A Trial to Assess the Amount of Insulin Antibodies in Diabetic Patients by Surface Plasmon Resonance

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Abstract

Objective To measure the amount and affinity of insulin antibodies, we performed a trial to establish a new method for quantitative and qualitative analysis of these antibodies by using surface plasmon resonance (BIAcore™ system).

Methods Real-time detection of insulin antibody interaction and kinetic analysis were performed using the BIAcore™ system.

Patients or Materials Eight diabetic patients with insulin antibodies and whose fasting total immunoreactive insulin levels were more than 100 $\mu\text{U/ml}$ were selected. The patients with and without recurrent hypoglycemia were classified into hypoglycemic episode-positive or hypoglycemic episode-negative groups, respectively. Seven diabetic patients without insulin antibodies were selected as controls.

Results In the 8 patients, the concentration of insulin antibodies ranged from 2.91 to 16.3 $\mu\text{g/ml}$ and insulin antibodies were not detected in the control group. The apparent KD (dissociation constant) and kd (the dissociation rate constant) values of the patients were much larger than those seen for the anti-human insulin monoclonal antibody. The KD values were significantly higher in the hypoglycemic episode-positive group than in the hypoglycemic episode-negative group ($p < 0.05$). No significant differences in the concentration, the ka (the association rate constant) and the kd values were noted between the groups.

Conclusion The data suggests that insulin antibodies of the patients have an apparently lower affinity status in sera as compared with that for the anti-human insulin monoclonal antibody, and dissociate easily from the immune-complex in the sera, especially in cases where there is recurrent hypoglycemia in the patients. Therefore in-

ulin antibody characteristics are one of the causative factors in hypoglycemic episodes.

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Key words: diabetes mellitus, insulin antibody, surface plasmon resonance, Scatchard analysis, hypoglycemia

Introduction

In diabetic patients treated with exogenous insulin, insulin antibodies are sometimes detected in the sera (1). Although the wide use of recombinant human insulin has reduced the production of insulin antibodies (2, 3), Fineberg et al (4) reported that insulin antibodies still appear in 40–60% of diabetic patients treated with human insulin resulting in problems such as insulin resistance or hypoglycemia.

High-affinity insulin antibodies frequently induce insulin resistance in patients (5, 6) and low-affinity antibodies induce hypoglycemia due to the dissociation of high amounts of insulin from the insulin antibody complex (4, 7). However the actual status and effects that can be attributed to the antibody affinities are still unknown.

Up until the present, the affinity of insulin antibodies has typically been measured by a traditional binding assay and analyzed by a Scatchard analysis (8). This method is widely used for affinity measurements in many facilities, but it is basically a method used to measure high-affinity, i.e., a one component (monoclonal) antigen-antibody reaction. If antibodies are found to be polyclonal, we usually hypothesize that there is the existence of more than two components, such as high-affinity and low-affinity antibodies, and fit the dissociation constant (KD) through calculations. However, the Scatchard analysis sometimes does not adequately mea-

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sure the low-affinity antibodies (KD less than about 10^{-4} M) because the low-affinity interaction is usually reversible and easily dissociates the ligand from the complex.

Since the introduction of the BIAcore™ system in 1991 (9, 10), the use of surface plasmon resonance technology has facilitated kinetic analysis of molecular interactions. This method is especially suitable for determining low-affinity antigen-antibody interactions such as lectin and sugar, and reversible protein interactions such as cellular signal transduction.

In the present study, we have developed a new method using the BIAcore™ system to assess the apparent affinity of insulin antibodies in patients with diabetes mellitus. It enables us to measure both the amount and the apparent affinity of insulin antibodies and may reveal the clinical significance of insulin antibodies.

Materials and Methods

Study subjects and sample collection

All patients gave prior informed consent. Eight diabetic patients with insulin antibodies in their sera and whose fasting total immunoreactive insulin (TIRI) levels, as measured by the polyethylene glycol (PEG) method, were more than 100 µU/ml were selected for the present study. Patients that exhibited recurrent hypoglycemia and unstable blood glucose levels, including unexpected hyperglycemia, were classified as hypoglycemic episode-positive patients (patients No. 1–No. 4). The other 4 patients (patients No. 5–No. 8) that had no recurrent hypoglycemia were classified as hypoglycemic episode-negative patients. Seven diabetic patients without insulin antibodies were selected as the corresponding

control group. Serum samples were immediately frozen and TIRI, free immunoreactive insulin (FIRI) and 125 I-labeled human insulin binding (normal range, <5%) were measured according to the method reported by Uchigata et al (11). FIRI was measured after the precipitation of antibody-bound insulin with PEG.

