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## Original article

# A study of annexin-V labeled-lymphocytes apoptosis in pediatric-onset systemic lupus erythematosus in comparison to juvenile rheumatoid arthritis

***Background:*** *In systemic lupus erythematosus (SLE), which is the prototype of autoimmune diseases, the autoimmune process seems to be antigen driven. Apoptosis is responsible for eliminating cells from the immune system that are autoreactive, and defects in apoptosis may contribute to autoimmune diseases such as SLE and juvenile rheumatoid arthritis (JRA).*

***Objective:*** *This work is aimed to study the apoptotic peripheral blood lymphocytes in patients with pediatric- onset SLE, to trace its correlations, if any, with the disease activity and clinical presentation, and to compare the apoptotic process to that in JRA, as an example of another rheumatologic disorder.*

***Methods****: The study was conducted on 32 patients with pediatric- onset SLE; their ages ranged between 5 and 25 years (mean ± SD = 15.5 ± 4.4). In addition to various laboratory investigations needed for diagnosis, assessment of different system involvement as well as disease activity, the percentage of early circulating apoptotic lymphocytes was measured by flowcytometry using Annexin –V. The results were compared to that of 20 age and sex matched clinically healthy children and adolescents as well as 10 JRA patients.*

***Results:*** *The percentage of circulating early apoptotic lymphocytes was significantly higher in SLE patients (mean ± SD = 7.02 ± 7.29 %) and JRA patients (mean ± SD=5.91± 6.00 %) as compared to healthy controls (mean ± SD = 1.89 ± 2.21 %; p=0.0003 and 0.023, respectively). The levels of apoptotic lymphocytes seemed higher in SLE patients than in JRA patients but the difference was statistically insignificant (p=0.58). There was no correlation between the percentage of circulating apoptotic lymphocytes and the disease activity markers (SLEDAI and ESR), different system involvement and the dose or duration of corticosteroids therapy.*

***Conclusion****: The general increase of circulating apoptotic lymphocytes seen in SLE patients may not be specific to SLE and could be seen with other autoimmune diseases. It seems that disturbance in the apoptotic process contributes more to the phenomenon of autoantigenicity rather than the prediction of the disease clinical activity or specific organ involvement.*

**Key words:** SLE, apoptosis, annexin V, autoimmune diseases, JRA, Pediatric.

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## INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibodies directed against self antigens[1]. Its clinical manifestations are extremely variable, and its natural history is unpredictable; untreated SLE is often progressive and leads to death[2]. The prevalence of SLE has been estimated to be between 4 and 250 per 100000 population, being more common in urban than in rural areas [3].

The disease mechanisms of SLE remain unknown. Researches suggest that many factors: genetic, hormonal and environmental contribute to immune dysregulation in lupus[1]. Investigations have recently focused on the normal phenomenon of programmed cell death (apoptosis), which is an important mechanism by which lymphocytes are removed from the site of inflammation [4]. Dysregulation of apoptosis is associated with the pathogenesis of a wide array of diseases: cancer,

neurodegeneration, autoimmunity, heart diseases and others [5]. Increased in vitro apoptosis and altered expression of apoptosis-related molecules have been reported in systemic lupus erythematosus (SLE) [6].It was speculated that autoantigens released from apoptizing cells may contribute to the etiopathogenesis of SLE by both activation of autoreactive lymphocytes and the formation of immune complexes[6].

This work is aimed to study the apoptotic peripheral blood lymphocytes in patients with pediatric- onset SLE, to trace its correlations, if any, with the disease activity and clinical presentation, and to compare the apoptotic process to that in JRA, as an example of another rheumatologic disorder.

## METHODS

The study was conducted on 32 patients with pediatric-onset SLE (onset of the disease before 16 years of age) recruited from those regularly attending the Pediatric Allergy and Immunology Outpatient Clinic, Children's Hospital, Ain Shams University, Cairo. All of them fulfilled at least four of the American College of Rheumatology Classification criteria for SLE[7]. They were twenty nine females (90.7%) and three males (9.3%); their age ranged between 5 and 25 years with a mean of 15.5 ± 4.46 years.

**For comparison of our results, two groups were included:**

-Ten age and sex-matched patients with history of juvenile rheumatoid arthritis, eight females (80%) and two males (20%); their age ranged between 8 and 17 years with a mean of 12.6 ±2.8 years.

- Twenty age and sex-matched clinically healthy subjects; sixteen females (80%) and four males (20%); their age ranged between 8 and 23 years with a mean of 16± 3.8 years.

