Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes

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Children at risk for type 1 diabetes can develop early insulin autoantibodies (IAAs). Many, but not all, of these children subsequently develop multiple islet autoantibodies and diabetes. To determine whether disease progression is reflected by autoantibody maturity, IAA affinity was measured by competitive radiobinding assay in first and subsequent IAA-positive samples from children followed from birth in the BABYDIAB cohort. IAA affinity in first positive samples ranged from less than 10⁸ l/mol to more than 10¹¹ l/mol. High affinity was associated with HLA DRB1*04, young age of IAA appearance, and subsequent progression to multiple islet autoantibodies or type 1 diabetes. IAA affinity in multiple antibody–positive children was on average 100-fold higher than in children who remained single IAA positive or became autoantibody negative. All high-affinity IAAs required conservation of human insulin A chain residues 8–13 and were reactive with proinsulin. In contrast, most lower-affinity IAAs were dependent on COOH-terminal B chain residues and did not bind proinsulin. These data are consistent with the concept that type 1 diabetes is associated with sustained early exposure to (pro)insulin in the context of HLA DR4 and show that high-affinity proinsulin-reactive IAAs identify children with the highest diabetes risk.

Introduction

During the natural history of childhood diabetes, insulin autoantibodies (IAAs) are often the first autoantibody detected early in infancy (1–5). Many, but not all, IAA-positive children also develop autoantibodies to other β cell antigens such as glutamic acid decarboxylase (GAD) and the protein tyrosine phosphatase–like proteins IA-2 and IA-2B (2–4). Children who also develop these antibodies usually progress to clinical type 1 diabetes mellitus (T1DM), whereas children who remain positive only for IAAs rarely develop T1DM (2). Development of multiple islet autoantibodies is, therefore, an important step in the pathogenesis of the disease.

There is no marker that distinguishes the IAA-positive children who will eventually become multiple antibody–positive. In a typical antibody response, exposure to antigen in the presence of B cell growth factors results in B lymphocyte expansion and IgM antibody production. Sustained or repeated antigen exposure results in a switch from IgM to IgG production, and subsequent exhaustion of antigen leads to the selection of clones that produce high-affinity antibodies to the antigen (6, 7). Affinity could, therefore, reflect a stage of antigen encounter and, in the case of IAAs, may be useful in staging the preclinical phase of T1DM. In order to address whether autoantibody affinity matures during preclinical diabetes and whether it predicts progression to multiple islet autoantibodies, IAA affinity was measured in IAA-positive children from the prospectively followed BABYDIAB cohort (8). The findings in these children who are followed during infancy indicate that IAA affinity is fixed relatively early in the autoimmune response, that it distinguishes IAAs with different epitope reactivity, and that it identifies IAA-positive children who will progress to multiple autoantibodies.

Results

IAA binding characteristics are consistent with a 1-site binding model and therefore relatively homogeneous within samples. IAAs are measured by radiobinding assay using [125I] insulin labeled at tyrosine at position 14 of the A chain. To determine whether iodine labeling at this position affected the binding of autoantibodies and therefore IAA affinity measurements, [125I] insulin labeled at 1 of 3 different tyrosine residues of the insulin A chain (Tyr14A, Tyr19A) or B chain (Tyr16B) were used to measure affinity in an IAA-positive relative. Although the nonspecific binding was increased when Tyr16B insulin was used as label, binding curves and IC₅₀ and Kₐ values were similar with the [125I] insulin labeled at residues Tyr14A, Tyr19A, or Tyr16B (Figure 1A), indicating that Tyr14A iodine labeling is unlikely to interfere with the binding of IAAs.

The IAA binding curve observed in the IAA-positive sample was consistent with a 1-site binding model (Figure 1, A and B) and IAAs of high affinity (1.7 × 10¹⁰ l/mol). In order to determine whether mixed IAA populations of discrete affinities could be identified by the experimental system, mixing experiments were performed by spiking of the high-affinity IAA-positive serum with serum containing low-affinity (2 × 10⁵ l/mol) or moderate-affinity (6.3 × 10⁷ l/mol) IAAs (Figure 1C). The competitive binding curves of the mixed sera were consistent with a 2-site binding model, and the calculated IAA affinities for each of the IAAs were similar to those determined in the original samples (high-low mix, 1.6 × 10¹¹ l/mol and 2.8 × 10⁸ l/mol; high-moderate mix, 1.2 × 10¹¹ l/mol and 6.2 × 10⁷ l/mol).

