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Early Autoantibody Responses in Prediabetes Are IgG1 Dominated and Suggest Antigen-Specific Regulation¹

Ezio Bonifacio,* Miriam Scirpoli,* Katharina Kredel,[†] Martin Füchtenbusch,[†] and Anette-G. Ziegler^{2†}

The islet autoimmunity of preclinical type 1 diabetes remains poorly characterized in humans. In this paper, the IgG subclass response to the islet autoantigens insulin, glutamic acid decarboxylase, and IA-2 was studied sequentially from birth to diabetes onset or current follow-up in 26 autoantibody positive offspring of parents with diabetes. Islet autoantibody appearance was characterized by an early IgG1 peak response to one or more Ags, most commonly to insulin, at a median age of 2.2 yr (interquartile range, 2–2.9 yr). In five offspring, an acute fulminant β -cell destruction and diabetes onset occurred during this initial Ab response. In the remainder, early Ab levels declined markedly, and Ab peaks against other β cell Ags arose sequentially over several years suggesting regulation and spreading of autoimmunity. Second peak Ab responses to the same Ag were observed in only two offspring, both developing diabetes at this time. Two others developed diabetes with declining Ab levels. Abs of IgG1 subclass dominated against each Ag, and other subclasses, were usually only detected during peak IgG1 responses. The IgG4 response to insulin was exceptional, being dominant over IgG1 in four offspring and in five others appeared and/or persisted after IgG1 levels declined. These Th2-associated IgG4 responses were not correlated with protection from diabetes. The presence of IgG1-restricted responses to DA2 were associated with diabetes development. These findings suggest that type 1 diabetes has an early acute destructive phase of β cell autoimmunity, which may be regulated and which spreads chronically until diabetes onset. *The Journal of Immunology*, 1999, 163: 525–532.

Type 1 diabetes is preceded by a preclinical phase in which autoimmunity to islet proteins is detected (1). This may start early in life (2–4) and can sometimes last decades (5, 6). Some researchers have suggested that there is a linear decline in β cell mass during the prediabetic period and have proposed mathematical models to estimate time to diabetes onset (7). These models assume that the rate of decline is proportional to active autoimmunity which can be assessed by circulating autoantibody levels and that these levels remain relatively stable over time. An alternative view is that β cell destruction is nonlinear with both latent and active phases of autoimmunity during the preclinical period (8, 9).

Studies in animal models of diabetes have shown that destructive autoimmunity is mediated through a Th1-dominated autoimmune response (10–13). This has been suggested by some to arise relatively late in the disease process after a latent period of non-aggressive Th2 islet autoimmunity (8, 9). Other researchers have proposed that the natural course is characterized by spontaneous Th1 autoimmunity against a primary autoantigen with spreading to other islet Ags in the progression to diabetes (14–16). In both

models, modulation of the autoimmunity to either maintain or initiate Th2 dominance, albeit by different strategies, impedes β cell destruction (16–19). Th1 and Th2 immunity in the mouse is associated with the generation of IgG2a and IgG1 Abs, respectively, and measurements of Ag-specific Ab subclasses have been used to identify periods of Th1- vs Th2-dominant immunity (20–24). In humans, the Ab responses of Th1 and Th2 immunity are not so distinct. There is some evidence that Th2 immunity is associated with IgG4 and IgE Ab responses, because both can be stimulated by the Th2 cytokine IL-4 (25, 26), and both are characteristic of the Th2-dominated response of atopy. The IgG subclass response found in Th1-dominated immunity appears inconsistent and can be IgG1 restricted or include most subclasses (27, 28).

A major limitation in our ability to characterize preclinical islet autoimmunity in humans is that subjects are usually identified when islet autoimmunity has already commenced, precluding the possibility of analyzing the early autoimmune response and subsequent changes in the progression to diabetes onset. To overcome this, we initiated the BABYDIAB study which investigates the temporal development of islet autoimmunity prospectively from birth in offspring of parents with diabetes (2). The study has shown that autoantibodies targeting insulin appear by 2 yr of age, and Abs to glutamic acid decarboxylase (GAD)³ and the protein tyrosine phosphatase IA-2 often appear simultaneously or soon after (29). In this paper, we have measured IgG subclass Abs to insulin, GAD, and IA-2 from initial autoantibody appearance until diabetes onset or current follow-up several years later in BABYDIAB offspring to determine whether there is regulation and change of autoantibody responses in the prediabetic period. The findings show heterogeneity, with an acute unregulated type of autoimmunity and

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³ Abbreviations used in this paper: GAD, glutamic acid decarboxylase; IAA, insulin autoantibodies; GADA, GAD Abs; IA-2, insulinoma-associated cDNA 2; IA2A, IA-2 Abs; SDS, SD score; IDDM, type 1 insulin-dependent diabetes mellitus; ICA, islet cell Abs.

rapid disease onset in some, and a slower “infectious” autoimmunity characterized by sequential IgG1-dominated autoantibody peaks to distinct islet autoantigens in others. Both support the presence of early destructive autoimmunity that may have the capacity for self-regulation. The infectious nature further suggests an ongoing Ag-specific regulation and spreading associated with periods of remitting and relapsing autoimmunity.

