Original Article

Association of Anti-Coxsackievirus-B IgG With IFN-α, IFN-β, IL-6 and TNF-α in Children With Type 1 Diabetes

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Abstract

Rapid Background: Type 1 Diabetes (T1D) is an auto-immune illness distinguished by the gradual and subtle loss of pancreatic beta cells over time. Coxsackievirus-B is an enterovirus that preferentially targets pancreatic cells. Cytokines are the primary inflammatory mediators which play an important function in preventing the death of beta cells. Aim: This study aimed to identify interferon alfa, interferon beta, interleukin-6, and tumor necrosis factor-alpha which are pro-inflammatory and stimulate the activation of immune cells and anti-cell autoreactive T lymphocytes. In addition, they have a substantial role in the development of type 1 diabetes in some viral infections. Materials and Methods: A total of 75 children with type 1 diabetes were enrolled in the present study between January and March of 2021. The levels of Coxsackievirus B IgG, interferon alfa, interferon beta, interleukin-6 and tumor necrosis factor-alpha in the serum of the participants were determined using the enzyme-linked immunosorbent assay. Results: The T1D patients had significantly greater levels of anti-Coxsackievirus-B IgG antibodies than controls. The results of interferon alfa, interferon beta, interleukin-6 and TNF-a were tested and correlated with Coxsackievirus IgG antibodies. Conclusion: Patients with anti-Coxsackievirus-B IgG antibodies were found to have higher levels of these pro-inflammatory cytokines The results showed that these cytokines have an important link between many viral and immune factors which are involved in the pathology of Type 1 diabetes.

Keywords: Coxsackievirus-B, Interferon-α, Interferon-β, Interleukin-6, Type 1 diabetes, Tumor Necrosis Factor-α

Introduction

Type 1 Diabetes (T1D) is an auto-immune illness distinguished by the gradual and subtle loss of pancreatic beta cells over time. Besides environmental elements, genetics play an important role in the incidence and progression of T1D, for instance, the gene variants of MHC class II, particularly the HLA-DR and HLA-DQ genes have been linked to T1D vulnerability.[1] The T helper 1 (Th1) cells, which are responsible for the breakdown of the immune system, are a major factor in the illness because they promote cell-mediated immune responses. Moreover, they are required for host defense against intracellular viral and bacterial pathogens, resulting in the development of insulitis.[2]

Initial step for the diagnosis of MPE is to confirm wheth Type 1 Diabetes has been linked to a number of environmental variables including enterovirus infection.[3] Coxsackievirus B (CVB), an enterovirus, preferentially targets pancreatic cells and is found in the pancreas of 60–70% of T1D patients. However, 6% of non-diabetic individuals' pancreases are also affected by this virus.[4]

Cytokines are the most important inflammatory mediators and are essential in preventing the death of β-cells from occurring.[5] Many novel cytokines have been studied in the non-obese diabetic (NOD) mouse model to counteract the immune-mediated β-cell destruction

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caused by T1D.[2] The pathophysiology of this auto-immune disorder gets more complicated as the disease progresses and the inflammation increases. This makes it hard to figure out which cytokines play a specific role in the progression of the disease.[5]

Type 1 Diabetes patients have elevated blood levels of Interferon-alpha/beta (IFN-α/β), as well as islet β cells that express the cytokine and plasmacytoid dendritic cells (pDCs) that produce it.[2] Following a viral infection, Interferon regulatory factor 7 (IRF7) is required for the production of IFN-α whereas, activation of IFN-β occurs immediately.[6] IFN-α causes endoplasmic reticulum stress, apoptosis and up-regulation of HLA-I molecule in human pancreatic cells indicating its involvement in the death of β-cells.[7] It has been proven that medications which target IFN-α and its signaling are effective in delaying the progression from prediabetes to T1D.[2]

A number of investigations in NOD mice have demonstrated a relationship between the elimination of pancreatic pro-inflammatory infiltrates and the generation of local Interleukin-6 (IL-6).[8] However, the evidence on IL-6 serum levels in T1D patients is inconclusive.[2] It has been shown that increased IL-6 expression in pancreatic beta cells is associated with severe insulitis, which is accompanied by the invasion of B cells, macrophages and T cells.[8]