IAA competitive binding curves to Tyr14A [125I] insulin were consistent with a 1-site model in the first IAA-positive sample from all but 1 of the 56 children tested, suggesting that IAAs were of...
IAA affinity is high in children who develop multiple islet autoantibodies. IAA affinity was analyzed with respect to progression to multiple islet autoantibodies and to diabetes (Figure 3A). IAA affinity in the first IAA-positive sample was significantly higher in the 38 children who developed multiple islet autoantibodies (median IAA affinity, 5.4 × 10^9 l/mol; interquartile range [IQR], 2.7 × 10^9 to 1.3 × 10^10 l/mol) than in the 18 children who did not develop multiple antibodies (median, 5.2 × 10^7 l/mol; IQR, 1.2 × 10^7 to 7.0 × 10^8 l/mol; P < 0.0001). Thirty-six of the 38 children who developed multiple islet autoantibodies and all 20 children who developed T1DM had IAA affinities greater than 10^11 l/mol, compared with only 2 of 18 of the children who did not progress to multiple islet autoantibodies, including none of 5 who later became IAA negative (transient IAA). IAA affinity in a second group of IAA-positive relatives (Munich family study) was also significantly higher in relatives who had or developed multiple islet autoantibodies (median affinity, 6.9 × 10^9 l/mol) than in relatives who did not progress to multiple islet autoantibodies (median affinity, 8.1 × 10^8 l/mol; P = 0.002). Progression to multiple antibodies in both cohorts was not related to IAA titer (Figure 3B).

In comparison, affinity of insulin antibodies in patients after treatment with subcutaneous insulin was high (median affinity, 2.0 × 10^10 l/mol) and remarkably consistent between patients (IQR, 1.7 × 10^10 to 2.2 × 10^10 l/mol; Figure 3A). In contrast, IAA affinities in 2 sera from blood donors found to be IAA positive in the Diabetes Autoantibody Standardization Program (9) were low (sample M66290, 2.0 × 10^10 l/mol; sample N05151, 9.2 × 10^9 l/mol).

High IAA affinity identifies individuals who later progress to multiple islet autoantibodies. Thirty-three of the IAA-positive BABYDIAB children tested did not have other islet autoantibodies in their first IAA-positive sample from blood donors found to be IAA positive in the Diabetes Autoantibody Standardization Program (9) were low (sample M66290, 2.0 × 10^10 l/mol; sample N05151, 9.2 × 10^9 l/mol). IAA affinity is high in children who develop multiple islet autoantibodies (Figure 3A).

IAA affinity varies between children. IAA affinity in the first positive sample from the BABYDIAB children varied substantially between children and ranged from less than 10^6 l/mol to more than 10^11 l/mol (Figure 2). Affinity was not correlated with IAA titer (r = −0.016; P = 0.91) but was associated with HLA DRB1*04 (median affinity, 5.4 × 10^9 l/mol in HLA DRB1*04 children vs. 9.3 × 10^8 l/mol in non-DRB1*04 children; P = 0.002) and the age of first IAA detection (P = 0.003, Kruskal-Wallis H test). IAA affinity was greater than 10^9 l/mol in all 16 children who had IAAAs at 9 months (median affinity, 6.8 × 10^9 l/mol). Only 4 of these 16 children were positive at birth, so the high-affinity IAAAs were not due to residual maternal insulin antibodies. The majority (69%) of children in whom IAAAs were first detected at age 2 years also had high-affinity IAAAs (median affinity, 3.6 × 10^9 l/mol). In contrast, only 3 of 14 children in whom IAAAs were first detected at age 5 or 8 years had affinities above 10^10 l/mol (median affinity, 2.7 × 10^8 l/mol; P = 0.004 vs. IAA affinity in children who developed IAAAs at age 9 months or 2 years).
IAA affinity is relatively stable during follow-up. The findings indicated that a high-affinity IAA response could occur early in the natural history of T1DM, and that this was predictive of who would develop multiple islet autoantibodies and diabetes. In order to determine whether the lower-affinity responses “matured” and became of higher affinity later in childhood, IAA affinity was determined in 92 follow-up sera from 31 children (Figure 5A). Changes that were greater than 1 log were observed in 4 of these 31 children. IAA affinity increased in follow-up in only 1 of the 11 children with initial IAA affinity less than $10^9$ l/mol. This child was remarkable in that IAA was positive at age 2 years with an affinity of $10^{8}$ l/mol, became negative at age 2.7 years, and returned to positive with increased affinity ($1.4 \times 10^{11}$ l/mol) together with GAD antibodies at age 5 years. IAA affinity in a second child increased from $2.7 \times 10^9$ l/mol to $7.6 \times 10^{10}$ l/mol. Two children had decreased IAA affinity on follow-up.

