*Original Article*

# GAD Autoantibody Affinity and Epitope Specificity Identify Distinct Immunization Profiles in Children at Risk for Type 1 Diabetes

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**OBJECTIVE—**Autoantibodies to insulin and GAD are features of preclinical type 1 diabetes in children. For insulin autoantibodies, the antibody affinity and epitope specificity predict which children progress to diabetes. We asked whether autoantibodies to GAD (GADAs) are heterogeneous in affinity and epitope recognition and whether diabetes-related GADA are restricted to high-affinity responses.

**RESEARCH DESIGN AND METHODS—**GADA affinity was measured by competitive binding experiments with [$^{125}$I]-labeled and -unlabeled recombinant human GAD65 in the first GADA-positive sample from 95 children with a type 1 diabetes family history who were prospectively followed from birth and in follow-up samples from 65 of these children.

**RESULTS—**At first GADA appearance, affinity ranged from $10^7$ to $10^{10}$ l/mol. Affinity was higher in multiple islet autoantibody-positive children ($P < 0.0001$) and in HLA DR3–positive children ($P = 0.006$). GADA affinities were $>10^9$ l/mol in 52 of 53 multiple autoantibody-positive children. In contrast, children who were single GADA positive often had lower affinity GADA and/or GADA with specificities that were restricted to minor $NH_2$-terminal GAD65 epitopes. At follow-up, affinity increased from low to high in 3 of 65 children. All 24 children who developed diabetes had high-affinity GADAs before diabetes onset.

**CONCLUSIONS—**Children develop discrete, heterogeneous antibody responses to GAD that could arise from distinct immunization events, only some of which are diabetes relevant. Subtyping the GADA responses using affinity measurement will improve type 1 diabetes risk assessment. ***Diabetes*** **56:1527–1533, 2007**

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GADA, GAD autoantibody; IA-2A, IA-2 autoantibodies; IAA, insulin autoantibodies; TBST, Tris-buffered saline with Tween; WHO, World Health Organization.



Childhood type 1 diabetes is preceded by the development of autoantibodies to multiple islet antigens (1). In young, genetically at-risk children, insulin autoantibodies (IAA) are typically the first islet autoantibodies to appear before clinical diabetes (2). The autoantibody response to insulin is heterogeneous with respect to its affinity for human insulin and its epitope specificity. High-affinity responses recognizing epitopes exposed on both insulin and proinsulin are associated with the HLA DR4-DQ8 haplotype and are characteristic of children who progress to diabetes (3,4). In contrast, low-affinity IAA recognize epitopes that are often not exposed in the conformation of proinsulin, are not associated with high-risk HLA haplotypes, and do not confer a high risk for diabetes. Based on these findings, we proposed that both the nature and intensity of the early immunizing event and the host HLA are critical in determining the subsequent disease pathogenesis in autoimmune diabetes (3).

Autoantibodies to GAD (GADAs) are also a prominent feature of pre–type 1 diabetes. GADAs often appear together with or after IAA in young children (2). GADAs can also occur without IAA, particularly in older children (5). Unlike IAA, GADAs are not inversely related to age, GADA titer does not predict progression to diabetes, and controversial reports exist with respect to relevance of GADA epitope reactivity to diabetes progression (6–12). IAA and GADAs also differ in their associations with HLA class II alleles—IAA being preferentially associated with HLA DR4-DQ8 haplotypes and GADAs preferentially associated with HLA DR3-DQ2 haplotypes (13–16). GADAs, therefore, represent a useful model to corroborate the initial findings on IAA and extend the concept that immunization can evoke heterogeneous autoimmune responses that determine disease pathogenesis. Here, we asked whether GADAs vary in their affinities between children at the time of first GADA appearance, whether GADA affinity changes over time, and whether affinity is associated with progression of islet autoimmunity.

## RESEARCH DESIGN AND METHODS

Samples analyzed in this study were obtained from individuals who participated in the German BABYDIAB (2) and BABYDIET studies (17). Both studies follow children from birth for the development of islet autoantibodies and diabetes. Participating children have a first-degree relative with type 1 diabetes, and, in the BABYDIET study, children also have an HLA DR-DQ genotype conferring susceptibility to type 1 diabetes. Children who partici-

pated in the BABYDIET study also underwent dietary intervention in the form of first introduction of gluten at 6 or 12 months of age. Children in the BABYDIAB study were routinely tested for IAA, GADA, and autoantibodies to IA-2 (IA-2A) at 9 months and 2, 5, 8, 11, 14, and 17 years of age, and children in the BABYDIET study were routinely tested for these autoantibodies at 3-month intervals beginning at 3 months of age. The BABYDIAB and BABYDIET studies were approved by the ethical committees of Bavaria, Germany (Bayerische Landesärztekammer, no. 95357), and the Ludwig-Maximilians-University Munich, Germany (no. 329/00), respectively. All participants in this study gave informed consent, and the study was carried out in accordance with the 2000 revised Declaration of Helsinki (http://www.wma.net/e/policy/b3.htm).

