Identification of a Dominant Epitope of Glutamic Acid Decarboxylase (GAD-65) Recognized by Autoantibodies in Stiff-Man Syndrome

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Summary

Glutamic acid decarboxylase (GAD) is the enzyme that synthesizes the neurotransmitter γ-aminobutyric acid (GABA) in neurons and in pancreatic β cells. It is a major target of autoimmunity in Stiff-Man syndrome (SMS), a rare neurological disease, and in insulin-dependent diabetes mellitus. The two GAD isoforms, GAD-65 and GAD-67, are the products of two different genes. GAD-67 and GAD-65 are very similar to each other in amino acid sequence and differ substantially only at their NH_2-terminal region. We have investigated the reactivity of autoantibodies of 30 Stiff-Man syndrome patients to GAD. All patient sera contained antibodies that recognize strongly GAD-65, but also GAD-67, when tested by immunoprecipitation on brain extracts and by immunoprecipitation or immunocytochemistry on cells transfected with either the GAD-65 or the GAD-67 gene. When tested by Western blotting, all patient sera selectively recognized GAD-65. Western blot analysis of deletion mutants of GAD-65 demonstrated that autoantibodies are directed predominantly against two regions of the GAD-65 molecule. All SMS sera strongly recognized a fragment contained between amino acid 475 and the COOH terminus (amino acid 585). Within this region, amino acids 475–484 and 571–585 were required for reactivity. The requirement of these two discontinuous segments implies that the epitope is influenced by conformation. This reactivity is similar to that displayed by the monoclonal antibody GAD 6, suggesting the presence of a single immunodominant epitope (SMS-E1) in this region of GAD-65. In addition, most SMS sera recognized at least one epitope (SMS-E2) in the NH_2-terminal domain of GAD-65 (amino acids 1–95). The demonstration in SMS patients of a strikingly homogeneous humoral autoimmune response against GAD and the identification of dominant autoreactive target regions may help to elucidate the molecular mechanisms of GAD processing and presentation involved in GAD autoimmunity. Moreover, the reactivity reported here of GAD autoantibodies in SMS partially differs from the reactivity of GAD autoantibodies in insulin-dependent diabetes mellitus, suggesting a link between the pattern of humoral autoimmunity and the clinical condition.

The enzyme glutamic acid decarboxylase (GAD)[1] catalyzes the conversion of L-glutamic acid to γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter (1). GAD is expressed in GABA-secreting neurons and in pancreatic β cells (2, 3), which may also secrete GABA as a paracrine signal molecule (4–6). In addition, GAD is expressed in the testis and in the epithelium of the fallopian tube (7, 8). GAD has been identified as a dominant autoantigen in two human diseases, Stiff-Man syndrome (SMS) (9, 10, 11) and insulin-dependent diabetes mellitus (IDDM) (12). SMS is a rare neurological condition characterized by progressive rigidity of the body musculature with superimposed painful spasms. It is thought to result from an impairment of GABA-ergic inhibitory inputs to α-motor neurons (13–15). Autoantibodies directed against GAD (GAD-Abs) were detected in 72 of 119 SMS patients we studied. A frequent occurrence of IDDM was observed in the GAD-Abs-positive SMS patients (10), an observation that led to the identification of the 64-kD antigen of IDDM as GAD (12). IDDM results from an autoimmune destruction of pancreatic β cells (16). GAD-Abs are present in the large majority of IDDM patients at the time of the diagnosis and are also present in a large fraction of prediabetic patients (17, 18).

GAD exists as two isoforms of 67 and 65 kD, respectively, commonly referred to as GAD-67 and GAD-65. The two

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes; Pt, patients; SMS, Stiff-Man syndrome; SMS-E1, Stiff-Man syndrome epitope 1.

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proteins are the products of two different genes that have been cloned and sequenced from several species (19–25). Both proteins are expressed in the brain while their expression in β cells varies among species (26, 27). GAD-67 and GAD-65 are highly conserved in evolution (97% identity between rat and human GAD-67; 96% identity between rat and human GAD-65) and are very similar to each other. They differ substantially only in the first 95 NH_2-terminal amino acids ($\sim$23% identity, $\sim$61% similarity), which are responsible for the different subcellular localization of the two molecules (28, 29). Over the remaining part of the molecule, which contains the catalytic portion of the enzyme, they are $\sim$73% identical and $\sim$95% similar.

Both autoantibodies of IDDM patients and of SMS patients were found to immunoprecipitate GAD-65 from pancreatic or brain extracts prepared in nondenaturing conditions (12). However important differences were observed between GAD-Abs in SMS and IDDM patients. In SMS, GAD-Abs have a much higher titer than in IDDM. In addition, GAD-Abs of most SMS patients react with GAD in Western blots, while GAD-Abs of the majority of IDDM patients do not (12). Elucidating the autoreactive epitopes of GAD-Abs in the two conditions represents a first step towards the elucidation of molecular mechanisms of GAD autoimmunity and may be useful for the design of optimal diagnostic assays for the two diseases.

In this study we have investigated the properties of GAD-Abs in a large group of SMS patients. An analysis was performed on 30 of the 72 GAD-Abs positive SMS sera of our case-load, all of which were GAD reactive in Western blots. All sera contained antibodies that independently recognized both GAD-65 and GAD-67 by immunoprecipitation. However, in Western blots all sera only reacted with GAD-65. This reactivity was accounted for primarily by antibodies directed against two regions of the GAD-65 molecule. One (SMS-Epitope-1 [SMS-E1]), encompassing the COOH-terminal 110 amino acids represents the dominant autoantigenic region. The other (SMS-E2) corresponds to the NH_2-terminal 95 amino acids, i.e., the region where GAD-65 differs substantially from GAD-67.

Materials and Methods

Antibodies and Synthetic Peptides. Human sera of SMS patients were from our collection of 72 SMS sera positive for GAD-Abs (9–11). Control human sera were from healthy subjects. Rabbit sera 7673 (raised against a peptide corresponding to the sequence 577–593 of rat GAD-67) and N65 (raised against peptide corresponding to the sequence 4–21 of rat GAD-65) were previously described (29). Rabbit serum 9056 was raised against recombinant mouse GAD-67 (clone 3b-3; kindly provided by Drs. Z. Katarova and G. Szabo, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary) (24). Rabbit serum 9057 was raised against rat brain GAD-65 obtained by affinity purification and preparative electrophoresis. Rabbit serum K2 (30) was purchased from Chemicon Intl., Inc. (Temecula, CA). The following antibodies were generous gifts: mAb GAD 6 (from Dr. D. Gottlieb, Washington University School of Medicine, St. Louis, MO) (31) and rabbit serum 6799 (from Drs. Z. Katarova and G. Szabo) (24). Synthetic peptides were synthesized at the Yale Protein Chemistry Facility (New Haven, CT).