IAA binding curves in follow-up samples from the child with concomitant high- and low-affinity IAAs were informative with respect to affinity maturation (Figure 5B). Samples were available at birth and at approximately 3-month intervals from ages 6 months to 21 months. Insulin antibodies at birth and at 6 months were high affinity ($7 \times 10^9$ l/mol) without a low-affinity component, consistent with the presence of maternally acquired antibodies to injected insulin. At age 9 months, insulin binding increased markedly. A high-affinity component was present with titer greater than that observed in the 6-month sample, indicating that this included de novo production of IAAs in the child. A predominant low-affinity IAA was also present. Subsequent samples at ages 12, 15, 18, and 21 months had decreasing amounts of both the high- and the low-affinity IAAs until the low-affinity component became undetectable at 18 months. None of the samples from this child contained IgM IAAs (data not shown). This child developed diabetes at age 2.8 years.

Lower-affinity IAAs show a less mature isotype and a restricted IgG subclass distribution. IAA IgG subclasses were measured in the first IAA-positive samples from 44 of the BABYDIAB children. These included 31 with IAA affinity greater than $10^9$ l/mol (30 of whom developed multiple islet autoantibodies) and 13 with IAA affinity less than $10^9$ l/mol. All 31 with high-affinity IAAs had IgG1 IAAs, and 19 of these also had IgG2, IgG3, or IgG4 IAAs. In contrast,
11 of the 13 children with low-affinity IAAs had IAAs consisting of only 1 IgG subclass \((P = 0.008\) vs. children with high-affinity IAAs), including 1 child with IgG2 IAAs, 1 with IgG3 IAAs, and 1 with IgG4 IAAs (data not shown). The restricted IgG subclass in the lower-affinity IAA samples was independent of IAA titer, which was similar in high- and low-affinity samples.

IgM antibodies are of lower affinity than IgG antibodies. Two children with high-titer low-affinity IAAs \((<10^6 l/mol)\) had high titers of IgM IAAs, and their IAA binding was abolished when the reaction was performed at room temperature, indicating that these were cold-reactive IgM antibodies to insulin. All the other sera tested had predominantly IgG component of IAAs, and were reactive both at 4°C and at room temperature (data not shown).

Affinity identifies IAAs with distinct insulin binding characteristics. The wide range of IAA affinity found between subjects suggested that there were substantial differences in the IAA-insulin interaction. We therefore examined binding to alternatively labeled insulin and insulin from different species or insulin analogs. Binding to Tyr19A \([^{125}I]\) insulin was markedly reduced relative to binding to Tyr14A \([^{125}I]\) insulin in some patients (Figure 6). Binding to Tyr19A \([^{125}I]\) insulin was significantly correlated to IAA affinity \((r = 0.57; P = 0.001)\), and IAAs of very low affinity did not bind to Tyr19A \([^{125}I]\) insulin, even when titers against Tyr14A \([^{125}I]\) insulin were high.

