Autoantibody Appearance and Risk for Development of Childhood Diabetes in Offspring of Parents With Type 1 Diabetes

The 2-Year Analysis of the German BABYDIAB Study

Anette-G. Ziegler, Michael Hummel, Michael Schenker, and Ezio Bonifacio

The temporal development of autoantibodies was studied in 1,353 offspring of parents with type 1 diabetes. Islet cell antibodies (ICAs) and autoantibodies to insulin (IAAs), glutamic acid decarboxylase, and IA-2 were measured at birth, 9 months, 2 years, and 5 years of age. At birth, no offspring had islet autoimmunity other than maternally acquired antibodies, which were shown to influence antibody prevalence up to age 6 months. Antibodies detected thereafter were likely to represent a true de novo production, since prevalences were the same for offspring from mothers and fathers with diabetes, antibodies detected at 9 months were almost always confirmed in the 2-year sample and were associated with an increased likelihood of having or developing other antibodies. By 2 years of age, autoantibodies appeared in 11% of offspring, 3.5% having more than one autoantibody. IAAs were detected most frequently, and few had autoantibodies in the absence of IAAs. In 23 offspring with multiple islet autoantibodies, IAAs preceded other antibodies in 10 cases and were first detected concurrently with other antibodies in 12 and after detection of other antibodies in 1. Development of additional antibodies and changes in levels, including decline of IAAs at older age, was frequent. Nine children, all with IAAs and ICAs, developed diabetes. Overall cumulative risk for disease by 5 years of age was 1.8% (95% CI 0.2–3.4) and was 50% (95% CI 19–81) for offspring with more than one autoantibody in their 2-year sample. Autoimmunity associated with childhood diabetes is an early event and a dynamic process. Presence of IAAs is a consistent feature of this autoimmunity, and IAA detection can identify children at risk. *Diabetes* 48:460–468, 1999

From the Diabetes Research Institute and 3rd Medical Department (A.-G.Z., M.H., M.S.), Krankenhaus München-Schwabing, Germany; and Institutio Scientifico San Raffaele (E.B.), Milan, Italy.

Address correspondence and reprint requests to Priv.-Doz. Dr. Anette-G. Ziegler, Diabetes Research Institute, Koelner Platz 1, D-80804 Munich, Germany. E-mail: anziegler@lrz.uni-muenchen.de.

Received for publication 29 May 1998 and accepted in revised form 2 November 1998.

BSA, bovine serum albumin; GADA, glutamic acid decarboxylase autoantibody; IA2A, antibody to the protein tyrosine phosphatase IA-2; IAA, insulin autoantibody; ICA, islet cell autoantibody; JDF, Junvenile Diabetes Foundation; PEG, polyethylene glycol.

Autoantibodies to β-cell antigens precede the development of type 1 diabetes and are commonly used as sensitive markers to identify the preclinical period of the disease (1–4). The age when autoantibodies first appear and the risk associated with their early appearance are still poorly defined. Both are important for understanding the pathogenesis of the disorder and for timing of first-line screening and intervention, and several studies are now underway to evaluate the development of autoimmunity from birth (5–9). BABYDIAB, a study from birth in children of parents with type 1 diabetes, was initiated in 1989 and has up to 8 years' prospective follow-up (5). Samples for islet autoantibody measurement are collected at birth, 9 months, 2 years, 5 years, and 8 years, and all children are followed for the development of diabetes to determine when autoimmunity commences, which are the initial targets of autoimmunity, what is the risk for disease, and what factors are associated with increased risk for autoimmunity and diabetes development in childhood.

Critical in determining when autoantibodies appear is appropriate measurement and accurate identification of positive signals. Measurement of islet autoantibodies has improved markedly since the introduction of antigen-specific radiobinding assays (10–12). Selection of thresholds useful for exclusion or prediction of disease has also been addressed (13). Difficulty remains, however, in determining thresholds that identify the presence of a humoral autoimmune response. Parametric and nonparametric methods have been used to determine when signals are outside normal limits, but several factors complicate the application of these traditional methods to sequential samples of a childhood cohort. First, it is unclear how age affects the distribution of islet autoantibody measurements, and the use of age-matched control populations is not always feasible, since sufficient numbers of such controls are not readily available, not least of all for ethical reasons. Second, measurement in control populations is not always performed at the same time as that in the cohort under analysis, and assay performance can change with time. Third, in the specific case of type 1 diabetes, autoantibodies appear many years before the onset of clinical disease, and without lifelong follow-up it is not possible to determine which control subjects are truly diabetes-free. An alternative is to use the cohort under analysis to define thresholds. This is inherent for studies of antibody

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

prevalences in the general population where thresholds must be selected based on the distribution of signals in that cohort (13). We now use a novel approach to analyze the BABYDIAB data with probability plots to define thresholds that identify signals associated with autoimmunity. Based on this analysis, we present the 2-year analysis of the BABYDIAB Study and examine risk estimates on the appearance of islet autoantibodies in early life, the sequence of antibody responses to the β-cell antigens insulin, GAD65, and IA-2, and the predictive value of antibody screening at 2 years of age for progression to type 1 diabetes in early childhood.

RESEARCH DESIGN AND METHODS

Offspring of parents with type 1 diabetes. BABYDIAB is a prospective German multicenter study that schedules for regular visits with venous blood sampling at birth (cord blood), and at 9 months and 2, 5, and 8 years of age in offspring of parents with type 1 diabetes (5,14). Recruitment into the study began in 1989 after all general practitioner pediatricians, hospital diabetes outpatient departments, and hospital obstetric departments in Germany were informed of the study by letter. Families entered into the study on a voluntary basis with written consent. Ethical committee approval for the study was granted by the Bayrische Landesärztekammer. At the time of this analysis (April 1998), 871 newborn children of a mother with type 1 diabetes, 467 newborn children of a father with type 1 diabetes, and 15 newborn children of two parents with type 1 diabetes had been recruited at birth and followed up to the age of 8 years (median follow-up, 0.9 years, range 0.1–8.5 years). In seven of the families, the newborn also had a sibling with type 1 diabetes. For analysis, children with two diabetic parents were assigned to the group of the parent with earlier age of onset (10 to children of diabetic mothers and 5 to children of diabetic fathers). Of the 1,353 recruited, samples from 986 children (695 of mothers, 291 of fathers) were obtained for their 9-month visit between 0.5 and 1.5 years (median age 0.85). For the 2-year visit, samples from 554 children (402 of mothers, 152 of fathers) were obtained between 1.5 and 2.5 years of age (median age 2.16). For the 5-year visit, samples from 114 children (99 of mothers, 15 of fathers) were obtained between 4.5 and 5.5 years of age (median age 4.93). Five participated at the 8-year visit. Details of follow-up ascertainment and dropout from the study are shown in Table 1. Blood samples were tested for islet cell antibodies (ICAs) and antibodies to insulin (IAAs), glutamic acid decarboxylase (GADAs), and the protein tyrosine phosphatase IA-2 (IA2As). From 3 years of age, an oral glucose tolerance test was performed yearly in those who were identified to have islet autoantibodies. Type 1 diabetes diagnosis was defined according to World Health Organization criteria, and treatment with insulin commenced on the day of diagnosis.

