Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using $^{35}$S or $^{3}$H recombinant human ligands *

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Abstract

Autoantibodies are an important marker of human autoimmune diseases and the development of simple, precise and reproducible immunoassays to detect autoantibodies is important to our understanding of human autoimmunity. GAD65 autoantibodies occur frequently in insulin-dependent diabetic patients and is a useful marker for IDDM. A RIA to detect immunoreactive GAD65 has not been described. In the present study we describe a semi-automated fluid-phase immunoassay for the rapid detection of GAD65 autoantibodies in human serum. We also developed a sensitive RIA to determine immunoreactive human GAD65 in biological fluids and in vitro cell systems. Using in vitro translated recombinant human GAD65 in a multiwell-adapted procedure, our GAD65Ab RIA combines high specificity and sensitivity with a high capacity to analyze a large number of samples. In this report the three critical steps in the GAD65Ab RIA, DNA preparation, in vitro translation and immunoprecipitation, have been optimized. In our RIA, GAD65Ab were detected in 116/155 (75%) new onset Swedish IDDM children and in 1/85 (1.2%) healthy controls. In an immunoassay to detect autoantibodies against the proinsulin converting enzyme 2 (PC-2) no such antibodies were detected in IDDM patients. In the GAD65 RIA the lower detection limit was 2 ng/ml (31 fmol/ml). Our data demonstrate that autoantigen radioligands produced by in vitro translation are useful in RIA for autoantibodies and autoantigens in studies of human autoimmunity.

Abbreviations: Ab, antibodies; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; GAD, glutamic acid decarboxylase; IAA, insulin autoantibodies; ICA, islet-cell antibodies; IDDM, insulin-dependent diabetes mellitus; IMP, immunoprecipitation; JDF, Juvenile Diabetes Foundation; LB, Luria-Bertani broth; PAGE, polyacrylamide gel electrophoresis; PC-2, proinsulin converting enzyme-2; PCIAM, phenol-chloroform-isomyl alcohol; RIA, radioimmunoassay; SDS, lauryl sulfate sodium salt; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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1. Introduction

Several autoantigens detected by autoantibodies in human autoimmune disorders have been cloned and sequenced. Numerous immunoassays with recombinant autoantigen have been developed to detect circulating autoantibodies as well as the autoantigen itself. Solid phase assays such as immunoblotting or enzyme-linked immunoassays are often used. This is possible when the autoantibodies are high affinity and high titer and not sensitive to conformational epitopes. In some autoimmune disorders such as insulin-dependent diabetes mellitus (IDDM) the loss of pancreatic beta cells is thought to be a chronic autoimmune process (Palmer and Lernmark, 1990) associated with autoantibodies against insulin (Palmer et al., 1983), glutamic acid decarboxylase (GAD) (Baekkeskov et al., 1982, 1990; Karlsen et al., 1992b) or islet cells (ICA) (Bottazzo et al., 1974; Bosi et al., 1994). Serum exchange analyses have demonstrated that solid phase assays are unreliable in the detection of insulin specific autoantibodies (IAN) (Greenbaum et al., 1992) or GAD autoantibodies (GAD-Ab) (Schmidli et al., 1994) in IDDM.

The observation that antibodies against the M, 65 000 isoform of glutamic acid decarboxylase (GAD65) (Baekkeskov et al., 1982; Karlsen et al., 1992a; Hagopian et al., 1993b) appear before ICA or IAA (Atkinson et al., 1990) and that, in first-degree relatives of diabetic patients, GAD65Ab correlate with progressive beta-cell dysfunction better than ICA or IAA (Bärmeier et al., 1991) suggests that GAD65Ab may have sufficient diagnostic specificity and sensitivity to predict IDDM. However, most IDDM patients occur in the general population where the predictive values of current immunological tests are low (Bingley et al., 1993).

The cloning of the M, 65 000 (GAD65) (Karlsen et al., 1991) and M, 67 000 (GAD67) (Michelsen et al., 1991) isoforms made it possible to develop recombinant antigen immunoassays to detect GAD autoantibodies (Hagopian et al., 1993a; Velloso et al., 1993; Seissler et al., 1993; Grubin et al., 1994; Petersen et al., 1994; Falorni et al., 1994). These novel assays showed that GAD65Ab, but not GAD67Ab, had a high diagnostic sensitivity for IDDM to support the hypothesis that GAD65 is a main autoantigen in IDDM.

