Impact of anti-glutamic acid decarboxylase-65, anti-insulin and anti-tyrosine phosphatase autoantibodies on disease activity in type 1 diabetes patients

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Abstract

Background: Type 1 diabetes mellitus (T1D) is a chronic autoimmune disease with autoantibodies against glutamic-acid-decarboxylase (GAD)65, insulin and tyrosine phosphatase (TP) as a feature of disease. The correlations of these autoantibodies with disease severity remain to be explored. Here we investigate the status and contribution of autoantibodies against GAD65, insulin and TP in T1D patients and to explore whether these antibodies have a role in T1D progression and in T1D associated neuropathy.

Methods: Sera from 57 T1D patients with varying levels of disease activities and 42 age- and sex-matched healthy controls were evaluated for anti-GAD65-antibodies, anti-insulin-antibodies and anti-TP-antibodies.

Results: Serum analysis showed T1D patients contain 42% of anti-GAD65-antibodies, 76% of anti-insulin-antibodies and 34% of anti-TP-antibodies. Interestingly, not only was there an increased number of subjects positive for these antibodies, but also levels of these antibodies were significantly higher among T1D patients whose ages were >35 years as compared with younger T1D patients (age ≤35 years). In addition, significant correlation was observed between the levels of these antibodies and glycated haemoglobin (HbA1c). Furthermore, T1D neuropathic patients had higher levels of these antibodies compared with T1D patients without neuropathy.

Conclusions: Our data support an association between these markers autoantibodies and severity of T1D. The stronger response observed in patients with uncontrolled T1D suggests that these antibodies may be useful biomarkers in evaluating the progress of T1D and in elucidating the mechanisms of disease pathogenesis.

Keywords: Diabetes type 1, autoimmunity, anti-GAD65-autoantibodies, anti-insulin-autoantibodies, anti-TP-autoantibodies, T1D progress, T1D associated neuropathy

Introduction

Type 1 diabetes mellitus (T1D) is an autoimmune disease characterized by the targeted destruction of the insulin-secreting β-cells within the pancreatic islet [1]. The precise etiology remains uncertain; however, there is a general consensus that T1D is a T-cell mediated disorder that results from immune dysfunctions, with subsequent loss of tolerance to β cell antigens and destructive lymphocytic infiltration of the islets. This, in turn, leads to hyperglycemia [2]. Although unlikely to be intrinsically diabetogenic, circulating autoantibodies targeting pancreatic β cell proteins have been defined [3]. Monitoring such systemic autoantibodies is currently the most reliable biomarker in the prodromal phase of T1D, since their appearance typically precedes overt T1D onset for years [1,2]. This provides a window for therapeutic intervention, and considerable effort has been devoted for identification of autoantigens as biomarkers of T1D. In the absence of reliable T-cell tests, dissection of autoantibody responses in subjects of genetic risk should prove useful in identifying triggers of islet autoimmunity by examining seroconversion and maturation of the autoantibody response that may mark time to onset of T1D [4]. The complexity of the disease process is exemplified by multiple clinical phenotypes, including autoimmune diabetes masquerading as type 2 diabetes in youth and adults [5]. Autoantibodies may also provide prognostic information in clinically heterogeneous patient populations when examined longitudinally [6]. The ability to measure autoantibodies in type 1 diabetes using recombinant autoantigens has paved the way for the identification of several different autoantigens detected by autoantibodies in a large number of other autoimmune disorders but the utility of these autoantigens is yet to be standardized [7]. Furthermore, the islet cell autoantibodies (ICA) reactivity does not always correlate toward defined autoantigens, suggests that additional specific autoantigens remain to be identified [8]. However, three major targets (GAD65, insulin and IA-2) have been confirmed, with approximately 94% of all subjects with a clinical diagnosis of T1D expressing autoantibodies.
The average (±SD) of post prandial blood sugar level for healthy subjects (27 male and 15 female), age range 18-48 years (mean ±SD years 49±15 years). The control group comprised 42 healthy subjects (27 male and 15 female), age range 21-52 years (mean ±SD years 43±17 years). The mean ages were not significantly different between the groups (p>0.05). The average (±SD) of post prandial blood sugar level for T1D patients was 345±87 mg/dl, while for 42 healthy subjects it was 109±9 mg/dl. The average (±SD) glycosylated hemoglobin (HbA1c) for patients was 8.0±1.5, whereas for normal subjects it was 4.7±0.4. Diabetic neuropathy was assessed by physical examination and nerve conduction studies. The clinical characteristics of the subjects recruited are summarized in Table 1. Venous blood from all studied subjects was collected, and sera were separated. All serum samples were heated in water bath at 56°C for 30 min to inactivate complement system and were stored in small aliquots at -80°C until analysed further.

