Development of an ultra-sensitive enzyme immunoassay for human insulin autoantibodies

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ARTICLE INFO

Article history:
Received 22 January 2012
received in revised form 24 May 2012
accepted 24 May 2012
Available online 2 June 2012

Keywords:
Enzyme immunoassay
Insulin antibodies
Diabetes
Graves’ disease
Dextran-charcoal

ABSTRACT

Objectives: We developed an ultrasensitive enzyme immunoassay (ICT-EIA) for insulin autoantibody (IAA) measurements to better understand the pathophysiology of diabetes.

Design and methods: We developed ICT-EIA for IAA and measured IAA in 24 patients with type 1 diabetes, 30 patients with type 2 diabetes, 30 patients with methimazole-treated Graves’ disease, 20 patients with Hashimoto’s disease, 9 patients with hyperinsulinemia, and 73 healthy control subjects.

Results: The conventional ELISA identified 3 patients with type 1 diabetes and 2 patients with type 2 diabetes as IAA positive, whereas 15 patients with type 1 diabetes, 7 patients with type 2 diabetes, and 4 patients with methimazole-treated Graves’ disease were identified as IAA positive using ICT-EIA.

Conclusions: The ICT-EIA is an ultrasensitive and specific assay for IAA, and its use may provide a better understanding of the role of IAA in diabetes onset and progression.

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Introduction

In the widely used conventional enzyme-linked immunosorbent assay (ELISA) of antibody IgG in serum, an antigen-coated solid phase is incubated with serum to trap antigen-specific IgG. The amount of antigen-specific IgG trapped on the solid phase is subsequently estimated by incubating with an anti-IgG-enzyme conjugate. However, non-specific IgG in serum samples is non-specifically absorbed on the solid phase. This absorption of non-specific IgG on the antigen-coated solid phase causes background noise in the ELISA during the reaction with the anti-IgG–enzyme conjugate leading to decreased assay sensitivity [1,2].

We previously developed a novel, highly sensitive enzyme immunoassay (immune complex transfer enzyme immunoassay, ICT-EIA) to help solve this problem. The advantage of ICT-EIA-I is that antibodies in the serum sample are simultaneously reacted with dinitrophenyl (DNP)–biotinyl-labeled antigen and an enzyme–antigen conjugate. The immune complex formed by these three components is first trapped onto a solid phase of anti-DNP IgG-coated polystyrene beads. This immune complex is then transferred to a second solid phase of streptavidin-coated polystyrene beads, followed by an assay of the conjugated enzyme. Thus, transfer of the immune complex from the first solid phase to the second solid phase minimizes the effect of non-specific IgG and other interfering substances. Consequently, detection of specific IgG in serum is much more sensitive than that achieved with conventional ELISA [3,4].

When this novel ICT-EIA-I assay was used to detect anti-insulin antibody in serum, the detection of guinea pig anti-insulin IgG in serum was 4000-fold more sensitive than that of conventional ELISA [3,4]. However, in this ICT-EIA-I assay, binding of the serum anti-insulin antibody to the epitope on insulin was affected by steric hindrance, because the relatively small insulin molecule (molecular weight, 6 kDa) was directly conjugated with the relatively large β-o-galactosidase (540 kDa). This steric hindrance resulted in a marked inhibition of the immunoreaction of the insulin conjugate with insulin antibodies.

In the present study, we report on the development of an improved ultra-sensitive enzyme immunoassay (ICT-EIA-II) for insulin autoantibody (IAA) levels in serum, which is negligibly affected by steric hindrance of insulin epitopes.

