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P-Glycoprotein Function in Aplastic Anemia: Role in Immune Etiology

IMAN M. MANSOUR, M.D.* and ILHAM Y. IBRAHIM, M.D.**

The Departments of Clinical and Chemical Pathology and Pediatrics**, Kasr El-Aini School of Medicine, Cairo University.*

ABSTRACT

The MDR1 gene-encoded P-glycoprotein (P-gp) participates in the cell secretion of cytokines and in the cytotoxic function of killer cells. Activated T cell plays an important role in the pathogenesis of aplastic anemia. The aim of the present study was to evaluate the function of P-gp in aplastic anemia and to study the effect of treatment on its activity.

Patients and Methods: The study group included 7 newly diagnosed AA cases and 20 cases under treatment with cyclosporine, in addition 10 age matched subjects were included as a control group. P-gp function was assayed using a rhodamine 123 efflux assay measured by flowcytometry.

Results: Percent of Rh123 effluxing cells was significantly higher in newly diagnosed and under treatment cases of AA compared to the control group (71.55±13.08, 72.92 ±18.45, 51.84±18.63 respectively). (*p*-value 0.024, 0.000). However, percent retention with verapamil was significantly higher in control group compared to that in newly diagnosed and under treatment cases. On comparing percent retention with and with out verapamil within each group, a significant increase in percent retention of Rh123 with verapamil was found in control group (*p*-value 0.004) and to a less extent within under treatment cases (*p*-value 0.024), and no significant change was observed within newly diagnosed cases

Conclusion: pgp function is deficient in newly diagnosed cases, with cyclosporine treatment there is an increase in cells expressing P-gp activity yet still less than that in normal subjects.

Key Words: *P-glycoprotein - Aplastic anemia - T lymphocytes.*

INTRODUCTION

The MDR1 gene-encoded P-gp, involved in the export of substances from the cell, is expressed by different normal tissues, such as the hematopoietic tissue **[1]**. Peripheral blood T, B and NK lymphocytes as well as hematopoietic stem cells are the major hematopoietic cells expressing P-gp, but the glycoprotein plays different physiological roles in these cells **[2]**. In lymphocytes, P-glycoprotein participates in the cell secretion of cytokines and in the cytotoxic function of killer cells **[3]**. P-gly expression is lineage specific with relatively high levels among CD56+ cells i.e natural killer cells **[4]**. Normal T lymphocytes express significant levels of a functional P-gp as determined by efflux of fluorescent dyes. Within the CD4 population, IL-4 producing T cells are almost entirely contained within the Rhodamine 123 (Rh123) high subset. Thus, differences in Rh123 extrusion, and by inference P-gp activity, distinguish functionally distinct groups of helper cells **[5]**.

Aplastic anemia, the paradigm of bone marrow failure syndromes, is defined as pancytopenia and an empty bone marrow **[6]**. Activated T cell plays an important role in the pathogenesis of aplastic anemia (AA) by infiltrating the bone marrow and secreting excessive levels of the anti-hemopoietic cytokines, interferon gamma and tumor necrosis factor alpha **[7]**. CD4+ T cells are divided into Th1 cells producing hematopoietic inhibitory cytokines like interferon-gamma and Th2 cells producing interleukin-4 **[8]**. The bone marrow failure in SAA might be caused not only by the increase of Th1 cells, Th1 type effector cells and cytokines, but also by insufficient compensation of Th2 cells and Th2 type cytokines, which shifts the balance of Th1/Th2 favorable to Th1 **[9]**. Immune- mediated stem cell damage has been postulated to be responsible for disease initiation and progression in AA.

In the present study the P-gp activity in peripheral blood lymphocytes in newly diagnosed aplastic anemia cases and cases under treatment, was studied in order to clarify the role of P-gp in immune mediated injury of stem cells. In addition, the effect of treatment with cyclosporine on P-glycoprotein function was evaluated.

SUBJECTS AND METHODS

Subjects:

The present study included 27 cases of aplastic anemia from the hematology clinic of the new pediatric Hospital, Cairo University. Seven newly diagnosed cases and 20 cases under treatment with cyclosporine. In addition, 10 healthy age matched subjects were included as a control group.

