GAD65 autoantibody responses in Japanese latent autoimmune diabetes in adults (LADA) patients.

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Running title: GAD65Ab epitope specificity distinguishes LADA.

Received 31 January 2008 and accepted 7 May 2008.
Objective: Determine whether development of insulin requirement in patients with latent autoimmune diabetes in adults (LADA) is accompanied with the emergence of a type 1 diabetes-like autoimmune response.

Research Design and Methods: We correlated beta-cell specific autoimmunity reflected in autoantibodies to the 65kDa isoform of GAD (GAD65) with insulin requirement. We determined GAD65Ab epitope specificities in type 1 diabetes patients, LADA patients without insulin requirement (non-progressed), and LADA patients that had developed insulin requirement (progressed).

Results: Recognition of a type 1 diabetes-specific GAD65Ab epitope was more pronounced in type 1 diabetes patients than in non-progressed (p<0.001) or progressed (p<0.01) LADA patients, with no significant differences between the two LADA cohorts. These differences were particularly pronounced in samples with GAD65Ab titers <1000 U/ml, with no differences in epitope specificities in samples with higher GAD65Ab titers. Disease duration (initial diabetes diagnosis until sample collection, or development of insulin requirement) in non-progressed and progressed LADA patients, respectively, was not correlated with epitope specificity, suggesting lack of epitope maturation. This was supported by epitope analyses of longitudinal samples from LADA patients during progression to insulin requirement.

Conclusions/interpretation: a) the GAD65Ab specific autoimmune reaction in type 1 diabetes patients with low and moderate GAD65Ab titers differs from that in LADA patients, irrespective of insulin requirement, b) the GAD65Ab specific autoimmune response in LADA patients does not change after their initial diabetes diagnosis, and c) LADA patients with high GAD65Ab titers resemble type 1 diabetes patients in their GAD65Ab epitope specificity.
Latent autoimmune diabetes in adults (LADA) consists of a sub-group (~10%) of adult patients initially diagnosed with type 2 diabetes who show signs of beta cell autoimmunity and eventually develop insulin requirement (1,2). Signs of beta cell autoimmunity, such as the well-characterized autoantibodies to insulin (IAA), glutamate decarboxylase (GAD65), and the tyrosine phosphatase-like protein IA-2, indicate significant damage of the beta cells and subsequent development of insulin requirement in these patients (1). While IAA and IA-2Ab are inversely correlated with age at onset, GAD65Ab show no, and in some studies even a positive correlation with age at onset and are therefore particularly attractive markers for autoimmune diabetes in the adult population (3,4). Moreover, GAD65Ab can be detected years after the clinical onset of the disease, indicating that these autoantibodies may be permanent markers for the autoimmune response (5,6).

Notably not all LADA patients progress to insulin requirement, raising the possibility that the autoimmune response in these patients resembles that in autoantibody-positive healthy individual, with no significant risk for development of insulin requirement (7,8). A better understanding of the autoimmune response is necessary to predict insulin requirement in LADA patients, which is important to prevent escalation of blood glucose levels and subsequent complications.

In previous studies we have investigated the humoral immune response towards GAD65 as a reflection of islet cell destruction (9). It remains unclear whether the autoimmune response in LADA patients and type 1 diabetes patients differs or whether only the duration of the prodromal period distinguishes between the two groups (10). Therefore we compared the GAD65-specific humoral autoimmune response in type 1 diabetes patients with that in LADA patients who had or had not progressed to insulin requirement.

**RESEARCH DESIGN AND METHODS:**

Patients and Sera: Sera of GAD65Ab-positive type 1 diabetes patients were collected at the Saitama Social Insurance Hospital, Japan (n=119). All type 1 diabetes patients required insulin treatment at the time of diabetes diagnosis. Sera were collected between 1989-2005 and were taken at various times after onset of disease (0-27 years (median: 1 year) of disease duration).

