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Assessment of Interleukin 18 in Children with Type 1 Diabetes and Their Relatives: Its Relation to Autoantibodies

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The clinical manifestation of type 1 diabetes mellitus is preceded by asymptomatic prodromal period called prediabetes or preclinical diabetes during which the autoimmune destruction of the insulin producing beta cell in the pancreas progresses. T cells are thought to be the effector cells for the β cells destruction. So we are aiming to study the circulating level of IL18 and antipancreatic autoantibodies in a group of Egyptian children with type 1 diabetes mellitus and their brothers/sisters (relatives) for early detection of the risk of development of type 1 diabetes. This study included 26 children with type 1 diabetes mellitus and 31 of their healthy relatives who are still normoglycaemic without clinical or laboratory criteria of diabetes Forty six age and sex matched subjects were served as a control group. History taking and clinical examination were performed for all children included in the study. Fasting serum samples from patients, their relatives and controls were taken for analysis of: Fasting Blood glucose, anti-glutamic acid decarboxylase antibodies (Anti- GAD65 Ab), tyrosine phosphatase antibodies (anti-IA2 Ab) and anti insulin antibodies (IAA Ab) and Interleukin 18. This study included 26 children with type 1 diabetes mellitus (12 male and 14 female), their mean age was 12±0.77 years (3.5-17 year) and mean duration of disease was 3.8±3.4 year (0.1-14 year). Frequency of anti-GAD antibody was significantly higher in patients with type 1 diabetes and their relatives than controls, while anti IA2 and IAA antibodies were significantly higher in patients with type 1 diabetes than controls. IL 18 was higher in relatives of diabetic patients than diabetic cases and controls but it was statistically not significant. The relatives of diabetic patients were classified as low and high risk for disease according to the results of the antipancreatic autoantibodies (including GAD Ab, IA2Ab and IAA Ab). Relatives with 2 or more positive antibodies were considered high risk for disease. IL 18 did not differ statistically between high and low risk relatives. However only one of the high risk relatives discovered accidently to have diabetes showed very high IL18 level. No significant correlation was found between IL18 and other parameters in diabetic patients and their relatives. Screening for signs of beta cell autoimmunity is crucial to avoid missing young children on route to overt diabetes mellitus. IL18 serum levels are seemed to be increased selectively during early clinical stage of type 1 diabetes. However, Serum IL 18 levels and at least two autoantibodies should be studied on larger scale to give more informations.

Key words: Interleukin 18, antipancreatic auto antibodies, type 1 diabetes mellitus

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INTRODUCTION

The clinical manifestation of type 1 diabetes mellitus is preceded by asymptomatic prodromal period called prediabetes or preclinical diabetes. It may last from a few months to several years, during which the autoimmune destruction of the insulin producing beta cell in the pancreas progresses. T cells are thought to be the effector cells for the β cells destruction. Glutamic acid decarboxylase, insulinoma-associated protein 2 and insulin represent the three major autoantigens. Autoantibodies are early detectable markers of an on going disease process and to diagnose prediabetes (Kulmala, 2003).

The autoimmune attack on pancreatic cells has two distinct stages insulinitis and diabetes and progression of the former to the later appear to be highly regulated. Identifying the factors controlling this transition has been difficult because it is a complex process that occurs non-universally and asynchronously. The interleukins (IL18, IL12) and Tumor necrosis factor alpha (TNF α) were pivotal, their induction occurring almost immediately and their coordinate action being required for the onset of aggression (Andro Schmutz *et al*., 1999).

Interleukin 18 (IL18) a recently identified proinflammatory cytokine, has been implicated in a variety of pathological conditions such as rheumatoid arthritis, insulin dependant diabetes mellitus type 1 and inflammatory liver disease (Suk, 2001).

IL18 is secreted by a variety of cells such as epithelial cells, macrophages and dendritic cells in the process of chronic inflammation (Gutzmen *et al*., 2003).

IL18 is produced not only by types of immune cells but also by non-immune cells. In collaboration with IL12, IL18 stimulates T-helper1 (Th1) mediated immune responses which play a critical role in the host defense against infection by intracellular microbes through the induction of interferon (IFN) γ (Kretowski *et al*., 2002). However, the overproduction of IL12 and IL18 induced severe inflammatory disorder, suggesting that IL18 is a potent proinflammatory cytokine that has pathophysiological roles in several inflammatory conditions. IL18 alone can stimulate Th2 cytokine production as well as allergic inflammation therefore functions of IL18 *in vivo* are very heterogeneous and complicated (Nakanishi *et al*., 2001).

Type 1 diabetes is believed to be a Th1 lymphocyte mediated disease and both environmental and genetic factors play a role in its pathogenesis.