Materials and Methods

Study subjects

BABYDIAB investigates the temporal development of islet autoimmunity from birth in offspring of parents with diabetes. It schedules regular visits with venous blood sampling at birth (cord blood), and at 9 mo and 2, 5, and 8 yr of age for measurements of autoantibodies to islets (ICA), insulin (IAA), GAD (GADA), and IA-2 (IA2A), and HLA class II typing. Since 1989, 2079 newborns have been recruited (1598 from a parent with type 1 diabetes and 481 from mothers with gestational diabetes). A total of 1391 have had a 9-mo visit; 1019 a 2-yr visit, and 298 a 5-yr visit. Twenty-six offspring have developed Abs to more than one islet autoantigen (median age of first islet autoantibody detection, 1.4 yr; range, 0.5–5.4 yr) and were included in this study. All 26 were followed with approximately yearly blood samples until the development of diabetes in 11 offspring (median onset age, 3.6 yr; range, 1.3–8.5 yr) or current age (median, 4.5 yr; range, 1.3–8.3 yr). In all offspring with elevated Ab levels repeated oral glucose tolerance tests were performed to monitor for elevated blood glucose levels. Type 1 diabetes diagnosis was defined according to World Health Organization criteria.

Autoantibody determination

IAA were measured by binding to ^{125}I -labeled insulin in protein A/G and polyethylene glycol (PEG) competition radio-binding assays as previously described (29–31). IgG Abs to GAD and to IA-2 were measured by binding to [^{35}S]methionine-labeled *in vitro* translated recombinant human Ag as previously described (29, 32). The upper limit of normal values was defined by the 99th percentile of Ab levels in nondiabetic control children (50 nU/ml IAA for the PEG assay and 4 units for the protein A/G assay, 3 units for GADA, and 5 units for IA2A).

IgG subclass and isotype autoantibodies

For IAA, GADA, and IA2A, the protein A/G radio-binding assays were used, substituting the addition of the protein A/G Sepharose with IgG subclass or isotype specific Ab bound Sepharose beads. Biotin-labeled mouse mAbs against human IgG1 (cat. no. 35052D), IgG2 (cat. no. 35072D), IgG3 (cat. no. 35082D), IgG4 (cat. no. 35092D), IgM (cat. no. 34152D), IgA1/IgA2 (cat. no. 35112D), IgE (cat. no. 34612D), and as a control for nonspecific binding against rat IgM (cat. no. 10062D) were obtained commercially (PharMingen, San Diego, CA). Sepharose 4B streptavidin beads (Zymed, San Francisco, CA) were prepared by washing once with ice-cold PBS (50 mM phosphate buffer and 150 mM NaCl (pH 7.4)), followed by incubation of beads with biotinylated mAb with rotation at 4°C for 18 h, washing twice in PBS, once in assay buffer, and resuspension in assay buffer. The quantity of antiserum and streptavidin beads required to completely capture Ig in the assay reaction was determined by checkerboard titration under assay conditions. For IAA, this corresponded to 10 μl of Sepharose 4B streptavidin beads with 10 μg of Ab per well of the assay reaction, and for GADA and IA2A, 10 μl and 5 μg of Ab per well in the assay reaction.

IgG subclass specific IAA were measured by incubating 5 μl of serum with 1159 nU ^{125}I -labeled insulin (Hoechst, Frankfurt, Germany; specific activity, 360 $\mu\text{Ci}/\mu\text{g}$) in 25 μl 50 mM Tris and 1% Tween-20 (pH 8) (TBT) at 4°C for 72 h before addition of 50 μl Ab-coated Sepharose bead suspension, incubation for 1 h at room temperature, washing in cold TBT, and counting for 10 min. For each serum, nonspecific binding was also measured using beads coated with anti-rat IgM mAb. Results were expressed as nU insulin bound/ml of serum calculated as: [(IgG subclass specific counts – anti-rat IgM counts)/(total counts per tube)] \times 1159 \times 200.

All samples from the same offspring were tested for all subclasses in the same assay. For each subclass the results from BABYDIAB offspring showed two populations. One had a relatively normal distribution around 0 nU/ml ranging from –250 to +250 nU/ml, and a second above 250 nU/ml. The threshold for each IgG subclass IAA was therefore placed at 250 nU/ml, which corresponded to \sim 200 counts after subtraction of the rat IgM control. No control subjects had levels above 250 nU/ml.

For GADA and IA2A, 0.5 μl of serum was incubated with 25,000 cpm of [^{35}S]methionine-labeled *in vitro* transcribed and translated Ag in 25 μl

of 50 mM Tris, 150 mM NaCl, and 1% Tween 20 (pH 7.4) (TBST) overnight at 4°C, before addition of 50 μl Ab-coated Sepharose bead suspension, incubation for 1 h at room temperature, washing in cold TBST, and counting. Results were expressed as delta cpm (IgG subclass or isotype specific cpm – anti-rat IgM cpm) and converted to a SD score (SDS) calculated as: [(IgG subclass or isotype-specific counts delta cpm – mean delta cpm of control subjects)/SD delta cpm of control subjects]. The mean delta cpm + SD of 44 control subjects was used as the threshold for detection for each of the subclasses and isotypes tested. For GADA this was: IgG1, 71; IgG2, 47; IgG3, 41; IgG4, 55; IgM, 122; IgA, 57; and IgE, 68; and for IA2A: IgG1, 47; IgG2, 33; IgG3, 46; IgG4, 106; IgM, 72; IgA, 115; and IgE, 22. None of the control subjects had values above 3 SDS for any of the subclass or isotype GADA or IA2A. The specificity of the IgG subclass and isotype specific methods were validated using the MICA4 human IgG1 GAD mAb (33). SDS for IgG2, IgG3, IgG4, IgM, IgA, and IgE GADA measurements of the MICA4 supernatant and of MICA4 supernatant diluted 1/5 in normal human serum ranged between –1.5 and 1.6.

