

# Maturation of the Humoral Autoimmune Response to Epitopes of GAD in Preclinical Childhood Type 1 Diabetes

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GAD is a major target of autoimmunity in preclinical type 1 diabetes. Here we examine the maturation of the humoral response to GAD epitopes sequentially from birth to diabetes onset or current follow-up in 29 GAD antibody (GADA)<sup>+</sup> offspring of parents with diabetes from the BABYDIAB Study. Antibodies were measured against GAD65, GAD67, and GAD65/67 chimeras by radiobinding assay. In 28 of 29 offspring, the first GADAs contained reactivity against epitopes within GAD65 residues 96–444, suggesting that the middle GAD65 region is a primary target of GAD humoral autoimmunity. In 7 of these 28 offspring, initial antibody reactivity was against all epitope regions tested (middle GAD65, COOH-terminal GAD65 residues 445–585, NH<sub>2</sub>-terminal GAD65 residues 1–95, and GAD67); in 16 offspring, reactivity was to middle and COOH-terminal GAD65 epitopes, and in 5 offspring, reactivity was only to the middle GAD65 epitopes. The single offspring without middle GAD65 reactivity had antibodies to the NH<sub>2</sub>-terminal epitopes in the absence of all other islet autoimmunity. Subsequent GADA epitope spreading was frequent and seen in 10 of 15 offspring with informative follow-up samples. Spreading was mostly (eight cases) to NH<sub>2</sub>-terminal GAD65 epitopes. In two offspring, spreading to new epitopes was found when antibody titers to GAD65 and early epitopes were declining, suggesting determinant-specific regulation of the humoral response. None of the GADA reactivities nor any changes in reactivity over time were specifically associated with diabetes onset. The findings suggest that the humoral autoimmune response to GAD found in childhood is dynamic, is initially against epitopes within the middle portion of GAD65, and spreads to epitopes in other regions of GAD65 and GAD67. *Diabetes* 49:202–208, 2000

**A**utoantibodies to islet proteins are detected before the onset of type 1 diabetes, and their measurement identifies individuals at high risk for developing the disease (1). Whereas the majority of genetically susceptible individuals who have islet antibodies develops disease, little is known of the manner in

which the immune response to islet autoantigens develops and behaves in the preclinical phase of diabetes. The identification of autoantigen targets and their major epitope-containing regions allows the maturation of the islet autoimmune response leading to diabetes to be studied.

GAD is one of the major autoantigens of type 1 diabetes (2). There exist two isoforms: 1) GAD65, which is 585 amino acids in length and the principal target of autoantibodies, and 2) GAD67, which is 593 amino acids in length and to which antibodies can be detected in a minority of patients (3–6). Autoantibodies most frequently bind to conformational epitopes of GAD65 contained within its central portion (amino acids 240–440) and within the COOH-terminal amino acids 440–585 (7–10). Other epitopes are found in NH<sub>2</sub>-terminal residues (11–14), while the GAD67 reactivity is thought to be mainly against epitopes shared with GAD65 (5). Reactivity to linear epitopes at both the COOH- and NH<sub>2</sub>-terminal portions is infrequent, but it may be a distinguishing feature of patients with neurological disorders associated with very high titers of GAD65 antibodies (11–14). Whereas the major autoantibody epitopes are mapped, no studies of the maturation or spreading of GAD autoantibody reactivity over time have been reported. Here we measure antibodies to the principal epitope regions of GAD in sequential samples from birth in the BABYDIAB cohort of offspring of parents with type 1 diabetes. This is a cohort of genetically susceptible children who have been followed from birth to the development of autoimmunity and subsequently type 1 diabetes, and it provides an opportunity to examine the maturation of autoimmunity associated with childhood disease. We examined which epitopes are first recognized by antibodies, whether there are changes over time in the epitopes recognized, and whether there are epitope-specific GAD antibodies (GADAs) or specific changes associated with progression to diabetes.

## RESEARCH DESIGN AND METHODS

**Study subjects.** BABYDIAB is a prospective German multicenter study in which offspring of parents with type 1 diabetes are scheduled for regular visits with venous blood sampling at birth (cord blood), 9 months, and 2, 5, and 8 years of age (15). Subjects in whom islet autoantibodies are found are subsequently requested to provide yearly blood samples. Ethical committee approval for the study was granted by the Bayrische Landesärztekammer. At the time of this study, 1,036 newborns of mothers with type 1 diabetes, 497 newborns of fathers with type 1 diabetes, 36 newborns of two parents with type 1 diabetes, and 602 newborns of mothers with gestational diabetes had been recruited at birth and followed up to the age of 9.6 years. From the 2,171 children recruited, 1,436 9-month samples, 1,121 2-year samples, and 300 5-year samples have been obtained. All offspring have been tested for insulin autoantibodies (IAAs), GADAs, protein tyrosine phosphatase IA-2 antibodies (IA-2As), and islet cell antibodies (ICAs). A total of 28 offspring of parents with type 1 diabetes and 1 offspring from a mother with gestational diabetes (case 1,085) have developed GADAs after birth. From these 29 offspring, samples were collected in intervals from birth up to the age of 7.7 years and were included

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GADA, GAD antibody; IA-2A, antibody to tyrosine phosphatase IA-2; IAA, insulin autoantibody; ICA, islet cell antibody.

