GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD(65) enzyme activity and humoral response

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GAD-alum treatment in patients with type 1 diabetes and the subsequent effect on GADA IgG subclass distribution, GAD65 enzyme activity and humoral response

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Abstract

We have previously shown that two injections of 20 μg GAD-alum to recent onset type 1 diabetic children induced GADA levels in parallel to preservation of insulin secretion. Here we investigated if boosted GADA induced changes in IgG1, 2, 3 and 4 subclass distributions or affected GAD$_{65}$ enzyme activity. We further studied the specific effect of GAD-alum through analyses of IA-2A, tetanus toxoid and total IgE antibodies. Serum from children receiving GAD-alum or placebo was collected pre-treatment and after 3, 9, 15 and 21 months. At 3 month a reduced percentage of IgG1 and increased IgG3/IgG4 were detected in GAD-alum treated. Further, IA-2A, IgE and tetanus toxoid antibodies, as well as GAD$_{65}$ enzyme activity, were unaffected confirming the specific effect of treatment. In the GAD-alum group, higher pre-treatment GADA were associated to more pronounced C-peptide preservation. The induced IgG3/IgG4 and reduced IgG1 suggest a Th2 deviation of the immune response.

Keywords: Immunotherapy; GAD$_{65}$; GAD-alum; GADA; Type 1 diabetes; T1D; IgG; IgG subclass

ClinicalTrials.gov identifier: NCT00435981

Abbreviations: GABA, gamma-aminobutyric acid; GADA, glutamic acid decarboxylase antibody; IA-2A, tyrosine phosphatase like protein IA-2 antibody; LADA, Latent Autoimmune Diabetes in Adults; SPS, Stiff person syndrome.
Introduction

In type 1 diabetes, autoantibodies against pancreatic antigens such as the 65 kD isoform of glutamic acid decarboxylase (GAD$_{65}$) [1], insulin and the tyrosine phosphatase-like protein IA-2 (IA-2) [2] may be detected long before clinical onset of the disease, and they are often present at diagnosis [3; 4; 5; 6; 7]. Even though autoantibodies are indicators of an ongoing immune process, it has not been established whether they contribute to the pathogenesis of the disease or if they are markers of an ongoing destructive process.

Specific immunomodulation with autoantigens aimed to halt the autoimmune process has been considered, both in the prevention and treatment of type 1 diabetes [8; 9; 10]. Studies using GAD$_{65}$ as a specific immunomodulatory agent aimed to halt the autoimmune process has shown promising results, not only in various studies using murine models, but also in clinical trials. In NOD mice, administration of GAD$_{65}$ prevented type 1 diabetes and also protected transplanted islets after onset of the disease [11; 12]. A dose-finding study in Latent Autoimmune Diabetes in Adults (LADA) patients supported the clinical effect and safety of subcutaneous administration of 20 µg alum-formulated recombinant human GAD$_{65}$ (GAD-alum) [13], and the 5-year follow up suggests a persistent effect [14]. We have conducted a phase II clinical trial, where we showed that two injections of 20 µg GAD-alum contributes to the preservation of residual insulin secretion in children with recent onset type 1 diabetes [15].

It has been suggested that the protective effect of antigen-based immunotherapies relies on the skewing of the immune response towards a Th2 associated phenotype [11; 16]. Nasal administration of GAD$_{65}$ to NOD mice induced high levels of the Th2 associated GADA IgG1 subclass [16]. In humans the antibody subclass association with Th1 or Th2 response is not
clearly defined, but IgG4 antibody production is characteristic for the Th2 predominant immune responses induced by the Th2 cytokines IL-4 and IL-13 [17; 18; 19].

