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High titre GAD65 autoantibodies detected in adult diabetes patients using a high efficiency expression vector and cold GAD65 displacement.

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Running title: High titre GAD65 autoantibodies in diabetes

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Abstract

Adult type 2 diabetes patients with GAD65 autoantibodies (GADA) are known as latent autoimmune diabetes in adults (LADA). It has been suggested that GADA in LADA patients preferentially bind to the N-terminal end of GAD65. Using the N-terminal end extension of ^{35}S-GAD65 generated by the pEx9 plasmid, we tested the hypothesis that GADA in LADA patients preferentially react with ^{35}S-GAD65 from the pEx9 plasmid compared to the normal length pThGAD65 plasmid. Healthy control subjects (n=250) were compared with type 1 (n=23), type 2 (n=290) and unspecified (n=57) diabetes patients. In addition, radiobinding assays for GADA with ^{35}S-GAD65 generated from both the pEx9 and pThGAD65 plasmids were used in displacement assays with an excess of recombinant human GAD65 (2 μg/mL) to correct for non-specific binding.

^{35}S-GAD65 produced by either pEx9 or pThGAD65 did not differ in binding among the healthy controls and among the type 1 diabetes patients. Among the type 2 and unspecified patients there were 4/290 and 3/57 patients, respectively, with binding to the pEx9 but not to the pThGAD65 generated ^{35}S-GAD65. In the displacement assay we discovered fourteen patients with very high titre GADA among the type 1 (n=3, 12,272-29,915 U/mL), type 2 (n=7; 12,398-334,288 U/mL) and unspecified (n=4; 20,773-4 053,580 U/mL) patients. All samples were fully displaced following appropriate dilution.

We conclude that pThGAD65 is preferred for the coupled *in vitro* transcription translation of ^{35}S-GAD65 and that displacement with recombinant GAD65 may detect very high titre GADA with possible clinical relevance.

INTRODUCTION

Type 1 diabetes is caused by a loss of the pancreatic islet ß cell function in association with islet autoimmunity. After a prodrome of islet autoimmunity of varying length (1-4), islet autoantibody positive subjects eventually develop type 1 diabetes. The patients become dependent of insulin in attempts to maintain normal glucose levels. In the majority of type 1 diabetes patients, autoantibodies against glutamic acid decarboxylase (GAD65), insulinoma-associated antigen 2 (IA-2) or insulin appear before clinical onset; insulin autoantibodies seem to predominate among the young while GAD65 autoantibodies (GADA) are more common in adolescence and adults (2, 3). It is not fully known what triggers first the appearance of islet autoantibodies and thereafter the clinical onset. Environmental factors, e.g. virus infections or infant diet, together with genetic predisposition are considered to be important etiological factors both to trigger islet autoimmunity but also for progression to diabetes (5).

As the pace of loss of ß-cell function differ markedly between islet autoantibody-positive individuals, it is not surprising that several intermediary phenotypes are observed in the clinic. GADA are often found in patients classified with type 2 diabetes (6, 7). As diabetes is classified based on clinical symptoms, it is not always possible to tell whether the disease is associated with islet autoimmunity or not. Because of an increasing use of GADA analyses in the clinic, patients who are classified with type 2 diabetes, but positive for GADA are more commonly reported. These patients are referred to as latent autoimmune diabetes in adults (LADA) (6-10) or Slowly Progressive Insulin Dependent Diabetes Mellitus (SPIDDM) (10). These clinical subtypes are of interest as these patients often become insulin dependent (reviewed in 11).

In type 1 diabetes, GADA are thought to be directed against epitopes located at the middle and C-terminal part of GAD65 (12, 13). High risk of progression to type 1 diabetes has been shown to

be associated with the emerge of GADA specific for epitopes at the N-terminal end and the middle region of GAD65 (14, 15). In contrast, LADA patients have been shown to have a higher frequency, compared to type 1 diabetes patients, of GADA directed against the N-terminal end of GAD65 (16).

In a previous study we compared the frequency of GADA detected after *in vitro* transcription/translation of GAD65 cDNA using the first generation pEx9 with that of the second generation pThGAD65 plasmid (17). Compared to the pThGAD65, the pEx9 plasmid generates a protein that has an additional 13 amino acids on its N-terminal end in about 50% of the *in vitro* transcription/translation product (17-19). Although the N-terminal extension of GAD65 has no resemblance to any known human sequence, we hypothesized that GADA in LADA patients may be directed against the N-terminal end of GAD65, because of epitope spreading (12, 15, 16, 20). The LADA patients may have been exposed to GAD65 for a prolonged period of time, which may result in the spreading of epitopes. Moreover it is unclear to what extent a prolonged exposure to endogenous GAD65 affects titres.

