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Original article

Lymphocyte apoptosis in the pathogenesis of type 1 diabetes mellitus

Background: *Beta cell apoptosis has been associated with insulin dependent diabetes mellitus (IDDM) onset in newly diagnosed diabetic patients. There is an emerging evidence that T cell-induced apoptosis is a dominant effector mechanism in diabetes mellitus type 1 (DM1). Pancreatic β-cells derived from newly diagnosed type 1 diabetics were found to have increased cell surface expression of Fas (CD95) compared to β-cells from healthy subjects.*
Objective: *The study investigates the spontaneous lymphocyte apoptosis via CD95 molecule expression to demonstrate activation induced cell death in children with high risk of DM1 and in type 1 diabetics under insulin therapy.*
Methods: *This study comprised 90 children and adolescents, divided into 3 groups. G(1) comprised 40 type-1 diabetics, their ages ranging from 8.0 to 17.0 years and disease duration between 2.0 and 12.0 years. G(2) (prediabetics) included 30 euglycaemic subjects who were first degree relatives of type 1 diabetics, with normal fasting blood glucose and positive first phase insulin release (FPIR) and/or positive islet cell (ICA) or glutamic acid decarboxylase (GAD) antibodies. G(3) comprised 20 healthy, age and sex matched subjects with no clinical or laboratory signs or family history of type-1DM. Patients were subjected to clinical evaluation with special emphasis on signs suggestive of microvascular complications. The study measurements included random blood sugar (RBS), glycosylated hemoglobin (HbA_{1c}), urinary microalbumin assay and flow cytometric assessment of apoptosis by measuring CD95 percentage expression on CD3 lymphocytes.*
Results: *The percentage of CD95 positive T-lymphocytes was significantly higher in prediabetics than in type-1 diabetics and controls (57.687±6.68, 45.01±6.648,16.75±4.98% respectively; p<0.001). CD3 positive lymphocytes were significantly lower in prediabetics than type-1 diabetics and controls (52.93±11.64, 66.23±7.04, 63.910±3.4% respectively; p<0.001). The percentage of CD95 on T-lymphocytes could not be correlated with age, insulin dose and RBS, but HbA_{1c} was positively correlated with both CD3 lymphocytes and CD95% expression. Complicated type-1 diabetics showed higher CD95% expression compared to non-complicated patients.*
Conclusion: *Peripheral blood lymphocytes with CD95 antigen expression are increased in prediabetics. As CD95 is an important receptor for activation-induced cell death, CD95 mediated apoptosis could play a potential role in the pathogenesis of DM1.*

Keywords: lymphocyte apoptosis; CD95 system; type 1 DM; prediabetes.

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INTRODUCTION

Type 1 diabetes mellitus (DM1) is the effect of T cell dependant autoimmune destruction of insulin producing beta cells in the pancreatic islets. T cells are activated in response to islet dominant autoantigens, the result being the development of type 1 diabetes mellitus.[1]

Apoptosis (a combination of the Greek word apo: off/ and Potosi: falling) is a highly regulated form of cell death defined by distinct morphological and biochemical features.[2] It is a coordinated series of events for the programmed cell death, and plays an important role in the maintenance of tissue homeostasis, embryonic development, and in the control of immune responses in humans.[3]

Fas (Apo-1/ CD95) is a 45-KDa surface receptor belonging to the nerve growth factor superfamily, which on binding by Fas ligand (FasL) induces translocation of phosphatidylserine from the inner to the outer leaflet of the cellular membrane and directly transduces the signal for programmed cell

death (apoptosis).[4] Mature T lymphocytes express Fas (CD95/Apo1) molecules. Their expression can be enhanced upon activation of the T cell by autoantigen or inflammation and these cells become more sensitive to FasL mediated apoptosis[3]. Defective regulation of leukocyte apoptosis may be a factor which contributes to the pathogenic mechanism of autoimmune diseases.[5] It was found that immunological, inflammatory and metabolic signals cause β-cell apoptosis, and that these signals converge toward a common β-cell death signaling pathway.[6]

METHODS

This study comprised 90 children and adolescents, who were divided into three groups.

Group (1): It comprised 40 type-1 diabetic children and adolescents recruited from the regular attendants of the Pediatric Diabetes Clinic, Children's Hospital, Ain Shams University.

Inclusion criteria:

a) Disease duration > 1 year
b) Regular insulin therapy

They were 17 males and 23 females. Their ages ranged from 8.0 to 17.0 years with a mean of 12.96±2.55 years. Their disease duration ranged between 2.0 and 12.0 years with a mean of 4.90±2.55 years. All the patients included were under human insulin therapy in a dose ranging from 1.1-3.0 U/Kg/day with a mean of 1.765±0.667 U/Kg/day. Twenty six were non-complicated and 14 had chronic microvascular complications.

Group (2): It comprised 30 euglycaemic prediabetic children and adolescents.

Inclusion criteria:

a. First degree relatives of type 1diabetic patients.
b. No clinical signs of the disease.
c. Normal fasting blood glucose level.
d. Positive First Phase Insulin Release (FPIR) and/or positive ICA or GAD antibodies.

They were 14 males and 16 females. Their ages ranged from 9.0 to 17.0 years with a mean of 12.90 ±2.48 years.

Group (3): This group comprised 20 healthy age and sex matched children and adolescents who served as a control group.

Inclusion criteria:

a- No clinical or laboratory signs of type-1 diabetes.
b- No family history of diabetes in their first or second degree relatives.

They were 9 males and 11 females. Their ages ranged from 9.0 to 17.0 years with a mean of 13.50±2.48 years.

All patients were subjected to the following:

A. History taking through a structured questionnaire planned to fulfill the following data: Demographic data, disease duration, insulin therapy (type, dose and frequency), history suggestive of acute metabolic complications and history suggestive of chronic diabetic complications. Their files were revised for the presence of hypertension, microalbuminuria and diabetic retinopathy.