We are aiming to study the circulating level of IL18 and antipancreatic autoantibodies in a group of Egyptian children with type 1 diabetes mellitus and their relatives for early detection of the risk of development of type 1 diabetes.

MATERIALS AND METHODS

This study included 26 children with type 1 diabetes mellitus (12 male and 14 female) and 31 of their brothers/sisters (relatives) who are not known to be diabetic were included in the study (19 male and 12 female). Forty six age and sex matched subjects were served as a control group. Non of the children included in the study suffer from infections, allergic or other autoimmune diseases 6 months before the sampling and they did not use any immunomodulatory drugs 3 months preceding the study. Patients were selected among those attending to the pediatric clinic (National Research Center) in the period between May 2004 and January 2005.

History taking and clinical examination were performed for all children included in the study.

Serum samples from patients, their relatives and controls were analyzed for:

- Fasting blood glucose
- Interleukin 18: Blood was collected in dry tubes, sera were separated from cells by centrifugation and kept frozen at -20° after aliquoting. IL18 was measured quantitatively using the Human IL18 ELIZA Kits* (From Medical and Biological Laboratories Co., LTD, Japan) It was based on sandwich ELIZA. The assay uses two monoclonal antibodies against two different epitopes of human IL18. The concentration of human IL18 was calibrated from a dose response curve based on reference standards. Results were expressed in pg mL^{-1}.
- Circulating anti-GAD-65, anti-IA2 and IgG class insulin autoantibodies (IAA) were detected in serum samples. Anti GAD was measured quantitatively using DRG ELIZA Kit from DRG International, Inc. USA. The GAD value of each patient's serum was determined using its absorbance value and extrapolating from a dose response curve on x-axis. Anti - IAA was detected qualitatively using Isletest-TM ELIZA Kit from Biomerica, Inc., USA.

Anti-IA2 was detected quantitatively by immunoradiometric assay. It was based on sandwich type assay using Immunotech SA, France. Samples and standards were incubated with I125 labeled recombinant IA2. This was followed by addition of protein A to

precipitate any I125 -IA2 / anti- IA2 complexes which were formed after centrifugation. The precipitates were counted for I125. Values were calculated by interpolation from the standard curve. The radioactivity was directly proportional to the concentration of anti- IA2 autoantibodies in the sample. Results were expressed in U mL^{-1}. (N.B) Assessment of antibodies tests can't be done for all cases and controls included in the study as the kits were too expensive so the numbers differ for different tests.

Consents were taken from the parents of the patients to share in the study.

Statistical methods: Statistical Package for Social Science (SPSS) program version 9 was used for analysis of data. Data were summarized as Mean±SE. Non parametric (Mann Whitney test) was used for analysis of two different quantitative samples and we used Kruskal-wallis-H test for analysis of more than two quantitative samples. Chi-Square was done for analysis of qualitative samples. Pearson's correlation was also done, r was considered weak if < 0.25, mild if ≥ 0.25 - < 0.50, moderate if ≥ 0.5-< 0.75 and strong if ≥ 0.75. p-value was considered significant if ≤ 0.05. Cut off of antibodies was calculated as mean±2 SD of all the control group.

RESULTS

This study included 26 children with type 1 diabetes mellitus (12 male and 14 female), their mean age was 12±0.77 years (3.5-17 year) and mean duration of disease was 3.8±3.4 year (0.1-14 year). Frequency of anti-GAD antibody was significantly higher in patients with type 1 diabetes and their relatives than controls, while anti IA2 and IAA antibodies were significantly higher in patients with type 1 diabetes than controls. Although IA2 and IAA were higher in relatives than controls, they were not statistically significant (Table 1).

One positive antibody was found in 10 (33.3%), two antibodies in 17 (56.7%) and three antibodies were found in 3 (10%) of the unaffected relatives of type 1 diabetic patients.

Table 2 showed that IL 18 was higher in relatives of diabetic patients than diabetic patients and controls but it was statistically not significant. The relatives of diabetic patients were classified as low and high risk for disease according to the results of the

Table 1: Percentage of anti insulin, anti IA2 and anti GAD 65 antibodies in patients with type 1 diabetes, their relatives and controls

	Diabetic patients		Sibs of diabetic patients		Controls		p-value	
							Sibs of diabetic Type 1 diabetes	patients
Variables	No.	%	No.	%	No.	%	Vs control	Vs control
Anti insulin (U mL^{-1}):								
High	17	68	3	10	1	7.1		0.6
Normal	8	32	27	90	13	92.9		
Total number	25		30		14		0.0001*	
Anti IA2(U mL^{-1}):								
High	6	31.6	2	11.8	1	2.2	0.002*	
Normal	13	68.4	15	88.2	45	97.8		0.2
Total number	19		17		46			
Anti GAD 65 (U mL^{-1}):								
High	12	46.2	18	62.1	1	2.2		
Normal	14	53.8	11	37.9	44	97.8	0.0001*	0.0001*
Total number	26		29		45			

p-value is significant if ≤ 0.05.