GAD65Ab screening of the general population requires an assay able to analyse several hundred samples in few days. Based on our previously described radioligand binding assay to detect GAD65Ab in human serum (Grubin et al., 1992, 1994), the aim of the present study was: (1) to optimize in vitro transcription and translation in order to radiolabel the recombinant human autoantigens GAD65 and proinsulin converting enzyme 2 (PC-2) (Smeekens and Steiner, 1990); (2) to develop a high capacity, semi-automated assay for autoantibodies; and (3) to develop a sensitive radioimmunoassay to detect immunoreactive GAD65.

2. Materials and methods

2.1. Human sera and rabbit antisera

Sera from 155 newly diagnosed Swedish insulin-dependent diabetic patients (male/female ratio: 84/71; age 1–18 years) and from 85 healthy Swedish individuals (male/female ratio: 37/48; age 8–25 years) were used in the study. The diabetic patients were diagnosed at the St. Görans Hospital, Stockholm, Sweden, between 1986 and 1992. The serum samples were kept frozen at −20°C until the analysis in 1993–1994. The study was approved by the ethics committee at Karolinska Institute, Stockholm, Sweden.

Sera from eight GAD65Ab positive IDDM patients (Grubin et al., 1994) already used extensively in the Immunology of Diabetes Workshop to standardize ICA (Bonifacio et al., 1988) were used, as in our first assay (Grubin et al., 1994), to validate the semi-automated assay.
Rabbits were immunized with synthetic GAD65 peptides as described (Li et al., 1995). Antiserum R7309 was raised against a synthetic peptide corresponding to amino acid 4–22 of human GAD65, antiserum R8100 was against amino acid 250–269 of human GAD65 and R10266 was against amino acid 2–19 of human GAD67.

A rabbit antiserum generated against human PC2-peptide 4 (a kind gift of Dr. Donald F. Steiner, Chicago, IL, USA) was used to test the immunoreactivity of the radiolabelled recombinant human PC2.

2.2. Plasmid DNA preparation

A full-length human GAD-2 cDNA clone (pEx9), derived from two overlapping cDNA clones (Grubin et al., 1994), inserted in the pCDNAII (Invitrogen, San Diego, CA, USA) plasmid was used to transform competent DH5aF' E. coli cells.

The pEx9 plasmid DNA was prepared from an overnight DH5aF' bacterial suspension using two different procedures. In the first procedure a 250 ml bacterial suspension in Luria-Bertani (LB) broth was used and the plasmid DNA extracted and purified using a commercially available kit (Maxi-kit, Qiagen, Chatsworth, CA, USA) following the manufacturer's instructions. The DNA was resuspended in 200 µl Tris 10 mM, EDTA 1 mM (TE) buffer, and the DNA concentration evaluated spectrophotometrically (Labarca and Paigen, 1980) (typically between 1.5 and 3 mg/ml).

In three separate experiments, small-scale preparations of plasmid DNA were obtained by lysing the cells from a 20 ml LB bacterial suspension in alkali and after overnight incubation at 37°C using previously described standardised procedures (Sambrook et al., 1990). DNA was purified by phenol:chloroform:isoamyl alcohol (25:24:1), precipitated in absolute ethanol (2 vols.) and 0.1 vols. 3 M sodium acetate pH 5.2, washed in 70% ethanol and resuspended in 50 µl of Tris-EDTA (TE) buffer. The DNA concentration was 320–500 µg/ml.

In both of the procedures the quality of the pEx9 plasmid preparation was evaluated by linearisation of the DNA from a 2 µl aliquot with XbaI (Pharmacia, Uppsala, Sweden) and analysis by standard agarose gel electrophoresis.

Full-length human PC2 cDNA clone (pPC2) inserted into a pBluescript vector (Stratagene Cloning Systems, La Jolla, CA, USA) was kindly donated by Donald F. Steiner, Chicago, IL, USA. The pPC2 plasmid DNA was prepared as described above using 250 ml of overnight bacterial suspension and the commercially available kit from Qiagen.

