High titers of autoantibodies to glutamate decarboxylase in Type 1 Diabetes Patients: Epitope Analysis and Inhibition of Enzyme Activity

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Abstract

Objective—Autoantibodies to glutamate decarboxylase (GAD65Ab) are found in patients with autoimmune neurological disorders and patients with type 1 diabetes. The correct diagnosis of GAD65Ab-associated neurological disorders is often delayed by the variability of symptoms and a lack of diagnostic markers. We hypothesize that the frequency of neurological disorders with high GAD65Ab titers is significantly higher than currently recognized.

Methods—We analyzed GAD65Ab titer, inhibition of GAD65 enzyme activity, and pattern of GAD65Ab epitopes in a cohort of type 1 diabetes patients (n=100) and correlated our findings with neurological symptoms and diseases.

Results—Fourty-three percent (43/100) of the patients had detectable GAD65Ab titers (median=400 U/ml, range: 142–250,000U/ml). The GAD65Ab titers in 10 type 1 diabetes patients exceeded the 90th percentile of the cohort (2,000–250,000 U/ml). Sera of these 10 patients were analyzed for their GAD65Ab epitope specificity and their ability to inhibit GAD65 enzyme activity in vitro. GAD65Ab of five patients inhibited the enzyme activity significantly (by 34–55%). Three of these patients complained of muscle stiffness and pain, which was documented in two of these patients.

Conclusions—Based on our findings we suggest that neurological disorders with high GAD65Ab titers are more frequent in type 1 diabetes patients than currently recognized.

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Keywords

GAD65; neurological disorder; epitopes; radioligand binding assay; Stiff Person Syndrome; cerebellar ataxia

Introduction

Autoantibodies to the smaller isoform of glutamate decarboxylase (GAD65) are found in the majority of patients with type 1 diabetes (frequency: 80%), patients with neurological disorders, such as Stiff Person Syndrome (frequency: 70–80%) (SPS), cerebellar ataxia (frequency: 30–60%), intractable epilepsy (frequency: 10–30%) (for review see (1)) and Batten disease (frequency: 100%) (2). GAD65Ab in these different diseases show distinct antibody titers, epitope specificities, tissue distributions, and capacities to inhibit GAD65 enzyme activity. Patients with GAD65Ab-associated neurological disorders often have GAD65Ab titers that exceed those found in type 1 diabetes patients by at least 100-fold (3). In patients with SPS and cerebellar ataxia GAD65Ab can be detected in the patients' peripheral blood and cerebrospinal fluid (CSF), while GAD65Ab in type 1 diabetes patients are restricted to the circulating blood levels (4, 5). GAD65Ab epitope pattern in GAD65-associated neurological disorders differ from those in type 1 diabetes patients (6), and 83% of GAD65Ab-positive SPS sera inhibit the enzyme activity of GAD65, an uncommon characteristic for type 1 diabetes patients (7).

GAD65-associated neurological disorders such as SPS are considered rare diseases affecting one person per million in the general population (8). SPS is often associated with other autoimmune diseases and 30% of SPS patients are also diagnosed with type 1 diabetes (9). However, the prevalence of SPS in type 1 diabetes patients is thought to be as low as 1/10,000 (10).

The identification of patients with GAD65Ab-associated neurological disorders is hampered by a wide range of mild and non-specific symptoms such as phobias (11, 12), depression (13), and muscle aches and pain during the early stages of the neurological disease. The variability of symptoms (14–16) hinders the correct diagnosis of these conditions and previously used strict diagnosis criteria are not always helpful in clinical practice (17). We hypothesize that the prevalence of GAD65Ab-associated neurological disorders is currently underestimated. The aim of this study was to establish the prevalence of GAD65Ab with antibody characteristics found in GAD65Ab-associated neurological diseases in a cohort of type 1 diabetes patients. We chose this cohort based on the known association of type 1 diabetes and other autoimmune diseases and the relatively higher frequency of autoimmune neurological disorders in patients with type 1 diabetes (18–20).

