Quantitation of Glutamic Acid Decarboxylase Autoantibody Levels in Prospectively Evaluated Relatives of Patients With Type I Diabetes

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In this study, we demonstrate that levels of glutamic acid decarboxylase (GAD) autoantibodies (GAAs) by radioassay differ between relatives with GAD-absorbable and GAD-nonabsorbable islet cell antibodies (ICAs). Extremely high levels of GAAs are often found in relatives with GAD-absorbable ICAs (>1,800 cpm, >9 SD above normal control subjects; mean = 1,991 cpm), and lower levels (mean = 1,078 cpm) of GAAs were present in relatives with nonabsorbable ICAs ($P < 10^{-5}$). The serum levels of GAAs were remarkably constant for relatives of both groups over time. The levels of GAAs were found to be inversely related to both the levels of insulin autoantibodies and the rate of loss of intravenous glucose-stimulated insulin secretion ($P < 10^{-5}$ and $P < 0.01$, respectively). Relatives with low positive levels of GAAs had more rapid loss of insulin secretion and were at high risk to become diabetic (50% diabetic at 4 years) compared with relatives with higher levels (1,800 cpm) of GAAs (10% diabetic at 4 years; $P < 0.05$). These data suggest that high levels of GAAs are associated with a decreased risk of progression to type I diabetes and extend the hypothesis that distinct subsets of ICAs and GAAs with differing prognostic significance can be identified. ***Diabetes*** **43:1229–1233, 1994**

Our interest in glutamic acid decarboxylase (GAD) autoantibodies (GAAs) was stimulated by the paradoxical finding of a subset of cytoplasmic islet cell antibodies (ICAs) among first-degree relatives of patients with type I diabetes, which was associated with reduced progression to diabetes (1). We termed this subset "restricted," because reactivity was restricted to rat and human pancreas (not reacting with mouse), and the islet staining by this form of ICA was restricted to β-cells (1). Further studies revealed that restricted ICA was identical in its reactivity (e.g., staining of GABA-ergic [γ-amino-*n*-butyric acid] neurons, able to Western blot GAD) to that of the ICAs from the rare patients with stiff-man syndrome, and for most sera, its reactivity could be removed completely by preincubation with affinity-purified GAD (GAD-absorbable ICAs). The great majority of ICA-positive sera from patients with newly onset diabetes, though reported to contain GAAs by immunoprecipitation assays, are unable to react with denatured GAD on Western blots or stain brain sections (nonrestricted ICAs), and their ICA reactivity could not be absorbed with GAD molecules (GAD-nonabsorbable ICAs). Bottazzo and coworkers (2) have described a similar subset of ICA (termed "selective") among patients with polyendocrine autoimmunity, which was also associated with reduced progression to diabetes.

The above studies and the lack of progression to diabetes of the great majority of patients with restricted ICAs led to the hypothesis that GAD-absorbable ICAs are a unique form of GAAs. This hypothesis has recently been challenged with the suggestion that GAD-absorbable ICAs (i.e., GAAs alone accounting for ICA staining) might simply be GAAs followed by the appearance of other anti-islet autoantibodies closer to the time of diabetes onset (3). It was hypothesized that with the appearance of other autoantibodies, a relative would be closer in time to overt diabetes. This implies that the GAAs of GAD-absorbable ICAs do not differ from the GAAs of GAD-nonabsorbable ICAs except for the presence of autoantibodies to other islet antigens. The study by Atkinson et al. (3) used absorption of diluted sera with recombinant GADs as the only criterion for characterizing ICAs. To further define the properties of the anti-GAD ICAs associated with progression to diabetes, we have quantitated GAAs over time in first-degree relatives of patients with type I diabetes.

