

Prevalence of Autoantibodies to the 65- and 67-kD Isoforms of Glutamate Decarboxylase in Insulin-dependent Diabetes Mellitus

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Abstract

We investigated the presence of autoantibodies to baculovirus-expressed human recombinant 65- and 67-kD isoforms of glutamate decarboxylase (GAD₆₅ and GAD₆₇) in insulin-dependent diabetes mellitus (IDDM). In the immunoprecipitation test using [³⁵S]methionine-labeled GADs antibodies to GAD₆₅ were detected in 13/15 (87%) islet cell antibody (ICA)-positive and in 1/35 (2.9%) ICA-negative first-degree relatives of patients with IDDM, in 6/11 (54.5%) ICA-positive nondiabetic schoolchildren, and in 35/50 (70%) patients with newly diagnosed IDDM. GAD₆₇ antibodies were positive only in five (33%) of the ICA-positive relatives ($P < 0.05$) and in nine (18%) IDDM patients at onset ($P < 0.00001$). After onset of IDDM antibodies to GAD₆₅ and GAD₆₇ declined but were still positive in 25 and 9.4% of subjects with long-standing IDDM (> 10 yr). In all study groups antibodies to GAD₆₇ were only detected in GAD₆₅ antibody-positive sera. An immunotrapping enzyme activity assay for GAD₆₅ antibodies was positive in 64/75 (85.3%) of sera that were GAD antibody positive in the immunoprecipitation test ($r = 0.870$, $P < 0.0001$). In two (2.7%) sera GAD₆₅ antibodies that block GAD enzyme activity were found. Our data suggest that antibodies to GAD₆₅ but not to GAD₆₇ represent sensitive markers for preclinical and overt IDDM. The immunotrapping assay here described represents a valuable technique for specific and sensitive screening for GAD antibodies. (*J. Clin. Invest.* 1993. 92:1394–1399.) Key words: glutamic acid decarboxylase • autoantibodies • insulin-dependent diabetes mellitus • islet cell antibodies

Introduction

Insulin-dependent diabetes mellitus (IDDM)¹ is strongly associated with the appearance of islet cell-specific autoantibodies, reflecting the autoimmune-mediated destruction of the pancre-

atic beta cells. Cytoplasmic islet cell antibodies (ICA) as well as antibodies to a 64-kD islet cell protein (64K) have been detected in 70–90% of individuals before and at the time of diagnosis of IDDM (1–4). In prospective studies only a subgroup of ICA-positive nondiabetic individuals, especially those with high ICA titers and complement-fixing ICA, developed IDDM (5, 6). In first-degree relatives and ICA-positive nondiabetic subjects antibodies to the 64K antigen were detected early in the preclinical course of IDDM and were strongly correlated with rapid progression to overt diabetes (7, 8). Therefore, the accurate determination of antibodies to the 64K antigen would be of particular interest to identify subjects at high risk for the development of IDDM. A large-scale detection of 64K antibodies has been hampered by the laborious technique of the immunoprecipitation of [³⁵S]methionine-labeled islet cells preparations.

The target antigen of the 64K antibodies has been identified as the enzyme glutamate decarboxylase (GAD), which synthesizes γ -aminobutyric acid (GABA) from glutamic acid (9). GAD exists at least in two different isoforms with molecular sizes of 65 kD (GAD₆₅) and 67 kD (GAD₆₇) encoded by two distinct genes (10, 11). It was shown that GAD₆₅ corresponds to the 64K antigen and both isoforms are recognized by sera from patients with IDDM (12, 13). Population-based data on the frequency of antibodies to human GAD₆₇ are scanty. Thus, the importance of antibodies to GAD₆₇ for IDDM is still unclear.

Because both isoforms of GAD are expressed in brain neurons (12, 13) several studies used brain preparations to determine antibodies to GAD by an immunotrapping enzyme activity assay (ITA) (14–16). In contrast to 64K antibodies, antibodies to GAD, as measured in the ITA, were detected in only 25–37% of newly diagnosed patients with IDDM. It has been suggested that these controversial data might be due to differences in the sensitivity of the assays or that only a part of the immunoreactivity to the 64K islet protein is directed to GAD. In none of these reports has a direct comparison of the immunoprecipitation test and the ITA been performed to address this question.