### Sandwich ELISAs

Overproduction of anti-GAD_65 antibodies or anti-TP antibodies in T1D patients were determined by Sandwich enzyme-linked immunosorbent assays (ELISA) established in our laboratory. Briefly, flat bottomed 96-well microtiter plates (polystyrene polysorp immunoplates, Nunc ImmunTM MicroWell, Sigma) were coated with GAD-65/67 (C-20) polyclonal antibodies (catalog # sc-7513, Santa Cruz Biotechnology, CA) or PRL-1/2/3 (C-14) antibodies (catalog # sc-49253; Santa Cruz Biotechnology) overnight at 4°C. The plates were washed with TBS-Tween 20 and the non-specific binding sites were blocked with TBS containing 1% BSA (Sigma) at room temperature for 1 h. After washing extensively with TBS-Tween 20, 100 µl of patient’s sera (1:100 diluted) were added to duplicate wells of the coated plate and incubated at room temperature for 2 h and overnight at 4°C. The plates were washed five times with TBS-Tween 20 and 100 µl of GAD-65 (H-95) monoclonal IgG (catalog # sc-5601, Santa Cruz Biotechnology; diluted 1:100) or PRL-1/2/3 (FL-173) antibodies (catalog # sc-33197, Santa Cruz Biotechnology) overnight at 4°C. The plates were washed extensively and 100 µl of anti-human IgG-horseradish peroxidase (catalog # sc-2769, Santa Cruz Biotechnology) was added and incubated for 2 h. After washing, 100 µl of 3,3′,5,5′-Tetramethylbenzidine peroxidase substrate (TMB, catalog # 206697A, Santa Cruz Biotechnology) was added to each well. The reaction was stopped after 10 minutes by adding 100 µl of stop solution (2M H_2SO_4) and OD was read at 405 nm on an automatic microplate reader (AnthosZenyth 3100 Multimode Detectors, Salzburg, Austria).

### Preparation of human insulin antigen for direct binding ELISA

An aqueous solution of human insulin (code # L1384-OL, Aventis Pharma Deutschland GmbH, Frankfurt, Germany) was diluted in phosphate buffer saline (10 mM; 150 mM NaCl) and pH was adjusted to 7.4 using 6 N NaOH (Sigma-Aldrich). This insulin solution was dialyzed extensively against PBS (150 mM NaCl, pH 7.4).

### Direct binding ELISA

Anti-insulin autoantibodies were detected by Direct...
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binding ELISA. It was performed on flat bottom 96-well, polystyrene maxiSorp immunoplates (Catalog # P8616; Nunc-ImmunoTM MicroWell, Sigma-Aldrich) as described previously by our published procedure [22]. Briefly, polystyrene maxisorp immunoplates were coated with 100 µl of insulin antigen (10 µg/ml) in carbonate buffer (0.05 M, pH 9.6). The plates were coated for 2 h at room temperature (RT) and overnight at 4°C. Each sample was coated in duplicate and half of the plates served as control devoid of only antigen coating. Unbound antigen was washed with PBS-T (10 mM, 150 mM NaCl, pH 7.4 containing 0.05% Tween-20) and unoccupied sites were blocked with block buffer (PBS containing 1% BSA) for 1-2 h at RT. After incubation, the plates were washed with PBS-T. The test serum (1:100) in PBS-T (100 µl/well) was adsorbed for 2 h at RT and overnight at 4°C. Bound antibodies were analysed with anti-human HRP linked conjugate (catalog # sc2769, Santa Cruz Biotechnology) using 3,3',5,5'-Tetramethylbenzidine substrate (TMB, catalog # 206697A, Santa Cruz Biotechnology). Reaction was stopped by stop solution (2M H2SO4) and absorbance of each well was recorded at 405 nm on an automatic microplate reader (Anthos Zenyth 3100 Multimode Detectors, Salzburg, Austria).

Statistical analysis
All measurements were performed in duplicates using age- and sex-matched diabetes or control samples. Comparisons were performed using Origin 6.1 software package (Northampton, MA, USA) and Graph Pad Prism-5 (San Diego, CA, USA) (one paired two tailed t-test with ANOVA analysis and Tukey’s post-hoc analysis). P values less than 0.05 were considered significant, and P values less than 0.001 were considered highly significant. Values shown as mean ± SEM (95% confidence level) unless stated otherwise.

