A novel antineuronal antibody in stiff-man syndrome

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Article abstract—Two-thirds of stiff-man syndrome (SMS) patients harbor an autoantibody specific for a 64-kD species of glutamic acid decarboxylase (GAD), the rate-limiting enzyme in GABA synthesis. We assayed SMS antisera from two patients with SMS for the presence of anti-GAD antibodies using Western blot, immunohistochemical, and enzymatic analyses. Both SMS antisera recognized an 80-kD antigen present in human and rat neuronal extracts, and failed to recognize the 64-kD GAD species. Immunohistochemistry demonstrated neuronal binding identical to that reported with anti-GAD antibodies. Both sera depleted GAD activity from brain extracts. Our analysis indicates that these SMS antisera differ from previously reported SMS antisera by recognizing a novel 80-kD antigen, and suggests that they contain antibodies directed against either a species of GAD different in size from the 64-kD enzyme, or a protein that co-immunoprecipitates with GAD.

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In 1956, Moersch and Woltman[1] described 14 patients as having "stiff-man syndrome" (SMS), the insidious onset of progressive stiffness of the axial musculature accompanied by intermittent painful muscle spasms and precipitated by startle responses. The first clue to the pathophysiology of the disease was pharmacologic; typical patients had such a remarkable response to benzodiazepines[2] that, without it, the diagnosis was questionable.[3] Since benzodiazepines are GABAergic agonists, and electrophysiologic studies show continuous motor activity suggestive of dysfunction of inhibitory neurons proximal to the anterior horn cell (ie, Ib spinal interneurons or suprasegmental descending inhibitory neurons),[4-6] it has been suggested that SMS is a disorder of central GABAergic pathways.

In 1988, Solimena et al[7] described an autoantibody in an SMS patient that bound to a 64-kD enzyme, glutamic acid decarboxylase (GAD). Because GAD is the rate-limiting enzyme in the production of GABA in neurons, an antibody directed against GAD was consistent with the hypothesis of GABAergic dysfunction in SMS. Anti-GAD antibodies, however, have not been detected in all patients with SMS.[8,9] Solimena et al,[8] reporting anti-GAD antibodies in 19 patients with SMS, were unable to identify any autoantibodies in 13 clinically similar patients. Furthermore, six of the 19 SMS antisera that did show anti-GAD antibodies by immunohistochemistry failed to detect the 64-kD GAD antigen on Western blot.[8] The results suggest that SMS represents a group of disorders, in which only some patients harbor anti-64-kD GAD antibodies. We describe two patients with SMS diagnosed by typical symptoms and EMG findings who displayed a different autoantibody.

Case report. Patient Be. A 37-year-old man developed episodic muscle tightness and stiffness of his left leg in June 1987. Examination elsewhere revealed increased tone and deep tendon reflexes in the left leg, without weakness or sensory or sphincter disturbance. Laboratory investigations, including brain and spinal imaging, were unremarkable. An EMG revealed motor units firing repeatedly at rest, but was otherwise unremarkable. Over the next 3 months, episodic left leg stiffness increased in frequency and severity. Awkward position or loud noises precipitated the episodes; between spasms, the leg remained persistently stiff. Baclofen, 80 mg/d, produced only minor relief. Diazepam 5 mg po tid was begun in March 1988 after an acute exacerbation. Dramatic improvement followed, but the right leg gradually became involved and he was referred to New York Hospital (NYH).