Purification of Rat Brain GAD. GAD was purified from rat brain using a GAD 6 affinity column as previously described (31) with some modifications. Approximately 100 g of rat brains (Pel-Freeze Biologicals, Rogers, AZ) were homogenized in 600 ml of 10-mM potassium phosphate buffer, pH 7.5, 0.2-mM EDTA, 1-mM 2-aminoethylisothiouronium bromide (AET), 0.2-mM pyridoxal 5-phosphate (PLP), pepstatin, aprotinin, leupeptin, antipain (each at 4 μg/ml; Sigma Chemical Co., St. Louis, MO), and 0.4 μM PMSF (buffer A) using an SDT Tissumizer (Tekmar Co., Cincinnati, OH) at maximum speed and clarified by centrifugation at 130,000 *g* for 1 h at 4°C. The supernatant was loaded on an affinity column prepared by conjugating the antibody GAD 6 to Affi-gel 10 (Bio-Rad Laboratories, Richmond, CA). The column was extensively washed with buffer A without EDTA (buffer B), and subsequently with 1.0 M NaCl in buffer B. GAD was eluted with high pH buffer (50 mM potassium phosphate, 10 mM diethylamine, and 20 mM glutamic acid adjusted to pH 11), neutralized, dialyzed against 2 mM Hepes, pH 7.4, at 4°C, concentrated by lyophilization, resuspended in SDS-PAGE sample buffer, and run on an 8% polyacrylamide preparative gel. The GAD-65 and GAD-67 bands, visualized by Coomassie blue staining, were separately excised from the gel and electroeluted with an Elutrap device (Schleicher and Schuell, Inc., Keene, NH).

GAD-65 and GAD-67 Fusion Proteins. A full-length rat brain GAD-65 cDNA clone was isolated as previously described (29). A full-length rat brain GAD-67 cDNA clone was a kind gift of Dr. D. Gottlieb (21). cDNAs encoding full-length GAD-67, full-length GAD-65, as well as GAD-65 fragments were subcloned into the bacterial expression vector, pmalc2 (New England Biolabs, Beverly, MA), by using synthetic oligonucleotide primers in PCR reactions. A specific restriction enzyme site was introduced into each primer to facilitate subcloning into the polylinker region of pmalc2. EcoRI and HindIII sites were added, respectively, to 5′ and 3′ primers of GAD-65, and XbaI and HindIII sites to 5′ and 3′ primers of GAD-67. Stop codons were engineered in the 3′ primers for those constructs missing the COOH terminus. Constructs yielded a recombinant protein composed of the maltose binding protein ($\sim$42,000 daltons) fused to the NH_2 terminus of GAD or GAD fragment. Isopropyl β-D-thiogalactopyranoside (IPTG)-induced *Escherichia coli* expressing the fusion protein were resuspended in SDS-PAGE sample buffer and used for Western blot analysis. In these lysates, the fusion protein represented a major protein band whose identity was confirmed by Western blotting, using an antiserum directed against the maltose binding protein (New England Biolabs). The electrophoretic mobilities of the fusion proteins approximately coincided with the expected mobilities based on the estimated molecular mass. However, because mobility on SDS gels does not correlate absolutely with molecular mass, constructs that proved critical in the definition of the epitopes were sequenced. These were H, D, E, F4, F5, and F14.

SDS-PAGE and Immunoblotting. SDS-PAGE and Western blotting were performed essentially as described by Laemmli (32) and by Towbin et al. (33). All protein samples were boiled 3 min in SDS-PAGE sample buffer in the presence of 20 mM dithiothreitol before loading on gels. Nitrocellulose blots were incubated in blocking solution of 20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (vol/vol) Tween-20, and 5% (wt/vol) dry milk (TBST/milk). All antibody dilutions, subsequent incubations and washes were performed in TBST/milk at room temperature. Primary antibodies to be used for immunoblotting of bacterial lysates were preadsorbed with a lysate of the host bacteria not expressing the fusion protein. Primary antibodies were used at the following final dilutions: GAD 6, 6799 and patient sera at 1:500; 7673 at 1:250; K2 and N65 at 1:100. SMS sera and GAD 6 were followed by rabbit anti-human

or rabbit anti-mouse IgG (1:1,000 dilution), respectively. Bound antibodies were detected by ^{125}I protein A (10^5 cpm/ml). For quantitation of the integrated optical density of individual autoradiographic bands, autoradiograms were scanned using the Bio Image program on a Visage 2000 scanner (Millipore Corp., Bedford, MA).

Miscellaneous Procedures. The preparation of Chinese hamster ovary (CHO) cells that stably express GAD-65 or GAD-67 was previously reported (29). For SDS-PAGE of these cells, SDS-PAGE sample buffer was added directly to the incubation flasks. Preparation of Triton X-100 extracts of rat brain and of CHO cells, as well as immunoprecipitation from such extracts, were performed as described (5, 29). In some cases, proteins from rat brain were denatured by SDS and boiling before being used for immunoprecipitation. The postnuclear supernatant (5) was solubilized in 1% SDS or in 1% SDS, 25 mM dithiothreitol, 5 mM iodoacetamide, and boiled for 3 min. A fivefold (wt/vol) excess of Triton X-100 was then added. Subsequently, the final concentration of this nonionic detergent was brought to 1% by dilution with 10 mM Hepes, pH 7.4, 150 mM NaCl, and protease inhibitors, and immunoprecipitation was then performed as described (5). Double-immunofluorescence was performed as described (34). Protein concentration was determined by the BCA reagent (Pierce, Rockford, IL).

