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Permalink
https://escholarship.org/uc/item/1kb932cs

Journal
The Journal of experimental medicine, 180(2)

ISSN
0022-1007

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Publication Date
1994-08-01

DOI
10.1084/jem.180.2.595

Peer reviewed
Higher Autoantibody Levels and Recognition of a Linear NH2-terminal Epitope in the Autoantigen GAD$_{65}$, Distinguish Stiff-Man Syndrome from Insulin-dependent Diabetes Mellitus

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Summary

The smaller form of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD$_{65}$) is a major autoantigen in two human diseases that affect its principal sites of expression. Thus, destruction of pancreatic $\beta$ cells, which results in insulin-dependent diabetes mellitus (IDDM), and impairment of GABA-ergic synaptic transmission in Stiff-Man syndrome (SMS) are both characterized by circulating autoantibodies to GAD$_{65}$. Anti-GAD$_{65}$ autoantibodies in IDDM are predominantly directed to conformational epitopes. Here we report the characterization of humoral autoimmune responses to GAD$_{65}$ in 35 SMS patients, of whom 13 (37%) also had IDDM. All SMS patients immunoprecipitated native GAD$_{65}$ and the main titers were orders of magnitude higher than in IDDM patients. Furthermore, in contrast to the situation in IDDM, autoantibodies in 35 of 35 (100%) of SMS patients recognized denatured GAD$_{65}$ on Western blots. Two major patterns of epitope specificity were identified on Western blots. The first pattern, detected in 25 of 35 SMS patients (71%), of whom 11 had IDDM (44%), was predominantly reactive with a linear NH2-terminal epitope residing in the first eight amino acids of GAD$_{65}$. Nine of nine individuals who were HLA-haplotyped in this group carried an IDDM susceptibility haplotype and HLA-DR3. DQw2 was particularly abundant. The second pattern, detected in 10 of 35 patients (29%) of whom two had IDDM (20%), included reactivity with the NH2-terminal epitope plus strong reactivity with one or more additional epitope(s) residing COOH-terminal to amino acid 101. The second epitope pattern may represent epitope spreading in the GAD$_{65}$ molecule, but may also include some cases of epitope recognition associated with IDDM resistant HLA-haplotypes. The principal NH2-terminal linear epitope in GAD$_{65}$ distinguishes the reactivity of SMS and IDDM autoantibodies and may be a determinant of pathogenicity for GABA-ergic neurons. The greater magnitude and distinct specificity of the humoral response to GAD$_{65}$ in SMS may reflect a biased involvement of the T helper cell type 2 (Th2) subset of CD4$^+$ T cells and antibody responses, whereas IDDM is likely mediated by the Th1 subset of CD4$^+$ T cells and cytotoxic T cell responses.

The synthesizing enzyme for the inhibitory neurotransmitter GABA, glutamic acid decarboxylase (GAD)$^1$, is encoded by two nonallelic genes, GAD$_{65}$ and GAD$_{67}$ (1), which share a similar exon-intron structure and are probably derived from a common ancestral gene (2). These two GAD proteins share extensive homology except in their first 95 amino

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1. *Abbreviations used in this paper: AET, aminoethylisothiouranium bromide; DTT, dithiothreitol; F/M, female/male; GAD, glutamic acid decarboxylase; HMA, Hepes/NaOH/MgCl$_2$; AET, PLP; ICA, islet cell cytoplasmic antibodies; IDDM, insulin-dependent diabetes mellitus; JDF, Juvenile Diabetes Foundation; PLP, pyridoxal 5’phosphate; PVDF, polyvinylidene difluoride; SMS, Stiff-Man syndrome; TBS, tris-buffered saline.
Acids (1). Both are synthesized as hydrophilic soluble molecules (3), but only GAD65, which is found in neurons and pancreatic β cells, undergoes posttranslational lipid modification in the NH2-terminal domain to become targeted and anchored to the membrane of synaptic-like microvesicles, which store and secrete GABA (3-5).

Autoantibodies to GAD65 are detected in ~80% of individuals who develop insulin-dependent diabetes mellitus (IDDM) (6, 7). Autoantibodies to GAD67 are detected in only a small fraction of IDDM patients, and seem to represent anti-GAD65 autoantibodies that crossreact with the larger isofrom (8, 9). T lymphocytes are implicated as the effectors of β cell destruction (10). GAD65 has been identified as the primary T cell autoantigen in the nonobese diabetic mouse model of IDDM, and induction of tolerance to GAD65 in young mice prevents the disease (11, 12).

Autoantibodies to GAD65 are also found in a rare neurological disorder called Stiff-Man syndrome (SMS) (13). Stiffness of axial muscles and superimposed painful spasms are characteristic of this disorder and are alleviated by benzodiazepines, a finding that is consistent with the hypothesis that SMS represents an impairment of GABA-ergic neurotransmission in the central nervous system (14). SMS is characterized by a 30-40% coincidence with IDDM. Conversely, however, SMS is diagnosed in <1/10⁴ IDDM patients and in <1/10⁶ individuals in the general population. If GAD is a primary autoantigen in both SMS and IDDM, the question is why the diseases do not always coincide. At least three possibilities can be suggested. First, neurons and β cells may express isoforms of GAD that differ antigenically. Second, the accessibility of β cell destruction (10). GAD65 has been identified as the primary T cell autoantigen in the nonobese diabetic mouse model of IDDM, and induction of tolerance to GAD65 in young mice prevents the disease (11, 12).

Autoantibodies to GAD65 are also found in a rare neurological disorder called Stiff-Man syndrome (SMS) (13). Stiffness of axial muscles and superimposed painful spasms are characteristic of this disorder and are alleviated by benzodiazepines, a finding that is consistent with the hypothesis that SMS represents an impairment of GABA-ergic neurotransmission in the central nervous system (14). SMS is characterized by a 30-40% coincidence with IDDM. Conversely, however, SMS is diagnosed in <1/10⁴ IDDM patients and in <1/10⁶ individuals in the general population. If GAD is a primary autoantigen in both SMS and IDDM, the question is why the diseases do not always coincide. At least three possibilities can be suggested. First, neurons and β cells may express isoforms of GAD that differ antigenically. Second, the accessibility to the immune system may differ in the two tissues because neurons, but not β cells, are protected by the blood brain barrier. Third, the humoral and/or cellular immune responses to GAD65 may differ in the two diseases. The demonstration that β cell and neuronal forms of GAD are identical with regard to antigenicity and sequence (3, 15-17) seems to exclude the first possibility, although minor differences in posttranslational modification have not been ruled out. The two tissues clearly do differ in their vulnerability to immune system interactions. In addition to their sequestration behind the blood brain barrier, neurons do not express MHC class I antigens (18). In contrast, β cells are readily accessible to circulating immunological effectors and express MHC class I antigens (19) that can target them for killing by cytotoxic T lymphocytes (20, 21). These differences can explain why SMS is a rare disease in individuals afflicted with IDDM, but not why only 30-40% of SMS patients develop diabetes. To explain the low penetrance of IDDM in SMS, we hypothesize that the immune mechanisms in SMS and IDDM reflect distinct recognition of GAD.

