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Preferential Expression and Activity of Multidrug Resistance Gene 1 Product (P-Glycoprotein), a Functionally Active Efflux Pump, in Human CD8+ T Cells: A Role in Cytotoxic Effector Function

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The multidrug resistance gene 1 (*mdr 1*) product, the P-glycoprotein (Pgp), is a 170-kD transmembrane transport protein, whose overexpression is associated with multidrug resistance in cancer cells and in chloroquine-resistant *Plasmodium falciparum* infection. In this study we show that normal freshly isolated human lymphocytes express low levels of *mdr 1* mRNA and membrane Pgp. Although Pgp is expressed in both CD4+ and CD8+ T cells, it is preferentially expressed in CD8+ T cells. Activation of T lymphocytes with phytohemagglutinin leads to an amplification of both *mdr 1* mRNA and membrane Pgp in T cells. P-glycoprotein in T cells is a functionally active efflux pump as demonstrated by decreased retention of rhodamine-123 and its increased accumulation by cyclosporin A, an inhibitor of Pgp function. In addition, MRK-16 antibody increased accumulation of Rh123 in CD8+ T cells. Furthermore, MRK16 anti-P-glycoprotein monoclonal antibody, in a concentration-dependent manner, inhibited T lymphocyte-mediated cytotoxicity. These data suggest a physiologic role of P-glycoprotein in cytotoxic T-lymphocyte effector function.

KEY WORDS: T-cell subsets; P-glycoprotein; multidrug resistance gene; cytotoxic T-cell function.

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[4]Abbreviations used: CsA, cyclosporin A; CTL, cytotoxic T lymphocyte; FITC, fluoresceine isothiocynate; HBSS, Hanks' balanced salt solution; MDR, multidrug resistance; *mdr 1*, multidrug resistance gene 1; MFC, mean fluorescence channel; MNC, mononuclear cells; PE, phycoerythrin; PCR, polymerase chain reaction; Pgp, P-glycoprotein; PHP, phytohemagglutinin P; PBS, phosphate-buffered saline; Rh123, rhodamine-123.

INTRODUCTION

P-glycoprotein (Pgp),[4] a 170-kD plasma membrane glycoprotein, is a member of a small, highly conserved multigene family with three genes in rodents and two genes in humans (1, 2). It shares extensive sequence homology with numerous bacterial and eukaryotic transport proteins (3–6). Pgp has been implicated in acquired multidrug resistance (MDR) in cancer (3) and in the chloroquine-resistant *Plasmodium falciparum* infection (7, 8) and shown to function as a metabolically active efflux pump responsible for rapid efflux of drugs (9–11). The gene for Pgp has been cloned (4–6, 12) and the sequence encoding Pgp reveals a tandemly duplicated molecule of about 1280 amino acids. Each half consists of a large hydrophobic domain containing six putative membrane-spanning domains and a highly conserved hydrophilic domain with an ATP binding site.

A number of normal tissues in human and rodents that have been shown to express high levels of Pgp include the adrenal cortex, liver, kidney, and intestine, a subset of muscle fibers, the gravid uterus, and the capillary endothelial cells of the brain (2, 13–19). Although the physiologic role of Pgp is presently unclear, its localization to the apical surfaces of renal proximal tubular cells, bile and pancreatic ductule cells, secretory epithelial cells of the gravid uterus, etc., suggests a secretory function for Pgp and perhaps its role to clear the cells of xenobiotics. The expression and function of Pgp in human T cells have not been explored. In the present investigation, we show that the Pgp is expressed preferentially in CD8+ T cells. P-glycoprotein in human T cells is a functionally active

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efflux pump and appears to play a role in effector cytotoxic T lymphocyte (CTL) function.

