Journal of
Medical Sciences

Research Paper

Implications of TNF-α, sFas and Apoptosis in Insulin Dependent Diabetes Mellitus

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The objective of this research is to study the apoptosis of lymphocytes, sFas, and mRNA expression and serum level of TNF-α in children with Insulin dependent diabetes mellitus (IDDM) in order to explain an aspect of disease development and pancreatic β-cell death. The study included 71 children suffering from IDDM and 60 healthy children enrolled in the following groups; G1: included 28 children with recent onset of the disease, divided into: G1a included 8 with ketoacidosis, and G1b included 20 with nonketotic hyperglycemia; G2: included 43 undertreatment for more than 6 months, divided into: G2a included 7 with controlled status and G2b included 36 uncontrolled; G3: 60 normal children as control. For all the subjects the following have been done: detailed history, clinical examination and diagnostic investigations; detection of apoptotic cells by giemsa, acridine orange, propidium iodide and DNA fragmentation; measurements of sFas; mRNA of TNF-α; and serum levels of TNF-α. The results showed that: sFas is decreased in patients with recent onset only. No significant difference between neither G1a and G1b nor between G2a and G2b. Low percentage of lymphocyte apoptotic cells detected by the three stains only in cases with recent onset; DNA fragmentation confirmed the apoptosis; mRNA and serum levels of TNF-α showed parallel results of increased levels in cases with recent onset. No significant difference neither between G1a vs G1b nor between G2a and G2b; positive correlation's between sFas and apoptosis and between the results of apoptosis by the three stains; and negative correlation between sFas and apoptosis on one side and serum level of TNF-α on the other side. In conclusion, the inhibition of apoptosis result in accumulation and migration of immune reactive cells to infiltrate the islet cells of the pancreas. This together with the increased level of TNF-α may lead to the process of insulitis, and β-cells death by either apoptosis or necrosis.

Key words: TNF-α, sFas, apoptosis, insulin dependent diabetes mellitus

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Introduction

In type 1 diabetes, an immune mediated process leads to the destruction of pancreatic B-cells. In the last decade, considerable progress has been made in understanding the cellular and biochemical pathogenic process of the disease. However, more needs to be known about the immune mechanisms leading to B-cell death (Mauricio and Mandrup- Poulsen, 1998).

Peripheral blood mononuclear cells from people with or at increased risk for type 1 diabetes showed increased reactivity to β cell autoantigens, such as insulin secretory granule proteins and glutamic acid decarboxylase (Klas *et al.*, 1993). Mononuclear cell infiltration into the pancreatic islets (insulitis) have been recognized together with reduction of β cells as pathological features of recent onset type 1 diabetes. The infiltrate found to be composed of CD8⁺ T, CD4⁺ T, B lymphocytes and macrophages (Suarez–Pinzon *et al.*, 2000).

Recent evidence indicates that alterations in cell survival contribute to the pathogenesis of a variety of diseases such as cancer, viral infections and autoimmune diseases including insulin dependent diabetes mellitus (IDDM) (Thompson, 1995). A direct linkage between the control of apoptosis and autoimmune diseases is yet to be demonstrated and will have obvious implications for the understanding of the pathogenesis of these diseases. One of the central signaling pathways of apoptosis in the immune system is the sFas (CD95). It is expressed by mature T-cells, but expression is enhanced upon activation by antigen, thus rendering T-cells more sensitive to Fas L-mediated apoptosis (Lee and Shacter, 2001).

Some reports defined proinflammatory cytokines which are known to be secreted from macrophages, as the candidate mediators of B-cell destruction by apoptosis in autoimmune diabetes (Rabinovitch, 1993; Dunger *et al.*, 1996; Delaney *et al.*, 1997). However, TNF is one of the most important physiological inducers of apoptosis. The critical role of TNF mediated apoptosis has been recognized in a wide variety of situations including immunomodulation and autoimmunity (Rath and Aggarwal, 1999). Sidoti-de Fraisse *et al.* (1998) and Natoli *et al.* (1998), reported that TNF regulates cell growth by apoptotic, nonapoptotoic, and antiapoptotic mechanisms. Also different cell types respond quite differently to TNF which kills some cell types and induces proliferation of others. This heterogeneity has been noticed even within the same cell type under different conditions (Burow *et al.*, 1998).

In this study, investigated apoptosis of lymphocytes by different methods, sFas and, semiquantitative detection of mRNA and serum levels of the cytokine tumor necrosis-alpha (TNF-α) in children with IDDM at the onset of the disease and after treatment for more than 6 months, inorder to explain an aspect of disease development and pancreatic B-cell death.

