T-Cells Proliferation and Serum Cytokine levels in Type 1 Diabetic Children

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Abstract

Background: There is plenty of evidence suggesting that involvement of several groups of viruses in the development and / or acceleration of Type 1 Diabetes Mellitus (T1DM).

Objective: To analyze the T- cell proliferation in the presence of Coxsackie virus B5 (CVB5), Polio and Adenovirus antigens in addition to assessment of Interferon- gamma (IFN-γ), Interleukins (IL-10 and IL-6).

Methods: In 60 Iraqi T1DM children with recent onset of T1DM, Lymphocyte proliferation was analyzed using Methylthiazol tetrazolium (MTT) assay by culturing Peripheral Blood Lymphocytes (PBLs) with Coxsackie Virus B_5 (CVB5), Adenovirus, and Polio vaccine. Serum Interferon-γ, IL-10 and IL-6 were quantified by sandwich ELISA.

Results: No significant differences were shown in the PBL proliferative percentage in response to Con-A mitogen and tested viruses (CVB_5 and Adenovirus) between T1DM and healthy controls, but it showed a significant decline in patients in response to Polio vaccine. Higher significant serum levels of IFN-γ, IL-10, and Il-6 were observed in the investigated patients compared to controls ($p<0.05$). Mean PBL proliferative percentage in response to tested viral antigens was correlated with the serum IFN-γ, IL-6 and IL-10 levels.

Conclusions: In children with new- onset diabetes, mean proliferative percentage of Peripheral Blood Lymphocytes was generally decreased. A significant elevation of serum levels of IFN-γ, IL-10 and IL-6 were observed, which is significantly correlated to mean proliferative responses of PBL to viral antigens.

Key Words: T1DM, Lymphocyte proliferation, IFN-γ, IL-10 and IL-6

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Introduction:

pidemiological studies indicate that autoimmune diseases such as type 1diabetes Emellitus (T1DM) have a strong environmental component to their pathogenesis [(1)]. There is a considerable evidence suggesting that involvement of several groups of viruses, but particularly those of the Enterovirus genus in the development and / or acceleration of T1DM [(2)]. Coxsackie virus B4 (CVB4) specific IgM antibodies are more common in newly diagnosed subjects with T1DM than in healthy control subjects [(3)]. Another study found that diabetic children vaccinated against Poliomyelitis had high levels of specific IgM antibodies to Poliovirus derived VP1 peptide at onset of T1DM [(4)]. The participation of viruses in causing T1DM could be through cross-reactivity that may involve molecular mimicry between viral and self antigens [(5)], or in addition, inflammatory cytokines secreted by mononuclear cells like IL-1α, IL-1β, TNF-α, TNF-β, IFN-α, IL-2, IL-12, and IFN-γ mediated death of islet cells [(6, 7)]. Lymphocytes can rapidly produce these cytokines on activation [(8)]. It was demonstrated that IL-10 was essential for an early phase of diabetes in NOD mice via CD_8^+ T-cell pathway [(9)]. Another study found that serum levels of IL-6 were elevated markedly in young T1DM patients without clinical evidence of microvascular and macrovascular complication [(10)]. Based on these considerations, the goal of the present study was to analyze the T- cell proliferation in the presence of CVB5, Polio and Adenovirus antigens in addition to assessment of IFN-γ, IL-10 and IL-6 in a population of children with T1DM and children who were healthy.

Subjects, Materials and Methods:

Sixty Iraqi T1DM children (28 males and 32 females) were subjected to this study. The patients were attending the National Diabetes Center at Al-Mustansiriya University during the period May 2004 - October 2005. Their ages ranged from 3 -17 years, and they were

new onset of the disease (diagnosis was from one week up to five months). Diagnosis of Diabetes Mellitus and selection of patients was accomplished with the assistance of the consultant medical staff in the National Diabetes Center. All the patients were treated with daily replacement doses of insulin at the time of blood sampling. The patients were divided into two groups according to their ages in order to assess the aggressive of immune responses: 36 children equal or less than 10 years and 24 children up to 10 years. For the purpose of comparisons, 50 healthy control subjects matched for age (4-17 years old) and sex (25 males and 25 females) were selected who have no history or clinical evidence of type 1 diabetes or any chronic diseases and obvious abnormalities as a control group.

