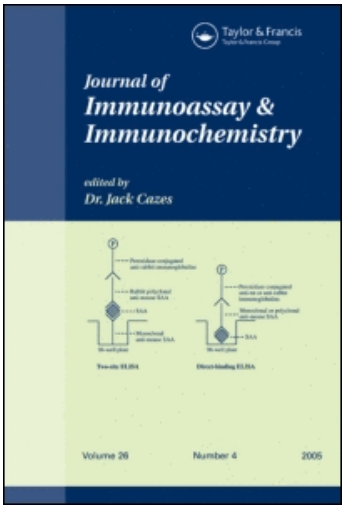

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Sensitive Monoclonal Antibody-Based Sandwich Elisa for Determination of the Diabetes-Associated Autoantigen Glutamic Acid Decarboxylase GAD65

Michael Schlosser[a]; Jens Hahmann[a]; Brigitte Ziegler[a]; Petra Augstein[a]; Manfred Ziegler[a]
[a] Ernst-Moritz-Arndt-University of Greifswald, Institute of Pathophysiology, Karlsburg, Germany

To cite this Article Schlosser, Michael , Hahmann, Jens , Ziegler, Brigitte , Augstein, Petra and Ziegler, Manfred(1997) 'Sensitive Monoclonal Antibody-Based Sandwich Elisa for Determination of the Diabetes-Associated Autoantigen Glutamic Acid Decarboxylase GAD65', Journal of Immunoassay and Immunochemistry, 18: 4, 289 — 307
To link to this Article: DOI: 10.1080/01971529708005823
URL: http://dx.doi.org/10.1080/01971529708005823

JOURNAL OF IMMUNOASSAY, 18(4), 289-307 (1997)

SENSITIVE MONOCLONAL ANTIBODY-BASED SANDWICH ELISA FOR DETERMINATION OF THE DIABETES-ASSOCIATED AUTOANTIGEN GLUTAMIC ACID DECARBOXYLASE GAD65

Michael Schlosser, Jens Hahmann, Brigitte Ziegler, Petra Augstein and Manfred Ziegler. Ernst-Moritz-Arndt-University of Greifswald, Institute of Pathophysiology, Greifswalder Str. 11a, D-17495 Karlsburg, Germany

ABSTRACT

Although various methods for the detection of autoantibodies against glutamic acid decarboxylase (GAD65-AAb) are known, no sensitive method for the quantification of GAD65 as autoantigen is available. We describe a sandwich ELISA based on monoclonal GAD65 antibodies (Mc-GAD65-Ab) of different epitope specificities to quantify GAD65 in pancreatic islets and in different organ/cell extracts and during the preparation of GAD from brain extracts. GAD65 was captured via solid phase coated Mc-GAD65-Ab and detected via a second biotin-labelled Mc-GAD65-Ab recognizing a NH_2-terminal epitope of the molecule. The detection limit was estimated to be 0.03 ng GAD65/ml using alkaline phosphatase (AP)-conjugated streptavidin. GAD65 contents in islets of neonatal BB/OK rats and Lewis rats amounted to 37.4 and 43.7 pg/islet, respectively. Furthermore, GAD65 was quantified in brain extracts of pig (55.1 ng/mg protein), mouse (39.5 ng/mg), rat (243.8 ng/mg) and pig cerebellum (514.8 ng/mg) and in different organ extracts of Lewis rat. (KEY WORDS: glutamic acid decarboxylase GAD65, monoclonal GAD65 antibodies, ELISA, biotin-streptavidin, pancreatic islets)

INTRODUCTION

Glutamic acid decarboxylase (GAD; EC 4.1.1.15) is an enzyme catalyzing the formation of gamma-aminobutyric acid (GABA), a neurotransmitter in central

nervous system but is also found in peripheral neurons and in the pancreatic islets, especially in Beta-cells (1, 2). It could be demonstrated that at least two main isoforms of GAD with the molecular weight of 65 kD (GAD65) and 67 kD (GAD67) exists which differ in their enzymatic kinetics and interactions with the cofactor pyridoxal-phosphate (3). GAD65 and GAD67 are encoded each by distinct genes (4). The sequence identity between these two isoforms is approximately 65% (5).

In 1990 a major component of the 64 kD autoantigen band recognized by autoantibodies (AAb) in insulin-dependent diabetes mellitus (IDDM) could be identified as GAD (6). Furthermore, it could be demonstrated that in sera of newly diagnosed patients with IDDM but also in prediabetic patients AAb to GAD65 (GAD65-AAb) occur in a higher prevalence than GAD67-AAb (7-10).

Several assays for measurement of GAD-AAb have been proposed such as measurement of enzyme activity after immunotrapping and immunoprecipitation assays using native GAD from brain extracts or human recombinant GAD (10-12). However, for determination of GAD itself only semiquantitive and time consuming enzymatic methods have been described by measuring $^{14}CO_2$ from ^{14}C-glutamate without distinction of the GAD isoforms (1, 13, 14). Furthermore, the application of GAD-specific antisera (15) and monoclonal antibodies with specific reactivity to the GAD isoforms (16-18) in immunohistochemical investigations allows only semiquantitive determinations of GAD-isoforms.

