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Cytofluorimetric Analysis of a Renal Tubular Cell Line and its Resistant Counterpart

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Abstract. *P-glycoprotein (P-gp) and multidrug resistance related protein (MRP) overexpression is often responsible of the development of multidrug resistance in cancer therapy. These proteins are also expressed in normal tissues, where their physiological role is related to the extrusion of endogenous toxins or to secretory function in liver and kidney. The LLC-PK$_1$ cell line is derived from normal pig proximal renal tubule and physiologically expresses low levels of P-gp and MRP. A resistant cell line (LLC-PK$_1$/ADR) has been established in our laboratory by chronic exposure to increasing doses of doxorubicin. Cytofluorimetric analysis of P-gp and MRP expression performed by C219 and MRPm6 immunofluorescence detection showed that these cells overexpress P-gp but not MRP. The uptake of doxorubicin and rhodamine 123 has been quantified in LLC-PK$_1$ and LLC-PK$_1$/ADR cells and compared with data obtained using other tumor cell lines commonly used as reference for studying P-gp or MRP overexpression. P388 sensitive cells and its resistant counterpart P388/ADR cells, which overexpress P-gp and PANC-1 cells, which express high levels of MRP were used. A lower fluorescence intensity was evident with both doxorubicin and rhodamine 123 in LLC-PK$_1$/ADR as well as in P388/ADR cells, that overexpresses P-gp, in comparison with the parental lines. The uptake was increased by a pretreatment with verapamil. Verapamil was completely ineffective on PANC-1 cells, confirming a selective effect of this inhibitor on P-gp. Propidium iodide staining, performed after doxorubicin treatment, confirmed a higher cytotoxicity of the antineoplastic drug in the LLC-PK$_1$ cells compared with the resistant counterpart.*

Acquired resistance to chemotherapeutic agents is a major cause of therapeutic failure in clinical settings. Resistance is often due to the overexpression of membrane energy-dependent transport proteins such as P-glycoprotein (P-gp) (1, 2) and multidrug resistance related protein (MRP) (2-4). P-gp and MRP are members of the ABC protein superfamily, that use the energy derived from ATP hydrolysis to extrude hydrophobic substances, including chemotherapeutics, from the cytoplasm (1-3). These proteins are present also in many normal tissues such as liver, kidney, intestine and endothelia of the blood brain barrier (5-9). Their physiological role, although not yet completely clear, is related to extrusion of endogenous toxins, steroid secretion in the adrenal gland, secretion of bile salt in the liver canaliculi and secretory function in the kidney (10).

It has been previously shown that the expression of these proteins can be modulated both *in vitro* and *in vivo* by chronic treatments with substances that are their substrates (11-15). Induction of these proteins, which can transport a wide range of structurally different substrates, could be responsible for pharmacokinetic alterations that could in turn be responsible for new toxicity.

Cultures of cells derived from normal tissues, that maintain *in vitro* the structure and function of the organ from which they are derived, could be particularly useful to clarify the role of multidrug resistance proteins and the effects of their induction in normal tissues. The LLC-PK$_1$ cells are derived from pig kidney tubular cells (16) and it has been shown that, similarly to the kidney proximal tubule, they physiologically express low levels of P-gp (17, 18). It seems therefore of particular interest to study, in a simple experimental system *in vitro* the possible induction of multidrug resistance proteins in cells derived from normal tissues.

The aim of this study was therefore to evaluate by means of cytofluorimetric analysis, the expression of multidrug resistance proteins, their function and the different toxicity of doxorubicin in LLC-PK$_1$ cell line and in its resistant counterpart LLC-PK$_1$/ADR, obtained by means of a chronic treatment with the antineoplastic drug.

Materials and Methods

Cell lines and establishment of doxorubicin resistant clones. The pig kidney epithelial cell line LLC-PK$_1$ obtained from the American Type Culture

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Collection (ATCC-CRL-1392) was grown in plastic bottles in medium 199 supplemented with 10% fetal bovine serum without antibiotics, in a 5% CO_2-95% air atmosphere at 37°C. The cells were selected for doxorubicin resistance by continuous stepwise increments of doxorubicin concentrations. Cells were seeded at a cell density of 5 x 10^4 cells/ml in 35 mm dishes, in 2 ml of complete culture medium and doxorubicin was then added for 48 hours. The initial concentration of doxorubicin to which cells were exposed was 0.1 μM, which was gradually increased to 20 μM. After incubation with doxorubicin, the cells were washed and cultured in drug free medium, that was changed every fourth day and were again treated with increasing concentrations of doxorubicin when cell number increased at a similar rate as parental cells. A resistant subline tolerating 20 μM doxorubicin, LLC-PK_1/ADR, was collected and stored in liquid nitrogen with 10% dimethylsulfoxide (DMSO) as the cryoprotective agent. The drug resistance phenotype was stable for several months without further selection.