The aim of the present study was to compare the reactivity between ^{35}S-labelled GAD65 generated from both the pEx9 and pThGAD65 plasmids, with sera from healthy subjects and patients with type 1, type 2 and unspecified diabetes. Furthermore, we also determined the specificity of the GADA using an excess of non-radioactive human recombinant GAD65 to correct for non-specific binding.

PATIENTS AND METHODS

Patients

Three-hundred-seventy adults with diabetes were identified through a local diabetes register (21) covering the county of Västerbotten in northern Sweden and 250 non-diabetic individuals, were selected at random from Västerbotten Intervention Programme (VIP) as controls. Diabetes was determined using the 1999 diagnostic criteria and the classification criteria of 2007 (ICD-10) of the World Health Organization (WHO). The diabetes subjects were classified with type 1 diabetes (n=23), type 2 diabetes (n=290), and unspecified diabetes (n=57) by a diabetologist. The mean age were 46.2 (range 30-60) for the type 1 diabetes, 54.9 (range 30-61) for the type 2 and 50.0 (range 30-60) for the unspecified patients. Among the controls the mean age were 52.6 (range 30-61). All subjects had participated in a health survey and donated a blood sample stored in the medical bio bank, Umeå University, at -80° C. Samples were coded and the code key was not opened until after analysis. All living participants provided written informed consent. This study was approved by the Regional Ethics Board, Umeå, Sweden.

Materials

Recombinant human GAD65 was kindly donated by Diamyd Medical AB, Stockholm, Sweden (Lot n: o 10-65702-16-01). EasyTag™ ^{35}S–methionine, (NEG709A, 500µCi) and OptiPhase Supermix Cocktail was from Perkin Elmer, Waltham, Massachusetts, USA, TnT® Coupled Reticulocyte Lysate Systems from Promega, Madison, Wisconsin, USA, NAP™-5 columns from GE Healthcare, Buckinghamshire, UK, Protein A-Sepharose, (Lot.Nr 60505544) from Zymed, South San Francisco, California, USA, and filtration plates, (MultiScreen HTS™, DV) from Millipore, Billerica, Massachusetts, USA

pEx9 And pThGAD65 Plasmid Preparations

The pEx9 plasmid containing the full length GAD65 cDNA, described in detail elsewhere (18) was previously made available in a modified pcDNAII vector (17). This vector contains a modified Kozak consensus sequence to enhance plasmid efficiency for coupled *in vitro* transcription translation of GAD65 (18). The Kozak consensus sequence leads to 13 additional amino acids in 50 % of the product. Recently we have recloned the human GAD65 cDNA into the pTnT™ Vector (Promega PN L5610) to improve the translation performance beyond that of pEx9. The complete pThGAD65 cDNA sequence is available at http://depts.washington.edu/rhwlab/resMat/MatTransfer/pThGAD65seq.htm.

Preparation Of ^{35}S-methione-labelled GAD65

The TnT® Coupled Reticulocyte Lysate System was used as described by the manufacturer for *in vitro* transcription/translation of GAD65 (18). The two different expression plasmids, pEx9 or pThGAD65 were added to the reaction mixture (about 100 microliter) to a final concentration of 0.02 µg/µL. The mixture was then incubated for 90 minutes at 30°C before ^{35}S-methionine incorporated in the translation product or not was separated on a NAP™ 5 column. Nine fractions were collected and an aliquot was used to determine the radioactivity. Fractions 1-4 consists of ^{35}S-labelled GAD65 protein and fractions 5-9 consists of unbound label.

