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Dear Colleagues:

It is our pleasure to introduce the first issue of the Journal of the Egyptian Society of Haematology & Research.

The Society was founded in June 2003 to promote the spread of knowledge and skill and promote research in the rapidly advancing field of Haematology and Haemato-oncology.

The society held two successful conferences in 2004 and 2006 and many working symposia around Eypgt (Tanta, Assiout .. etc) that attracted young doctors working in its field.

At present the society counts around 300 members and is becoming affiliated to the International Society of Haematology.

The journal of the Egyptian Society of Haematology & Research was registered and accepted in 2005 by the Egyptian supreme press council.

Articles accepted in the journal were reviewed by member of the journal board and new articles are welcomed to be peer reviewed prior to publication in our semester volumes.

The Editorial Board

The Journal of the Egyptian Society of Haematology & Research

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Conferences and Workshop Allerts:

1- Meeting of the Thrombosis and Haemostasis group. Symiramis Intercontinental, Cairo, 3rd of May, 2006.

2- International Conference on Thalassaemia 8-9th of May 2006-04-04. Nile Hilton, Contact thalass-eg@yahoo.com 0101418408 -0123124674.

3- European Society for Haematology, Amsterdam, 15-18 June, 2006.

4- XXXI world Congress of International Society of Haematology 9-12 August, 20076 San Juan, Puerto Rico. www.ish2006.org.

5- The 6th Flow cytometery workshop BMT lab unit, Clinical Pathology Department, NCI, Cairo University 11-16 November, 2006 Contact: 3664656.

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

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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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Clonal Rearrangement of Immunoglobulin Heavy Chain Gene (IgH) and Detection of Bone Marrow Involvement in B- Cell Non-Hodgkin's Lymphoma

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ABSTRACT

Objective: The evaluation of staging or post-therapy bone marrow biopsies for involvement by B cell lymphoma has traditionally been based on morphologic findings. To improve sensi-tivity, morphologic examination is now augmented by ad-vances in molecular technology that detect clonal lym-phoid populations by immunoglobulin gene rearrange-ments and lymphoma associated chromo-somal translo-cations.

The purpose of the present study is to correlate the sensitivity of morphologic and histochemical assessment of bone marrow lymphomatous involvement and PCR-based molecular studies in pa-tients with B-lineage lym-phomas.

Patients and Methods: This study was conducted on 49 patients with histopathological diagnosis or clinical suspicion of NHL that were considered for bone marrow examination either for diagnosis, staging or post therapy re-evaluation. Bone marrow biopsies were evaluated for presence or absence of infiltration using conventional morphologic examination, and immunohistochemical staining using CD 79a and CD 20. Detection of clonal IgH gene rearrangement in paraffin embedded biopsy sections using PCR was performed.

Results: Of the 49 cases studied, 19 cases (39%) had evident morphological B.M. involvement, 11 cases (22%) showed minimal infiltration and 19 cases (39%) showed no marrow infiltration. Using immunohistochemical stain-ing, 25 cases (51%) were positive for the monoclonal CD79a, while only 15 cases (30%) were positive for the monoclonal CD20. Using PCR technique, 35 cases showed clonal IgH rearrangement (71%), 31 cases using FRIIIA (88.6%), seven cases using FRII (20%) and three cases with both FRIIIA and FRII (9%).

Conclusion: In histopathologically diagnosed cases of B-NHL, presence of morphological evidence of bone marrow infiltration requires confirmation of a clonal B-cell nature where demonstration of clonally rearranged IgH gene may be considered a valuable test. Yet, in histopathologically diagnosed cases of B-NHL showing minimal non-conclusive or absent morphological evidence of infiltratrion, demonstration of clonal rearrangement of IgH using PCR represents a solid evidence of marrow involvement. In absence of a histopathological diagnosis of B type-NHL, presence of a clonal rearrangement of IgH, is not alone sufficient to document a B-cell lymphoma.

Key Words: *B-NHL - Minimal residua l - Disease - Clonal IgH rearrangement.*

INTRODUCTION

B cell lymphomas constitute about 85% of NHL. Most patients suffering from intermediate and high grade B cell NHL achieve a complete remission following conventional chemotherapy. However, 50% of these patients relapse within 2 years. The relapse is presumably caused by the persistence of a small number of lymphoma cells that are below the limit of detection of standard diagnostic procedures. Unlike more aggressive NHL histiotypes, indolent lympho-mas (SLL, CLL , FCL, MCL) are disseminated disorders with frequent microscopic bone mar-row and peripheral blood involvement **[1]**.

The evaluation of staging or post-therapy bone marrow biopsies for involvement by B cell lymphoma has traditionally been based on morphologic findings. To improve sensi-tivity, morphologic examination is now augmented by ad-vances in molecular technology that detect clonal lym-phoid populations by immunoglob-ulin gene rearrange-ments and lymphoma asso-ciated chromosomal translo-cations. Develop-ment of the polymerase chain reaction (PCR) presumably detects clonal populations below the morphologic threshold. Although clone-

specific PCR methods are specific and sensitive to a level of one in 107 cells, these methods are too inef-ficient for routine testing because unique primers are required for each patient and may fail in some leukemias and lymphomas as a result of clonal evolution **[2]**.

The development of consensus primers for the hyper variable region of the immunoglobulin heavy chain (IgH) permits routine use of gene rearrangement studies in the evaluation of lymphocytic disorders. These assays achieve excellent specificity, although they have demonstrated a lower sensitivity than the clone-specific primers. The specific sensitivity depends on the primer set, lymphoma subtype, degree of competitive amplifi-cation between monoclonal and polyclonal lymphocyte DNA, and tissue fixation **[3]**. With a multiple primer panel, the clonality detection rate in primary diagnostic tissue is approximately 80% overall, varying with the type of non-Hodgkin's lymphoma **[2]**.

The purpose of the present study is to correlate the sensitivity of morphologic and histochemical assessment of bone marrow lymphomatous involvement with PCR-based molecular studies in pa-tients with B-lineage lymphomas.

SUBJECTS AND METHODS

This study was conducted on a total of 49 patients who were admitted to Kasr Al-Aini hospitals with a histopathological diagnosis or clinical suspicion of lymphoma. Patients were candidates for bone marrow examination either for staging, re-evaluation, or primary diagnosis.

Patients comprised 32 males and 17 female with an age range of one year to seventy years.

All histopathological data were obtained for all patients

Patients were classified according to their pathology as follows: (Fig. 1).

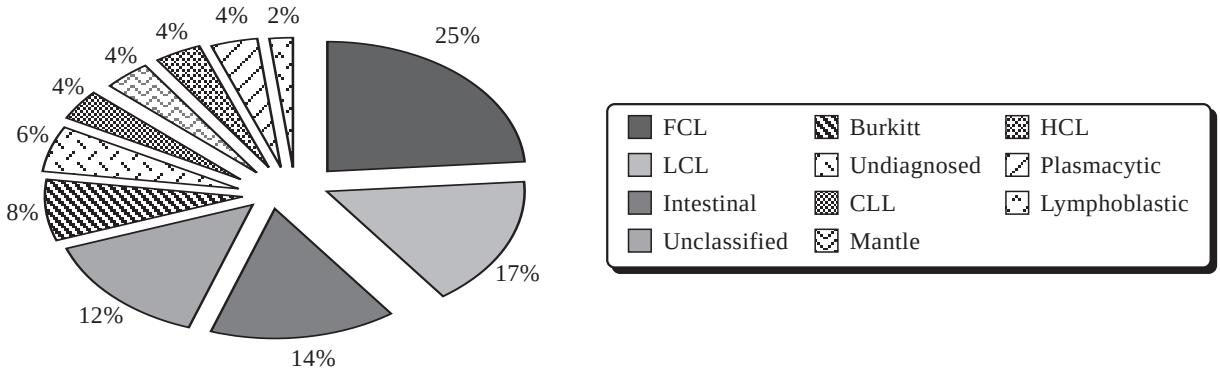

Fig. (1): Distribution of cases among various histopathologic types.

For each patient a unilateral or bilateral iliac crest bone marrow biopsy was obtained together with an aspirate for cytological evaluation of smears.

Paraffin embedded reactive and normal lymph node sections from archival blocks were used as a negative control for the molecular study.

Bone Marrow Evaluation:

Sample Processing:

1- *Bone marrow core biopsy:* Cores were fixed in a mercurial fixative (Zenker solution) for 24 hours, washed in running tap water, dehydrated in ethanol (70%, 95%, 100%), and embedded in paraffin. 3-5mm serial sections were cut on glass slides. For each patients 8 slides were prepared (2 on ordinary glass slides for routine examination, and four on positively charged slides for IHC. Prior to staining, samples were deparaffinized by immersing in two changes of xylene and rehydrated by dipping in decreasing concentrations of ethanol (100%, 95%, 70%) and finally in distilled water.

2- *Bone marrow aspirate smears:* Spread and stained with leishman -Giemsa.

Morphologic Examination:

Morphological examination of stained aspirate smears and routinely stained biopsy sections

(Geimsa and hematoxylin and eosin stains) was done for each case.

Immunohistochemical Staining Procedure:

Peroxidase block was done using 3% H_2O_2 in methanol. This was followed by heat retrieval using EDTA buffer pH 8.6 and pressure cooker heating for 1.5 min for CD79a antibody while CD20 required no antigen retrieval **[4]**.

Protocol:

Dako Envision immunostaining Kit was used (DakoCytomation Denmark A/S, DK-2600 Glostrup, Denmark). The kit utilizes an immunoperoxidase staining technique. CD20: prediluted (Monoclonal Mouse Human B-cell, CLASS/SUBCLASS: lgG2a, kappa) and CD79a in a dilution of 1/10 (Dako, Monoclonal mouse Anti-Human CD2O, clone L26 and CD79a, B Cell Clone JCB117) were used as primary antibodies.

Sections of reactive lymph nodes were similarly treated and used as controls.

Molecular Studies:

Fixed Tissue DNA Extraction (DNAzol method)

DNA was directly extracted from fixed, paraf-fin - embedded trephine biopsies of all patients. Approximately 50 4-mm trephine sections were twice incubated with xylene at room temperature, centrifuged at 12000rpm, and decanted: After ethanol washing, evapo-ration, and proteinase K treatment, the supernatant was decanted and bone marrow biopsy pellet was used for DNA extraction using DNAzoL Reagent (Genomic DNA isolation Reagent).

Determination of DNA Concentration:

1- 5 µl of the DNA solution were diluted to about 50 times with 245 µl of water and mixed well by vortexing (this DNA is to be discarded and can therefore be treated in this way).

2- The absorbance (A) was read in a spectrophotometer (Pharmacia LKB-Ultraspec II, Pharmacia) at 260 nm against a water blank.

3- Calculations: A solution of DNA at a concentration of 50 pg/ml, gives an absorbance of 1.0 at 260 nm. Therefore the absorbance reading at 260 nm (A) multiplied by 50 gives the concentration, of the prepared diluted DNA, solution and multiplied by the dilution factor (50) gives the concentration of the original DNA solution.

The following equation was used: Concentration of DNA in pg/ml = absorbance reading at 260 x 2500.

Determination of the Purity of DNA Solution:

1- The absorbance reading at 260nm was taken (A260). The absorbance reading at 280nm was also taken (A280). The ratio of A260 to A280 should be in the range of 1.7 **[5]**.

2- If it is less than 1.7, the DNA solution is contaminated with other proteins.

Analysis of Immunoglobulin Heavy Chain Genes Rearrangement By PCR **[6]***:*

Polymerase Chain Reaction Methods:

Primers: (R & D Systems, Inc., Minneapolis, MN 55413, USA) (Table 1).

Table (1): Primers specifications.

VH-FRIIIA	5` ACACGGC(C/T) (G/C) TGTATTACTGT3
VH-FRII	5`GTCCTGCAGGC(C/T)(C/T)CCGG (A/G)AA(A/G)(A/G) GTCTGGAGTGG 3`
Anti sense VJH	5` GTGACCAGGGT (A/G/C/T) CCTTGGCCCCAG 3`
Anti sense JH	5` TGAGGAGACGGTGACC 3`

For each sample two amplifications were performed for each sense primer using a DNA ther-mocycler (Perkin Elmer Cetus 480, Norwalk, CT). For the first amplificaltion a standard 50µl reaction mixture was used that con-sisted of 2µl extracted DNA, 25µl master mix prediluted (Taq DNA polymerase, PCR buffer containing 3mM MgCl2, dNTP mix (Qiagen), 2µl VH-FRIIIA or VH-FRII, 2µl Anti sense JH and 19 ml distilled water. 30 PCR cycles were performed consisting of denaturation for 1 min at 94ºC annealing at 60ºC for 1 min, extension for 1 min at 73ºC.

For the second amplification a standard 50µl reaction mixture consisting of 2µl of amplified DNA from first amplification, 25µl master mix prediluted, 2µl VH-FRIIIA or VH-FRII, 2µl Anti sense VJH, 19 ml distilled water. 20 PCR cycles were performed consisting of denaturation for 1 min at 94ºC annealing at 60ºC for 1 min, extension for 1 min at 73ºC.

Amplified Material Analysis:

15µl of PCR reaction product together with a molecular weight marker were electrophoresed in a 6% polyacrylamide gel stained with ethidium bromide and photographed under ultraviolet light.

A PCR was considered positive when a sharp monoclonal band resided within the appropriate primer size range. The primer VH-FRIIIA gave the monoclonal band between 50 and 150 Kb while the VH-FRII primer gave the monoclonal band between 150 and 300 Kb.

RESULTS

Results of Bone Marrow Aspirate and Biopsy Examination:

Twenty eight cases (57%) showed no evidence of infiltration on aspirate examination, however biopsy examination revealed minimal infiltration in 9 of these cases (35%). In the remaining 21 cases (43%), examination of both bone marrow aspiration and biopsy revealed infiltration, thus bone marrow biopsy revealed infiltration in 61.2% of cases (30/49) (Fig. 2).

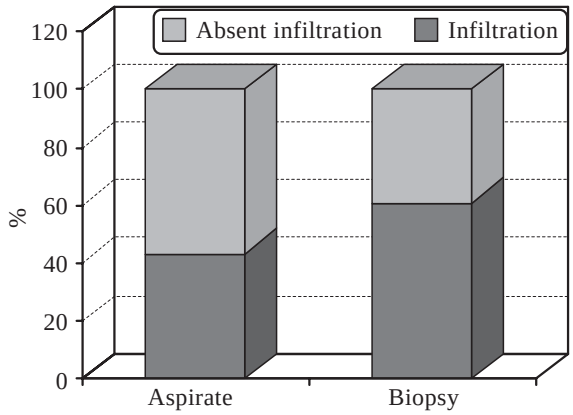

Fig. (2): Infiltration incidence according to aspirate and biopsy morphological examination.

According to morphological infiltration of bone marrow aspirate and biopsy, cases were classified into three groups as shown in the following table (2).

Table (2): Morphological classification of cases.

Group	No. of cases	%	Description
Group A	19	39	Marrow infilt. >10% of marrow cellularity
Group B	11	22	Minimally infilt. <10% (suspicious involve.)
Group C	19	39	No marrow infiltration

IHC staining & PCR results: (Table 3) (Figs. 3,4,5)

In all the studied cases (49):

Immunohisto-chemical staining with monoclonal CD79a showed positivity in twenty five cases (51%) (Fig. 3), while CD20 was positive in fifteen cases only (30%) (Fig. 4).

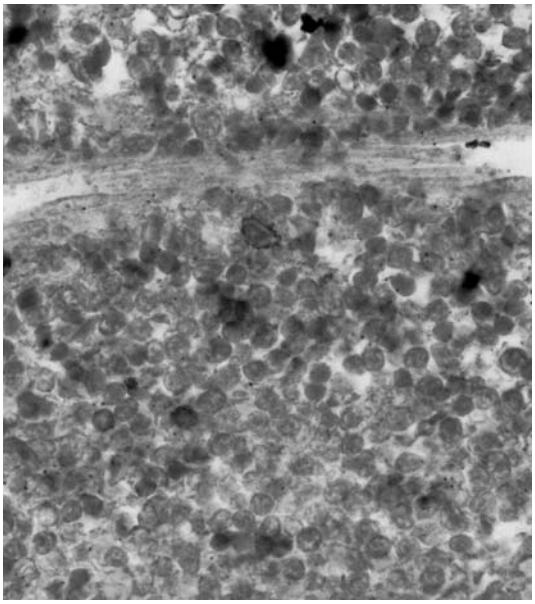

Fig. (3): CD20+ staining in an infiltrated case of FCL. Immunoperoxidase staining.

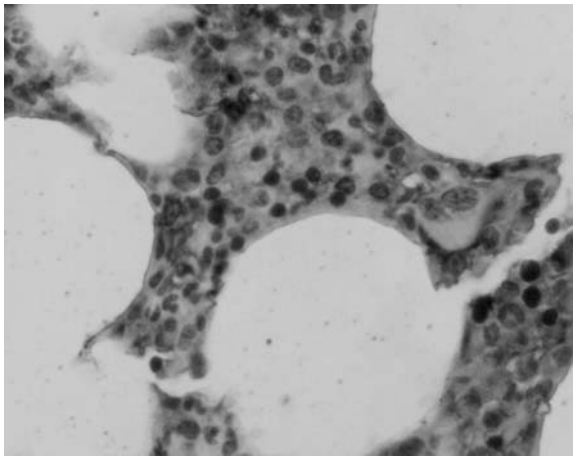

Fig. (4): CD79a showing few scattered positive cells. Immunoperoxidase staining.

Polymerase Chain Reaction:

PCR for immunoglobulin heavy chain gene rearrangement showed clonal rearrangement in thirty five cases (71%) (Fig. 5).

Table (3): IHC and PCR results of all the cases.

	CD20		CD79a		PCR	
+ve	15	31%	25	51%	35	71%
-ve	34	69%	24	49%	14	19%

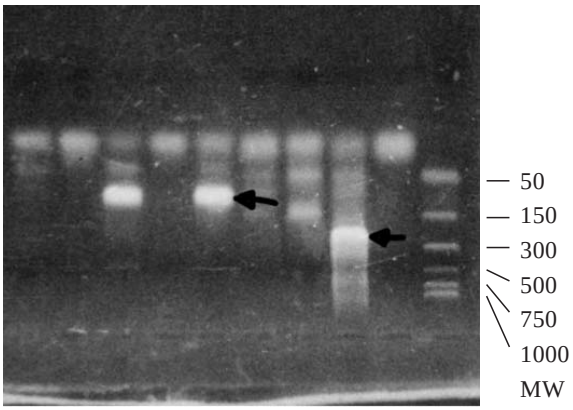

Fig. (5): Ethidium bromide stained agarose gel electrophoresis of PCR product obtained using the FRIIIA (solid arrow) & FRII (dotted arrow) consensus primers sets.

Distribution of PCR positivity among FRIIIA and FRII primers: (Fig. 6).

The majority of cases showing clonal gene rearrangement were detected by FRIIIA primer set alone [31/35 cases (88.8%)]. A clone was detected in 7/35 cases (20%) using FRII primer set alone, while three cases (9%) showed a clone using both FR IIIA and FRII primer sets.

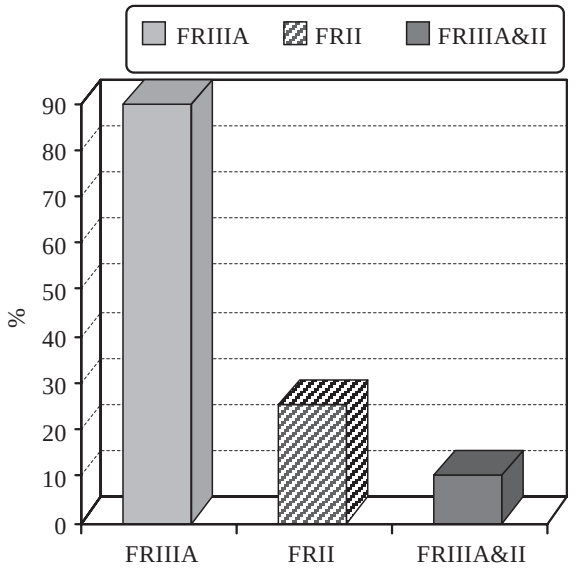

Fig. (6): Distribution of PCR positivity among FRIIIA and FRII.

Distribution of frame positivity among the FCL and LCL:

Distribution of frame positivity among the FCL and LCL revealed a clonal rearrangement of IgH gene in 67% of FCL cases (8/12) using both primer sets. FRIIIA primer set detected 6 of these cases (75%), FRII detected 3 cases (37.5%) while one case was detected using both primer sets (12%). Clonal rearrangement of the immunoglobulin heavy chain gene was detected in 75% of our LCL cases (6/8), including all 3 minimally infiltrated cases and 3/5 of the morphologically non-infiltrated cases. FRIIIA primer set detected all 6 (100%) cases while clonality was revealed in only one case using FRIIIA and FRII primer sets (Fig. 7).

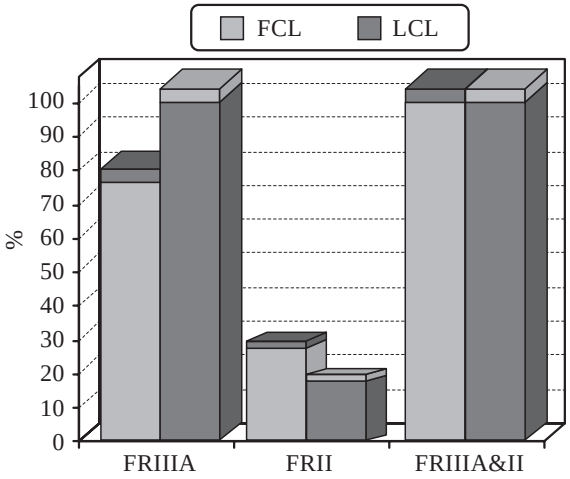

Fig. (7): Distribution of frame positivity among the FCL and LCL subgroups.

Distribution of CD 79a,CD20 and PCR results according to pathology (table 4a and b):

Pathologically Diagnosed Cases (40):

In the 24 cases with minimal or no morphological evidence of lymphomatous involvement (groups B & C), IHC staining using CD79a revealed an infiltrate in 5 cases while demonstration of clonal IgH rearrangement using PCR revealed an infiltrate in 14 cases. The combined use of IHC and PCR detected infiltration in a total of 16 cases in this group.

Thus the sensitivity of the IHC in detecting minimal marrow involvement in our cases; using CD79a was found to be 31.25% (5/16), while that of PCR was 87.5% (14/16).

Pathologically unconfirmed cases (9): Table (4-b).

Table (4-A): Distribution of CD79a, CD20 and PCR results among the different groups and pathological subtypes.

Pathology	No.	Group A: >10% infiltration				Group B: Minimally infiltrated <10%				Group C: Not infiltrated			
		No.	CD79a	CD20	PCR	No.	CD79a	CD20	PCR	No.	CD79a	CD20	PCR
FCL	12	7	7	7	7	1	0	0	1	4	1	0	0
LCL	8	–	–	–	–	3	1	0	3	5	1	0	3
Burkitt	4	2	2	2	2	1	0	0	1	1	1	0	0
SLL	2	2	2	2	2	–	–	–	–	–	–	–	–
MCL	2	2	2	2	2	–	–	–	–	–	–	–	–
LPL/L	2	1	1	0	1	–	–	–	–	1	0	0	1
HCL	2	1	1	1	0	1	0	0	1	–	–	–	–
L/L	1	1	1	0	1	–	–	–	–	–	–	–	–
Extra-nodal	7	–	–	–	–	1	0	0	1	6	1	0	3
Total no. of B-NHL cases	40	16	16	14	15	7	1	0	7	17	4	0	7
Percent	100	40%	100%	87.5%	93.8%	17.5%	14.3%	0%	100%	42.5%	23.5%	0%	41.2%

FCL = Follicular cell lymphoma. CLL = Chronic lymphocytic leukemia. LPL/L = Lympho-plasmacytic lymphoma/leukemia.
LCL = Large cell lymphoma. MCL = Mantle cell lymphoma. L/L = Lymphoblastic lymphoma/leukemia.

Table (4-B): Distribution of CD79a, CD20 and PCR results in pathologically unconfirmed cases.

Pathol.		No.	CD79a	CD20	PCR
Group A > 10% infilt.	Unclassified	3	2	1	3
	Undiagnosed	—	—	—	-
	Total no.	3	2	1	3
Group B < 10% infilt.	Unclassified	1	0	0	0
	Undiagnosed	3	0	0	3
	Total no.	4	0	0	3
Group C not infilt.	Unclassified	2	2	0	0
	Undiagnosed	—	—	—	—
	Total no.	2	2	0	0

DISCUSSION

The evaluation of staging or post therapy bone marrow biopsies for involvement by lymphoma has traditionally been based on morphologic findings. To improve sensi-tivity, bilateral and double bilateral trephine biopsies have been performed to reduce sampling error. Morphologic examination is augmented by advances in molecular technology that detect clonal lym-phoid populations by immunoglobulin gene rearrange-ments and lymphoma associated chromosomal translo-cations. In our study, Bone marrow biopsy infiltration was studied using different techniques in an attempt to establish a protocol for detecting and confirming lymphomatous involvement of the bone marrow in cases with B-NHL.

This study was conducted on 49 patients with histopathological diagnosis or clinical suspicion of NHL that were considered for bone marrow examination either for diagnosis, staging or re-evaluation post therapy. Nine of these cases had their bone marrow examined for a clinical suspicion of lymphoma without a documented pathological examination of another tissue. Bone marrow biopsies were evaluated for presence or absence of infiltration using

Conventional morphologic examination, and immunohistochemical staining using CD 79a and CD 20 which cover the whole spectrum of B lymphoid neopla **[7]**. Detection of clonal IgH heavy chain gene rearrangement, an early event in B-cell development, in paraffin embedded biopsy was performed to confirm the clonal nature of the infiltrate **[8]**.

