Associations of Anti-GAD Antibodies with Islet Cell Antibodies and Insulin Autoantibodies in First-Degree Relatives of Type I Diabetic Patients

URSULA ROLL, MICHAEL R. CHRISTIE, EBERHARD STANDL, AND ANETTE-G. ZIEGLER

Sera from 114 first-degree relatives of insulin-dependent diabetes mellitus (type I diabetes) patients and 81 healthy individuals living in Germany were analyzed for antibodies to rat brain glutamic acid decarboxylase (GAD-ab) using an immunoprecipitation assay. The determination of GAD-ab in the 81 islet cell antibody (ICA) and insulin autoantibody (IAA) negative healthy individuals established a normal range (mean ± 2 SD); 2 healthy individuals (2.5%) possessed GAD-ab levels above this range, but became negative on follow-up. None of 86 ICA^-/IAA^- first-degree relatives had GAD-ab; whereas, 42.9% of 28 ICA^+ and/or IAA^+ relatives were positive for GAD-ab. Presence of GAD-ab was negatively correlated with IAA ($P = 0.02$) and positively with ICA ($P = 0.0006$). Follow-up samples were obtained from 25 of 28 ICA^+ and/or IAA^+ relatives with a mean (± SD) follow-up period of 20.6 ± 12.1 months. In these 25 relatives, GAD-ab were positive in 70% ICA^+/IAA^-, 0% ICA^-/IAA^+, and 57.1% ICA^+/IAA^+ relatives in the first sample and in 57.1% ICA^+/IAA^-, 0% ICA^-/IAA^+, and 70% ICA^+/IAA^+ relatives in the last sample. GAD-ab, once detected, persisted in 9 of 11 GAD-ab^+ relatives. Of the relatives, 2 converted to GAD-positivity, concomitant with the appearance of ICA, and 2 others lost GAD-ab during follow-up. Of the 28 ICA^+ and/or IAA^+ relatives, 6 progressed to overt type I diabetes on follow-up, and GAD-ab were detectable in 4 of these relatives. In conclusion, this study indicates that GAD-ab in first-degree relatives are significantly correlated with ICA, are independent of the appearance of IAA, and are present in 66.7% of relatives who subsequently developed type I diabetes.
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Insulin-dependent diabetes mellitus (type I diabetes) is suggested to be an autoimmune disease caused by an organ-specific destruction of pancreatic β-cells (1,2). Islet cell autoantibodies (ICA) (3) and insulin autoantibodies (IAA) (4) are observed in preclinical subjects and recent-onset diabetic patients (5). It is estimated that ~40% of ICA^+/IAA^- and 90–100% of ICA^+/IAA^+ relatives will develop type I diabetes during 5 years of follow-up (6). One major limitation of screening with ICA and IAA alone is that not all relatives who develop diabetes are positive for both antibodies. Screening for additional markers for diabetes may improve our ability to predict disease development. A membrane-associated protein of 64,000 M_r expressed by pancreatic islets was found to be a major autoantigen in type I diabetes (7–12). The recent identification of this 64,000 M_r islet protein as the enzyme glutamic acid decarboxylase (GAD) (13), which synthesizes the inhibitory neurotransmitter γ-aminobutyric acid (GABA), has allowed the development of simple assays for the detection of GAD-ab. In previous studies, GAD-ab have been found in 22–69% of recently diagnosed diabetic individuals and 67–89% ICA^+, first-degree relatives of type I diabetic patients using different methods for antibody determination (14–19).

Previously, we screened sera from 795 first-degree relatives of type I diabetic patients for ICA and IAA and found 3.5% positive for one or both of these antibodies. In this study, an immunoprecipitation assay with rat brain extract was used (14) to determine the presence of GAD-ab in relatives who have high or low risk for diabetes development, respectively. These studies have allowed us to test the prevalence of GAD-ab and assess

From the Diabetes Research Institute and Third Medical Department, Schwabing-City-Hospital, Munich, Germany (U.R., E.S., A.G.Z.); and the Nuffield Department of Clinical Biochemistry, John Radcliffe Hospital, Headington, Oxford, United Kingdom (M.R.C.).

Address correspondence and reprint requests to Ursula Roll, Diabetes Research Institute, Schwabing-City-Hospital, Koelner Platz 1, D-80804 Munich, Germany.

