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A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM

C. E. Grubin[1], T. Daniels[1], B. Toivola[2], M. Landin-Olsson[3], W. A. Hagopian[1], L. Li[1], A. E. Karlsen[4], E. Boel[5], B. Michelsen[4], Å. Lernmark[1,6]

[1] Department of Medicine, University of Washington, Seattle, Washington, USA
[2] Department of Laboratory Medicine, University of Washington, Seattle, Washington, USA
[3] Department of Medicine, University of Lund, Lund, Sweden
[4] Hagedorn Research Institute, Gentofte, Denmark
[5] Novo Nordisk A/S, Bagsvaerd, Denmark
[6] Department of Endocrinology, Karolinska Hospital, Stockholm, Sweden

Summary Insulin-dependent diabetes mellitus (IDDM) is associated with autoreactivity against GAD but the diagnostic sensitivity (positivity in disease) and specificity (negativity in health) of isoform-specific GAD antibodies have yet to be defined in assay systems suitable for screening large number of samples. One set of IDDM patient ($n = 10$) and control ($n = 50$) standard sera were used to develop quantitative antibody assays with in vitro synthesized recombinant ^{35}S-methionine-labelled GAD65 and GAD67, respectively, and protein A-Sepharose to separate free from antibody-bound ligand. Binding levels were not normally distributed ($p < 0.0001$) and therefore, the diagnostic accuracy of GAD antibodies was analysed by the ROC plots in population-based, consecutively-diagnosed, recent onset, 0–14 year-old patients ($n = 105$), and matched, healthy control subjects ($n = 157$). The ROC plots showed that the diagnostic sensitivity of GAD65 antibodies was 77 % and the specificity 92 % compared with 8 % and 98 %, respectively for GAD67 antibodies. In the IDDM sera, GAD65 and GAD67 antibodies were concordant in 7 % (6 of 81) and GAD65 antibodies and ICA in 89 % (72 of 81) without a correlation between the autoantibody levels. Autoantibodies to recombinant human islet GAD65 are specific and sensitive markers for childhood IDDM in this immunoassay with in vitro synthesized ^{35}S-methionine-labelled recombinant GAD. [Diabetologia (1994) 37: 344–350]

Key words Glutamic acid decarboxylase, receiver-operating characteristic plot, diagnostic accuracy, islet cell antibodies, autoimmunity, diabetes mellitus

Serum samples from recent onset patients with IDDM [1–3] or first degree relatives later developing this disease [4, 5], have been shown to immunoprecipitate a M_r 64,000 (64K) islet cell protein, later identified as GAD [6]. The human islet GAD was discovered to represent a new isoform, GAD65, which is coded for by a previously unidentified gene (GAD2) on chromosome 10p11.3–p12 [7]. GAD65 shows 65 % amino acid identity with GAD67, the isoform coded for by the GAD1 gene on chromosome 2q31 [7–9]. The molecular cloning of full-length human islet GAD65 [7] and rat islet GAD67 [10] cDNA has made it possible to demonstrate autoreactivity in diabetes to the recombinant proteins in both eukaryotic [11, 12] and bacterial [13] expression systems. GAD65 (but not GAD67) is expressed in human islets [11, 14], however, variable reactivity of patient sera has been reported [12, 13, 15–19]. GAD65 specificity of IDDM sera was first demonstrated in our immunoprecipitation assay with recombinant GAD expressed in transfected cells [12] and recently confirmed in other assays with recombinant antigens [18, 20]. The use of different assay systems and species-specific GAD65 and GAD67 may explain the lower frequency of GAD67 antibodies in these compared to previous reports [13, 16]. We now report the

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Corresponding author: Professor Å. Lernmark, Karolinska Institute, Department of Endocrinology, Karolinska Hospital, L1:02, S-171 76 Stockholm, Sweden

Abbreviations: IDDM, insulin-dependent diabetes mellitus; GAD, glutamic acid decarboxylase; ROC, receiver-operating characteristic; ICA, islet cell antibodies; JDF, Juvenile Diabetes Foundation

use of GAD65 [7, 21] and GAD67 [10] cDNA to synthesize by in vitro translation and transcription specific radioactive ligands used in a radioimmunoassay of GAD65 and GAD67 antibodies and the use of the ROC plot [22–25] to determine the diagnostic accuracy of the GAD antibody test.

