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Research Article

Increased incidence of Anti - IgG of Coxsackievirus and Cytomegalovirus among diabetic children in Egypt.

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Abstract: Cytomegalovirus (CMV) and Coxsackievirus (CV) are included in the environmental factors potentially relevant to the pathogenesis of type 1 diabetes (T1D). Thus, this study aimed to detect the prevalence of both anti-IgG for each of CV and CMV in diabetic children with (EV^+) or without (EV^-) enteroviruses infection. The current study revealed that the T1D-EV^+ and T1D-EV^- groups had marked elevations in hemoglobin A1c (HbA1c), C-reactive protein (CRP) and glutamic acid decarboxylase autoantibodies (GADA) as compared to non-diabetic control. Moreover, anti-IgG for the CV and CMV groups were detected in three groups; diabetic infected (T1D-EV^+), diabetic non-infected (T1D-EV^-) and control group. Detection of anti-CV IgG achieved 22.7%, 6.7% and 64% in control, T1D-EV^- and T1D-EV^+, respectively. However, detection of anti-CMV IgG revealed 23.4%, 50% and 40% in control, T1D-EV^- and T1D-EV^+, respectively. In conclusion, the prevalence of the antibodies of CV-IgG and CMV-IgG in the sera of diabetic children more than that in healthy control children may give a role of these viruses in the etiology and progression of T1D.

Keywords: Type 1 diabetes, Enteroviruses, Coxsackievirus, Cytomegalovirus, IgG

Introduction

T1D was one of the most common pediatric chronic diseases, particularly in children under 5 years of age (Patterson *et al.,* 2012). T1D was characterized by dysregulated blood glucose caused by β-cell insufficiency accompanied by increased hemoglobin A1C (Xiao *et al.,* 2014; Ismail *et al.,* 2016). Elevated C-reactive protein (CRP) is known to be a marker of inflammation. Increased CRP levels have been described in people with diabetes (Pradhan *et al.,* 2001). Immunological and metabolic biomarkers were used widely in the diagnosis of T1D or b cell destruction (Lebastchi and Herold, 2012). Immunological markers included islet cell cytoplasmic autoantibodies (ICA), glutamic acid decarboxylase autoantibodies (GADA), insulin autoantibodies (IAA) and pancreatic b cell-specific zinc transporter (ZnT-8) autoantibody and the measurement of these autoantibodies can be of a significant value to the clinician in the prediction, diagnosis, and management of patients suffering from diabetes (Kawasaki *et al.,* 2004).

Cytomegalovirus (CMV) is included amongst the environmental factors potentially relevant to the pathogenesis of T1D (Aarnisalo *et al.,* 2008). CMV infection has been also linked to the induction of new onset post-transplantation diabetes (Hjelmesaeth *et al.,* 2004; Leung *et al.,* 2008) as an independent risk factor. Post-transplantation diabetes is a common complication of organ transplantation, recognizing multiple causes, including the effects of immunosuppressive drugs, increasing age, ethnicity, post-transplantation weight gain (Bonatti *et al.,* 2008). The pathogenetic mechanisms underlying CMV-induced diabetes remain unclear, but several hypotheses have been discussed (Ercolini and Miller, 2009). This association raises the possibility that CMV prophylaxis may be a useful and cost-effective strategy for management of new-onset post-transplantation diabetes.

Also, one of the most widespread and effective environmental factors is the infection with enteroviruses (EVs) which accelerate β-cell destruction in type 1 diabetes (T1D). The Enterovirus genus of the Picornaviridae family consists of small, non-enveloped, positive, single-strand RNA viruses, including polio viruses, Coxsackievirus (CV) A and B and echoviruses (Bergaminand and Dib, 2015). Transmission methods were identified to be faecal–oral or, less commonly, respiratory routes. Data from initial

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epidemiological studies suggested a possible link between EVs, in particular, CVB4, and T1D (Jaïdane and Hober 2008). The aim of this study is to detect anti-IgG for each of CVs and CMV in sera of diabetic children with and without enteroviruses infection and in healthy control children.

