Assessment of Islet Cell Autoantibodies (ICA and GADA) in patients with Type 1 Diabetes Mellitus

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Abstract
Background: Type 1 Diabetes Mellitus (DM) is an autoimmune disease characterized by insulin deficiency resulting from cell-mediated and humoral autoimmune destruction of islet beta cells. The aim of the present study was to assess the occurrence of glutamic acid decarboxylase autoantibodies (GADA), islet cell antibodies (ICA), and antinuclear antibodies (ANA) diabetic patients of AL-Najaf city in Iraq.

Introduction
Type 1 Insulin Dependent Diabetes Mellitus (IDDM) is an autoimmune disease characterized by insulin insufficiency that result from a progressive immunological destruction of insulin-secreting islet B cells by reactive leukocytes and their mediators. Although the exact nature of the inducing agents and the sequence of events leading to the autoimmune destruction of islet B cells and subsequently hyperglycemia are currently not completely understood, it is well established that genetic, non genetic, and immunologic factors contribute to the pathogenesis of type 1 diabetes Atkinson and Maclaren (1994). The initial interaction of genes and environmental factors, such as viral infection, trigger an immune response to islet autoantigens, with the emergence of autoantibodies as the first sign of beta cells destruction, followed by progressive loss of the first phase insulin secretion. Bach, (2002). To date, autoantibodies, called islet cells antibodies (ICAs) are detected in individuals with type 1 diabetes and allow the clinical course of diabetes to be studied in human subjects. Levinson and Jawetz, (1998). Many autoantibodies against B-cells have been identified. The most important ones are: islet cell antibody (ICA), Potter and Wilkin (2000),anti-insulin Bruining et al., (1989), anti-glutamic acid decarboxylase (GAD) Bingley, et al. (1994) and the antibody (Ab) against the tyrosine phosphatase (PTP)-like protein known as ICA-512 (IA-2), Bach (2000)). In the very early 1990s, the nature of the 65kD autoantigen was revealed as glutamic acid decarboxylase (GAD) leading to the recognition of glutamic acid decarboxylase autoantibodies (GADAs) Kimpimaki, et al., (2000). New autoantibodies associated
with type 1 diabetes continue to be discovered, but the most important autoantibodies are: islet cell antibodies (ICA), anti-insulin antibodies, anti-glutamic decarboxylase (GAD) Leiva, et al., (2007). However, evidence supporting the autoimmune component of T1D is inconsistent. It is unclear whether this is due to an inconsistency in techniques or an inconsistency arising from an underlying genetic variability in different populations. Studies in Caucasian populations have revealed an 80–90% occurrence of autoantibodies in patients newly diagnosed with T1D Notkins and Lernmark (2001). Recent reports from Asia have demonstrated an 83.3% – 90% occurrence of glutamic acid decarboxylase autoantibodies (GADA) in Japanese patients with T1D Kawasaki and Eguchi (2004). However earlier studies in the 1980's reported an occurrence as low as 7% of islet cell autoantibodies (ICA) in Japanese patients with T1D Kobayashi et al.,(1981) and around a 40% occurrence in a mixed Asian population with T1D in Singapore. Todd et al., (2004). Knowing the frequency of these autoantibodies in a population is an important step for a better understanding and diagnosis of type 1 diabetes. The aim of the present study was to assess the occurrence of autoantibodies glutamic acid decarboxylase 65 autoantibodies (GAD65As) and Islet Cells Antibodies (ICA) among patients of Type 1 diabetes in AL-Najaf city and to compare these results with the prevalence of autoantibodies reported in other studies from different countries and investigate about relation between diabetes mellitus and antinuclear antibodies (ANA).

Materials and methods

Study Design and Patients

During the period from July /2009 to October /2009, seventy five individual were included in the present study. They were divided into two groups: patients and control groups.

Patients group

Fifty diabetic patients from AL-Hakeem Centre for Researches and Treatment of Diabetic Mellitus in AL-Sader Teaching Hospital have been recruited as case and control study. Patients were clinically checked as type 1 diabetic from both sexes, their ages ranged between 10-57 years. The disease duration period between one month to seventeen years. Diabetes was diagnosed according to World Health Organization criteria (1985). All of them were insulin dependent.

Control group

Twenty five apparently healthy volunteers have been chosen in this study, this group was collected from people not suffering from diabetic, through measuring Fasting plasma glucose (≤ 111 mg/dl) and Random plasma glucose (≤ 135 mg/dl), Their ages ranged between 13-55 years from both genders.