Methods

Patient cohort

Samples from patients with type 1 diabetes analyzed in this study (n=100) were collected at the University of Washington in Seattle, Washington between 2007 and 2009. Along with the blood samples, surveys were conducted regarding age at onset of diabetes, duration of

disease, and diabetes management. Inclusion criteria for this study were determined by a clinical diagnosis of type 1 diabetes with subcutaneous insulin treatment. Exclusion criteria were: ages younger than 18 years at sampling, serious illnesses affecting the immune system, and medications suppressing the immune system. Median age at onset was 16 years (range: 2–62), median duration of disease was 25 years (range: 2–60), and median age at sampling time was 42 years (range: 18–85). Fifty-six patients were female. Local institutional ethics committee approval and subjects' consent was obtained prior to collection of all serum samples.

GAD65Ab Radioligand binding assay (RBA)

Recombinant human [^{35}S]-GAD65 was produced in an in vitro coupled transcription/translation system with SP6 RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI, USA) as described previously (21). The in vitro translated [^{35}S]-GAD65 was kept at −70°C and used within 2 weeks.

Sera (2.5μl) were incubated with [^{35}S]-GAD65 (25,000 of TCA precipitable radioactivity). After an overnight incubation at 4°C, antibody-bound [^{35}S]-GAD65 was separated from unbound antigen by precipitation with Protein A Sepharose (Invitrogen, Carlsbad, CA). The immunoprecipitated radioactivity was counted using a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences, Inc, Boston, MA). A standard curve consisting of dilutions of the international WHO standard for GAD65Ab was included for the calculation of the antibody levels. The cut-off value for positivity (135 U/ml) was determined as the 99th percentile of a healthy control cohort (n=50).

Epitope-specific Radioligand Binding assay (ES-RBA)

The capacity of the recombinant Fab (rFab) to inhibit GAD65 binding by human serum GAD65Ab was tested in a competitive ES-RBA as described (22). Sera were analyzed at a dilution representing half maximal binding to GAD65 as established by serial dilutions. All sera and a positive control were analyzed for their binding to GAD65 in the presence of rFab b96.11 and b78. b96.11 shares its GAD65Ab epitope specificity with the majority of type 1 diabetes patients (22), while b78 is a prototypical monoclonal antibody for GAD65Ab epitope specificities in SPS patients (6).

Non-competed GAD65 binding was established by no addition of rFab. The cutoff for specific competition was determined as >15% by using a control rFab. Each sample was measured in triplicates, and the mean value was calculated. Binding of GAD65Ab to GAD65 in the presence of rFab was expressed as follows: GAD65Ab cpm in the presence of rFab (competed) / GAD65Ab cpm in the absence of rFab (non-competed) x100. A higher binding to GAD65 in the presence of an rFab indicates a lower proportion of GAD65Ab binding to the respective epitope.

Definition of high GAD65Ab titer

Sera with high GAD65Ab levels were defined by us by two criteria: 1) their GAD65Ab titer exceeded that of the median GAD65Ab titer of the entire cohort (119U/ml) by at least 10-fold, and 2) successful displacement of binding to radiolabled GAD65 by 100μg/ml

recombinant GAD65 was observed only when serum samples containing GAD65Ab that were not displaced in the presence of 100μg/ml recombinant GAD65 following the procedure previously described (23) (data not shown). Each serum sample with a GAD65Ab binding level of 135 U/ml or higher was analyzed for binding to radiolabeled GAD65 in the presence of 100μg/ml recombinant human GAD65 to determine the level of unspecific binding. We found that binding to radiolabeled GAD65 in all but ten samples was successfully displaced (binding was reduced by more than 50%) with 100μg/ml GAD65. These ten samples had GAD65Ab levels of 2000 U/ml and were subsequently reanalyzed at their half-maximal binding concentration, at which their binding to radiolabeled GAD65 was successfully displaced. The cut-off for high GAD65Ab titer of 2000 U/ml is in agreement with other reports (24).

GAD65 enzyme activity assay

GAD65 enzyme activity was measured by the $^{14}CO_2$-trapping method described previously (25). Recombinant human GAD65 (100 ng) was incubated with or without the indicated amounts of serum for 1 hr at room temperature. The enzymatic reaction was initiated by the addition of 0.56 mM L-glutamate and 0.018 μCi ^{14}C-glutamate (Amersham Life Science Inc, Arlington Heights, IL) and allowed to continue for 2 hours at 37°C with gentle agitation. During incubation, released $^{14}CO_2$ was captured on filter paper (Kontes, Vineland, NJ) soaked in 50 μl 1 M NaOH. After the incubation, the absorbed radioactivity was determined in a Wallac Microbeta Liquid Scintillation Counter. The results are presented as follows: % residual activity = cpm in the presence of serum/cpm in the absence of serum × 100.