The aim of this study was to establish a quantitative sandwich ELISA using monoclonal antibodies of different epitope specificities to GAD65 for the sensitive and specific determination of the autoantigen in pancreatic islets, various tissue and cell extracts as well as during the preparation and purification of GAD65-enriched fractions from brain extracts.

MATERIALS AND METHODS

Generation of Monoclonal GAD Antibodies, Antibody Screening and Characterization

The GAD65-specific monoclonal antibody (Mc-GAD65-Ab) M63/2H3D9 (isotype IgG_{2A}) was obtained after repeated immunization of a female Balb/c mouse with a GAD65 sequence of 91 amino acids from the NH_2-terminus (19). The monoclonal autoantibody M61/7E11 (isotype IgG_1) was generated from a non-obese diabetic (NOD) mouse by treatment with a single intraperitoneal injection of a non-diabetic dose of 80 mg/kg body weight of the Beta-cell toxin streptozotocin as previously detailed (17). Further Mc-GAD65-Ab were generated after three immunizations of an 8-week-old female Balb/c mouse with full-length human recombinant GAD65 (18-20).

3×10^7 mouse splenoytes were immortalized with 1×10^7 cells of the myeloma cell line SP2/0 according to the method of Lane et. al. (21) as detailed previously (17, 18). After two weeks the supernatants were screened for antibodies against GAD by an ELISA using chromatographically purified natural GAD from rat brain extract for coating microtiter plates or by coating with GAD-AAb containing immunoglobulins of a patient suffering from Stiff-man syndrome for capturing natural GAD as described in detail previously (17, 22). The specificity of the monoclonals to both GAD isoforms was detected by an ELISA-kit (elias, Freiburg, FRG) using human recombinant GAD65 and GAD67. Results were confirmed by fluid-phase radioimmunoassay using ^{125}I-labelled human recombinant GAD65 and GAD67 (10).

Immunoglobulin (Ig) concentrations and isotypes were determined by ELISA standard procedures as described elsewhere (23). Hybridoma supernatants of

monoclonals contain concentrations of 45.2±4.0 mg Ig/l for M63/2H3D9 and 23.3±3.0 mg Ig/l for M61/7E11 (mean±SD; n=3).

Epitope specificity of Mc-GAD65-Ab was tested by an ELISA using human recombinant solid-phase coated GAD65 (0.03 µg/ml; Synectics Biotechnology, Stockholm, Sweden). Two different Mc-GAD65-Ab of equal Ig concentrations were incubated single and both together. Bound Ab were detected by incubation of POD-conjugated rabbit anti-mouse IgG (Fc-specific; Jackson Immunores. Lab., Wilmington, USA) according to ELISA standard procedure (23). In comparison to single incubation combinated incubation will result in an increase of optical density (O.D.) if different epitopes are detected. Combinated incubation of two monoclonals but bearing identical epitope specificity will not achieve increased O.D. in comparison to single incubation. Calculation of addition indices (A.I.) was performed according to the formula of Friguet et al. (24):

$$A.I. = \left[\frac{2 \times O.D._{1+2}}{O.D._1 + O.D._2} - 1 \right] \times 100$$

If the A.I. will be significant greater than 50%, different epitopes are recognized by the Mc-GAD65-Ab tested.

Antibody Purification and Biotin-Conjugation

Mc-GAD65-Ab were purified after fractionated precipitation of Ig of ascites fluids at concentrations of 28% and 45% of saturated ammonium sulphate solution followed by extensively dialysis against phosphate-buffered saline containing 0.1% sodium azide (PBSA) and stored at -20°C until use. Biotin-conjugation was performed by incubation of 1 mg/ml of purified Mc-GAD65-Ab, dialysed against coupling buffer (0.1 M sodium hydrogencarbonate buffer, pH 7.7), with 50 µg of biotin-X-NHS (Calbiochem GmbH, Frankfurt, FRG) in 50 µl of dimethyl

formamide for 30 min as detailed elsewhere (25). Separation of biotin-conjugated M61/7E11 from free biotin was performed by gelchromatography using Sephadex G 25 (diameter: 1.5 cm, length: 20 cm) in elution buffer (0.1 M sodium acetate / 0.2 M sodium chloride, pH 5.0) and controlled by the Bio-Rad ECONO system. Pooled Ab fractions were dialyzed aginst PBSA and stored at -20°C until use.