To assess possible differences in recognition of GAD, we have begun to characterize the target epitopes of the humoral autoimmune response in the two diseases. The predominant epitopes for GAD65 autoantibodies associated with IDDM are conformational and localized to the COOH-terminal and middle regions of the GAD65 molecule (22). In rare cases where IDDM autoantibodies recognize the denatured GAD65 molecule on Western blots, the epitope has been localized to the COOH-terminal domain and may represent the phenomenon of restricted epitope spreading (22, 23). We and others have shown that GAD autoantibodies in SMS react with the denatured antigen on Western blots (7, 13, 24) suggesting that the humoral autoimmune response to GAD65 differs in IDDM and SMS. In the present study we have characterized an epitope that clearly distinguishes SMS autoantibodies from those of IDDM.

Materials and Methods

Antisera. Sera were obtained with informed consent from 33 SMS patients identified at the Mayo Clinic (Rochester, MN) during the period of 1989-1992, and from two patients who attended the University of California, San Francisco (UCSF) Neurology Clinic during the period of 1991-1993. The patients were diagnosed using uniform standardized criteria developed at the Mayo Clinic and adapted at UCSF (14). The age, sex, age at onset of SMS, and the concurrence with other autoimmune disorders, including IDDM, are shown in Table 1. Control human sera included: (a) Sera from 11 newly diagnosed IDDM patients. This group included a serum (no. 675, "IDDM serum 2") in this study, female aged 11 years) that is used as a standard for quantitative analyses of GAD65 autoantibodies by several laboratories (9, 25, 26) and has an arbitrary value of 10 in our GAD65 antibody assay (26). The remaining sera in this group were from 10 individuals selected for having the highest GAD65 antibody reactivity amongst 143 newly diagnosed IDDM patients collected from the USA and Scandinavia (Table 2). (b) Sera from 10 healthy controls (female/male [F/M] = 3:7; mean age ± SD = 29.8 ± 4.1; range 22-34). (c) Sera from 10 patients diagnosed at UCSF with polyendocrine autoimmune disorder but not SMS (F/M = 9:1; mean age ± SD = 45.7 ± 19.5; range 31-75). Each autoimmune disorder coexisting in the SMS patients was represented in at least one of these control patients. Eight of the polyendocrine control patients had IDDM (duration 0-31 yr; mean ± SD 12.6 ± 12.1) and six of those were GAD65 antibody positive (Table 2).

Rabbit antisera 1267 and 1701 were raised against a 19-amino acid peptide derived from the COOH terminus of rat GAD65. Both sera react equally well with rat and human GAD65 and GAD67 (3, 27). Rabbit antiserum 2001 was raised against a peptide corresponding to residues 83-93 of human GAD65. The mouse monoclonal antibody GAD26 (28) is specific for GAD67 (3, 27). Rabbit antisera 1267 and 1701 were kindly donated by Dr. D. Gottlieb (Washington University, St. Louis, MO). The mouse hybridoma GAD1 (28) was obtained from the American Type Culture Collection (Rockville, MD).

Immunoprecipitation Analyses. GAD65 autoantibodies were analyzed by a quantitative immunoprecipitation assay using [35S]methionine labeled rat GAD65 transiently expressed in COS-7 cells as described in detail elsewhere (26). Human sera were analyzed at dilutions of 1:10, 1:100, 1:1000, 1:10,000, and 1:100,000, and resulting immune complexes were isolated on protein A-Sepharose (Pharmacia LKB, Piscataway, NJ) and analyzed by SDS-PAGE. Seropositivity was scored on the basis of a visible GAD65 signal obtained by analyses using a model 425 Phosphor-Imager (Molecular Dynamics, Inc., Sunnyvale, CA). Thus, each immunoprecipitate was quantitated by phosphorimaging with IDDM serum 2 (no. 675) serving as a positive standard. GAD65 antibody values were calculated using the formula: 10 × (value for GAD65 immunoprecipitate band in an unknown serum subtracted by value for background area of identical size)/(value for IDDM serum 2 subtracted by value for background area of identical size)). This
Table 1. *GAD<sub>65</sub> Antibodies and ICA in 35 SMS Patients*