In July 1989, 1 week after diazepam had been discon-

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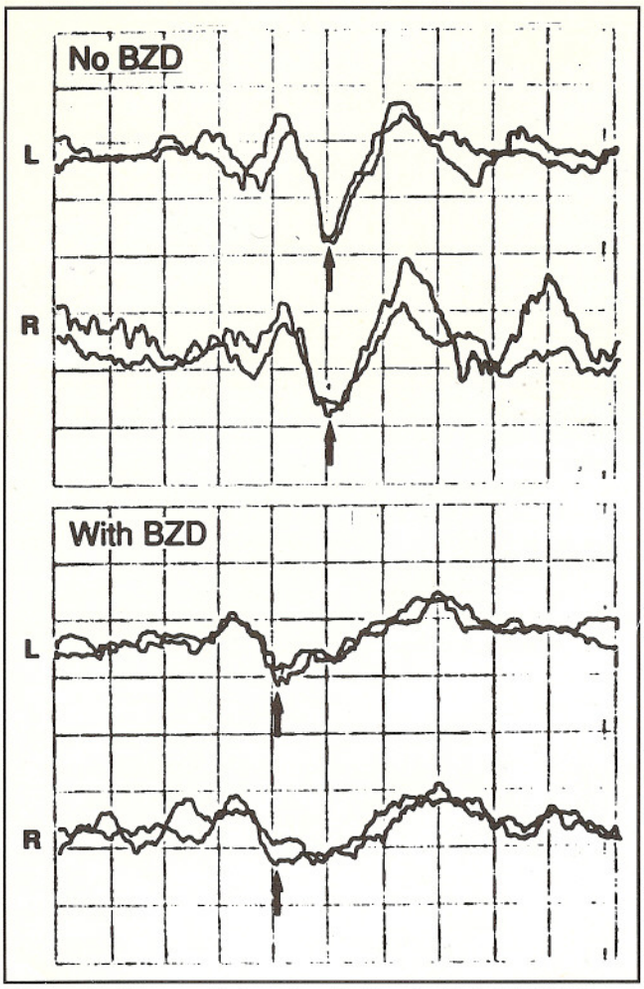

Figure 1. Abnormal visual evoked potentials (VEPs) in SMS patient Be normalize with benzodiazepines (BZD). (Top panel) VEPs 1 week after tapered withdrawal of diazepam. P100 (arrow) latencies are more than 2 SD above normal (123 msec OS and OD; normal population mean, 109 msec; upper limits of normal [mean + 2 SD], 117 msec). (Bottom panel) VEPs 48 hours later, after resumption of diazepam (10 mg po tid). P100 latencies (101 msec OS, 107 msec OD) are within the normal range. Scale = 3 μV (vertical), 25 msec (horizontal).

tinued, the patient was admitted to the Cornell-New York Hospital Clinical Research Center. Neurologic examination revealed normal mental status, cranial nerves, and upper extremities. Examination of the legs revealed muscular hypertrophy and marked bilateral muscular hypertonia throughout the range of passive movement at the hips, knees, and ankles. Stimulus-induced muscle spasms threw both legs into rigid, immovable extension for periods lasting up to 3 minutes. There was mild weakness of right ankle dorsiflexion and knee flexion. Deep tendon reflexes were hyperactive, but no abnormal reflex or sensory abnormalities were found. CBC, electrolytes, including glucose, sedimentation rate, CK, hepatitis serologies, B_{12}, folate, serum protein electrophoresis, growth hormone, ACTH, prolactin, ceruloplasmin, acetylcholine receptor antibody, HTLV-I titer and 24-hour urine methylmalonic acid, methoxyhydroxyphenylglycol, vanillylmandelic acid, homovanillic acid, catecholamines, creatinine, and protein were all normal. CSF had a protein of 49 mg/dl, glucose of 75 mg/dl, one WBC, a negative cytology, and no oligoclonal bands.

Visual evoked potentials (VEPs) performed when the patient was not taking benzodiazepines were abnormal bilaterally (figure 1). Forty-eight hours after reinstituting the patient's diazepam, the P100 was normal (figure 1). The results suggested an asymptomatic deficit in visual processing; its reversal with diazepam suggested that it involved GABAergic pathways.

EMG prior to re-starting diazepam revealed continuous muscle activity. Appropriate pharmacologic manipulations of the motor system (see Methods) resulted in expected loss of muscle activity in all instances except with saline infusion, which resulted in no change. Despite increasing doses of valium (80 mg/d), the patient was readmitted to NYH in April 1991 with a gradual worsening in motor stiffness. A course of five plasma exchanges resulted in no significant change in symptoms.