Islet-infiltrating dendritic cells (DCs) and macrophages present the exogenous islet antigens to cytotoxic T lymphocytes through an alternative CD40/CD154-independent pathway to MHC-I expression, which has been shown to expedite the death of beta cells.[9] Activation of pancreatic lymphocytes by Tumor Necrosis Factor-alpha (TNF-α) has been previously shown to enhance the expression of dendritic cell maturation markers in the CD-$11b^{+}CD11c^{+}$ fraction. These findings suggest that TNF-α has a significant impact on the development of T1D.[2]

The goal of this study was to examine the relationship between anti-Coxsackievirus-B IgG and T1D disease. Furthermore, the researchers sought to identify if there is any link between the cytokines IFN-α, IFN-β, IL-6 and TNF-α and anti-Coxsackievirus-B IgG by investigating their levels in Iraqi children with this autoimmune disease. .

Materials and Methods

Specimen collection

After the approval from the Institutional Review Board (IRB) of Al-Nahrain University (approval declaration number 2/3/191), a total of 75 children diagnosed with T1D from January to March, 2021 were recruited in this study. These patients were diagnosed with T1D at the Thi-Qar governorate diabetic and endocrine glands center. In addition, the left over blood samples of 75 apparently healthy children who visited Bint Al-Huda Children's Hospital and had no family history of T1D were included as control samples. The age of patients and controls ranged from one month to 15 years.

Before taking the blood samples, written agreement was obtained from the children's guardian. A disposable syringe was used to draw 5ml of blood from the patient's venous system. In order to separate the serum from the blood, each sample was placed in a sterile plain tube and allowed to stand at room temperature for 20 minutes. Later, the samples were centrifuged for 5 minutes at 3000 revolutions per minute., and the

Serology

An enzyme linked immunosorbent assay (ELISA) was used to measure the levels of Coxsackievirus B IgG, IFN-α, IFN-β, IL-6 and TNF-α in the serum. Then the cut-off value was used to detect the quantitative measurement of Coxsackievirus B IgG.

Statistical analysis

Data was analyzed using statistical software SPSS version 25. The mean and standard deviation of the data were calculated on the basis of the assumption that the data had a normal distribution. After determining if the data had a non-normal distribution that was statistically significant for the study, the Mann-Whitney U test was used to determine the median and range of each variable. The receiver operating characteristic curve (ROC) was used to investigate the diagnostic efficacy of various cytokines and anti-Coxsackievirus IgG. P- value of less than 0.05 was considered statistically significant.

Results

Cytokine levels among type 1 diabetes patients

With respect to the IFN-α concentration, highly significant difference was observed between patients and controls. In the former group, the median IFN-α concentration was found to be 0.5 picograms per milliliter (pg/ml) (range = 0.04–15.64 pg/ml), while that in controls was 0.63 pg/ml (range = 0.39–0.86 pg/ml).. Similar significant results were observed in both groups when the level of cytokines were observed in them. These included higher levels of IL-6 (2.05 pg/ml) and TNF-α (15.67 pg/ml) in patients as compared to controlsi.e. 1.75 pg/ml and 6.98 pg/ml (Table-1).

Cytokines' Diagnostic Values

To determine the diagnostic value of an immunocytokine, the data was analyzed using a receiver operating characteristic (ROC) curve. The area under the curve (AUC) obtained for IFN-α was 0.645, with a 95 percent confidence interval (CI) of 0.562–0.746 (P = 0.001).

When the test was performed in the presence of IFN-α at a cutoff value of 0.54 pg/ml, the specificity of the test came out to be 71% whereas its sensitivity was found to be 61% (Figure-1).