| SMS Patient No. | Sex | Age at serum sampling | SMS age at onset | IDDM/age at onset | ICA | Other auto-antibodies/disorders | GAD<sub>65</sub>Ab IMP titer |
|-----------------|-----|-----------------------|------------------|-------------------|-----|--------------------------------|----------------------------|
| 1*              | F   | 45                    | 34               | no<sup>1</sup>     | 8,620| yes                            | 5,260                      |
| 2               | F   | 48                    | 43               | no<sup>1</sup>     | 819 | yes                            | 495                        |
| 3               | F   | 40                    | 36               | no<sup>1</sup>     | 3,940| yes                            | 10,150                     |
| 4               | F   | 41                    | 39               | no                | 8,620| yes                            | 13,310                     |
| 5               | F   | 51                    | 48               | 49                | 819 | yes                            | 2,920                      |
| 6               | F   | 35                    | 34               | 34<sup>4</sup>     | 8,620| yes                            | 5,440                      |
| 7               | F   | 34                    | 26               | no<sup>4</sup>     | 1,610| N/A                            | 7,040                      |
| 8               | M   | 40                    | 27               | no                | 1,800| yes                            | 930                        |
| 9               | M   | 40                    | 36               | no<sup>5</sup>     | >10,000| N/A                            | 16,620                     |
| 10              | M   | 64                    | 56               | no<sup>5</sup>     | 171 | N/A                            | 578                        |
| 11              | F   | 34                    | 29               | no                | 1,800| yes                            | 3,460                      |
| 12              | F   | 43                    | 40               | no                | 8,620| yes                            | 11,140                     |
| 13              | M   | 57                    | <47              | no                | 1,087| yes                            | 1,166                      |
| 14              | F   | 38                    | 34               | no<sup>5</sup>     | 33<sup>4</sup> | 554 | yes                            | 1,260                      |
| 15              | F   | 59                    | 50               | 57                | 2,660| yes                            | 2,870                      |
| 16*             | F   | 44                    | 42               | no                | 7,570| yes                            | 3,380                      |
| 17              | F   | 36                    | 32               | 16                | 253 | yes                            | 444                        |
| 18              | F   | 39                    | 37               | 35                | 3,940| N/A                            | 2,920                      |
| 19              | F   | 54                    | 44               | no<sup>5</sup>     | 345 | yes                            | 4,100                      |
| 20*             | M   | 36                    | 35               | no                | >20,000| N/A                            | 65,700                     |
| 21              | M   | 63                    | 59               | 63                | 1,800| yes                            | 1,654                      |
| 22              | F   | 65                    | 64               | 55<sup>4</sup>     | 374 | yes                            | 1,551                      |
| 23              | F   | 56                    | <52              | Pre-DM            | >10,000| yes                            | 10,660                     |
| 24              | M   | 68                    | 61               | Pre-DM            | Neg. | yes                            | 44                         |
| 25              | F   | 39                    | 39               | No                | 1,210| yes                            | 3,450                      |
| 26              | M   | 49                    | 47               | Pre-DM            | 115 | None                           | 173                        |
| 27              | F   | 50                    | N/A              | no                | 3,110| yes                            | 6,090                      |
| 28              | F   | 40                    | 39               | no                | 2,370| N/A                            | 2,600                      |
| 29              | M   | 50                    | 47               | no                | 2,370| N/A                            | 1,338                      |
| 30              | F   | 50                    | 39               | 39                | >10,000| yes                            | 9,720                      |
| 31              | M   | 70                    | 67               | 70                | 5,110| yes                            | 1,561                      |
| 32*             | F   | 47                    | 43               | no                | >10,000| yes                            | 20,280                     |
| 33              | F   | 45                    | 37               | no                | >10,000| yes                            | 9,500                      |
| 34*             | F   | 50                    | 46               | no                | >10,000| N/A                            | 1,561                      |
| 35              | F   | 60                    | 48               | no                | 5,110| N/A                            | 752                        |

* African Americans, the remaining patients are caucasian.
† Father had NIDDM.
$ IDDM in first degree relative(s). N/A, information not available.
IMP, immunoprecipitation.
Table 2. Summary of GAD65 Antibody Titers in SMS, IDDM, and Polyendocrine Autoimmune Disorder (PE) Patients

|     | n   | Sex | Age mean ± SD | Age range | GAD65 Ab IMP titer median | GAD65 Ab IMP titer range |
|-----|-----|-----|---------------|-----------|--------------------------|--------------------------|
| SMS | 35  | 25/10 | 48 ± 10.1     | 34–70     | 2920                      | 44–65,700                |
| IDDM| 10* | 2/8  | 4.6 ± 5.0     | 0.9–15    | 20.3                      | 14.7–36.6                |
| PE  | 6‡  | 5/1  | 48 ± 19       | 11–75     | 8.2                       | 1.3–15.9                |

* Selected for having the highest GAD65 antibody titer amongst 143 newly diagnosed IDDM patients, 115 (80%) of whom were positive for GAD65Ab with a median titer of 1.7.
‡ From a group of 10 PE patients, 6 of whom were GAD65Ab positive.
IMP, immunoprecipitation.

assay was used in the first GAD antibody workshop (Orlando, FL, April 1993) and yielded 100% sensitivity and 100% specificity.

Expression Systems and Protein Synthesis. The generation of recombinant baculoviruses harboring either rat GAD65 (4), human GAD65, or human GAD67 (22) was described earlier. A recombinant baculovirus for expressing an NH2-terminal deletion mutant of rat GAD65 lacking the first 101 amino acids was generated by first making a 2-kb deletion mutant by oligonucleotide-directed mutagenesis (5), cloning this mutant into the EcoRI site of phbluescript (Stratagene, La Jolla, CA), from which it was released by Smal and NotI digestions, and finally inserting the mutant cDNA into the Smal and NotI sites of the baculovirus expression vector pVL1393 (Invitrogen, San Diego, CA). A clone containing the sense orientation for the A1-101 mutant of rat GAD65 was used in a calcium phosphate cotransfection with wild-type Autographa californica nuclear polyhedrosis virus. Recombinant viruses were isolated as described (30). Recombinant proteins were expressed from the baculovirus constructs in Sf9 cells. Generation of NH2-terminal deletions of a rat GAD65 mutant in which cysteines at positions 30 and 45 have been substituted with alamines, and insertion of the mutants into the COS-cell expression vector pSVSPORT (GIBCO BRL, Gaithersburg, MD) were described earlier (5). Deletion mutants lacking the first NH2-terminal 8 (Δ1-8), 15 (Δ1-15), and 23 (Δ1-23) amino acids of rat GAD65, respectively, were produced by transient transfection of COS-7 cells using lipofectamine (GIBCO BRL).

For Western blot analyses, Sf9 or COS-7 cells expressing GAD65, GAD67, or NH2-terminal deletion mutants of GAD65 were washed twice in cell harvest buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl, 0.1 mM PMSF), and lysed in HMAP buffer (10 mM HEPES/NaOH, pH 7.4, 1 mM MgCl2, 1 mM aminooethylisothiouranium bromide hydrobromide [AET], 0.2 mM pyridoxal 5′-phosphate [PLP]) containing 1% Triton X-114, and 0.1 mM PMSF at 4°C. After centrifugation at 264,000 g, 4°C, for 30 min, the supernatant was collected and prepared for SDS-PAGE by boiling for 3 min in SDS sample buffer at a final concentration of 8 mM Tris/HCl, 15% sucrose, 2% SDS, 5% β-mercaptoethanol, and 0.006% bromophenol blue.

A 58-kD tryptic fragment containing the COOH terminus was generated from rat or human GADs produced in Sf9 cells. Approximately 60 × 106 Sf9 cells were harvested 72 h after infection with the appropriate baculovirus. The cell pellet was extracted in 4 ml of HMAP buffer supplemented with 1% Triton X-114 and 0.1 mM PMSF for 1 h on ice. The lysate was diluted with 21.6 ml of HEMAP (HMAP supplemented with 1 mM EGTA) and treated with 5.4 ml of Ntosyl-l-phenylalanine-chloromethylketone (TPCK)-treated trypsin (2 mg/ml; Sigma Chemical Co., St. Louis, MO) for 20 min at 0°C. The reaction was quenched by adding 400 μl of 100 mM PMSF and 6.8 ml 5× SDS-sample buffer. This material was used for Western blot analysis.