Patient Rm. A published abstract[10] provides a full clinical description of the second SMS patient, Rm. Briefly, the patient is a 65-year-old man with a 4-year history of severe stiffness, limitation of motion, and muscular hypertrophy in his neck, trunk, and proximal extremities. EMG revealed continuous motor activity at rest in the neck and shoulder muscles. The patient had mild clinical improvement after taking benzodiazepines.

Methods. *Serum and cerebrospinal fluid.* Samples of serum and CSF were obtained following informed consent. Anti-GAD-64 (SMS-64) antiserum was kindly provided by Dr. Pietro De Camilli (Yale University Medical School); serum from patient Rm was provided by Dr. Mark Gordon (Columbia-Presbyterian Medical Center).

Neurophysiology. To specify the patient's disorder and outline optimal therapy, several studies were performed, all after informed consent had been obtained. Motor and sensory nerve conduction studies in the right arm and leg, repetitive nerve stimulation of the left median-thenar system, blink reflexes, concentric needle EMG, motor unit potential analysis, and VEPs were performed using standard techniques. The stimulus for the VEPs shown in figure 1 was a contrast-reversing checkerboard, ¼-degree checks, 100% contrast, 80 candles/m^2 luminance, 1.88-Hz reversal rate, 15-degree field; the recording was Cz to Oz, with 1-Hz to 250-Hz filters and 100 averages per trace. VEP latencies elicited by ½-degree checks showed a similar, though less marked, change.

The following pharmacologic manipulations were performed with sufficient time between studies for the effects of previous medication to disappear: left femoral nerve block using 3% chloroprocaine; lumbar epidural anesthesia at L2-3 using 3% chloroprocaine resulting in a T-10 level to pin and cold testing; spinal anesthesia using 3% chloroprocaine resulting in a T-4 spinal level to sensation; intravenous injection of saline, performed on three occasions in a blinded fashion with the patient expecting curare or saline; infusion of 3 mg of D-tubocurarine; and general anesthesia using sodium thiopental.

Immunoblotting. Purkinje cell and cortical neuronal extracts were prepared from human autopsy specimens from neurologically normal individuals, and purified through Ficoll gradients as previously described.[11] Protein concentrations were determined by Bradford's assay.[12] Equal volumes of loading buffer were added to each lane before denaturation and electrophoresis through 8% SDS-PAGE and electrophoretic transfer to nitrocellulose as described.[13] Filters were blocked in 5%