Statistical analyses

Binding of GAD65Ab to GAD65 in the presence of rFab was expressed as follows: cpm of [^{35}S]-GAD65 bound in the presence of rFab/cpm of [^{35}S]-GAD65 in the absence of rFab ×100.

All samples were analyzed in triplicate determinations and the intra-assay average coefficient of variation was 5% with the highest value of 13% and the lowest being 0.04%.

Results

We found detectable GAD65Ab in 43% (43/100) of the patients (median titer of GAD65Ab-positive patients=400 U/ml, range: 142–250,000U/ml). Ten of these patients had GAD65Ab titers 2,000 U/ml (2,000–250,000 U/ml) (Figure 1). No significant correlations between GAD65Ab titer, age at onset, duration, gender, or age at sampling were observed (data not shown). Presence of other autoimmune diseases, as reported by the patients, in these two groups is shown in Table 2.

The ten patients with GAD65Ab titers 2,000 U/ml were further evaluated for their GAD65Ab epitope pattern and their serum's ability to inhibit GAD65 enzyme activity.

Epitope pattern

Patients' sera were diluted to their half-maximal binding activity and analyzed for binding to GAD65 in the presence of rFab b96.11 or b78 (Table 3). Six of the 10 sera showed drastically reduced binding to radiolabeled GAD65 in the presence of rFab b96.11 (26–75% inhibition). Four sera showed substantially reduced GAD65 binding in the presence of rFab b78 (16–42% inhibition).

Enzyme inhibition

Patients' sera were tested for their capacity to inhibit GAD65 enzyme activity (Table 3). GAD65 enzyme activity was significantly inhibited (inhibition by 34–55%) for 5/10 patients. Three of these five sera also showed significant reduction in binding to GAD65 in the presence of rFab b78.

Clinical evaluation of neurological symptoms

Three of the ten patients complained of generalized muscle stiffness and pain, and/or arthritis pain. One of the patients (#9) was a 54-year-old woman with type 1 diabetes for >27 years. Approximately 20 years ago, she developed diffuse body aches and was subsequently diagnosed with fibromyalgia. About four years ago she developed increased stiffness, particularly in her neck. She was found to have cervical stenosis and her symptoms improved for a few months following surgical decompression. Subsequently, diffuse discomfort in her hands, legs, neck, and back recurred. She could not "straighten" her shoulders or back and felt that her neck was chronically flexed forward. The intensity of her symptoms waxed and waned. Overall, she had diminished mobility and was no longer able to participate in vigorous exercise. She was started on 5 mg of diapzepam (Valium) and immediately felt an improvement in her sleep and the stiffness affecting her neck and shoulders. Her stiffness continues to be improved by low doses of diazepam (4 to 6 mg per day). Her examination was normal except for tightness in her trapezius and cervical muscles with slight reduction in neck mobility, mild symmetrical reduction in sharp-dull sensation to her mid-calves and decreased sharp-dull sensation in her left hand in a median nerve distribution suggestive of a differential diagnosis that would include carpal tunnel syndrome.

Another patient was a 44-year-old man (#5). He developed type 1 diabetes >25 years ago. Subsequently, he noticed a tendency to have cold hands and feet. In his mind, he was always tensing in an effort to combat this cold feeling. He gradually noticed a chronic stiffness affecting shoulders, forearms, posterior thighs, low back, and, to a lesser extent, his calves. This tightness was nearly always present, and the patient recalled it to have been most severe when he was in his late 20s to, early 30s. He thought the tension was less severe more recently but also wondered if this was because he had simply learned how to manage it more effectively. His muscular tightness resulted in difficulties with competitive athletic activities such as playing soccer. He could do this at a slow pace, but if he competed more vigorously he developed severe spasms in his abdominal region, which could compromise his ability to breathe freely. He thought that his symptoms were better during sleep. He did not have pain with his muscular stiffness except very rarely when he might have brief lower calf pain. He had no symptoms in his hands and his handwriting and ability to play the piano were unaffected. He was tried on diazepam at night (5 mg), and noticed that he slept much easier

for the first time in years and was somewhat less stiff during the day. His examination was normal except for a slightly stiff gait with decreased associated movements, and slightly tense trapezius and cervical muscles.