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Type I diabetes, insulin-dependent diabetes mellitus; GAD, glutamic acid decarboxylase; GAD-ab, GAD antibodies; ICA, islet cell antibody; IAA, insulin autoantibody; AET, 2-amino-ethyl-isothiouronium bromide; JDF U, Juvenile Diabetes Foundation Units; GABA, γ-aminobutyric acid; IVGTT, intravenous glucose tolerance test; OGTT, oral glucose tolerance test; ELISA, enzyme-linked immunosorbent assay.

their relationship to ICA, IAA, and the development of diabetes.

RESEARCH DESIGN AND METHODS

Since 1989, serum samples of 795 nondiabetic first-degree relatives living in Germany have been analyzed for autoantibodies to ICA and IAA, including 163 parents (20.5%), 233 siblings (29.3%), and 399 children (50.2%) of type I diabetic patients. In 28 of 795 (3.5%) relatives, one or two antibodies (ICA and/or IAA) were detectable: that is, of the 28 relatives, 11 were ICA^+/IAA^- and 9 were ICA^-/IAA^+, whereas 8 had both ICA and IAA. For this study, all sera of these antibody-positive relatives and sera from 86 ICA^-/IAA^- relatives were screened for (anti-)GAD-ab (ICA^+ and/or IAA^+ relatives: 19 women, 9 men; mean age 20.0 ± 14.6 years of age, range 3–60 years; ICA^-/IAA^- relatives: 47 women, 39 men, including 54 children, 9 parents, and 23 siblings; mean age 17.5 ± 13.2 years of age, range 2–43 years). Follow-up samples were available for 25 of 28 antibody-positive relatives, which allowed more detailed study.

In addition, 81 healthy ICA^-/IAA^- control individuals (42 women, 39 men; mean age 27.6 ± 6.2 years of age, range 20–51 years) with no known family history of type I diabetes in first- and second-degree relatives were included to establish a normal range for GAD-ab in the German population.

Assay for (anti-)GAD-ab. The presence of (anti-) GAD-ab was determined using an immunoprecipitation assay with rat brain extracts as described previously (14). For each serum, GAD-ab were determined in two independent assays, including initial and follow-up samples when available in the same assays.

Preparation of GAD. Whole fresh brains from adult male or female Wistar rats were homogenized in a 10-fold volume of 1 mM 2-amino-ethyl-isothiouronium bromide (AET), 0.2 mM pyridoxal phosphate, 1 mM benzamidine, 25 mM potassium phosphate, pH 7.0 (homogenization buffer), and centrifuged twice at 100,000 *g* for 30 min. The supernatant (cytosolic extract) was diluted to a GAD activity of 2.5×10^{-9} mol · ml^{-1} · h^{-1}.

Immunoprecipitation. Aliquots (50 μl) of the extract were incubated with 12.5 μl test serum for 5 h at 4°C. Immune complexes were isolated on 12.5 μl preswollen protein A Sepharose beads and washed three times in 1 ml 10 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM Benzamidine, 1 mM AET, 0.2 mM pyridoxal phosphate, 0.5% Triton X-114, 0.1 mg/ml bovine serum albumin, and one time in 1 ml homogenization buffer.

GAD-enzyme activity assay. Protein A-Sepharose pellets were incubated for 16 h at 37°C with 12.5 μl 5 mM L-glutamic acid and 0.125 μCi L-[1-^{14}C]glutamic acid in homogenization buffer. The $^{14}CO_2$ was formed using GAD and, after release, was absorbed on Whatman filter papers (Bender & Hobein, Bruschal, Germany) (3 MM), soaked with 50 μl 1 M hyamine, and quantified by liquid scintillation spectrometry (LKB Wallac, Turku, Finland). Blanks were performed with protein A-Sepharose only. GAD-activity was expressed as a percentage of the antibody-positive serum included in each assay and was calibrated against a reference standard used in other determinations of GAD-ab and 64 kD antibodies (10,11,14,15).

Sera were regarded as positive if the GAD activity in immunoprecipitates was above mean + 2 SD of the activity in the 81 nondiabetic, control sera. Negative percentages of GAD-ab are computational products, in which the counts of the blanks (without enzyme) were greater than in the corresponding test serum. In the first international standardization of GAD-ab (anti-GAD autoantibody workshop, Orlando, FL), our immunoprecipitation assay (lab identification no. 23) reached a validity of 87.5%, a sensitivity of 83.3%, and a specificity of 100%.

