Characterization of the humoral immune response to glutamic acid decarboxylase in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) and/or type 1 diabetes

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Abstract
Objective: A humoral autoimmune response to glutamic acid decarboxylase (GAD65) is common both in patients with type 1 diabetes and in those with the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome, while overt type 1 diabetes is relatively rarely diagnosed in APECED patients. The aim of this study was to assess whether this difference in the incidence of type 1 diabetes is associated with variability in the humoral immune response to GAD65, one of the major autoantigens in type 1 diabetes.

Methods: Epitope- and isotype-specific GAD65 autoantibodies were analysed in 20 patients with APECED and 20 patients with newly diagnosed type 1 diabetes alone by radiobinding assays.

Results: GAD65 autoantibodies targeted the middle and carboxy-terminal regions of GAD65 and occasionally the amino-terminal region in the APECED patients and comprised mainly the IgG1 subclass and less frequently the IgG2 and IgG4 subclasses. The profile of epitope- and isotype-specific GAD65 autoantibodies was similar in type 1 diabetes and APECED, except that IgG2 subclass antibodies were observed more often and at higher levels in the patients with type 1 diabetes alone (P < 0.05). None of the measured parameters separated APECED patients with type 1 diabetes from those without type 1 diabetes.

Conclusion: APECED-associated humoral autoimmunity to GAD65 does not differ markedly from that observed in type 1 diabetes; only IgG2-GAD65 antibodies may be more closely associated with the latter entity.

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Introduction
Autoantibodies to the gamma-aminobutyric acid (GABA) synthesizing glutamate decarboxylase (GAD) occur frequently in type 1 diabetes, polyendocrine autoimmune diseases and some neurological diseases (1–4). Two isoforms of GAD are expressed in man, with molecular masses of 65 000 and 67 000Da (5, 6). Antibodies to GAD65 (GAD65Abs) occur at diagnosis in up to 80% of patients with type 1 diabetes, whereas antibodies to GAD67 (GAD67Abs) are detected infrequently and probably as a result of epitopes shared with GAD65 (7). Autoantibodies to GAD65 are also common in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also known as autoimmune polyendocrine syndrome type 1), a rare autosomal recessive disease linked to mutations in the AIRE gene on chromosome 21 (2, 8–10). Type 1 diabetes is a component of APECED in up to 23% of patients (11). Since GAD65Abs occur in some 40% of APECED patients, however, their positive predictive value for future type 1 diabetes is low in these cases (8, 12). This is in contrast to persons with preclinical isolated type 1 diabetes, whose GAD65Abs are closely associated with progression to diabetes (13). Even so, some of these susceptible individuals fail to progress, as is also the case with the majority of GAD65Ab-positive patients with APECED. Such GAD autoimmunity may, at least partly, represent non-destructive islet cell autoimmunity. Autoantibodies to GAD65 target mainly conformational antigenic determinants located in the middle
and C-terminal regions of GAD65 (14–17) and are composed of the IgG1 subclass and to a lesser extent other IgG subclasses in cases of type 1 diabetes (18). In patients with APECED, GAD65Abs are present at higher levels (8), more frequently share epitopes with GAD67 (8), and may more often recognize linear epitopes and be reactive to epitopes other than those observed in classical type 1 diabetes (19). Since there is limited information available on the detailed characteristics of humoral autoimmunity to GAD in patients with APECED, we set out to characterize reactivity to three major epitope clusters of GAD65 and the isotype distribution within the humoral response to GAD65 in a series of GAD65Ab-positive patients with APECED, comparing them with patients with newly diagnosed type 1 diabetes alone.

