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*J Immunol* 2005; 175:7755-7762; ;  
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# Analysis of GAD65 Autoantibodies in Stiff-Person Syndrome Patients<sup>1</sup>

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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 rFab-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  $\gamma$ -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. *The Journal of Immunology*, 2005, 175: 7755–7762.

Stiff-Person syndrome (SPS)<sup>3</sup> is a rare autoimmune neurological disease that is characterized by rigidity, episodic spasms of musculature, and continuous motor unit activity (1–3). The disorder is thought to be the result of an immune-mediated deficiency of  $\gamma$ -aminobutyric acid (GABA), a major inhibitory neurotransmitter in the CNS (4). The majority of SPS patients have high titers of autoantibodies directed against both isoforms of glutamate decarboxylase, GAD65 and GAD67 (5), the enzymes that catalyze the conversion of glutamate to GABA (6, 7). The two isoforms coded by genes on two different chromosomes differ in their subcellular location and their interaction with the cofactor pyridoxal 5'-phosphate (PLP) (6–8). Although most of GAD67 is constantly saturated with PLP, a great proportion of GAD65 is present as the inactive apoenzyme (8–10). GAD67 has been sug-

gested to regulate the basal levels of GABA (for review, see Ref. 10) and GAD67-knockout mice die shortly after birth (11, 12). Reduced GABA levels were found in the brain of these animals (12). GAD65 is seen as a regulatory enzyme that adapts the GABA level in situations of sudden demand. GAD65-knockout mice may suffer from epilepsy and stress-induced seizures but survive to adulthood (13). Autoantibodies to GAD65 (GAD65Ab) are also found in patients with type 1 diabetes and in other rare disorders such as Batten disease and autoimmune polyendocrine syndrome (APS) type 1 (14–17). Interestingly, GAD65Ab are also found in patients with neurological diseases, such as cerebellar ataxia (14–16), drug-refractory epilepsy (17, 18), and palatal myoclonus (19, 20).

Analysis of GAD65Ab in SPS and type 1 diabetes has shown similarities and differences in Ag recognition (21, 22). The GAD65Ab titer in SPS patients was found to be 100-fold greater than in type 1 diabetes (23). GAD65Ab in type 1 diabetes recognize conformational epitopes (24), while GAD65Ab in SPS recognize GAD65 in Western blot analysis, suggesting the presence of linear epitopes (21). Furthermore, GAD65Ab-positive sera of SPS patients have been demonstrated to block the enzymatic activity of GAD65 (21, 25). This GAD65Ab-induced inhibition has also been reported for GAD65Ab found in patients with APS-1 and type 2 diabetes (26) but has not been observed for patients with type 1 diabetes (21, 25). However, the mechanism of this inhibition is not understood.

In patients with neurological diseases, GADAb are found in both blood and cerebrospinal fluid (CSF) (27–29), while in patients with type 1 diabetes, these autoantibodies are present only in blood circulation (27). The presence of GADAb in the CSF together with their interference with GABA-mediated synaptic transmission (30–32) have led to the suggestion of a pathogenic role of GADAb in neurological disease. In this study, we analyzed the mode of enzymatic inhibition and the GAD65Ab epitope specificities in SPS patients.

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Received for publication July 22, 2005. Accepted for publication September 19, 2005.

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<sup>1</sup> This study was supported by a Career Development Award from the American Diabetes Association (to C.S.H.). C.J.P. received travel awards from Novo-Nordisk (administered by the Society for Endocrinology, Metabolism, and Diabetes of South Africa) and the National Health Laboratory Service.

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<sup>3</sup> Abbreviations used in this paper: SPS, Stiff-Person syndrome; GABA,  $\gamma$ -aminobutyric acid; PLP, pyridoxal 5'-phosphate; APS, autoimmune polyendocrine syndrome; CSF, cerebrospinal fluid.