By the end of 2005, a total of 99 children from both the BABYDIAB ($n = 84$) and the BABYDIET ($n = 15$) study developed GADAs that were confirmed positive in a subsequent antibody test. Ninety-five of these 99 children were selected for our study on the basis of having sufficient serum available for GADA affinity measurement. Samples with suspected maternally acquired GADAs were not included. We used the first GADA-positive sample from 88 children and the next available GADA-positive sample from 7 children. The study cohort consisted of 49 boys and 46 girls. The median age of the 95 children at time of first sample used in this study was 3.8 years (interquartile range 2.1–5.4). GADAs developed together with or after IAA and/or IA-2A in 53 children and without IAA or IA-2A in 42 children. Of the 42 single GADA-positive children, 9 developed IAA and/or IA-2A in follow-up samples, and 33 either remained single GADA positive ($n = 24$) or became GADA negative in follow-up samples ($n = 9$). Twenty-four of the 95 children developed diabetes. GADA affinity was also measured in follow-up samples from 65 of the 95 GADA-positive children.

**Islet autoantibody measurements.** IAA, GADA, and IA-2A were measured by protein A/G radiobinding assays as previously described (2,18) using [$^{125}$I]recombinant human insulin labeled at tyrosine A14, and [$^{35}$S]methionine labeled in vitro translated recombinant human GAD65 and IA-2, respectively. For each antibody, results were expressed as arbitrary units that were derived from a standard curve. GADA results were converted into World Health Organization (WHO) units. The thresholds for positivity in each assay corresponded to the 99th percentile for titer in control subjects.

GADA epitope specificity was determined using a radiobinding assay on [$^{35}$S]methionine-labeled GAD65/67 chimeric proteins as previously described (10). Thresholds for positivity were defined as the upper limit of 50 control subject sera. Chimeric proteins used were $GAD65_{1–95}/GAD67_{101–593}$, $GAD67_{1–243}/GAD65_{235–444}/GAD67_{453–593}$, and $GAD67_{1–452}/GAD65_{445–585}$. These could distinguish GADA to GAD65 $NH_2$-terminal (residues 1–95), GAD65 middle (residues 235–444), and/or GAD65 COOH-terminal (residues 445–585) epitopes. GADA were classified as *1*) reactive to middle and/or COOH-terminal epitopes, *2*) reactive to $NH_2$-terminal epitopes only, or *3*) reactive to none of the epitopes represented in the chimeric proteins.

**GADA affinity measurement.** GADA affinity was measured by competitive binding experiments. Serum (2 μl) was incubated in duplicate for 48 h in Tris-buffered saline with Tween (TBST) buffer (50 mol/l Tris, 150 mmol/l NaCl, 1% Tween 20, pH 7.4) in the presence of 4.7 femtomoles [$^{125}$I]recombinant human GAD65 (0.1 nmol/l) (Dr. Schlosser, Greifswald, Germany [19]) and five increasing quantities of unlabeled human GAD65 ($1.5 \times 10^{-15}$, $1.5 \times 10^{-14}$, $1.5 \times 10^{-13}$, $1.5 \times 10^{-12}$, and $1.5 \times 10^{-11}$ moles, respectively) (RSR, Cardiff, U.K.) or TBST buffer only in a final volume of 47 μl. Immune complexes were precipitated for 60 min with 1.5 mg protein A Sepharose (Amersham, U.K.) preswelled in 50 μl TBST. Protein A Sepharose was subsequently washed five times with 1.8 ml ice-cold TBST buffer. Bound [$^{125}$I]GAD65 was measured using a Gamma counter (Packard, Frankfurt, Germany). Results were expressed as counts per minute. Nonspecific binding was determined as the binding of a GADA-negative control serum to [$^{125}$I]GAD65 in presence of 15 pmol unlabeled human GAD65. $IC_{50}$ and $K_d$ values were calculated by nonlinear regression analysis using the GraphPad Prism3 program (GraphPad Software, San Diego, California), and GADA affinity was expressed as reciprocal $K_d$ value (l/mol). Displacement curves were computed directly from the counts per minutes for each competition reaction, and background binding or maximal displacement of radio-labeled ligand was not subtracted from bound counts per minute. Samples with very high GADA titers that showed binding to [$^{125}$I]GAD65 of >50% of the total [$^{125}$I]GAD65 activity added were diluted until the noncompeted binding fell <50% of the total activity. For such samples, GADA affinity was calculated from the competitive binding curve obtained with the diluted sample. The reproducibility of GADA affinity measurements was determined from replicates of a GADA-positive serum that was included in each experiment. The coefficient of variation at a mean affinity of $7.1 \times 10^9$ l/mol was 3% ($n = 23$ experiments).

