6$ cells/mL. PBMC subsets were identified by flow cytometry after treatment with murine monoclonal antibodies against their differentiation markers, which were conjugated with their respective fluorochromes.

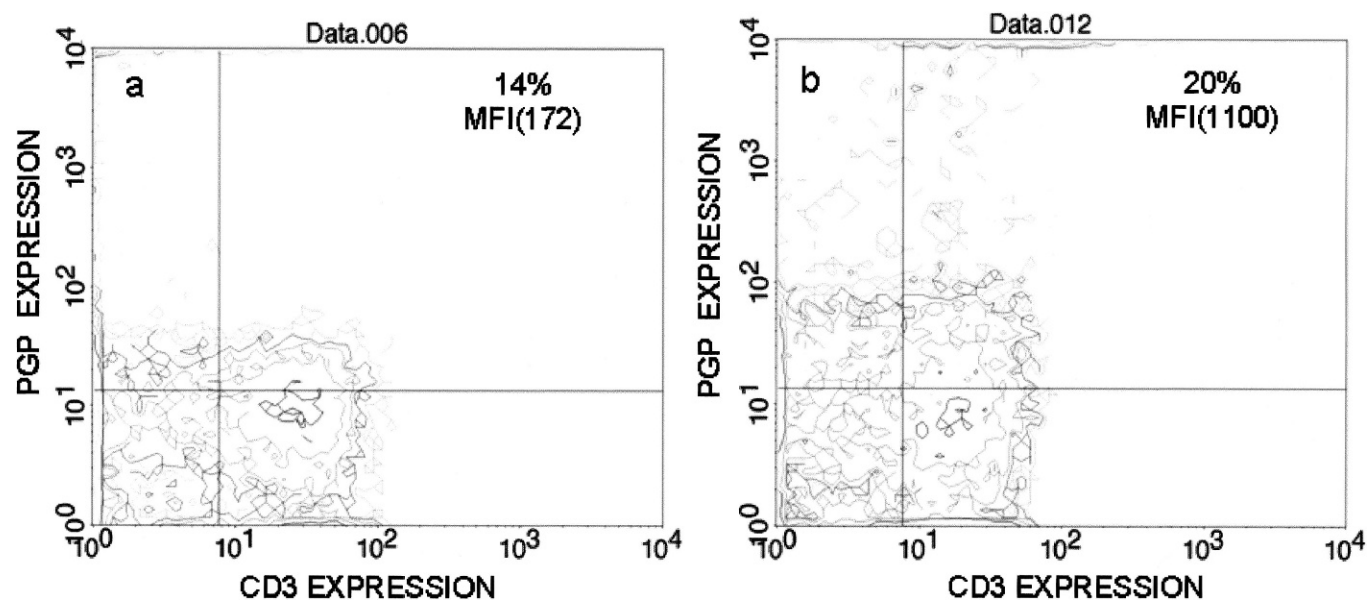


Fig 1. P glycoprotein (P-gp) expression on normal peripheral blood mononuclear cells (PBMC) activated *in vitro*. P-gp expression of CD3-positive and CD3-negative cells (x-axis) is shown by a contour plot using the program software. Quantity of P-gp expressed on the cells is expressed as the fluorescence intensity (APC: FL-4) (y-axis). Figures A and B represent the P-gp expression profiles of normal PBMC before and after activation, respectively. Percentage of P-gp expressing CD3-positive T lymphocytes (upper right) and the average quantity of P-gp expressed, as assessed by the mean fluorescence intensity (MFI), are shown in each figure: 14% and 20% of cells, before and after activation by (PMA+IM) for 24 hours, expressed 172 and 1100 MFIs of P-gp, respectively.

Assessment of P-gp Expression by Lymphocyte Subsets

In this study, lymphocytes were identified by flow cytometry according to their forward and side scatter characteristics (lymphocyte gate). Lymphocytes were only classified in this study as CD3-positive T lymphocytes and CD3-negative non-T lymphocytes. One of the existing technical problems to assess efflux pump activity associated with P-gp-positive T cells (or non-T cells) was the inability of the phycoerythrin-conjugated anti-human P-gp monoclonal antibody (clone 17F9; BD Biosciences, San Jose, Calif) to detect P-gp expressed on human PBMC. While the same monoclonal antibody conjugated with fluorescein-isothiocyanate (FITC) could distinguish P-gp-positive—albeit of low density—from P-gp-negative PBMC, the emission spectrum of FITC significantly overlapped with that of Rho123, the previously mentioned fluorescent P-gp substrate that was used in most reports.⁶⁻⁸ Allophycocyanin (APC) conjugate of the same antibody was,

therefore, custom-ordered and found to be satisfactory for P-gp detection in PBMC, with negligible overlapping of the emission curve with that of Rho123.