To examine how long after birth maternally acquired antibodies remain detectable in offspring serum, samples obtained in the first 6 months after birth from 114 offspring of parents with type 1 diabetes were also analyzed together with 9-month samples from BABYDIAB offspring. These additional offspring were not recruited at birth and were not part of the BABYDIAB Study.

Autoantibody determination

IAAs. Autoantibodies to insulin were measured with a competitive fluid-phase radiobinding assay. Serum (150 µl) was incubated in duplicate with 50 µl of assay buffer (0.04 mol/l phosphate buffer, pH 7.5, containing 0.05% bovine serum albumin [BSA], 0.025% bovine γ-globulin, and 150 mmol/l NaCl) for at least 1 h at 4°C with and without the addition of unlabeled human insulin (H-Insulin 100; Hoechst, Frankfurt, Germany) at a concentration of 9 mU/ml (360 ng/ml; equivalent to 3 mU/ml or 120 ng/ml serum). Assay buffer (200 µl) containing 7 µU/ml (0.3 ng/ml; equivalent to 10 µU/ml or 0.4 ng/ml serum) of ^{125}I-labeled human insulin (Hoechst; specific activity 360 mCi/mg) was added, and tubes were incubated for 6 days at 4°C before the addition of 1.5 ml of 14.3% polyethylene glycol (PEG), centrifugation, washing in 11% PEG, centrifugation, and counting of the pellets in a gamma counter for 9 min. The counts per minute (cpm) specifically precipitated after subtraction of cpm obtained in tubes containing excess cold insulin were expressed as nanounits of bound insulin per milliliter of serum. Negative IAA levels were obtained when the counts with cold displacement were slightly greater than counts in the absence of unlabeled insulin.

GADAs and IA2As. Antibodies to in vitro translated [^{35}S]GAD65 or [^{35}S]IA-2ic were measured by immunoassay as previously described (11). cDNA for GAD65 was cloned into the pEx9 in vitro transcription vector (gift from Dr. A. Lernmark, Seattle, WA), and IA-2ic was cloned in the vector pGEM-4Z (gift from Dr. M. Christie, London, U.K.). GAD65 and IA-2_{ic} were transcribed and translated in vitro using commercial kits (Promega, Madison, WI) in the presence of [^{35}S]methionine (Amersham, Aylesbury, U.K.), and 15,000 cpm [^{35}S]GAD65or [^{35}S]IA-2ic in 50 µl immunoprecipitation buffer (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.15% Tween 20, 0.1% aprotinin, 10 mmol/l benzamidine, 0.1% BSA) were incubated with 2 µl serum overnight at 4°C. Immune complexes were isolated on 50 µl of 50% protein A Sepharose (Pharmacia, Uppsala, Sweden) in BSA-coated, prewashed 96-well filtration plates (Multiscreen-DV; Millipore, Eschborn, Germany), and immunoprecipitates were washed extensively with washing buffer (20 mmol/l Tris, pH 7.4, 150 mmol/l NaCl, 0.15% Tween 20, 0.1% BSA). After adding scintillation fluid (Microscint; Packard, Meriden, CT), the plates were counted in a scintillation counter (Top Count; Packard). Results were expressed as units relative to a positive serum with an arbitrary value of 100 U. Units were calculated as follows:

$$\frac{[\text{mean cpm}_{\text{sample}} - \text{mean cpm}_{\text{neg.control}}] \times 100}{[\text{mean cpm}_{\text{pos.sample}} - \text{mean cpm}_{\text{neg.control}}]}$$

ICA. ICAs were measured by indirect immunofluorescence and end-point titers of test samples converted to Juvenile Diabetes Foundation (JDF) units (15).

Thresholds and assay specificity and sensitivity. The previously determined 99th percentile of antibody levels in nondiabetic control children was 50 nU/ml for IAAs, 13 U for GADAs, 5 U for IA2As, and 5 JDF units for ICAs (14). Antibody assays were entered into the Antibody Proficiency Program (Immunology of Diabetes Society), and using these thresholds, the IAA assay achieved a specificity of 100% and a sensitivity of 100%, the GADA assay 100 and 94%, the IA2A assay 100 and 100%, and the ICA assay 100 and 67%, respectively. The interassay coefficient of variation for samples with 168 nU/ml IAA was 11%, those with 13 U of GADA 18%, and those with 14 U of IA2A 16%.

Data analysis. Q-Q probability plots were used to analyze the distribution of IAA, GADA, and IA2A measurements for normality and the presence of outliers (16). For each observation, observed antibody values were plotted along the horizontal axis against expected standard deviation values under normality on the vertical axis using Blom's proportion estimation formula (r – [3/8]/n + [1/4]). This was not possible for ICAs because of the semiquantitative measurement of the assay, and the threshold of 5 JDF units was used to define positivity. Kaplan-Meier life-table analysis was used to determine the cumulative risk of antibody development and progression to type 1 diabetes. For the appearance of antibodies, follow-up started at birth and ended when antibodies were first detected or the day of the most recent blood sample. In children from mothers with diabetes, antibodies detected before age 0.5 years were excluded because of potential interference from antibodies acquired transplacentally, and in children from fathers with diabetes, IAAs detected at birth were excluded because of potential artifact in IAA measurement in cord blood (14,17). Antibody events were experienced at intervals between 0.5 and 1.5 years (9-month visit), 1.5 and 2.5 years (2-year visit), and 4.5 and 5.5 years (5-year visit). For progression to diabetes, follow-up started at birth and ended with diabetes onset or with the day of last contact with the family. Differences in the probability of antibody appearance or diabetes-free survival between groups were determined by the log-rank test. 95% CIs for the cumulative risk were calculated from the standard error thereof. For all statistical methods, the Statistical Package for Social Sciences (SPSS, Chicago, IL) was used.

RESULTS

Islet autoantibody distribution and age. The distribution of IAAs, GADAs, and IA2As in offspring are shown in Fig. 1 as Q-Q probability plots, where for each observation, observed antibody values were plotted against expected standard deviation values under normality. At each age and for each antibody,

TABLE 1
Follow-up of offspring recruited into the BABYDIAB Study

	Number of offspring from parents (mothers/fathers) with type 1 diabetes			
Sample visit age	Reached follow-up visit age	Participated in follow-up visit	Dropped out from study	Dropout rate (%)
9 Months	1,031 (733/298)	986 (695/291)	45 (38/7)	4.4
2 Years	599 (434/165)	554 (402/152)	45 (32/13)	7.5
5 Years	126 (110/16)	114 (99/15)	12 (11/1)	9.5