MATERIALS AND METHODS

Materials

MRK-16 monoclonal antibody (specific for human Pgp and directed against a surface membrane component of Pgp) was prepared by Hakada and Tsuruo (20). The *mdr 1*-specific primers for polymerase chain reaction (PCR) designed to produce 167-bp DNA (21) were synthesized by ChemGene, Waltham, MA. Chemical grade of cyclosporin A (CsA) was a gift from Dr. David Winters, Sandoz Pharmaceuticals, East Hanover, NJ. Rhodamine 123 (Rh123) was purchased from Molecular Probes, Eugene, OR. Direct fluorescein isothiocynate (FITC) or phycoerythrine (PE)-conjugated anti-CD3, anti-CD4 (Leu 3), and anti-CD8 (Leu 2) monoclonal antibodies were purchased from Becton-Dickinson, San Jose, CA. Phytohemagglutinin-P (PHA-P) was purchased from Calbiochem, La Jolla, CA.

Isolation of Mononuclear Cells and Activation

Mononuclear cells from healthy volunteer donors were separated from peripheral venous blood by Ficoll–Hypaque density centrifugation. Cells were washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) and resuspended in culture medium (RPMI-1640; 100 U/ml penicillin, 100 μg/ml streptomycin, 2 m*M* L-glutamine, 25 m*M* HEPES, and 10% fetal calf serum) at a desired concentration. Cells (2×10^6/ml) in 12 × 75-mm Falcon test tubes were activated with 5 μg/ml PHA-P for various lengths of time. Cells were washed three times with HBSS and examined for the expression of Pgp using MRK-16 monoclonal antibodies and FACScan.

Membrane Expression of Pgp

Cells (1×10^6/ml) were washed with phosphate-buffered saline (PBS) and were incubated with 20 μg of MRK-16 monoclonal antibody (optimal concentration of antibody determined by titeration) for 30 min on ice. Cells were then washed three times with PBS and counterstained with FITC-conjugated goat IgG $(Fab')_2$ anti-mouse antibody for 30 min on ice. Cells were washed three times with PBS, resuspended in PBS, and analyzed with FACScan (Becton-Dickinson, San Jose, CA). As a control, cells were incubated with mouse IgG2a (same isotype as the MRK-16 monoclonal antibody) and FITC goat anti-mouse IgG antibody. Ten thousand cells were analyzed using both forward and side scatter display. To determine whether the expression and amplification of membrane Pgp following PHA activation were on T cells and preferential to any of the two major subset of T cells, cells were analyzed by dual-color analysis. Cells were first stained with 20 μg MRK-16 (optimal concentration determined by titeration curve) and second antibody as described above, washed with PBS, and then stained with PE-labeled anti-CD3, anti-CD4, or anti-CD8 monoclonal antibody for 30 min on ice. Cells were washed with PBS and analyzed with FACScan.

mdr 1 *mRNA in T Cells*

RNA was extracted from MNC stimulated with PHA for various time intervals. Semiquantitative PCR was done using a RNA PCR kit and a thermal cycler from Perkin-Elmer Cetus (Norwalk, CT). Five hundred nanograms of RNA was converted to cDNA by reverse transcriptase using random hexamer as primer, and PCR was done with *mdr 1*-specific primers (21) for 42 cycles at 55°C annealing temperature. PCR with primers for the β-actin gene (Clontech, Palo Alto, CA) was done to confirm equal amounts of template RNA used. Ten microliters of PCR solution was analyzed by 2% agarose gel electrophoresis.

Rh123 Accumulation in T-Cell Subsets

In order to determine whether Pgp in T cells functions as an efflux pump, accumulation of Rh123 was examined in the presence and absence of 1 μg/ml CsA. Rh123 is a substrate for enhance outward drug transport system associated with multidrug resistance, the Pgp (22). Cyclosporin A binds to Pgp (23). Mononuclear cells were incubated with Rh123 (100 ng) for 1 hr, washed with PBS, resuspended in Rh123-free medium, and incubated at 37°C for 40 min, and dye retention was measured by FACScan. To determine the relative accumulation of Rh123 dyes in T-cell subsets, cells were first loaded with the dye for 1 hr at 37°C, washed, resuspended in Rh123-free medium for 40 min, and then stained with PE-labeled anti-CD4 or anti-CD8 monoclonal antibodies. The accumulation of Rh123