GADA Radiobinding Assay

^{35}S-labelled GAD65 was diluted in antigen buffer (150mM NaCl, 20 mM Tris, 0.15% Tween 20, 0.1% BSA; pH 7.4) to a final radioactivity of 400 cpm/µL. A total of 60 µL representing 24 000 cpm were added to duplicates of the samples (2.5 µL serum) and incubated overnight at 4°C. Filtration plates were coated overnight in 200 µL antigen buffer at room temperature, and then replaced with 50 µL of 20% (v/v) Protein A-Sepharose. From the samples incubated with the ^{35}S-labelled GAD65 protein, 50 µL were added to the filtration plates. The plates were incubated at

4°C for 60 minutes and thereafter washed eight times with washing buffer (150mM NaCl, 20 mM Tris, 0.15% Tween 20; pH 7.4). The samples were allowed to dry at room temperature and 50 µL of OptiPhase Supermix Cocktail was added. The amount of radioactivity was then measured on a ß-counter.

When analyzing the samples, a standard curve and positive and negative quality controls were used in each run. The level of antibodies in the samples was determined from a standard curve. It consisted of an international WHO standard for GAD65 and IA-2 antibodies, which defines levels of GAD65 antibodies in Units/mL (U/mL) (22-24). The standard was diluted in serum from GAD65 antibody-negative healthy subjects. All samples with a CV% greater than 20% were reanalyzed.

GADA Competitive Analysis

Five different concentrations of non-radioactive human recombinant GAD65 from Diamyd were used to determine the amount needed to completely displace the ^{35}S-GAD65. The concentrations of recombinant GAD65 in each well were 0.02, 0.1, 0.2, 1 and 2 µg/mL, respectively. Samples used for this optimization were the WHO standard and positive quality controls. Each sample was analyzed in duplicates, simultaneously either with or without human recombinant GAD65. These samples were adjacent each other on the microtiter plate. To the samples 30 µL of either antigen buffer or recombinant GAD65 were added. Thereafter, 30 µL of ^{35}S-labelled GAD65 protein representing 24 000 cpm (800 cpm/µL) was added to each sample. Displacing the WHO-standard as well as our in-house GADA positive quality control samples (n= 15) with different concentrations of recombinant GAD65 showed that all but two of the samples were completely displaced in the presence of 1 µg/mL GAD65 (data not shown). The concentration of recombinant GAD65 chosen for the subsequent analyses was therefore 2 µg/mL. Each patient sample previously analyzed for GADA with a binding level of 25 U/mL or higher was also

analyzed in the presence of 2 µg/mL human recombinant GAD65 to determine the level of unspecific binding. The samples were analyzed in parallel with and without recombinant GAD65 on the same assay plate. Care was taken to re-analyze high binders at increasing dilutions to ensure displacement by recombinant GAD65.

Statistical Analysis

Differences in frequencies between groups were tested using chi-square tests. Correlation in antibody analysis between ^{35}S-GAD65 generated by pEx9 and pThGAD65 was tested using Spearman's correlation. Graphs were drawn on PRISM 4.03 and statistical analysis performed using SPSS version 14. P-values less than 0.05 were considered significant.

RESULTS

^{35}S-GAD65 Generated From pEx9 Or pThGAD65

The pEx9 expression plasmid showed less incorporation of ^{35}S-labelled methionine than the incorporation achieved with pThGAD65 (Figure 1). The radioactivity (cpm) in the first peak, which shows the labeled protein, was higher for pThGAD65 compared to pEx9. Consequently, the second peak of unbound label was almost five times lower for pThGAD65. When comparing binding percentage to the WHO-standard curve, pEx9 generated ^{35}S-GAD65 revealed a decreased binding at the three highest concentrations of the standard curve (data not shown).

It is interesting to note that the use of pThGAD65 was a major saving. One *in vitro* transcription/translation with ^{35}S-methionine and pEx9 would at 19% incorporation yield enough ^{35}S-GAD65 for 300 samples. In comparison, pThGAD65 at 69% incorporation yield labeled GAD65 enough for 1000 samples. The cost per sample including labor is reduced to half with pThGAD65 compared to pEx9.

GADA Cut-off level Analysis With ^{35}S-GAD65 From Either pEx9 Or pThGAD65

The binding on a log scale in the 250 control samples of ^{35}S -GAD65 generated by the pEx9 plasmid showed a near linear increase (Figure 2a). The 97.5th percentile in binding was reached at 34 U/ml and the 99th percentile at 55 U/ml. In contrast the binding of ^{35}S -GAD65 generated by pThGAD65 was curvilinear suggesting less non-specific binding at levels below the 70th percentile (Figure 2b). The 97.5th percentile for pThGAD65 was reached at 55 U/ml and the 99th percentile at 87 U/ml. In analyzing the patient samples we therefore used 55 U/mL for pEx9 and 87 U/mL for pThGAD65, respectively as the cut-off level at the 99th percentile for both plasmids.