Activation of T Lymphocytes

Since normal PBMC expressed very little P-gp, PBMC were first activated by the addition of 1 $\mu\text{g}/\text{mL}$ of phorbol 12-myristate 1,3-acetate (PMA) (Sigma) and 40 ng/mL of IM (IM) (Sigma) for 24 hours at 37°C (98°F). Activated PBMC were washed twice with the RPMI medium and then treated with 10 μL of APC-conjugated murine anti-human P-gp monoclonal antibody (clone 17F9) for 20 minutes at room temperature.

Assessment of P-gp Efflux Pump Activity

Rho123 was loaded by the method described by Calado et al,⁶ with minor modifications. Briefly, PBMC were treated with 1 mM verapamil (Sigma) for one hour at 37°C (98°F) before the loading of .5 $\mu\text{g}/\text{mL}$ Rho123 for

30 minutes at 37°C (98°F). Cells were washed twice with phosphate buffered saline (Sigma), and the FL1 (Rho123) profile was acquired by the flow cytometer, BD FACSAria Cell Sorting System. The Rho123 profile of loaded T lymphocytes was obtained as the FL1 histogram of events logically gated for the lymphocyte gate events that were also CD3 (FL3) positive and P-gp (FL4) positive, according to BD CellQuest software. The post-efflux Rho123 (FL1) profile was similarly obtained after incubating the cells for 150 minutes at 37°C (98°F). The culture was incubated similarly but in the presence of 1 mM of verapamil, which was used to adjust for possible leakage or non-P-gp-mediated efflux of the fluorescent dye.