Results

GAD-Abs of SMS Patients Recognize Selectively GAD-65 in Western Blots. The reactivity of SMS sera was first tested by Western blotting on full-length GAD using both purified GAD from rat brain, as well as recombinant rat GAD. We had previously shown that SMS sera react similarly with rat and human GAD in Western blots (9, 10). Rat brain GAD was purified by affinity chromatography using the mAb GAD 6. As previously reported (31), this procedure results in the purification of both GAD-65 and GAD-67 (see also Fig. 1). To facilitate the analysis of the reactivity of the two isoforms the purified material was run on a preparative SDS polyacrylamide gel and the gel regions corresponding to GAD-65 and GAD-67 were separately excised and electroeluted. Protein staining of electrophoretically-purified GAD-65 and GAD-67 is shown in Fig. 1 *A*. Nitrocellulose strips containing samples identical to those shown in Fig. 1 *A* were immunoblotted with several anti-GAD animal sera (Fig. 1 *B*) and SMS sera (Fig. 1 *C*).

Rabbit serum 7673, which was raised against the 17 COOH-terminal amino acids of rat GAD-67, recognized both GAD isoforms (Fig. 1 *B*). GAD-65 differs from GAD-67 in this region by a single amino acid (serine 577 of GAD-67 is replaced by a glutamine in GAD-65). Rabbit sera 6799 and K2, which were raised to recombinant GAD-67 from mouse and cat, respectively (24, 30), recognized primarily, but not exclusively, GAD-67 (Fig. 1 *B*). The mAb GAD 6 (Fig. 1 *B*) and all 30 SMS sera only reacted with GAD-65 (Fig. 1 *C* and not shown).

Identical results were obtained when the same animal antibodies and SMS sera were reacted by Western blotting with full-length GAD-65 and GAD-67 expressed as fusion proteins in bacteria (Fig. 2) or independently in CHO cells (not shown).

GAD-Abs of SMS Patients Recognize Both GAD-67 and GAD-65 by Immunoprecipitation and Immunocytochemistry. All SMS sera immunoprecipitated both GAD-67 and GAD-65 from Triton X-100 brain extracts prepared in nondenaturing conditions (Fig. 3 *A*). For comparison, immunoprecipitates obtained with several anti-GAD and control animal antibodies are shown in Fig. 3 *B*. These findings confirmed our previous

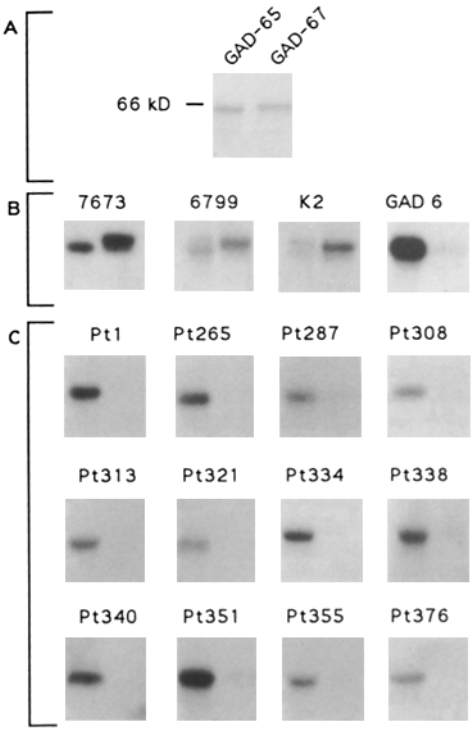


Figure 1. SMS patient sera recognize selectively GAD-65 in Western blots of brain GAD. Coomassie blue staining of GAD-65 and GAD-67 purified by affinity chromatography and preparative electrophoresis from brain (*A*). Western blot of nitrocellulose strips identical to those shown in *A* with rabbit sera 7673, 6799, K2, and mAb GAD 6 (*B*). Western blot of strips identical to those shown in *A* with sera of SMS patients (Pt, followed by patient code number) (*C*). A slight cross-contamination of the two isoforms is not visible by protein staining but is apparent from the Western blots shown in *B* and *C*.

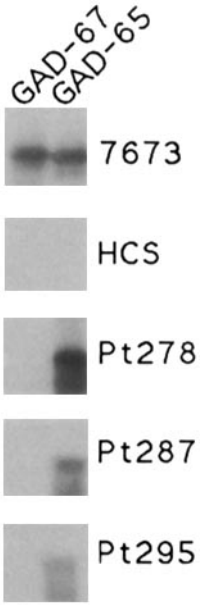


Figure 2. SMS patient sera recognizes selectively recombinant GAD-65 in Western blots. Bacterial lysates expressing full-length GAD-65 and GAD-67 were reacted by Western blotting with rabbit serum 7673, a human control serum (HCS), and three sera of SMS patients (Pt, followed by patient code number).

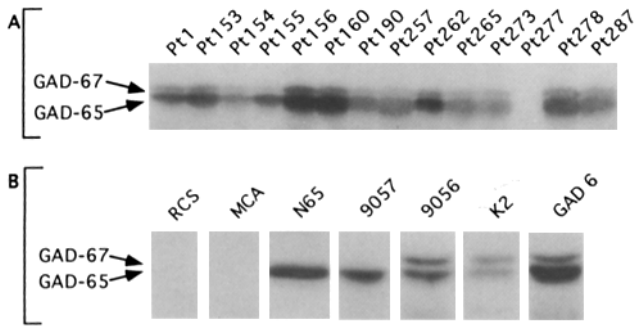


Figure 3. SMS patient sera immunoprecipitate both GAD 65 and GAD 67 from a brain extract. Triton X-100 extracts of rat brain were immunoprecipitated with sera of SMS patients (Pt followed by patient code numbers) (*A*) and several anti-GAD animal antibodies (*B*). GAD present in the immunoprecipitates was revealed by immunoblotting with serum 7673, which reacts with both GAD isoforms. Rabbit sera N65 and 9057 immunoprecipate selectively GAD-65; rabbit sera K2, and 9056 as well as mAb GAD 6 immunoprecipitate both GAD isoforms but in different relative ratios. RCS, rabbit control serum; MCA, mouse control ascites. Note the similar ratio of GAD-67 to GAD-65 immunoprecipitated by all SMS sera. Pt227 is a control patient negative for GAD-Abs.