The last patient (#10) reported neurological symptoms, but did not undergo neurological evaluation or treatment. As such, we presume that this patient had SPS.

The clinical parameters of these three patients are summarized in Table 1.

None of the other patients in this cohort reported neurological autoimmune diseases or associated symptoms.

Discussion and Conclusions

We analyzed GAD65Ab in 100 type 1 diabetes patients to identify GAD65Ab specificities in patients with high GAD65Ab titers. Although the median duration of disease in our cohort was 25 years, we were able to detect significant GAD65Ab titers in 43% of the patients, confirming earlier observations of persistent GAD65Ab titers in long-standing diabetes (26, 27). Ten of these patients had GAD65Ab exceeding the typical GAD65Ab titer in type 1 diabetes patients by 10-fold, a characteristic of patients with GAD65-associated neurological disorders (3). We identified three patients with high GAD65Ab titers and inhibition of GAD65 enzyme activity, who suffer neurological symptoms, such as muscle stiffness and pain.

It is of importance to note that neither assay alone could positively identify these patients. While all three patients had high GAD65Ab titer (inclusion criteria), and inhibited GAD65 enzyme activity, GAD65Ab of only 2 of the 3 patients recognized the b78-defined GAD65Ab epitope, which has been associated with SPS (6). Moreover, one patient, who met all three criteria (high GAD65Ab titer, recognition of the b78-defined epitope, and inhibition of GAD65 enzyme activity), had no neurological symptoms. We hypothesize that in such patients, GAD65 enzyme inhibiting antibodies are present only in the periphery and not in the CSF. CSF was not available for any of the patients to test our hypothesis. In a previous study we did not observe significant differences in GAD65Ab titers in CSF obtained from SPS patients with or without type 1 diabetes (unpublished results).

High GAD65Ab titers have been described in other cohorts of diabetes patients. In a recent report very high GAD65Ab titers (above 20,000 U/ml) were detected in 3/23 type 1 diabetes patients, 7/290 type 2 diabetes patients, and 4/57 patients with unspecified diabetes (23). The authors suggested that the prolonged exposure of these patients to GAD65 was involved in these uncharacteristically high GAD65Ab titers. The neurological status of these patients was not evaluated. In another study, GAD65Ab titers above 2,000 U/ml were detected in only 0.8% of analyzed type 1 diabetes patients (24). None of these patients showed neurological symptoms. Neither study investigated the epitope pattern of the associated GAD65Ab or enzyme inhibition. It is unclear what caused the difference in frequencies of high GAD65Ab samples in these studies.

The lack of a detailed neurological examination of the patients presents a major flaw of our study. In future studies we plan to include thorough neurological exams evaluating stiffness, rigidity or increased tone and painful spasms and electromyography (EMG) performed by a physician familiar with the findings of SPS to validate our hypothesis of higher prevalence of neurological symptoms in patients with increased GAD65Ab titers. However our data suggest that measurement of GAD65Ab in type 1 diabetes patients in conjunction with neurological exams may identify patients with underlying neurological diseases such as SPS and aid in their successful treatment.

Acknowledgments

Abbreviations

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cpm	counts per minute
CSF	cerebrospinal fluid
EMG	electromyography
ES-RBA	Epitope-specific Radioligand Binding assay
GAD65	glutamate decarboxylase
GAD65A	autoantibodies to GAD65
RBA	Radioligand binding assay
SPS	Stiff Person Syndrome
TCA	Trichloroacetic Acid
WHO	World Health Organization
U/ml	Units per ml
rFab	recombinant Fab