Results
Recognition of GAD65 by Type 1 diabetes autoantibodies
In an attempt to find out the role of GAD65 in pathogenesis of type 1 diabetes (T1D), this report determines the serum levels of GAD65 specific antibodies in patients with T1D. Sera from 57 T1D patients and 42 normal human (NH) subjects were tested for the detection of anti-GAD65 autoantibodies by GAD65-specific sandwich ELISAs. Data showed that T1D patient’s serum autoantibodies showed strong binding to GAD65 at 1:100 serum dilutions. To validate our hypothesis, we assessed the increases in serum levels of anti-GAD65 antibodies as a function of the disease progress (Figure 1).

As evident in Figure 1a, levels of anti-GAD65 antibodies in all of the diabetes patients (both those with age ≤35 years and those with age >35) were significantly higher in comparison with healthy controls (p<0.0001). Interestingly, levels of anti-GAD65 antibodies in diabetes patients with age >35 years were significantly higher than those age ≤35 years (p<0.001). To further investigate the relation of T1D patient’s age and the occurrence of autoantibodies, T1D patients were divided into different age groups in accordance with the guidelines on age-wise distribution of diabetes and pre-diabetes [23,24]. Our data showed that autoantibodies against GAD65 increase with the patient’s age (Table 2). In addition, remarkably increase in levels of anti-GAD65 antibodies in patients with higher HbA1c (Figure 1b) in comparison with those in patients with lower HbA1c suggesting a positive association between the increase in anti-GAD65 antibodies and HbA1c levels. Not only this, our data also showed that levels of anti-GAD65 antibodies were significantly higher in those diabetic neuropathy patients as compared to those T1D patients who were not associated with neuropathy (Table 3).
Anti-insulin autoantibodies in Type 1 diabetes patients
Sera from 57 diabetes patients and 42 normal human subjects were screened for the detection of anti-insulin antibodies (IAA). Our results showed that 76% T1D patient’s sera showed strong binding human insulin at 1:100 serum dilutions (Table 3). To validate our hypothesis, we assessed the increases in serum levels of IAA antibodies as a function of the disease activity. As evident in Figure 2a, levels of IAA in diabetes patients (both those with age≤35 years and those with age>35) were significantly higher in comparison with healthy controls (p<0.0001). Interestingly, levels of IAA antibodies in diabetes patients with age>35 years were significantly higher than those ages≤35 years (p<0.001). Our results also showed that autoantibodies against human insulin increase with the patient’s age (Table 2). In addition, remarkably increased levels of IAA antibodies in patients with higher HbA1c (Figure 2b) in comparison with those in patients with lower HbA1c suggest a positive association between the increase in IAA and disease activity. Our results also showed that levels of IAA were higher in diabetic neuropathy patients as compared to those patients which were not having any secondary complications (Table 3).

Table 2. Age wise distribution of serum autoantibodies in type 1 diabetes patients.

| Age years | n  | Anti-GAD65 antibodies (normalized OD units) | Anti-insulin antibodies (normalized OD units) | Anti-TP antibodies (normalized OD units) |
|-----------|----|--------------------------------------------|---------------------------------------------|------------------------------------------|
| 10-20     | 07 | 0.014                                      | 0.021                                       | 0.007                                    |
| 21-30     | 05 | 0.018                                      | 0.033                                       | 0.011                                    |
| 31-40     | 23 | 0.021                                      | 0.039                                       | 0.013                                    |
| 41-50     | 11 | 0.025                                      | 0.048                                       | 0.015                                    |
| 51-60     | 07 | 0.031                                      | 0.059                                       | 0.019                                    |
| >61       | 04 | 0.032                                      | 0.053                                       | 0.018                                    |
| 49±15     | 57 | 0.024±0.007                               | 0.042±0.014                                 | 0.014±0.005                              |

Table 3. Levels of anti-GAD65, anti-insulin antibodies and anti-TP antibodies in patients with type 1 diabetes and diabetic neuropathy.

| Parameters | T1D (mean±SEM) | T1D neuropathy (mean±SEM) | Healthy controls (mean±SEM) |
|------------|----------------|---------------------------|-----------------------------|
| n          | 57             | 13                        | 42                          |
| Anti-GAD65 antibodies (normalized OD units) | 0.024±0.003* | 0.038±0.005               | 0.004±0.020                |
| Anti-insulin antibodies (normalized OD units) | 0.033±0.023* | 0.045±0.007               | 0.0037±0.0019              |
| Anti-TP antibodies (normalized OD units) | 0.014±0.003* | 0.019±0.001               | 0.0035±0.0021              |

Abbreviations: T1D, type 1 diabetes; GAD65, glutamic acid decarboxylase; TP, tyrosine phosphate; n, number of serum samples tested, SD, standard error; SEM, standard error of mean; TS, total number of samples.