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

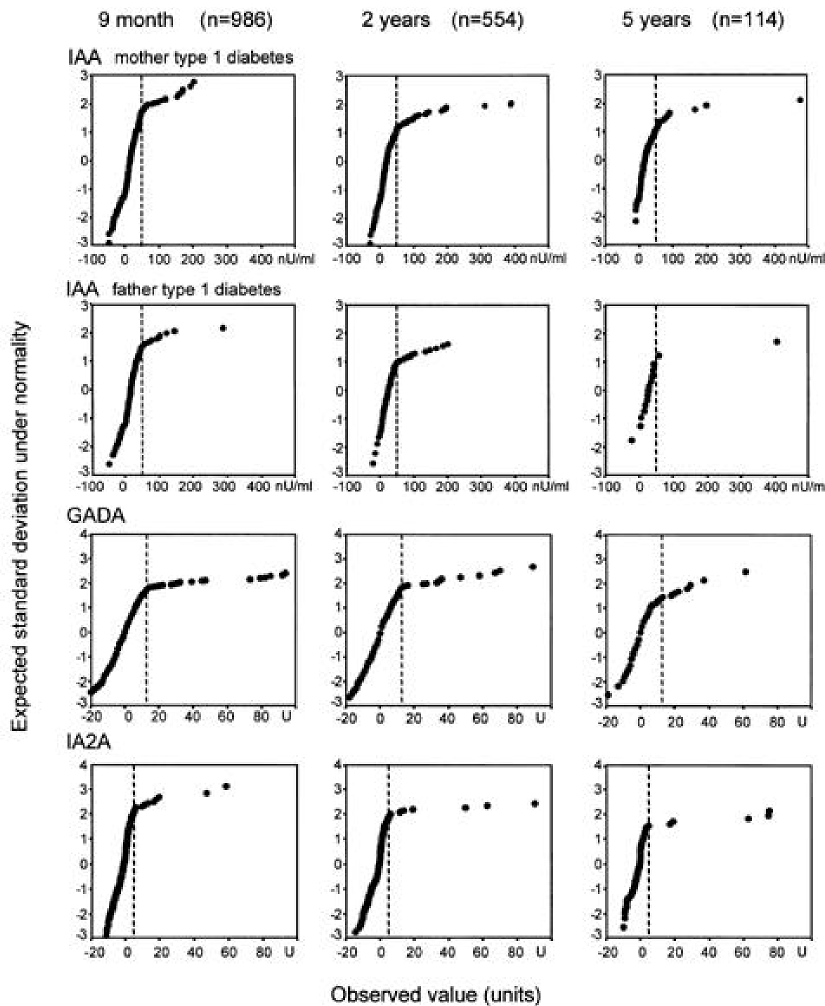

FIG. 1. Normal plots of IAAs, GADAs, and IA2As in offspring of parents with type 1 diabetes at 9 months, 2 years, and 5 years of age. Observed antibody values (abscissa) are plotted against the expected standard deviation under normality (ordinate). If the observation data follow a normal distribution, the plot lies close to a straight line. Inflection indicates deviation from normality and, in this case, identifies two distinct populations of signals in the antibody assays. The vertical broken line indicates the assay thresholds, which were previously determined from the 99th centile of control subjects.

the Q-Q plot shows two distinct populations that meet at an inflexion point indicating deviation from normality. The population on the left of the inflexion point is interpreted to be a close-to-normal distribution of signals around zero or undetectable antibody, and that on the right a population with distinctly different signals in the antibody measurement and therefore not zero. For antibody distributions at 9 months and 2 years, the point of inflexion for individual antibodies was remarkably similar between children from mothers with diabetes and those from fathers with diabetes and between boys and girls (data not shown). This suggests that the signals obtained in the antibody assays are not affected by age over the range 9 months to 2 years or by sex of offspring or proband. At 5 years, the points of inflexion are less clear, probably as a result of fewer samples in the distributions. The inflexion points at 9 months and 2 years were around 50 nU/ml for IAAs, 13 U for GADAs, and 5 U for IA2As and corresponded to previously determined 99th centiles of antibody levels in control sera (14). The Q-Q plots therefore support these thresholds for distinguishing offspring with specific antibody signals at each age group under investigation, and the thresholds were used to define positivity in the remainder of the analysis.

Likelihood of antibody persistence or appearance of a second antibody. To further validate the selected thresholds, the likelihood of antibody persisting or of the appearance of a second antibody at 2 years was examined over the range of measurement in all offspring in whom samples were obtained from both the 9-month and 2-year visits (Fig. 2). For IAA measurements at 9 months, the majority (60%) of offspring with levels >60 nU/ml had antibody (IAAs, GADAs, IA2As, or ICAs) in their 2-year sample, 40% of offspring with levels between 50 and 60 nU/ml had antibody in their 2-year sample, and 11% of offspring with levels <50 nU/ml had antibody in their 2-year sample. At 2 years, only IAA levels >80 nU/ml were associated with the detection of a second antibody (GADAs, IA2As, or ICAs) in the same sample. For GADAs, 60% of children with levels >13 U at 9 months and 17% with levels <13 U had antibody at 2 years. All those with GADAs >13 U and only 10% with GADAs <13 U at 2 years had a second antibody in the same sample. For IA2As, 75% of children with levels >5 U at 9 months and 16% with levels <5 U had antibody at 2 years; 83% with IA2As >5 U and 11% with IA2As <5 U at 2 years had a second antibody in the same sample. For each antibody, the correspondence of an increased likelihood of either antibody persisting or of the appearance of a second antibody at 2 years with the point of inflexion in the Q-Q plot was remarkable and further supported the use of those levels as thresholds for antibody appearance.

Influence of maternal antibodies. It is not certain how long maternal antibodies remain in the circulation of offspring and how they affect antibody measurement in the 9-month sample. To identify a time point at which antibody signals in offspring were no longer likely to be due to circulating maternal antibodies, the prevalence of IAAs with respect to age