Specimen Collection

Three ml of venous blood were drawn from each patients and controls were put in plain tubes (without anticoagulant) which were separated by centrifugation at 3000 rpm for 10 minutes. Part of separated sera was used for Fasting plasma glucose and Random plasma glucose analysis, and the remaining amount was stored in two separated plain tubes at deep freeze before testing, all serum tubes allowed to be thawed once (repeated thawing should be avoided).

Autoantibodies detection: Anti-GAD65, anti-IC and anti-AN autoantibodies were detected with commercial assays using Quantities Enzyme Linked Immune Sorbent Assay test for detection of circulating autoantibodies against Glutamic Acid Decarboxylase antigens (GAD65). (EUROIMMUN / Germany indirect
immunofluorescence test (EUROIMMUN/Germany) to detection islet cell antibodies and Anti Nuclear Antibody (ANA) in human serum.

**Detection islet cell antibodies by Indirect immunofluorescence test in sera patients**

Islet cell antibodies are detected by indirect immunofluorescence (IIF). Aid in the diagnosis insulin dependent diabetes mellitus (IDDM). In this method, patient sera are incubated on section of monkey pancreas to allow binding of antibodies to the tissue substrate. Bound antibodies of the IgG class are detected by incubation of this section with fluorescein-labeled conjugates. Reaction is observed under a fluorescence microscope equipped filter.

**Quantitive Enzyme Linked Immune Sorbent Assay test for detection of circulating autoantibodies against Glutamic Acid Decarboxylase antigens.**

A purified GAD antigen is immobilized onto microwells. GAD specific IgG antibodies present in the patient's serum sample are allowed to react with the antigen. The excess/unbound serum proteins are washed-off from the microwells. Add biotin-labelled GAD to bind with specific antibody. An enzyme (Peroxidase-labelled avidin) labeled goat-antibody; specific to human IgG is added to the GAD antibody complex. After washing off excess unreacted enzyme conjugate from the microwells, a substrate (TMB/H2O2) is added and the color generated, then stop solution (sulphuric acid) is added to stop the reaction & the color measured by reader is a spectrophotometrically. The intensity of the developed color gives directly the concentration of GAD autoantibodies in the test serum sample. GAD positive and negative controls serve as an internal quality control to ensure valid result.

Normal value: The upper limit of the normal range (cut-off value) recommended by EUROIMMUN is: < 10 IU/ml: Negative / ≥ 10 IU/ml: Positive.

**Anti Nuclear Antibody (ANA) indirect immunofluorescent test (Qualitatively determination of anti-human IgG, IgM and IgA attach against tissue antigen)**

This test kit is determination of human antibodies in serum or plasma. The determination can be performed qualitatively. In this method, diluted patient sera are covering Hep-2 cells and incubated to allow binding of specific antibodies of classes IgA, IgG and IgM attach to the tissue substrate (antigen). Any antibodies not bound are removed by rinsing. The attached antibodies are stained with fluorescein-labeled anti-human antibodies and visualized using fluorescence microscopy.

**Statistical Analysis**

Data were transformed into codes using a specially designed coding sheet, and then converted into a computerized database structure. Statistical analysis was done using SPSS version 17 computer software (Statistical Package for Social Sciences). Results were expressed using simple statistical parameters such as mean, percentage and standard deviation. Analysis of qualitative data was done using t-test and Chi-square test($X^2$); Correlation was done using to determine the difference in the characteristics between patients with type 1 and controls. Acceptable level of significance was considered to be below 0.05. Sorlie, (1995)

**Results**

**Demographic and clinical characteristics of study groups**

Fifty type 1 diabetic subject "24(48%) Females and 26(52%) Males" who had been diagnosis with type 1 Diabetes Mellitus. The mean over all duration of diabetes was (5.22) years (range from less than 1 to 17years).Patients group were compared to 25 subjects (11 females and 14 males) with normal fasting & random glucose and no family history of type 1 diabetes i.e apparently healthy, (table-1).
Table (1): Demographic and clinical characteristics of Diabetes Mellitus type 1 and healthy control subjects

