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GAD autoantibody epitope pattern after GAD-alum treatment in children and adolescents with type 1 diabetes

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Abstract

Aims—We have previously shown that two injections of glutamic acid decarboxylase formulated in alum (GAD-alum) preserved residual insulin secretion in children and adolescents with recent onset type 1 diabetes (T1D), and was accompanied by increased GAD autoantibody (GADA) titers. The aim of this study was to investigate whether GAD-alum treatment affected the GADA epitope pattern.

Methods—Serum samples from patients treated with GAD-alum (n = 33) or placebo (n = 27), at baseline, 1, 3, 9, and 15 months after the initial injection, were tested for their binding capacity to specific GADA epitopes in an epitope-specific radioligand binding assay with six recombinant Fab (rFab) (b96.11, DPA, DPD, MICA3, b78, and N-GAD₆₅ mAb).

Results—No significant differences in variability of binding to any of the tested rFab were observed from baseline to 15 months. There was a sustained low binding of GADA to the b78- and N-GAD₆₅ mAb-defined epitopes, often recognized by GADA in patients with stiff person syndrome (SPS) and seldom in T1D patients. However, binding of GADA to the T1D-associated b96.11-defined epitope increased between baseline and 3 months in GAD-alum (−8.1%, min −72.4%, max 39.6%) compared to placebo patients (1.5%, min −28.3%, max 28.6%) (p = 0.02). Subsequently, the b96.11-defined epitope recognition returned to levels similar to that observed at baseline.

Conclusions—GAD-alum injections did not affect binding of GADA to SPS-related epitopes, further supporting the safety of the treatment. There were no changes in GADA epitope specificity to the T1D-related epitopes, except for a temporarily increased binding to one of the tested epitopes.

Keywords

autoantibody; epitope; GAD; T1D

Type 1 diabetes (T1D) is an autoimmune disease resulting from destruction of the insulin producing beta cells in the pancreas (1). The patients need life-long insulin treatment, complications are common and to date there is no cure for the disease. A number of strategies for treatment or prevention of T1D have been investigated and others are currently

ongoing (2, 3). Specific immunomodulation with autoantigens has been considered, both in prevention and treatment of T1D (4). This approach, which aims to selectively target self-reactive T cells, could potentially constitute the most specific and safe treatment for autoimmune disease (5, 6). Indeed, numerous studies have demonstrated that immunomodulatory therapies with antigens can prevent and suppress autoimmunity in murine models (7–9). However, attempts in humans have shown varying success (2, 10, 11).

Glutamic acid decarboxylase 65 (GAD₆₅) is one of the major antigens targeted by self-reactive T cells in T1D (7, 12). T lymphocytes specific for GAD₆₅ are among the first to enter inflamed islets and may be important for initiation of autoimmune diabetes (13), and it has been shown that T cells mediating beta cell destruction in recent onset T1D patients are predominantly of T helper (Th) 1 cell phenotype secreting large amounts of IFN- γ (14).

Administration of GAD₆₅ has been tested in human clinical trials based on the successful outcome of animal studies in the Non-obese Diabetic mouse (15, 16). In a dose-finding study in Latent Autoimmune Diabetes in Adults (LADA) patients, GAD autoantibody (GADA) levels did not increase after injection of 20 μ g of GAD-alum, the dose that resulted in beneficial clinical effects (17). However, in the same study, increased GADA levels were observed in patients treated with 500 μ g of GAD-alum, and were accompanied by a temporary increase in GADA binding to one T1D-related epitope specificity (18). We have previously shown that two injections of 20 μ g GAD-alum contributed to the preservation of residual insulin secretion in both children and adolescents with recent onset T1D (19). The treatment of T1D patients was accompanied by several GAD-specific effects on the immune system (20–22), including a significant increase of GADA titers (19). Together with the increased GADA levels, an early Th2 response was induced after GAD-alum injections, with increased levels of IL-5 and IL-13 already after 1 month, followed by induction of a broader range of cytokines at 3 and 9 months after initiation of treatment (20). Furthermore, a reduction in the proportion of the predominantly occurring IgG1 subclass together with increased IgG3 and IgG4 at 3 months after the first injection of GAD-alum suggested a temporary switch towards a protective Th2 response (21). Other results from the study indicated that the GAD-alum treatment induced GAD₆₅-specific T cells with regulatory features (22).

