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Immunoglobulin Subclass Profiles of Anti-idiotypic Antibodies to GAD65Ab Differ Between Type 1 Diabetes Patients and Healthy Individuals

S. Oak[*], **J. Radtke**[*], **C. Törnt**[†], **M. Landin-Olsson**[‡], and **C. S. Hampe**[*]

[*]Department of Medicine, University of Washington, Seattle, WA, USA

[†]Department of Clinical Sciences, Lund University, Skåne University Hospital, Malmö, Sweden

[‡]Department of Endocrinology and Diabetology, Lund University Hospital, Lund, Sweden

Abstract

Previously we reported the presence of anti-idiotypic antibodies (anti-Id)-specific to autoantibodies against GAD65 (GAD65Ab) in healthy individuals while the activity of anti-Id directed to GAD65Ab in type 1 diabetes (T1D) patients was significantly lower. These anti-Id recognize the antigen-binding site of GAD65Ab, thus preventing their binding to GAD65. Here, we characterized the IgG subclass profile of these anti-Id (GAD65Ab specific) and of the associated GAD65Ab themselves. The IgG subclass response of anti-Id in healthy individuals (n = 16) was IgG3-dominated, while in T1D patients (n = 8) IgG1 was the major IgG subclass. The GAD65Ab bound by anti-Id in both healthy individuals (n = 38) and GAD65Ab-negative T1D patients (n = 35) showed a predominant rank order of IgG1 > IgG2 > IgG4 > IgG3. However, the frequency of GAD65Ab of the IgG4 subclass was significantly higher in T1D patients ($P < 0.05$). We conclude that the IgG subclass profile of anti-Id (GAD65Ab specific) in healthy individuals differs from that in T1D patients. These differences may provide insights into the development of these antibodies.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin producing pancreatic beta cells. Autoantibodies to islet cell antigens including the 65 kDa isoform of glutamate decarboxylase (GAD65) are observed months to years before the clinical onset of diabetes and, together with autoantibodies to other islet cell antigens, serve as markers in the prediction of T1D [1, 2]. Recently, we demonstrated that GAD65Ab can be detected in the majority of healthy individuals after removal of anti-idiotypic antibodies (anti-Id) specific to GAD65Ab. These anti-Id bind to the antigen-binding site of GAD65Ab, thus preventing the latters' detection in conventional screening assays. Anti-Id to GAD65Ab are absent in T1D patients, resulting in readily detectable GAD65Ab [3, 4]. Anti-Id may have regulatory functions in the immune system, as first postulated in the Network Hypothesis [5]. The presentation of antibody-derived Id-peptides on MHC class II molecules stimulates Id-specific T cells [6, 7]. Presentation of endogenous Id-peptides can result in Id-driven T-B cell collaboration [8, 9] and antigen-independent autoantibody secretion as observed in autoimmune diseases [10] including the continuous GAD65Ab presence years after the clinical diagnosis of T1D [11].

T1D is a T cell-mediated autoimmune disease and the T cell responses are reflected in isotypes and IgG subclasses of the autoantibodies. The Th1-dominated T cell response in T1D patients is accompanied by a IgG1/IgG3-dominated GAD65Ab response [12], while the Th2 associated IgG subclasses IgG2 and IgG4 are found less frequently [13]. The

different IgG subclasses serve specific biological effector functions. IgGl and IgG3 are part of the cellular immune response and activate compliment (predominantly IgG3), enhance phagocytosis, mediate antibody-dependent cytotoxicity and opsonization (primarily IgG1) [14]. IgG4 appears to protect against the biological effects of IgG1 and IgG3, possibly through its inhibition of the binding of IgG1 to complement factor C1q [15].

We analyzed the IgG subclass profile of anti-Id specific to GAD65Ab in T1D patients and healthy individuals.

Materials and methods

Serum samples

Healthy individuals—Healthy controls (n = 38) were collected in Seattle in 2002 and contain 19 men, age at sampling was >18 years (mean age: 25.5 years).

T1D patients—GAD65Ab-negative new onset T1D patients (n = 35) [mean age 27.7 years (range 15–34 years), 27 men] were selected from the Diabetes Incidence Study in Sweden [16]. The GAD65Ab-negative T1D patients tested also negative for autoantibodies to insulin and IA-2 (data not shown). GAD65Ab-positive new onset T1D patients (n = 9) were selected from the same cohort.

All subjects or their legal guardian gave informed consent. Local institutional ethics committee approval was obtained prior to collection of all serum samples.

Antibodies

Human mAb b96.11 specific to GAD65 was derived from a patient with autoimmune polyendocrine syndrome type 2 [17]. B96.11 recognizes an epitope that is specifically bound by patients with T1D [18, 19].

