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Annexin V and Apoptosis in Peripheral Blood Lymphocytes of Children with Down's Syndrome

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ABSTRACT

Down's syndrome (DS) is the most common and best-known chromosomal disorder and is associated with several other pathologic conditions including immunodeficiency which makes a significant contribution to morbidity and mortality. Various immunological theories and observations to explain the predisposition of individuals with DS to various infections have been published, one of which is increased apoptotic cells.

The aim of this study was to identify the phenotype of apoptotic immune cells (T and B lymphocytes) in children with DS using Annexin V staining of phosphatidyserine (PS) as a specific marker of early apoptosis.

The study included 17 children with karyotypically ascertained DS (7 males and 10 females). Their ages ranged from 4 months to 14 years with mean age of 5.7± 4.35 and a median of 7 years. Seventeen age matched healthy children were included in the study as controls. Complete blood picture, relative and absolute number of CD3+ (T-cells) and CD19+ (B-cells) and analysis of apoptosis using Annexin V were done to all children included in this study using flow cytometry.

Complete blood picture, relative and absolute number of CD3 and CD19 did not show significant differences between DS children and control group. However, the relative and the absolute number of early apoptotic CD3 positive T lymphocytes were significantly higher (p=0.001) in DS children which may explain the functional impairment of these cells and the defect in adaptive immunity in theses patients. On the other hand, the relative number of early apoptotic CD19 positive B lymphocytes was significantly higher in DS children but no significant difference was detected when the absolute number was compared (p=0.022 and 0.286 respectively).

In conclusion, increased apoptotic CD3 cells may contribute to the impaired cellular immunity in DS children in spite of the normal absolute number of these cells. Further studies on apoptotic cellular phenotype in DS children and its correlation with the infection episodes in these children are recommended.

Key Words: *Annexin V – Apoptosis – Down's syndrome.*

INTRODUCTION

Down syndrome (DS) is the most common and best-known chromosomal disorder and is associated with several other pathologic conditions including immunodeficiency which makes a significant contribution to morbidity and mortality **[1]**.

Various immunological theories and observations to explain the predisposition of individuals with DS to various infections has been published **[2,3,4]**. Neutrophil and monocyte dysfunction is well documented in DS. Impaired function of chemotaxsis, phagocytosis, and oxidative response has been reported **[5]**. Additionally aspects of humoral immunity have been examined. Despite normal levels of circulating B-cells, immunoglobulin levels of DS may differ from normal values with tendency towards high immunoglobulin G (IgG) and low IgM serum levels **[6]**. Although the circulating numbers of cells bearing the T-cell marker CD3 are generally normal, the ratio of CD4 to CD8 cells is decreased in DS. In addition, the proportion of cells expressing the α, β chains of T-cell receptor (TCR) is decreased in peripheral blood (PB) of patients with DS and the proportion of γ, δ chains is increased. Another lymphocyte subset, natural killer (NK) cells, shows abnormalities in DS; circulating numbers of NK cells are increased while functional activity is impaired **[7]**.

On the other hand, DS patients show signs of precocious aging of various organs and tissues, one of which is the immune system and

according to some investigations, DS ranks first among human "segmental progeroid syndromes" defined as those genetic disorders in which multiple major aspects of the senescent phenotype appear **[8,9,10]**.

Apoptosis is a mechanism of programmed cell death, which plays an important role in a number of biological processes. Increased apoptosis has been suggested to be responsible for many aspects of DS pathologic condition; as it has been detected in cell lines obtained from neurons of patients with DS **[11]**. Also the percentage of apoptotic granulocytes from DS was found to be significantly higher than that from healthy subjects and this may contribute to the risk of infections in these patients **[12]**.

Apoptosis in the peripheral blood of Down syndrome have been studied before by means of electron microcopy, in situ nick translation (ISNT), and DNA electrophoresis **[13]**. However the previous methods do not identify the phenotype of apoptotic cells.

The aim of this study is to identify the phenotype of apoptotic immune cells (T and B lymphocytes) in children with DS using Annexin V staining of phosphatidyserine (PS) as a specific marker of early apoptosis.

During apoptosis, externalization of PS and phosphatidylethanolamine is a hallmark of the changes in the cell surface. These phospholipids are normally sequestered within the cell surface on the cytoplasmic side of the plasma membrane. This occurs relatively early just after segmentation of the nucleus during which the cell membrane remains intact **[14]**.