High GADA levels, exceeding those typically found in T1D patients by 100- to 1000-fold, have been associated with certain neurological diseases such as stiff person syndrome (SPS), raising the concern that induction of high GADA titers by GAD-alum treatment might cause adverse events, especially neurological symptoms. However, in our study, no treatment-related adverse events have been reported following GAD-alum injection (19, 23). GADA in SPS patients differ from those present in T1D patients, both in their epitope pattern and their capacity to inhibit the enzymatic activity of GAD₆₅ *in vitro* (24). We have previously shown that the GAD₆₅ enzyme inhibition by sera from GAD-alum injected T1D patients was not affected by the GAD-alum treatment (21). The aims of this study were to investigate whether GAD-alum treatment in children and adolescents with recent onset T1D induced changes in GADA epitope pattern and to further confirm the safety of the treatment.

Materials and methods

Study population

The phase II clinical trial was a randomized, double blind, placebo-controlled clinical trial in which recent onset T1D patients were treated with 20 μ g recombinant aluminum formulated human GAD₆₅ (GAD-alum) (Diamyd®, Diamyd Medical, Stockholm, Sweden) subcutaneously in a prime and booster injection 4 wk apart (19). The participants (n = 70) were 10- to 18-yr old at diagnosis of T1D and had disease durations of <18 months, fasting

c-peptide levels of >0.1 nmol/L and tested positive for GADA at the initial screening. Serum samples taken before the first injection (baseline) and at 1, 3, 9, and 15 months after the initial injection were analyzed in this study. Nine patients (seven from the placebo group and two from the GAD-alum group) with baseline GADA levels <60 U/mL were excluded because epitope mapping based on competition with recombinant Fab (rFab) in samples with low GADA levels is unreliable. One additional patient in the placebo group was excluded from our analysis because this individual dropped out after the first injection. Thus, GADA epitope specificity was analyzed in 27 patients from the placebo group and 33 from the GAD-alum group using GAD₆₅-specific rFab as described below.

rFab used in this study

Six GAD₆₅-specific rFab were used to determine the GADA epitope specificity in serum samples. The monoclonal antibodies DPA and DPD were isolated from a patient with T1D (25). The monoclonal antibodies b96.11 and b78 were similarly derived from a patient with autoimmune polyendocrine syndrome type 2 (26). To date, this patient has not developed T1D. The monoclonal antibody MICA3 was isolated from a patient with T1D (27). The monoclonal antibody N-GAD₆₅ mAb was raised in mice and recognizes a linear epitope representing amino acids 4–22 of human GAD₆₅ (28). The human monoclonal antibodies recognize independent conformational epitopes located at the N-terminus (DPD), middle region (b96.11), and C-terminus (DPA, b78, and MICA3). More detailed information about these conformational epitopes has been published previously (29). All antibodies are specific to GAD₆₅ and do not bind to GAD₆₇ (28, 29). The insulin-specific rFab (CG7C7) (30) was included on each plate to determine non-specific competition. The rFab were cloned, expressed, and purified as described previously (31).

Radioligand binding assay (RBA)

GADA titers in serum samples were measured by RBA as described (32). Briefly, recombinant human [³⁵S] GAD₆₅ was produced in an *in vitro* coupled transcription and translation system with SP6 RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI, USA) (33). The *in vitro* translated [³⁵S] GAD₆₅ was kept at -70°C and used within 2 wk of preparation. Serum samples were incubated overnight at 4°C with [³⁵S] GAD₆₅. Free [³⁵S] GAD₆₅ was separated from the antibody-bound tracer by precipitation with Protein A Sepharose (Invitrogen, Carlsbad, CA, USA). The immunoprecipitated radioactivity was counted on a Wallac Microbeta Liquid Scintillation Counter (Perkin Elmer Life and Analytical Sciences, Inc, Boston, MA, USA). A standard curve consisting of serial dilutions of the World Health Organization (WHO) standard for GADA (34), GADA negative controls, and a buffer blank were included in each assay to correct for interassay variation and to express GADA levels as a relative unit. In the Diabetes Antibody Standardization Program (DASP) workshop 2005 the GADA analysis ranked at 80% sensitivity and 91% specificity.