The approximate location of the tryptic cleavage site was estimated based on the relative molecular weight of the fragment obtained by SDS-PAGE, followed by sequencing of the NH2 terminus of the isolated peptide. Approximately 300 × 106 Sf9 cells infected for production of human GAD65 were harvested and suspended in HMAP buffer containing 1% Triton X-114 and 0.1 mM PMSF, and incubated for 1 h on ice. The cells were then homogenized using a Tissumizer homogenizer (Tekmar Co., Cincinnati, OH) for 30 s on ice. The cell lysate was centrifugated at 100,000 g and the supernatant was applied to a phenyl-Sepharose CL-4B column (Pharmacia LKB) preequilibrated in buffer A (50 mM potassium phosphate, pH 7.0, 10 mM diithiothreitol [DTT], 1 mM AET, 0.2 mM PLP) containing 2 mM ammonium sulfate, then washed with 5 column volumes of buffer A containing 2 mM ammonium sulfate. The protein was eluted using buffer A and a gradient with initially 0% Triton X-100 and 1.5 mM ammonium sulfate, and finally 2% Triton X-100 and 0 mM ammonium sulfate. Fractions containing GAD activity were pooled and subjected to immunoblot analysis using the GAD1 monoclonal antibody as described (27) except that the gel matrix was washed sequentially with 10 volumes of buffer B (10 mM HEPES/NaOH, pH 7.4, 10 mM DTT, 1 mM AET, 0.2 mM PLP), 10 volumes of buffer B with 0.5 M NaCl and 10 volumes of buffer B before elution of GADs in 1-ml aliquots of buffer A at pH 11 and neutralized in 100 μl 1M potassium phosphate pH 6.5, 0.2 mM PLP. Protein concentrations were determined using Coomassie Plus protein reagent (Pierce, Rockford, IL), using BSA as a standard. Purified human GAD65 (81 μg/ml, 100 μl) was treated with 5 μl of 0.2 mg/ml TPCK-treated trypsin (1 μg in 5 μl) for 1 h at 0°C. The reaction was quenched by adding 10 μl of 100 mM PMSF and freezing at −20°C. The sample (13 pmol of the 58-kD protein) was subjected to preparative SDS-PAGE and blotted to Pro Blott polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, Inc., Foster City, CA). A slice of PVDF paper containing the 58 kD fragment was used for gas phase sequencing using a gas phase sequencer (model 470A; Applied Biosystems, Inc.). The phenylthiohydantoin (PTH)-derivatives were identified by reverse-phase HPLC using an on-line PTH analyzer (model 120A; Applied Biosystems, Inc.). Electrophoresis, blotting, and sequencing were carried out at the Biomedical Resource Center, UCSF. The results were consistent with the generation of two fragments by cleavage at either position 69 (lys) or 70 (arg).

Western Blot Analyses. To compare serum IgG specificities for GAD65, GAD67, the 58-kD fragment of GAD65, and deletion mu-

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tants of GAD65, it was essential to load equal amounts of each protein on the gel. A preparation of full-length rat or human GAD65 was chosen as an arbitrary standard and titrated on Western blots. Each protein preparation was titrated and calibrated against this standard to obtain an identical signal on Western blots immunostained with rabbit 1267 antisera, which is equally reactive with both GAD65 and GAD67 (27). Finally, the concentrations of different proteins which consistently gave a signal equivalent to that of the GAD65 standard were tested several times on the same blots, to ensure equivalent signal intensities. Equal amounts of full-length GAD65, GAD67, 58-kD trypsin fragment of GAD65, or deletions mutants of GAD65 were then subjected to SDS-PAGE, blotted to Immobilon PVDF transfer membrane (Millipore Corp., Bedford, MA) and analyzed for binding of human IgG and animal antiserum IgG after dilution of sera as described earlier (27). In some experiments, human sera were preabsorbed using a homogenate of noninfected Sf9 cells as indicated. Sf9 cells were homogenized in HMAP buffer (107 cells/ml) using a Tissumizer homogenizer (Tekmar Co.) for 1 min on ice. The homogenate was centrifuged at 264,000 g. Sera were incubated with 0.2 vol of Sf9 cell supernatant for 16 h at 4°C, diluted, and used for immunostaining of Western blots (27).

Synthesis of Peptides for Blocking Studies. Peptides of 20 residues, spanning amino acids 1-119 of the human GAD65 molecule (17), and each overlapping the next sequence by 9 amino acids, were synthesized by a multiple peptide synthesizer (Advanced Chemtech, Louisville, KY), using 4-fluorophenylethylcarbonyl (FMOC)--protected amino acids and amino acid resins (Bachem California, Torrance, CA). Side chain deprotection and cleavage from the resin were performed with trifluoroacetic acid/anisole/dimethyl sulfoxide (9:0.5:0.5 vol/vol), and the peptides were extracted with ether. Peptide identity was confirmed by protein sequence analysis with a Procise protein sequence analyzer (Applied Biosystems, Inc.). For blocking studies, the peptides were solubilized in TBS/Tween-20 buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) to a concentration of 0.5 mg/ml. All peptides, except no. 8, dissolved readily in this buffer. Because peptide 8 (residues 78-97) was only partially soluble, it was used as a saturated solution which effectively blocked the binding of rabbit antisera 2001 (reactive with residues 83-93 in human GAD65).

Dot Blot Analyses. Human GAD65 was extracted from Sf9 cells and isolated by 95% purity by immunoaffinity chromatography as described previously (27). Purified GAD65 at a concentration of 55 μg/ml in 100 mM potassium phosphate, 0.2 mM PLP, 1 mM AET, 0.1% SDS, was boiled for 3 min before spotting (0.2 μg) on Immobilon PVDF transfer membrane (Millipore Corp.) using a BRL HybriDot manifold (GIBCO BRL). The PVDF membrane was blocked with TBS/Tween-20 buffer containing 3% milk protein for 1 h at room temperature. The membranes were incubated with sera (diluted in TBS/Tween-20 buffer containing 2% BSA) for 1.5 h at room temperature; after three washes of 5 min in TBS/Tween-20, they were incubated with alkaline phosphatase-conjugated goat anti-human IgG (Zymed Laboratories, Inc., South San Francisco, CA) or anti-rabbit IgG (Zymed Laboratories, Inc.) at a dilution of 1:500 in 3% milk/TBS/Tween-20 buffer for 1 h at room temperature. After two washes of 5 min in TBS/Tween-20 and one in TBS/Tween-20 buffer, the blots were developed by incubation for 3 min in 0.4 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mM nitro blue tetrazolium in 150 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl2.