Table 1 shows the demographics and medical characteristics for the patients with the insulin antibody and the controls that lacked the insulin antibody. Although the sex ratios varied somewhat, the age ranges and body mass index (BMI) for the patients were not grossly different. All subjects in the control group were treated with human insulin alone. In 8 patients with insulin antibodies, 4 had been treated with human insulin alone and 4 had been treated with both animal and human insulin in the past. The duration of the insulin therapy was longer in patients with the insulin antibody than in the control group but there was no difference between the groups with regard to current insulin dosage. There was no significant difference observed between the hypoglycemic episode-positive and -negative groups for age, sex, BMI, insulin species, duration of insulin treatment and current insulin dosage. The hemoglobin A_{1c} (HbA_{1c}) levels shown in the table are for the data obtained at the time when insulin antibodies were measured.

Insulin antibody interactions (BIAcore™ analysis)

Real-time detection of insulin antibody interaction and kinetic analysis were performed using the BIAcore™ system (BIAcore 2000, Biacore AB, Uppsala, Sweden). This instrument detects changes in the refractive index of the solution close to the surface of the sensor chip. To perform the analysis, one reactant (the ligand) is immobilized in a dextran ma-

Table 1. Summary of Clinical Characteristics of the Patients

Patient no.	Age (years)	Sex (M/F)	BMI (kg/m ²)	Insulin species	Duration of insulin treatment (year)	Current insulin dosage (/day)	Hypoglycemic episode	GADA (U/ml)	HbA _{1c} (%)	TIRI (µU/ml)	FIRI (µU/ml)	¹²⁵ I- insulin binding (%)	HLA	
													DR	DRB1
1	60	F	24.1	human	21	R 28U, NPH 10U	+	9.5	7.4	153	5.0	49.6	DR4 DR9	0405 0901
2	69	F	20.1	bovine and human	20	Mix 30U	+	0.4	10.1	678	25	65.4	DR8 DR9	0803 0901
3	85	F	19.4	bovine and human	15	Mix 14U	+	0.4	14.7	1000	5.0	70.0	DR4 DR9	–
4	60	F	23.6	human	2	Mix 16U	+	0.4	7.8	1250	8.2	76.9	DR4 DR6	0405 1401
5	74	F	25.2	human	25	R 66U	–	0.4	9.4	2500	230	70.8	DR2 DR6	–
6	74	F	23.8	human	2	NPH 14U	–	0.4	6.8	100	5.0	76.5	DR4	0401 0405
7	64	F	20.5	bovine and human	16	NPH 24U	–	0.4	6.8	449	7.0	75.3	N.D.	N.D.
8	78	F	24.1	bovine and human	17	Mix 28U	–	0.4	12.1	4100	7.0	85.4	DR9	0901 0901
Control														
9	42	F	29.0	human	1	Mix 28U	–	0.4	9.9	30.4	30.2	2.0	N.D.	N.D.
10	49	M	19.3	human	1	Mix 12U	–	0.4	11.0	3.2	3.0	2.0	N.D.	N.D.
11	70	F	28.8	human	1	Mix 20U	–	0.4	7.2	5.6	5.4	2.0	N.D.	N.D.
12	62	F	27.0	human	1	Mix 24U	–	0.4	9.0	5.6	5.2	2.0	N.D.	N.D.
13	69	M	27.7	human	25	NPH 30U	–	0.4	8.0	14.8	14.6	2.0	N.D.	N.D.
14	87	F	21.8	human	1	R 24U, NPH 6U	–	0.4	12.0	5.6	5.4	2.0	N.D.	N.D.
15	75	F	20.9	human	1	R 12U, NPH 4U	–	0.4	7.9	3.0	3.0	2.0	N.D.	N.D.