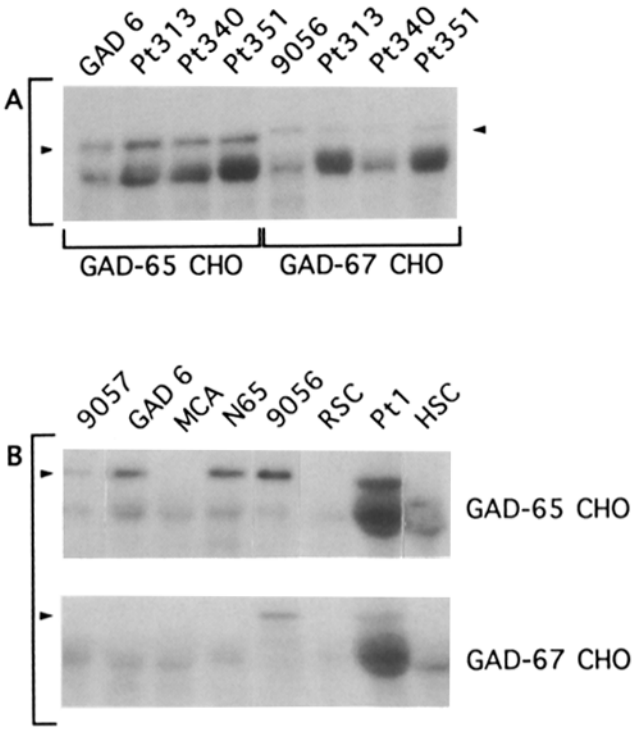


Figure 4. SMS patient sera immunoprecipitate both GAD-65 and GAD-67 from CHO cells independently transfected with the two GAD isoforms. Triton X-100 extracts of cells that stably express either GAD-65 or GAD-67 were immunoprecipitated with SMS sera and anti-GAD animal antibodies. GAD present in the immunoprecipitates was revealed by immunoblotting with rabbit serum 7673, which recognizes both GAD isoforms. The GAD bands are indicated by arrowheads. The heavy band just below GAD in each panel represents the heavy chain of IgG. Antibody and patients codes are indicated at the top of each lane.

immunoprecipitation results obtained with a small group of SMS sera (12). The discrepancy between the reactivity displayed by SMS sera in Western blots and immunoprecipitations may have at least two explanations. One is that SMS sera contain antibodies that recognize both proteins in their native state. The other is that SMS sera contain only antibodies directed against GAD-65, but immunoprecipitate GAD-67 because the two proteins form heterodimers in the brain and in the pancreas. On the other hand, two rabbit sera, N65 and 9057, can immunoprecipitate selectively GAD-65 (Fig. 3 *B*). N65 was raised against a GAD-65–specific sequence (amino acids 4–21), and 9057 was raised against purified rat brain GAD-65. Note that two rabbit sera (9056 and K2) raised against GAD-67 immunoprecipitated both isoforms but with an higher relative ratio GAD-67/GAD-65 compared with SMS sera and to GAD6.

Establishing whether autoantibodies recognize GAD-67 as well as GAD-65 is of considerable interest for the elucidation of mechanisms of autoimmunity. Therefore we addressed this question in more detail. SMS sera were tested for their capacity to recognize in immunoprecipitation experiments GAD-65 or GAD-67 independently expressed by transfection in CHO cells. Fig. 4 shows results of immunoprecipitation performed on Triton X-100 extracts of CHO cells expressing either GAD-65 (GAD-65–CHO cells) or GAD-67 (GAD-67–CHO cells) with animal and human sera. In each gel, the lower band represents immunoglobulin heavy chain. The ability of four SMS sera to immunoprecipitate both proteins is shown by the figure. Identical results were obtained with all the other SMS sera tested. Therefore, SMS sera do recognize both GAD-65 and GAD-67 and their failure to react with GAD-67 in Western blots may be due to irreversible loss of GAD 67 epitope(s) by SDS-induced denaturation. In fact, SMS sera immunoprecipitated only GAD-65 when tested against brain extracts that had been denatured by SDS and boiled before immunoprecipitation (not shown).

Immunoprecipitates obtained with several animal anti-GAD antibodies are shown in Fig. 4 *B* as controls. Rabbit serum 9056 immunoprecipitated both GAD-67 and GAD-65. Similar results were obtained with serum K2 (not shown). However, rabbit sera 9057 and N65 as well as mAb GAD 6 immunoprecipitated GAD only from GAD-65–CHO cells, confirming their specificity for GAD-65. The capacity of GAD 6 to immunoprecipitate both GAD isoforms from brain Triton X-100 extracts supports the concept that GAD-65/GAD-67 heterodimers can be precipitated by GAD-65–specific sera. When GAD 6 was tested against brain extracts pretreated with SDS and boiling, only GAD-65 was immunoprecipitated (not shown). A possible explanation for the lack of GAD-67 in immunoprecipitates produced by sera N65 and 9057 (Fig. 3 *B*) is that the epitope(s) recognized by these antibodies is (are) hidden in the heterodimer. As discussed below, serum 9057 reacts selectively with the region of GAD-65 that contains the peptide recognized by N65.

Immunocytochemistry of formaldehyde-fixed transfected CHO cells with the same set of human and animal antibodies confirmed immunoprecipitation results. SMS sera stained both GAD-67– and GAD-65–CHO cells, and produced the staining pattern expected (29) for the two GAD isoforms (Fig. 5), i.e., a diffuse and a particulate pattern respectively. Sera N65 and 9057 as well as mAb GAD 6 stained only GAD-65–CHO