Collection of Blood Samples

Eight milliliters of venous blood were collected from each subject. Five milliliters of blood were put in heparinised test tube (10 U/ml) and used for the lymphocyte proliferation. The remaining blood was drawn into plain test tube and the serum was separated by centrifugation at 2500 rpm for 10 min., divided into aliquot and kept at-20°C until used.

Lymphocyte Proliferation using Methyl-thiazol tetrazolium (MTT) assay:

Peripheral Blood Lymphocytes (PBLs) were isolated using Ficoll-isopaque gradient centrifugation (Flow-Laboratories, UK). The washed PBLs were resuspended in complete RPMI- 1640 medium (Euroclone, UK) supplemented with 10% heat inactivated human AB serum; Hepes; crystalline penicillin (1000000 IU) and streptomycin (1gm) (Pharma-intersprl, Belgica), and the final lymphocyte concentration was adjusted to 1-2x10^6 cells / ml. Triplicate incubations of 100 µl of cell suspension with antigen(s) in 96 flat-bottom microculture plates for 3 days at 37°C in a humidified 5% CO_2 incubator. Then 20 µl of 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, Germany) working solution was added to each culture well and the culture were incubated for further 4 hrs. The converted dye was solubilized by adding acidic isopropanol. The absorbency was read using microculture plate reader using a wave length of 570 nm [11].

The antigens were: CVB5 antigen solution (1:5 dilution) (KBR-CF antigen Vero, France), Poliovirus Trivalent Vaccine (1:5 dilution) (Polioral Trivalent; Chiron), and Adenovirus type 3, 4, 7 solution (1:10 dilution) (KBR-CF antigen type 3, 4, 7, Vero). The final concentration or dilution for the three viral antigens was achieved according to the result of MTT serial dilution run of these antigens. Concanvalin-A (100 µg/ml), was used as a mitogen positive control [11].

Interferon-γ, IL-10 and IL-6 were quantified in serum by sandwich ELISA using human IFN-γ kit (Immunotech Beckman Coulter), human IL-10, and human IL-6 kits (Mabtec).

Statistical analysis:

Student t-test was used to measure the differences between two means, the results were expressed as means ± standard error (SE), and the Pearson Correlation (R) was employed.

Results:

Lymphocyte Proliferation: The results of mean proliferative percentage in response to Con-A were represented in table (1). The newly diagnosed T1DM patients tended to have a lower non significant proliferative percentage in response to Con-A than control subjects ≤10 years old (83.33 *vs.* 85.93%, P_1=0.82) and in >10 years old group (86.04 *vs.* 92.7%, P_1= 0.62)

Considering the response to different viral antigen, a lower mean proliferative percentage was seen among patients ≤10 years old in response to CVB_5 compared to controls (36.67 *vs.* 49.16%) and among patients >10 years old than controls (38.87 *vs.* 51.20%). Those differences failed to reach significant levels in both age groups (P_1=0.061, and 0.14 respectively), (Table - 1). Significant decline of proliferative response against Polio vaccine was seen in T1DM patients compared to controls (34.44 *vs.* 47.38%, P_1= 0.045) in ≤10 years old group and >10 years old group (28.30 *vs.* 40.86%, P_1= 0.004) (Table -1). A non significant (P_1= 0.82) proliferative percentage decline in response to Adenovirus

was observed in ≤10 years old patients (19.97%) compared to controls (20.67%) and also in patients >10 years old (23.02%) in comparison with controls (28.61%) (P_1=0.23). No statistical differences appeared in the mean lymphocyte proliferative percentage between patients in both age groups against CVB_5 (P_2=0.57), Polio vaccine (P_2=0.14) and Adenovirus (P_2=0.57).

Serum Level of hIFN-γ: Higher means of serum levels of IFN-γ were observed in the investigated patients ≤10 years and >10 years old (75.60 and 70.78 pg/ml respectively) than controls (42.66 and 40.39 pg/ml respectively). Out of 29 healthy controls in the age group >10 years old, only one of them had serum IFN-γ less than standard level (0.095 pg/ml). The statistical analysis revealed a significant difference between patients and controls (P_1= 0.005 and 0.006 respectively), while between patients no statistical difference appears (P_2= 0.73), table (2).

Serum Level of hIL-10: Table (3) demonstrated the mean serum levels of IL-10 in the studied groups. The mean value of serum IL-10 for patients group ≤10 years old was significantly higher than controls (104.92 *vs.* 57.01 pg/ml, P_1= 0.003). Patients >10 years old showed also significant elevation in IL-10 serum levels (84.22 pg/ml) compared with controls (59.50 pg/ml) (P_1=0.037). A statistically difference of mean IL-10 concentration appears between patients in both age groups (P_2= 0.04).