BMI: body mass index, Insulin species: insulin species used in the past treatment, Hypoglycemic episode: hypoglycemic episode-positive patients exhibited recurrent hypoglycemia and unstable blood glucose levels including unexpected hyperglycemia, GADA: anti-glutamic acid decarboxylase antibody, TIRI: total immunoreactive insulin, FIRI: free immunoreactive insulin, 125 I- insulin binding: 125 I-labeled human insulin binding, R: human regular insulin, NPH: human neutral protamine Hagedorn insulin, Mix: 30% regular and 70% NPH insulin, N.D.: not determined.

trix on the sensor chip. The sample containing the other reactant (the analyte) is injected over the CM5 sensor chip (Biacore AB) surface using a controlled flow. Any change in surface concentration resulting from the interaction is detected as a surface plasmon resonance signal, and is expressed in resonance units (RU). One RU corresponds to approximately 1 pg/mm² of any biomolecule, as the specific response is based on the refractive index increment, which is similar for peptides, proteins, glycoproteins, lipids and nucleic acids. Interactions take place in a cell of approximately 60 nl volume. The continuous display of RU as a function of time gives a plot that is called a sensorgram, which provides a complete record of the progress of association and dissociation between the two molecules. After analyzing one interaction, the surface can be regenerated using an appropriate eluant solution that does not affect the activity of the immobilized ligand.

Purified human insulin (Peptide Institute, Osaka, Japan) was immobilized on a CM5 sensor chip surface using standard amine coupling in HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20 at pH 7.4) running buffer (flow rate: 5 µl/min) at 25°C. The carboxyl group at the CM5 sensor chip surface was activated with EDC/NHS (0.2 M N-ethyl-N-(3-diethylaminopropyl) carbodiimide and 0.05 M N-hydroxysuccinimide) over a 7 minutes injection period and HBS buffer flow was maintained after the activation. Human insulin was dissolved in 10 mM acetate buffer at pH 4.8 and was injected during 16 minutes to achieve immobilization. The activated residue was then blocked with 1 M ethanolamine hydrochloride (pH 8.5) injection. The immobilized surface was washed twice for 30 minutes by injections of 10 mM glycine-HCl (pH 1.5) after specimen exposure. The association rate constant (k_a) and the dissociation rate constant (k_d), and the KD were obtained by the analysis of the sensorgrams using BIA evaluation (ver. 3.1) software. The procedure was based on the following formula:

$$dR/dt = k_a C R_{\max} - (k_a C + k_d) R_t$$

where $R_{\max}$ is the maximum analyte binding capacity in RU and R_t is the response in RU at time t and C is the concentration of injected analyte.

The k_a and k_d were calculated from the $-(k_a C + k_d)$ slope, which was given by the plot of $dR/dt = -k_d + R_t$ plot. The KDs were determined by k_d/k_a .

Deinsulinization of the insulin antibody complex in patient's serum

Deinsulinization was carried out according to the methods of Eguchi et al (12) and Dixon et al (13). To separate and remove insulin from the insulin antibody complex in the serum, 125 µl of 0.12 N HCl and 50 µl of dextran-coated charcoal were added to 100 µl of serum at 4°C. After shaking for 5 minutes, an additional 125 µl of 0.12 N HCl and 600 µl of HBS were added and then centrifuged at 3,000 rpm for 30 minutes at 4°C. The supernatant was filtrated with a

0.22 filter (Millipore Co., Ltd) and the filtrate was frozen at -80°C until required for laboratory analysis.

For affinity analysis of insulin antibodies, CM-dextran sodium salt (Fluka Chemie AG, Switzerland) was added to each sample in order to eliminate the nonspecific reaction. CM-dextran was dissolved in HBS buffer and the final concentration was 10 µg/ml. We confirmed that over 97.8% of the insulin was removed from the serum by this method (data not shown).

Quantitation of insulin antibody in the serum

A standard curve (RU vs. antibody concentration) was prepared by using an anti-human insulin monoclonal antibody (Japan Biotest Laboratory, Tokyo, Japan). The concentrations of anti-human insulin monoclonal antibody used were 1.00×10^{-2} , 5.00×10^{-3} , 2.50×10^{-3} , 1.25×10^{-3} , and 6.25×10^{-4} mg/ml, respectively.

To measure the concentration of insulin antibodies in sera, we prepared CM5 sensor chips that immobilized human insulin and the patient's serum, which was deinsulinized as mentioned above. The patient's serum was injected over 180 seconds and the binding level was measured after 150 seconds in HBS running buffer (flow rate: 10 µl/min). The immobilized surface was washed twice for 30 minutes by injections of 10 mM glycine-HCl (pH 1.5). Using the difference between the binding levels of the serum and the control, the concentration of the patient's insulin antibodies was assessed using a standard curve.