The affinity-related interference with IAA binding caused by labeling at residue A19 could be explained by steric hindrance of sufficient magnitude to reduce binding of the lower-affinity IAAs, or by IAA epitope differences. In order to determine whether the lower-affinity IAAs were directed against distinct epitopes, we performed competition studies using modified insulin in 65 IAA-positive subjects from the BABYDIAB \((n = 54)\) and Munich family study \((n = 11)\) cohorts (Figure 7, A and B, and Table 1). The majority of subjects \((n = 46)\) had IAAs that bound equally well to human, porcine, and human B28lysB29pro insulin, bound less to sheep A8his insulin and human A13trpB28lysB29pro insulin, and did not bind fish insulin. This pattern corresponded to binding that required conservation of the human sequence within A chain residues 8–10 and 13, but not B chain residues 28–30 (A8–10/B28–30–independent binding). A second group of subjects \((n = 6)\) had IAAs that bound equally well to all insulins and insulin analogs except fish insulin, which indicated that they were unaffected by changes at A chain residues 8–10 or 13 or B chain residues 28–30 (A8–10/B28–30–independent binding). A third group of...
subjects (n = 13) had IAAs that were affected by changes to insulin B chain residues 28, 29, or 30 (B28–30–dependent binding). These included 3 subjects with IAAs that did not bind to porcine insulin, 3 with IAAs that did not bind to human B28lysB29pro insulin (but bound well to fish insulin), and 7 with IAAs that bound neither human B28lysB29pro nor porcine insulin.

All 44 subjects with high-affinity IAAs had the A8–10/13–dependent binding pattern. In contrast, of the 21 subjects with lower IAA affinity, 13 had IAAs with a B28–30–dependent binding pattern (P < 0.0001 vs. high-affinity IAAs), 6 had IAAs with A8–10/13/B28–30–independent binding (P = 0.0007), and only 2 had IAAs with the A8–10/13–dependent binding pattern (P < 0.0001).

Figure 6
Relationship between IAA affinity and relative binding to Tyr19A [125I] insulin. Percent binding to Tyr19A [125I] insulin relative to binding to Tyr14A [125I] insulin (abscissa) is shown in relation to IAA affinities (ordinate axis) for individual sera. Children who had or developed multiple islet autoantibodies are indicated by circles and those who did not develop multiple islet antibodies by crosses. Filled symbols represent children who developed diabetes.

Figure 7
Epitope analysis of IAA. (A) Differences in amino acid sequences in the A and B chains of the insulin molecules used for competition studies of IAA binding. (B) Competitive inhibition of IAA binding to Tyr14A [125I] human insulin using human insulin (open circles), human B28lysB29pro insulin (open triangles), human A13trpB28lysB29pro insulin (shaded squares), porcine insulin (shaded circles), sheep A8his insulin (filled diamonds), and fish insulin (crosses). Five patterns were discernible and are shown by representative sera. Forty-six subjects had IAAs with the A8–10/13–dependent binding pattern represented in the top left panel. Six subjects had IAAs with the A8–10/13/B28–30–independent binding pattern represented in the middle left panel. Three subjects had IAA with the B30–dependent binding pattern represented in the top right panel. Three subjects had the B28/29–dependent binding pattern represented in the bottom panel. Seventeen subjects had the B28–30–dependent binding pattern represented in the middle right panel. The dotted line represents competition with human insulin.

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Relationship between IAA affinity and relative binding to Tyr19A [125I] insulin. Percent binding to Tyr19A [125I] insulin relative to binding to Tyr14A [125I] insulin (abscissa) is shown in relation to IAA affinities (ordinate axis) for individual sera. Children who had or developed multiple islet autoantibodies are indicated by circles and those who did not develop multiple islet antibodies by crosses. Filled symbols represent children who developed diabetes.