Preparation of Tissue Homogenates and Calibration Standard

Heart, liver, kidney, spleen, muscle, testis and pancreas from five male adult Lewis rats were freshly prepared and separately homogenized with an Ultra-Turrax (TP 18/10, 100 W) 5 x 15 sec on ice in lysis buffer (1/5; wt/wt) containing 0.2 mM pyridoxal-5'-phosphate (Merck, Darmstadt, FRG), 1 mM ethylenediamine tetraacetic acid-disodium salt (EDTA; Berlin Chemie AG, Berlin, FRG), 1 mM phenylmethyl-sulfonylfluoride (PMSF; Sigma, St. Louis, USA), 1 mM (2-aminoethyl)-isothiouroniumbromid (Sigma, St. Louis, USA) and 1% Triton X-100 in 25 mM sodium phosphate, pH 7.4. After sonication for 3 x 15 sec on ice (SONOPLUS HD70, MS 73; 156 W/cm^2) the homogenates were precleared by centrifugation at 3,000 x g for 15 min and the supernatants were ultracentrifuged twice at 100,000 x g for 45 min at 4°C. Islets of neonatal NOD mice, Balb/c mice, Lewis rats and BB/OK rats were prepared by fractionated collagenase digestion of pancreata as described detailed (26). 1,000 rat islets and 1,000 mouse islets were sonicated on ice and ultracentrifuged at 100,000 x g as described above. Furthermore, 100,000 x g supernatants of 2 x 10^6 cells of different cell lines, i.e. rat insulinoma cells (RIN5AH-T2, -T3) and neuroblastoma cells (Neuro 2A) grown in RPMI 1640 supplemented with 7.5% fetal calf serum (FCS), pig cerebellum cells (CBS-R) and mouse pituitary cells (ATT) grown in DMEM/7.5% FCS, and baby-hamster-kidney cells (BHK-21) grown in MEM/7.5% FCS and neonatal Lewis rat Beta-cells separated by FACS were dispensed in 1 ml of lysis

buffer, sonicated and centrifuged for 15 min at 100,000 x g. Whole brain extracts from Lewis rat, Balb/c mouse and pig and pig cerebellum were prepared by the same procedure described above for Lewis rat organ extracts. A 4-fold concentrated 100,000 x g supernatant of rat brain was chromatographically purified by Sepharose CL-6B as matrix and was used as calibration standard for the quantitative GAD65-ELISA. Protein concentrations of all 100,000 x g supernatants of cell and organ extracts were determined according to the method of Lowry et. al. (27).

Quantitative GAD65-ELISA

Flat-bottomed microtiter plates of high binding capacity (Greiner, Nürtingen, FRG) were coated with the purified Mc-GAD65-Ab M63/2H3D9 (25 µg/ml in 50 mM sodium carbonate buffer, pH 9.6, 50 µl/well) overnight at 4°C. After washing twice with phosphate-buffered saline containing 0.05% Tween 20 (PBST), nonspecific binding sites were blocked by 100 µl/well PBST supplemented with 20% neonatal calf serum (NCS) for 1 h at room temperature (RT). After three washings with PBST, dilutions of the calibration GAD65-standard and samples diluted in PBST containing 5% NCS were incubated for 2 h at RT by shaking. After washing four times with PBST the biotin-labelled Mc-GAD65-Ab M61/7E11 was added (dilution 1/1,000 in PBST with 5% NCS, 50 µl/well) and incubated for 2 h at RT under shaking. Bound Ab was visualized after incubation of alkaline phosphatase (AP)-conjugated streptavidin (1/1,000; Jackson Immunores. Lab, Wilmington, USA) for 1 h at RT followed by addition of the chromogenic substrate solution containing 2.7 mM p-nitrophenyl phosphate-disodium salt (p-NPP; Sigma, St. Louis, USA) in 1M diethanolamine/0.5 mM $MgCl_2$ buffer (pH 9.8). After 1 h at RT O.D. at 405 nm (test wave length) and 630 nm (reference

wave length) was measured by a Dynatech reader MRX Revelation and GAD65 contents of samples were calculated using cubic spline fitting. Alternatively, POD-streptavidin (1/5,000; Jackson Immunores. Lab, Wilmington, USA) was used and colour development (0.5 h at RT) was performed as described elsewhere (23). The chromatographically purified rat brain extract used as GAD65 standard was calibrated by full-length human recombinant GAD65 (purity: 95%) expressed via infection of *Spodoptera frugiperda* cells (Synectics Biotechnology, Stockholm, Sweden).

RESULTS

The mc-GAD-Ab M63/2H3D9 and M61/7E11, used in standard assay, specifically recognize GAD65 (O.D.490nm±SD: 0.58±0.09 and 2.71±0.08; n=3) without any cross-reaction to GAD67 (O.D.490nm±SD: 0.08±0.01 and 0.04±0.01) in an ELISA-kit using human recombinant GAD65 and GAD67 solid-phase coated.

Both Mc-GAD65-Ab recognize distinct epitopes of the molecule as demonstrated by an ELISA using solid-phase coated human recombinant GAD65. The GAD65 binding of M61/7E11 and M63/2H3D9 alone amounted to 0.837±0.02 (mean O.D.490nm±SD; n=4) and 0.345±0.03 (n=4), respectively, and for the simultaneous incubation of both Ab 1.069±0.05 (n=4; A.I.=80.8%).