Subjects and methods

Patients

Two sets of patients and control subjects were studied (Table 1). The first was used to validate the assays and represented sera obtained by plasmapheresis from 10 recent onset IDDM patients [26, 27] and from 50 healthy, 20–49-year-old volunteers (top panel, Table 1). The IDDM sera were the very same as those previously used to identify antibodies to the 64K protein [2]. These sera have been used extensively in the Immunology of Diabetes Workshop to standardize ICA [28, 29] and one of the samples is used as the worldwide standard for expression of ICA levels in JDF units [28].

The second set of subjects comprised 105 consecutively diagnosed IDDM patients and 157 age-, sex- and geographically-matched healthy control subjects (lower panel, Table 1). These Swedish patients and control subjects have previously been described [30, 31] and represent 0–14-year-olds with a IDDM incidence of 25 per 100,000 and prevalence rate of 0.15 % [32].

Preparation of recombinant GAD

Two overlapping human islet GAD65 cDNA clones [7] were spliced [11] into a full-length expression clone, pEx9 in pcDNAII (Invitrogen, San Diego, Calif., USA). A full-length rat islet GAD67 cDNA clone [10] was similarly inserted into the pGEM4 vector (Promega, Madison, Wis., USA) to generate pEX12. The pEX9 plasmid DNA was linearized with XbaI and pEx12 with Hind III. Both enzymes cut in the respective 3′-untranslated region. After phenol:chloroform:isoamyl alcohol (50:48:2) extraction, the cDNA was precipitated in ethanol, dried and resuspended in H_2O. RNA was transcribed using 5 µg linearized cDNA in 50 µl final volume containing 40 U SP6 polymerase (BRL, Gaithersburg, Md., USA) in transcription buffer (40 mmol/l Tris, pH 7.9, 6 mmol/l $MgCl_2$, 2 mmol/l spermidine, 10 mmol/l dithiothreitol, 0.1 mg/ml bovine serum albumin 1 U/ml RNAsin) supplemented with 0.5 mmol/l each of ATP, CTP, and UTP, 50 mmol/l GTP, and 0.5 µmol/l m7GpppG. After incubation for 60 min at 37 °C, an additional 50 µl of transcription buffer supplemented with 0.5 mmol/l each of ATP, CTP, UTP and GTP was added, and the incubation continued for another 45 min at 37 °C. The reaction was stopped by addition of 5 U RNase-free DNase and incubation for 15 min at 37 °C. RNA was phenol:chloroform:isoamyl alcohol extracted, precipitated with 0.5 volume 7.5 mol ammonium acetate and 2.5 volumes ethanol, and collected by centrifugation for 30 min at 4 °C. The precipitate was washed in 70 % ethanol and resuspended in distilled H_2O. Denaturing agarose gel electrophoresis using 3-(M-morpholino) propane sulphonic acid/formaldehyde, and visualization with ethidium bromide under ultraviolet light was used to check the presence of a single band of RNA.

In vitro translation was carried out with nuclease treated rabbit reticulocyte lysate (Promega) according to the manufacturer's instructions. Briefly, 2 µg in vitro synthesized mRNA was heated at 67 °C for 10 min and immediately put on ice prior to the addition of translation mixture and 5 µCi ^{35}S-methionine (Amersham Int., Amersham, Bucks., UK; > 1000 Ci/mmol) and incubation for 90 min at 30 °C. Translation products were analysed by SDS-PAGE and autoradiography to demonstrate the presence of M_r 65,000 and M_r 67,000 components for GAD56 and GAD67 (Fig. 1, panel A). Typically, 5×10^6 cpm of trichloroacetic acid precipitable radioactivity was obtained, representing 6.5 % of the total radioactivity added.