In this study consensus primers instead of clone-specific primers were used. Although clone-specific PCR methods are specific and sensitive to a level of one in l06 cells, these methods are too inef-ficient for routine testing because unique primers are required for each patient and may fail in some leukemia and lymphomas as a result of clonal evolution **[2]**. It has been reported by Abdel Rehim et al. **[9]** that the primers with the highest detection rate for the immunoglobulin heavy chain gene rearrangements are directed against the framework (FR) III region of the various VH genes. The addition of other framework regions, particularly

FRII primers, increases the detection rate of this test. We therefore included both primer sets in this study.

On morphological examination, twenty eight cases (57.2%) showed no evidence of infiltration on aspirate examination, however biopsy examination revealed minimal infiltration in 9 of these cases (35%). In the remaining 21 cases (42.8%), examination of both bone marrow aspiration and biopsy revealed infiltration. Thus bone marrow aspiration examination detected infiltration in 42.8% while bone marrow biopsy revealed infiltration in 61.2% of cases (30/49).

Following bone marrow biopsies examination, patients were classified into three groups; Group (A): Cases that showed marrow infiltration constituting more than 10% of marrow cellularity. This group included 19 cases (39%). Group (B): cases that were minimally infiltrated (suspicious) 11 cases (22%) Group (C): cases with no marrow infiltration (19 cases (39%). As regards infiltration incidence in the different histopathological subtypes, it was only feasible to analyse cases with FCL (12) and LCL (8), as we had only small number of cases in other pathologic categories. Among the follicular lymphoma group, seven cases (58%) were morphologically infiltrated, a single case was minimally infiltrated and four cases (33%) showed no evidence of infiltration. For large cell lymphoma, none of the 8 cases studied showed evident marrow involvement on morphological and/or immunohistochemical examination, while a minimal marrow involvement was detected in 3 cases on morphological examination, which is similar to the incidence observed by Kroft et al. (2001) **[10]**.

PCR amplification of IgH chain genes from DNA obtained from paraffin embedded biopsy sections detected a clonal rearrangement in a total of 35 cases. 88.6% of these cases were detected by FRIIIA primer set (31/35). A clone was detected in 7 cases (20%) using FRII primer set. Three cases (9%) showed a clone using both FR III and FRII primer sets. Thus the use of FRIIIA and FRII primer sets together detected all clonally rearranged IgH genes. In accordance with this finding, Coad et al. **[2]** detected PCR positivity in 37% of cases using single primer set while the percentage increased to 80% using multiple primer sets. Crotty et al. and **[11]**, Lehann et al. **[12]** detected a clone in 67.5% and in 66% of B-NHL cases respectively. Distribution of frame positivity among the FCL and LCL revealed a clonal rearrangement of IgH gene in 67% of FCL cases (8/12), with 75% of cases detected by FRIIIA primer set alone. FRII primer set detected the remaining 2 cases. In LCL cases Clonal rearrangement of the immunoglobulin heavy chain gene was detected in a total of 75% using FRIIIA primer set, FRII detected 1 case while both primer sets revealed clonality in one case. In a similar study a combination of FRII and FRIIIA primers detected 79 to 98% of B cell neoplasms depending on the type of disease **[13]**.

In group (A), CD79a was positive in all cases with a histopathological diagnosis of NHL while CD20 was positive in only 87.5% of these cases. A clonal rearrangement of the IgH was detected in 93.7% of histopathologically diagnosed cases. In a study by Kremer et al. **[14]** PCR was 100% positive in 12 patients with evident marrow involvement with B-NHL. Krober et al. **[15]** detected a clone in 77% of 529 patients in the same category. Thus the more the number of examined cases, the higher the possibility of evaluating the sensitivity of the technique. Failure to demonstrate a clonal IgH gene rearrangement in the case with HCL, in spite of an evident marrow involvement on morphological and immunohistochemical examination, may be due to the use of consensus primers which are not a perfect match to the sequence being amplified and result in less efficient amplification. In addition, somatic mutations of the immunoglobulin heavy chain gene of some mature B disorders might alter the sequence of the region amplified by the primers so that primer hybridization is sub optimal or does not occur **[16]**.

In group (B), PCR revealed a clonal rearrangement in 100% of cases, CD 79a detected only one case (14.28%), while CD 20 detected 0%. Failure to demonstrate cases with minimal infiltration by IHC staining may be attributed to the loss of lymphomatous tissue on serial sectioning due to their small size. Coad et al. 1997 had a lower percentage (25%) of detection of clonal rearrangement in minimally infiltrated NHL cases than in the present study, yet this could be attributed to the use of bone marrow aspiration rather than bone marrow biopsy, which decreases the amount of clonal tissue

available. In similar studies using PCR amplification, Kremer et al. **[14]**, detected a clone in 20% of 5 cases with minimal bone marrow infiltration. Using laser micro-dissection technique, which greatly increased the amount of clonal tissue in the sample, they could detect clonality in 70% of their cases. The higher sensitivity in clonal detection in this group in the present study might also be attributed to the criteria used to define minimal infiltration. In our study cases showing small lymphomatous collections that do not meet the criteria of morphological classification as involvement were considered minimally infiltrated.

Seventeen cases were morphologically uninvolved with a lymphomatous infiltrate (group C). Immunohistochemical staining of these cases using CD 79a revealed scattered positive cells in 4 cases (23.5%), however only 2 of these cases showed a clonal rearrangement of the IgH gene. CD 20 failed to reveal any positivity. PCR revealed a clonal rearrangement in 41.2%of cases (7cases). Cases with interstitially scattered lymphomatous involvement are difficult to diagnose on morphological basis alone but require confirmation by IHC staining or demonstration of a clonal proliferation. Coad et al. **[2]** detected a clone in 13 cases (11%) out of 129 cases of B-NHL which were morphologically non-infiltrated. The lower incidence of infiltration detection in their study could be attributed to the use of bone marrow aspiration rather than bone marrow biopsy which decreases the amount of clonal tissue available. In the same study, DNA extracted from sections provided results more concordant with morphology, as PCR detected a clone in 10 out of 11 DNA specimens extracted from trephine biopsies with positive morphologic findings and negative PCR aspirates.

In the 24 cases with minimal or no morphological evidence of lymphomatous involvement, the sensitivity of the IHC staining in detecting minimal marrow involvement, in this study, using CD79a was found to be 31.25% (5/16), while that of PCR was 87.5% (14/16) Similarly, in a multicenter study, a PCR sensitivity of 83.5% in non follicular lymphomas was found **[17]**. Also, Uchiyama et al. **[18]** reported that PCR has been used with increasing frequency to detect clonal rearrangements of IgH in formaldehyde fixed, paraffin wax embedded tissues with a sensitivity ranging between 50 and 80%.

Nine cases were undiagnosed by histopathology, 6 were reported as unclassified and 3 had no accessible tissue for examination. Of the 6 unclassified cases, 3 belonged to group (A), two showed positive staining with CD 79a and one with CD20. All three cases showed a clonal IgH gene rearrangement. The remaining three cases, one belonged to group (B) and two to group (C), showed no staining with either CD79a or CD20 and no clonal rearrangement on PCR examination. The three cases with no accessible tissue but performed the biopsy based on clinical suspicion belonged to group B and all showed a clonal rearrangement of IgH gene. A follow-up of two of these 3 cases revealed a leukemic phase of a lymphoblastic lymphoma confirmed by immunohistochemistry, in one case. The other case was pathologically diagnosed as T cell lymphoma following surgical removal of a mediastinal lymph node. IgH gene aberrant rearrangements have been detected in 16% of T-cell neoplasms **[19]**. Follow-up of the third case was not feasible.

From the above results, we suggest the following scheme for studying and interpreting clonality in B-NHL, involving the bone marrow:

I- In histopatholgically diagnosed cases of B-NHL

• A positive PCR bone marrow result in a case of known B-NHL is a confirmatory test for the presence of a clonal involvement of the bone marrow in presence of evident morphological and/or immunohistochemical infiltration.

• A positive PCR bone marrow result in a case of known B-NHL is mandatory and an indispensable proof for the presence of a clonal involvement of the bone marrow in presence of minimal morphological and/or immunohistochemical evidence or absence of either.

II- In histopathologically non-diagnosed cases of B-NHL a clonal IgH gene rearrangement, in absence of morphological and immunohistochemical evidence of infiltration, though confirming the presence of a clonal disorder, yet is non-conclusive for diagnosing a B type-NHL in the bone marrow. Further confirmation by demonstration of Ig light chain gene rearrangement is recommended.

Thus, in morphologically minimally-infiltrated or non-infiltrated bone marrows of

histopathologically documented cases of B-NHL; positive IHC and/or PCR findings are strong suggestive evidence of involvement.

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Ultrastructural Alterations of Inflammatory Cells in Bladder Cystitis Versus Bladder Carcinoma

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ABSTRACT

Chronic inflammatory cystitis, caused either by chemical irritants or bacterial, viral and parasitic infections, has long been known to play a crucial role in the development of bladder cancer. However, two opposing actions have been suggested to be produced by inflammation as it may either enhance cancer development or destroy malignant cells. An ultrastructural comparative study between inflammatory cells in bladder cystitis and bladder cancer is conducted in a trial to verify the anti-tumour and tumorigenic role played by such inflammatory cells during the development of tumor on top of cystitis. Urine cytology samples were obtained from three groups of patients, three patients each: bladder cystitis, transitional cell carcinoma [TCC] and squamous cell carcinoma [SqCC] and examined by electronmicroscopy using the agarose cell block technique. Most inflammatory cells seen in bladder cystitis (with predominance of granulocytes) revealed features of activity as indicated by presence of intracytoplasmic phagocytic vacuoles, cytoplasmic villous projections and hypogranularity (denoting a degranulation process). However, in cancerous conditions, inflammatory cells showed two main entities with distinct morphological features . Some cells appeared with reactive features similar to those of cystitis and others represented abnormal forms with marginated and condensed nuclear chromatin, bizarre-shaped granules, disrupted cytoplasm, abnormal undefined inclusions, intense vacuolation and occasional phagocytic vacuoles. It is hence suggested that, in cases of bladder cancer, the inflammatory cells with reactive features could be assigned to a protective antibacterial (similar to cases of uncomplicated cystitis) or tumoricidal /tumoristatic action. The distorted population, on the other hand, could be assigned to a different function with a tumorigenic action. Therefore the appearance of the latter population in the urine of patients suffering from cystitis could be regarded as an early warning sign to the urine cytologist for cancer development.

Key Words: *Bladder cancer - Bladder cystitis - Urine cytology - Ultrastructure - Inflammatory cells.*

INTRODUCTION

In Egypt, bladder carcinomas account for about 25-50% of all malignancies **[1-3]**. They are regularly associated with schistosomiasis that results in chronic inflammation in which infiltrating cells such as neutrophils, macrophages and eosinophils are sources of mediators (Oxygen radicals, cytokines…etc) known to induce DNA damage of the bladder epithelium **[1]**. However, other factors are implicated in carcinogenesis in these patients e.g. smoking, bacterial bladder infections or exposure to environmental carcinogens **[4]**.

Hence an intricate relationship exists between inflammatory reaction and cancer development (carcinogenesis). In fact, inflammation may produce two opposing effects depending on the state of activation of inflammatory cells. It may either enhance cancer development or may destroy malignant cells (tumoricidal action) or arrest their growth (tumorstatic action).

This concept has been evidenced by many in vivo and in vitro studies dealing with various cancers. Macrophages and neutrophils are regarded as components of inflammatory infiltrates commonly observed in stroma of tumors. They seem to play a dualistic role in human development either producing cytotoxic effects or enhancing tumor growth **[5-11]**.

The significance of natural killer cell and lymphocyte infiltrates in cancer was related to lysis and killing of tumor cells (12; cited by 13).

On the basis of such association of inflammation with bladder cancer, the pursuit of inflammatory cells in various bladder lesions could provide a valuable predictive marker for development, growth, invasiveness and aggressiveness of bladder carcinomas.

However, morphology of inflammatory infiltrates, histopathologically notable in tumors, cannot distinguish inflammatory cells that cause tumor destruction from those that promote tumor growth **[13]**.

On the other hand, electronmicroscopy (EM) in urine cytology by showing more details in the cellular infiltrates could demonstrate the definite differential morphological features of this double action.

It is aimed in the present work to use the alternative non-invasive urine cytology technique for electronmicroscopic examination of exfoliated inflammatory cells in urine samples of both bladder cystitis and bladder cancer. The inflammatory cell ultrastructural alterations in bladder cystitis as compared to urothelial cancers could be of value in verifying their differential role in both conditions. They could also be important in deciding the therapeutic effectiveness of eliminating the chronic irritation in reducing the incidence of bladder cancer.

MATERIAL AND METHODS

Patients:

Three groups of patients (three in each) were selected: A group with bladder cystitis (diagnosed by urine analysis showing 100 pus cells/HPF); a second group with transitional cell carcinoma (TCC) and third group with squamous cell carcinoma (SqCC) (as diagnosed by histopathological examination of cystoscopic biopsy).

Specimen Preparation:

Voided urine was centrifuged at 1500 rpm for 10 min., supernatant decanted and the deposit fixed in 2% glutaraldehyde in cacodylate buffer for one hour and after centrifugation processed for agarcyto cell block formation.

Agarcyto Cell Block Preparation:

This was performed according to a technique first reported by Kerstens et al. **[14]** for diagnosis of cervical cancer and modified by Mansy **[15]** to suit urine cytology samples.

The fixed cells were centrifuged for 10 minutes at 2000 rpm, supernatant decanted and deposit transferred to 1.5ml Eppendorf tube and spun down. The deposit was resuspended in 1ml of 2% liquid agarose at 65ºC and concentrated by centrifugation in the agarose which is solidified at 4ºC for 30 minutes. The other agarose block was processed for EM examination where the specimen was refixed in buffered glutaraldehyde for two hours after being sectioned into small pieces (1x1 mm in size) and processed according to Clement et al. **[16]**. After rincing, the specimen was postfixed in osmium tetroxide (1% in cacodylate buffer) for one hour at 4ºC, dehydrated in ascending grades of alcohol, substituted in a mixture of Epon and absolute alcohol in equal volumes and lastly infiltrated in three baths of epon resin. The specimen was then embedded in a mixture of Epon resin and tri-dimethylamino ethyl phenol accelerator (DMP30) in capsules. Polymerization then follows in an oven for 12 hours at 37ºC and then for 2 or 3 days at 60ºC. Ultrathin sections were prepared and examined by Philips electron microscope (EM 208S).

RESULTS

The urine cytology of the majority of bladder cystitis cases showed abundant neutrophils, some phagocytic mononuclear cells, many bacteria (intra- and extracellular) and some urothelial cells. The phagocytic cells appeared scattered and actively engulfing bacterial particles (Fig. 1). Neutrophils showed nuclei with fine chromatin (Figs. 2,3). The cytoplasm was of normal density and amount but showed many projections (Fig. 3). Intracytoplasmic granules displayed the normal heterogenous structure with variation in size, shape and density with predominance of large electron-dense primary granules (Fig. 2). Frequent phagocytic vacuoles were apparent with or without bacterial contents. No abnormal inclusions were present. Occasional apoptotic and degenerating neutrophils were encountered.

Patients with transitional cell carcinoma had a more aggressive cellular infiltration in their urine cytology with many malignant degenerated urothelial cells and neutrophils but fewer bacterial particles. Most neutrophils appeared in aggregates or adherent to the tumor cells (Fig. 4,5). The phenomenon of phagocytosis was less

prominent than in cystitis cases. Many degenerated neutrophils were encountered with membrane lysis and extracellular release of granules (Fig. 6). A detailed examination of the neutrophil infiltrate revealed many cells with gross cellular abnormalities, viz. chromatin margination or condensation, bizarre-shaped granules, disrupted cytoplasm, abnormal undefined inclusions, intense vacuolation and occasional phagocytic vacuoles containing debris (Figs. 6,7). Some other less distorted cells are encountered showing fine nuclear chromatin but their granules were more or less bizarre and the cytoplasm was slightly vacuolated (Figs. 4,5).

Most patients with SqCC presented with hematuria and so their urine contained many red blood cells. Malignant urothelial cells and eosinophils were rarely seen. Unlike TCC, eosinophils in SqCC urine cytology constituted the main component of the inflammatory infiltrate. Most of these cells appeared degenerated with disintegrated nuclei, lysed membrane and cellular fragmentation. Intact cells showed bizarre-shaped granules in their cytoplasm (Fig. 8).

DISCUSSION

The functional relationship between inflammation and cancer has long been established in many studies. Moreover, a dualistic role of inflammatory cells has been assumed **[1,2,5,7, 11,10,17,18,19]**. Although inflammation may enhance cancer development by helping to add mutations or support malignant growth, inflammation may also destroy malignant cells or arrest their growth. Investigators **[20]** explained these opposing actions by pointing out that intense inflammation leading to destruction of the initiated cells could decrease tumor development but that nondestructive inflammatory responses might promote tumor growth.

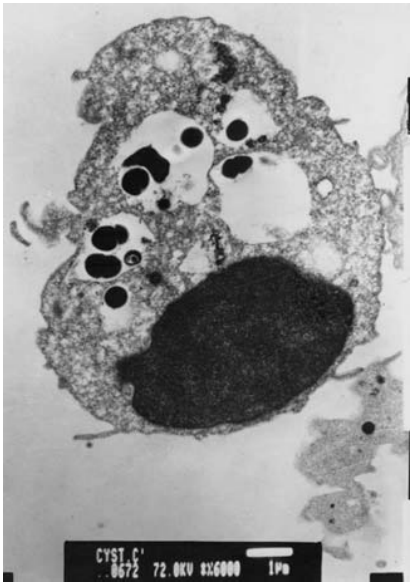

Fig. (1): A phagocytic cell from a case of bladder cystitis engulfing many bacterial cocci enclosed in large phagocytic vacuoles in the cytoplasm X6000.

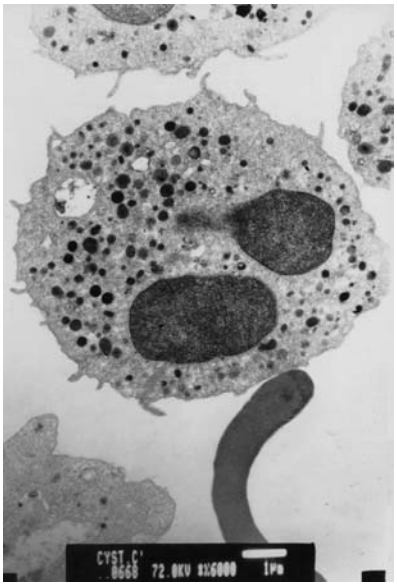

Fig. (2): Urine cytology from a case of bladder cystitis showing a neutrophil with bilobed nucleus and relatively fine chromatin. The cytoplasm appears studded with rounded electron-dense primary granules over-numbering the less dense small elongated secondary granules. Few phagocytic vacuoles are also encountered and the surface membrane shows projections X6000.

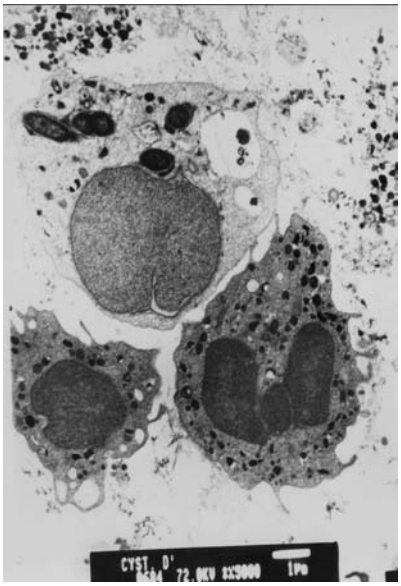

Fig. (3): Two neutrophils in a bladder cystitis case juxtaposed to a urothelial cell. They display phagocytic vacuoles, many granules and cytoplasmic projections X5000.

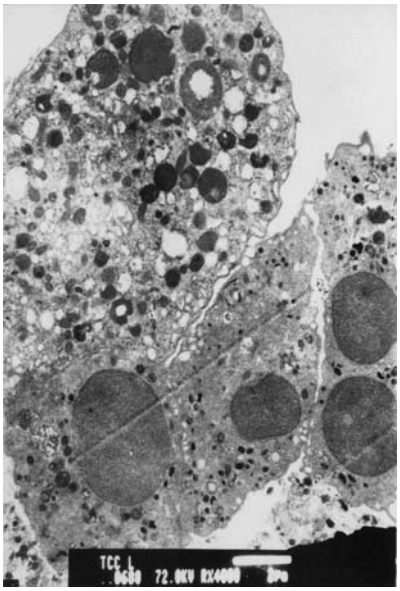

Fig. (4): A photomicrograph showing a collection of adherent neutrophils from a patient with TCC (grade II) showing nuclei with fine chromatin and few scattered bizarre-shaped granules. A nearby malignant cell (k) is also encountered X4000.

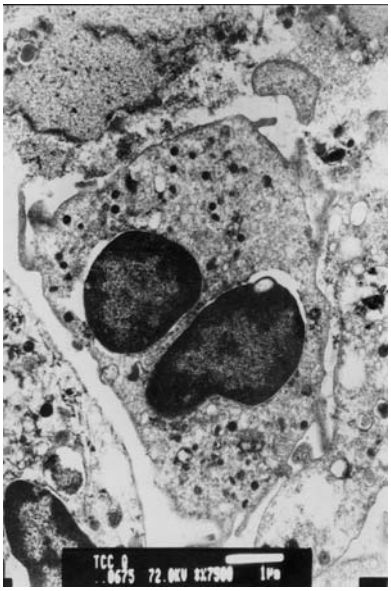

Fig. (5): A photomicrograph of a neutrophil from urine cytology of a patient with TCC revealing a bilobed mature nucleus. The cytoplasm contains few granules and some vacuoles. Some projections are seen extending towards nearby cells X7500.

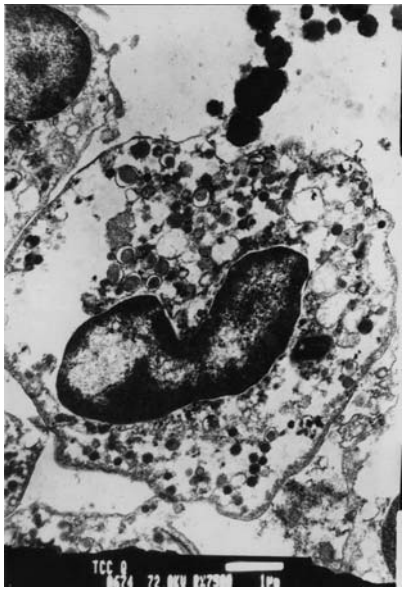

Fig. (6): A phagocytic cell in urine cytology of a TCC patient showing disintegrated cytoplasm and including many vacuoles and distorted granules X7500.

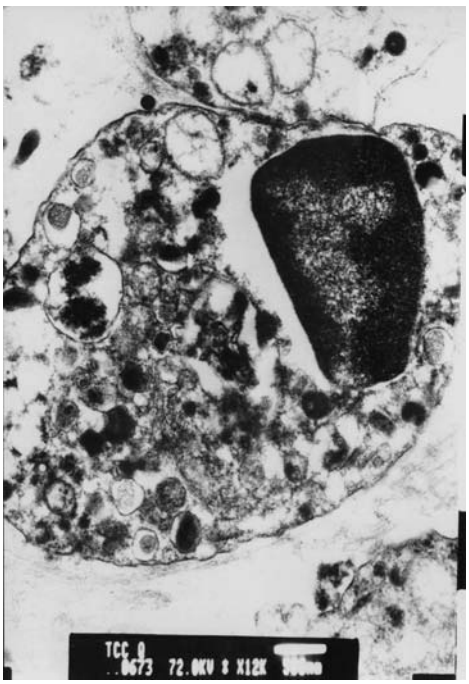

Fig. (7): A photomicrograph of a phagocytic cell in urine of a patient with TCC (stage II). The nucleus appears mature but unilobular with condensed marginal chromatin and central less dense euchromatin. The disrupted cytoplasm shows markedly distorted granules. Engulfed particles are seen in large phagocytic vacuoles X12,000.

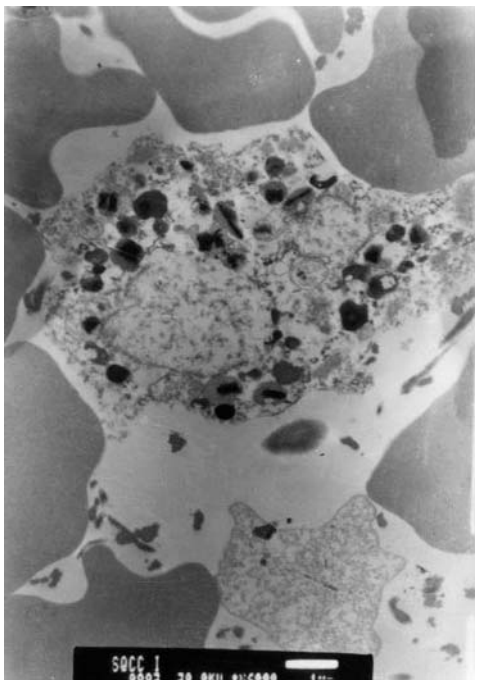

Fig. (8): A photomicrograph of urine cytology of a patient with SqCC showing many red cells and an eosinophil (Eo) with a disintegrated nucleus and disrupted cytoplasm studded with many distorted granules at various stages of degranulation. The cytoplasmic membrane appears lysed in many regions X6000.