Table 2: Comparison between IL18 in patients with type 1 diabetes, their relatives and controls

Variables	Diabetic patients N = 24	Relatives of diabetic patients N = 28	N = 14	Controls p-value
IL 18 (pg mL^{-1})	214.9±43.8	262.4±32.4	235.4±25.5	0.6

p-value is significant if ≤ 0.05.

Table 3: Comparison between IL18 in relatives of diabetic patients in relation to antibodies (high and low risk)

Variables	High risk N = 9	Low risk N = 19	p-value
IL 18 (pg mL^{-1})	255.3±60.9	265.8±39.2	0.8

p-value is significant if ≤ 0.05, High risk = Two or more positive antibodies, Low risk = No or only one positive antibody

Table 4: Correlation between IL18 and other parameters in patients with type 1 diabetes and their relatives

	Diabetic patients		Relatives	
Variables	r	p-value	r	p-value
Age (year)	- 0.3	0.2		
Duration of disease (year)	-0.2	0.3		
Anti GAD antibodies (U mL^{-1})	0.1	0.7	0.02	0.9
Anti IA2 antibodies (U mL^{-1})	0.3	0.3	0.03	0.9
Anti IAA antibodies (U mL^{-1})	-0.1	0.5	-0.3	0.1

Anti GAD antibodies: Antiglutamic acid decarboxylase antibodies, IA2 antibodies: Tyrosine phosphatase antibodies, IAA antibodies: Insulin antibodies

antipancreatic autoantibodies GAD Ab, IA2Ab and IAA. Relatives with 2 or more positive antibodies were considered high risk for disease.

Table 3 showed that IL18 was lower in high risk relatives but it was statistically not significant. No significant correlation was found between IL18 and other parameters in diabetic patients and their relatives (Table 4).

DISCUSSION

Prediction of type 1 diabetes mellitus (IDDM) and its identification in pre clinical period is one of the central problems in modern medicine. They are based on comprehensive genetic, immunological and metabolic evaluation (Kulaeva *et al*., 2003).

First degree relatives at risk for insulin dependent diabetes mellitus can be predicted by using a combination of immunological markers in prediabetic stage (Mrena *et al*., 2003).

In the present study, according to mean levels for all controls ±2 SD for each antibody: the cut off points for positivity was found to be 1.1 U mL^{-1} for anti GAD65 antibodies and 0.70 U mL^{-1} for IA2 antibodies. Kit used for assessment of anti IAA was qualitative. Prevalence of positivity in patients with type 1 diabetes and their sibs was found to be 68 and 10% for anti insulin antibodies, 46.2 and 62.1% for anti GAD 65 antibodies and 31.6 and 11.8% for anti IA2 antibodies, respectively. In this study the cut off level was similar to that of the kits as we use mean±2 SD as most of the literature use.

Levels and prevalence of auto antibodies against islet antigens reported in various studies vary widely according to ethnic and racial differences in their ranges and cut off limits of positivity, calculated from control subjects. Seissler *et al.* (1996) in Germany defined a cut off limit for IA2 positivity as 3 U mL^{-1} and for GAD Ab as 7 U mL^{-1} calculated according to mean±4 SD of controls, while Kulmala *et al.* (2000) in Finland defined the cut off limit of IA2 antibodies as 0.43 U/Ab and GAD Ab as 6.6 U mL^{-1} calculated according to the 99th centile of controls. On the other hand, Bingley *et al.* (2001) in Italy determined a cut off limit of 1.4 U mL^{-1} for IA2 antibodies and 2.7 U mL^{-1} for GAD antibodies which was the 99.5th centile of control subjects.

The percentage of positive anti GAD antibodies, anti IA2 antibodies and anti IAA antibodies in type 1 diabetic patients were significantly higher than controls ($p = 0.0001$, 0.002 and 0.0001, respectively).

Also anti GAD antibodies was significantly higher in relatives than controls ($p = 0.0001$). Although percentage of positive anti IAA and anti IA2 antibodies was higher in relatives than controls, it was statistically not significant.

In the current study, one positive antibody was found in 10 (33.3%), two antibodies in 17 (56.7%) and three antibodies were found in 3 (10%) of the unaffected sibs of type 1 diabetic patients. So two antibodies at least are recommended especially anti- GAD and anti IA2.