Radioligand binding assay

In each analysis, 0.5 µl translation mixture of ^{35}S-methionine-labelled GAD65 or GAD67, (corresponding to 50,000 cpm and 70,000 cpm of trichloroacetic acid-precipitable material, respectively) were separately diluted to a final volume of 50 µl in immunoprecipitation buffer (20 mmol/l Tris, 150 mmol/l NaCl, 1 % Triton X-100, 0.1 % aprotinin (Novo Nordisk A/S, Bagsvaerd, Denmark) and 10 mmol/l benzamidine, (pH 7.4)), 2 µl (in the GAD65 antibody assay) or 4 µl (in the GAD67 assay) of either human serum, ascites of a GAD65-specific monoclonal antibody (GAD-6), [33] or a GAD67-specific rabbit antiserum (R9886) prepared against a synthetic heptadecapeptide representing the N-terminus of GAD67 were used per assay tube. After 60-min incubation on ice or overnight at 4 °C, antibody-bounded labelled GAD was separated from free GAD using 50 µl 50 % (v/v) Protein A-Sepharose (Zymed, South San Francisco, Calif., USA) in immunoprecipitation buffer incubated for 60 min at 4 °C on a rotating platform. After four washes by centrifugation in immunoprecipitation buffer (the third in immunoprecipitation buffer with 400 mmol/l NaCl), the beads were boiled in SDS sample buffer (62.5 mmol/l Tris, 2 % SDS, 10 % glycerol, pH 6.8) and either analysed by SDS-PAGE, and fluorography, or carefully transferred to vials for scintillation counting. The intra-assay coefficient of variation in duplicate or triplicate determinations was 11 %.

Statistical analysis

Antibody levels were expressed either as percent of total recombinant GAD radioactivity (cpm) precipitated or as a GAD antibody index defined as: cpm in the unknown sample – negative standard/positive standard – negative standard. Antibody positive and negative samples were included in every assay to correct for inter-assay variation. The JDF ICA standard [34, 35], which is also GAD65 antibody positive as verified by immunoprecipitation [2–4, 12] was used as the GAD65 antibody positive standard. One of the 105 IDDM patients was found to be GAD67 antibody positive as verified by immunoprecipitation [12]. This serum sample was subsequently used as the positive standard in the GAD67 antibody assays. A randomly selected control serum from one of the healthy volunteers was used as the negative standard in both assays. The intra-assay coefficient of variation for duplicate and triplicate samples was 10 % for GAD65 and 15 % for GAD67 antibodies. Samples considered positive based either on percent precipitated radioactivity or GAD index were confirmed by gel electrophoresis and fluorography.

The Pearson chi-square distribution analysis showed that both GAD65 and GAD67 antibody indices were not normally distributed in both test groups ($p < 0.0001$). The GAD antibody indicies were therefore analysed with the receiver-operating ROC plot [22–24] which is a powerful means to describe diagnostic accuracy (reviewed in [25]). Diagnostic accuracy is measured as diagnostic sensitivity (true positives/[true positives + false negatives]) and diagnostic specificity (true negatives/[true negatives + false positives]). The ROC plot is replac-

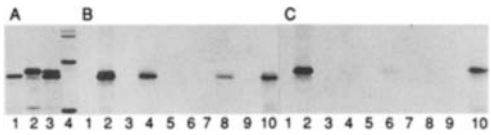

Fig. 1. SDS gel electrophoresis and autoradiography of recombinant GAD65 and GAD67 after in vitro translation and immunoprecipitation. Panel A: Analysis of in vitro translated GAD65 (lane 1), GAD67 (lane 2) and a mixture of both (lane 3). Molecular mass markers, M_r 92.5K, 69K and 46K are in lane 4. Panel B: Immunoprecipitation of ^{35}S-methionine-labelled recombinant GAD65 by an irrelevant IgG mouse monoclonal antibody (lane 1), the GAD65-specific monoclonal GAD-6 IgG antibody (lane 2), four sera from healthy control subjects (lanes 3, 5, 7, and 9) and IDDM patients (lane 4, 6, 8, and 10). Panel C: Immunoprecipitation of ^{35}S-methionine-labelled recombinant GAD67 by normal rabbit serum (diluted 1:100) (lane 1), the N-terminal synthetic peptide GAD67-specific rabbit antiserum (R9886) (diluted 1:100), as well as the four sera from healthy control subjects (lanes 3, 5, 7, and 9) and four diabetic patients (lanes 4, 6, 8, and 10) used in panel B. While all controls are negative, note that diabetic serum in lane 4 is strongly positive for GAD65 but only weakly reactive with GAD67, lane 6 diabetic serum is GAD67 but less GAD65 reactive, lane 8 is GAD65 but not GAD67 reactive and the lane 10 serum is positive for both

ing the conventional cut-off judged by standard deviation – which is applicable to normally distributed samples only – and is a graphical representation of all the sensitivity/specificity pairs to depict the overlap between the two distributions by plotting the sensitivity vs 1 – specificity for the complete range of decision thresholds [25].