Table I. Clinical characteristics of SPS patients

Patient	Sex	Duration of SPS (years)	Age (years) at SPS Onset	Other Autoimmune Diseases
1	F	5	30	Type 1 diabetes
2	F	14	26	Pernicious Anemia, thyroiditis
3	M	9	41	None
4	M	2	55	Thyroiditis
5	F	15	45	Type 1 diabetes, Pernicious Anemia, thyroiditis
6	F	7	33	None
7	F	6	57	None
8	F	20	24	Type 1 diabetes
9	M	3	50	Type 1 diabetes
10	M	10	32	Type 1 diabetes
11	M	24	43	Type 1 diabetes
12	M	3	29	Graves' disease, cerebellar Ataxia

## Materials and Methods

### SPS patients sera

We studied sera and CSF from 11 well-characterized SPS patients with high GAD65Ab titers, evaluated under Institutional Review Board-approved protocols at the Neuromuscular Diseases Section, National Institutes of Health (Bethesda, MD), from 1997 to 2004. Another SPS patient was evaluated at King's College Hospital (London, U.K.) with full ethical approval from King's College Hospital National Health Service Trust and consent from the patient. The age, gender, age at onset of SPS, and the presence of other autoimmune diseases are shown in Table I.

### 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).

GAD65Ab-positive newly diagnosed 0- to 18-year-old type 1 diabetes patients ( $n = 23$ ) were part of a study conducted at the St. Görans Children's Hospital (Stockholm, Sweden) during 1993–1995. The serum samples of all diabetes patients in this study were obtained at the clinical diagnosis of diabetes. All subjects or their parents or legal guardians gave informed consent. The Karolinska Institute's institutional ethics committee approval was obtained before collection of the serum samples.

### Polyclonal and mAbs

Rabbit antisera were raised by immunization with synthetic peptides corresponding to the indicated amino acid residues of GAD65 (38). Antiserum 7996 was raised toward amino acid residues 570–585, antisera 5581, 5576, and 5052 were raised to amino acid residues 390–404, including the site of PLP attachment, and antiserum 5571 was raised to amino acid residues 405–418.

mAbs N-GAD65 mAb and 221–442 were raised in mice and recognize epitopes representing amino acids 4–22 and 221–442, respectively (39, 40). Human mAbs DPA, DPC, and DPD specific for GAD65 were isolated from a patients with type 1 diabetes (41) and recognize epitopes located at amino acid residues 483–585, 195–412, and 96–173, respectively (42, 43). Additional human mAbs b96.11 and b78 specific to GAD65 were similarly derived from a patient with APS-1 (44), and recognize epitopes located at amino acid residues 308–365 and 451–585, respectively (43, 44). All of the tested rFab were GAD65-specific and showed no reactivity to GAD67.

### 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 [ $^{35}$ S]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  $^{35}$ S-Ag was kept at  $-70^{\circ}\text{C}$  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 PAS 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 [ $^{35}$ S]GAD65 bound in the presence of rFab/counts per minute of [ $^{35}$ S]GAD65 bound in the absence of rFab  $\times$  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  $\mu\text{g}/\text{ml}$ .

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  $^{14}\text{CO}_2$ -trapping method described previously (49). Recombinant human GAD65 (a donation by Amgen, Seattle, WA) (100 ng) was incubated with reaction buffer (50 mM  $\text{K}_2\text{HPO}_4$ , 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  $\mu\text{Ci}$  of  $^{14}\text{C}$ -glutamate (Amersham Biosciences) and allowed to continue for 2 h at 37°C with gentle agitation. During incubation, released  $^{14}\text{CO}_2$  was captured on filter paper (Kontes) soaked in 50  $\mu\text{l}$  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  $\times$  100.