TABLE 1  
Offspring of parents with type 1 diabetes who developed GADs in BABYDIAB

Case	Total antibodies	Samples tested (age [years])	Age at first GADA (years)	Age at diabetes or last contact (years)
4849	IAA, GADA, IA-2A, ICA	0, 0.8, 1.3	0.8	1.3 D
2277	IAA, GADA, IA-2A, ICA	0.8, 1.7	1.7	1.8 D
4262	IAA, GADA, IA-2A, ICA	0.6, 0.8, 2.2	0.6	2.2 D
1628	IAA, GADA, IA-2A, ICA	0.7, 2.0, 3.0	2.0	3.0 D
4005	IAA, GADA, IA-2A, ICA	0.8, 2.1, 3.1	2.1	3.1 D
3941	IAA, GADA, IA-2A, ICA	0.7, 2.0	2.0	3.8 D
1085	IAA, GADA, IA-2A, ICA	0.8, 2.3, 3.0, 3.8, 6.1	2.3	6.1 D
1032	IAA, GADA, IA-2A, ICA	0.8, 1.9, 2.4, 2.9, 3.5, 4.0, 5.0, 6.1	1.9	7.1 D
1088	IAA, GADA, IA-2A, ICA	0.9, 2.1, 2.9, 3.5, 3.9, 5.0, 5.7, 7.0	2.8	8.5 D
5006	IAA, GADA, ICA	0.8, 2.2, 3.3	0.8	3.9
1649	IAA, GADA, IA-2A, ICA	0.9, 2.1, 2.2, 2.3, 3.5, 4.9	0.9	7.0
6746	IAA, GADA, IA-2A, ICA	0.8, 2.0	2.0	2.3
2223	IAA, GADA, IA-2A, ICA	0.8, 2.1, 2.5, 3.6, 4.6	2.1	6.6
4050	IAA, GADA, IA-2A, ICA	0.9, 2.1, 3.2	2.1	4.6
4204	IAA, GADA, IA-2A, ICA	1.0, 2.1, 3.0	2.1	4.4
6354	IAA, GADA, IA-2A	1.5, 2.1	2.1	3.3
6226	IAA, GADA, ICA	0.9, 2.2	2.2	3.2
2160	GADA	1.0, 2.4	2.4	3.8
3975	IAA, GADA, ICA	0.8, 2.4	2.4	3.2
3322	IAA, GADA, ICA	2.6	2.6	3.1
3929	IAA, GADA, IA-2A, ICA	0.9, 2.1, 3.1, 3.2, 4.2	3.1	5.6
4215	IAA, GADA, IA-2A, ICA	0.8, 2.1, 3.8, 4.8	3.8	5.3
4000	IAA, GADA, ICA	0.8, 3.8	3.8	4.8
4453	IAA, GADA	0.8, 3.8	3.8	4.3
1068	IAA, GADA	0.9, 1.8, 3.2, 4.2, 5.3	4.2	8.6
1103	GADA	0.8, 1.9, 5.2, 6.4, 9.0	5.2	9.5
1948	IAA, GADA, ICA	5.4, 6.3	5.4	8.6
1063	GADA, IA-2A	0.9, 2.1, 5.8	5.8	6.4
2721	IAA, GADA	0.9, 3.5, 6.5	6.5	8.0

D, offspring who have developed type 1 diabetes.

in this study (Table 1). The median follow-up time (time to diabetes or last contact) of the offspring from birth was 4.6 years (range 1.3–9.6 years). A total of 99 samples from the 29 offspring were available for this study. These included 24 samples at time points before that of the first GADA<sup>+</sup> sample from 23 offspring; the remaining 65 samples had GADs above the threshold for positivity. Nine of the offspring developed overt type 1 diabetes during their participation in the study (median age at onset was 3.1 years, range 1.3–8.5 years); two of the GADA<sup>-</sup> cohort have also developed diabetes. Diabetes onset was defined as a 2-h blood glucose value >11.4 mmol/l in the oral glucose tolerance test. Insulin treatment commenced on the day of diagnosis.

GAD constructs. The full-length human GAD65 cDNA (accession no. M81882) was previously cloned into the pGEM3 vector (Promega, Madison, WI) under the control of the SP6 promoter (16). A full-length human GAD67 cDNA (accession no. M81883) was amplified from brain mRNA by reverse transcriptase–polymerase chain reaction and cloned into the pGEM-T Easy vector (Promega). To detect GADs reactive with epitopes contained within the NH<sub>2</sub>-terminal, middle, and COOH-terminal regions of GAD65, GAD65/GAD67 chimeric constructs were prepared. First, both GAD65 and GAD67 cDNAs were mutagenized by site-directed mutagenesis (Stratagene, La Jolla, CA), respectively, to introduce a BglII restriction site at position 336 of the GAD65 sequence, to introduce a StuI site in nucleotide position 1902, and to abolish a BglII site in nucleotide position 687 of the GAD67 sequence. The GAD65<sub>1–95</sub>/GAD67<sub>102–593</sub> chimeric construct (NH<sub>2</sub>-terminal GAD65 epitopes) was made by subcloning an EcoRI–BglII fragment from GAD67 into the corresponding sites of the mutagenized GAD65. The GAD67<sub>1–101</sub>/GAD65<sub>96–444</sub>/GAD67<sub>453–593</sub> chimeric construct (middle GAD65 epitopes) was made by subcloning a partial BglII–StuI restriction fragment from the mutagenized GAD65, and containing the nucleotides coding for amino acids 96–444, into the corresponding sites of the mutagenized GAD67. The GAD67<sub>1–452</sub>/GAD65<sub>445–585</sub> chimeric construct (COOH-terminal GAD65 epitopes) was made by subcloning a Sall–StuI fragment from the mutagenized GAD67 into the corresponding sites of the full-length GAD65 clone. All constructs were under the control of the SP6 promoter and correctly corresponded to expected chimeric sequences as verified with an automated sequencer (Applied Biosystems, Foster City, CA).