HLA typing

HLA-DR and -DQ alleles were determined using PCR-amplified DNA and nonradioactive sequence-specific oligonucleotide probes (34).

Data analysis

Linear regression analysis was used to correlate total and IgG subclass Ab responses. Kaplan-Meier life-table analysis was used to determine the cumulative risk of progression to type 1 diabetes. For all statistical methods the Statistical Package for Social Sciences (SPSS, Chicago, IL) was used.

Results

Islet autoantibodies in prediabetes show early peak responses

Considerable changes in Ab titers for IAA, GADA, and IA2A were found. In each offspring, and for each autoantibody, a peak titer was reached soon after first detection (see examples in Fig. 1). In most cases, decline was marked and rapid after this peak. Peak responses to each Ag often occurred sequentially in an almost infectious manner, suggesting continuous regulation and spreading to new determinants. In case 1032, sequential peaks in GADA, IAA, and IA2A were found, whereas in case 1649 the peak sequence was IAA, GADA, IA2A. In case 1032 diabetes onset was concurrent with a second rise in Abs to insulin and IA-2. In case 4005, all Abs rose simultaneously with rapid diabetes onset. Case 1872 was unique, developing both IAA and ICA with simultaneous peaks, but no subsequent responses to either GAD or IA-2, and developing diabetes when Abs had reached almost undetectable levels.

Earliest peak is commonly an IgG1 response

In each of the cases shown in Fig. 1, the peak responses corresponded to major rises in the IgG1 Abs (Fig. 1, B–D). This result was a consistent finding in all offspring. The magnitude of peaks and decline of Ab and specifically IgG1 responses was often very large, ranging from 184 to 7773 nU/ml (37–99% decrease; median, 90%) for IAA, from 5 to 67 SDS (19–99% decrease; median, 73%) for GADA, and from 3 to 124 SDS (35–99% decrease; median, 59%) for IA2A.

Fig. 2 shows the age and sequence of IgG1 peak responses for each offspring. The median age of the first IgG1 peak for any of the Abs was 2.2 yr (interquartile range, 2–2.9 yr). A first peak response to insulin (15 cases) was more frequent than to GAD (6 cases, $p < 0.01$) or IA-2 (3 cases, $p < 0.001$). The IgG1-IAA peak preceded other islet Ab peaks in 13 offspring and was concomitantly first with a peak response to GAD in one case (4204) and to IA-2 in another (1088). In five offspring a peak response to GAD was found before IAA, and in one of these concomitantly with an IA2A peak (1032). In the remaining six offspring the peak sequence could not be determined: in five offspring, the highest Ab titers to each Ag were found in the last sample obtained before