Methods:

All cases were subjected to thorough history taking, clinical and laboratory evaluation. Estimation of drug efflux function using rhodamine 123-efflux assay was performed on peripheral blood mononuclear cells.

Sample:

9 ml were withdrawn from each case using a sterile syringe and divided as follows: 5ml in a sterile vaccutainer containing lithium heparin as anticoagulant for determination of P-gp function, 2ml on EDTA for performing a complete hemogram and 2ml were left to clot for routine biochemical profile estimation.

Determination of Functional Activity of P-Gp by Flow Cytometry:

Isolation of Peripheral Blood Lymphocytes by Ficoll Hypaque Density Gradient Centrifugation:

Heparinized blood was layered on ficoll hypaque and centrifuged for 30 minutes at 1600 r.p.m. differential migration during centrifugation results in the formation of layers containing different cell types. Lymphocytes are found at the top layer coming at the interface between the ficoll and plasma with some other slowly migrating particles. Lymphocytes are then recovered from the interface and washed Hank's solution (GIBCO). After the third wash the supernatant is removed and cells are resuspended in a known volume of Hank's solution.

Reagent Preparation: Rhodamine 123 (Rh123) (Sigma): the dye is dissolved in absolute ethanol for the preparation of a stock solution of 5mM. Before use, Rh123 was diluted at 1/100 in absolute ethanol, then at 1/10 in water.

Verapamil (Sigma): 0.01gm verapamil powder is dissolved in 10 ml water (stock), then before use 100 µl of stock solution is added to 300 µl distilled water

Rhodamine Retention Assay Using Flowcytometry:

Flowcytometric analysis of variation in the percent of cells effluxing rhodamine 123, allows studying of the functional activity of P-gp in aplastic anemia samples. Blocking of Rh123 efflux by the P-gp inhibitor verapamil suggests that Rh123 efflux is likely due to P-gp **[10]**.

Procedure:

Fresh mononuclear cells were adjusted at 2 x 106 / cells/ ml in serum free RPMI (GIBCO). 250µl of the cell suspension (5 x 105 cells/tube) were distributed in 6 test tubes: 2 to evaluate cell autofluoresence: uptake control (UC) (Tube 1) and efflux control (EC) (Tube 2), 2 to evaluate Rh123 uptake (UR) (Tube 3) and efflux (ER) (Tube 4), and 2 to evaluate verapamil effect on Rh123 uptake (UV) (Tube 5) and efflux (EV) (Tube 6). 10 µl of PBS were added in tube UC and EC. Five µl of verapamil at 500 µM were added in tubes UV and EV. 5 µl Rh123 were added in tubes UR, ER, UV and EV.

All tubes were incubated for 1 hr. at 37°C, avoiding light exposure, at the end of the incubation the uptake tubes (1, 3, 5) were kept on ice a few minutes until assayed by flowcytometry. Two ml of cold RPMI (4°C) were added to the efflux tubes (2, 4, 6), then tubes were centrifuged for 5 min. at 450 g at 4°C and washed again with 2 ml cold serum - free RPMI. After two washes the cells were diluted in 250µl of serum -free RPMI at 37°C. Five µl of PBS were then added to tube 2 and 4 and 5 µl of verapamil were added to tube 6. All tubes were incubated in the dark at 37°C for 1 hr.

Interpretation: Samples were analyzed on Coulter EPICS flowcytometer and results were expressed as Rh123 fluorescence intensity (FI) either after loading or after 1 hr. efflux in Rh 123 free medium with or without verapamil.

Percent efflux was calculated using the following formula:

$$\frac{\text{A-B x 100}}{\text{A}}$$

A- % of positive Rh123 lymphocytes during influx phase

B- % of positive Rh123 lymphocytes during efflux phase **[11]**.

$$\% \text{ Retention} = \frac{\% \text{ of cells showing Rh123 fluorescence during efflux}}{\% \text{ of cells showing Rh123 fluorescence during influx}} \times 100$$

Verapamil blocking effect was defined by the percent of cells retaining the Rh123 fluorescence in presence of verapamil compared to that without.