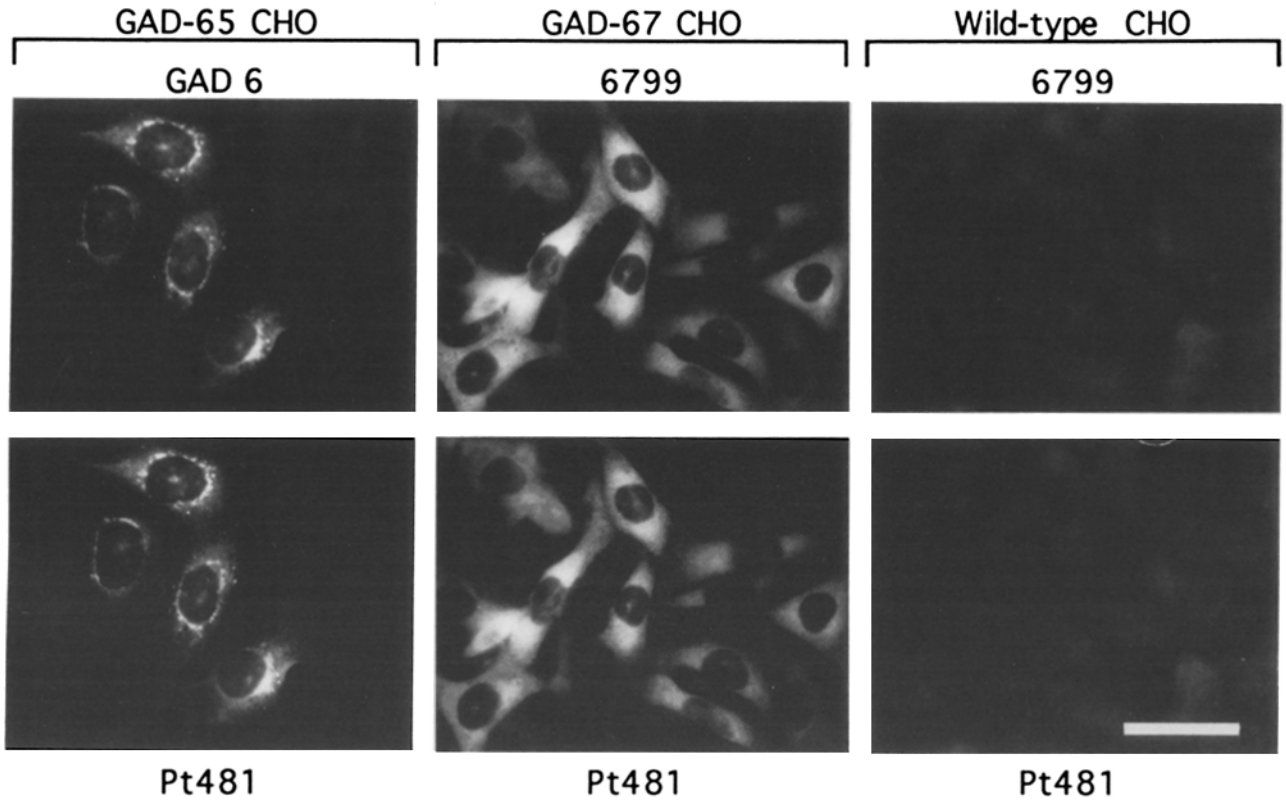


Figure 5. SMS sera stain both GAD 65 and GAD 67 in formaldehyde-fixed transfected CHO cells. GAD-65 and GAD-67 CHO cells, as well as wild type CHO cells, were stained by immunofluorescence with the serum of SMS patient 481 and counterstained with mAb GAD 6 and rabbit serum 6799 (which recognizes both isoforms). (*Calibration bar*, 31 μm).

cells, while sera 9056 and K2 stained GAD-67 CHO cells more intensely than GAD-65–CHO cells (not shown).

Identification of Two Dominant Autoreactive Regions in GAD-65. The property of GAD-Abs to react with GAD-65 in Western blots is a characteristic of the large majority of SMS sera, but is only seldomly observed in IDDM sera (12, 35, 36). It was of interest to identify the GAD-65 autoepitope(s) responsible for this reactivity. SMS sera were tested by Western blotting against deletion mutants of GAD-65 expressed as fusion proteins in bacteria. Initial experiments carried out with a few SMS sera on a variety of GAD-65 fragments in which progressively longer portions of the NH_2-terminal region had been deleted, demonstrated that autoantibodies of different patients had similar patterns of reactivity and were primarily directed against the COOH-terminal region. Therefore immunoreactivity of the COOH-terminal region was mapped further.

Several deletion mutants of GAD-65 were constructed as fusion proteins (Figs. 6 *D* and 7 *D*, and not shown), and lysates containing approximately equal amount of fusion proteins (Figs. 6 *A* and 7 *A*) were analyzed by Western blots with animal GAD antibodies (Figs. 6 *B* and 7 *B*) and with all 30 human SMS sera (Figs. 6 *C* and 7 *C*, Table 1, and not shown).

As expected, rabbit serum 7673 directed against the COOH terminus of both GAD-67 and GAD-65 labeled all GAD-65 fragments including the COOH terminus of the protein, confirming the identity of the constructs (Figs. 6 *B* and 7 *B*). Deletion of the last four amino acids was sufficient to greatly reduce labeling by this serum. All SMS sera reacted very strongly with a fragment (fragment F), composed of the region between amino acid 445 and the COOH terminus (amino acid 585) (Fig. 6 *C*). Reaction with this fragment appeared to be even stronger than with full-length GAD-65, as if the NH_2-terminal region of the molecule interfered with GAD-Ab binding to this GAD-65 region (Fig. 7 *C*). Progressive deletions of up to 30 amino acids residues from the NH_2-terminal side of this fragment (fragments F2, F3, and F4) did not significantly change its reactivity (Figs. 6 *C* and 7 *C*). An additional 10–amino acid deletion at the NH_2 terminus of F4 (amino acids 475–585) to create fragment F5 (amino acids 484–585) drastically reduced immunoreactivity (Figs. 6 *C* and 7 *C*). This suggested that the epitope lies between amino acids 475 and 484.

However, quite unexpectedly, fragment F1 (amino acids 445–515) resulting from a COOH-terminal deletion of fragment F, was also unreactive (Fig. 6 *C*). A similar reactivity towards fragment F4, but not towards fragments F5 and F1, was observed with the mAb GAD 6 (Figs. 6 *B* and 7 *B*). Further deletion analysis of the COOH-terminal side of fragment F4 (Fig. 7 *C*) indicated that removal of the last 14 amino acids (fragment F12; amino acids 475–571) was sufficient to abolish reactivity with all SMS sera and with GAD 6. Moreover, deletion of only the last four amino acids (frag-