Table 1: Cytokines levels among type 1 diabetes patients and healthy controls

Cytokines	Type 1 Diabetes population (n=75)	Healthy control population (n=75)	P value*
IFN-α, pg/ml			
Mean±SD	1.86±3.76	0.62±0.14	
Median	0.5	0.63	0.001
Range	0.04±15.64	0.39-0.86	
IFN-β, pg/ml			
Mean±SD	3.5±10.18	0.73±0.12	
Median	0.71	0.75	0.326
Range	0.11- 66.76	0.41- 0.97	
IL-6, pg/ml			
Mean±SD	5.13±8.37	1.75±0.61	
Median	2.05	1.75	0.007
Range	1.03-36.96	0.38-2.87	
TNF-α, pg/ml			
Mean±SD	21.87±21.94	7.06±1.57	
Median	15.67	6.98	< 0.001
Range	3.29-150.51	4.0-9.84	

*Mann Whitney U test

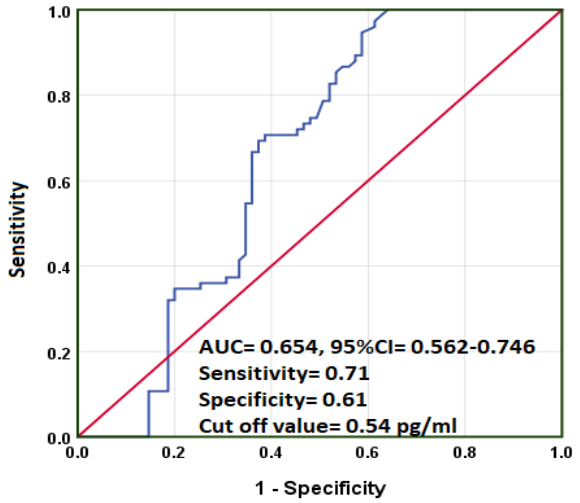

Figure 1: The IFN-α ROC curve in relation to type 1 diabetes patients and controls

The AUC for IL-6 was found to be 0.627 with 95% CI of 0.537-0.716 (P = 0.007). At the cut-off value of 1.81pg/ml for IL-6, the sensitivity and specificity of the test were 58% and 53% respectively, which indicated that the test was highly sensitive and specific. In case of TNF-α, the AUC was 0.968 with 95 percent CI of 0.931-01.0 (P = 0.001).For TNF-α, the cut-off value was 9.43pg/ml and the test's sensitivity and specificity were found to be 96% and 97% respectively (Figure-2).

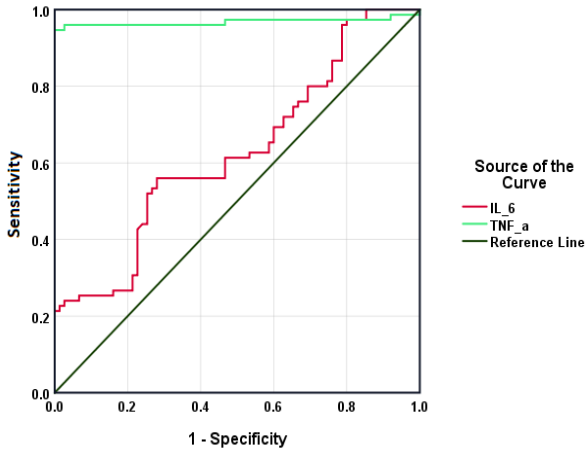

Figure 2: IL-6 and TNF-α receiver operating characteristic curves for T1D patients and controls.

Detection of anti-Coxsackievirus-B IgG Antibody

The median level of anti-Coxsackievirus-B IgG antibodies in patients was found to be 0.85 pg/ml (range 0.25–3.3 pg/ml), which was significantly greater than that in controls i.e. 0.71 pg/ml (range 0.47–0.97 pg/ml) (P = 0.005). Of all the patients, 13.3% (n=10) were tested positive for anti-Coxsackievirus-B IgG antibodies, whereas none of the children in the control group tested positive.

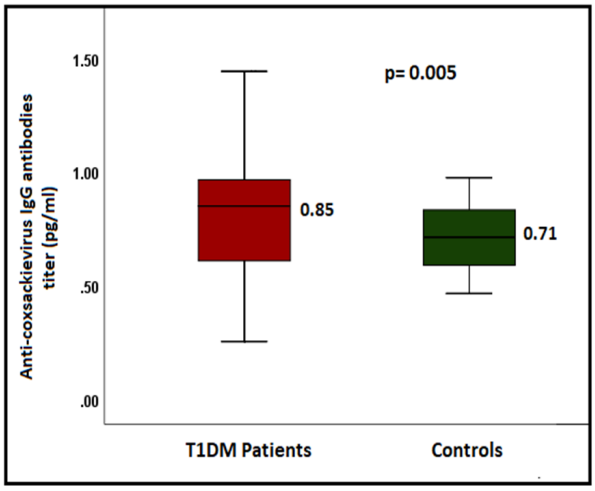

Figure 3: The median levels of anti-Coxsackievirus-B IgG antibody titer in T1D patients and controls