B. Physical examination with particular emphasis on:

1- Anthropometric measurements: The weight (Kg) and height (cm) values were plotted against percentiles for age and sex according to Egyptian growth charts. The body mass index was calculated.

2- Assessment of sexual maturity according to the Tanner's classification.

3- Neurological examination for evidence of peripheral neuropathy.

4- Fundus examination using direct ophthalmoscopy to detect diabetic retinopathy.

C. Laboratory investigations:

Fasting blood glucose was measured for all prediabetics and controls to exclude the possibility of being diabetics. The sibs were followed up throughout the study for the development of DM.

a. Random blood sugar (RBS): performed on CX9 system (Beckman Corporation Brea, California, USA).
b. Glycosylated hemoglobin (HbA1c): using quantitative calorimetric determination of glycohemoglobin in whole blood by Teco Diagnostics 1268 N. LAKEVIEW AVE USA–ANAHEIM, CA 92807, 1-800-222-9880. It was measured as a reflection of long term glycemic control over the preceding 12 weeks.
c. Quantitative determination of urinary microalbumin as a predictor of diabetic nephropathy. Microalbuminuria was defined as excretion rate of albumin 30-300 mg/gm urinary creatinine. Calculation of mean random blood sugar, mean HbA_{1c} in the last year prior to the study was done retrospectively from the patients' files.
d. Flow cytometric assessment of CD3 lymphocytes and CD95 percentage expression on peripheral lymphocytes as a measure of apoptosis. Evaluation of the surface expression of CD3 and CD95 T cell subsets on gated lymphocytes was performed by flowcytometry (Coulter electronics EPICS-XL, FT, USA). Two ml of venous blood were drawn from each patient into a tube containing K-EDTA solution. Monoclonal antibodies were used included CD3 [fluorescene

isothiocyanate (FITC) labeled, CD95 (phycoerythrin (PE) labeled] (coulter). Isotypic negative controls FITC, PE, labeled were used to determine the non specific binding (coulter), and Lysing solution (NH4Cl buffered with $KHCO_3$ at PH 7.2).

For each sample, one assay and one control tube were used 50 μL whole blood were delivered in each tube, monoclonal antibodies and isotypic controls were added in the test tube and control tube respectively. The tubes were vortexed and incubated for 15 minute at room temperature in the dark cells were then washed in P.B.S. Stained samples were then treated with lysing solution, then incubated for 37^{o}C and washed again prior to flow cytometric analysis. Analysis of the results was done on flow cytometer (coulter electronics Epics-XL, FT, USA) equipped with 480 nm air-cooled Argon Laser. The data was plotted on 3 histograms. The first histogram was based on forward scatter (FS) versus side scatter (SS) where lymphocytes are gated. The second histogram measures the percentage expression of CD3. The third histogram measures the percentage of CD95 expression as mean florescence intensity or percentage of gated positive cells for antibody used.

e. Glutamic acid decarboxylase autoantibodies: These were measured by radiological based assay (CentAKr anti-GAD65, Medpan Diagnostica, Entwicklungs-undvertriebs GmbH. Germany) for the prediabetics and controls. A cut off values of antibody positivity were determined using mean values of controls + 2SD.
f. Islet-cell autoantibodies (ICA): They were assessed using the indirect immuno-fluorescence (IIF) technique, (Medica/ APICA kit) for the prediabetics and controls.
g. First Phase Insulin Release (FPIR): This was performed for prediabetics who had one positive antibody assay and controls.

A solution of 25% dextrose (0.5 g/kg body weight up to a maximum of 35g) was infused over 3 minutes±15 seconds. Blood samples for determination of glucose and insulin level were drawn at 1, and 3 minutes after the end of glucose infusion. The insulin values at 1 and 3 minutes after the end of glucose infusion were added to determine the first phase insulin release which is termed (FPIR). Enzyme Amplified Sensitivity Immunoassay method was used on microtiter plate MEDGENI INS-ESIA. Several monoclonal antibodies (mabs) directed against specific epitopes of insulin are used, which allow highly sensitive assays and avoid hyperspecificity. Standard or samples containing insulin INS react with capture antibodies (mabs1) coated on a plastic wells and with a second monoclonal antibodies labeled with horseradish peroxidase (HRP) (conjugate) An incubation period is given to allow the formation of a sandwich: coated (mabs1) - INS- (mabs2) in HRP. Washing step was done to remove unbound enzyme labeled antibodies. A substrate was added tetramethylbenzydine (TMB H2O2) was added then followed by a second incubation period. Optical density (OD) of the studied samples and standards were read using the specific wave length. A standard curve was plotted using standards concentrations and OD readings. Insulin concentrations in samples were determined by interpolation from the curve. According to Chase et al.[7] FPIR was classified with modification into low risk group (>80-100mu/L), intermediate risk group (65-80mu/L) and high risk group (<65-48mu/L) less than 5th percentile and (<48mu/L) less than first percentile.

Statistical analysis:

Standard computer program SPSS for Windows, release 10.0 (SPSS Inc, USA) was used for data entry and analysis. All numeric variables were expressed as mean ± standard deviation (SD). Comparison of different variables in various groups was done using Mann Whitney test for nonparametric variables. Chi-square (χ^2) test was used to compare frequency of qualitative variables among the different groups. Spearman's correlation test was used for correlating the non-parametric variables. For all tests a probability (p) less than 0.05 was considered significant.