Epitope-specific radioligand binding assay

The capacity of the rFab to inhibit GAD₆₅ binding by human serum GADA was tested in a competitive epitope-specific radioligand binding assay (ES-RBA) as previously described (31). Serum samples were first tested at a dilution of 1/25. Samples with a GADA titer exceeding 1000 Units/mL were diluted to <1000 Units/mL and reanalyzed. The seven rFab were added to separate wells at a concentration sufficient to compete binding of the originating intact mAb to GAD₆₅ by at least 80%, resulting in 4, 0.6, 1.7, 1, 14, 7, and 2 $\mu\text{g/mL}$ for rFab DPA, b96.11, DPD, MICA3, N-GAD₆₅ mAb, b78, and CG7C7, respectively. Non-competitive GAD₆₅ binding was established by no addition of rFab. The cutoff for specific competition was determined as $>15\%$ by using a negative control rFab CG7C7

specific to insulin (30), at 2 µg/mL. Each sample was measured in triplicates, and the mean value was calculated. The intra-assay average coefficient of variation was 4% with the highest value at 13% and the lowest being 0.03%. A control serum was included on each plate to correct for intra-plate variations. Binding of GADA to GAD₆₅ in the presence of rFab was expressed as follows: Ratio = GADA cpm in the presence of rFab (competed)/GADA cpm in the absence of rFab (non-competed). A higher binding to GAD₆₅ in the presence of an rFab indicates a lower proportion of GADA binding to the respective epitope.

Statistical analyses

As the immunological markers were not normally distributed according to the D'Agostino & Pearson omnibus normality test, non-parametric tests corrected for ties were used. Unpaired analysis was performed using Mann–Whitney *U* test and correlations with Spearman's rank order correlation test. The sums of absolute differences between consecutive measurements were compared using Mann–Whitney *U* test to investigate differences in variability of the ratios over time. Dot plots were used to visualize the data. Calculations were made with PASW Statistics 18 for Windows. A probability level of <0.05 was considered statistically significant.

Results

GADA titers at baseline

We compared the GADA titers at baseline in patients assigned to GAD-alum treatment with those in the placebo group. We found significantly lower GADA titers in patients assigned to GAD-alum (median 633 U/mL) than those in the placebo group (median 946 U/mL) ($p = 0.04$). This apparent contradiction to our previous finding for the entire study population (19) was caused by the exclusion of eight patients from the placebo group and two patients from the GAD-alum group (outlined in the Materials and Methods section).

GADA epitope pattern at baseline

Our comparison of GADA epitope specificity at baseline revealed a significantly higher median binding to GAD₆₅ in the presence of rFab b96.11 in patients assigned to GAD-alum treatment (80%) compared to the placebo group (68%) ($p = 0.04$) (Fig. 1; Table 1), indicating a lower proportion of GADA binding to the b96.11-defined epitope. No differences for GADA binding to the DPD- and MICA3-defined epitopes were detected.

Binding levels to the DPA-, N-GAD₆₅ mAb-, and b78-defined epitopes were very low throughout the study (Table 1) and were therefore excluded from the statistical analyses.

Longitudinal changes in GADA epitope pattern

To investigate possible longitudinal changes in GADA binding specificity due to the treatment, longitudinal samples from baseline and 1, 3, 9, and 15 months were analyzed for GAD₆₅ binding in the presence of different rFab. When analyzing the entire 15-month period, we found no statistical differences in variability (sums of absolute differences between consecutive measurements) in epitope binding between the placebo and GAD-alum treated groups (data not shown). However, a trend towards a higher variability for the b96.11-defined epitope was observed ($p = 0.07$).

