

Early steps of the P-glycoprotein expression in cell cultures studied with vital fluorochrome

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This study shows that flow cytometry analysis of the rate of fluorochrome Rh123 efflux may be used for detection of cells at initial steps of P-glycoprotein expression and of minor subpopulations of multidrug-resistant (MDR) variants in human cell lines. This method also evaluates the fraction of low-level MDR cells among peripheral blood leukocytes of patients with chronic myeloid leukemia. The alterations in Pgp function were revealed in rat hepatoma cells after short treatment with colchicine.

Multidrug resistance; P-glycoprotein; Rhodamin 123

1. INTRODUCTION

The 170–180 kDa transmembrane glycoprotein (P-glycoprotein or Pgp) associated with multidrug resistance (MDR) is involved in drug transport across the plasma membrane of cells [1]. This protein is an energy-dependent efflux pump for anticancer drugs, various fluorescent dyes and some other substances [2,3]. Pgp is expressed in some normal (mainly secretory) tissues, in cell lines selected *in vitro* by drugs, and in drug-resistant cancers. It is usually detected by indirect immunofluorescence using anti-Pgp monoclonal antibodies (Mab) and by mRNA assay [1,4]. However, it is necessary not only to reveal the Pgp-positive cells and Pgp mRNA, but also to test Pgp-mediated efflux. Human malignancies often exhibit low levels of Pgp. Therefore it should be possible to detect early stages of Pgp activity. A simple test for Pgp-mediated efflux was elaborated. It evaluates the efflux of Pgp-transported fluorescent dyes from the cells [3]. Here we study whether this technique can assess low rates of Pgp-mediated efflux in tumor cell lines and in human PBL (in CML patients). We also studied Pgp function alterations in cells treated with the MDR-inducing drug colchicine (CH).

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Abbreviations: CH, colchicine; CML, chronic myeloid leukemia; Mab, monoclonal antibodies; MDR, multidrug resistance; PBL, peripheral blood lymphocytes; PE, Phycoerythrin; Pgp, P-glycoprotein; Rh123, Rhodamin 123.

2. MATERIALS AND METHODS

Cells used in the study: human melanoma mS and its drug-resistant sub-lines mS-0.02 and mS-0.5 (2- and 130-fold resistant to CH, respectively); rat hepatoma McA RH 7777 and a 17-fold CH-resistant derivative McA RH 7777-0.4. Cell lines and culture conditions were as described previously [5]. For Pgp assay the cells were detached from the substratum by 0.02 M EDTA. PBL of the patient with CML, blast crisis, were prepared as described in [6].

Pgp was evaluated by staining the cells with anti-Pgp Mab UIC2 as in [7]. To measure Pgp function we have used the fluorescent dye Rhodamine 123 (Sigma) (Rh123) and the technique described in [3]. For double labeling of the cells by Rh123 and Mab UIC2 the Rh123-stained cells were treated as in [2]. Mab UIC2 (Ig 2a) was the kind gift of Dr. E.B. Mechetner (Department of Genetics, Univ. of Illinois, Chicago, USA). Conjugates of Ig 2a labeled by Phycoerythrin (PE) were the kind gift of Dr. T.M. Thischmann (Johns Hopkins Oncology Center, Pediatric Oncology, Baltimore, USA). The cells were analyzed in the flow cytometer FACScan (Becton Dickinson). Data were analyzed by the Hewlett Packard program.

3. RESULTS AND DISCUSSION

We checked whether Rh123 technique can detect cells with initial levels of drug resistance and to discriminate cells with various drug-resistance levels. The double staining of the cells by Rh123 and by UIC2 antibodies (Fig. 1) demonstrates Pgp on the 2-fold CH-resistant cells and the correlation between the level of Pgp expression and the retention of the fluorescent dye. Cells with a 2-fold level of drug resistance (Fig. 1b) differ from the wild-type cells as well as from more resistant counterparts (Fig. 1a,c). This demonstrates that the first steps of selection of human melanoma cells for MDR lead to isolation of the cells with altered Pgp function.