Materials and Methods

This study was conducted on 71 patients diagnosed after detailed history, clinical examination and laboratory investigations as IDDM according to the consensus guidelines reported by the International Society for Pediatric and Adolescent Diabetes (2000). They were taken up from the Endocrine and Diabetes Unit and the research investigations have been done in the Pediatric Genetics Unit, the University Children Hospital at Mansoura. In addition to the patients, 60 completely healthy children have been included in the study as a control group. The research has been carried out through the period from March 2000 to November 2001. For the purpose of interpretation and analysis (the demographic and clinical data are presented in Table 1), all the subjects were enrolled into the following groups:

Group 1 (G1) : Included 28 children with recent onset of IDDM. They were admitted to the hospital, diagnosed and investigated immediately. They were 12 males and 16 females with mean age of 6.3 years ± 4.6. They were divided into 2 subgroups; G1a: included 8 patients presented with diabetic ketoacidosis (DKA).

They were 3 males and 5 females with mean age 5.2 years± 4.1, and G1b: included 20 patients with nonketotic hyperglycemia (NKHG). They were 9 males and 11 females with mean age 6.5 years± 5.2.

Group 2 (G2): Included 43 old diabetic children who were previously diagnosed and under treatment for 6 months and more before they entered the study. They were taken up from the follow up outpatient clinic. They were 20 males and 23 females with mean age 6.5 years ± 3.2. After clinical examination and laboratory investigations they entered the study. They were divided into 2 subgroups according to the status of the disease; G2a: included 7 patients with controlled status of the disease as indicated by HbA1c < 7.6. They were 3 males and 4 females with mean age 5.5 years ± 3.4, and G2b : included 36 patients with uncontrolled status of the disease (HbA1c > 7.6).

Group 3 (G3): Included 60 healthy children as control group. They were 26 males and 34 females with mean age 6.4 ± 4.5. They have no family history of diabetes, other autoimmune disease or any other disorder that may alter the immunologic status.
All subjects were subjected into the following:

Detection of apoptotic cells by giemsa stain, acridine orange stain (AO), propiduim iodide stain (PI) and DNA fragmentation.
Lymphocyte separation: Cells were obtained from whole EDTA-blood using lymphocyte-separating solution (Ficoll-Paque; Pharmacia, Uppsala, Sweden) by the method of Boyum (1968). Lymphocytes were washed twice with sterile PBS pH 7-6.

Culture of lymphocyte: Cells were suspended at 107/ml in RPMI 1640 (GIBCO) containing 25 mM glucose 20 mML-glutamine, 92 μm gentamicin 20 μg ml⁻¹ (Walkersvlle MD) and 25 mM HEPES buffer (pH 7.3: GIBCO). Cells were suspended in sterile PBS (0.05$_M$PO$_4$, 0.15M NaCl, pH 7.2). The cell suspension were divided into 4 aliquots to measure the apoptosis at zero time (base lime), then 24, 48 and 72 h, cells were incubated (at 37 °C in 5% carbon dioxide). After incubation, cells were centrifuged (200xg 5min), resuspended in ice-cold PBS and analyzed on a coulter counter.

Assessment of cell viability: At time zero and then at subsequent times, cells were removed from culture and counted on a hemocytometer. Cell viability was determined by trypan blue dye exclusion test, one volume of trypan blue (0.4% GIBCO) was added to 5 volumes of cells at room temperature for 5 minutes.

Detection of apoptotic cells
Propidium iodide stain: Cells were stained with Hoechst 33342 and propidium iodide (PI) and visualized using fluorescence microscopy as described by Lee and Shacter (1998). A minimum of 200 cells were counted per sample and were classified as follows:

a) Live cells (normal nuclei, blue chromatin with organized structure),
b) Membrane intact apoptotic cells (bright blue chromatin that is highly condensed, marginated, or fragmented) and
c) Membrane permeable apoptotic cells (bright red chromatin, highly condensed or fragmented).

The extent of apoptosis was calculated as the percentage of total apoptosis divided by the total number of cells counted.

Giemsa stain: At time zero and at subsequent times, cells were removed from each culture, fixed in methanol, harvested on slides and slides were stained with May Grunwald Giemsa and examined by oil immersion light microscope (Wyllie and Morris, 1985). For assessment of the percentage of cells showing morphology of apoptosis 500 cells/slide were examined for each case at different times (O, 24, 48 and 72 hours). Lymphocyte were considered apoptotic if they exhibited the highly characteristic morphological