RESULTS

Table (1) summarizes the demographic and clinical data of the studied sample. The studied groups were comparable in terms of sex and age distribution although a higher percentage of positive consanguinity and positive family history of type-1 DM were found in the diabetics and prediabetics compared to controls (P <0.05). Non-significant differences in weight, height and BMI were found between the studied groups. Although the mean weight and height percentiles were lower in type-1 diabetic patients, the difference did not reach a statistical significance. No significant difference could be elicited between the studied groups in puberty staging. Higher mean heart rate, systolic and diastolic blood pressure values were found in diabetics when compared to prediabetics and controls although statistically non significant.

Table (2) shows the metabolic parameters and complications of the studied diabetic patients. Forty diabetic patients were included with a mean disease duration of 4.90±2.55 years. Their mean RBS was 248.38±39.4 mg/dL, mean HbA_{1c} was 8.58±1.18% and their mean insulin dose was 1.76±0.66 u/kg/day. The frequencies of occurrence of chronic microvascular complications were as follows; 15% had nephropathy, 12% had neuropathy and 7.5% had diabetic retinopathy. Regarding the acute diabetic complications, 5% had history of DKA, 13% experienced attacks of minor hypoglycemia and 7% had major hypoglycemia in the last year prior to the study.

When we compared complicated and non complicated diabetic patients in terms of the metabolic and laboratory parameters; we found highly significant increase in disease duration, mean RBS and mean insulin dose in complicated compared to non-complicated cases (P <0.001). Also, a significant increase in the mean HbA1c was detected in complicated compared to non-complicated diabetics (p<0.05). Both complicated and non-complicated type-1 diabetic patients were comparable regarding their percentage of CD3 lymphocytes but a significant increase in CD 95% expression were found in complicated diabetics (Table 3).

Thirty sibs of patients with type I DM were included in this study. They were derived from 38 families. Eighteen (60%) of them (prediabetics) were positive for GAD65 antibodies, 26.7% were positive for ICA antibodies and 13.3% were positive for both antibodies (ICA, GAD) (table 4). The cut off values for antibodies positivity were determined using the mean value of the controls + 2 SD. Comparison between mean level of GAD65 Abs among prediabetics and controls revealed a higher level of GAD Abs in prediabetics (2.15±3.83 U/ml) compared to controls (0.497±0.526 U/ml, p<0.05). Prediabetics with positivity for one antibody (either ICA or GAD) were subjected to FPIR. There was a highly significant decrease in the mean level of FPIR in prediabetics compared to controls (table 5). Grading of decreased levels of FPIR among the studied prediabetics was presented in table (6).

The percentage of CD3 lymphocytes was significantly decreased in prediabetics compared to type-1 diabetics and controls (52.9±11.6, 66.2±7.04, 63.9±3.4%; P <0.001). Also, there was a highly significant increase in CD95 molecule percentage expression in peripheral blood T and B lymphocytes in prediabetics as compared to the type-1 diabetics and controls (57.68±6.6, 45.0±6.6, 16.7±4.9% respectively; P < 0.001), figure (1).

Table 1. Demographic and clinical data of the studied groups (one-way ANOVA test)

Variable	Diabetics (n=40)	Prediabetics (n=30)	Control subjects (n=20)	p-value
Age (years) Range Mean±SD	 8-17 12.96±2.55	 9-17 12.9±2.48	 9-17 13.5±2.48	0.667
Positive consanguinity n (%)	24 (65)	20 (66.7)	4 (20)	< 0.05
Family history of type I DM n (%)	22 (55)	30 (100)	0.0 (0)	< 0.05
Sex M/F	17/23	14/16	9/11	> 0.05
Weight percentile (mean ± SD)	37.12±28.9	39.44±29.78	39.63±36.77	> 0.05
Height percentile (mean ± SD)	25.11±27.28	27.75±24.56	27.15±56	> 0.05
BMI percentile (mean ± SD)	40.36±28.15	42.59±41.35	42.31±73.61	> 0.05
Puberty o Normal o Delayed o Prepubertal	 36 3 1	 26 2 2	 18 1 1	> 0.05
Basal heart Rate (b/m) (mean ± SD)	86.4±13.3	86.3±9.5	82±71	> 0.05
Systolic blood pressure (mean ± SD)	106±13.2	102±11.8	97.3±8.85	> 0.05
Diastolic blood pressure (mean ± SD)	72±1008	67±12.2	64.6±12.2	> 0.05

M: Male; F: Female; BMI: Body mass index; SD: Standard deviation; b/m: beat/minute

Table 2. Metabolic parameters, chronic microvascular and acute complications of the studied type 1 diabetes patients

Variable	Type 1 diabetic patients (n = 40)
Disease duration (yr)	
Range	2-12
(mean ± SD)	4.90±2.55
Mean RBS (mg/dl)	
Range	203-375
(mean ± SD)	248.38±39.42
Mean insulin dose (U/K/d)	
Range	1.1-3.0
(mean ± SD)	1.76±0.66
Mean HbA1c (%)	
Range	6.9-11.2
(mean ± SD)	8.58±1.182
Chronic microvascular complications	
Nephropathy n (%)	6 (15)
Neuropathy n (%)	5 (12)
Retinopathy n (%)	3 (7.5)
Acute complications	
DKA n (%)	2 (5)
Hypoglycemia	
minor n (%)	5 (13)
major n (%)	3 (7)

RBS: Random blood sugar; HbA_{1c}: haemoglobin A_{1c}; SD: Standard deviation; DKA: Diabetic Ketoacidosis; n: Number.