In the present study human recombinant GAD expressed in the baculovirus system was used to determine autoantibodies to GAD₆₅ and GAD₆₇. We analyzed for the first time the prevalence of antibodies to GAD₆₅ and GAD₆₇ in individuals at high risk for IDDM, at the onset of the disease, and in patients with a long duration of IDDM. In our approach we compared the results of the conventional immunoprecipitation test with the GAD immunotrapping enzyme activity assay in individual sera. This made it possible for us to determine antibodies which block GAD enzyme activity and evaluate the diagnostic value of the different immunoassays regarding the detection of antibodies to GAD.

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1. Abbreviations used in this paper: AET, 2-aminoethylisothiuronium bromide; GAD₆₅, GAD₆₇, 65- and 67-kD; glutamate decarboxylase; ICA, islet cell antibodies; 64K, 64-kD islet cell protein; IDDM, insulin-dependent diabetes mellitus; IPT, immunoprecipitation test; ITA, immunotrapping assay; PLP, pyridoxal 5-phosphate.

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Methods

Human sera. We studied sera from 50 patients with IDDM, obtained within the first month of diagnosis, and 77 sera from patients with a duration of IDDM from 0.5 to 40 yr. In addition, subjects at high risk for the development of IDDM were evaluated for GAD antibodies. We assayed GAD antibodies in 11 persistently ICA-positive nondiabetic schoolchildren without a family history of IDDM from the Ulm-Frankfurt population study (8, 17, 18) and 50 nondiabetic first-degree relatives of patients with IDDM. Among the 50 relatives, 15 were positive for circulating ICA at high level (> 40 JDF-U). 60 ICA-negative sera from normal individuals without a family history for IDDM were used as control samples. The sera were coded, stored at -20°C , and tested in a blinded way in each of the assays. Informed consent was obtained from all patients and control subjects.

Detection of cytoplasmic ICA. Cytoplasmic ICA were analyzed by the indirect immunofluorescence tests on cryostat sections of human pancreas from an organ donor with blood group O (19). ICA were expressed in Juvenile Diabetes Foundation (JDF) Units (IDW ICA Proficiency Program, Lab ID No 116). The detection limit of the assay in our laboratory was 5 JDF-U.

Antigen expression and preparation. GAD_{65} and GAD_{67} cDNA clones were constructed by inserting full-length human GAD_{65} and GAD_{67} cDNAs into the baculovirus vector pVL1393 (Invitrogen, San Diego, CA). The cDNA clones were cotransfected with the wild-type *Autographa californica* virus and the recombinant baculovirus clones were isolated as described (20). *Spodoptera frugiperda* (Sf9) cells were infected with the recombinant baculovirus clones and cultured in SF900 medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.04% FCS for 48 h. Then cells were harvested and homogenized in 20 mM potassium phosphate, pH 7.0; 2 mM EDTA; 2 mM PMSF; 5 $\mu\text{g}/\text{ml}$ Leupeptin; 1 mM 2-aminoethylisothiuronium bromide (AET); and 0.2 mM pyridoxal 5-phosphate (PLP) (buffer A). For immunoprecipitation experiments infected Sf9 cells were cultured in Grace's medium (Gibco Laboratories) for 36 h, labeled with [^{35}S]-methionine (200 $\mu\text{Ci}/5 \times 10^6$ cells) for 6 h, and homogenized in buffer A. The homogenates were centrifuged at 33,000 g for 30 min to separate the soluble cytosolic fraction in the supernatant and the particulate fraction in the pellet. Cell pellets were stored at -80°C until further preparation.

Immunoprecipitation test (IPT). The IPT was performed with minor modifications as described previously (8). The particulate fractions of [^{35}S]-methionine-labeled Sf9 cells were resuspended in 20 mM potassium phosphate, 150 mM NaCl, pH 7.2; 2 mM EDTA; 2 mM PMSF; 1% Trasylol; and 1% Triton X-100 for 2 h at 4°C followed by centrifugation at 33,000 g for 30 min to obtain the membrane fraction (MF) in the supernatant. MFs containing equal amounts of radiolabeled GAD_{65} or GAD_{67} were incubated with a pool of normal human serum (25 $\mu\text{l}/100$ μl lysate, equivalent to 5×10^5 infected Sf9 cells) for 6 h and then preabsorbed with protein A-Sepharose (Pharmacia Inc., Freiburg, Germany). 100 μl of the precleared extracts were precipitated with 25 μl test serum, followed by an adsorption of the immunocomplexes on protein A-Sepharose. After extensive washing the bound proteins were eluted with 65 mM Tris/HCl, pH 6.8; 2% SDS; and 5% mercaptoethanol and analyzed by fluorography. Fluorograms were analyzed by densitometry (LKB Ultrosan; Pharmacia Inc.). In each experiment a positive and a negative reference serum were included as internal controls. Peak areas compared with the positive serum were taken as a measure of the GAD antibody level and expressed in percent of the positive standard serum. Values above mean $+ 3$ SD of 60 control subjects were considered positive. Using this assay in the First GAD Antibody Workshop, we achieved a specificity of 100%, a sensitivity of 83.3%, and a validity of 87.5%.