In human malignancies only a part of the cells may express Pgp. To determine whether comparatively small portions of the cells with Pgp hyperfunction may be

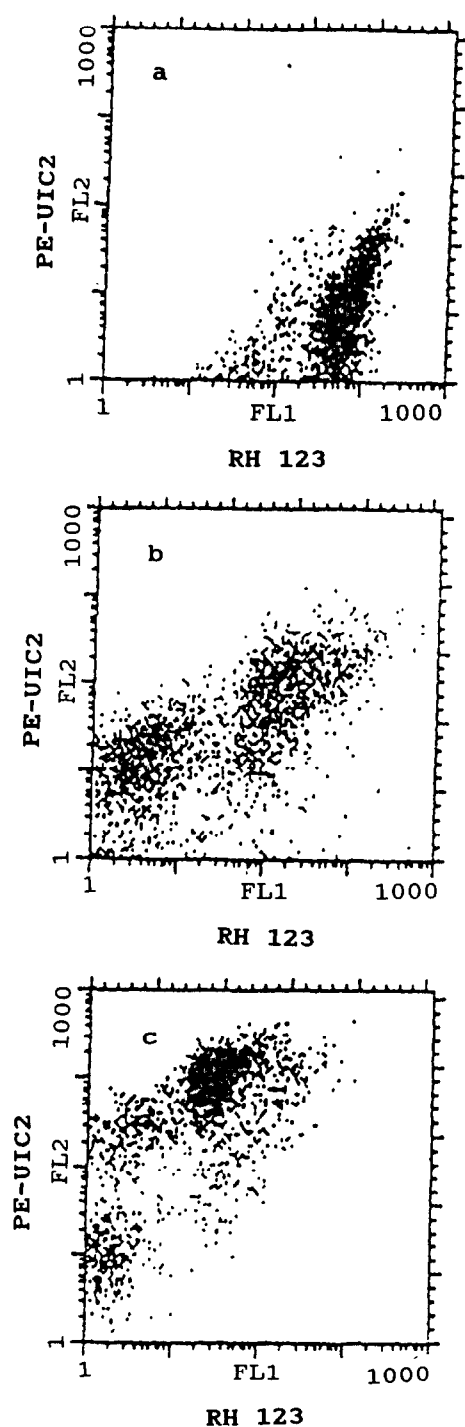


Fig. 1. Correlation between the retention of Rh123 and expression of Pgp in (a) human melanoma mS; (b) 2-fold CH-resistant mS-0.02; (c) 130-fold CH-resistant mS-0.5. Dot density maps of the cells stained with UIC2 anti-Pgp Mab indirectly labeled with PE (FL2 axis = fluorescence intensity of PE) and with Rh123 (FL1 axis = fluorescence intensity of Rh123). Incubation time in dye-free medium was 30 min.

found in heterogeneous cell populations by Rh123 staining we studied mixtures of mS and mS-0.5 cells (Fig. 2). Flow cytometry shows that the intensity of

signals both for CH-sensitive and -resistant cells correlates with the fraction of the cells in cell mixtures (Fig. 2). 10% of mS-0.5 cells are clearly recognizable.

We used the cytometric technique to find MDR cells in blood and bone marrow of patients with CML. It is known that after chemotherapy MDR cells may appear. In the blood of patient 'K' a part of the cell population exhibited Pgp (Fig. 3). The quantity of Pgp-positive cells detected by the immunofluorescence method and Rh123 staining was approximately the same (Figs. 3 and 4); nearly half of the cell population was UIC2-reactive and excluded Rh123 (Fig. 4a,b). The Pgp function in these cells was relatively weak since the dye was