Association of anti-Coxsackievirus-B IgG antibodies positivity with Cytokine tests

Strong correlation was observed between anti-Coxsackievirus-B IgG antibodies and cytokines. The median concentration of IFN-α, IFN-β, IL-6 and TNF-α in patients positive for anti-Coxsackievirus-B IgG antibodies was 10.74 pg/ml, 14.5pg/ml, 23.5pg/ml and 62pg/ml respectively. Table-2 shows median concentration of these cytokines in patients positive and negative for anti-Coxsackievirus-B IgG antibodies.

Table 2: Association of anti-Coxsackievirus-B IgG antibody positivity with cytokine tests

Laboratory Parameters	IgG-positive (10)	IgG-negative (65)	p- value
IFN-α, pg/ml			
Median	10.74	0.43	**< 0.001***
Range	8.43-15.64	0.04-0.9	
IFN-β, pg/ml			
Median	14.5	0.66	**< 0.001***
Range	6.44-66.76	0.11-1.0	
IL-6, pg/ml			
Median	23.19	1.83	**< 0.001***
Range	14.41-36.96	1.03-14.88	
TNF-α, pg/ml			
Median	62.93	15.21	**< 0.001***
Range	50.0-150.51	3.29-18.83	

*Mann Whitney U test

The levels of anti-Coxsackievirus IgG antibody and cytokines in patients were compared using Spearman's correlation to determine whether there was any probable link between them. Significant correlations were found with IFN-α (r = 0.404), IL-6 (r = 0.394) and TNF-α (r = 0.397). In each case, the P value obtained was < 0.001.

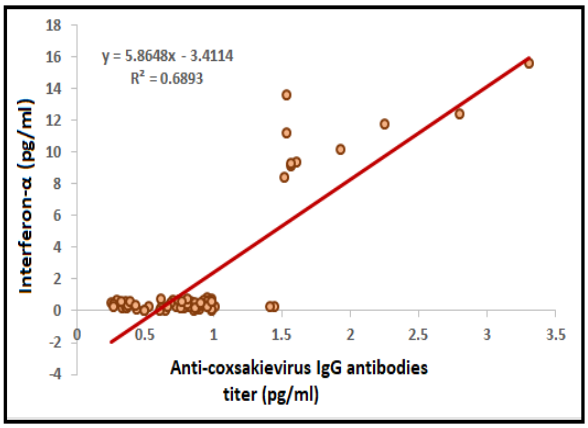

Figure 4: Regression line between anti-coxsackievirus IgG antibodies and IFN-α in T1D patients

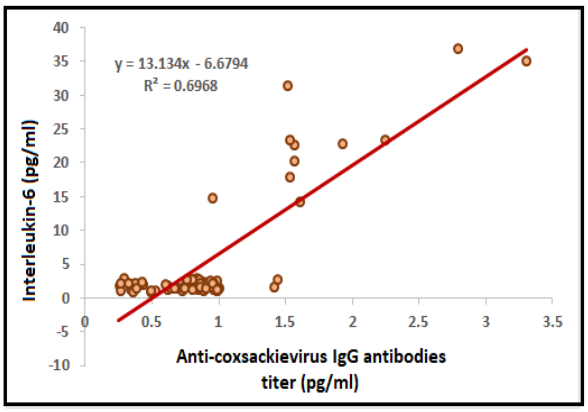

Figure 5: Regression line between anti-coxsackievirus IgG antibodies and IL-6 in T1D patients