ICA assay. Sections of blood group 0 cryofixed human pancreas were used for screening sera in dilutions 1:2/1:4 with peroxidase conjugated protein A. Results were expressed in Juvenile Diabetes Foundation Units (JDF U). Our peroxidase-protein A assay had a detection limit of 20 JDF U, a specificity of 100%, and a sensitivity of 75% (third ICA proficiency test). In the fourth international immunology of diabetes serum exchange workshop, our laboratory (lab identification no. 25) was 1 of 6 that again reached 100% specificity (20).

IAA assay. Sera were tested for IAA as previously described (6,21) with a competitive fluid phase radiobinding assay and expressed in nU/ml of insulin precipitated (cutoff at 49 nU/ml = 4 SD above normal mean).

Intravenous glucose tolerance test (IVGTT). After 3 days of unrestricted diet and at least 10 h of fasting, IVGTT was tested by infusing dextrose 0.5 g/kg of body weight in a 25% solution over a period of 3 min. Blood samples were collected before (0 min) and 1,3, and 5 min after the end of the rapid intravenous infusion and were assayed for insulin (22). Insulin levels were determined by a double antibody radioimmunoassay. The sum of 1 + 3 min insulin values were used as index of the early phase insulin response; the results were expressed as a percentile of the response of these 81 normal individuals.

Statistical analysis. Results were expressed as mean SD or median values. Statistical analyses used the two-tailed Student's *t* test, Wilcoxon rank test, and Fisher's exact test. Differences were considered significant if $P < 0.05$. The correlation between GAD levels and age of first-degree relatives was calculated with linear regression analysis.

RESULTS

GAD-ab in healthy control subjects. The serum determination of 81 ICA^-/IAA^- healthy subjects from Germany resulted in an average of GAD-activity of 1.56 ± 10.76% (mean ± SD; range, −15.0 to 48.0%; median 1.1) relative to a positive reference serum included in each assay. In agreement with the British studies (14,15), positivity was defined as mean + 2 SD of the 81 control sera, and a cutoff of 23% GAD-ab activity was obtained. Two of 81 (2.5%) control sera were found to have elevated GAD-ab activities of 45.1% (mean + 4.05 × SD) and 48.0% (mean + 4.32 × SD), respectively (dot-plot analysis Fig. 1). Follow-up samples of these 2 positive control subjects were obtained two years

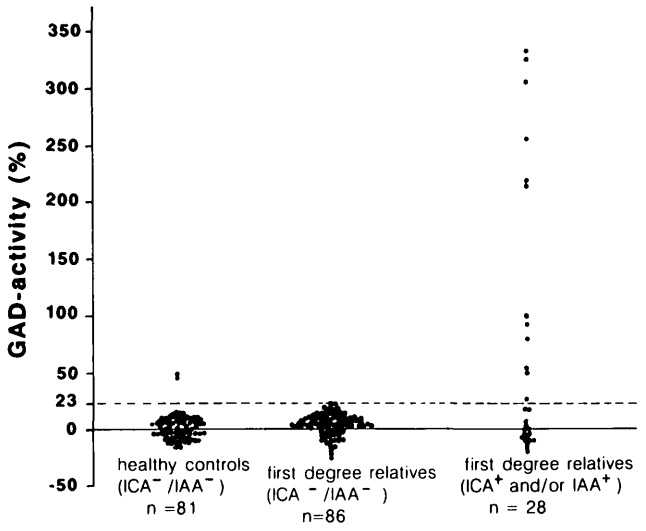

FIG. 1. Activity of GAD-ab (%) was significantly higher in ICA^+ and/or IAA^+ first-degree relatives of type I diabetic patients than in ICA^-/IAA^- healthy individuals ($P < 10^{-6}$) and in ICA/IAA relatives ($P < 10^{-6}$). The dashed line represents the upper limit (23%) of our normal range (mean + 2 SD).

later and tested together with the first samples in the same assay. GAD-ab positivity of the first samples was confirmed, whereas the later samples from both subjects were negative (−19.9% and 2.3%, respectively). An IVGTT was performed in all control subjects. The 1 + 3 min insulin secretion values for the 2 control subjects with elevated GAD-ab activities were 70.5 μU/ml (25th percentile) and 23.7 μU/ml (0.5 percentile), respectively.