Radioligand binding assay (RBA)

GAD65Ab were determined in a RBA as previously described [3, 20]. Briefly, sera were incubated with recombinant $^{[35]}$S-GAD65 for 18 h at 4 °C. Antibody-bound $^{[35]}$S-GAD65 was precipitated by Protein A-Sepharose (PAS) (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).

Coupling of antibody to PAS

Purified mAb was cross-linked to PAS using the Dimethylpimelimidate method [21] as previously described [3]. Cross-linked antibody-PAS was subjected to 0.2 M glycine, pH 2.0 and pH 11 to ensure complete removal of non-cross-linked antibody. The efficiency of the coupling was 50%.

Absorption of anti-Id to b96.11-PAS beads

To dissociate complexes of GAD65Ab and their bound inhibitors in serum samples, we followed the heat dissociation method as described earlier [3, 22]. Briefly, sera were incubated with antibody-PAS for 10 min at 55 °C followed by stepwise cooling to room temperature. The supernatant was analyzed for GAD65Ab by RBA.

Analysis of IgG subclasses of GAD65Ab-specific anti-Id

Anti-Id were absorbed to b96.11-PAS as described above. For the isolation of anti-Id, 1.5–2 ml serum was absorbed to b96.11-PAS (200 μl of 50% slurry) to obtain sufficient amounts

of anti-Id. The anti-Id bound to b96.11-PAS were eluted by incubation with 0.2 M glycine, pH 10.0. The beads were then centrifuged at 20, 200 *g* for 5 min. The neutralized supernatant was analyzed for IgG subclasses by ELISA following manufacturer's instructions (Invitrogen).

Assay for GAD65Ab IgG subclasses

GAD65Ab IgG subclasses in sera were measured as previously described [23], with some modifications. Briefly, biotinylated antibodies specific to human IgG1, IgG2 (BD Pharmingen, San Jose, CA, USA), IgG3 and IgG4 (Southern Biotech, Birmingham, AL, USA) were coupled to streptavidin agarose (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The assay followed the same procedure as described for the RBA, with the substitution of PAS with 50 μl of 50% slurry of subclass-specific beads.

GAD65Ab-negative samples were first absorbed to b96.11-PAS to remove anti-Id (GAD65Ab specific). The resulting free GAD65Ab were analyzed for their IgG subclass profile. Each assay included the World Health Organization (WHO) standard [24] that was not absorbed to b96.11-PAS, and a blank control, which consisted of PBS that had been absorbed to b96.11-PAS. Interassay variation on this sample was approximately 10%. The iso-type-specific GAD65Ab levels were expressed as percentage of the total GAD65Ab precipitated by biotinylated IgG isotype subclass-specific antibodies bound by streptavidin PAS beads.

Total human IgG subclasses were determined by ELISA following manufacturer's instructions (Invitrogen).

Statistical analysis

All samples were analyzed in triplicate determinations. Our assay for GAD65Ab showed good sensitivity (86%) and high specificity (97%) in the 2007 Diabetes Antibody Standardization Program Workshop [25] Positive and negative controls were included for each assay. Binding levels between different treatments within one serum group were adjusted to the untreated positive and negative controls to correct for interassay variations. Median GAD65Ab levels between groups were analyzed using the non-parametric analysis of variance (Kruskall–Wallis test) followed by Dunn's multiple comparisons test. Frequency distribution between groups was analyzed using Fisher's exact test. Significance was defined as $P < 0.05$.

Results

Frequency of anti-Id (GAD65Ab specific) IgG subclasses in healthy controls and GAD65Ab-negative T1 D patients

The anti-Id IgG subclasses in a random subset of healthy controls ($n = 16$) and GAD65Ab-negative T1D patients ($n = 8$) were analyzed to determine whether the anti-Id IgG subclass profile differed between healthy individuals and T1D patients (Fig. 1). The median protein concentration of the isolated anti-Id was significantly higher for healthy controls [0.6 μg/ml (range: 0.3–0.78 μg/ml)] as compared for GAD65Ab-negative T1D patients [0.4 μg/ml (range: 0.24-0.5 μg/ml)] ($P = 0.005$) (data not shown). In GAD65Ab-negative T1D patients, the prevalent rank order was IgG1 > IgG3 > IgG2 > IgG4 (63%), while in healthy individuals the prevalent rank order was IgG3 > IgG1 > IgG2 > IgG4 (75%). Consequently, anti-Id of the IgG3 subclass were significantly more frequent in healthy individuals (75%) than in T1D patients (37%, $P = 0.02$).