Autoantibody measurement. Purified plasmid DNA of the constructs was obtained by Quantum Prep spin column preparation (Bio-Rad, Hercules, CA) and in vitro transcribed and translated using the TnT SP6 Coupled Rabbit Reticulocyte Lysate System (Promega) in the presence of [<sup>35</sup>S]methionine (Amersham International, Bucks, U.K.), according to the manufacturer's instructions. Unincorporated [<sup>35</sup>S]methionine was removed by gel chromatography on a NAP5 column (Pharmacia, Uppsala, Sweden). The correspondence of the molecular size for all translated proteins to that predicted from amino acid sequence was verified by SDS-PAGE and autoradiography. Radioimmunoassay was carried out as previously described (17). Results were expressed as arbitrary units relative to a standard curve prepared by measurement in each assay of a serum from a patient with stiff-man syndrome with high autoantibody levels serially diluted in normal serum. The upper value of 50 control sera was used as the threshold of autoantibody detection for each construct. This was 3 U for GAD65, 5 U for GAD67, 6 U for GAD67<sub>1–101</sub>/GAD65<sub>96–444</sub>/GAD67<sub>453–593</sub>, 8 U for GAD67<sub>1–452</sub>/GAD65<sub>445–585</sub>, and 4 U for GAD65<sub>1–95</sub>/GAD67<sub>101–593</sub>. Conformation of the GAD67<sub>1–101</sub>/GAD65<sub>96–444</sub>/GAD67<sub>453–593</sub> and the GAD67<sub>1–452</sub>/GAD65<sub>445–585</sub> products was validated using the human monoclonal antibodies MICA4, -6, -10, and -3, which were provided by Dr. Wiltrud Richter (18), and conformation of the GAD65<sub>1–95</sub>/GAD67<sub>101–593</sub> product was validated using sera from patients with stiff-man syndrome after competition with unlabeled GAD67 to remove reactivity to GAD67. In sera with reactivity to GAD67, the reactivity to the GAD65 portion of the chimeric constructs was verified by competition with unlabeled GAD67. Competition experiments in the study were performed by the addition of either purified baculovirus human GAD65 (1 µg), produced and purified in our laboratory in a manner similar to previously described procedures (19), or 15 µl of an in vitro translated unlabeled GAD67 reaction to 2 µl of serum for 1 h before performing the radiobinding assay.

Statistical analysis. Linear regression was used to correlate antibody titers to GAD65/67 chimeras and GAD67 with those against full-length GAD65. Spreading was defined as the detection of epitope binding above thresholds for positivity in follow-up samples but not in the initial GADA<sup>+</sup> sample in individual offspring. The difference between the frequency of detecting binding to chimeras in initial sam-

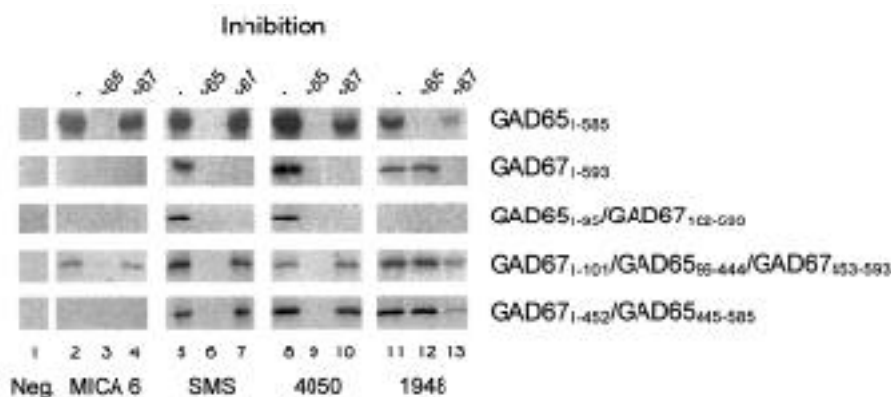


FIG. 1. Immunoprecipitation of GAD65, GAD67, and GAD65/67 chimeras. Direct binding (lanes 2, 5, 8, and 11) and binding after competition with unlabeled GAD65 (lanes 3, 6, 9, and 12) or unlabeled GAD67 (lanes 4, 7, 10, and 13) to the labeled proteins indicated on the right of the figure are shown for the MICA6 human monoclonal antibody (lanes 2–4), a serum from a patient with stiff-man syndrome (SMS) (lanes 5–7), a serum from BABYDIAB offspring 4050 (lanes 8–11), and a serum from offspring 1948 (lanes 12–14). Binding by a serum from a control subject is shown in lane 1.

ples and follow-up samples was determined using McNemar's test for matched samples. For all statistical methods, the Statistical Package for Social Sciences (SPSS, Chicago) was used.

## RESULTS

Epitopes recognized by offspring with GADAs. Antibody reactivity to the constructs used in the study is shown in Fig. 1. Antibodies to each of the constructs were detected. Binding to GAD65 was always completely inhibited by competition with unlabeled GAD65, but not with unlabeled GAD67. Binding to GAD67 was completely inhibited by both unlabeled GAD65 and GAD67 in some sera (Fig. 1, lanes 5–10), but by only GAD67 in others (Fig. 1, lanes 11–13), indicating the presence of antibodies that are against epitopes shared between GAD65 and GAD67 and antibodies that are specific for GAD67, respectively. Binding to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> and GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> chimeras was completely inhibited by GAD65 and not GAD67, indicating that antibodies against the GAD65 portions of the chimeras were present in all samples except the 6.3-year sample of offspring 1948. This sample (Fig. 1, lanes 11–13) has GAD67-specific antibodies that bound the GAD67 portion of these chimeras. Binding was only marginally inhibited by GAD65, and antibodies to the GAD65 portion of the chimeras was indicated by incomplete inhibition with GAD67. The lack of binding to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera in this sample indicates that this GAD67 reactivity requires residues within NH<sub>2</sub>-terminal amino acids 1–100 of GAD67. Antibody reactivity to the GAD65 portion of the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera was indicated either by the absence of GAD67 reactivity or, in the case of samples with GAD67 reactivity, incomplete inhibition by GAD67.

The level of antibody binding to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> and to the GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> chimeras correlated with that against the full-length GAD65 molecule ( $r = 0.5$  and  $0.8$ , respectively). A total of 5 sera from 3 offspring had antibodies to full-length GAD65 without detectable binding to GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> and 13 sera from 6 offspring bound full-length GAD65 and not the GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> chimera. Antibody levels to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera and to GAD67 correlated weakly with that against the full-length GAD65 molecule ( $r = 0.2$  and  $0.4$ , respectively), and reactivity to these constructs was not confined to those sera with the highest GAD65 antibody titers.