Removal of IgG

IgG sorb (Enzyme Center, Malden, MA, U.S.A.) was used to remove IgG from the serum. Deinsulinized serum was incubated for 10 minutes with IgG sorb, and centrifuged at 10,000 rpm for 1 minute at 4°C to remove material that bound non-specifically to the adsorbent. CM-dextran (40 µl) was added to the collected supernatant and then assayed by BIAcore™. The control consisted of serum diluted with the same amount of HBS buffer and 40 µl of CM-dextran.

Scatchard analysis

The patient's serum that had been deinsulinized was used for the Scatchard analysis (8), which was performed as per the method previously reported by Eguchi et al (12). Briefly, the serum was incubated with 100 µl of ¹²⁵I-human insulin in the presence of 100 µl of serial concentrations of human insulin solution and after precipitation with PEG 6,000, the radioactivity of the pellets was counted with an automatic gamma counter. We performed Scatchard analysis in 5 of 8 patients exhibiting the insulin antibodies.

Statistical analysis

Data are presented as the mean ± standard deviation (M±SD). Differences between groups were evaluated by the Mann-Whitney U-test for comparison of the hypoglycemic episode-positive and -negative groups. P values less than 0.05 were regarded as significant.

Results

Immobilization

To measure the concentration of insulin antibodies, purified human insulin was immobilized on a CM5 sensor chip by the amine coupling procedure. The immobilized quantity was 1,760 RU. In order to measure the affinity of insulin antibodies, it was necessary to prepare a sensor chip with less immobilized quantity. The immobilized quantity for this measurement was 360 RU.

Identification of specific binding for insulin on the sensor chip

Figure 1 shows a representative example (patient No. 5) that was analyzed by BIAcore™. Figure 1A shows the pattern of a deinsulinized sample analyzed with a sensor chip to measure the concentration of insulin antibodies. The difference between the sample (continuous line) and the control (HBS buffer, broken line) reveals that insulin antibodies bound insulin on the sensor chip (specific binding).

To verify the insulin antibodies shown in Fig. 1A, we analyzed the patient sample after adding an excess amount of insulin under the same conditions. As shown in Fig. 1B, specific binding almost disappeared after the addition of the excess amount of insulin.

Removal of IgG

A representative example is shown in Fig. 1C. The continuous line represents the results of the sample assay after IgG sorb and the broken line represents the corresponding control by BIAcore™. After removal of IgG by the treatment with IgG sorb, the specific binding of insulin was almost diminished. IgG sorb was examined in another sample (patient No. 4), and the results were found to be similar to the first case.

Standard curve of insulin antibody

Figure 2 shows the standard curve for insulin antibody using anti-human insulin monoclonal antibody as a standard. The standard curve was constructed by using the BIAcore™ software. It was thought that the standard curve was representative of a good correlation.

Concentration of insulin antibodies in the sera

Table 2 shows the concentration of insulin antibodies in the sera as measured by BIAcore™. In 8 patients, the concentration ranged from 2.91 to 16.3 µg/ml. On the other hand, insulin antibodies were not detected in any of the sera of the corresponding controls.

Affinity of patient's insulin antibodies

To measure the affinity of the patient's antibodies and mouse monoclonal antibody, a sensor chip with a less immobilized quantity was prepared as described previously. As shown in Table 2, a 1 : 1 (Langmuir) binding was used for the fitting model. In patients with hypoglycemic episodes,

the apparent affinity of the insulin antibody ranged from 5.53 to $2.49 \times 10^4 \text{ Ms}^{-1}$ for the k_a , from 2.62×10^{-4} to $1.15 \times 10^{-3} \text{ s}^{-1}$ for the k_d and from 1.05×10^{-8} to $2.09 \times 10^{-4} \text{ M}$ for the K_D . In the patients without hypoglycemic episodes, the apparent affinity of the insulin antibody ranged from 1.35×10^3 to $1.52 \times 10^6 \text{ Ms}^{-1}$ for the k_a , from 1.91×10^{-4} to $2.47 \times 10^{-3} \text{ s}^{-1}$ for the k_d and from 1.62×10^{-9} to $1.41 \times 10^{-7} \text{ M}$ for the K_D . The K_D values were significantly higher in the hypoglycemic episode-positive group ($5.44 \pm 10.3 \times 10^{-5} \text{ M}$; $p < 0.05$) than in the hypoglycemic episode-negative group ($3.73 \pm 6.91 \times 10^{-8} \text{ M}$). No significant differences in the concentration, the k_a and the k_d values were noted between the groups. The concentration, k_a , k_d , and K_D of insulin antibodies measured by BIAcore™ did not correlate with TIRI, FIRI or ^{125}I -labeled human insulin binding.

