Early epitope- and isotype-specific humoral immune responses to GAD65 in young children with genetic susceptibility to type 1 diabetes

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Abstract

Objective: The pattern of the humoral immunity to disease-associated autoantigens may reflect the severity of the autoimmune disease process. The purpose of this study was to delineate the maturation of the humoral immunity to one of the main autoantigens in type 1 diabetes (T1D), glutamic acid decarboxylase (GAD65).

Design and methods: Serum samples were obtained for the detection of epitope- and isotype-specific antibodies sequentially with short intervals from 36 young children with HLA-conferred genetic susceptibility to T1D starting from the first appearance of GAD65Ab. During prospective observation, ten children developed T1D. Antibodies were analyzed using biotinylated anti-human immunoglobulin (Ig) antibodies and chimeric GAD molecules in radio-binding assays.

Results: The immune response to GAD65 started as reactivity to the middle region and spread rapidly to the C-terminal region. IgG1 antibodies dominated among the isotypes from the first appearance of GAD65Ab, while other IgG subclasses were observed to a lesser extent. IgG4 antibodies emerged clearly as the last IgG subclass. A broad initial response comprising three to four IgG subclasses and the lack of an emerging IgG4 response during follow-up was associated with increased risk for progression to clinical diabetes (P<0.05).

Conclusions: The humoral response to GAD65 epitope clusters is relatively uniform in young children, whereas there is conspicuous individual variation in IgG subclass responses except for IgG1. A narrow initial IgG subclass response to GAD65 and the emergence of IgG4 antibodies were characteristic of those who remained non-diabetic over the first few years of GAD65 autoimmunity.

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin-producing β-cells in the pancreatic islets. The actual destruction of the β-cells is considered to be mediated by cell-mediated immunity (1). However, autoantibodies to β-cell antigens are commonly observed in the preclinical period and at clinical diagnosis, and are useful markers for the identification of individuals at risk of T1D (2). The character of the immune response is considered to be regulated by cytokines secreted from two CD4+ T-helper (Th) lymphocyte subsets: cytokines secreted from Th1 cells predominantly support cell-mediated immunity, whereas those produced by Th2 cells mainly activate humoral immunity (3). Thus, the balance between these two cell types may affect the risk of developing overt autoimmune disease. Evidence from non-obese diabetic (NOD) mice studies, a murine model for autoimmune diabetes, supports the view that β-cell destruction is mediated by a Th1-dominated immune response (3).

As cytokines regulate the generation of different immunoglobulin (Ig) isotypes and IgG subclasses, the distribution of various isotype-specific antibodies may reflect whether the immune response is Th1- or Th2-biased (4). However, the pattern is complex and poorly defined in humans. There is evidence that the Th2 cytokine IL-4 induces the synthesis of IgG4 and IgE (5), and the Th1 cytokine interferon γ (IFN) stimulates IgG1 and IgG3 production (6).

Glutamic acid decarboxylase (GAD) is one of the major autoantigens involved in T1D. Two isoforms of GAD are expressed in man, GAD65 and GAD67 (7, 8). Although these are quite homologous in their amino acid sequence, the humoral autoimmune response...
characteristic of T1D is mainly directed at GAD65 (9). The GAD65 antibodies (GAD65Ab) associated with T1D target principally the middle and C-terminal domains of the molecule, whereas a response to the N-terminal domain is rarely seen (10–13).

In this study, the maturation of the humoral immune response to GAD65 was explored by analyzing epitope- and isotype-specific GAD65Ab in young children genetically susceptible to T1D, starting from the first appearance of GAD65 autoimmunity with the follow-up samples taken as frequently as three to four times per year. Furthermore, the intention was to identify potential surrogate markers of disease development, since some of the children presented with overt T1D during prospective observation.