**Exclusion criteria:**

i- Infection till cured.
ii- Active smoking

**All patients underwent the following:**

1- History taking including:

- Age of onset and duration of the disease.
- Presenting symptoms.
- Different system involvement.
- Therapeutic modalities.

Patients received treatment according to the disease activity. Patients' records of corticosteroids doses (prednisone or its equivalent in milligrams per kilograms body weight) were used to calculate the current dose and the duration of steroids therapy during the whole course of disease, and the duration of the last steroid regimen.

**Data for each patient were collected from:**

- Clinical and laboratory information recorded at the time of the study.
- Medical records of the Pediatric Allergy and Immunology Outpatient Clinic.
- Personal interviews with each patient and parent (s) to obtain informations, e.g., events that were not available from the medical records.

2- Clinical examination:

- System involvements were sought for.
- The disease activity was considered clinically according to SLEDAI [8], and the patients was considered to have low activity of SLE if SLEDAI was ≤ 15 and to have highly active disease if SLEDAI was > 15 [9].

3- The following laboratory investigations were done:

a. CBC using coulter counter (T660, USA).
b. ESR was determined by the Westergren method.
c. Kidney function tests (serum creatinine and BUN) were determined by Synchron CX5 Clinical System (Beckman, USA).
d. Antinuclear antibodies (ANA) by immuno-fluorescent microscopy (using commercially available slides coated with mouse kidney stomach tissue, obtained from Kallestad, Germany) and anti-native DNA antibodies (using commercially available slides coated with crithedia Luciliae substrate, obtained from Kallestad, Germany).
e. Routine urine analysis (macroscopic and microscopic).
f. Flowcytometric detection of apoptotic lymphocytes using fluorecein isothiocyanate-labeled Annexin –V.

*Principle of the assay:*

Annexin V-FITC Apoptosis Detection Kit (Trevigen, Inc. RD Systems, Minneapolis, USA) uses Annexin V-FITC conjugates for flowcytometric detection of cell surface changes that occur early in the apoptotic process. The Annexin V-FITC conjugate facilitates rapid fluorimetric detection of apoptotic cells. Annexin V is an anticoagulant protein that preferentially binds negatively charged phospholipids. Early in the apoptotic process, phospholipids asymmetry is disrupted leading to the exposure of phosphatidylserine (PS) on the outer leaflet of the cytoplasmic membrane. This event is thought to be important for macrophage recognition of cells undergoing apoptosis [10].

**Different systems involvement:**

From the previous information collected as history, data records, examination, and investigations, organ involvement was determined according to the definitions provided by the American College of Rheumatology [11- 13].

**Statistical analysis:**

Data were analyzed with statistical software package v.5 (StatSoft, Tulsa, OK, USA). All numeric data were expressed as mean ± standard deviation (SD). Data were analyzed using student t-test to compare mean values of different variables. Person r correlation coefficient was used to determine the relationship between different quantitative variables. Mann Whitney U test was used to compare non parametric data. For all tests, probability of less than 0.05 was considered significant.

## RESULTS

According to the report of the Second Task Force on Blood Pressure Control in Children (1987) [14], 24 (75%) patients were considered hypertensive and 8 patients were normotensive. Renal involvement was encountered in the course of the disease in 14 (43.75%) of our patients, 11 of whom had active renal disease during inclusion. Neuropsychiatric lupus was diagnosed in 40.6% of patients where manifestations varied between lupus headache, organic brain syndrome, seizures and psychosis. Nine (28%) of the studied patients had evidence of mucocutaneous involvement which included malar or discoid rash, mucosal ulcers and/or alopecia. On the other hand, nine patients were diagnosed as having secondary antiphospholipid antibody syndrome, where thrombotic evidence in the form of hemiparesis, deep venous thrombosis or myocardial infarction was associated with laboratory increased level of $\beta_2$ glycoprotein- I antibody and / or Lupus anticoagulant (Table 1).

Thirty patients have already received steroid therapy for a mean total duration of 25.4±14.05 weeks. The current dosage ranged between 0.1 and 1.8 mg/kg (mean 0.61 $\pm$ 0.379 mg/kg) for a period ranging between 2 and 54 weeks (mean 11.74+13.2 weeks). Four patients had received cytotoxic therapy; three of them received cyclophosphamide (700 mg/ m2 body surface area) and one received azathioprine (50 mg).