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

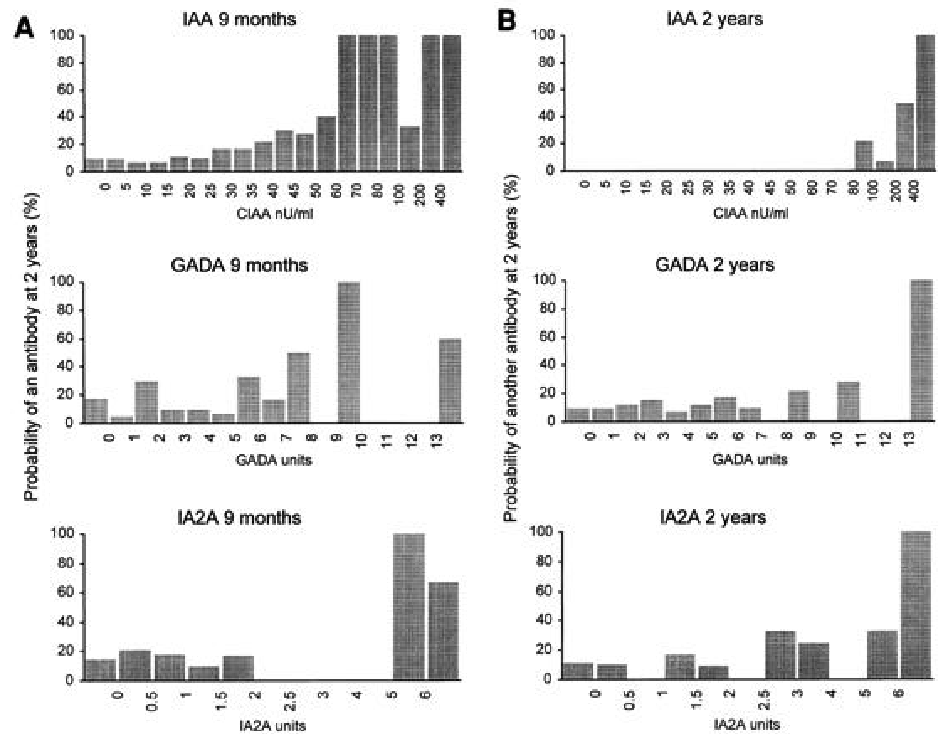

FIG. 2. Likelihood of antibodies persisting or of having a second antibody over the range of measurement for IAAs, GADAs, and IA2As. *A*: Proportion of subjects having an antibody (IAAs, GADAs, IA2As, or ICAs) detected at 2 years for increasing levels of IAAs, GADAs, and IA2As measured at 9 months. *B*: Proportion of subjects having a second antibody detected at 2 years for increasing levels of IAAs, GADAs, and IA2As measured at 2 years.

was compared between offspring from mothers and fathers with type 1 diabetes (Fig. 3). Samples after birth were obtained from day 1 to 1.5 years in 787 offspring of mothers with type 1 diabetes and 317 offspring of fathers with type 1 diabetes. Offspring from fathers with type 1 diabetes showed a high antibody prevalence (59%) in samples collected within the first 0.1 years of age, confirming the altered distribution of IAA measurements in samples close to birth (14,17). IAA prevalences at every interval after 0.1 years of age were <10%. Offspring from mothers with type 1 diabetes had high prevalences of IAAs up to 0.5 years of age, and a lower stable prevalence similar to that in offspring from fathers with diabetes after this age. This suggests that maternal antibodies are likely to be detected up to 6 months, and that signals obtained after that age are due to autoantibodies from the child.

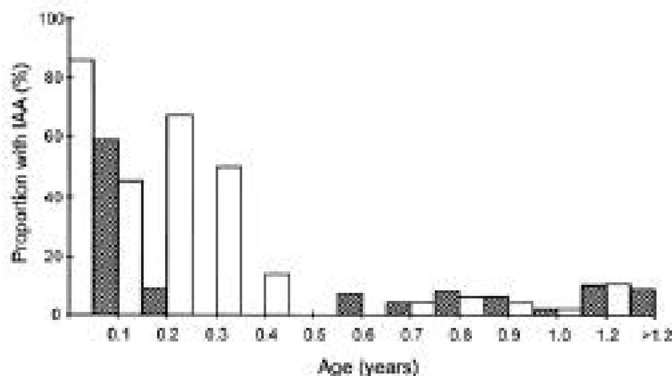

FIG. 3. Interference of maternal antibodies in early autoantibody measurements. The prevalence of IAAs in offspring from mothers (□) and fathers (▨) with type 1 diabetes is shown relative to the age of blood sampling from day 1 until 1.5 years of life. IAA prevalence in offspring from mothers decreases to that in offspring from fathers with type 1 diabetes at around 0.5 years of age.

Appearance of elevated islet autoantibodies. Table 2 summarizes the probability of antibody development calculated by Kaplan-Meier life-table analysis in offspring. The probability to develop at least one antibody (IAAs, GADAs, IA2As, or ICAs) was 4.4% by the 9-month sample, 11.1% by the 2-year sample, and 14.6% by the 5-year sample. No significant difference between offspring of mothers and fathers with type 1 diabetes was observed. The probability to develop more than one elevated autoantibody was 0.6% by 9 months, 3.5% by 2 years, and 4.4% by 5 years. IAAs were detected earlier and more frequently than any other autoantibody marker (Fig. 4). Most IAA events occurred by the 2-year sample visit, and the probability of developing IAAs by this age was 10.9% (95% CI 8.5–13.3). For GADAs, IA2As, and ICAs, a consistent increase in the cumulative probability of events at each of the 9-month, 2-year, and 5-year sample visits was observed. The probability of developing GADAs by 2 years was 2.9% (95% CI 1.7–4.1), IA2As 2.6% (95% CI 1.4–3.8), and ICAs 2.1% (95% CI 1.0–3.2).

Offspring with elevated islet autoantibody levels. In total, 87 offspring had antibodies in their 9-month, 2-year, or 5-year sample. IAAs were found in 77, GADAs in 27, IA2As in 21, and ICAs in 16 cases. In 36, a follow-up sample was obtained after the visit in which antibodies were first detected; 19 of those had more than one elevated β-cell autoantibody in the first or follow-up sample. A further 7 had one elevated antibody (in all cases IAAs) in the first and follow-up samples, and another 10 showed transient elevations of antibody, being below the threshold in the most recent blood sample (8 with IAAs, 1 with GADAs, and 1 with IAAs and GADAs). The remaining 51 offspring with elevated antibodies were not yet tested in a follow-up sample (38 with IAAs only, 6 with GADAs only, 3 with IA2As only, and 4 with multiple autoantibodies).

Sequence of appearing antibodies. IAAs were detected in the first sample with islet autoantibodies in 76 (87%) of the 87 offspring, GADAs in 17 (20%), IA2As in 13 (15%), and ICAs in

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

TABLE 2
Probability of autoantibody development in offspring of parents with type 1 diabetes

	Sample visit age		
	9 Months	2 Years	5 Years
n	985	554	110
Probability of first antibody event			
Mother with diabetes	4.4 (2.9–6.1)	11.6 (8.7–14.5)	15.5 (10.9–20.1)
Father with diabetes	4.2 (1.1–7.3)	9.7 (4.4–14.0)	9.7 (4.4–14.0)
Total	4.4 (3.1–4.7)	11.1 (8.7–13.5)	14.6 (10.6–18.6)
Probability of first multiple-antibody event			
Mother with diabetes	0.4 (0.0–1.4)	3.4 (1.7–5.1)	4.4 (1.9–6.9)
Father with diabetes	0.9 (0.1–1.7)	3.6 (0.8–6.4)	3.6 (0.8–6.4)
Total	0.6 (0.1–1.1)	3.5 (2.0–5.0)	4.4 (2.1–6.7)

Data are % (95% CI).

5 (6%). In the 23 offspring with multiple islet autoantibodies (Table 3), IAAs preceded the development of other antibody markers in 10 cases (43%), and in a further 12 (52%) offspring, IAAs were detected in the first sample, together with other antibody markers. In only one case (4%), GADAs preceded the development of IAAs. Other antibodies preceded the development of GADAs in 10 offspring, IA2As in 8, and ICAs in 11. In the 9-month sample, 11 (48%) of those in the group with multiple antibodies had IAAs, 5 (22%) had GADAs, and 2 (9%) had IA2As. By 2 years, all had IAAs, 15 (65%) had GADAs, and 16 (70%) had IA2As.