Human sera (SMS, IDDM, polyendocrine, and normal sera) were diluted 1:100, 1:200, and 1:500 in TBS/Tween-20 buffer, 2% BSA. Rabbit antisera 1701 and 2001 were diluted in the same buffer, 1:200,000 and 1:2,000, respectively. For peptide blocking experiments, diluted sera were incubated overnight at 4°C with either a mixture of peptides (0.3 μg/μl each), an individual peptide (0.3 μg/μl), human GAD65 (5.5 μg/ml), or no additive, before incubation with dot blots of purified human GAD65.

**Analyses of Islet Cell Cytoplasmic Antibodies (ICA).** ICAs were analyzed by indirect immunofluorescence using frozen sections of human pancreas from blood group O-positive donors of cadaveric kidneys (31). Serum samples were titrated and end point titers were defined as the highest dilution of detectable ICA-staining. Positive samples were expressed in Juvenile Diabetes Foundation (JDF) units by comparing their end point dilution to a standard calibration curve using the international JDF reference serum provided by the Immunology of Diabetes Workshops. This assay has performed in category A in the ICA proficiency program conducted under the auspices of the Immunology of Diabetes Workshops (32).

**HLA-typing.** DRB1, DQBI, and DQA1 typing was performed with a PCR dot blot technique as described (33, 34).

### Results

**SMS Sera Contain Higher Levels of Immunoprecipitating GAD65 Autoantibodies than IDDM Sera.** Sera from 35 SMS patients were analyzed for GAD65 autoantibodies by immunoprecipitation of [35S]methionine-labeled Triton X-114 detergent phase extracts of COS-7 cells expressing rat GAD65 (Table 1). Anti-GAD65 IgG titers, quantified in serially diluted sera by phosphorimager analyses, were orders of magnitude higher in SMS sera (Tables 1 and 2) than in IDDM sera and sera from patients with polyendocrine autoimmune disorder (Table 2). Thus, the GAD65 binding capacity of SMS IgG is orders of magnitude higher than that of IDDM IgG. Titers of ICA in the SMS patients were also higher (Table 1, median titer 2660 JDF units) than in 327 ICA-positive newly onset IDDM sera analyzed by the same assay (median titer 110 JDF units) (31). The correlation coefficient between ICA and GAD65 autoantibody titers in the 35 SMS patients was 0.77.

**SMS Epitopes Recognized in Denatured GAD65, Are Absent in GAD67, and Distinct from IDDM Epitopes.** The 35 SMS sera were analyzed by immunoblotting for reactivity with equal amounts of denatured GAD65 and GAD67. Additional sera were tested from 11 IDDM patients, 10 healthy individuals, and 10 patients with polyendocrine autoimmune disorder without SMS. No serum IgG bound to human or rat GAD67 on Western blots (Fig. 1 A, lanes 3, 5, 7, 9, 11). IgG in all SMS sera reacted specifically with GAD65 (Fig. 1 A, lanes 4, 6, 8), but no reactivity was found in the sera from patients with IDDM (Fig. 2, lanes 22 and 25), polyendocrine autoimmune (Fig. 1, lane 10), or healthy control subjects (Fig. 1, lane 12; Fig. 2, lanes 28 and 31) at the same dilution. To analyze whether the lack of reactivity of IDDM sera with GAD65 on Western blots was due to low titers rather than differences in epitope recognition, the IDDM sera used in this study were also analyzed at lower dilutions to obtain a final GAD65 antibody titer of 4 and compared with SMS sera analyzed at the same titer. To avoid nonspecific binding of Sf9 cell proteins at high IgG concentrations, the sera were preabsorbed with noninfected Sf9 cell lysates.
the low dilutions, IDDM sera still failed to react with denatured GAD65 on Western blots (Fig. 1 B). The results demonstrate that SMS autoantibodies are directed at epitope(s) in the GAD65 molecule that are resistant to denaturation by SDS and are therefore likely to be continuous. Reactivity with these GAD65-specific epitope(s) qualitatively distinguishes SMS autoantibodies from anti-GAD autoantibodies found in IDDM and polyendocrine autoimmune disorder in the absence of SMS.

**SMS Autoantibodies Recognize Epitopes in the NH2-terminal Region of Denatured GAD65.** Since GAD65 differs most significantly from GAD67 in the first 95 amino acids, we speculated that this region might harbor GAD65-specific SMS epitopes recognized by immunoblotting. To address this possibility, all sera were analyzed in parallel for reactivity with equal amounts of full-length rat GAD65, a 58-kD tryptic fragment of rat GAD65 lacking the first 69 or 70 amino acids, and a 55-kD deletion mutant lacking the first 101 amino acids of rat GAD65 (GAD65Δ1-101) (Fig. 2 and Table 3). Among the SMS sera, two major patterns of reactivity were observed. The majority (25 of 35, or 71%) of SMS sera exhibited pattern I, in which reactivity with full-length GAD65 was much stronger than with both the 58- and 55-kD fragments. This serological pattern suggests that the NH2-terminal 69 or 70 amino acids in rat GAD65 harbor a major linear epitope(s) (Fig. 2, lanes 4–15, and Table 3). Among the pattern I sera, IgG in 9 (pattern IA) reacted strongly with the full-length GAD65 protein, but did not recognize the 58- and 55-kD fragments, demonstrating that the recognition of denatured rat GAD65 was limited to the first 69 or 70 amino acids (Fig. 2, lanes 4–9). IgG in the remaining 16 SMS sera of the pattern I group (pattern IB) reacted strongly with the full-length protein, and were significantly less reactive with the 58- and 55-kD fragments. This result again suggested IgG that recognized a major epitope in the first 69 or 70 amino acids but, in addition, minor epitope(s) further downstream in GAD65 (Fig. 2, lanes 10–15, and Table 3). Thus for 71% of the SMS sera (pattern IA and B), the major epitope recognized in denatured rat GAD65 resides in the first 69 or 70 amino acids. Pattern II reactivity, found in 10 of 35 SMS sera (29%, pattern II), was distinctly different in that reactivity with both the 58-kD tryptic fragment and the 55-kD deletion mutant was only minimally less than reactivity with the full-length protein (Fig. 2, lanes 16–21, and Table 3). Thus 29% of the SMS patients had autoantibodies that recognized an additional major epitope(s) further downstream in the denatured GAD65 molecule. In sum, residues 1–69 or 1–70 in rat GAD65 contribute to a linear epitope(s) that is recognized by IgG in serum of all SMS patients, and residues COOH terminal to amino acid 101 in rat GAD65 contribute to a second antigenic region that is recognized by IgG in only some SMS patients.