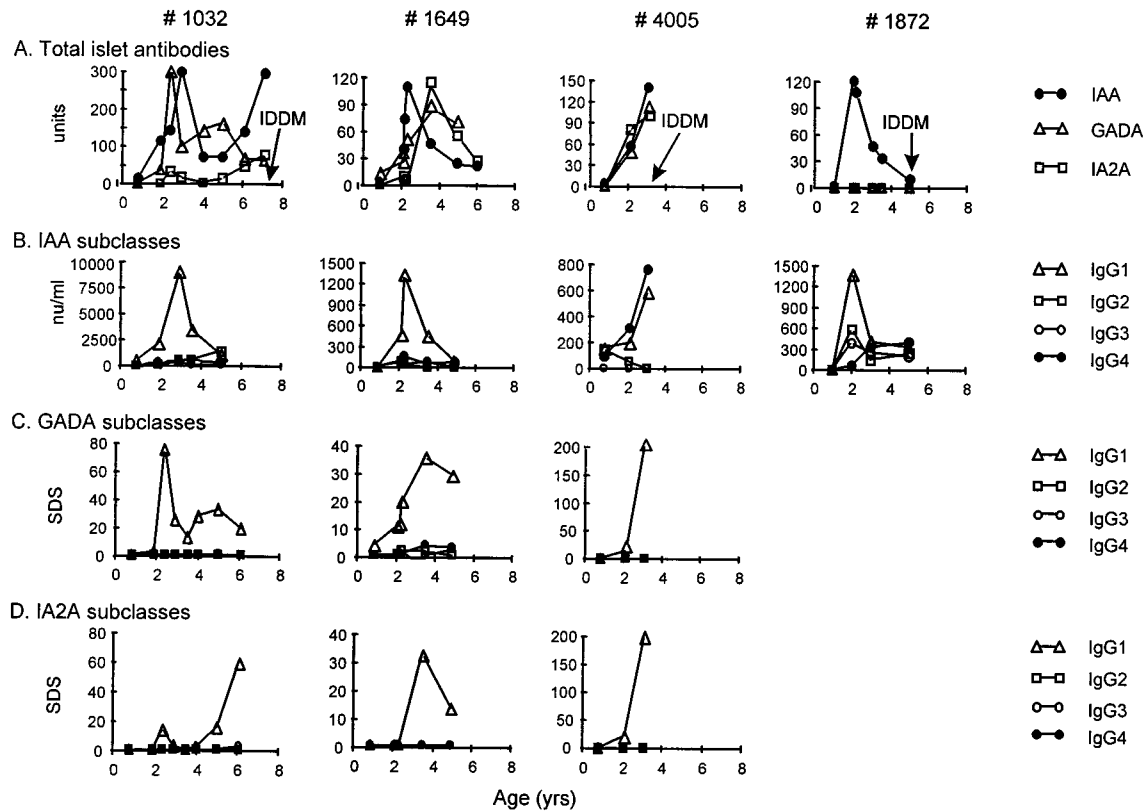


FIGURE 1. Representative course of islet Ab levels (A) and their subclasses (B–D) in four BABYDIAB offspring. The y-axis in A corresponds to Ab units of GADA (Δ) and IA2A (\square) and to nU/ml for IAA (\bullet). For subclasses, the y-axis corresponds to nU/ml of IAA subclasses (B) or SDS of GADA (C) or IA2A (D) subclasses. IDDM indicates type 1 diabetes onset.

onset of diabetes which in all five was before age 4 yr, and in one offspring (3975) only one sample with islet Abs has been tested. In two offspring a second rise in the total and IgG1 Ab response to either insulin (1032) and/or IA-2 (1032, 1088) was found before diabetes onset. Of interest, in case 1032 the first IA2A peak was due to Abs against epitopes in the juxtamembrane region of IA2, whereas in the second IA2A peak the Abs were against distinct epitopes in the IA-2 tyrosine phosphatase domain (35).

The presence of a first IAA peak or GADA peak appeared HLA linked. Eight of 10 offspring HLA typed in whom the first Ab peak included IAA had HLA DR4, whereas 4 of 5 with an initial GADA peak had HLA-DR3, consistent with insulin autoimmunity being associated with HLA-DR4 (36) and GAD autoimmunity with HLA-DR3 (37).

Distribution of autoantibody isotype and IgG subclass in prediabetes

For IAA, GADA, and IA2A, total Ab titers correlated with IgG1 Ab levels ($r = 0.8, 0.73, \text{ and } 0.57$, respectively; Fig. 3), but only slightly or not at all with IgG2, IgG3, and IgG4 Ab levels (data not shown). Highest levels for all Abs were seen for IgG1 (Fig. 4). For IAA, IgG1 responses were frequently accompanied or followed by rises in other IgG subclass responses, but for GADA and IA2A, other subclass responses were less frequently found. In particular, IgG4 were often elevated during the response to insulin (Fig. 4A) and in four offspring (4005, 5006, 4050, 4215) IgG4-IAA levels were as high or higher than IgG1-IAA (Table I). Low levels of IgG4-GADA were found in only five offspring, and no offspring had IgG4-IA2A. Table I shows which subclasses were detected in each offspring. Seven offspring had IAA of all subclasses, one GADA of all subclasses, and none IA2A of all subclasses. Exclusively IgG1 Abs were found for IAA in eight offspring, for GADA

in eight offspring, and for IA2A in nine offspring. None, however, had exclusively IgG1 Abs to all three Ags, and with the exception of few cases, the subclass response to one Ag was not usually paralleled with similar subclass usage to other Ags. An exception is case 4204 who had IgG1, IgG3, and IgE Abs to GAD and IgG1, IgG2, IgG3, and IgE Abs to IA-2. Two offspring (4050, 4215) with strong and dominant IgG4 responses to insulin had relatively high IgG2 titers to both GAD and IA-2. Of note is offspring 1948 in whom IgA-GADA was the first and major autoantibody response.

IgG subclass changes in islet autoantibody responses

In most offspring there were minimal or no changes in the IgG subclass islet Abs detected, with either a consistent presence of IgG1 responses only or the detection of additional subclass responses only when IgG1 levels were at their peak. In a few cases, however, there was later recruitment of other subclasses that did not parallel IgG1. These are shown for IAA in Table II. Most noticeably is the appearance of IgG4-IAA after first detection of IgG1-IAA, and a persistence of the IgG4 response after significant declines in the IgG1-IAA titer (1032, 1872, 2223, 6226, 6637). This was also found for IgG2-IAA in three offspring (1032, 4204, 6637). In these examples, IgG2-IAA were most similar to IgG4-IAA responses, whereas IgG3-IAA paralleled the IgG1-IAA response. The development of IgG4-IAA and their persistence was not associated with similar changes in the subclass response to GAD or IA-2. Moreover, the predominant subclass Ab against GAD or IA-2 did not change over time in all offspring.

Progression to diabetes

Diabetes onset usually occurred concurrent with a rise in islet Ab titer. In five offspring diabetes developed at 1.8, 2.4, 3.2, 3.3, and 3.8 yr when all detectable islet Abs were at their highest levels.