Positivity for islet cell autoantibodies have been regarded as inclusion criterion in previous intervention studies [20; 21]. However, there has been some concern that induction of high autoantibody levels, following administration of autoantigens as immunomodulatory agents, could result in adverse events. In trials using either intranasal insulin in individuals at high risk for developing type 1 diabetes [22], or insulin B-chain given intramuscularly to patients with the disease [23], insulin antibodies were boosted without any adverse events. In LADA patients, GADA levels increased after injection of 500 µg GAD-alum but not after the lower dose, which yielded beneficial clinical effects [13]. However, administration of GAD-alum to type 1 diabetes children enhanced GADA levels at the clinically effective dosage [15]. High GADA levels have been associated with Stiff Person Syndrome (SPS), a rare neurological disorder [24]. In SPS patients, the enzyme activity of GAD$_{65}$ is inhibited, leading to a reduced $\gamma$-aminobutyric acid (GABA) synthesis and clinical manifestations [25; 26]. Even though high levels of GADA are commonly detected in both SPS and type 1 diabetes patients, it is estimated that 1/10 000 diagnosed with type 1 diabetes is affected by SPS [27].

Thus, this study was aimed to analyse changes in the GADA IgG subclass distribution, and if the effect of GAD-alum treatment was antigen specific in the humoral immune responses. We also studied whether the increase in GADA levels, observed upon GAD-alum injection, was associated with changes in the enzymatic activity of GAD$_{65}$ similar to that observed in patients with SPS.
Materials and methods

Study population
A detailed description of the trial has been published elsewhere [15]. Briefly, 70 type 1 diabetic individuals (10-18 years old), diagnosed within the previous 18 months, with fasting C-peptide levels above 0.1 nmol/L and presence of GADA at a screening visit were included in a double-blind, randomised, controlled trial. Randomly, 35 patients were assigned 20 µg GAD-alum (Diamyd®, Diamyd Medical, Stockholm, Sweden) treatment and 35 patients to placebo (alum-formula alone). Patients were given a primary injection on day 0 and a booster injection after four weeks.

Approval for the study was obtained from the national regulatory authorities and the Research Ethics Committee, Linköping. Written informed consent was obtained from participants and their parents according to the Declaration of Helsinki. Adverse events were regularly registered throughout the study, and neurological examination was performed by a paediatrician at regular intervals. Throughout the trial, all analyses were performed in a blinded manner.

Sample collection
Blood samples from 69 patients (one placebo patient dropped out after the first injection) were collected before the first injection (day 0) and after 1, 3, 9, 15, 21 and 30 months, in addition, a Mixed meal Tolerance Test (MMTT) was performed at all time points as previously described [15]. To avoid time-of-day differences, sample collection was performed during the morning hours. All samples were transported to Linköping within 24 hours, and serum samples were stored at −70°C for simultaneous analysis to avoid interassay variation.
The IA-2A and tetanus toxoid titers were measured at baseline, 3 and 9 months. Enzymatic activity and subclass analysis were performed at baseline, 3 and 15 months. Total IgE was analysed at baseline and at 21 months.

**GADA IgG subclass assay**

The GADA IgG 1, 2, 3 and 4 subclasses were measured using a modification of the conventional GADA assay and all samples were analysed in duplicates. Briefly, 5 µL serum and 60 µL human-recombinant \(^{35}\text{S}\)-labeled GAD\(_{65}\) (hr\(^{35}\text{S}\)-GAD\(_{65}\)) were incubated in a 96-well plate (Nunc 96 MicroWell plate, Nunc A/S, Roskilde, Denmark) under vigorous shaking at 4°C over night. In parallel, biotinylated monoclonal mouse anti-human IgG1, IgG2 and IgG4 (BD PharMingen, San Diego, CA, USA) and IgG3 (Southern Biotech, Birmingham, AL, USA) were incubated with streptavidin agarose beads (Thermo Scientific, Rockford, IL, USA) under vigorous shaking at 4°C over night. After incubation, 50 µL of the hr\(^{35}\text{S}\)-GAD\(_{65}\)-GADA complex was incubated with 50 µL of the biotinylated anti-human IgG antibody coupled to streptavidin agarose beads in a 96-well filtration plate (Millipore, Bedford, MA, USA). After incubation under vigorous shaking at 4°C for 2 h, samples were washed 8 times with 150 µL/well assay buffer using a vacuum device (Millipore). Then, scintillation liquid (OptiPhase Supermix, Perkin-Elmer Life Sciences Wallac) was added to the wells, and the activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, Perkin-Elmer Life Sciences, Wallac). The cut-off value for each subclass was determined using a GADA negative control, which was run in duplicate in each assay. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control.
**GAD\textsubscript{65} enzymatic activity assay**