Antigen spreading and decline of IAAs. Spreading of antibody reactivity to other antigens was observed in 14 offspring (Tables 3 and 4). In some (cases 1032, 1649, and 1088), spreading was rapid, while in others (case 1724), additional antibodies did not develop until several years after initial autoantibody appearance. In 12 offspring, multiple antibodies were found in the first sample with autoimmunity. Antibody values of five cases with multiple antibodies and a follow-up of at least 5 years are shown in Table 4. In cases 1872, 1032, and 1724, IAAs were the first detectable antibodies. Reactivity to ICAs (case 1872) or GADAs (cases 1032 and 1724) and IA2As (cases 1032 and 1724) developed on follow-up. In case 1649, GADAs preceded the development of IAAs, IA2As, and ICAs, and in case 1088, IAAs and IA2As appeared at the same age and GADAs developed later. In three of these five cases, IAA levels decreased remarkably during follow-up, from peak values of 1,185, 974, and 599 nU/ml to values of 87, 199, and 50 nU/ml in the last sample before diagnosis or the sample of the most recent follow-up.

Diabetes development. Nine of 1,353 offspring developed type 1 diabetes during childhood (life-table risk at 5 years of age 1.8%, 95% CI 0.2–3.4) (Fig. 5*A*). As shown in Table 2, all 9 (100%) had IAAs and ICAs in at least one serum sample before diagnosis of diabetes, and 7 (78%) had all four autoantibodies (IAAs, GADAs, IA2As, and ICAs) before diagnosis. By 9 months, 2 had multiple antibodies, 4 had a single antibody, and 3 had no islet antibodies. By 2 years, all but one had at

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

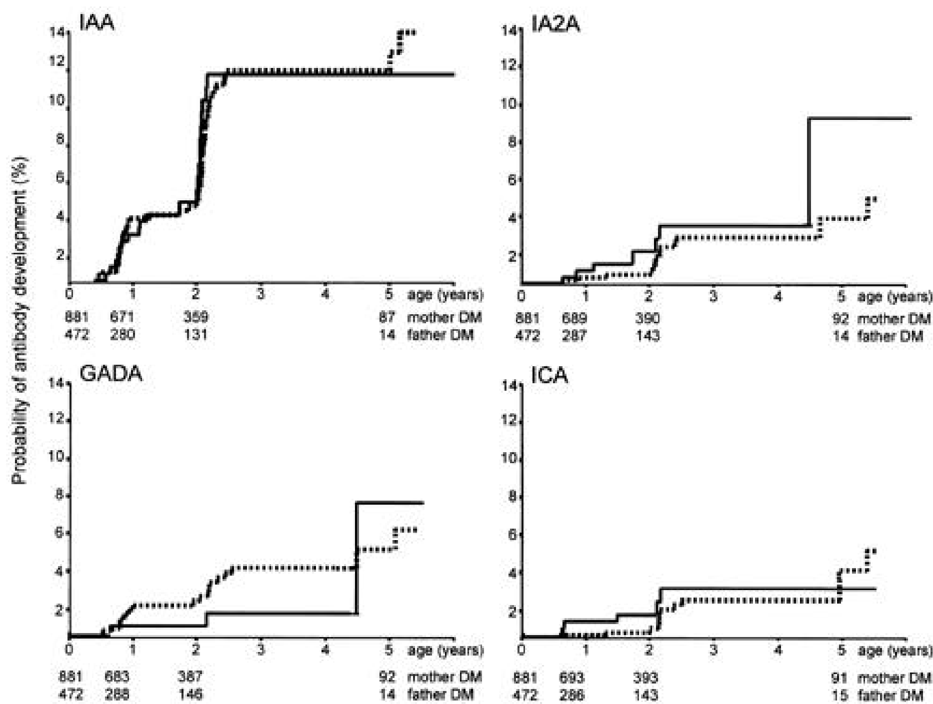

FIG. 4. Kaplan-Meier analysis: probability of developing IAAs, GADAs, IA2As, and ICAs in offspring of mothers (- - -) and fathers (——) with type 1 diabetes. IAAs are detected earlier and at a higher proportion than GADAs, IA2As, or ICAs. No significant difference in the probability to develop any respective marker was observed between children of mothers versus fathers with type 1 diabetes.

TABLE 3
BABYDIAB offspring with multiple islet autoantibodies

Case number	Proband	Age at first antibody (years)	First antibody	Total antibodies	Age at most recent sample (years)	Age at diabetes onset (years)
5006	Mother	0.8	IAA	IAA, GADA	2.2	—
1068	Mother	0.9	IAA	IAA, GADA	5.4	—
1872	Mother	2.0	IAA	IAA, ICA	—	4.9
4161	Father	0.9	IAA	IAA, ICA	—	2.4
1032	Mother	0.8	IAA	IAA, GADA, IA2A, ICA	—	7.1
4050	Mother	0.9	IAA	IAA, GADA, IA2A, ICA	3.2	—
4005	Mother	0.8	IAA	IAA, GADA, IA2A, ICA	—	3.3
2223	Mother	2.1	IAA	IAA, GADA, IA2A, ICA	4.6	—
1724	Father	1.1	IAA	IAA, GADA, IA2A, ICA	5.6	—
1628	Father	2.0	IAA	IAA, GADA, IA2A, ICA	3.9	—
1649	Mother	0.9	GADA	IAA, GADA, IA2A, ICA	4.9	—
4849	Mother	0.8	IAA, GADA	IAA, GADA, IA2A, ICA	—	1.3
6499	Mother	0.5	IAA, GADA	IAA, GADA	0.5	—
4460	Mother	2.0	IAA, IA2A	IAA, IA2A	3.0	—
4603	Mother	2.1	IAA, IA2A	IAA,IA2A	2.1	—
1088	Mother	2.1	IAA, IA2A	IAA, GADA, IA2A, ICA	—	8.5
5686	Mother	0.9	IAA, GADA, IA2A	IAA, GADA, IA2A	0.9	—
2905	Mother	2.4	IAA, GADA, IA2A	IAA, GADA, IA2A	2.4	—
3929	Father	2.1	IAA, IA2A, ICA	IAA, GADA, IA2A, ICA	4.2	—
2277	Father	1.7	IAA, GADA, IA2A, ICA	IAA, GADA, IA2A, ICA	—	1.8
3941	Mother	2.0	IAA, GADA, IA2A, ICA	IAA, GADA, IA2A, ICA	—	3.8
4204	Father	2.2	IAA, GADA, IA2A, ICA	IAA, GADA, IA2A, ICA	3.0	—
4262	Both parents	0.6	IAA, GADA, IA2A, ICA	IAA, GADA, IA2A, ICA	—	2.2

least 2 islet autoantibodies. The cumulative risk to develop type 1 diabetes by age 5 years was 50% (95% CI 19–81) for offspring with more than one elevated antibody by the 2-year sample, compared with 14% (95% CI 0–40) for offspring with one and 0% for offspring with no elevated antibodies by 2 years ($P < 0.001$ for >1 antibody vs. 0 antibody and >1 antibody vs. 1 antibody, Fig. 5*B*).