Table 1: Age, sex, duration of disease at time of sampling and level of HbA1c in the studied groups and subgroups

| Groups | Sub-groups | N | Age (years) | | Sex | | Duration of disease at time of sampling | | Hb/A1c |
			Range	Mean± SD	Male	Female	Range	Mean± SD	
G1		28	1.75-12.5	6.3+ 4.6	12	16	1-90 days	14.2± 6.3	
	1a	8	1.75-7.5	5.2+ 4.1	3	5	1-10 days	3.7± 2.4	ND
	1b	20	3.5-12.5	6.5+ 5.2	4	11	7-90 days	24.6± 6.1	ND
G2		43	3.5-12.5	6.5+ 3.2	20	23	0.5-8years	2.4± 1.8	
	2a	7	3.7-7.0	5.5+ 3.4	3	4	0.5-3years	1.2± 0.6	< 7.6
	2b	36	6.0-12.5	8.4+ 4.3	17	19	0.5-8years	2.8± 1.6	> 7.6
G3		60	3.5-13	6.4+ 4.5	26	34			

ND: Not done   N= No. of patients

features of chromatin aggregation, nuclear pyknosis and cytoplasmic vacuolation. The apoptotic lymphocyte percentage at different times was calculated.

Acridine orange stain (AO): One drop of cell suspension was added to one AO solution (10μg ml⁻¹ in PBS), mixed gently on a slide, and immediately examined with an olympus HB-2 microscope with fluorescence attachment. Green fluorescence was detected between 500 and 535nm cells exhibit bright fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic cells and expressed as a percentage of the total number viable cells, which exhibited a green, diffusely stained intact nucleus (Kumagai et al., 1995)

Assessment of apoptotic index: For this analysis in each case the mean number of monocytic cells per field was registered in 15 randomly chosen fields. The apoptotic bodies were then counted in a greater number of fields and expressed as percentages monocytic cells per case (Del Vecchio et al., 1991).

DNA fragmentation: Total DNA was extracted with 0.5 ml saturated phenol followed by 0.5 ml chloroform:isoamyl alcohol (24:1) before centrifugation at 7000g, 4 °C for 10 min. DNA in the supernatant was precipitated by adding 0.02 ml of 5 m NaCl and 1 ml of absolute ethanol. After centrifugation at 9500g for 15 min, the pellets were air-dried prior to suspending in 0.1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA was eliminated by Rnase digestion (0.1 mg ml⁻¹ Rnase, incubated at room temperature for 1h). The DNA was electrophoresed using 1.8% agarose gel and visualized by ethidium bromide staining (Maruyama et al., 2000).

Measurement of sFas: The levels of sFas were determined by the following enzyme linked immunosorbent assay (ELISA) using two anti-human Fas monoclonal antibodies, DX-2 (IgG1) and DX-3 (IgG2a), that were generated as described previously by Cifon et al. (1994). These antibodies react with distinct epitopes in the extracellular domain. Anti-Fas antibody (DX-3; 10 μg ml⁻¹ in 0.01μm phosphate buffered saline, pH 7.2 (PBS), was coated onto 96 well microliter plates (Sumililon multiplate MS-8496 F, Sumitomo Co, Ltd., Japan) and incubated at 37 °C for 3 hr. Blocking performed with 1:2 dilution with PBS of Block Ace (Dai Nihon Seiyaku Co, Ltd., Japan) at 37 °C for 2 hr. Then sera were added and incubated at room temperature for 1 hr. After washing five times with 0.05% tween / PBS, 10 μg ml⁻¹ biotin conjugated anti-Fas antibody (DX-2) in 0.05% tween / PBS containing 5% mouse serum was added and incubated at room temperature for 1 hr. After washing five times, 1:150 diluted ABC solution (Vectastain ABC kit Elite pK-6100, Vector Laboratories Inc., Burlingame, CA) was added and incubated at room temperature for 1 hr. After washing five more times, O-phenylenediamine (OPD) and 0.03% H₂O₂ in citrate buffer (pH 5.0) were added and incubated at room temperature for 30 min. OD490 was measured on an automated plate reader (Model 3550-UV Microplate Reader, Bio-Red, Hercules, CA) and the levels of sFas were determined by comparison to the standard curve obtained using recombinant

sFas (19, 9.5, 4.75, 2.37, 1.18. 0.59. 0.29, and 0.14 ng ml⁻¹). The lower limit of detection was 0.59 ng ml⁻¹ (Tokano et al., 1996).

mRNA detection and measurement of serum levels of TNFα
Measurement of TNF-α concentrations: Serum was obtained by centrifugation of blood sample at 2500 g for 15 minutes, then aliquoted and immediately used for functional assay or stored at −80 °C until assayed. Before each assay, frozen samples were brought slowly to room temperature and gently mixed. Tumor necrosis factor-alpha (TNF-α) was measured by commercially available enzyme linked immunosorbent assays (R and D System Europe, Oxon, UK). According to the manufacturer's procedure, a standard curve is generated for each set of samples assayed by standards of various concentration. Analyze concentration found in patients was in the range of linearity in each assay. The development of a color reaction caused by the conversion of chromogenic substrate (teramethy-1 benzidine), directly proportional to the amount of analyze assayed, was followed within 5 to 20 minutes with an enzyme linked immunosorbent assay plate reader (ETI-System, Sorin Biomedica, Saluggia, Italy) at 450 nm.