Samples collected at baseline and at 3 and 15 months, were included in the analysis of GAD\textsubscript{65} enzymatic activity. GAD\textsubscript{65} enzyme activity was measured by a $^{14}$CO\textsubscript{2}-trapping method based on the enzymatic reaction of glutamate to GABA. GAD\textsubscript{65} works as an enzyme together with the coenzyme pyridoxal 5-phosphate (PLP). GADA-positive serum from SPS patients have shown to inhibit this reaction [26]. Thus, 150 µL of a solution containing K\textsubscript{2}HPO\textsubscript{4} (50 mmol/L), PLP (3 mmol/L; Fisher Scientific, Göteborg, Sweden) and GAD\textsubscript{65} (0.2 mg/mL; Diamyd Diagnostics AB, Stockholm, Sweden) was mixed with 15 µL serum, and incubated for 1 hour at room temperature. Subsequently, 28 µL of a mix containing K\textsubscript{2}HPO\textsubscript{4} (50 mmol/L), L-glutamic acid (5 mmol/L; Fisher Scientific, Göteborg, Sweden) and L-$[^{14}$C-(U)]-glutamic acid (0.4 µCi; Perkin Elmer, Boston, USA) was added to each tube. All tubes were sealed with a rubber stopper attached with a center well (Kimble Chase Kontes, Vineland NJ, USA) in which a NaOH-soaked (50 µL of 1 mol/L NaOH) filter paper (Camlab Ltd, Cambridge, UK) was placed and incubated in a water bath at 37°C for 1 hour with gentle agitation. The reaction was stopped by placing the tubes on ice for 10 minutes. The radioactivity of $^{14}$CO\textsubscript{2} captured on the filter papers was measured in a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences, Inc, Boston, MA, USA). Serum from one SPS patient was included in all the assays as a positive control for inhibition.

**Determination of GADA titers**

Serum GADA titers were determined using a radio-binding assay employing $^{35}$S-labelled recombinant human GAD\textsubscript{65} produced by in vitro transcription/translation (pEx9 vector kindly supplied by Prof. Åke Lernmark, University of Washington, Seattle, WA, USA). Sepharose protein A was used to separate free from antibody bound labelled GAD\textsubscript{65} [28].
A standard curve, consisting of serial dilutions of GADA positive serum, was included on each plate. Wells containing only buffer were included as blanks. Positive and negative controls were also included in each plate. The immunoprecipitated radioactivity was counted in a Wallac 1450 Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences), and the results were expressed as units/mL in relation to the standard curve. The cut-off for positivity was regarded as 67.3 u/mL (corresponding to 23.1 WHO units) based on the 95th percentile of measurements from 1700 children, aged 5-6 years, participating in the ABIS (All Babies of Southeast Sweden) study. Our assay is validated through the Diabetes Autoantibody Standardization Program (DASP) workshop; in 2007 our assay had 94% specificity and 82% sensitivity.

To ensure the accuracy of the results, all samples were tested in four replicates by analyses of duplicated wells in two plates that were simultaneously assayed. To exclude inter-assay variation, samples from each patient collected before the first injection and after 1, 3, 9 and 15 months were run simultaneously. Samples collected at the 21 and 30 month controls were subsequently analysed in parallel with samples from the 15 month control to further minimise inter-assay variation.

**Determination of IA-2A**

Measurements of antibodies to tyrosine phosphatase-like protein IA-2 (IA-2A) were performed in serum as described for GADA. A plasmid (pSP64poly A, kindly supplied by Prof. Åke Lernmark) containing cDNA for the cytoplasmic portion of islet antigen 512 was used. In the 2007 DASP workshop, our IA-2A assay had 65% sensitivity and 98% specificity.
Analysis of tetanus toxoid antibodies

Determination of tetanus toxoid antibodies in serum was performed using an Immunozym Tetanus Ab ELISA according to manufacturer’s instructions (IBL, Hamburg, Germany). The optical density was measured at 450 nm using a VersaMax microplate reader (MDS, Inc., Sunnyvale, CA, USA). Cut-off levels were determined at > 0.070 IU/mL.