The affinity of anti-human insulin antibodies (mouse monoclonal) as measured by the BIAcore™ system was $4.31 \times 10^5 \text{ Ms}^{-1}$ (k_a), $5.21 \times 10^{-8} \text{ s}^{-1}$ (k_d) and $1.21 \times 10^{-13} \text{ M}$ (K_D).

Scatchard analysis

As shown in Table 2, although the k_1 values tended to be higher in the hypoglycemic episode-positive than in the hypoglycemic episode-negative group, there were no differences between the groups for the b_1 , k_2 and b_2 values.

Discussion

In this study, we describe a new method for quantitative and qualitative analysis of insulin antibodies in the sera of diabetic patients using the BIAcore™ system.

To calculate the apparent affinity of the insulin antibody, the concentration of insulin antibodies is a very important factor for the BIAcore™ system software. In this study, we first tried to measure the concentration of the insulin antibody in diabetic patients. We decided to use mouse monoclonal anti-human insulin antibody as the control for this experiment because there is no standard human anti-insulin antibody available. Since the antibodies to the exogenous insulin are generally considered to be polyclonal and consist of various components with different affinities, the mouse monoclonal antibody we selected in this study might show different time constants for the antigen-antibody interaction compared with those of patients. Our results indicated that the k_d and the K_D values for the patients that possessed the insulin antibody in their sera were more than ten thousand times larger than that for mouse anti-insulin monoclonal antibody. Additionally the K_D of the hypoglycemic episode-positive group was significantly higher than that for the hypoglycemic episode-negative group. These results suggest that the patients with recurrent hypoglycemic episodes may have insulin antibodies that dissociate more easily from the immune-complex in the sera. In other words, the existence and the characteristics of the insulin antibodies have important causative roles in hypoglycemic episodes.

Berson et al (5, 6) demonstrated that almost all insulin-treated patients possess insulin-binding immunoglobulins.

Figure 1. Example of BIAcore™ analysis (patient No. 5). A: The pattern for a deinsulinization sample. The difference between the sample (continuous line) and the control (broken line) reveals that insulin antibodies bound insulin on the sensor chip (specific binding). B: The results of the sample assay after adding an excess amount of insulin. The specific binding almost disappeared. C: The results of the sample assay after IgG sorb. After removal of IgG the specific binding was abated.

These antibodies were later shown to be polyclonal IgG (14). In our study, the insulin antibody that presented in the patients with exogenous insulin treatment may also belong to the IgG immunoglobulin class because we found that the specific binding was almost completely diminished in the

serum of patients after the removal of IgG by IgG sorb. However, in this study we only examined two samples. Even so, it did seem that these reactions were associated with IgG class immunoglobulins.

Since patients with insulin autoimmune syndrome (IAS)

Figure 2. A standard curve (RU vs. antibody concentration) was prepared by using an anti-human insulin monoclonal antibody. The concentrations of anti-human insulin monoclonal antibody used were 1.00×10^{-2} , 5.00×10^{-3} , 2.50×10^{-3} , 1.25×10^{-3} and 6.25×10^{-4} mg/ml, respectively.

also have insulin antibodies and show frequent hypoglycemic episodes, it is necessary to be able to distinguish between the two types of patients. IAS can be characterized by spontaneous hypoglycemia that occurs without any evidence of exogenous insulin administration. Additionally, HLA-DR4 and DRB*0406 genotypes are common in patients with IAS (1, 15). However, there was no history of spontaneous hypoglycemia before insulin administration in any of our patients and none of the subjects was treated with insulin until after the diagnosis of diabetes mellitus. And since our patients had different HLA typing from that which is seen in IAS (Table 1), they can be distinguished from those patients with IAS.

To date few papers describe the insulin antibodies analyzed by Scatchard analysis (1, 12, 16). One of these reports using IAS patients showed that the insulin antibodies of insulin-treated diabetic patients with hypoglycemia exhibit a much smaller high-affinity k_1 value and a much larger low-capacity b_1 value than the k_1 and b_1 values seen for insulin antibodies in patients without hypoglycemia (12). In the Scatchard analysis that was performed in 5 of our patients, the results that were found indicated that the k_1 values tended to be even higher in the hypoglycemic episode-positive group than in the hypoglycemic episode-negative group. Additionally, there was no difference found for the b_1 values between the groups (Table 2). On the contrary, in the

hypoglycemic episode-positive group, the KD values were significantly larger and the k_a values tended to be smaller than those in the hypoglycemic episode-negative group. These results also corresponded with the clinical features observed.