RNA isolation: Following sorting, cells were lysed with guanidinium isothiocyante as described (Chomzynsky and Sacci, 1987) and stored at 70 °C prior to preparation of cDNA.

cDNA synthesis: Total RNA was incubated for 30 min at 37 °C in a solution containing Tris–HCl pH 7.5, 10 mM dithiothreitol and 2.5 U of RQI Dnase to remove any contaminating genomic DNA from the preparation. After phenol chloroform genomic DNA from the preparation. After phenol chloroform extraction and ethanol precipitation, RNA pellets were resuspended in water. cDNA was synthesized from RNA by priming 1μg of total RNA at 37 °C for 1h in a 50 μl total volume containing 1μg of an oligo (dT) primer, 200 nmol of each deoxynucleoside triphosphate (Pharmacia), and 200 U of Moloney murine leukaemia virus reverse transcriptase (RT; Gibco BRL); 20 mM dithiothreitol and 5x Moloney murine leukaemia virus RT buffer were added as recommended by the manufacturer (Gibco BRL).

Amplification of cDNA: cDNAs were tested for the presence of cytokine gene sequences by polymerase chain reaction (PCR) performed in a 30 μl total volume with specific primer pairs. Two microlitres of the relevant cDNA were amplified by PCR in the presence of 0.2 μM sense antisence oligonucleotide primers. 2 mM deoxynucleoside triphosphate (Pharmacia. BRL Technologies Inc. Gaithersburg, MD). 0.5 U of Ampli Taq polymerase (Perkin Elmer, Branchbureg, NJ) and 10xPCR buffer. The amplification was performed in a thermal cycler from Perkin Elmer, running a programme of 40 cycles with denaturation at 96 °C for 30s, annealing at 60 °C for 10 min, and extension at 37 °C for 1.5 min. oligonucleotide primers specific for β −actin were used to normalize the results. The antisense primer of β actin was 5'-CTGGTGCCTGGGGCG, and the sence primer was 5'-AGCTCGCCTTTGCCGA as described previously (Kreuzer 1999). Sense TNF-α 5'primer 5'- TTC TGT CTA CTG AAC TTC GGG GTG

ATC GGT C −3′ antisense TNF- α 3′primer 5′- GTA TGA GAT AGC AAA TCT GGC CTA CGG TGT GGG-3′PCR amplification products were visualized in 1.5% agarose gels after staining with ethidium bromide. The density of the PCR products for various cytokines was compared, with β-actin used as an internal control.
Quantification was achieved by comparing the intensities of bands due to cytokine mRNAs with those of the corresponding synthtic mRNA control (Wang *et al.*, 1989).

Statistical analysis: Statistical analysis was performed by means of student-t-test to compare between serum levels of sFas,percentages of apoptotic lymphocytes and serum levels of TNF-α, chi-square test for comparison between the grades of mRNA expression and Pearson correlation test for the correlation between serum level of sFas and percentage of apoptotic lymphocytes, and between serum level of TNFα and each of serum level of sFas and percentage of apoptotic lymphocytes (Armitage, 1989).

Results
Analysis of the results revealed important findings. Significant decrease of sFas in the serum (ng ml^{-1}) of recent onset cases with IDDM compared to control (P< 0.01) and cases under treatment for more than 6 months (P< 0.01). No significant difference between those presented with diabetic ketoacidosis and others with nonketotic hyperglycemia (Table 2).

Fig. 2: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patients under treatment more than 6 months, stained with propidium iodide (original magnification x 100). The arrows point to the apoptotic cells, showing within normal count

Fig. 1: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patients with recent onset IDDM, stained with propidium iodide (original magnification x 100). The arrows point to the apoptoctic cells, showing low count
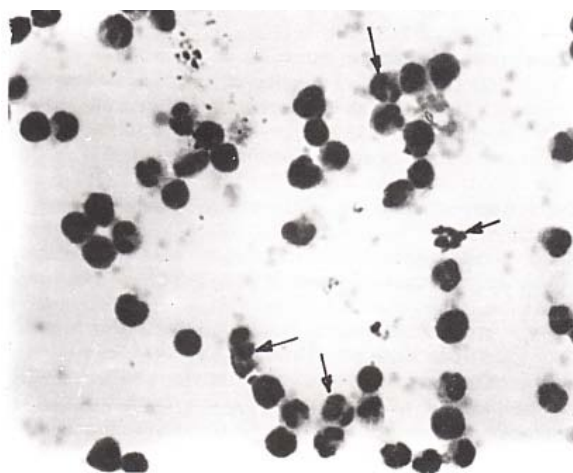