Patients classified as LADA patients were admitted to the Saitama Social Insurance Hospital, Japan. Diagnosis of LADA was according to the commission of Immunology of Diabetes Society (2), (patients were diagnosed with type 2 diabetes and tested positive for GAD65Ab with an onset age ≥ 30 years). None of these patients required insulin treatment within the first six months after the initial diagnosis.

We differentiated two groups of LADA patients, based on their insulin requirements. Non-progressed LADA patients (n=56) did not require insulin treatment for over five years after diagnosis with type 2 diabetes. Six of these samples were collected at Keio University, Japan. Some of the samples were taken earlier (see Table 1), however, all patients were followed to ensure that they did not require insulin treatment for over five years past type 2 diabetes diagnosis. Progressed LADA patients (n=58) developed insulin requirement after the initial LADA classification and had low fasting serum c-peptide levels (≤0.4ng/ml). Insulin treatment was started at HbA1c of ≥ 8% despite usage of maximum dose of glibenclamide (5mg) and observation of strict diet.

Longitudinal samples were obtained from nine individuals (5 males, median age 34 years) who were classified as LADA...
GAD65Ab epitope specificity distinguishes LADA patients and developed insulin requirement during follow up. Local institutional ethics committee approval was obtained prior to collection of all serum samples.

Informed consent was obtained from all patients or their legal guardians. The age at onset of diabetes (type 1 diabetes or type 2 diabetes), GAD65Ab titer, duration of diabetes, requirement of insulin and other clinical relevant information are summarized in Table 1.

**GAD65Ab titer determination:** GAD65Ab-positivity of the serum samples was initially evaluated using a commercial radioimmunoprecipitation assay (Cosmic Co, Tokyo, Japan) and the manufacturer’s suggested cut-off level of 1.5 U/ml. GAD65Ab-positivity was confirmed using a radioligand binding assay (RBA) (described below). The World Health Organization (WHO) standard for GAD65Ab (11) and negative samples were included in every assay to correct for inter-assay variation and to express immunoglobulin binding levels as Units/ml (U/ml). Cut-off levels for positivity (34 U/ml) were calculated as the 98th percentile from a healthy control group (n=50). Samples with a GAD65Ab U/ml >1000 in the initial screen were diluted to determine their half-maximal binding concentration. Subsequent epitope mapping experiments were carried out at this half maximal binding concentration. In the Diabetes Antibody Standardization Program (DASP) 2005 workshop the GAD65Ab analysis ranked at 80% sensitivity and 91% specificity.

**rFab used in this Study:** Monoclonal antibodies used in this study were previously described (12). Recombinant Fab (rFab) were produced in *E. coli* 25F2 cells as previously described (9). Briefly, DPA and DPD were derived from a type 1 diabetes patient and recognize epitopes located at amino acids (483-499+556-586) and 96-173, respectively. Monoclonal antibody b96.11, derived from a patient with Autoimmune polyendocrine syndrome type 2, recognizes a conformational epitope involving amino acids located both in the middle and the C-terminus of the molecule (13). Monoclonal antibody MICA-3, isolated from a patient with type 1 diabetes, recognizes an epitope located at amino acid residues 451-585.

**Epitope-specific Radioligand Binding assay (ES-RBA):** Recombinant human [35S]-GAD65 was produced in an *in vitro* coupled transcription/translation system with SP6 RNA polymerase and nuclease treated rabbit reticulocyte lysate (Promega, Madison, WI, USA) as described previously (14). The *in vitro* translated [35S]-antigen was kept at -70°C and used within 2 weeks.

The capacity of the rFab to inhibit GAD65 binding by human serum GAD65Ab was tested in a competitive ES-RBA using Protein A Sepharose (PAS) (Zymed Laboratories) as described (9). The rFab were added at a concentration sufficient to compete binding of the originating intact mAb to GAD65 by at least 80% (0.7-1µg/ml). The background competition for each rFab was established in competition experiments with normal control sera. The background was subtracted prior to calculation of percent binding. The cutoff for specific competition was determined as >10% by using a negative control rFab D1.3 (a kind gift from Dr. J. Foote, Arrowsmith Technologies, Seattle), specific to an irrelevant target, hen-egg lysozyme, at 5 µg/ml.