With regard to those points, the apparent affinity, as measured by the BIAcore™ system, seems to more closely reflect the situation of the serum than the data obtained from a Scatchard analysis since the BIAcore™ system can easily measure low-affinity interactions that are less than 10^{-4} M k_d . The affinity of exogenous insulin antibodies is one of the causes of hypoglycemia. Since hypoglycemia has been considered to result from low-affinity insulin antibodies, the BIAcore™ system can measure the k_d regardless of the state of the affinity in the sera. Based on these results, the BIAcore™ system can more extensively evaluate insulin antibodies than the Scatchard analysis.

The mechanism of the immunogenicity of exogenous human insulin antibody is still unknown. Several factors are considered to influence the production of insulin antibodies, such as genetic factors, mode of insulin administration, and degree of purity and species of insulin. In the future, we will be able to identify the epitopes and the mechanism of insulin antibody production by using the BIAcore™ system.

In diabetic patients treated with human recombinant insulin, we need to pay more attention to the presence of anti-

Table 2. Comparison of Concentration, Affinity and Scatchard Analysis of Insulin Antibodies between Hypoglycemic Episode-positive and -negative Group

Patient no.	Hypoglycemic episode	BIAcore™				Scatchard analysis			
		Concentration (μg/ml)	ka (M ⁻¹ s ⁻¹)	kd (s ⁻¹)	KD (M)	k1 (l/mol)	b1 (l/mol)	k2 (l/mol)	b2 (l/mol)
1	+	4.81	5.53	1.15×10 ⁻³	2.09×10 ⁻⁴	N.D.	N.D.	N.D.	N.D.
2	+	4.10	4.59×10 ⁵	8.02×10 ⁻⁴	1.75×10 ⁻⁷	2.17×10 ⁹	1.91×10 ⁻¹⁰	2.53×10 ⁵	1.16×10 ⁻⁷
3	+	2.91	64.1	5.32×10 ⁻⁴	8.30×10 ⁻⁶	2.66×10 ⁹	1.46×10 ⁻⁹	2.94×10 ⁶	1.33×10 ⁻⁸
4	+	10.8	2.49×10 ⁴	2.62×10 ⁻⁴	1.05×10 ⁻⁸	N.D.	N.D.	N.D.	N.D.
M±SD		5.65±3.51	7.39±11.9×10 ⁴	6.87±3.80×10 ⁻⁴	5.44±10.3×10 ^{-5*}	—	—	—	—
5	—	16.3	4.20×10 ⁵	8.35×10 ⁻⁴	1.99×10 ⁻⁹	1.65×10 ⁷	2.68×10 ⁻⁹	9.88×10 ⁴	2.40×10 ⁻⁷
6	—	4.15	1.83×10 ⁵	8.37×10 ⁻⁴	4.59×10 ⁻⁹	2.44×10 ⁶	1.16×10 ⁻⁸	8.32×10 ⁴	1.73×10 ⁻⁷
7	—	3.38	1.35×10 ⁵	1.91×10 ⁻⁴	1.41×10 ⁻⁷	3.66×10 ⁷	7.85×10 ⁻¹⁰	2.87×10 ⁵	3.67×10 ⁻⁸
8	—	7.71	1.52×10 ⁶	2.47×10 ⁻³	1.62×10 ⁻⁹	N.D.	N.D.	N.D.	N.D.
M±SD		7.90±5.94	5.31±6.81×10 ⁵	10.8±9.73×10 ⁻⁴	3.73±6.91×10 ⁻⁸	—	—	—	—
Mouse monoclonal antibody			4.31×10 ⁵	5.21×10 ⁻⁸	1.21×10 ⁻¹³	N.D.	N.D.	N.D.	N.D.

Data are presented as mean (M)±SD. *p<0.05 compared with hypoglycemic episode-negative group. N.D.: not determined.

insulin polyclonal antibodies. We strongly recommend that the measurement of serum insulin antibodies should be done in patients who develop insulin resistance and hypoglycemia.

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