Epitopes recognized by early GADAs. In all but 1 of the 29 offspring, the first serum with detectable GAD65 antibodies showed binding to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/

GAD67<sub>453-593</sub> chimera (middle GAD65 epitopes), suggesting that the middle GAD65 epitopes were very early (Fig. 2 and Table 2). The single offspring without middle GAD65 reactivity (case 1103) had persistent high-titer antibodies to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera (NH<sub>2</sub>-terminal epitopes) in the absence of all other islet autoimmunity. Of the 28 offspring with middle GAD65 reactivity, 7 had antibody reactivity to all constructs (cases 4849, 4262, 6746, 3322, 4000, 1068, and 1063). Of the remaining 21 offspring, 16 showed binding also to the GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> chimera (COOH-terminal GAD65 epitopes), 1 also to GAD67, and none to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera (NH<sub>2</sub>-terminal epitopes) in their first GADA<sup>+</sup> sample. The prevalence of antibodies to each epitope region was significantly increased in the first GADA<sup>+</sup> sample compared with the prior GADA<sup>-</sup> sample ( $P < 0.01$ ). The proportion of offspring with antibodies binding the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera (8/29) or GAD67 (8/29) were significantly less than those with binding to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> chimera ( $P < 0.0001$ ) and to the GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> chimera ( $P < 0.0005$ ).

Spreading of GADA reactivity. In 18 of the offspring, one or more follow-up samples more than 1 year after first detection of GADAs and before or at diabetes onset were available for testing (Table 2 and Fig. 3). Three of these had reactivity to all constructs already in their first GADA<sup>+</sup> sample, and one offspring had persistent binding to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera only. Four had initial reactivity to the middle GAD65 epitopes only, and follow-up samples from

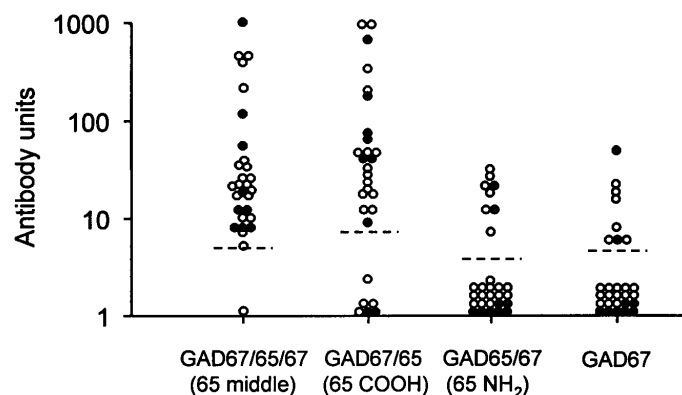


FIG. 2. Antibody titers to the GAD65/67 chimeras and to GAD67 in the first sample with GADAs in each of the 29 BABYDIAB offspring. ●, Offspring who have developed type 1 diabetes.

TABLE 2  
First GADA epitope reactivity and spreading in BABYDIAB offspring

Case	Age at first GADA (years)	First epitopes				Total epitopes				Age at spreading (years)	Age at diabetes (years)
		Middle	COOH	NH <sub>2</sub>	67	Middle	COOH	NH <sub>2</sub>	67		
4849	0.8	+	+	+	+*	+	+	+	+*		1.3
2277	1.7	+	+	-	-			NT			1.8
4262	0.6	+	+	+	+†	+	+	+	+†		2.2
1628	2.0	+	-	-	-	+	+	-	-	3.0	3.0
4005	2.1	+	+	-	-	+	+	+	-	3.1	3.1
3941	2.0	+	+	-	-			NT			3.8
1085	2.3	+	+	-	-	+	+	+	-	6.1	6.1
1032	1.9	+	+	-	-	+	+	+	-	2.9	7.1
1088	2.8	+	-	-	-	+	-	+	-	5.0	8.5
5006	0.8	+	+	-	-	+	+	-	-	None	
1649	0.9	+	+	-	-	+	+	+	-	2.1	
6746	2.0	+	+	+	+			NT			
2223	2.1	+	+	-	-	+	+	-	-	None	
4050	2.1	+	+	-	+†	+	+	+	+†	3.2	
4204	2.1	+	+	-	-	+	+	-	-	None	
6354	2.1	+	+	-	-			NT			
6226	2.2	+	+	-	-			NT			
2160	2.4	+	+	-	-			NT			
3975	2.4	+	-	-	-			NT			
3322	2.6	+	+	+	+			NT			
3929	3.1	+	-	-	-	+	+	+	-	4.2	
4215	3.8	+	+	-	-	+	+	-	-	None	
4000	3.8	+	+	+	+			NT			
4453	3.8	+	+	-	-			NT			
1068	4.2	+	+	+	+*	+	+	+	+*		
1103	5.2	-	-	+	-	-	-	+	-	None	
1948	5.4	+	-	-	-	+	+	-	+*†	6.3	
1063	5.8	+	+	+	+*			NT			
2721	6.5	+	+	-	-	+	+	+	-	7.7	

\*Antibodies recognizing GAD67-specific epitopes; †antibodies recognizing GAD67 epitopes shared with GAD65. NT, no follow-up sample >1 year after first GADA detection available.

three of these (cases 1628, 3929, and 1948) had additional binding to the GAD67<sub>1-452</sub>/GAD65<sub>444-585</sub> chimera (COOH-terminal GAD65 epitopes); follow-up samples from one (case 1948) also had binding to GAD67 epitopes, and those from the fourth (case 1088) had binding to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera (NH<sub>2</sub>-terminal GAD65 epitopes). From the remaining 10 offspring with both middle and COOH-terminal reactivity in their first GADA<sup>+</sup> sample, 6 showed additional NH<sub>2</sub>-terminal GAD65 reactivity, but no additional GAD67 reactivity in follow-up samples (cases 4005, 1085, 1032, 1649, 4050, and 2721). Antibodies to the GAD65<sub>1-95</sub>/GAD67<sub>101-593</sub> chimera were significantly more frequent in follow-up than in the initial GADA<sup>+</sup> samples ( $P < 0.01$ ). Whereas large changes (>100 U) in GAD65 antibody levels were usually concurrent with similar changes in antibody levels to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> and/or GAD67<sub>1-452</sub>/GAD65<sub>444-585</sub> chimeras, changes were not always unidirectional for all epitope regions (Fig. 3). Spreading to new epitopes occurred concurrently with a rise in GAD65 antibody titer in five offspring, in three offspring (cases 4005, 1088, and 1948) in a period of stable GAD65 titers, and in two offspring (cases 1032 and 1085), spreading to NH<sub>2</sub>-terminal epitopes occurred when titers to GAD65 and the other epitopes were declining (Figs. 3 and 4). In two offspring (cases 1088 and 5006), reactivity to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> chimera

was not detectable in follow-up samples, despite the presence of GAD65 antibodies and binding to other chimeras. No other loss of reactivity was observed.