GAD-ab in ICA^- and IAA^- first-degree relatives. Sera from 86 ICA^-/IAA^- relatives were selected and analyzed for GAD-ab. All 86 relatives (mean, 17.5 ± 13.2 years of age) were GAD-ab^- with a GAD-activity of 4.05 ± 9.98% (mean ± SD; range, −26.7% to 19.8%; Fig. 1). GAD-ab levels were not significantly different from the control population ($P = 0.4$).

GAD-ab in ICA^+ and/or IAA^+ first-degree relatives. Overall, 12 (42.9%) of the 28 high-risk relatives had GAD-ab levels above mean + 2 SD (39.3% were above

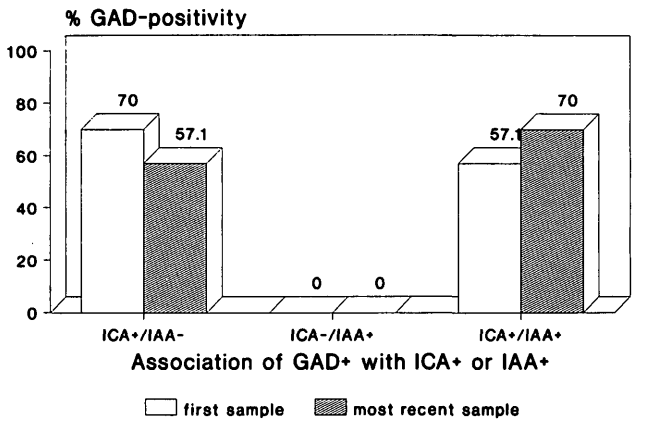

FIG. 2. Prevalence of GAD-ab (%) in relatives with ICA^+/IAA^-, ICA^-/IAA^+, or ICA^+/IAA^+ in first and most recent follow-up samples. GAD-ab was found to have a positive association with ICA ($P = 0.0006$) and a negative association with IAA ($P = 0.02$).

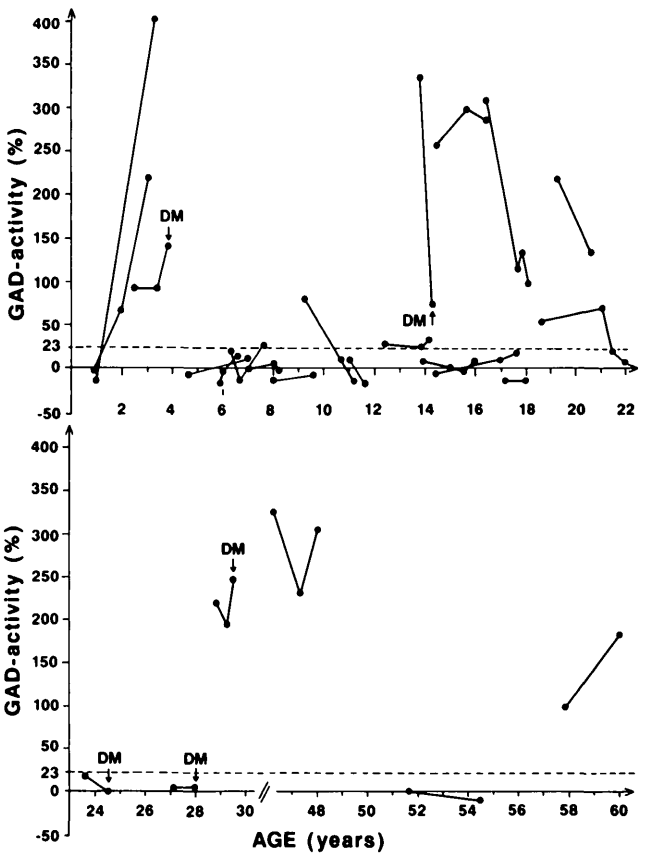

FIG. 3. Activity of GAD-ab (%) in 25 ICA^+ and/or IAA^+ relatives during follow-up. The dashed line represents the upper limit (23%) of our normal range (mean + 2 SD). DM = development of type I diabetes.

mean + 4 SD) of our normal control population (Fig. 1). The mean GAD activity (± SD) of these 28 first-degree relatives was significantly different from that of the 81 control subjects (72.3 ± 114.8% vs. 1.6 ± 10.8%; $P < 10^{-6}$; Fig. 1) and from 86 ICA^-/IAA^- relatives (72.3 ± 114.8% vs. 4.1 ± 10.0%; $P < 10^{-6}$; Fig. 1).