The percentage of circulating early apoptotic lymphocytes was significantly higher in SLE patients (mean ± SD = 7.023±7.293 %, median = 4.25%) and in JRA patients (mean ± SD = 5.907±6.0008 %, median =3.24%) compared to healthy controls (mean ± SD = 1.878 ±2.207%, median =1.05%) [z=3.60 and 2.27, p=0.0003 and 0.023 respectively]. However, there was no significant difference in the percentage of apoptotic lymphocytes in SLE patients compared to JRA patients [z=-0.546, p=0.585] (Figure 1). The percentage of circulating early apoptotic lymphocytes was not significantly different in SLE patients with each system involvement (whether CNS, renal, mucocutaneous, antiphospholipid syndrome … etc.), compared to those without affection of that system. For instance, patients with active renal disease were comparable to those without nephropathy in terms of circulating early apoptotic lymphocyte percentage values.

As to the effect of the disease activity on apoptosis, we found that the circulating early apoptotic lymphocytes were not significantly different in SLE patients with SLEDAI >15 compared to those with low activity of the disease i.e. SLEDAI $\leq$15 (z= 0.226, p= 0.82), yet the values of both groups were higher than the healthy controls ( z= 2.98 and 3.108, p= 0.0028 and 0.008, respectively) (Figure 2). Furthermore, the levels of circulating early apoptotic lymphocytes were not significantly correlated to either the laboratory (ESR) or clinical (SLEDAI) markers of disease activity (Table 2).

As to the effect of steroids therapy on the degree of apoptosis, the levels of circulating early apoptotic lymphocytes were not significantly correlated to either the dose or the duration of steroids therapy (Table 3).

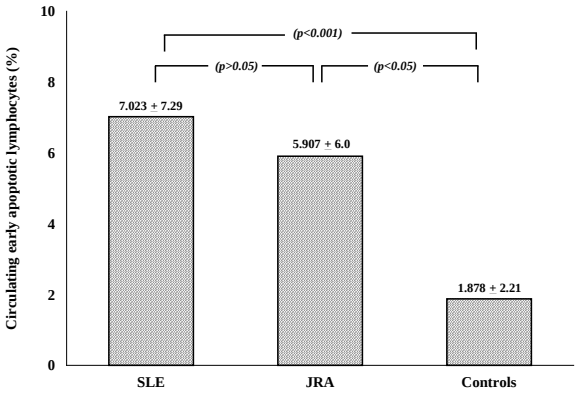


**Figure (1): Percentage values of circulating early apoptotic lymphocytes in the studied sample.**

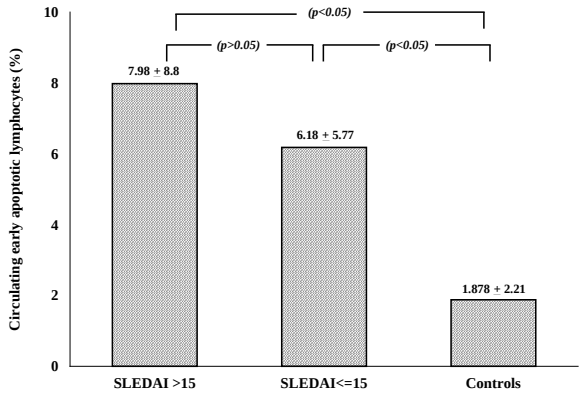


**Figure (2): Comparison between SLE patients with SLEDAI >15 to those with SLEDAI ≤ 15 in the percentage of circulating early apoptotic lymphocytes.**

**Table (1): Descriptive clinical and laboratory data of SLE patients**

| **SLE patients (n= 32)** | |
|---|---|
| • **Blood Pressure values :**<br>- Normotensive<br>- Hypertensive | <br>24 (75%)<br>8 (25%) |
| • **CNS**<br>- Lupus headache<br>- Seizures<br>- Organic brain syndrome<br>- Psychosis | 13 (40%)<br>8 (61.5%)<br>2 (15.4%)<br>4 (30.77%)<br>1 (7.7%) |
| • **Active renal involvement** | 11 (34.38%) |
| • **Arthritis** | 9 (28.13%) |
| • **Mucocutaneous affection:** | 9 (28.13%) |
| • **Cardiac involvement:**<br>- Pericardial effusion<br>- Wall motion abnormality<br>- Thickened valve | 5 (15.63%)<br>1 (20%)<br>2 (40%)<br>2 (40%) |
| • **Pleural involvement** | 4 (12.5%) |
| • **Antiphospholipid antibody syndrome** | 9 (28.13%) |
| • **ESR (mm/hr)**<br>- Mean ± SD<br>- Range | <br>23.3± 22.5<br>5- 96 |
| • **SLEDAI**<br>- Mean ± SD<br>- Range | <br>17.03± 6.9<br>6 - 32 |