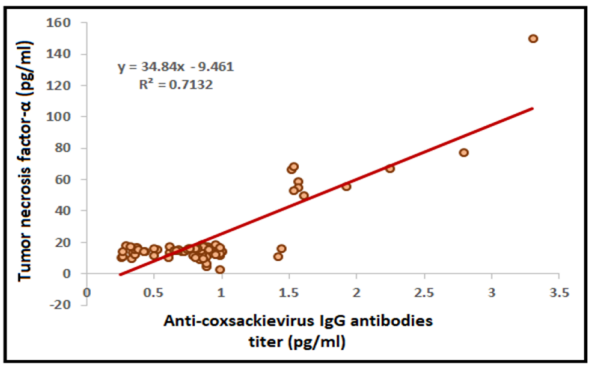

Figure 6: Regression line between anti-coxsackievirus IgG antibodies and TNF-α in T1D patients

Discussion

The findings of this study demonstrate that the T1D group has a considerably higher level of IFN-α than the control group. These findings are consistent with previous studies conducted in the United States by Lombardi *et al.*[10] and Devendra *et al.*[11]. IFN-α is a crucial modulator of innate and adaptive immunity in both mice and human models of infection and infection-related illness. The results of this study have gained new insights into the possible immune-mediated pathways through which IFN-α may induce autoimmunity in a variety of physiological and pathological situations.[10] In response to interferon binding to its receptor, both HLA class I proteins and chemokines are produced which attract macrophages, T cells and natural killer cells to the infected area. Because of this immunological cascade, autoimmunity may develop in people who are genetically susceptible to it. Another early T1D pancreatic islet feature is the presence of Endoplamsic Reticulum stress-modified autoantigens.[11]

The non-significant increase in the level of IFN-β as compared to IFN-α is because most of the T1D patients in this study are from non-viral causes. The expression of IFN-β is regulated by promotor binding of NF-κB, ATF-2/c-Jun (ATF-2/c-Jun) and IRF3 whereas IFN-α expression is regulated by IRF7. In parenchymal cells, all of these transcription factors are constitutively expressed except IRF7, which is induced into a positive feedback loop by IFN-α. This reliance on IRF7 delays the expression of IFN-α after a viral infection. This is in contrast to the expression of IFN-β, which is immediate upon a viral infection. In DCs, however, IRF7 is constitutively expressed and they can rapidly express large amounts of IFN-α.[12]

This study also depicted significantly higher level of IL-6 in T1D group than in control group. These results are in accordance with other studies.[13-15] A study found association between overexpression of IL-6 in beta cells with severe pancreatic insufficiency and infiltration of macrophages and T cells. Another study indicated that

STAT3 activation is associated with increased IL-6 receptor expression in $CD4^+$ and $CD8^+$ T cells from T1D patients, providing further support to the medicinal importance of the IL-6 signaling pathway.[13] This might change how T1D effector T cells move or how they respond to IL-6.[14]

Interleukin-6 is a very effective cytokine that is involved in a variety of biological processes common to all cytokines, including inflammatory and immunological responses. Furthermore, IL-6 not only promotes the production of insulin but also controls its secretion. Some studies have revealed that a low dosage of IL-6 can stimulate insulin secretion, whereas its high concentration can block insulin production. IL-6 may also play a role in the risk of developing T1D in people who have a family history of this disease.[16]

In the present study, significantly higher levels of TNF-α were observed in T1D patients than in control group. These findings are similar to the results reported by other studies conducted in different countries[14,15,17,18] According to a study, TNF-α is involved in the development and operation of the immune system. In response to TNF-α, adhesion molecules are upregulated and macrophages are activated, both of which are necessary in developing T1D.[17] Alterations in regulatory T cells and an imbalance between $CD4^+$ T-helper 1 and 2 cells may contribute to ineffective suppression of pro-inflammatory cytokines in T1D. Infiltrative autoreactive T cells cause pancreatic islet inflammation and subsequent beta cell destruction. Cytokine profile in T1D patients is characterized by significantly increased TNF-α, which has a strong correlation with patient's age and blood glucose level.[19] Another study has showed higher TNF-α level in pediatric T1D patients also. They also reported tendency for higher TNF-α gene expression and methylation levels in the promoter region of this gene.[20]

The current study revealed a significant increase in the level of anti-CoxV-B IgG within T1D patients in comparison with the control group. Although these results are in accordance with various studies[21,22], yet they are also contradictory to some other studies which revealed no differences between anti-CoxV-B IgG in T1D patients and the control group.[23] A negative result for anti-CoxV-B IgG does not rule out the possibility of ongoing or recent infection with the virus. IgG antibodies may not have been present at this point in the disease's progress.