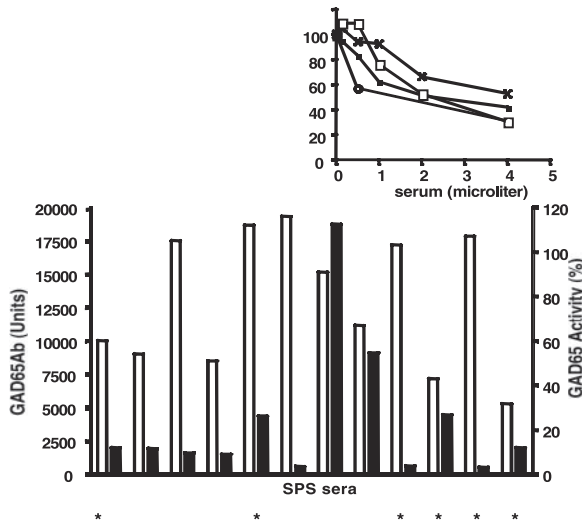
### Humanization of N-GAD65 mAb

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

Table II. Kinetic parameters for the GAD65/rFab interactions

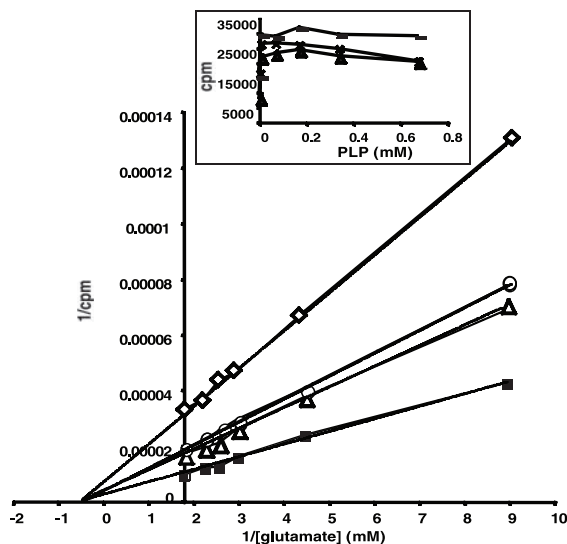
rFab	Density (RU)	$k_a$ ( $\text{M}^{-1}\text{s}^{-1}$ )	$k_d$ ( $\text{s}^{-1}$ )	$K_D$ (nM)
hN-GAD65 mAb	320	$2.03 \pm 0.02 \times 10^5$	$2.79 \pm 0.02 \times 10^{-4}$	$1.37 \pm 0.02$
N-GAD65 mAb	400	$1.989 \pm 0.01 \times 10^5$	$6.239 \pm 0.07 \times 10^{-5}$	$0.313 \pm 0.004$

<sup>a</sup> The SE of fit for the individual  $k_a$  and  $k_d$  values from each binding assay is reported.  $K_D$  was obtained from  $k_d/k_a$ , the error is reported.

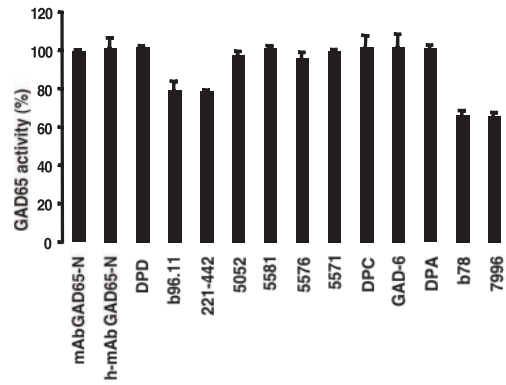


**FIGURE 1.** SPS sera inhibit GAD65 enzymatic activity in a dose-dependent manner. SPS sera were tested for their capacity to inhibit GAD65 enzymatic activity. Patients with both SPS and type 1 diabetes are indicated by an asterisk. Results are reported as percentage related to GAD65 enzymatic activity in the absence of serum (100%) (□). GAD65Ab titer is reported as units (■). Different serum dilutions of four sera (no. 1 ×, no. 2 ■, no. 4 ◇, and no. 12 ○) were tested for their inhibition of enzymatic activity (*inset*).

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  $\mu$ l of annealing buffer (4 mM Tris and 0.8 mM MgCl<sub>2</sub> (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



**FIGURE 2.** GAD65 enzymatic inhibition by SPS sera cannot be overcome by high concentrations of glutamate or PLP. GAD65 enzymatic activity was tested at different concentrations of glutamate and PLP (*inset*) in the absence (■) and in the presence of SPS sera (◇, ○, and △). The effect of different glutamate concentrations is shown in a Lineweaver-Burk graph. The effect of different PLP concentrations is shown as a saturation curve (*inset*).



**FIGURE 3.** Polyclonal and mAbs and their inhibition of GAD65 enzymatic activity. Purified monoclonal and polyclonal Abs specific to GAD65 were tested for their capacity to inhibit GAD65 enzymatic activity. Results are shown as percent enzymatic activity related to GAD65 enzymatic activity in the absence of any Ab (100%). The figure represents three independent experiments, each conducted in duplicates. The error bars represent SEM.