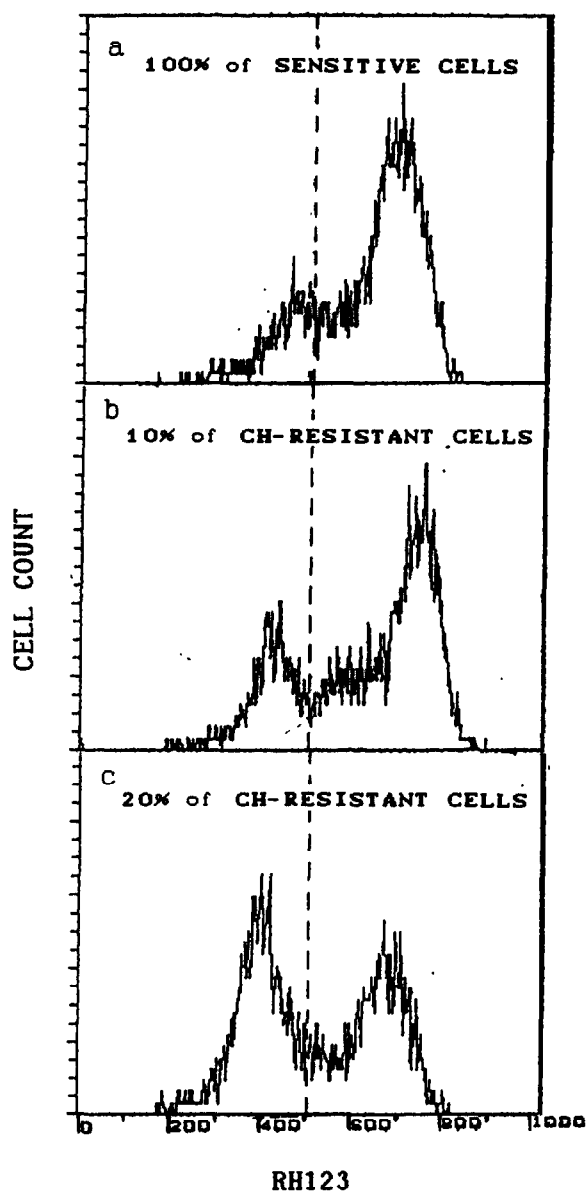


Fig. 2. Evaluation of minor CH-resistant cell populations. Logarithmic fluorescence intensity of Rh123 treated (a) mS cells; (b) mS (90%) + mS-0.5 (10%); (c) mS (80%) + mS-0.5 (20%). Incubation time of the dye-stained cells in dye-free medium was 15 min.

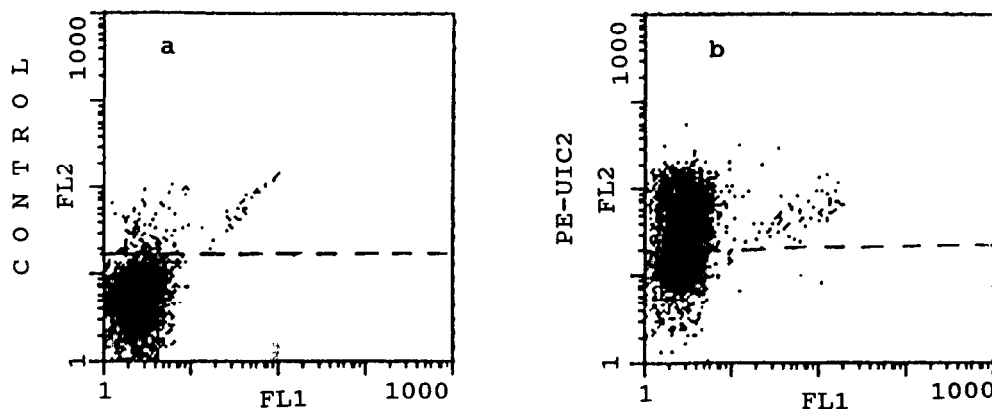


Fig. 3. Evaluation of the Pgp expressing cells in the blood of CML patient 'K'. Dot density maps of the cells stained with (a) mouse IgG2 isotype control antibodies; (b) UIC2 Mab indirectly labeled with PE (FL2 axis = fluorescence intensity of PE).

