Detection of autoantibodies to the 65-kD isoform of glutamate decarboxylase by radioimmunoassay

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Autoantibodies (AAB) to glutamate decarboxylase (GAD) occur with a high prevalence in sera of newly diagnosed type 1 (insulin-dependent) diabetic patients. The aim of this study was to establish a GAD-AAB radioimmunoassay using 125I-labelled GAD65 and to evaluate this assay in a cross-sectional study with newly diagnosed type 1 diabetic patients and individuals suffering from other autoimmune diseases were examined in this assay. For GAD-AAB detection, 125I-labelled GAD65 was incubated with 10 μl of human serum overnight on ice. Thirty of 51 (59%) type 1 diabetic patients but none of the 54 healthy blood donors tested were found to be positive. A displacement step using 100 000 g supernatant from rat brain containing or not containing GAD showed the specificity of the binding of 125I-GAD65. Concerning the individuals at high risk of developing diabetes, 9/12 (75%) islet cell antibody (ICA)-positive non-diabetic and 4/34 (12%) ICA-negative subjects with metabolic abnormalities were GAD-AAB positive. These results show the association between type I (insulin-dependent) diabetes mellitus and the occurrence of GAD65-AAB, which possibly predicts a risk of developing the disease.

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Type I (insulin-dependent) diabetes mellitus is an organ-specific autoimmune disease resulting from the specific destruction of pancreatic beta cells. In addition to beta cell-reactive T-cell clones (1), autoantibodies (AAB) reactive with insulin and islet cell antibodies (ICA) or surface islet cell antigens (ICSA) were detected in newly diagnosed type I diabetic patients (reviewed in Ref. 2).

These disease-relevant autoantigen(s) include a 64-kD islet cell protein detected by immunoprecipitation from pancreatic islets (3) by sera from type I diabetic patients and identified as the gamma-aminobutyric acid (GABA)-synthesizing enzyme glutamate decarboxylase (GAD) (4). There are at least two isoforms of GAD, which differ in their molecular size (65 and 67 kD, respectively) and amino acid sequence and were derived from two genes located on two different chromosomes (5-7). The homology between these two isoforms is approximately 65%; the differences are mainly found at the N-terminus (5).

Autoantibodies against the 64-kD (GAD65) autoantigen occur years before clinical onset of type I diabetes mellitus (8) and seem to have a predictive value for the onset of the disease in the range of that of ICA (9-11). However, all of these studies included only a small number of probands and were done using the material- and time-consuming method of autoradiography after immunoprecipitation of metabolically labelled islet proteins. Recently, two radioimmunoassays using native GAD from pig brain (12. 13) and an ELISA using recombinant GAD67 (14) were established for the detection of autoantibodies to GAD.

The aim of our study was to establish a radioimmunoassay as a rapid and sensitive method for the detection of autoantibodies to GAD using 125I-labelled recombinant human GAD65 and to evaluate this assay in a cross-sectional study that includes healthy blood donors and newly diagnosed type I diabetic patients. Additionally, we investigated individuals at high risk of developing type I diabetes.

Materials and methods

Patients

Sera from 54 healthy blood donors (18 male, 36 female; mean age 35.8 ± 11.2 years, ranging from 18 to 58 years) without a family history of diabetes and from 51 newly diagnosed (< 6 weeks after diagnosis) type I diabetic patients (27 male, 24 female; mean age 13.2 ± 5.5 years, ranging from 2 to 24 years) were
tested for GAD-AAb in this study. Additionally, sera from 46 individuals at risk of developing type I diabetes (39 first-degree relatives of type I diabetic patients and seven individuals with metabolic abnormalities but without family history of diabetes in the first degree) were investigated (23 male, 23 female; mean age 17.2 ± 7.8 years, ranging from 4 to 35 years). Twelve of these 46 subjects (six male, six female; mean age 13.5 ± 7.6 years, all first-degree relatives of type I diabetic patients) are ICA-positive in the range of 10–80 JDF units. Three of the 12 have a diminished insulin secretion in the first phase (15) (1- and 3-min insulin response below the fifth percentile of healthy subjects after intravenous glucose stimulation of 0.5 g/kg body wt), one has an impaired oral glucose tolerance (according to the prescription and criteria of the National Diabetes Data Group (16)) and one has both of these. Seventeen of 34 ICA-negative subjects (17 male, 17 female; mean age 18.6 ± 7.5 years) have an impaired oral glucose tolerance, 14 show a diminished insulin secretion in the first phase after intravenous glucose stimulation and three have both of these. In addition, 10 sera of patients with other autoimmune diseases were investigated (two male, eight female). mean age 44 ± 18 years; seven suffer from autoimmune hyperthyroesia, two from autoimmune thyroiditis and one from systemic lupus erythematosus).