**Table (2): Correlation of the percentage of circulating early apoptotic lymphocytes to clinical and laboratory markers of activity in SLE patients**

| **Variable** | **ESR** | **SLEDAI** |
|---|---|---|
| **Apoptotic lymphocytes %** | r = -0.30 (p>0.05) | r = -0.115 (p>0.05) |

p>0.05 insignificant

**Table (3): Correlation of the percentage of circulating early apoptotic lymphocytes to corticosteroid dose and duration.**

| **Variable** | **Current dose of steroids** | **Duration of current treatment** | **Total duration of steroid treatment** |
|---|---|---|---|
| **Apoptotic lymphocytes %** | r = -0.12 (p>0.05) | r = -0.03 (p>0.05) | r = -0.18 (p>0.05) |

p>0.05 insignificant

## DISCUSSION

The pathogenesis of most autoimmune diseases is unclear. Recently, much more attention has been devoted to the role of apoptosis in the pathogenesis of these diseases [15]. The present study measured the levels of circulating early apoptotic lymphocytes in SLE patients compared to patients with JRA and to normal healthy controls. Experimental design with FITC-Annexin-V staining of freshly isolated lymphocytes was used to detect early apoptotic cells in circulation to avoid in vitro artifacts.

Pediatric-onset SLE patients showed a significantly higher levels of circulating early apoptotic lymphocytes compared to normal healthy controls. Our results are in agreement with the study of Pernoik et al.9, and as well with the study of Courtney et al. [16], who found that the percentage of apoptotic lymphocytes was significantly

increased in the peripheral blood of SLE patients compared to normal healthy controls. Similar results were reported by Funauchi et al [17] and Grondal et al. [18], who studied early apoptosis in lymphocytes of SLE patients and found that the levels of apoptotic T lymphocytes were significantly increased compared to the healthy controls.

To explain the relationship between SLE and the dysregulation of apoptosis, Emlen et al [19] and Lorenz et al [15] proposed that under physiologic conditions, cellular constituents are not released from apoptosis and therefore cannot activate immunocompetent cells. However, in theory, an increased rate of apoptosis could lead to an overflow of the phagocytic system with apoptotic cell bodies. Thus, intracellular constituents, such as apoptotic DNA fragments, would be presented to and recognized as non self antigens by immunocompetent cells leading to the formation of autoantibodies against intracellular particles such as ds-DNA.

In agreement with the previous suggestion, are the results of the work of Casciola-Rosen et al [20], who reported that after ultra-violet (UV) irradiation-induced in vitro apoptotic cell death of keratinocytes, most of the known SLE autoantigens are clustered within surface blebs of apoptotic cells. In another study, the same group found that after induction of apoptosis by Sindbis virus infection, viral antigens and autoantigens blebs form antigenic structure of mixed viral and self antigens and could define a novel immune context. Thus, an immune response originally directed against the virus could easily react with apoptotic particles. This could lead to formation of autoantibodies against intracellular constituents and initiation of autoimmune disease[21].

The increased amounts of circulating cells in the early phase of apoptotic cell death of SLE may be due to accelerated rate of apoptosis or defective clearance. It seems more likely that impaired clearance of apoptotic material contributes to the accumulation of apoptotic cells in SLE patients, as suggested by Herrmann et al [22], and Mevorach et al [23]. Defective clearance mechanisms may cause a flood of autoantigenic material from apoptotic bodies, leading to permanent challenge to natural tolerance. Opsonization of apoptotic material by autoantibodies and complement could then lead to antigenic spread of the inflammatory response [9]. On the other hand, Gergely et al [24], in their study on peripheral blood lymphocytes from SLE patients, concluded that T cells are sensitized for apoptosis, which may significantly contribute to the increased spontaneous apoptosis. In contrast to our study results, the study done by Lorenz et al [15], and that by Bijl et al [6], failed to show significant difference in circulating apoptotic lymphocytes in the peripheral blood of SLE patients compared to healthy controls.