Frequency of GAD65Ab IgG subclasses in healthy controls and GAD65Ab negative T1D patients after removal of anti-Id

GAD65Ab IgG subclasses of sera of healthy controls (n = 38) and GAD65Ab-negative T1D patients (n = 35) were analyzed after removal of anti-Id from the sera. In both study cohorts, we observed more than one rank order of IgG subclasses.

In both cohorts, the prevalent rank order was IgG1 > IgG2 > IgG4 > IgG3 (81% of healthy individuals and 60% of T1D patients) (Fig. 2A, C). The rank order of IgG1 > IgG4 > IgG2 > IgG3 was observed in 13% of healthy individuals and 10% of T1D patients (Fig. 2B). While the rank order IgG2 > IgG1 > IgG4 > IgG3 was observed in 14% of T1D patients (Fig. 2D), it was present in <4% of healthy individuals. IgG3 was in both study groups the least frequent subclass (only 22% of individuals had detectable IgG3). IgG4 was more frequently detected in T1D patients as compared to healthy controls ($P < 0.05$).

Frequency of GAD65Ab-IgG subclasses in GAD65Ab-positive T1D patients

The GAD65Ab subclasses in GAD65Ab-positive T1D patients were analyzed to determine possible differences between overt GAD65Ab and GAD65Ab bound by anti-Id. For overt GAD65Ab, we found the predominant rank order of IgG1 > IgG2 > IgG4 > IgG3 (data not shown).

Total levels of IgG subclasses

Total circulating IgG subclass levels were determined by ELISA in a random subset of healthy controls (n = 7) and GAD65Ab-negative T1D patients (n = 7). The predominant total IgG subclass profile in both groups was IgG1 > IgG2 > IgG3 > IgG4. No correlation between the subclass profile and gender or age was observed.

Discussion

Our recent finding of anti-Id that specifically inhibit the binding of GAD65Ab to GAD65 [3] prompted us to investigate the IgG subclass profile of these anti-Id and the GAD65Ab bound by them.

Our major findings from this study are that (1) anti-Id IgG subclasses in T1D patients differ from those in healthy individuals and (2) IgG subclasses differ between anti-Id and GAD65Ab.

These differences were mainly seen in the distribution of IgG3 and IgG1. The anti-Id IgG subclass profile in healthy individuals was IgG3-dominated, while IgG1 was the most prevalent subclass of anti-Id in T1D patients. Together with IgG4, IgG3 is the least frequent IgG subclass in humans. Our observation of IgG3 as the major IgG subclass in anti-Id in healthy individuals is therefore of interest and we will discuss the potential causes and consequences in the following.

High IgG3 autoantibody levels have been reported in autoimmune diseases such as SLE [26] and are generally found in the early stages of the immune response [27]. A shift from an IgG3- to an IgG1-dominated immune response is observed during extended periods of autoimmunity [27]. Indeed, GAD65Ab of the IgG3 subclass characteristically appears during the first phase of the immune response to GAD65 [28], and decline to lower levels at clinical onset [23]. It is possible that the IgG1-dominated GAD65 response present a longer standing immune response, while the IgG3-dominated anti-Id response is caused by a more recent antibody exposure. This is further supported by our observation that GAD65Ab IgG3 levels in T1D patients were lower when compared to the overall IgG subclass profile, and a

sizeable portion of T1D patients (14%) displayed an IgG4 dominated IgG subclass profile. IgG4 antibody responses have been associated with long-term antigenic stimulation with T cell-dependent antigens [15].

IgG1 and IgG3 have similar effector functions and are both effective complement activators. While IgG3 is the most efficient subclass in regard of Clq binding, it is less efficient than IgGl in complement–dependent cell lysis and ADCC [29, 30]. Moreover, IgG3 has a shorter half-life compared with the other IgG subclasses (7 versus 21 days). Whether these differences in biological function are of clinical relevance needs to be established.

We need to emphasize that the majority of T1D patients have little or no anti-Id bound GAD65Ab and display overt GAD65Ab [3, 4]. Therefore, this analysis represents only a subgroup of T1D patients.

We focused our analysis on anti-Id directed towards the GAD65Ab epitope that is recognized by the human monoclonal antibody (b96.11). While this clearly limits our study, we selected this GAD65Ab epitope because it is recognized by GAD65Ab in the majority of T1D patients [18]. Moreover, anti-Id activity specific to b96.11 was significantly lower in T1D patients as compared to healthy individuals, while anti-Id to other GAD65Ab showed no differences [3]. These results suggest that this GAD65Ab epitope specificity and its anti-Id are specific to the T1D pathogenesis.