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IAA epitope and affinity are related to proinsulin binding. The proinsulin molecule includes a connecting peptide that alters the conformation of COOH-terminal residues of the insulin B chain (10). We therefore asked whether binding to proinsulin could distinguish affinity- and epitope-related IAA reactivity (Figure 7C and Table 1). All B28–B30–independent IAAs, including all high-affinity IAAs, were completely inhibited by both insulin and proinsulin. In marked contrast, all but 1 of the B28–B30–dependent IAAs were inhibited with proinsulin (P < 0.0001). The one exception (case 42b in Table 1) is striking, since this is the low-affinity IAA component in the child with mixed IAA populations. The low-affinity IAAs in this child were uninhibited with porcine insulin and sheep A8–13 insulin, both of which have residues at position B30, but, unlike all other B30–dependent IAAs, were readily inhibited with human proinsulin. The high-affinity IAA component in this child had A8–13–dependent proinsulin binding. Also of note is that the low-affinity IAAs in the first positive sample of the child who subsequently developed multiple islet autoantibodies (case 47) were inhibited with proinsulin. Finally, 2 children had IAAs with affinity less than 10^5 l/mol with A8–13–dependent and proinsulin binding, and in both cases IAAs were transient (cases 45 and 46).

**Discussion**

The affinity of IAAs was found to vary considerably among IAA-positive relatives. Although IAAs within individual subjects appeared relatively homogeneous, they ranged from very high-affinity IgG in most individuals to low-affinity, cold-reactive, IgM antibodies in others. High-affinity IAA responses were most frequent when IAAs developed at a very young age and in relatives who had the DRB1*04 allele. IAAs were of high affinity already in the first IAA-positive sample in almost all IAA-positive relatives who developed multiple islet autoantibodies and all who developed T1DM. In contrast, relatives who remained positive only for IAAs or became islet autoantibody–negative usually had lower-affinity IAAs that did not increase in affinity on follow-up. High-affinity IAAs differed from lower-affinity IAAs in their insulin binding characteristics in a manner consistent with distinct epitope recognition and, in contrast to the lower-affinity IAAs, always bound strongly to proinsulin. The findings indicate that the nature of early exposure to (pro)insulin is relevant to disease pathogenesis, and that IAA affinity and epitope reactivity can classify stages of autoimmunity against islet cells and stratify diabetes risk.

This is the first study to examine islet autoantibody affinity over time and, in particular, from a very young age close to the development of islet autoimmunity. The affinity of IAAs was relatively fixed from the time of first detection and was already very high in children who were IAA positive at age 9 months, indicating that the IAA response can mature quickly.

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**Table 1**

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<th>Case no.</th>
<th>Affinity^4</th>
<th>A8–10/13 dependent</th>
<th>B28/29 dependent</th>
<th>B30 dependent</th>
<th>Proinsulin binding</th>
<th>Autoantibody on follow-up</th>
<th>HLA DR4–positive</th>
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<td>No</td>
<td>Yes</td>
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<td>3/8 (37%)</td>
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<td>No</td>
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^4High affinity is greater than 10^5 l/mol. ^5Child that developed high-affinity IAAs and multiple islet autoantibodies on follow-up. ^6Low-affinity IAA component in child with mixed IAA populations. ^7Unable to determine whether residue changes in A8–10 or A13 affect binding. ^cOnly child that had GAD antibodies prior to developing IAAs. ^HLA typing was not performed in 4 subjects. Underlining indicates characteristics associated with multiple islet autoantibodies and diabetes.
The findings of this study are relevant to both pathogenesis and prediction of T1DM. With respect to pathogenesis, they indicate that the encounter between antigen and the immune system that gives rise to diabetes-relevant IAAs is likely to be of sufficient duration to evoke a high-affinity IgG response. IAAs that were of affinity less than 10^8 l/mol rarely led to multiple islet autoantibodies, even when their epitope reactivity was the same as that of the diabetes-relevant IAAs. The findings also indicate that there are probably distinct interactions between the immune system and antigen that evoke IAAs that are of lower affinity or predominantly IgM, and that these events are of insufficient magnitude or quality to start the cascade of multiple islet autoantibody responses that are associated with the development of T1DM. These low-affinity responses appear more likely to occur in the absence of the HLA DRB1*04 allele and in children over the age of 2 years. Moreover, some of these lower-affinity IAA responses became seronegative, as might be expected if the immunizing event was relatively brief and of low intensity.