Relationship of autoantibody epitope reactivity to progression to type 1 diabetes. So far, nine of the offspring with GADAs have developed type 1 diabetes, and their GAD epitope reactivity is shown in Table 2 and Fig. 4. Two of these (cases 4849 and 4262) had antibody reactivity against all constructs very early (ages 0.8 and 0.6 years) and developed diabetes at 1.3 and 2.2 years of age. One offspring (case 2277) developed GADA reactivity shortly before diabetes onset at age 1.8 years; another three offspring (cases 1628, 4005, and 1085) showed spreading of GADA reactivity in the diabetes onset sample, indicating spreading shortly before disease onset; and in the remaining three offspring, a sample at onset or within 1 year before onset was unavailable for testing. Neither broad reactivity nor spreading of reactivity were, however, specific indicators of rapid progression to diabetes: six offspring (cases 6756, 3322, 4050, 4000, 1068, and 1063) had reactivity against all constructs later in life (2.0, 2.6, 3.2, 3.8, 4.2, and 5.8 years) and have not yet developed diabetes; and spreading also occurred without rapid disease progression in three others (case 1032: diabetes onset 4.2 years after first spreading; case 1088: diabetes onset 3.5 years after first spreading; and case 1649: no diabetes 4.2 years after first

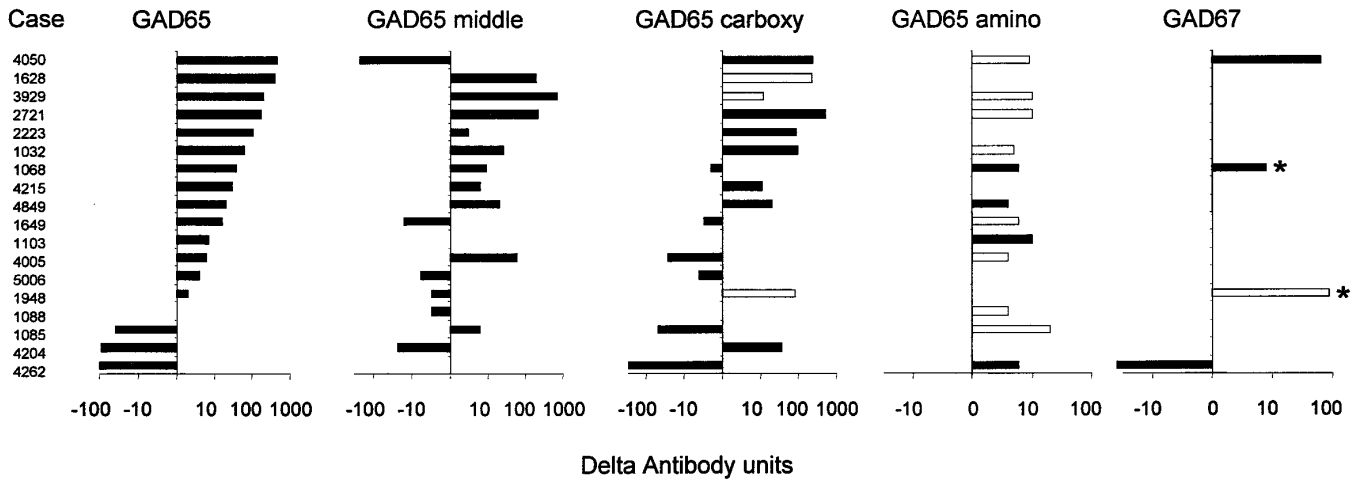


FIG. 3. Changes in antibody levels to epitope regions relative to changes in overall GAD65 levels. For each of the 18 offspring in whom follow-up samples were available, the difference in antibody units between the first GADA+ sample and the follow-up sample in which spreading occurred (10 offspring) or the first follow-up sample tested (8 offspring) is shown for each of the epitope regions. □, Antibodies detected for the first time in the follow-up sample. \*Reactivity against GAD67-specific epitopes. Differences are plotted on a logarithmic scale. The scales used for the GAD65 NH<sub>2</sub>-terminal epitope chimera and GAD67 differ from the remainder. Units are arbitrary and cannot be compared between epitope regions.

spreading). Of note is that the four cases in whom no spreading has been observed (cases 5006, 2223, 4204, and 4215) have not developed diabetes 2.9, 4.7, 2.4, and 1.5 years after first detection of GADAs (Table 2).

DISCUSSION

Autoantibodies to GAD often occur concomitantly or soon after the first detectable autoantibody response in infants who develop diabetes-associated autoimmunity (15). Here