The frequency of GAD-ab was found to be 72.7% in ICA^+/IAA^- and 50% in ICA^+/IAA^+ relatives. Interestingly, GAD-ab were not detected in any sera from ICA^-/IAA^+ relatives. GAD-ab were positively correlated with presence of ICA (ICA^+/GAD^+ versus ICA^-/GAD^-; $P = 0.0006$) and negatively correlated with IAA (IAA^+/GAD^- versus IAA^-/GAD^+; $P = 0.02$). However, no correlation was observed between activity of GAD-ab and titers of ICA ($r = 0.27$; $P = 0.059$).

GAD-ab in ICA^+ and/or IAA^+ relatives with follow-up. One or more follow-up samples were available from 25 ICA^+ and/or IAA^+ relatives (Fig. 1). The mean duration of follow-up (± SD) between the first and most recent sample was 20.6 ± 12.1 months. Of both the first and last samples from these 25 relatives, 11 (44%) were GAD-ab^+. In the first samples, the frequency of GAD-ab was found to be 70% in ICA^+/IAA^-, 0% in ICA^-/IAA^+, and 57.1% in ICA^+/IAA^+ relatives (Fig. 2). In the most recent sera, GAD-ab were present in 57.1% of ICA^+/IAA^-, 0% in ICA^-/IAA^+, and in 70% of ICA^+/IAA^+ relatives. GAD-ab were again strongly negative when associated

TABLE 1
Presence of ICA, IAA, and GAD-ab in 25 first-degree relatives of type 1 diabetic patients with follow-up

Subject number	Age (years)	Follow-up (months)	First sample (+/−)			Last sample (+/−)		
			ICA	IAA	GAD-ab	ICA	IAA	GAD-ab
1	10	3	−	+	−	−	+	−
2	6	12	−	+	−	−	+	−
3	27	10*	−	+	−	−	+	−
4	51	38	−	+	−	−	+	−
5	7	43	−	+	−	−	+	−
6	5	7	−	+	−	+†	+	−
7	13	28	−	+	−	+†	+	−
8	1	28	−	+	−	+†	+	+†
9	1	31	−	+	−	+†	+	+†
10	17	3	+	−	−	+	−	−
11	23	10*	+	−	−	+	−	−
12	7	16	+	−	−	+	−	−
13	28	6*	+	−	+	+	−	+
14	46	21	+	−	+	+	−	+
15	14	23	+	−	+	+	−	+
16	57	36	+	−	+	+	−	+
17	18	39	+	−	+	+	+†	−
18	13	4*	+	−	+	+	+†	+
19	16	16	+	−	+	+	+†	+
20	4	32	+	+	−	−	+	−
21	14	37	+	+	−	−	+	−
22	2	16*	+	+	+	+	+	+
23	12	17	+	+	+	+	+	+
24	21	18	+	+	+	+	+	+
25	9	21	+	+	+	−	−	−

*Developed overt type I diabetes.
†Antibodies appeared on follow-up (see Fig. 4).

with the presence of IAA ($P = 0.04$) and positive when correlated with ICA ($P = 0.0005$).

Of 25 relatives available for follow-up (mean GAD activity ± SD: first sample, 90.8 ± 123.0%, last sample 95.0 ± 126.4%), GAD-ab persisted in 9 of 11 GAD-ab$^+$ relatives (2 of 11 lost GAD-ab), whereas 12 of 14 were consistently negative for GAD-ab (2 of 14 developed GAD-ab) (Fig. 3). The presence of all antibodies (ICA, IAA, and GAD-ab) in the first and most recent samples of these 25 relatives is shown in Table 1. In 9 IAA$^+$/ICA$^-$/GAD$^-$ relatives (subjects 1–9 in Table 1), IAA were the first detected antibodies and preceded the development of only ICA in 2 cases (subjects 6 and 7) and both ICA and GAD-ab in an additional 2 cases (subjects 8 and 9; Fig. 4*A*). The latter were the 2 youngest relatives (age 2 and 3 yr) in this study. Of the 7 ICA$^+$/IAA$^-$/GAD$^+$ relatives (subjects 13–19), 3 (subjects 17–19, Fig. 4*B*) also developed IAA on follow-up.