Table I. P-Glycoprotein Expression in Human T Cells and T-Cell Subsets[a]

Subject	PHA	CD3+MRK16+ (%)	CD4+MRK16+ (%)	CD8+MRK16+ (%)
1	−	5.0 (8%)	ND	ND
	+	35.0 (50%)	12.0 (25%)	22.0 (80%)
2	−	6.0 (10%)	1.5 (3%)	4.6 (20%)
	+	43.0 (75%)	16.0 (40%)	27.0 (100%)
3	−	3.5 (5%)	1.0 (2%)	2.4 (10%)
	+	24.0 (35%)	7.0 (16%)	15.0 (54%)

[a]Freshly isolated MNC and MNC stimulated with PHA for 48 hr were analyzed with dual-color analysis using PE-anti-CD3, anti-CD4, or anti-CD8 monoclonal antibodies and MRK16 monoclonal antibody and FITC goat anti-mouse IgG2a. Isotype control for MRK-16 and goat anti-mouse antibody were simultaneously performed. Data are expressed as percent MRK-16 positive cells over the isotype and second antibody control. Numbers in parentheses represent Pgp+ cells as percent of CD3+, CD4+, and CD8+ T cells in the preparations. ND, not done.

in T-cell subsets was measured by FACScan. In order to determine whether MRK-16 monoclonal antibody inhibits Pgp function, cells were incubated with 50 μg of MRK-16 or mouse IgG2a (as isotype control) and Rh123 accumulation was measured in CD8+ T cells by dual-color analysis using FACScan.

Cytotoxic T-Cell Function

Cytotoxic T lymphocytes (CTL) were generated in a mixed lymphocyte reaction (24). Briefly, MNC (2×10^6 cells/ml) from a normal donor were mixed with equal number of mitomycin (50 μg/ml)-treated stimulator cells from another normal donor. Cells were incubated for 5 days at 37°C in a humidified CO_2 incubator. The target cells were prepared from 3-day PHA-stimulated blast cells from the stimulator donor. The PHA-activated blasts were labeled with 100 μCi of $^{51}NaCrO_4$ (NEN, Boston, MA) for 1 hr at room temperature, washed three times with HBSS, and resuspended at 1×10^5 cells/ml in culture medium. Target cells (1×10^4) in 0.1 ml were dispensed in a 96-well tissue culture plates. Effector CTL were incubated in the presence or absence of various concentrations of MRK16 monoclonal antibody or mouse IgG2a for 1 hr at 37°C, washed three times, and resuspended at 2×10^6 cells/ml. MRK16- or mouse IgG2a-treated and untreated effector cells were added to the target cells to yield an effector-to-target cell ratio of 20:1 and incubated for 5 hr at 37°C. The plates were centrifuged, and 0.1 ml of supernatants was collected and counted in a gamma counter. The percentage inhibition of CTL function by MRK16 antibody was calculated by the following formula:

$$\% \text{ Inhibition} = 1 - \frac{\text{cpm of CTL treated with MRK16/mouse IgG2a} - \text{cpm of spontaneous release}}{\text{cpm of untreated CTL} - \text{cpm of spontaneous release}} \times 100$$