Analysis of IgE

Total serum IgE was quantified using the ImmunoCap100 system (Phadia AB, Uppsala, Sweden). The measuring range for the assay was 2-50000 kU/L, and calibrators were run in duplicate to obtain a full calibration curve. Levels of total IgE ≥ 85 kU/L were regarded as positive.

Criteria for Responder, Intermediate responder and Non-responder

Clinical effect of the treatment was determined by changes in stimulated C-peptide, as measured by Area Under the Curve (AUC) from baseline until month 15. Based on this measurement, patients receiving GAD-alum were stratified in three subgroups: Responders (patients with a loss of AUC less than 10%, n=8), Intermediate responders (AUC loss between 10% and 65%, n=18) and Non-responders (AUC loss more than 65%, n=9).

C-peptide measurement

Laboratory analyses were performed at the Paediatric Research Laboratory, Linköping University, Sweden. C-peptide levels were measured in serum samples with a time-resolved fluoroimmunoassay (AutoDELFIA™ C-peptide kit, Wallac, Turku, Finland). Results in each assay were validated by inclusion of a C-peptide control module containing a high, a medium and a low-level control (Immulite, DPC, UK). A 1224 MultiCalc® program (Wallac) was
used for automatic measurement and result calculation, with measurements expressed in pmol/mL.

Statistics

As the immunological markers were not normally distributed, non-parametric tests corrected for ties were used. Unpaired analysis was performed using Kruskal-Wallis followed by the Mann-Whitney $U$-test. Differences within the GAD-alum and placebo groups were analysed by Friedman’s test followed by Wilcoxon-signed rank test; p-values <0.05 were considered statistically significant. The statistical analyses were performed using SPSS version 16.0 software package for Windows (SPSS Inc., Chicago, IL, USA).
Results

GAD-alum treatment reduced GADA IgG1 while IgG3 and 4 subclasses increased

To clarify if induction of GADA levels following GAD-alum injection had an effect on the frequency of IgG subclasses, IgG1, 2, 3 and 4 were measured. Frequencies of the different IgG subclasses were calculated with respect to total IgG in each sample at every time point. The predominant IgG subclass for GADA was IgG1, both in the GAD-alum and placebo cohorts (Table 1). At baseline the percentage of IgG1 was similar for both groups (GAD-alum: 95.8%, placebo: 95.5%). However, in samples collected at 3 months, the frequency of IgG1 decreased significantly in GAD-alum treated (89%) compared to the frequency observed in placebo patients (95%) (p=0.005) (Fig 1A). At this time-point the percentages of IgG3 and IgG4, in relation to total IgG, increased in the GAD-alum treated patients (2.2-5%, p=0.01; and 1-2.8%, p=0.048, respectively), whereas the distribution remained unaffected in the placebo group. Differences between the groups after 3 months were explained by the longitudinal reduction in the IgG1 frequency and an increase of IgG3 and IgG4 in samples from GAD-alum patients (Fig. 1B), while the frequency of IgG1, 2, 3 and 4 did not change at any time point in samples from patients receiving placebo (Fig. 1C).