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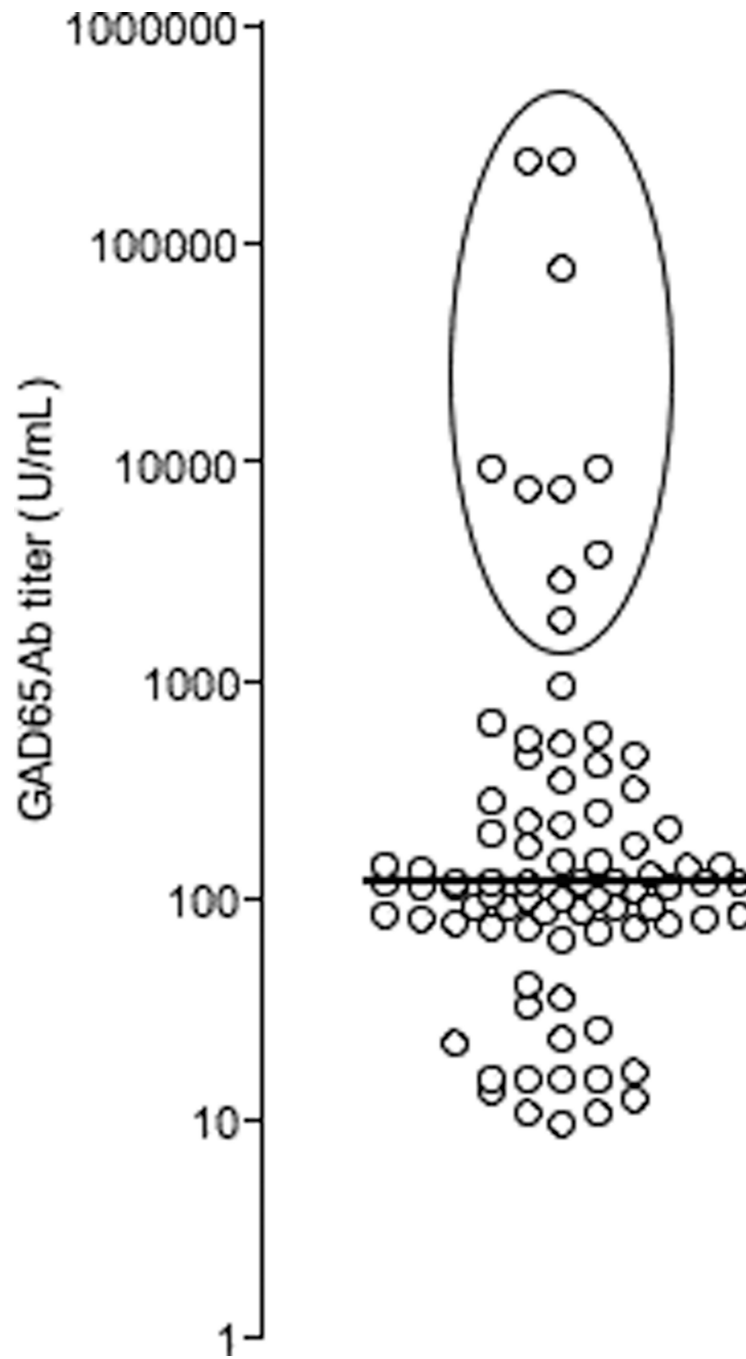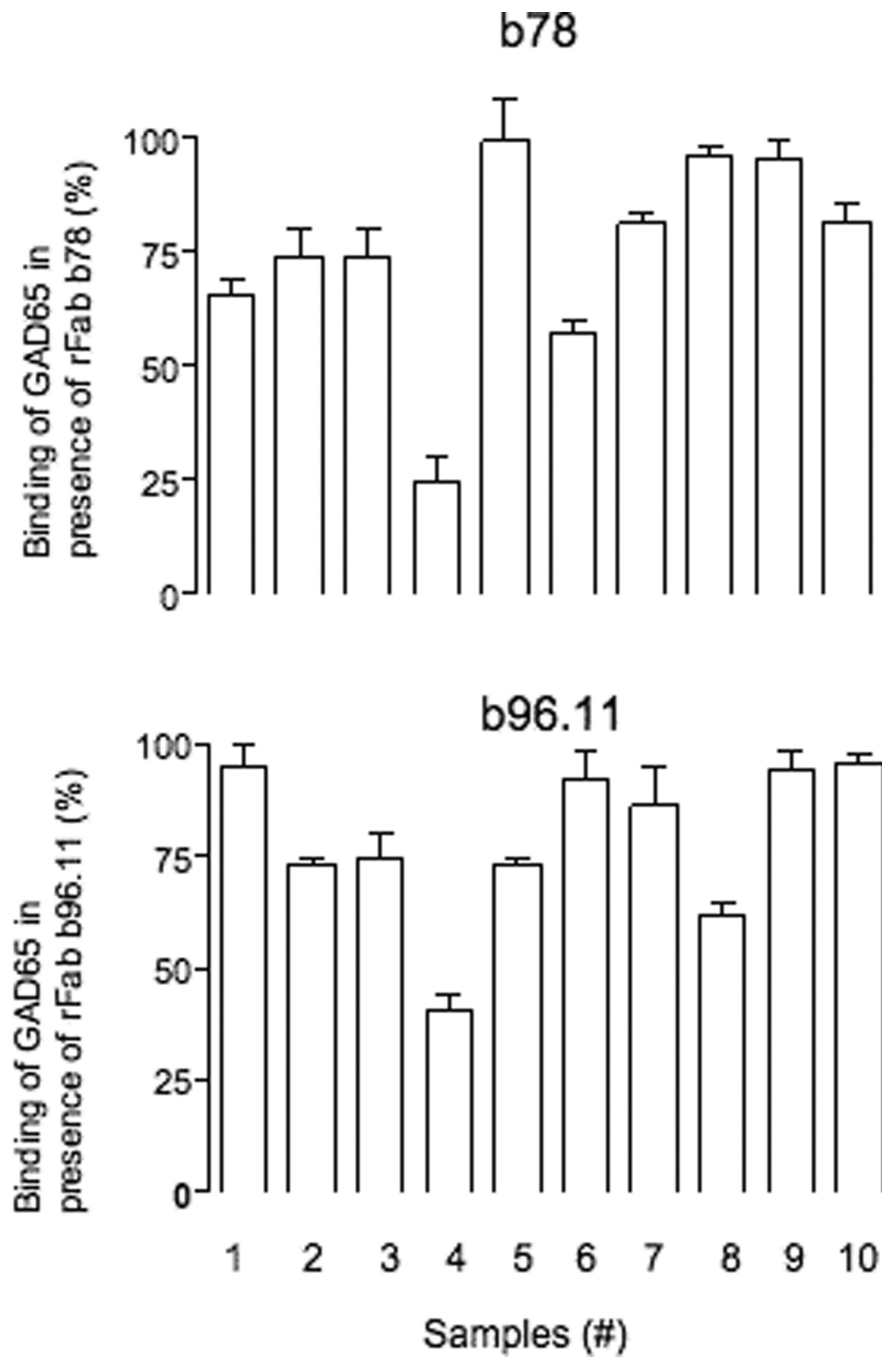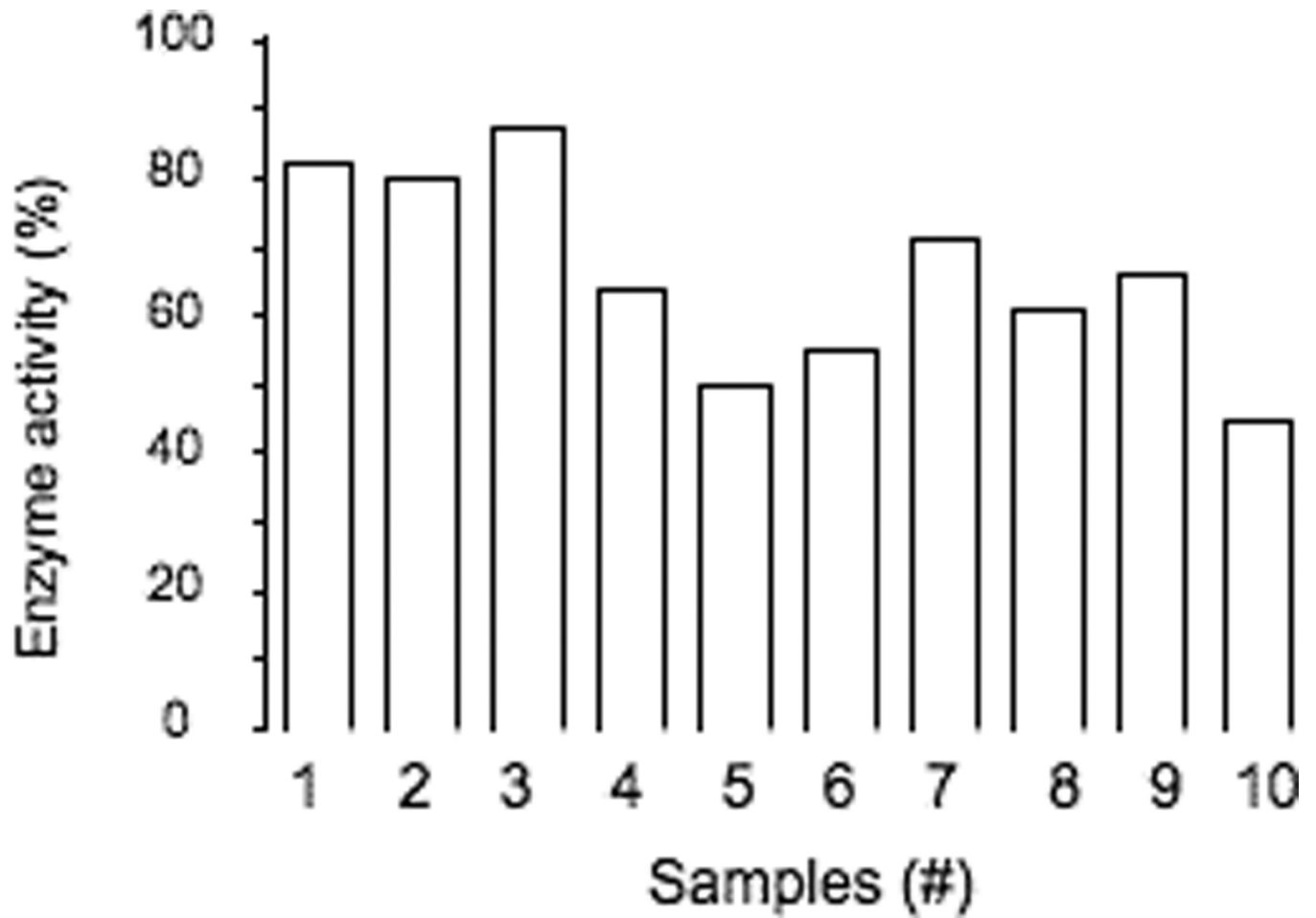
Figure 1. Analysis of GAD65Ab in 100 type 1 diabetes patients
GAD65Ab titers are shown on a logarithmical scale, patients, whose sera exceeded a
GAD65Ab titer of 2,000U/ml, are presented in the circled area. Median GAD65Ab titer is
indicated.