Enteroviruses, like Coxsackievirus, have the potential to initiate or accelerate the disease process, ultimately leading to clinical T1D through different pathways. When diabetogenic $CD8^+$ T lymphocytes cause planned cell death-1, the first mechanism prevents their proliferation by increasing the expression of programmed cell death-1 ligand 1 on lymphoid cells. Second benefit of increasing the number of regulatory T cells which produce transforming growth factor-beta (TGF-beta) cytokine through the $CD4^+CD25^+Foxp3^+$ pathway is that it aids in the maintenance of immunological tolerance in peripheral tissues. The virus-induced cytolysis of pancreatic beta-cells has the potential to cause direct cell death.[23]

Coxsackievirus-B antibodies are frequently reported in newly diagnosed patients with T1D. Therefore, the relationship between this autoimmune disease and Coxsackievirus-B has been investigated in many regions. These studies have reported the presence of IgM and IgG antibodies against Coxsackievirus B3 and B4 in serum samples of patients with T1D. Based on these results, the researchers in the present study proposed that children are at a higher risk of developing T1D if they have been exposed to Coxsackievirus B4.[24]

The current study also found significant association between positive anti-Coxsackievirus-B IgG antibodies and IFN-α, IFN-β, IL-6 and TNF-α in T1D patients in comparison to the results of negative anti-Coxsackievirus-B IgG antibodies. The current findings are consistent with other studies with respect to both IFN-α and IFN-β.[25-30] IFN-α/β stimulates the activation of immune cells, the production of cytokines and the transcription of interferon-stimulated genes, all of which are boosted in an autocrine and paracrine fashion.[30]

IFN-α has the ability to increase inflammation and the activation of anti-cell autoreactive T lymphocytes, which, if left untreated, can lead to the development of autoimmune diabetes.[28] In addition to this, peripheral tolerance may also be compromised by incorrect IFN-alpha/beta synthesis. On the other hand, studies have also shown that in NOD mice, persistent IFN-β production impairs the anti-inflammatory IL-10 signaling. Hence, elevated IFN-α/β expression has been linked to the development of T1D in both mice and humans.[31]

The results with respect to the levels of IL-6 in the present study are also consistent with the findings of other studies.[16,29,32,33] In case of viral infection, there are two plausible explanations for the upregulation in IL-6 production: (i) increased viral load has increased ability to evade the immune response, and (ii) polymorphisms in the IL-6 gene promoter are associated with increased IL-6 production after viral infection.[16]

The results related to TNF-α in the present study are also similar to other studies.[29,33] Several studies have shown that infection with the Coxsackievirus B4 (CVB4) may lead to the generation of pro-inflammatory cytokines like IL-1β and TNF-α. Other studies have postulated that immune-mediated cytokines produced in response to the virus may have a substantial role in the development

of T1D in some individuals. According to a study[34], TNF-α, in particular, has been involved in the destruction of insulin producing cells.

The strong evidence for the role of Coxsackievirus-B in the pathogenesis of T1D is originated from a plethora of studies including human studies and *in vitro* / *in vivo* mechanistic animal studies. Since T1D clearly involves autoimmune phenomenon, the present research focused on the activation of autoimmune responses against β-cell proteins and investigating the antibodies against Coxsackievirus-B which may play a role in T1D development. Other possible mechanisms include bystander activation of autoreactive T-cell clones driven by virus-induced inflammation and the secretion of pro-inflammatory cytokines. Because Coxsackievirus-B can infect and damage human β-cells *in vitro,* therefore, virus-induced apoptosis or necrosis is also possible.

Conclusion

Patients with anti-Coxsackievirus-B IgG antibodies had higher levels of the cytokines IFN-α, IFN-β, IL-6 and TNF-α in their blood than the control group. The presence of these cytokines in CVB-T1D patients shows that CVB viruses may play an important role in the development of autoimmune illnesses such as type 1 diabetes.

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Author Contributions

The study's planning, design, analysis and interpretation were completed by all of the authors.

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Nil

Conflicts of Interest

There are no conflicts of interest.

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