Increase in GADA levels was not associated with inhibition of GAD_{65} enzymatic activity:

High GADA levels leading to neurological symptoms have been associated to a decreased production of GABA in the nervous system of SPS patients [24; 29]. Serum analysis demonstrated that there were no longitudinal changes in the capacity for inhibiting the enzymatic activity of GAD_{65}, neither in samples from GAD-alum treated (n=35, Fig. 2A) nor from placebo patients (n=34, Fig. 2B). Further, there were no differences between the groups at any time point (data not shown). In contrast, the SPS serum included as positive control
The effect of GAD-alum in GADA subclass distribution. Differences in IgG 1, 2, 3, 4 subclasses between GAD-alum treated (black circles) and placebo patients (white circles) (Fig. 1A). Changes in subclasses within the GAD-alum (Fig. 1B) and placebo (Fig. 1C) groups from baseline to 15 months. Frequencies of the different IgG subclasses were calculated with respect to the combined sum of all four subclasses in each sample (i.e. total IgG). The median percentage of total IgG is shown for each respective subclass. Results were expressed as cpm, and positivity of each sample was calculated by subtraction of the mean cpm value plus three times the standard deviation (SD) obtained for the negative control. P values < 0.05 were regarded as statistically significant.
clearly inhibited the GAD$_{65}$ enzymatic activity compared to patients receiving GAD-alum or placebo injections ($p<0.001$) (Fig. 2A-B).

**Fig. 2 A-B**
GAD$_{65}$ enzymatic activity assay for serum obtained from patients treated with GAD-alum (Fig 2A) or placebo (Fig 2B). GAD$_{65}$ enzyme activity is reported as radioactivity from $^{14}$CO$_2$ released from $^{14}$C-glutamic acid (cpm). Serum samples obtained at baseline and after 3 and 15 months were analysed for their inhibition of GAD$_{65}$ enzyme activity. A control sample from one SPS patient (white triangles) was included in each run. Horizontal lines represent the median. $P$ values $<0.05$ were regarded as statistically significant.

IA-2A titers are unaffected by GAD-alum treatment

To assess whether the immunomodulatory effect of GAD-alum treatment on the humoral immune response was specific to GAD$_{65}$, IA-2A was measured prior treatment and in samples collected 3 and 9 months later. Analysis of IA-2A levels revealed no longitudinal changes in either GAD-alum or placebo patients (Fig. 3). Pre treatment levels of IA-2A were higher in the group that randomly received placebo compared to individuals treated with GAD-alum ($p<0.05$) (Fig. 3). This difference persisted at 3 and 9 months, and a gradual decrease in antibody titers was observed in both groups. After correcting for the IA-2A baseline level, no significant difference in the decrease in IA-2A titers was observed between the groups (data not shown).
Effect of GAD-alum treatment on IA-2 autoantibody (IA-2A) levels in treated children. IA-2A was determined by RIA in serum samples collected prior to treatment and at 3 and 9 months after the first injection. Individual levels for the GAD-alum treated (n=35, black circles) and placebo (n=34, white circles) groups are shown as dot plots. Horizontal lines represent the median. P values < 0.05 were regarded as statistically significant.

Tetanus antibody and IgE titer analysis

To further assess the specific effect and to search for a possible allergy-associated effect in response to injection of GAD-alum, tetanus toxoid and total IgE antibodies were measured. Tetanus toxoid titers were analysed in samples collected prior to treatment and after 3 and 9 months (Fig. 4). Total IgE was analysed in samples collected pre-treatment and 15 months after the first injection (Fig. 5). There were no significant differences between or within the groups at any time point for any of the respective analytes.

Response to GAD-alum in relation to baseline GADA

To determine whether baseline levels of GADA were related to the effect of treatment, children receiving GAD-alum were stratified into Responders, Intermediate and Non-
Tetanus toxoid antibody levels in children who received GAD-alum or placebo. Antibodies were determined by ELISA in serum samples collected prior to treatment, and 3 and 9 months after the first injection. Antibody levels for each patient in the GAD-alum (n=35, black circles) and placebo (n=34, white circles) groups are shown as dot plots. Horizontal lines represent the median. P values < 0.05 were regarded as statistically significant.

