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Analysis of GAD65 Autoantibodies in Stiff-Person Syndrome Patients

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Autoantibodies to the 65-kDa isoform of glutamate decarboxylase GAD65 (GAD65Ab) are strong candidates for a pathological role in Stiff-Person syndrome (SPS). We have analyzed the binding specificity of the GAD65Ab in serum and cerebrospinal fluid (CSF) of 12 patients with SPS by competitive displacement studies with GAD65-specific Fab-derived from a number of human and mouse mAbs specific for different determinants on the Ag. We demonstrate considerable differences in the epitope specificity when comparing paired serum and CSF samples, suggesting local stimulation of B cells in the CSF compartment of these patients. Moreover, these autoantibodies strongly inhibit the enzymatic activity of GAD65, thus blocking the formation of the neurotransmitter γ-aminobutyric acid. The capacity of the sera to inhibit the enzymatic activity of GAD65 correlated with their binding to a conformational C-terminal Ab epitope. Investigation of the inhibitory mechanism revealed that the inhibition could not be overcome by high concentrations of glutamate or the cofactor pyridoxal phosphate, suggesting a noncompetitive inhibitory mechanism. Finally, we identified a linear epitope on amino acids residues 4–22 of GAD65 that was recognized solely by autoantibodies from patients with SPS but not by serum from type 1 diabetes patients. A mAb (N-GAD65 mAb) recognizing this N-terminal epitope was successfully humanized to enhance its potential therapeutic value by reducing its overall immunogenicity.

Table I. Clinical characteristics of SPS patients

<table>
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<tr>
<th>Patient</th>
<th>Sex</th>
<th>Duration of SPS (years)</th>
<th>Age (years)</th>
<th>Other Autoimmune Diseases</th>
</tr>
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<tr>
<td>1</td>
<td>F</td>
<td>5</td>
<td>30</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>14</td>
<td>26</td>
<td>Pernicious Anemia, thyroiditis</td>
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<tr>
<td>3</td>
<td>M</td>
<td>9</td>
<td>41</td>
<td>None</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>2</td>
<td>55</td>
<td>Thyroiditis</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>15</td>
<td>45</td>
<td>Type 1 diabetes, Pernicious Anemia, thyroiditis</td>
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<td>F</td>
<td>7</td>
<td>33</td>
<td>None</td>
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<tr>
<td>7</td>
<td>F</td>
<td>6</td>
<td>57</td>
<td>None</td>
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<tr>
<td>12</td>
<td>M</td>
<td>3</td>
<td>29</td>
<td>Graves’ disease, cerebellar Ataxia</td>
</tr>
</tbody>
</table>

Type 1 diabetes patients

Sera were obtained from eight children who were diagnosed with diabetes at age 7–12 years (median, 10 years) and subjected to plasmapheresis (33). These samples have been used in all Immunology of Diabetes Workshops to standardize islet cell Abs (34) and GAD65Ab (35). One sample from this set is serving as the 97/550 World Health Organization standard for islet cell Abs and GAD65Ab (36, 37).

Expression and purification of rFab

Recombinant Fab were produced in E. coli 25F2 cells using a phoA-based expression vector (45). Briefly, bacteria containing the recombinant plasmids were grown for 16 h at 30°C in complete MOPS medium (46). Cells were then subcultured and grown in the absence of phosphate at 30°C for 4 h. The rFab were isolated from the bacteria as previously described (45) and purified by two subsequent affinity chromatography steps on Ni-NTA agarose (Qiagen) and protein G-Sepharose (Zymed Laboratories). Fractions were examined by immunoblot for the presence of rFab and by RIA for GAD65 binding. Active fractions were pooled, and the protein concentration was determined. The yield of functional purified rFab was typically 0.5–1 mg/L bacterial culture.

Radioimmunoassay

Recombinant [125I]GAD65 was produced in an in vitro-coupled transcription/translation system with SP6 RNA polymerase and nuclease-treated rabbit reticulocyte lysate (Promega) as described previously (47). The in vitro-translated [125I]-Ag was kept at −70°C and used within 2 wk. Binding of rFab to radiolabeled Ag was determined as previously described (51, 52), using protein G-Sepharose as the precipitating agent.