ITA. The particulate fraction of Sf9 cells expressing GAD_{65} was resuspended in buffer A, pH 7.0, supplemented with 1% Triton X-100 for 2 h at 4°C . After centrifugation at 33,000 g for 30 min, the supernatant was used to perform the ITA. 100 μl of serum were incubated with 200 μl reconstituted protein A-Sepharose beads for 2 h at 20°C . The

beads were washed four times with 50 mM potassium phosphate, pH 7.0; 1 mM EDTA; 1 mM AET; 0.2 mM PLP; and 0.5% Triton X-100, 400 μl of MF (corresponding to a GAD activity of 0.5 mU) was added for 12 h at 4°C . After another washing step, 400 μl 50 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA, 1 mM AET, 1 mM glutamic acid, 0.2 mM PLP (buffer B) was added, and the samples were divided into two aliquots and the precipitated enzymatic GAD activity was determined. The immunotrapped GAD activity was calculated by subtracting the background counts using PBS instead of serum and was expressed in μU immunotrapped by 50 μl serum. Sera were considered GAD antibody positive if the immunoprecipitated GAD activity was above the mean $+ 3$ SD of 60 control subjects.

Determination of GAD enzyme activity. GAD activity was analyzed using a modification of the protocol described by Miller et al. (21). Aliquots of homogenates or immunocomplexes were diluted in buffer B to a total volume of 200 μl with a final concentration of 1 mM/liter L-glutamic acid and a specific radioactivity of $1[1-^{14}\text{C}]$ glutamic acid (Amersham Corp., Braunschweig, Germany) of 500 $\mu\text{Ci}/\text{mmol}$ for assaying of GAD activity in the cell extracts and 1,000 $\mu\text{Ci}/\text{mmol}$ in the ITA, respectively. After adding the reaction mixture into glass tubes, a filter paper soaked with 50 μl 1 M hyamine hydroxide was placed into the tubes. The tubes were closed and incubated for 1 h at 37°C . The reaction was stopped by injecting 1 ml 5 N sulphuric acid followed by an equilibration period of 1 h to allow complete adsorption of released $^{14}\text{CO}_2$. Then the $^{14}\text{CO}_2$ trapped to the filter paper was measured in a liquid scintillation counter. GAD enzyme activity was expressed in U/mg protein. One unit was defined as the formation of 1 μmol CO_2/min under standard assay conditions.

Statistical analysis. The significance of differences between observations was tested using the Wilcoxon test, the chi-square test with Yates' correction, or the Fisher's exact test, where appropriate. The correlations of antibody levels were tested by linear-regression analysis.

Results

Autoimmunity to GAD in IDDM is mainly directed to GAD_{65} . Human recombinant GAD_{65} and GAD_{67} were highly expressed in a baculovirus system. The enzymatic activity of