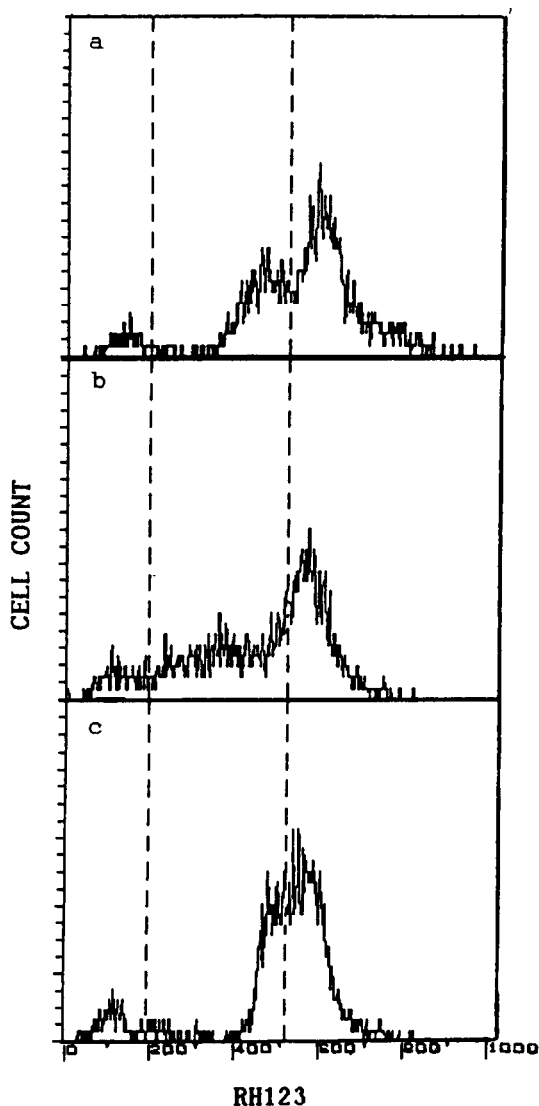


Fig. 4. Efflux of Rh123 from the blast cells of CML patient 'K' (logarithmic fluorescence intensity). Note the part of the histograms between the dotted lines. Exposure in dye-free medium for: (a) 15 min; (b) 60 min; (c) in Verapamil-supplemented medium (10 µg/ml).

excluded from the cells slowly (for 60 min; Fig. 4a,b). Rh123 efflux was Verapamil-sensitive (Fig. 4c). Thus it is possible to determine by the rate of Rh123 efflux both the portion of cells with functioning Pgp and the rate of Pgp-mediated efflux.

It was shown that cytotoxic drugs induce elevation of *mdr1* (Pgp) mRNA in rodent cells [8,9]. We studied alterations of Pgp function in response to CH treatment. Treatment of rat hepatoma McA Rh 7777 cells with 0.01 µg/ml of CH for 24 h increased the fraction of Rh123-dull cells in the cell population (Fig. 5a,b). Thus CH-treated cells were more effective in dye efflux than untreated cells. As Fig. 5c,d show, treatment of CH-resistant McA Rh 7777-0.4 by 0.01 µg/ml of CH for 3 h also resulted in an increase in the proportion of dye-excluding cells. It is noteworthy that CH-resistant McA Rh 7777-0.4 was selected and propagated continuously in the medium supplemented with 0.4 µg/ml of CH. Before the experiment CH was omitted from culture medium for 48 h. Stimulation of Pgp function by a CH concentration 40-fold less than the selective dose clearly demonstrates the mechanism of drug resistance of these cells. These data also show that using the fluorescence method it is possible to detect initial alterations in Pgp function induced by cytotoxic drugs.

Our data and the data of other authors [2,3,7] show that initial alterations in Pgp function and very early stages of MDR can be detected by measurement of Rh123 exclusion from the cells. This technique would provide adequate information about Pgp-mediated mechanisms of cellular drug resistance and the rate of Pgp function in the cells, as well as on the proportion of MDR cells in heterogeneous cell populations and agents influencing Pgp function in the cells.