Levels Of GADA With ^{35}S-GAD65 From Either pEx9 Or pThGAD65 In Control, Type 1, Type 2 Or Unspecified Diabetes Subjects

Controls: GADA levels (on a log scale) showed two individuals (0.8%, 2/250) with increased binding for both plasmids (Figure 3a).

Type 1 diabetes patients: We observed 19/23 (82, 6 %) GADA positive patients, two of whom (8.7 %) showed GADA levels higher than 20,000 U/mL (Figure 3b). One individual was positive with the pEx9, but not with the pThGAD65 product.

Type 2 diabetes patients: 40/290 (13.8 %) were positive for both pEx9 and pThGAD65 ^{35}S-GAD65 (Figure 3c). Remarkably, no patient showed binding to the pThGAD65 product without also being positive to the pEx9-produced ^{35}S-GAD65. In contrast, there were two patients (0.7 %) who reacted with pEx9, but not pThGAD65 generated ^{35}S -GAD65. These two patients were 60 and 50 years of age at diagnosis and their c-peptide levels were 0,75 and 0,36 nmol/L, respectively. A total of 248 patients scored negative for both types of ^{35}S -GAD65.

Unspecified diabetes patients: A total of 32/57 (56 %) were positive for both pEx9 and pThGAD65 ^{35}S -GAD65 (Figure 3d). Again, no patient showed binding to the pThGAD65 product without also being positive to the pEx9-produced ^{35}S-GAD65. Two patients (3.5 %) reacted with pEx9 ^{35}S -GAD65 but not pThGAD65. These two patients were 23 and 51 years of

age at diagnosis and their c-peptide levels were 0,02 and 0,004 nmol/L, respectively. They were both treated with insulin after diagnosis. A total of 23 patients were negative for both types of ^{35}S -GAD65. We found a strong correlation between samples positive with pEx9 and pThGAD65 generated ^{35}S -GAD65 in type 1 ($r = 0.995$, $p<0.0001$), type 2 ($r = 0.982$, $p<0.0001$) as well as in unspecified diabetes subjects ($r = 0.997$, $p<0.0001$) (Figure 3).

Competition Analysis And Levels Of GADA With ^{35}S-GAD65 From pThGAD65

All samples with a concentration above 25 U/mL (a total of 126 samples) were analyzed with both pEx9 and pThGAD65 produced ^{35}S-GAD65, in the presence of 2 μg/mL recombinant GAD65. In ^{35}S-GAD65 generated by pEx9, nine samples were not displaced by the recombinant GAD65. Two of these showed a higher concentration of GADA when the recombinant GAD65 was added and therefore attained a negative value after correcting for the non-specific binding in the presence of recombinant GAD65 (data not shown). Using ^{35}S-GAD65 generated by pThGAD65, ten samples were not displaced by the recombinant GAD65. Three of these showed a higher concentration of GADA when the recombinant GAD65 was added and therefore attained a negative value after correcting for the non-specific binding in the presence of recombinant GAD65 (Figure 4, left panel). We therefore analyzed all patient samples a second time at an appropriate dilution (to fall within the span of the standard curve) with or without 2 ug/mL recombinant GAD65 (Figure 4, right panel).

All samples tested at the standard 1:25 dilution with a resulting level above 25 U/mL were tested at various dilution (1:100 – 1:640,000) to establish the U/mL. Once all samples – controls as well as patients – above 25 U/mL had been properly diluted, we analyzed the correlation between GADA U/mL determined with ^{35}S-GAD65 produced by pEx9 and pThGAD65. In contrast to low level GADA (Figure 2) were pEx9 tended to show increased non-specific binding, there was a significant correlation between the two labeled GAD65 at high levels ($r^2 = 0.9989$; $p<0.0001$). In

the subsequent analyses we therefore compared the levels of GADA determined with ^{35}S -GAD65 from pThGAD65 (Figure 4). The analyses were carried out without and with recombinant GAD65 first at the 1:25 standard dilution (Figure 4, left panel) and then at the final U/ml after appropriate dilution (Figure 4, right panel). It was found that as many as four type 1 (17.4 %), 10 type 2 (3.4 %) and 12 unspecified diabetes (21 %) patients had levels above 5000 U/mL, which meant that they had to be diluted at least 1:2000 to fall within the span of the standard curve. At the 1:25 dilution, four type 1 diabetes patients samples were not fully displaced by recombinant GAD65 as indicated by the arrows in Figure 4 a. At the appropriate final dilution all samples were displaced (Figure 4b).