Table 1

Clinical parameters of three type 1 diabetes patients meeting both physical and immunological criteria for GAD65Ab-associated autoimmune neurological disorders.

Sample (#)	Age at onset	Age at sampling (years)	Gender	Related diagnosis	Complaints about pain and muscle stiffness
5	20	45	Male		Yes
9	14	51	Female	Fibromyalgia	Yes
10	62	85	Female		Yes

Table 2

Self-reported autoimmune diseases besides type 1 diabetes in patients with GAD65Ab <2000 U/ml and 2000U/ml.

	GAD65Ab titer (U/ml)
GAD65Ab titers 2000U/ml	
Hypothyrodism	2897
Hypothyrodism	7020
Hashimoto's thyroditis, Pernicious Anemia, Fibromyalgia	11279
GAD65Ab <2000U/ml	
Graves' disease	1421
Hashimoto's thyroditis	364
Hashimoto's thyroditis, celiac disease	115
Hashimoto's thyroditis	161

Table 3

Summary of GAD65Ab characteristics in samples with high GAD65Ab titer.

Sample (#)	Inhibition by rFab b78 (%)	Inhibition by rFab b96.11 (%)	Enzyme inhibition (%)	GAD65Ab titer (U/ml)
1	26 (±2)	28 (±4)	39 (±4)	250000
2	0 (±5)	0 (±5)	29 (±1)	10000
3	4 (±0)	38 (±3)	36 (±7)	10000
4	16 (±0)	13 (±2)	45 (±3)	8000
5	3 (±2)	56 (±5)	50 (±7)	4000
6	0 (±2)	26 (±0)	13 (±1)	3000
7	1 (±3)	75 (±5)	20 (±5)	2000
8	4 (±4)	26 (±2)	18 (±9)	8000
9	42 (±2)	7 (±6)	55 (±6)	250000
10	34 (±4)	7 (±4)	34 (±5)	80000