The permeability of the plasma membrane is a central difference between necrosis and apoptosis. Large molecular DNA binding dyes, such as propidium iodide (PI), can not enter intact cells because of their large size and without permebilization treatment, do not label apoptotic cells until the final lysis stage. Annexin V, a Ca^{2}- dependent phospholipids binding protein, which possesses high affinity for PS can be used specifically for detecting early apoptotic cells. When used with promidium iodide (PI), Annexin V staining allows the quantitation of cells at early stages of apoptosis and the simultaneous identification of cell surface markers **[15]**.

PATIENTS AND METHODS

The study included 17 children with karyotypically ascertained DS (7 males and 10 females) from the genetics clinic, Ain Shams University. Their ages ranged from 4 months to 14 years with mean age of 5.7±4.35 and a median of 7 years. Seventeen age matched healthy children were included in the study as controls. The following was done to all children included in this study:

1- *Complete blood count and immunophenotyping:*

The relative and the absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). The region of lymphocyte population (lymphocyte gate), was set manually, based on the forward-scatter and side-scatter characteristics (Beckman Coulter flow cytometer, USA). The relative count of each lymphocyte subpopulation was expressed as a percentage within the total lymphocyte population. The absolute count of each lymphocyte subpopulation (CD3+ and CD19+ lymphocytes) was calculated from the relative count of the total lymphocyte subpopulation (% of CD3+ and CD19+ lymphocytes), the relative count of the total lymphocyte population (%), and the absolute leukocyte count **[16]**.

2- *Apoptosis in peripheral T and B lymphocytes:*

Apoptosis in T and B lymphocytes was measured by staining with Fluorescein isothiocyanate (FITC) conjugated annexin V and Propidium iodide (PI) using IQ products Phosphatidyl Serine Detection Kit (IQP-116F) following manufacture instructions. Indotricarbocyanine (Cy5) coupled to Phycoerythrin (PE) conjugated anti-CD3 (PE-Cy5) IQP-519 and anti-CD19 (PE-Cy5) Dako-C7066 antibodies were used to identify apoptotic cell phenotype.

Aliquots of whole blood (50µl) were lysed using IQ lyse (IQP-199 ready to use), then washed with calcium buffer (1x stored at 4ºC). Cy5-labelled monoclonal antibodies and FITC labeled annexin V were incubated at the same time for 20 minutes on ice and in the dark. The cells were washed with calcium buffer for another time, then 10µl of PI were added and incubation for 10 minutes on ice was done. The cells were kept on ice till analyzed by flow cytometer **[14]**.

Three-color flow cytometry analysis was performed on a BECKMAN Coulter equipped with a single 488 nm argon ion laser. At least 10,000 events were acquired for each sample. The voltages and compensation were set according to the standard procedure, using negative controls and tested cells stained in a single color or a combination of colors. The proportion of FITC+/PI-, corresponding to early apoptosis in T and B lymphocytes, was evaluated by gating for CD3-PE-Cy5 and CD19-PE-Cy5.Cells that are FITC+/PI+ which are late apoptotic and those who are FITC-/PI+ necrotic cells were excluded.

Statistical methods:

Data management and analysis were performed using Statistical Analysis Systems. Numerical data were summarized using means and standard deviations. Categorical data were summarized as percentages. Comparisons between two groups with respect to numeric variables were done using the Mann-Witney nonparametric test, Kruskal-Wallis test was used for more than two groups. Comparisons between categorical variables were done by the chisquare test or Fisher's exact for small sample size. All *p*-values are two-sided. *p*-values < 0.05 were considered significant **[17]**.

RESULTS

1- *Complete blood picture:*

Red blood cells (RBCs) count, haemoglobin level (Hb), heamatocrite (Hct) level, mean corpuscular volume (MCV), mean corpuscular heamoglobin (MCH), platelets and total leucocytic counts did not show significant difference between DS children and control group (Table 1).

No significant difference was detected between the percentage and the absolute values of neutrophils, lymphocytes, monocytes or eosinophils between DS and normal children. The percentage of basophils was significantly higher in DS children but no significant difference was detected when absolute values were compared (Table 2).

2- *Immunologic markers:*

Although the absolute value of CD19+ B lymphocytes was lower in DS children, no significant difference was detected. On the other hand, both the relative and the absolute values of CD3+ T lymphocytes showed no significant difference between DS and normal children (Table 3).

3- *Apoptosis in peripheral blood T and B lymphocytes:*

The relative and the absolute number of early apoptotic CD3 positive T lymphocytes were significantly higher (p=0.001) in DS children than age matched controls. On the other hand, the relative number of early apoptotic CD19 positive B lymphocytes was significantly higher in DS children but no significant difference was detected when the absolute number was compared (p=0.022 and 0.286 respectively) Table (4), Figs. (1,2).