Abbreviations: ICT-EIA, immune complex transfer enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DNP, 2,4-dinitrophenyl; FI, fluorescence intensity; GAD, glutamic acid decarboxylase; IA-2, insulinoma-associated antigen-2; LADA, latent autoimmune diabetes in adults.
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Materials and methods

Buffers

The following buffers were used in the present study: i) buffer A: 10 mM sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl, 1.0 mM MgCl2, 1.0 g/L NaN3, and 1.0 g/L bovine serum albumin (BSA); ii) buffer B: 10 mM sodium phosphate buffer (pH 7.0), containing 0.4 M NaCl, 1.0 mM MgCl2, 1.0 g/L NaN3, and 1.0 g/L BSA; and iii) buffer C: 10 mM sodium phosphate buffer (pH 7.0), containing 0.1 M NaCl, 1.0 mM MgCl2, 1.0 g/L NaN3, and 0.1 g/L BSA. BSA (fraction V). All buffers were obtained from Intergen Co. (Purchase, NY).

Antibodies

Rabbit (anti-DNP–BSA) serum was obtained from Shibayagi Co., Ltd. (Gunma, Japan). Rabbit (anti-human-IgG γ-chain) IgG was obtained from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan).

Antigen and ELISA

Recombinant human insulin was obtained from Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan). The ELISA kit for insulin antibodies was obtained from ORGENTEC Diagnostika GmbH (Mainz, Germany) (Fig. 1).

Subjects

Serum was obtained from patients with type 1 diabetes (n = 24), type 2 diabetes (n = 30), Graves’ disease treated with methimazole for more than 6 months (n = 30), Hashimoto’s disease with or without levo-thyroxine-replacement (n = 20), and hyperinsulinemia associated with obesity (n = 9), as well as from non-obese, healthy control subjects (n = 73). Sera from patients with auto-immune diseases associated with diabetes were analyzed. Patients with type 1 or type 2 diabetes were diagnosed based on the clinical and laboratory findings defined by the Japan Diabetes Society (http://www.jds.or.jp/). Patients with hyperinsulinemia were defined as obese healthy individuals with a BMI greater than 30 kg/m² and high fasting plasma insulin levels (over 30 μU/mL). Patients with Graves’ or Hashimoto’s disease were diagnosed based on the clinical and laboratory findings defined by the Japan Thyroid Association (http://www.japanthyroid.jp/). These patients were either receiving antithyroid therapy with methimazole or replacement therapy with levo-thyroxine for more than 3 months, and showed euthyroidism. The study protocol was approved by the ethics committee of Tokushima Bunri University, and all participants provided written informed consent.

Blood sampling

Blood samples were drawn early in the morning from the antecubital vein of subjects who fasted overnight. Samples were then transferred into a chilled glass tube, and kept on ice for 30 min. Serum was prepared from all blood samples by centrifugation at 1500 × g for 15 min at 4 °C, and kept frozen at −30 °C until analysis.

Preparation of protein-coated polystyrene beads

Polystyrene beads (6.4-mm diameter, Immunochemical Inc., Okayama, Japan) were coated with affinity-purified rabbit (anti-DNP-BSA) IgG (0.01 g/L), affinity-purified rabbit (anti-human-IgG γ-chain) IgG (0.1 g/L), and biotinyl–BSA (0.01 g/L) by physical adsorption [5,6]. Biotinyl–BSA-coated polystyrene beads were then coated with

Fig. 1. Protocols for the conventional ELISA (a), ICT-EIA-I (b), and ICT-EIA-II (c) for the detection of anti-insulin antibodies. ICT-EIA, immune-complex transfer enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; DNP, 2,4-dinitrophenyl.
streptavidin in 100 mM sodium phosphate buffer (pH 7.5), containing 1.0 g/L NaN₃ (0.01 g/L) [6].

**Preparation of dextran–charcoal**

Dextran–charcoal was prepared by using the Dixon method with the following substitutions [7]. BSA and Norit A (Nacalai Tesque Inc., Kyoto, Japan) were substituted for human serum albumin and Norit NK, respectively. The dextran–charcoal suspension contained 60 mg of charcoal in dry weight per milliliter.