2.3. In vitro coupled transcription / translation of human radiolabelled GAD65 and PC2

Recombinant human 35S-GAD65 or 35S-PC2 was produced in an in vitro coupled transcription/translation system with SP6 (35S-GAD65) or T3 (35S-PC2) RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI, USA). Plasmid DNA (2 µg) was incubated for 90 min at 30°C in a 50 µl reaction mixture containing 25 µl rabbit reticulocyte lysate (Promega, Madison, WI, USA), 1 µl RNA polymerase (40 U/µl) (Promega), 1 µl 1 mM amino acid mixture (minus methionine) (Promega), 1 µl RNasin (20 U/µl) (Promega) and 4 µl translation grade [35S]methionine (> 1000 Ci/mmol) at 10 mCi/ml (NEN Research Products, Boston, MA, USA). After completion of the reaction, 2 µl of the translation product were incubated for 10 min at 37°C with 1 M NaOH/2% H2O2 and precipitated with 25% trichloroacetic acid (TCA) for 30 min on ice. TCA precipitated proteins were collected on a Whatman GF/A glass fiber filter (Whatman, Maidstone, UK) and washed five times with 5% TCA. Total precipitable radioactivity was evaluated in a liquid scintillation analyzer (Tri-Carb 1550, Packard Instrument Company, Meriden, CT, USA).

The in vitro translated 35S-GAD65 and 35S-PC2 were kept at -70°C until used in the radiomunoassays, within 5 weeks.

In three separate experiments, the in vitro transcription and translation of 35S-GAD65 were performed following a two step procedure as previously described (Grubin et al., 1994). In another three experiments, 3H-GAD65 was pro-
duced by coupled in vitro transcription/translation using 1 μl 1 mM amino acid mix without leucine and 5 μl [3H]leucine (150 Ci/mmol) at 5 mCi/ml (Amersham) and following the procedure described above.

2.4. Radioimmunoassays for GAD65 and PC2 antibodies

Multiscreen-DV 96-well filtration plates, 0.65 μm hydrophilic PVDF (Millipore AB, Västra Frölunda, Sweden) were pretreated with 200 μl/well of Tris/NaCl buffer (150 mM NaCl, 20 mM Tris, pH 7.4) for 1 h at 4°C, and then incubated for 2 h at room temperature with 1% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO, USA) in Tris/NaCl buffer. After the BSA coating, the buffer was removed and the filters washed twice with 200 μl 0.05% Tween 20 (Sigma) in Tris/NaCl buffer, and stored for 2-3 days at 4°C in the same buffer. The use of Triton X-100 (Sigma) instead of Tween 20 was associated with a higher non specific binding of labelled GAD65.

In both the GAD65 antibody (GAD65Ab) and the PC2 antibody (PC2Ab) specific RIAs, the in vitro translated 35S-radiolabelled recombinant human antigen was directly immunoprecipitated with human serum. In previous studies (Grubin et al., 1994; Falorni et al., 1994) we showed that using our cDNA clone (pEx9), more than 95% of the translated GAD65 was full-length. Preliminary results (data not shown) indicated that the use of more than 20000 cpm of TCA precipitable 35S-GAD65 was associated with a higher non specific binding of labelled GAD65.

In two experiments, 20000 cpm of TCA precipitable 3H-GAD65 was immunoprecipitated, in quadruplicate, with scalar dilutions (1/25-1/250) of the JDF positive and one healthy negative serum, following the procedures described above.

2.5. Expression of GAD65 and PC2 antibody levels and determination of an upper level of normal values

To control for inter-assay variation, GAD65 antibody levels were expressed as a relative index (GAD65 index) using a positive and three negative control sera in each assay. The positive control serum was the Juvenile Diabetes Foundation world standard (JDF standard) for islet cell antibodies (ICA). This serum has been previously shown to be positive for GAD65Ab (Grubin et al., 1994; Hagopian et al., 1993a). The sera from three healthy Swedish individuals served as negative controls. A GAD65Ab index (cpm of the
unknown sample – average cpm of three negative standards)/(cpm of the positive standard – average cpm of three negative standards) was calculated.