RESULTS

P-gp Expression on Normal PBMC Activated *in vitro*

As shown in Figures 1a and 1b, normal PBMC did not consistently

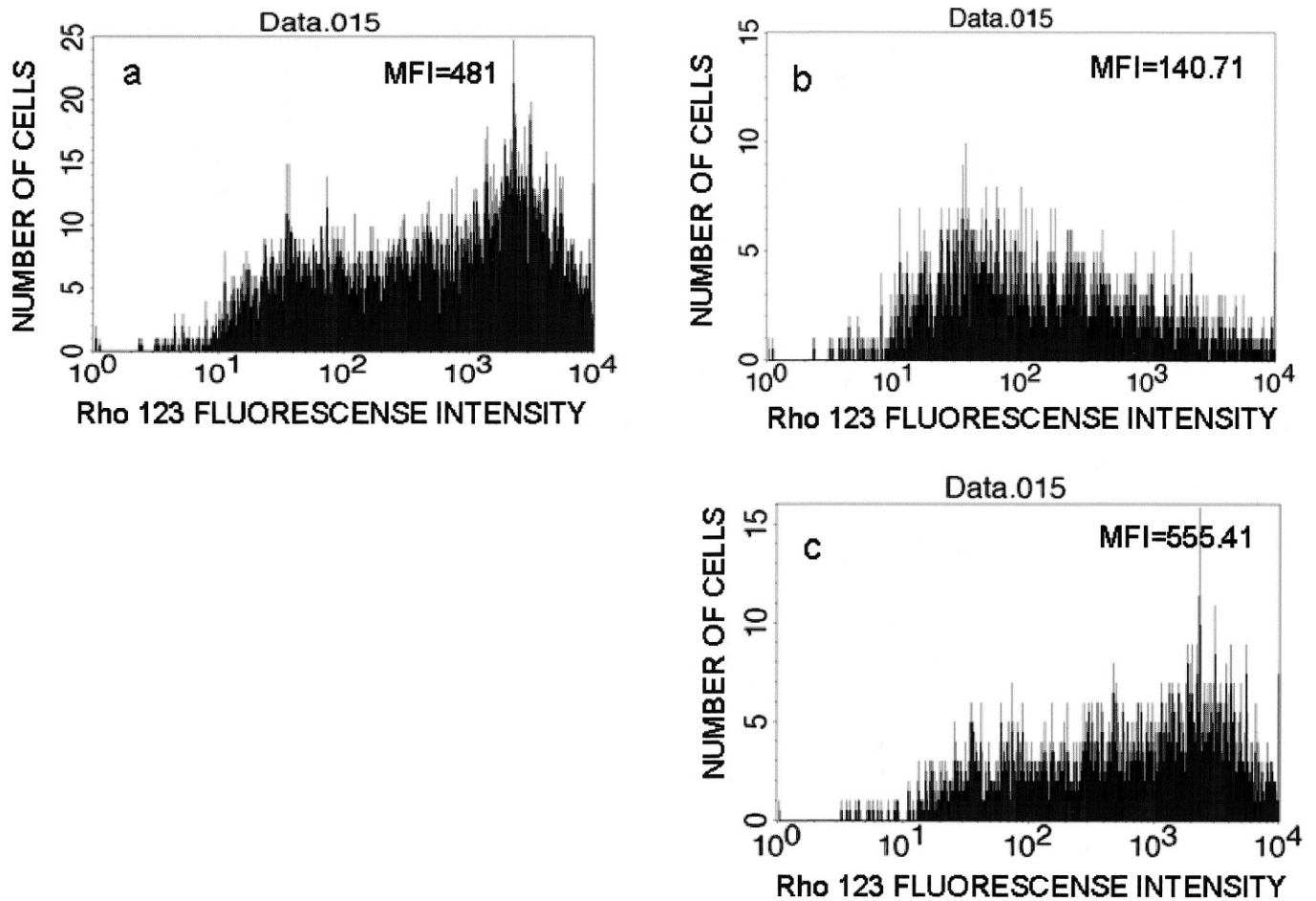


Fig 2. Use of verapamil to enhance the Rho123 loading. Rho123 was loaded, as described in the text, in the presence of 1 mM verapamil, for 30 minutes. Figures 2a, 2b, and 2c represent the Rho123 retention profiles of the total, P glycoprotein (P-gp)-positive, and P-gp-negative T-cell populations. Horizontal (x) axis represents the quantity (fluorescence intensity) of the dye retained and y-axis the number of cells retaining each given quantity of the dye.

express enough P-gp to be clearly distinguishable from P-gp-negative cells (Figure 1a). Activation by a combination of PMA and IM for 24 hours markedly increased the P-gp expression on PBMCs, and $\approx 20\%$ of activated CD3-positive T lymphocytes could be classified as P-gp-positive (Figure 1b), as opposed to only $\approx 14\%$ of non-activated cells. Activation of T lymphocytes increased not only the number of cells expressing P-gp but also the density of P-gp expressed in each cell, eg, 172 mean fluorescence intensity (MFI) of P-gp on T lymphocytes before activation vs 1100 MFI after activation. Treatment with PMA and IM similarly

activated both T cells and non-T (CD3-negative) lymphocytes (Figure 1b). In this study, however, we only described the assessment of the efflux pump associated with T lymphocytes.

Use of Verapamil to Enhance the Retention of Rho123

Figure 2 shows a typical FL1 histogram of the total T-lymphocyte population (2a) and its P-gp-positive (2b) or P-gp-negative (2c) subsets, which were loaded with Rho123 in the presence of verapamil, a P-gp inhibitor. Two peaks in Figure 2a actually consisted of the dye-loading profiles of P-gp-negative (Figure 2c: MFI=555.41) and P-gp-

positive (Figure 2b: MFI=140.71) cell populations. The use of verapamil helped to obtain a higher and more uniform dye-loading profile and therefore it was included in all dye-loading procedures. However, cells continued to partially efflux the dye even at the highest nontoxic verapamil concentration, which was incorporated in all dye-loading procedures. Cell cultures that included verapamil during dye loading and efflux were used as the controls to specifically calculate the P-gp-mediated efflux pump activity (data not shown). Other cellular pumps besides P-gp may have pumped Rho123. Rho123 loading did not significantly alter P-gp expression of the cells.