The murine lymphoid neoplasm P388 and its counterpart resistant to doxorubicin, P388/ADR, were obtained from the NCI Frederick Cancer Research Facility/DCT Tumor Repository, (Bethesda, MD, USA) and grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum, without antibiotics.

PANC-1 cells, derived from human pancreas epithelioid carcinoma, obtained from the American Type Culture Collection (ATCC-CRL-1572) were grown in plastic bottles in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum without antibiotics.

Staining with rhodamine 123 and doxorubicin. Cells (1x10^6 cells/ml) were incubated for 120 minutes (P388 and P388/ADR cell lines) or for 240 minutes (LLC-PK_1 and LLC-PK_1/ADR) with different concentrations of rhodamine 123 (Sigma-Aldrich S.r.l., Italy) (from 0.1 μM to 10 μM) in culture medium, in 5% CO_2-95% air atmosphere at 37°C. Similar experimental conditions were adopted when the cell lines P388, P388/ADR, LLC-PK_1, LLC-PK_1/ADR and PANC-1 were incubated for 240 minutes with doxorubicin (Adriblastina, Pharmacia S.p.A) from 10 μM to 100 μM). Some samples were pretreated with 100 μM verapamil for 1 hour at 37°C before the addition of the fluorescent drugs. After incubation, the cells were centrifuged, resuspended in PBS and stored at 4°C until the FACS analysis.

Staining with propidium iodide. After flow cytometric analysis of the uptake of doxorubicin, LLC-PK_1 and LLC-PK_1/ADR cells were centrifuged at 400 xg for 10 minutes at 4°C, resuspended in 1 ml of a staining solution containing RNAse 1 mg/ml, propidium iodide 0.05 mg/ml and Nonidet P-40 0.1% (all obtained from Sigma-Aldrich S.r.l., Italy), then incubated at 37°C for 30 minutes. The cells were again analysed by FACS to determine their viability and the cell cycle stage.

Immunofluorescence staining with C219 and MRPm6 antibodies. For immunofluorescence staining with the murine monoclonal antibody C219, specific for P-gp detection (19), cells (5x10^6/ml) were fixed in 0.2% (vol/vol) paraformaldehyde solution in 100% acetone for 30 seconds. A fixation with 100% ethanol at -20°C for 15 minutes (20) was adopted when the murine monoclonal antibody MRPm6 was used to specifically detect MRP (21). After the fixation step the cells were centrifuged at 400 xg at 4°C for 10 minutes, washed with PBS added with 3% BSA and resuspended in the same buffer. After 15 minutes at room temperature, the primary antibodies C219 (5-10 μg/ml) and MRPm6 (1.25-2.5 μg/ml) were added to aliquots of 200 μl of cells (about 1x10^6 cells). To determine the aspecific staining, the respective isotypic controls IgG2a (5-10μg/ml) and IgG1 (1.25-2.5μg/ml) were also used. After 1 hour at room temperature the cells were washed twice with PBS-3%BSA and finally resuspended in PBS-3%BSA containing the FITC conjugated antimouse IgG secondary antibody (Sigma 1 :100). After 30 minutes of incubation at room temperature in the dark, the cells were washed in PBS, then resuspended in a final volume of 500 μl PBS and analysed by FACS.

Flow cytometric analysis. Flow cytometric analysis was performed by an EPICS ELITE ESP Coulter equipped with an argon/ion laser excitation source emitting at 488 nm. FITC fluorescence was collected through a 525 nm band pass filter. Propidium iodide (λ EX max: 540 nm, λ EM max: 625 nm), Rhodamine 123 (λ EX max: 505 nm, λ EM max: 534 nm) and doxorubicin (λ EX< max: 471 nm, λ EM max: 556 nm) fluorescence was collected with a 575 nm band pass. At least 20.000 cells were analysed and gated on the basis of the forward and sideward scatter.

The green and red fluorescence amount was measured using log mode amplification. Propidium iodide staining was registered in linear mode amplification.

As measure of intensity of the staining, the fluorescence index (FI) was calculated, which represents the ratio between the mean fluorescence intensity of cells stained with the specific antibodies and that of cells stained with the isotypic control antibody in immunofluorescence experiments. In drug uptake experiments, the FI was the ratio between the fluorescence of dyes and the fluorescence of untreated cells.