Materials and Methods

Study population:

The study population consisted of 100 children with T1D and 50 healthy controls. Children with T1D ranged from 2 to 16 years, while healthy controls ranged from 3 to 14. All of these samples were assayed previously for enteroviruses detection using reverse transcriptase- PCR. In the present study the samples were divided into three groups, T1D- EV+, T1D- EV+ and control (where n=50 in each group).

Laboratory assessment

HbA1c ELISA kit was purchased from MyBiosource (California, USA). CRP ELISA kit was purchased from R&D Systems (Minneapolis, Canada). GADA was determined using ELISA kits purchased from MyBiosource (California, USA). ELISA classic CV and CMV IgG kit were purchased from Serion GmbH (Würzburg, Germany). Procedure was performed according to the kit instructions.

PCR of Enterovirus

Viral RNA extraction was performed using BIOZOL® Total RNA Extraction Reagent (Bioflux, Tokyo, Japan) and according to the manufacturer's instructions. RT- PCR was performed as previously described (Puig *et al.,* 1994). Extracted samples (10 µl) were heated to 99^{0}C for 5 min. Salts, nucleotides, primers and 100 U of reverse transcriptase (Thermoscientifc Fisher, Massachusetts, USA) were added in 5µl final volume, and 0.3 µM of primer. The samples were incubated for 60 min at 42˚C. Five µl of the RT product were added to a final volume of 50 µl of the PCR reaction mix After a denaturation step of 94°C for 4 min, 40 cycles of amplification at 92°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2 min were performed with a final extension of 72°C for 10 min. The nested PCR involved adding 2 µl of first-round PCR product to a 48µl PCR mix. Cycling conditions for nested PCR were the same as first PCR. PCR products (10 µl) were analyzed by electrophoresis on 1.5% agarose gels (Panreac-spain). Marker V (Roche Molecular Biochemicals, Indianapolis IN) was used as molecular weight standard. The presence of DNA bands was determined under ultraviolet light.

Statistical analysis

Analysis of data was carried out using an IBM computer utilizing statistical program for social science (version 23.0; Chicago, Illinois, USA). Description of quantitative variables was given as mean and SD. The ANOVA test was used for comparison of quantitative data among different time points in the same group. P values less than 0.05 were considered significant and P values less than 0.01 were considered highly significant.

Results

The mean levels of HbA1c in children (T1D -EV+ and T1D -EV-) were significantly higher than control ($p< 0.001$; Figure 1). CRP (as a marker of inflammation) induced a highly significant increase in both T1D -EV+ and T1D -EV$^-$ groups as compared to control ($p< 0.001$; Figure 2), however, a significant increase was observed in T1D -EV+ values than that of T1D - EV$^-$ group. GADA increased ($p< 0.001$) in T1D - EV$^-$ compared to control but showed further increase ($p< 0.001$) in T1D – EV+ compared to T1D - EV$^-$ (Figure3).

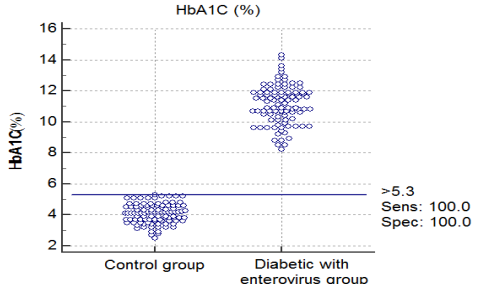

Figure 1. Scatter diagram of HbA1C (%) in diabetic patients and healthy control group.

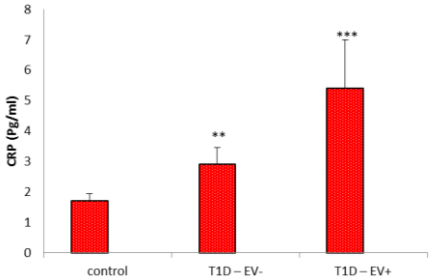

Figure 2. Determination of and CRP in control, EV$^-$ and EV$^+$ groups. Means ± SD are represented. ** $p< 0.001$, when compare between control and T1D EV$^+$, T1D EV$^-$.