**Dextran–charcoal treatment of serum**

Free- and IAA-bound insulin in serum were removed as follows. Serum (50 μL) was diluted with 150 μL of buffer A, and 13.6 μL of 0.4 M HCl was added. Subsequently, dextran–charcoal (72.8 μL), as described above, was added and shaken for 6 min. The acidity of this dextran–charcoal-serum mixture was neutralized with 13.6 μL of 0.4 M NaOH. The pH-neutralized serum containing dextran–charcoal was centrifuged at 1500 × g for 15 min, and the supernatant fluid was then re-centrifuged at 1500 × g for 15 min in a new tube. The final supernatant fluid was stored at 4 °C.

**Serum treatment with inactive β-d-galactosidase**

Inactive β-d-galactosidase was used to eliminate interference with an anti-β-d-galactosidase antibody in serum [8]. Dextran–charcoal treated serum (90 μL) was incubated with 10 μL of 1.0 μM inactive β-d-galactosidase ([β-d-galactosidase-mutein, Roche, Mannheim, Germany]) in buffer A at room temperature for 1 h.

**ICT-EIA-I assay for insulin antibodies**

An aliquot (100 μL) of dextran–charcoal treated serum with inactive β-d-galactosidase was incubated overnight at 4 °C with 100 μL of buffer B containing 100 fmol of a DNP–biotinyl–BSA–insulin conjugate and 100 fmol of a β-d-galactosidase–insulin conjugate (formation step). Thereafter, one polystyrene bead coated with affinity-purified IgG (anti-DNP-BSA) was added to the mixture and incubated at room temperature for 25 °C) for 30 min with shaking (210 strokes/min; entrapment step). After removal of the incubation mixture, the polystyrene bead was washed twice with the addition and aspiration of 2.0 mL of buffer C, and incubated with 150 μL of buffer A containing 2.0 mM DNP-lysine at room temperature for 30 min while shaking (210 strokes/min; elution step). After removal of the polystyrene bead, one streptavidin-coated polystyrene bead was added to the eluate and incubated for 30 min while shaking (210 strokes/min; transfer step). The bead was then washed, and the bound β-d-galactosidase activity was assayed fluorometrically using 4-methylumbelliferyl-β-d-galactoside (0.2 mM) as the substrate, following an incubation of 20 h at 30 °C. The fluorescence intensity (FI) was measured relative to 1.0 × 10⁻⁸ M 4-methylumbelliferone in 0.1 M glycine–NaOH buffer (pH 10.3) using a spectrofluorophotometer (F-3010, Hitachi, Ltd., Tokyo, Japan) with 360 nm for excitation and 450 nm for emission analysis [9] (Fig. 1).

**ICT-EIA-II assay for insulin antibodies**

An aliquot (100 μL) of dextran–charcoal treated serum was incubated overnight at 4 °C with 50 μL of buffer A containing 100 fmol of a DNP–insulin conjugate, 100 fmol of a recombinant biotin–insulin conjugate, and 10 pmol of inactive β-d-galactosidase-mutein (formation step). Thereafter, one polystyrene bead coated with affinity-purified IgG (anti-DNP-BSA) was added to the mixture and was incubated at room temperature (25 °C) for 15 min while shaking (210 strokes/min; entrapment step). After incubation, a streptavidin–β-d-galactosidase conjugate (100 fmol) in 50 μL of buffer A was added to the mixture, and was incubated at room temperature for 15 min while shaking (210 strokes/min; reaction step). After removal of the incubation mixture, the polystyrene bead was washed twice with the addition and aspiration of 2.0 mL of buffer C, and incubated with 150 μL of buffer A containing 2.0 mM DNP-lysine at room temperature for 30 min while shaking (210 strokes/min; elution step). After removal of the bead with tweezers, one polystyrene bead coated with affinity-purified IgG (anti-human-lgG γ-chain) was added to the eluate and was incubated for 30 min while shaking (210 strokes/min; transfer step). Bound β-d-galactosidase activity was then assayed, as described above for ICT-EIA-I (Fig. 1).