RESEARCH DESIGN AND METHODS

Serum samples. From the Joslin and Barbara Davis family studies, 265 first-degree relatives of patients with type I diabetes (from 1 to 66 years of age, mean = 15 ± 12) were analyzed for levels of GAAs and insulin autoantibodies (IAAs). Individuals with diabetic glucose tolerance tests at the time of the first study were excluded from analysis. Forty-one relatives were subsequently followed until onset of diabetes as defined by National Diabetes Diagnosis Group criteria with either fasting hyperglycemia or diabetic oral glucose tolerance test, and 224 relatives with at least one autoantibody positive (ICA, IAA, or GAA) have not yet developed diabetes. A subset of sera from 93 relatives followed at the Joslin Diabetes Center with more than one intravenous glucose tolerance test (IVGTT) was analyzed for the correlation of their GAA levels with both the rate of loss of IVGTT and the survival distribution function. Sixty-two relatives (with 33 relatives having two time points for analyzing GAA stability), who were defined as ICA-positive among 93 Joslin relatives, were evaluated for GAD absorbability: 16 with GAD-absorbable ICAs and 46 with GAD-nonabsorbable ICAs. The definition of GAD-absorbable ICAs was that all ICA reactivity of 2 μl serum was removed by absorption with 7 μg affinity-purified GAD in a total volume of 20 μl. In addition, sera from 68 control subjects without a family history of diabetes were analyzed for GAA levels, and the limit of normal

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Received for publication 7 September 1993 and accepted in revised form 23 June 1994.

GAD, glutamic acid decarboxylase; GAA, glutamic acid decarboxylase antibody; ICA, islet cell antibody; IAA, insulin autoantibody; GABA, γ-amino-*n*-butyric acid; IVGTT, intravenous glucose tolerance test; FPIR, first-phase insulin release; JDF, Juvenile Diabetes Foundation.

was defined as 790 cpm (mean = 572 + 2 SD). Two other levels of GAAs for comparisons were defined based on a comparison of levels of GAAs for GAD-absorbable and non-GAD-absorbable sera (Fig. 1): 1,800 cpm (a level exceeded by only two GAD-nonabsorbable sera but by one-half of GAD-absorbable sera) and 900 cpm (a level exceeded by all GAD-absorbable sera). To define the frequency of GAA levels >1,800 cpm, sera from 851 sequential relatives seen at the Barbara Davis Center for the first time were analyzed for GAAs.

GAD purification. GAD molecules used in our GAA assay were purified from porcine brain. Fresh pig brain was homogenized in ice-cold 10 mm potassium phosphate buffer (pH 7.5) containing 0.2 mmol/l EDTA, 1 mmol/l aminoethylisothiouronium bromide, 0.2 mmol/l pyridoxal-L-phosphate, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 1 ml of proteinase inhibitors containing 1 μg antipain, 1 μg leupeptin, 2 μg aprotinin, and 1 μg pepstatin. The homogenate was centrifuged at 1,500 *g* to remove cell debris and then centrifuged at 100,000 *g*. The supernatant was passed over a GAD-1 monoclonal antibody affinity column, and GAD was eluted with 100 mmol/l ammonium acetate (pH 11.5). The fraction was neutralized with 1 mol/l HCl, dialyzed into 1 mmol/l potassium phosphate buffer (pH 7.5), and analyzed by sodium dodecyl sulfate-gel electrophoresis.

GAD radioassay. Radioassay for GAAs was performed by methods similar to those described by Zimmet and coworkers (4). Briefly, the purified porcine GAD was labeled with ^{125}I followed by Sephadex G-100 chromatography. Specific activities of iodinated GAD were 85–100 μCi/μg protein. Nonspecific reactivity was reduced by preincubating 5 million cpm of labeled GAD with one normal control serum (250 μl) for 2 h on ice, followed by precipitation with 500 μl 50% protein A-Sepharose. Then 50,000 cpm of this preabsorbed labeled GAD supernatant was incubated overnight at 4°C with duplicate 50-μl aliquots of serum, precipitated with 100 μl 50% protein A-Sephrose, washed three times with washing buffer (20 mmol/l Tris-HCl, pH 7.5, 150 mmol/l NaCl, 0.5% Triton X-100), and counted in a γ-counter. The interassay coefficient of variation, determined by testing three samples in six different assays, was 6.5% for a serum with a high level of GAAs (mean = 2,697 cpm), 9.0% for a serum with a low level of GAAs (mean = 1,166 cpm), and 5.0% for a normal control serum (mean = 699 cpm). During the International GAA Assay Workshop of 1993 (Orlando, FL), sensitivity of our assay was 72% and specificity was 100%.