The EM examination of the urine cytology in the present work has revealed quite distinguishing characteristics of the inflammatory infiltrates on comparing bladder cystitis with bladder carcinomas. In bladder cystitis, neutrophils show an activated reactive status as demonstrated by internalized bacterial particles in phagocytic vacuoles, many villous projections and cytoplasmic hypogranularity indicative of a degranulation process (Figs. 1,2,3). Marmont et al. **[21]**, by electronmicroscopic analysis of the cells fixed during phagocytosis, have shown that neutrophils incubated with Escherichia coli depicted numerous microorganisms within phagocytic vacuoles and the cell was almost completely degranulated.

In the urine of transitional cell carcinoma cases, the main inflammatory cell component was the neutrophil as with cases of cystitis. However, neutrophils show more propensity to aggregation as well as degeneration (Figs. 4,5,6, 7). The morphology also varies since some cells were demonstrated having reactive features similar to those of cystitits though with less phagocytic capacity as indicated by decreased intracytoplasmic phagolysosomes (Figs. 4,5). Other cells represented an abnormal form with marginated condensed chromatin, disrupted vacuolated cytoplasm including distorted granules and abnormal inclusions (Figs. 6,7).

It is suggested that the cells with reactive features could be assigned to a protective function either against a superimposed bacterial infection (similar to cases of uncomplicated cystitis) or against the tumor cell itself (tumoricidal or tumoristatic action). The distorted population with bizarre-shaped granules and inclusions and weak phagocytic ability could be assigned to a different function such as supporting tumor growth as assumed by the in vitro and in vivo function studies. Dallegri and Ottonello **[7]** have reported a neutrophil destructive action on human and urine tumor cells. This was supported in vivo by an IL4-induced neutrophil tumoricidal effect **[8]**. Furthermore, neutrophils may attract antigen-presenting cells to the inflammatory site generating tumor-specific T-cell response by the host **[18,19]**. Other lines of evidence, however have suggested a growth enhancing effect of neutrophils on cancer cells as granulocyte infiltration occurred in proximity to highly proliferative areas of the growth in murine squamous cell carcinomas **[6,9]**.

The main inflammatory cell associated with squamous cell carcinoma (SqCC) was the eosinophil. This could be explained by the fact that SqCC is common among patients with schistosoma infestation **[22]**. However, the eosinophil exhibits degenerative changes in the nucleus and cytoplasm (Fig. 8). Also Bizarre-shaped granules are demonstrated (Fig. 8). These changes are different from those encountered in the inflammatory eosinophilic reactions described in other conditions as allergy or parasitic infestations **[23,24]**. Hence, they could be regarded as tumor growth promoters rather than reactive antitumor effectors.

In conclusion, the distinguishable morphological alterations encountered in inflammatory cells associated with tumors but absent in bladder cystitis, though could not be diagnostic, yet could act as a warning sign to the urine cytologist when starting to appear in the urine of patients suffering from chronic irritation or inflammation of the bladder epithelium.

Further EM investigations of the features exhibited by the inflammatory cells in premalignant states and in various grades of bladder cell carcinoma could be of predictive value in tumor aggressiveness and invasiveness.

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The Apoptotic Effect of PTX on Inflammatory Cells in the Liver of Mice Infected by Schistosoma mansoni: An Ultrastructural Study

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ABSTRACT

Pentoxifylline (PTX) is known for its suppressor effect in various inflammatory lesions. It has been recently suggested that PTX may play a role in downregulating the migration and participation of inflammatory cells in the granuloumatous lesions in murine models of schistosomiasis. This could help to alleviate the injurious effect exerted by inflammation on the host tissue. Accordingly, PTX was administered in Schistosoma mansoni-infected experimental mice (at 7 weeks & 15 weeks stages) and its effect on inflammatory cells was investigated by transmission electronmicroscopic ultrastructural study of the migrating inflammatory cells in the intravascular compartment and those residing in the extravascular granulomatous lesion. The inflammatory cells in the PTX-treated groups showed obvious apoptotic morphological changes (chromatin condensation and margination, shrinkage of the nucleus, widening of the perinuclear space, cytoplasmic condensation, intact organelles and cytoplasmic membrane) during both early and late stages of the disease. These results denote that the induction of apoptosis of inflammatory cells by PTX could be regarded as one mechanism whereby this drug produces its antiinflammatory action. Thus the administration of PTX could be a beneficial therapeutic approach in ameliorating the noxious effect produced by the full blown inflammatory process.

Key Words: *Pentoxifylline - Schistosomiasis - Inflammatory cells - Hepatic granulomatous lesions - Ultrastructure - Apoptosis.*

INTRODUCTION

Pentoxifylline (PTX), a methyl xanthine derivative, has recently been shown to have an antiinflammatory effect by interfering with migration and adherence of immune cells **[1-5]**.

Many studies have addressed the mechanisms underlying such effect and suggested the inhibition of production of the proinflammatory cytokine tumor necrosis factor -alpha (TNF-α) and the subsequent suppression of ICAM-1 (intercellular adhesion molecule-1) expression on the recruited cells **[5-11]**.

Many therapeutic trials using PTX have been conducted to evaluate its antiinflammatory potentiality in various inflammatory diseases **[12-15]**.

Studies have been performed to investigate effects of PTX on hepatic periovular granuloma, an inflammatory injurious lesion responsible for morbidity and mortality in schistosomiasis, which is usually superceded by mobilization and accumulation of inflammatory cells in the hepatic tissue. In one study using an experimental model **[16]**, PTX was found to produce significant reduction of the expression of the adhesion molecule LFA-1 (lymphocyte function-associated molecule-1) on leucocytes infiltrating the liver in both intra- and extarvascular compartments during the vigorous stage of granuloma. This indicated an antiinflammatory effect of PTX through inhibiting LFA-1- mediated adherence and subsequent migration of inflammatory cells forming the granulomatous lesion.

Another evidence for PTX antiinflammatory potency was presented by Reis et al. **[17]** who showed decrease in the intragranulomatous eosinophil accumulation due to interference with cellular recruitment and/or differentiation.

In the present work, a detailed ultrastructural study of the migrating inflammatory cells was conducted in order to demonstrate the possible PTX -induced alterations in the integrity and activity of the inflammatory cells. This would,

in turn, throw more light on the therapeutic efficacy of PTX in ameliorating the murine Schistosoma mansoni granulomatous lesion during the acute vigorous and chronic stages of the infection.

MATERIAL AND METHODS

Animals:

Twelve white albino mice were infected with Schistosoma mansoni by SC injection of 60 cercariae/ mouse. They were then equally divided into 4 groups:I. The first group was sacrificed at 7 weeks post infection, II. The second group was sacrificed 15 weeks after infection, III. The third group was treated with pentoxifylline (Sigma) 3 weeks before sacrifice and then sacrificed at 7 weeks postinfection, IV. The fourth group was treated with PTX for 3 weeks using the same dose level as in group III before sacrifice and then sacrificed at 15 weeks postinfection.

The therapeutic dose was calculated first by transforming the human dose of trintal, the commercial name for PTX (400 mg three times daily), into a murine dose (3.12 mg three times daily) using special tables devised by Paget and Barnes **[18]**. The murine dose was further multiplied by 1.5 (4.68 mg daily) in order to ensure higher PTX effect. The treatment was continued for three weeks until the date of sacrifice.

Transmission Electronmicroscopy:

Liver samples (1mmX1mm in size) were prepared for transmission electronmicroscopy according to Clement et al. **[19]** as follows:

1- Fixation in 2.5% glutaraldehyde (Merk) in cacodylate buffer [0.2M, pH=7.4] (Electronmicroscopy Sciences) for 2 hours at 4ºC.
2- Post fixation in 1% OsO4 (Electronmicroscopy Sciences) in cacodylate buffer [0.3M, pH=7.4] for 1 hour at 4ºC.
3- Dehydration in ascending grades of ethanol (30%, 50%, 70%, 90% and 100%).
4- Substitution in a mixture of epoxy resin (Electromicroscopy Sciences) and absolute ethanol.
5- Inclusion in three washes of epoxy resin at room temperature twelve hourly.
6- Embedding in epoxy resin capsules.
7- Polymerization of the resin at 60ºC for three days.
8- Ultrathin sectioning of capsules and examination of sections under the electronmicroscope, Joel 1200 EX II.

RESULTS

In the 7 weeks S.mansoni-infected untreated animals:

By EM, the inflammatory cells are seen both intravascular and intragranulomatous (Fig. 1). Most granuloma cells and those present in the lumen of blood vessels are composed of lymphocytes, plasma cells, monocytes, some neutrophils and very few eosinophils (Fig. 2).

In the intravascular compartment, the cells are abundant and some appear adherent to the endothelial cells lining the vessel. Most of the cells exhibit normal morphology with intact nuclei displaying normal size, shape and chromatin pattern. Also the cytoplasm density is normal and contains normal intact well-defined granules and other cellular organelles such as mitochondria and endoplasmic reticulum. Slight collagen deposition is encountered (Fig. 1).

In the 7 week S.mansoni-infected PTX- treated mice:

The same types of inflammatory cells are present in the treated and untreated groups of the corresponding stage and they are still represented inside the lumen of blood vessels and in the granulomata (Fig. 3). Also the relative number of cells to fibrous tissue is not affected by treatment. However, EM revealed morphological alterations of many cells with appearance of apoptotic and degenerative elements as evidenced by intranuclear chromatin condensation and margination, shrinkage of the nucleus, widening of the perinuclear space as well as condensation, shrinkage and budding of the cytoplasm (Figs. 3,4).

In the 15 weeks S.mansoni-infected untreated animals:

As the disease passes from the 7 to 15 weeks stages, the inflammatory cells start to disappear from the lumen of the blood vessels but continue to be present in the granulomata, though reduced in number relative to the amount of collagen deposits. The differential counts start to divert from the pattern mentioned during the 7-weeks stage towards predominance of eosinophils and fibroblasts (Figs. 5,6). Also some morphological changes are observed in some cells reflecting

a mild degree of degeneration (Fig. 7). This is evidenced by partial disintegration of cytoplasm and rupture of cytoplasmic membrane.

In the 15 weeks S.mansoni-infected PTX treated mice:

The inflammatory cells have disappeared from the intravascular compartments as in the15 weeks untreated group (Fig. 8). Cells also continue to appear in the same relative proportions in the granuloma as in the untreated 15 weeks stage. Furthermore, deposits and fibroblasts are increased (Fig. 9). A shift towards the increase of eosinophils is also encountered. Morphological apoptotic alterations have been observed (Fig. 10).

DISCUSSION

The inflammatory reaction incited in the liver of Schistosoma mansoni-infected mice is a double-edged weapon exerting both a beneficial role in combating the parasite and a noxious effect on the host tissue. The antiinflammatory agent PTX was thus used in this study in a therapeutic attempt to alleviate such injurious inflammatory reaction.

The present work has introduced a morphological analysis of inflammatory cell types in the murine hepatic granuloma revealing the characteristic chronological events that occur during the progression of the granuloma from the seventh week to the fifteenth week post-infection in the infected untreated group of animals. The 7 week granuloma was composed mainly of lymphocytes and plasma cells followed, in the 15 weeks, by their retreat and replacement by eosinophils, fibroblasts and collagen. Also abundant inflammatory cells that appeared in the intravascular compartment during the 7 weeks stage were significantly decreased during the 15 weeks. These findings agree with previous ultrastructural studies of Schistosoma mansoni granulomatous reactions **[20,21]**.

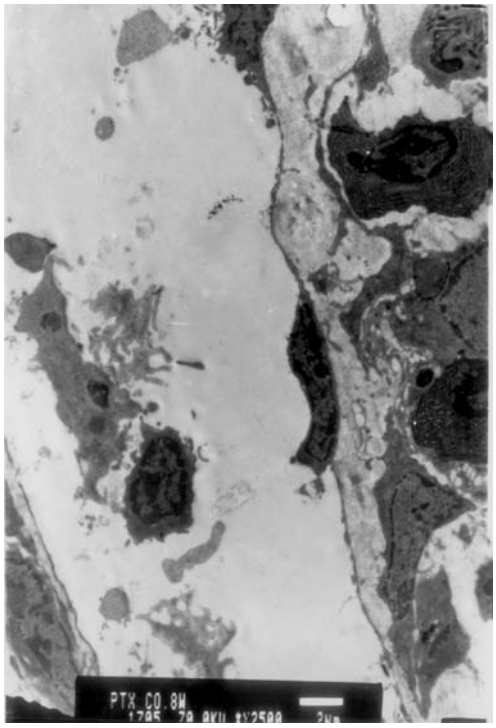

Fig. (1): TEM photo of a 7 wks S. mansoni- infected mouse showing a longitudinal section of a blood vessel in the liver lined by well-defined endothelial cells (EC). The inflammatory cells seen within the vessel are a lymphocyte (LY) and an activated neutrophil (NE) displaying many villous projections. Many inflammatory cells are seen recruited extravascularly in close contact to the vessel. X 2500.

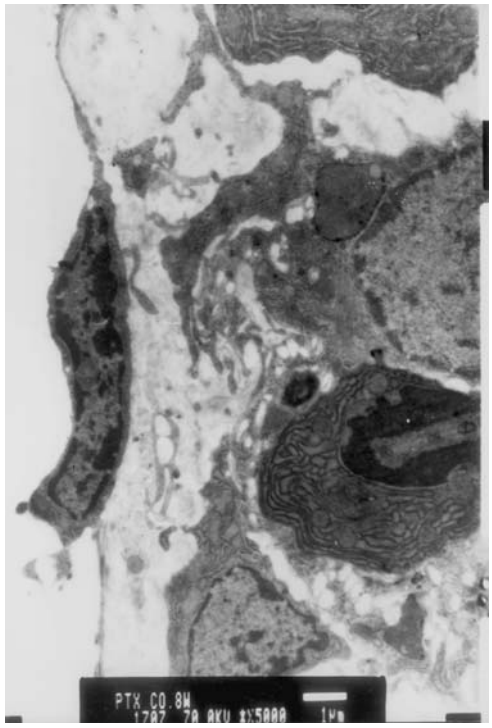

Fig. (2): A granulomatous inflammatory reaction in proximity to the same blood vessel shown in the previous photo. The granuloma is composed mainly of plasma cells (PC) exhibiting the specific onion-like appearance of endoplasmic reticulum in the cytoplasm. X5000.

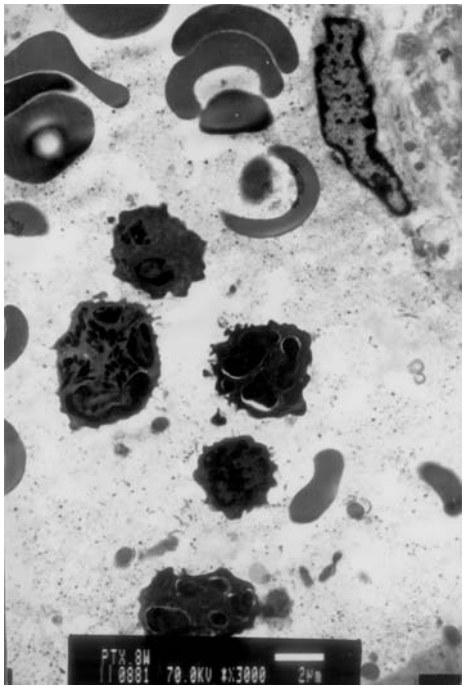

Fig. (3): A micrograph of a liver section of a 7wks PTX-treated S. mansoni- infected mouse showing a collection of apoptotic inflammatory cells in the lumen of a blood vessel. The cells show condensation and pyknosis of their nuclei with widening of perinuclear space as well as condensation and shrinkage of the cytoplasm. X3000.

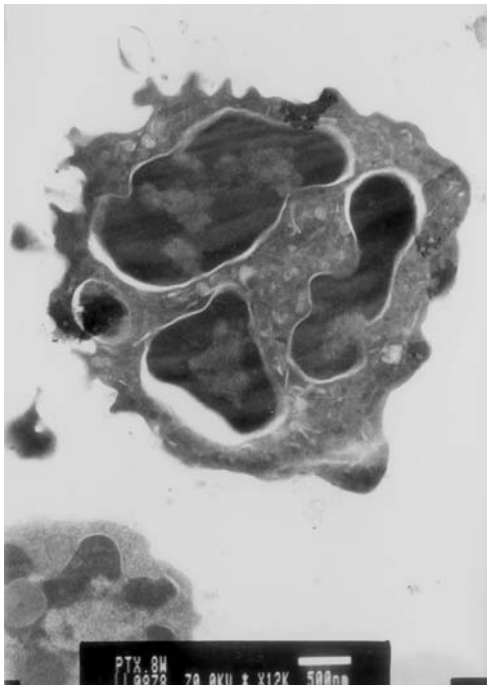

Fig. (4): An apoptotic neutrophil (in a 7wks p.i. PTX-treated mouse) with shrunken and condensed nuclear lobes surrounded by widened perinuclear spaces. The cytoplasm appears condensed but studded by intact granules. X12,000.

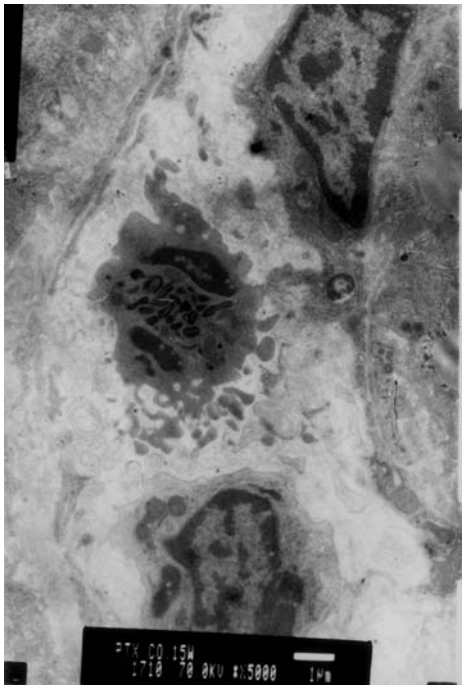

Fig. (5): A TEM photograph in the liver of a 15 wks S. mansoni-infected mouse revealing a blood vessel lined by an endothelial cell (EC) which is seen throwing some projections to reach villi of an eosinophil lying in the lumen of the vessel. A lymphocyte (LY) is also encountered inside the lumen. X5000.

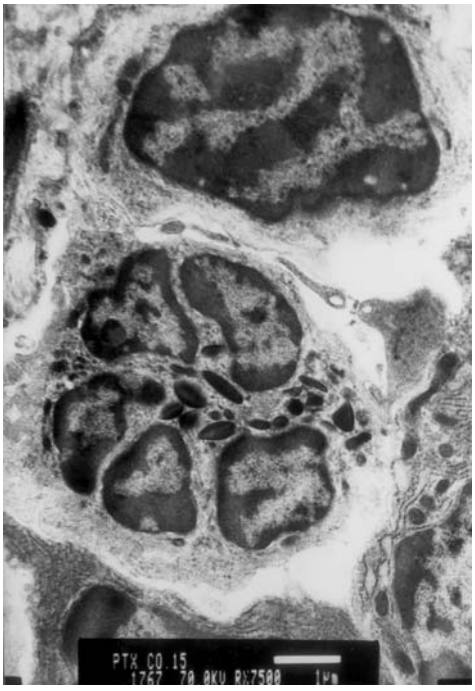

Fig. (6): A cross section in a 15wk hepatic granuloma of a S. mansoni-infected mouse showing an eosinophil (EO) and a lymphocyte (LY) with normal morphology. X7500.

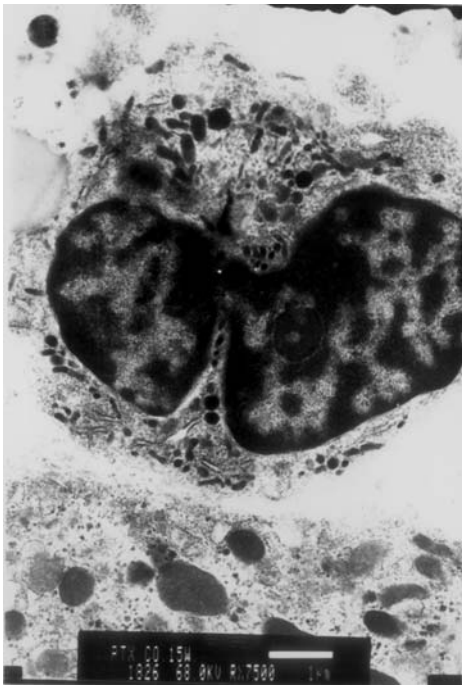

Fig. (7): A photograph of a 15 wk granuloma showing a degenerating eosinophil with disappearance of its plasma membrane and partial disintegration of the cytoplasm. X7500.

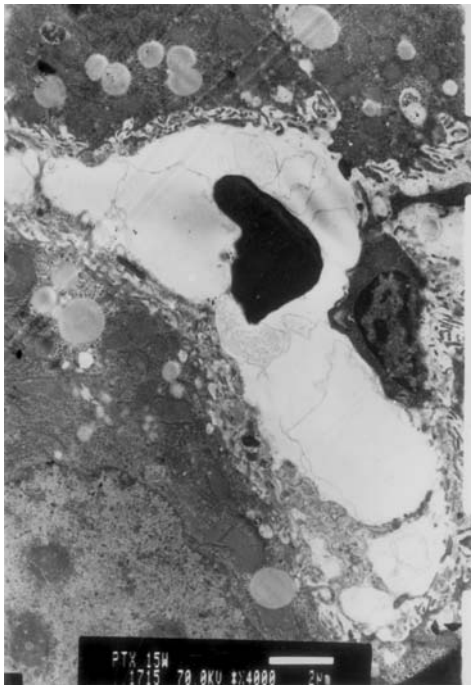

Fig. (8): A blood vessel in the liver of a mouse infected with S. mansoni (15 wks p.i.) and treated with PTX. The vessel is empty from inflammatory cells. Only mature red cells can be seen. X4000.

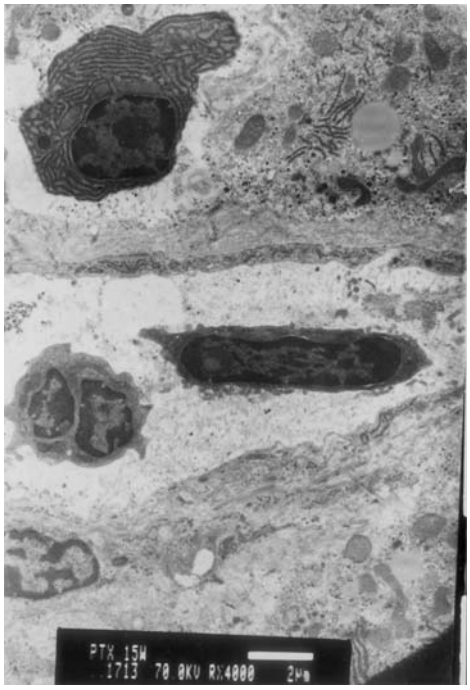

Fig. (9): TEM micrograph of a liver section from a PTX-treated mouse after 15wks infection with S. mansoni. A blood vessel is revealed with a lymphocyte (LY) and a fibroblast (FB) in its lumen. An extravascular plasma cell (PC) is seen nearby the blood vessel wall. X4000.

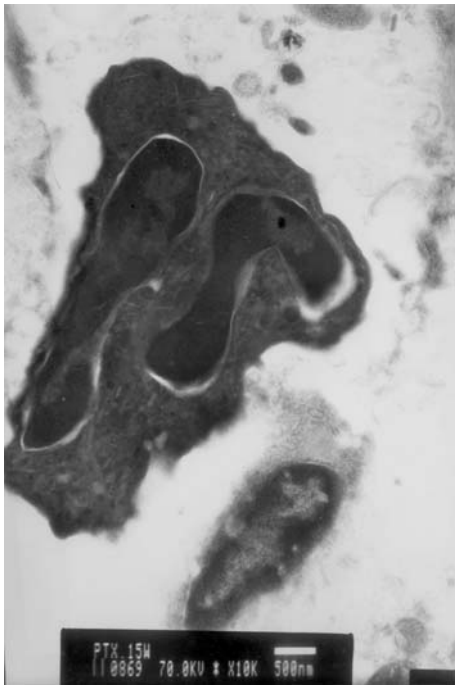

Fig. (10): A neutrophil showing apoptotic features with chromatin condensation and shrinkage of the nucleus leaving a space surrounding it. The cytoplasm also appears condensed and shrunken yet the granules are intact. X10,000.

Ultrastructurally, most cell types were of normal morphology in the 7th week granuloma except for appearance of pronounced cytoplasmic projections. The latter might subserve either adherence of inflammatory cells to endothelial lining for their extravasation into the hepatic tissue or contact of inflammatory cells to each other for cellular interactions. In the 15th week stage, some degenerative changes were observed in a small population of granulomatous cells explaining the demolishment of cellular elements in the downregulated granuloma.

In the PTX- treated group, the 7th week stage showed apoptotic morphological features in both intravascular and granulomatous inflammatory cells (Figs. 3,4) while disintegration and degeneration of the cytoplasm were also observed in other cells. During the 15 weeks stage, PTX rendered the apoptotic changes more pronounced than in the untreated group. The apoptotic effect of PTX probably presents a mechanism for alleviating inflammation in Schistosoma mansoni infection.

Another mechanism of PTX antiinflammatory effect was shown by Ghanem et al. **[16]** to be through the reduction of the expression of the adhesion molecule LFA-1 on the intravascular and granulomatous inflammatory cells, thus hindering LFA-1 mediated adherence and migration of inflammatory cells. Reis et al. **[17]** also showed a decrease in the intragranulomatous eosinophil accumulation by PTX. However, they related this effect to the possible immunosuppressive properties of PTX being capable of inhibiting proliferation of mononuclear cells and lymphocytes induced by T- and B- cell mitogens **[22]** reducing indirectly the number of eosinophils which are strongly dependent on T-cells cytokines such as IL-3, IL-5 and GM-CSF **[23]**.