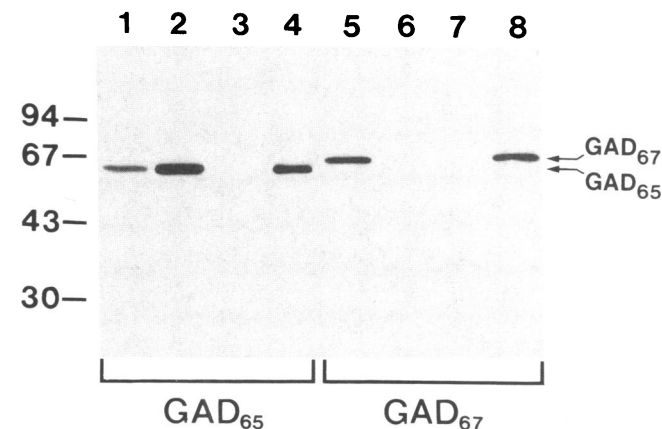


Figure 1. Immunoprecipitation of [^{35}S]-methionine-labeled human recombinant GAD_{65} and GAD_{67} . Membrane fractions of GAD_{65} (lanes 1–4) and GAD_{67} (lanes 5–8) were immunoprecipitated with two sera from newly diagnosed IDDM patients (patient 1, lanes 1 and 6, patient 2, lanes 2 and 5) and a serum from a control subject (lanes 3 and 7). Patient 1 was positive for antibodies to GAD_{65} but negative for antibodies to GAD_{67} , patient 2 had antibodies to GAD_{65} and GAD_{67} . As positive controls the immunoprecipitation with the mouse monoclonal antibody GAD 1 (lane 4) and the rabbit anti-serum K-2 (lane 8) are shown. Molecular weight markers ($M_r \times 10^{-3}$) are indicated on the left margin.

Table I. Characterization of Study Groups

Study groups	Number of sera tested	Sex		Age		
		Female	Male	Mean	Median	Range
Control subjects	60	34	26	23.5	22	8-38
High risk subjects						
First degree relatives						
ICA negative	35	17	18	20.0	21	4-37
ICA positive	15	9	6	25.0	23	15-59
ICA + schoolchildren	11	4	7	12.0	13	8-15
Patients with IDDM						
New onset IDDM	50	23	27	25.1	25	5-41
Duration of IDDM						
0.5-2 yr	21	9	12	22.2	24	6-38
2-10 yr	24	12	12	26.0	29	3-47
>10 yr	32	15	17	35.1	36	18-61

crude lysates of Sf9 cells expressing GAD₆₅ and GAD₆₇ was 155 and 169 mU/mg protein, respectively. Both GADs were immunoprecipitated by IDDM sera. Recombinant GAD₆₅ was recognized by the mouse monoclonal antibodies GAD1 (ATTC no. HB184; reference 22) and recombinant GAD₆₇ was precipitated by the K-2 antiserum (23), which binds to the 67-kD isoform of GAD (Fig. 1). The characterization of study groups and the prevalences of antibodies to GAD₆₅, GAD₆₇, and ICA in the different study groups are summarized in Ta-

bles I and II. In the immunoprecipitation test, antibodies to GAD₆₅ were present in 35 (70%) of 50 newly diagnosed IDDM patients, whereas GAD₆₇ antibodies were detected in only 9 (18%) subjects ($P < 0.00001$). In addition, antibodies to GAD₆₇ were observed only in GAD₆₅-positive sera. At the onset of IDDM, ICA were positive in 38 (76%) sera (mean ICA level 157 ± 304 JDF-U, range 10-1,280). No correlation could be demonstrated between the levels of ICA and antibodies to GAD₆₅ or GAD₆₇. Out of 12 ICA-negative sera from patients

Table II. Prevalence of Antibodies to GAD₆₅ and GAD₆₇ and Islet Cell Antibodies within the Study Groups

Study groups	Number of sera tested	ICA	Immunoprecipitation test		Immunotrapping assay
			GAD ₆₅ antibody	GAD ₆₇ antibody	GAD ₆₅ antibody
		<i>JDF-U</i>			
Control subjects	60	0	0	0	0
High risk subjects					
First degree relatives					
ICA positive	15	15 (331±211)	13 (86.7%) (143±85)	5 (33.3%) (102±105)	12 (80.0%) (117±89)
ICA negative	35	0	1 (2.9%) (23)	0	0
ICA + schoolchildren	11	11 (52±45)	6 (54.6%) (34±20)	0	6 (54.6%) (19±15)
Patients with IDDM					
New onset IDDM	50	38 (76.0%) (157±304)	35 (70.0%) (74±66)	9 (18.0%) (64±72)	27 (54.0%) (56±61)
Duration of IDDM					
0.5-2 yr	21	8 (38.1%) (117±103)	7 (33.3%) (131±90)	3 (14.3%) (14/23/210)	6 (28.6%) (146±86)
2-10 yr	24	6 (25.0%) (90±56)	5 (20.8%) (76±99)	2 (8.3%) (16/100)	5 (20.8%) (88±90)
>10 yr	32	2 (9.5%) (60/160)	8 (25.0%) (82±79)	3 (9.4%) (13/67/136)	8 (25.0%) (50±60)