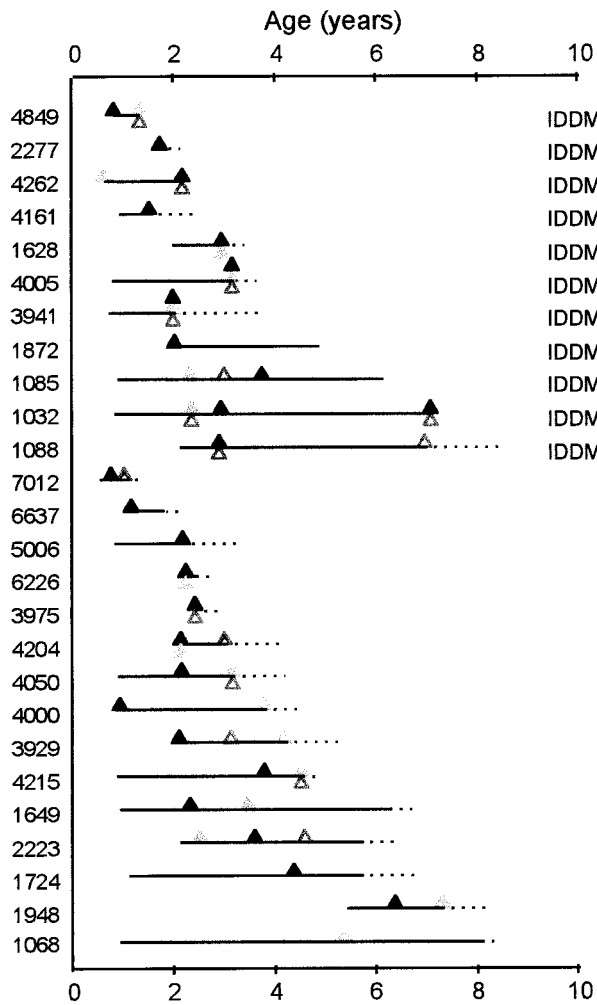


FIGURE 2. Schematic representation of peak IgG1 responses in BABYDIAB offspring with multiple islet autoantibodies. For each offspring (shown on the y-axis) the period in which islet Abs are detected is shown by solid lines; dotted lines indicate additional time of follow-up after last Ab measurement. Triangles indicate the age when peak IgG1 responses were observed for IAA (▲), GADA (△), and IA2A (△). Eleven offspring shown as IDDM developed type 1 diabetes at the end of follow-up.

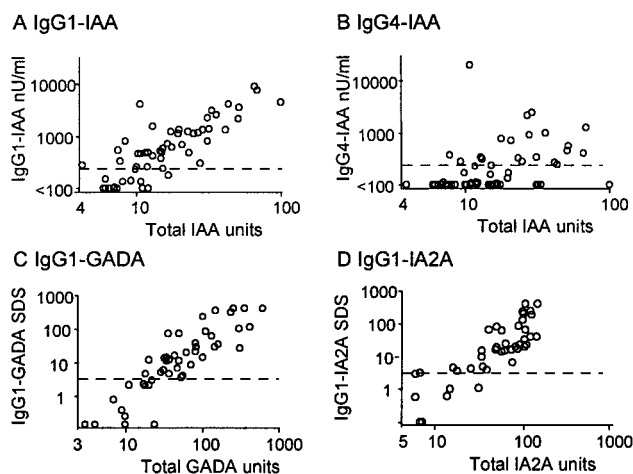


FIGURE 3. Correlation of IgG1 (A) and IgG4 (B) subclasses with IAA (protein A/G-IAA assay) and of IgG1 with GADA (C) and IA2A (D). Dotted lines indicate thresholds for positivity in the subclass Ab assays.