Results

Staining with rhodamine 123 and doxorubicin. The cells were used in the exponential growth phase. To study the function of P-gp and MRP, the uptake of two fluorescent substances (rhodamine 123 and doxorubicin) was evaluated in a proximal tubular renal cell line (LLC-PK_1) and in its resistant counterpart (LLC-PK_1/ADR). Differences between resistant and sensitive cells were observed with both substances (Figures 1A and B). A 1 hour pretreatment with verapamil, a reverting agent that is reported to competitively inhibit the P-gp mediated extrusion of several substrates, significantly increased the intracellular fluorescence only in the resistant cell line, suggesting a major role of P-gp in the extrusion of these fluorescent substrates in LLC-PK_1/ADR cells.

To confirm data P388 and P388/ADR cells, which are known to overexpress P-gp, and PANC-1 cells that express high levels of MRP were used. Results obtained with P388 and P388/ADR cells were similar to those obtained in the renal cell lines, showing an higher ability to extrude the dyes in the resistant counterpart. Verapamil was effective in its reverting effect in the resistant line (Figures 2A and B). On the contrary, when PANC-1 cells were similarly treated, no effect of verapamil was evident (Figure 3).

Staining with propidium iodide. The viability of LLC-PK_1 and LLC-PK_1/ADR cells was evaluated in cells exposed for 4 hours to the antineoplastic drug doxorubicin by staining with propidium iodide. While the cell cycle of viable cells showed no difference in the two lines, the reduction of the mean peak corresponding to DNA staining in LLC-PK_1 cells and the observation of the morphology of the two cell lines, confirmed a higher resistance of the LLC-PK_1/ADR cells to doxorubicin treatment (Figure 4).

Immunofluorescence staining with C219 and MRPm6 antibodies. P-gp expression was measured in sensitive and resistant cells with the monoclonal antibody C219 directed against an intracellular epitope of P-gp. A fluorescence signal was

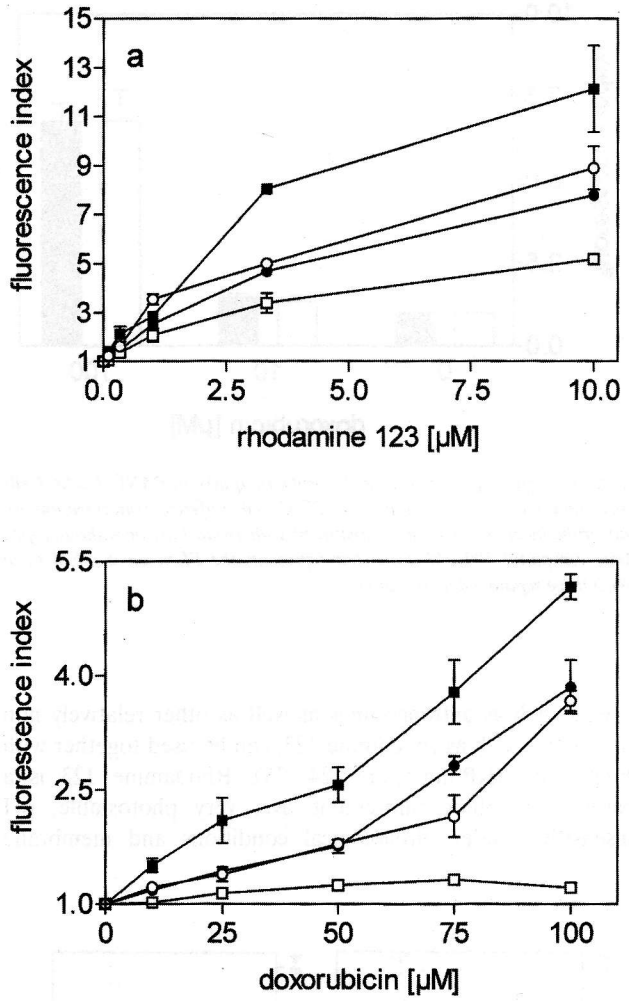

Figure 1. *Rhodamine 123 (A) and doxorubicin (B) uptake in LLC-PK$_1$ (○) and LLC-PK$_1$/ADR (□) cells. Cells were incubated with different doses of the fluorescent drugs in complete medium for 240 minutes at 37°C. The effect of a 1 hour pretreatment with 100 μM verapamil is also shown in LLC-PK$_1$ (●) and LLC-PK$_1$/ADR (■) cells. Each point represents the fluorescence intensity (FI) mean ± SEM of at least three separate experiments. FI was calculated as the ratio between the mean peak of fluorescence showed by cells treated with drugs and that of untreated cells.*