Results obtained with the ITA are expressed in μ U of enzyme activity immunotrapped by 50 μ l serum; values $> 6.7 \mu$ U are taken as positive. GAD antibody levels in the IPT are demonstrated as % of the GAD antibody positive reference serum. Antibody levels (mean + SD) are given in parentheses (if less than four subjects were positive the measured values are indicated).

with new onset of IDDM, GAD₆₅ and GAD₆₇ antibodies were found in 7 (58.3%) and 1 (8.3%) subjects, respectively. Thus, 26/50 (52%) of the patients had ICA as well as antibodies to GAD₆₅ and 45/50 (90%) were found to be positive for one of these markers. Among the 60 controls neither antibodies to GAD₆₅, antibodies to GAD₆₇, or ICA were detected.

The prevalence of each of the antibody specificities tested declined after the onset of IDDM (Table II). After 0.5–2 yr, antibodies to GAD₆₅ as well as ICA were decreased compared with newly diagnosed patients with IDDM ($P < 0.05$). IDDM patients tested up to 10 yr after onset of IDDM exhibited a highly significant reduction of antibodies to GAD₆₅ and ICA ($P < 0.0005$). In long-term diabetic patients (11–40 yr), the frequency of antibodies to GAD₆₅ (8/32, 25.0%) was slightly increased compared with the IDDM patients 2–10 yr after onset of IDDM. The prevalence of antibodies to GAD₆₇ declined from 18% at diagnosis to 9.4% after long duration of IDDM, but this did not reach the level of significance.

High prevalence of GAD antibodies in subjects with increased risk for IDDM. 6 of 11 (54.5%) nondiabetic individuals with persistent ICA (mean 52 ± 45 JDF-U, range 10–160) without a family history of IDDM possessed antibodies to GAD₆₅. All of these six sera had been found 64K antibody positive in the IPT using labeled islets (8). 13 (86.7%) of 15 first-degree relatives with ICA levels > 40 JDF-U (mean 331 ± 211 JDF-U, range 80–640) had antibodies to GAD₆₅, whereas only 5 (33.3%) of them were GAD₆₇ antibody positive ($P < 0.05$). The level of the GAD₆₅ antibodies (143±85%) was significantly increased compared with new onset IDDM patients (74±66%) ($P < 0.005$). In 1 of 35 (2.9%) ICA-negative relatives, antibodies to GAD₆₅ were observed. Antibodies to GAD₆₇ were only detected in GAD₆₅ antibody-positive sera. Antibodies to GAD₆₇ were negative in all ICA-negative first-degree relatives and in 11 ICA-positive schoolchildren.

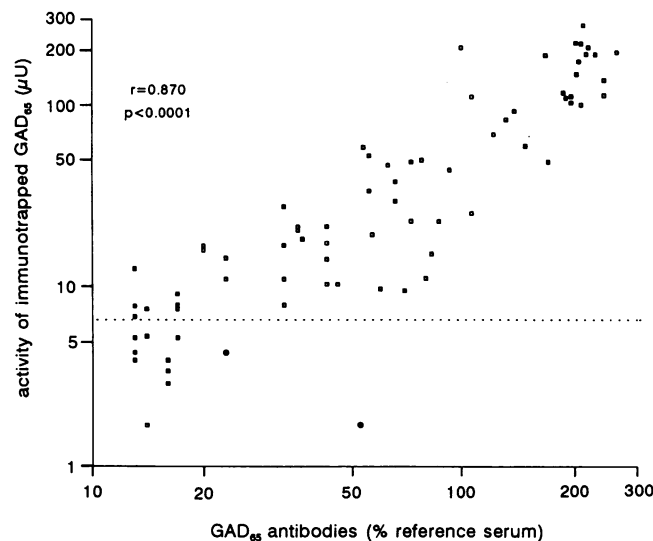


Figure 2. Correlation of the ITA and the conventional IPT for the detection of antibodies to human GAD₆₅. The results of 75 GAD₆₅ antibody-positive subjects are demonstrated. The levels of GAD antibodies in the ITA are expressed as immunotrapped enzyme activity (μ U) per 50 μ l serum. Results of the immunoprecipitation test are given in % of the GAD antibody-positive reference serum. The dashed line represents the mean + 3 SD of healthy controls (6.7 μ U). (●) GAD blocking antibodies.