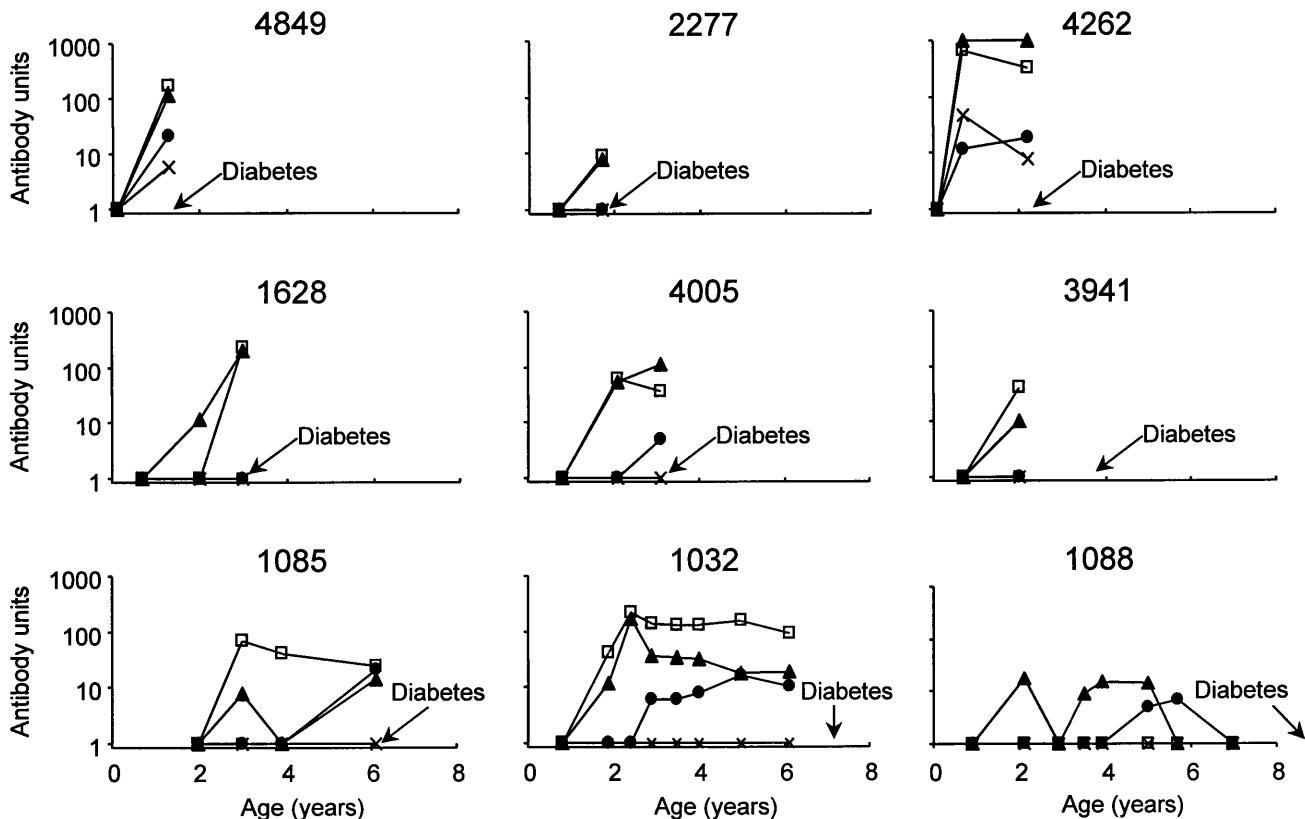


FIG. 4. Antibody titers to the GAD67<sub>1-101</sub>/GAD65<sub>96-444</sub>/GAD67<sub>453-593</sub> (middle GAD65 epitopes; ▲), GAD67<sub>1-452</sub>/GAD65<sub>445-585</sub> (COOH-terminal GAD65 epitopes; □), and GAD65<sub>1-95</sub>/GAD67<sub>102-593</sub> (NH<sub>2</sub>-terminal GAD65 epitopes; ●) chimeras and GAD67 (×) in sequential samples from birth in the nine GADA+ BABYDIAB offspring who developed type 1 diabetes. An arrow indicates the age at diabetes onset in each case.

it is shown that the first GADA reactivity almost always includes binding to epitopes contained within the central portion of GAD65, which corresponds to the previously described MICA4/6 reactivity (9) in the IDDM-E1 region (8). Binding to the COOH-terminal region corresponding to MICA1/3 reactivity in the IDDM-E2 region was also found in the majority of cases, indicating either a rapid spreading of reactivity or simultaneous immunization against distinct GAD65 regions, and showing the polyclonal nature of the GAD autoimmune response. Reactivity to NH<sub>2</sub>-terminal GAD65 epitopes was less common, weaker, and usually appeared after that against central and COOH-terminal epitopes, suggesting that these autoantibody epitopes are secondary in the diabetes-associated autoimmunity. GAD67 antibody reactivity was also relatively uncommon. Spreading was frequent, sometimes occurring several years after the first detection of GADAs, and was not always concurrent with increasing GADA titers. The few cases that showed stability in antibody reactivity did not develop disease, but diabetes development could not be predicted by antibody reactivity to GAD epitopes or by changes over time in this reactivity.

Whether there is a primary epitope is relatively important for understanding potential mechanisms of immunization and propagation of diabetes autoimmunity. In the nonobese diabetic mouse model of diabetes, it is reported that T-cell reactivity initiates against a COOH-terminal GAD65 peptide and subsequently spreads to other regions and other autoantigens (20). Others have also shown COOH-terminal and middle region T-cell epitopes in the mouse (21,22). In humans, T-cell clones derived from peripheral blood at onset of disease have been found to respond to epitopes in both the middle and COOH-terminal GAD65 regions (23–25), but studies in the early prediabetic phase are absent. Material in which the early responses can be studied in humans is scarce.

The German BABYDIAB Study is the first that examines the early phase of autoimmunity in children of parents with type 1 diabetes with sequential serum samples from birth (26), and allows analysis of the earliest epitopes recognized by the humoral arm of the autoimmune response. In this cohort, the early GADA response was against epitopes within GAD65 amino acids 235–444 in all but one case (see NOTE ADDED IN PROOF). Additional early antibody binding to epitopes in the GAD65 COOH-terminal region in the majority of the offspring indicates that both the middle and COOH-terminal regions of GAD65 are dominant early targets of the humoral GAD autoimmunity. However, no offspring had COOH-terminal GAD65 antibodies alone, while five offspring had antibodies to only the middle GAD65 epitopes, suggesting that the latter may be the principal primary humoral GAD target. Consistent with this hypothesis is the rapid spreading of antibody reactivity to the COOH-terminal epitopes in two of these offspring. Rapid spreading of GAD humoral reactivity is also suggested by the initial presence of antibodies to all four GAD specificities in seven offspring, two of these having such reactivity together with antibodies to other islet autoantigens already at 0.7 years and 1.3 years of age, respectively. Spreading, especially to minor epitopes, was not, however, always so rapid, and in two cases was seen 3 years after initial GADAs. Overall, the data are consistent with an initial humoral response to the middle region of GAD65 with rapid spreading to the second dominant region found in the

COOH-terminal of GAD65, and less frequently to GAD67, and subsequently to NH<sub>2</sub>-terminal GAD65 epitopes. One case was exceptional with reactivity to only the stiff-man syndrome-associated NH<sub>2</sub>-terminal GAD65 epitopes (13). Interestingly, this offspring had no other islet autoantibodies, and such reactivity may be atypical of type 1 diabetes. Similar studies of IA-2A reactivity in this cohort differed, with no single common early target region and independent reactivity to distinct portions of the protein (27). The epitope specificity of the early IA-2 humoral response was HLA linked. No HLA association was found for antibody reactivity to GAD epitopes (data not shown).