Figure 2. Detection of anti-insulin antibodies (IAA) in type 1 diabetes patients.
(a) Age-wise detection of IAA in type 1 diabetes (T1D) patients. *p<0.01 versus IAA detected in T1D patients of age>35; $p<0.001 versus IAA detected in normal human subjects (NHS).
(b) Glycosylated hemoglobin (HbA1C)-wise detection of IAA in T1D patients. $p<0.0001 versus IAA detected in T1D patients with HbA1C>7.2; $p<0.001 versus IAA detected in NHS. The number of T1D serum samples was 57 and NHS was 42. Insulin-specific direct binding ELISAs were used and serum samples were 1:100 diluted. Results show mean with 95%CI.
Levels of anti-tyrosine phosphatase autoantibodies in patients with type 1 diabetes

Sera from 57 diabetes patients and 42 normal human subjects were screened for the detection of anti-tyrosine phosphatase (TP) antibodies by TP-specific sandwich ELISAs. Our data showed that 34% T1D patients’ sera showed strong binding human insulin at 1:100 serum dilutions. Figure 3a shows that the levels of anti-TP-antibodies in all tested diabetes patients (both those with ages35 years and those with age>35) were significantly higher in comparison with healthy controls (p<0.001). In contrast to our central hypothesis that levels of anti-TP-antibodies in diabetes patients with age>35 years were higher than those ages35 years, but this difference in the occurrence of anti-TP-antibodies in these groups was not statistically significant (p>0.05) (Figure 1a). Not only this, we also determined the levels of anti-TP autoantibodies in different age groups, our results showed that autoantibodies against tyrosine phosphatase increase with the patient’s age and the levels of these autoantibodies were saturated after 60 years of patients age (Table 2). Furthermore, levels of serum anti-TP-antibodies were increased with the increased of HbA1C (Figure 3b). In addition, we also determined that levels of anti-TP antibodies in T1D neuropathic patients were found to be higher as compared with patients without neuropathy (p<0.05; Table 3).

Comparative assessment of anti-GAD65-antibodies, anti-insulin-antibodies and anti-TP-antibodies in patients with type 1 diabetes

The occurrence of antibodies against GAD65, insulin and tyrosine phosphatase in type 1 diabetes patients has been shown in Figure 4. Our novel data showed that 42% T1D patient’s sera showed strong binding with GAD65, 76% T1D patient’s sera showed strong binding with human insulin, whereas 34% of patient’s sera strongly recognized tyrosine phosphatase.

Discussion

Type 1 diabetes (T1D) is a chronic autoimmune disease in which insulin-producing β-cells are located in the pancreatic islets of Langerhans, which are gradually destroyed by autoreactive T cells [2,3,9]. Prospective studies with pre-T1D patients have shown that the process of β-cell degeneration can take more than 3 years before the clinical manifestation and the disease progression occurs in waves or in cycles [24]. Despite the power of modern molecular and immunological approaches and persisting investigative efforts, T1D remains an enigmatic disorder and the agent (or agents) triggering this autoimmune disorder remains to be identified [25,26]. Current understanding of
the aetiology and pathogenesis of T1D show that disease most likely results from an unfortunate combination of genetic susceptibility and exposure to an environmental trigger [27,28]. The main effector mechanism is clearly an autoimmune reaction, which is also evident at time of clinical diagnosis. This implies two concepts for clinical treatment: the first one is the knowledge of the cause is more critical for prevention than for at-onset therapy, and the other is at/near-onset therapy requires an immune silencing [2,10,11]. All these debates clearly indicate that several silent immune events occur before the appearance of clinical symptoms in T1D, which strongly suggest that immunological events play a fundamental role in the onset as well as in the progression of T1D. In view of these, we believe that the factors responsible for T1D include the appearance and spreading of antigenic determinants. The speed of T1D progression could depend on the degree of epitope spreading, which is an intrinsic to the immunological process that leads to T1D [29]. If the regulatory response is insufficient, the recurring immune events could eventually lead to the destruction of the majority of the islet β-cell mass and thereby reduce the body’s capacity to produce insulin [30-32]. Understanding and mapping the precise kinetics of the immune response to the disease has implications for the design of immunomodulatory therapeutics, and the regulatory immune components of T1D could be therapeutically exploited to ultimately suppress the progression of T1D [33-35]. However, the temporal and compositional details of the immune regulation that are associated with the onset and progress of T1D remain controversial [2,36].