**Table 3. Distribution of SMS-Patients According to Patterns of Epitope Recognition in Denatured GAD65**

| Pattern | Linear NH2-terminal epitope | NH2-terminal epitope predominant | Epitope in a Δ101 mutant | SMS-patients no. |
|---------|-----------------------------|---------------------------------|--------------------------|------------------|
| IA      | yes                         | yes                             | no                       | 1,2,5,8,17,      |
|         |                             |                                 |                          | 22,27,28,31      |
| IB      | yes                         | yes                             | weak                     | 7,10,11,13       |
|         |                             |                                 |                          | 14,15,16,19      |
|         |                             |                                 |                          | 21,23,24,25      |
|         |                             |                                 |                          | 26,29,30,35      |
| II      | yes                         | no                              | strong                   | 3,4,6,9,12       |
|         |                             |                                 |                          | 18,20,32,33,      |
|         |                             |                                 |                          | 34               |

**Figure 1.** SMS sera recognize GAD65 but not GAD67 on Western blots and this reactivity distinguishes SMS from IDDM and polyendocrine autoimmune in the absence of SMS. (A) Lysates of Sf9 cells expressing human GAD67 (lanes 1, 3, 5, 7, 9, 11) or GAD65 (lanes 2, 4, 6, 8, 10, 12) produced from a baculovirus expression system were subjected to SDS-PAGE, blotted to a PVDF membrane, and immunostained using the human sera indicated (PE, polyendocrine patient serum; NHS, normal human serum) at dilutions of 1:500 and the rabbit anti-GAD antiserum 1267 at dilution 1:100,000. (B) Lysates of Sf9 cells expressing human GAD67 (lanes 1, 3, 5) or GAD65 (lanes 2, 4, 6) immunostained with SMS and IDDM sera preabsorbed to a Sf9-cell lysate and diluted (SMS22, final dilution 1:388; IDDM1, final dilution 1:9) to obtain a titer of approximately 4 for GAD65 antibodies. Immunostaining with the 1267 antiserum (dilution 1:20,000) is shown in lanes 1 and 2.
Figure 2. SMS sera show two patterns of reactivity with 58- and 55-kD COOH-terminal fragments of rat GAD65. Full-length GAD65, a 58-kD tryptic fragment of rat GAD65, and a 55-kD NH2-terminal deletion mutant of rat GAD65 lacking amino acids 1-101, were subjected to SDS-PAGE, blotted to PVDF membrane, and immunostained using the sera at the following dilutions: SMS 1, 12, 18, 30 at 1:500, SMS 2, 21 at 1:200, and IDDM and NHS at 1:500. SMS sera 1, 2, 21, and 30 (pattern I) recognize a major epitope NH2-terminal to amino acid 69/70, whereas SMS sera 12 and 18 (pattern II) also recognize a strong epitope COOH-terminal to amino acid 101. Among pattern I sera, two subpatterns are observed. In pattern IA, the sera only recognize the full-length protein. In pattern IB, the sera recognize a weaker epitope COOH-terminal to amino acid 101.

The Principal NH2-terminal Epitope Resides in the First Eight Amino Acids. To further localize the SMS epitope(s) in the NH2-terminal region, we synthesized ten peptides, each 20 amino acids long, spanning residues 1-119 in the GAD65 molecule and overlapping each other by 9 amino acids. We tested the ability of the peptides to block the dot blot reactivity of SMS IgG with denatured GAD65. The first series of experiments tested three mixtures of peptides, peptides 1-3 (spanning residues 1-42), peptides 3-6 (residues 34-75), and peptides 7-10 (residues 67-119). The mixture of peptides 1-3 completely blocked the reactivity of pattern IA sera, significantly blocked pattern IB reactivity, and partially blocked pattern II reactivity. These results are consistent with recognition of an epitope in the first 42 amino acids by both pattern I and pattern II sera. The mixture of peptides 4-6 did not affect the binding of SMS IgG to denatured GAD65 (not shown). The mixture of peptides 7-10 partially blocked the reactivities of both pattern I and pattern II sera. We next analyzed the activity of individual peptides 1, 2, 3, 7, 8, 9, and 10 to block the binding of SMS IgG to denatured GAD65. These experiments revealed that only peptide 1 of the 1-3 mixture inhibited the binding of SMS IgG to human GAD65 (see Fig. 3), suggesting that the first 11 residues of the NH2 terminus of GAD65 contribute to an SMS epitope. This blocking was specific, because peptide 1 did not block

Figure 3. An NH2-terminal peptide of GAD65 specifically blocks the binding of SMS sera. Human GAD65 purified from a baculovirus expression system was spotted on a PVDF membrane and incubated with SMS sera (all at 1:500 dilution) or serum 1701 (raised to peptide 170, corresponding to 19 COOH-terminal amino acids in GAD, at a dilution of 1:200,000) that had been incubated overnight with or without the indicated peptides or human GAD65. Peptide 1 completely blocks the reactivity of serum 1 (pattern IA), significantly inhibits the reactivity of serum 11 (pattern IB), partially inhibits the reactivity of serum 12 (pattern II), but fails to inhibit serum 1701. Peptide 2 does not inhibit binding of any of the sera and peptide 170 only inhibits binding of the serum 1701, which was raised against it.
the binding of rabbit IgG raised against either a COOH-terminal peptide (serum 1701, Fig. 3, panels 25/26) or a peptide encompassing residues 83–93 in the NH2-terminal region (serum 2001, not shown). Each of these antisera were blocked by the peptide against which they were raised (Fig. 3, panels 23/24 and results not shown). Only peptide 7, in the mixture 7–10, caused any block of the reactivity of SMS sera. However, blocking by peptide 7 was not specific because it also inhibited the reactivity of several rabbit antisera raised against irrelevant peptides (not shown). The reason for nonspecific inhibition by peptide 7 of IgG binding to GAD65 on dot blots is unclear. This activity was not found with peptides 6 and 8, which together account for all but two of the amino acids in peptide 7. Thus, among the NH2-terminal peptides spanning the first 119 amino acids, only peptide 1 specifically blocked the pattern I reactivity of SMS IgGs and partly inhibited the reactivity of pattern II SMS IgGs. This result is consistent with a major linear NH2-terminal SMS epitope residing in the first 11 amino acids of GAD65.