**HLA typing.** HLA-DRB1-DQB1 alleles were typed using PCR-amplified genomic DNA and nonradioactive sequence-specific oligonucleotide probes (20).

**Statistical analysis.** The Mann-Whitney *U* test was used to compare GADA affinities and titers between groups. Fisher's exact test was used to compare frequencies between groups. Spearman's correlation was used to determine the correlation between variables. Life table analysis was used to determine cumulative risk to develop multiple islet autoantibodies and/or diabetes in children who developed GADAs without prior or concomitant IAA or IA-2A. The time to event was defined from the first GADA-positive sample, and the end of follow-up was defined as the date of the first multiple autoantibody-positive sample and/or diagnosis of diabetes or the date of last contact. Survival between groups was compared using the log rank test. For all analyses, a two-tailed *P* value of 0.05 was considered significant. Statistical analyses were performed using SPSS (SPSS 14.0; SPSS, Chicago, IL).

## RESULTS

**GADA affinity is homogeneous within samples and varies between children.** GADA-competitive binding curves to [$^{125}$I]GAD65 were consistent with a one-site binding model in the first GADA-positive sample from all 95 children tested, suggesting that GADAs were of relatively homogeneous affinity within each sample. GADA affinities varied between children and ranged from $4.1 \times 10^7$ l/mol to $2.6 \times 10^{10}$ l/mol. Example binding curves are shown in Fig. 1*A*. Affinity was relatively constant when measured at different serum dilutions (Fig. 1*B*).

Affinity was not significantly correlated with the age at first GADA appearance ($r = -0.085$; $P = 0.4$; Fig. 2*A*) but showed a significant positive correlation with GADA titer ($r = 0.32$; $P = 0.002$; Fig. 2*B*). GADA affinity was higher in children with HLA DRB1*03 alleles ($P = 0.006$; Fig. 2*C*). GADA with a restricted binding to the $NH_2$-terminal region of GAD65 (residues 1–95) were of lower affinity than GADA that bound to the middle (residues 235–444) and/or the COOH-terminal (residues 445–585) region of GAD65 ($P = 0.003$; Fig. 2*D*). Seven of the 12 children with $NH_2$-terminal–restricted GADAs became GADA negative at follow-up.

**GADA affinity is related to autoantibody status.** GADA affinity was analyzed with respect to the presence of other islet autoantibodies (Fig. 3). Affinity was significantly higher in the 53 children who also had IAA and/or IA-2A at or before their first GADA-positive sample than in the 42 children who were IAA and IA-2A negative ($P < 0.0001$). Fifty-two of the 53 multiple islet autoantibody-positive children already had GADA affinities $>10^9$ l/mol at first GADA appearance. In comparison, 22 of the 42 single GADA-positive children had GADA affinities $>10^9$ l/mol ($P < 0.0001$).

**GADA affinity and epitope specificity identify distinct GADA profiles.** Stratification of children on the basis of whether GADA appeared with or without other islet autoantibodies, GADA epitope specificity, GADA affinity, and titer showed relatively discrete early GADA profiles (Fig. 4). The predominant initial GADA response in the 53 children who developed GADA as part of an immune response to multiple islet antigens (multiple autoantibody-positive children) was high-affinity GADAs against the common middle and/or COOH-terminal GAD65 epitopes (Fig. 4*A*). The remainder ($n = 12$) had high-affinity GADAs that did not bind epitopes contained within the middle, COOH-, and $NH_2$-terminal GAD65 epitopes represented in the chimeric proteins used (not shown). The latter group of children was distinguished by a lower GADA titer ($P < 0.0001$). Almost half (24/52) of the children with multiple autoantibodies at first GADA detection had the HLA DR3 allele.