<table>
<thead>
<tr>
<th>Variables</th>
<th>Type 1 DM</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.(Female/Male)</td>
<td>50 (24/26)</td>
<td>25 (11/14)</td>
</tr>
<tr>
<td>Age range (mean) years</td>
<td>10-57 (28.3)</td>
<td>13-55 (24.6)</td>
</tr>
<tr>
<td>Family history (+ve No.) %</td>
<td>(6) 12%</td>
<td>-----</td>
</tr>
<tr>
<td>Smoking of diabetes (+ve No.)%</td>
<td>(5) 10%</td>
<td>(2) 8%</td>
</tr>
<tr>
<td>Fasting glucose / range (mean) mg/dl</td>
<td>71-213 (142.3)</td>
<td>68-111 (83.1)</td>
</tr>
<tr>
<td>Random glucose / range (mean) mg/dl</td>
<td>79-492 (201.6)</td>
<td>72-135 (102.8)</td>
</tr>
<tr>
<td>Onset of disease / range (mean) years</td>
<td>1-17 (5.22)</td>
<td>-------</td>
</tr>
</tbody>
</table>

Percentage of ICA & GADA in study groups

In type 1 DM patients, 32 (64%) were seropositive for ICA, while control group showed no positive result in ICA test. Regarding GADA, nineteen of type 1 diabetic patients which represent (38%) GADA were seropositive in comparison to control group. Data illustrated in table (2) revealed the significant difference (P < 0.05).

Table (2): Positivity percentage of ICA & GADA in studied groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Study groups</th>
<th>Type 1DM</th>
<th>Healthy control</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICA+ve(No.)%</td>
<td></td>
<td>(32/50) 64%</td>
<td>(0) 0.0%</td>
<td>$X^2=5.5, df=1$ $P&lt;0.05$</td>
</tr>
<tr>
<td>GADA+ve(No.)%</td>
<td></td>
<td>(19/50) 38%</td>
<td>(0) 0.0%</td>
<td>$X^2=4.2, df=1$ $P&lt;0.05$</td>
</tr>
</tbody>
</table>

Positivity percentage of ICA & GADA in type 1 DM according to the gender:

When gender was taken in consideration, the males showed more positive results 17/26 (53.1%) percent of ICA than females 15/24 (46.9%), in the studied group. These differences were statistically no significant (P> 0.05), when gender was taken in consideration, females showed (57.9%) more effected than males (42.1%) in GADA assay this study. Table (3).

Table (3): distribution of ICA & GADA in type 1 DM according to the gender

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Study</th>
<th>Type 1DM</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICA+ve(No.)%</td>
<td>Females</td>
<td>(15/24) 46.9%</td>
<td>$X^2=1.28, df=1$ $P&gt;0.05$</td>
</tr>
<tr>
<td>GADA+ve(No.)%</td>
<td>Males</td>
<td>(17/26) 53.1%</td>
<td>$X^2=2.8, df=1$ $P&gt;0.05$</td>
</tr>
</tbody>
</table>

The distribution of positivity ICA&GADA according to age of disease onset:

Islet cells antibodies appeared to be prevalent in those who developed the disease at young ages compare with older ages; Type 1 diabetic patients were (43.8%,34.4%and21.8%)with age of onset 10-25 years, 26-41 years and 42-57 years, respectively. However the data illustrated significant difference (P< 0.05). It was found from present results that GAD65AAs were prevalence in patients who developed the disease during childhood and early puberty and prevalence began to
decrease as the age of onset increase, The seropositivity of GADA were (63.2%), (31.6%) and (5.2%) with age of onset 10-25 years, 26-41 years and 42-57 years respectively. Statistical analysis revealed significant difference (P < 0.05), Table (4).

Table (4): ICA & GADA positive distribution in relation to the age of onset disease among diabetic patients

<table>
<thead>
<tr>
<th>Age of onset</th>
<th>ICA +ve No.(%)</th>
<th>GADA +ve No.(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 -25 years</td>
<td>14(43.8)</td>
<td>12(63.2)</td>
</tr>
<tr>
<td>26-41 years</td>
<td>11(34.4)</td>
<td>6(31.6)</td>
</tr>
<tr>
<td>42-57 years</td>
<td>7(21.8)</td>
<td>1(5.2)</td>
</tr>
<tr>
<td>Total</td>
<td>32(100%)</td>
<td>19(100%)</td>
</tr>
<tr>
<td>Statistic</td>
<td>$X^2=4.33$, df=2, p&lt;0.05</td>
<td>$X^2=6.4$, df=2, p&lt;0.05</td>
</tr>
</tbody>
</table>

Prevalence of islet cells autoantibodies among type 1 diabetic patients

From fifty type 1 diabetic patients, there were 32 (64%) of ICA positive, 19 (38%) of GADA positive, where as the percent of positivity increase to 38 (76%) when seropositive ICA and/or GADA are taken together. Data illustrated in table (5) revealed that a significant difference (P< 0.05) in ICA and the ICA and/or GADA, while no significant (P>0.05) in GADA.