The effects of serum titration on the final GAD65 index was evaluated in the eight positive standard sera. From this analysis it was found that sera with a GAD65 index above 0.75 had to be reanalysed at a dilution of 1/250. The final GAD65 index was evaluated using similar serum dilutions of the positive and the three negative standard sera. The upper level of normal of the assay was calculated using the average +3 SD of the healthy Swedish individuals.

Similarly, PC2 antibody levels were expressed as a relative index (PC2 index) using the specific rabbit antiserum as a positive and three sera from healthy individuals as negative controls. The upper level of normal for the assay was calculated using the average +3 SD of a population of 50 healthy Swedish individuals.

2.6. Radioimmunological determination of the concentration of GAD65 in the in vitro translation reaction and in human serum

The concentration of recombinant GAD65 at the end of the in vitro translation reaction, and in 15 sera from healthy individuals was evaluated. Non radiolabelled in vitro translated recombinant GAD65 was prepared, in three different experiments, in the presence of cold 1 mM methionine (Sigma).

In the GAD65 RIA, in vitro translated $^{35}$S-GAD65 served as tracer and the serum from a GAD65Ab-positive patient with so-called Stiff-Man Syndrome (a kind gift of Tiinamaija Tuomi, Helsinki, Finland) served as antiserum. As a standard, recombinant human GAD65, expressed in and purified from a baculovirus system (a kind gift from John Robertson, Synectics Biotechnology, Stockholm, Sweden) was used. The purity of the standard GAD65, as evaluated by SDS-polyacrylamide gel electrophoresis, was more than 98% and the concentration was determined by total aminoacid composition.

In the GAD65 RIA, 12,000 cpm of TCA precipitable $^{35}$S-GAD65, in triplicate, was diluted in 51 $\mu$l of IMP buffer. Before addition of 3 $\mu$l of a 1/5000 dilution of the Stiff-Man serum (final serum dilution 1/100,000), 6 $\mu$l of a scalar dilution of standard GAD65 in IMP buffer (corresponding to an original concentration ranging from 0 to 130 ng/ml) or 6 $\mu$l of a 1/100 or a 1/200 dilution in IMP buffer of the cold in vitro translated GAD65 were added. The serum of the patient with Stiff-Man Syndrome was negative for GAD67Ab when tested at a 1/100,000 dilution.

To determine the concentration of GAD65 in human serum, the standard GAD65 was diluted in GAD65-depleted human serum (by preadsorption on a column of GAD6 monoclonal antibody) instead of IMP buffer. 6 $\mu$l of human serum, in triplicate, were used in the GAD65 RIA.

After overnight incubation at 4°C, on a rotating platform, the immunoprecipitated radioactivity was evaluated by separating the antibody bound $^{35}$S-GAD65 using the procedures described above for the GAD65Ab RIA.

The final GAD65 concentration in both the in vitro translation reaction and in human serum was estimated by constructing a semi-logarithmic standard curve using the immunoprecipitated radioactivity in the presence of scalar dilutions of the standard GAD65.

2.7. Statistical analysis

The intra- and inter-assay coefficients of variation of the GAD65Ab and the PC2Ab assay and the percent $^{35}$S-GAD65 or $^{35}$S-PC2 immunoprecipitated were calculated for the positive and the three negative control sera.

Difference in GAD65Ab or PC2Ab levels between IDDM patients and healthy controls was evaluated using the non-parametric Mann-Whitney test.

In the GAD65 RIA, the standard curve was tested by linear regression analysis and the intra-assay and the inter-assay coefficients of variation calculated for five dilutions of the standard GAD65 and 3 analyses of the in vitro translated GAD65, respectively.
3. Results

3.1. In vitro translation of radiolabelled recombinant human GAD65 and PC2

The efficiency of translation was evaluated by measuring the TCA precipitated radioactivity (cpm/μl of translated GAD65 or PC2). A comparison between the two-step and the coupled transcription/translation procedures, as well as a comparison between the two plasmid preparation techniques was carried out for 35S-GAD65 as shown in Table 1. Large-scale preparation of plasmid DNA/QIAGEN purification and use of the coupled in vitro transcription/translation procedure resulted associated with the highest efficiency of in vitro translation of 35S-GAD65.