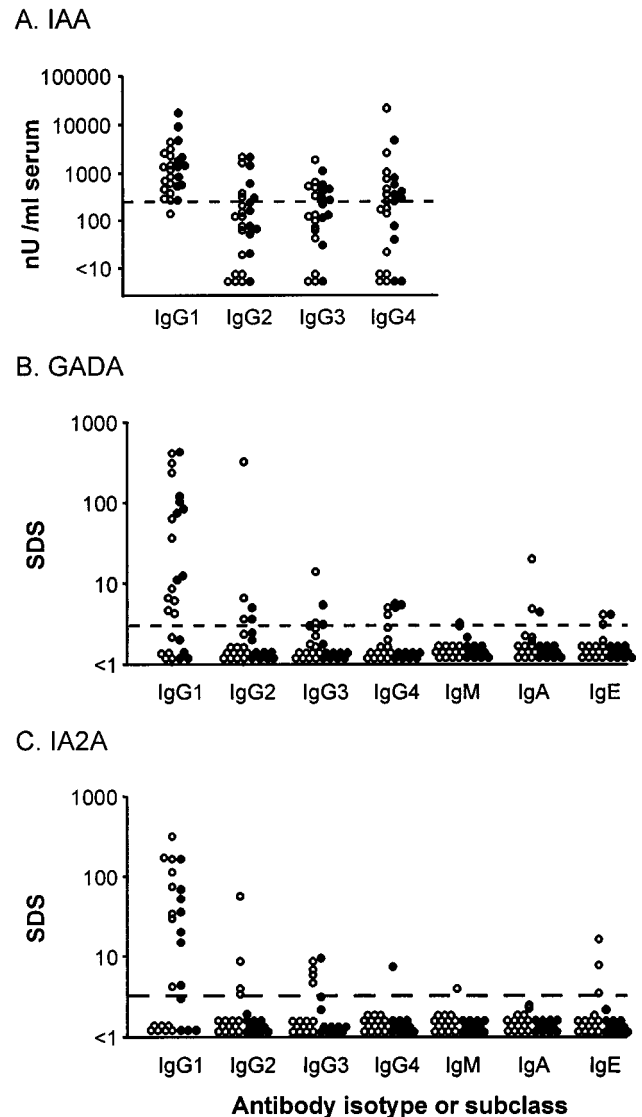


FIGURE 4. Peak levels of each Ab subclass or isotype of IAA (A), GADA (B), and IA2A (C) in BABYDIAB offspring with multiple islet autoantibodies. Filled circles indicate offspring who have developed type 1 diabetes. The dotted lines represents the threshold for positivity.

Another four offspring had an early peak Ab response to one or more Ags before diabetes, but either had peak responses to other Ags (4849, 4262, 1032) and/or second peak responses to the same Ags (1032, 1088) at diabetes onset. Only two offspring (1872, 1085) developed diabetes without rising Ab titers and after significant decline. Neither the presence or absence nor the titers of specific IgG subclass Abs were associated with progression to diabetes. A change to, or persistence of, an IgG4-IAA response was not associated with protection from diabetes. Specifically, cases 4005 (see Fig. 1) and 1872 (see Fig. 1 and Table II) developed diabetes when IgG4-IAA levels were at their peak and higher than IgG1-IAA, cases 1032 (see Fig. 1 and Table II) and 3941 (data not shown) developed diabetes with rising IgG4-IAA, and case 4161 (data not shown) with persistent high levels of IgG4-IAA. By life table analysis, risk of diabetes progression was elevated in offspring with an exclusively IgG1 response to IA-2 compared with offspring who had other subclass Abs to IA-2 (diabetes risk by age 5 yr, 56% (95% CI: 24–88) vs 0%, $p = 0.03$), but exclusively IgG1 responses against insulin or GAD were not associated with rapid diabetes development.

Table I. Autoantibody subclass and isotype response in BABYDIAB offspring with multiple islet autoantibodies

Case No.	Age at First Ab (yr)	Islet Antibodies ^a	IgG Subclass and Isotype Response			Age (yr) at Diabetes Onset or Last Sample	HLA DR
			IAA	GADA	IA2A		
4849	0.8	IAA, GADA, IA2A	IgG1 ^b	IgG1, 2, 4, IgA	IgG1	1.3 IDDM ^c	4/9
2277	1.7	IAA, GADA, IA2A	IgG1	None detected	None detected	1.8 IDDM	3/4
4262	0.6	IAA, GADA, IA2A	IgG1, 2, 3	IgG1, 2, 3, 4, IgE	IgG1	2.2 IDDM	3/1
4161	0.9	IAA	IgG1, 2, 3, 4			2.4 IDDM	NT ^d
1628	2.0	IAA, GADA, IA2A	IgG1, 3	IgG1	IgG1	3.2 IDDM	11/12
4005	0.8	IAA, GADA, IA2A	IgG1, 4	IgG1, 4	IgG1	3.3 IDDM	3/4
3941	2.0	IAA, GADA, IA2A	IgG1, 4	IgG1	IgG1	3.8 IDDM	4/4
1872	2.0	IAA	IgG1, 2, 3, 4			4.9 IDDM	1/1
1085	0.9	IAA, GADA, IA2A	IgG1, 2, 3, 4	IgG1	IgG1	5.9 IDDM	3/4
1032	0.8	IAA, GADA, IA2A	IgG1, 2, 3, 4	IgG1	IgG1	7.1 IDDM	11/2
1088	2.1	IAA, GADA, IA2A	IgG1	None detected	IgG1, 2, 3	8.5 IDDM	3/4
7012	0.5	IAA, GADA, IA2A	IgG1, 3	None detected	IgG1, 2, 3, IgM	1.0	NT
6637	1.0	IAA	IgG1, 2, 3, 4			2.0	NT
5006	0.8	IAA, GADA	IgG1, 4	None detected		2.2	4/1
6226	2.2	IAA, GADA	IgG1, 4, 2	IgG1		2.4	NT
3975	2.4	IAA, GADA	IgG1	None detected		2.7	4/4
4204	2.1	IAA, GADA, IA2A	IgG1, 2	IgG1, 3, IgE	IgG1, 2, 3, IgE	3.0	4/1
4050	0.9	IAA, GADA, IA2A	IgG1, 2, 3, 4	IgG1, 2, 4, IgM	IgG1, 2	3.2	4/4
4000	0.8	IAA, GADA	IgG1	IgG1, 2, 3, IgA		3.8	3/1
3929	2.1	IAA, GADA, IA2A	IgG1, 4	IgG1	IgG1, 3, IgE	4.2	4/11
4215	2.1	IAA, GADA, IA2A	IgG1, 4, 2	IgG1, 2	IgG1, 2, 3	4.5	3/4
1724	1.1	IAA, IA2A	IgG1		IgG1	5.6	3/4
2223	2.1	IAA, GADA, IA2A	IgG1, 2, 3, 4	IgG1	IgG1, IgE	5.7	3/4
1649	0.9	IAA, GADA, IA2A	IgG1	IgG1, 4	IgG1	6.3	3/4
1948	5.4	IAA, GADA	IgG1	IgG1, IgE, IgA		7.3	4/6
1068	0.9	IAA, GADA	None detected	IgG1		8.1	3/1

^a All had also ICA.