Serum Level of hIL-6: The levels of IL-6 in sera of the patients were higher than control group (147.6 *vs.* 80.4 pg/ml, P_1= 0.036) in ≤10 years old group table (4). Out of 36 patients, 4 patients had serum levels of IL-6 out of standard level; three were less (0.097, 0.098 and 0.098 pg/ml), while the fourth one was high (2224.29 pg/ml) than the standard. In the same age group, out of 21 controls, 2 individuals had serum IL-6 levels less than standard (0.082 pg/ml).

The mean levels of serum IL-6 were also significantly elevated in the patients >10 years old compared to controls (171.8 *vs.* 81.6 pg/ml respectively, P_1= 0.04). Out of 24 patients, 2 patients had serum IL-6 concentration less than standard (0.082 and 0.092 pg/ml) and another 2 patients had high levels (1410.86 and 1654.20 pg/ml) than standard. Concerning the healthy controls, 12 individuals had serum IL-6 level less than the standard levels. No significant difference appear in the serum IL-6 concentration between the two age groups of patients (P_2=0.70).

Correlation between Lymphocyte Proliferation and Serum Cytokines Levels in T1DM Patients : It has been found that cell mediated immunity level presented by proliferative percentage in response to CVB_5 was directly positive correlated with the serum IFN-γ level (r =0.332, P<0.05) figure (1), and inversely correlated with the serum IL-6 levels (r = -0.326, P<0.05) figure (2), while a weak inversely correlation has been gotten with IL-10 level (r = -0.18). Concerning the proliferative percentage in response to Polio vaccine, it has been found that there was a direct positive correlation with the serum IFN-γ and IL-10 levels (r =0.332 figure (3), r = 0.619 figure (4) respectively, P<0.05), while a weak inversely correlation was found with the IL-6 serum level (r = -0.134). The proliferative percentage in response to Adenovirus was also found in direct positive correlation with the serum IFN-γ level (r = 0.54, P<0.05) and with IL-10 level (r = 0.25) and inversely correlated with the IL-6 levels (r = -0.27)

Table- 1: Comparison of mean proliferation percentage of PBL in response to Con- A, CVB_5, Polio vaccine and Adenovirus between controls and T1DM patients.

Mitogen	≤10 years					>10 years					
	Groups	No.	Mean	SE	P_1	Groups	No.	Mean	SE	P_1	P_2
Con-A	Controls	21	85.93	10.60	0.82	Controls	29	92.70	10.2	0.62	0.57
	T1DM	36	83.33	5.60		T1DM	24	86.04	8.27		
Viral antigens											
CVB_5	Controls	21	49.16	5.88	0.061	Controls	29	51.20	5.97	0.14	0.57
	T1DM	36	36.67	3.08		T1DM	24	38.87	5.08		
Polio vaccine	Controls	21	47.38	5.83	0.045 (S)	Controls	29	40.86	3.28	0.004 (S)	0.14
	T1DM	36	34.44	2.79		T1DM	24	28.30	3.28		
Adeno-virus	Controls	21	20.67	2.24	0.82	Controls	29	28.61	3.73	0.23	0.35
	T1DM	36	19.97	1.61		T1DM	24	23.02	3.27		

P_1: T1DM patients *vs.* control
P_2: T1DM patients ≤10 years *vs.* patients >10 years old

Table 2: Mean concentration of serum hIFN-γ in healthy subjects and T1DM patients groups.

Parameters	≤10 years							>10 years							P_2
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	
hIFN-γ (pg/ml)	Controls	21	42.66	3.95	12.67	81.33	0.005 (S)	Controls	28	40.39	1.19	10.83	90.37	0.006 (S)	0.73
	T1DM	36	75.60	10.3	30.1	345.5		T1DM	24	70.78	9.78	30.94	203.93		

P_1: T1DM patients *vs.* controls
P_2: T1DM patients ≤10 years *vs.* patients >10 years old.

Table 3: Mean concentration of serum hIL-10 in control and T1DM patients group.

Parameters	≤10 years							>10 years							
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	P_2
hIL-10 (pg/ml)	Controls	21	57.01	9.92	20.97	97.62	0.003 (S)	Controls	29	59.50	12.6	20.43	81.37	0.037 (S)	0.04 (S)
	T1DM	36	104.92	8.81	57.63	360.0		T1DM	24	84.22	4.67	61.86	141.36		

P_1: T1DM patients *vs.* controls
P_2: T1DM patients ≤10 years *vs.* patients >10 years old.