After optimizing the dilutions of capture Ab (M63/2H3D9) and detector Ab (M61/7E11) the GAD65 standard was calibrated using human recombinant GAD65 (first dilution: 25 ng/ml). The GAD standard from rat brain contains 127 ng GAD65/ml and was used in every test. By application of POD-strepavidin to detect GAD65-bound biotin-M61/7E11, the detection limit (mean O.D.+3SD of a GAD-free rat brain extract; n=20) was 0.3 ng/ml (15 pg/well or 0.23 fmol/well;

Fig. 1). Using AP-streptavidin as conjugate, the sensitivity of the assay could be significant enhanced up to 10-fold (detection limit: 0.03 ng GAD65/ml, i.e. 1.5 pg GAD65 or 0.023 fmol per well of a microtiter plate; Fig. 1). Other mc-GAD-Ab recognizing conformational GAD65 epitopes were tested either solid-phase coated as capture Ab or biotinylated as detector Ab. However, all other antibody pairs show less sensitivity ranging from 0.045 up to 0.94 ng GAD65/ml although the determined GAD65 contents of unknown samples were comparable (data not shown). The inverse combination of M61/7E11 solid-phase immobilized and the biotinylated M63/2H3D9 as detector results in a 36-fold higher detection limit of 1.08 ng GAD65/ml compared to the standard assay.

As shown in Tab. 1A the GAD65 content of islets of BB/OK rats (37.4 ng GAD65/1,000 islets, i.e. 19.3 ng GAD65/mg protein) and Lewis rats (43.7 ng/1,000 islets, i.e. 20.4 ng/mg) was comparable whereas GAD65 was not detectable in mouse islets of both Balb/c and NOD mice.

Analyzing the GAD65 content of cell homogenates the highest amount was determined in rat Beta-cells (6.27 ng/1 x 10^6 cells corresponding to 13.75 ng GAD65/mg protein; Tab. 1B). GAD65 contents of 1 x 10^6 RIN 5AH-T2 (1.58±0.24 ng) and RIN 5AH-T3 cells (1.55±0.41) was comparable but 4-fold lower vs. rat Beta-cells. CBS-R and ATT cells contain low but significant detectable amounts of GAD65 whereas no GAD65 was detectable in Neuro 2A and BHK-21 cells (Tab. 1B).

The highest GAD65 concentration was detected in brain extracts of different species (Fig. 2, left part). The GAD65 content of pig cerebellum (514.8 ng GAD65/mg protein) was about 10-fold higher compared to whole pig brain extracts (55.1 ng/mg; mean of three speparate experiments). Except brain, pancreas and heart extracts no GAD65 was detectable in 100,000 x g supernatants of liver, kidney, muscle, spleen, testis extracts of Lewis rats (Fig. 2, right part).

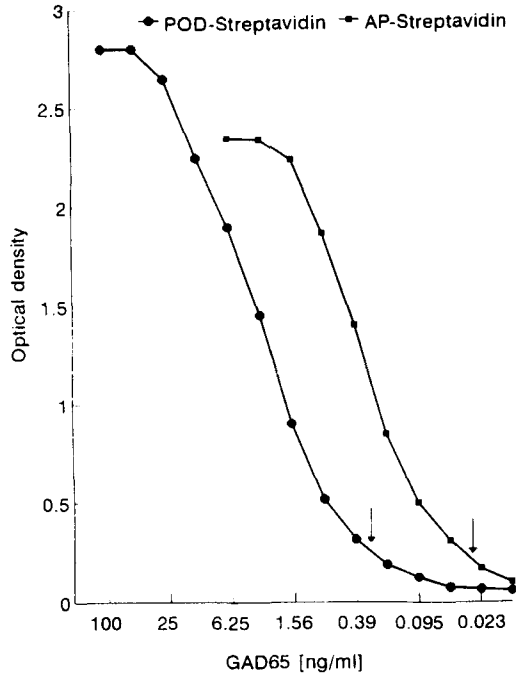

FIGURE 1 Standard dilution curves of human recombinant GAD65 using POD-conjugated streptavidin (●) vs. AP-conjugated streptavidin (■) according to the standard assay protocol. The application of AP-streptavidin enhances the sensitivity of the assay by factor 10 resulting in a detection limit of 0.03 ng GAD65/ml compared to 0.3 ng/ml for the POD-streptavidin. The detection limit (indicated by the arrows) was calculated from the mean O.D. + 3xSD of a GAD-free brain extract.

The GAD65 content per mg protein amounted to 243.8±28.8 ng/mg for rat brain compared to rat pancreas 0.29±0.06 ng/mg and heart 0.11±0.01 (data are given as mean±SD of 5 rats). Thus the GAD65 concentration in rat brain was approximately 1,000-fold higher than in rat pancreas and 3,000-fold higher than in rat heart. GAD65 content of rat liver, kidney, muscle, spleen and testis was below the detection limit of this sandwich ELISA. A nonspecific binding of streptavidin

TABLE 1:

GAD65 Contents of pancreatic Islets [A] and different Cell Lines [B] determined by GAD65-ELISA (Data are given as Mean±SD of three Determinations; n.d.: not detectable)

[A]	animal strain	GAD65/1,000 islets [ng]	GAD65/protein [ng/mg]
	BB/OK rat	37.4 ± 5.9	19.28 ± 3.6
	Lewis rat	43.7 ± 5.1	20.44 ± 3.4
	NOD mouse	< 0.015	< 0.016
	Balb/c mouse	< 0.015	< 0.016