Competition studies of rFab

The capacity of the rFab to inhibit GAD65 binding by human serum GAD65Ab was tested in a competitive RIA using protein A-Sepharose (Zymed Laboratories) as the precipitating agent (48). Fab lack the CH2 domain of the Fc region and do not bind protein A. In control experiments, we did not observe GAD65 binding of any of the rFab when using PAA as the precipitating agent. Serum samples were tested at their half-maximal binding concentration (indicated in the figures and text). The rFab concentration necessary to compete its intact mAb was used in the competition assays (48).

Statistical analyses

Binding of GAD65Ab to GAD65 in the presence of rFab was expressed as follows: (counts per minute of [125I]GAD65 bound in the presence of rFab) / (counts per minute of [125I]GAD65 bound in the absence of rFab) × 100.

The cutoff for specific competition was determined as >10% by using as a negative control rFab D1.3, specific to an irrelevant target, anti-hen egg white lysozyme, at 5 μg/ml.

All samples were analyzed in triplicate determinations and the intra-assay average coefficient of variation was 5% with the highest value of 9% and the lowest being 0.04%. Competition levels within each group were analyzed using the nonparametric Wilcoxon-matched pair test. A value of p < 0.05 was considered significant.

GAD65 enzymatic activity assay

Glutamate decarboxylase activity was measured by the 14CO2-trapping method described previously (49). Recombinant human GAD65 (a donation by Amgen, Seattle, WA) (100 ng) was incubated with reaction buffer (50 mM KH2PO4, 0.03 mM PLP, and 0.1 mM DTT (pH 6.8)) for 1 h at room temperature with or without the indicated amounts of serum, CSF, or Ab. The enzymatic reaction was initiated by the addition of 0.56 mM L-glutamate and 0.018 μM of 14C-glutamate (Amersham Biosciences) and allowed to continue for 2 h at 37°C with gentle agitation. During incubation, released 14CO2 was captured on filter paper (Kontes) soaked in 50 μL of 1 M NaOH. After the incubation, the absorbed radioactivity was determined in a Beckman scintillation counter. The results are presented as follows: percentage of residual activity = counts per minute in the presence of rFab or Ab / counts per minute in the absence of rFab or Ab × 100.

Humanization of N-GAD65 mAb

Design of a humanized version of murine N-GAD65 mAb is described in the result section. The amino acid sequence was translated to generate the
nucleotide sequence, while optimizing the codons of the designed sequence for *Escherichia coli* expression (see Fig. 6). We generated humanized constructs by overlap extension of a set of 12 and 11 oligonucleotides (40–61 nucleotides) encoding variable domains of the humanized L and H chains, respectively. The oligonucleotides encoding each chain (50 pM each) were combined in 50 μl of annealing buffer (4 mM Tris and 0.8 mM MgCl₂ (pH 8)) and heated to 80°C for 5 min, 67°C for 30 min, and allowed to cool slowly to room temperature. The annealing reaction mix was then diluted 1:20 in TE buffer and cloned into a pUC-derived vector containing the respective constant regions. Subsequently, the Fab H and L chain genes were subcloned into the expression vector pAK19 (45). An additional six histidine residues were introduced at the C terminus of the H chain to enable purification of the rFab on Ni-NTA agarose.

**BIACore analysis**

The kinetic constants for the interaction between GAD65 and both rFab were determined by surface plasmon resonance (BIACore 2000). All analyses were conducted on a BIACore 2000. Biotinylated rFab was immobilized at a density of 300–400 resonance units on a previously prepared streptavidin surface (~3000 resonance units of streptavidin) using a CM4 sensor chip. GAD65 binding was measured at 25°C using a running buffer consisting of 10 mM HEPES buffered saline (pH 7.4), with 0.05% Tween 20. Binding reactions were monitored at GAD65 concentrations, spanning 11–100 nM. Associations were monitored for 2 min and dissociations for 10 min. To determine the kinetic rate constants, each rFab data set was double referenced, and a 1:1 interaction model was fit by allowing a global value for the rate constants and floating the individual binding capacity of the rFab surface to account for the response obtained at surface density. The data were normalized by dividing data points of individual GAD65 responses by the respective surface capacity estimate and multiplying the normalized data by 100. Finally, all data obtained per rFab surface (initial responses and normalized responses) were fit to obtain the global kinetic constants reported in Table II.