Fig. 3: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patientswith recent onset IDDM, stained with geimsa (original magnification x 100). The arrows point to the paoptotic cells, showing low count

The percentage of apoptotic lymphocytes detected by PI, Geimsa and AO are parallel and the apoptosis was found to be inhibited only in the recently diagnosed patients compared to G2 and G3 (P< 0.01 and < 0.01 respectively) and no significant difference either between the subgroups G1a and G1b or G2a and 2b both at 0 and 72 hours (Table 2 and Fig. 1-6). Agarose gel electrophoresis for DNA of apoptotic lymphocytes showed fragmentation of DNA (Fig. 7) Semiquantitative detection of mRNA of TNF-α (Table 3 and Fig. 8) revealed significant increase of mRNA production of TNFα only in cases with recent onset (P< 0.001 between G1 and each of G2 and G3 respectively). No significant difference neither between G1a (with ketoacidosis) and G1b (without ketoacidosis), nor difference between G2a and G2b. Serum levels of cytokine are mirror images of mRNA (Table 4 and Fig. 9). They are significantly high only in G1 compared to G2 and G3 (P < 0.001) and no significant difference has been found neither between G1a and G1b nor between G2a and G2b.

For patients with recent onset of IDDM, Pearson correlation test between sFas and percentage of apoptotic lymphocyte cells detected by the different types of stains and between the results of the different stains with each others revealed highly significant positive correlation in every test (P < 0.001) (Table 5). Also applying the test between the serum level of TNF-α and sFas level and the percentage of apoptotic lymphocytes by the different stains at 72 hours in patients with recent onset showed highly significant negative correlation in all tests (P < 0.001). The same results have been found in patients with diabetic ketoacidosis and those with nonketotic hyperglycemia (Table 6).

Discussion
One of the important factors regulating cell death in lymphocytes is the cell surface receptor Fas (CD95), a member of TNF receptor family (Scaffidi *et al.*, 1998). Stimulation of Fas receptor on activated lymphocytes can induce apoptosis. The death-inducing signal complex (DISC) is formed by interaction of FADD/MORT-1
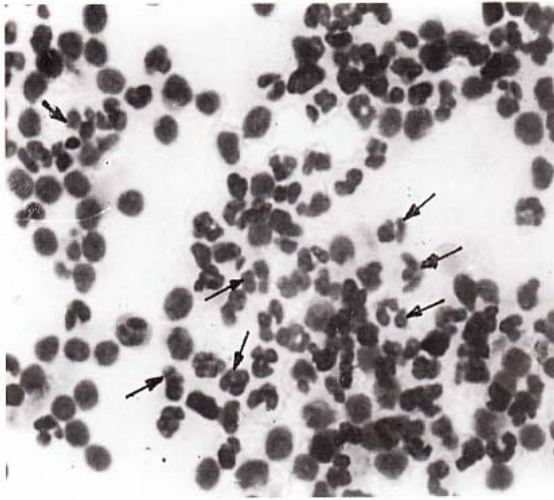
Fig. 4: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patients under treatment more than 6 months, stained with geimsa (original magnification x 100). The arrows point to the paoptotic cells, showing low count
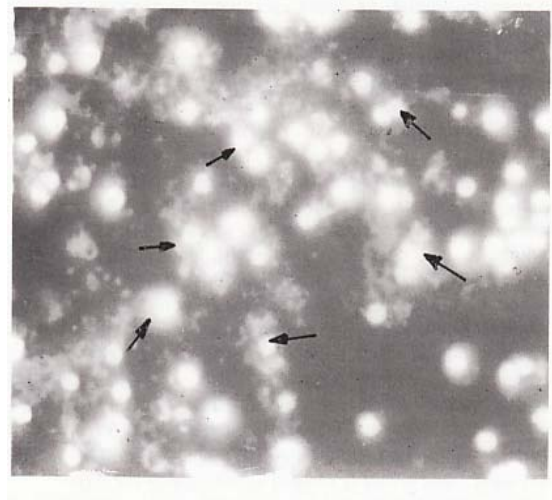