Differences in levels of autoantibodies were tested with the Mann-Whitney U-test, and the Spearman rank correlation analysis was used to compare levels of different antibodies with age.

Results

GAD antibody radioligand binding assays

The in vitro cDNA-directed synthesis of recombinant GAD produced stable, ^{35}S-methionine-labelled ligands which were easily and reproducibly separated from antibody-bound ligand in a single immunoabsorption step with protein A-Sepharose. SDS-PAGE and fluorography (Fig. 1), revealed that the GAD65-specific monoclonal antibody GAD-6 (Fig. 1 B, lane 2) and the GAD67-specific peptide antiserum (Fig. 1 C, lane 2), respectively, immunoprecipitated its relevant but not irrelevant ligand (data not shown). The validation sera in Figure 1 illustrate negative immunoprecipitates in four healthy volunteers (Fig. 1 B and C, lanes 3, 5, 7 and 9). Among the IDDM sera, the JDF ICA standard precipitated the recombinant GAD65 (Fig. 1 B, lane 4) while only weak activity was observed for recombinant GAD67 (Fig. 1 C, lane 4). The IDDM serum in lane 6 readily precipitated recombinant GAD67 but less recombinant GAD65, the serum in lane 8 recombinant GAD65 but not recombinant

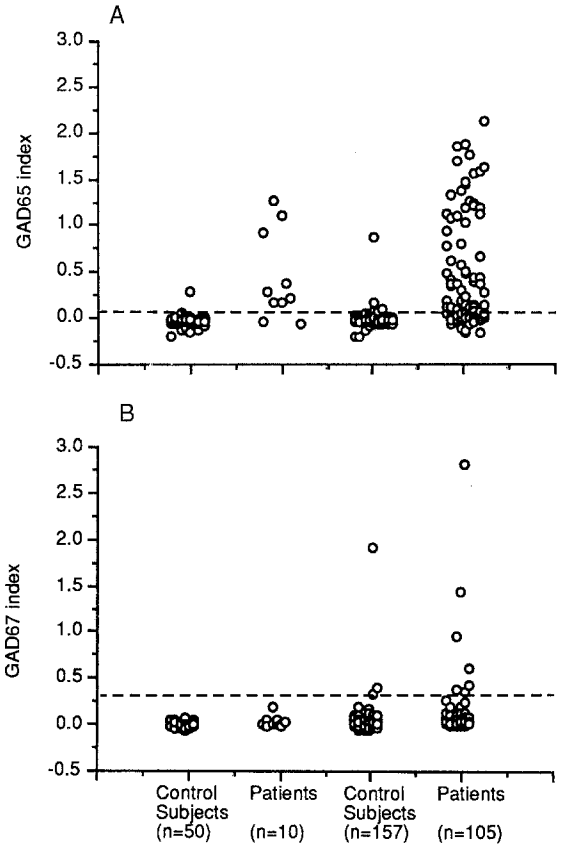

Fig. 2 A, B. Difference in recombinant GAD antibody index for GAD65 (**A**) and GAD67 (**B**) in control subjects and patients with recent onset IDDM. The data in **A** show the GAD65 antibody index in the validation sera used to standardize the radioligand binding assay in healthy volunteers ($n = 50$) and ICA standard samples ($n = 10$) as well as in control children ($n = 157$) compared with recent onset diabetic patients ($n = 105$). The data in **B** show GAD67 antibody levels in the same serum samples. The dotted line shows the GAD index level which indicates a diagnostic specificity of 98 %

GAD67 while in lane 10, the serum precipitated both ligands. The IDDM serum used for validation with the highest GAD65 titre precipitated 33 % of the total radioactivity at the 1:25 dilution which was the dilution found (in separate experiments, not shown) to obtain maximal precipitation and therefore used throughout the investigation. The GAD67 antibody standard also precipitated about 30 % of the total recombinant GAD67 radioactivity; however, in the GAD67 assay optimal precipitation occurred at a 1:12.5 dilution which was used with all samples.