The ITA reveals GAD antibodies of high specificity and sensitivity. All sera from the different study groups were tested in our newly developed ITA using recombinant GAD₆₅. The mean + 1 SD of the immunotrapped GAD activity of 60 control subjects was $2.32 + 1.46$ (range 0.44–5.77 μ U). Binding of > 6.7 μ U (mean + 3 SD) was considered as immunotrapped GAD₆₅ antibody positive. The intra- and interassay coefficients of variation were 10.2% ($n = 8$) and 15.7% ($n = 8$), respectively. Antibodies to GAD₆₅, detected by the ITA were found in 64 of 75 (85.3%) of the GAD₆₅ antibody-positive subjects, as characterized in the IPT (Table 1). The mean level of the positive subjects was 71 ± 75 μ U (range 6.8–277.0 μ U). There was a strong correlation between the levels of GAD₆₅ antibodies determined in the IPT and the level of immunotrapped GAD activity (Fig. 2) ($r = 0.870$, $P < 0.0001$). None of the GAD₆₅ antibody-negative subjects or of the normal controls exceeded the normal range (mean + 3 SD of controls). Compared with the conventional IPT as the gold-standard technique, the ITA achieved values of 100% for specificity and 87.2% for sensitivity.

GAD-blocking antibodies appear in only a minority of individuals. GAD antibodies that block the enzyme activity were detected by analyses of the results of the IPT compared with the ITA in individual sera. Regarding 11 GAD₆₅ antibody-positive sera, which were negative in the ITA, 9 turned out to be just weakly GAD₆₅ antibody positive (GAD₆₅ antibody level 13–17% of the reference serum) (Fig. 2). Thus, only 2 of 75 (2.7%) GAD₆₅ antibody-positive sera may possess high titers of GAD antibodies that inhibited the enzyme activity.

Discussion

Antibodies to the 64K islet cell protein have been described as an early and reliable serological marker to predict future development of IDDM in nondiabetic individuals. The identification of the 64K protein as GAD and the recognition of at least two isoforms have raised the question as to which isoform would be the major antigen in the natural history of IDDM. To address this question we expressed human GAD₆₅ and GAD₆₇ in the baculovirus system to produce a homogeneous source of native antigens. This allowed us to overcome the disadvantages of the antigen shortage, differences in the cell preparation, and antigen purity, which were critical to the conventional IPT using isolated islets.

The observed prevalence of 86.7% of antibodies to GAD₆₅ in ICA-positive relatives and 70% in patients with recent onset of IDDM was similar to the frequency of 64K antibodies using labeled islets (4, 24). In addition, we detected antibodies to GAD₆₅ in all 64K antibody-positive schoolchildren (8). These findings support the contention that GAD₆₅ is identical to the 64K islet cell protein (9, 13). Consistent with studies suggesting that antibodies to the 64K antigen indicate an increased risk for the development of IDDM (7), we found that three of the six GAD₆₅ antibody-positive schoolchildren developed overt IDDM after a follow-up of 16–30 mo (8). In our study the prevalence and levels of antibodies to GAD₆₅ were higher in ICA-positive relatives compared with patients with new onset IDDM. This suggests that the autoimmune reaction to GAD appears at an early stage preceding the diagnosis of IDDM.

Until now, only limited data are available on the presence of antibodies to GAD₆₇ in IDDM. We detected antibodies to GAD₆₇ in only 33.3% of ICA-positive relatives and in 18% of