^b The dominant subclass or isotype responses are shown in larger font.

^c IDDM indicates diabetes onset.

^d NT, not tested

Discussion

Islet autoantibodies are indirect markers of type 1 diabetes associated autoimmunity. Although fluctuations of islet autoantibodies have been reported (38), it is generally considered that they rise to a level which remains relatively stable until diabetes onset and decline thereafter. In contrast, when studying the maturation of the humoral autoimmune response from birth in relatives of patients with diabetes, we find that Ab levels rise rapidly and markedly and decline soon after reaching a peak; that peak responses are IgG1 dominated and often sequential in Ag specificity; and that childhood diabetes onset is usually concurrent with rising or peak Ab levels.

The consistent early burst of Ab activity, with peak responses to at least one Ag seen by 2–3 yr of age was a surprising finding. The relatively homogeneous timing of this appearance is consistent with programmed spontaneous islet autoimmunity in these children. Neonates acquire their ability to produce IgG responses after birth, are comparatively hypogammaglobulinaemic in the first months of life, and do not mount effective responses until late into the first year of age (39). The islet autoantibody responses found in the BABYDIAB offspring correlate well with this physiological maturation and may therefore be a visible expression of a defect in tolerance during immune repertoire formation. An alternative to defective tolerance is that islet autoimmunity in the offspring is a consequence of early β cell damage. Maternal Ab is unlikely to cause damage because only a minority of those with maternally transferred Ab develop autoimmunity, and islet autoimmunity is at least as frequent in offspring of fathers with type 1 diabetes. Damage through environmental agents is possible, especially as the neonatal period is marked by a high susceptibility to infection (39). Increased infection in animal models, where insulinitis appears uni-

formly already at around 4 wk of age (40), is however associated with protection from diabetes. Moreover, the relative absence of an initial IgM response to autoantigen in the offspring more likely speaks against direct immunization induced as a result of β cell damage.

Although the timing of Ab appearance was homogeneous, the Ab sequence was not. In some there was an acute simultaneous autoantibody response to all or most diabetes-associated autoantigens. These offspring developed diabetes very early in life, suggesting a rapid unregulated form of destructive autoimmunity. Only some of these had the high risk HLA *DRB1*03/DRB1*04* genotype. In the majority of offspring, however, Ab responses occurred in a wave of sequential peaks to distinct Ags. The first peak most frequently included IgG1-IAA. In a minority an IgG1-GADA peak preceded the IAA peak, whereas in most offspring IgG1-IA2A peaks followed, suggesting IA-2 as a secondary autoantigen target. The changes in Ab levels seen were striking, with a significant decline in Ab titer after peak if disease did not occur. This wave of Ab responses suggests either a correlate with Ag expression/presentation and/or regulatory mechanisms. In general, only one Ab peak to each Ag was found, and sequential peaks were against new distinct Ags when Ab response to previous Ags was declining. This is evident in case 1032 even at the epitope level, where two distinct Ab responses to IA-2 four to five years apart were against diverse epitopes (35). These data might argue for an induction of regulation which is Ag-specific, with subsequent, almost infectious, spreading of the autoreactivity to other Ags. Dampening of Ab titer could be achieved either by specific regulatory T cells (41) or may merely reflect exhaustion and apoptosis of autoreactive cells to early Ag (42) with spreading to new specificities.

Table II. Autoantibody subclass changes over time in BABYDIAB offspring

Offspring	Age (yr)	IAA ^a				GADA ^b		IA2A ^b	
		IgG1	IgG2	IgG3	IgG4	IgG1	Other	IgG1	Other
1032	0.8	+	-	-	-	-		-	
	1.9	+++	-	-	+	+		-	
	2.4					+++		++	
	2.9	++++	+	+	+	++		+	
	3.5	+++	+	-	+	++		-	
	5.0	++	++	-	+	++		++	
	6.1	++	++	++	+	++		+++	
1872	2.0	++	+	+	-	-		-	
	3.0	+	-	+	+	-		-	
	5.0	+	-	-	+	-		-	
2223	2.1	++	-	+	-	-		-	
	3.6	+++	-	+	+	+		++	
	4.6	-	-	-	+	-		++	
	5.7	+	-	-	-				
4204	2.1	++	-	-	-	+++	IgG3	+++	
	3.0	+	+	-	-	++		++++	IgG2, 3
6226	2.2	+	-	-	-	-		-	
	2.4	-	-	-	-	-		-	
	2.7	-	-	-	+	+		-	
6637	1.0	+++	+	+	+	-		-	
	1.8	+	++	-	+	-		-	
	2.0	+	++	-	+	-		-	

^a IAA; -, <250; +, 250-1000; ++, 1001-2000; +++, 2001-5000; +++++, >5000 nU/ml.

^b GADA and IA2A; -, <3, +, 3-10; ++, 10.1-50; +++, 50.1-100; +++++, >100 SDS.