**Statistical analysis**

The detection limit of the ICT-EIA or ELISA for anti-insulin antibody was defined as the maximal dilution of serum containing anti-insulin IgG (diluted with pooled healthy control serum), which produced bound-enzyme activity levels that were significantly greater than that with healthy control serum (background). A significant difference from the background was confirmed by Student's t-test (P < 0.01).

Statistical analysis was performed using SPSS version 20.0.0. The ICT-EIA method was compared with the ELISA method by correlation analysis using Spearman's correlation coefficient.

**Results**

The ICT-EIA-II was developed to improve the sensitivity of the previous ICT-EIA-I for the detection of IAA from the serum of patients with autoimmune diseases. In this novel and improved immunoassay, as outlined in Fig. 1, a 20-μL aliquot of test serum containing IAA was treated with dextran–charcoal to remove both free insulin and insulin that was bound to IAA in serum. After centrifugation, the supernatant containing IAA was incubated simultaneously with DNP–insulin and biotinyl–insulin to form an immune complex of these three components. These immune complexes were then trapped onto a polystyrene bead coated with anti-DNP IgG. After washing to remove the excess biotinyl–insulin, streptavidin–labeled β-d-galactosidase was attached to the immune complexes. Subsequently, the immune complexes comprising these four components were transferred in the presence of an excess of DNP-lysine to a second polystyrene bead that was coated with anti (human-lgG γ-chain) IgG, and β-d-galactosidase activity was then measured. Thus, direct conjugation of β-d-galactosidase to insulin, which sterically hinders antibody–insulin interaction in the ICT-EIA-I assay, was prevented.

**Sensitivity and specificity of the ICT-EIA-II for insulin antibodies**

In order to compare the sensitivity of IAA detection via the ICT-EIA-II assay with that of the previous ICT-EIA-I and conventional ELISA, we assayed anti-insulin antibody levels in dextran charcoal-treated serum samples from patients with type 1 diabetes that were serially diluted with dextran charcoal-treated serum from healthy control subjects, using the three different assay methods. The detection of IAA serum levels using ICT-EIA-I and -II was found to be 100-fold and 1000-fold more sensitive, respectively, than with using conventional ELISA (Fig. 2).

In order to compare the specificity of insulin antibody detection of the two ICT-EIA methods, insulin (10 pmol) was added to serum samples from patients with type 1 diabetes, and the samples were then analyzed using ICT-EIA-I and -II. The specific signals of both assays, measured as the fluorescence intensity of bound β-d-galactosidase activity, were significantly decreased (97–99% decrease).
and a patient with Hashimoto’s disease, the fluorescence signals were higher than 25.5, but the decrease induced via pre-incubation with insulin was not as significant as observed in other patients. Using this ICT-EIA-II assay, we detected insulin antibodies in 15 out of 24 (63%) patients with type 1 diabetes, 7 out of 30 (23%) patients with type 2 diabetes, 4 out of 30 (13%) patients with methimazole-treated Graves’ disease, none of the 20 (0%) patients with Hashimoto’s disease, and none of the 9 (0%) patients with hyperinsulinemia (Fig. 3 and Table 1).

Detection of human anti-insulin antibodies using the ICT-EIA-I

Anti-insulin antibodies in dextran charcoal-treated serum samples obtained from healthy control subjects and patients were also analyzed using ICT-EIA-I. Using a cutoff value of 8.7 FI (mean ± 2 standard deviations), which was determined based on data obtained from healthy control subjects, positive fluorescence signals, i.e., signals that were higher than 8.7, were confirmed as insulin-antibody specific signals following the induction of a significant decrease in the signal (53.6–92.7%) via pre-incubation with excess insulin. In a patient with hyperinsulinemia, the fluorescence signal was higher than 8.7, but the decrease induced via pre-incubation with insulin was not as significant as observed in other patients. Using the ICT-EIA-I assay, insulin antibodies were detected in 2 out of 4 (50%) patients with type 1 diabetes, 3 out of 23 (13%) patients with type 2 diabetes, and none of the 9 (0%) patients with hyperinsulinemia (Fig. 4 and Table 1).