The very early appearance of high-affinity IAAs with uniform binding characteristics in almost all children who subsequently developed multiple autoantibodies or diabetes suggests a consistent mode of immunization. The binding characteristics of the high-affinity diabetes-relevant IAAs were very similar to those previously described (11) and indicated that autoantibody binding is affected by changes in regions A8–A13 and unaffected by changes at the COOH-terminal end of the B chain. These autoantibodies also bound to proinsulin with high affinity. In contrast, the majority of the IAAs seen in children who did not progress to multiple islet autoantibodies bound poorly to insulin molecules altered in the B28–30 residues, and to proinsulin. It is conceivable, therefore, that the early immunizing event that precipitates the autoimmunity leading to T1DM involves the exposure of proinsulin to the immune system, and that exposure to proinsulin preferentially results in IAAs with A8–13–dependent binding. From a viewpoint of structure, both the A8–13 and the B29–30 sites are accessible on the surface of monomeric and hexameric insulin (20, 21), whereas residues B29 and B30 are conformationally altered in proinsulin because of covalent bonding between B30 and A1 (10). Also consistent with a proinsulin immunization model, autoreactive T cells against proinsulin-specific peptides have been detected in patients with T1DM or preclinical T1DM and in HLA DR4 transgenic mice (22–26). Proinsulin-specific antibodies, however, were rarely found in our cohort and were never found prior to the appearance of IAAs (27). Moreover, insulin antibodies generated after insulin treatment in patients with T1DM, type 2 diabetes, or gestational diabetes were similar to the high-affinity IAAs (A13 dependent, proinsulin reactive), suggesting that exposure to proinsulin is not essential in order to generate diabetes-relevant IAAs (K. Koczwarza et al., unpublished observations). Regardless of whether exposure to proinsulin or proinsulin leads to diabetes-relevant autoimmunity, it is likely that some of the lower-affinity IAAs result from immunization to a molecule(s) other than insulin or proinsulin. Persistence of the response without transition to high-affinity IAAs is likely to reflect either prolonged presence of antigen that is not insulin (IAAs resulting from cross-reactivity) or continuous exposure to insulin that does not become limiting for clonal antigen competition. The rare occurrence of transition from low-to-high-affinity IAAs indicates that the presence of B lymphocytes that have low-affinity receptors for insulin is unlikely to directly lead to higher-affinity IAAs through antigen presentation or affinity maturation. Exceptions to this were observed in 2 children who had low-affinity IAAs that were proinsulin reactive.

With respect to prediction, the findings indicate that the measurement of IAA affinity in children who are only IAA-positive will be helpful in distinguishing who is more likely to develop multiple islet autoantibodies and to progress to T1DM. Identification of the low-affinity IAAs could be achieved by competitive binding with a few critical concentrations of cold insulin, insulin analog, or proinsulin, or, for some sera, simply by measurement of IAAs at room temperature or against alternatively labeled insulin. In view of the relatively high frequency of IAAs that is detected in young children, these modifications to the IAA assay will prove useful for improving specificity.

Methods

Study cohort. BABYDIAB prospectively follows offspring of parents with T1DM from birth with venous blood sampling and collection of questionnaire data at birth (cord blood) and at ages 9 months and 2, 5, 8, and 11 years (8). A total of 1,610 children were recruited at birth and followed at least until the 9-month visit. The median follow-up time was 6.5 years, range 9 months to 12.5 years, for a total of 9,480 subject years. Sera from all participants of the BABYDIAB study were tested for the presence of IAA, GAD antibodies, and IA-2 antibodies at follow-up visits, and frequent blood samples (3–12-month intervals) were subsequently obtained from antibody-positive children. All children were prospectively monitored for the development of diabetes, which was diagnosed using oral glucose tolerance tests by WHO/American Diabetes Association criteria (28). Monitoring in autoantibody-positive children was performed by oral glucose tolerance tests every 6–12 months and measurement of monthly random blood glucose values. Written informed consent was obtained from the parents. The study was approved by the ethical committee of Bavaria, Germany (Bayerische Landesarztekammer no. 95357).