It has been discussed whether IgG subclasses correlate with Th1 and Th2 immune responses. Some consider the early spontaneous autoimmunity in animal models of diabetes nondestructive with a Th2 component (8, 9), whereas others suggest that it is typically a Th1 autoreactivity from initiation (15). Regardless of the phenotype of initial autoimmunity, diabetes onset is most likely to be concurrent with β cell destruction. In the BABYDIAB offspring, diabetes onset per se and thus β cell destruction often occurred during an early or subsequent IgG1-dominated peak Ab response, suggesting that the IgG1 islet autoantibody response in these BABYDIAB offspring is characteristic of destructive autoimmunity. Because autoimmune β cell destruction associated with type 1 diabetes is thought to be Th1 mediated, it follows that Th1 islet autoimmunity includes the production of IgG1 autoantibodies. This finding would be consistent with the subclass responses seen in infections inducing Th1 immunity (27). In this study we also show that the subclass distribution of the initial peak Ab responses is similar to that seen at diabetes onset, suggesting that a destructive, potentially Th1 autoreactivity may be present from initiation in these offspring.

The strongest evidence for an Ab subclass response being characteristic of a Th phenotype in humans is the link between IgG4 and Th2 immunity. IgG4 shares features of the Th2-associated mouse IgG1 and is stimulated by the Th2 cytokine IL-4 (25, 26). In the BABYDIAB offspring, IgG4 responses were most frequently found against insulin, IgG4-IAA detected in around half of the offspring. In some of these, IgG4-IAA was clearly not synchronous with IgG1-IAA, supporting the possibility that they are influenced by different factors. In four offspring, IgG4-IAA were the predominant IAA from initial detection, suggesting the presence of concurrent Th2 autoimmunity. The observation that persistent IgG4-IAA appeared after peak IgG1-IAA titers in a minority of offspring also suggests a recruitment of, or switch to, Th2

immunity. Importantly, however, the presence of or switch to IgG4-IAA, even when dominant, was not associated with protection from diabetes. Moreover, the detection of IgG4-IAA was not followed by an IgG4 response to GADA and/or IA-2, indicating that if IgG4 is a marker of Th2 autoimmunity in these offspring, then this phenotype exhibits Ag specificity.

IgG2 and IgG3 Abs to all three Ags could be detected, but were usually of low titer, and particularly for IgG3, found predominantly when IgG1 Ab levels were at their peak. Indeed, when detected, IgG3 responses generally paralleled IgG1, whereas IgG2 levels followed the IgG4 response in some individuals. The IgG1 dominance seen for GADA and IA-2A is consistent with previous reports examining ICA at onset of diabetes (43, 44), whereas IgG4-IAA have been reported (45). The subclass distribution of GADA found in BABYDIAB offspring differs, however, from that recently reported for older first degree relatives, where using ELISA for Ab measurement, IgG2- and IgG4-GADA were frequently found in the absence of IgG1-GADA, particularly in relatives who had not progressed to disease (24). Although the discrepancies could be explained by differences in the cohorts tested and methods used for Ab measurement, they are also suggestive of eventual switching in the subclass distribution of GADA. We found no evidence of a deviation away from an IgG1-dominated GADA response over time in BABYDIAB offspring, but we cannot exclude such changes in individuals with long-standing islet autoimmunity.

Diabetes onset occurred during a peak Ab response in most of the offspring who developed disease. The only other correlate to diabetes development was a significantly higher progression in offspring who had exclusively IgG1 Ab responses to IA-2, suggesting that a subclass restricted, potentially Th1-biased autoreactivity to late Ag could represent a more aggressive pathology. Changes in

the subclass of autoantibodies did not correlate with protection or progression, and unlike what was reported for GADA (24), there was no evidence for slower progression if IgG2 or IgG4 autoantibody responses could be detected within the BABYDIAB offspring. Because IgG4 autoantibody responses may reflect an influence by IL-4, and a defect in the number and quality of IL-4 secreting NK-T cells together with low serum IL-4 levels has been reported in diabetes (46, 47), it will be relevant to measure serum IL-4 concentration in offspring before and after the development of autoimmunity to determine whether IL-4 concentrations are a better marker for disease protection.

The findings reported in the BABYDIAB offspring reflect the early islet autoimmunity associated with childhood type 1 diabetes in genetically susceptible individuals. We cannot predict whether autoimmunity arising later in life or in subjects with no family history of diabetes will be different, nor how the autoantibody response will behave in those BABYDIAB offspring who have autoimmunity but do not develop diabetes in childhood. Of interest is the IgA-dominated GADA response in offspring 1948 who developed islet autoimmunity relatively late. This indicates first, that other nonspontaneous forms of islet autoimmunity are likely, and second, that some of these forms could involve the mucosa.

In conclusion, the study of islet autoantibody titer, specificity, subclass, and previously epitope spreading in the BABYDIAB offspring cohort shows a very dynamic and active autoimmunity within the early pre-clinical phase of diabetes. We suggest that the relatively homogeneous timing of initial autoantibody responses in these offspring could be consistent with a spontaneous development of autoimmunity, and that this autoimmunity and the primary target may be genetically determined. The initial autoantibody response has characteristics of an early destructive autoimmunity which either results in rapid progression to disease or proceeds to a chronic autoimmune disease with remitting and relapsing activity.

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