To further analyze the reactivity of SMS IgG with the NH2-terminal region of GAD65, deletion mutants lacking the first 8 and 15 amino acids of rat GAD65, respectively, were analyzed by immunoblotting for interaction with SMS IgG (Fig. 4). These experiments demonstrated that pattern I sera reacted either very weakly (pattern IB) or not at all (pattern IA) with both the Δ1-8 and Δ1-15 deletion mutants (Fig. 4, lanes 4–15). In contrast, pattern II sera recognized both deletion mutants (Fig. 4, lanes 16–21). All of the SMS sera either failed to react with both of the short deletion mutants or showed equal reactivity to both of them (Fig. 4), indicating that the NH2-terminal linear epitope recognized by SMS sera resides in amino acids 1–8 and that amino acids 9–15 do not contribute to this or other SMS epitopes.

The results of the peptide blocking experiments and analyses of deletion mutants localize the NH2-terminal epitope recognized by SMS sera to the first 8 amino acids.

**HLA-DRBI*0301, DQBI*0201 Is the Predominant Haplotype among Patients of Pattern I Seropositivity.** Many autoimmune diseases, including IDDM, are strongly associated with certain MHC class II haplotypes. Thus ≥95% of IDDM patients carry the HLA-DRBI*0301, DQBI*0201 (DR3, DQw2) and/or DRBL1*0401, DQBI*0302 (DR4, DQ8) susceptibility haplotypes (35). To assess whether SMS and IDDM have similar HLA-association, we obtained lymphocytes from 12 of the SMS patients, 9 with serological pattern I and 3 with serological pattern II (Table 4, and Lennon, V., S. Baekkeskov, T. Bugawan, and H. Erlich, manuscript in preparation). 9 of the 12 SMS patients carried the IDDM associated allele DQw2 (DQBI*0201), 8 on the DR3 (DRBL1*0301) and one on the DR7 (DRBL1*0701) haplotype. Amongst the DQw2 positive patients, 8 (4 of whom were homozygous) had serological pattern I reactivity and one had pattern II reactivity. The only non-DQ2 positive patient with pattern I was positive for the IDDM associated haplotype DR4, DQ8 (Table 4). Thus all nine patients with serological pattern I but only one of the three patients of pattern II carried an IDDM associated haplotype (Table 4). One of the pattern II patients carried the DRBL1*1501, DQBI*0603 (DR2, DQw6) haplotype, which is protective for IDDM (36). The DR3, DQw2 IDDM-associated haplotype is particularly abundant, whereas the DR4, DQw8 IDDM-associated haplotype is rare in the pattern I SMS patients (Lennon V., S. Baekkeskov, T. Bugawan, and H. Erlich, manuscript in preparation).

**Table 4. DR and DQ Haplotypes of SMS Patients**

| SMS patient no. | DRBI   | DQBI   | Western blot pattern |
|-----------------|--------|--------|----------------------|
| 1               | 0701, 1302 | 0201, 0604 | I                    |
| 2               | 0301, 0301 | 0201, 0201 | I                    |
| 3               | 1601, 1401 | 0502, 0503 | II                   |
| 4               | 1501, 1301 | 0603, 0602/03 | II                   |
| 6               | 0301, 0901 | 0201, 0303 | II                   |
| 7               | 0301, 0401 | 0201, 0301/02 | I                    |
| 8               | 0404, 1103 | 0302, 0301 | I                    |
| 11              | 0301, 0301 | 0201, 0201 | I                    |
| 14              | 0301, 0401 | 0201, 0302 | I                    |
| 15              | 0301, 0400 | 0201, 0301 | I                    |
| 19              | 0301, 0301 | 0201, 0201 | I                    |
| 30              | 0301, 0301 | 0201, 0201 | I                    |

**Figure 4.** SMS sera recognize an epitope located in the first eight NH2-terminal amino acids. Full-length rat GAD65 and short deletion mutants lacking 8 and 15 amino acids, respectively, were subjected to SDS-PAGE, blotted to PVDF membranes, and immunostained with SMS sera at the following dilutions: SMS 1, 7, 9, 12, 30 at 1:500 and SMS 22 at 1:120. All the sera show identical signal or lack of signal with the 1–8 and 1–15 deletion mutants suggesting that they react equally well with both mutants and that the NH2-terminal epitope resides in the first eight residues, whereas residues 9–15 do not contribute to the epitope.
epitope further downstream in denatured rat GAD6s on Western blots. The difference between our results and those of Butler et al. (24) is not readily explained. Several possibilities can be suggested. First, we have analyzed all SMS patients at the Mayo Clinic and UCSF over the last 5 yr. The study by Butler et al. (24) used sera from 30 patients which were selected from a cohort of 72 SMS patients by unknown criteria; the selection procedure may have favored pattern II sera. Second, their immunoblotting protocol may enhance renaturation of the amino acid 475–585 epitope on Western blots. A third possibility is that the coupling of the 475–585 fragment to a maltose binding protein enhances its refolding on nitrocellulose.

In addition to linear epitopes associated with SMS and IDDM respectively (Fig. 5), Ujihara et al. (36) have identified a third linear epitope in residues 421–442 in the GAD6s protein. This epitope is recognized by IgG in a rare group of IDDM relatives that develop high titers of GAD6s autoantibodies yet carry the HLA-DRB1*1501, DQB1*0603 (DR2, DQw6) haplotype which is protective for diabetes. Interestingly, one of the three SMS patients of pattern II who was HLA-haplotyped carried this haplotype. We speculate that additional pattern II patients, amongst whom IDDM was rare (20%) compared with pattern I patients (44%), may carry alleles that are protective for IDDM and that pattern II may in some cases include recognition of the epitope described by Ujihara et al. (36). In comparison, none of the 9 pattern I patients who were HLA-haplotyped were DR2, DQw6 positive. Rather they all carried one or two IDDM susceptibility alleles and the DR3, DQw2 allele was particularly abundant (7/9).