DISCUSSION

The aim of this study was to determine autoantibody distributions and the chronology of antibody appearance from birth in offspring of parents with type 1 diabetes and to present risk estimates for progression to type 1 diabetes in childhood associated with the appearance of islet autoantibodies. At least one autoantibody was found by age 2 years in 11.1% of offspring, 3.5% having more than one islet autoantibody. Islet autoantibodies most frequently appeared in the 2-year sample. IAAs were the most frequently detected autoantibodies and were almost always detected in the first sample with islet autoantibodies. Risk for type 1 diabetes was highest in those with more than one islet autoantibody, and all those developing diabetes had IAAs. The data suggest that screening for islet autoantibodies, and in particular IAAs, at an early age will be a useful strategy for identifying relatives of patients who are at risk for type 1 diabetes in childhood.

In analyzing antibody appearance, it is necessary to identify thresholds in antibody measurement that distinguish the presence of antibody. We describe a novel approach that appears useful, particularly when suitable control populations may not be available. We argue that there is currently no satisfactory way to identify this threshold, and we suggest that this point may correspond to where there is change in the shape of the frequency distribution in the cohort under analysis. We have used the normal plot to aid in the identification of this point. The normal plot is most commonly used to determine if a distribution fulfills criteria of normality. Clearly, the distributions of the islet autoantibody measurements in offspring of parents with type 1 diabetes were not normal, and with the numbers tested, the Q-Q plot could identify the point at which the distribution changed. We interpreted signals above this point as being different from those below, which followed a close-to-normal distribution around zero. Remarkably, for each of the islet autoantibodies tested, this point was relatively consistent at ages 9 months and 2 years. The points were also similar for antibody measurements in offspring from mothers versus fathers with diabetes and girls versus boys, indicating that factors associated with maternal transmission or sex do not affect islet autoantibody measurements at these ages. Moreover, we tested when signals due to maternal antibodies in the circulation of the offspring cease to affect measurement and found that such effects are likely up to almost 6 months of age, but not thereafter. The thresholds identified using the Q-Q plots were the same as, and therefore support those previously derived from, 99th centile values from a separate control cohort (14). To further test these threshold values, we examined the likelihood of there being a second islet autoantibody detected over the range of measurement, arguing that true positive autoantibodies were more likely to be associated with the detection of a second autoantibody than would be false positives. Again, the point of change in the Q-Q plot corresponded well with those at which there was an increased likelihood of a second antibody. We therefore conclude that the approach we have used is valid and that, while there will inevitably be some imprecision in measurement, the thresholds selected are likely to closely reflect measurements that

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

TABLE 4
Course of antibody titers in five children with multiple antibodies

Case number and age (years)	IAA (nU/ml)	GADA (U)	IA2A (U)	ICA (JDF units)
1872				
0.96	Negative	Negative	Negative	<5*
2.0	1,185	Negative	Negative	<5
3.1	439	Negative	Negative	160
3.5	342	Negative	Negative	80
5.0†	87	Negative	Negative	80
1032				
0.8	159	Negative	Negative	<5
1.9	1,174	35	Negative	<5
2.9	3,077	118	18	160
4.0	733	77	7	80
5.0	736	37	74	80
6.1	1,165	59	57	160
7.1†	2,978	67	80	80
1724				
1.1	72	Negative	Negative	<5
2.1	166	Negative	Negative	<5
3.1	247	Negative	Negative	<5
4.4	605	29	32	<5
5.6	470	Negative	26	20
1649				
0.9	Negative	32	Negative	<5
2.1	387	33	12	160
2.3	974	58	7	160
3.5	407	65	103	160
4.9	199	61	57	40
1088				
0.9	Negative	Negative	Negative	<5
2.1	96	Negative	34	<5
2.9	315	26	119	160
3.9	599	Negative	86	160
5.0	165	20	75	80
5.7	343	Negative	35	80
7.0	50	20	103	80

*Titers <5 were considered negative. †Age when type 1 diabetes developed.

distinguish true autoantibody. It should be noted, though, that levels below the threshold do not necessarily imply the absence of antibody, since it cannot be assumed that any assays are sufficiently sensitive to exclude antibody. This approach to distinguish normality from nonnormality by normal plots offers the possibility for future prospective studies to determine thresholds without using control populations. This may be particularly valuable in general population studies or populations of young age.

Early appearance of islet autoantibodies in offspring was frequent. By the 2-year sample visit, >10% had already developed one islet autoantibody, and 3.5% had more than one antibody, which is similar to antibody prevalences reported in 1st-degree relatives of older age (18). The presence of islet autoantibodies other than those from the maternal circulation in the birth sample was rare or nonexistent. In three offspring whose mothers did not have diabetes or antibodies and who were previously found to have ICAs (14), antibodies were not detected in follow-up samples, nor were the ICAs confirmed in the birth sample when subsequently repeated on a different pancreas, suggesting that antibodies detected at birth may be nonspecific or unstable. ICAs in those who had

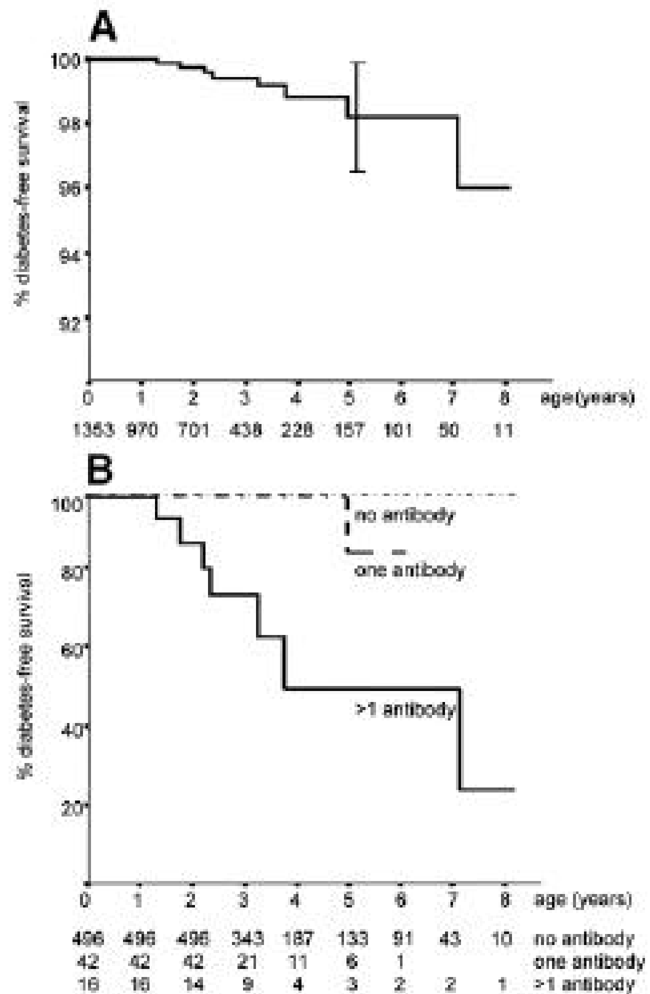

FIG. 5. *A*: Diabetes-free survival of 1,353 offspring of type 1 diabetic parents. The overall diabetes risk by 5 years of age in the BABYDIAB cohort was 1.8% (95% CI 0.2–3.4). *B*: Diabetes-free survival in relation to the antibody status at 2 years of age. Offspring with multiple antibodies by 2 years had a higher risk to develop type 1 diabetes in childhood (50% by 5 years) than offspring with one or no antibodies (14 and 0%, $P < 0.001$).