The coupled transcription/translation was also evaluated using [3H]leucine. The percentage incorporation of [3H]leucine in the translated GAD65 was similar to that observed using [35S]methionine (Table 1). In vitro translation of 35S-PC2 resulted in 154600 ± 23400 cpm of TCA precipitable protein/μl of reaction, corresponding to 13.4 ± 1.6 percentage incorporation of [35S]methionine.

3.2. Validation of the GAD65 and PC2 antibody assays

The immunoreactivity of the in vitro translated recombinant human 35S-GAD65 and 35S-PC2 was tested using the specific rabbit antisera. 35S-GAD65 was immunoprecipitated by the GAD65-specific R7309 and R8100 antisera but neither by the GAD67-specific R10266 nor the PC2-peptide specific rabbit antisera. As a negative control, the percentage immunoprecipitation obtained with healthy human serum is shown.

In 84 healthy Swedish individuals, the GAD65 index was -0.0065 ± 0.0105 (mean ± SD) and was normally distributed (Fig. 2). The upper level of normal (mean + 3 SD) was estimated to be 0.025. In one healthy serum, the GAD65 index (0.825) was significantly higher than the upper level of normal. This sample was not considered in the above analysis.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Small-scale DNA preparation</th>
<th>Large-scale DNA preparation</th>
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<tbody>
<tr>
<td></td>
<td>PCIAA purification</td>
<td>QIAGEN purification</td>
</tr>
<tr>
<td>Coupled transcription</td>
<td>45 670 ± 3 300</td>
<td>567 250 ± 46 620</td>
</tr>
<tr>
<td>translation of 35S-GAD65</td>
<td>(4.8 ± 0.9%)</td>
<td>(48.9 ± 3.9%)</td>
</tr>
<tr>
<td>Two steps transcription</td>
<td>95 180 ± 7 740</td>
<td>109 875 ± 6 890</td>
</tr>
<tr>
<td>translation of 35S-GAD65</td>
<td>(9.9 ± 1.4%)</td>
<td>(11.4 ± 1.2%)</td>
</tr>
<tr>
<td>Coupled transcription</td>
<td>ND</td>
<td>180 750 ± 7 330</td>
</tr>
<tr>
<td>translation of 3H-GAD65</td>
<td></td>
<td>(33.3 ± 1.4%)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM cpm/μl of translated GAD65 (percentage incorporation) of ten reactions. ND: not determined.
The average percentage $^{35}$S-GAD65 immunoprecipitation in five assays was 53% with the positive and 1.9%, 2.1%, and 2.7%, respectively, with the three negative standard sera. The ratio between the immunoprecipitation of the positive and the average of the three negative standard sera ranged between 20:1 and 25:1.

The intra-assay and inter-assay coefficients of variation were 3% and 9% respectively for the positive, and 11–17% and 10–14% respectively for the negative standards.

When the GAD65 index was plotted as a function of the serum dilution in the eight GAD65Ab positive IDDM standard sera linear dilution curves were obtained for low level antibody positive sera and saturated binding for the high level sera (Fig. 3). Using the JDF standard serum as the GAD65Ab positive standard we detected GAD65Ab in all of the seven positive standard sera tested (GAD65 index 0.05–1.08). At increasing serum dilutions, the frequency of GAD65Ab decreased as the IDDM sera gradually overlapped within the range of the negative healthy controls. The GAD65 index was found to be a linear function of the serum dilution in five IDDM sera with an index between 0.05 and 0.62, but not in two sera with an index above 1.00.

In 50 healthy Swedish individuals, the PC2Ab index was $-0.005 \pm 0.008$ (mean ± SD) and the upper level of normal (mean + 3 SD) was estimated to be 0.020.

The average $^{35}$S-PC2 immunoprecipitated with the rabbit serum in five assays was 65% with the positive standard sera and 1.3%, 1.2%, and 1.4%, respectively with the three negative standard sera. The ratio between the immunoprecipitation of the positive and the average of the three negative standard sera ranged between 35:1 and 45:1.

The intra-assay and inter-assay coefficients of variation were 2% and 10% respectively for the positive, and 12–15% and 9–15% respectively for the negative standards. The sensitivity of the PC2Ab RIA was tested with serial dilutions of the rabbit PC2-peptide 4 antiserum. The end-point dilution titre of this antiserum, as evaluated in the RIA, was 1/20,000.