This middle portion of GAD65 contains both the pyridoxyl phosphate-binding domain and the region of GAD65 (amino acids 250–273), which has homology with the Coxsackie PC-2 protein (3). It was previously demonstrated that T-cell reactivity of patients can be detected against the corresponding peptides of both proteins (28). Cross-reactivity of GADAs with this region of the Coxsackie protein was, however, excluded (29), and we therefore predict that the major determinants reactive with these early middle GAD65 autoantibodies most likely correspond to the dominant conformation-dependent MICA4/6 specificity contained within GAD65 amino acids 245–440 (9).

For the first time, GAD67 has been shown as a unique autoantigen of the humoral diabetes-associated autoimmune response and not just as a consequence of cross-reactivity with GAD65. GAD67-specific antibodies were found in addition to antibodies against the GAD65 middle and COOH-terminal epitopes in the first GAD humoral response in three offspring and soon after middle GAD65 epitope reactivity in a fourth, indicating that GAD67-specific autoimmunity can appear early in the GAD response. Moreover, GAD67 binding in two of these offspring was not inhibited by competition with GAD65, suggesting the absence of antibodies cross-reactive with GAD65 and, therefore, a mechanism of antibody spreading that may not be via shared epitopes. T-cell reactivity to GAD67 has been reported and is likely to include recognition of GAD67-specific epitopes (30). The previous inability to identify GAD67-specific antibodies may have been due to the use of rat GAD67 as antigen in most studies. Similarly, binding to human and not rat protein has been reported for the diabetes-associated IA-2 $\beta$ -specific autoantibodies (17).

This study failed to identify GAD epitope-specific antibody reactivity that could be used as a marker for progression to disease. Neither the GADA reactivities nor any changes in reactivity over time were specifically associated with disease onset. However, while humoral intramolecular determinant spreading was seen in offspring with and without disease development, the absence of spreading was only found in offspring who did not develop disease and, therefore, may be an indicator of relative stability in the disease process. Previously reported IA-2A reactivity showed that spreading was limited to the autoimmune response found in early life, and that the autoimmunity seen in older subjects was stable with few or no changes in the autoantibody epitope repertoire (27). Such stability in later stages of the prediabetic period, also evidenced by relatively few changes in autoantibody titer (31,32), suggest that the humoral autoimmune response may be dampened either through self-regulation or reduced stimulus. The observation here for GAD and previously for IA-2 that spreading to some epitopes can

occur concurrently with a fall in antibody titer to others is consistent with a presence of determinant-specific regulatory mechanisms.

In conclusion, the humoral GAD autoimmunity associated with childhood type 1 diabetes appears to be first directed against epitopes within the middle portion of GAD65, with a rapid spreading to epitopes in other regions of GAD65 and less frequently GAD67. This dynamic early response is consistent with the previously observed marked intramolecular spreading of antibody reactivity against the IA-2 and IA-2 $\beta$  autoantigens (27) and intermolecular spreading of reactivity among the various islet autoantigens (15), and it suggests an active and aggressive autoimmunization in the early period of diabetes-associated autoimmunity in children.

Note added in proof. All 28 offspring with antibodies to middle GAD65 epitopes were also positive against a GAD67<sub>1-243</sub>/GAD65<sub>235-444</sub>/GAD67<sub>453-593</sub> chimera in their first GADA<sup>+</sup> sample, suggesting that GAD65<sub>235-444</sub> contains the early GADA epitope.