Human recombinant GAD_{65} radioassay. The radioassay used for GAD_{65} autoantibodies was described previously (5) with some modifications. In brief, [^{35}S]GAD_{65} was in vitro translated from human recombinant GAD_{65} cDNA, and radioassay was performed by immunoprecipitation with patient serum at a dilution of 1:25–100,000 according to the antibody positivity of the serum. Immunocomplex was precipitated by protein A-Sepharose, and radioactivity was counted.

IAA assay and IVGTT. IAAs were determined with a radioimmunoassay as described previously (6). The relatives were followed over time, with a serum sample and an IVGTT (6) at each evaluation. The first-phase insulin release (FPIR), defined as the sum of the insulin concentrations at 1 and 3 min from the end of glucose infusion (0.5 g/kg infused over 3 min), was determined from each IVGTT.

ICA assay. ICAs were detected in patient sera by indirect immunofluorescence on an unfixed cryostat section of human pancreas. Quantification in Juvenile Diabetes Foundation (JDF) units was performed using a serial dilution in parallel with standard samples. Our low limit of ICA positivity is defined as ≥20 JDF units. In the ICA proficiency tests of 1993, organized by the University of Florida, sensitivity of our assay was 100% and specificity was 100%.

Statistical analysis. Statistical methods used included χ^2 analysis, rank-sum test, linear regression analysis, and product-limit survival analysis. Statistical analysis was performed with Epistat (Richardson, TX) and SAS (SAS Institute, Cary, NC) software.

RESULTS

As Fig. 1 illustrates, 100% of relatives (16 of 16) with GAD-absorbable ICAs were GAA-positive, and as a group, those with GAD-absorbable ICAs had higher GAA levels (median: 1,991 cpm) compared with relatives with GAD-nonabsorbable ICAs (median: 1,078 cpm; $P < 10^{-6}$). Half of the relatives' sera (8 of 16) with GAD-absorbable ICAs precipitated >1,800 cpm of labeled GAD and none was below 900 cpm. Among relatives with GAD-nonabsorbable

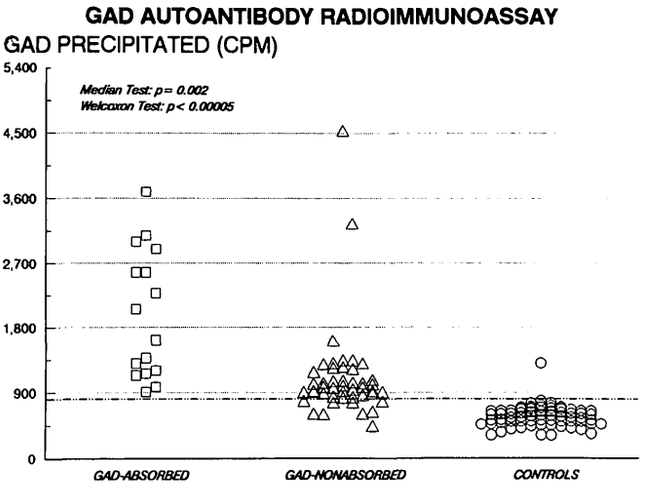

FIG. 1. Levels of autoantibodies to GAD in 16 relatives with GAD-absorbable ICAs, 46 relatives with GAD-nonabsorbable ICAs, and normal subjects (68 individuals). - ·· - ·· -, Upper limit of the reference range (790 cpm).

ICAs, 80% (37 of 46) were GAA-positive, only 2 were >1,800 cpm, and 46% (21 of 46) were <900 cpm.