RESULTS

Membrane Pgp and mdr 1 *mRNA in T Lymphocytes*

Mononuclear cells were stimulated in the presence or absence of PHA-P for 48 hr and Pgp expression was measured with MRK-16 monoclonal antibody, using FACScan. IgG2a isotype was used as monoclonal antibody control. To define the Pgp expression on T cells and T-cell subsets, dual-color analysis with PE-labeled anti-CD3, anti-CD4, or anti-CD8 monoclonal antibodies was done. Table I shows the data from three representative normal controls for the presence of Pgp in freshly isolated and PHA-activated T cells. Approximately 3.5 to 6.0% of freshly isolated T cells expressed Pgp. Dual-color analysis for T-cell subsets shows that the expression of Pgp was predominantly on CD8+ T cells; approximately two-thirds of Pgp-positive T cells belonged to CD8+ T cells, whereas only one-third of Pgp-positive T cells were contained in CD4+ T cells. Following PHA activation, approximately 24–43% of T cells expressed membrane Pgp and the Pgp expression was predominantly in CD8+ T cells. When data were analyzed for Pgp+ cells as a percentage of each population, 5–10% of freshly isolated T cells, 3% of CD4+ T cells, and 10–20% of CD8+ T cells expressed Pgp. Following PHA activation, 35–75% of CD3+, 16–40% of CD4+, and 54–100% of CD8+ T cells expressed Pgp. Figure 1 shows a representative

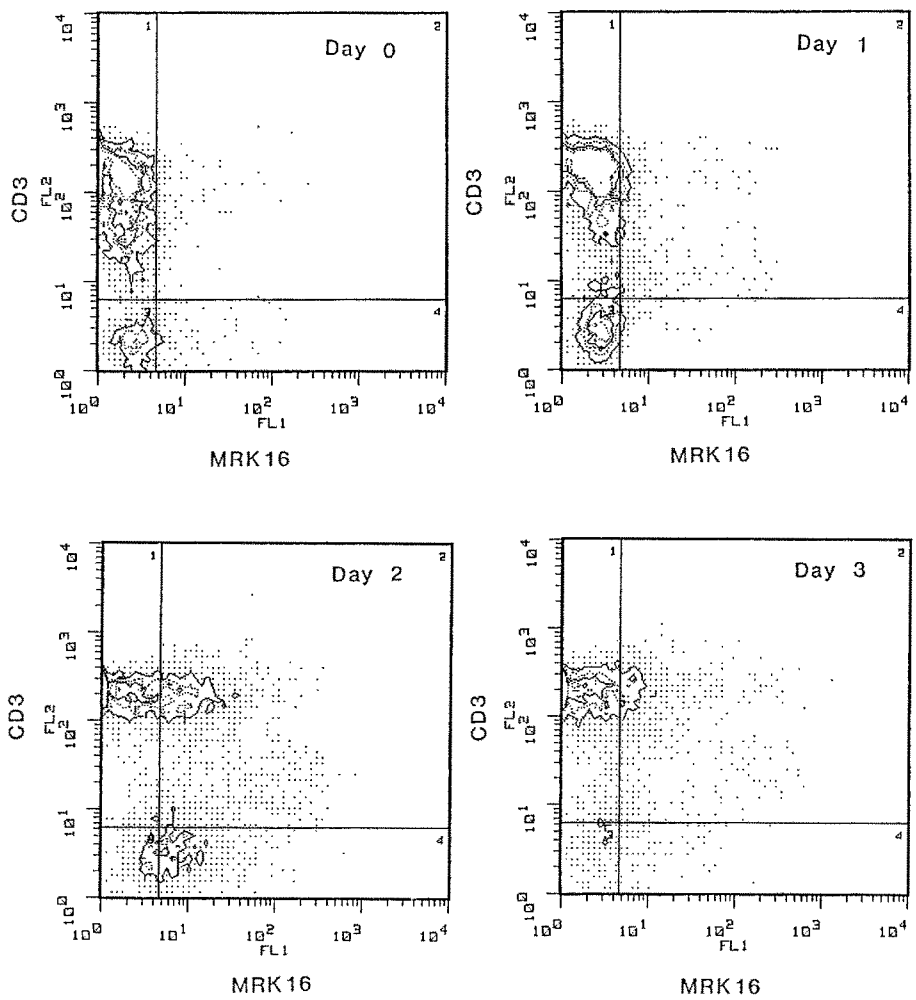

Fig. 1. Dual-color analysis of time kinetics of Pgp expression on T cells following PHA activation of mononuclear cells. The proportions of MRK16+ CD3+ T cells were as follows: day 0, 4.9%; day 1, 9.5%; day 2, 35.3%; and day 3, 26.6%.