[B]	cell line	GAD65/1 x 10^6 cells [ng]	GAD65/protein [ng/mg]
	CBS-R	0.49 ± 0.09	3.03 ± 0.28
	Neuro 2A	n.d.	n.d.
	ATT	0.68 ± 0.08	4.14 ± 0.47
	RIN 5AH-T2	1.58 ± 0.24	6.79 ± 0.38
	RIN 5AH-T3	1.55 ± 0.41	5.48 ± 0.49
	BHK-21	n.d.	n.d.
	Beta cells (rat)	6.27 ± 0.71	13.75 ± 0.95

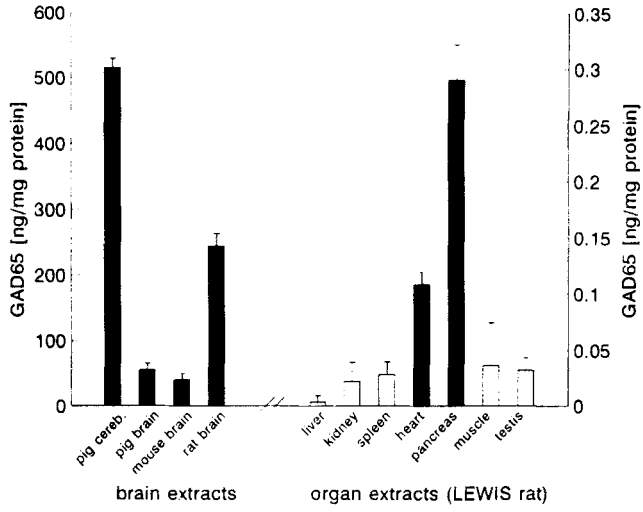

FIGURE 2. GAD65 contents per protein [ng/mg] of 100,000 x g supernatants of whole brain extracts of pig, mouse, rat and pig cerebellum extract quantified by the GAD65-ELISA (left part; data are given as mean±SD of three separate experiments). Comparison of GAD65 contents of 100,000 x g supernatants of different organ extracts of Lewis rats (right part; data are given as mean±SD of 5 Lewis rats). GAD65 could only be detected in brain, pancreas and heart of Lewis rats. In rat brain the GAD65 concentration was approximately 1,000-fold higher compared to pancreas and 3,000-fold higher compared to heart.

conjugates to the plates (which could not be blocked) was detectable in liver and kidney extracts. To verify positive results of tissue extracts investigated, modified ELISA technique was performed by means of absence and presence of biotin-conjugated detector antibody.

Fig. 3 demonstrates a further application of the GAD65-ELISA, i.e. the determination of GAD65-containing fractions during the chromatographic purification of 4-fold concentrated 100,000 x g supernatant of rat brain extract to prepare the calibration standard used in every assay.

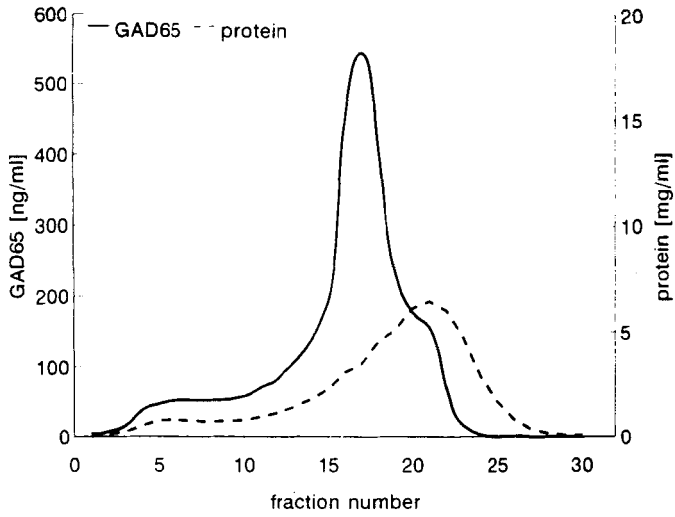

FIGURE 3. Determination of GAD65 (ng/ml; solid line) in relation to the protein content (mg/ml; dotted line) in fractions eluted by gel chromatography of concentrated (4 fold) 100,000 x g supernatant of rat brain extract using Sepharose CL-6B as matrix.

Nonionigenic detergents such as Triton X-100 and Triton X-114 could influence the assay only in concentrations higher than 0.25% by decreasing sensitivity.

The reliability of the GAD65-ELISA characterized by the coefficients of variation (CV) at 0.39 ng/ml was 3.8% for the intraassay (i.e. 20 determinations per plate) and 9.8% for interassay (i. e. 20 determinations over 20 days).

DISCUSSION

Although various methods for detection and differentiation of GAD-AAb in sera of IDDM and prediabetic patients are known (28), sensitive assays for the quantification of GAD as autoantigen have not yet been described.

Common assays for quantitative GAD enzyme acitivity measurement via ^{14}C-glutamate are time consuming and fail to differentiate GAD isoforms unless additional tests for pyridoxal-5'-phosphate dependency of enzymatic activity are performed (3, 29, 30). Immunoprecipitation of labelled GAD, Western Blotting or immunohistochemical analysis are only semiquantitative but allow to discriminate between GAD65 and GAD67 (31, 32).