Therefore the above mechanisms suggestesd by various studies could together explain the potentiated antiinflammatory action of PTX in hepatic schistosomiasis.

On the other hand, the direct morphological evidence of apoptotic effect of PTX on inflammatory cells, as encountered in the present study, needs further dissection of the underlying mechanisms through the analysis of apoptotic pathways in the in vitro and/or in vivo granuloma.

It is also important in future studies to standardize the dose and the stage of administration of the drug in order to obtain an appropriate antiinflammatory therapeutic effect without possible interference with the defensive mechanisms of the body.

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MPO Antigen Negative HLA-DR Negative Acute Myeloid Leukemia: Is it a Separate Clinical Entity?

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ABSTRACT

Background and Purpose: Immunophenotyping is an important tool to assign acute leukemia blast cells to myeloid lineage. The pattern of marker expression in AML is quite heterogeneous even within the same FAB subtype. The whole mark for diagnosis of AML is being MPO positive; the introduction of the MPO antigen (Ag) detection by flow cytometry proved superior to both cytochemistry and electron microscopy. However, lack of MPO Ag in cases which are morphologically and cytochemically proven AML has been reported. The choice of 10% as a cutoff in flow cytometry compared to 3% in cytochemistry may, partly, explain this phenomenon. DR class II MHC is another marker that is supposed to be expressed in most AML cases, except M3. DR negative cases have been previously reported; its impact on prognosis is controversial. Leukemic cells in AML can also express lymphoid-lineage antigens. Studies addressing the prognostic value of immunophenotyping in AML are limited and not conclusive. Up to our best knowledge, there are no studies addressing the prognostic relevance of MPO Ag negative HLA DR negative AML.

The aim of this work is to analyze detailed immunophenotyping in 193 newly diagnosed adult AML patients excluding M3. The prognostic significance of the different markers is verified with special emphasis on MPO Ag negative and HLA-DR negative cases.

Patients and Methods: Excluding M3, one hundred and ninety three newly diagnosed adult AML patients presenting to Medical oncology department of the National Cancer Institute, Cairo University were included in the study. Immunophenotypic analysis was assessed by multicolor flow cytometry. According to MPO Ag% positivity, patients were classified into 3 groups: Group I: MPO Ag negative ≤3%, Group II: MPO Ag weak positive >3<10%, Group III: MPO Ag positive ≥10%. Treatment and follow up: Induction therapy for patients ≤60 years included 2 regimens: Regimen 3 & 7 and HAM regimen. Evaluation of response was done after 2-3 weeks to determine cases in complete remission (CR) or refractory.

Results: Marker expression showed that CD13 and CD33 were the most frequently expressed (89% and 86.5%); 2 cases were CD13 negative and CD33 negative. No significant differences were encountered between MPO Ag weak and MPO Ag positive cases; only CD33 showed near significance association with MPO Ag positive group (p0.06) which attained significance when both positive groups were pooled (p0.02). CD34 and lymphoid marker expression were significantly associated with MPO Ag negative group (p<0.01 and p0.013). The significance was attained even at single marker level namely CD5, CD19 and CD22 and increased when MPO Ag weak positive and MPO Ag positive groups were pooled (p0.008, p0.007 and p0.002). MPO Ag negative DR negative cases (10 patients, 5.2%) had special features: Female predominance (M:F 1:2.33 versus 1.28:1 in the whole group), statistically significant lower peripheral blood blast percent (p0.04) and lower frequency of CD13 and CD34 (p0.004 and p0.025). Out of seventy-eight evaluable patients, CR was achieved in 56. CD14 showed significant association with CR rate (p0.04). No significant association with MPO Ag expression was encountered.

Conclusion: The study emphasizes that >3% should be the cutoff for MPO Ag expression by flow cytometry. A small subset of AML cases (MPO Ag negative DR negative AML patients) apparently showed special characteristics which need collection of a larger number of cases to verify. It seems that the combination rather than a single marker expression would make the difference. Apparently a new era is just starting to stratify AML cases according to immunophenotyping besides the standard FAB categorization.

Key Words: *Immunophenotyping - AML - MPO - HLA DR.*

INTRODUCTION

Acute myeloid leukemia (AML) in adults is a heterogeneous disease, with a variable response to therapy with anticancer agents **[1]**. Using combination chemotherapy protocols,

approximately 60% of patients achieve complete remission (CR) but only a minority remains leukemia free **[2]**.

A variety of clinical and biological parameters have been examined for potential value in predicting treatment response and survival, including age, gender and cytogenetics **[2]**.

Although immunophenotyping is an important tool to assign acute leukemia blast cells to myeloid lineage, its role has been largely confined to differentiate it from ALL and to confirm the diagnosis of M0, M6 and M7 **[3]**. Studies addressing the prognostic value of immunophenotyping in AML are not conclusive. Leukemic myeloblasts express a variety of leucocyte differentiation antigens, which reflect commitment to the myeloid lineage as well as level of differentiation **[4,5]**. These antigenic phenotypes have been proven very useful in the diagnosis of AML, but the prognostic value has remained uncertain and unclear **[5]**. Initial reports suggested a relationship between patterns of myeloid lineage differentiation antigens and patient prognosis but subsequent studies have produced conflicting and inconsistent results **[4,5,6]**. Some markers showed controversial significant prognostic association in more than one study; CD13 **[7,8]**, CD14 **[9,10]**, CD15 **[11]** as well as CD34 which is claimed to be associated with poor clinical outcome in AML **[5,10]**.

Leukemic cells in AML can also express lymphoid-lineage antigens **[12,13]**. The prognostic significance of this phenomenon in AML has also been examined, but with high conflicting claims of poorer or unaltered prognosis **[5,12,14]**.

The pattern of marker expression in AML is quite heterogeneous even within the same FAB subtype **[15]**. This observation has urged trials to develop immunological classifications that could possibly have a prognostic significance **[16,17]**.

The whole mark for diagnosis of AML is being MPO positive; the introduction of the MPO antigen (Ag) detection by flow cytometry proved superior to both cytochemistry and electron microscopy **[18]**. However, lack of MPO Ag in cases which are morphologically and cytochemically proved AML has been reported **[16,17]**. The choice of 10% as a cutoff in flow cytometry compared to 3% in cytochemistry may, partly, explain this phenomenon **[19]**. Another marker that is supposed to be expressed in most AML cases, except M3, is DR class II MHC. DR negative cases have been previously reported; its impact on prognosis is controversial **[6,10,16,20,21]**. Up to our best knowledge, there are no studies addressing the prognostic relevance of MPO Ag negative AML.

In this study, we are analyzing detailed immunophenotyping of 193 newly diagnosed adult AML patients excluding M3. The prognostic significance of the different markers is verified with special emphasis on MPO Ag negative and HLA-DR negative cases.

PATIENTS AND METHODS

Patients:

One hundred and ninety three newly diagnosed adult AML patients presenting to Medical Oncology Department of the National Cancer Institute, Cairo University in the period from 2000 to 2003 were included in the study. Written informed consent was obtained from the patients and the protocol was approved by the Institution Research Board. The age ranged from 18 to 74 years with a median of 31 and a mean of 34.55±31.87 years. They were 108 male and 84 female.

Diagnosis of AML was performed according to standard criteria including clinical, morphological and cytochemical examination. The FAB subtype was determined **[22]**. M3 cases were excluded from the study.

Immunophenotypic Analysis:

Immunophenotypic analysis was performed on mononuclear cells from fresh peripheral blood or bone marrow samples taken at the time of diagnosis. It was assessed by multicolor flow cytometry (Coulter Epics XL, Hialeh). A wide panel of FITC (fluorescin) or PE (phycoerythrin) conjugated monoclonal antibodies (Mo Abs) was used (Table 1). Double and Triple marker labeling was performed, including proper isotype controls.

Detection of Surface Markers by Direct Staining:

The whole blood staining method was performed. In short, 10μl labeled Mo Ab was added to 100μl whole blood, incubated in the dark for

20 minutes then processed by the Q prep system (Coulter Corp, Hialeh, Fl) where immunoprep reagent A for lysing, B as stabilizer and C as fixative were consecutively added. The samples were analyzed on the flow cytomter.

Detection of Intracellular Markers:

Hundred µl of whole blood was lysed using lysis solution (Becton & Dicknson) for 10 minutes. Cells were washed once and re-suspended in 1ml PBS. A mixture of 500µl 4% paraformaldehyde as fixative, 500µl PBS and 5µl tween 20 as detergent was added to the cells and incubated for 10min. The cells were washed and 10µl Mo Ab was added and incubated for 30min at 4°C. Cells were washed, suspended in 500µl PBS and analyzed **[23]**.

Any antigen was considered positive when ≥20% of blast cells were stained above the negative control except for CD34 and CD10 where ≥10% was considered positive. According to MPO Ag% positivity, patients were classified into 3 groups:

1- Group I: MPO negative ≤3%.

2- Group II: MPO weak positive >3<10%.

3- Group III: MPO positive ≥10%.

In an attempt to consider MPO Ag cutoff positivity at a lower percentage (3% instead of 10%), group II and group III were compared to each other and were considered as one group in the statistical analysis thereafter.

Each group was further divided according to DR expression into DR negative (a) and DR positive (b). Fig. (1) shows an AML case MPO Ag +/DR+.

Fig. (2) shows an AML case MPO Ag -/DR.

Treatment and Follow up:

Induction therapy for patients ≤60 years included 2 regimens:

1- Regimen 3 and 7 was given as Duanorubcin 45mg/m^2 or Doxorubicin 40mg/m^2, IV, from day 1 to day 3 and Cytosine arabinoside 100mg/m^2, by continuous infusion, from day 1 to day 7.

2- HAM regimen: By high dose Cytosine arabinoside 1gm/m^2/12 hours from day 1 to day 3 by infusion over 3 hours and Mitoxantrone 12mg/m^2 from day 3 to day 5 by short infusion.

Patients above the age of 60 years (4 cases) received non-anthracyclin containing regimen.

Evaluation of response has been done after 2-3 weeks. Complete remission (CR) was defined as cellular marrow with less than 5% blasts, no circulating blasts, no evidence of extramedullary leukemia and recovery of granulocytes ≥1.5x10^9/L and platelet ≥100x10^9/L.

Patients who attained CR were considered for post remission therapy while those who failed to respond to induction therapy were evaluated as refractory. Post remission therapy was risk adapted: Patients with unfavorable risk and having HLA-identical donor were subjected to high dose therapy and peripheral stem cell transplantation. Those with no available donor or with contraindication for transplantation as well as patients with favorable prognosis were treated with 3 more cycles of HAM regimen and then kept under follow up. DFS and OS were evaluated for all patients in addition; OS was evaluated for the three MPO groups.

Supportive Care:

Blood components transfusion was given to keep the hemoglobin level at 8gm/dl or higher. Theraputic platelet transfusion was given to patients with bleeding manifestation and thrombocytopenia. Prophylactic platelet transfusion was given when platelet count <10x10^9/L or at a higher level if patients had complications or planned for invasive procedure.

Evaluation and management of infection was applied according to the rules recommended for infection management in the immunocompromised patients **[24]** and according to the ongoing institutional protocols.

Statistical Analysis:

Statistical package for social sciences (SPSS) version 9 was used. Quantitative variables were summarized using mean and SD, median, minimum and maximum values. Qualitative data were summarized using frequencies and percentage.

The relation between quantitative variables was tested by Spearman Correlation. Chi or Fisher's exact tests were used whenever appropriate to test the association between the different qualitative variables. Differences were considered significant at a *p* value of ≤0.05 and highly significant at a *p* value of ≤0.01 **[25]**.

RESULTS

The study included 193 newly diagnosed adult AML cases, (excluding M3). FAB classification was available for 105 cases. M1 was the predominant FAB subtype (p0.02). The results are summarized in (Table 2). Peripheral blood blast median was 65% with a range of 20-96% and a mean of 53.74±31.79%.

Marker Expression:

CD13 and CD33 were the most frequently expressed; 2 cases were CD13 negative, CD33 negative; one was M1 (MPO Ag negative, DR negative and CD5 positive) and the other was M5 (MPO Ag positive, DR negative, CD14 negative, CD2 and CD7 positive).

CD14 was significantly associated with M4 and M5 (p<0.01) being expressed in 61.5% and 46% respectively vs. 0% in M0 and M7, 7.3% in M2 and 7.4% in M2.

MPO Ag expression was 61.1% (118/193 cases). According to MPO Ag % positivity cases were divided into 3 groups:

- Group I (MPO Ag negative ≤3%): 47 cases.
- Group II (MPO Ag weak positive >3% and <10%): 28 cases.
- Group III (MPO Ag positive ≥10%): 118 cases.

With regards to myeloid markers expression in context of MPO Ag expression, only CD33 showed near significant association with MPO Ag positive group (p0.06), which attained significance when MPO Ag weak positive and MPO Ag positive groups were, pooled (p0.02).

CD34 showed statistically significant association with MPO Ag negative group (p<0.01). The significance increased when MPO antigen weak positive and MPO Ag positive groups were pooled (p0.005).

Lymphoid marker expression was significantly associated with MPO Ag negative group (p0.013). The significance was attained even at single marker level namely CD5, CD19 and CD22 (p0.03, 0.03 and 0.009 respectively). The significance increased when the MPO Ag weak positive and MPO Ag positive groups were pooled (p0.008, 0.007 and 0.002 respectively).

HLADR Status:

Each of the 3 MPO groups was further divided according to the DR status into DR negative (a) and DR positive (b).

Group I: 47 cases including 10 DR negative (Ia) and 37 DR positive (Ib). Group Ia showed female predominance (M: F 1:2.33), though not statistically significant (p0.08), statistically lower peripheral blood blast percent (p<0.04), lower frequency of CD13 and CD34 (p0.004 and 0.025 respectively). The 2 CD5 positive cases lied in this group (p0.006). Table (3) shows marker expression in the 10 MPO Ag negative DR negative AML cases.

Group II: 28 cases including 7 DR negative (IIa) and 21 DR positive (IIb). None of the tested markers showed any significant association with either group.

Group III: 118 cases, including 35 DR negative (IIIa) and 83 DR positive (IIIb). Group IIIa showed significantly lower frequency of CD13 expression (p<0.01).

Clinical Outcome:

Seventy-eight patients were evaluable while 31 cases showed early death during induction and were considered non-evaluable. The main cause of death was infection, bleeding and organ failure.

Complete remission (CR) was achieved in 56 patients (71.1%). Out of the 56 patients, 6 were subjected to peripheral blood stem cell transplantation as post remission therapy. No significant association was encountered between CR on one hand and age, sex, TLC, or surface markers expression on the other hand except for CD14. CR in CD14 negative cases was 76.5% vs. 50% in CD14 positive AML cases (p0.04) table (4). When cases were stratified according to MPO antigen status, CD14 retained its significance only for the MPO antigen positive group (p0.05). With regard to MPO, no significant impact was encountered but there was grading of the CR rate in the three groups being 59% in MPO Ag –ve, 75% in MPO Ag weak and 83.3% in MPO Ag +ve.

DFS was 44% for all patients with a median observation of 12 and range of 1-33 months. OS was 32% (Fig. 3). OS was 24.2% versus 35.6% in MPO Ag negative group and MPO Ag positive patients respectively (Fig. 4).

Table (1): Panel of monoclonal antibodies (mAbs).

Monoclonal Ab	Clone	Source
Myeloid Markers:		
CD13	My7 - PE	Coulter Hialeah, FL
CD14	RmO52 PE	Coulter Hialeah, FL
CD33	M9 - PE	Coulter Hialeah, FL
CD41	P2 - FITC	Coulter Hialeah, FL
Glycophorin A	11E4B.7.6 (KC16)	Coulter Hialeah, FL
Myeloperoxidase	MPO7 FITC	DAKO
Lymphatic Markers:		
B Lineage:		
CD19	BL6 - FITC	Immunotech Marseille, France
CD22	Sd10 PE	Immunotech Marseille, France
T Lineage:		
CD1	BL6	Coulter Hialeah, FL
CD2	39C1.5 FITC	Coulter Hialeah, FL
CD3	UCHT1 FITC	Coulter Hialeah, FL
CD4	13B8.2 - FITC	Immunotech Marseille, France
CD5	BL1A - PE	Coulter Hialeah, FL
CD7	3A FITC	Coulter Hialeah, FL
CD8	B9.11 - PE	Immunotech Marseille, France
NK:		
CD16	3G8 FITC	Coulter Hialeah, FL
CD56	N901 (NKH-1) PE	Coulter Hialeah, FL
Others:		
CD45	Immu 19.2 - FITC	Coulter Hialeah, FL
HLA-Dr	B8.12.2 FITC	Immunotech Marseille, France
CD10	d5 FITC	Coulter Hialeah, FL
CD34	581	Immunotech Marseille, France
Isotypic Controls:		
IgG1 (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG1 (Mouse)	PE	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	FITC	DAKO/COULTER/DIACLONE
IgG2a (Mouse)	PE	DAKO/COULTER/DIACLONE

Table (2): Characterization of 193 AML cases according to MPO antigen percent expression.

Parameters	AML cases	Group I (MPO Ag -ve ≤3%)	Group II (MPO Ag weak +ve>3 %< 10%)	Group III (MPO Ag +ve ≥10%)
Age: (years)*	31 (18-74)	26 (18-70)	38 (18-74)	30 (18-70)
M:F	1.28:1	1.19:1	2.5:1	1.14:1
TLC:X109/L*	36 (0.54-236)	35 (0.54-194)	25.6(2.4-78)	36.9(0.6-236)
FAB:	(105 cases)	(29 cases)	(14 cases)	(62 cases)
M0	2 (1.9%)	2 (6.9%)	0	0
M1	47 (44.8%)	14 (48.3%)	4 (28.6%)	29 (46%)
M2	29 (27.6%)	5 (17.2%)	5 (35.7%)	19 (30%)
M4	13 (12.4%)	4 (13.8%)	3 (21.4%)	6 (9.7%)
M5	13 (12.4%)	4 (13.8%)	1 (7.1%)	8 (12.9%)
M7	1 (1%)	0	1 (7.1%)	0
Marker expression:	193 cases	47 cases	28 cases	118 cases
Myeloid lineage:				
CD13	172 (89%)	41 (87%)	24 (85.7%)	107 (90.7%)
CD33	166 (86.5%)	36 (76.6%)	24 (85.7%)	107 (90.7%)
CD14	42 (21.8%)	8 (17%)	6 (21.4%)	28 (23.7%)
CD41	1 (0.5%)	0	1 (3.5%)	0
Lymphoid markers:	73 (37.3%)	27 (55.3%)	8 (32.1%)	38 (31.4%)
CD19	10 (5.2%)	6 (12.8%)	1 (3.6%)	3 (2.6%)
CD22**	3 (1.6%)	3 (6.4%)	0	0
CD24	2 (1%)	1 (2.1%)	0	1 (1.2%)
CD2	12 (6.2%)	2 (4.3%)	4 (14.3%)	6 (5.1%)
CD5	2 (1%)	2 (4.8%)	0	0
CD7	22 (11.4%)	7 (14.9%)	3 (10.7%)	12 (10.3%)
CD4	3 (1.6%)	1 (2.1%)	0	2 (2.4%)
CD16(NK)	9 (4.7%)	2 (4.5%)	2 (7.1%)	5 (4.3%)
CD56(NK)	19 (9.8%)	6 (13%)	1 (3.6%)	12 (10.2%)
Other markers:				
HLADR	141 (73.1%)	37 (78.7%)	21 (75%)	83 (70.3%)
CD34	50 (26.7%)	18 (43.9%)	8 (28.6%)	24 (20.3%)
CD10	1 (0.5%)	0	0	1 (1.2%)

* Median (range). ** Cytoplasmic CD22.

Table (3): Marker expression in MPO antigen negative DR negative AML cases.

Cases	FAB	CD13	CD33	CD14	CD34	CD19	CD22	CD2	CD5	CD7	CD16	CD56
Case 1	M0	–	+	–	+	–	–	–	+	–	–	–
Case 2	M4	–	+	+	–	+	–	–	–	–	–	–
Case 3	M1	+	–	–	–	–	–	–	–	+	–	–
Case 4	M2	+	+	+	–	–	–	–	–	–	+	–
Case 5	M1	+	+	–	–	+	+	–	–	–	–	–
Case 6	M0	+	+	–	–	–	–	–	–	+	–	–
Case 7*	M1	–	–	–	–	–	–	–	+	–	–	–
Case 8	M1	+	+	–	–	–	–	–	–	–	–	–
Case 9	M1	+	+	–	–	–	–	–	–	–	–	–
Case 10	M2	–	+	–	–	–	–	–	–	–	–	–

–: Negative.
+: Positive.
*: Sudan black B positive.

Table (4): CR rate in relation to surface marker expression in 78 evaluable AML cases.

Surface markers	Positive cases out of 78 evaluable patients		CR rat		*p*-value*
	Number	Percent	Number	Percent	
CD13	70	89.7	50	71.4	0.8
CD33	61	78.2	45	73.8	0.46
CD14	14	17.9	7	50	0.045
HLA-DR	54	69.2	39	72.2	0.9
CD34	28	35.9	19	67.8	0.54
CD2	5	6.4	4	80	0.5
CD7	10	12.8	8	80	0.6
CD16	3	3.8	2	66.7	0.8

* Significance $p \leq .0.05$.

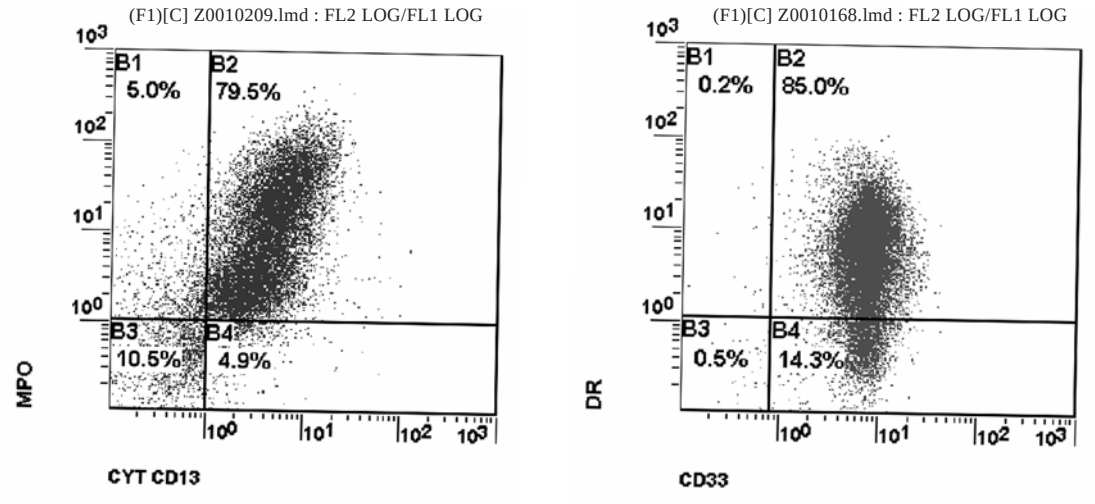

Fig. (1): An AML case MPO Ag +/DR+.

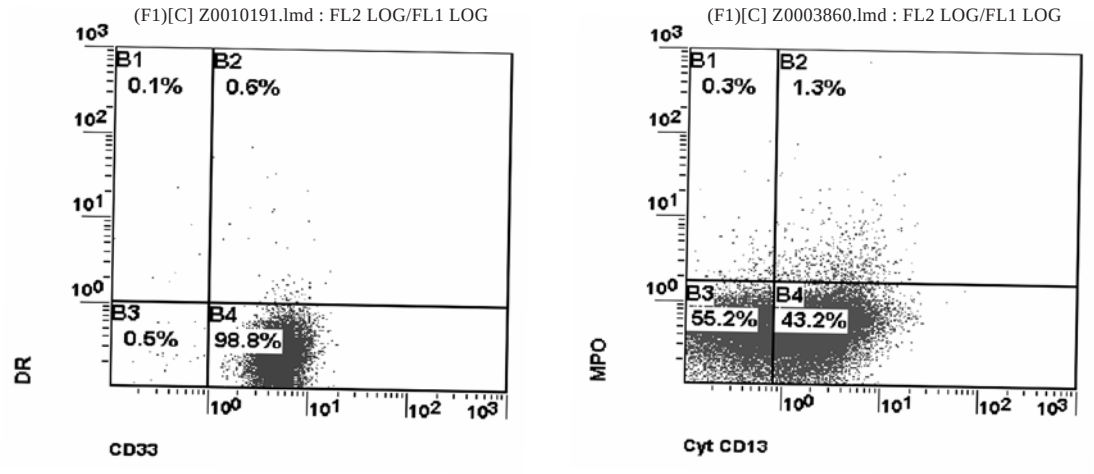

Fig. (2): An AML case MPO Ag -/DR-.

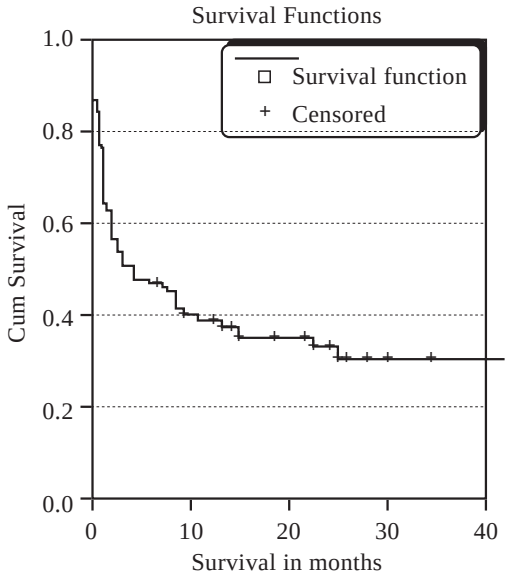

Fig. (3): Overall survival (O.S) in AML cases.