Fig. (1): Flow cytometric analysis of apoptotic CD3+ T-lymphocytes in a healthy control.

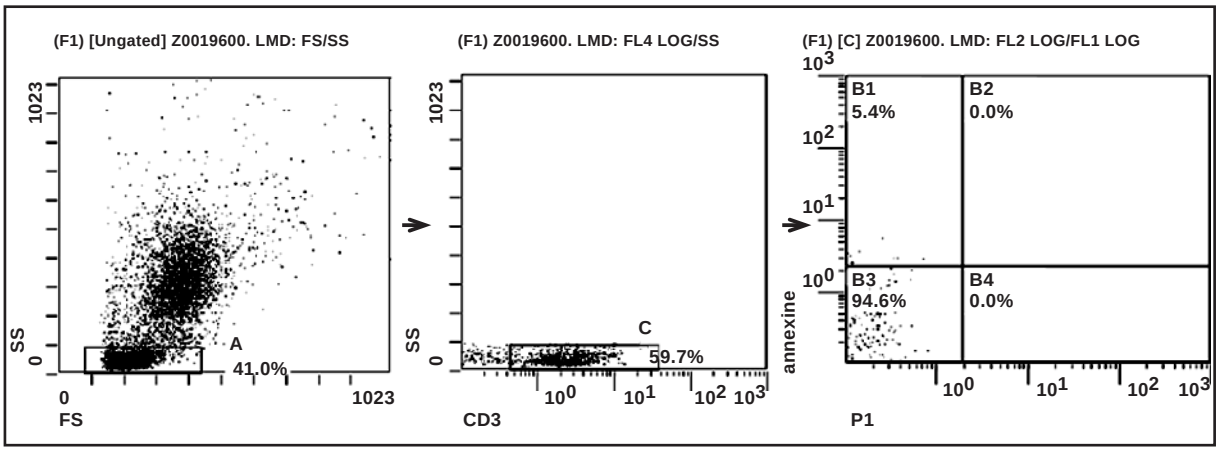

Fig. (1): From left to right: Dot plot showing gating on total lymphocytes, dot plot showing gating on CD3+ T-lymphocytes, and in the right is a dot plot showing the proportion of early apoptotic cells in the upper left quadrant of a control.

Fig. (2): Flow cytometric analysis of apoptotic CD3 positive T-lymphocytes in a patient with down's syndrome.

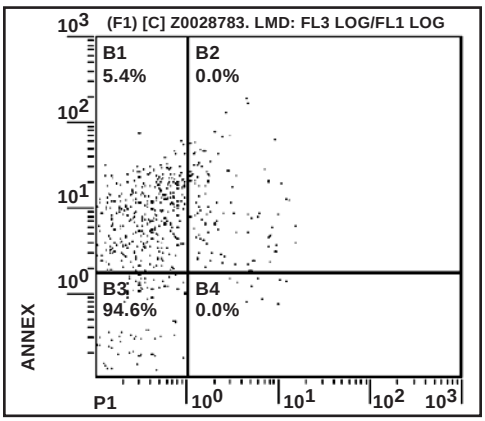

Fig. (2): Showing the percentage of FITC+/PI-in the upper left quadrant (early apoptotic cells), and the FITC+/PI+ in the upper right quadrant (late apoptotic cells) as well as the FITC-/PI+ (necrotic) cells in the lower right quadrant after gating on CD3 cells in a DS child.

Table (1): Hematological values for 17 DS patients and control group.

Variable	DS (Mean ± SD)	Controls (Mean ± SD)	*p*-value
RBC (x10^{12}/L)	4.4±0.48	4.3±0.58	0.534
HGB (g/dl)	11.71±1.70	11.47±1.21	0.856
HCT (%)	34.75±3.88	32.92±3.60	0.115
MCV (fl)	72.75±14.92	75.07±12.08	0.463
MCH (pg)	26.24±4.09	26.64±2.30	0.478
PLT (x10^9/L)	398.82±171.73	348.02±132.15	0.942
TLC (x10^9/L)	7.85±6.17	10.34±13.94	0.868

Table (2): The Differential and absolute counts of white blood cells for the 17 DS patients and age matched control group.

Variable	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
Neutrophils:			
Percentage	44.94±19.04	42.78±14.20	0.758
Absolute (x10^9/L)	4.26±5.59	4.43±5.06	0.850
Lymphocytes:			
Percentage	40.30±16.65	38.06±15.28	0758
Absolute (x10^9/L)	2.55±1.05	3.16±1.92	0.320
Monocytes:			
Percentage	8.25±4.27	7.74±4.44	0.950
Absolute (x10^9/L)	0.59±0.39	0.53±0.32	0.776
Eosinophils:			
Percentage	4.01±6.11	4.81±3.38	0.113
Absolute (x10^9/L)	0.25±0.41	0.62±1.17	0.066
Basopils:			
Percentage	1.89±1.07	0.96±0.76	0.007
Absolute (x10^9/L)	0.20±0.42	0.06±0.07	0.12

* Standard deviation (SD)

Table (3): The relative and absolute counts of CD3 and CD19 of 17 DS patients and age matched control group.