One relative, a young girl (subject 25), was initially positive for all three antibodies (ICA$^+$/IAA$^+$/GAD$^+$), but then lost both IAA and GAD-ab after 17 months and, finally, after another 4 months was negative for all antibodies (Fig. 5). Her IVGTT was always below the first percentile from first sample until current follow-up, but she has remained nondiabetic. Since recruitment into the study, her β-cell function has been studied in 5 OGTTs. All tests were normal; in the most recent follow-up, the OGTT was 5.1 (0 min), 7.7 (30 min), 7.8 (60 min), 4.3 (90 min), and 6.1 mM (120 min). The mean GAD-ab activity of all available follow-up samples of each relative was compared with the mean ages. No significant correlation was observed ($P = 0.15$; $r = 0.32$; $r^2 = 0.10$; Fig. 6).

Of the ICA$^+$ and/or IAA$^+$ first-degree relatives available for follow-up, 21.4% (6 of 28) subsequently progressed to overt type I diabetes (5 women, 1 man; mean age ± SD at diabetes manifestation was 20.0 ± 11.1 years of age, range, 4–30 years). The mean duration from first sample to diabetes onset was 11.2 ± 5.6 months; range, 6–18 months. Of the 6 relatives, 2 were positive for one antibody (ICA$^+$/IAA$^-$/GAD$^-$ and ICA$^-$/IAA$^+$/GAD$^-$), 3 were ICA$^+$/IAA$^-$/GAD$^+$, and only 1 was positive for ICA$^+$/IAA$^+$/GAD$^+$. Altogether, of the 6 relatives who developed diabetes, ICA was present in 5, GAD-ab was present in 4, and IAA was present in 2.

DISCUSSION

In this study, antibodies to GAD were determined in 114 first-degree relatives of type I diabetic patients, of which 86 were ICA$^-$/IAA$^-$ and 28 were ICA$^+$ and/or IAA$^+$ and considered to have a high risk for development of type I diabetes. Antibodies to GAD were absent in all 86 antibody$^-$ relatives, but present in 42.9% of the high-risk relatives with ICA$^+$ and/or IAA$^+$. The most striking results were that GAD-ab were *1*) positively correlated with ICA ($P = 0.0006$), but *2*) were negatively correlated with IAA ($P = 0.02$).

A similar positive correlation between GAD-ab and high titers of ICA was previously reported by Thivolet et al. (19), who used the same assay format and found GAD-ab in 89% of ICA$^+$ first-degree relatives. In another