Children who developed GADAs without prior or concomitant antibodies to insulin or IA-2 (single GADA-

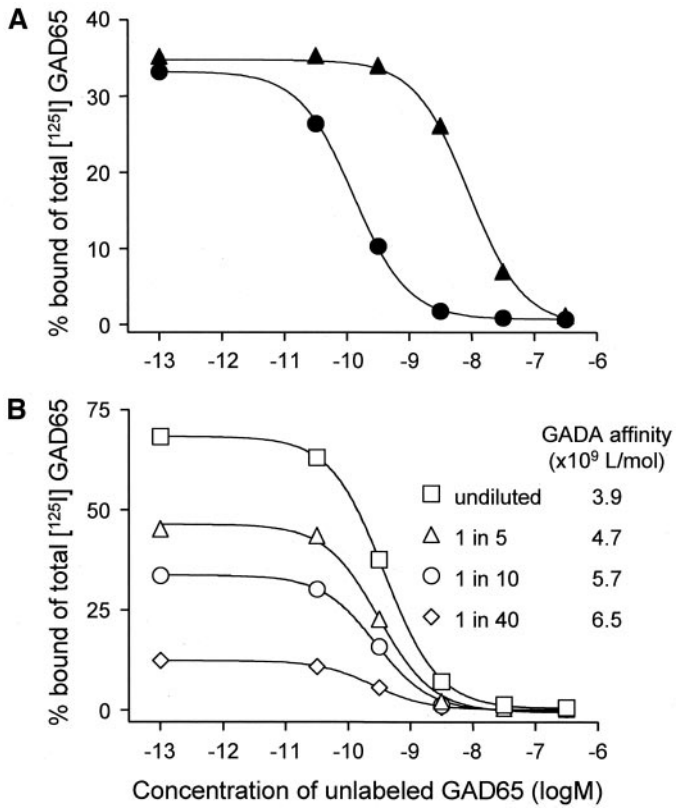


**FIG. 1. Example of competitive GAD65 binding curves obtained from children's sera at the time of first GADA appearance. The percent of GADA binding to human [$^{125}$I]GAD65 is shown relative to the total amount of human [$^{125}$I]GAD65 per test tube (ordinate scale) at increasing concentrations of competitive unlabeled human GAD65 (abscissa). *A*: Displacement curves for a sample with high-affinity GADAs of 1.5 × $10^{10}$ l/mol (●) and a sample with low-affinity GADAs of 8.5 × $10^{7}$ l/mol (▲). Both curves are consistent with a one-site binding model. The shift of the second curve to the right indicates lower-affinity GADAs. *B*: Displacement curves for an undiluted sample with very-high GADA titer (>1,000 WHO units) (□) and for 1:5 (△), 1:10 (○), and 1:40 (◇) dilutions in sample buffer. GADA affinity is calculated for each dilution (*inset*).**

positive children) had heterogeneous GADA profiles (Fig. 4*B* and *C*). Twelve of these 42 children (29%) had profiles typical of the predominant multiple antibody associated response: high affinity, relatively high titer, and middle and/or COOH-terminal GAD65 epitope reactivity (Fig. 4*B*). These 12 children had an excess of HLA DR3 (75 vs. 30% in the remainder of single GADA-positive children; $P = 0.01$). Unique to the single GADA-positive children was a group of 11 children who had GADAs that recognized the middle and/or COOH-terminal epitopes but with affinities $<10^9$ l/mol (Fig. 4*B*). HLA DR3 was infrequent (18%) in this group of children. GADAs that bound only the $NH_2$-terminal epitopes were also unique to the single GADA-positive children (Fig. 4*C*). These 12 children did not have an excess of HLA DR3 and had GADAs of intermediate/low affinities. Finally, seven of the single GADA-positive children had GADAs that did not bind to the middle, COOH-, or $NH_2$-terminal epitopes (not shown). Among the single GADA-positive children, progression to multiple islet autoantibody status or diabetes was frequent in children who had the high-affinity middle and/or COOH-terminal GAD65 epitope profile (8/12 vs. 3/30 in children with other profiles; $P = 0.0005$). The 5-year risk for progression to multiple islet autoantibodies or diabetes in these children was 54% (95% CI 22.6–85.4) compared with 11% (0–22.8; $P = 0.007$) in children with other profiles (Fig. 5).

**Changes in GADAs affinity are infrequent but are related to change in autoantibody status.** GADA affinity was measured in follow-up sera from 65 children (Fig. 6). Affinity changes >1 log were observed in only three children. All three had low-affinity GADAs that became high-affinity GADAs at follow-up. One of these children had multiple antibodies and developed diabetes (Fig. 6*A*), and the other two had single GADAs and became multiple islet autoantibody positive at or soon after the rise in GADA affinity (Fig. 6*B*).

**GADA affinity and progression to diabetes.** Twenty-three of the 24 GADA-positive children who developed diabetes had high-affinity middle GAD65 epitope–reactive GADA before diabetes onset. Affinity was $>10^9$ l/mol in the first GADA-positive sample in 23 children and in follow-up samples in the remaining child. A further eight children with single GADA who had ($n = 6$) or developed ($n = 2$) high-affinity GADAs progressed to having multiple islet autoantibodies but not yet to diabetes. None of the children who have remained low-affinity GADA positive and none of the children who developed GADA reactive to $NH_2$-terminal epitopes only have developed multiple islet autoantibodies or diabetes.