The inability of recombinant GAD65 to fully displace the binding of ^{35}S-GAD65 at the 1:25 dilution was also clearly shown in seven type 2 (Figure 4c) and four unspecified diabetes patients (Figure 4e). At the appropriate final dilution all of these samples were also displaced (Figure 4d and f, respectively). The levels of GADA binding varied markedly and two groups of patients were observed (Figure 4 b, d and f, respectively). The first group from 25 to 60,000 U/mL included all type 1 diabetes patients (Figure 4b), all type 2 diabetes patients except two (Figure 4d) and all unspecified diabetes patients except three (Figure 4f). There were no patients with levels between 60,000 and 150,000 U/mL. While none of the type 1 diabetes patients had levels above 150,000 U/mL (Figure 4b) there were two type 2 diabetes patients (Figure 4d) and three unspecified diabetes patients (Figure 4f) with GADA levels above 150,000 U/mL. The samples with high non-specific binding before dilution were shown to have very high levels of GADA (above 12,000 U/mL). One patient had GADA levels as high as 4 000,000 U/mL (Figure 4f). This sample had to be diluted 1:640,000 to fall within the span of the standard curve. Identical results were obtained when GADA levels were determined with ^{35}S-GAD65 generated with the pEx9 plasmid (data not shown)

DISCUSSION

The present study uncovered two major findings. The first was that ^{35}S-GAD65 produced by pEx9 showed comparable binding as the label produced by pThGAD65 in serum samples from controls and type 1 diabetes patients. Elevated binding to the pEx9 generated ^{35}S-GAD65 product among type 2 diabetes and unspecified diabetes patients only occurred in low level sera and in no instance was there evidence for positivity in the autoantibody test to be specific to pEx9 product. Despite the fact that pEx9 has an alternative splice site generating an additional 13 amino acid extension at the N-terminal end in about 50% of the product, there was not an apparent major increase in specific binding to sera from any of the patients. These data therefore do not support the hypothesis that LADA patients have GADA recognizing GAD65 extended at the N-terminal with 13 amino acids. In order to increase the transcriptional efficiency of pEx9, the nucleotide sequence upstream of the ATG start site of GAD65 was modified (18, 19). The aim was to use a so-called Kozak consensus sequence to enhance but not alter transcription. However, these manipulations resulted in an ATG sequence at position -39 from the ATG start site. Hence, in about 50% of the transcripts, a non-sense N-terminal end amino acid sequence was also generated, which increased the number of amino acids in GAD65 from 585 to 598 amino acids. The sequence of the 13 additional amino acids, M H Q A W Y R A R I Q F T, was used to search the National Center for Biotechnology Information (NCBI) database to reveal that this sequence is not known among human proteins, but may be present in Drosophila. It is speculated that the N-terminal extension would therefore not immediately contribute to a marked non-specific binding. The later use of pThGAD65 took advantage of a commercially available expression plasmid, pTnT™ developed by the Promega company, which we confirmed had a marked increase in transcription translation efficiency.

The increased binding below the 90th percentile (Figure 2a) in the 250 controls for the ^{35}S-GAD65 product generated by pEx9 suggests that there was some non-specific binding for the pEx9 product. It is suggested that the increased non-specific binding was due to a lower rate of incorporation of ^{35}S-methionine using pEx9. As an equal amount of radioactivity (400 cpm/µL reaction mixture) was used in all assays, more pEx9 product than pThGAD65 had to be added to the samples. This may have affected low level binding. However, it can not be excluded that the 13 amino acid extension influenced low level binding by the N-terminal end altering the conformation of GAD65 or by an increase in the possible binding of polyreactive autoantibodies (20).