RESULTS

Rh123 Efflux Without Verapamil:

Percent efflux of Rh123 was significantly higher in newly diagnosed AA cases and in cases under treatment as compared to control group (p-value .0002 and 0.006 respectively). No statistically significant difference was found between newly diagnosed and under treatment cases of AA (Table 1,2).

Rhodamine 123 Retention in Presence of Verapamil:

As regards Rh123 efflux in presence of verapamil, control group showed a statistically significant higher percent retension compared to both newly diagnosed AA cases (p-value 0.024) and cases under treatment (p-value 0.000). No significant difference was found on comparing percent retention in newly diagnosed AA cases to cases under treatment (Table 3, 4).

Effect of Blocking Using Verapamil:

On comparing % Rh123 retention in presence and absence of verapamil among studied groups, a statistically significant difference (p-value 0.024) was found in AA cases under treatment, however no statistically significant difference was found in newly diagnosed cases (p-value 0.063).

Control group showed a highly statistically significant difference (*p*-value 0.004) (Table 5).

Table (1): Results of flowcytometric analysis of Rh123 retention assay.

		% of cells showing Rh123 fluorescence (at loading) (A)	% of cells showing Rh123 fluorescence (after efflux) (B)	% efflux
Newly diagnosed AA cases (7)	Range	50.3-81%	4-35.2%	56.5-93.8%
	Mean	67.85%	18.52%	71.55%
	SD	11.5%	12.01%	13.08%
AA Cases under treatment (20)	Range	37.8-88.5%	2.5-52.4%	36.39-96.04%
	Mean	69.02%	19.24%	72.95%
	SD	12.52%	15.36 %	18.45%
Control group (10)	Range	69.3-91.7%	14.6-63.7%	25.14-78.8%
	Mean	79.82%	40.28%	51.84%
	SD	8.51%	17.58%	18.63%

Table (2): Statistical analysis of flowcytometric results as regards to % efflux.

	Newly diagnosed AA cases vs Control group	AA cases under treatment vs Control group	AA cases under treatment vs Newly diagnosed AA
p-value	0.030	0.006	0.855
Significance	S	HS	NS

Table (3): Flowcytometric data of percent Rh123 fluorescence in the 3 groups using verapamil.

		% of cells showing Rh123 fluorescence		% Retention
		At loading with verapamil	After 1hr efflux with verapamil	
Newly diagnosed AA	Mean	59.05714	23.62857	39.01857
	Range	25.1-80.1	7.9-50.1	16.2-62.6
	SD	21.18276	17.7	18.33427
AA Cases under treatment	Mean	70.26	23.2475	33.7
	Range	49.9-85.6	7.6-58.4	10.3-68.2
	SD	8.342497	15.03949	16.4
Control group	Mean	82.58	50.82	60.885
	Range	73.6-96.2	25.9-76.8	32.3-83.8
	SD	7.578596	18.1	17.09237

Table (4): Statistical comparison of percent retention of Rh123 in presence of verapamil.

	Newly diagnosed AA cases vs Control group	AA cases under treatment vs Control group	AA cases under treatment vs Newly diagnosed AA
p-value	0.024	0.000	0.476
Significance	S	HS	NS

Table (5): Statistical comparison of percent Rh123 retention in the three groups with and without the use of verapamil.

	Percent Rh 123 retention					
	Newly diagnosed AA		AA under treatment		Control group	
	Without verapamil	With verapamil	Without verapamil	With verapamil	Without verapamil	With verapamil
Range	6.2-62.6		4-63.6	10.3-68.2	21.2-74.9	32.3-83.8
Mean	28.44714	39.01857	27.045	33.7	48.154	60.885
SD	±13.07962	±18.33427	±18.45271	±16.4	±18.62856	±17.09237
p-value	0.063		0.024		0.004	

DISCUSSION

Acquired aplastic anemia in childhood is characterized by bone marrow failure in which there is reduction in the effective production of mature erythrocytes, granulocytes and platelets by the bone marrow which is hypocellular leading to peripheral blood pancytopenia. Immune mediated suppression of hemopoiesis has been considered to play an important role in most cases of acquired aplastic anemia. Inhibition of hemopoietic cell growth by patient lymphocytes and their overproduction of myelosuppressive cytokines, have supported this hypotheisis **[12]**.