3.3. GAD65Ab and PC2Ab in IDDM patients and healthy controls

The levels of GAD65Ab, expressed as the GAD65 index, were significantly higher in IDDM patients (median 0.297; range $-0.020$–2.402) than in healthy individuals (median $-0.007$; range $-0.035$–0.825) ($p < 0.005$) (Fig. 4). GAD65Ab were found in 75% (116/155) IDDM patients and in 1.2% (1/85) healthy individuals. In IDDM
patients, GAD65Ab occurred more frequently in female (81.7%, 58/71) than in male (67.9%, 57/84) subjects ($\chi^2 = 3.86; p = 0.049$). Furthermore, the median GAD65 index was significantly higher ($p = 0.048$) in GAD65Ab positive female (median 0.405, range 0.028–2.289) than in male (median 0.223, range 0.026–2.402) IDDM patients. The occurrence of GAD65Ab in male IDDM patients was related to the age at onset (Table 2), being higher among those patients more than 12 years old than among those less than 12 years old.

The levels of PC2Ab, expressed as the PC2 index, did not differ between IDDM patients (median: −0.004; range −0.025–0.016) and healthy control subjects (median −0.005; range −0.027–0.015). None of the sera from the 50 IDDM patients tested had a PC2 index above the upper level of normal.

### 3.4. GAD65 radioimmunoassay

A representative standard curve from the GAD65 RIA is shown in Fig. 5. The lower detection limit of the RIA was 2 ng/ml (31 fmol/ml) and the intra-assay and inter-assay coefficients of variation were 9.2% and 11.1%, respectively.

Analysis of the in vitro translated cold GAD65, at dilution of 1/100 and 1/50, resulted in a GAD65 concentration of 7.5 ± 0.9 µg/ml. This concentration was similar to that (9.2 ± 1.1 µg/ml) predicted from the percentage incorporation (49%) of $[^{35}S]$methionine into the in vitro translated GAD65, the concentration (1.1 ± 0.1 µg/ml) and specific activity (1000 Ci/mmol) of the $[^{35}S]$methionine used, and the total number of methionine residues in the human GAD65 ($n = 25$).

The GAD65 concentration in healthy human sera varied between 2 and 6 ng/ml in ten samples, 12.5 ng/ml in one sample and undetectable (concentration < 2 ng/ml) in four samples.

### 3.5. Immunoreactivity of $^3H$-GAD65

Immunoreactivity of $^3H$-GAD65 was tested by immunoprecipitation with the JDF positive stan-
Table 3

Immunoprecipitation of \(^{3}\text{H}\)-GAD65 and \(^{35}\text{S}\)-GAD65 with the JDF positive and one healthy negative standard serum

<table>
<thead>
<tr>
<th>Serum (dilution)</th>
<th>Immunoprecipitation of (^{3}\text{H})-GAD65 (%)</th>
<th>Immunoprecipitation of (^{35}\text{S})-GAD65 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy negative (1/25)</td>
<td>3.0 \pm 0.1</td>
<td>2.1 \pm 0.2</td>
</tr>
<tr>
<td>JDF positive (1/25)</td>
<td>50.7 \pm 1.5</td>
<td>53.2 \pm 0.9</td>
</tr>
<tr>
<td>JDF positive (1/32)</td>
<td>45.0 \pm 1.0</td>
<td>49.3 \pm 1.2</td>
</tr>
<tr>
<td>JDF positive (1/50)</td>
<td>32.7 \pm 2.1</td>
<td>35.5 \pm 2.3</td>
</tr>
<tr>
<td>JDF positive (1/100)</td>
<td>18.6 \pm 0.9</td>
<td>19.6 \pm 1.9</td>
</tr>
<tr>
<td>JDF positive (1/250)</td>
<td>9.5 \pm 0.5</td>
<td>10.1 \pm 1.0</td>
</tr>
</tbody>
</table>

Values are mean \pm SD of quadruplicates.

standard serum at different dilutions, and with a negative standard serum. As shown by the comparison with the values obtained when immunoprecipitating \(^{35}\text{S}\)-GAD65 with the same sera (Table 3), \(^{3}\text{H}\)-GAD65 was immunoreactive and potentially useful for the detection of GAD65Ab.