The 10 IDDM sera (about 2–33 % of total recombinant GAD65 radioactivity was precipitated) used for validation confirmed eight positive samples (Fig. 2 A). Among the 50 controls (48 samples precipitated less than 2 %, while only two samples precipitated 5 % and 13 %, respectively of total GAD65 radioactivity) only two samples were positive (Fig. 2 A). In the GAD67 antibody assay only 2 of 10 diabetic samples were weakly positive (3 % and 6 % precipitated and con-

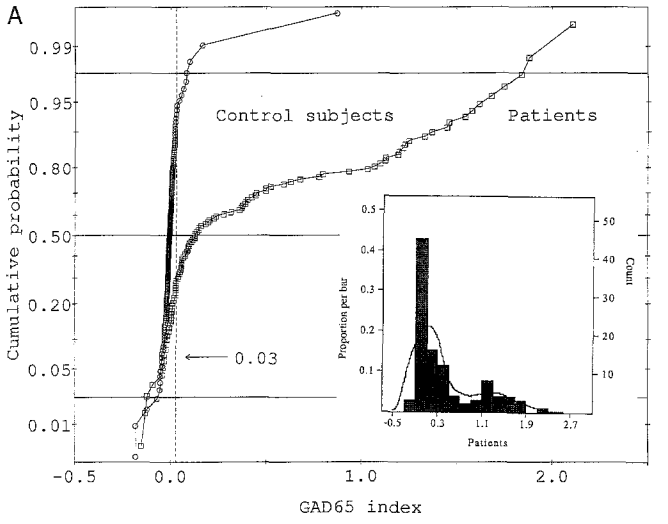

Fig. 3 A, B. Cumulative probability of antibodies in relation to antibody index in control children (○ , $n = 157$) compared with recent onset IDDM patients (□ , $n = 105$). The data in **A** demonstrate the high frequency of sera with a elevated GAD65 index. The dotted line at a GAD65 index of 0.03 corresponds to the level where sera begin to immunoprecipiate recombinant GAD65 detected by fluorography. The insert shows the the distribution of GAD65 indicies among the patients. The data in **B** illustrate the few high GAD67 antibody index sera among both control subjects and patients. The dotted line at a GAD67 index of 0.3 corresponds to the level where sera begin to immunoprecipiate recombinant GAD67 detected by fluorography. The insert shows the distribution of GAD67 indicies among the patients

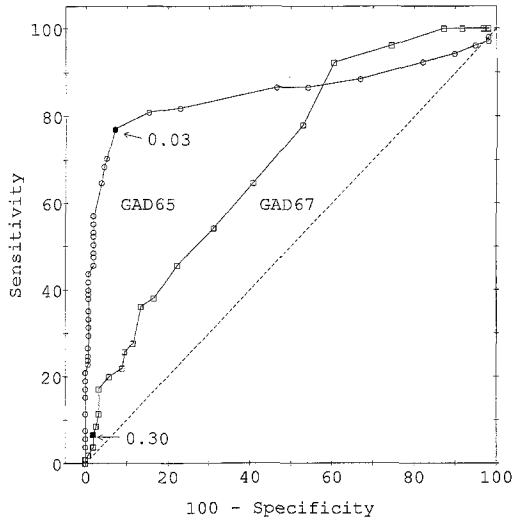

Fig. 4. The ROC curve analysis of GAD65 and GAD67 antibodies. The analysis demonstrates that at a GAD65 index of 0.03 (as can be seen in Fig. 3 A) the diagnostic sensitivity (positivity in disease) is 77% and diagnostic specificity (negativity in health) is 92%. The GAD67 antibody test is not useful for IDDM since at a GAD67 index of 0.3 (see Fig. 3 B), the diagnostic specificity is 98% but the diagnostic sensitivity only 8%. While a perfect test would show a ROC plot that passes through the upper left corner, the dotted line (45° diagonal line) represents a test with no discrimination

firmed by fluorography). The former sample was the JDF standard (Fig. 1 C, lane 4) and the latter, one of the GAD65 antibody weak sera (Fig. 1 C, lane 6). All of the 50 validation control sera were negative for GAD67 antibodies (Fig. 2 B).