## DISCUSSION

The affinity of GADAs was found to vary up to 1,000-fold in GADA-positive children. Children who had multiple islet autoantibodies or developed diabetes consistently had high-affinity GADAs. In contrast, children who remained single GADA positive often had lower-affinity GADAs and/or GADAs with specificities that were restricted to minor GAD65 epitopes. These findings suggest the GADAs can result from distinct immunization events, only some of which are relevant to type 1 diabetes.

The current study includes the largest number of GADA-positive children studied around sero-conversion. Children were identified by prospective follow-up and measurement of autoantibodies from birth, with sample intervals ranging from 3 months to 3 years. Despite the relatively narrow intervals, it is possible that the antibody response had already undergone substantial maturation when first detected in our cohort, and much shorter time intervals between sampling would show different findings at first immunization. Because the data were obtained in children who have an affected family member, they may not be representative of GAD autoimmunity that first occurs in older individuals or in patients with neurological disease (21) or autoimmune polyendocrine disorders (22,23).

Consistent with what was observed for IAA (3), this study describes relatively discrete GADA profiles that are distinguished by their affinity (high or low) and epitope specificity. GADA profiles were relatively homogeneous in children who had multiple islet autoantibodies or developed diabetes and were heterogeneous in children who developed GADAs in the absence of other islet autoantibodies. High-affinity GADAs against epitopes located in the middle and COOH-terminal of GAD65 were frequent in multiple islet autoantibody-positive children. A similar or identical GADA profile was also found in a subset of single GADA-positive children. Not surprisingly, this GADA profile in single GADA-positive children was associated with later progression to multiple islet autoantibodies. Lower-affinity GADAs against epitopes in the middle and COOH-