This paper describes a sensitive sandwich ELISA to quantify the 65 kD isoform of the enzyme GAD, which is the major autoantigen targeted by AAb of newly diagnosed IDDM and prediabetic patients (6, 8, 10). The assay is based on two monoclonal antibodies specifically recognizing GAD65 by two different epitopes without any cross-reaction to GAD67. Only the combination of the immobilized capture antibody M63/2H3D9, generated after immunization with a GAD65 sequence of the first 91 amino acids from the NH_2-terminus (19), and the biotin-conjugated detector antibody M61/7E11, derived from a non-obese diabetic (NOD) mouse (17) and recognizing a linear epitope located in the NH_2-terminal region (epitope 4-17), and the application of the biotin-streptavidin system (33) enables to quantify GAD65 with the described high sensitivity. Using POD-streptavidin the detection limit was 0.3 ng GAD65/ml which could be further enhanced by factor 10 to 0.03 ng/ml using AP-streptavidin corresponding to 1.5 pg or 0.023 fmol GAD65 per well of a microtiter plate. This improvement is due to reduced non-specific binding and improved colour stability. All other capture/detector pairs of Mc-GAD65-Ab tested, for example Mc-GAD65-Ab recognizing conformational GAD epitopes, result in a reduced sensitivity of the assay, although the determined GAD65 contents of different organ extracts are identical with results obtained with the standard assay (data not shown). Also the inverse Ab combination, i.e. the M61/7E11 as capture and the biotin-labelled M63/2H3D9 as detector results in a 36-fold loss of sensitivity (detection limit: 1.08

ng GAD65/ml). Moody et al. (34) using POD-streptavidin in a GAD65-ELISA with the monoclonal GAD-1 (16) solid-phase coated for capturing GAD and the biotin-labelled monoclonal GAD-6 as detector reported a detection limit of 5 ng GAD65/ml. Thus affinity of monoclonals, valency of antigen-antibody interactions and non-specific binding are of importance for sensitivity of a sandwich ELISA. Furthermore, biotin conjugation could influence behaviour of antigen binding of certain monoclonals resulting in lower sensitivity of immunoassays.

Our quantitative data with islets of NOD and Balb/c mouse confirm immunohistochemical findings that GAD65 is either not or to a very small amount expressed in mouse pancreas (17, 31, 32). Regarding to the sensitivity of our ELISA, the GAD65 content in one mouse islet is lower than 0.015 pg. Thus, compared to one BB/OK rat islet (37.4 pg GAD65) the GAD65 content of one mouse islet is about 2,500 fold lower. Contrary to our findings GAD65 could be detected by other groups on a very low level in mouse islets (32, 35). Furthermore, the generation of specific GAD65 monoclonal antibodies (as our detector antibody M61/7E11) by treatment of a NOD mouse with streptozotocin and complete Freund's adjuvant (17) suggests the expression of GAD65 in mouse islets. However, this Mc-GAD65-Ab did not detect GAD in cryosections of mouse pancreas as also shown for GAD65-AAb from IDDM patients which will immunostain human and rat islets but not mouse islets (35).

As demonstrated by this study our monoclonals are reactive with GAD65 of human, rat, mouse and pig. This is in accordance with the described high identity of amino acid sequences (greater than 95%) of brain GAD65 of different species such as human, pig, rat or cat (1, 29, 30, 36). Therefore it is justified to use monoclonals raised against GAD65 of different species to quantify GAD65 of different species. In addition, it could be demonstrated that the homology of GAD65 in brain and pancreatic islets is 99.4 to 100% (37). Our results have shown

high GAD65 contents in brain of rat, mouse and pig. The low GAD65 content of mouse brain compared to rat brain might be due to a higher GAD67 expression in brain of NOD and Balb/c mouse as also demonstrated for mouse islets (no GAD65 detectable). The amount of GAD65 in pig cerebellum was found to be 10-fold higher compared to whole pig brain.

Analyzing the 100,000 x g supernatants of different organ extracts of Lewis rats, GAD65 was only detectable in brain, pancreas and heart. The GAD65 concentration in brain was found to be 1,000-fold higher compared to pancreas and about 3,000-fold higher than in heart of rats. In liver, kidney, spleen, muscle and testis of animals no GAD65 could be detected. This discrepancy to the reported detection of GAD in liver, kidney, spleen, testis (GAD67 only) or oviduct (2, 30) can be interpreted either by very low levels of GAD65 expression in these tissues. On the other hand an inability to distinguish between GAD67 and GAD65 using enzymatic assays, the use of not exclusively GAD65-specific antisera for immunohistochemical investigations or the occurrence of other GAD isoforms like a recently described new integral membrane protein from pig brain with a native molecular weight of 120 kD (homodimer of 60 kD) might explain these differences (38). Furthermore, a non-specific binding of tissue extracts containing high protein concentrations which might cause false positive results must be taken into consideration. This was also observed in our ELISA in rat liver and kidney extracts inducing high non-specific binding (not GAD65) of streptavidin conjugates.