Variable	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
CD3:			
Percentage	67.19±11.81	62.11±10.31	0.163
Absolute (x10^9/L)	2.44±3.05	2.16±1.17	0.687
CD19:			
Percentage	13.20±5.74	17.39±7.27	0.136
Absolute (x10^9/L)	0.40±0.31	0.67±0.61	0.076

* Standard deviation (SD)

Table (4): The relative and absolute number of annexin V positive CD3 and CD19 in 17 DS patients and age matched control group.

Annexin V positive cells	DS (Mean ± *SD)	Controls (Mean ± SD)	*p* value
CD3:			
Percentage	32.11±13.57	9.65±8.04	0.001
Absolute (x10^9/L)	0.79±1.19	0.18±0.13	0.001
CD19:			
Percentage	27.85±16.68	13.54±12.36	0.022
Absolute (x10^9/L)	0.10±0.07	0.08±0.11	0.286

* Standard deviation (SD)

DISCUSSION

An impairment of both specific and non-specific defence mechanisms has been documented in patients with DS. Neutrophil chemotaxis, leucocytes opsonization, and phagocytosis as well as leucocytes bactericidal activity were found to be decreased in children with DS **[18]**.

Several studies have focused their attention on the role of the thymus, and have described a variety of structural and anatomic alterations present in DS **[19]**. Although studies of T-cell phenotype and function have frequently resulted in conflicting results, the overall evidence strongly points to a primary and profound impairment of T-cell mediated immunity in DS individuals **[20]** Quantitative studies of peripheral blood T lymphocytes reveal a reduction, often quite small, in the percentage and/or absolute number of T lymphocytes, although normal proportions or numbers of T and B lymphocytes in DS children have also been reported **[2]**.

Other authors found that, there was no early expansion of T and B lymphocytes in the peripheral blood of children with DS in the first

year of life. The T-lymphocyte subpopulation gradually approaches those of normal children over time contradicting the theory of precocious aging and do not explain the observed disturbance in the adaptive immune system in DS **[15]**.

In this study, both the relative and the absolute number of apoptotic (CD3 positive annexin V cells) were significantly higher in DS than that of normal children. This is in spite of the absence of significant difference in their number which may explain the functional impairment of these cells and the defect in adaptive immunity in these patients after the first year of life.

Also, T cells were more severely affected than B cells which may further support the impairment of cellular immunity in these children. The higher percentage of apoptotic CD19 positive B lymphocytes in DS children should not be ignored as it may indicate impairment of humoral immunity as well, although not as severe as that of the cellular immunity.

Although, a direct evidence is lacking for a major intrinsic defect of the B-cell compartment. Some, if not all, of the deficit of the humoral response might be explained by a lack of proper T-cell helper activity **[21]**.

Previous investigators evaluated apoptosis in peripheral blood of patients with DS by different methods such as electron microscopy, (ISNT) and agarose gel electrophoresis of DNA **[13]**. But unlike our study they did not identify the cellular phenotype of apoptotic cells in these patients.

Another unique advantage of the method used in this work is that it was both sensitive and specific for detecting very early cellular apoptotic changes as it was able to discriminate between three different types of cells, early apoptotic (Annexin V positive PI negative), late apoptotic (Annexin V positive PI positive), and necrotic cells (Annexin negative PI positive). Both late apoptotic and necrotic cells were excluded during analysis. None of the methods used before could differentiate between early and late apoptotic changes.

Although ISNT is considered a good method for the detection of early stages of apoptosis, Annexin V staining was found to detect apoptosis at an earlier stage **[15]**.

The most specific assay for the detection of apoptosis is perhaps the oldest, which is the detection of nuclear shape changes in the early stages of apoptosis using microscopy **[22]**. But unlike, the annexin V staining it can not identify the cellular phenotype of apoptotic cells and unlike gel electrophoresis, the method used in this study is rather simple and does not requires the extraction of large number of cells **[22]**.

In conclusion, increased apoptotic CD3 cells may contribute to the impaired cellular immunity in DS children with normal absolute number of these cells. Further studies on apoptotic cellular phenotype in DS children and its correlation with infection episodes in these children are recommended.

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