All blood samples were drawn with informed consent of the patients and stored at −20°C until use.

Cloning, expression and purification of recombinant GAD65

A recombinant full-length cDNA clone was isolated from a human pancreatic carcinoma cDNA library by oligonucleotide screening and polymerase chain reaction (PCR) amplification as described previously (17). For purification purposes, a histidine hexapeptide coding sequence was added to the 3'-end by site-specific mutagenesis (18). The modified cDNA was inserted into the baculovirus transfer vector pVL 1393 (Invitrogen). The recombinant transfer vector and linearized wild-type Autographa california (Ac NPV) DNA (19) were introduced into Spodoptera frugiperda SF9 cells (20) by lipofection. Recombinant viruses were isolated by plaque purification and selected for expression by western blot analysis (21). After amplification, one of these recombinant viruses was used to infect 500-ml suspension cultures of SF9 cells (1.6 × 10^6 cells/ml) in serum-free SF-900 medium (Gibco) at a multiplicity of infection of five (22). Cells were harvested by centrifugation at 500 g and immediately frozen in liquid nitrogen. Recombinant GAD was extracted from SF9 cell pellets by resuspension in chromatography buffer (40 mmol/l HEPES–KOH (pH 7.5), 0.5 mol/l NaCl, 0.2% (w/v) Lubrol, 0.1 mmol/l phenylmethylsulphonyl fluoride (PMSF) and 2 mg/l of the following protease inhibitors: sprotonin, leupeptin, bestatin and pepstatin), followed by homogenization with 20 strokes of a tight-fitting teflon pestle homogenizer. After centrifugation (100 000 g, 30 min), the supernatant was applied to a chelating Sepharose FF column loaded with Ni^{2+} and equilibrated with chromatography buffer. The column was washed with the same buffer until the effluent absorbance had returned to its baseline value, and was then step-eluted with chromatography buffer containing 10, 30, 100 or 500 mmol/l imidazole. Column fractions were tested for GAD content by SDS-PAGE and western blotting, using serum of a newly diagnosed type I diabetic patient previously characterized as being GAD-AAb positive by immunoprecipitation and autoradiography (10).

Iodination of GAD65

Iodination was performed by the chloramine T method (23). A 5-µg aliquot of GAD65, diluted in 100 µl of 0.4 mol/l phosphate buffer (pH 7.4), 5 µl of NaI (125I) (0.5 mCi) (Amersham and Buchler GmbH, Braunschweig, Germany) and 10 µl of chloramine T (40 mg/l in 0.04 mol/l phosphate buffer, pH 7.4) were mixed. After shaking for 20 s, 30 µl of hydrolyzed lactalbumin (10% in bidistilled water) (Difco Laboratories, Detroit, IL) was added. The 125I-labelled GAD was purified by gel filtration on a Sephadex G-10 and a Sephadex G-100 column. The specific activity of the labelled protein was about 0.01 µCi/mg. After SDS-PAGE and autoradiography, two bands were visible: one in the molecular weight range of about 62 kDa and one at about 54 kDa (not shown). The latter band is considered to be a degradation product of GAD65.

Radioimmunoassay

A 20-µl aliquot of 125I-labelled recombinant GAD65 (about 20 000 cpm) diluted in standard buffer (PBS/1% Tween 20/0.1% sodium acide/0.5% human serum albumin) was incubated with 10 µl of undiluted serum sample overnight on ice. For dilution curves, the serum samples were diluted in standard buffer. Then, 100 µl (diluted 1/5) of protein G plus/protein A agarose (Oncogene Science, New York, NY) was added and incubated under shaking for 15 min at 4°C. The samples were centrifuged for 10 min at 2000 g, and the pellets were washed eight times in 1 ml of standard buffer containing 1% Triton X-114 (Fluka, Buchs, Switzerland) and counted for radioactivity in a gammacounter (Multi-crystal counter LB 2104, Berthold, Wildbach, Germany). A Stiff-man syndrome serum as positive control and serum from a healthy blood donor as negative control were tested in each assay. The intra-assay cv of duplicates was 8.9%, and the interassay cv for the positive control was 12.2% (N = 14).
Displacement test

A 100 000 g supernatant from rat brain (15 g protein/l) containing 38 mg GAD65/l was used for displacement experiments. For direct comparison and to avoid unspecific protein effects, GAD was removed (<0.15 mg/l GAD65) from the 100 000 g supernatant by adjusting to pH 2.8 for 2 h followed by neutralization. A 25-µl aliquot of GAD-containing or GAD-depleted 100 000 g supernatant was added to each serum sample, and the radioimmunoassay was carried out as described above.