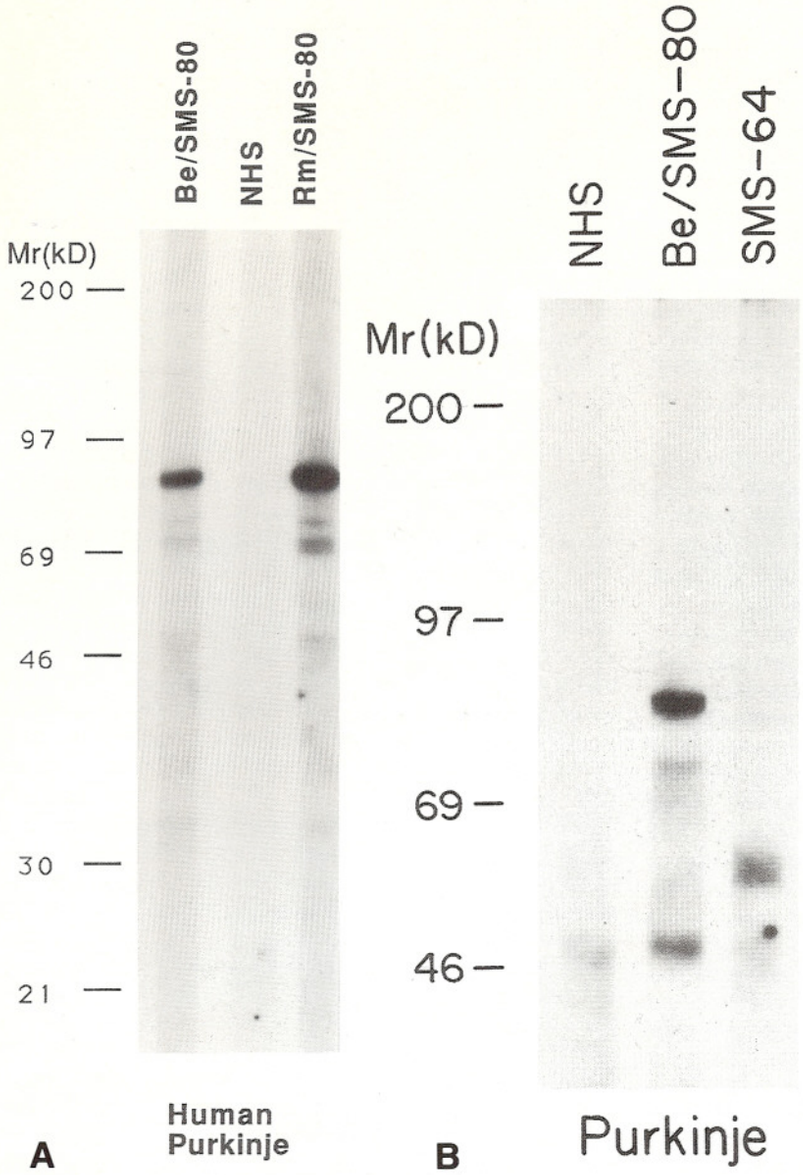

Figure 2. Western blot analysis of antigens recognized by SMS antisera. (A) Human Purkinje cell neuronal extracts reacted with two SMS antisera recognize an 80-kD antigen. Thirty micrograms of Purkinje cell extracts per lane were run on an 8% SDS-PAGE and reacted with Be antiserum (Be/SMS-80), NHS, or Rm antiserum (Rm/SMS-80), each at 1:500 dilution. Both SMS antisera recognize a major antigen of M_r 80 kD, whereas normal human serum does not. Both SMS antisera showed reactivity to serum titers of up to 1:5,000; Be CSF showed reactivity to titers of up to 1:500 (data not shown). (B) Reactivity of SMS-80 and SMS-64 antisera with human Purkinje neurons. Thirty micrograms per lane of Purkinje cell neuronal extracts were run on SDS-PAGE and transferred to nitrocellulose; strips were incubated with antiserum from patient Be (Be/SMS-80) at 1:500 dilution; antiserum from SMS patient 1 described by Solimena et al (SMS-64)[7,8] at 1:500 dilution; or NHS at 1:500 dilution. Serum from patient Be recognizes a major antigen of 80 kD. Serum from SMS patient 1 of Solimena et al (SMS-64) recognizes an antigen of 64 kD as reported.[8] Smaller-molecular-weight antigens identified with Be antiserum (see also A) do not comigrate with the 64-kD antigen identified by SMS-64 antisera. NHS is nonreactive with any of these species.

milk in phosphate-buffered saline (PBS), washed, incubated with primary antibodies at the indicated dilutions, and washed; bound antibody was detected with ^{125}I-labeled protein A and autoradiography.

Immunoprecipitation and GAD enzyme assay. GAD from pig brain or rat brain extracts was used. Partially purified GAD from pig brain, prepared as described,[14] was kindly provided by Dr. J.Y. Wu. Crude rat cortex containing GAD was prepared as described.[15] Crude rat brain extracts for GAD assay were prepared by homogenization at 4 °C in five volumes of PEA buffer (2 mM pyridoxal-5′-phosphate [Sigma], 1 mM 2-amino-ethyl-isothiouronium bromide hydrobromide [AET; Sigma], and 50 mM potassium phosphate pH 7.2) in a hand-held dounce homogenizer, followed by centrifugation at 3,000 *g* to remove cell debris.