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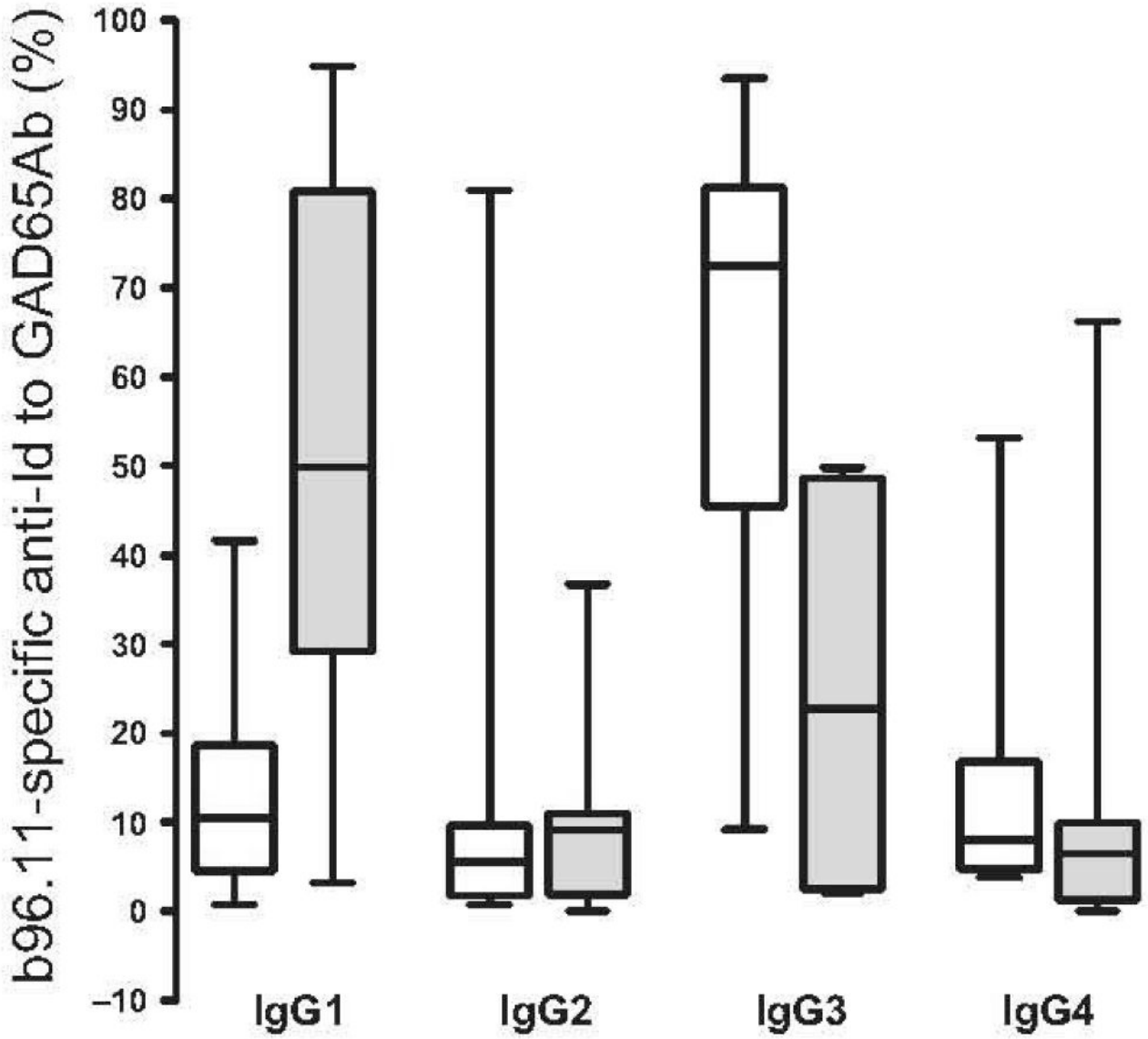

Figure 1.

Frequency of b96.11-specific anti-idiotypic antibodies (anti-Id) IgG subclasses in healthy controls and GAD65Ab-negative type 1 diabetes (T1D) patients. The b96.11-specific anti-Id IgG subclasses in sera of healthy controls (n = 16, white box) and antibody-negative T1D patients (n = 8, grey box) were analyzed. The b96.11-specific anti-Id IgG subclasses are presented as percentage of the total b96.11-specific anti-Id (set as 100%) detected by the human IgG subclass ELISA. The results are shown as a box and whiskers plot with median, interquartile range, and upper and lower extremes.