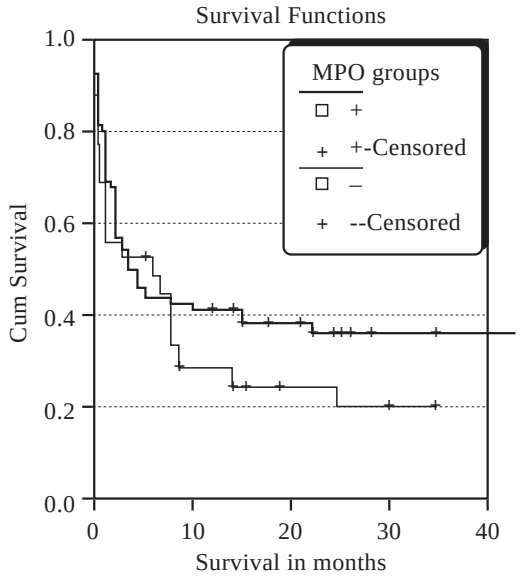

Fig. (4): Overall survival (O.S) in MPO Ag –ve vs. MPO Ag +ve cases.

DISCUSSION

In this work we have studied 193 newly diagnosed AML cases. Their age ranged from 18-74 with a median of 31 years. This is in agreement with previous Egyptian studies that reported a similar median age **[26,27]** but it is much lower than what is reported in Western series with a median of 64 years **[28]**. The male: female ratio in this study was 1.28:1 which is comparable to previous Egyptian reports **[26,27]** and to Western data **[17]**. TLC median in this study was $36x10^9$/L which is higher than corresponding figures in Western series being $15x10^9$/L in the largest series available comprising 909 patients **[17]**. In the present study, M1 was significantly the predominant FAB subtype being 44.8% of our cases. This is comparable to results reported by a previous Egyptian study which was 47.1% **[27]** compared to about 27% in the largest Western series so far reported **[17]** denoting a relatively more immature nature of our cases. The incidence of M4 and M5 in the present study is comparable to Casasnovas' report **[17]**.

The present study emphasizes the marked heterogeneity of AML immunophenoytypes reported in the literature **[1]**. As previously reported, CD13 and CD33 were the most frequently encountered. CD13, CD33 and CD117 combined were claimed to be superior in the

diagnosis of AML **[17]** than the unique detection of MPO antigen **[18]**. Lack of expression of both CD13 and CD33 was encountered in 2 cases of our series. CD13 and CD33 negative AML cases have been previously reported and were viewed as possible examples of extreme asynchrony in sequence of morphologic and immunologic maturation or abnormal epitope expression in leukemic cell molecules **[8]**. CD14 was encountered in 21.8% of our cases; figures in the literature varied between 9% and 35% probably reflecting the relative incidence of M4 and M5 **[5,10,16,17]**. Another factor, however, is the known epitopic variability of the antigen **[5]**. The frequency of CD34 expression in our series was 26.7%. A similar figure was reported **[16]**. However this figure is much lower than that reported by most workers with a frequency of 42-65% **[5,10,17]**. The lymphoid marker expression encountered in our series was 37.3%; the most frequent being CD7 (11.4%) followed by CD2 (6.2%), CD19 (5.2%) CD22 (1.6%) and CD5 (1%); other markers were very low. A comparable figure was reported **[14]**. We have previously reported an incidence of 20% lymphoid marker expression being 23.8% in children and 15.4 in adults **[29]**. The two cases expressing cyt CD22 are considered biphenotypic; they were not excluded on account of their classical morphological and cytochemical patterns; they were both M1. Variable incidence of lymphoid marker expression was reported with figures as low as 15% **[16]** and as high as 44-50% **[5,10,17,30]**. A lower frequency of CD7 (9%) was reported in a large series of 909 studied by Casasnovas et al. **[17]** but higher figures varying from 15 to 32% were reported by others **[5,6,10,16,30]**. Expression of other lymphoid markers were also variable **[5,6, 10,17,30]**.

DR was expressed on 73.1% of our cases. The figures previously reported varied between 65% and 90% **[10]**. Our figures for the various maker expression were nearest to those of Casasnovas et al. **[17]** in their 909 patient series. In this work we categorized our patients according to MPO Ag expression into MPO Ag negative group <3% (29.4%), a group with weak MPO Ag expression of >3 <10% (14.5%) and a MPO Ag positive group with ≥10% MPO Ag +ve blast cells (61.1%). Excluding MPO Ag negative AML has been previously reported **[16]** in 49/325 (15%) of their cases and by Casanovas et al. **[17]** in a special subset of their series (CD13-ve, CD33-ve) to be 39%. The standard cutoff for MPO Ag expression by Flow Cytometry is ≥10%. However, this has been recently criticized and the cutoff of >3% used for cytochemistry was adopted **[19]**.

The cutoff for cytochemistry was determined on the assumption that the bone marrow could, normally, contain up to 3% myeloblasts; any extra blast cell(s) would belong to the leukemic population. When Flow cytometry was used for detection of MPO Ag, this fact was ignored and a cutoff similar to that adopted for other markers e.g. CD10 was automatically applied. In fact, we would go further and suggest that any MPO Ag positive blast cells in the peripheral blood whether detected by cytochemistry or by Flow cytometry should be taken as an indication of the myeloid nature of the leukemia provided that the cell is definitely documented as a blast and not one of the more mature myeloid series namely a promyelocyte or a myelocyte. In all previous reports on MPO Ag negative AML, the 10% cutoff level was used and neither MPO or DR status were considered in the two available trials to establish an immunological classification of AML **[16,17]**.

In this work, Lack of MPO Ag (≤3%) showed significant association with CD34 and lymphoid markers expression while MPO Ag expression showed nearly significant association with CD33 (p0.06) but no association with FAB. This denotes a less differentiated phenotype of the MPO negative AML expressing the stem cell marker (CD34), lacking the myeloid marker (CD33) and having aberrant expression of lymphoid markers; the cell has not yet frankly adopted the myeloid differentiation pathway from the point of view of marker expression even though it is morphologically and cytochemically documented as AML. Taking in consideration that the majority of our cases were M1 which is still early in the pathway of differentiation we may speculate that some of them have not yet acquired the full array of myeloid markers and that the malignant transformation had occurred in a relatively immature cell.

In this work the group with weak MPO Ag expression (>3 <10%) showed no significant association with any other markers, probably on account of its small number. When the two positive groups were pooled together the sig-

nificant associations remained the same and the nearly significant association with CD33 (p0.06) acquired statistical significance (p0.02) which is in harmony with the assumption that the more differentiated the cells, the more myeloid markers they express. It also emphasizes the suggestion that the cutoff should be >3 rather than ≥10.

In this work we have further subdivided our AML cases according to the DR status. The MPO –ve DR –ve group showed significant lower frequency of other markers namely CD13 and CD34 (p=0.004 and 0.002 respectively). Thus we, apparently, have a subgroup lacking many markers denoting relative immaturity. This group showed female predominance though insignificant (p=0.08) against male predominance in the whole series. It also showed significantly lower percentage of peripheral blood blasts (p=0.04) and the 2 CD5 positive cases lied in this group (p=0.006). Thus, in spite of the small number being only 10 cases, this group has a certain pattern, the significance of which awaits studying of a larger number.

Within the MPO Ag +ve group, there was significant association between CD13 and DR which further emphasizes the findings in the MPO Ag –ve group. There was, as well, significant negative association with CD14; this is an expected finding as CD14 +ve monocytic leukemia usually lack MPO. The same applies to the significant positive association between CD14 and DR, where monocytic cells are expected to express class II MHC.

In the present study, CR was attained in 56/78 evaluable cases (71.7%). The corresponding figures in the literature ranged from 60-80% **[17,31,32]**. DFS was 44% at a median observation period of 12 months. Reported DFS at 4 years was 30-40% in patients less than 60 years **[32]**. In the present study, 6 of our patients received post remission allogenic peripheral blood stem cell transplantation (SCT). This was not based on randomization but rather on availability of a matched donor and fulfilling specific eligibility criteria such as age. Comparative analysis in AML patients achieving CR consistently showed markedly reduced relapse rate following allogenic SCT **[33,34]**.

We have studied the impact of various marker expression on CR. CD14 was associated with a lower CR (50%vs.76.6%, p=0.045). When analyzed in context of MPO Ag expression, it retained its significance only in the MPO Ag positive group (p<0.05) while the significance was lost for the MPO Ag –ve group (p=0.329). None of the other markers showed impact on CR including CD34 and CD7. None of the markers studied showed an impact on survival. Previous reports have addressed the prognostic relevance of various markers. CD14 was reported to be associated with low OS **[17,30]** especially when associated with CD7 **[30]**. CD7 is one of the most extensively studied markers with a lot of controversy. In the aforementioned study, it showed an adverse effect on CR (p<0.002), CCR (p<0.001) and OS (p<0.001) which was confirmed by multivariate analysis. The adverse effect has been reported by other workers **[35,36,37]**. On the other hand this was denied by Tien et al. **[13]**. With regard to CD34 expression, the majority of reports documented its adverse effect as an independent prognostic factor **[17,38]** which we failed to detect in this series and in a previous one as well **[39]** Sporadic reports are available for other markers including a high CR rate in CD2+ CD19+ AML cases with superior survival **[14]**, lack of prognostic impact of HLA DR –ve cases, if M3 is excluded **[21]**. The marked controversy in the reports on the prognostic value of different markers has urged the search for an immunological classification of AML that might have a prognostic relevance. Two main proposals for such a classification are available in the literature. In the first **[16]**, the authors concentrated on developing a scoring system for better discrimination between M0 and ALL. The second **[17]** comprised 909 AML cases studied in two stages, 176 as a test series and 733 as a training series to validate the findings obtained from studying the first group. They classified the cases according to the pattern of marker expression into five groups (MA-ME), taking in account the expression of 4 groups of markers namely CD13 or CD33 or CD117, CD7, CD35 or CD36, and CD15. They tested the prognostic impact of other markers in context of these five groups (MA-ME). Specific independent prognostic factors were related to poor overall survival in each of these groups namely CD34+ in MA and MD, CD7+ in MB-non- APL, and CD14 in MD and ME. The study emphasizes the findings obtained in this work that it is the combination rather than a single marker expression which might make the difference, though we used two different markers

namely MPO and DR for categorization of our cases. It seems that a new era is just starting to evaluate the prognostic value of immunophenotyping of AML which was previously confined, mostly, to diagnose M0, M6 and M7.

In Conclusion: The role of immunophenotyping in AML is not, any more, confined to diagnosis. Characterization of specific subsets is required to establish an immunological classification with potential prognostic relevance. In this study, as a first step, we have indicated a potentially distinct subtype namely MPO Ag –ve HLA DR –ve. A large number of this apparently infrequent phenotype is needed to verify its significance.

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Bone Marrow Microenvironment; Assessment of Changes in Acute Leukemia at Diagnosis and in Remission

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ABSTRACT

This study included 40 patients with Acute Leukemia (15 Acute Lymphoblastic and 25 Acute Myeloid) at diagnosis comprising Group I and during remission comprising Group II. 15 bone marrow donors for transplantation were taken as control comprising Group III.

All cases were subjected to morphological, cytochemical, and immunophenotypic analysis for diagnosis and typing of acute leukemia.

All cases were examined for assessment of different bone marrow microenvironment (BMM) elements: Fibronectin by Radial Immunodiffusion, TNFα and L-selectin by ELISA technique in plasma samples of bone marrow aspirate. Bone marrow biopsies were done for 10/15 (66%) of ALL cases in Group I and II, and 9/25 (36%) of AML cases in Group I and 5/25 (20%) during remission in Group II as well as for controls.

Results: Fibronectin mean level in plasma of BM in Group I at diagnosis was found to be significantly decreased ($p<0.05$) as compared to control Group III i.e. Group III was 2.5 folds higher frequencies of Group I, while during remission, it was more than its level at diagnosis but still lower than control.

L-selectin mean level of BM plasma in acute leukemic patients at diagnosis was found to be markedly increased as compared to control i.e. Group I was 25 folds of Group III, while during remission, it was less than its level at diagnosis but still higher than control i.e. Group II was nearly 5 folds of Group III.

TNFα mean level in plasma of BM was significantly increased at diagnosis reaching 9 folds the mean levels of controls, while it was decreased markedly after chemotherapy, i.e group II was nearly 2 folds of group III. There was no difference in TNFα levels between ALL and AML.

As regard stromal cell pattern: There was an increase in reticular cells in variable degrees, in addition to fibroblasts and macrophages which showed elevation in 90% of cases. Adipocytes were reduced in only 25% of cases. In Group II during remission only few cases showed minimal increase in reticular cells and fibroblasts while all the remaining cases showed normal reticular cells, fibroblasts, macrophages and adipocytes.

Conclusion: The study of bone marrow microenvironment in acute leukemia showed how the malignant process can cause a significant disturbance in the equilibrium of this microenvironment. Also, the use of L-selectin in BM plasma is recommended as a useful prognostic marker in acute leukemia.

Key Words: *BM microenvironment - Fibronectin - L-selectin - TNFα.*

INTRODUCTION

Normal hematopoiesis takes place in the bone marrow and is the result of interaction between hematopoietic progenitor stem cells and the surrounding microenvironment **[1]**.

The bone marrow microenvironment (BMM) plays an important role in promoting hematopoietic progenitor cell proliferation and differentiation as well as the controller progress of these developing hematopoietic cells **[2]**.

The normal hematopoietic microenvironment (HM) in the bone marrow consists of a heterogeneous population of hematopoietic and nonhematopoietic stromal cells, their extracellular biosynthetic products, and hematopoietic cytokines **[3]**.

The cells include myofibroblasts, other fibroblastoid cells, endothelial cells, osteogenic precursors, adipocytes and macrophages. These cells produce a complex array of extracellular matrix (ECM) molecules consisting of proteoglycans and their constituent sulfated gly-

cosaminoglycans, chondroitin, heparin, fibronectin, thrombospondin and glycoprotein **[4]**.

In addition, hematopoietic progenitors express cell adhesion molecules (CAMs) classified into six superfamilies: Integrins, selectins, sialomucins, immunoglobulins, cell surface proteoglycans and cadherins **[5]**. Cells comprising the HM also provide a source of many hematopoietic cytokines, either secreted or membrane bound, including GM-CSF and stem cell factor **[6]**.

Fibronectin is a two-subunit, multidomain subendothelial extracellular matrix protein that is also found in platelet alpha-granules and plasma. Fibronectin extracellular matrix plays a critical role in the microenvironment of cells. Loss of this matrix frequently accompanies oncogenic transformation, allowing changes in cel1 growth, morphology, and tissue organization **[7]**.

L-selectin is a member of selectin family adhesion molecules; it is expressed on surface of all classes of leucocytes and plays an important role in homing and function of these cells. L-selectin dependant attachment to endothelium is observed in vivo as well as in vitro under rotating or flow conditions, suggesting that L-selectins is involved in the initial attachment of leucocytes to the endothelium **[8]**.

The effect of TNFα on hematopoiesis can be either directly mediated or indirectly by inducing other cells to produce growth factor (GFS) such as GM-CSF from fibroblast and G-CSF and CSF-1 from monocytes **[9]**.

This study was carried out in order to investigate BMM of acute leukemia patients (at diagnosis and at remission) through assessing the BM levels of fibronectin and levels of L-selectin and TNFα, in addition to microscopic examination of BM biopsies to assess the stromal cells.

MATERIAL AND METHODS

Materials:

Study Samples were divided into three groups:

Group (I): 40 patients with de novo diagnosis of acute leukemia (24 males and 16 females) with a mean age 35 years (range 8-70). Patients of this group were subdivided according to FAB Classification into: (a) ALL (6 cases L_1 and 9 cases L_2) (b) AML (4 cases M_1 - 7 cases M_2 - 3 cases M_3 - 4 cases M_4 - 3 cases M_5 and - 4 cases M_7).

Group (II): The same 35 patients out of 40 during remission, after induction course of chemotherapy (21 males and 14 females), age range 8-60 years. 5 cases died during follow up (2 cases M_7 & 3 cases M_3).

Group (III) Control group: 15 healthy subjects (7 males and 8 females) with age range 18-36 years selected from bone marrow transplantation donors. All the cases were selected from National Cancer Institute and Nasser Institute from 2002 to 2003.

Sample Collection:

1- Peripheral blood samples were collected for routine lab investigations.

2- Bone marrow sample: This was done for all patients and controls by the standard technique **[10]**. Diagnosis was done based on morphology, cytochemistry and immunophenotyping. The remaining aspirate was transferred into a tube containing heparin and centrifuged at 1000 x g for 10min and plasma was divided into 6 sterile aliquots of 250-500ul and stored at -70ºC till use.

3- Bone marrow biopsy: BM biopsy was done only to fulfil the diagnosis whenever indicated (done only for 10 cases of ALL and 9 cases of AML) to study the stromal cells, using the standard technique according to Williams and Nicholson, 1963 **[11]**.

Methods:

Clinical examination was performed for all patients with emphasis on fever, pallor, purpura, ecchymosis, lymph node enlargement, hepatomegaly, splenomegaly and palpable masses. Full laboratory investigations: included CBC, ESR, bone marrow aspiration, routine laboratory and radiological investigations and bone marrow trephine biopsy for ALL cases and for some cases of AML. These investigations were done for leukemic patients at time of diagnosis and during remission.

Assay of Fibronectin:

Fibronectin in BM plasma samples was measured by Radial-Immunodiffusion technique by Mancini et al. **[12]**. The kit was manufactured

by BINDARID, NANORID kits. (The Binding site Ltd., R&D, Birmingham, UK).

Assay of SCD 62 L (L-Selectin) and TNFα:

L-Selectin and TNFα were measured by a two step sandwich ELISA technique by Diaclone Research, FRANCE **[13]** for in-vitro quantitative determination in bone marrow plasma. L-selectin is expressed in ng/ml and TNFα in pg/ml. Sensitivity of L-selectin <1ng/ml and TNFα <25pg/ml.

All assays were performed as per the manufacture's instructions. Each sample was assayed in duplicate. The plates were read at 450nm wavelength.

Statistical Analysis:

The collected data were tabulated and statistically analyzed (Minitab statistical software version, 1998):12-1. Chi-square as a non parametric test was used to assess the statistical significance of associations among categorical variables when assumptions for its application were fulfilled.

Pearson correlation coefficient (*r*) was used to assess the statistical significance of correlation among normally distributed quantitative variables. The value of *r* ranges from –1 to +1, if the value of r positive then the correlation is positive, whereas negative values of *r* indicate inverse or negative correlation.

Difference, associations and correlations were considered significant when the *p*-value of the corresponding test is less than or equal to 0.05.

RESULTS

Characteristic and Clinical Data of Patients and Controls: Pallor was the most common finding present in 80% of cases, 56% of cases were presented by fever, 48% were presented by purperic rashes and 32% were presented by ecchymosis. Splenomegaly was felt in 60% of cases hepatomegaly in 44% of cases and lymphadenopathy in 60% of cases.

Laboratory Investigations:

Hematological tests were performed for all cases. In patients at diagnosis; 82% of patients presented by anaemia and leucocytosis, while 18% of patients presented by leucopenia and 90% patients presented by thrombocytopenia.

Specific Investigations:

1- *Assay of bone marrow plasma level of Fibronectin (mg/L):*

Table (1) shows the bone marrow plasma levels of Fibronectin (mg/l) in all groups. The mean level of Fibronectin in acute leukemia patients at diagnosis (67±10.58) was found to be significantly decreased as compared to the mean level in control (173±67.5) i.e Group III was 2.5 folds higher frequencies of Group I.

During remission, the mean level of Fibronectin (117±27.13) was more than its level at diagnosis but still lower than control i.e. Group III was nearly 1.5 folds of Group II. There was no significant difference in the mean levels of Fibronectin in ALL & AML neither at diagnosis nor during remission (Table 2).

2- *Assay of Bone Marrow Plasma Level of L-selectin (ng/ml):*

Table (3) shows the BM plasma levels of L-selectin (ng/l) in all groups: The mean level of BM plasma L-selectin in acute leukemic patients at diagnosis (107.87±133.28) was found to be markedly increased as compared to the mean level in controls (4.29±1.82) i.e. Group I was 25 folds of group III.

During remission, the mean level of BM plasma L-selectin (29.91±13.08) was less than its level at diagnosis but still higher than in controls i.e. Group II was nearly 5 folds of Group III.

Table (4) shows comparison in BM plasma L-selectin levels between ALL and AML cases at diagnosis and during remission identifying no significant statistical difference neither at diagnosis nor during remission.

3- *Assay of Bone Marrow Plasma Level of TNF-α (pg/ml):*

Table (5) shows the BM plasma levels of TNFα (mg/L) in all groups. The mean level of BM plasma TNF-α in acute leukemic patients at diagnosis (388.63±189.83) was found to be significantly increased as compared to the mean levels in controls (42.13±10.56) i.e. Group I was 9 folds higher frequencies of Group III.

During remission, the mean level of TNF-α (94.84±33.91) was less than its level at diagnosis but still higher than its level in controls i.e. Group II was nearly 2 folds of Group III.

Comparison of BM plasma TNF-α level between ALL and AML at diagnosis and during remission identified no significant statistical difference (Table 6).

Evaluation of BM Stromal Cells: BM Trephine biopsies were performed for 10/15 (66%) of ALL cases at diagnosis and during remission, and 9/25 (36%) of AML cases at diagnosis and 5/25 (20%) during remission.

Microscopic findings of BM trephine biopsies in Group I at diagnosis:

- Bone marrow was hypercellular in 5/19 cases (26%), hypocellular in 6/19 cases (32%) and normocellular in 8/19 cases (42%).
- The stromal cell pattern of BM biopsies demonstrated an increase in reticular cells in variable degrees (slight, moderate, marked) in all examined cases. Also, fibroblasts and macrophages showed variable degrees of elevation in 90% of cases. Adipocytes were reduced in only 25% of cases.
- Fibrosis was present only in AML M_7 cases (4 cases).

Microscopic Findings of BM Trephine Biopsies in Group II During Remission:

- One case showed hypocellularity while all the remaining cases were normocellular.
- The stromal cell pattern of BM biopsies showed minimal increase in reticular cells and fibroblasts in few cases while all the remaining cases showed normal reticular cells, fibroblasts, macrophages and adipocytes.

Table (7) illustrates coefficient correlation between the studied levels of FN, L-Selectin, and TNFα and total leucocytic count, % of blasts in peripheral blood and in bone marrow.

- There was significant positive correlations between L-Selectin levels and total leucocytic count, % blasts in both peripheral blood and BM ($p<0.05$).
- There was no significant correlation between levels of FN as well as TNFα and any of the studied parameters ($p>0.05$).

Table (1): Bone marrow plasma levels of fibronectin in all groups (normal mean value = 213mg/l).

Fibronectin	Group I	Group II	Group III	*t* value	*p* value
Range	40-90	58-150	100-290	t_1=12.99	*
Mean	67.75	117.31	173	t_2=2.18	*
± SD	10.58	27.13	67.50	Paired$_t$=17.55	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value ($p<0.05$).

Table (2): Comparison between ALL and AML patients versus control as regard fibronectin levels (expressed in mg/L).

Fibronectin levels	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	55-90	90-150	40-88	58-135	100-290
Mean	68.53	124.13	67.28	119.7	173
± SD	11.34	21.76	10.31	24.03	67.50
t value at diagnosis			0.16		
During remission			2.11 (NS)		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (3): Bone marrow plasma levels of L-selectin in all groups (normal mean value = 3.0±0.9ng/ml).

L-Selectin	Group I	Group II	Group III	*t* value	*p* value
Range	28-889	8-65	2-8	t_1=7.70	*
Mean	107.87	29.71	4.15	t_2=5.06	*
± SD	133.28	13.08	1.53	Paired$_t$=7.99	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value (p<0.05).

Table (4): Comparison between ALL and AML patients versus control as regard L-selectin levels in BM plasma (expressed in ng/ml).

L-Selectin	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	28-180	11-53	36-889	8-65	2-8
Mean	109.41	30.27	118.94	29.30	4.15
± SD	49.09	12.62	164.68	13.73	1.53
t value at diagnosis			0.41		
During remission			0.58 NS		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (5): BM plasma levels of TNF-α (pg/ml) in all studied groups.

TNF-α	Group I	Group II	Group III	*t* value	*p* value
Range	175.00-760.0	145.00-175.80	30.00-65.00	t_1=7.52	*
Mean	388.63	94.840	42.133	t_2=3.58	*
± SD	189.83	33.911	10.855	Paired$_t$=8.37	*

SD = Standard deviation.
t_1 = Control versus leukemic patients at diagnosis.
t_2 = Control versus leukemic patients during remission.
Paired t = Leukemic patients at diagnosis vs themselves during remission.
* = Statistically significant *p*-value (p<0.05).

Table (6): Comparison between ALL and AML patients versus control as regard TNFα levels in BM plasma.

TNFα level	ALL		AML		Control
	Diagnosis	Remission	Diagnosis	Remission	
Range	235-760	29.2-175	175-750	46-135	30-65
Mean	368.27	104.06	400.84	87.93	42.13
± SD	190.66	40.88	192.19	26.63	10.86
t value at diagnosis			0.07		
During remission			0.15 NS		

SD = Standard deviation.
NS = Nonsignificant statistically.

Table (7): Correlation coefficient (*r*) of Group I (acute leukemic patients diagnosis).