To estimate the frequency of GAAs at the initial determination of GAA levels >1,800 cpm, 851 individuals with initial screening at the Barbara Davis Center were sequentially evaluated for GAAs and ICAs. Of seven relatives with GAAs >1,800 cpm, four were ICA-positive (57%). Three of 786 ICA-negative relatives had levels >1,800 cpm (0.38%) versus 4 of 65 (6.2%, $P < 0.001$) ICA-positive relatives.

The GAA levels were remarkably constant over time for most of the relatives studied at more than one time point (Fig. 2). The correlation between first and subsequent GAA levels is shown in Fig. 3 ($r = 0.91$, $P < 10^{-4}$).

As illustrated in Fig. 4, an inverse correlation exists between GAA and IAA levels. Only 3 of 14 relatives with GAA levels >1,800 cpm (from 3 to 64 years of age, mean = 22 ± 14) were IAA-positive (>39 nU/ml), and none of them had a level >150 nU/ml, an IAA level we have previously reported to be associated with more rapid progression to diabetes (6). Among those relatives with GAA levels <1,800 cpm, IAA positivity was a frequent finding (GAA 900–1,800 cpm: from 2 to 66 years of age, mean = 14 ± 11, 40% IAA-positive [52 of 129]; GAA <900 cpm: from 1 to 58 years of age, mean = 15

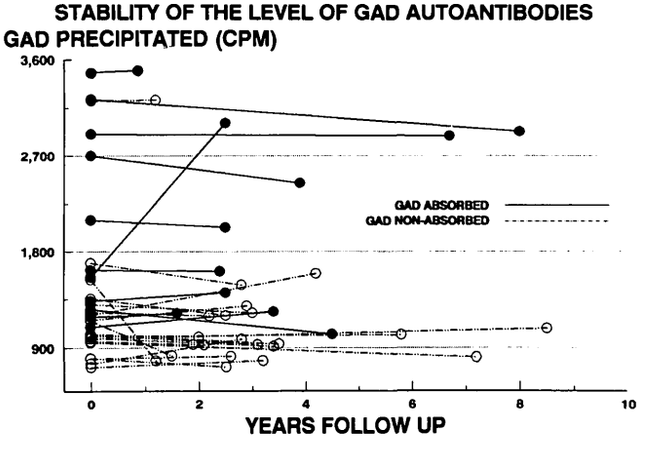

FIG. 2. Levels of autoantibodies to GAD during follow-up in relatives with GAD-absorbable ICAs (11 individuals) and relatives with GAD-nonabsorbable ICAs (22 individuals).

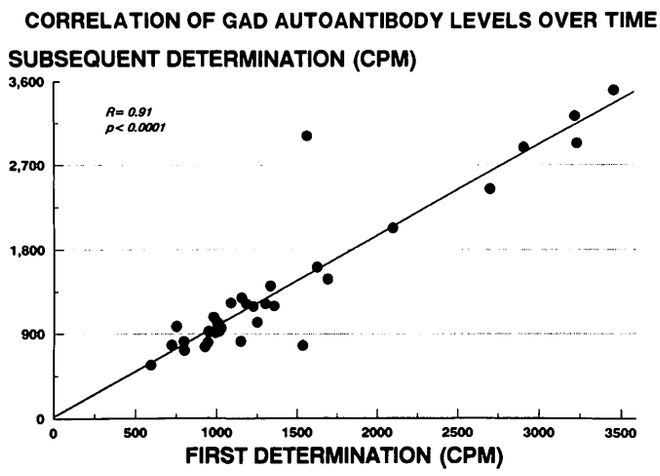

FIG. 3. Time concordance of GAD autoantibody levels between first and subsequent determinations in ICA-positive relatives (n = 33) including relatives with either GAD-absorbable ICAs or GAD-nonabsorbable ICAs.

± 12, 67% IAA positive [85 of 122]; $P < 10^{-5}$). There was no significant correlation between levels of GAAs and ICAs as shown in Fig. 5.