of three separate experiments for the time kinetics of the expression of Pgp in T cells following PHA activation of MNC for various time intervals. A time-dependent increase in membrane Pgp was observed in CD3+ T cells that peaked at 48 hr. The *mdr 1* mRNA was also examined in both unstimulated cells and cells stimulated with PHA, using PCR technique. PCR analysis shows low levels of *mdr 1* mRNA in unstimulated cells and a time-dependent increase in *mdr 1* mRNA up to 24 hr (Fig. 2). These results show that PHA augments both *mdr 1* mRNA and membrane Pgp. In all these experiments, positive control included the MCF7/ADR cell line, which overexpresses P-glycoprotein, and the HL60 and HL60/AR cell lines, which lack P-glycoprotein and *mdr 1* mRNA (data not shown).

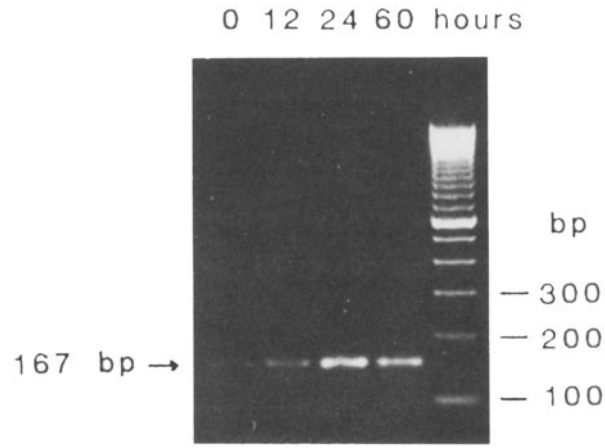

Fig. 2. Semiquantitative PCR analysis of time kinetics of *mdr 1* mRNA of MNC following stimulation with PHA. A time-dependent increase in *mdr 1* mRNA was observed, with a peak at 24 hr.