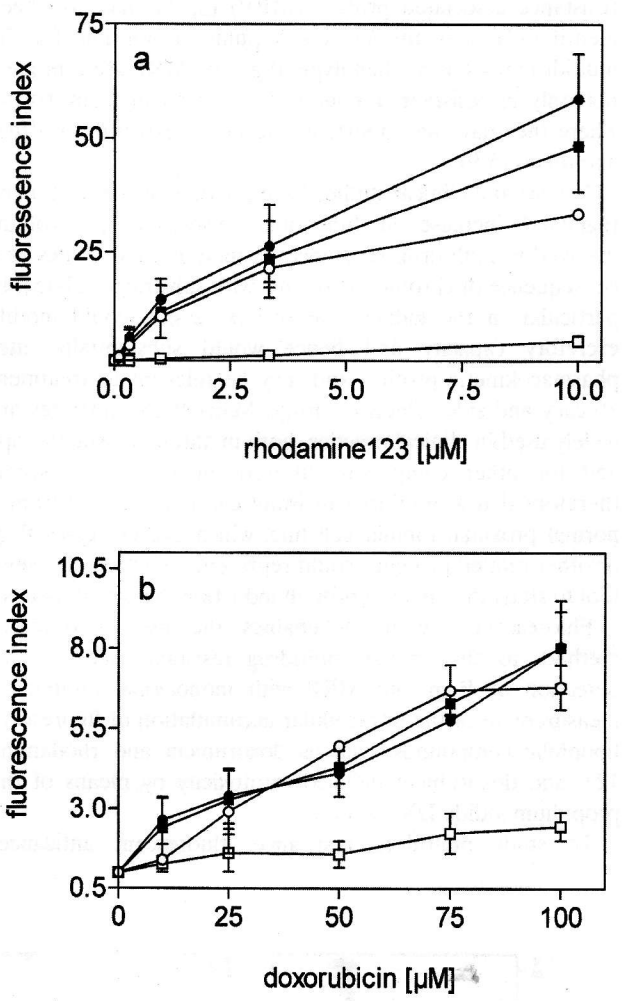

Figure 2. *Rhodamine 123 (A) and doxorubicin, (B) uptake in P388 (○) and P388/ADR (□) cells. Cells were incubated with different doses of rhodamine 123 in complete medium for 120 minutes at 37°C or with doxorubicin for 240 minutes at 37°C. The effect of a 1 hour pretreatment with 100 μM verapamil is also shown in P388 (●) and P388/ADR (■) cells. Data are reported as the FI mean ± SEM of at least three separate experiments.*

obtained in LLC-PK$_1$ and LLC-PK$_1$/ADR cells; the signal was stronger in the resistant line. Slight reaction was observed with PANC-1 cells; as expected P388/ADR cells showed a strong signal, higher than its sensitive counterpart (Figures 5A,B,C and D).

When the MRPm6 monoclonal antibody, directed against an internal epitope of MRP was used, the strongest signal was obtained with PANC-1 cells according to the reported high levels of MRP in this cell line. No signal was evident in P388/ADR cells, while a slight reaction in the LLC-PK$_1$ and LLC-PK$_1$/ADR cells indicated the presence of low levels of MRP in these cells, with no apparent difference of expression between the sensitive and the resistant line (Figures 6A,B,C and D).

Discussion

Acquired or intrinsic resistance to cytotoxic agents is one of the major causes of failure in cancer chemotherapy. The multidrug resistance phenotype is associated with the presence of a phosphorylated transmembrane glycoprotein of 170 kDa (P-gp) (22) that pumps drugs outside the resistant cells by an energy-dependent mechanism. In addition, another, structurally related protein, the 190 kDa multidrug

resistance associated protein (MRP) (3, 23) has also been identified as a membrane efflux pump responsible for the multidrug resistance phenotype. P-gp and MRP are expressed not only in resistant tumour cells but also in many tissues where they have an important role in the extrusion of drugs and toxins (5-9).

In vitro and clinical studies have provided evidence for the regulated increase in functional expression of proteins involved in multidrug resistance in many normal tissues as a consequence of chronic treatments with substrates (11-15). In particular in the kidney, the overexpression could modify excretory capacity and hence would substantially alter pharmacokinetic profiles and may be relevant to treatment efficacy and side effects of drugs. Many P-gp substrates are widely used in clinical practice, both in antineoplastic therapy and for other completely different indications. It seems therefore that a multidrug resistant cell line, derived from a normal proximal tubular cell line, which overexpresses P-gp or other related proteins, could represent an extremely useful tool to study the effect of protein induction in normal tissues.

Fluorescence cytometry enables the use of different methods to characterize multidrug resistant cells, such as detection of P-gp and MRP with monoclonal antibodies, measurement of the intracellular accumulation of fluorescent lipophilic compounds such as doxorubicin and rhodamine 123, and doxorubicin induced cytotoxicity by means of the propidium iodide DNA staining.