Islet cell antibodies

Islet cell antibodies were detected on cryostat sections of blood group O human pancreas as substrate, according to the method of Pilcher and Elliot (24), with the exception that peroxidase-conjugated rabbit anti-human IgG (gamma chain-specific) (Dakopatts, Glostrup, Denmark) was used. Sections were incubated with sera diluted in 0.05 mmol/l TRIS buffer containing 0.01% BSA. The chromogenic reaction was initiated by the addition of 3,3′-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) containing 0.001% hydrogen peroxide. After three washings, the slides were mounted with DPX mounting medium (25). Conversion of end-point titres was accomplished by using serially double dilutions of the JDF standard (80 U) in normal human serum, as recommended by the Second International ICA Workshop. The detection limit of our method was <5 JDF units. Our laboratory participated in the ICA Workshops and the 8th IDW ICA proficiency programme. The results for laboratory performance were as follows: validity 88%, consistency and specificity 100%, sensitivity 75%.

Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed using the paired or unpaired Student’s two-tailed t-test. The normal distribution of samples was analysed using the Kolmogorov–Smirnow goodness-of-fit test: linear regression analysis was used to calculate the correlation between levels of GAD-AAb and ICA.

Results

The antibody binding of the 125I-labelled GAD65 preparation used by the test sera was expressed as a percentage of total radioactivity. The mean binding of sera from the 54 healthy blood donors was 2.56 ± 0.53%. The cut-off for positivity was defined to be 3.92% (mean +2.57 SD; this is the t value for 1% probability with regard to the normal distribution of binding levels). Thirty out of 51 (59%) newly diagnosed type I diabetic patients and none of the 54 healthy blood donors exceeded this cut-off (Fig. 1). The mean binding of sera from type I diabetic patients was 4.39 ± 1.46% (range 1.8–7.4%) and was enhanced significantly (p < 0.01) compared to that of healthy blood donors. Twenty-two out of the 30 GAD65-AAb-positive sera and 14 GAD65-AAb-negative sera from type I diabetic patients were found to be ICA-positive. There was no correlation between levels of GAD-AAb and ICA (r = 0.183; NS).

Nine of 12 (75%) of the ICA-positive and 4/34 (12%) of the ICA-negative non-diabetic subjects with metabolic abnormalities such as diminished insulin secretion in the first phase and/or impaired oral glucose tolerance had autoantibodies to recombinant GAD65 (Fig. 1).
subjects (4.98 ± 1.63%) was increased significantly (p < 0.01) compared to that of healthy blood donors, while the mean binding of sera from ICA-negative non-diabetic subjects (2.63 ± 0.98%) was not. None of the 10 patients with other autoimmune diseases (Morbus Basedow, autoimmune thyroiditis or systemic lupus erythematosus) were found to be GAD65-AAb-positive (Fig. 1). The mean binding of 2.25 ± 0.6% was not different from that of healthy blood donors (Fig. 1).

The binding of the GAD65-AAb-positive sera of 125I-GAD65 was closely associated with the end-point dilution, which was still detected to be positive, as shown in the dilution curves of 5 AAb-positive sera (Fig. 2).

The addition of 25 µl of GAD-containing in contrast to GAD-depleted 100 000 g supernatant from rat brain resulted in a significant reduction in the binding of GAD65-AAb-positive sera (N = 30) from type 1 diabetic patients of 125I-GAD65 (mean binding 5.4 ± 0.96% vs 2.06 ± 0.78%, p < 0.01) (Fig. 3). No serum exceeded the cut-off after displacement. The mean binding of GAD65-AAb-negative sera (N = 21) from type 1 diabetic patients was unaffected by the displacement step (mean binding 1.82 ± 0.4% vs 1.54 ± 0.29%; NS) (Fig. 3).

There was no correlation between the prevalence of GAD65-AAb and sex in all groups studied (Table 1).

Of the 12 ICA-positive non-diabetic subjects, only those with additional metabolic abnormalities and/or ICA of > 20 JDF units were found to be GAD65-AAb-positive.

**Discussion**

Previous studies have suggested that autoantibodies to

**Table 1.** Results of glutamate decarboxylase (GAD) autoantibody detection by radioimmunoassay regarding the sex.

<table>
<thead>
<tr>
<th>Group studied</th>
<th>Sex</th>
<th>Subjects tested</th>
<th>GAD65-AAb-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newly diagnosed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>type 1 diabetic patients</td>
<td>M</td>
<td>27</td>
<td>16 (59%)</td>
</tr>
<tr>
<td>ICA-positive non-diabetic subjects</td>
<td>F</td>
<td>24</td>
<td>14 (58%)</td>
</tr>
<tr>
<td>ICA-negative subjects with metabolic abnormalities</td>
<td>M</td>
<td>6</td>
<td>5 (83%)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>6</td>
<td>4 (67%)</td>
</tr>
</tbody>
</table>

*ICA: islet cell antibody
a 64-kD islet cell protein occurs in newly diagnosed type I diabetic patients with high prevalence (26, 27), and that this antigen is identical to GAD (4). However, these studies lacked a simple and rapid method for autoantibody detection. Recently, immunoassays using native GAD from pig brain (12, 13) or recombinant GAD67 from mouse brain (14) were established for GAD autoantibody detection. It has been reported, however, that the 65-kD GAD isofrom is the predominant form in the human pancreas (28). Furthermore, this isoform is connected with the membrane of synaptic-like small microvesicles (29), which are secretory in nature. It has been suggested that this GAD isoform could play a more important role in autoimmune than the GAD67 isoform (30).