Subjects and methods

Subjects

The population was derived from the Type 1 Diabetes Prediction and Prevention (DIPP), project in Finland (14). In DIPP, children with increased genetic susceptibility to T1D (DQB1*02/0302, DQB1*0302/x (x≠DQB1*0301, DQB1*0602 or DQB1*0603) and males born in one of the three study centers with DQA1*05-DQB1*02/x) from the background population are observed from birth by taking blood samples at intervals of 3–6 months up to the age of 2 years and subsequently, 6–12 months. Islet cell antibodies (ICA) are used for the primary screening of β-cell autoimmunity. If ICA are detected, antibodies to GAD65, the protein tyrosine phosphatase-related IA-2 protein and insulin are analyzed in all the subsequent and the previous samples from that individual. Sequential samples are obtained from autoantibody-positive subjects at 3-month intervals. Children who persistently have autoantibodies (positive in at least two consecutive samples) are invited to take part in a randomized placebo-controlled intervention trial to explore whether it is possible to delay the manifestation of clinical diabetes by daily administration of nasal insulin. The protocol has been approved by the local ethics committees and the parents of the children have given their written informed consent to participate in the study.

The present series comprised the first 36 children in whom GAD65Ab had been detected in at least two samples during the prospective follow-up. All were ICA positive according to the screening protocol applied in the DIPP study. During observation, ten children presented with clinical T1D. The median age at diagnosis was 2.2 years (range 0.9–4.3 years), whereas the median age at the last sampling among those who remained unaffected was 3.4 years (range 2.0–5.0 years). The median number of samples analyzed per child was 7 (range 4–16) and 11.5 (range 7–18) among the progressors and non-progressors respectively. The mean interval between the last GAD65Ab-negative sample and the first GAD65Ab-positive one was 4.9 and 5.1 months in the progressors and non-progressors respectively, and between all samples it was 3.4 months in both the groups.

None of the children had transplacentally transferred GAD65Ab at the age of 3 or 6 months. Six children had a father with T1D (cases 1, 12, 16, 20, 21, and 33) and one child had a brother (case 18, Table 1) with T1D, whereas none of the children had an affected mother. In our series, 24 children (67%) had been randomized for the placebo-controlled intervention trial with intranasal insulin offered to those who persistently tested positive for two or more diabetes-associated autoantibodies. The median age was 2.0±0.5 (s.d.) years and the median time from the first appearance of GAD65Ab was 8 months at the time point when the insulin/placebo treatment had been initiated.

Antibodies to GAD65 epitope clusters and GAD67 were measured in all the follow-up samples available, and isotype- and IgG subclass-specific responses were determined starting from the last negative sample preceding the appearance of GAD65Ab. The samples taken after the diagnosis of T1D were not analyzed in this study. As far as possible, all samples from the same subject were analyzed in the same assay run.