To study multidrug resistance, fluorescent anticancer agents such as anthracyclines as well as other relatively non toxic dyes, such as rhodamine 123, can be used together with P-gp and MRP-inhibitors (24, 25). Rhodamine 123 is a cationic lipophilic fluorescent dye, very photostable, pH insensitive under physiological conditions and membrane

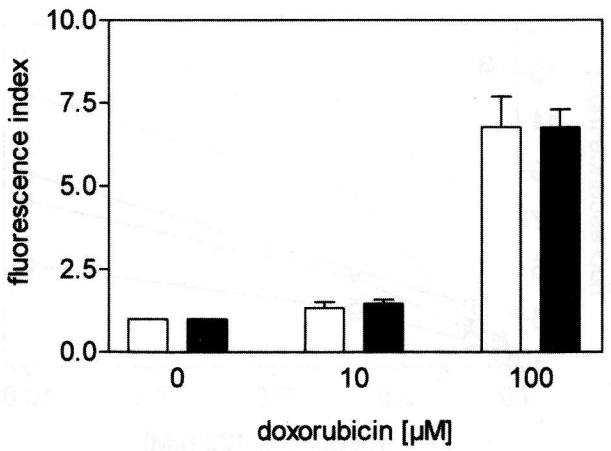

Figure 3. *Effect of verapamil on doxorubicin uptake in PANC-1 cells. Cells were incubated for 240 minutes at 37°C with different concentrations of doxorubicin after a 1 hour pretreatment with (solid bar) or without (open bar) verapamil 100 μM. Each bar represents the FI mean ± SEM of at least three separate determinations.*

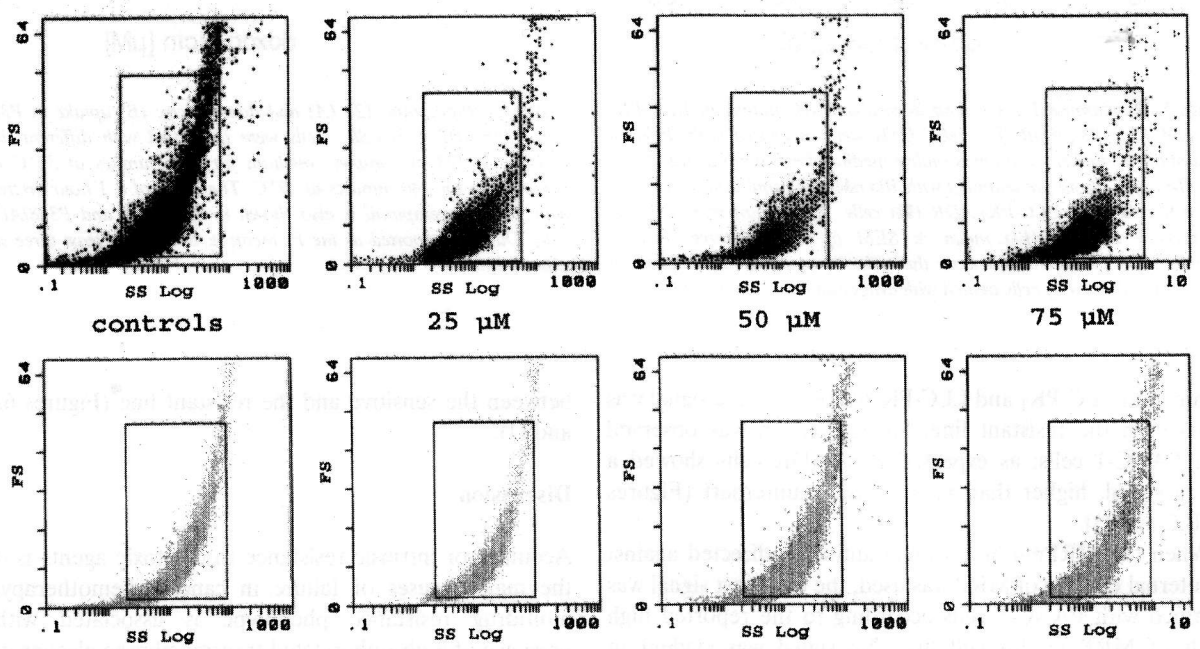

Figure 4. *Representative analysis by two parameters Forward scatter (FS)/Side scatter (SS) flow cytometry from LLC-PK$_1$ (upper panel) and LLC-PK$_1$/ADR (lower panel) cells after a treatment with increasing doses of doxorubicin for 240 minutes at 37°C. 20.000 cells were analysed by propidium iodide DNA staining after doxorubicin staining.*