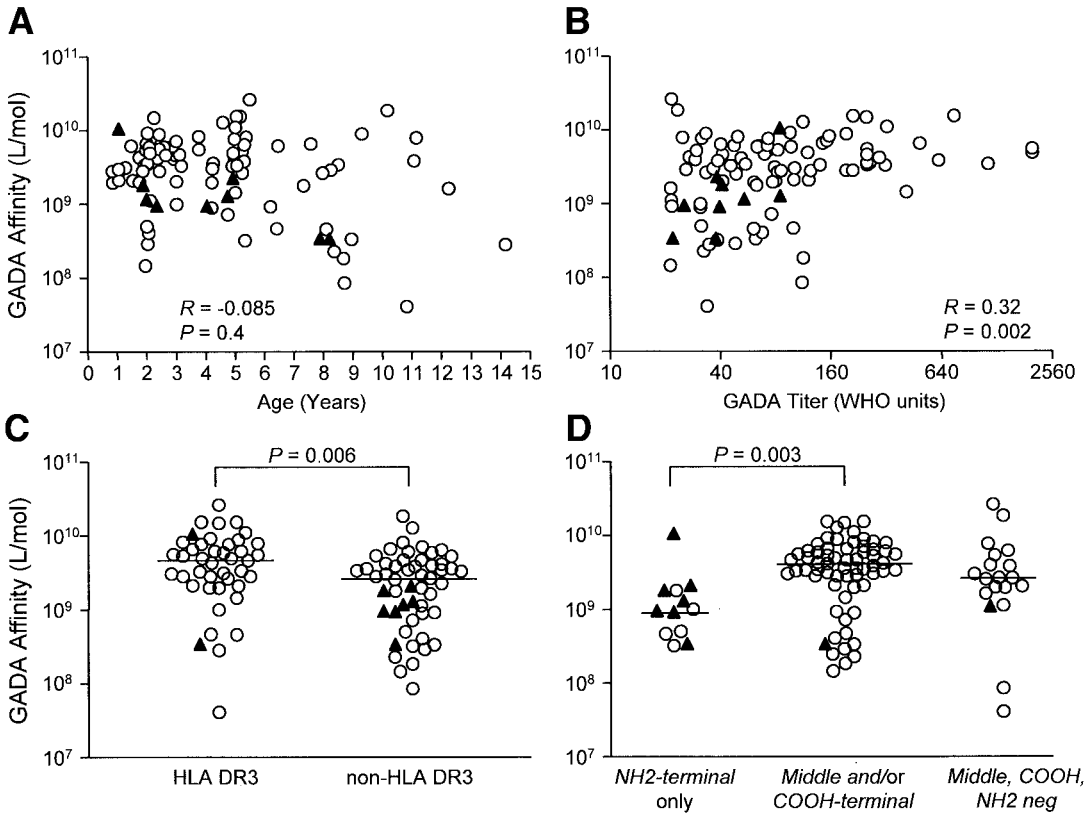


**FIG. 2. Relationship between GADA affinity and the age of GADA appearance, GADA titer, HLA, or GADA epitope binding in the first GADA-positive samples of children. *A* and *B*: GADA affinity (ordinate scale) plotted against the age of the child at the time of blood sampling (*A*) or the titer of GADA (*B*) (abscissa) for all 95 children. *C*: GADA affinity in children who had the HLA DRB1*03/DQB1*02 haplotype (HLA DR3; $n = 42$) compared with those who did not have this haplotype (non–HLA DR3; $n = 52$). *D*: Affinity of GADA that only bound epitopes contained within residues 1–95 of GAD65 ($NH_2$-terminal; $n = 12$) compared with GADA that bound epitopes within residues 235–585 (middle and/or COOH-terminal; $n = 64$), and GADA in which binding to the $NH_2$-, middle, or COOH-terminal epitope constructs could not be detected (middle, COOH, $NH_2$ neg; $n = 19$). Children who only had transient GADAs are identified (filled triangles). In *C* and *D*, the median affinity is indicated for each group.**

terminal of GAD65 were also found in the single GADA-positive children. A conspicuous lack of HLA DR3 distinguished these children from those with high-affinity GADAs. This suggests that the HLA *DRB1**03-*DQB1**02 haplotype favors the maturation and expansion of the humoral immune response to GAD.

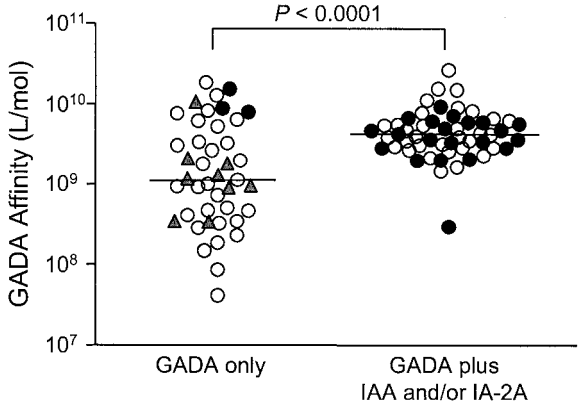


**FIG. 3. Relationship between GADA affinity, islet autoantibody status, and diabetes. The first GADA-positive samples from 95 children are classified as having developed GADAs without prior or concomitant IAA or IA-2A (GADA only; $n = 42$) or having developed GADAs after or concomitantly with IAA and/or IA-2A (GADA plus IAA and/or IA-2A; $n = 53$). Children who developed type 1 diabetes at follow-up (●) and children who only had transient GADAs (▲) are identified. The median affinity is indicated for each group.**

Unique in the single GADA-positive children were GADA reactive only to $NH_2$-terminal epitopes. None of the children with this GADA profile progressed to multiple islet autoantibodies or diabetes in childhood. The intermediate affinity of these $NH_2$-terminal–reactive GADA is consistent with their transitory nature. GADAs that include reactivity to epitopes in the $NH_2$-terminal region have been previously described in patients with stiff man syndrome, in Japanese patients with slowly progressive type 1 diabetes, and in a minority of patients with type 1 diabetes (24–27). In these studies, antibodies to the $NH_2$-terminal epitopes occur together with antibodies to the more common middle and/or COOH-terminal epitopes. Moreover, $NH_2$-terminal reactivity was usually associated with high-titer GADAs (25,28). It is possible, therefore, that the epitopes bound by antibodies in our $NH_2$-terminal only reactive to GADAs are not the same as the $NH_2$-terminal epitopes bound by GADAs in disease. A number of GADA-positive patients with neurological diseases or polyendocrine autoimmunity also develop diabetes. Like the children in our cohort, progression to diabetes in these patients is associated with multiple islet autoantibodies (22). The ability of GADA affinity to further discriminate progression to diabetes in single GADA-positive patients with diseases other than type 1 diabetes or in subjects with GADAs responsible for the restricted islet cell autoantibody staining (29–31) should be investigated.