or developed multiple antibodies were always confirmed on repeat testing. In one offspring from a father with type 1 diabetes, GADAs and ICAs were already present at birth, but both ICAs and GADAs were also detected in the mother's serum (14). Almost half who developed islet autoantibodies had at least one islet autoantibody in the 9-month sample, although in many of these, further islet autoantibodies appeared only in later samples, and in the majority, the first islet autoantibodies appeared at the 2-year sample. A few offspring had transient appearances of antibodies, which, in one case, reappeared at 5 years (data not shown). In offspring with multiple antibodies, we could distinguish two different patterns of antibody appearance. One group developed one single antibody, and spreading to other antigens occurred almost sequentially in follow-up samples. The other group already had multiple antibody responses in the first sample with islet autoantibodies, suggesting either rapid amplification and spreading of autoimmunity from one antigen to other antigens or simultaneous activation of immune responses to multiple antigens. The observations indicate that autoimmunity at

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

this young age is a dynamic process that is spreading and increasing rapidly in some offspring, more slowly in some, and declining in others and is not always as stable as in relatives of older age.

Of the islet autoantibodies measured, IAAs were the most frequently detected. IAAs were almost always present in the first sample showing islet autoimmunity, and their detection often preceded that of other antibody markers. Moreover, they were detected before diabetes in all nine offspring who have developed disease. This argues for insulin as a candidate early antigen in the pathogenesis of human type 1 diabetes. While IAA signals detected at birth are likely to be artifact (17) or maternally acquired, those after 6 months of age are not. To confirm this, IAAs in those who developed multiple islet antibodies were also measured using a protein-A precipitable assay (19) and were shown to be true immunoglobulin G (data not shown). It has been reported by us (20) and others (21) that IAAs are frequently detected in patients who develop type 1 diabetes in childhood. The levels of IAAs correlate inversely with the age of diabetes onset, and IAAs are significantly less prevalent in adult patients with newly diagnosed type 1 diabetes. It remains unclear if this lower prevalence in older patients is because IAAs were never present or because they have disappeared from the circulation. In this study, we followed five offspring with multiple islet autoantibodies up to the age of 5 years. In three, IAA levels showed a peak response >500–1000 nU/ml at an early age and, remarkably, declined thereafter to below or around levels of 100 nU/ml. These preliminary observations indicate that the antibody status at onset or during late stage of prediabetes does not always reflect the magnitude of responses during early stages of islet autoimmunity. They also suggest that the lower prevalence of IAAs in older subjects is in part due to loss of antibody from the circulation, opening the possibility that insulin also plays a role in the early immune response in these subjects.

By life-table analysis, 1.8% of the offspring were predicted to develop type 1 diabetes by 5 years of age. Previous studies in the United States (22,23) and Finland (24) have shown that the cumulative incidence of type 1 diabetes by age 20 years is 6 and 7.6%, respectively, for offspring of fathers and 2 and 3.5% for offspring of mothers with type 1 diabetes, and the incidence up to the age of 5 years is around 1%. In our study, all children who developed diabetes had autoimmune diabetes with more than one autoantibody present in the circulation before or at clinical diagnosis. IAAs and ICAs were found in 100% and GADAs and IA2As in 75% of cases. Although the numbers developing disease are not large, the detection of multiple antibodies by the age of 2 years in offspring of patients was associated with a relatively high risk (50% by 5 years) of diagnosed diabetes during early childhood. The high risk and very early development may in part reflect earlier diagnosis as an inevitable result of more intensive monitoring by parents of children with islet antibodies. This is also evidenced by the fact that all children were diagnosed with oral glucose tolerance and none presented with ketoacidosis. A high risk in offspring with multiple antibodies is consistent with various other studies in 1st-degree relatives which have shown that risk for diabetes is correlated with the number of islet autoantibodies (2,3,25). Earlier diagnosis in relatives with antibodies is a potential bias of this and all prospective diabetes family studies. Diabetes risk in this study is unlikely to be biased significantly by possible follow-up differences since, apart from a small number of families who could be not traced because of a change of address, all were contacted for diabetes onset at the time of their scheduled 9-month, 2-year, and 5-year visits. Since this is not a population-based study with complete ascertainment of all cases, there may be recruitment bias that has not been determined.

The proportion of offspring from mothers with diabetes with multiple islet autoantibodies was similar to the previously reported incidences of type 1 diabetes by 20 years (22,23). Unlike the marked differences reported in the prevalence of diabetes in offspring from mothers versus fathers with type 1 diabetes, and what we reported in the earlier analysis in which offspring from mothers with gestational diabetes were also included (14), we found no difference in autoantibody frequencies between the two groups up to age 5 years, and the proportion of offspring from fathers with multiple islet antibodies was around half the previously reported 20-year diabetes incidences. Warram and colleagues (22,23) in fact also observed no major differences in diabetes risk during the first decade of life, although the same group reported a three- to sixfold increase in risk for children of diabetic fathers by the age of 20 years. If the differences observed in disease incidences are also true for autoantibody prevalences, then it must be expected that antibodies will appear after 2 or even 5 years of age in offspring from fathers with type 1 diabetes.

Regardless of ethical considerations as to whether screening in families where there is an affected patient is justified, such screening is being offered and indeed requested by these families. The findings of this study have practical implications for future screening for preclinical diabetes in childhood. They indicate that screening with islet autoantibodies could be started in relatives of patients with type 1 diabetes as early as 2 to 3 years of age, since all those who developed IDDM in this study had at least one islet antibody at this age. Earlier screening would seem relatively insensitive. In this study, 2 subjects who developed type 1 diabetes at 3.8 and 4.9 years and 12 (52%) of those with multiple antibodies would not have been identified by a screen at 9 months. However, 2 relatives who may have presented with type 1 diabetes before age 2 would inevitably be lost in a screening strategy that commences at or after age 2. The findings also indicate that initial screening at 2 to 3 years of age can be performed using IAA measurement, with GADAs, IA-2A, and ICAs measurement only in those identified to have IAAs. IAAs were present at 2 years in all who developed type 1 diabetes or multiple antibodies, while GADAs were not in 2 and 8 cases, respectively, and IA2As were not in 3 and 7 cases. This is in contrast to what we and others have suggested for relatives of older age in whom the combined testing for GADAs and IA2As is a sensitive screen to identify at-risk subjects who should be further tested with IAAs and/or ICAs (18). Therefore, practical screening needs to be tailored to the population tested. Accordingly, the findings of this study should be confirmed in the general population before children with no family history of type 1 diabetes are screened. These studies are in progress (26).