Assays for antibodies to GAD65, GAD67, and GAD65 epitope clusters

Antibodies to GAD65 and GAD67, and GAD65 epitope clusters were analyzed with a radio-binding assay using 35S-labeled full-length proteins or GAD65/67 chimeric molecules produced in an in vitro transcription/translation system (Promega, Madison, WI, USA) as previously described (15, 16). The chimeric proteins were GAD65_1-95/GAD67_102-593 for N-terminal antibodies (GAD65-N-Ab), GAD671-101/GAD65_96-444/GAD67_451-593 for middle region antibodies (GAD65-M-Ab), and GAD67_1-453/GAD65_445-585 for C-terminal antibodies (GAD65-C-Ab) (12). The chimeric constructs were kindly donated by Ezio Bonifacio (Milan, Italy). The conformations of the chimeric proteins have been validated previously using monoclonal antibodies and competitive inhibition tests (12). The conformation of the GAD65 molecule was confirmed by monoclonal antibodies recognizing conformational epitopes in the middle domain (MICA4) and the C-terminal domain (MICA3) of GAD65. The results were expressed in relative units (RU) based on a standard curve run on each plate. The standard curves were constructed from serial dilutions of a pool of GAD65Ab-positive serum samples. The cut-off limit for positivity was set at the 99th percentile in 373 non-diabetic young Finnish subjects (mean age 10.5 years, range 0–19 years) in the GAD65Ab assay (5.35 RU) and in 104 subjects (mean age 10.9 years, range 0.5–18.2 years) in the assays.
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N, N-terminal Ab; M, middle region Ab; C, C-terminal Ab; IgA, IgE and IgM, isotype-specific Ab; the numbers indicate IgG subclass-specific Ab; ICA, islet cell antibodies; IAA, insulin autoantibodies; IA–2A, antibodies to IA-2 protein. Italic font indicates antibody positivity in a single sample.
for GAD65-N-Ab (0.86 RU), GAD65-M-Ab (1.51 RU), GAD65-C-Ab (1.59 RU), and GAD67Ab (0.91 RU). In order to eliminate false-positive samples resulting from GAD67 antibodies that are reactive with the GAD67 portion of the chimeric N-terminal molecule, a sample was considered not to have GAD65-N-Ab if the counts obtained from the GAD67 antibody assay exceeded the non-specific binding. The extremely low levels of GAD67 antibodies observed in eight subjects were not considered to affect the analysis of the middle and the C-terminal antibodies, the titers of which were many times higher than the levels of GAD67 antibodies. The disease sensitivity of the GAD65Ab assay was 82% and the specificity 98% in the 2002 Diabetes Antibody Standardization Program (DASP) workshop. In all assays, low and high standard serum samples were included on each plate to monitor assay precision. The coefficients of inter- and intra-assay variation were <20% in most assays.

**Assays for isotype-specific GAD65Ab**

IgG subclass- and isotype-specific GAD65Ab were measured using a modification of the conventional GAD65Ab assay (15). Briefly, 2 μl serum was incubated with 35S-labeled full-length GAD65 diluted in 50 μl assay buffer, after which biotinylated monoclonal mouse anti-human IgG1 (clone G17-1), IgG2 (G18-21), IgG3 (G18-3), IgG4 (JDC-14), IgA (G20-359), IgE (G7-26), or IgM antibodies (G20-127), and anti-rat IgM antibody (G53-238; all from BD PharMingen, San Diego, CA, USA) for unspecific binding were added at concentrations of 5 μg for IgG1, IgG3, IgE, IgM, and anti-rat IgM assays, and 8 μg for IgG2, IgG4, and IgA assays diluted in 25 μl assay buffer and incubated by shaking gently at +4 °C for 2 h. The immune complexes were separated by adding 15 μl biotin-specific streptavidin agarose beads (Pierce, Rockford, IL, USA) suspended in 50 μl assay buffer to each well and incubating with vigorous shaking at +4 °C for 1 h. After incubation, the reaction volume was transferred to a 96-well filtration plate (Millipore, Bedford, MA, USA) and the samples were washed 12 times with 150 μl assay buffer using a vacuum device (Millipore). The scintillation liquid (OptiPhase Supernix, Perkin-Elmer Life Sciences Walck, Turku, Finland) was added to the wells and the activity measured in a liquid scintillation counter (1450 Microbeta Trilux, Perkin-Elmer Life Sciences, Wallac, Finland). The results were expressed in S.DS as previously defined (17). The SDS was calculated from the equation: SDS = [delta c.p.m. (IgG subclass- or isotype-specific c.p.m. – unspecific anti-rat IgM c.p.m.)/mean delta c.p.m. of control subjects]/S.D. delta c.p.m. of control samples.

As controls, 22 non-diabetic Finnish children (mean age 9.2 years, range 0.7–16.6 years) were used. The cut-off limit for positivity was set at 3 SDS. None of the control samples exceeded the cut-off value in any isotype or subclass assay. The precision of the assays was monitored by re-analyzing 10% of the samples and including the assay on a regular basis, an internal standard known to be positive for the antibodies measured, except for the IgE-specific assay. Mean SDS ± S.D. of the internal standard samples were as follows: IgG1 85 ± 10, IgG2 9.8 ± 4.8, IgG3 4.2 ± 1.0, IgG4 3 ± 1.2, IgA 3.1 ± 1.5, and IgM 4.7 ± 1.8. We participated in the serum-exchange workshop carried out between laboratories known to measure isotype-specific responses to GAD65 (results presented at the 5th International Congress of the Immunology of Diabetes Society in Chennai, India in 2001), and our results were highly concordant with those laboratories using a similar method.