Detection of human anti-insulin antibodies using a conventional ELISA

Serum samples were collected from healthy control subjects and from patients with type 1 diabetes, type 2 diabetes, Graves’ disease, Hashimoto’s disease, or hyperinsulinemia, and anti-insulin antibodies were analyzed using a conventional ELISA. In three patients with type 1 diabetes and one patient with insulin-treated type 2 diabetes, absorbance signals were higher than 0.136 (an arbitrary cut-off value) and were significantly decreased following a pre-incubation with excess insulin. In three healthy control subjects and two patients with hyperinsulinemia, absorbance signals were higher than 0.136, but the decrease induced via pre-incubation with insulin was not as significant as observed in other patients (Fig. 5 and Table 1). Using this conventional ELISA, we detected IAA in 3 out of 24 (13%) patients with type 1 diabetes, 2 out of 30 (7%) patients with type 2 diabetes, and none of the 30 (0%) patients with methimazole-treated Graves’
Table 1
Comparison of human insulin antibody detection via conventional enzyme-linked immunosorbent assay (ELISA) and immune-complex transfer enzyme immunoassays (ICT-EIA-I and ICT-EIA-II).

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Insulin treatment</th>
<th>Immunoassay</th>
<th>Detection of insulin autoantibodies (positive subjects/total)</th>
<th>Positive rate (%)</th>
</tr>
</thead>
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<tr>
<td>Healthy control subjects</td>
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<td>ICT-EIA-II</td>
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<td></td>
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<tr>
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<tr>
<td></td>
<td></td>
<td>ELISA</td>
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<tr>
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<tr>
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<tr>
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<td>ICT-EIA-II</td>
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ICT-EIA, immune-complex transfer enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay.

disease, 20 (0%) patients with Hashimoto’s disease, or 9 (0%) patients with hyperinsulinemia.

Correlation between ICT-EIA-II and ELISA

The results obtained with the ICT-EIA-II and ELISA in 24 patients with type 1 diabetes and 30 patients with type 2 diabetes were compared with regression analysis. There was a significant correlation between ICT-EIA-II and ELISA data (r = 0.53, P < 0.001) (Fig. 3 and Fig. 5). Interestingly, only five of the samples (i.e., 3 from patients with type 1 diabetes and 2 from patients with type 2 diabetes) that were positive in ELISA were also positive in ICT-EIA-II. However, 17 samples from patients with type 1 and 2 diabetes were negative in ELISA, but positive in ICT-EIA-II.

Discussion

We developed a novel and improved ultra-sensitive enzyme immunoassay (ICT-EIA-II) for the detection of IAA. The sensitivity for detecting IAA with the ICT-EIA-II assay was compared with that of the ICT-EIA-I and conventional ELISA. Detection of IAA serum levels using the ICT-EIA-II was found to be 1000-fold and 10-fold more sensitive than conventional ELISA and ICT-EIA-I, respectively.

We compared the detection of anti-insulin antibodies in dextran-charcoal-treated serum samples from patients with type 1 and 2 diabetes and other autoimmune diseases using ICT-EIA-II, ICT-EIA-I, and a conventional ELISA. In serum samples from patients with type 1 diabetes, anti-insulin IgG antibodies were detected in 63% and 50% of the samples via ICT-EIA-II and ICT-EIA-I, respectively, but in only 13% of the samples using a conventional ELISA. In serum samples from patients with insulin-treated type 2 diabetes, anti-insulin IgG antibodies were detected in 23% and 13% of the samples via ICT-EIA-II and ICT-EIA-I, respectively, but in only 7% of the samples using a conventional ELISA. In the analysis of serum samples from patients with other autoimmune diseases, including Graves’ disease and Hashimoto’s disease, anti-insulin IgG antibodies were only detected using ICT-EIA-II, which detected these antibodies in 13% of samples from Graves’ disease patients. These data clearly indicate that the ICT-EIA-II assay is more sensitive than previous assays for detection of anti-insulin antibodies in serum.