GAD enzyme activity was measured radiometrically as described previously.[14] Each 100-μl reaction contained 0.5 μCi L-[1-^{14}C] glutamic acid (Amersham; 55 mCi/mmol), 50 μl enzyme mixture (supernatant or pellet in figure 4), and 40 μl PEA buffer in a disposable glass culture tube capped with rubber stoppers holding center wells containing 200 μl hyamine hydroxide. After 30 minutes at 37 °C, reactions were terminated by injection of 200 μl of 0.5 N H_2SO_4 through the rubber stopper. $^{14}CO_2$ was allowed to evolve for an additional 2 hours, center wells were transferred to vials containing 5 ml Permafluor V liquid scintillation fluid (Packard), samples were allowed to quench overnight in the dark, and $^{14}CO_2$ counts were determined in a liquid scintillation counter. Background counts for reactions with no enzyme added were in the range of 20 to 50 disintegrations per minute (DPM); absolute counts for enzyme reactions from supernatants of samples in which no immunoprecipitate was performed were 11,000 DPM, which corresponded to 40 μmol/g/min of GAD activity.

Immunohistochemistry. Six-micron-thick frozen sections were cut on a cryostat, fixed in cold acetone for 10 minutes, and washed in PBS. Sections were treated in 0.3% H_2O_2 at room temperature for 5 minutes to inactivate endogenous peroxidase activity, washed in PBS, and preincubated with normal goat serum (NGS) (diluted 1:10) for 10 minutes to suppress nonspecific binding. NGS was removed, antiserum was added at the appropriate dilution (in PBS with 10% NGS), and sections were incubated overnight at 4 °C. After a washing with PBS, sections were incubated with avidin-biotin peroxidase complex (Vectastain ABC complex, Vector Labs) for 30 minutes, washed in PBS, and developed for 2 minutes with 0.05% diaminobenzidine-HCl.[16] Each finding reported was repeated in several independent experiments; findings did not vary with different batches of antiserum.

Results. *Characterization of a novel SMS autoantibody Western blot analysis.* Several assays were performed with the serum from each patient to detect the presence of antineuronal antibodies. Sera from patients Be and Rm recognize a major band of Mr 80 kD in Purkinje neurons (figure 2A) or cortical neurons (data not shown) by Western blot analysis; identical results were seen with CSF (data not shown). There was no reactivity with normal human serum (NHS; figure 2A) or normal CSF (data not shown). Minor bands of lower molecular weight were observed on longer exposures (see below). Variation in the intensity of these lower bands between neuronal extracts suggests that they may represent proteolytic fragments of a larger species. Similar bands, interpreted as proteolytic fragments, have been reported with the 64-kD GAD antigen.[15]

Prior reports have found that SMS antiserum reacts with a protein of Mr 64 kD. Because we used a different and more purified source of antigen than that used in the earlier studies (which used crude[7] or partially purified[15] rat brain extracts), we obtained SMS antisera from the index SMS case reported in 1988,[7] and repeated our analysis with two different neuronal extracts. Figure 2B shows the results of a Western blot in which human Purkinje cell neurons were blotted against different SMS antisera or NHS. Our assay conditions reproduced reactivity with an antigen of Mr 64 kD (GAD-64) using the SMS antiserum of Solimena et al[7] (figure 2B). In the same Western blot, serum from patient Be reacted with an antigen of Mr 80 kD (figure 2B). Serum from 10 normal individuals and from eight patients with unrelated neurologic disorders (idiopathic peripheral neuropathy [3], idiopathic cerebellar degeneration [4], or Sjögren's syndrome [1]) showed no reactivity with either protein species (data not shown). We conclude that antisera from our SMS patients Be and Rm react with a neuronal antigen distinct in molecular weight from that recognized by previously reported SMS antisera. At the same time, these SMS antisera are unable to detect the 64-kD antigen on Western blot, even in overexposed autoradiographs