Table (5): Frequency of anti-islet cells autoantibodies among type 1 diabetic patients

<table>
<thead>
<tr>
<th>Anti-Islet cells Abs.</th>
<th>Positive No.(%)</th>
<th>Negative No. (%)</th>
<th>Total</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ICA</td>
<td>32 (64%)</td>
<td>18 (36%)</td>
<td>50</td>
<td>P&lt; 0.05</td>
</tr>
<tr>
<td>Anti-GADA</td>
<td>19 (38%)</td>
<td>31 (62%)</td>
<td>50</td>
<td>P&gt;0.05</td>
</tr>
<tr>
<td>Anti-ICA &amp; or / Anti-GADA</td>
<td>38 (76%)</td>
<td>12 (24%)</td>
<td>50</td>
<td>P&lt; 0.05</td>
</tr>
</tbody>
</table>

Relation between anti-nuclear antibodies and Type 1 DM

Results of recent research revealed a low seropositive percent for ANA, the data obtained from table (6), demonstrated there was no significant difference (P > 0.05) of ANA with DM type 1.

Table (6): Distribution of ANA in type 1 DM & Control group

<table>
<thead>
<tr>
<th>Results</th>
<th>+ve No. (%)</th>
<th>-ve No. (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1 DM</td>
<td>1 (2%)</td>
<td>49 (98%)</td>
<td>50</td>
</tr>
<tr>
<td>Control</td>
<td>0(0.0%)</td>
<td>25 (100%)</td>
<td>25</td>
</tr>
</tbody>
</table>

$X^2 = 1.13$, df =1  
P > 0.05
Discussion

Type 1 DM is an autoimmune metabolic disorder characterized by the presence of activated, self-reactive lymphocytes that invade the pancreas and destroy the insulin producing beta cells found there in. Although the etiology of type 1 DM is incompletely understood, it is recognized to be due to both genetic and environmental determinants. DM is growing, a modern epidemic due to rapidly increasing prevalence in recent decades worldwide. Zaman, (2009).

In recent study showed that type 1 DM distribution among different age stages. Type 1 diabetes (formerly known as childhood) is not exclusively a childhood problem, the adult incidence of type 1 is not worthy-many adults who contract type 1 diabetes due to misdiagnosed with type 2 Abel M, and krokowski, M (2001).

Patients with type 1 DM who had a positive family history of DM represent (12%). The risk of type 1 DM associated with its occurrence in first-and second-degree relatives, in other words it increased with a positive family history, Atoblli, et al., (1998). The present result showed small percentage may be related to small number of personal involved in the present study.

Percentage of ICA& GADA in study groups

Type 1 DM is a multifactorial disease resulting from destruction of islet beta cells that leads to an absence of intrinsic insulin secretion, one theory regarding the etiology of type 1 DM is that it results from damage to pancreatic beta cells by infectious or environmental agents that triggers the immune system in genetically susceptible individual to develop an autoimmune response against altered pancreatic beta cell antigens. Currently, autoimmunity is considered the major factor in pathophysiology of type 1 DM, Winter, et al., (2002). Current result is in agreement with Borg et al., (2002).who found approximately similar range of this percentage among type 1 diabetic patients in different localities. The above findings demonstrate the important role of islet cell autoantibodies in pathogenesis of the disease and clarify that autoimmune diabetes (type 1a) which is still more prevalent than idiopathic (type 1b).However, these results seem to be higher than that detected by Samuelsson et al, (2001) in a study from Sweden where type 1 diabetic patients showed (40%) for ICA, the reason for such difference in the prevalence of this antibodies may be to attributed to difference in assay used, difference in sensitivity levels of procedure used and difference in patients genetics and environmental characters. There is a fluctuation in the percentage of ICA in different area from 7% in Sub-Saharan Africa to 36% in Nigeria and Tanzania, Lutale, et al., (2007). Present data revealed 38% of diabetics patients positivity with GADA. However, these results agreed with at least one thesis but seem less than that reported by Rasheed et al.(2008), in Iraq that reported 62.5% of type 1 DM patients with positive GAD antibodies. In a study from Saudi Arabia on type 1 diabetic patients, which showed 54% ,Damanhour, et al., (2005) , also in a study from Taiwan as type 1 diabetic patients showed 47% in their serum Tsai and Lan, (2004).GAD65AAs are present in most patients with type 1DM years before the clinical manifestation of the disease appeared Marcovina, et al., (2000)).The autoantibodies often have predictive value. For instance, individuals testing positively for antibodies to both GADA and insulin have a high risk of developing type 1 DM .Roitt, et al., (2001).The reason for such difference in these results may be attributed to the difference in sensitivity procedure assay used, genetic factor and environmental characters.
Effect of gender on ICA & GADA percentage among type 1 DM