In conclusion, our data demonstrate that autoimmunity in children of parents with type 1 diabetes is initiated very early in life, with a first peak of antibody appearance by the age of 2 years, that IAAs are present in the majority of these children in the first sample with autoantibodies, and that the detection of multiple antibodies at 2 years of age in offspring of parents

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023

with type 1 diabetes is associated with a high risk of progression to disease by age 5 years. These findings need to be confirmed in studies from the general population, but with this knowledge, new concepts of screening programs and intervention strategies aiming to modulate the early stage of the disease process may be considered.

ACKNOWLEDGMENTS

This work was supported by grants from the Bundesministerium für Forschung, Bildung und Technologie (BMBF 01KD89030 and 1KD9601). M.H. and M.S. are supported by the BMBF.

We thank M. Füchtenbusch, H. Naserke, U. Mollenhauer, M. Dübell, J. Dittler, K. Ferber, A. Schimmel, and J. Vordemann for data collection and expert technical assistance. We also thank all obstetric departments, pediatricians, and family doctors in Germany that participated in the trial for the recruitment and follow-up of families in BABYDIAB.

REFERENCES

1. Gorsuch AN, Spencer KM, Lister J, McNally J, Dean BM, Bottazzo GF, Cudworth AG: Evidence for a long pre-diabetic period in type 1 (insulin-dependent) diabetes mellitus. *Lancet* 335:147–149, 1981
2. Bingley PJ, Christie MR, Bonifacio E, Bonifanti R, Shattock M, Fonte MT, Bottazzo GF, Gale EAM: Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 43:1304–1310, 1994
3. Verge CF, Gianani R, Kawasaki E, Yu L, Pietropaolo M, Jackson RA, Chase HP, Eisenbarth GS: Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 45:926–933, 1996
4. Schatz D, Krischer J, Horne G, Riley R, Spillar R, Silverstein J, Winter W, Muir A, Derovanesian D, Shah S, Malone J, MacLaren N: Islet cell antibodies predict insulin-dependent diabetes in United States school age children as powerfully as in unaffected relatives. *J Clin Invest* 93:2403–2407, 1994
5. Ziegler AG, Hillebrand B, Rabl W, Mayrhofer M, Mollenhauer U, Vordemann J, Lenz A, Standl E: On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 36:402–408, 1993
6. Rewers M, Bugawan TL, Norris JM, Blair A, Beaty B, Hoffman M, McDuffie RS, Hamman RF, Klingensmith G, Eisenbarth GS, Erlich HA: Newborn screening for HLA markers associated with IDDM: Diabetes Autoimmunity Study in the Young (DAISY). *Diabetologia* 39:807–812, 1996
7. Guaita G, Shattock M, Cossu E, Sepe V, Songini M, Cirillo R, Bottazzo GF, and the SNI study group: The Sardinian newborn-IDDM (SNI) study. North to south gradient for ICA in 2933 cord blood samples from Sardinian newly born children (Abstract). *Diabetologia* 38 (Suppl. 1):A86, 1995
8. Martikainen A, Saukkonen T, Kulmala PK, Reijonen H, Ilonen J, Teramo K, Koskela P, Knip M, Akerblom K: Disease-associated antibodies in offspring of mothers with IDDM. *Diabetes* 45:1706–1710, 1995
9. Galloway TS, Millward BA, Fewster D, Wilkin TJ: Binding specificity of IAA in cord blood. The EarlyBird study (Abstract). *Diabetologia* 39 (Suppl. 1):A96, 1996
10. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL: Insulin autoantibodies in insulin-dependent diabetes before insulin-treatment. *Science* 222:1337–1339, 1983
11. Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A: A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. *Diabetologia* 37:344–350, 1994
12. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E: Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 155:5419–5426, 1995
13. Bingley PJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM: Prediction of IDDM in the general population. Strategies based on combinations of autoantibody markers. *Diabetes* 46:1701–1710, 1997
14. Roll U, Christie MR, Füchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG: Perinatal autoimmunity in offspring of diabetic parents: the German multicenter 'BABY-DIAB' study. Detection of humoral immune responses to islet antigens in early childhood. *Diabetes* 45:967–973, 1996
15. Bonifacio E, Lernmark A, Dawkins RL: Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. *J Immunol Methods* 106:83–88, 1988
16. Bland M: The normal distribution. In *An Introduction to Medical Statistics*. 2nd ed. Bland M, Ed. Oxford Medical Publications, Oxford, 1995, p. 101–118
17. Bilbao JR, Calvo B, Urritia I, Linares A, Castano L: Anti-insulin activity in normal newborn cord-blood serum. Absence of IgG-mediated insulin binding. *Diabetes* 46:713–716, 1997
18. Seissler J, Morgenthaler NG, Achenbach P, Lampeter EF, Glawe D, Payton M, Christie MR, Scherbaum WA, and the DENIS Study Group: Combined screening for autoantibodies to IA-2 and antibodies to glutamic acid decarboxylase in first degree relatives of patients with IDDM. *Diabetologia* 39:1351–1356, 1996
19. Naserke HE, Dozio N, Ziegler AG, Bonifacio E: Comparison of a novel microassay for insulin autoantibodies with the conventional radio binding assay. *Diabetologia* 41:681–683, 1998
20. Vardi P, Ziegler AG, Mathews JH, Dib S, Keller RJ, Ricker AT, Wolfsdorf JI, Herskowitz RD, Rabizadeh A, Eisenbarth GS, Soeldner JS: Concentrations of insulin autoantibodies at onset of type 1 diabetes. Inverse log-linear correlation with age. *Diabetes Care* 11:736–738, 1981
21. Gorus FK, Vandewalle CL, Dorchy H, Van Crombrugge P, Schuit FC, Pipeleers DG, and the Belgian Diabetes Registry: Influence of age on the association among insulin autoantibodies, islet cell antibodies, and HLA DQA1*0301-DQB1*0302 in siblings of patients with type 1 (insulin-dependent) diabetes mellitus. *J Clin Endocrinol Metab* 78:1172–1178, 1994
22. Warram JH, Krolewski AS, Gottlieb MS, Kahn CR: Differences in risk of insulin-dependent diabetes in offspring of diabetic mothers and diabetic fathers. *N Engl J Med* 311:149–152, 1984
23. El-Hashimy M, Angelico MC, Martin BC, Krolewski AS, Warram JH: Factors modifying the risk of IDDM in offspring of an IDDM parent. *Diabetes* 44:295–299, 1995
24. Tuomilehto J, Podar T, Tuomilehto-Wolf E, Virtala E: Evidence for importance of gender and birth order for risk of IDDM in offspring of IDDM parents. *Diabetologia* 38:975–982, 1995
25. Christie MR, Roll U, Payton MA, Hatfield ECI, Ziegler AG: Validity of screening for individuals at risk for type 1 diabetes by combined analysis of antibodies to recombinant protein. *Diabetes Care* 20:965–970, 1997
26. Rewers M, Eisenbarth GS, Norris J, Erlich HA, Beaty B, Klingensmith G, Elliott RB: Population and family risk of β-cell autoimmunity in the first two years of life: diabetes and autoimmunity study in the young (Abstract). *Diabetologia* 39 (Suppl. 1):A15, 1996

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/48/3/460/364133/10078544.pdf by guest on 30 November 2023