patients with newly diagnosed IDDM. The surprising difference in the antibody reactivity to GAD₆₅ and GAD₆₇ emphasizes the relevance of the diversity of the two isoforms of GAD. The amino acid sequence identity between human GAD₆₅ and GAD₆₇ is ~ 65%, with the highest diversity for the amino-terminal 120 amino acids (11). This may either suggest that the majority of antibodies to GAD are primarily directed to the amino terminus or the diversity of the amino acid sequence between GAD₆₅ and GAD₆₇ leads to conformational changes of the GAD protein. The importance of the three-dimensional structure of GAD is emphasized by the fact that most of the IDDM sera do not react with denatured GAD₆₅ (9). In contrast to the present data, Kaufman et al. (13) and Deaizpurua et al. (25) reported on antibodies to GAD₆₇ in 9 of 12 and in 7 of 9 preclinical subjects as well as in 3 of 3 and in 6 of 13 patients with IDDM, respectively. Various explanations could be found for this disagreement. First, these authors used bacterially expressed rat or mouse GAD₆₇. Despite a high homology of rat, mouse, and human GAD₆₇ (11) the discrepancy could be due to the species differences, since different antigenic enzyme forms of GAD have been described (26, 27). Second, Kaufman et al. (13) used a mixture of [³⁵S]methionine-labeled GAD₆₅ and GAD₆₇ in the IPT, which could lead to unspecific precipitation of GAD isoforms. There is evidence that monoclonal GAD antibodies that recognize only GAD₆₅ coprecipitate both isoforms from brain preparations (24, 28). This suggests that GAD₆₅ and GAD₆₇ can tightly associate, probably by forming heterodimers. The higher frequency of antibodies to GAD₆₇ observed by Deaizpurua et al. (25) may be explained by the application of an ELISA for the detection of the autoantibodies. International workshops on the standardization of insulin autoantibody measurement have clearly shown that solid-phase and fluid-phase assays detect different antibody populations with different affinities (29). Insulin autoantibodies were more frequently detectable by ELISA but antibodies measured by fluid-phase assays were much better correlated with IDDM (29). According to our data, GAD₆₇ is only a minor target antigen in IDDM. This is in line with data suggesting that human islets express only GAD₆₅ but not GAD₆₇ (11). The autoimmune reaction in IDDM may be primarily directed against GAD₆₅ and subgroups of the polyclonal natural autoantibodies could recognize common epitopes of the two isoforms of GAD. This assumption is supported by the fact that our human monoclonal GAD antibodies (MICA 1-6) derived from a patient with newly diagnosed IDDM all recognized only the GAD₆₅ isoform (30). Furthermore, in the present study, antibodies to GAD₆₇ were restricted to patients with GAD₆₅ immunoreactivity. Direct epitope studies will be required to fully support this hypothesis.

Analogous to ICA, the prevalence of antibodies to GAD₆₅ and GAD₆₇ declined in the first years after the onset of IDDM. This has also been shown for 64K antibodies (31). However, in our study, antibodies to GAD₆₅ were still detectable in 25% of subjects with a long duration of IDDM. As initially reported by Kaufman et al. (13), we here observed that six of eight patients with long-standing IDDM and antibody reactivity to GAD₆₅ had a peripheral diabetic neuropathy (data not shown). The high frequency of GAD antibodies in these patients could be explained by the reappearance of antibodies to GAD caused by a repeated presentation of GAD to the immune system by the affection of GABA synthesizing neurons during the development of diabetic neuropathy (32).

After the identification of GAD as a target antigen in IDDM, antibodies to GAD were detected in several studies by measuring the immunotrapping enzyme activity. The prevalence of antibodies to GAD was reported to be as low as 25–38% using rat (14), pig (15), or human brain preparations (16). On the basis of the studies mentioned above, it could not be decided whether the low prevalence of antibodies to GAD in the ITA compared with the IPT is due to differences in the source of antigen or to the lower sensitivity of the ITA. In our newly developed ITA using human GAD₆₅, we can detect antibodies in > 85% of sera that were positive in the IPT. For the first time we demonstrate a strongly positive correlation between the level of antibodies in both assays. With this new assay the ITA achieved a 100% specificity and a high sensitivity that was only slightly decreased compared with the standard IPT. It is important to emphasize that out of 75 GAD antibody positive sera only 2 sera had high levels in the IPT, being repeatedly negative in the ITA. This suggests the presence of GAD binding antibodies inhibiting the GAD enzyme activity. Furthermore, nine sera with low antibody levels in the IPA were negative in our ITA. These negative results may be explained by the lower sensitivity of the measurement of enzyme activity in the ITA compared with the fluorography technique. As we cannot exclude the presence of antibodies that block GAD enzyme activity in these sera, our data at least indicate that GAD-blocking antibodies are only present in a minority of patients with IDDM.

In conclusion, antibodies to GAD₆₅ are valuable serological markers for preclinical and overt IDDM, whereas antibodies to GAD₆₇ may be of minor importance. Because GAD-blocking antibodies appear only in a minority of patients with IDDM, the ITA represents a valuable tool for the detection of antibodies to GAD with the advantage of easy performance and quantitative measurement of the autoantibodies. The availability of human recombinant GAD facilitate specific and sensitive screening for antibodies to GAD on large scale and may be useful to determine the role of GAD in the development of IDDM.

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