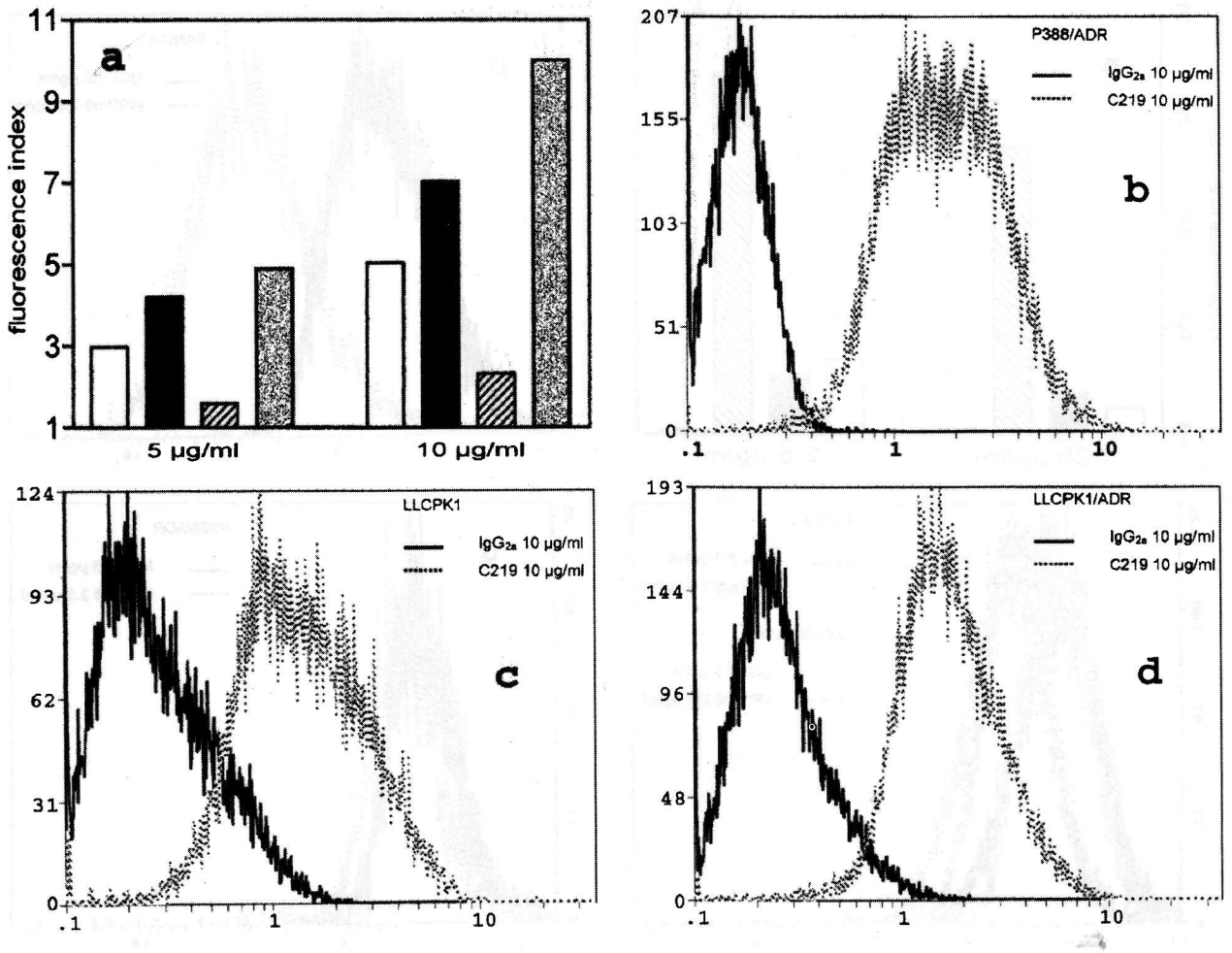

Figure 5. *Representative experiment of immunofluorescence detection of P-gp with monoclonal antibody C219. In A are reported the FI values obtained with two doses of antibody in LLC-PK1 (open bar), LLC-PK1/ADR (solid bar), PANC-1 (right-rising fine bar) and P388/ADR (dotted bar) cells. Each bar represents the FI calculated as the ratio between the fluorescence intensity obtained by the specific antibody staining and that obtained with the isotypic control antibody. In the figure are also reported the representative histograms (B,C,D) (counts versus fluorescence intensity) obtained with the higher dose of the antibody.*

permeable, which shows a rapid uptake by mitochondria of living cells (26). This substance was proposed to be a reliable marker for identifying multidrug resistant cells on the basis of reduced accumulation in these cells and of inhibition of efflux by verapamil (27). In addition rhodamine 123 has been recently shown to be a substrate not only for P-gp but also for MRP (28). Therefore, these functional tests did not allow discrimination regarding which mechanism of multidrug resistance was involved.