Summarizing our results, a sensitive assay for detection and quantification of GAD65 in various tissue and cells extracts as well as during the preparation of GAD65-enriched fractions was established. The assay is technically easy to perform, highly reproducible and allows to quantify small amounts of this important autoantigen IDDM.

ACKNOWLEDGMENTS

We are greateful to Mrs. H. Kenk and Mrs. S. Tietz for excellent technical assistance. This study was supported by the WIP project 018305/M KAI e.V. and the BMFT project 07NBL02/98064. The cell line CBS-R was a kind gift of Dr. R. Riebe, Friedrich-Loeffler-Institute, Riems, Germany.

Correspondence to: Dr. Michael Schlosser, Institute of Pathophysiology
University of Greifswald, D-17495 Karlsburg, Germany
Phone: +49 383 556 8178
Fax: +49 383 556 8111

REFERENCES

1. Legay, F., Pelhate, S. and Tappaz M.L. Phylogenesis of brain glutamic acid decarboxylase from vertebrates: Immunochemical studies. J. Neurochem. 1986; 46: 1478-86.

2. Erdö, S.L., and Wolff, J.R. Gamma-aminobutyric acid outside the mammalian brain. J. Neurochem. 1990; 54: 363-72.

3. Martin, D.L., Martin, S.B., Wu, S.J. and Espina, N. Regulatory properties of brain glutamate decarboxylase (GAD): The apoenzyme of GAD is present principally as the smaller of two molecular forms of GAD in brain. J. Neurosci. 1991; 11: 243-9.

4. Erlander, M.G., Tillakaratne, N.J.K., Feldblum, S., Patel, N. and Tobin, A.J. Two genes encode distinct glutamate decarboxylases. Neuron 1991; 7: 91-100.

5. Bu, D.F., Erlander, M.G., Hitz, B.C. et al. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. Proc. Natl. Acad. Sci. USA 1992; 89: 2115-9.

6. Baekkeskov, S., Aanstoot, H.J., Christgau, S. et al. Identification of the 64 K antigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990; 347: 151-6.

7. Kaufmann, D.L., Erlander, M.G., Clare-Salzler, M., Atkinson, M.A., Maclaren, N.K. and Tobin, A.J. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. J. Clin. Invest. 1992; 89: 283-92.

8. Hagopian, W.A., Michelsen, B., Karlsen, A.E. et al. Autoantibodies in IDDM primarily recognize the 65,000-M_r rather than the 67,000-M_r isoform of glutamic acid decarboxylase. Diabetes 1993; 42: 631-6.

9. Velloso, L.A., Kämpe, O., Hallberg, A., Christmanson, L., Betsholtz, C. and Karlsson, F.A. Demonstration of GAD_{65} as the main immunogenic isoform of glutamate decarboxylase and determination of autoantibodies using a radioligand produced by eucaryotic expression. J. Clin. Invest. 1993a; 91: 2084-90.

10. Lühder, F., Schlosser, M., Mauch, L. et al. Autoantibodies against GAD65 rather than GAD67 predict the onset of Type I diabetes. Autoimmunity 1994; 19: 71-80.

11. Martino, G.V., Tappaz, M.L., Braghi, S. et al. Autoantibodies to glutamic acid decarboxylase (GAD) detected by an immuno-trapping enzyme activity assay. J. Autoimmunity 1991; 4: 915-23.

12. Lühder, F., Woltanski, K.-P., Hamann, J., Klöting, I., Ziegler, B. and Ziegler, M. Detection of antibodies against both isoforms of glutamate decarboxylase in BB/OK rats by western blotting and immunotrapping enzyme activity assay. Diabetes Res. 1993; 20: 97-107.

13. Albers, R. and Brady, R.O. The distribution of glutamate decarboxylase in the nervous system of the rhesus monkey. J. Biol. Chem. 1959; 234: 926-8.

14. Wu, J.-Y. and Roberts, E. Comparative studies of L-glutamate decarboxylases from mouse brain and kidney. Am. Soc. Neurochem. 1973; 4: 70-9.

15. Oertel, W.H., Schmechel, D.E., Tappaz, M.L. and Kopin, I. Production of a specific antiserum to rat brain glutamic acid decarboxylase (GAD) by injection of an antigen antibody complex. Neurosci. 1981; 6: 2715-35.

16. Gottlieb, D.I., Chang, Y.-C. and Schwob, J.E. Monoclonal antibodies of glutamic acid decarboxylase Proc. Nat. Acad. Sci. USA 1986; 83: 8808-12.

17. Ziegler, B., Augstein, P., Lühder, F. et al. Monoclonal antibodies specific to the glutamic acid decarboxylase 65 kDa isoform derived from a non-obese diabetic (NOD) mouse. Diabetes Res. 1994; 25: 47-64.

18. Ziegler, B., Augstein, P., Schröder, D. et al. Glutamate decarboxylase (GAD) is not detectable on the surface of rat islet cells examined by cytofluorometry and complement-dependent antibody-mediated cytotoxicity of monoclonal GAD antibodies. Horm. Metab. Res. 1996; 28: 11-5.