P-gp plays a role in cytolytic activity and cytokine secretion by lymphocytes. The blockade of P-gp function by the MRK-16 monoclonal antibody inhibits T cell-mediated cytotoxicity. MRK-16 and UCI2 (monoclonal antibodies directed against Pgp) and other drug-pump inhibitors are able to inhibit the transport of interleukin-2 (IL-2), IL-4 and interferon-_ in T lymphocytes **[13]**.

The function of P-glycoprotein in aplastic anemia in newly diagnosed and in cases under cyclosporine treatment and its role in the immune etiology of aplastic anemia was investigated.

In the present study, aplastic anemia cases under treatment showed a significantly higher hemoglobin level, leucocytic count and platelet count compared to newly diagnosed cases. All cases under treatment received cyclosporine.

Correction in hematological parameters were considered as a favorable response to therapy.

P-glycoprotein functional analysis was performed by flowcytometry using rhodamine 123 efflux assay on peripheral blood lymphocytes. The study included 7 newly diagnosed cases and 20 cases under treatment, in addition to 10 age matched control group. Functional assay of P-gp activity provides the advantage of directly quantifying the potential to transport substances out of the cells. They don't depend on a correlation between the amount of protein or RNA and transport activity. In the present study verapamil was used as a modulator of P-gp function. Verapamil is a calcium channel blocker which inhibits P-gp function **[14]**.

In the present study, percent efflux of Rh123 was significantly higher in newly diagnosed AA cases and in cases under treatment as compared to control group. Such finding could have been interpreted as an increase in P-gp pump activity. However, percent retention of Rh123 fluorescence in the presence of verapamil was statistically significantly higher in control group compared to either newly diagnosed or under treatment cases of aplastic anemia. In addition, on comparing % Rh123 retention in presence and absence of verapamil among studied groups, a statistically significant difference was found in control group and in AA cases under treatment, however no statistically significant difference was found in newly diagnosed cases. Verapamil is a specific inhibitor of P-gp, that completely blocks the efflux of Rh123 via P-gp, and thus confirming that the efflux of Rh123 is effected by P-gp **[15]**. In addition, since it has been reported that sensitivity to doxorubicin was enhanced by treatment with the P-gp inhibitor, verapamil, in proportion to the P-gp expression level **[16]**. Thus it could be concluded that P-gp pump activity is present in control group, deficient in newly diagnosed cases and appeared with treatment yet didn't reach control level. In concordance with the present study findings Calado et al. **[17]** reported a statistically significant decrease in pump activity in the newly diagnosed aplastic anemia cases compared to normal cases.

Thus the significant increase in percent of Rh123 effluxing cells in newly diagnosed cases and in cases under treatment may be effected through an efflux pump other than P-gp namely multidrug resistant associated protein-1 (MRP1) as several studies have suggested that Rh123 is a substrate for MRP1 **[18-20]** and the presence of cyclosporin A and verapamil does not modify MRP1 activity **[21]**.

The therapeutic effect of cyclosporine is achieved by correcting a Th1/Th2 imbalance (a shift of Th1 type to Th2 type) **[22]**. Within the CD4 population, IL-4 producing T cells (Th2) are almost entirely contained within the Rh123 high subset and thus the increase in pump activity in cases under treatment in the present study may be attributed to correction of the Th1/Th2 **[5]**. Similarly, Witkowski and Miller [23] reported that T cells differ in R123 extrusion most likely due to differences in the functional activity of P-gp. Thus, differences in Rh123 extrusion, and by inference P-gp activity, distinguish functionally distinct groups of helper cells **[5]**.

As majority of peripheral blood lymphocytes are T cells, it could be concluded from the present study that T lymphocytes with low P-gp function predominate in newly diagnosed cases. With treatment T cells with increased P-gp function (Th2) appear producing more IL-4 thus restoring the Th1/Th2 balance.

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