Responders. The patients who responded to the treatment with a better preservation of residual insulin secretion at 15 months, had higher baseline GADA levels compared to those regarded as Intermediate-responder (p<0.05) and Non-responder patients, although the latter difference did not reach statistical significance (Fig. 6). In the placebo group no association between baseline GADA levels and preservation of C-peptide was observed (data not shown). In contrast, no difference in baseline IA-2A levels was observed regardless of the response to treatment.
Total IgE antibody levels were determined by Multicap technique (Phadia AB, Uppsala, Sweden) in serum from patients receiving GAD-alum or placebo, collected prior to treatment and at 21 months after the first injection. Individual antibody levels for the GAD-alum (n=35, black circles) and placebo (n=34, white circles) groups are shown as dot plots. Horizontal lines represent the median IgE for the group. P values < 0.05 were regarded as statistically significant.
Baseline GADA levels in serum samples from GAD-alum treated. Patients were stratified as responders, intermediate and non-responders, according to the preservation of beta cell function, defined as changes in the stimulated C-peptide (AUC) from baseline until 15 months. Responder: patient with a loss of AUC of less than 10%. Intermediate responder: AUC loss between 10% and 65%. Non-responder: AUC loss more than 65%. Horizontal lines represent the median. P values < 0.05 were regarded as statistically significant.
Discussion

We have previously reported that administration of GAD-alum induced GADA levels in type 1 diabetic children [15]. It has been suggested that the protective effect of antigen-based immunotherapies in type 1 diabetes relies on skewing the Th1 predominant immune response towards a Th2 associated phenotype. As cytokines regulate the generation of different IgG subclasses, distribution of subclass specific antibodies may reflect whether the immune response is Th1 or Th2 skewed [30]. As previously described for type 1 diabetes patients, we found that GADA antibodies were mainly composed of the IgG1 subclass, while the other subclasses are less frequent [31; 32]. The IgG subclass distribution prior initiation of the treatment was similar in both groups. However, 3 months after the first injection, a significant reduction in the IgG1 frequency accompanied by an increase in IgG3 and IgG4 subclasses was detected in samples from patients treated with GAD-alum. These changes were evident at the same time point where the highest levels of GADA induced by the treatment were observed [15]. In the Prediction and Prevention study in Finland, emergence of GADA of the IgG4 subclass was observed in genetically susceptible children who remained non-diabetic [32]. Moreover, higher GADA of the IgG4 subclass were observed in LADA as compared to type 1 diabetes patients, which was interpreted as an indication of a more balanced immune response in the pancreatic tissue [33]. Thus, increased IgG4 after administration of GAD-alum might further support the notion, here at the level of a humoral response, that a predominant protective Th2 response was induced as a result of the treatment. It is noteworthy that after 9 months, the IgG subclass distribution in GAD-alum treated returned to those observed prior treatment. Simultaneously, the levels of GADA boosted by treatment started to decrease [15]. Thus, it is possible that an additional booster injection of GAD-alum, able to
strengthen the early induced Th2 responses, might maintain a predominant IgG4 subclass response.

There has been some concern that high GADA levels following injection of GAD-alum, could result in adverse events due to a possible effect on the nervous system [7; 26; 29]. In our study, no treatment-related adverse events were observed and higher GADA levels were not associated with any clinical neurological symptoms [15]. To further clarify the safety of GAD-alum, serum samples were analysed for their capability to inhibit the enzymatic activity of GAD_{65}. It should be noted that the GAD_{65} enzymatic activity measurement is an *in vitro* assay. Whether this *in vitro* assay reflects the *in vivo* status is unclear, considering that we assess GADA present in peripheral blood, while the neurological symptoms associated with SPS most likely originate from the CNS. However, the present study indicates that the enzymatic activity of GAD_{65} was unaffected by serum samples obtained from GAD-alum treated, while in contrast, SPS serum clearly inhibited the activity, as previously reported [25].

We also investigated if the effect of GAD-alum treatment on the humoral response was antigen specific. Additional analysis of autoantibodies to another type 1 diabetes related autoantigen, IA-2, revealed that IA-2A levels were unaffected by GAD-alum injections, corroborating the specific effect of treatment. Higher IA-2A prior treatment in the placebo group should be considered as a selection bias, since inclusion criteria for the trial only required measurement of GADA but not IA-2A. However, IA-2A levels were not associated to C-peptide preservation at any time-point in either treatment group, suggesting that IA-2A levels have no influence on the effect of GAD-alum. In concordance with previous findings in type 1 diabetes patients [5], a general decrease of IA-2A was observed in both groups over time. The specific immunomodulatory effect of GAD-alum was further confirmed by analysis
of tetanus toxoid antibodies, as no differences in titers were observed between the groups after treatment. The absence of allergic reactions following GAD-alum injections, demonstrated in the clinical trial, was confirmed by our analyses of total IgE.