**Statistical analyses:** Binding of GAD65Ab to GAD65 in the presence of rFab was expressed as follows:

\[ \text{cpm of } [35S]-\text{GAD65 bound in the presence of rFab/ cpm of } [35S]-\text{GAD65 bound in the absence of rFab x 100} \]

All samples were analyzed in triplicate determinations and the intra-assay average coefficient of variation was 5% (13 - 0.04%). Median ages, GAD65Ab titers and competition levels between groups were
analyzed using the non-parametric analysis of variance (Kruskal-Wallis test) followed by Dunn's multiple comparisons test. Competition levels within each group were tested for significance using the non-parametric Wilcoxon matched-pair test. A p-value <0.05 was considered significant.

RESULTS:

Autoantibody status and clinical parameters: The type 1 diabetes cohort had a significantly lower median age compared with the non-progressed and progressed LADA patients (p<0.0001) (Table 1). No significant difference between the median ages of the non-progressed LADA patients and the progressed LADA patients was observed. We wish to emphasize that samples from progressed LADA patients were taken after they developed insulin-requirement. No significant differences in GAD65Ab levels between the three groups were observed.

GAD65Ab response in relation to insulin requirement: All serum samples were analyzed for their binding to GAD65 in the presence of GAD65-specific rFab DPA, b96.11, DPD, and MICA-3 (Figure 1).

We observed significant reduction in median binding to GAD65 in the presence of rFab DPA, DPD, b96.11, and MICA-3 in all groups. No correlation between GAD65Ab titer and reduction of binding conferred by any of the rFab was observed in the type 1 diabetes and progressed LADA patients. In the non-progressed LADA patients we observed a significant correlation of GAD65Ab titer and reduction of binding conferred by rFab b96.11 (p=0.005) and DPD (p=0.004) (data not shown). No correlation between GAD65Ab epitope specificity and gender or age was observed in any of the groups.

To determine whether the epitope recognition differed between the groups, we compared the differences in reduction in median binding to GAD65 conferred by the different rFab (Figure 1). We found that the reduction in binding conferred by rFab b96.11, was significantly more pronounced in type 1 diabetes patients as compared to progressed and non-progressed LADA patients (p<0.01 and p<0.001, respectively).

GAD65Ab response in relation to GAD65Ab titer: Based on our above findings of a correlation between GAD65Ab titer and epitope recognition in the non-progressed LADA patients, and our previous observation that high GAD65Ab titers predict progression to insulin requirement (15), we divided the analysis between samples with GAD65Ab titers above and below 1000 U/ml (Figure 1, inset). We found that sera exhibiting high GAD65Ab titer samples in both LADA groups showed strong inhibition of GAD65 binding by rFab b96.11, similar to that observed in sera obtained from type 1 diabetes patients. For both insulin requiring patient groups (type 1 diabetes and progressed LADA), no significant differences in inhibition levels observed in the presence of rFab b96.11 were observed when comparing sera with high and low GAD65Ab titers. However in non-progressed LADA patients binding levels in the presence of rFab b96.11 in sera with high GAD65Ab titers were significantly lower as compared with sera with low GAD65Ab titers (p<0.001). Consequently, samples with GAD65Ab titers below the 1000 U/ml cut-off showed significant differences in the GAD65 binding in the presence of rFab b96.11 between type 1 diabetes patients and progressed (p<0.01) and non-progressed LADA patients (p<0.001).

GAD65Ab response in relation to disease duration: We tested whether the GAD65Ab epitope specificities may change longitudinally towards progression to insulin requirement. Therefore we correlated epitope specificities with disease duration (initial diabetes diagnosis) in non-progressed LADA patients, and time from initial diabetes diagnosis to insulin requirement in progressed
LADA patients. No correlation with GAD65Ab titer or GAD65Ab epitope specificity was observed, indicating no longitudinal changes over time.