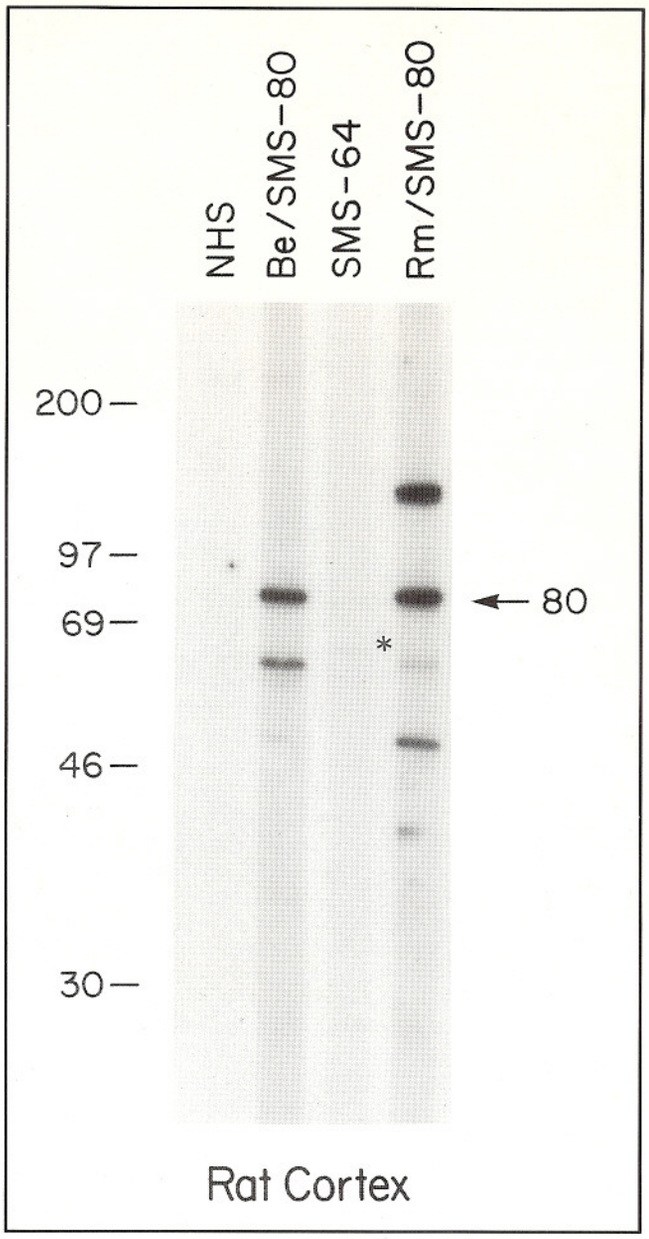

Figure 3. Reactivity of SMS sera with rat cortical extracts. Thirty micrograms per lane of a crude rat cortical extract was run on SDS-PAGE and transferred to nitrocellulose. Individual strips were incubated with antisera as indicated at dilutions of 1:500—normal human serum (NHS), serum from SMS patient Be (Be/SMS-80), serum from patient 1 of Solimena et al (SMS-64),[7,8] and serum from SMS patient Rm (Rm/SMS-80). Both SMS-80 antisera reacted with a major rat cortical antigen of 80 kD (arrow). Anti-Rm antiserum also reacted with a higher molecular-weight antigen of Mr 120 kD; this antigen was not identified in human Purkinje neuronal extracts blotted with anti-Rm antiserum, although an 80-kD antigen of intensity equal to or greater than that seen with anti-Be antiserum was identified (data not shown). The intensity of the smaller-molecular-weight antigens varied between extracts; the largest of two small-molecular-weight antigens common to both SMS-80 antisera seen here is 58 kD (asterisk), distinct from a weakly reactive band at 64 kD seen with SMS-64 antisera).

(data not shown).