Non-specific antibodies, which are present in large quantities in blood, bind non-specifically to the solid phase of the conventional ELISA, thereby creating background noise and making it difficult to improve the sensitivity of this assay. In contrast, such background noise has been dramatically reduced in both of the ICT-EIA (I and II) assays. These assays reduce background noise because only the immune complex consisting of the conjugates and the insulin antibodies from the first solid phase are transferred to the second solid phase. Therefore, any non-specific antibodies and antigen–enzyme conjugate that were non-specifically bound to the first solid phase were removed, resulting in a marked decrease in non-specific signals (noise) (Fig. 1).

Additionally, ICT-EIA-II was improved over ICT-EIA-I, in that insulin was labeled with the low molecular weight compounds DNP and biotin, and streptavidin, rather than insulin, was labeled with β-D-galactosidase. These modifications confer the advantage that labeling of insulin with the relatively very large β-D-galactosidase molecule, which may result in steric hindrance of IAA-insulin recognition, is not required for this assay. This modified step, therefore, facilitates the immunoreaction of insulin antibodies with epitopes of the insulin conjugates, and thereby enhances the formation of immune complexes, leading to increased assay sensitivity (Fig. 1).

ICT-EIA-II is the only assay that was able to detect insulin antibodies in the serum of patients with methimazole-treated Graves’ disease. It has been reported that methimazole, which is a therapeutic drug for hyperthyroidism, induces the occurrence of serum insulin antibodies [10]. The presence of insulin antibodies in the four patients with Graves’ disease is, therefore, likely to be a result of methimazole treatment.

It was recently reported that 5–30% of patients that were originally regarded as having type 2 diabetes, actually had type 1 diabetes [11–14]. Autoantibodies against an islet-cell antigen are usually found in type 1 diabetes, but autoantibodies against at least one of the islet-cell antigens, insulin, glutamic acid decarboxylase (GAD), or insulinoma-associated antigen-2 (IA-2), are found in approximately 10% of patients with type 2 diabetes, often referred to as latent autoimmune diabetes in adults (LADA) [15]. LADA is generally found in...
Acknowledgments

The ICT-EIA-II assay for insulin antibodies may be useful for the detection of insulin antibodies than the previous ICT-EIA-I and conventional ELISA. Furthermore, ICT-EIA-II detected insulin antibodies in a greater number of patients than when ICT-EIA-I or conventional ELISA. Additionally, ICT-EIA-II detected insulin antibodies, or IA-2 antibodies, is deemed necessary. The design of the ultrasensitive assay described herein may also prove useful for analysis of the serum levels of such antibodies.

In summary, we developed an improved and novel ultra-sensitive enzyme immunoassay for IAA (ICT-EIA-II). The ICT-EIA-II was more sensitive for the detection of insulin antibodies than the previous ICT-EIA-I and conventional ELISA. Furthermore, ICT-EIA-II detected insulin antibodies in a greater number of patients than when ICT-EIA-I or conventional ELISA was used. The more effective measurements of anti-insulin antibodies via the ICT-EIA-II assay appear to be a result of the labeled antigen of low molecular weight (i.e., that is not conjugated with a high molecular weight label), which facilitates antibody-antigen binding. Thus, the ICT-EIA-II assay for insulin antibodies may be useful for the early detection and diagnosis of type 1 diabetes or LADA.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (C), 2009, 21500701, from the Ministry of Education, Science, Sports and Culture of Japan. The authors thank T. Tominaga (University Tokushima, Tokushima, Japan) for assistance in blood sampling.

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