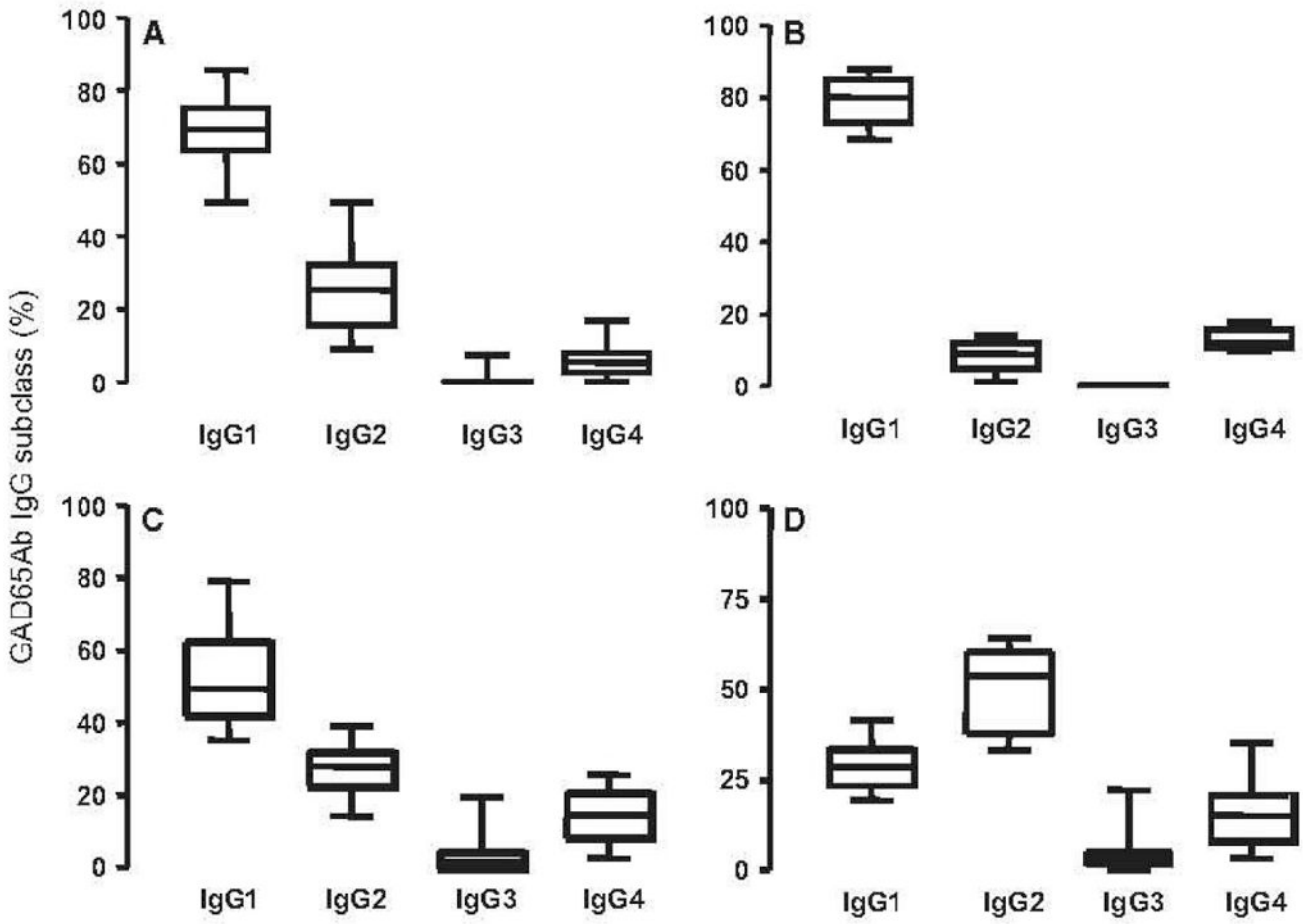

Figure 2.

Frequency of GAD65Ab IgG subclasses in healthy controls (A and B) and GAD65Ab negative type 1 diabetes (T1D) patients (C and D) after removal of b96.11-specific anti-idiotypic antibodies (anti-Id). GAD65Ab IgG subclasses of sera of healthy controls (n = 38) and GAD65Ab negative T1D patients (n = 35) were analyzed after removal of b96.11-specific anti-Id. The isotype-specific GAD65Ab levels are presented as percentage of the total GAD65Ab (set as 100%) precipitated by biotinylated IgG isotype subclass-specific antibodies bound by streptavidin PAS beads after removal of anti-Id. Dominant rank orders in healthy individuals (81%) and T1D (60%) patients are shown in A and C, respectively. Box and whiskers plots with median, interquartile range, and upper and lower extremes are shown.