**Other assays**

Islet cell antibodies were quantified by a standard indirect immunofluorescence method using sections of human pancreas. The sensitivity and the specificity of the ICA assay were 100 and 98% respectively in the most relevant standardization workshop. Antibodies to IA-2 (IA-2A) and insulin (IAA) were analyzed with specific radio-binding assay. In the 2002 DASP workshop, the sensitivity and specificity of the IA-2A and IAA assays were 62 and 100%, and 44 and 100% respectively. More detailed information on the assays has been published previously (18).

**Genotyping**

The presence of HLA-DQB1 alleles *02, *0301, *0302, *0602, *0603, and *0604, and DQA1 alleles *02 and *05 was analyzed as described elsewhere (19, 20).

**Data handling**

In order to compare the antibody levels between the progressors and non-progressors, each progressor was matched as closely as possible with two non-progressors for sex (concordance 85%), genotype (85%), and GAD65Ab-positive observation time (1.43 vs 1.44 years respectively). The follow-up time was calculated from the last sample that preceded the first GAD65Ab-positive one to the time of diagnosis in the progressors, and the temporally corresponding samples in the non-progressors. Integrated antibody levels over the observation period were calculated for each antibody by area under the curve (AUC) analysis (21).

**Statistical analysis**

Differences in the frequencies of autoantibodies were tested with cross-tabulation and Chi-squared statistics or the Fisher’s exact test. The Mann–Whitney U-test was used to compare the AUC levels of antibodies
between the groups. Correlations were analyzed using the Spearman’s non-parametric rank correlation test ($r_s$). Kaplan–Meier life-table survival analysis and log-rank statistics were used to assess progression to clinical diabetes in relation to various epitope- and isotype-specific GAD65 antibodies. The statistical analyses were performed using the SPSS statistical software package, version 9.0 (SPSS, Chicago, IL, USA).

**Results**

**Antibodies to GAD65**

Antibodies to GAD65 appeared at the median age of 1.4 years (range 0.8–4.0 years) and remained detectable after their initial appearance in all but five subjects. In the latter subjects, GAD65Ab were either fluctuating (case 8) or transient (cases 3, 7, 9, and 12, Table 1). The median concentration of GAD65Ab increased as a function of the duration of the response. Antibodies to GAD67 were observed at an extremely low level in eight subjects.

**Epitope-specific responses to GAD65**

The first antibodies against GAD65 were mainly directed to the middle region of GAD65 (GAD65-M-Ab), 32 out of 36 children (89%) having GAD65-M-Ab in the first epitope-specific positive sample. The initial epitope-specific response comprised antibodies to the C-terminal region (GAD65-C-Ab) in 15 children (42%), always together with GAD65-M-Ab, and antibodies to the N-terminal region (GAD65-N-Ab) in three cases (8%) either alone or together with other specificities. Two children showed no reactivity to any of the epitope clusters at any time during the observation period (cases 7 and 36).

Antibodies to the middle region of GAD65 were later found in all children in whom antibodies to GAD65 epitope clusters were observed. Out of 17, 12 children (71%) with an initial response directed exclusively to the middle region developed subsequently GAD65-C-Ab (Fig. 1). When the antibody response spread to the C-terminal region, this occurred rapidly, so that the frequency of GAD65-C-Ab was equal to that of GAD65-M-Ab by 12 months after the initial GAD65 response (Fig. 1). Except one child (case 16, Table 1), the other children lost C-terminal GAD65Ab only if GAD65Ab were transient or fluctuating. The median levels of GAD65-M-Ab and GAD65-C-Ab increased as a function of the duration of the GAD65Ab positivity. The spreading of the immune response to the N-terminal region did not follow any uniform pattern. In general, the responses to the N-terminal region were weak and transient, and disappeared in all children within 22 months after their appearance.