Table 3. Variations of disease duration, metabolic control and lymphocyte percentage among complicated and non-complicated cases of type-1 diabetes mellitus

Variable	*Complicated* n=14 (mean ± SD)	*Non-complicated* n=26 (mean ± SD)	p-value
Disease duration (years)	7.97±2.72	3.35±1.35	0.000
Mean RBS (mg/dl)	357.64±75.63	248.38±39.42	0.000
Mean insulin dose (U/kg/day)	2.407±.612	1.419±.378	0.000
Mean HbA1c (%)	9.971±.841	7.976±.825	0.018
CD3 (%)	68.143±6.140*	65.212± 7.396*	0.138
CD95 (%)**	44.635±6.477	39.635± 6.477	0.05

Mann- Whitney test was used in the analysis
HbA_{1c}: haemoglobin A_{1c} ; RBS: Random blood sugar; SD: Standard deviation
* Data presented as percent of labeled cells; ** CD95 antigen expression on peripheral blood lymphocytes

Table 4. Distribution of GAD65 antibodies and ICA positivity among prediabetics

High risk group n=30	No	%
-GAD65- Ab positive.	18	60
-ICA positive.	8	26.7
-Both (GAD-Ab+ICA) positive.	4	13.3

GAD: Glutamic acid Decarboxylase; ICA: Islet cell antibody; Ab: Antibody

Table 5. Mean levels of FPIR in prediabetics and controls

FPIR (mU/L)	**Prediabetics n=26***	**Control subjects n=20**
Range	38.9-270	255-313
Mean±SD	130.67±99.5	284.76±21.76
t	8.528	P < 0.01

FPIR: First phase insulin release. P <0.05 significant; P <0.01 highly significant
*Prediabetics who were positive for one antibody

Table 6. Grading of decreased levels of FPIR among the studied prediabetic patients (n=26)

Grading of decreased levels of FPIR	Number	Frequency
I. Low risk group		
1. Preserved FPIR (>100 mU/L)	12	46.2%
2. Non preserved FPIR (>80 – 100 mU/L)	-	-
II. Intermediate risk group		
3. Borderline FPIR (>65 - <80 mU/L)	-	-
III. High risk group		
4. Less than 5th percentile (<65 – 48 mU/L)	5	19.2%
5. Less than 1st percentile (< 48 mU/L)	9	34.6%
Total	26	100%

FPIR: First phase insulin release.

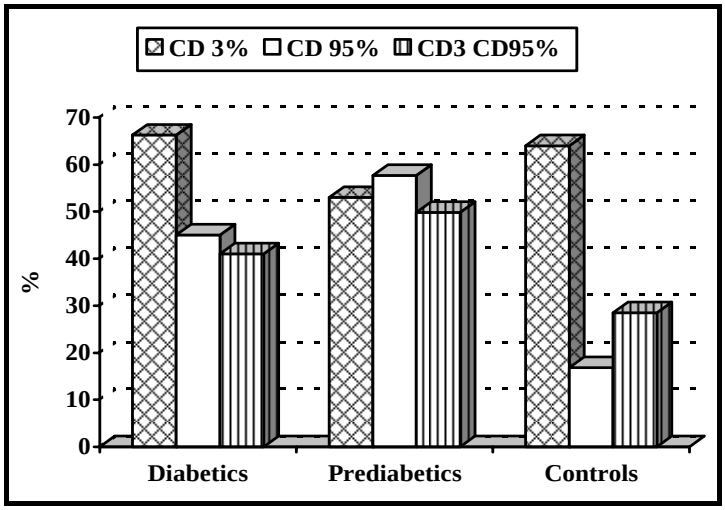


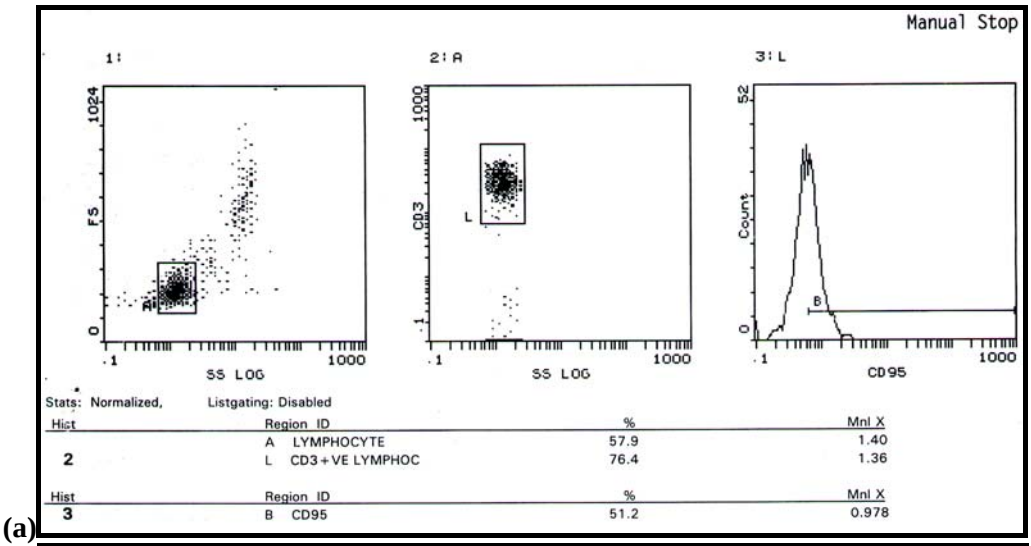
Figure 1. Comparison between the studied groups in terms of their mean percentage of CD3 lymphocytes and CD95 antigen percentage expression on peripheral lymphocytes. (p<0.001).