**Subjects and methods**

**Patients**

The subjects were derived from a series of patients with APECED diagnosed in Finland up to 1988 (10). The patients whose sera were available and who had been observed to be GAD65Ab-positive were retested in our laboratory in Oulu, and 20 of them (13 male, 65%) were confirmed positive. They all had chronic mucocutaneous candidiasis together with hypoparathyroidism, seven and/or primary adrenocortical failure. Altogether, thirteen of them (65%) had hypoparathyroidism, seven (35%) adrenocortical failure and six (30%) clinical type 1 diabetes alone. Their mean age at the first sample with detectable GAD65Abs was 22.4 years (±11.4 (S.D.), range 4.3–47.9). Serum samples were available, 1–8 for each patient, taken at irregular intervals and stored at −20°C. Two patients had one sample with detectable GAD65Abs, four had two samples, two had three samples, five had four samples, three had five samples, two had seven samples, and two had eight samples. The median duration of the GAD65Ab-positive period was 10.4 years (range 1.1–23.8) in those with more than one sample positive for GAD65Abs. The six cases of clinical type 1 diabetes had been diagnosed at age 19.3, 19.6, 23.5, 35.9, 43.3, and 45.3 years, the individual sample closest to the diagnosis having been obtained at age 34.1, 19.2, 23.5, 36.2, 42.3, and 42.7 years respectively. The patients with type 1 diabetes alone were selected from a series of 352 newly diagnosed patients admitted to the Departments of Paediatrics and Internal Medicine, Oulu University Hospital, in 1988–1995 (20). These were matched with the APECED patients for age (mean ± S.D., 21.4 ± 11.6 vs 22.4 ± 11.4), gender (13 male and 7 female) and levels of total GAD65Abs (median 100.1 vs 132.1 relative units (RU)). One inhibition analysis was not performed in one patient with type 1 diabetes alone due to insufficient serum volume, and this APECED–type 1 diabetes pair was excluded from the statistical analysis. Two subjects with type 1 diabetes alone were re-matched with each of the six diabetic APECED patients based on their age at the sample closest to the diagnosis of diabetes (mean ± S.D., 24.8 ± 11.6 vs 32.1 ± 10.9; median levels of GAD65Abs, 115.8 vs 87.2 RU). When the APECED patients with diabetes were compared with those without, the first available GAD65Ab-positive sample was used. All the APECED patients had been examined at the Hospital for Children and Adolescents in Helsinki, and the study was approved by the local Ethical Committees. The patients gave their written informed consent.