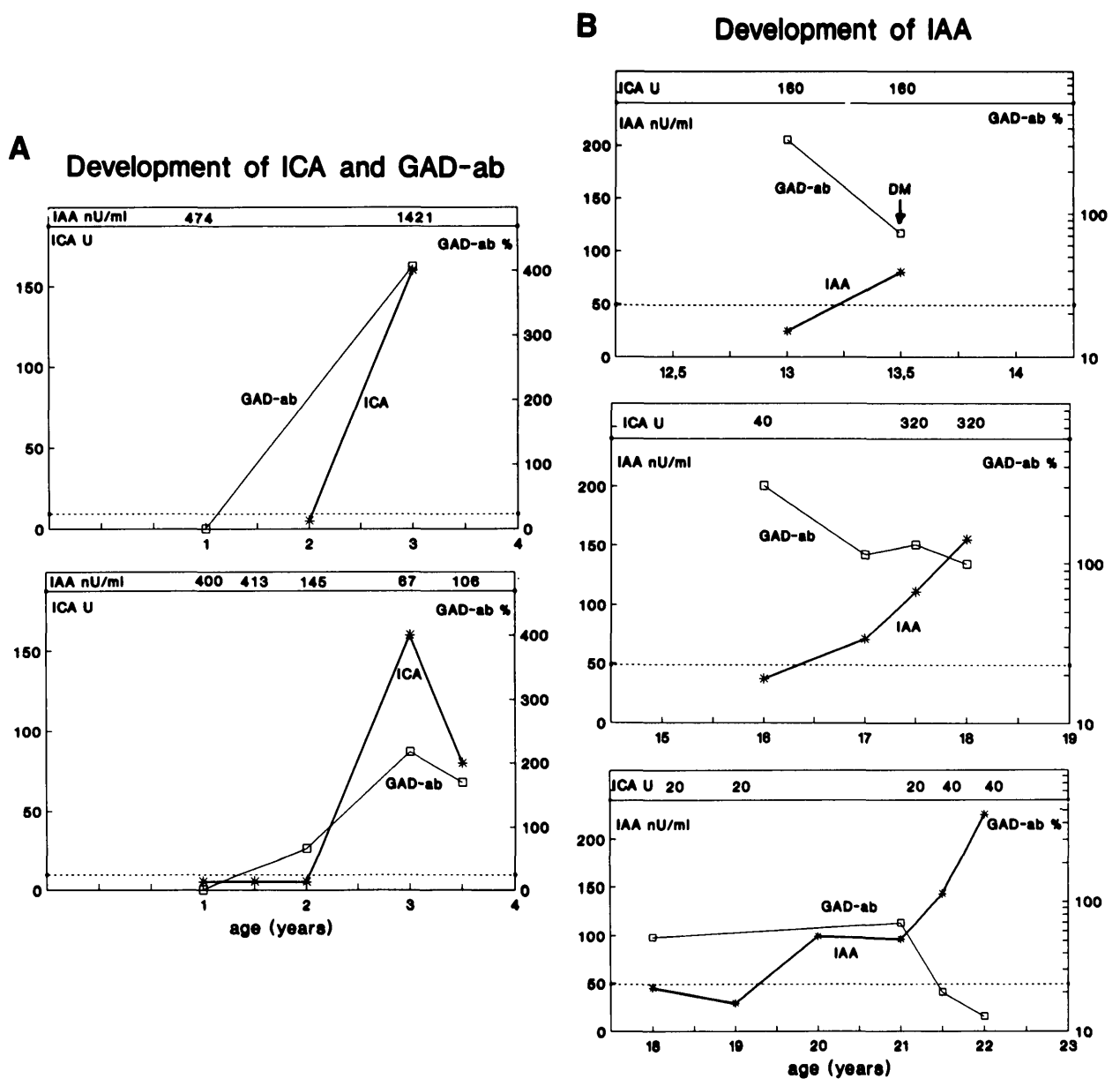

FIG. 4. Time course of ICA, IAA, and GAD-ab during follow-up in 5 relatives with either development of ICA and GAD-ab (n = 2) (*A*) or development of IAA (n = 3) (*B*). The dashed line represents the cutoff for both IAA and GAD-ab.

study, 78% of preclinical ICA$^+$ subjects were found to be GAD-ab$^+$ in an enzyme-linked immunosorbent assay (ELISA), using recombinant GAD peptides (16). Rowley et al. (18) also demonstrated some concordance between GAD-ab and ICA in newly diagnosed type I diabetic patients by the use of a radioimmunoprecipitation assay. In addition, 2 subjects who developed GAD-ab in our study also developed ICA. These data suggest that GAD-ab may represent a subset of ICA, as indicated by some studies (23–27), or that some unidentified events can induce an immune response simultaneously to GAD and to other islet cell antigens detected as ICA.

In contrast with this overall agreement about the relationship between ICA and GAD-ab, few data are available on the association of GAD-ab with IAA. In this study, a clear negative association of IAA and GAD-ab was observed (P = 0.02), and GAD-ab were not detectable in IAA$^+$/ICA$^-$ relatives. Three subjects who developed IAA on follow-up showed decreasing levels of GAD-ab. Thus, in contrast with the correlation observed between GAD-ab and ICA, the lack of positive associations between GAD-ab and IAA in this population indicates that induction of autoimmune responses to GAD and insulin are independent events.

In addition, our data indicate that most relatives, once found to have elevated GAD-ab, are likely to remain positive, at least for some months of follow-up. Nevertheless, fluctuations in GAD-ab were observed. The 2 youngest (IAA$^+$/ICA$^-$) first-degree relatives in our study developed GAD-ab with very high titers during follow-up (Fig. 4*A*) at 1 and 2 years of age. This indicates that