Measurement of P-gp Efflux Pump Activity of Activated Normal T Lymphocytes

Figure 3 shows the typical FL1 histograms of Rho123 retained by P-gp-positive T lymphocytes after efflux (at 37°C for 150 minutes with verapamil) (3a) and after efflux (without verapamil) (3b). In each histogram, two distinct populations were identified, ie, those cells that effectively pumped Rho123 (M1) showing MFI=33.63 (Figure 3a) and MFI=10.14 (Figure 3b) and those that did not (M2) with MFI=740 (Figure 3a) and MFI= 730 (Figure 3b). The P-gp-mediated efflux pump activity of each culture was calculated as $(A-B)/A$, where A and B were the quantities of Rho123 remaining without P-gp efflux activity (in the presence of verapamil) (Figure 3a) and after P-gp-mediated activity (Figure 3b). Empirically, therefore, A and B were calculated as the addition of $(MFI) \times (\% \text{ cellular events})$ of M1 and M2 populations, in respective sets of assays. The percentage of cellular events rather than the actual cell event number was used to correct for the fluctuation in the total number of cell events acquired in each flow cytometric run.

In this particular set of data, for example, the culture without P-gp efflux (ie, with verapamil) (Figure 3a) retained $A = (30.09 \times 33.63) + (69 \times 739.84) = 52,060$ arbitrary units of Rho123; while the culture with P-gp-mediated efflux (ie, without verapamil) (Figure 3b) had $B = (78.84 \times 10.14) + (20.6 \times 730.31) = 15,839$ Rho123 units. The amount of Rho123 pumped by the P-gp pump was thus calculated as $[(52,060 - 15,839) / 52,060] \times (100) = 69.5\%$.

Validation of this New Procedure Using T Lymphocytes with Elevated P-gp *in vivo*

In chronic intravenous cocaine users, P-gp is highly upregulated on T cells (Y. Yamamura, unpublished data). Figure 4a shows an example of P-gp expression in a chronic cocaine user's T