Table 7. Correlation between CD3 lymphocytes and CD95 antigen percentage expression and some other studied variables

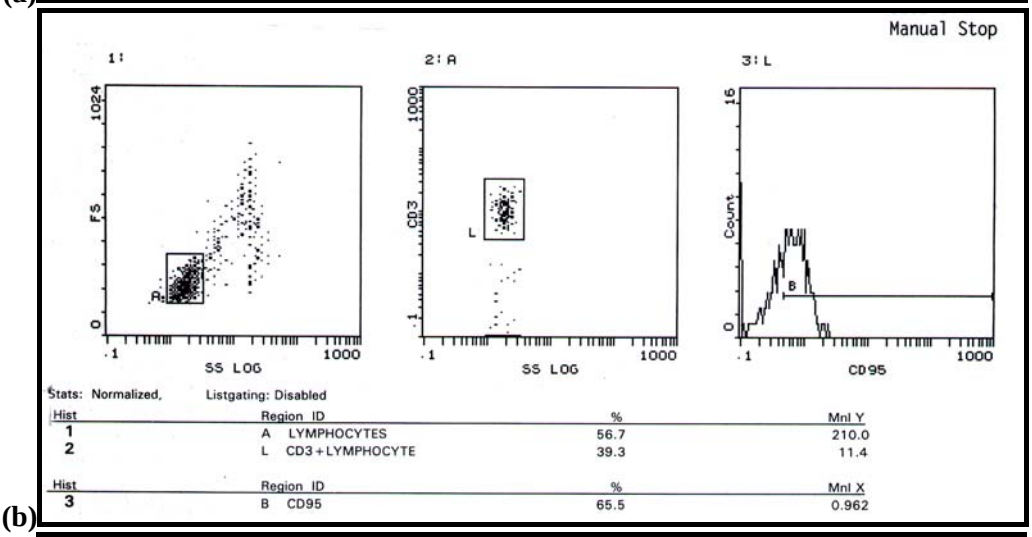
The percentage of CD 3 lymphocytes	**r**	**p**
Age (years)	0.166	0.686 (NS)
Disease duration (yrs)	0.010	0.952 (NS)
Random blood sugar (mg/dL)	0.037	0.819 (NS)
Mean insulin dose (U/kg/day)	0.034	0.835 (NS)
HbA1c (%)	0.669	0.015 (S)
The percentage of CD 95 molecule expression	**r**	**p**
Age in (yrs)	0.112	0.493 (NS)
Disease duration (yrs)	-0.003	0.987 (NS)
Random blood sugar (mg/dL)	0.097	0.550 (NS)
Mean insulin dose (U/kg/day)	0.071	0.663 (NS)
HbA1c (%)	0.639	0.04 (S)

NS: Non significant; S: Significant; P<0.05 Significant

A significant positive correlation was found between the percentage of CD3 lymphocytes and HbA1c (p<0.05). Attempts to correlate it with age, RBS, insulin dose and disease duration did not reach a statistical significance (P>0.05). The CD95% expression on peripheral lymphocytes could not be correlated to age, disease duration, RBS, insulin dose (P>0.05). HbA1c was positively correlated with CD95% molecule expression on peripheral blood lymphocytes (P<0.05) table (7).



(a)



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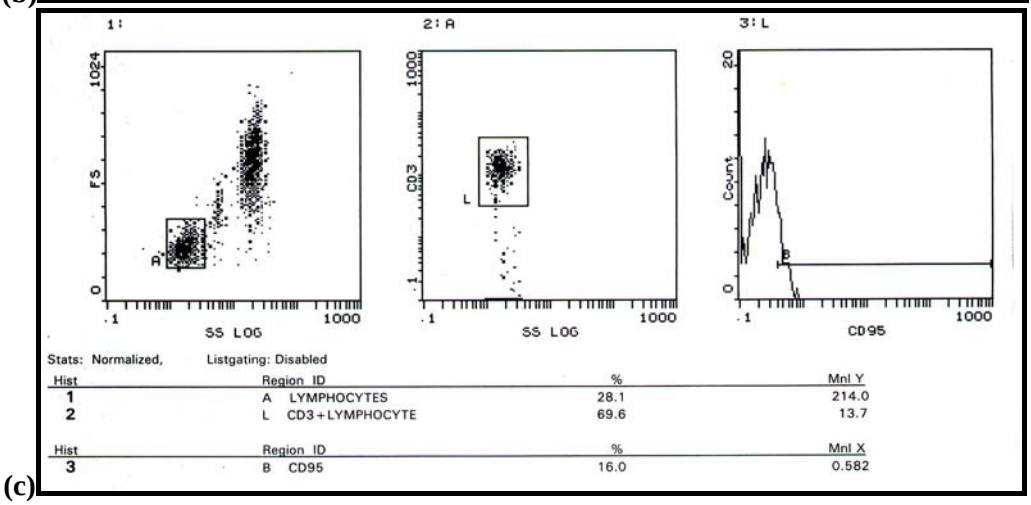

(c)

Figure 2. Flow cytometric assessment of CD_3 lymphocytes and analysis of CD95 antigen expression on lymphocytes from peripheral blood of one diabetic patient (a), one prediabetic subject (subject with high risk of DM1) (b) and in one of the controls (c). There was considerable increase in CD95 molecule expression on lymphocytes of prediabetics.

DISCUSSION

Much attention has been paid to Fas/Fas L-mediated cell death and to the possibility that the triggering of death receptors on β cells might be the conduit for β cell destruction[8,9]. A high proportion of apoptotic lymphocytes in diabetic states may explain the impaired immune function in poorly controlled diabetic patients.[10,11,12]

The present data demonstrate significant increase in the positive consanguinity and family history for DM1 in the studied diabetic and prediabetic groups compared to the control group. These results are in agreement with Lambert et al.[13] who reported that human type I diabetes requires a genetic background of susceptibility based on inheritance patterns and family studies.