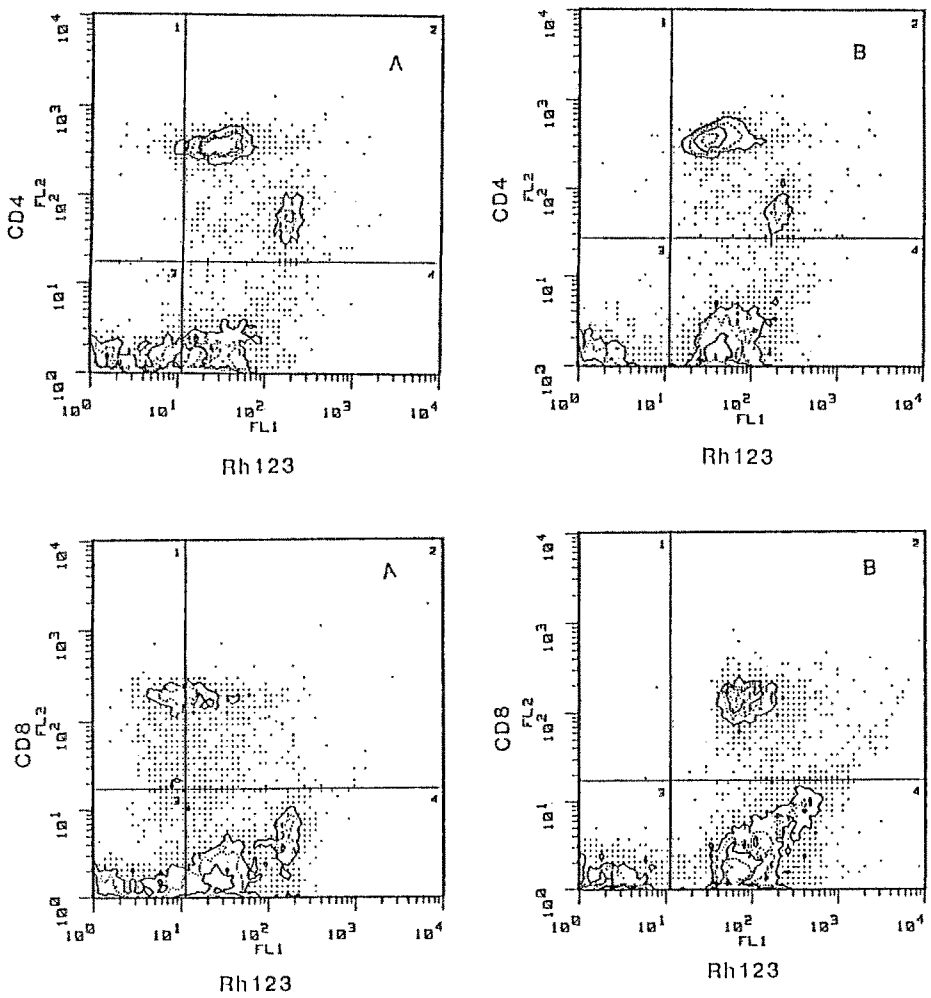

Fig. 3. Dual-color analysis of the retention of Rh123 dye in T-cell subsets in the presence or absence of CsA. MNC stimulated with PHA for 48 hr were incubated with Rh123 for 1 hr, washed, and resuspended in Rh123-free medium in the presence or absence of CsA for 40 min. Cells were counterstained with PE-labeled anti-CD4 and anti-CD8 monoclonal antibodies, and dye accumulation, as an indication of dye retention, was measured with FACScan. The relative fluorescence is indicative of Rh123 dye accumulation. Dye retention was less in CD8+ T cells (bottom, A) as compared to CD4+ T cells (top, A). In the presence of CsA (bottom, B) a greater increase in Rh123 accumulation was observed in CD8+ T cells (over baseline value), as evidenced by the shift in the contour graph to the right, compared to CD4+ T cells.

Membrane Pgp in T Cells Is a Functionally Active Efflux Pump

In order to determine whether the Pgp in T cells is a functionally active efflux pump, PHA-activated MNC were loaded with Rh123 dye in the presence and absence of CsA, and Rh123 accumulation was measured with FACScan, by dual-color analysis, using anti-CD4 or anti-CD8 monoclonal antibodies. Figure 3 shows the retention of Rh123 in CD4+ and CD8+ T cells, in the presence or absence of CsA. The retention of Rh123 in CD8+ T cells was significantly less compared to CD4+ T cells (Fig. 3A), suggesting a greater efflux of dye from CD8+ T cells. Furthermore, CsA significantly and preferentially increased Rh123 accumulation in CD8+ T cells compared to CD4+ T cells (Fig. 3B). This is consistent with the data on preferential expression of Pgp in CD8+ T cells.

MRK16 Monoclonal Antibody Inhibits Cytotoxic Effector T-Cell Function

Because of preferential expression of Pgp in CD8+ T cells, we investigated whether Pgp plays a role in CTL function. The CTL effectors generated