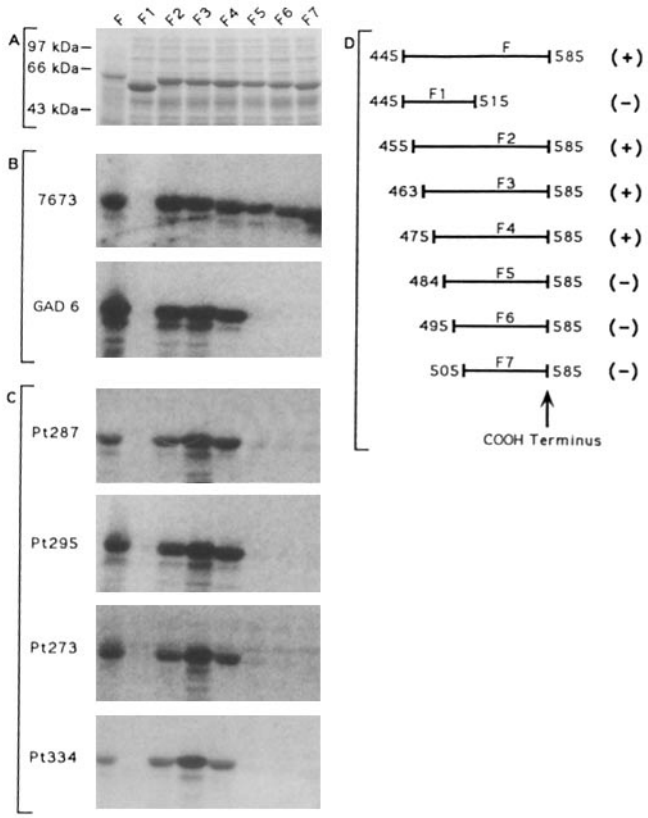


Figure 6. Identification of the COOH-terminal GAD epitope in SMS sera. Deletion mutants of GAD-65 were constructed as fusion proteins and lysates containing approximately equal amounts of fusion proteins were analyzed by SDS-PAGE and Western blotting. The boundaries of the fragments are shown schematically in *D*. Numbers represent amino acid position in GAD-65. (*A*) Protein staining of bacterial lysates. Note that the fusion proteins appear as major protein bands. (*B*) and (*C*) Immunoblots of gels identical to those in *A*. Blots were incubated with serum 7673, with mAb GAD 6 (*A*), and with several SMS patient sera (*C*).

ment F14, amino acids 475–581) produced a major loss of reactivity in the case of 14 SMS sera and GAD 6, as well as a complete loss of reactivity in the other 16 SMS sera (see Fig. 7 and Table 1). Reactivity of fragment F14 was similar to that of fragment M (amino acids 190–581), and reactivity of fragment F12 was comparable to that of fragment N (amino acids 190–571), demonstrating that most of the immunoreactivity present in the COOH-terminal two-thirds of the molecule is attributable to an epitope contained in fragment F4 (henceforth defined as SMS-Epitope 1 = SMS-E1). In only a few patients a weak immunoreactivity was observed in fragment E (amino acids 190–375) which did not contain SMS-E1. In these cases, fragments M and N were slightly more immunoreactive than fragments F12 and F14 (i.e., patient 338 in Fig. 7 *C*). The importance of the COOH terminus of GAD-65 to the reactivity of SMS sera was emphasized by the observation that deletion of the last 14 COOH-terminal amino acids from full-length GAD resulted in a dramatic loss of reactivity of those sera that reacted exclusively or almost exclusively with SMS-E1 (not shown). In summary, SMS-E1 is contained within the COOH-terminal 110 amino acids of GAD-65 and both amino acid segments 475–484 and 571–585 are crucially required for reactivity.

The retention of some reactivity towards COOH-terminal–deleted GAD-65 constructs implied the presence of some immunoreactivity also in the NH_2-terminal region of GAD-65. This was confirmed by analyzing the reactivity of SMS sera with NH_2-terminal GAD-65 fragments (Fig. 7 *C* and Table 1). In all cases intensity of immunoreactivity against fragment D (amino acids 1–190) was very similar to immunoreactivity against fragment H (amino acids 1–95), suggesting that the NH_2-terminal autoantigenic region (SMS-Epitope 2 = SMS-E2) is contained within amino acids 1–95, i.e., the region of diversity between GAD-65 and GAD-67. Rabbit serum N65 (Fig. 7 *B*), raised against an NH_2-terminal GAD-65–specific region (amino acids 4–21), recognized selectively, and with equal intensity, fragments D and H. A similar selective reactivity with fragments D and H was observed with serum 9057 raised against rat brain GAD-65 (not shown) suggesting that this serum as well is primarily directed against its NH_2-terminal 95 amino acids.

Table 1 summarizes the reactivity of the 30 SMS sera against the fragments that define epitopes SMS-E1 and SMS-E2 as determined by scanning of the corresponding autoradiograms. The table clearly shows the predominant reactivity against SMS-E1.

Discussion

In this study we have investigated the antigenic targets of GAD-Abs in SMS. A remarkable degree of similarity in the immune response of these patients was observed. Antibodies recognize both GAD isoforms, but selectively recognize GAD-65 after SDS-induced denaturation. Within GAD-65, two main autoreactive regions which are located in the COOH-terminal and NH_2-terminal regions, respectively, were identified. The COOH-terminal region contains the main autoreactive epitope (SMS-E1) which is recognized by all SMS sera.

The ability of SMS sera to immunoprecipitate GAD-67 in addition to GAD-65 from brain extracts prepared in nondenaturing conditions confirms our previous results (12) and extends them to a large group of SMS sera. In those previous results it could not be discerned whether GAD-67 was coprecipitated with GAD-65 simply due to heterodimer formation (as it is probably the case for the mAb GAD 6 [this study]) or due to the presence of antibodies that directly recognize GAD-67. This study conclusively establishes that both proteins are recognized by SMS GAD-Abs.

The GAD-65 domain, which was recognized in Western blots by all SMS sera tested, and which accounted for most of the immunoreactivity in nearly all of them, was mapped to the COOH-terminal 110 amino acids of GAD-65 (fragment F4), i.e., a region where GAD-65 and GAD-67 are similar to each other (75% identical, 81% similar) (20–23). Short deletions at either the NH_2-terminal or COOH-terminal region of fragment F4 were sufficient to abolish reactivity. Fragment F4 is recognized similarly by the mAb GAD 6 suggesting that GAD 6 and SMS GAD-Abs recognize a single immunodominant epitope (SMS-E1) in this fragment. The observation that COOH-terminal deletions of only 14 amino acids completely abolish reactivity of most SMS