The findings for GADAs and previously for IAA suggest

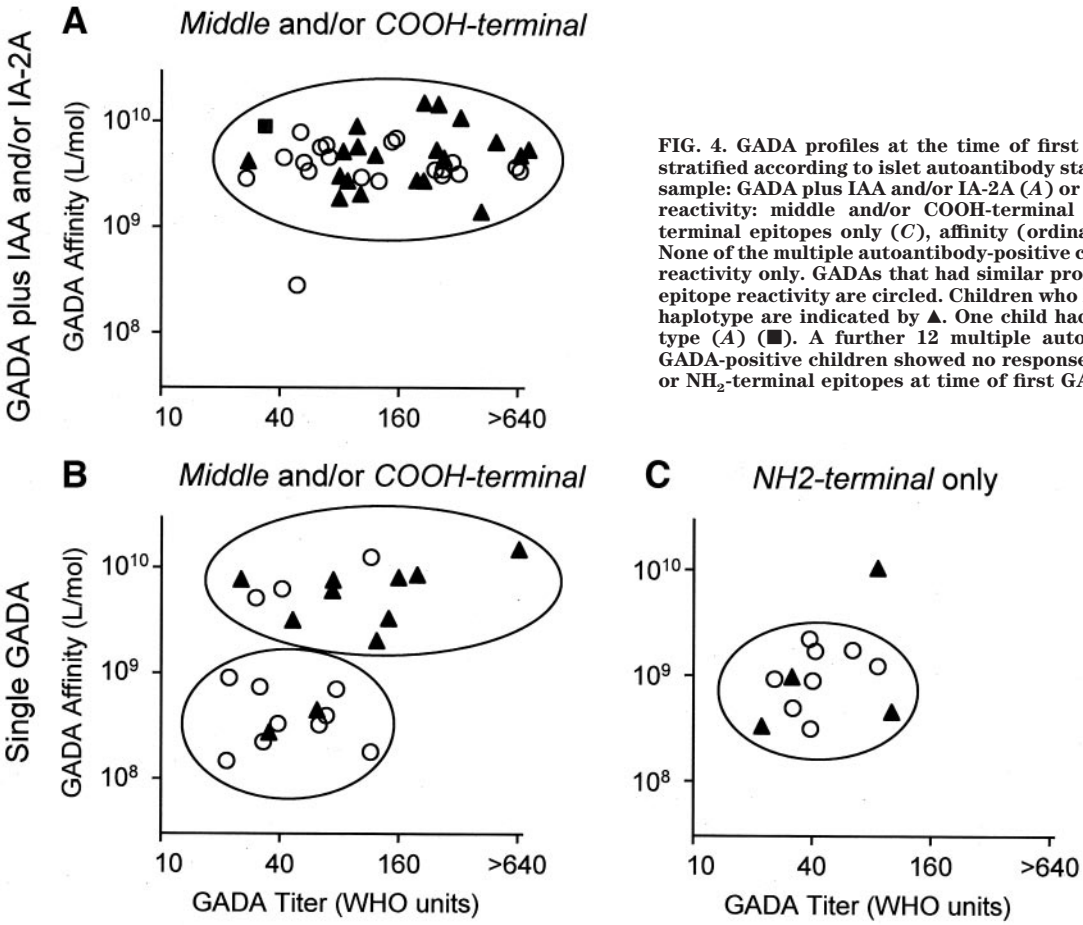


**FIG. 4. GADA profiles at the time of first GADA appearance. GADAs are stratified according to islet autoantibody status in their first GADA-positive sample: GADA plus IAA and/or IA-2A (*A*) or single GADA (*B* and *C*); epitope reactivity: middle and/or COOH-terminal epitopes (*A* and *B*); or $NH_2$-terminal epitopes only (*C*), affinity (ordinate scale), and titer (abscissa). None of the multiple autoantibody-positive children had $NH_2$-terminal GADA reactivity only. GADAs that had similar profiles on the basis of affinity and epitope reactivity are circled. Children who had the HLA DRB1*03/DQB1*02 haplotype are indicated by ▲. One child had no information on HLA haplotype (*A*) (■). A further 12 multiple autoantibody-positive and 7 single GADA-positive children showed no response to the middle, COOH-terminal, or $NH_2$-terminal epitopes at time of first GADA appearance (not shown).**

that islet autoantibodies can arise from what appear to be distinct immunization events with diverse etiologies. We postulate that at least three islet autoantibody responses can occur. The first type of response is seen to multiple β-cell autoantigens, is against epitopes typically found in patients with type 1 diabetes, and probably results from acute or chronic immunization of sufficient duration to evoke high-affinity antibodies to multiple autoantigens. The second type is against similar epitopes, but has a stunted maturation, and has a limited number of targets. This could result from similar immunization event(s) as in the corresponding high-affinity antibodies, but with weaker intensity or reduced expansion in part due to the absence of HLA DR3. The third type is restricted to atypical epitopes such as the $NH_2$-terminal epitopes of GAD65. This type of responses could arise from immunizing events that are unrelated to the pathogenesis of type 1 diabetes.