Longitudinal samples obtained from LADA patients (n=9) during their progression to insulin requirement were analyzed for their epitope specificities (Figure 2). While some patients showed longitudinal changes over time (a, b, c), no overall trend in the change of epitope specificities was obvious.

**rFab concentration needed for maximal inhibition is identical in the three groups:** Disparities in inhibition levels of GAD65 binding exerted by a rFab could be caused by different affinities, or differences in binding specificities. Therefore we established the rFab b96.11 concentration necessary to achieve maximal inhibition in all three groups (Figure 3). The median rFab concentration to reach 50% inhibition (EC50) was 0.39 nM for all three groups. Serum samples whose GAD65 binding was not inhibited by rFab concentrations of 2.5 nM, were also not inhibited at 12.5 nM rFab. This confirms that the assay conditions are optimal, as the rFab concentration used (12 nM) exceeded the rFab concentration necessary to achieve maximal competition. These results also suggest that the observed differences between the groups were not caused by different binding capacities to the GAD65Ab epitope defined by b96.11.

**CONCLUSIONS**

Our results confirmed previous observations of different GAD65-specific humoral immune responses in type 1 diabetes patients and non-progressed LADA patients (9,16). Progressed LADA patients exhibited a GAD65Ab epitope pattern intermediate between type 1 diabetes patients and non-progressed LADA patients. This may indicate that the GAD65Ab response matures in patients as they progress towards insulin requirement. We tested this possibility by analyzing longitudinal samples obtained from a small group of LADA patients during their development of insulin requirement. While some of the patients showed changes in their epitope binding specificities, no overall trend was observed.

In the progressed LADA patients the disease duration before insulin requirement varies from 0.5-27 years. We analyzed whether LADA patients who progressed faster to disease showed different GAD65Ab epitope specificities from patients that progressed slower. However, no correlation between GAD65Ab epitope specificities and length of the prodromal period was observed. These findings together with our earlier observations of longitudinal changes in GAD65Ab epitope specificities in adult healthy individuals during their progression to type 2 diabetes (17) lead to our hypothesis that the autoimmune response in LADA patients remains constant after type 2 diabetes onset.

Some of the non-progressed LADA patients showed a very long disease duration without developing insulin requirement (up to 27 years since initial diabetes diagnosis). While the presence of GAD65Ab in LADA patients is considered as a risk factor for subsequent insulin requirement (1), one autoantibody alone confers only a low risk for progression in the general population (7). One could therefore assume that some LADA patients show signs of beta cell autoimmunity, but are unlikely to develop insulin requirement. To test this hypothesis, we analyzed GAD65Ab epitope specificities in correlation with disease duration. However, no correlation between disease duration and epitope specificity was observed. These data are in agreement with the longitudinal study of LADA patients in the UKPDS 77 study reporting stagnant GAD65Ab epitope reactivities (18).

The observed differences in GAD65Ab epitope specificities were
particularly pronounced in the samples with medium-low GAD65Ab titers, while high GAD65Ab titer samples in the three groups recognized the type 1 diabetes-associated b96.11-epitope to similar degrees. This may indicate that the type 1 diabetes-associated autoimmune response is more emphasized in LADA patients with high GAD65Ab titers. While these unexpected findings need to be confirmed in a larger study cohort, previous studies report that LADA patients with high GAD65Ab titers progress to insulin-requirement more often as compared to LADA patients with low GAD65Ab titers (15,19). Moreover, a recent study reported that LADA patients with high GAD65Ab titers resemble type 1 diabetes patients in respect to clinical characteristics, genetic susceptibility, and other autoimmune components (20). However, no correlation between GAD65Ab titer and aggressiveness of beta cell autoimmunity was found in the recent UKPDS 77 study (18). These differences may be caused by different distribution of GAD65Ab titers, as our LADA cohorts included serum samples with very high GAD65Ab titers, while the sera in the UKPDS 77 study cohort showed more moderate GAD65Ab titers.