**Methods**

Antibodies to human full-length GAD65 and its three major epitope clusters and to human full-length GAD67 were determined with a radiobinding assay using 35S-labelled full-length proteins or GAD65/67 chimaeric molecules (21, 22). Radiolabelled antigens were produced in an in vitro transcription/translation system (Promega, Madison, WI, USA). The chimaeric molecules used were GAD651–9 5/GAD67102–593 for N-terminal GAD65 epitopes, GAD671–101/GAD6596–444/GAD67453–593 for epitopes in the middle region of GAD65 and GAD671–453/GAD65445–585 for C-terminal GAD65 epitopes (17). cDNAs of the chimaeric constructs were gifts from Ezio Bonifacio (Milan, Italy). The conformations of these chimaeric proteins have been validated using monoclonal antibodies and competitive inhibition tests (17), and that of the GAD65 molecule by monoclonal antibodies recognizing conformational epitopes in the middle domain (MICA4) and the C-terminal domain (MICA3) of GAD65. The levels of antibodies were expressed as RU based on a standard curve run on each plate. The cut-off limit for antibody positivity was set to represent the 99th percentile in 373 non-diabetic Finnish children and adolescents in the GAD65Ab assay or 104 subjects in the epitope-specific GAD65Ab and GAD67Ab assays. These limits were 5.35 RU for GAD65Abs, 0.86 RU for N-terminal antibodies (GAD65-N-Abs), 1.51 RU for middle region antibodies (GAD65-M-Abs), 1.59 RU for C-terminal antibodies (GAD65-C-Abs) and 0.91 RU for GAD67Abs. Since the levels of GAD67Abs were high in many samples, inhibition experiments were performed to confirm specific binding. All initially GAD65Ab-positive samples were re-analysed for specific GAD65Abs in an inhibition assay with unlabelled GAD67, all initially GAD67Ab-positive samples for specific GAD67Abs in an inhibition assay with unlabelled GAD65, and all samples initially positive for GAD65-N-Abs, GAD65-M-Abs and/or GAD65-C-Abs for specific GAD65 epitope binding were re-analysed in inhibition assays with unlabelled GAD67. The unlabelled antigens were produced by the same method as the labelled antigens but in the absence of 35S-methionine. The serum
samples were diluted for the inhibition assays to provide an uninhibited binding of approximately 1000 c.p.m. If the binding was inhibited by 80% or more upon adding the unlabelled antigen, the sample was considered to lack specific binding for the labelled antigen. In all assays, low and high standard serum samples were included on each plate to monitor the precision of the assay. The coefficients of interassay and intra-assay variation in our assays were maximally 25%. The disease sensitivity of the GAD65Ab assay was 82% and the specificity 98% in the 2002 Diabetes Antibody Standardization Programme (DASP) workshop. Isotype-specific GAD65Abs were analysed with a methodology similar to that used for the assay of full-length GAD65Ab except that the protein A Sepharose precipitation was replaced by monoclonal anti-human Ig antibodies linked to streptavidin agarose. Briefly, the streptavidin agarose beads were washed thoroughly with buffer before use, and the biotinylated monoclonal anti-human IgG1, IgG2, IgG3, IgG4, IgA, IgE and IgM antibodies were linked to them by joint incubation. The beads were then washed with buffer. Finally, they were suspended in buffer and used for the precipitation. A more detailed procedure has been published recently (22). The results were expressed as s.d. scores (SDS) calculated as: SDS = \[\frac{\text{delta c.p.m.} - \text{mean c.p.m. of control subjects}}{\text{s.d. c.p.m. of control samples as described previously}}\] (18). Forty non-diabetic young Finnish subjects (mean age 9.9 ± 4.0 (S.D.), range 0.6–16.6 years) served as controls. The threshold for positivity was set at 3 SDS. Matched sample pairs were run as far as possible in the same isotype-specific GAD65Ab assays to eliminate the effect of interassay variation. Samples were re-analysed if the coefficient of variation in parallel samples exceeded 20%. A series of other samples were re-analysed for quality control purposes. We participated in the serum exchange workshop carried out between the laboratories known to measure isotype responses to GAD65 (results presented at the 5th International Congress of the Immunology Diabetes Society 2001, Chennai, India). Our results were highly concordant with those of other laboratories using a similar method.

Statistical analysis

Chi-square statistics and Fisher’s exact test were used as appropriate to evaluate differences in the frequencies of antibodies, and the Mann-Whitney U-test was used to compare antibody levels between the patient groups. The statistical analyses were performed using the SPSS statistical software package, version 10 (SPSS, Chicago, IL, USA) or Arcus QuickStat Biomedical Statistical Software (Addison Wesley Longman, Research Solutions, Cambridge, Cambs, UK).