Previously, we have shown a significant increase in GADA titers reaching its maximum level 3 months after the initial injection in the GAD-alum group (19). We next investigated whether this increase in GADA titer was accompanied by any change in GADA epitope recognition. For this analysis, GADA binding to rFab-defined epitopes was compared in samples collected at two time points (baseline to 1 month, baseline to 3 months, and 3

months to 15 months). During the period between baseline and 3 months, binding to the b96.11-defined epitope increased in the GAD-alum group compared to placebo, shown by a decreased median binding to GAD₆₅ in the presence of rFab b96.11 in the GAD-alum group (−8.1%, min −72.4%, max 39.6%) compared to that of patients in the placebo group (1.5%, min −28.3%, max 28.6%) ($p = 0.02$) (Table 2; Fig. 1). This development was reversed during the period between 3 and 15 months, when the median binding to GAD₆₅ in the presence of rFab b96.11 increased significantly in the GAD-alum group (8.3%, min −17.1%, max 36.7%) compared to the placebo group (−2.4%, min −32.8%, max 30.1%) ($p < 0.05$) (Table 2; Fig. 1). No differences for binding to the b96.11-defined epitope between baseline and 1 month, or between baseline and 15 months were observed. Furthermore, no differences in binding to epitopes defined by DPD and MICA3 between GAD-alum and placebo groups were observed.

Changes in GADA epitope pattern in GAD-alum treated patients that showed a >100% increase in GADA titer (high GADA increasers)

Based on the increase in GADA titer, we identified patients with an increase in titer of more than 100% from baseline to 3 months (high GADA increasers). In the GAD-alum group, 82% (27/33) of the patients were high GADA increasers, whereas in the placebo group no patient fulfilled this criterion. In the GAD-alum group, binding to the b96.11-defined epitope increased in the high GADA increaser samples from baseline to 3 months, shown by a decreased median binding to GAD₆₅ in the presence of rFab b96.11 in the high GADA increasers (−10.8%, min −72.4%, max 30.5%) compared to the six patients who were not high GADA increasers (3.1%, min −7.9%, max 39.6%) ($p = 0.04$). No differences in binding responses for the other epitopes were observed when comparing the same groups (data not shown). Furthermore, no changes in epitope binding response were observed when samples from any other time points were compared (data not shown).

Correlation of epitope binding and GADA titer

At baseline GADA levels correlated with binding to the b96.11- and DPD-defined epitopes, as indicated by the inverse correlations of GADA titer and the median response for binding to GAD₆₅ in the presence of b96.11 ($p = 0.001$, $r = -0.423$) and DPD ($p = 0.003$, $r = -0.377$) (Table 3). When dividing the samples into GAD-alum and placebo groups, baseline GADA levels correlated with recognition of the b96.11- ($p = 0.02$, $r = -0.400$) and DPD- ($p = 0.01$, $r = -0.436$) defined epitopes only in patients assigned to GAD-alum treatment. GADA titer and binding to the MICA3-defined epitope did not correlate. In contrast, when analyzing the samples collected at 3 months, the correlation of GADA binding to b96.11- ($p = 0.005$, $r = -0.524$) and DPD- ($p = 0.004$, $r = -0.538$) defined epitopes was observed only in the placebo group. In the same group, GADA levels also correlated with GADA binding to b96.11- ($p = 0.019$, $r = -0.448$) and DPD- ($p = 0.048$, $r = -0.385$) defined epitopes at 15 months, with no correlation in the GAD-alum group (Table 3).