This controversy may be explained by the difference in methodology and the selection of the patients Lorenz et al., detected increased in vitro apoptosis, most likely reflecting apoptosis induced by non specific activation as characterized by increased Apo1/fas and Bcl2 expression. Analysis of apoptosis in vitro does not reflect the situation in living tissues as apoptotizing cells are usually cleared effectively in vivo by phagocytes [9]. On the other hand, Bijl et al [6], included in their study clinically quiescent SLE patients (SLEDAI score < 4 for at least 4 months prior to blood sampling), on low dose corticosteroids with no immuno-modulating drugs.

A question needed to be addressed:, if these data were specific for SLE or can it also be seen in cells from patients with other autoimmune diseases. Comparing apoptotic lymphocytic cell counts from patients with SLE to those from patients with JRA, we found no significant difference though both were significantly higher than values of the healthy control group. Similar results were reported by Courtney et al.[16], who found that the percentage of apoptotic lymphocytes and lymphocyte Fas expression in SLE were not significantly different from that of patients with rheumatoid arthritis, and concluded that increased lymphocytes apoptosis may not be specific to SLE.

Furthermore, in their study, Lorenz et al. [15] found that in vitro apoptosis and apoptosis-related molecules in lymophocytes from SLE patients were not significantly different from patients with other autoimmune diseases (e.g., mixed connective tissues disease, polyarteritis nodsa, Takayasu arteritis and Wegener's granulomatosis), and they concluded that the accelerated apoptosis represents a feature that is not specific for SLE, but can also be seen in autoimmune diseases characterized by a different pattern of autoantibodies. However, the same study showed that, the percentage of early apoptotic lymphocytes and apoptosis-related molecules from SLE patients were higher than that of rheumatoid arthritis (RA) patients. They proposed that T-cell migration in RA to the inflamed joints, which is not present in the peripheral vessels, might explain their findings. Their proposal was supported by Emlen et al. [19], and Nishimura- Morita et al. [25]. It is also supported by Nakajima et al.[26], who demonstrated typical apoptotic changes in rheumatoid arthritis synovial

cells in a significantly higher number than control. However, this was not the case in our study, where the levels of circulating apoptotic lymphocytes were not significantly increased in SLE compared to JRA patients.

Concerning the correlation of circulating apoptotic cells with activity markers, whether clinical (SLEDAI) or laboratory (ESR), apoptotic cell counts revealed no significant correlation, and in a subgroup analysis of low active patients (SLEDAI ≤15) compared to highly active SLE patients (SLEDAI >15), there was no significant difference in circulating early apoptotic cells between the two SLE groups. In concordance to our results, Pernoik et al. [9], found no correlation between disease activity as measured by SLAM score, ESR, or ds-DNA antibody titer and apoptosis. Also, Grondal et al. [18], found no correlation between disease activity markers (SLEDAI and ESR) and apoptosis. The present study suggests that the activation-induced cell death may not be the only major factor in the high level of apoptotic cells. The subgroup analysis of low active patients with likewise elevated levels of early apoptotic cells implies a more generally disturbed removal of apoptotic material. By contrast, Emlen et al. [19] found only a weak positive correlation between clinical activity and the level of apoptosis in vitro culture. However, our experimental design of apoptosis detection in freshly isolated lymphocytes might better reflect in vivo conditions as the afore-mentioned study was designed to record apoptosis under in vitro, non-inflammatory conditions and growth factor withdrawal.

Focusing on results concerning correlation of circulating apoptotic cells with corticosteroids therapy, the total received dose of corticosteroids (in mg/kg), the total duration of corticosteroids therapy and the duration of current corticosteroids therapy showed no significant correlation to the level of apoptotic lymphocytes. Similarly, Perniok et al.[9], reported that apoptotic cell count revealed no correlation to corticosteroid doses, and concluded that the increase in apoptosis in SLE is independent on the therapeutic intervention.

From former studies, data indicate that surface blebs of apoptotic cells constitute an important immunogenic particle in SLE. The phosphatidyl-serine expressed on the outside of these blebs can induce the production of antiphospholipid antibodies. It was suggested that this could explain the increased incidence of pathological intravascular coagulation events that occur in some lupus patients who have antiphospholipid antibodies [21,27]. Nevertheless, in our study, we found no significant difference in apoptotic cell count between patients with and without antiphospholipid antibody syndrome.

In conclusion, the general increase of circulating apoptotic lymphocytes seen in SLE patients may not be specific to SLE and could be seen with other autoimmune diseases. It seems that disturbance in the apoptotic process contributes more to the phenomenon of autoantigenicity rather than the prediction of the disease clinical activity or specific organ involvement

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