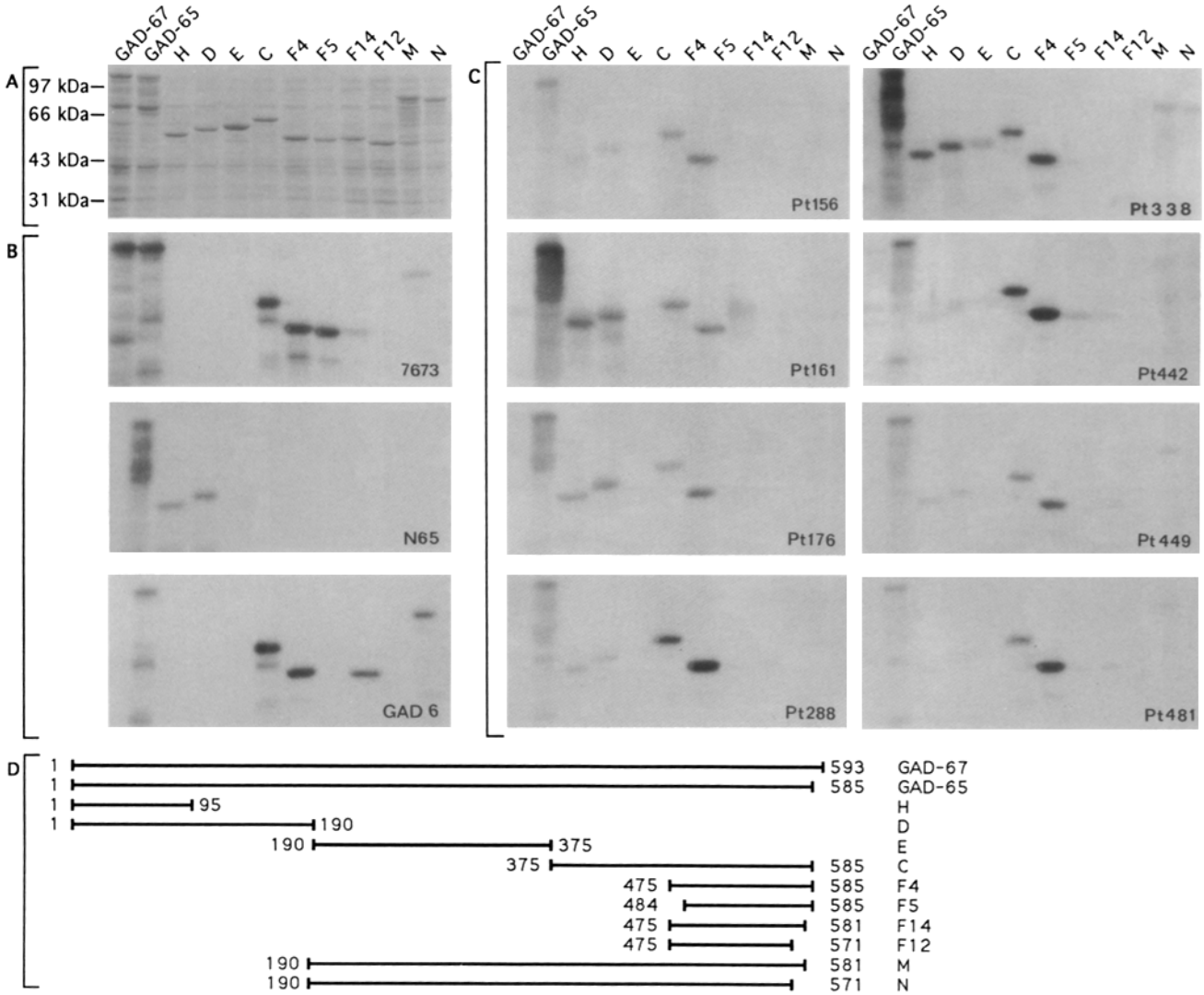


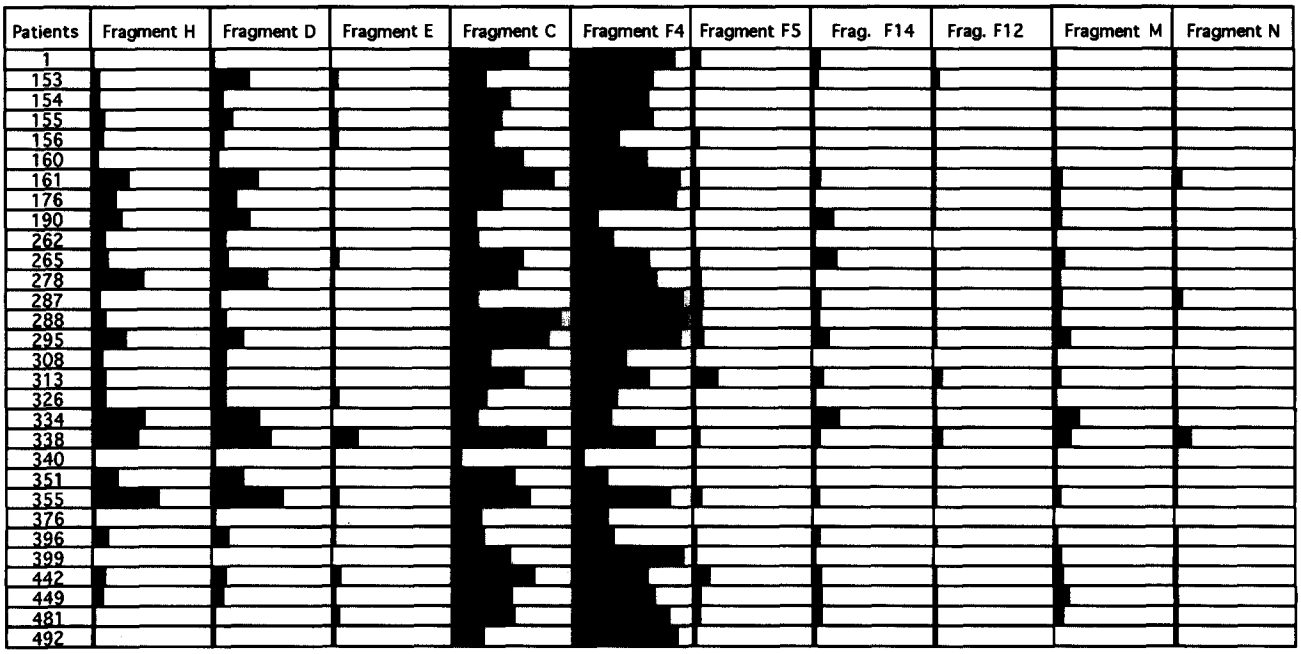
Figure 7. Reactivity of SMS sera with GAD fragments expressed as fusion proteins. (*A*) Protein staining of bacterial lysates expressing the constructs shown in *D*. In all lanes the fusion protein is represented by a major band. *B* and *C* show Western blots of gels identical to that shown in *A* labeled with animal antibodies (*B*) and sera of SMS patients (*C*) as indicated on each gel.

sera, while few of these sera as well as GAD 6 are still partially reactive after COOH-terminal deletions of only four amino acids, suggests some heterogeneity in the binding properties of different antibodies to this epitope. The possibility that our results may be due to abnormal folding of fragment F4 due to its being part of a fusion protein seems unlikely, because COOH-terminal deletions had similar effects on F4, on GAD constructs that included much longer portions of the molecule, and on full-length GAD-65.