Autoantibodies are currently the most robust biomarkers of T1D and are frequently used to establish entry criteria for the participation of genetically at-risk individuals in secondary prevention/intervention clinical trials. Since their original description almost 40 years ago, considerable efforts have been devoted toward identifying the precise molecular targets that are recognized. Such information can have significant benefit for developing improved metrics for identifying/stratifying at-risk subjects, developing potential therapeutic targets, and advancing understanding of the pathophysiology of the disease [27]. Currently, three major molecular targets (insulin, GAD$_{65}$ and IA-2) have been reported, with approximately 94% of all subjects with a clinical diagnosis of T1D expressing autoantibodies to at least one of these molecules at clinical onset [2,3,9-11]. However, the precise interrelation of these markers autoantibodies with the extent of T1D progress is largely unknown. Therefore, in the present study we have investigated the levels of autoantibodies against GAD$_{65}$, IA-2A and IAA in T1D patients and to explore whether these markers autoantibodies have a role in disease induction and progression. Our data demonstrate that 42% T1D patient’s serum autoantibodies showed strong binding with GAD$_{65}$, 76% T1D patient’s autoantibodies showed binding with human insulin, whereas 34% autoantibodies from patient’s showed recognition of tyrosine phosphatase. This clearly indicated that autoimmunity against insulin is much stronger than GAD$_{65}$ or tyrosine phosphatase. For the validation of our central hypothesis, we assessed the increases in serum levels of anti-GAD$_{65}$ antibodies, IA-2A and IAA as a function of the disease activity in different T1D patients groups. Analysis of Age-wise distribution anti-GAD$_{65}$ antibodies, IAA or IA-2A show that levels of anti-GAD$_{65}$ antibodies, IAA or IA-2A in T1D patients with age>35 years were significantly higher than those age≤35 years. This indicating that age plays a role in the occurrence of these markers autoantibodies. HbA$_{1c}$ wise distribution of autoantibodies show high levels of anti-GAD$_{65}$ antibodies, IAA or IA-2A in patients with higher HbA$_{1c}$ in comparison with those in patients with lower HbA$_{1c}$, suggesting a positive association between the increase in anti-GAD$_{65}$ antibodies, IAA or IA-2A and disease progress.

The prevalence of T1D associated secondary complications is rapidly increasing worldwide [37], but the treatment of such complications is limited. Diabetic neuropathy represents its major complication with multiple clinical manifestations, which is detected in up to 57% of T1D patients [38,39]. The pathogenesis of diabetic neuropathy is still not fully understood and may involve multiple mechanisms. In the literature, controversial data regarding the association markers autoantibodies and diabetic neuropathy have been reported [12,19-21,40]. To verify whether autoimmune markers of T1D are associated with subclinical neuropathy, we examined the levels of anti-GAD$_{65}$ antibodies, IAA and IA-2A in T1D neuropathic patients. Our data show that these autoantibodies were found to be higher in diabetic neuropathy patients as compared to non-neuropathic T1D patients. These data clearly pointed out that these autoantibodies not only play a role in autoimmune diabetes, but also in autoimmune diabetic neuropathy.

**Conclusions**

This is the first study that compares the levels of occurrence of autoantibodies against GAD$_{65}$, insulin and tyrosine phosphatase in type 1 diabetes patients. Our results demonstrate the role of anti-GAD$_{65}$ antibodies, anti-insulin antibodies and anti-tyrosine phosphatase antibodies in type 1 diabetes and in diabetic neuropathy, which might play an active part in the progression of disease. Our findings support an association between these autoantibodies and type 1 diabetes. The stronger response observed in serum samples from patients with higher age and higher HbA$_{1c}$ scores suggests that these autoantibodies may be useful in evaluating the progression of type 1 diabetes and in elucidating the mechanisms of disease pathogenesis.

**Competing interests**

The authors declare that they have no competing interests.
Authors’ contributions

| Authors’ contributions | JF | AA | UZ | AMAM | ZR |
|------------------------|----|----|----|------|----|
| Research concept and design | √ | √ | -- | -- | √ |
| Collection and/or assembly of data | √ | -- | √ | √ | √ |
| Data analysis and interpretation | √ | -- | √ | √ | √ |
| Writing the article | √ | √ | √ | -- | √ |
| Critical revision of the article | √ | √ | -- | -- | √ |
| Final approval of article | √ | √ | √ | √ | √ |
| Statistical analysis | -- | -- | √ | √ | -- |

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