The second major finding was that the use of an excess of non-radioactive GAD65 detected sera with very high titre GADA in the type 2 diabetes and unspecified diabetes patients. Our initial design was to use recombinant GAD65 in the displacement assay to uncover non-specific binding to the ^{35}S-GAD65 product generated by the pEx9 plasmid. Undisputable prozone effects (25) were revealed in as many as 11 patient's sera, all from type 2 diabetes and unspecified diabetes patients. The prozone effect was detected by the inability of the excess of GAD65 to displace ^{35}S-GAD65 whether it was generated by pEx9 or pThGAD65. **In 2 % type 2 and 7 % unspecified diabetes patients, the prozone effect was revealed either by no displacement or on occassion by an increased binding in the displacement assay.** After proper dilution to reveal very high titre GADA (> 20,000 U/mL), all these samples were effectively fully displaced by the recombinant GAD65. The discovery of these very high titre sera is important and we believe that such patient sera may often be overlooked.

This type of very high titre sera have previously only been reported in patients with Stiff Person Syndrome (SPS), previously known as Stiff Man Syndrome (26, 27). It is notable that less than

30% of SPS patients also have diabetes (26). In Sweden, it has been estimated that one patient per year is diagnosed with SPS (28).

In conclusion, pThGAD65 is preferred for the coupled *in vitro* transcription/translation of ^{35}S-GAD65. The use of this plasmid for *in vitro* transcription/translation has not only economical advantages, but also less background binding compared to pEx9. Furthermore, displacement with an excess of recombinant GAD65 combined with serum dilutions in type 2 diabetes and unspecified diabetes patients would be useful to discover LADA patients with very high titres of GADA. Further studies are needed to determine the possible clinical significance and importance of the newly discovered extraordinarily high titre GADA in LADA patients.

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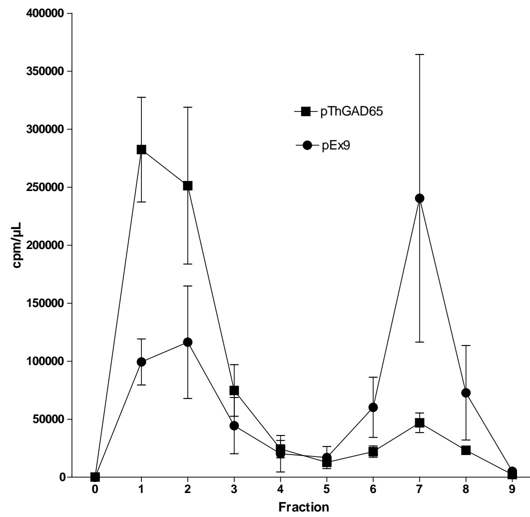

Figure 1. Mean difference (SD) between pThGAD65 and pEx9 to incorporate ^{35}S-methionine into GAD65 by coupled in vitro transcription/translation. NAP-5 purification, pEx9 compared to pThGAD65. The radioactivity (counts per minute/µL) of ^{35}S-GAD65 or unincorporated ^{35}S-methione was determined in nine fractions collected by hand from the NAP-5 column. The first peak is the labeled protein and the second is unbound label.

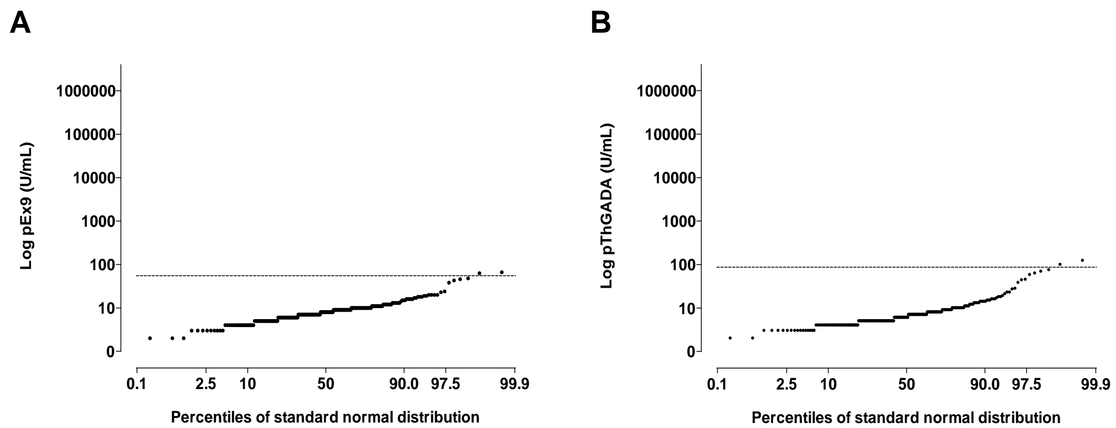

Figure 2. Distribution and cut-offs using pEx9 (A) and pThGAD65 (B) GAD65 cDNA plasmids to determine GAD65 autoantibodies. The line represents cut-off at 99th percentile.