In this study, we employed both rhodamine 123 and doxorubicin to study P-gp and MRP function in the LLC-PK_1 cell line and its resistant counterpart LLC-PK_1/ADR, as well as in some cell lines used as reference: P388 and P388/ADR cells, that overexpress P-gp (29) and PANC-1 cells that show high levels of MRP (30).

The cytofluorimetric analysis, reported in this study, demonstrated the MDR phenotype of resistant cells by measurement of intracellular accumulation of rhodamine 123 and doxorubicin. For both substances the uptake was lower in the resistant LLC-PK_1/ADR cells than in LLC-PK_1 cells as well as in P388/ADR cells compared to P388 cells, used as controls.

Several substances such as calcium channel blockers, cyclosporin A, calmodulin inhibitors and the recently tested PSC883 and MK571 (25) were experimentally used to verify the specificity of the drug transport by P-gp or MRP. In fact, these substances, employed in functional studies in which the uptake of drugs was evaluated, increased the intracellular level of drugs by inhibiting the efflux pump activity of P-gp or MRP. Many of these substances are not specific and do not permit differentiation between P-gp or MRP activity. It has however been reported that MRP is not strongly inhibited by

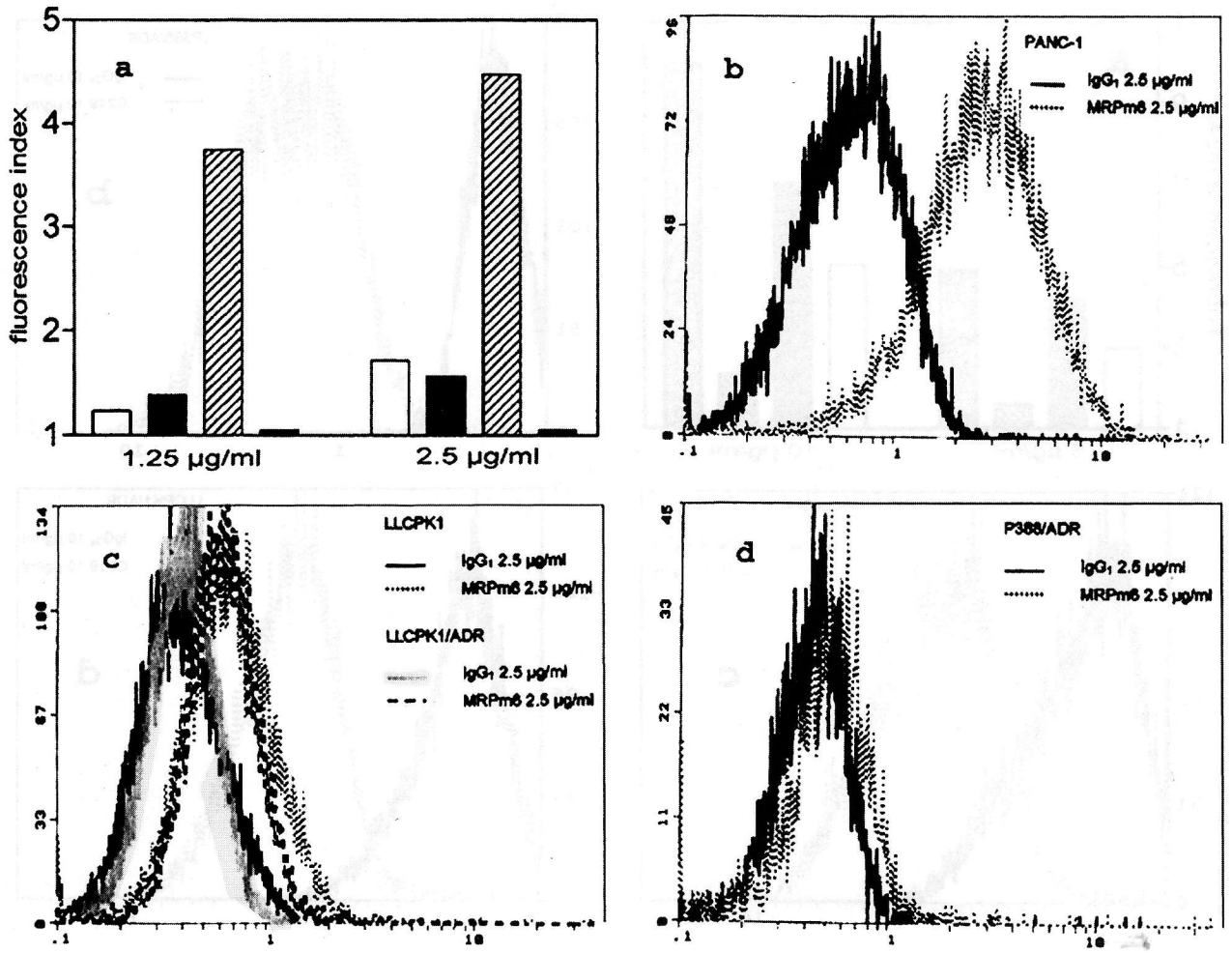

Figure 6. *Representative experiment of immunofluorescence detection of MRP with monoclonal antibody MRPm6. In A are reported the FI values obtained with two doses of antibody in LLC-PK1 (open bar), LLC-PK1/ADR (solid bar), PANC-1 (right-rising fine bar) and P388/ADR (dotted bar) cells. Each bar represents the FI calculated as the ratio between the fluorescence intensity obtained by the specific antibody staining and that obtained with the isotypic control antibody. In the figure are also reported the representative histograms (B,C,D) (counts versus fluorescence intensity) obtained with the higher dose of the antibody.*

verapamil, hence it has been considered a useful tool to identify the mechanism of drug resistance (31). From our results, verapamil caused an increase of drug accumulation in the resistant P388/ADR cell line to a level similar to that of the sensitive P388 cell line, indicating a complete block of P-gp efflux. In our experimental condition, verapamil was not able to modify the rhodamine 123 and doxorubicin accumulation in the PANC-1 cells, confirming specificity for P-gp but not for MRP. In LLC-PK_1/ADR cells pretreatment with verapamil led to an increase of intracellular level of both dyes tested, mimicking the situation observed with P388/ADR cells, again suggesting the prevalent role of P-gp in the MDR phenotype of these normal renal cells.

We have previously demonstrated by RT-PCR analysis that LLC-PK_1 and LLC-PK_1/ADR express both MDR1 and MRP genes, the former being in addition overexpressed in the LLC-PK_1/ADR cells. Western blot analysis, using C219 monoclonal antibody specific for P-gp, confirmed the overexpression of this protein on the membrane surface of LLC-PK_1/ADR cells (32).