**Isotype-specific responses to GAD65**

The humoral immune response to GAD65 was mainly composed of IgG1 antibodies, as the initial subclass-specific response comprised IgG1-GAD65Ab in all subjects (Table 1), and IgG1-GAD65Ab were dominant in the subsequent samples. There was also a close correlation between the levels of IgG1-GAD65Ab and GAD65Ab, and IgG1-GAD65Ab was almost always present when GAD65Ab were detected (Fig. 2). Antibodies of subclasses IgG2 and IgG3 often appeared together with IgG1 in the first isotype-specific positive sample or soon after the initial IgG1 response (Table 1, Fig. 2). Antibodies of subclass IgG3 were present mostly at low levels for a short time over the initial 3–12 months, after which there was a steep drop in their prevalence (Fig. 2). The occurrence of IgG2-GAD65Ab was more...
stable than that of IgG3-GAD65Ab; the IgG2 response lasted longer than a year in eight children and was present in the last sample analyzed in 14 cases, while the IgG3 response remained detectable for more than a year only in three children and was observed in the last sample analyzed in seven children. Antibodies of subclass IgG4 were clearly the last IgG antibodies to appear; only three children tested positive for IgG4 at the time of the appearance of GAD65Ab and their prevalence increased progressively during the follow-up (Table 1, Fig. 2). The IgG4 antibodies were found to be present for a year or longer in four children and were detected in the last sample analyzed in 11 cases. The median levels of antibodies of the IgG subclasses followed the trend observed in their frequencies. Antibodies of IgA, IgE, and IgM classes were rarely seen, and mostly in single samples only and at very low levels.

**Antibodies to GAD65 in the progressors and the non-progressors**

Antibodies to GAD65 appeared earlier in those who progressed to overt T1D (progressors) than in those who did not develop clinical disease (non-progressors, median age 1.1 vs 1.5 years; \( P < 0.05 \)), and the response in the progressors was often transient or fluctuating. No significant difference was seen in the appearance of epitope-specific antibodies between the groups (Fig. 3). The progressors more often had three or more IgG responses in their initial GAD65 response \( (P < 0.05) \), especially IgG2 and IgG3 responses than did the non-progressors (Table 1, Fig. 4). However, isotype-specific responses were observed equally over the whole follow-up in both the groups (Fig. 4). According to the life-table analysis, multiple IgG subclasses in the initial GAD65Ab response and the absence of IgG4 response during the observation time predicted more rapid progression to clinical T1D (Fig. 5A and B).

There was no significant difference in the integrated antibody levels based on AUC-matched observation time between the groups. The non-progressors had a trend towards increased AUC levels of GAD65Ab \( (P = 0.082) \), GAD65-M-Ab \( (P = 0.055) \), and IgG1-GAD65Ab \( (P = 0.091) \). Interestingly, the levels of IgG4-GAD65Ab correlated with those of IgG2 and IgG65Ab among the non-progressors \( (r = 0.46 \text{ and } 0.52; P < 0.05) \), whereas no significant correlation was seen among the progressors.

The levels of insulin autoantibodies (IAA, \( P < 0.05 \)) but not ICA or IA-2 antibodies (IA-2A) were higher in the progressors. No difference in the frequencies of these antibodies was observed between the groups. The progressors tend to be positive for all four major antibody specificities, i.e. ICA, IAA, IA-2A, and GADA, more frequently than the non-progressors but not significantly \( (70 \text{ vs } 30\%, P = 0.09) \). Multiple antibody specificities were not found to be associated with any epitope- or isotype-specific response or with a broad IgG subclass response to GAD65.