To evaluate the influence of GAA levels on loss of β-cell function, we analyzed both FPIR and progression to overt diabetes. The relationship between the levels of GAAs and IVGTT insulin secretion is illustrated in Fig. 6. Only 2 of 14 relatives with GAA levels >1,800 cpm at their last test had an IVGTT <48 μU/ml (first percentile of normal), which is associated with high diabetes risk (one of these two relatives is now diabetic) (6). With GAA levels <1,800 cpm, severely impaired insulin secretion was frequently observed (<48 μU/ml). To analyze the relationship between levels of GAA and the rate of the loss of IVGTT (shown in Fig. 7), we analyzed loss of IVGTT versus time. Because FPIR (sum of 1 + 3 min insulin) has a log-normal distribution, the formula for rate of loss of IVGTT was as follows: rate = (ln [last IVGTT insulin] − ln [first IVGTT insulin])/time. Subdividing relatives by levels of GAAs, those with the highest levels of GAAs (>1,800 cpm) had no significant change in IVGTT insulin secretion over time (Δ = 0.00 ± 0.06) in marked contrast to relatives with lower GAA levels, 900–1,800 cpm and <900 cpm (Δ = −0.19 ± 0.28, P = 0.035 and −0.29 ± 0.38, P = 0.01, compared with relatives with GAA >1,800 cpm).

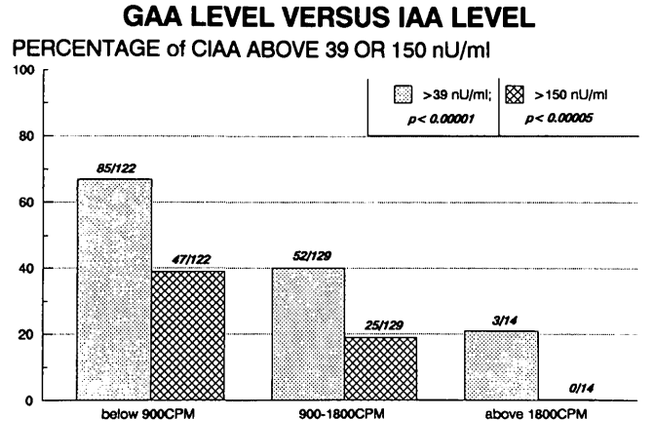

FIG. 4. Relationship between GAD autoantibody level and insulin autoantibody level in relatives (n = 265) with at least one autoantibody positive ICA, IAA, or GAA.

Figure 8 illustrates life table analysis of relatives subdivided by GAA levels (> and <1,800 cpm). The greater progression to diabetes of relatives with GAA levels <1,800 cpm in contrast with those with GAA levels >1,800 cpm is apparent.

We randomly selected 11 sera with GAA levels >1,800 cpm and 20 sera with GAA levels <1,800 cpm and performed a human recombinant GAD_{65} radioassay with titrations. The two radioassays (Fig. 9) were well correlated (regression analysis r = 0.89, $P < 0.001$). Ten of 11 sera with previous GAA levels >1,800 cpm reacted at high titers with human recombinant GAD_{65}, remaining positive at sera dilutions >1:10,000 versus only 3 of 20 sera with GAA levels <1,800 cpm. Two of the three sera from the group with GAA levels <1,800 cpm, which reacted at >1:10,000 titer with the recombinant GAD assay, were in the restricted ICA category and had porcine GAD radioassay levels between 1,500 and 1,700 cpm.

DISCUSSION

This study of first-degree relatives of patients with type I diabetes reveals very different GAA levels in two subsets of ICA-positive relatives. Half of the relatives with GAD-absorbable ICA have extremely high levels of GAAs. In contrast, lower levels of GAAs were present in relatives with GAD-nonabsorbable ICAs. Comparison between this affinity-purified porcine GAD radioassay and the in vitro translated human recombinant GAD_{65} radioassay indicated that the two assays were highly correlated (r = 0.88, $P < 0.001$) for GAA levels. The levels of GAAs were stable over time for all but two relatives tested. Of note are eight relatives with GAD-absorbable ICAs who had lower GAA levels in the range of relatives with GAD-nonabsorbable ICAs. Two of these relatives have progressed to overt diabetes. Recently, Richter et al. (7) reported their GAD absorption data on 25 new-onset diabetic patients, and 24 of 25 showed GAD-nonabsorbable ICAs. One relative in their report showed ICAs on mouse pancreas, switching from negative to positive when pathological results on an IVGTT were found.