Because the original analysis[7] of autoantibodies in SMS used crude rat cortex extracts for Western blot analysis, we compared reactivity of our SMS-80 antisera with that of SMS-64 antisera in rat cortical extracts prepared as described[7] (figure 3). Both antisera reacted with a major protein species of Mr 80 kD, as well as with several minor smaller molecular weight bands similar to those seen with human neuronal extracts (figure 2). In addition, Rm antiserum reacted with a larger antigen of Mr 120 kD (figure 3); this antigen was not identified with Rm antisera in human Purkinje neuron extracts nor with Be/SMS-80 antisera. Several smaller molecular-weight bands could be detected with Be and Rm antisera in rat cortex; these bands migrated at a distinctly lower molecular weight than GAD-64 (figure 3). Reactivity with GAD-64 was not detectable with Be or Rm antisera even at longer exposures; however, because the SMS-64 reacts so faintly with GAD-64 in these crude rat cortical extracts, the possibility of a low-titer antibody against rat GAD-64 in SMS-80 antisera cannot be excluded. There was no reactivity when NHS were blotted against rat cortical extracts. Thus the two different SMS sera react with a major antigen of 80 kD present in human and rat brain that is not recognized by SMS-64 antisera. By all measures, SMS-80 and SMS-64 antisera appear distinct.

Identification of anti-GAD antibodies in SMS sera. The ability of sera from the two SMS patients to immunoprecipitate GAD activity was tested as follows. Whole-brain extracts from pig (figure 4) or rat (data not shown) were incubated with antisera, bound antibody was immunoprecipitated, and GAD enzyme activity was assayed in both the supernatant and the pellet. NHS did not precipitate any GAD activity (figure 4); all enzyme activity remained in the supernatant. Both SMS-80 and SMS-64 antisera, however, depleted enzyme activity completely and with equal efficiency from the supernatant (figure 4). The immunoprecipitate obtained with both SMS antisera contained equivalent amounts of assayable enzyme activity, although recovery was not quantitative at high antibody concentrations. These results were comparable to those published by Baekkeskov et al using SMS-64 antisera.[15]

Immunohistochemistry with SMS antisera. Serum from both patients Be and Rm reacted with what appeared to be an interneuronal antigen, giving a granular staining pattern in the gray matter surrounding neurons (data not shown); this pattern is characteristic of that seen with SMS antiserum or purified anti-GAD antibodies.[17,18] This staining pattern has been interpreted to reflect the presence of GAD in synaptic terminals.[18] No staining was seen in serial sections blotted with NHS (data not shown).

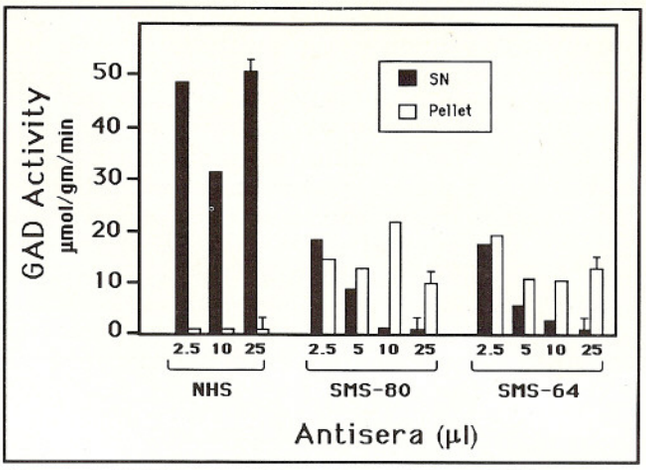

Figure 4. SMS antisera immunoprecipitate GAD enzyme activity. Seventy-five micrograms of GAD extract of pig brain was incubated for 12 hours with the specified type and amount of antisera, and immunoprecipitated with protein-G Sepharose. Enzyme assays of pellets and supernatants (SN) were performed in a 100-µl reaction containing 0.5 µCi ^{14}C-glutamic acid; the $^{14}CO_2$ produced, a measure of GAD activity, was measured (see Methods). Conditions were established in which a linear yield of $^{14}CO_2$ was obtained as a function of input enzyme concentration. Similar results were obtained for both SMS-80 antisera, and in several independent experiments. Error bars indicate 2 SD for samples run in triplicate from a single experiment. Both SMS-80 antisera and SMS-64 antisera, but not normal human sera, were also able to immunoprecipitate GAD from crude extracts of rat brain, prepared as described in Methods (data not shown).