Using C219 monoclonal antibody, specific for detection of P-gp (19) and an FITC-conjugated secondary antibody, P-gp expression on cell membranes was quantified by cytofluorimetric measurement. This analysis indicated a high level of specific fluorescence in the P388/ADR and LLC-PK_1/ADR cells confirming an overexpression of P-gp, while low levels were detected in LLC-PK_1 and PANC-1 cells. PANC-1 is a tumor pancreatic cell line that expresses high levels of MRP and is often used to study the role of this transporter; however recently it has been demonstrated that these cells also express low levels of P-gp (30).

MRPm6, a monoclonal antibody specific for MRP

detection was also used in our experiments. The results indicated a high level of expression in the PANC-1 cells, a lower level in LLC-PK_1 and LLC-PK_1/ADR and complete absence of any signal in P388 cells. No significant difference in fluorescence intensity with MRPm6 staining could be demonstrated between the two renal lines.

The results from drug uptake measurement and from protein immunodetection taken together indicated that LLC-PK_1/ADR resistance was mainly due to an overexpression of P-gp. The role of MRP in LLC-PK_1/ADR and a possible involvement of this protein in development of the resistant phenotype have to be demonstrated.

Doxorubicin was used in this study to functionally evaluate the activity of transport proteins; it should however be noted that this anticancer agent is highly cytotoxic. The cytofluorimetric technique allows one to study at the same time and on the same sample the uptake of the drug, the modulation of the efflux pump activity, the characterization of the transport protein involved and, finally, the toxic effect on cells, commonly determined by the MTT cytotoxicity assay that gives results after longer incubation periods and requires high cell number. Propidium iodide staining, performed immediately after the cytofluorimetric measurement of doxorubicin uptake, confirmed the resistance of the LLC-PK_1/ADR cell line in comparison with LLC-PK_1 cells after a 4 hour treatment with this drug.

In conclusion, in this study, we examined by means of cytofluorimetric techniques the characteristics of LLC-PK_1/ADR cells, a resistant cell line derived from normal renal proximal tubular cell line. The resistance phenotype was demonstrated by functional studies and was related to the overexpression of P-gp. The other extrusion pump, MRP, was also expressed in these cells, as well as in the sensitive counterpart, but was not responsible for the multidrug resistant phenotype. Flow cytometry provides a useful tool to evaluate, quickly and simultaneously, different aspects of the multidrug resistance phenotype.

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