The 10 IDDM validation sera, which had been kept frozen for 10 years, showed GAD65 antibody reactivity identical to our first demonstration of this antigen in human islet detergent extracts [2].

Diagnostic sensitivity and specificity of antibodies to recombinant GAD65

The validation sera (Fig. 2) showed that 2 of 50 healthy volunteer and 8 of 10 IDDM sera had GAD65 antibodies. A GAD65 index of 0.03 used at the cut-off. We then used the assay in a population-based study, using consecutively diagnosed IDDM children and control children.

As shown by the cumulative probability of the 105 IDDM patients and 157 control children (Fig. 3) the GAD65 antibody index was not normally distributed. Analysis of the control samples indicate that the 95th percentile in cumulative probability was reached at a GAD65 index of 0.03 (Fig. 3 A). The ROC curve demonstrated that the 0.03 GAD65 index was the most efficient accurate measurement, yielding 77% sensitivity and 92% specificity (Fig. 4). The ten control sera at or above the 0.03 GAD65 index cut-off were checked by fluorography which demonstrated that only 2 of 10 sera precipitated a band. The frequency of true positive controls would therefore be 2% (3 of 157). The median GAD65 index value in the patients was 0.13 which significantly different from −0.003 in the controls ($p < 0.001$) (Table 1).

There was no correlation between GAD65 antibodies and age, sex, or ICA levels (data not shown). Levels of GAD65 antibodies and ICA in 89% (72 of 81) double positive patients did not correlate ($r = 0.16$; $p > 0.05$).

Table 1. Patients with IDDM and control subjects studied to compare recombinant GAD65 and GAD67 antibody reactivity

	Patients	Control subjects
IDDM (ICA standards) and healthy volunteers validation sera		
n	10	50
Male/female ratio	6/4	23/27
Age (years) median (range)	12.5 (10–16)	27.5 (20–49)
Recombinant GAD65 antibodies:		
% cpm ppt; range	2–33 %	1–13 %
GAD65 index; median (range)	0.25 (– 0.046–1.3)	– 0.016 (– 0.19–0.30)
Antibody positivity	8/10	2/50
Recombinant GAD67 antibodies:		
% cpm ppt; range	1–6 %	1–3 %
GAD67 index; median (range)	– 0.0002 (– 0.024–0.17)	– 0.0064 (– 0.060–0.066)
Antibody positivity	2/10	0/50
Recent onset IDDM children and matched controls		
n	105	157
Male/female ratio	56/49	80/77
Age (years) median (range)	10 (0–14)	10 (0–14)
Duration of IDDM in days: median (range)	1 (0–64)	N/A
Recombinant GAD65 antibodies:		
% cpm ppt; range	1–54 %	1–15 %
GAD65 index: median (range)	0.13 (– 0.15–2.11)	– 0.003 (– 0.18–0.87)
Antibody positivity (%)	81/105 (77 %)	3/157 (2 %)
Recombinant GAD67 antibodies:		
% cpm ppt; range	1–72 %	1–36 %
GAD67 index: median (range)	0.027 (– 0.026–2.75)	0.002 (– 0.064–1.87)
Antibody positivity (%)	8/105 (8 %)	3/157 (2 %)
ICA:		
JDF Units: median (range)	30 (0–5520)	265 (30–410)
Frequency of positive samples (%)	92/105 (88 %)	3/157 (2 %)
Antibody concordance rates:		
GAD65 and GAD67	6/81 (7 %)	2/3
GAD65 and ICA	72/81 (89 %)	1/3
GAD67 and ICA	8/8 (100 %)	1/3

cpm ppt is radioactivity precipitated of total trichloroacetic acid precipitable radioactivity added in each assay tube