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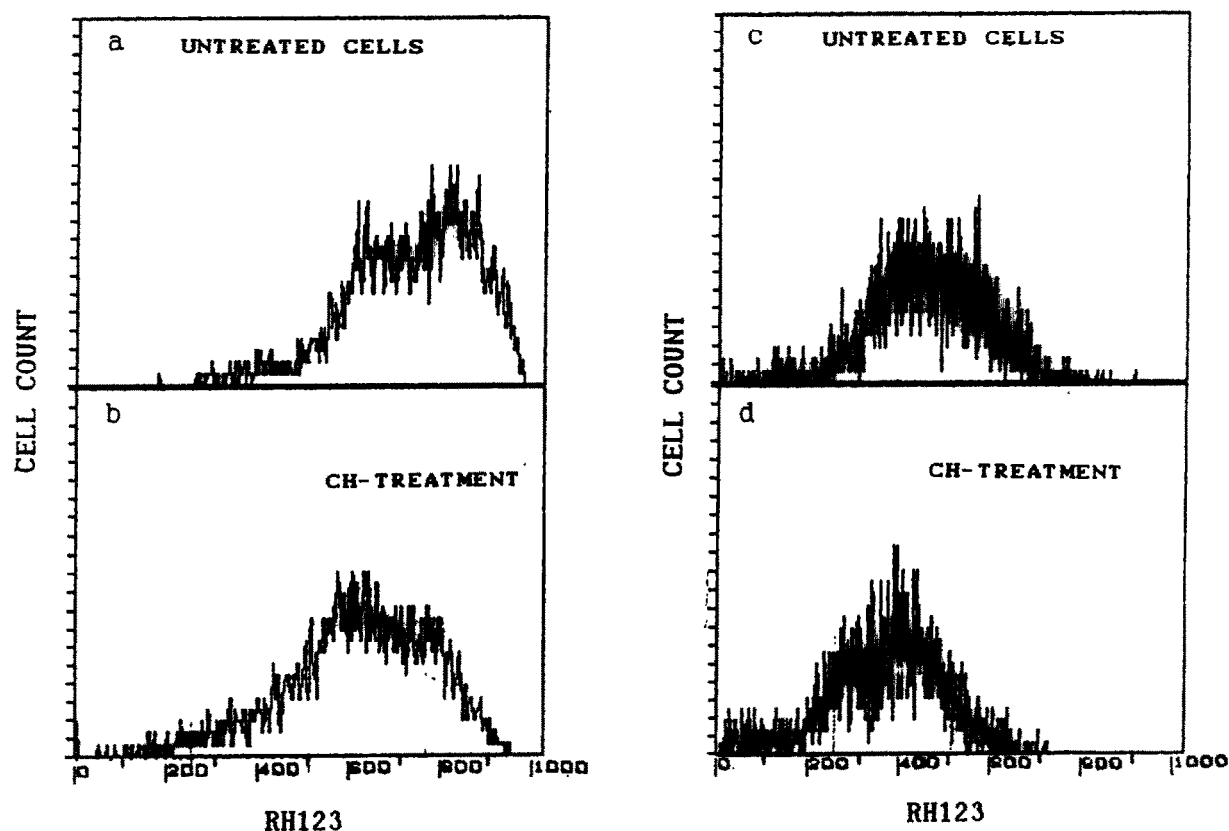


Fig. 5. Influence of CH treatment on Pgp-mediated efflux of Rh123. Logarithmic fluorescence intensity of the cells: (a) of CH untreated rat hepatoma McA 7777; (b) rat hepatoma McA 7777 treated with 0.01 $\mu\text{g/ml}$ CH for 24 h; (c) CH untreated 17-fold CH-resistant rat hepatoma McA 7777-0.4; (d) McA 7777-0.4 treated with 0.01 $\mu\text{g/ml}$ CH for 3 h. All cells were stained with Rh 123 and then incubated in the dye-free medium for 60 min. One of two independent experiments is shown.

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