Although GADA positivity was an inclusion criterion in the trial, pre treatment levels differed considerably among the patients. Therefore, we explored whether GADA titers prior treatment were associated with the clinical outcome in patients receiving GAD-alum. Our analysis revealed that baseline GADA levels were higher in patients with a better C-peptide preservation 15 months after the first injection. In the Diabetes Prevention Trial (DPT-1), oral insulin did not prevent type 1 diabetes. However, a beneficial effect of treatment was observed in individuals with high levels of insulin autoantibodies pre treatment [34]. It cannot be excluded that GADA levels simply reflect the current immunological status, and that patients with higher autoantibody levels might have more residual beta cells. However, this explanation seems unlikely, as the GADA titers after onset of type 1 diabetes is independent of C-peptide levels [35]. Although this is an interesting observation, considered by others as an outstanding issue for antigen based therapies in type 1 diabetes [36], this analysis was based on a small number of individuals, and our results need to be assessed in the ongoing phase III GAD$_{65}$-intervention trial including a larger study population with shorter disease duration.

In summary, promotion of the Th2 associated IgG4-subclass as part of the boosted GADA response is an interesting observation to be confirmed in ongoing trials. In addition, analyses of the effect of serum on the enzymatic activity of GAD$_{65}$ and the antigen-specific effect of treatment on the humoral immune response, further supports the safety of GAD-alum. Understanding the immunomodulatory effect of antigen-specific immunotherapy in humans
with type 1 diabetes is a key issue for the development of effective intervention treatments, alone or in combination with other approaches.

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References


Table 1

GADA subclass distribution

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th></th>
<th>IgG2</th>
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<th>IgG3</th>
<th></th>
<th>IgG4</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>GAD-alum</td>
<td>Placebo</td>
<td>GAD-alum</td>
<td>Placebo</td>
<td>GAD-alum</td>
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<td>GAD-alum</td>
<td>Placebo</td>
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<tr>
<td>Baseline</td>
<td>3394 (110-12199)</td>
<td>3754 (97-11515)</td>
<td>50 (4-240)</td>
<td>35 (4-291)</td>
<td>80 (1-735)</td>
<td>90 (3-2714)</td>
<td>47 (3-688)</td>
<td>65 (1-1099)</td>
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<td></td>
<td>35/35</td>
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<td>24/34</td>
<td>31/35</td>
<td>29/34</td>
<td>22/35</td>
<td>24/34</td>
</tr>
<tr>
<td>3 months</td>
<td>7420 (760-12569)</td>
<td>3024 (119-10811)</td>
<td>96 (18-937)</td>
<td>47 (3-252)</td>
<td>425 (15-4086)</td>
<td>87 (1-2094)</td>
<td>231 (5-3017)</td>
<td>67 (6-668)</td>
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<td>24/34</td>
<td>32/35</td>
<td>31/34</td>
<td>29/35</td>
<td>27/34</td>
</tr>
<tr>
<td>15 months</td>
<td>6063 (265-11311)</td>
<td>3468 (65-9795)</td>
<td>61 (5-1646)</td>
<td>40 (5-696)</td>
<td>237 (13-2186)</td>
<td>109 (7-1016)</td>
<td>103 (6-2762)</td>
<td>57 (7-542)</td>
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<td>28/35</td>
<td>27/34</td>
</tr>
</tbody>
</table>

The median levels (range) of IgG are represented in cpm. The numbers of individuals with detectable levels are shown for each time point. To determine positivity for every sample, the mean cpm value for a GADA negative control and 3xST (standard deviation) of the same control was subtracted from each sample. All values above 0 after this calculation were regarded as positive.