Diagnostic sensitivity and specificity of antibodies to recombinant GAD67

The GAD67 radioligand binding assay revealed only two weakly positive samples among the IDDM but none of the control validation sera (Fig. 2 B). Analysis of the 157 control and 105 IDDM sera revealed equally low frequencies of positive sera (Fig. 2 B). Since the two IDDM validation sera with low level GAD67 antibodies were not useful, we selected one of the GAD67 antibody positive patient samples as a positive control and re-analysed all samples to calculate the GAD67 antibody index (Table 1). The cumulative distribution of the GAD67 index, showed that patients deviated minimally from the control curve (Fig. 3 B). The ROC curve indicated that the most efficient GAD67 index cut-off was 0.3. At this index, the sensitivity of GAD67 antibodies for IDDM is only 8 % (8 of 105) and the specificity 98 % (154 of 157). The median GAD67 antibody index was 0.027 among the patients which was different from 0.002 among the control subjects ($p < 0.003$). The eight GAD67 antibody positive patients precipitated a M_r 67,000 band and six of eight were also positive both for GAD65 antibodies and for ICA. One of the GAD67 antibody positive healthy controls also had GAD65 antibodies as well as ICA, and this individual developed IDDM 8 months after the sample was obtained [31].

Discussion

The same 10 IDDM sera used in the present study to validate our novel radioligand binding assays with recombinant GAD, were also those used in the first study to demonstrate the 64K human islet antigen [2] recently identified as GAD [6]. Subsequent molecular cloning showed human islet GAD [7] to represent GAD65, a novel isoform different from the previously identified GAD67 [8, 10, 36]. The availability of cDNA sequences for human islet [7] and brain [8] GAD65 enabled their amino acid sequence comparison and showed these to

be identical. The present study supports previous demonstrations that the GAD65 mRNA [7] codes for the 64K antigen [12, 21, 37]. The results of this study also confirm other studies [12, 18, 20] but fail to support investigations showing that IDDM sera have significant reactivity to GAD67 [13] or GAD67 peptide fragments [16]. This discrepancy remains to be explained but may be due to the formation of GAD65/GAD67 heterodimers [38] in extracts of brain or rat islet [14] or recombinant mixtures which contain both isoforms. A distinct advantage of the present assays is the use of a radioligand which is translated from the transcript of the cDNA genes for GAD. The assay type may also be critical. Our cDNA directed in vitro-synthesized proteins retain full antibody reactivity not only with IDDM sera but also with the GAD65 monoclonal antibody (GAD-6) and our own GAD67-specific N-terminal synthetic peptide antiserum. However, it cannot be assumed that recombinant GAD or GAD fragments will yield the same information when adsorbed to plates for ELISA. The present assay is a liquid phase radioimmunoassay which allows the ligand to remain in solution and also alleviates the cumbersome SDS-PAGE analysis of numerous samples. It is possible that IDDM antibodies recognize a subtle tertiary polypeptide structure as the vast majority of IDDM sera are not reactive on immunoblots containing either native [6] or recombinant [21] GAD65. The present study also suggests that the recombinant GAD65 and GAD67 produced by in vitro translation is sufficiently folded and that little or no post-translational modifications are necessary for IDDM serum reactivity.

The present analysis has implications for studies on the prediction of IDDM using GAD antibody assays. Our GAD65 and GAD67 antibody assays allow the diagnostic accuracy (for review see [25]), i.e. sensitivity and specificity to be defined in attempts to predict IDDM in the general population. Large population screenings have been initiated; however, there are several complicating factors. First, although certain HLA alleles such as DQB1*0302-DQA1*0301(DQ8)-DR4 DQB1*0201-DQA1*0501(DQ2)-DR3 are found among more than 90% of IDDM children (sensitivity is high), about 50% of the healthy population are positive for these alleles (poor specificity). Second, ICA and insulin autoantibodies, alone or in combination show 88% sensitivity and 96% specificity, but the predictive value of finding a child with the disease was only 3% since the prevalence rate is as low as 0.15% [39]. Third, not all ICA, insulin autoantibodies or GAD antibody-positive individuals (such as healthy first degree relatives) progress to diabetes [40]. Fourth, the frequency of ICA (2–4%), insulin autoantibodies (1–2%) and now GAD65 antibodies (2%) among healthy schoolchildren exceeds the prevalence of the disease by a factor of 10–20. This discrepancy between marker positivity and clinical onset may be explained by GAD antibodies that develop irrespective of the HLA type and that conversion from marker positivity to disease onset requires the presence of susceptible HLA alleles. The availability of the present simple, precise and reproducible radioligand recombinant GAD antibody binding assays suitable for small blood samples, (2–4 µl) will make it possible to identify GAD antibody-positive individuals and factors which determine progression to or protection from IDDM.

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