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#### REFERENCES

- Bonifacio E, Bingley P: Islet autoantibodies and their use in predicting insulin-dependent diabetes mellitus. *Acta Diabetologica* 34:185-193, 1997
- Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, DeCamilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347:151-156, 1990
- Kaufman DL, Erlender MG, Clare-Salzer M, Atkinson MA, Maclaren NK, Tobin AJ: Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 89:283-292, 1992
- Velloso LA, Kampe O, Hallberg A, Christmansson L, Betsholtz C, Karlsson FA: Demonstration of GAD65 as the main immunogenic isoform of glutamate decarboxylase in type 1 diabetes and determination of autoantibodies using a radioligand produced by eukaryotic expression. *J Clin Invest* 91:2084-2090, 1993
- Hagopian WA, Michelsen B, Karlsen AE, Larsen F, Moody A, Grubin CE, Rowe R, Petersen J, McEvoy R, Lernmark A: Autoantibodies in IDDM primarily recognize the 65,000-M<sub>r</sub> rather than the 67,000-M<sub>r</sub> isoform of glutamic acid decarboxylase. *Diabetes* 42:631-636, 1993
- Seissler J, Amann J, Mauch L, Haubruck H, Wolfjart S, Bieg S, Richter W, Holl R, Heinze E, Northemann W: Prevalence of autoantibodies to the 65- and 67-kD isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 92:1394-1399, 1993
- Richter W, Shi Y, Baekkeskov S: Autoreactive epitopes defined by diabetes-associated human monoclonal antibodies are localized in the middle and C-terminal domains of the smaller form of glutamate decarboxylase. *Proc Natl Acad Sci U S A* 90:2832-2836, 1993
- Daw K, Powers AC: Two distinct glutamic acid decarboxylase auto-antibody specificities in IDDM target different epitopes. *Diabetes* 44:216-220, 1995
- Syren K, Lindsay L, Stoehrer B, Jury K, Luhrer F, Baekkeskov S, Richter W: Immune reactivity of diabetes-associated human monoclonal autoantibodies defines multiple epitopes and detects two domain boundaries in glutamate decarboxylase. *J Immunol* 157:5208-5214, 1996
- Fallorni A, Ackefors M, Carlberg C, Daniels T, Persson B, Robertson J, Lernmark A: Diagnostic sensitivity of immunodominant epitopes of glutamic acid decarboxylase (GAD65) autoantibodies in childhood IDDM. *Diabetologia* 39:1091-1098, 1996
- Butler MH, Solimena M, Dirckx R, Hayday A, De Camilli P: Identification of a dominant epitope of glutamic acid decarboxylase (GAD65) recognized by autoantibodies in stiff-man syndrome. *J Exp Med* 178:2097-2106, 1993
- Li L, Hagopian WA, Brashear R, Daniels T, Lernmark A: Identification of autoantibody epitopes of glutamic acid decarboxylase in stiff-man syndrome patients. *J Immunol* 152:930-934, 1993
- Kim J, Namchuk M, Bugawan T, Fu Q, Jaffe M, Shi Y, Aanstoot HJ, Turck CW, Erlich H, Lennon V, Baekkeskov S: Higher autoantibody levels and recognition of a linear NH<sub>2</sub>-terminal epitope in the autoantigen GAD65 distinguish stiff-man syndrome from insulin-dependent diabetes mellitus. *J Exp Med* 180:595-606, 1994
- Daw K, Ujihara N, Atkinson M, Powers AC: Glutamic acid decarboxylase autoantibodies in stiff-man syndrome and insulin-dependent diabetes mellitus exhibit similarities and differences in epitope recognition. *J Immunol* 156:818-825, 1996
- Roll U, Christie MR, Fuchtenbusch M, Payton MA, Hawkes CJ, Ziegler AG: Perinatal autoimmunity in offspring of diabetic parents: the German multicenter BABYDIAB Study: detection of humoral immune responses to islet antigens in early childhood. *Diabetes* 45:967-973, 1996
- Bonifacio E, Genovese S, Braghi S, Bazzigalupi E, Lampasona V, Bingley PJ, Rogge L, Pastore MR, Bognetti E, Bottazzo GF, Gale EAM, Bosi E: Islet autoantibody markers in insulin-dependent diabetes: risk assessment strategies yielding high sensitivity. *Diabetologia* 38:816-822, 1995
- Bonifacio E, Lampasona V, Bingley PJ: IA-2 is the primary phosphatase-like autoantigen in type 1 diabetes. *J Immunol* 161:2648-2654, 1998
- Richter W, Endl J, Eiermann TH, Brandt M, Lientsch-Engel R, Thivolet C, Jungfer H, Scherbaum WA: Human monoclonal islet cell antibodies from a patient with insulin-dependent diabetes mellitus reveal glutamate decarboxylase as the target antigen. *Proc Natl Acad Sci U S A* 89:8467-8471, 1992
- Moody AJ, Hejnaes KR, Marshall MO, Larsen FS, Boel E, Svendsen I, Mortensen E, Dyrberg T: Isolation by anion-exchange of immunologically and enzymatically active human islet glutamic acid decarboxylase 65 over-expressed in Sf9 insect cells. *Diabetologia* 38:14-23, 1995
- Tian J, Olcott AP, Hanssen LR, Zekzer D, Middleton B, Kaufman DL: Infectious Th1 and Th2 autoimmunity in diabetes-prone mice. *Immunol Rev* 164:119-127, 1998
- Quinn A, Sercarz EE: T-cells with multiple fine specificities are used by non-obese diabetic (NOD) mice in the response to GAD(524-543). *J Autoimmun* 9:365-370, 1996
- Chao CC, McDevitt HO: Identification of immunogenic epitopes of GAD65 presented by Ag7 in nonobese diabetic mice. *Immunogenetics* 46:29-34, 1997
- Lohmann T, Leslie RD, Londei M: T-cell clones to epitopes of glutamic acid decarboxylase 65 raised from normal subjects and patients with insulin-dependent diabetes. *J Autoimmun* 9:385-389, 1996
- Endl J, Otto H, Jung G, Dreibusch B, Donie F, Stahl P, Elbracht R, Schmitz G, Meini E, Hummel M, Ziegler AG, Wank R, Schendel DJ: Identification of naturally processed T-cell epitopes from glutamic acid decarboxylase presented in the context of HLA-DR alleles by T-lymphocytes of recent-onset IDDM patients. *J Clin Invest* 99:2405-2415, 1997
- Bach JM, Otto H, Jung G, Cohen H, Boitard C, Bach JF, van Endert PM: Identification of mimicry peptides based on sequential motifs of epitopes derived from 65-kDa glutamic acid decarboxylase. *Eur J Immunol* 28:1902-1910, 1998
- 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
- Naserke HE, Ziegler AG, Lampasona V, Bonifacio E: Early development and spreading of autoantibodies to epitopes of IA-2 and their association with progression to type 1 diabetes. *J Immunol* 161:6963-6969, 1998
- Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK: Cellular immunity to a determinant common to glutamate decarboxylase and Coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 94:2125-2129, 1994
- Richter W, Mertens T, Schoel B, Muir P, Ritzkowski A, Scherbaum WA, Boehm BO: Sequence homology of the diabetes-associated autoantigen glutamate decarboxylase with Coxsackie B4-2C protein and heat shock protein 60 mediates no molecular mimicry of autoantibodies. *J Exp Med* 180:721-726, 1994
- Honeyman MC, Cram DS, Harrison LC: Glutamic acid decarboxylase 67-reactive T-cells: a marker of insulin-dependent diabetes. *J Exp Med* 177:535-540, 1993
- Ziegler AG, Ziegler R, Jackson RA, Eisenbarth GS: Type 1 diabetes: testing the "linear" destruction hypothesis. In *Immunotherapy of Type 1 Diabetes*. Andreani D, Kolb H, Pozzilli P, Eds. New York, Wiley and Sons, 1989, p.155-168
- Bingley PJ, Williams AJK, Gale EAM: Stability of autoantibody combinations over time (Abstract). *Diabetes* 47 (Suppl. 1):A226, 1998