Discussion

We investigated whether GAD-alum treatment had an effect on GADA epitope specificity, by competing GADA binding to GAD₆₅ with six rFab recognizing different epitopes on GAD₆₅. Four of the epitopes tested in our study (defined by rFab b96.11, DPA, DPD, and MICA3) have previously been described to be recognized by GADA in T1D patients (31). In contrast, it has been reported that the two epitopes defined by rFab N-GAD₆₅ mAb and b78 are only rarely recognized by T1D patients, but are associated with the GAD-specific immune response in SPS patients (24). The sustained low binding to the b78- and N-GAD₆₅ mAb-defined epitopes found in our study indicates that these antibody specificities do not contribute to the increased GADA levels after GAD-alum treatment. It is unclear if GADA

with an epitope profile as the one observed in SPS patients but not in patients with T1D (24, 31) contributes to the development of the neurological symptoms that are found in the SPS disease. However, as it has been shown that binding of GADA to SPS-related epitopes is uncommon in T1D (24), increased binding to these epitopes may indicate an increased risk for developing SPS. Thus, the results from this study speak in favor of the previously observed safety of the GAD-alum treatment.

Overall, we found no differences in epitope binding pattern of GADA between baseline and 15 months in the group that received GAD-alum compared to placebo. As the increase in GADA levels observed after treatment with GAD-alum peaked at 3 months after the first injection, we studied the GADA epitope specificity before and after this time point. We observed a temporary increased binding of GADA to the b96.11-defined epitope between baseline and 3 months, and decreased binding between 3 and 15 months, reaching levels that did not differ significantly from those observed at baseline. This result is in accordance with the previous dose-finding study in LADA patients, where an increase in GADA titer and a transient increase in the binding to the b96.11-defined epitope were observed for the patients receiving 500 µg GAD-alum (18). The change in GADA levels and b96.11 epitope recognition after treatment with 20 µg GAD-alum found in our study was not seen in the group of LADA patients receiving the same dose. Notably, in both studies the increase in GADA titer was accompanied by a transient increase in binding to the b96.11-defined epitope, in agreement with previous studies reporting that an increase in GADA was associated with increased binding to conformational epitopes (35, 36). In our study, binding to the b96.11- and DPD-defined epitopes correlated with GADA titer at 3 and 15 months in the placebo group, but not in the GAD-alum group. We cannot explain the lack of correlation in the GAD-alum group, but the treatment induced GADA titers much higher than the ones commonly detected in the majority of GADA positive diabetic patients. Thus, it appears that the binding to the b96.11-defined epitope increased at a higher frequency compared to the overall GADA titer. Our results suggest that the increase in GADA after GAD-alum treatment could be due to, at least in part, the activation of GADA-specific memory B cells with the b96.11-defined antibody specificity. However, it cannot be excluded that GAD-alum might also induce memory B cells producing GADA with epitope specificities other than b96.11. Other epitopes or epitope regions not included in this study might be part of the epitope binding pattern of the induced GADA.

The monoclonal antibody b96.11 recognizes a conformational epitope in the middle region of GAD₆₅, a region that is recognized early in the immune response to GAD₆₅ (37). Epitope spreading from an antibody response directed toward the middle region to the C- and N-terminal regions has been described both in children with increased risk of T1D (38, 39) and in children after onset of T1D (36). The T1D children with increasing GADA levels specific to the middle epitope needed a lower amount of exogenous insulin and had lower HbA_{1c} (36). In our study, we found no correlation between c-peptide levels and binding of GADA to the b96.11-defined epitope (data not shown). Whether the increased binding to the b96.11-defined epitope observed by us is associated with the clinical outcome in our study is unknown.

In conclusion, the epitope binding pattern was only to a small extent affected by the treatment, with minor changes in binding to one T1D-related epitope. Furthermore, the sustained low binding of GADA to SPS-related epitopes supports the previously observed safety of the GAD-alum treatment.

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Fig. 1.

Binding to GAD₆₅ in the presence of rFab b96.11 at baseline, 1, 3, 9, and 15 months for GAD-alum and placebo groups, presented as ratio of competed/non-competed. A higher binding to GAD₆₅ in the presence of rFab b96.11 indicates a lower proportion of GADA binding to the epitope. $p < 0.05$ were regarded as statistically significant. #These p values show the significant comparison for the difference between two time points in the GAD-alum group and the difference between the same time points in the placebo group.