The observed differences in GAD65Ab epitope specificities between high and low GAD65Ab titer samples within the non-progressed LADA patients suggest a heterogeneous autoimmune response in this group. The disease progression in high titer LADA patients with type 1 diabetes-like GAD65Ab epitope specificity needs to be analyzed in future studies to address this hypothesis.

We conclude that the autoimmune responses in LADA and type 1 diabetes patients show different GAD65-specific immune responses, particularly in the samples with moderate GAD65Ab titers. The particular GAD65Ab characteristics remain stable and do not mature during progression to insulin requirement, which may suggest a distinct autoimmune response in the pathogenesis for LADA patients.

ACKNOWLEDGMENTS:
The study was performed as independent research sponsored by the National Institutes of Health (DK53456), as well as DK53004, DK26190, to Dr. Åke Lernmark, and DK17047.
REFERENCES


**Table**

<table>
<thead>
<tr>
<th>Type</th>
<th>Age at Onset (years)</th>
<th>GAD65Ab titer (WHO U/ml)</th>
<th>Duration since diabetes onset (years)</th>
<th>Duration until insulin requirement (years)</th>
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<td>type 1 diabetes</td>
<td>26 (6-84)</td>
<td>489 (52-4668)</td>
<td>1 (0-12)</td>
<td>NA</td>
<td>114 (61 female)</td>
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<td>non-progressed LADA</td>
<td>49 (32-78)</td>
<td>176 (54-129334)</td>
<td>7 (0.5-27)</td>
<td>NA</td>
<td>56 (35 female)</td>
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<tr>
<td>progressed LADA</td>
<td>45 (30-67)</td>
<td>657 (55-150990)</td>
<td>7 (0.5-26)</td>
<td>2 (0-18)</td>
<td>58 (27 female)</td>
</tr>
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Median and range of values are indicated when appropriate. NA: not applicable.
FIGURE LEGENDS:

**Figure 1:** GAD65Ab epitope specificities in patients with different forms of autoimmune diabetes.

Sera of type 1 diabetes patients, progressed LADA, and non-progressed LADA patients were analyzed for their capacity of binding to GAD65 in the presence of the indicated rFab. Results are presented as binding to GAD65 in the presence of rFab related to uncompeted binding (set at 100%). Median, interquartile range, and upper and lower extremes are shown. P-values are indicated.

Inset: GAD65Ab epitope specificities in LADA patients with high GAD65Ab titers resemble those in type 1 diabetes patients.

Serum samples of type 1 diabetes, progressed LADA, and non-progressed LADA patients with GAD65Ab titers > 1000U/ml (left panel) and GAD65Ab titers < 1000U/ml (right panel) were analyzed for their binding to GAD65 in the presence of rFab b96.11. Results are presented as binding to GAD65 in the presence of rFab related to uncompeted binding (set as 100%). Median, interquartile range, and upper and lower extremes are shown. P-values are indicated.

**Figure 2:** No overall longitudinal trend in GAD65Ab epitope specificities suggests lack of epitope maturation.

GAD65Ab epitope specificity was analyzed in longitudinal samples from LADA patients who progressed to insulin requirement in the follow-up period. Longitudinal samples obtained from LADA patients (n=9) were analyzed for their GAD65Ab epitope specificities. GAD65 binding in the presence of rFab DPA (circles), b96.11 (black triangles) and MICA-3 (squares) is reported in relation to uncompeted binding (set as 100%). The zero time point indicates time of insulin requirement, negative values refer to time before insulin requirement, positive values to samples obtained after initiation of insulin requirement.

**Figure 3:** Binding capacity of the three patient cohorts to the b96.11-defined epitope.

Binding to GAD65 by serum samples of type 1 diabetes, progressed LADA, and non-progressed LADA patients in the presence of the indicated concentrations of rFab b96.11 was analyzed. Results are presented as binding to GAD65 in the presence of rFab related to uncompeted binding (set as 100%). Median, interquartile range, and upper and lower extremes are shown.
GAD65Ab epitope specificity distinguishes LADA

Figure 1.

Figure 2
Figure 3