	TLC	% PB Blasts	% BM Blasts
Fibronectin:			
r	-0.065	0.021	0.026
p	0.692	0.897	0.884
L-Selectin:			
r	0.610*	0.560*	0.653*
p	0.016	0.030	0.008
TNF-α:			
r	0.136	-0.002	0.090
p	0.403	0.989	0.607

DISCUSSION

Leukemias are a group of disorders of uncertain etiology characterized by an abnormal proliferation of the leucopoietic tissue of the body, they are almost invariably fatal although remission may occur **[3]**.

This study was conducted to evaluate some elements in plasma of BMM of acute leukemia patients (at diagnosis and during remission) through assessing the levels of fibronectin by radial immuno-diffusion technique and levels of L-selectin and TNFα by Elisa technique in addition to microscopic examination of BM trephine biopsies to assess the stromal cells.

The present study was conducted upon 40 patients; 15 cases acute lymphoblastic leukemia (ALL) and 25 cases acute myeloid leukemia (AML) in addition to 15 healthy subjects of matched age (donors of BM transplantation) as control group. In this study, we found that the levels of plasma BM Fibronectin (FN) in acute leukemic patients (both ALL & AML) were lowered than control group. During remission, the FN levels were increased but still lower than its level in normal control subjects.

In agreement with our findings, Gee et al. **[14]** demonstrated that blood plasma fibronectin was markedly reduced in newly diagnosed acute leukemic patients (ALL and AML) than in controls. And its level was corrected to near normal level with treatment. So it predicted the onset of relapse in these patients and follow up till complete remission.

Ariel et al. **[15]** reported that in acute leukemia cases, low blood plasma level of FN was detected at diagnosis and relapse and returned to normal range after achieving chemotherapy. This level was not related directly to tumour load or neutropenia but correlated well with episodes of intercurrent infection. Also, they found that in severe infections, FN fell rapidly to very low levels and was sometimes not restored to normal up to two weeks later.

Brenner et al. **[7]** stated that decreased blood plasma fibronectin levels compared to the controls and to its levels after treatment, may be attributed to increased consumption of FN in expanded mononuclear phagocytic system present in the liver and spleen, and also to reduced hepatic synthesis.

It was concluded that measurement of plasma fibronectin may help in early detection of infection in acute leukemia and may be considered of prognostic value in follow up of treatment. However, as its level is influenced by sepsis, blood transfusion and chemotherapy, it can not serve as a treatment marker at least as a single isolated measurement **[14]**.

In the present study, there were no significant correlations between levels of fibronectin and any of the studied parameters of acute leukemia at diagnosis ($p>0.05$).

L-Selectin, a member of selectins family recognizes mucins. L-selectin found on all mature leucocytes, marrow progenitor cells and stroma express various combination of L-selectin and mucin. Leukemic blasts express a wide range of L- selectin which is important in homing of the blasts to the bone marrow **[16]**.

As regard L-selectin in the present study, the estimated BM levels of L-selectin in acute leukemic patients (both ALL & AML) at diagnosis were found to be significantly higher than its levels in controls ($p<0.05$). Also, we found that BM levels of L-selectin in leukemia patients who achieved complete remission (CR) were significantly lowered than their levels at diagnosis before starting chemotherapy ($p<0.05$), and did not significantly differ from that of controls ($p>0.05$).

There was no statistical significant difference in L-selectin levels between ALL and AML cases neither at diagnosis ($p>0.05$) nor during remission ($p>0.05$).

In agreement with our study, Olejnik et al. **[17]** demonstrated that serum L-selectin decreased significantly from diagnosis to the end of intensive chemotherapy and increased in relapse. These results suggested that monitoring of L-selectin may be useful for evaluating leukemic activity.

In the present study, there were significant positive correlations between L-selectin levels and total leucocytic count (r=0.610, p=0.016), percentage of blasts in peripheral blood (r=0.560, p=0.030) and percentage of blasts in bone marrow (r=0.653, p=0.008), while there was no significant correlation between levels of FN as well as TNF-α and any of the studied parameters (p>0.05).

In the present work; plasma BM levels of TNFα were significantly higher in acute leukemic patients at diagnosis than healthy controls. TNFα levels during remission were noticeably lowered but still slightly more than normal controls.

This is in agreement with Gessner et al. **[18]** who reported that 80% of plasma BM sample of acute leukemia patients had markedly higher TNF-α than control group during remission. It was concluded that the elevated plasma BM levels of TNFα are a useful marker to assess the disease activity, but not prognosis of acute leukemia.

Stromal cells are important elements of BMM that influence the development of hematopoietic cells through the production of cytokines and through the signals mediated by direct contact of progenitor cells with stromal cells **[19]**.

It is evident that leukemia cells interact with the BMM at many levels and mimic the action of normal early precursors to a variable extent **[4]**.

In the present study, microscopic examination of BM biopsies of cases included in the study, revealed disturbed BM cellularity in 58% of cases, (26% of cases were hypercellular and 32% were hypocellular) while the remaining 42% were normal in cellularity.

Also, we found disturbance is stromal cell pattern in BM biopsies: As regard reticular cells, we demonstrated increase in its number by variable degrees (slight, moderate, marked) in all examined cases in relation to normal control subjects. Also, fibroblasts and macrophages showed variable degrees of elevation in most cases (90%). Adipocytes were in normal distribution in 75% of examined biopsies and were reduced in the remaining 25%.

Our findings were in agreement with a previous study done by Giles et al. **[3]** comparing bone marrow biopsies, obtained from different categories of leukemia cases, with those from normal donors. They revealed normal number of macrophages but increased number of fibroblast and reticular cells.

In conclusion, we found that the different components of BMM are greatly affected by malignant transformation of the hematopoietic cells in acute leukaemia. L-selectin can be used as a prognostic marker in acute leukemia, while Fibronectin and TNFα can be used as markers for the disturbance of the hematopoietic microenvironment. Also, bone marrow trephine biopsies may be used as a tool to asses the BMM in acute leukaemia as stromal elements are essential factors in the development of haemopoetic cells.

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Endothelial Apoptosis and Arterial Thrombosis: Expression of CD31 and CD146 in Recent and Old Thrombotic Events

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ABSTRACT

Background: Apoptosis (programmed cell death) of vascular lining endothelium is one of the causes of increased thrombogenicity. The luminal release of apoptotic endothelium-derived microparticles can cause the activation of tissue factor (TF).

Subjects and Methods: This study included 76 cases of recent and old arterial thrombosis and 15 age and sex-matched controls. Endothelial-derived CD146 positive microparticles were statistically higher among cases of old thrombosis vs. controls ($p<0.001$). They were also statistically higher among old thrombosis than recent thromosis cases ($p<0.05$). No statistical difference was found between recent thrombosis cases and controls regarding CD146. CD31 was found to be insignificantly raised among recent and old thrombotic cases in comparison to controls.

Results: Nineteen diabetics were among the patients' group but no statistical difference was detected regarding CD31 or 146 levels in comparison to non-diabetic patients. Similarly, 8 hypertensive cases were included with no statistical difference regarding CD31 or CD146 in comparison to normotensive cases. Endothelial microparticle detection among old cases of arterial thrombosis may indicate the persistence of the danger of rethrombosis.

Key Words: *Apoptosis - Thrombosis - Endothelial-derived CD146, CD31.*

INTRODUCTION

Clinical manifestations of atherosclerosis are the consequences of atherosclerotic plaque rupture that triggers thrombus formation. Tissue factor (TF), the most potent known initiator of the blood clotting system, is highly dependent on its activity on the presence of phosphatidyl serine (PS) (Mallat et al., 2000). Luminal endothelial cell apoptosis with shedding of PS rich microparticles might be responsible for thrombus formation on eroded plaques without rupture (Farb et al., 1998). These circulating microparticles can be identified by anti-CD31 and CD146 antibodies (Tegui and Mallat, 2001). So, the aim of this work was to study the presence of thrombogenic circulating microparticles in cases of recent and old arterial thrombosis as evidenced by circulating CD31 and CD146 exhibiting particles.

SUBJECTS AND METHODS

This study included 76 patients with arterial thrombosis above the age of 25 years with no history of anemia, infection, auto-immune disease, malignancy, hormonal therapy for females, renal problems or trauma related thrombosis. These included: Thirty nine cases of recent thrombosis (within 48 hours of acute attack) and thirty seven cases of old thrombosis (three or more months after acute attack).

Cases were Sub-Grouped as Follows:

Eleven cases of recent peripheral artery thrombosis as evidenced by cold pulseless limb and Duplex study.

Twelve cases of recent cerebral thrombosis as evidenced clinically by signs of lateralization, CT scan and/or MRI. Embolic cases were excluded.

Sixteen cases of acute myocardial infarction with acute chest pain, ECG findings, raised cardiac enzymes.

Eight cases of old peripheral thrombosis as evidenced clinically and by Duplex, history of amputation or recent graft surgery (within one week of surgery).

Sixteen cases of old cerebral thrombosis as evidenced clinically and by CT scan.

Thirteen old myocardial infarction as evidenced clinically, by ECG and normalization of cardiac enzymes.

All Cases were Subjected to:

Full history taking including reference to special habits and clinical exam Blood count-lipid profile-liver and kidney functions-uric acid-Hb A1c-Serum soluble CD31 and CD146 level estimation:

Venous sample was collected and serum was separated. Annexin V was used to capture PS containing particles. CD31 and CD146 levels were estimated in nmol/L PS equivalent by ELISA using anti-CD31 and CD146 antibodies respectively.

Cases were compared to 15 age-matched healthy controls.

RESULTS

This study included 76 patients with an age range of 39 and 74 years with a mean of 51.1 years ±8 and a male to female ratio of 2.67:1. Recent thrombosis was found in 39 cases while old thrombosis was found in 37 cases. Fifteen controls were included with a mean age of 47.3±9.1 years.

A mean platelet count of 250000/cmm was found among recent thrombosis group, and 265000/cmm among the old thrombosis group. Neither differed from a mean of 234500/cmm count (±4690) among the control group with no statistical difference (p=0.05).

Total cholesterol mean of 146.8 (±103.6) mg/dl was detected among the recent group. This did not differ significantly neither from the old group (mean of 130.6±45.2mg/dl) nor from the control group (mean of 208.1± 87.5mg/dl).

LDL cholesterol levels still did not show any significant difference among the three groups (mean of 148.7±41.5mg/dl) in the recent group 32.6±29.6mg/dl among the old group and 134.8±34.6mg/dl among the control group).

HDL levels showed a mean of 45.5±14.2 mg/dl among the recent group, 41.6±14.6mg/dl among the old group and 49.8±13.9mg/dl among the control group with no statistical difference between the groups (p>0.05).

Serum triglycerides still did not differ significantly among the groups with a mean of 162.3±88.6mg/dl among the recent group, 137.7±73.7mg/dl among the old group and 116.6±84.3mg/dl among the controls.

Among our patients, 50 cases were smokers, half of whom smoked two or more packs per day. Duration of smoking showed a mean of 15±4.3 years.

Nineteen cases were known diabetics, eight receiving insulin and 11 on oral therapy with a mean Hb A1c of 9%. Eight cases were known hypertensive, four of whom were controlled on therapy.

CD31 level among the recent group showed a mean of 13.1±6.4nmol/L, among the old group showed a mean of 12±6.6mol/L and 11.9± 5.4nmol/L among the control group with no statistical difference. CD31 levels were not statistically different among patients who were smokers and patients who were not diabetic and non-diabetic cases, nor were they different among hypertensive vs, normotensive cases.

CD146 showed a mean of 428.7±121.9 nmol/L among the recent thrombosis group, 675±223.4nmol/L among the old thrombosis group and 382.7±120.2nmol/L among the control group. Difference between the old group and controls was highly significant (p<0.01) and was also significant between the old and the recent group (p<0.05).

CD146 levels did not show a significant difference between smoking and non-smoking cases, diabetic and non-diabetic cases, nor was a significant difference found between hypertensive and non-hypertensive cases.

DISCUSSION

Apoptosis, through its procoagulant ad proadhesive potentials, may play a critical role in both plaque and blood thrombogenicity and may be an important step in the transition from stable to unstable atherosclerotic disease. Circulating apoptotic microparticles could be a valuable marker in thrombus formation and hence the instability of the atherosclerotic plaque. All cell types involved in atheromatous

plaque are involved in apoptosis, particularly apoptotic macrophages. Vascular endothelial cell apoptosis promotes the coagulation process (Iwakura et al., 2000).

Among the first morphological changes after initiation of apoptosis of the endothelium is membrane blebing with shedding of microparticles, loss of focal adhesion sites and retraction from the matrix followed by detachment from arterial wall. A significant number of these shed cells results (Mallat and Tedgui, 2000).

CD146 (S-Endo1-associated antigen) (Mel-CAM) (MUC 18) is a transmembrane glycoprotein that is constitutively expressed in the whole of the human endothelium, some dendritic cells and smooth muscle cells (Bardin et al., 1996). It is localized at areas of cell-cell junction (Bardin et al., 2001). The enumeration of circulating endothelial cells released in the blood after vascular injury represents a direct exploration of this process of apoptosis and shedding. Monoclonal antibody that recognizes CD146 allows the detection of high numbers of these cells or smaller particles of their residues in thrombotic, infectious, or immunological disorders with insignificant levels in normal subjects, thus a useful marker for vascular wall injury (Dinat-George and Sampol, 2000).

CD31 (Platelet Endothelial Cell Adhesion Molecule 1) (PECAM1) is a cell adhesion molecule of the Ig superfamily expressed on vascular endothelium, platelets, monocytes, neutrophils and subsets of T lymphocytes. It has been implicated in leukocyte-endothelial cell interactions and monocyte and neutrophil recruitment. Simon et al., 2002, showed that apoptosis disabled CD31-mediated cell detachment from phagocytes.

The presence of these antigens in blood in the context of arterial thrombosis implicates the existence of apoptotic endothelial-derived microparticles with potential thrombogenic activity (Tedgui and Mallat, 2001). Tissue factor may be found encrypted in these microparticles resulting in the enhancement of Factor VIIa enzymatic activity (Morrissey, 2001).

Multivascular atherosclerosis reduces life expectancy. After an initial myocardial infarct, life expectancy is 13.9 years, after an initial stroke, it is 8.8 years, and after peripheral arterial disease it is 16 years, going down to 1.5 to 1.8 years if later complicated by myocardial infarction (Habrel and Dembowski, 1999).

Our results revealed a highly significant elevation of the soluble CD146 in arterial thrombosis as compared to controls ($p<0.01$). We also found a highly statistical difference when comparing CD146 levels between controls and old peripheral thrombosis group ($p<0.01$) and between controls and old coronary thrombosis group (0.01) with a very high significant difference between controls and old thrombosis group ($p<0.001$). Among the recent thrombosis patients, a significant difference was detected between controls and recent cerebral thrombosis group ($p<0.05$).

Our findings are in line with the detection of elevated levels of circulating procoagulant microparticles 8 days after acute coronary ischemia reported by Van Belle et al., 1998, who observed persistent intracoronory thrombi 24 hours to 30 days after the ischemic episode. They propose that persistence of these high levels may be a useful indicator of the persistence or recurrence of ischemic events.

Soejima et al. (1999), reported that plasma tissue factor antigen levels are significantly elevated in patients with unstable angina compared to stable angina cases and was associated with poor prognosis.

Mallat et al. (1999), analyzed the presence of shed membrane apoptotic microparticles in extracts form 6 human atherosclerotic plaques and 3 underlying arterial walls with detection of marked tissue factor expression in close proximity to apoptotic cells with significant tissue factor thrombogenic activity.

Mallat et al. (2000) found high levels of CD146 positive microparticles in acute coronary syndrome cases in comparison to stable angina and non coronary chest pain patients. They collected their samples on days 0 to 8 of diagnosis but no follow up of samples on later dates was performed.

In our study, soluble CD31 levels did not differ from patients to controls thus contradicting findings by Mallat et al. (2000) detecting high levels of CD31 positive microparticles in acute coronary cases. However, Seebruany and Gerbel (1999), found that soluble PECAM1 plasma

levels was identical between acute myocardial infarction cases and controls, with significant rise 3 hours after thrombolysis (p=0.02) followed by significant decrease 24 hours after attempted reperfusion.

Our results depended on samples taken very early after diagnosis and at least three months after acute event.

A statistically significant difference (p<0.05) was found comparing soluble CD31 levels among controls vs. thrombosis patients when grouped according to affected arterial bed. Cerebral thrombosis group was higher vs. coronary thrombosis group (p<0.01) and still higher when compared to peripheral thrombosis group (p<0.05). Zaremba and Losy, (2002), found increase in soluble PECAM1 level in serum and CSF within 24 hours of the onset of stroke which is confined to the endothelial cells of the blood brain barrier. Thus, PECAM1 may play a role in regulation of inflammatory cell migration in CNS (Qing et al., 2001).

In addition to their direct effect in promotion and amplification of the coagulation cascade, endothelial derived microparticles may be responsible for dissemination of the procoagulant and pro-inflammatory potentials to sites remote from the micro environment of their formation (Satta et al., 1994).

Synthesis of TF is increased in vitro by several factors including shear stress, hypoxia, oxidized lipoproteins and anionic phospholipids (Conner, 1994).

Among our patients, 50 cases were smokers forming a hypoxic stress. However, no significant difference between smoking and non-smoking cases. Hypertension can form a shear stress (Tricot et al., 2000). PECAM1 endothelial cell expression was found to be upregulated in response to experimentally induce hypertension with endothelial cell injury as reported by Suzuki et al. (2001). However, no difference as detected between our hypertensive patient group (eight cases) and our non-hypertensive group (68 cases) neither in CD31 nor in CD146 levels.

Diabetes increases oxygen intermediates with activation of protein kinase C, a major intracellular intermediate in cell apoptosis pathway (Schwartz et al., 1992).

No difference was found in our study between the nineteen diabetic patients included in the study and the non-diabetic cases. Thus the vascular endothelium can no longer be viewed as a static physical barrier. Prolonged and exaggerated endothelial activation leads to dysfunction. Apoptosis of the vascular endothelium is an important factor in thrombogonicity. Detection of circulating procoagulant micro-particles as a result of apoptosis is suggested to be done repeatedly to detect its power in the prediction of persistence or recurrence of ischemic events.

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Inherited Thrombotic Risk Factors in Non-Selected Group of Egyptian Population

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ABSTRACT

In recent years, knowledge concerning inherited and acquired causes of thrombophilia has greatly increased. The association of of venous thromboembolism is reported to be different according to the genotype, being higher among the carriers of natural anticoagulant deficiencies and homozygotes for factor V Leiden.

The aim of this study was to evaluate the incidence of five inherited thrombotic risk factors (FV1691A, FV4070G, PT20210A, EPCR 23 bp insertion and ACE 300bp deletion) non-selected group of healthy individuals.

The study included one hundered and twenthy randomly selected healthy individual with age ranged from one and 62 years. This included 32 children (15 males and 17 females with age rang between 1 and 18 years with mean of 13.29 years) and 90 adults (32 males and 58 females with age range between 18.5 years and 62 years with mean of 33.52 years).

The study revealed that eighteen individuals (15%) had FV1691 Amutation with a frequency of 0.075. R2 and R3 haplotypes were found in 15 (12.5%) and 2 (1.6%) individuals respectively. Two of them carried FV1691A and R2 haplotype at the same time. Only one individual had EPCR 23bp insertion in heterozygous form (0.83%). None of the 120 individuals had PT 20210A mutation. The distribution of angiotensin converting enzyme -300bp del in homozygous state was present in 61 (50,8%) individuals. The frequency of DD allele was 0.508. Neither age nor sex was found to affect the distribution of this mutation.

Conclusion: Our preliminary data revealed that FV1691A mutation is an important risk factor for thrombosis in Egyptians and further studies on a larger scale population is needed.

Key Words: *Factor V leiden - Thrombotic risk.*

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INTRODUCTION

The term thrombophilia describes a tendency to develop thrombosis on the basis of inherited or acquired disorders of blood coagulation or fibrinolysis leading to a prothrombotic state. Familial thrombophilia was first described in 1956 on a clinical basis by Jordan and Nandroff **[1]**. Inherited thrombophilia is defined as a genetically determined tendency to thromboembolism. Dominant abnormalities or combinations of less severe defects may be clinically apparent from early age of onset, frequent recurrence or family history. Milder traits may be discovered only by laboratory investigations **[2]**. Thus venous thromboembolic disease is now viewed as a multicausal model, the thrombotic event being the result of gene-gene and gene - environment interactions **[3]**.

In recent years, two common gene polymorphisms were recognozed as important causes of hypercoagulability: Factor V leiden and prothrombin G20210A mutations. Factor V Leiden thrombophilia is the most common inherited form of thrombophilia. The mutant factor V Leiden is inactivated at an approximately tenfold slower rate than normal and persists longer in the circulation, resulting in increased thrombin generation and a mild hypercoagulable state, reflected by elevated levels of prothrombin fragment and other activated coagulation **[4]**. Prothrombin 20210 G-A change of the prothrombin gene was reported to be an important genetic risk factor for thrombosis by increasing the plasma levels of prothrombin **[5]**.

After the determination of these two coagulation factors gene defects, endothelial protein C receptor (EPCR) gene polymorphisms and angiotensin converting enzyme (ACE) gene defects were recognized. EPCR slows down protein C activation via thrombomodulin-thrombin at the endothelial surface. ACE gene defects are associated with venous thromboembolism (VTE) by affecting the vascular tone **[6,7]**. EPCR gene has a 23bp insertion into a normal DNA sequence in exon 3 that causes stop amino acid sequence into the EPCR protein which causes a truncated protein at the end **[8]**. ACE gene constitutes an insertion/deletion polymorphism of a 300bp fragment and the possible loss of function in the enzyme was suggested to be responsible for the ACE levels in circulating blood **[7]**.

The aim of this study was to investigate two common mutations of the factor V gene, (1691 G-A and 4070 A-G), prothrombin gene 20210 G-A alteration, EPCR 23bp insertion and ACE ins/del polymorphisms in a sample of Egyptian population. The Egyptian population has a mixed genetic background with an ethnic heterogeneity. The analyzed risk factors influence both the coagulation pathway and the endothelial changes in the case of vascular damage.

SUBJECTS AND METHODS

The study included 120 randomly selected healthy Egyptian individuals. Their age ranged between one and 62 years. This included 32 children (15 males and 17 females with age range between 1 and 18 years with mean of 13.29 years) and 90 adults (32 males and 58 females with age range between 18.5 years and 62 years with mean of 33.52 years).

Factor V 1691 G-A and Prothrombin 20210 G-A mutations were analysed with previously described techniques and real-time PCR method using Light Cycler (Roche Diagnostics, Germany) **[9]**. For the detection of insertion/deletion polymorphisms of ACE gene and EPCR gene and HR2 haplotype of factor V gene was determined according to previously described techniques **[10,11]**.

RESULTS

The frequencies of the five thrombophilic genetic factors are shown in Table (1). Both mutations in the factor V gene were found to have a high frequency of 15 % for 1691 G-A mutation and 12.5 % for the factor V 4070 A-G. Prothrombin 20210 G-A mutation could not be detected in any of the individuals. Only one individual carried the EPCR 23bp insertion making its frequency very low. ACE 300bp ins/del polymorphism was found to be frequent like other factor V gene changes. D allele of the ACE gene polymorphism was found to be more frequent, whereas I allele was obtained in 5 individuals with its homozygous state. Of the 120 individuals we found fifteen individuals carrying R2 haplotype (12.5%) and two carrying R3 haplotype. We also found that two individuals have both the FV1691A mutation and R2 haplotype at the same time (1.66%).

Table (1): Frequency of the five risk factors in the studied group.

Genetic changes	No.	%	Allele frequency
Factor V 1691 G-A	18	15	0.075
Factor V 4070 A-G:			
R2 haplotype	15	12.5	0.062
R3 haplotype	2	1.6	0.008
Prothrombin 20210 G-A	0	0	0
EPCR 23bp insertion	1	0.83	0.004
ACE ins/del:			
I/I	5	4.1	0.041
I/D	54	45	0.225
D/D	61	50.8	0.508

DISCUSSION

Our study is the first report informing five thrombosis-related risk factors in 120 healthy Egyptians. Althogh Prothrombin 20210 G-A mutation was not found in our studied group, both factor V gene mutations were found to be very frequent. It was reported that the highest heterozygosity rate of factor V Leiden is found in Europe with a prevalence of 10-15% in southern Sweden and Greece. In the US, heterozygosity for factor V Leiden was found in 5.2% of Caucasian American, 2.2% of Hispanic Americans, 1.2% of African Americans, 0.45% of Asian Americans, and 1.25% of Native Americans **[12]**. In contrast to what was claimed before, that Factor V Leiden mutation is not found in populations with African origin, Factor V 1691 G-A and 4070 A-G mutations were found to have high frequency in our studied group of Egyptian population. Further studies on a larger

scale population is needed especially that it is known that our population has different ethnic background. The determination of the R3 haplotype of factor V gene may confirm the suggestion that the mutation is of African origin and older than factor V Leiden **[13]**.

It is worth noting that most of our understanding of inherited risk factors for thrombosis is derived from the study of largely white populations. Although some attribute the presence of factor V Leiden and prothrombin mutation in some African and Asian populations to migration or colonization, other have arrested that the presence of these mutations in small genetic isolates may argue for multiple origins for at least the factor V Leiden mutation **[14]**. Thus, although studies have begun to elucidate the basis of familial thrombophilia in white populations, a great deal remains to be learned in other populations like ours.