Table 4: Mean concentration of serum hIL-6 in control and T1DM patients groups.

Parameters	≤10 years							>10 years							
	Groups	No.	Mean	SE	Min.	Max.	P_1	Groups	No.	Mean	SE	Min.	Max.	P_1	P_2
hIL-6 (pg/ml)	Controls	19	80.4	56.4	0.1	524.10	0.036 (S)	Controls	17	81.6	69.0	0.1	499.61	0.04 (S)	0.70
	T1DM	32	147.6	45.0	0.1	1018.70		T1DM	20	171.8	80.2	0.1	981.30		

P_1: T1DM patients *vs.* controls
P_2: T1DM patients ≤10 years *vs.* patients >10 years old.

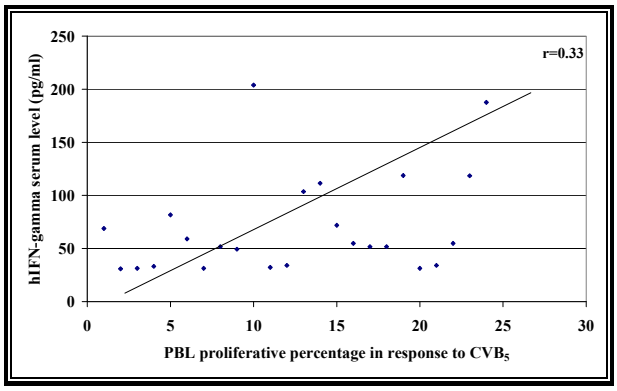

Figure 1: Direct linear regression and correlation between proliferative percentage of PBL in response to CVB5 and serum level of IFN-γ.

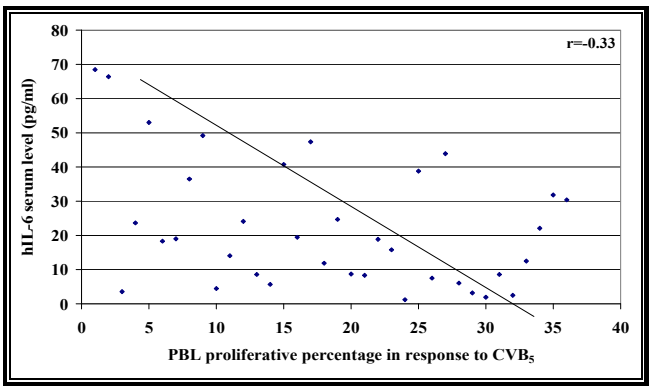

Figure 2: Inverse linear regression and correlation between PBL proliferative percentage in response to CVB_5 and serum level of IL-6.

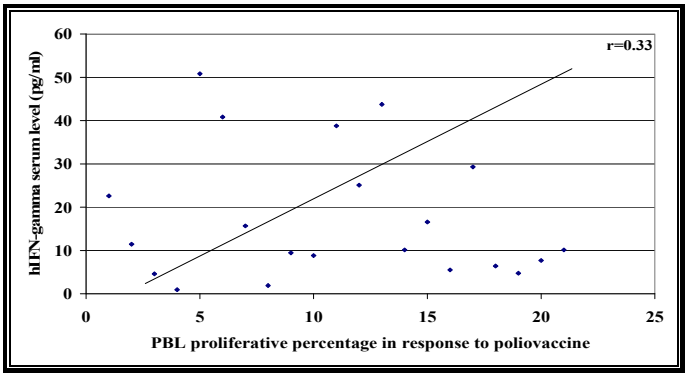

Figure 3: Direct positive linear regression and correlation between PBL proliferative percentage in response to Polio vaccine and serum level of IFN-γ.

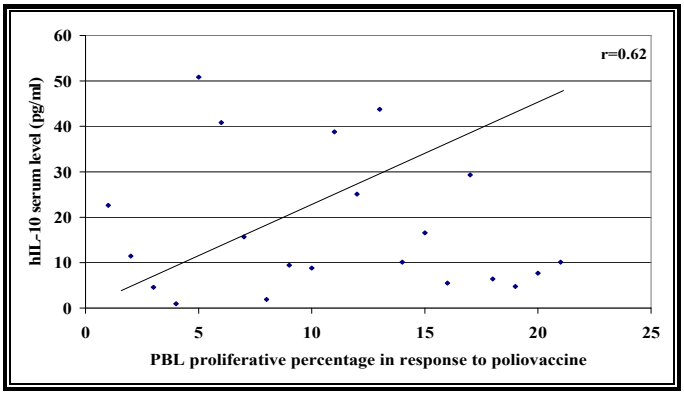

Figure 4: Direct linear regression and correlation between PBL proliferativ percentage in response to polio vaccine and serum level of IL-10.