The requirement of two amino acid segments that are separated by ~100 amino acids for the reactivity of fragment F4 may have at least two explanations. One is that epitope SMS-E1 is conformational and requires the two amino acid segments to be in close proximity for reactivity with SMS sera. The renaturation of such an epitope so that it would be detected by Western blot is consistent with the partial folding and renaturation of other proteins after SDS-PAGE and transfer to nitrocellulose (37, 38). Alternatively, the antibodies recognize a short linear epitope within fragment F4, which becomes cryptic following short deletions from either side of the fragment. Possibly, either of the two amino acid stretches that are crucially required for the reactivity of fragment F4 (amino acids 475–484 and 571–585) may be part of the epitope. To test this possibility we examined whether reactivity of SMS sera and of the mAb GAD 6 with the epitope SMS-E1 was abolished by their preincubation with synthetic peptides. Neither a peptide represented by amino acids 473–486 of GAD-65, nor the 17-mer peptide used to raise rabbit serum 7673 (which contains amino acids 571–585 of GAD-65), abolished or reduced the reactivity of SMS sera or GAD 6 on immunoblots. As expected, the latter peptide completely abolished reactivity of serum 7673 (not shown). Lack of inhibition was also observed when similar peptide competition experiments were performed by immunocytochemistry on GAD-65–CHO cells and by Western blotting on rat brain, i.e., on full-length GAD-65 (our unpublished observations).

A second autoantigenic region of GAD-65 was mapped to the NH_2-terminal portion of the molecule (epitope SMS-

Table 1. *Reactivity of SMS-Abs with GAD-65 Fragments*



The table was constructed by analyzing autoradiograms like those shown in Fig. 7 *C*. Individual autoradiographic bands corresponding to GAD-65 fragments and full-length GAD-65 were quantified as described in Materials and Methods. The ratio of the OD of each fragment to the OD of full-length GAD-65 was calculated. Each ratio is represented by the filled-in black areas. Thus, the size of the black area directly reflects the reactivity of each GAD fragment. Fragment boundaries are shown in Fig. 7 *D*.

E2), which is quite divergent in amino acid composition from GAD-67 (20–23). Whether SMS-E2 represents the collection of several epitopes remains to be elucidated. In the case of most SMS sera, reactivity with this region is much less intense than reactivity with SMS-E1.

Our study demonstrates some important similarities between the humoral response to GAD in SMS and IDDM. In IDDM as well, GAD-Abs are primarily directed against GAD-65 (35, 36, 39–41) and predominantly against the region of greatest similarity between GAD-65 and GAD-67 (12, 36, 42–45). Moreover, the COOH terminus of GAD-65, which strongly influences SMS reactivity towards GAD, is also critical for reactivity of several IDDM antibodies (36, 46). In spite of these similarities, GAD-Abs found in SMS and IDDM display potentially important differences. GAD-Abs are present in SMS sera with a much higher titer (12). Furthermore, and possibly related to the titer, the majority of IDDM sera are nonreactive, or only weakly reactive, in Western blots and in immunocytochemistry of fixed brain and pancreas (10, 12, 46, 47). Recently, the identification of the autoepitopes recognized in GAD-65 by five human mAbs derived from lymphocytes of one newly diagnosed diabetic patient was reported (36). Three epitopes, which were all localized in the middle and COOH-terminal region, were identified. Two of these epitopes are crucially dependent upon distant regions of the molecule, not reactive in Western blots, and require either the middle and COOH-terminal domain or a middle domain of GAD-65. The reactivity of the human mAbs that recognize these epitopes appeared to reflect the reactivity of several IDDM patients. The third epitope was defined as a linear epitope located close to the COOH terminus. A similar GAD-65 COOH-terminal linear epitope recognized by a few IDDM sera was also reported by Mouch et al. (46). None of these epitopes appear to coincide with the epitope SMS-E1 identified by us.

These differences are not likely to be accounted for by differences between human (36, 46) and rat (this study) GAD, because rat and human GAD react equally well with SMS sera (9, 10) and because the COOH-terminal region of GAD-65 (fragment F4) is almost identical in rat and human (97% identity and 100% similarity). From the data shown by Richter et al. (36) it cannot be excluded that the COOH-terminal linear epitope might coincide with epitope SMS-E1 because this epitope is recognized by Western blot, is contained within amino acids 423–585, and is disrupted by COOH-terminal deletions. However, only a few IDDM antibodies appear to recognize this epitope.

Our findings do not exclude the possibility that sera of SMS patients might contain autoantibodies similar to those present in IDDM in addition to the dominant antibodies investigated in this study. The epitope mapping performed in this study was aimed at characterizing the autoantibodies that react with GAD even after SDS denaturation, i.e., the antibodies characteristic of SMS.

The different reactivity of autoantibodies in IDDM and SMS is intriguing. Such a difference suggests a possible link between epitope specificity of the autoantibodies and clinical condition. The difference in humoral autoimmunity towards

GAD in SMS and IDDM may correlate with the fact that IDDM, when present in SMS patients, has usually a late onset (reference 11 and our unpublished observations). It was reported that the epitope specificity of the antibodies that internalize a protein in an antigen-presenting cell may affect the pattern of proteolysis of the antigen and the nature of fragments presented to helper T cells (48). Whether this phenomenon is relevant for the apparent correlation between antibody response to GAD and clinical symptoms in human patients (SMS and/or IDDM) remains to be determined.

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