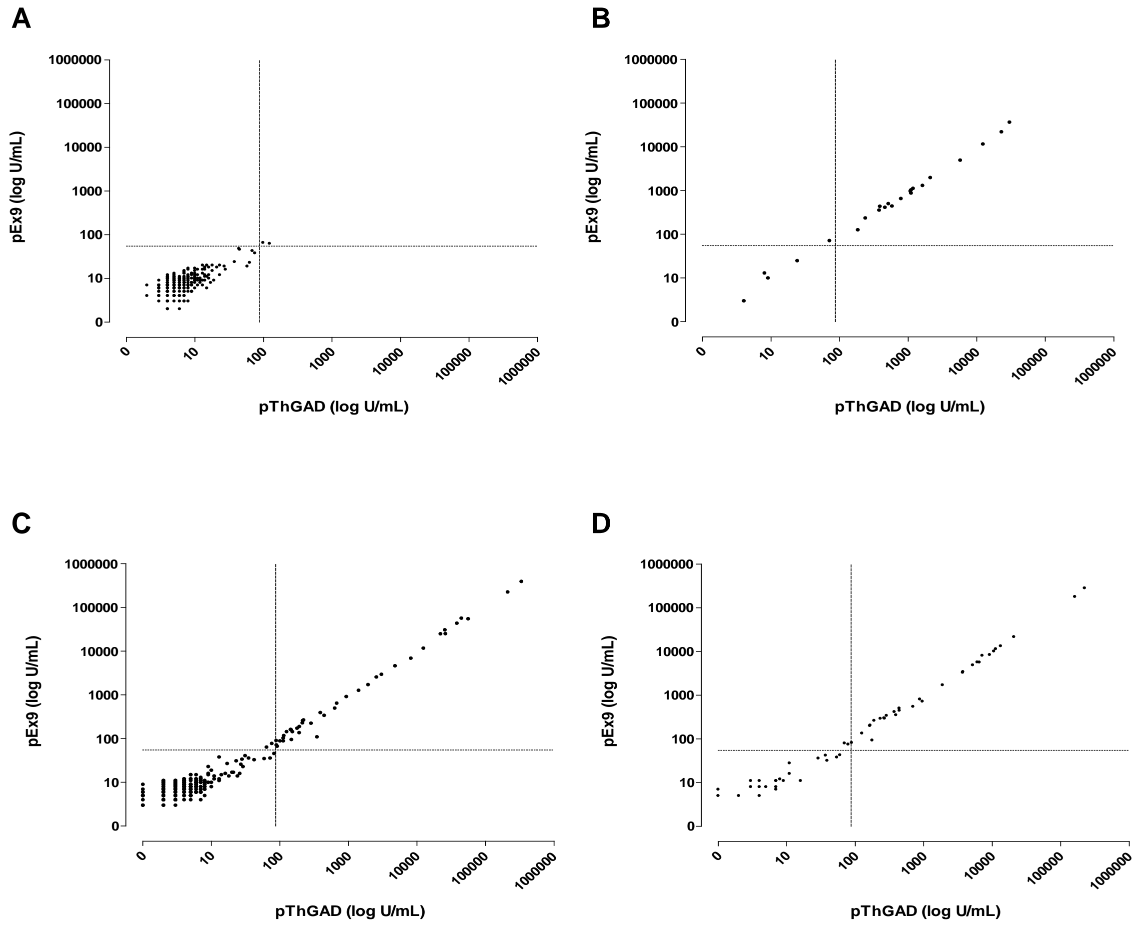

Figure 3. Comparison between GAD65 autoantibodies analyzed with labeled antigen produced by either pThGAD65 or pEx9. The panels are a) Controls n=250; b) Type 1 diabetes n=23; c) Type 2 diabetes n=290 and d) Unspecified diabetes n= 57. The lines represent cut-off at the 99th percentile.