Fig. 6: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patients under treatment more than 6 months, stained with acridine orange (original magnification x 100). The arrows point to the paoptotic cells, showing low count
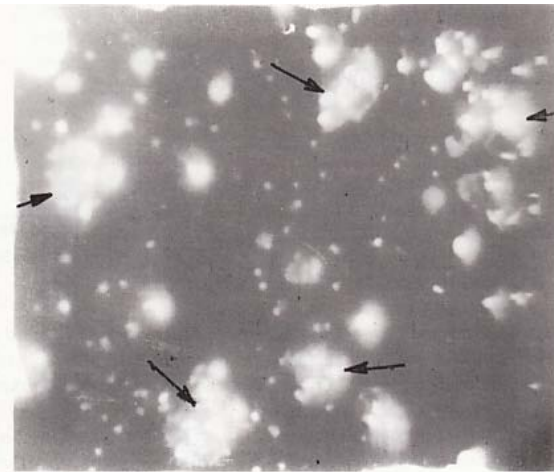

Fig. 5: A photomicrograph of peripheral blood lymphocyte cells (PBLC) of patients with recent onset IDDM, stained with acridine prange (original magnification x 100). The arrows point to the paoptotic cells, showing low count
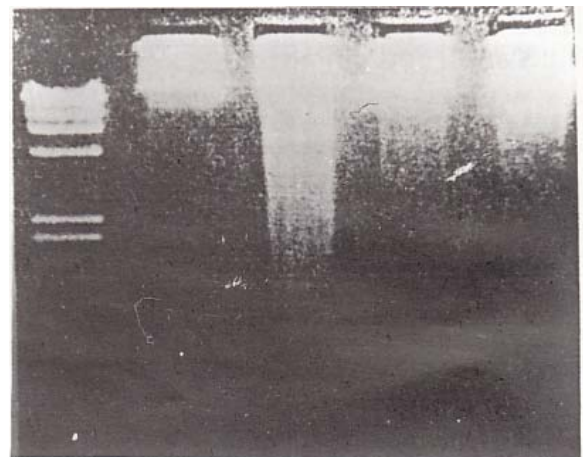

Fig. 7: Gel electrphoresis for DNA. Lane 1; Marker; Lanes 2-; DNA fragmentation in apoptotic perideral blood lymphocytes

with the death domain of Fas. This interaction activates caspase-8 and caspase-3 which proceed the process of apoptosis (Lee and Shacter, 2001). In the study found a decreased sFas expression in cases with recent onset of IDDM, which is in agreement with the report of Giordano *et al.* (1995), who found that B-and-T cell from subject with IDDM and those at risk for the disease are highly defective in the surface expression of Fas.

Present finding of increased production of mRNA and consequently high level of circulating TNF-α in children with recent onset IDDM can explain the decreased sFas level. Zhang *et al.* (2000) reported that stimulation of the cells with TNFα has been shown to generate 2 signals, one that initiates programmed cell death and another that leads to activation of the transcription factor $_k$B (NF-$_k$B), which induces inhibitors of apoptosis and promotes the production of proinflammatory factors. TNF-α strongly activates NF-$_k$B through a second class of adaptor protein

TNFR-associated factors, and this transcriptional factors regulates the expression of antiapoptotic gene products (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996), such as antiapoptotic members of the Bcl-2 family and the inhibitor of apoptosis c-1AP1 and c-1AP2. TNF-α also activates sphingosine kinase which converts sphingosine to sphingosine 1-phosphate, which is a lipid-derived mediator which is shown to prevent the cytotoxic action of TNF-α (Osawa *et al.*, 2001). Nicholson and Thomberry (1997) suggested that TNF-α may be cytotoxic to B-cells by activating cysteine proteases (caspases) that initiate the proteolytic cascade. Andersen *et al.* (2000) postulated that there is synergistic effect of TNF-α , IL-β , and IFN-γ in the functional inhibition and induction of cell death in pancreatic β cells through a synergistic activation of mitogen-activated protein kinase activity. However, this is highlighted by our finding of strongly negative correlation between the serum level of TNF-α and the level of sFas on one side and the percentage of apoptotic cells by the different

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Table 2: Mean± SD of the level of sFas (CD95) (ng ml⁻¹) and percentage of apoptotic PBLCs by the different types of stains at 0 time and at 72 hours