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*).

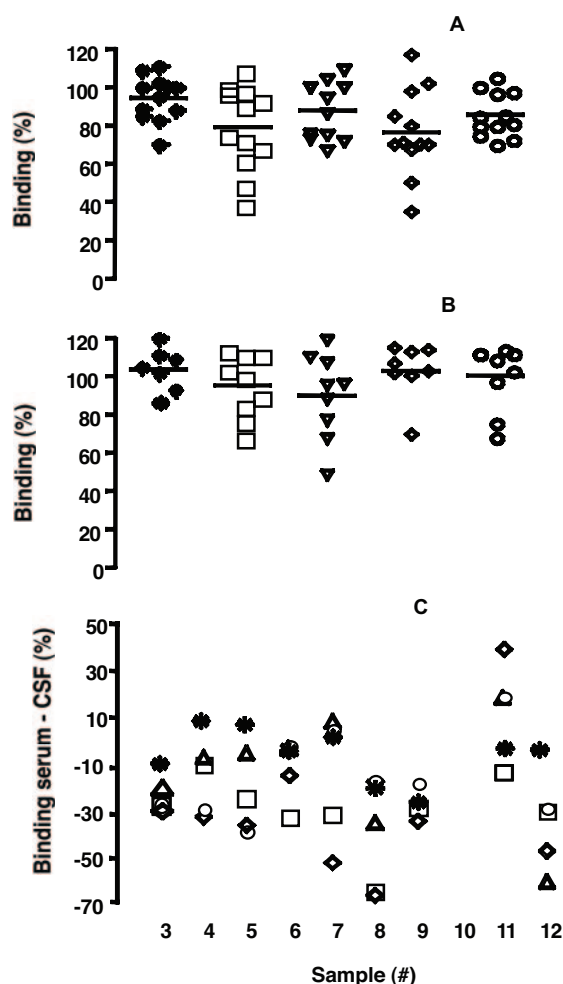
### SPS sera inhibition of GAD65 enzymatic activity cannot be overcome by high concentrations of glutamate or PLP

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





**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 uncompetitor binding (100%). Difference between GAD65 binding of serum and CSF are demonstrated (C).

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  $\mu\text{g}/\text{ml}$ , respectively (data not shown).

#### Epitope mapping of SPS sera and CSF

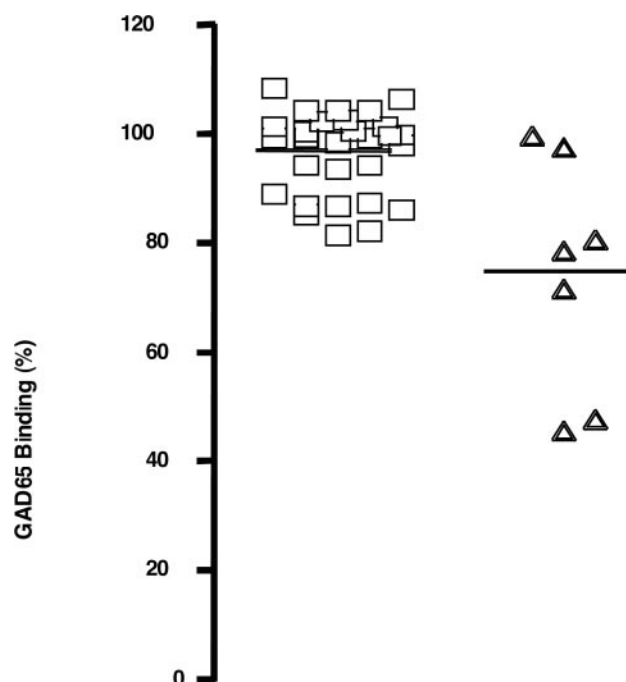
We analyzed the GAD65Ab binding specificities present in sera of SPS patients using rFab derived from GAD65-specific mAbs (Fig.

4A). Most of the sera were competed by rFab DPD (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, class1, and CDR2, class 2A. We then scored residue-to-residue homology between the CDRs of N-GAD65 mAb and those of germline 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 5.** Binding to b78-defined epitope is specific to SPS patients. Recombinant Fab b78 competition of GAD65 binding by sera from SPS patients (right panel) and type 1 diabetes patients (left panel). The median for both groups is indicated in the figure.