There are different approaches for identification of individuals at risk for development of type-1 diabetes, during the asymptomatic preclinical period which may last for years during which progressive beta cell destruction occur. These approaches are based on the presence of positive family history of type-1 diabetes, genetic, autoimmune or metabolic markers. These alternatives may also be combined in various ways to improve the predictive characteristics of the screening strategy.[11,14]

Autoantibodies to various beta cell antigens have proved to be an early marker of ongoing-β-cell destruction and were used to assess the risk of future manifestations of clinical disease in first degree relatives of patients with type-1 diabetes[12,15]. Among first-degree relatives of patients with type-1 diabetes, the risk for clinical disease can be graded from <5% in those with one or no antibodies to >90% in individuals who carry the risk of genotype and are positive for multiple auto-antibodies[6,16]. In the present study 60% of the first degree relatives (prediabetics) were positive for GAD 65 antibodies, 26.7% were positive for ICA antibodies and 13.3% were positive for both. Studies in the first-degree relatives of patients with type-1 diabetes have shown conclusively that autoantibodies to islet antigens precede the onset and can be used to predict clinical disease**.**[17]

Our study demonstrated no significant difference between the studied groups in terms of weight, height and BMI percentiles although short stature and under weight were more frequently observed in type-1 diabetics compared to prediabetics and controls**.** This might reflect the notion that controlled diabetes does not significantly affect growth[17].

We studied 40 type-1 diabetics; 14 were complicated (35%) and 26 were non- complicated (65%). A highly significant increase was found in disease duration and mean random blood sugar in complicated compared to non-complicated diabetic patients (p value <0.001). The DCCT[18] revealed that diabetes duration is clearly involved in the causation of microvascular complications and has shown that the more time individuals are exposed to chronically elevated plasma glucose levels, the greater their risk of developing diabetic microvascular complications. In the same way, ADA[19] reported that postprandial hyperglycemia is a direct and independent risk factor for cardiovascular disease (CVD).

The present study demonstrated that the percentage of CD3 lymphocytes was significantly lower in patients with a high risk of DM1 (prediabetics) compared to both type 1 diabetics and controls (p <0.001). This is in agreement with Tchórzewski et al.[1] who reported that, the percentage of CD3 lymphocytes was decreased in children with a high risk for type-1 diabetes compared to type-1diabetics and controls; and they explained this finding by the decreased percentage of CD3 in peripheral blood of patients with a high risk for DM1 (prediabetics) which reflects the involvement of T-cells in the local immune reaction. Also, it was reported by Barbeau et al.[20] that apoptosis is important in removing autoreactive T-cells and thus prevents the occurrence of autoimmune disease in prediabetics because if these autoreactive T-cells are not efficiently eliminated, the progress to autoimmune type-1 DM is enhanced due to destruction of pancreatic islets by autoreactive T-cells.

In the present study, a significant increase in CD95 percentage expression was found in children with high risk for type-1 diabetes (prediabetics) in comparison to type-1 diabetics and controls (p<0.001). In concordance, Tchórzewski et al.[1] found that there was higher CD95 percentage expression in 12 prediabetics and in only 2 diabetics although the percentage expression of CD95 in controls was below the sensitivity of the assay. They also found that, the CD95 molecule expression was increased in all populations of peripheral blood T and B lymphocytes in prediabetics when measured immediately after blood collection. Expression of both the percentage of CD95 molecule labeled cells and CD95 mean fluorescence intensity was elevated.

Mauricio and Mandrup-Poulsen[21] also reported that a Fas (CD95) is a potential mechanism of pancreatic beta cell death in DM1. In the same way, Chowdhry et al.[22] reported that emerging evidence has begun to unify the genetic susceptibility and

genes in the apoptosis signaling machine, as increasing numbers of apoptosis regulatory genes have recently been linked to the pathogenesis of diabetes. Sharma et al.[23] reported that CD95 receptor expression on resting lymphocytes obtained from prediabetics was increased in comparison to healthy controls and patients with DM1 and they found that insulin lead to decreased CD95 receptor expression on lymphocytes obtained from prediabetics. They concluded that failure of autoreactive T-cells apoptosis is responsible for the early processes of diabetogenesis and disease progression. It was reported that the specific susceptibility of T lymphocytes from children with high risk for DM1 to insulin induced inflammatory cytokine production and anti-CD95 dependent apoptosis supports the clinical observation that small doses of insulin may inhibit disease progression in these individuals.[8]

The CD95 molecule is responsible for increased apoptosis of T lymphocytes after exposure to anti-Fas antibody. This hypothetical mechanism is responsible for selective elimination of specific autoreactive T-cells but it was not confirmed in large clinical trials.[24] Moreover, soluble Fas ligand is not detected in the sera of healthy individuals, but it was more frequent in sera of prediabetics.[25] On the other hand, Green[2] reported a reduced expression of the apoptosis-inducing CD95 receptor on T and B lymphocytes of individuals with clinical and preclinical type-1 diabetes, and this defective expression may impair the capacity of autoreactive lymphocytes to undergo CD95-mediated apoptosis.

The current study revealed a significant increase in CD95 percentage expression in DM1 in comparison to controls ($p < 0.000$). Kohler et al.[4] reported that immunological, inflammatory, and metabolic signals leading to β-cell apoptosis were increased in diabetic patients, and they proposed that these signals converge toward a common β-cell death signaling pathway.

The increased generation of free radicals in the hyperglycemic state may lead to the production of advanced glycation end products and the peroxidation reaction in lipids and protein. Moreover DNA is also vulnerable to the action of free radicals. Thus, chronic hyperglycemia at the onset of diabetes may be associated with increased genotoxicity and apoptosis, thus has an impact on DNA repair machinery[26,27].