**Results**

**Inhibition of GAD65 enzymatic activity by SPS sera**

We tested 12 sera obtained from patients with SPS for their capacity to inhibit the enzymatic activity of GAD65 (Fig. 1). Significant inhibition (40–65%) was observed for 6 of 12 of the sera. No correlation between GAD65Ab titer and inhibition efficiency was observed. The inhibition was dose dependent as verified in dilution experiments (Fig. 1, inset).

**SPS sera inhibition of GAD65 enzymatic activity cannot be overcome by high concentrations of glutamate or PLP**

To further investigate the inhibition mechanism of the sera, we conducted classical kinetic studies, varying glutamate (Fig. 2) and PLP (Fig. 2, inset) concentrations. We established that the inhibition of the tested SPS sera could not be overcome by high glutamate or PLP concentrations.

**Inhibition of GAD65 enzymatic activity by rabbit antisera and mAbs**

To identify possible epitope specificities involved in the inhibition of GAD65 enzymatic activity, we analyzed a number of both
polyclonal and monoclonal GAD65-specific Abs (Fig. 3). As anticipated from the noncompetitive inhibition by SPS patient sera, we did not observe any inhibitory effect when testing polyclonal Abs raised to the cofactor binding site of GAD65, indicating that the inhibition does not occur through steric hindrance of the active site. Significant reduction in GAD65 enzymatic activity was observed in the presence of mAbs b96.11, 221-442, b78, and polyclonal Ab 7996. Inhibition by b78 and 7996 was significantly stronger than by b96.11 and 221-442 (p < 0.01). Abs b78 and 7996 recognize epitopes located at the C terminus of GAD65. However, other Abs to the C-terminal region (mAb DPA) did not affect the enzymatic activity. Both b78 IgG and rFab inhibited the activity in a concentration-dependent fashion with maximal inhibition to 54 and 42% of uninhibited activity at 2.8 and 2.3 μg/ml, respectively (data not shown).

Epitope mapping of SPS sera and CSF

We analyzed the GAD65Ab binding specificities present in sera of SPS patients using rFab derived from GAD65-specific mAbs (Fig. 4A). Most of the sera were competed by rFab DPA (8 of 12), recognizing a conformational epitope located in the N-terminal region of GAD65. Some sera (nos. 2, 10, and 12) showed little competition with any of the rFab, while other sera were competed with a wide range of rFab (nos. 3, 5, 7, 8, and 9). Competition of GAD65 binding with rFab b78 was significantly stronger for sera obtained from SPS patients with rFab b78 than for sera obtained from type 1 diabetes patients (p = 0.003) (Fig. 5). The binding to the b78-defined epitope and the serum’s ability to inhibit GAD65 enzymatic activity correlated significantly (p = 0.02) (data not shown).

No correlation between the epitope specificity of matched serum/CSF pairs was observed (Fig. 4C). Competition observed for the CSF samples was in general lower as compared with the matched serum samples. Competition with rFab DPA, DPD, and 144 was significantly stronger in serum samples than in CSF samples (p = 0.03, 0.02, and 0.04, respectively).

Construction of a humanized rFab

Murine N-GAD65 mAb was humanized by the method of Hwang et al. (50). Applying the Chothia guidelines (51), we assigned the following canonical classes for the CDRs of N-GAD65 mAb: L chain: CDR1, class 4 as identified by the five residue insertion; CDR2, only one class exists; and CDR3, class 1; and H chain: CDR1, class 1, and CDR2, class 2A. We then scored residue-to-residue homology between the CDRs of N-GAD65 mAb and those of germine genes that used the same canonical structures. Kabat positions 27–35 and 52–60 in the H chain and 26–32, 50–56, and 89–95 in the L chain were used for scoring. Based on homology matching, we chose VK2-30*01 (52) to be the best human germline match for the L chain and VH1-46*01 (53) for the H chain. For the J segment, we chose JK3 for the L chain and JH3 for the H chain. The framework of the chosen human germline sequences was then