Discussion

Peripheral Blood Lymphocyte Proliferation: The use of lymphocyte proliferation technique is based on the capability of the lymphocytes for responding to an antigen (specific response) which has induced memory lymphocyte, either by vaccination or by natural infection. These lymphocytes, when they are repeatedly contacted with antigens, have a blastogenic transformation [12].

The proliferative percentage of PBLs has been found lower in T1DM patients than in healthy controls in response to Con-A. Considering the responses to viral antigens, proliferative responses against CVB_5 and Adenovirus were tended to have a lower percentage in T1DM patients than controls, but these values were not statistically different, while the proliferative responses against Polio vaccine were significantly lower in patients especially in >10 years old group than controls. No differences in immune responses were found between patients in the two age groups The low proliferative responses against CVB_5 antigen at disease onset is in agreement with other studies showing reduced T-cell proliferation against CVB_4 [13]. Juhela *et al.,* 2000 found that PBLs of the children at onset of T1DM had significant weaker responses to purified CVB_4 and non-significant decrease in response to Poliovirus type 1 and 3 than healthy children, while the responses to Adenoviruses did not differ between patients and controls [14].

The decreased responses of PBLs in the present study may be due to redistribution of virus-specific T-cells, with virus-responder cells presumed to have homed to the pancreas and therefore unavailable for detection in peripheral blood [15], and so T-cell responses to various viral antigens may be suppressed at the onset of the disease. On the other hand Varela-Calvino *et al.,* indicated abundance of circulating primed CVB_4 specific responder T-cells that secretes IFN-γ in patients with relative lack of proliferation [13].

IFN-γ

The present data demonstrated that serum IFN-γ concentration were higher in patients with T1DM compared to its concentration in the healthy controls. These data were in common with other studies which stated that proinflammatory cytokines IFN-γ may play an important role in the pathogenesis of T1DM, and its concentration was higher in patients [13, 16]. Many studies largely support the concept that β-cell destructive insulitis is associated with increased expression of Th_1 cytokines (IFN-γ, TNF-β, IL-2) and IL-12 [17].

Mechanically, proinflammatory and Th_1 cytokines including IFN-γ induced and accelerated β-cell destruction through different mechanisms, by exerting their effects primarily at the level of macrophages, enhancing infiltration of these cells in the islet, thus accelerating β-cells destruction through the release of synthesized cytotixc mediators like nitric oxide and oxygen radicals [18], or by induction T-cells infiltrate the islets (MHC class I restricted CD_8^+ T-cells) which could bring about extensive tissue damage on β-cells [17, 19, 2], or by rendering β-cells susceptible to T-cell mediated killing via induction of Fas (CD_{95}) receptor on their surface [20, 7]. A direct linear correlation was found between IFN-γ and PBL proliferative percentage in response to CVB_5 ($r = 0.33$), Polio vaccine ($r = 0.33$) and Adenovirus ($r = 0.54$). This might indicate a previous exposure of lymphocytes to tested viruses and might enhance the release of IFN-γ by effectors memory subsets in response to viruses. Heitmeir *et al*., 2001 proposed a model for the effects of dsRNA (the viral replicative intermediate) plus IFN-γ induced β-cell damage [21] (Figure -5). In the course of viral infection, dsRNA, the active component that activates the antiviral response, stimulates IL-1β expression by β-cells and IL-1α and IL-1β expression by macrophages. IL-1β requires proteolytic

processing for activation, an event that appears to be mediated by IFN-γ induced ICE (IL-1β-converting enzyme) activation in β-cell. Active ICE cleaves inactive pro-IL-1β to the active mature cytokine resulting in the release of IL-1β by β-cells followed by autocrine or paracrine stimulation of adjacent β-cells to express iNOS (nitros oxide sysnthase) and produce nitric oxide (NO). The local release of IL-1 by resident macrophages has also contributed to the IFN-γ induced iNOS expression by the islet results in a potential inhibition of insulin secretion and islet degeneration. However, in the presence of inflammatory T-cells capable of producing IFN-γ, viral infection would be predicted to induce islet cell necrosis in addition to apoptosis, and the necrotic events may increase islet inflammation because the necrosis of β-cells and release of β-cell antigens and induction of autoimmunity directed against remaining β-cells [22].