A specific factor V gene haplotype (HR2) is present in 8% of normal subjects and is an additional cause of resistance to activated protein C **[15]**. Its role as an independent risk factor for VTE is uncertain, yet double carriers of the HR2 haplotype and factor V Leiden have an increased plasma resistance to activated protein C and an increased risk of VTE in comparison with heterozygotes for factor V Leiden, the rare state of homozygosity for HR2 produces a 5.5 fold increase in the risk of VTE **[16]**. Lunghi et al., reported that in the exon 13 of the factor V gene there is a different restriction pattern with Rsa I digestion. They found that the haplotype have 3935 A-G (His 1254 Arg) polymorphism instead of 4070 G-A (His 1299 Arg) and referred the haplotype as R3 **[17]**. In the present study, fifteen individuals were found to carry R2 haplotype (12.5%), two of them carried FV1691A and R2 haplotype at the same time (1.66%) and R3 haplotype was present in two individuals. The former result is also higher than that reported before **[15]**.

The distribution of the 23 bp insertion polymorphism in the EPCR gene was very rare with a frequency between 0-3% in different case and control groups **[18]**. The ins/del polymorphism of the ACE gene showed a variation in the Egypt population. The influence of ACE gene defect was not clear in the issue of the levels in ACE enzyme in plasma. Recently, a quantitative trait locus was identified that is claimed to be linkage disequilibrium with APCR levels **[19]**. There is a great variation in ACE ins/del polymorphism too. Studies concerning ACE gene defects in different populations show a great variation.

From this study we revealed the genetic tendency and the frequency of the five thrombotic risk factors in a group of Egyptian population. Further studies are needed in Egyptian patients with thrombosis, as testing may be used for better understanding of the origin of these mutations and better evidence based management of these patients.

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Effect of L-Carnitine on the Physical Fitness of Thalassemic Patients

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ABSTRACT

Introduction: Poor physical fitness is a common problem among thalassemic patients. Many of these patients complain of muscular weakness and myalgia. Reduction of serum carnitine levels might play an important role in the appearance of muscular dysfunction. Moreover, cardiopulmonary diseases secondary to chronic anemia and hemosiderosis still remain a major cause of morbidity and mortality in these patients and contribute to their poor physical fitness. Chronic anemia and tissue hypoxia result in impairment of free fatty acid oxidation and ATP production. L-carnitine, a butyric acid derivative, acts as an essential cofactor in the β-oxidation of long-chain fatty acids which results in production of ATP.

Objective: To study the effect of L-carnitine therapy on exercise tolerance and physical fitness in thalassemia major patients.

Patients and Methods: Thirty patients attending the Hematology Clinic of the New Cairo University Children Hospital were included in this study. The mean age was 14.6±2.7 years. Clinical, laboratory and cardiopulmonary exercise testing were performed before and after 6 months of oral L-carnitine therapy (50mg/kg/day).

Results: In our study oxygen consumption (VO_2 max), cardiac output and oxygen pulse at maximal exercise increased significantly after L-carnitine therapy ($p<0.001$, $p<0.002$ and $p<0.001$ respectively). However, there was no significant change in minute ventilation and ventilatory equivalent of carbon dioxide ($p>0.05$). The degree of improvement in exercise parameters was higher for the younger patients. Our results also showed a significant increase in weight and height after L-carnitine therapy ($p=0.04$ and $p=0.03$ respectively). A significant increase in the interval of blood transfusion after therapy ($p=0.008$) was observed, but there was no increase in hemoglobin concentration ($p>0.05$).

Conclusion: L-carnitine seems to be an effective therapeutic approach in thalassemic patients. It improves their cardiac performance, physical fitness and general activity. Its effect on physical growth is worthy for further studies.

Key Words: *Physical fitness - L-carnitine - Thalassemia major.*

INTRODUCTION

Poor physical fitness is a common feature among thalassemic patients. It is known that children with homozygous β-thalassemia manifest a decrease in muscular mass and many of them complain of muscular weakness and myalgia **[1]**. Several interpretations have been given to justify these symptoms, including tissue anoxia, peripheral nerve disorders and abnormal calcium metabolism attributed to decreased hemoglobin and systemic hemosiderosis **[2,3]**. Moreover, cardiopulmonary affection and growth retardation still remain major problems for these patients in spite of the progress in management of β-thalassemia with chronic transfusion therapy and iron chelation **[4]**. All of the aforementioned abnormalities contribute to the poor physical fitness in these patients. Parameters of poor physical fitness include chronic ventilatory and cardiopulmonary abnormalities **[5]**.

Chronic anemia and subsequent tissue hypoxia impairs free fatty acid oxidation which is the major energy providing pathway of myocardium and its inhibition has been shown to impair myocardial function **[6]**.

L-carnitine, a butyrate analogue (3-hydroxy-4-N-trimethyl-aminobutyric acid) is a well tolerated and safe physiological compound. It

plays an essential role in fatty acid oxidation, glucose metabolism and energy production **[7]**. It is crucial to the shuttle mechanism of long chain fatty acid across the inner mitochondrial membrane, thus providing substrate for oxidation and subsequent energy production especially in those organs and tissues that preferentially use fatty acid for their energy needs as the myocardium and the skeletal muscle **[6]**.

We hereby report our results regarding the effect of L-carnitine therapy on exercise tolerance and physical fitness in 30 patients with thalassemia major followed up for 6 months in our center.

PATIENTS AND METHODS

Patients:

Thirty patients, 19 males and 11 females, with homozygous β-thalassemia attending the Hematology Clinic of the New Cairo University Children Hospital were included in this study. Their age ranged between 10-20 years with a mean of 14.6±2.7 years. All patients were on regular blood transfusion. They were all on desferoxamine chelation therapy (20-40mg/kg/day). None of the enrolled patients had chest wall deformity, cardiac, muscle, metabolic or neurological disorders.

The patients were evaluated before and 6 months after oral L-carnitine therapy (50mg/kg/day). The drug was supplied by the Hematology Clinic on an outpatient basis for free for each patient on each visit.

Clinical study of the patients included full history taking, focusing on general activity indicators as exercise tolerance, hours of sleep, school performance and sharing in social activities. Frequency of blood transfusion and chelation therapy were also recorded. Physical examination was done focusing on weight, height, heart rate, liver and spleen status.

Evaluation of Physical Fitness Using Cardiopulmonary Exercise Test:

Each patient performed an incremental cycle ergometer exercise during which work load of pedaling was increased at one minute interval.

Patient breaths room air through a pneumotachometer and expired air is continuously sampled and analyzed while heart rate is continuously recorded by a cardiac monitor. The following measurements were taken:

- Maximal oxygen consumption (VO_2 max) that is the oxygen consumption at maximal exercise.
- Minute ventilation (Ve) that is the volume of air respired per minute.
- Ventilatory equivalent for CO_2 (Ve/V_{CO2}).
- Oxygen pulse (O_2 pulse) that is the ratio of VO_2/heart rate.
- Cardiac output calculated from oxygen consumption and heart rate according to Striger et al. (1997) **[8]**.

All measurements were expressed as percentage of normal predicted values for each subject according to Wasserman et al. (1999) **[9]**.

The increment size was adjusted to each subject according to his/her age, height and weight so as patient reaches his/her maximal exercise level in around ten minutes as follows:

$$\text{Size of work load increment (watt/min)} = \frac{\text{Predicted } VO_2 \text{ max} - VO_2 \text{ of unloaded pedaling}}{100}$$

where, VO_2 of unloaded pedaling = 150+6 x weight in kg **[9]**.

Statistical Methods:

The numerical data is presented as mean ± standard deviation. Student's *t*-test (paired *t*-test) was used to compare numerical data between groups. Coefficient of correlation (*r*) was used to indicate the degree of correlation between different variables.

RESULTS

Exercise Findings:

A significant increase in VO_2 max, cardiac output and O_2 pulse after 6 months of L-carnitine therapy was found ($p<0.001$, <0.002 and <0.001 respectively). No significant changes were found in minute ventilation or in ventilatory equivalent of carbon dioxide ($p>0.05$) (Table 1, Fig. 1).

The correlation between the age of patients and the percentage change in VO_2 max, cardiac output and O_2 pulse was negative (r=0.49, 0.49 and 0.43 respectively).

Clinical and Laboratory Findings:

There was a significant increase in the mean weight and height of the patients 6 months after L-carnitine therapy (p=0.04 and 0.03 respectively). A significant increase in the interval of blood transfusion was also observed after therapy (p=0.008). However, there was no significant change in hemoglobin concentration (p>0.05) (Table 2).

Table (1): Cardiopulmonary exercise parameters before and after 6 months of L-carnitine therapy.

Parameter*	Before therapy	After therapy (50mg/kg/day)	*p* value
VO_2 max	39.1±11.6	56.7±17.1	<0.001
Cardiac output	56.6±12.1	73.2±15.1	<0.002
O_2 pulse	3.8±1.0	5.4±1.6	<0.001
Minute ventilation	56.0±15.9	60.0±16.8	>0.05
Ve/V_{CO2}	26.6±2.7	27.4±2.7	>0.05

*Expressed as percent of predicted value.

Table (2): Clinical and laboratory parameters before and after 6 months of L-carnitine therapy.

Parameter	Before therapy	After therapy (50mg/kg/day)	*p* value
Weight (Kg)	32.3±7.15	33.5±6.74	0.04
Height (cm)	140.8±15.18	141.7±15.3	0.03
Interval of blood transfusion (days)	28.6±9.27	37.3±14.89	0.008
Hemoglobin (g/dl)	7.8±0.79	7.3±0.95	>0.05

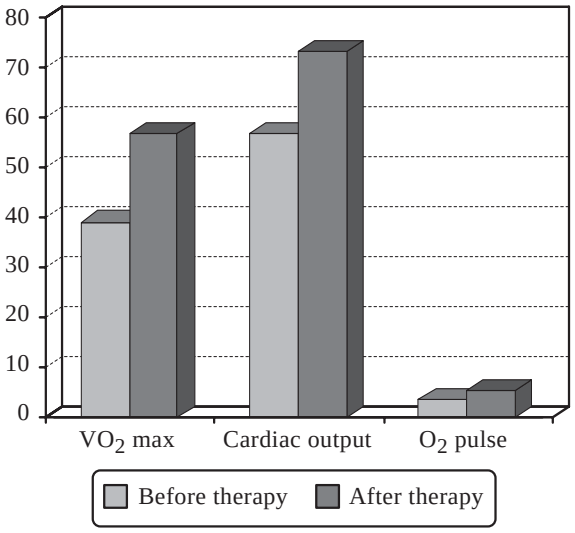

Fig. (1): Cardiopulmonary exercise parameters in thalassemic patients before and 6 months after L-carnitine.

DISCUSSION

Although current therapy for thalassemia, in the form of blood transfusion and iron chelation, has improved the prognosis of the disease, yet cardiac affection is still a major problem for these patients and muscular symptoms remain unaffected **[1,4]**.

Mitochondrial oxidation of long-chain fatty acids provides an important source of energy for the heart as well as for skeletal muscle during prolonged aerobic work. The carnitine is responsible for transferring long-chain fatty acids across the barrier of the inner mitochondrial membrane to gain access to the enzymes of beta-oxidation. Because of its key role on fatty acid oxidation, there has long been interest in the possibility that carnitine might be of benefit in energy production **[10]**.

VO_2 max is the VO_2 at which the performance of increasing levels of supra maximal work fails to increase the O_2 uptake further and this represents the highest VO_2 attainable for a given form of exercise **[11]**. There are many factors affecting VO_2 max as age, sex, physical activity, weight and height. Reduction of VO_2 max occurs in conditions with impaired O_2 flow to tissues as cardiac disease, anemia and pulmonary diseases **[9]**. In thalassemic patients, all the above mentioned conditions might contribute to a low VO_2. Anemia and cardiac dysfunction are common problems in thalassemia major. Moreover, reduction in pulmonary function tests in these patients has been reported. It was suggested that iron deposition due to repeated blood transfusion may play a central role in determining lung alterations **[12]**.

In our study, VO_2 max of thalassemic patients (expressed as percentage of their normal predicted value) was low before starting therapy. This was neither related to the degree of anemia as the hemoglobin concentration showed no significant change after treatment nor to the pulmonary state as there was no change in minute ventilation or Ve/V_{CO2} before or after therapy. However, cardiac output and O_2 pulse were low at maximum exercise. L-carnitine therapy for 6 months led to increase in VO_2 max, cardiac output and O_2 pulse. Thus improvement of VO_2 max can be attributed to improvement in cardiac function. Echocardiographic evidence of improvement of systolic

and diastolic cardiac function was shown in a study on thalassemic patients receiving L-carnitine for 2-18 months **[13]**. In another study a significant improvement of diastolic function demonstrated by MUGA was reported after 6 months of L-carnitine therapy **[14]**. Improvement in exercise capacity and VO_2 max in patients with heart failure treated with L-carnitine was reported. L-carnitine therapy appears to be particularly effective in correcting the exertion fatigue and shortness of breath that many patients continue to experience despite optimal treatment of heart failure **[15,16]**.

The most important factors that contribute to cardiac complications in thalassemic patients are chronic hypoxia and iron overload. Cardiac ischemia or hypoxemia causes striking changes in free fatty acid metabolism, inhibition of β oxidation and reduced transport of free fatty acid into the mitochondria. Inhibition of lipid metabolism results in accumulation of oxidative intermediates such as long chain acyl CoA esters that impair cardiac metabolic and mechanical function **[17]**. Iron overload leads to increased lipid peroxidation, mitochondrial membrane damage, lysosomal fragility and release of lysosomal enzymes. On the long run, fibrosis of the myocardium and specialized conductive system occurs **[18]**. Hypoxia and hemosiderosis cause myocardial damage and reduce myocardial carnitine. Exogenous carnitine therapy restores free carnitine in the myocardium and thus improves its metabolism **[19]**.

The observed improvement of cardiac function in our patients can therefore be explained by the effect of L-carnitine on myocardial energy production that is mainly covered by free fatty acids oxidation (60-90% of ATP production) **[6]**. L-carnitine therapy restores free carnitine in myocardial tissues with subsequent inhibition of accumulation of mitochondrial long chain acyl carnitine with concomitant improvement of myocardial metabolism **[20]**.

However, it has been reported that the benefit of L-carnitine therapy on myocardial mechanical function can be ascribed to the increase in overall glucose utilization rather than normalization of fatty acid metabolism **[21]**.

Moreover, L-carnitine therapy was found to strongly reduce the elevated plasma renin activity in patients with heart failure **[15]**.

On the other hand, L-carnitine improves oxidative metabolism of skeletal muscles that may indirectly improve cardiac performance as a result of a lower myocardial oxygen demand at sub-maximal exercise workload **[20]**.

In our study, all our patients showed improvement in their general activity in the form of increased exercise tolerance, decreasing sleep hours, better scholastic achievement and better sharing with social activity after therapy. Similar findings were reported in a study on the effect of L-carnitine (100mg/kg/day) for 1 month on 54 thalassemic patients, 23 of whom had myalgia and easy fatigability. L-carnitine therapy had caused remission of muscular symptoms and improvement of their quality of life **[22]**. Reduction of serum carnitine levels in thalassemic patients was also reported and it was suggested that this might play an important role in the appearance of muscular dysfunction and the clinical symptoms as myalgia and muscle weakness. L-carnitine administration in these patients might improve or even resolve these symptoms **[1]**. It has been found that it improves the skeletal muscle metabolism, increases pyruvic acid consumption and decreases lactic acid production **[23]**.

The improvement in cardiac function in response to L-carnitine therapy was higher in younger patients in our study; this finding may point out the importance of starting therapy at an early age.

We observed that administration of L-carnitine (50mg/kg/day) for 6 months has significantly increased the weight and height of our patients. In a study on the effect of L-carnitine on growth in 18 patients for duration of 6 months, a significant increase in growth velocity and a proportionate increase in the upper and lower segments were found. The increase in height was more than the increase in weight among our patients. It was concluded that carnitine has a positive effect on growth of the patients even on low hemoglobin level and bad chelation therapy **[24]**.

The positive effect of L-carnitine on growth can be explained by its effect on energy production in vital organs that depend on free fatty acid metabolism as the liver, heart and brain **[25]**. Improvement in erythrocytes survival and decreased iron deposition in different tissues can also be contributing factors **[26]**.

In this study a significant increase in blood transfusion interval after L-carnitine therapy was also observed. This was previously reported **[14,26]** and can be explained by the protective effect of L-carnitine on the erythrocytes from oxidative stress and its stabilization to the red cell membrane in which latent peroxidative damage has been produced **[27]**. Increased in vivo lipid peroxidation in children with β-thalassemia major has been shown **[28]**. The autoxidaton of globin chains and iron overload were the suggested mechanisms for the increased oxidative stress. The counteracting effect of antioxidants on lipid peroxidation processes and their protective effect against oxidative damage of red cells in β-thalassemia patients were also confirmed **[29]**.

However, in our study there was no statistically significant increase in hemoglobin concentration after L-carnitine therapy.

In conclusion, L-carnitine seems to be an effective therapeutic approach in thalassemic patients. It improves their cardiac performance, physical fitness and general activity. The effect of L-carnitine on growth of thalassemic patients is worthy for further studies.

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Detection of BCL-2 Family Proteins in Lymphoid Malignancies: An Immunocytochemical and Immunoelectron Microscopic Study

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ABSTRACT

Background Methods and Purpose: The apoptotic process is under the control of many regulatory genes that have been identified to be involved in inducing apoptosis e.g. p53, Bax and c-myc or inhibiting it e.g. Bcl-2 and Bag. This work aimed at studying the expression of Bcl-2, Bax, Bcl-x and p53 in various lymphoid malignancies using immunohistochemistry inorder to find out whether these oncoprotiens/tumour suppressor gene products have a possible influence on disease progression and outcome as regards to relapse and survival. Additionally, an immune-electron microscopic study was done for proper intracellular localization of Bcl-2.

Patients: The study was carried out prospectively on 60 patients with lymphoid malignancies of which 39 (31 Denovo and 8 cases in relapse) were acute lymphoblastic leukaemia (ALL), and 21 were chronic lymphocytic leukaemia (CLL).

Results: Results revealed a statistically significant higher expression of Bcl-2 and Bax as compared to the control group in ALL (p=0.03 & 0.045 respectively). No correlation was found between Bcl-2 and response to treatment or overall survival in both groups. Bcl-2 expression was significantly higher in stages III and IV as compared to stages I and II in the CLL, thus suggesting a possible role played by Bcl-2 in the poor outcome of advanced disease. Bax showed no correlation to the response of the cells to chemotherapy in both groups of lymphoma studied. Bcl-x showed no statistically significant difference as compared to the control group in both groups. Expression of p53 was significantly higher in the CLL as compared to the control group (p=0.015). There was a significant positive correlation between Bcl-2/Bax ratio and BM lymphocytes i.e. tumour burden (r=0.778, p< 0.01) in the CLL group. In the ALL, the eight relapsed cases showed a statistically significant higher expression of Bcl-2, and Bcl-x (p=0.006, and 0.013) and a statistically lower expression of Bax (p=0.005) when compared to the de novo ones. Immune electron microscopic localization of Bcl-2 and the two-step indirect immune peroxidase method was done. Bcl-2 was visualized in the outer and inner mitochondrial membranes, rough and smooth endoplasmic reticulum and the nuclear envelop.

Conclusion: From this study it can be concluded that: First, Egyptian CLL (chronic lymphocytic leukaemia) patients might be having a different disease biology since they are having more Bcl-2 expression (81% compared to the 73% in the Western CLL), less Bax (48% compared to 96%), more p53 (43% compared to 15%), present at younger age (age range in this study was 37-70y compared to 60-90y in the Western CLL), and present at a more advanced stage (Rai stages III & IV more than stages I & II). Second, Bcl-2/Bax ratio can be used to monitor tumour burden in chronic lymphocytic leukaemia owing to the significant (r=0.778, p<0.01) positive correlation found between this ratio and bone marrow lymphocytes. Third, because Bcl-2 is expressed in 81% of CLL and because all nine cases with positive Bcl-2 expression showed evidence of apoptosis using the electron microscopy, the mechanism of this apoptosis is suggested to be away from the mitochondrial pathway, either through an extrinsic pathway by passing the mitochondrial pathway or through an alternate pathway. Fourth, having a statistically higher expression of the prosurvival Bcl-2, and Bcl-x, and a statistically lower expression of the proapopotic Bax, in relapsed ALL compared to the denovo cases, might explain the cause of relapse as a result of emergence of a resistant clone expressing more prosurvival (Bcl-2 & Bcl-x) and less pro-apoptotic (Bax) markers. Finally, Bcl-2 expression in most of our CLL cases makes it an attractive target for the therapeutic treatment, by using Bcl-2 oligonucleotide antisense or the naturally available Genasense (Bcl-2 antisense).

Key Words: *Bcl-2, Bax - Bcl-x - p53 - ALL & CLL - IHC & immune-electron microscopy.*

INTRODUCTION

The control of cell number and type in multicellular biological system is extremely necessary for maintaining quantitative and functional homeostasis at the cellular level. This control of cell number is maintained through a balance

between cell proliferation and cell death. Each cell possesses its own self-induced destruction which is called programmed cell death **[1]**. Apoptosis- a matter of programmed cell death- is an active, gene-directed, self-inflicted process. It is associated with characteristic morphological & biochemical changes that ends in dissociation of a cell into membrane- bound apoptotic bodies, which are then engulfed by the local phagocytic system **[2]**.

Failure of the cells to undergo apoptosis leads to many diseases e.g. viral infections, autoimmune diseases and cancer. CLL is considered a disease with progressive accumulation of lymphocytes failing to undergo apoptosis. ALL, on the other hand, is a disorder due to uncontrolled increase in proliferation **[3]**. The apoptotic process is under the control of many regulatory genes, some are involved in inducing apoptosis e.g. p53, Bax and c-myc, others are inhibiting apoptosis e.g. Bcl-2 and Bag **[4]**.

This work aimed at studying the expression of Bcl-2, Bax, Bcl-x and p53 in the two distinct groups of lymphoid malignancies, one with increased proliferation and the other with defective apoptosis using immunohistochemistry, in order to find out whether these oncoprotiens/tumour supperssor gene products have a possible influence on disease progression and outcome as regards to relapse and survival. Additionally, immune-electron microscopic study was done for proper intracellular localization of Bcl-2.

PATIENTS AND METHODS

Patients:

The study was carried out prospectively on 60 patients with lymphoid malignancies of which: 39 were acute lymphoblastic leukaemia and 21 were chronic lymphocytic leukaemia. The age range was from 3-80 years in ALL group and 37-70 years in the CLL group. Male: female ratio was 1.5: 1.0 in both groups. Additionally, age- and sex-matched subjects were taken as controls. The patients presented to the National Cancer Institute, Cairo University and subjected to full clinical examination, CBC and BM examination. Immunophenotyping was done for proper lineage affiliation as well as immunohistochemical detection of Bcl-2, Bax, Bcl-x, and p35 using their corresponding monoclonal antibodies. Immune-electron microscopy was done for selected cases (nine CLL and two ALL) with high Bcl-2 expression.

Methods:

Mononuclear cell separation and test for viability were done according to Perper et al. **[5]** and Weir **[6]** respectively.

Immunohistochemistry was done according to Bisgaard and Pluzek **[7]**. The monoclonal antibodies used were as follows:

Bcl-2, Boehringer Mannheeim, Cat. No1624 989.

Bcl-x, Pharmingen BD, Cat. No 66461 A.

Bax, Zymed Laboratories Incorporation, Cat. No 18- 0218.

P53, Dako, Denmark, Cat. No M 7001.

The detection system was Dako Envision+ system, peroxidase, for mouse monoclonal antibodies and DAB as a chromogen, Cat. No 4007.

Interpretation of results:

At least 500 cells were counted for each monoclonal antibody. Negative cells appear homogeneously green while positive cells appear as brown black positivity on the cell. The results are expressed as % of positivity. A cut-off ≥ 5% of the tumour cells with unequivocal positivity in the cytoplasmic membrane and nuclear periphery was used for Bcl-2, Bax, and Bcl-x while a cut-off of ≥ 1% of the lymphoid cells with strong nuclear staining was used for p53 **[8]**.

Bcl-2/ Bax ratio was calculated by dividing the number of cells positive for Bcl-2 by the number of cells positive for Bax.

Immune-electron Microscopy: **[9]**

Immunoelectron Microscopy:

Mononuclear cells were fixed with 4% paraformaldhyde in PBS for 20 min., centrifuged at 200xg for 5 min., washed three times in PBS and stained using two-step indirect immunoperoxidase method. Following two washes, cells were fixed with 2% glutaraldehyde in PBS for 30 min and post fixed with 1% osmium tetroxide in PBS. After 2 washes, cells were stained with 1% uranyl acetone in PBS for 10 min, dehydrated and embedded in Epon 812 according to

routine procedures. Ultra thin sections were prepared and examined in a Joel electron microscope.

Statistical analysis was done according to Ingelfinger et al. **[10]** and Knapp and Miller **[11]**. Survival analysis and curves were done according to Kaplan Miere method. Log rak test was used for comparing survival. Significance level of 0.05 and 0.01was used throughout all statistical tests in the study.

RESULTS

Survival:

Median overall survival for two years of the CLL group was studied among Bcl-2, Bax, Bcl-x and p53 positive cases as compared to negative ones. Bcl-2 positive cases had a significantly shorter survival as compared to Bcl-2 negative ones (p=0.049). Bax, Bcl-x and p53 had no impact on the survival (p=0.46, 0.84, and 0.87 respectively). These data are shown in Fig. (1).