19. Mauch, L., Abney, C.C., Berg, H., Scherbaum, W.A., Liedvogel, B. and Northemann, W. Characterization of a linear epitope within the human pancreatic 64kDa glutamic acid decarboxylase and its autoimmune recognition by sera from insulin-dependent diabetes mellitus patients. Eur. J. Biochem. 1993; 212: 597-603.

20. Augstein, P., Schlosser, M., Ziegler, B., Hahmann, J., Mauch, L. and Ziegler, M. Comparison of the islet cell antibody pattern of monoclonal glutamic acid decarboxylase antibodies recognizing linear and conformational epitopes. Acta histochem. 1996; 98: 229-41.

21. Lane, R.D. A short-duration PEG fusion technique for increasing production of monoclonal antibody secreting hybridomas. J. Immunol. Meth. 1985; 81: 223-8.

22. Hamann, J., Lühder, F., Schlosser, M., Vieregge, P., Ziegler, B. and Ziegler, M. Specific screening of monoclonal antibodies to glutamic acid decarboxylase by a sandwich assay using the natural target antigen plate-bound via autoantibodies. Diabetes Res. 1993; 24: 57-66.

23. Schlosser, M., Witt, S., Ziegler, B. and Ziegler, M. Influence of target cell preparation on binding of monoclonal islet cell reactive antibodies (mc-ICRA) in cellular enzyme-linked immunosorbent assay (CELISA). J. Immunol. Meth. 1991; 140: 101-9.

24. Friguet, B., Djavadi-Ohaniance, L., Pages, J., Bussard, A. and Goldberg, M. A convenient enzyme-linked immunosorbent assay for testing whether monoclonal antibodies recognize the same antigenic site. Application to hybridomas specific for the ß-subunit of Escherichia coli tryptophan synthase. J. Immunol. Meth. 1983; 60: 351-8.

25. Guesdon, J.L., Ternynck, T. and Avrameas, S. The use of avidin-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem. 1979; 27: 1131-9.

26. Ziegler, B., Kohnert, K.-D., Noack, S. and Hahn, H.J. Effects of 3-isobutyl-1-methylxanthine on secretory response, cAMP accumulation and DNA synthesis of islets from postnatal and adult Wistar rats. Acta Biol. Med. Germ. 1982; 41: 1171-7.

27. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with Folin phenol reagent. J. Biol. Chem. 1951; 193: 265-75.

28. Schmidli, R.S., Colman, P.G., Bonifacio, E. and Participating Laboratories. Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. The Second International GADAb Workshop. Diabetes 1995; 44: 636-40.

29. Spink, D.C. and Martin, D.L. Multiple forms of glutamate decarboxylase in dog, rat, and human brain. In: Hertz, L., Kvamme, E., McGeer, E.G. and Schousboe, A. eds. Glutamate, glutamine and GABA in the central nervous system. New York: Liss, 1983: 129-43.

30. Tillakaratne, N.J.K., Erlander, M.G., Collard, M.W., Greif, K.F. and Tobin, A.J. Glutamate decarboxylases in nonneuronal cells of rat testis and oviduct: Differential expression of GAD_{65} and GAD_{67}. J. Neurochem. 1992; 58:618-27.

31. Petersen, J.S., Russel, S., Marshall, M.O. et al. Differential expression of glutamic acid decarboxylase in rat and human islets. Diabetes 1993; 42: 484-95.

32. Kim, J., Richter, W., Aanstoot, H.J. et al. Differential expression of GAD_{65} and GAD_{67} in human, rat and mouse pancreatic islets. Diabetes 1993; 42: 1799-1808.

33. Costello, S.M., Felix, R.T. and Giese, R.W. Enhancement of immune cellular agglutination by use of an avidin-biotin system. Clin. Chem. 1979; 25: 1572-80.

34. Moody, A.J., Hejnæs, K.R., Marshall, M.O. et al. Isolation by anion-exchange of immunologically and enzymatically active human islet glutamic acid decarboxylase 65 overexpressed in Sf9 insect cells. Diabetologia 1995; 38: 14-23.

35. Velloso, L.A., Kämpe, O., Eizirik, D.L., Hallberg, A., Andersson, A. and Karlsson, F.A. Human autoantibodies react with glutamic acid decarboxylase in human and rat but not mouse pancreatic islets. Diabetologia 1993b; 36: 39-46.

36. DeAizpurura, H.J. and Harrison, L.C. Glutamic acid decarboxylase in insulin-dependent diabetes mellitus. Diabetes Metab. Rev. 1992; 8: 133-47.

37. Lee, D.S., Tian, J., Phan, T. and Kaufmann, D.L. Cloning and sequence analysis of a murine cDNA encoding glutamate decarboxylase (GAD_{65}). Biochim. Biophys. Acta 1993; 1216: 157-60.

38. Nathan, B., Bao, J., Hsu, C.-C. et al. An integral membrane protein form of brain L-glutamate decarboxylase: purification, characterization and its relationship to insulin-dependent diabetes mellitus. Brain Res. 1994; 642: 297-302.