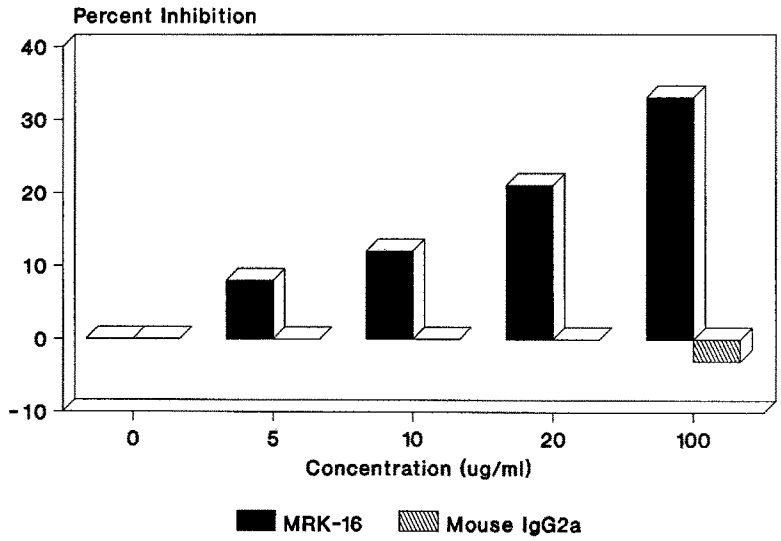

Fig. 4. Effect of MRK16 monoclonal antibody and mouse IgG2a on CTL effector function. Cytotoxic effector T cells generated in MLR were incubated with various concentrations of MRK16 antibody or mouse IgG2a for 1 hr, washed, and incubated with ^{51}Cr-labeled PHA blasts for 5 hr. Percentage inhibition of cytotoxicity was calculated by the formula given in Materials and Methods. A concentration-dependent inhibition of cytotoxicity was observed with MRK16 and not with mouse IgG2a.

in mixed lymphocyte culture reaction were treated with various concentrations of MRK16 antibody and similar concentrations of mouse IgG2a isotype control, washed, and incubated with target cells and cytotoxicity was measured in a 5-hr ^{51}Cr release assay. Figure 4 shows that MRK16, in a concentration-dependent manner, inhibited T cell-mediated cytotoxicity, whereas isotype control had no significant effect on T cell-mediated cytotoxicity.

MRK16 Monoclonal Antibody Inhibits Pgp Function

In order to determine whether the effect of MRK16 on CTL function was due to specific inhibition of Pgp function, mononuclear cells were incubated with 50 μg of MRK16 monoclonal antibody or mouse IgG2a, and Rh123 accumulation was observed in CD8+ T cells with dual-color analysis using FACScan. MRK16 increased Rh123 accumulation (mean fluorescence channel numbers 144 to 277) in CD8+ T cells, whereas mouse IgG2a had no effect on Rh123 accumulation (Fig. 5). These data indicate that MRK16 monoclonal antibody inhibits CTL function by inhibiting Pgp function.

DISCUSSION

P-glycoprotein is a member of a superfamily of transport proteins whose overexpression is associated with acquired MDR in cancer cells (25–28). Using

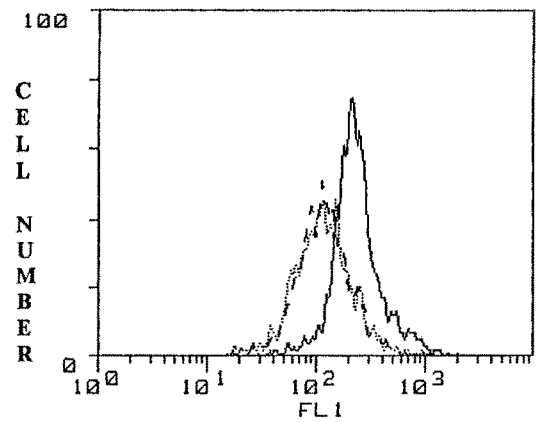

Fig. 5. Effect of MRK16 monoclonal antibody (——) and mouse IgG2a (- - - -) on basal (· · · ·) Rh123 accumulation in gated CD8+ T cells with dual-color analysis using FACScan. A significant increase in RH123 accumulation was observed as demonstrated by the increased RH123 fluorescence.