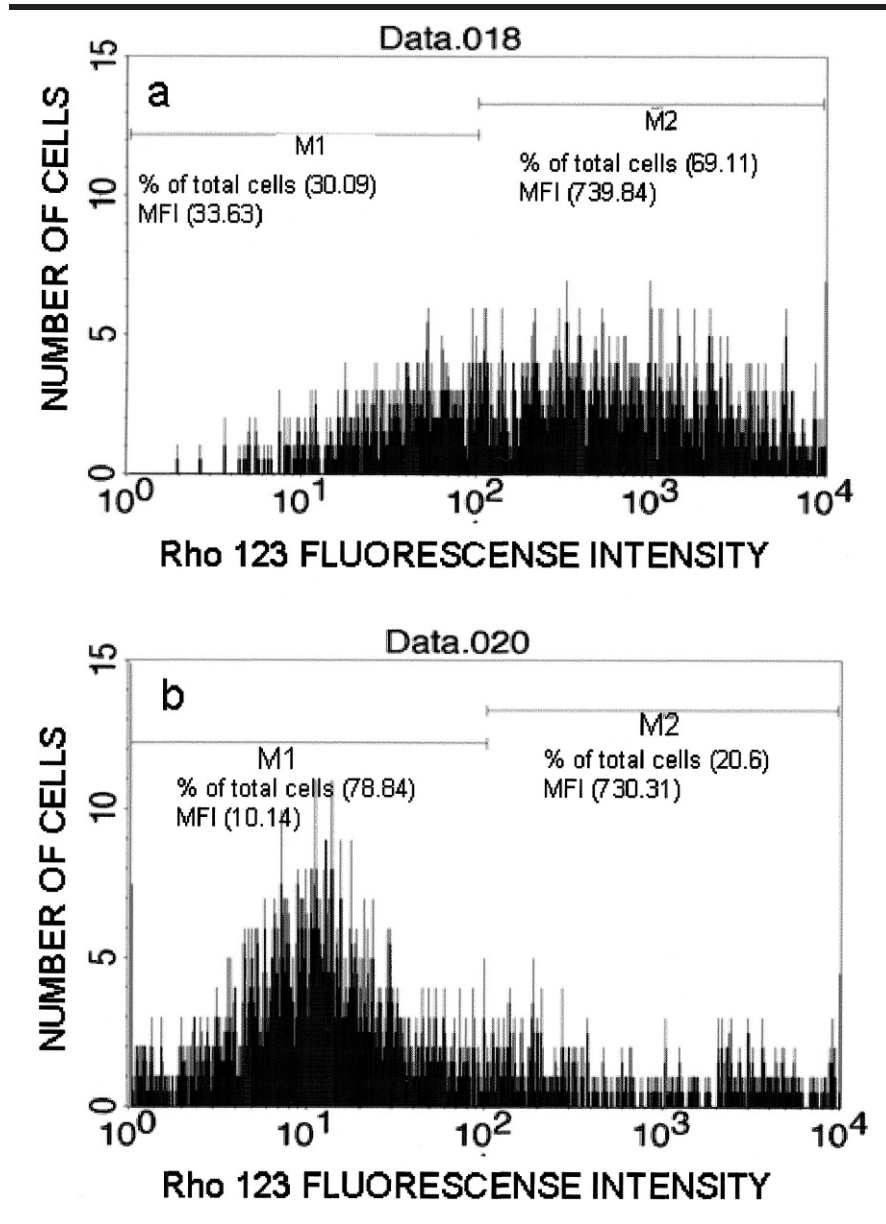


Fig 3. Measurement of P-glycoprotein (P-gp) efflux pump activity of activated normal T lymphocytes. Rho123-loaded T cells were allowed to efflux at 37°C for 150 minutes either with (3a) or without (3b) 1 mM verapamil, as described in the text. The histogram was obtained, as also described in the text, by selecting the population of P-gp-positive T cells by using a logical gating strategy.

lymphocytes (15% were P-gp positive, with MFI=270.76). Figure 4b shows Rho123 profile of the P-gp-positive T cells. Figures 4c and 4d represent the FL1 (Rho123) histograms after the cells were allowed to efflux with and without verapamil, respectively. Using the same formula described above, $A = 223,303$ and $B = 61,833$. Thus, the upregulated

P-gp pump of the T lymphocytes from a chronic drug user was able to efflux 72.3% of the substrate dye.