Immunoelectron Microscopy:

Immunoelectron microscopy was carried out for intracellular localization of Bcl-2 protein. Lymphoid cells from an acute lymphoblastic leukaemia case showed lymphoblastoid features characterized by a large nucleus & scarce cytoplasm. In the cytoplasm, abundant mitochondria, smooth & rough endoplasmic reticulum were observed. This is different from the chronic lymphocytic leukaemia case in which cell had sparse intracellular organelles. In both cases, immunoreactivity with anti-bcl-2 antibody was detected prominently in the mitochondria in both outer & inner mitochondrial membrane i.e. in between cisternea, smooth & rough endoplasmic reticulum membrane & in the nuclear membrane. These findings are shown in Figs. (4-6). Some apoptotic changes in the form of increased vesicles, dilated endoplasmic reticulum and condensation of heterochromatin were seen in Fig. (4).

Table (1): Expression of different markers among ALL, CLL and control cases.

Marker	ALL (n=39)	Control (n=10)	*p* value
Bcl-2	23 (59%)	4 (40%)	0.47
Bax	7 (18%)	1 (10%)	0.47
Bcl-x	8 (21%)	2 (20%)	0.67
P53	11 (28%)	0	*0.06
Marker	**CLL (n=21)**	**Control (n=10)**	***p* value**
Bcl-2	17 (81%)	4 (40%)	*0.03
Bax	10 (48%)	1 (10%)	*0.045
Bcl-x	7 (33%)	2 (20%)	0.38
P53	9 (43%)	0	*0.015

* Significant.

Table (2): Expression of different markers in relation to response to treatment among ALL cases and CLL cases respectively.

	CR+PR Mean% ± SD	Resistant Mean% ± SD	*p*-value	CR+PR Mean% ± SD	Resistant Mean% ± SD	*p*-value
Bcl-2	0.112±0.172	0.136±0.225	0.805	0.375±0.227	0.220±0.309	0.265
Bax	0.080±0.164	0.062±0.093	0.820	0.098±0.142	0.034±0.076	0.359
Bcl-x	0.013±0.046	0.096±0.169	0.090	0.111±0.219	0.038±0.085	0.489
P53	0.038±0.064	0.068±0.102	0.442	0.046±0.072	0.066±0.064	0.595
Bcl-2/Bax	5.32±9.29	0.27±0.42	0.377	8.581±11.31	0.059±0.001	0.507

Table (3): Expression of different markers in de novo and relapsed cases among ALL group.

	Denovo n=31	Relapse n=8
Bcl-2	7/31	8/8
Bax	5/31	3/8
Bcl-x	3/31	5/8
P53	12/31	4/8

Table (4): Expression of different markers in relation to disease stage among CLL cases.

	I & II		III & IV		*p*-value
	Mean%	SD	Mean%	SD	
Bcl-2	0.11	0.10	0.38	00.25	0.019*
Bax	0.12	0.08	0.07	00.13	0.450
Bcl-x	0.15	0.22	0.09	00.20	0.534
P53	0.16	0.24	0.04	00.07	0.080
Bcl-2/Bax	1.05	1.53	14.5	17.45	0.121

* Significant.

Table (5): Expression of different markers in Egyptian and Western CLL cases.

	Egyptian CLL	Western CLL
Bcl-2	81%	*73%
Bax	48%	*96%
Bcl-x	33%	?
P53	43%	**15%

* Logdon et al. (1993). ** Cordone et al. (1998).

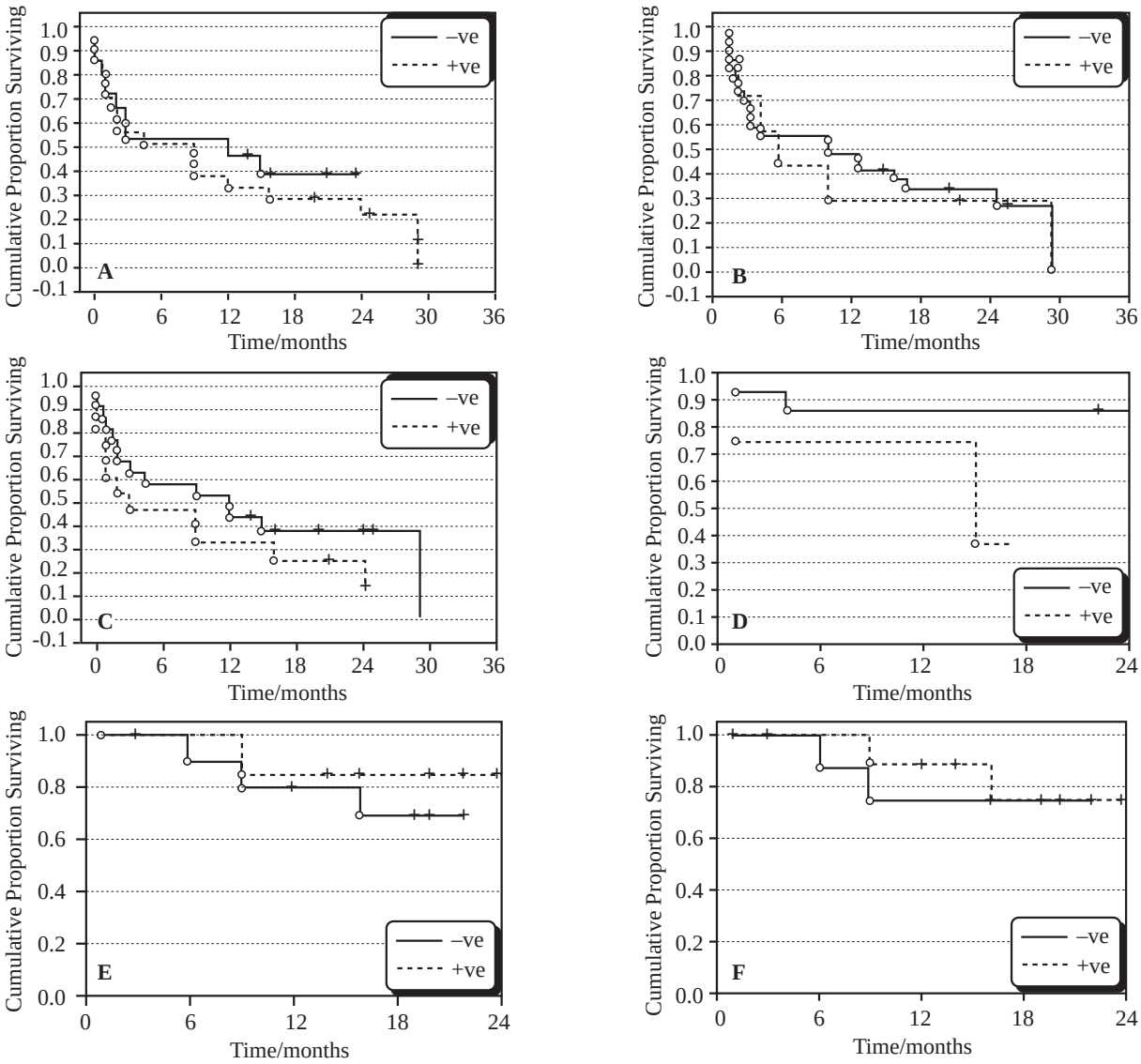

Fig. (1): (A, B, C) Overall survival of the ALL group in relation to Bcl-2, Bax and p53. ºComplete + censored *p* value = 0.54, 0.9 and 0.7 respectively. (D,E,F) Overall survival of the CLL group in relation to Bcl-2, Bax and p53 º Complete + Censored *p* value = 0.049*, 0.46 and 0.87 respectively.

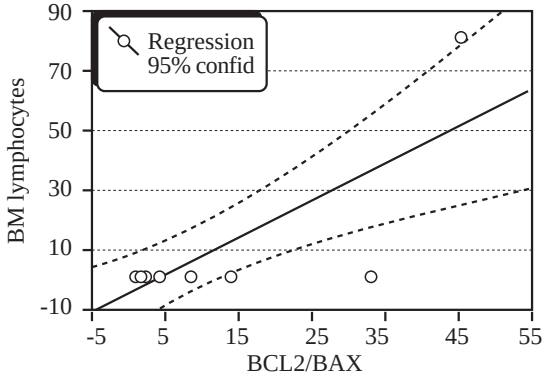

Fig. (2): Correlation between Bcl-2/Bax ratio vs BM lymphocytes in CLL cases Correlation: *r*=0.77800 *p*-value <0.01.

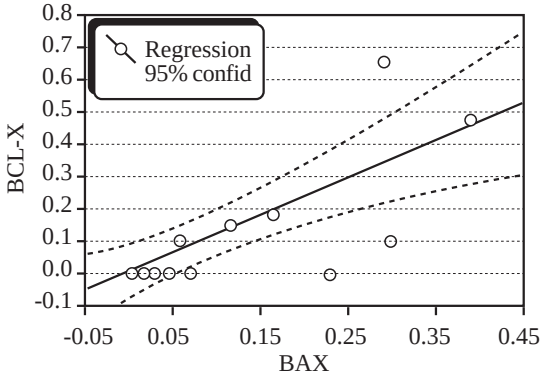

Fig. (3): Correlation between Bax vs. Bcl-x in CLL cases. Correlation: *r*=0.69 *p* value <0.05.

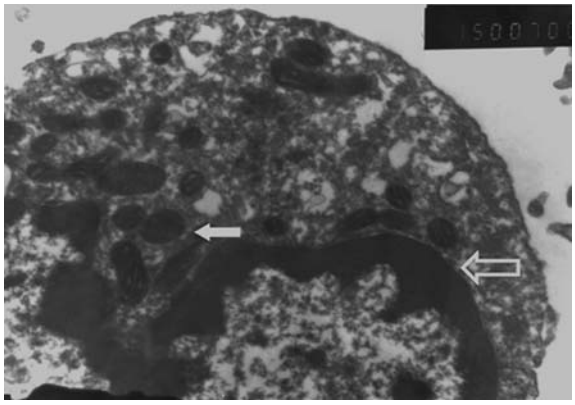

Fig. (4): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 in mitochondria and nuclear membrane (empty arrow: Nuclear membrane, white arrow: Mitochondria). Mild apoptotic changes in the form of condensation of heterochromatin and increased vesicles. X15 000.

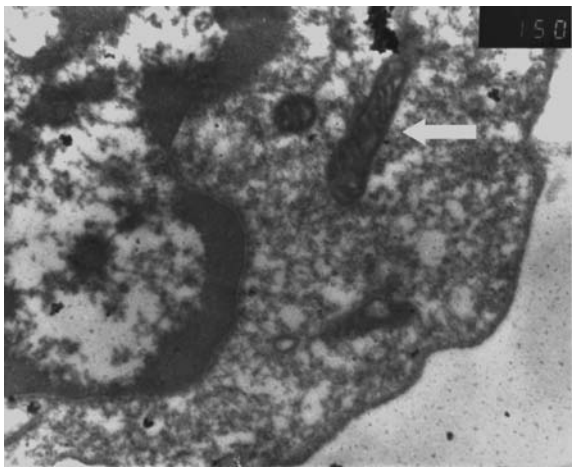

Fig. (5): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 inside the mitochondrial cisternea (white arrow: Mitochondria) X20 000.

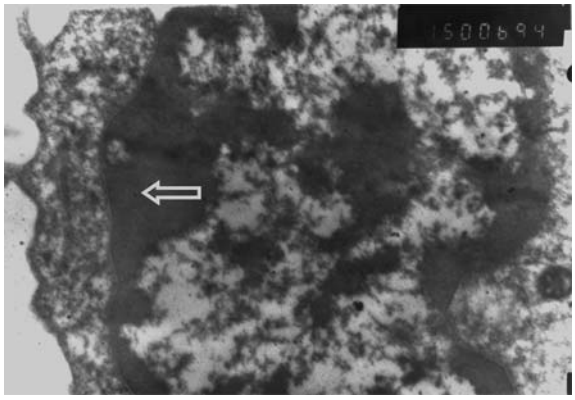

Fig. (6): Immunoelectron micrograph for Bcl-2 in a lymphocyte showing the distribution of Bcl-2 in the nuclear membrane and endoplasmic reticulum (empty arrow: Nuclear membrane) X15 000.

DISCUSSION

Cell division and survival are tightly linked to assure genomic integrity and tissue homeostasis. Failure to apoptose results in propagation of the damaged DNA and the survival of superfluous and potentially harmful cells **[12]**. Apoptosis or programmed cell death is a physiological, active, gene-directed, self-inflicted process by which normal and tumour cells are believed to die **[1]**.

Apoptosis occurs in 3 phases: Initiation, effector phase, and degradation. Susceptibility of a cell to apoptotic signals appears to be regulated, in part, by the relative levels and dimerization between Bcl-2 family members, which act on the effector stage of apoptosis, as well as on the competition for other cellular factors involved in the death pathway **[13]**. Bcl-2 family members can be divided into 3 groups: anti-apoptotic as Bcl-2 and Bcl-xL, pro-apoptotic as Bax and BH3 only proteins as Bid **[14]**. Another protein involved in apoptosis is the tumour suppressor gene, p53, which is involved in cell cycle arrest and induction of apoptosis in genetically damaged cells **[15]**.

In our study, Bcl-2 was expressed in 23/39 (59%) in ALL, in 17/21 (81%) in CLL and in 4/10 (40%) in the control group. A statistically significant higher expression of Bcl-2 as compared to the control group was found in the ALL (p=0.03) but not in the CLL group (p= 0.47). This might be attributed to the relatively small number of CLL subjects studied, increasing the number of CLL cases would bring such borderline difference to the statistical level. Our results are in agreement with Papakonstantinon et al. **[16]** who found a higher expression of Bcl-2 in mature B-cells of CLL/SLL and follicle center cell lymphoma (FCC) than in the immature B-cells of diffuse large B-cell lymphoma.

In our results, Bcl-2 expression of 81% in the CLL group is slightly higher than Logsdon et al. **[17]** who found Bcl-2 in 73% of FCC lymphoma by IHC. However, both Johnston et al. **[18]** and Gaballa et al. **[19]** found Bcl-2 in all cases of B-CLL/SLL by Western Blot (WB) technique.

Bcl-2 was detected in 59% of our ALL cases. This was higher than Gascoyne et al. **[20]** who reported an incidence of 24% using IHC and

lower than both Campana et al. **[21]** who detected Bcl-2 in all their adult ALL cases, Hogarth and Hall **[22]** who detected Bcl-2 in all their cases by WB technique, and Papakonstantinou et al **[16]** who found gene rearrangement in 85% of disseminated lymphoblastic lymphoma by PCR. In the latter study, they found no correlation between gene rearrangement and protein expression as some Bcl-2 translocations may fail to express Bcl-2 protein as a result of mutations in the open reading frame of the translocated Bcl-2 gene leading to absent or diminished Bcl-2 production. On the other hand, Bcl-2 protein expression may be absent in some cases with translocation, suggesting that mechanisms other than translocation can lead to Bcl-2 overexpression. This suggestion was also emphasized by Almasri et al. **[23]** in their study on 28 Jordanian NH lymphoma cases. Accordingly, Bcl-2 protein expression (by either Western Blot or Immunocytochemistry) is far more important than gene rearrangement analysis. Variations in the level of Bcl-2 expression in different studies may be due to different cut-offs used as well as variations among techniques in terms of sensitivity and specificity, its detection signifies its overexpression regardless of the underlying molecular mechanism.

Although Uckum et al. **[24]** found a significant association between high Bcl-2 and low WBC count and Gascoyne et al. **[20]** with high S.LDH, our results showed no significant correlation as regards age, gender, lab. prognostic features (Hb, Platelet count, S.LDH) and immunophenotype. However, both Salomon et al. **[25]**, Srinnivas et al. **[8]**, and Campos et al. **[26]** found similar results.

No correlation was found between Bcl-2 and response to treatment in ALL or CLL groups. This was consistent with that of Logsdon et al. **[17]** and Mekki et al. **[27]** who found no correlation between Bcl-2 and drug resistance or treatment outcome. In contrast to these studies, Baghdassarian et al. **[28]** found that high Bcl-2 expression was linked to poor response to cytotoxic drugs and both Maung et al. **[29]** and Tang et al. **[30]** reported a greater intensity of Bcl-2 staining in cells from ALL patients who failed to achieve complete remission than those who responded to treatment. Other studies concluded that low level of Bcl-2 was related to poor response and shortened survival in childhood ALL **[31]** and myelodysplastic syndrome **[32]**. The discrepancy between the results may be related to the presence of other Bcl-2 family proteins as Bax & Bcl-x modulating the effect of Bcl-2. Variability to the response to treatment could also be attributed to the variability in the drugs used or the presence of drug resistance. Moreover, Alderson et al. **[33]** reported that Bcl-2 expression may be associated with a mutant p53 gene, thereby conferring the resistant phenotype.

In our study, no statistically significant difference was found between Bcl-2 expression and overall survival in the ALL group. Similar results have been reported by many authors **[26,34,35,36]**. In contrast, Gascoyne et al. **[20]** reported a significant difference in overall survival in DL cases. Variations among studies may be due to differences in the uniformity of the patients selected, tumour bulk, or treatment given. A longer follow-up may also reveal more significant results.

Bcl-2 expression has a statistically worse survival in our CLL group. This is not in agreement with both Prowit-MacDonald et al. **[31]** and Lepelley et al. **[32]** as both reported that low Bcl-2 level was associated with shortened survival or Robertson et al. **[37]** who reported that high Bcl-2 expression is associated with adverse outcome.

Bcl-2 expression is significantly higher in stages III and IV as compared to stages I and II in the CLL suggesting a possible role played by Bcl-2 in poor prognosis and advanced disease.

The conformational changes of Bcl-2 and interaction between different family member may contribute to the different levels of Bcl-2 protein expression and the different effects and correlation of Bcl-2 protein with the clinical and laboratory prognostic factors. There is an evidence that post-transcriptional modifications regulate the function of Bcl-2 and possibly other family members **[20]**. This post-transcriptional modification might be the cause of discrepancy between Bcl-2 gene rearrangement and protein expression. Several studies have suggested that phosphorylation of Bcl-2 affects its function, although there have been conflicting reports that this may enhance **[38,39]** or reduce **[40]** its ability to suppress apoptosis. It is also possible

that mutations may alter the function of these proteins by, for example, affecting protein stability **[20]**. Also, Bcl-2 expression may be affected by infection with EBV **[41]** and human CMV **[42]**. IL-5 **[43]** and IL-10 **[44]** have also been reported to induce Bcl-2 expression.

Bax was expressed in 7/39 (18%) of the ALL, 10/21 (48%) of the CLL, and in 1/10 (10%) of the control group. Bax expression was significantly higher than the control group (p= 0.045) in the ALL.

Bax had a range of 0-53% and this was comparable to that of Srinivan et al. **[8]** who reported a range of 1-60%. Bax expression in the CLL was lower than that reported by Logsdon et al. **[17]** who detected an expression of 96% in FCC by IHC.

In the present study, Bax expression in the ALL was lower than Hogarth & Hall **[20]** who detected Bax in all their childhood ALL cases by WB technique. Differences in the level of expression of Bax may be due to the cut-off used, sensitivity of the monoclonal antibody and the sensitivity of the WB technique used to assess the level of Bax.

Bax showed no correlation to the response of the cells to chemotherapy in both groups of lymphoid malignancies studied and this was in agreement with both Hogarth & Hall **[22]** and Srinivas et al. **[8]** on their paediatric ALL cases.

Bcl-x was expressed in 8/39 (21%) of the ALL, 7/21 (33%) of theCLL, and in 2/10 (20%) of the control group. Bcl-x showed no statistically significant difference as compared to the control group both in the CLL and ALL.

Bcl-x used in this study had a common epitope for both Bcl-xl (anti-apoptotic) and Bcl-xs (pro-apoptotic). So, our results were difficult to interpret as due to presence of Bcl-xl or Bcl-xs. However, and in view of the significant positive correlation between Bcl-x and Bax in the homogenous group of CLL, a possible predominance of Bcl-xs (pro-apoptotic) effect could be elucidated in the antibody used (r= +0.58, p< 0.05). Simonian et al. **[13]** found that in FL5.12 lymphoid cell lines, the expression of both Bcl-2 and Bcl-xl has been implicated in the drug resistance of the tumour to therapy and Bcl-xl was better able than Bcl-2 to protect the cells from death induced by certain chemotherapeutic drugs. The study concluded that Bcl-xl expressing tumour cells may be more likely to escape initial destruction by certain chemotherapeutic agents than neoplasms expressing Bcl-2 and thus increasing the likelihood of relapse. In view of the above, and in view of the presence of complete remission in 5 of the relapsed ALL cases expressing Bcl-x i.e. none was resistant to chemotherapy (data not shown) provide a 2nd support of a Bcl-xs effect (pro-apoptotic) of the Bcl-x used in the present work.

P53 was expressed in 11/39 (28%) of the ALL, 9/21 (435%) of the CLL and in none of the control group. Expression of p53 was significantly higher in the CLL as compared to the control group (p=0.015).

The level of p53 expression was variable in the different reports. While it was 43% of our CLL cases, Cordone et al. **[15]** reported a 15% expression in CLL using IHC. Similarly, p53 was expressed in 28% of our ALL as compared to the 38% expression reported by Srinivas et al. **[8]** using ELISA. This variability may be due to different cut offs used and/ or sensitivity of the methods used for p53 detection as ELISA is known to be a more sensitive quantitative technique.

Bcl-2/Bax Ratio:

In the CLL, there was a significant positive correlation between Bcl-2/Bax ratio and BM lymphocytes i.e. tumour burden (r= 0.778, p< 0.01). This finding may correlate with both Thomas et al. **[45]** and Hogarth & Hall **[22]** who reported that increased Bcl-2/Bax ratio correlates with tumour burden and in-vitro resistance to drug-induced apoptosis. However, and in contrast to their results, we could not find any significant correlation between Bcl-2/Bax ratio and in vivo response to therapy.

Expression of the Different Markers in Relapsed Cases:

In the ALL group, the eight relapsed cases showed a statistically significant higher expression Bcl-2, Bax, and Bcl-x as compared to the de novo ones (p= 0.006, 0.005, and 0.013 respectively).

Our finding of higher Bcl-x in relapsed cases (5/8, 63%) than de novo ones (3/31, 10%) is in agreement with Findley et al. **[46]** Additionally,

Datta et al. **[47]** and Minn et al. **[48]** found that Bcl-xl over expression in murine cells and histiocytic lymphoma cells was associated with increased resistance to chemotherapeutic agents in vitro. However, and in contrast to his results, our cases were not drug resistant (five of eight entered complete remission) and the only one with partial remission, showed resistance to chemotherapy and was negative for Bcl-x. This could be attributed to-as mentioned previously-to a Bcl-xs rather than a Bcl-xl effect.

Correlation between Bcl-2 and p53:

Expression of Bcl-2 is down-regulated by the tumour suppressor gene p53. The relative expression and function of these molecules may therefore determine the extent of apoptosis **[49]**. Although, such correlation in our cases was found insignificant, yet it may give an attention to the relationship between Bcl-2 and p53 because only those with high level of Bcl-2 are associated with mutant p53 expression. Several authors reported similar results; Findley et al. **[46]** on paediatric ALL, Pezzela et al. **[50]** on NHL, and Hader et al. **[51]** on cancer breast. These findings could be attributed to the fact that there are p53-dependent and p53-independent apoptotic pathways as well as the heterogenity of the lymphoma group as regards apoptosis. It is worth mentioning that our p53 negative cases were either wild-type or null-type further explaining the insignificant correlation. Also Hussein et al. **[52]** reported that lymphoproliferative lesions had some peculiar clinicopathologic features and that Bcl-2 and p53 proteins are altered in the lymphoproliferative lesions in upper Egyptain cases.

Bcl-2 Localization by Immune Elelctron Microscopy:

Immune electron microscopic localization of Bcl-2 and the two-step indirect immune peroxidase method was done. Bcl-2 was visualized in the outer and inner mitochondrial membranes, rough and smooth endoplasmic reticulum and the nuclear envelop. This coincides with the findings previously reported by Yurihiro et al. **[53]** using the same method and by Lombardi et al. **[54]** using immune gold electron microscopy method on lymphoblastoid cell line.

The pleotropic distribution of Bcl-2 protein argues against the likelihood that mitochondrial function per se is involved in the apoptosis-blocking activity of Bcl-2. Additionally, Bcl-2 may also protect different organelles in the cytoplasm by the same mechanism. Bcl-2 has been suggested to be involved in the antioxidant pathway **[55]** as most of the reactive oxygens are produced in the mitochondria, nuclear envelop and endoplasmic reticulum **[56]**. Luciano et al. **[57]** suggested-through their work on lymphoblastoid cell line using immune gold technique- that Bcl-2 is associated with the nuclear pore and mitochondrial functional complexes; where the inner and outer membranes of these DNA containing organelles come into contact. This location of the Bcl-2 protein suggests that Bcl-2 - at least in part- acts as an adapter protein that targets cytosolic Raf kinase and p23-R-Ras to critical substrates. This is based on the reported data that revealed the association between Bcl-2 and p27 raf-1, **[58]** a serine/ threonine-specific protein kinase and with p23- R- Ras, **[59]** a Gtpase member of the Ras family.

Conclusion:

- Egyptian CLL cases might be having a different disease biology when compared to the Western ones.
- Bcl-2/ Bax ratio had a significant positive correlation with BM lymphocytes in CLL possibly a reflection of tumour burden.
- Because Bcl-2 is expressed in 81% of the CLL cases and because all nine cases with positive Bcl-2 expression showed evidence of apoptosis using EM, the mechanism of such apoptosis is suggested to be away from the mitochondrial pathway i.e. extrinsic or alternate pathways.
- In relapsed ALL, Bcl-2 and Bcl-x were significantly higher while Bax was significantly lower as compared to the de novo ones, possibly explaining the emergence of relapse from a resistant clone expressing the prosurvival markers (Bcl-2 and Bcl-x).
- Bcl-2 expression in most Egyptian CLL cases makes it an attractive target for Bcl-2 oligonucleotide antisense or the naturally available Genasense (Bcl-2 antisense).

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