The NH2-terminal linear epitope identified in our study is identical in rat and human GAD6s, which overall share >95% sequence homology (17). Although it has been reported that SMS sera recognize rat and human GAD6s equally well (24), our preliminary results suggest that the downstream epitope(s) may differ between the rat and human autoantigen. Thus a 58-kD tryptic fragment of the human protein is recognized more strongly on Western blots by some pattern IB and pattern II sera than its rat counterpart. Generation of mutants of the human protein and expression in cellular expression systems will be important for characterizing the SMS epitope(s) contained in the 58-kD tryptic fragment.

**SMS Sera Have an Increased GAD Epitope Recognition Compared with IDDM Sera.** Anti-GAD6s autoantibodies differ significantly in SMS and IDDM patients with regard to titers measured by immunoprecipitation. Titers of autoantibodies associated with SMS are orders of magnitude higher than in IDDM sera. Thus SMS is characterized by a much stronger humoral autoimmune response to GAD6s than is IDDM.

Is the strong antibody response to GAD6s in SMS a result of (a) an increased humoral epitope repertoire in this disease compared with IDDM; (b) an enhanced immune response to certain epitopes, or (c) a combination of both? Since no monoclonal autoantibodies are yet available from SMS patients, this question is difficult to address directly. However, considerable indirect evidence suggests that the humoral response in SMS sera is more broad than in IDDM. By immunoprecipitation assay, both SMS and IDDM sera recognize...
both in immunoprecipitation and Western blot assays. Our clinical diagnostic criteria similar to those of the Mayo Clinic Woltman (38). Two patients were diagnosed at UCSF using diagnosed at the Mayo Clinic in Rochester, where the dis-

It was suggested that SMS patients can be divided into two groups based on whether they have GAD autoantibodies or not (13). Most of the patients analyzed in our study were diagnosed at the Mayo Clinic in Rochester, where the disease was discovered and first described by Moersch and Woltman (38). Two patients were diagnosed at UCSF using clinical diagnostic criteria similar to those of the Mayo Clinic (14). All 35 patients were positive for GAD65 autoantibodies both in immunoprecipitation and Western blot assays. Our results suggest that a diagnosis of SMS using standardized clinical criteria identifies a uniform group of patients with strong humoral autoimmunity to GAD65. A SMS-like disorder associated with breast carcinoma was, however, recently described in four patients who were seronegative for anti-GAD65 antibodies but positive for autoantibodies to a second synaptic vesicle protein, amphiphysin (39). Thus anti-GAD65 autoantibodies may only be a universal characteristic of SMS in the absence of a paraneoplasia.

Role of B Cells and GAD65 Autoantibodies in IDDM and SMS. In spontaneous rodent models of IDDM, disease can be transferred by T cells but not B cells (10) and immuno-

electronmicroscopic studies suggest that β cell destruction is mediated by cytotoxic T cells (40). The primary Th responses to GAD65 in the nonobese diabetic mouse are of the Th1 subset (12), which is consistent with induction of cytotoxic T cell responses. Although autoantibodies to GAD65 have not been implicated as effectors of β cell destruction, it is conceivable that B cells, expressing GAD65-specific IgG on the surface, play an important role in antigen presenta-

in IDD. Activated B cells are particularly efficient presenters of low abundance antigens by virtue of antigen capture by their surface immunoglobulins (41) and may well be the principal antigen presenting cells that can maintain chronic autoimmunity to a rare antigen like GAD65 over the several years of progressive β cell destruction. The antibody binding to an antigen can influence its processing in endosomal compartments, and consequently the presentation of epitopes to T cells. Thus, antibody binding can suppress some epitopes while boosting others (42). The identification of humoral autoimmune epitopes in the GAD65 molecule which are associated with either IDDM susceptibility (22) or protec-

tion (36) may provide an example of how specificity of autoantibodies can influence autoimmune T cell responses resulting in either β cell destruction and disease, or absence of disease.

Do autoantibodies to GAD65 have a pathogenic role in SMS? Although the strong humoral autoimmune GAD65 responses in SMS may suggest a more direct role of autoanti-

bodies in impairment of GABA-ergic neurons than in de-

struction of β cells, the pathogenicity of SMS antibodies re-

mains to be shown. It is noteworthy that in the absence of MHC class I expression (18) autoimmune effector mech-

nisms directed at neurons would be limited to antibody re-

sponses. Furthermore, an involvement of a Th2 subset of CD4+ cells and strong antibody responses to GAD65 in the pathogenicity of SMS, in contrast to a requirement for a Th1 subset and cytotoxic T cell responses for β cell destruction, is consistent with the dichotomy of the two diseases. Thus SMS without IDDM would ensue in the absence of Th1 helper cell and cytotoxic T cell responses to GAD65.

An autoantibody-mediated impairment of GABA-ergic neurons would, however, require antigen exposure at the cell surface. Although there is some evidence to suggest that GAD can appear on the surface of neurons (43), the mechanisms of such exposure are unclear. GAD65 is believed to anchor at the cytosolic face of synaptic vesicle membranes and sur-

face expression during exocytosis of GABA would require a membrane translocation step. The identification of am-

phiphysin as a target of autoantibodies in a paraneoplastic SMS-like syndrome (39) does suggest that a localization of a protein to the cytosolic face of synaptic vesicle membranes can predispose it for autoantigenicity and that such proteins may reach the cell surface by an as yet unknown mechanism. Alternatively, SMS autoantibodies may not be involved in pathogenicity but rather be only a marker of disease.
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We are grateful to Dr. K. M. McEvoy and colleagues in the Mayo Clinic's Movement Disorder Clinic for their cooperation in identifying SMS patients; Dr. R. Layzer (Department of Neurology) and K. Fye (Department of Medicine) from the University of California, San Francisco, for providing SMS patient material and for valuable discussions; and Dr. A. Tobin (University of California, Los Angeles) and Dr. D. Gottlieb (Washington University, St. Louis, MO) for donation of research materials.

This study was supported by grants from the National Institutes of Health (NIH) (DK-41822-01 and CA-37343). M. Jaffe was supported by a NIH training grant in endocrinology; Y. Shi was supported by a Juvenile Diabetes Foundation fellowship; and H. J. Aanstoot by the Ter Meulen Foundation and by an Albert Renold Fellowship of the European Association for Study of Diabetes.

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Received for publication 3 February 1994 and in revised form 6 May 1994.

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