For this reason, we used the full-length recombinant human GAD65 isoform to establish a radioimmunoassay for GAD autoantibody detection. With this assay, we found a GAD autoantibody prevalence of 59% in newly diagnosed type I diabetic patients. This is comparable to the other immunoassays, which found a prevalence of GAD autoantibodies in newly diagnosed type I diabetic patients of 69% (12) and 59% (13) for GAD65-AAb and 46% (14) for GAD67-AAb. The specificity of our assay system is comparable to the other immunoassays (0% (12), 2.5% (13) and 0% (14) GAD autoantibody positivity of healthy blood donors, respectively). None of the 54 sera from healthy blood donors exceeded the cut-off. Additionally, none of 10 sera from patients with other autoimmune diseases have been found to be positive. The 125I-GAD65 binding of all AAb-positive sera from newly diagnosed type I diabetic patients could be displaced by the addition of GAD-containing in contrast to GAD-depleted 100 000 g supernatant from rat brain, showing the specificity of this binding.

Nine of 13 GAD65-AAb-positive sera from newly diagnosed Type I diabetic patients also precipitated GAD enzyme activity from rat brain extract (F Lühder, unpubl. obs.), suggesting that they contain AAb that are also reactive with native GAD. However, the sensitivity of such an immuno-trapping enzyme activity assay is much lower compared with radio- or enzyme-linked immunosorbent assays. Using this method, Martino et al. (31) found only 22% of newly diagnosed type I diabetic patients to be antibody-positive.

Only 22 out of 30 GAD65-AAb-positive sera from type I diabetic patients were found to be ICA-positive. This shows the higher sensitivity of the radioimmunoassay compared to the conventional ICA detection, because it is generally accepted that GAD is one of the ICA antigens. But ICA also detect antigens other than GAD, such as gangliosides (32). Thereby, the presence of GAD65-AAb-negative but ICA-positive sera from type I diabetic patients is not surprising.

The ICA-positive first-degree relatives of type I diabetic patients are considered to be high-risk individuals for developing the disease (33). We found a high prevalence of GAD65-AAb in this group, comparable to that of newly diagnosed type I diabetic patients. Interestingly, only subjects with additional metabolic abnormalities and/or ICA > 20 JDF units were found to be antibody-positive. This is in agreement with other studies suggesting that GAD autoantibodies are associated with persistent high-titre ICA in patients at high risk of developing diabetes (10, 11). This shows that GAD65-AAb are possibly a marker for a high risk of developing the disease. Additionally, we found 12% (4/34) of the subjects (all four first-degree relatives of type I diabetic patients) with metabolic abnormalities to be positive (two with impaired glucose tolerance and two with diminished insulin secretion in the first phase). There was no association of GAD autoantibodies with the two kinds of metabolic abnormalities studied here. Thivolet et al. (11) also have reported on GAD autoantibodies in ICA-negative first-degree relatives of type I diabetic patients. The prognostic value of the GAD autoantibodies found in these individuals should be evaluated in further studies.

We could not find a sex-dependent prevalence of GAD autoantibodies as described by Martino et al. (31), who found an association of GAD autoantibodies with the female sex. However, in diabetes-prone BB rats we did find an increase of the GAD autoantibody prevalence in female animals (34).

Altogether, these results, obtained with the established radioimmunoassay, characterize the assay as a useful tool for detecting autoantibodies to GAD. The assay can be used for screening a large group of individuals with good reproducibility. Longitudinal studies are necessary to evaluate the predictive value of GAD autoantibodies detected by this assay for the development of type I diabetes. To examine the prevalence and the predictive value of GAD isoform-specific autoantibodies, it is necessary to establish an immunosassay for GAD65-AAb. This will make it possible to differentiate between GAD isoform-specific autoantibodies and autoantibodies that react with both isoforms.

Acknowledgments. We are grateful to Priv. Doz. Dr W Northemann and Dr NI Cook for GAD purification, to Ms C Blumentritt for excellent technical assistance, to Mr P Heinke for statistical analysis and to Ms K Spleith for language revision of the manuscript. This work was supported by WIP Project 018305 KAI e.V. and BMFT project 07NBL02/98064.

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Received August 4th, 1993
Accepted February 8th, 1994