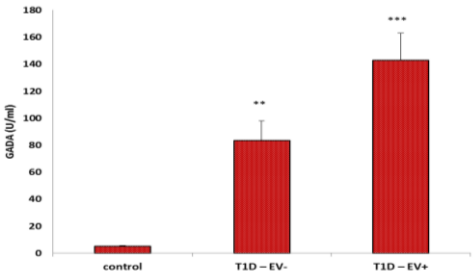

Figure 3: Determination of GADA in control, EV$^-$ and EV$^+$ groups. Means ± SD are represented. ** $p< 0.001$, when compare between control and T1D EV$^+$, T1D EV$^-$.

Frequency of anti-CV and anti- CMV IgGs

Detection of anti-CV IgG and anti-CMV IgG revealed 22.7% positivity in control, 6.7 % in T1D - EV^- and 64% in T1D – EV^+ groups for anti-CV IgG, while anti-CMV IgG revealed 23.4% in control, 50% in T1D - EV^- and 40% in T1D – EV^+ groups. T1D - EV^- group showed no change ($p > 0.05$) in anti-CV IgG levels compared to control, while T1D – EV^+ showed an increase ($p < 0.001$) versus T1D - EV^-, while anti-CMV IgG in T1D - EV^- group showed an increase ($p < 0.001$) compared to control and T1D – EV^+ (Figure4).

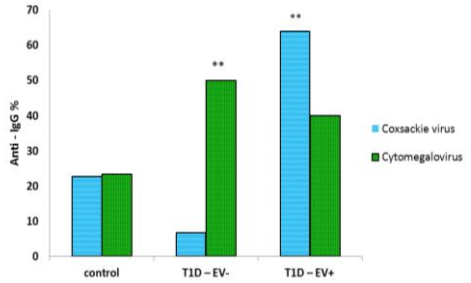

Figure 4: Determination the percentage of anti-IgGs for CV and CMV in EV^-, EV+ and control groups. ** $p < 0.001$, when compare between control and T1D EV^+, T1D EV^-. No change ($p > 0.05$) in anti-CV and CMV IgG levels in control group.

Discussion

Type 1 diabetes (T1D) is a chronic heterogeneous disease characterized by the progressive autoimmune destruction of pancreatic b-cells. The incidence of T1D is rising by an average of 3–5% in recent years, which cannot be fully explained by genetic predisposition alone (Onkamo *et al.,* 1999). Moreover, the concordance rate for developing T1D among monozygotic twins is about 66%, lower than that for type 2 diabetes (Redondo *et al.,* 2008). Hence, it is likely that environmental factors play a significant role during T1D development (Regnéll and Lernmark, 2013). The classical form of autoimmune type 1 diabetes usually presents with a markedly elevated HbA1c value, because autoantibodies against islet beta cells are present for months or even years prior to the development of marked hyperglycemia, and the beta cells are damaged gradually (Eisenbarth, 2005). This was observed in our study and especially increased in the presence of EVs.

Many viruses have been implicated in T1D in both animal models and humans with varying levels of evidence. Historically, the prevalence of viral infections in T1D was explored either by genomic approaches (which work if the viral nucleic acids remain present at the time of assay) or immunological approaches that only evaluated one viral protein or one type of virus at a time (Banatvala *et al.,* 1985). Viral DNA or mRNA were detected by PCR or in situ hybridization in a relatively low-throughput manner (Foy *et al.,* 1995). Enteroviruses (EV) are found to play a major role in induction of the disease based on three possible mechanisms (Jun and Yoon, 2003). The first is the invasion of pancreatic cells by viruses through cell surface receptors and replication followed by induction of innate immunity against the cells leading to destruction (Zanone *et al.,* 2007). The second is altered immunity as a result of viral infection leading to autoimmunity. The third is the induction of cross-reactive immunity between EV and pancreatic b cell antigens as a result of antigen mimicry. EVs are the most likely viruses to be linked with the onset of T1D (Varela-Calvino and Peakman, 2003).