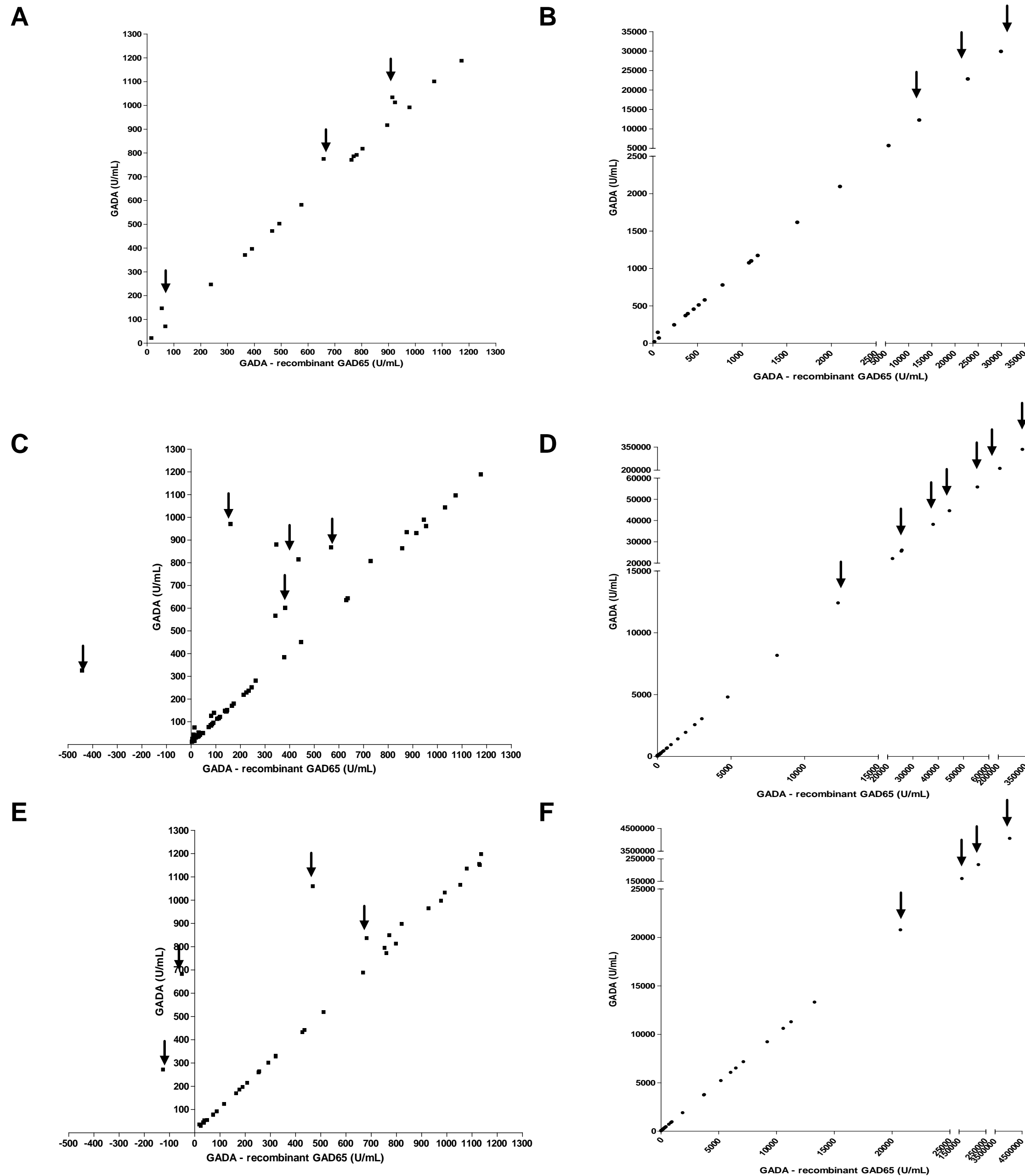

Figure 4. Detection of high titer GADA serum samples by failure of displacement (indicated by arrows) in the presence of recombinant human GAD65 (cold GAD65) in Panels A, C and E and displacement with cold GAD65 after these serum samples were diluted (1:2,000 – 1:640,000) in Panels B, D and F). Results from displacement for pThGAD65 produced ^{35}S-GAD65 before and after dilution. The arrows indicate the samples which were not displaced to the line of perfect fit. Concentration without recombinant GAD65 plotted against concentration corrected for unspecific binding. The panels are: a) Type 1 diabetes before dilution n=20; b) Type 1 diabetes after dilution n=20: c) Type 2 diabetes before dilution n=57; d) Type 2 diabetes after dilution n=57; e) Unspecified diabetes before dilution n= 40 and f) Unspecified diabetes after dilution n= 40.