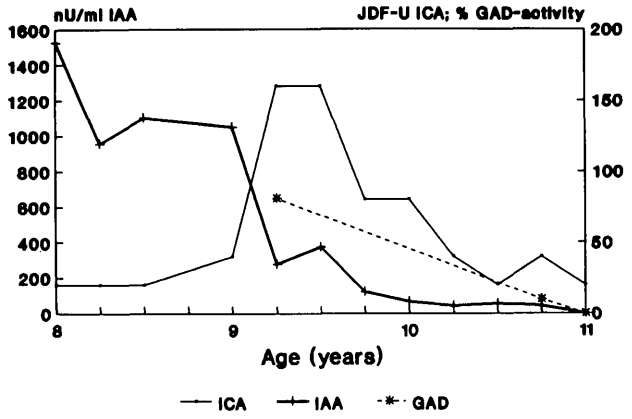

FIG. 5. Titers of ICA, IAA, and GAD-ab of a young girl during a follow-up period of 3 years shows loss of both IAA and GAD-ab after 17 months and loss of ICA after an additional 4 months. IVGTT was always <1 percentile, but OGTT was normal at all times.

GAD-ab responses can occur at a very young age and that further prospective studies in young relatives are warranted. However, no obvious correlation between age and the presence of GAD-ab was found.

Two relatives with initially elevated GAD-ab (81 and 53% GAD-activity, respectively, in the first sample), became GAD-ab$^-$ (−13% and 20% GAD-activity, respectively, in the last sample). One of them also lost ICA and IAA during follow-up (Fig. 5). Although first-phase insulin secretion is still below the first percentile, the subject has not developed diabetes (OGTT is still normal) and, with the loss of autoimmune phenomena associated with disease, may not do so.

It is still not clear how specific GAD-ab are as a marker for subsequent development of diabetes. A number of studies have identified GAD-ab in nondiabetic subjects with no known family history of diabetes. In a recent study in the normal British population by Christie et al., GAD-ab were detected in 2 control subjects of 80 analyzed. In two American studies, 1 of 33 (3%) and 2 of 51 (3.9%) control subjects were GAD-ab$^+$ (28,29). Consistent with these observations, in this study of the German population, we found that 2 of 81 (2.5%) healthy ICA$^-$/IAA$^-$ subjects had

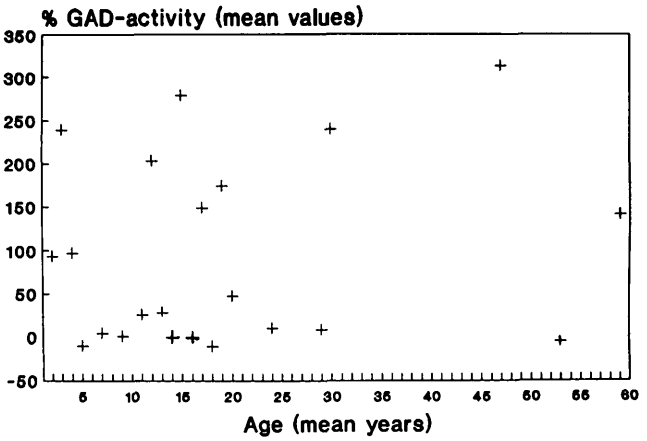

FIG. 6. Activities of GAD-ab (mean values of all serum samples of each relative) correlated with age (mean years) (P = 0.15).

elevated GAD-ab activity. In contrast, Rowley et al. (18) found that none of 42 nondiabetic subjects had elevated GAD-ab using immunoprecipitation of iodinated pig brain GAD (30). Thus, good evidence now exists that a significant proportion of the normal population can possess antibodies to GAD. However, in our study, follow-up samples of the GAD-ab$^+$ control subjects were negative, which suggests that the autoimmune response to GAD in these individuals is only transient.

Of our antibody-positive relatives (including IAA$^+$, ICA$^+$, GAD$^+$ relatives), 21.4% have developed overt type I diabetes during follow-up. Of these relatives, 4 of 6 had elevated GAD-ab, but no significant association between GAD positivity and disease development could be established in our study (diabetes development in 2 of 16 GAD-ab$^-$ vs. 4 of 12 GAD-ab$^+$). This is in contrast with the observations of Thivolet et al. (19), who reported a higher prevalence of diabetes in ICA$^+$/GAD$^+$ than in ICA$^+$/GAD$^-$ relatives.

Clearly, analyses of GAD-ab in larger groups of healthy individuals are required to determine the true prevalence of these antibodies in the background population. Studies in different laboratories and in a large number of first-degree relatives might clarify, in the future, whether the simultaneous presence of GAD-ab in antibody-positive first-degree relatives will increase the predictive value as a possible marker for diabetes progression.

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