Males showed more positive ICA than females; these results of research agreed with Eskola and his colleagues (2003), and disagreed with Demaine et al, who found that islet cell autoantibodies positivity was seen in girls at younger age, suggesting that beta cell destruction is faster and the total destruction occur earlier in girls, as the genetic controls autoantigen presentation to T-lymphocyte by specialized cells might affected by sex hormones which seems to be the same cause that explain affection of females gender more than males. Demaine et al, (1989). The difference in present results may be due to the small number of patients enrolled in this study. The analysis of data concern with GADA percentage demonstrated no significant (P>0.05), this result agree with Lindholm et al (2004). The explanation of this finding is the fact that autoimmune disease are more common in females than males and the logical causes for this difference would be the sex hormone, females might respond more to conventional antigen due to sex hormone Denman, (1991). However, no correlation between gender and autoimmunity in type 1 diabetic patients.

ICA and GADA positive distribution in relation to the age of onset in type 1 DM

Islet cells autoantibodies showed to be prevalent in those who developed the disease at young ages compare with older ages this result agreed with Sabah et al., (2000) and Leslie, and Castelli, (2004) Demonstrated decrease in the ICA prevalence with increase age of onset and also they concluded that islet cell autoantibodies are more common in those who develop Diabetic Mellitus type 1 in childhood and puberty, Kyvik, et al.,(2004). Environmental factors have been implicated in etiology of autoimmune diabetes (age-related non genetic factors), these factors include early exposure to cow's milk, reduced rates or duration of breast feeding and vitamin D consumption (Hypponen et al., (2001) Kimpimaki, et al.,(2001) . Results of GADA positive is agreement with Muir and his co-workers, Muir, et al.,(1998), and Rasheed et al, (2008) who recommended that GAD65 AAs were found in 75% and 62.5% respectively of new-onset type 1 DM patients.

Prevalence of islet cells autoantibodies among type 1 diabetic patients

These results are agreement with Rasheed et al (2008) and ADA (2002), which found harmony range of these percentages among type 1 diabetic patients in different localities. The above finding demonstrated the important role in the diagnosis and high-titer ICA identify a group of type 1 diabetic patients at high risk of rapidly losing residual beta-cell function Rasheed et al (2008) and American Diabetes Association, (2009) respectively. So it may be used ICA as a predictor investigation for type 1 DM.

Relation between anti-nuclear antibodies and Type 1 DM

There is a lot of autoantibodies association with type 1 DM like ICA, GADA and IA2-A (protein tyrosine phosphatase) are detected in 95% of type 1 DM patients at the time of diagnosis in diabetes Borg, et al; (2009), law association 2%, which agreed with Vahasalo et al, (1996) and Elkahi and his colleague (2002).

In Conclusions present results provide strong evidence that autoimmunity plays a major role in the pathogenesis of type 1 DM. Islet cell autoantibodies were more prevalent in those affected by the disease earlier, and those autoantibodies decrease and disappear with time. With no correlation between gender and diabetes type1. There are many cases of Diabetes Mellitus detected by laboratory investigation only; whom are asymptomatic, screening tests had helpful for early detection and treatment. ANA titer showed to have low association percentage in the test patients.
Farther studies worthwhile to be hold in a role of other marker like (C-peptide, IA-2, Insulin Autoantibodies (IAAs) & cytokines Farther studies need to be done in extensive workup in type 1 DM. And also to be applied in type 2 DM. ICA & GAD need to be done not only in patient with type 1 DM but even in general population, who has risk factor.

References
Eskola, V; Vaahasalo, P; Akerbolm, H.K. et al. (3003). Increased frequency of islet cell Antibodies in unaffected Brothers of children with type 1 Diabetes; Horm. Res, 59 (4); 195-200.


Vahasalo, P; Petays, T; knip, M; et al (1996) relation between Antibodies to islet cell antigens, other autoantigens and cow's milk proteins in diabetic children and unaffected siblings at the clinical manifestation of IDDM Autoimmunity 32 (3) 165-174.