Relatives with high levels of IAAs and rapid loss of IVGTT insulin are at high risk to become diabetic (6). This study indicates that relatives with higher levels of GAAs have lower levels of (or are negative for) IAAs. Relatives with high levels of GAA also have less rapid loss of insulin secretion after intravenous glucose. These data raise the hypothesis that higher levels of GAAs are associated with reduced progression to diabetes. We suggest that high levels of GAAs in relatives are inversely related to the risk of progression to type I diabetes, and this information will aid in the prediction of type I diabetes among first-degree relatives.

GAAs can contribute to ICA assay positivity. For those sera with a restricted ICA pattern, ICA reactivity can be totally removed by preincubation with brain GAD (2). Current ICA assays using human pancreas and immunofluorescence are time-consuming, often suffer from lack of human pancreas, and are difficult to standardize. In comparison, GAA radioassays, even among patients with GAD-absorbable ICAs, appear to correlate with disease progression. As illustrated by the current study and prior studies of insulin autoantibodies (6), specific autoantibodies are likely to reveal marked stability over time, and quantitative autoantibody levels may have prognostic significance. With the

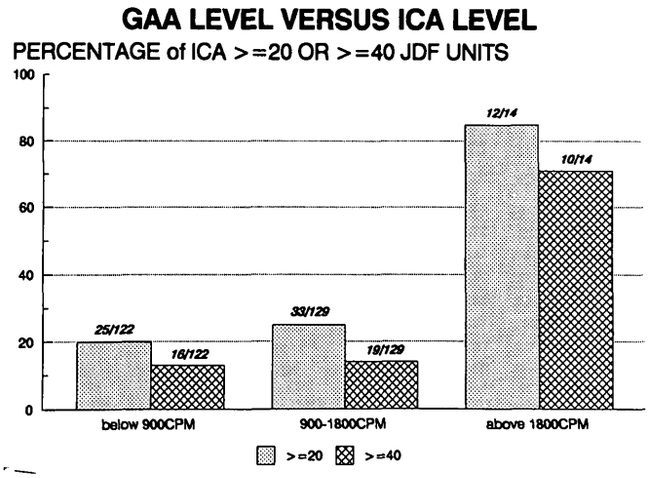

FIG. 5. Correlation between GAD autoantibody level and ICA titer in relatives (n = 265) of the same group as in Fig. 4.

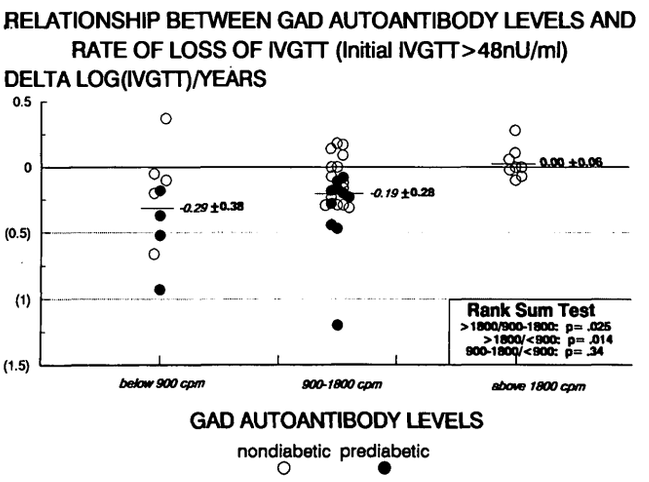

FIG. 7. Relationship between GAD autoantibody levels and the rate of loss of IVGTT in the same group of relatives as in Fig. 6 with their initial IVGTT >48 nU/ml (n = 40). The IVGTTs were followed up to 1–9 years (n = 40).

characterization of a family of defined autoantigens, we believe that such quantitative analysis will be readily applicable to other molecules (ICA69, GM2–1) (8,9).