Groups	Sub-groups	sFas 0	sFas 72 hours	PI stain 0	PI stain 72 hours	Geimsa stain 0	Geimsa stain 72 hours	AO stain 0	AO stain 72 hours
G1		1.32	8.31	2.16	8.54	4.39	11.17	7.90	16.92
		± 0.41	± 0.31	± 0.53	± 0.81	± 0.94	± 0.71	± 0.79	± 1.33
	1a	1.29	8.52	2.14	8.71	4.52	11.42	7.72	16.98
		± 0.62	± 0.42	± 0.36	± 0.84	± 0.82	± 0.81	± 1.31	± 1.12
	1b	± 1.33	± 8.21	± 2.71	± 8.42	± 4.26	± 0.89	± 8.31	± 6.76
		± 0.52	± 0.33	± 0.41	± 0.76	± 0.63	± 0.82	± 1.20	± 1.32
G2		5.36	32.91	15.7	49.72	24.5	61.91	31.62	61.73
		± 1.45	± 3.62	± 2.71	± 4.72	± 2.11	± 6.31	± 3.89	± 5.57
	2a	5.82	33.81	15.60	49.2	24.12	62.42	32.11	61.92
		± 1.61	± 4.22	± 3.41	± 4.13	± 2.62	± 7.31	± 3.14	± 5.12
	2b	5.13	21.80	15.94	49.92	24.81	60.83	30.93	61.43
		± 1.28	± 3.36	± 3.81	± 3.96	± 2.34	± 6.82	± 4.21	± 6.22
		5.22	33.17	15.50	49.52	25.82	63.34	30.16	60.31
		± 1.49	± 3.87	± 2.53	± 3.81	± 2.23	± 8.42	± 4.45	± 4.17

PBLCs: Peripheral blood lymphocyte cells, PI: Propidium iodide stain, AO: Acridine orange stain
G1 vs G2 and G1 vs G3 highly significant at P< 0.01   G2 vs G3, G1a vs G1b and G2a vs G2b non-significant

Table 3: mRNA expression of TNF-α in the studied groups and subgroups

Grade of expression	Groups and Subgroups						
	G1	G1a	G1b	G2	G2a	G2b	G3
-	-	-	-	21	4	25	44
+	12	3	9	8	3	11	16
+ +	15	5	10	-	-	-	-
+ + +	1	-	2	-	-	-	-

G1 vs G2 and G1 vs G3 P< 0.001   G1a vs G1b, G2a vs G2b and G2 vs G3 non-significant

Table 4: Serum levels of TNF-α in the studied groups and subgroups

Levels of TNF-α	Groups and subgroups						
	G1	G1a	G1b	G2	G2a	G2b	G3
Mean	73.51±	74.12±	73.24±	35.42±	36.62±	35.20±	34.71±
± S.D	9.12	8.83	7.23	4.33	3.21	3.96	8.43

G1 vs G2 and G1 vs G3 P < 0.001   G2 vs G3, G1a vs G1b and G2a vs G2b non-significant

Table 5: Correlation (r) between the level sFas (CD 95) (ng ml⁻¹) and the percentage of lymphocyte apoptotic cells detected by PI, Geimsa and AO stains and between the results of the different stains with each other at 0 time and at 72 hours

	At 0 time			At 72 hours		
	sFas	A0	Gimsa	sFas	A0	Gimsa
PI	0.86*	0.76*	0.78*	0.81*	0.77*	0.79*
Gimsa	0.83*	0.73*		0.87*	0.84*	
A0	0.77*			0.85*		

*: P< 0.001

Table 6: Pearson correlation (r) between serum levels of TNF-α (pg/ml) and sFas and the percentage of apoptotic lymphocytes detected by the three stains in G1, G1a and G1b at 72 hour

Percentage of apoptotic cells at 72 hours	Serum levels of TNF-α (Pg ml⁻¹)		
	G1	G1a	G1b
sFas	-0.62	-0.64	-0.58
PI	-0.52*	-0.69*	-0.53
Geimsa	-0.60*	-0.62*	-0.52*
AO	-0.53*	-0.58*	-0.56*

*: P< 0.001

methods of stain on the other side at 72 hours. The development of IDDM which is due to interaction between additive variance of some genes and environmental factors is strongly associated with HLA-class II , which is close to the genes of TNF and sFas within the area of HLA-class III on the short arm of chromosome number 6, may help to understand this relation. However the characterization of mRNA and their agreement with the changes in the circulating TNF-α , identify TNF-α-regulated gene, which points to normal transcription and translation.