4. Discussion

In the present paper we describe in detail a simple and semiautomated radioimmunoassay to detect GAD65 autoantibodies and to determine GAD65 concentration in a large number of samples. Additionally, we have developed a RIA to determine the levels of autoantibodies to the converting enzyme PC2 and we demonstrate that no such autoantibodies could be detected in 50 IDDM sera.

A large body of evidence indicates that GAD65 autoantibodies are strongly associated with IDDM and might be potentially useful for the diagnosis and identification of subjects at risk of developing IDDM.

Several methods have been proposed for the detection of GAD65 autoantibodies in human serum. Our group has previously suggested (Grubin et al., 1992,1994) an alternative strategy based on the in vitro transcription and translation of recombinant human GAD65 radiolabelled with \[^{35}\text{S}\]methionine. The efficiency of this approach was recently confirmed (Petersen et al., 1994; Falorni et al., 1994). All these studies demonstrated that in vitro translated recombinant GAD65 is immunoreactive and indistinguishable from the native protein. Moreover, the fact that radiolabelled GAD65 can be produced in an in vitro translation system permits less highly specialized laboratories to repeat the procedure without the need to express and purify GAD65 from eukaryotic cells.

In our RIA we found GAD65Ab in 75% IDDM patients (sensitivity of the assay) and in only 1.2% healthy controls (specificity of the assay 98.8%). The adaptation of the assay to a multiwell system (Millipore) represents a major improvement over our previous radiobinding assay and allowed a single person to analyze more than 400 samples per week. The average work time for a single assay (one multiwell plate: 28 samples plus 4 standards) was 2 h. A major improvement was achieved by optimizing the purification of plasmid DNA which was critical for the efficiency of the in vitro translation. The combination of a highly pure plasmid DNA preparation and of a coupled transcription/translation procedure increased 5–10-fold the efficiency of the translation (evaluated as percentage incorporation of the tracer). Moreover, we had previously demonstrated that the use of our cDNA clone, containing an appropriate 5' untranslated sequence was associated with high quality of in vitro translated GAD65, as evaluated by SDS-PAGE analysis (Grubin et al., 1994; Falorni et al., 1994). Contamination products of a lower molecular weight were minimal in our system but represent a major problem in other similar assays (Petersen et al., 1994). As a consequence, purification of the in vitro translated GAD65 was not required in our system. The adaptation of the procedure to a coupled transcription/translation system represents a major improvement because of the improved purity of the autoantigen and the reduction in work time with no need to handle mRNA.

Furthermore, we demonstrated that labelling GAD65 with \[^{3}\text{H}\]leucine generated similar results thereby confirming the efficiency of our transcription/translation system and opening up interesting possibilities of combining different autoantigens in a single assay.

Little information is available on the concentration of GAD65 in biological fluids and in vitro
cell systems. In the present paper we describe a sensitive RIA to determine GAD65 in biological samples and we have used this to determine the concentration of GAD65 produced in our in vitro translation system. Subsequently, we focused our attention on the concentration of GAD65 in human serum. However, from the preliminary results we obtained we can conclude that the sensitivity of our RIA was probably not adequate and only in a few samples could the concentration of GAD65 be estimated.

Using coupled in vitro transcription/translation we were able to produce radiolabelled recombinant human PC2 and to develop a PC2Ab RIA. Interestingly, none of the tested diabetic sera proved positive for PC2Ab suggesting that the autoimmune reaction against the islet molecules is restricted to specific targets, such as GAD65.

In conclusion we have developed a sensitive and semiautomated RIA to detect GAD65 autoantibodies. In particular, we have optimized our previously described procedure (Grubin et al., 1994; Falorni et al., 1994), both increasing the capacity of the assay and reducing the costs and the work time/sample. The resulting semiautomated and standardized GAD65Ab RIA with in vitro translated $^{35}$S-GAD65 is highly sensitive, highly specific, has a high capacitance, is easy to perform, and is economical. The large scale application of assays similar to that described will make possible the screening of the general population in order to identify subjects at risk of autoimmune disease.

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