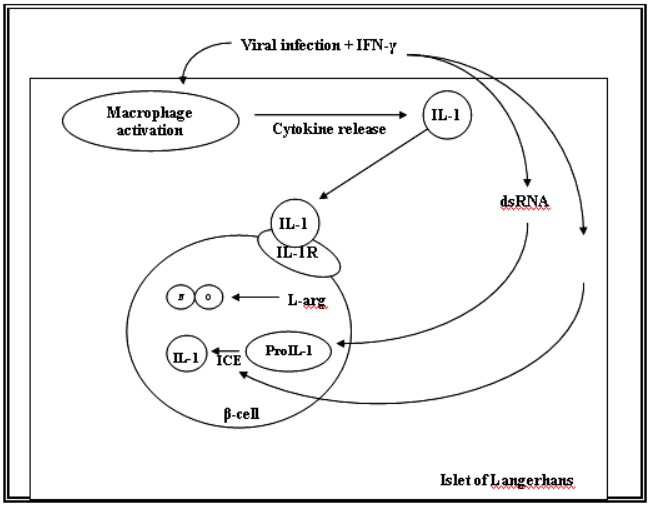

Figure 5: Schematic model of viral infection + IFN-γ induced beta-cell damage IL-10 [21]

The results of this study indicated a high level of serum IL-10 in T1DM patients compared to healthy controls. This result was encountered to many reports which found that T1DM could be prevented by induction of Th_2 cells or by treatment with Th_2 cytokines which in turn blocked the production of Th_1 cytokines [23]In contrast, other reports pointed against the anti-inflammatory action of Th_2 cytokines. Th_2 cytokines (IL-10 but not IL-4) were shown to be involved in T1DM pathogenesis through facilitation of pancreatic mononuclear cells infiltration [24] and producing intense and generalized pancreatitits and insulitis associated with islet cell necrosis in NOD mice [25]. This promoted the conclusion that T1DM is a Th_1 and Th_2 mediated autoimmune disease.

Functionally, Th_2 cytokines, in particular IL-10, may promote necrosis through occlusion of the microvasculature, thereby resulting in hypoxia and reducing the viability of the larger islets [26]. IL-10 is a potent B-cell activator, enhances MHC class II expression on B-cells, thus promoting peri-insulitis and insulitis [6]. Due to its role as cytotoxic T-cell stimulatory factor, IL-10 may stimulate activated T-cells and it's essential for an early phase of diabetes [9]. In any event, Th_2 cytokines can no longer be viewed as "protective" of T1DM.

IL-6: The present results indicated elevated serum levels of inflammatory IL-6

in T1DM patients as compared to controls that added to the evidence that the disease is an immunoinflammatory disorder. This result is in common with other reports [27, 10]. Interlukin-6 is a powerful inducer of hepatic acute phase protein (C-reactive protein) which is known to increase inflammation and the development of vascular disease and atherosclerosis [28]. Elevated C-reactive protein level detected in infants and young children before the onset of T1DM [29] may provide an additional marker for risk of progression to T1DM. Mechanically, IL-6 may exert its effect by inducing a condition with increased energy expenditure in the islet, through elevated glucose oxidation and oxygen uptake accompanied by a partial inhibition of the glucose stimulated insulin release and lowering the islet cellular ATP contents [30].

Viruses can also interact with and modulate the cytokine responses to IL-6. A possible link between virus infection and elevated IL-6 was postulated by Kishimoto, 1992. A nuclear factor controlling IL-6 gene expression (NF-IL-6) was also involved in the transcriptional regulation of various acute-phase protein genes. The NF-IL-6 was shown to recognize the enhancer core sequence of several viruses, suggesting a possible relationship of virus infection and IL-6 expression and possible autoimmune clinical outcome [31]. In this process it might be postulated that, following viral infection and local cytokine secretion immune reactivity undergoes dysregulation and produce organ specific autoimmune disease.

Conclusions:

The present results show that T- cell proliferation in response to viral antigens were decreased in T1DM patients, with a significant elevation of serum levels of IFN-γ, IL-10 and IL-6 in those patients.

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