In this research work studied the percentage of apoptotic cells in the peripheral blood by different methods of stains to endorse the assessment. Mc Gahon *et al.* (1995) and Maruyama *et al.* (2000), reported that the order of apoptotic features such as morphological changes or nuclear condensation is not the same in different types of cells, and several methods are needed. The results are the same with the different stains and revealed low count of apoptotic lymphocytes in patients with recent onset IDDM, compared to control and to patients undertreatment for more than 6 months. This is confirmed by the DNA fragmentation and found in the lymphocytes of such patients. In the process of apoptosis, cell nuclei and cytoskeleton are changed first, then membrane blebbing and caspase-3 activation occur, and fragmentation of chromosomal DNA is last (Maruyama *et al.*, 2000). Juntti-Berggren *et al.* (1993) reported that, apoptosis has been shown to occur in normal pancreatic B-cells after exposure to serum from patients with IDDM, an effect that was found to be IgM mediated. Lally *et al.* (2001) examined pancreatic biopsies from 30 diabetes-prone and 6 diabetes resistant BB/S rats matched for age. They found significant islet cell apoptosis in the pancreas of diabetic prone BB/rats, which coincide with the appearance of insulitis and the onset of diabetes. They also
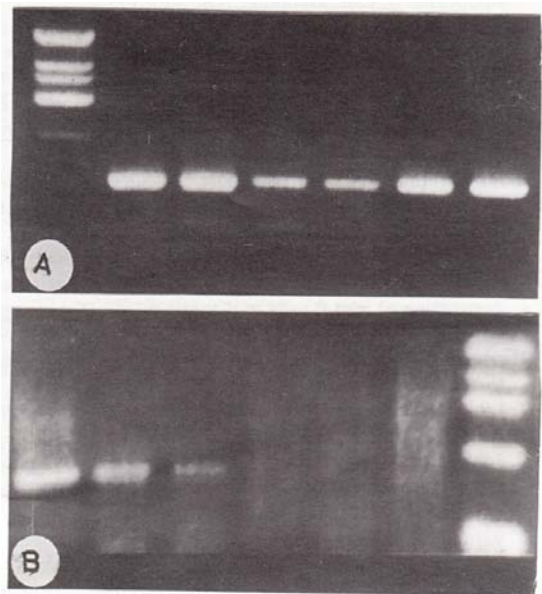
Fig. 8: Expression of mRNA of TNF-α. (A) Lane 1; Marker; Lanes 2-7: mRNA of cases with recent onset.(B) Lanes 1-6: mRNA of cases under treatment for more than 6 month. There is increased expression in the first group(A) compared to the secound group (B)
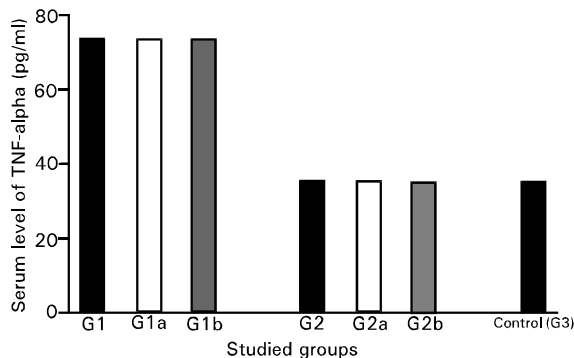
Fig. 9: Histogram of the serum levels of TNF-α

detected differences in the levels of apoptosis between diabetic and non-diabetic animals and suggest that such differences could be an important determinant in disease progression in this animal model Type I diabetes.

However, an autoimmune disease can result from failure to remove autoimmune cells that arise during immune response to an antigen (Thompson, 1995). The migration of autoreactive lymphocytes and other leukocytes from the blood stream into the target organ is very important in the pathogenesis of many organ-specific autoimmune-inflammatory disorders. In IDDM this migration results in lymphocytic invasion of the islets leading to the formation of insulitis (Yang *et al.*, 1996). This migration is controlled in part by selective expression and functional regulation of cell adhesion molecules on the surface of lymphocytes and vascular endothelium cells or in the extracellular matrix (Bargatze *et al.*, 1995).

The insignificant difference between the findings in children with recent onset IDDM presented with ketoacidosis and those with non-ketotic hyperglycemia, indicates that the associated metabolic

and biochemical changes in the first subgroup, have no influence on either the process of apoptosis or the increased production of mRNA of TNF-α. Also, the insignificant changes in patients under treatment for more than 6 months whatever, the status is controlled or uncontrolled, means that the pathological process ends mostly in the first six months, and hence the uncontrolled status is due to factors other than the process of insulitis, as therapeutic or dietetic factors.

In conclusion, the accumulation and migration of the immune reactive cells to the pancreas infilterating the islet cells together with increased TNF-α lead to the process of insulitis that may be accompanied by local increased production of other proinflammatory and/or antiinflammatory cytokines (Rabinovitch *et al.*, 1998). This may affect the β cells by either induction of apoptosis or direct cytotoxic effect to destroy the B cells. However, further investigations are needed at the level of the area of pancreatic islet cells. This may help in the early treatment of this disease aiming to stop the progress of the B-cell damage, since trials still underway through manipulation of the programmed cell death through the action of bcl2, p53 or c-myc, and by the use of specific anticytokines (Kroemer and Martinez, 1994; Thompson; 1995, Mauricio and Mandrup-Poulsen, 1998).

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