The expression of dominant-negative Fas(CD95) or neutralizing antibodies to Fas (CD95)L significantly blocks apoptosis, maintain adequate beta cell function, blocks transfer of diabetes by primed T cells, and retards the course of diabetes development.[28] This is also supported by Su et al.[11] who found that; apoptosis is highest in recent-onset type-1 diabetic subjects followed by high risk subjects. It was found that apoptosis reaching the highest level in subjects at the onset of the disease staying high during the period immediately following diagnosis, including the period in which the requirements for exogenous insulin drop dramatically and beta cell function improves "honey moon phase". Later on, when the autoimmune destruction is complete and insulin requirements increase, T-cell apoptosis goes back to the level detected in healthy control subjects[29,30]. The interaction of Fas(CD95/Apo-1) with its ligand promotes the deletion of potentially harmful, damaged, or unnecessary cells during the immune response. This interaction also regulates tissue remodeling and homeostasis. Impaired Fas-induced apoptosis results in abnormal cell proliferation and accumulation, whereas inappropriate expression or excessive Fas activity causes tissue damage and Fas L system among the most important cell death receptors comprising the tumor necrosis factor receptor super family, CD95/APO-1(Fas)[31,32].

Our data showed some increase in CD95% expression with age but this relation did not reach statistical significance ($p > 0.05$). This is supported by the results of Schindowski et al.[33] who revealed that higher susceptibility to apoptosis with aging could be due to an enhanced production and unsatisfactory elimination of reactive species leading to enhanced apoptosis.

In the present study, the CD95 expression decreased with the increase in disease duration. Glisic-Milosavljevic[34] found that higher level of apoptosis was in recent onset DM1 patients and apoptosis was similar among both control and long standing cases. Also, Allison et al.[28] revealed that apoptotic beta cell death was detected in the islets of female none obese diabetic (NOD) mice from the age of 3 weeks, and the highest level of beta cell apoptosis was observed at week 15, which coincidence with the earliest onset of diabetes.

We could not elicit a correlation between CD95 expression and insulin dose ($p > 0.05$). Otton et al.[10] reported that insulin therapy reduces the occurrence of apoptosis in lymphocytes from diabetic rats as compared with untreated cells. This is opposite to the data of Glisic-Milosavljevic et al.[34] that revealed that insulin plays only a minor role in the outcome of apoptosis, and the levels of apoptosis are not changed greatly by administration of exogenous insulin.

Our study showed a significant correlation between CD95 expression and HbA1c ($p<0.05$).

Many studies considered involvement of apoptosis in diabetic complications especially nephropathy and showed that complicated diabetics usually have higher HbA1c reflecting the bad metabolic control.[36] On the other hand, Tchórzewski et al.[1] reported that there was no correlation between glycated hemoglobin level and CD95 percentage expression.

In the present study there was higher CD95 expression in complicated compared to non complicated cases of DM1. Our results were in agreement with Baba et al.[35] who reported that apoptosis is involved in the advancement of diabetic nephropathy, and that CD95 expression might be a predicting factor for its prognosis.

In conclusion; the decreased percentage of CD3 lymphocytes in the peripheral blood of patients with high risk of DM1 is suggestive of the involvement of T cells in the local immune reactions. Increased peripheral blood T lymphocyte percentage with CD95 antigen expression was observed in children at high risk of DM1 (prediabetes) and this event may be critical for the early process of diabetogenesis and the mechanism responsible for disease progression. Susceptibility to apoptosis may be suggested as potential element for disease activity assessment. It is recommended to estimate CD95% expression on lymphocyte in peripheral blood as an additional biomarker of autoimmunity in the identification and monitoring of DM1 in high risk groups. CD95% could have a diagnostic role and may pave the way for the possible development of antiapoptotic therapy for prevention of DM1.

REFERENCES

1. **Tchórzewski H, Glowacka E, Banasik M, Lewkowicz P, Szalapska-Zanodmiak M.** Activated T lymphocytes from patients with high risk of type I diabetes mellitus have different ability to produce interferon-γ, interleukin-6 and interleukin-10 and undergo anti-CD95 induced apoptosis after insulin stimulation. Immunnol Lett 2001; 75: 225-34.
2. **Green, DR.** Apoptotic pathways: Ten minutes to dead. Cell 2005; 121: 671-4.
3. **Petrovsky N, Silva D, Socha L, Slattery R, Charlton B.** The role of Fas ligand in beta cell destruction in autoimmune diabetes of NOD mice. Ann NY Acad Sci 2002; 958: 204-8.
4. **Kohler M, Zaitsev SV, Zaitseva II, Leibiger B, Leibiger IB, Turunen M, et al.** ON line monitoring of apoptosis in insulin secreting cells. Diabetes; 2003; 52 (12): 2943-50.
5. **Sanlioglu AD, Griffith TS, Omer A, Dirice E, Sari R, Altunbas HA, et al.** Molecular mechanisms of death ligand mediated immune modulation: A gene therapy model to prolong islet survival in type 1 diabetes. J Cell Biochem 2008; 2 (4): 145-56.
6. **Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL.** Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005; 54 (Suppl 2): S97-107.
7. **Chase HP, Cuthbertson DD, Dolan LM, Kaufman F, krischer JP, Schatz PA, et al.** First phase insulin release during the intravenous glucose tolerance test is a risk factor for type I diabetes. J Pediatr 2001; 138 (2): 244-9.
8. **Lee SC, Pervaiz S.** Apoptosis in the pathophysiology of diabetes mellitus. Int J Biochem Cell Biol 2007; 39 (3): 497-504.
9. **Chen M, Wang J.** Initiator caspases in apoptosis signaling pathways. Apoptosis 2002; 7 (4): 313-9.
10. **Otton R, Soriano FG, Verleengia R, Curi R.** Diabetes induces lymphocyte apoptosis. J Endocrinol 2004; 182 (1):145-56.
11. **Su Xm, Hu O, Kristan JM, Costa C, Shen Y, Gero D, et al.** Significant role for Fas in the pathogenesis of autoimmune diabetes. J Immunol 2000; 164 (5): 2523-32.
12. **Kuzuya T, Shawkatova I, Fazekasova H, Michalkova D, Martinka F.** Association of type 1 DM with HLA alleles. Diabetes Care 2002; 20: 219-20.
13. **Lambert AP, Gillespie KM, Thomson G, Cordell JH, Todd JA, Gale EA, et al.** Absolute risk of childhood onset type 1 diabetes defined by human leukocyte antigen class II genotype: a population based study in the United Kingdom. J Clin Endocrinol Metab 2004; 89 (8): 4037-43.
14. European Nicotinamide Diabetes Intervention Trial Group. Intervening before the onset of type 1 diabetes. Baseline data from the ENDIT. Diabetologia 2003; 46 (3): 339-46.
15. **Ziegler AG.** Stratification of type I diabetes risk on the basis of islet autoantibody characteristics. Diabetes 2004, 53 (2): 384-92.
16. **Couper J, Donaghue K.** International Society for pediatric and adolescent diabetes Phases of diabetes. Pediatr Diabetes 2007; 8 (1): 44-7.
17. **Keskinen P, Korhonen S, Kupila A, Veijola R, Erkkila S, Savolainen H, et al.** First phase insulin response in young healthy children at genetic and immunological risk for type I diabetes. Diabetologia 2002; 45 (12): 1639-48.
18. DCCT (The Writing Team for the Diabetes Control and Complications Trial). Epidemiology of Diabetes Interventions and Complications Research Group Effect of Intensive therapy on the microvascular complications of type 1 diabetes mellitus. JAMA 2002; 45 (12): 287: 2563-9.
19. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 2005; 28: S37–S42.