monoclonal antibodies and cDNA probes, several investigators have examined the presence of Pgp in normal tissues and organs in human and experimental animals (2, 13–19); however, they failed to detect Pgp in thymus, spleen, and lymph nodes (13, 14, 17, 18). Thiebaut *et al.* (14) and Sugawara *et al.* (18), using MRK16 monoclonal antibody and immunohistochemical technique, failed to demonstrate membrane Pgp in spleen and lymph nodes. Cordon-Cardo *et al.* (17), using Hyb241, Hyb 612, and C-219 monoclonal antibodies, did not find membrane Pgp in lymph node T and B cells, spleen, and thymocytes. Fojo *et al.* (16) observed low levels of *mdr 1* mRNA in bone marrow and spleen. However, no cell types were identified and the expression of membrane Pgp was not analyzed. In the present study, using the optimum concentration of MRK16 monoclonal antibody (20 μg as determined by titeration curve) and FACS, we observed the presence of membrane Pgp in T cells and T-cell subsets. In addition, using the PCR technique, we showed the presence of *mdr 1* mRNA in MNC cells. Following PHA activation, augmentation of both *mdr 1* mRNA and membrane Pgp was observed. The augmentations of Pgp (over baseline levels) were of similar proportions in both CD4+ and CD8+ T cells. Because the proportions of Pgp+ CD8+ cells at the basal levels were two to three times those of CD4+ T cells, Pgp+ PHA-activated CD8+ T cells were three times greater than Pgp+ PHA-activated CD4+ T cells. We have shown that PHA-induced augmentation requires both transcription and translation events and PKC isoforms are involved (28). Using similar approaches (MRK16 monoclonal antibody, FACS, and PCR), Chaudhary and Roninson (29) have demonstrated the presence of *mdr 1* mRNA and membrane Pgp in CD34+ hematopoietic stem cells in human bone marrow. It is highly unlikely that Pgp+ cells in the present study are CD34+ because CD34+ stem cells lack CD4 and CD8 antigens and they represent less than 1% of peripheral mononuclear cells.

In the present investigation we show that Pgp is expressed predominantly on human CD8+ T cells. Furthermore, the accumulation/retention of Rh123 dye was significantly less in CD8+ cells compared to CD4+ T cells, indicating a greater efflux of dye from CD8+ T cells. In addition, the accumulation of Rh123 in CD8+ T cells was increased by CsA, an inhibitor of Pgp function (23). These observations suggest that the Pgp in T cells is a functionally active efflux pump. Neyfakh *et al.* (30) and Coon *et al.* (31) also demonstrated a preferential loss of Rh123 from murine and human CD8+ T cells. This efflux was sensitive to verapamil and other MDR reversing agents. Neyfakh *et al.* (30), however, did not examine the presence of Pgp or *mdr 1* mRNA using antibodies or probes. Coon *et al.* (31) mentioned in their discussion (data not shown) that they did not observe Pgp+ cells with MRK-16 monoclonal antibody. This could be due to the suboptimal concentration of MRK-16 monoclonal antibodies used. In the present study, we used an optimal concentration of MRK-16 monoclonal antibody, as determined by titration curve. It is interesting to note a discrepancy between MRK-16+ freshly isolated T cells and T cells that show rapid efflux of Rh123. This suggests either that Rh123 is a more sensitive technique than MRK-16 staining to detect Pgp or that there are additional mechanisms, other than Pgp, for the efflux of Rh123. A similar lack of correlation has been observed in multidrug resistance tumor cell lines between the number of Pgp-expressing cells and the number of cells that rapidly efflux DiOC5 dye (31).

Because Pgp is preferentially expressed in CD8+ T cells and CD8+ T cells play a role in cytotoxic effector function via transport of cytotoxic molecules (32, 33), we studied the effect of MRK16 monoclonal antibody on CTL effector function. The MRK-16 monoclonal antibody, in a concentration-dependent manner, inhibited T lymphocyte-mediated cytotoxicity. In order to determine whether the effect of MRK16 on CTL function was due to specific inhibition of Pgp function or due to nonspecific modulation of T-cell function, the effect of MRK16 and mouse IgG2a on Rh123 accumulation was examined. MRK16, and not the mouse IgG2a, increased Rh123 accumulation in CD8+ T cells. These data strongly suggest a role of Pgp in CTL function. Although the mechanism(s) by which MRK16 inhibits CTL function remains to be determined, it is possible that cytotoxic molecules (e.g., perforin, granzymes, etc.) may be substrates for Pgp. Therefore, MRK16 could block the transport of one or more of these molecules from effector CTL to target cells.

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