![Figure 4](image-url)

**FIGURE 4.** Epitope analysis of SPS sera and CSF using rFab. Samples at concentrations of half-maximal binding were analyzed for their binding to GAD65 in the presence of rFab DPA (+), b96.11 (squares), DPC (triangles), DPD (diamonds), and N-GAD65 mAb (circles). Median binding is indicated by a horizontal line. Binding of sera (A) or CSF (B) to GAD65 in the presence of competitor is presented as percentage related to uncompe ted binding (100%). Difference between GAD65 binding of serum and CSF are demonstrated (C).
combined with mouse CDR sequences in a CDR grafted molecule. An alignment of the sequences is shown in Fig. 6.

**Binding specificity of the humanized rFab**

To confirm whether the binding characteristics of the humanized rFab was preserved, we examined its binding to GAD65. A concentration-dependent binding was observed (Fig. 7, inset). The epitope specificity of the humanized rFab was confirmed in competition assays with the original mouse IgG (Fig. 7).

**BIACore analysis**

The results of kinetic analysis of binding of the mouse and humanized rFab are shown in Table II. The humanized rFab showed faster off-rates than the original mouse rFab while the on-rates are comparable. This resulted in a $K_d$ of the humanized rFab $\sim 4.4$ times higher than the mouse rFab.

**Humanized rFab competes specifically with sera of patients with SPS for GAD65 binding**

The humanized rFab was used in competition assays to compete binding to radioactive-labeled GAD65 by GAD65Ab present in the sera of patients with SPS or type 1 diabetes. The sera were incubated at their half-maximal binding concentration with mouse and humanized rFab N-GAD65 mAb at different concentration (2.4–150 ng/ml). Although significant competition with binding to GAD65 was observed for the sera obtained from patients with SPS (Fig. 8), no significant competition was seen when competing binding of sera from patients with type 1 diabetes (median remaining percent binding: 99%, data not shown). This is consistent with our previous data reporting no significant competition between sera of type 1 diabetes patients and rFab N-GAD65 mAb (48).

In contrast to the BIACore data, the humanized rFab competed better than mouse rFab for binding to GAD65. The rFab concentration required to achieve maximal competition varied (5–20 ng/ml). This may be a function of the different affinity these sera have toward the N-terminal epitope. Similar results were obtained in competition experiments with CSF (data not shown).

**Humanized rFab does not interfere with the inhibition of GAD65 enzymatic activity by SPS sera**

We next tested whether humanized rFab would itself inhibit GAD65 enzymatic activity or conversely interfere with the inhibition of SPS sera.