DISCUSSION

A number of published reports clearly demonstrate that either malignant

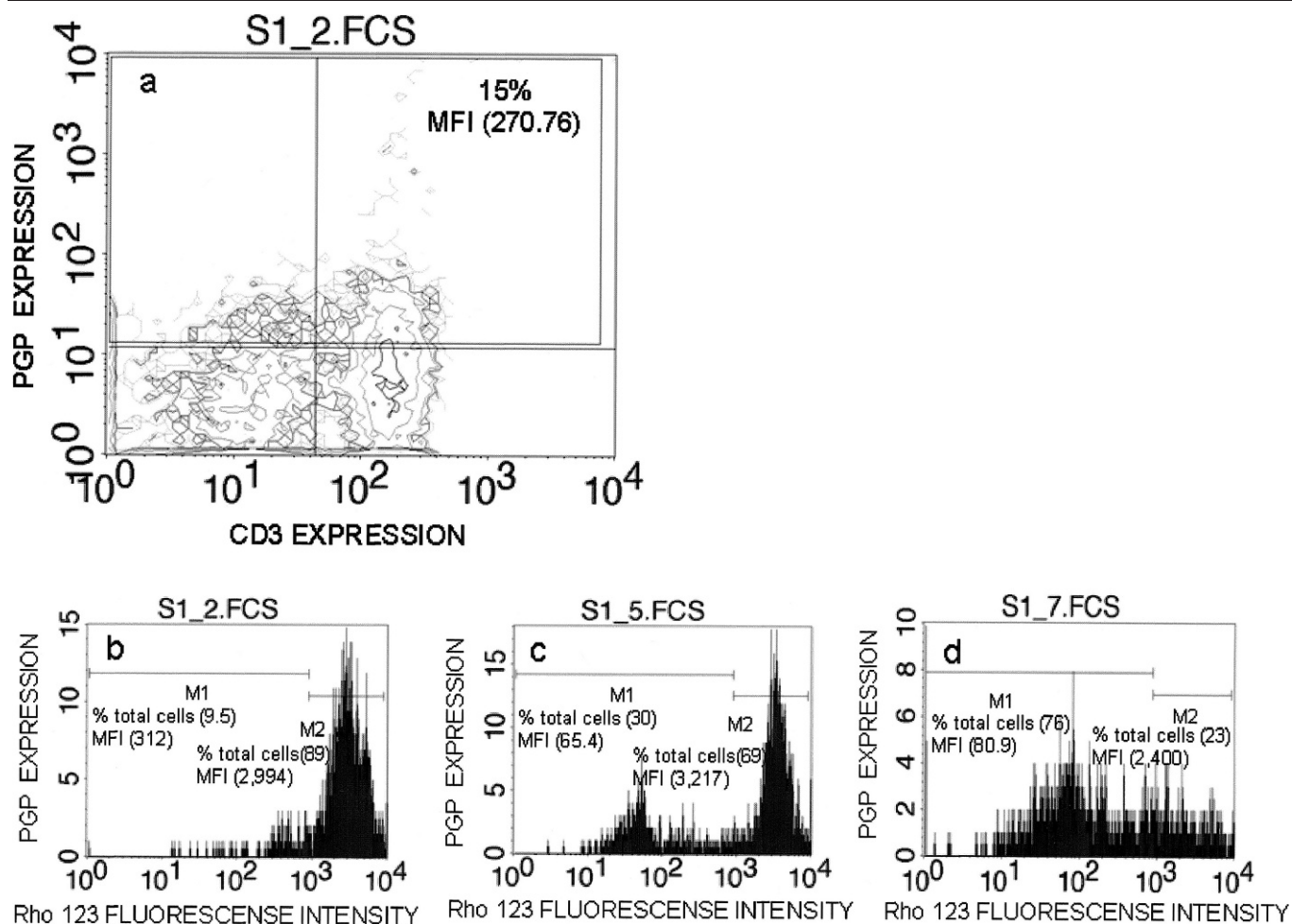


Fig 4. Validation of this new procedure using T lymphocytes with elevated P-glycoprotein (P-gp) *in vivo*. Figure 4a shows an example of P-gp expression by T lymphocytes of a chronic cocaine user. Figures 4b, 4c, and 4d represent the Rho123 retention profiles of the P-gp-positive T cells before efflux, efflux with verapamil (a P-gp inhibitor), and efflux without verapamil, respectively.

leukemia cells or other types of cell lines with high levels of P-gp expression are capable of effectively pumping the P-gp substrate, Rho123.¹ Further, certain clinical conditions, including the HIV-1 infection, cause P-gp expression to be upregulated on mononuclear cells or their subsets. Such mononuclear cells with upregulated P-gp *in vivo* may be involved in regulating the intracellular bioavailability of certain drugs, such as HIV-1 protease inhibitors. However, two serious obstacles prevented an accurate, quantitative assessment of P-gp efflux function of PBMC and PBMC subsets: 1) P-gp is expressed only by a small proportion of normal PBMC and only at a very low density (Figures 1a

and 1b) and 2) the P-gp substrate dye most commonly used, Rho123, could not be used to measure P-gp-associated efflux pump function in combination with FITC-conjugated anti-human P-gp monoclonal antibody because of a significant overlapping of the emission curves of Rho123 and FITC. The FITC conjugate of the BD monoclonal antibody (clone 17F9) efficiently identifies the presence of P-gp—albeit at a very low level—on human PBMC. However, a phycoerythrin conjugate of the same monoclonal antibody—for unexplained reasons—failed to demonstrate P-gp on the same PBMC. After close discussion with the BD technical team, an APC conjugate was custom made for the

study. The use of APC conjugate provided the means of identifying P-gp-positive cells. The use of verapamil during dye loading increased the MFI of the loaded cells, and the use of a logical gating strategy enabled us to assess Rho123 (FL1) efflux by P-gp-positive PBMC, a severe minority. A simple formula was developed to quantitatively assess the proportion of the substrate dye that was pumped specifically through the P-gp-mediated pump.

In conclusion, modification of several key steps in the procedure that has been used widely (mostly with established cell lines with high P-gp expression) has made it possible for us to quantitatively assess the P-gp efflux

pump activity of PBMC and T lymphocytes. The procedure was used to assess the P-gp efflux pump function of T lymphocytes from chronic drug addicts, who have highly upregulated P-gp expression in vivo. It was thus possible to correlate the phenotypic expression of P-gp molecules and its functional efflux pump activity in normal T lymphocytes as well as in cells taken from patients suffering from certain clinical abnormalities.

Implications for the Reduction of Health Disparities

P-gp affects some drugs and a number of pharmaceutical compounds; however, no reliable assay was available for use with PBMCs. This new assay allows for the correlation of P-gp expression and functionality in PBMCs, which—by taking into consideration the impact of the protein's function on

the drug's bioavailability—provides a new tool to improve patient therapy.

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