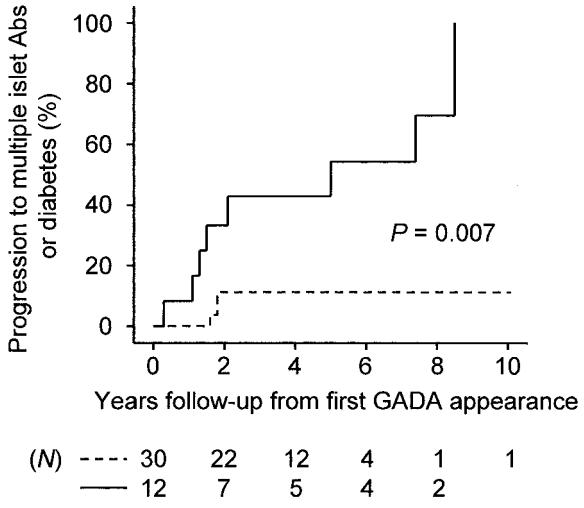


**FIG. 5. Cumulative risk for progression to multiple islet autoantibodies (Abs) and/or type 1 diabetes in 42 children who developed GADA without prior or concomitant IAA or IA-2A. Children were followed from the time of first GADA-positive sample and stratified by GADA profile of this sample. Children with high-affinity (>$10^9$ l/mol) and middle and/or COOH-terminal reactive GADA (solid line) are compared with children who had GADAs of lower affinity and/or were nonreactive with the middle or COOH-terminal GAD65 epitopes (broken line).**

These data have practical implications with respect to prediction and pathogenesis. Using affinity and epitope specificity, it should be possible to stratify short- to moderate-term diabetes risk in single GADA-positive children. Our study did not follow individuals into adulthood; therefore, it cannot estimate if affinity can stratify long-term diabetes risk. Nevertheless, GADA affinity could have a major impact on prediction if the findings from this study extend to older individuals, because single GADA-positive individuals make up a large number of cases in adult autoimmune diabetes (32–34). We also suggest that phenotyping islet autoantibody responses in the manner described here will be important for investigations into the etiological cause(s) of islet autoimmunity.

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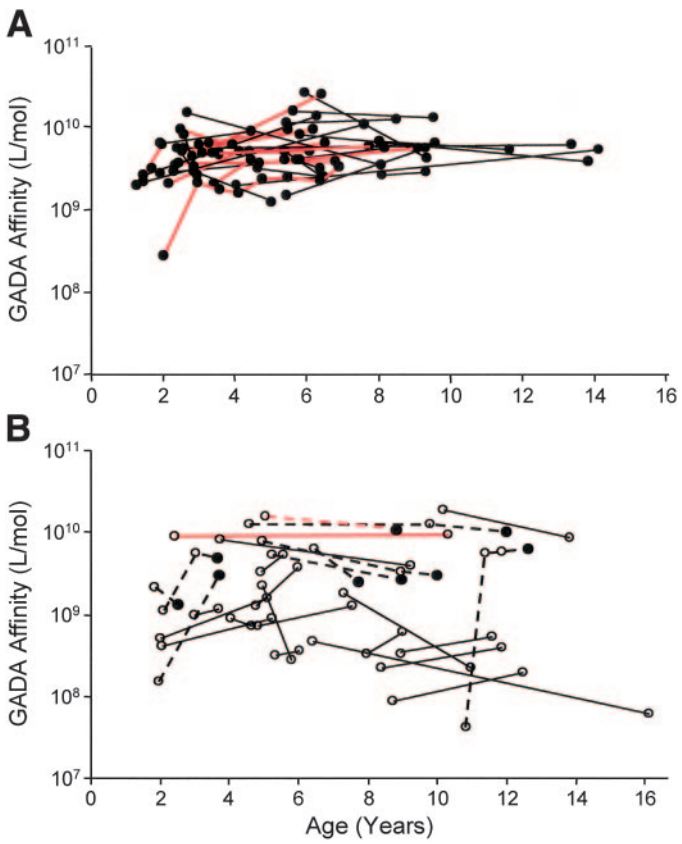


**FIG. 6. GADA affinity over time in 141 follow-up samples from 65 children. *A*: GADA affinity on follow-up for 38 children who were also IAA- and/or IA-2A positive at or before the first GADA-positive sample. *B*: GADAs affinity on follow-up for 27 children who did not have IAA or IA-2A in their first GADA-positive sample. Follow-up samples from individual children are connected by lines. Children who progressed to diabetes are identified by red lines (*A* and *B*), and nine children who progressed from single GADA to multiple islet autoantibodies are identified by broken lines (*B*). The multiple autoantibody-positive samples are indicated (●). One child developed multiple autoantibodies and progressed to diabetes (broken red line).**

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