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).

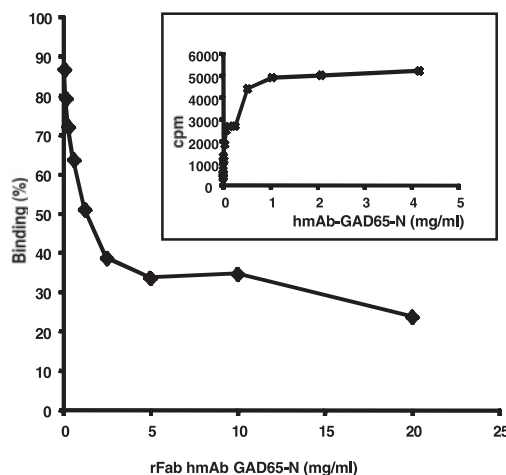
In contrast to the BIAcore data, the humanized rFab competed better than mouse rFab for binding to GAD65. The rFab concentration required to achieve maximal competition varied (5–20 ng/ml). This may be a function of the different affinity these sera have toward the N-terminal epitope. Similar results 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.

VH1-46	QVQLVQSGAE	VKKPGASVKV	SKASGYTFT	SYMHVWRQA	PGQGLEWMGI
hN-GAD65	QVQLVQSGAE	VKKPGASVKV	SKASGYRFS	SYNMHWVRQA	PGQGLEWMGA
N-GAD65	.QQLPQGAE	LVKPGASVKM	SKASGYRFS	SYNMHWVKQT	PGQGLEWIGA
	INPSGGSTSY	AQKFQGRVTM	TRDTSTSTVY	MELSSLRSED	TAVYYCARSY
	IYPRSGDTSY	AQKFQGRVTM	TRDTSTSTVY	MELSSLRSED	SAVYYCVRSY
	IYPRSGDTSY	NQKFKGKATL	TADKSSSTAY	MQLGSLTSED	SAAYYCVRSY
	DYDAPFAFWG	QGTITVTSS			
	DYDAPFAFWG	QGTITVTSS			
	DYDAPFAFWG	QGTITVTSSA			
A17	.DVVMTQSP	SLPVTLGQPA	SISCRSSQSL	VYSDGNTYLN	WFQQRPGQSP
hN-GAD65	.DVVMTQSP	SLPVTLGQPA	SISCRSSQSL	EHNNNGNTYLN	WFQQRPGQSP
N-GAD65	DAVVLQTPL	SLPVS.GDQA	SISCRSSQSL	EHN..NTY.N	WYLQKPGQSP
	RLRIYKVSNR	DSGVPDRFSG	SGSGTDFTLK	ISRVEAEDVG	VYYCMQGTHTP
	RLRIYRVSNR	FSGVPDRFSG	SGSGTDFTLK	ISRVEAEDVG	VYYCLQVTHVP
	QLLIY...NR	FSGGLDRFSG	SGSGTDFTLK	ISRVEAEDLG	VYFCLQVTHVP
J3	FTFGPGTKV	DIKR			
	FTFGPGTKV	DIKR			
	FTFGSGTKL	EIKR			

**FIGURE 6.** Amino acid sequence alignment of germline sequences, N-GAD65 mAb, and hN-GAD65 mAb. CDRs used for homology comparison are indicated in bold sequences.