**Discussion**

The present data provide a detailed characterization of the humoral immune response to GAD65 in
preclinical T1D. We also had the opportunity to compare antibody responses between children who progressed to clinical T1D and those who remained unaffected during the follow-up. The data are unique from an immunological perspective as well as in relation to study design: both the isotype and the epitope profiles of the immune response to a distinct antigen were studied in the same children and the frequent sampling interval starting from birth made it possible to detect even minor changes in the dynamics of the immune response.

The present study indicates that the early phase of the humoral autoimmune response to GAD65 is a highly dynamic process reflected by isotype switching from the IgG1 subclass to other IgG subclasses and rapid epitope spreading from the middle to the C-terminal region. Frequent sampling intervals made it possible to observe that the IgG3 antibodies were often characteristic of the first phase of the humoral immune response to GAD65, while IgG4 antibodies appeared clearly as the last IgG subclass, being initially nearly undetectable. In this study, rapid epitope spreading from the middle to the C-terminal region was reliably documented in more than 40% of the young children. Evidence for epitope spreading has previously been based mainly on the findings related to the occurrence of GAD65-C-Ab together with GAD65-M-Ab, while spreading as such has been seen only in single individuals (12). The immune response was not observed to spread to the N-terminal region to such an extent as described previously (12, 22), possibly due to the younger age of the children in the present study or the HLA selection criteria used. As a whole, the response to GAD65 was confirmed to be predominantly composed of IgG1 antibodies and antibodies targeting the middle region of GAD65. Other IgG subclasses and antibodies to the C-terminal region were also present often, while other isotype-specific antibodies were rarely detectable, which is consistent with previous reports (10, 12, 17, 23–25).

None of the epitope-specific response was found to be associated with progression to T1D. This is in accordance with the earlier observations based on prospectively monitored individuals in the German BabyDiab study (12), the Finnish DiMe study (16), or a combined cohort of the Bart’s Oxford and the Munich family studies (26). Neither the spreading of the reactivity from the middle domain to the C-terminal region nor the GAD65-C-Ab titer was related to a higher risk of progression to clinical disease in the present study. In contrast, high risk of progression to clinical T1D has been reported to be related to the emergence of antibodies specific for certain N-terminal and middle region epitopes in older children when using a recombinant fraction antigen-binding (rFab) inhibition method for the analysis of epitope-specific responses (22). However, one has to keep in mind that blocking with rFabs does not necessarily correspond to the presence of antibodies to that specific epitope. A positive blocking by rFab simply reflects impaired antibody binding possibly as a consequence of steric hindrance but potentially as a result of conformational changes. Accordingly, our observations are not directly comparable with those obtained with the technique using rFabs.

Some reports based on cross-sectional study cohorts have shown that patients with newly diagnosed T1D are discriminated from healthy GAD65Ab-positive children by increased reactivity to the C-terminal region (10) and from both GAD65Ab-positive adult first-degree relatives of patients with T1D (27) and patients with slow-onset autoimmune diabetes (28) by reduced reactivity to the N-terminal region or GAD67. In addition, antibodies directed to a linear N-terminal GAD65 epitope have been reported to be more closely associated with slowly progressive T1D than to acute-onset disease in Japanese patients (29). The discrepancies may be explained by natural variation in the character of the humoral immune response to GAD65 between children and adults, and by different genetic
populations, but may also be partly due to the differences in the chimeric constructs used in the various studies (30).

We could not observe any statistically significant differences in the levels or frequencies of the various isotype-specific responses between progressors and non-progressors, a finding consistent with previous reports (16, 17, 26). However, the present study shows that a broad IgG subclass-specific response in the first GAD65Ab-positive sample was associated with an increased risk of progression to T1D. Previously it has been reported that a broad-isotype response to insulin and IA-2 but not to GAD65 is associated with progression (26).