We realize that it is paradoxical that low levels of GAAs exceeding our normal range are associated with progression to diabetes, while extremely elevated levels are much less associated with progression. Only one relative to date with a GAA level >1,800 cpm has progressed to diabetes despite expressing high levels of ICAs (1 of 14) versus 20 of 46 relatives with GAA levels of 900–1,800 cpm and 20 of 33 relatives with GAA levels <900 cpm (P = 0.02 and 0.001, separately, Fisher's exact test). This lack of progression to diabetes is consistent with lack of loss of FPIR to an IVGTT of relatives with GAA levels >1,800 cpm (Fig. 7). At present, we cannot distinguish between two alternative hypotheses: *1*) high levels of GAAs as determined with a radioassay format protect from (or slow) progression to diabetes among certain autoantibody-positive relatives of patients with type I diabetes; or *2*) high levels of GAAs are associated with other variables (e.g., genetic or immune) that limit progression to diabetes. Recently, Harrison et al. (10) reported an inverse relation between humoral and cellular immunity to GAD in relatives at risk of developing type I diabetes. They concluded that the response to GAD is bipolar in vivo, turning toward either humoral or cellular immunity. Type I diabetes is likely to be an autoimmune disease in which cell-mediated immunological events play a critical role (11). If we assume that GAD is a pathogenic autoantigen, patients progressing to diabetes may have a higher T-cell response than relatives with high levels of antibodies to GAD. In other words, high levels of GAAs will be associated with a slower progression to clinical disease with less T-cell-mediated destruction of pancreatic islet β-cells. Two recent reports strengthen the hypothesis that T-cell responses to GAD may be pathogenic by showing a proliferative response to GAD and by preventing diabetes by injection of GAD (12,13). Further study of larger numbers of relatives and characterization of the GAD epitopes the antibodies recognize, as well as antibody levels and relation to T-cell response, should aid in exploring the paradox of a high GAA level associated with limited progression to diabetes.

Recently, Atkinson et al. (3) analyzed sera from three groups of ICA-positive individuals for absorption by recombinant GAD. The absorption protocol they used involved extensive dilution of sera before absorption, presumably to enhance the ability to absorb ICA reactivity. Consistent with our present study, they found the highest percentage of GAD-absorbable sera in ICA-positive relatives followed for

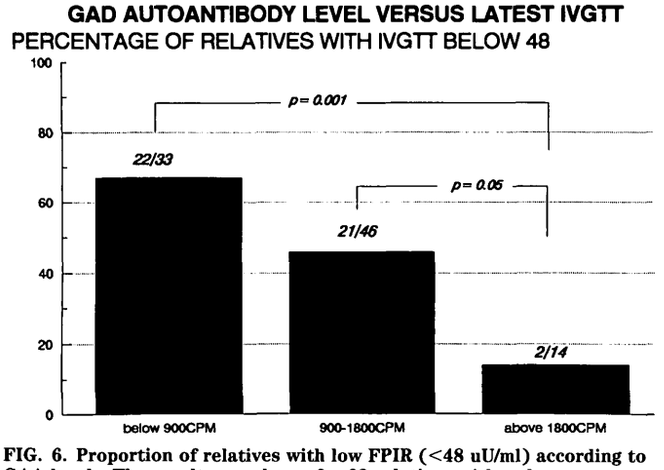

FIG. 6. Proportion of relatives with low FPIR (<48 uU/ml) according to GAA levels. The results are shown for 93 relatives with at least one autoantibody positive ICA, IAA, or GAA.