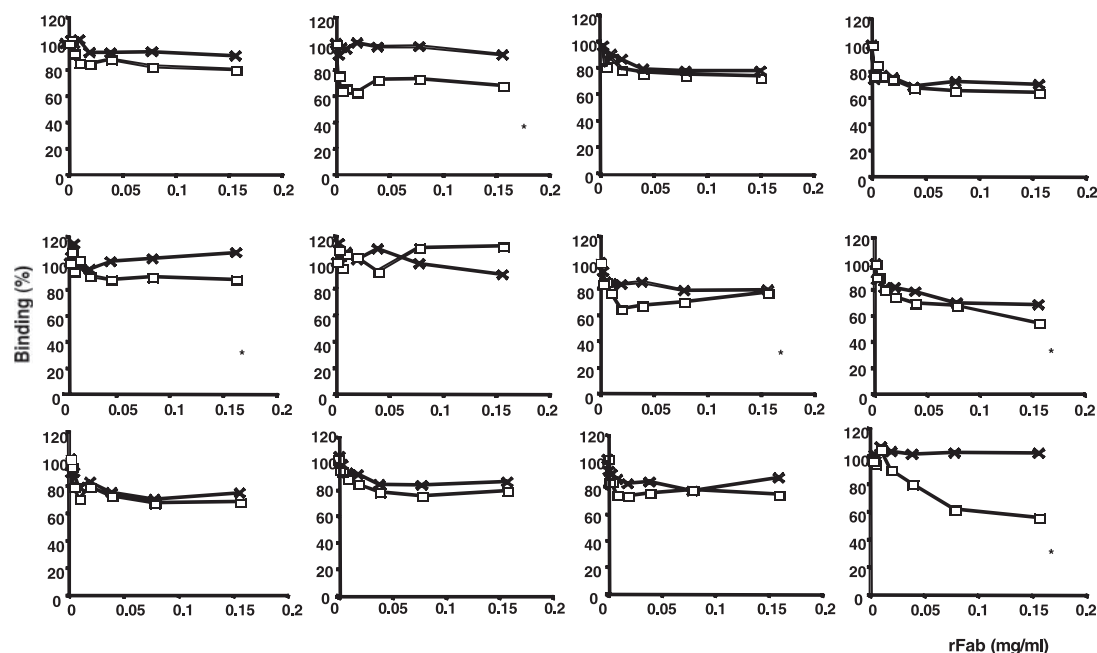
**FIGURE 7.** Binding characteristics of hN-GAD65 mAb to GAD65. N-GAD65 mAb binding to GAD65 was competed with the indicated concentrations of rFab hN-GAD65 mAb. Results are presented as percentage related to binding in the absence of competitor (100%). *Inset*, Dose-dependent binding of hN-GAD65 mAb to GAD65.

Like the original N-GAD65 mAb, the humanized construct showed no inhibition of the enzymatic activity of GAD65 (Fig. 3). We next incubated SPS sera with and without the hN-GAD65 mAb and recombinant human GAD65. No interference with the sera's ability to inhibit GAD65 activity was observed (data not shown). This indicates that the binding site of hN-GAD65 mAb to GAD65 is independent of the binding site of the potential pathogenic SPS GAD65Ab that inhibit its enzymatic activity.

## 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 specificities also inhibited the GAD65 activity, suggesting that binding to this C-terminal epitope affects the enzyme's activity. Recognition of the b78-defined epitope is more frequently observed in SPS sera as compared with sera from type 1 diabetes patients. The observation that not all SPS sera recognize this epitope or inhibit GAD65 enzymatic activity may be a reflection of the heterogeneity of the disease.

We found no correlation between the patient's clinical symptoms, the serum's inhibition kinetics, GAD65Ab titer, or GAD65Ab epitope pattern. Earlier reports show that the GAD65Ab titer cannot be correlated with the severity or duration of the disease (54). Although this could suggest that GAD65Ab may not be involved in the pathogenesis of SPS, it could also be due to different patient-specific thresholds for GAD65Ab levels.



**FIGURE 8.** hN-GAD65 mAb competes with binding to GAD65 by SPS sera. Sera from SPS patients ( $n = 11$ ) were incubated at their respective half-maximal binding with different concentrations of hN-GAD65 mAb ( $\square$ ) and N-GAD65 mAb ( $\times$ ). Patients with SPS and type 1 diabetes are indicated by an asterisk. Results are shown as percentage related to uncompleted binding to GAD65 (100%).

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  $\beta$  cells of pancreatic islets (55), testes (56), and oviducts (57, 58). In type 1 diabetes, the initiating Ag appears to originate from the pancreatic  $\beta$  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.

## Acknowledgments

We gratefully acknowledge Dr. Åke Lernmark for helpful advice and critical reading of the manuscript. We thank Dr. David Myszka at the Center for Biomolecular Interaction Analysis for running the BIAcore analyses.

## Disclosures

The authors have no financial conflict of interest.

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