20. **Barbeau WE, BAssaganya-Riera J, Hontecilas R.** Putting the pieces of the puzzle together-a series of hypotheses on the etiology and pathogenesis of type 1 diabetes. Med Hypotheses 2006; 68 (3): 607-19.
21. **Mauricio D, Mandrup-Poulsen T.** Apoptosis and the pathogenesis of IDDM: a question of life and death. Diabetes 1998; 47 (10): 1537-43.
22. **Chowdhry MF, Vohra HA, Galinanes M.** Diabetes increase apoptosis and necrosis in both ischemic and nonischemic human myocardium: role of caspases and poly-adenosine diphosphate ribose polymerase. J Thorac Cardiovasc Surg 2007; 134 (1): 124-31.
23. **Sharma K, Wang RX, Zhang LY, Yin DL, Luo XY, Solomon JC, et al.** Death the Fas way: regulation and pathophysiology of CD95 and its ligand. Pharmacol Ther 2000; 88 (3): 33-47.
24. **Darwiche R, Chong MM, Santamaria P, Thomas HE, Kay TW.** Fas is detectable on beta cells in accelerated, but not spontaneous, diabetes in nonobese diabetic mice. J Immunol 2003; 170 (12): 6292-7.
25. **Hayashi T, Faustman DL.** Role of defective apoptosis in type 1 diabetes and other autoimmune diseases. Recent Prog Horm Res 2003; 58: 131-53.
26. **Michalkova D, Mikulecky M, Tibenska E.** Alterations in lymphocyte subpopulations I peripheral blood at manifestation of type 1 diabetes mellitus in childhood. Bratisl-Lek-Listy 2000; 101 (7): 365-70.
27. **Hiromine Y, Ikegami H, Fujisawa T, Nojima K, Kawabata Y, Noso S, et al.** Trinucleotide repeats of programmed cell death-1 gene are associated with susceptibility to type 1 diabetes mellitus. Metabolism 2007; 56 (7): 905-9.
28. **Allison J, Thomas HE, Catterall T, Kay TW, Strasser A.** Transgenic expression of dominant negative Fas-associated death domain protein I beta cells protects against Fas ligand induced apoptosis and reduces spontaneous J Immunol 2005; 1; 175 (1): 293-301.
29. **Kadziela K, Kowalska H, Rymkiwicz-Kluczynska B, Kowalska M, Miszkurka G, Rybczynska J.** Changes in lymphocyte subsets in children with newly diagnosed type 1 diabetes mellitus. J Pediatr-Endocrinol Metab 2003; 16 (2): 185-91.
30. **Kim S, Kim KA, Hwang DY, Lee TH, Kayagaki N, Yagita H, et al.** Inhibition of autoimmune diabetes by Fas ligand: The paradox is solved. J Immunol 2000; 164 (6): 2931-6.
31. **Trudeau JD, Dutz JP, Arany E, Hill DJ, Fieldus WE, Finegood DT.** Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes 2000; 49 (1): 1-7.Review.
32. **Wilkin TJ.** The accelerator hypothesis: a unifying explanation for type -1 and type -2 diabetes. Nestle Nutr Workshop Ser Clin Perform Programme 2006; 11: 139-50.
33. **Schindowski K, Leutner S, Muller WE, Eckert A.** Age related changes of apoptotic cell death in human lymphocytes. Neurobiol Aging 2000; 21 (5): 661-70.
34. **Glisic-Milosavljevic S, Waukau J, Jailwala P, Jana S, Khoo HJ, Albertz H, et al.** At risk and recent onset type 1 diabetic subjects have increased apoptosis in the CD4+CD25+ T-cell fraction. PLoS ONE 2007; 2 (1): e146.
35. **Baba K, Minatoguchi S, Sano H, Kagawa T, Murata I, Takemura G, et al.** Involvement of apoptosis in patients with diabetic nephropathy: A study on plasma soluble Fas levels and pathological findings. Nephrology (Carlton) 2004; 9 (2): 94-9.
36. **Protopsaltis J, Kokkoris S, Nikolopoulos G, Spyropoulou P, Katsaros T, Salvanos L, et al.** Correlation between increased serum sFas levels and microalbuminuria in type 1 diabetic patients. Med Princ Pract 2007; 16 (3): 222-5.