Results

The 20 APECED patients previously documented as having tested positive for GAD65Abs were confirmed to be positive in our laboratory in Oulu. Sixteen of them were positive from the first available sample to the last follow-up sample. three seroconverted to stable GAD65 positivity during observation, and one was positive for GAD65Abs in the first two samples, seroconverted to negativity and then re-converted to positivity later. Antibodies to GAD67 were observed in 16 of the 20 patients during follow-up. Antibodies to the GAD65 construct, GAD671–101/GAD6596–444/GAD67453–593, were seen in 19 of the 20 APECED patients. GAD67 did not inhibit the binding totally in any of the samples, confirming the presence of specific GAD65-M-Abs. Similarly, specific C-terminal GAD65Abs were observed in all the samples that showed binding to the GAD671–453/GAD65445–585 molecule. Altogether, 18 of the 20 APECED patients had GAD65-C-Abs. When an epitope-specific response appeared, it tended to remain: GAD65-M-Abs and GAD65-C-Abs disappeared along with the disappearance of total GAD65Abs and re-appeared later in one patient, while the disappearance of the specific C-terminal and N-terminal responses was seen in one patient. IgG1-GAD65Abs were detected in 19 of the 20 patients and their levels were higher than those of any other IgG subclass or isotype. If GAD65Abs were present, an IgG1 response was detectable except in one sample with low levels of total GAD65Abs. IgG4-GAD65Abs were observed in 17 patients and IgG2-GAD65Abs in 15, whereas IgG3-GAD65Abs were detectable in only two patients. An IgG4 response was present or appeared as a continuous response in eleven patients, fluctuated in four and seroconverted to permanent negativity in two initially positive patients. IgG2 responses were more variable, a continuous response or seroconversion to permanent positivity occurring in five patients, fluctuating or single responses in five and inverse seroconversions in five. Both of the IgG3 responses detected were present in the first samples included and disappeared thereafter. There were no differences in the distribution of epitope-specific and isotype-specific GAD65Abs between the patients with APECED and those with type 1 diabetes alone except for the higher prevalence and levels of Ig2-GAD65Abs in the latter (P < 0.05, Fig. 1, Table 1). The finding of higher titres of IgE class antibodies in patients with type 1 diabetes alone is controversial, since the levels of IgE-GAD65Abs were in most cases below the cut-off limit.
for antibody positivity. Antibodies to GAD67 were more common and showed higher levels in the patients with APECED than in those with type 1 diabetes alone \( (P < 0.01, \text{Fig. 1, Table 1}) \). The distribution of epitope- and isotype-specific antibodies did not differentiate APECED patients with type 1 diabetes from non-diabetic patients or from those with type 1 diabetes alone (Figs 2 and 3). No apparent changes occurred in the epitope or isotype profile at the time of diagnosis of type 1 diabetes in the patients with APECED. Antibodies to GAD67 were more frequent \( (P < 0.05) \) and present at higher levels \( (P < 0.01) \) in the APECED patients with type 1 diabetes than in the patients with type 1 diabetes alone (Fig. 2). Antibodies to GAD67 were observed in the APECED patients irrespective of whether type 1 diabetes was present (Fig. 3).

**Discussion**

The aim of this study was to characterize the humoral immune response to GAD in APECED patients in detail by analysing antibodies to three major epitope clusters and isotype-specific responses. We observed that the humoral immune response to GAD mainly targets the middle and C-terminal regions of the GAD65 molecule, while a response to the N-terminus was seen in about half of the patients. The response comprised IgG1 subclass antibodies in almost all patients, whereas IgG2 and IgG4 subclass antibodies occurred in 60–80% of them. We also confirmed previous reports that antibodies to GAD67 cross-reacting with GAD65 are frequent in patients with APECED \((8, 19)\). In the present study, the APECED patients were matched with type 1 diabetic patients according to, among other things, total GAD antibody levels. The same criterion was applied when matching APECED patients affected by type 1 diabetes to those unaffected. The matching was relatively crude, as it was based on GAD antibody levels obtained without diluting strongly positive samples. The crude matching might potentially result in false positive differences due to unrevealed differences in total GAD antibody levels between the

**Table 1** median levels (RU or SDS) of antibodies in patients with type 1 diabetes \((n = 20)\) and APECED \((n = 20)\).

<table>
<thead>
<tr>
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<th>Type 1 diabetes</th>
<th>APECED</th>
<th>(P)-value (Mann-Whitney U)</th>
</tr>
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<tr>
<td>GAD65</td>
<td>100.1</td>
<td>132.1</td>
<td>1.000*</td>
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<tr>
<td>GAD67</td>
<td>0.2</td>
<td>2.4</td>
<td>0.002*</td>
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<td>338.6</td>
<td>681.2</td>
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<td>IgG2-GAD65</td>
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</tr>
<tr>
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<td>0.1</td>
<td>0.43</td>
</tr>
<tr>
<td>IgG4-GAD65</td>
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<td>0.84</td>
</tr>
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<tr>
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<td>0.1</td>
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</tr>
<tr>
<td>IgM-GAD65</td>
<td>7.8</td>
<td>13.3</td>
<td>0.16</td>
</tr>
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</table>