Subjects and samples. A total of 68 BABYDIAB children developed IAAs that were confirmed positive in a consecutive (n = 66) or subsequent follow-up sample. Fifty-six of the 68 children were selected for our study on the basis of having sufficient serum available from their first positive sample for IAA affinity measurement. Of these 56 children, 16 were IAA positive at age 9 months, a further 26 became IAA positive at age 2 years, and another 14 became IAA positive at age 5 or 8 years. Twenty-three also had antibodies to GAD or IA-2 in their first antibody-positive sample (1 had GAD antibodies prior to IAAs), another 15 developed GAD and/or IA-2 antibodies in follow-up samples, and 18 either remained IAA positive only (n = 13) or became islet autoantibody negative (n = 5) on follow-up. Twenty of the 56 children developed diabetes (median follow-up after first autoantibody-positive sample for all 56 children, 3.3 years; IQR, 1.9–5.2 years).

In order to verify the findings from the BABYDIAB cohort, IAA affinity was measured in a second group of 16 IAA-positive nondiabetic relatives selected from the Munich family study cohort (median age, 9.1 years; IQR, 6.0–15.1 years) (29). Eleven of these 16 relatives developed multiple islet autoantibodies, and 8 developed T1DM.

IAA affinity was also determined in sera from 11 patients with T1DM (median age, 13.1 years; IQR, 11.6–16.3 years) who had been treated with insulin injections for a median duration of 3.1 years (IQR, 2.8–3.3 years). All patients had high-titer insulin antibodies (median IAA titer, 70.8 units; IQR, 42.2–184.2 units) consistent with the presence of exogenously induced insulin antibodies.

Islet autoantibody measurements. IAAs, GAD antibodies, and IA-2 antibodies were measured by protein A/G radiobinding assays as previously described (2, 30) using Tyr4A[125I]-labeled human insulin (Aventis Pharma Deutschland GmbH, Bad Soden, Germany) and [35S] 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. For IAAs, there was a linear relationship between the units and the immunoprecipitated counts per minute (cpm) that extended beyond 800 units (around 5,000 cpm). No samples had IAAs above 800 units. The thresholds for positivity in each assay corresponded to the 99th percentile of control subjects. These assays had sensitivities (positivity in 50 patients with new-onset TIDM) and specificities (negativity in 100 blood donors) of 84% and 96%, respectively, for GAD antibodies; 66% and 100%, respectively, for IA-2 antibodies; and 64% and 99%, respectively, for IAAs in the Third Diabetes Autoantibodies Standardization Proficiency Workshop. For some experiments, [125I] insulin labeled at amino acid 19 of the insulin A chain (Tyr[14]A; Aventis Pharma Deutschland GmbH; kindly provided by Raymond Oekonomopulos) was used to determine IA A binding.

IGG subclasses and isotypes of IAAs were determined by radiobinding assays as previously described (31) using IGG subclass– or isotype-specific biotin-labeled mouse anti-human mAb’s (BD, San Diego, California, USA) bound on Sepharose 4B streptavidin beads (Zymed Laboratories Inc., San Francisco, California, USA). The antibodies used were mouse mAb’s against human IgG1 (clone G17-1), IgG2 (clone G18-21), IgG3 (clone HP6047; Zymed Laboratories Inc.), IgG4 (clone JDC-14), and IgM (clone G20-127). Nonspecific binding was determined for each serum using beads coated with anti-rat IgM mAb (clone G20-127). Results for IAAs subclasses were expressed as nanounits insulin bound per milliliter after subtraction of nonspecific binding. The cutoff for positivity for each IAA IGG subclass and isotype was 150 nU/ml (mean plus 3 SD of IAA-negative control subjects).

Cytoplasmic islet cell autoantibodies were detected by the indirect immunofluorescence test on unfixed cryostat sections of human pancreas from an organ donor with blood group 0 as previously described (2).