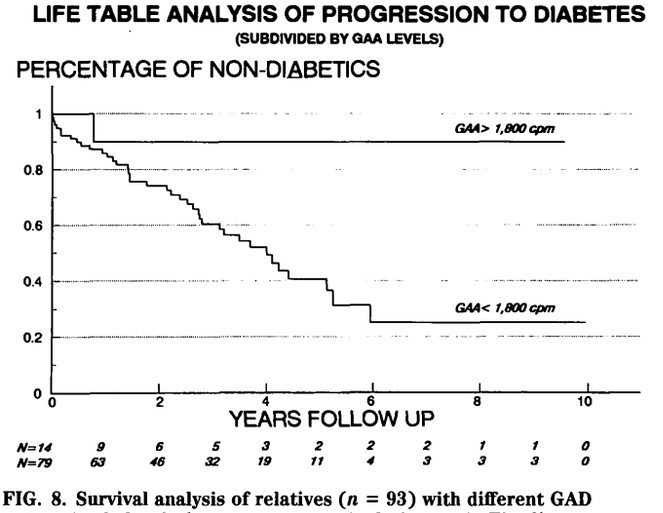

FIG. 8. Survival analysis of relatives (n = 93) with different GAD autoantibody levels (the same group of relatives as in Fig. 6).

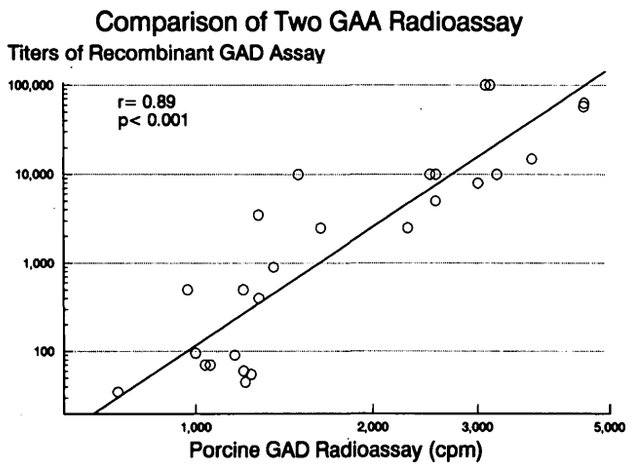

FIG. 9. Comparison of purified porcine GAD radioassay with human recombinant in vitro transcribed and translated GAD_{65} radioassay. The *x* axis represents GAA levels of porcine GAD assay (cpm) and the *y* axis represents the titers of sera showing a 50% decrease in cpm for the human recombinant GAD-65 assay.

more than 5 years without development of diabetes (3). They also reported switching of patterns (absorbable to nonabsorbable) in three relatives. They concluded that their observations were consistent with the concept that GAD-absorbable ICAs represented the presence of GAA before appearance of other autoantibodies, which was an event closer to the time of diabetes onset. By implication, GAD-absorbable ICAs were not unique or associated with a lack of progression to diabetes. As our present study indicates, as well as our prior studies (e.g., Western blotting of GAD and staining of GABA-ergic [γ-amino-*n*-butyric acid] neurons by GAD-absorbable and GAD-nonabsorbable ICAs), GAD-absorbable ICAs appear to be an unusual subset of both ICAs and GAAs, even with quantitative analysis. This subset has limited association with progression to overt diabetes or loss of FPIR over the time period studied (up to 10 years).

The identification of a major subset of the 64K antigen as GAD now allows biochemical determination of antibodies to GAD. To date, assays for GAAs have not been applied to large numbers of nonselected relatives of patients with diabetes (e.g., not already identified as ICA-positive). As the current study indicates, the highest levels of GAAs among ICA-positive relatives are minimally predictive of future diabetes. Although lower levels of GAAs in this study were associated with diabetes risk, relatives were selected for study because of expression of anti-islet autoantibodies. Further studies quantitating GAAs should aid in defining both their predictive potential and role in pathogenesis.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK-32083 and DK-43279, grants from the Blum-Kovler Foundation, the Juvenile Diabetes Foundation, the American Diabetes Association, and the Children's Diabetes Foundation at Denver. The Joslin Diabetes Center Clinical Research Center was essential to the described studies.

We thank Terry Smith for excellent nursing assistance and Dennis Dunlop for secretarial assistance.

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