Discussion. This study identifies two patients with clinical SMS whose disease differs from previously described SMS by the presence of an autoantibody that recognizes a distinct 80-kD neuronal protein (figures 2 and 3). Although our analysis of anti-GAD antibodies is consistent with previous reports of anti-GAD antibodies in SMS,[7,8] it distinguishes these patients as a related but distinct group. The immunoprecipitation of GAD enzyme activity (figure 4) and immunohistochemistry (data not shown) with SMS-80 antisera provide strong evidence for the presence of anti-GAD antibodies in these antisera, whereas Western blot analysis discriminates them from SMS-64 antisera.

The findings suggest three possible interpretations. First, the SMS-80 antisera may recognize a variant GAD enzyme that is distinct in size from the prototypic 64-kD enzyme. Although the best-characterized GAD enzyme is a 64-kD protein, several distinct molecular-weight species have been biochemically characterized or cloned as variant GAD isoenzymes.[19-22] In particular, Wu et al[14] and Denner et al[23] reported the purification of an 80-kD protein to homogeneity from rat brain that harbors GAD activity, and Huang et al[24] recently reported a distinct cDNA clone thought to encode this protein.

Second, the SMS-80 antigen might not itself har-

bor GAD activity, but could be associated with the GAD enzyme as part of a multisubunit complex. Such a protein would be expected to co-localize immunohistochemically and co-precipitate with the native GAD enzyme. We observed multiple immunoreactive bands in human and rat brain, including one of a higher molecular weight than 80 kD; whether these represent cross-reacting GAD species, proteolytic fragments, GAD-associated-subunits, or unrelated proteins is uncertain. Finally, SMS-80 antisera might harbor a form of anti-64-kD GAD antibody different from the antibody present in SMS-64 patients in that it recognizes only the native form of the 64-kD GAD enzyme. Such an antibody could potentially detect GAD by immunohistochemical and enzymatic assay but not by Western blots. In this case, an anti-80-kD antibody would be an additional component of SMS-80 antisera.

Our observations may at least partially explain the results of groups who have been unable to detect GAD-64 antibodies in patients with SMS.[8,9] In the largest published series[8] of SMS patients, an autoantibody directed against GAD-64 could be detected in only 19 of 32 SMS patients; in six of these 19 patients, Western blots failed to detect the 64-kD antigen whereas sera detected an antigen by immunohistochemistry that localized with GAD. Our study makes a similar observation: two SMS antisera detected an antigen that co-localizes with GAD in human brain sections but fails to detect a 64-kD antigen; furthermore, we detected a novel 80-kD antigen on Western blot. The results suggest that our Western blot technique, particularly the use of purified human Purkinje or cortical neurons, may provide a more sensitive assay than previously employed to detect SMS-associated antigens. Whether the 80-kD or other variants of the GAD enzyme might be detected if "negative" SMS antisera were probed against more purified antigens remains to be to determined.

The interest in detecting novel antineuronal antibodies in SMS extends beyond the characterization of GAD in the brain. Recently, GAD-64 antibodies were reported in patients with autoimmune diabetes,[15] in some cases prior to the development of clinical disease.[25] The relationship between SMS-80 antisera and autoimmune diabetes is unclear but deserves exploration, since one of our two patients[10] was diabetic.

One of the two patients has an abnormal visual evoked potential (a delayed P100) that reverted to normal with benzodiazepines (figure 1), consistent with central GABAergic dysfunction. Prior work has established evidence that GABAergic connections, which mediate local circuitry in the occipital (striate) cortex,[26-29] contribute to the P100 in the cat.[30] Indirect evidence of central GABAergic dysfunction in SMS has been previously noted from the observation that 10% or more of SMS patients are reported to have epilepsy.[1,7,8,31,32]

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