**Discussion**

GAD65Ab in SPS patients can be found in both CSF and blood and are characterized by their ability to inhibit GAD65 enzymatic activity. This makes them potential candidates in the pathogenesis of the disease. However, it remains unclear how these autoantibodies interfere with the enzymatic activity. Our analysis of the mechanism of inhibition clearly shows that SPS sera do not interfere with the binding of glutamate or PLP to GAD65 because the sera-imposed inhibition cannot be overcome by increased concentrations of either of these reagents. Moreover, polyclonal Abs raised toward the active site of GAD65 did not inhibit the enzymatic activity, weakening the argument that steric hindrance is the major pathway of inhibition. Our data suggest that SPS sera inhibit GAD65 enzymatic activity in a noncompetitive manner, perhaps via changes in the enzyme’s conformation. Testing mAbs and polyclonal Abs raised to specific GAD65 peptides, we observed that the C terminus-specific Abs b78 and 7996 resulted in significant inhibition. All SPS sera that showed b78-defined epitope inhibition also inhibited the GAD65 activity, suggesting that binding to this C-terminal epitope affects the enzyme’s activity. Recognition of the b78-defined epitope by SPS sera is more frequently observed. This makes them potential candidates in the pathogenesis of the disease. However, it remains unclear how these autoantibodies interfere with the enzymatic activity. Our analysis of the mechanism of inhibition clearly shows that SPS sera do not interfere with the binding of glutamate or PLP to GAD65 because the sera-imposed inhibition cannot be overcome by increased concentrations of either of these reagents. Moreover, polyclonal Abs raised toward the active site of GAD65 did not inhibit the enzymatic activity, weakening the argument that steric hindrance is the major pathway of inhibition. Our data suggest that SPS sera inhibit GAD65 enzymatic activity in a noncompetitive manner, perhaps via changes in the enzyme’s conformation. Testing mAbs and polyclonal Abs raised to specific GAD65 peptides, we observed that the C terminus-specific Abs b78 and 7996 resulted in significant inhibition. All SPS sera that showed b78-defined epitope inhibition also inhibited the GAD65 activity, suggesting that binding to this C-terminal epitope affects the enzyme’s activity. Recognition of the b78-defined epitope by SPS sera is more frequently observed.
The observed differences between epitope specificities in CSF and serum support earlier reports indicating that the GAD65Ab in the CSF are produced intrathecally by active B cells in the CNS compartment (27). The source of the initiating Ag in SPS remains unclear. The major location of GAD is the CNS, and it is also expressed in nonneuronal tissues such as the β cells of pancreatic islets (55), testes (56), and oviducts (57, 58). In type 1 diabetes, the initiating Ag appears to originate from the pancreatic β cells, while in SPS patients with GADAb in both serum and CSF, the Ag source could be neurological. It would be of major interest to investigate whether different sources of Ag influence the epitope specificity and pathogenicity of the autoantibodies.

We observed differences in binding between sera from SPS patients and type 1 diabetes patients when using rFab N-GAD65 mAb. This Ab recognizes a linear epitope located at aa 4–22 of GAD65 (39). We have shown in the past that this epitope is only present in 5% of type 1 diabetes patients (48). This is in agreement with other reports that found GAD65Ab in SPS sera to bind linear epitopes located at the N terminus of GAD65 (59). In a recent study, recognition of a N-terminal epitope represented by a GAD65/67 fusion protein was shown to correlate with a high GAD65Ab titer in type 1 diabetes patients (60). Such a correlation was not found in our study. This may be due to the use of different epitope mapping methods.

The high specificity and affinity of mAbs makes them attractive candidates in therapeutic applications. As most therapeutic mAbs are generated in mice, one major requirement is to reduce their immunogenicity by humanization (61). To enable the potential use of the N-GAD65 mAb in human therapy, we constructed a humanized rFab with similar binding affinity. By grafting the CDR regions onto the framework on the closest related human germline sequences, we were able to humanize the Ab while preserving the rFab’s ability to specifically recognize the Ag. The humanization was accompanied with a moderate loss of affinity, clearly demonstrating our success in transferring the binding structure onto a human background.

Although our competition data of the humanized rFab and the original mouse rFab of N-GAD65 mAb IgG suggested a weaker binding of the humanized rFab, we found that the humanized rFab was more efficient in competing binding for GAD65 with GAD65Ab from patients with SPS. This may be due to the changed framework of the humanized rFab that may change the binding specificity slightly.

Our humanized rFab may be useful in the characterization of SPS patients because it specifically competes with GAD65 binding in SPS patients but not in sera from type 1 diabetes patients. Furthermore, N-GAD65 mAb might be used to remove GAD65 and GAD65/GAD65Ab immunocomplexes from the patient. The clinical symptoms of patients with SPS can improve upon plasmapheresis together with a marked reduction in GAD65Ab titer (62–64). However, plasmapheresis removes Abs without selection, which can compromise the patient’s immune system. Furthermore, the GAD65Ab reappear within 2 wk (62), necessitating repeated treatment. hN-GAD65 mAb competes the binding to GAD65 only partially (maximal inhibition is 40%) and does not interfere with the serum’s ability to inhibit GAD65 enzymatic activity. We conclude that hN-GAD65 and the patients’ pathogenic GAD65Ab can bind simultaneously to the same Ag molecule. Therefore, a tagged version of hN-GAD65 mAb could be used for removal of GAD65 and endogenous GAD65 immune complexes by extracorporeal affinity adsorption.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