Specific anti-CMV IgG antibodies in 169 serum samples from children who had developed the first T1D-associated autoantibody by the age of 2 years and in parallel, 791 serum control from healthy children (Aarnisalo *et al.,* 2008). No association between perinatal CMV infection and progression to T1D was observed. This study concluded that perinatal CMV infections were not particularly associated with early serological signs of β-cell autoimmunity or progression to T1D in children with diabetes risk-associated HLA genotype (Aarnisalo *et al.,* 2008). However, serological, immunological, histological signs of autoimmunity and allograft rejection appeared concomitantly with early CMV infections in one T1D patient receiving pancreas allograft. This observation suggests that persistent CMV infections might be relevant to the pathogenesis of T1D (Zanone *et al.,* 2010).

The prevalence of EV infection among T1D children revealed 26% positive cases based on EV RNA detection in serum. This finding indicated a probability of infection with other environmental viruses which could be a cause of T1D. It was necessary of investigation of the role of other viral infections among T1D patients (Komulainen *et al.,* 1998). Indeed, Rubella virus, rotavirus, cytomegalovirus and mumps virus were found to be associated previously with children with T1D (Goto *et al.,* 2008). Some clinical studies relate CMV to the development of T1D in humans. The CMV specific viral genome was found in 22% of diabetic patients, correlating with the presence of islet cell autoantibodies in their serum, suggesting that persistent CMV infections may be relevant to pathogenesis in some cases of T1D (Pak *et al.,* 1988). Several hypotheses have been proposed to explain the link between CMV and islet autoimmunity (Ercolini and Miller, 2009). These include direct viral destruction of b cells, induction of virus-specific cytotoxic T cells killing virus-infected b cells, enhancement of antigen presentation, bystander activation of autoreactive T cells caused by the inflammatory environment during infection, and destruction by cross-reacting T cells. Notably, GAD65-specific T cells cross-

react with a peptide of the major DNA-binding protein of CMV, suggesting a mechanism of molecular mimicry (Hiemstra *et al.,* 2001).

In our study, the detection of anti-CV IgG revealed 22.7%, 6.7% and 64% in control, T1D-EV- and T1D-EV+ respectively, while detection of anti-CMV IgG revealed 23.4%, 50% and 40% in control, T1D-EV- and T1D-EV+ respectively. ELISA test for anti-CV IgGs gave 64% positivity. This is in turn might also imply the involvement of other EVs like echoviruses in the disease (Klemola *et al.,* 2008). As most of cases were anti-CV IgG positive, it could be realized that CV invaded β-cells through specific receptors, replicated and led to autoimmunity and cell destruction (Oikarinen *et al.,* 2008). This cell destruction was widespread as a result of highly exposed auto-antigens and activation of bystander T cells (Bergaminand and Dib, 2015). ELISA test for anti-CMV IgG revealed 50% in T1D-EV- is in turn might also imply the involvement of CMV in T1D that were negative for enteroviruses.

Aarnisalo *et al.,* (2008) indicated that specific anti-CMV IgG antibodies in 169 serum samples from children who had developed the first T1D-associated autoantibody by the age of 2 years and in parallel, 791 serum control from healthy children. This study concluded that perinatal CMV infections were not particularly associated with early serological signs of β-cell autoimmunity or progression to T1D in children with diabetes risk-associated HLA genotype (Aarnisalo *et al.,* 2008). However, serological, immunological, histological signs of autoimmunity and allograft rejection appeared concomitantly with early CMV infections in one T1D patient receiving pancreas allograft. This observation suggests that persistent CMV infections might be relevant to the pathogenesis of T1D (Zanone *et al.,* 2010).

In conclusion, the high prevalence of the antibodies; anti-CV and CMV IgG in the sera of diabetic children can indicate a role of these viruses in the etiology of T1D.

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