IAA affinity measurement. Affinity was measured by competitive binding experiments. Briefly, 5 μl serum was incubated in duplicate for 72 hours in TBT buffer (50 mM Tris, 1% Tween-20, pH 8.0) in the presence of 7.85 femtomoles of human [125I] insulin (Aventis Pharma Deutschland GmbH) labeled at the tyrosine at position 14 of the A chain of the insulin molecule (Tyr[14]A) (10 μCi/ml; 0.143 nM) with or without increasing quantities of unlabeled human insulin (2.6 × 10−17 to 1.7 × 10−6 mol; Aventis Pharma Deutschland GmbH) in a final volume of 55 μl immunocomplexes were precipitated using protein A/G-Sepharose, and washed as previously described for the IAA assay (30). Bound [125I] insulin was measured using a gamma counter (Packard Instrument Co., Meriden, Connecticut, USA). Nonspecific binding determined as the binding of an IAA-negative control serum to [125I] insulin in presence of 1.38 nmol of unlabeled human insulin was subtracted for each experiment, and results were expressed as counts per minute (cpm). IC50 and IC90 values were calculated using the GraphPad Prism 3 program (GraphPad Software Inc., San Diego, California, USA), and IAA affinity was expressed as reciprocal IC50 values (l/mol). The reproducibility of IAA affinity measurements was determined from replicates of an IAA-positive serum that was included in each experiment. The mean ± SD affinity of this sample was 1.69 × 10−11 ± 0.28 × 10−11 l/mol (interassay coefficient of variation, 16.2%; n = 10 experiments). In some experiments, [125I] insulin labeled at position 19 of the A chain (Tyr[19]A; Aventis Pharma Deutschland GmbH) or at position 16 of the B chain of insulin (Tyr[14]B; Aventis Pharma Deutschland GmbH; kindly provided by Raymond Oekonomopulos) was used instead of Tyr[14]A [125I] insulin.

Epitope analysis. In order to determine whether IAAs from different subjects bound similar epitopes, binding to Tyr[14]A [125I] insulin was competed with increasing amounts of cold insulin from different species (human [Insuman Rapid; Aventis Pharma Deutschland GmbH], porcine [Aventis Pharma Deutschland GmbH], and salmon [coho salmon, Oncorhynchus kisutch; a gift from Erika M. Plietskaya, University of Washington, Seattle, Washington, USA], with insulin analogs [human B28lysB32por [Humalog; Eli Lilly and Co., Indianapolis, Indiana, USA], human A13trpB28lysB29por [a gift from Panayotis G. Katsoyannis, Mount Sinai School of Medicine, New York University, New York, New York, USA], and sheep A8his [from P.G. Katsoyannis]), and with human proinsulin (Eli Lilly and Co.). The competing antigens were added to the standard IAA radiobinding assay at increasing concentrations ranging from 1.7 × 10−16 to 6.8 × 10−6 M.

HLA typing. HLA DRB1/DQB1 alleles were typed using PCR-amplified genomic DNA and sequence-specific oligonucleotide probes (32).

Statistical analysis. The Mann-Whitney U test or the Kruskal-Wallis H test was used to compare IAA affinities between groups. Multivariate analysis for significant variables was performed by multiple regression. Fisher’s exact test was used to compare prevalence between groups. Spearman’s correlation was used to determine the correlation between variables. Life table analysis was used to compare outcome (multiple-autoantibody status and diabetes status) for children with different IAA affinity categories (<10−6 l/mol or >10−6 l/mol). The time between first IAA-positive sample and first multiple islet autoantibody–positive sample or last sample was defined as the time to event for multiple-autoantibody status. The time between first antibody-positive sample and diagnosis of diabetes or last contact was defined as the time to event for diabetes status. Significance of differences between groups was determined using the log rank test. For all analyses, a 2-tailed P value of 0.05 was considered significant. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 11.0, SPSS Inc., Chicago, Illinois, USA).

Acknowledgments

This work was supported by grants from Deutsche Forschungsgemeinschaft (ZI 310/12-5), the Juvenile Diabetes Research Foundation (JDRF no. 1-2003-646), and the Eli Lilly Research Foundation. The authors thank Raymond Oekonomopulos for providing radiolabeled insulin, Panayotis G. Katsoyannis for providing insulin analogs, Erika M. Plietskaya for providing fish insulin, Ulrike Mollenhauer and Karolina von Dalwigk for technical support, and Markus Walter and Michael Hummel for clinical assistance.

Received for publication February 11, 2004, and accepted in revised form June 22, 2004.

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