The present study shows that an IgG3 response is frequently a component of the initial response to GAD65. In fact, the strongest initial IgG3 response to GAD65 was observed in single progressors. Similarly, the frequency of the IgG3 subclass of IAA was observed to be significantly higher in the progressors than the non-progressors among DIPP children in the first IAA-positive sample according to our earlier study (18). In addition, high levels of IgG3 subclass IAA were associated with progression to T1D. Since IgG3 subclass antibodies are perceived to reflect a Th1-biased immune response (6) and are induced most efficiently by virus infections (31), one may hypothesize that the immunization to a new autoantigen is induced by a virus infection and occurs in a Th1-cytokine milieu. This is supported by the fact that enterovirus infections have been shown to be associated with seroconversion to positivity for diabetes-associated autoantibodies (32). It has been shown in NOD mice that the Th1-biased immune response dominates in the initiation phase of the diabetic disease process, while the induction of a Th2-biased response later dampens the autoimmune response (33).

The late appearance of an IgG4 response to GAD65 mainly in those who remained non-diabetic might be interpreted as a change of the immune response towards less invasive Th2-immunity. In NOD mice, the switch of a Th1-polarized response towards a Th2-biased response has been reported to induce the production of antibody subclasses characteristic of Th2 responses (33). The present finding may be parallel to the observation on a reduced production of IgG2- and IgG4-specific antibodies to tetanus vaccine in young ICA-positive children at risk for future T1D (34). The increase observed in the IgG4-GAD65Ab levels hardly reflects the overall increase in IgG subclass concentrations during early childhood, since in that case one would expect to see a similar increase in the responses of the other GAD65Ab subclasses.

However, IgG4-GAD65Ab did not discriminate non-progressors from progressors prospectively; only a retrospectively performed life-table analysis indicated that progression to clinical T1D was significantly less common among those with an IgG4 response during the observation. Notably, the AUC analysis reflects the cumulative antibody response during the follow-up and accordingly it may not provide any information on possible late changes, e.g. the emergence of a growing IgG4 response in non-progressors. It has been reported that increased levels of IgG and IgM-GAD65Ab or IgG2, and/or IgG4-GAD65Ab characterize non-diabetic GAD65Ab-positive first-degree relatives of patients with T1D (23, 24). Unfortunately, the specificity of the assays employed in those two studies has not been confirmed in the recent workshop on the analysis of isotype-specific GAD65Ab.

The present study shows that frequent sampling is needed for a detailed characterization of the dynamic immune response, since isotype- and epitope-specific responses seem to appear sequentially with short intervals and isotype responses, especially the IgG3 response to GAD65, lasted often for less than a year. This may explain why e.g., IgG3 responses have been observed infrequently in other studies.

Some children followed here were treated with intranasal insulin. The administration of intranasal insulin has not been observed to affect first-phase insulin response and the levels of GAD65Ab in autoantibody-positive subjects at risk for T1D (35). Similarly, the initiation of exogenous insulin treatment in patients with newly diagnosed T1D has not been observed to induce any isotype-specific responses to GAD65 although induction of an Ig4 response to insulin was observed (36). This indicates that intranasal insulin treatment hardly had any impact on the present observations, and consistently, no immediate change was observed in isotype- or epitope-specific responses after the initiation of the intervention trial in the present study.

It has to be emphasized that the progressors in our cohort represent young children who develop clinical diabetes rapidly, while the non-progressors likely include both children with a more prolonged preclinical disease process and true non-progressors. The progressors in the present series often had a transient and fluctuating response to GAD65, which may reflect regulatory events in the disease process leading to overt T1D.

We conclude that the early humoral immune response to GAD65 usually starts as a response to the middle region of GAD65, then spreads to the C-terminal region within a year in ICA-positive young children. The response to GAD65 is mainly composed of antibodies of the subclass IgG1, but continuous IgG2 and IgG4 responses are frequently observed, whereas continuous IgG3 responses are rarely seen. A narrow initial IgG subclass response to GAD65 and the emergence of IgG4 antibodies were characteristic of those who remained non-diabetic over the first few years of GAD65 autoimmunity.
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