* \(P\) values are significant.
groups. The major finding of this study is, however, that the humoral immune response to GAD was surprisingly similar in patients with APECED and/or type 1 diabetes. No detailed epitope- and isotype-specific information has been available on the humoral immune response to GAD in APECED patients. It has been stated that mainly conformational epitopes located in the middle and C-terminal region of GAD65 are immunogenic in type 1 diabetes (16), and we similarly observed a strong response to both of these regions in APECED patients. We also detected epitope spreading from the middle region to the C-terminus in two patients, again an observation that is consistent with those observed in patients with type 1 diabetes (14, 15, 17). Sera from APECED patients have been observed to bind denatured GAD65 and to inhibit the enzymatic activity of GAD65 but not sera from patients with type 1 diabetes alone (19). This may be reflected by different patterns of epitope recognition. In several patients with APECED we observed reactivity to the N-terminus that was not inhibited completely by GAD67. Similarly, GAD65Abs recognize linear epitopes in the N-terminus of the molecule in the stiff-man syndrome, a rare neurological disease characterized by exceptionally high titres of GAD65Abs and the presence of type 1 diabetes in one-third of GAD65Ab-positive patients (23, 24). However, we observed no statistical difference in the frequency of N-terminal responses between the matched series of patients with APECED and type 1 diabetes alone.

To summarize, the humoral immune response to the three major epitope clusters of GAD65 is surprisingly similar in patients with APECED and patients with type 1 diabetes alone, in spite of the fact that strong cross-reactive responses with GAD67 are observed only in patients with APECED. The high GAD67Ab titres may indicate that sensitization to GAD67 occurs independently of GAD65 in patients with APECED, possibly reflecting a different pathogenic mechanism, as proposed by Björk et al. (19). This is supported by the fact that HLA-conferred susceptibility to type 1 diabetes has not been observed in patients with APECED (8). The isotype pattern of GAD65 antibodies in young children at risk of developing type 1 diabetes comprises a dominant IgG1 response, more infrequent IgG2 and IgG4 responses and rarely an IgG3 response (18, 22), although the last-mentioned subclass response may be somewhat more frequent in the initial phase (M Ronkainen, unpublished data). In the present series, a similar pattern apart from the absence of an IgG3 response was observed in the samples taken at the time of diagnosis from the patients with type 1 diabetes alone. The isotype profile seen in APECED patients, irrespective of the presence of concomitant type 1 diabetes, was very similar to that of the patients with type 1 diabetes alone but only the latter had IgG2 antibodies frequently. An IgG3 response was seen initially in only two non-diabetic APECED patients, one of whom was one of the youngest patients in the series. This is also consistent with our previous observation of the occurrence of IgG3 subclass antibodies during the initial GAD response. The apparently increased frequency of IgG4 responses among the non-diabetic APECED patients relative to the diabetic ones (79% vs 50%) could be interpreted as a sign of non-destructive autoimmunity. This would be in line with our observations in a prospective study of young GAD65Ab-positive children that those who remained non-diabetic were characterized by an emerging IgG4 response over the first 18 months after seroconversion to GAD65Ab positivity (M Ronkainen et al., unpublished observations). Previously reported findings are not unequivocal in this respect, however (18, 22, 25–27).

In conclusion, GAD65Abs in APECED patients target the middle and C-terminal regions and occasionally also the N-terminal region and comprise mainly the IgG1 subclass, although IgG2 and IgG4 subclass antibodies are also observed relatively frequently. The profile of their humoral immune response to GAD65 does not differ markedly from that of patients with type 1 diabetes alone, as only GAD65Abs of the IgG2 subclass are associated more closely with isolated type 1 diabetes. The antibody response to GAD is similar in diabetic APECED patients and in patients with type 1 diabetes alone. Our findings imply that it is not possible to discriminate APECED patients affected by type 1 diabetes from unaffected ones by means of epitope- and isotype-specific GAD antibodies. A strong humoral immune response to GAD67 characterizes APECED-related GAD autoimmunity and differentiates patients with type 1 diabetes alone from APECED patients, the former having, in addition, an increased IgG2 subclass response to GAD65.

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