Table 1

Median binding of GADA to GAD₆₅ in the presence of rFab b96.11, DPD, MICA3, DPA, b78 and N-GAD₆₅ mAb in T1D children treated with GAD-alum or placebo, at baseline and at 1, 3, 9, and 15 months after the first injection, reported as a percentage

rFab	Treatment	Baseline	1 month	3 months	9 months	15 months
b96	GAD-alum	80	79	72	73	74
	Placebo	68	70	70	69	69
DPD	GAD-alum	87	85	82	91	86
	Placebo	85	89	83	90	80
MICA3	GAD-alum	79	83	81	85	75
	Placebo	81	83	81	81	77
DPA	GAD-alum	91	88	84	87	88
	Placebo	89	86	89	86	83
b78	GAD-alum	99	95	97	95	95
	Placebo	97	98	94	99	101
N-GAD ₆₅ mAb	GAD-alum	94	92	90	92	93
	Placebo	87	93	89	90	92

Table 2

Differences in epitope binding between placebo and GAD-alum patients

Epitope	Months compared	<u>Difference in median response (min, max)</u>		p value
		GAD-alum	Placebo	
b96.11	0–1	–2.8 (–54.4, 52.6)	0.5 (–28.4, 28.2)	0.45
	0–3	–8.1 (–72.4, 39.6)	1.5 (–28.3, 28.6)	0.02*
	3–15	8.3 (–17.1, 36.7)	–2.4 (–32.8, 30.1)	<0.05*
	0–15	–5.6 (–62.5, 62.4)	1.6 (–25.6, 35.7)	0.24
DPD	0–1	0.9 (–24.1, 23.3)	3.2 (–15.3, 32.5)	0.59
	0–3	–1.8 (–43.6, 41.5)	2.4 (–28.6, 32.7)	0.87
	3–15	3.2 (–15.9, 24.3)	2.6 (–37.4, 26.7)	0.66
	0–15	–2.5 (–42.3, 42.1)	–0.1 (–49.8, 37.3)	0.85
MICA3	0–1	5.6 (–21.8, 51.7)	1.2 (–9.5, 49.4)	0.30
	0–3	3.1 (–31.1, 56.8)	0.1 (–36.6, 25.4)	0.18
	3–15	5.3 (–34.3, 20.7)	–0.9 (–42.1, 36.1)	0.20
	0–15	0.1 (–33.9, 39.2)	–0.1 (–36.2, 25.3)	0.89

Comparison of changes in median binding for the different epitopes was calculated by measurement of GADA in the presence of b96.11, DPD, and MICA3. Differences in epitope binding from baseline to 1 month, baseline to 3 months, 3 months to 15 months, and baseline to 15 months are represented for GAD-alum and placebo groups. A positive difference in median response indicates an increase in ratio between the two time points.

* indicates a p-value of <0.05.

Table 3

Correlations between GADA titer and the ratio of binding to the b96.11- and DPD-defined epitopes for all individuals, and for the GAD-alum and placebo groups, at baseline, 3 months, and 15 months after the first injection

Epitope	Time point	Individuals	Correlation with GADA titer	
			p value	r
b96.11	Baseline	All individuals	0.001**	-0.423
		GAD-alum	0.021*	-0.400
		Placebo	0.073	-0.350
	3 months	All individuals	0.018*	-0.305
		GAD-alum	0.688	-0.073
		Placebo	0.005**	-0.524
	15 months	All individuals	0.017*	-0.308
		GAD-alum	0.283	-0.193
		Placebo	0.019*	-0.448
DPD	Baseline	All individuals	0.003**	-0.377
		GAD-alum	0.011*	-0.436
		Placebo	0.088	-0.335
	3 months	All individuals	0.075	-0.232
		GAD-alum	0.840	0.036
		Placebo	0.004**	-0.538
	15 months	All individuals	0.025*	-0.289
		GAD-alum	0.254	-0.205
		Placebo	0.048*	-0.385

* indicates a p-value of <0.05,

** indicates a p-value of <0.01.