Mrena *et al.* (2003) reported that unaffected siblings were graded into four stages of pre clinical type 1 diabetes based on the initial number of disease associated autoantibodies detectable close to the time of diagnosis of index case: no pre diabetes (no antibodies), early (one antibody specificity), advanced (two antibodies) and late pre diabetes (three or more antibodies). Autoantibodies alone were more sensitive in the prediction of future diabetes in siblings than when combined with genetic susceptibility.

The long preclinical period of type 1 Diabetes Mellitus (DM) make it possible to identify individuals at increased risk for clinical DM before the beta cells destructive process has reached the point of no return. Autoimmunity may be initiated early in life and therefore early screening for signs of β cells autoimmunity is crucial to avoid missing young children on route to overt DM to start intervention. Young age positivity for at least two antibodies and low first phase insulin response are highly predictive for progression to clinical disease in initially unaffected sibling of children with type 1 DM (Kimpimaki and Knip, 2001). Many studies have confirmed that screening for antibodies to IA2 in combination with GAD antibody represent a powerful strategy for routine screening to identify subjects at increased risk for type 1 diabetes with a sensitivity similar to ICA and with improved specificity (Kulmola *et al*., 1998; Kulmola *et al*., 2000; Bingley *et al*., 2001).

As regards serum level of IL18, it was found to be higher in relatives of diabetic patients than diabetic

patients and controls, but it was statistically not significant. Nicoletti *et al.* (2001) reported that IL18 in relatives was detected higher in 14 out of 16 when sampled during pre diabetic stage.

However, during our study period a brother of one of our diabetic patients was discovered accidently to have diabetes (fasting blood glucose = 215 mg dL^{-1}, 3 antibodies were positive and IL18 level was very high. This coincided with Nicoletti *et al.* (2001) who reported that IL18 appears to increase during early subclinical stage of type 1 diabetes mellitus.

We subdivided the relatives of our diabetic patients into high and low risk groups according to number of positivity of antibodies (two or more positive antibodies were considered high risk).

There was no statistical difference between the serum IL18 levels of high and low risk groups. This could be related to the low percentage level of IA2 antibodies in the sibs of our diabetic patients, as positivity of IA2 antibodies was 11.8% only in relatives of diabetic patients.

This coincided with Decochez *et al.* (2002), who reported that IA2 antibodies positivity in sibling of type 1 diabetic patients is a more direct predictor of impending clinical onset than multiple antibodies positivity per se. Assessment of IA2 antibodies status allows us to select subjects with homogeneously high risk of diabetes for participation in prevention trial.

Cloned cytokine IL18 has been shown to be an enhancer to a Th1 cell immune response which may be prerequisite for development of type 1 DM (Knooke *et al.*, 1999).

IL18 induces natïve T cells to develop into Th2 cells, also IL18 induces IL13 /or IL4 production by Natural killer (NK) cells, mast cells and basophiles. So IL18 is a unique cytokine that enhances innate immunity and both Th1,Th2 derived immune responses (Nakanishi *et al.*, 2001). Rothe *et al.* (1999) reported that development of type 1 diabetes in animal models is T cell and macrophage dependent, islet cell inflammation begins as peripheral benign Th2 type insulinitis and progresses to destructive Th1 type insulinitis which is driven by the innate immune system via secretion of IL12 and IL18.

So administration of IL18 as a mediation of the innate immune system appears to suppress autoimmune diabetes in non obese diabetic mice by targeting the Th1/Th2 balance of inflammatory immune reactivity in the pancreas. Also Lukic *et al.* (2003) had shown that IL18 deficient mice were significantly more resistant to the induction of diabetes and did not develop the typical mononuclear cell infiltration of the islets. IL15 and IL18 are essential for the development of diabetes and are more important targets in prevention and early treatment of autoimmune diabetes. Also Oikawa *et al.* (2003) suggested that IL18 production has a promoting role as an enhancer of Th1 cells. Contrary to previous reports, the incidence of diabetes development was significantly increased in non Obese Diabetic (NOD) mice injected with IL18 compared with that in control.

Generally accepted concepts of the development of type 1 diabetes indicate that the pathogenic contribution of IL18 occurs through its ability to stimulate T cellsynthesis of IFN gamma. Increased production of IFN gamma has been observed in peripheral blood mononuclear cells from high risk first degree relatives of patients of type 1 diabetes (Karlsson *et al.*, 2000).

Autoimmunity may be initiated early in life and therefore early screening for signs of beta cell autoimmunity is crucial to avoid missing young children on route to overt diabetes mellitus. Thus it will be helpful to start intervention, when clinically applicable preventive modalities become available, before the disease process has advanced too far.

However, Serum IL18 levels and autoantibodies should be studied on larger scale to give more informations.

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