

## SERUM ANTIBODIES TO L-TYPE CALCIUM CHANNELS IN PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS

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**Abstract Background and Methods.** Sporadic amyotrophic lateral sclerosis is a chronic, progressive degenerative disease of the motor neurons of the spinal cord and motor cortex. The cause is unknown. Recent electrophysiologic studies in animals indicate that immunoglobulins from patients with this disease alter presynaptic voltage-dependent calcium currents and calcium-dependent release of neurotransmitters. To determine whether similar interactions might be identified biochemically, we used an enzyme-linked immunosorbent assay (ELISA) to detect the reaction of serum IgG with purified complexes of L-type voltage-gated calcium channels from rabbit skeletal muscle. The results from patients with amyotrophic lateral sclerosis were compared with those obtained from patients with other types of motor neuron disease, patients with autoimmune and non-autoimmune neurologic diseases, and normal subjects.

**Results.** Serum samples from 36 of 48 patients with

sporadic amyotrophic lateral sclerosis (75 percent) contained IgG that reacted with L-type calcium-channel protein, and serum reactivity on ELISA correlated with the rate of disease progression (Spearman rank-correlation coefficient, 0.62). Reactive serum was present in only 1 of 25 normal subjects and 1 of 35 control patients with no motor neuron disease. Antibodies to L-type voltage-gated calcium channels were identified in 6 of 9 patients with Lambert-Eaton syndrome, and in 3 of 15 patients with Guillain-Barré syndrome.

**Conclusions.** Antibodies to L-type voltage-gated calcium channels are present in the serum of patients with amyotrophic lateral sclerosis, and antibody titers correlate with the rate of disease progression. Together with previous data, these results suggest a role for autoimmune mechanisms in the pathogenesis of sporadic amyotrophic lateral sclerosis. (N Engl J Med 1992; 327:1721-8.)

AMYOTROPHIC lateral sclerosis is a neurodegenerative disease of unknown cause<sup>1-4</sup> that compromises motor neurons, produces progressive weakness and paralysis, and culminates in respiratory failure and death. Studies attempting to implicate viruses, toxins (especially excitotoxins), and the loss of trophic influences in this disease have not provided strong evidence of causation.<sup>4-6</sup> Familial amyotrophic lateral sclerosis accounts for less than 10 percent of diagnosed cases.<sup>7,8</sup>

There is some evidence implicating autoimmunity in the pathogenesis of sporadic amyotrophic lateral sclerosis. There are increases in the incidence of autoimmune disorders<sup>3</sup> and paraproteinemias among patients with amyotrophic lateral sclerosis.<sup>9,10</sup> Immune complexes are present in serum from these patients,<sup>11,12</sup> IgG is present in upper and lower motor neurons, and inflammatory foci of T cells and reactive microglia are found within the ventral horn of the spinal cord.<sup>13</sup> However, direct investigations that used tissue culture<sup>14,15</sup> and immunoblotting techniques<sup>16,17</sup> have not confirmed a role for humoral autoimmunity and have failed to demonstrate specificity or sensitivity of antibodies to the antigens tested.<sup>18-21</sup>

Two guinea pig models of immune-mediated motor neuron disease<sup>22-24</sup> provide circumstantial evidence of the potential role of autoimmunity. Immunoglobulins

from affected guinea pigs and from patients with amyotrophic lateral sclerosis passively transfer physiologic abnormalities to the neuromuscular junctions of injected mice.<sup>25</sup> These injected immunoglobulins increase the release of acetylcholine from motor-neuron terminals, possibly by modulating presynaptic voltage-dependent calcium currents and the release of calcium-dependent neurotransmitters. A test of this hypothesis assayed the effects of IgG from patients with amyotrophic lateral sclerosis on L-type voltage-gated calcium channels (VGCCs) in skeletal myotubules and demonstrated specific decreases in peak amplitude of the calcium current and in charge movement.<sup>26-28</sup>

The simplest explanation for these electrophysiologic data is a direct binding of these immunoglobulins to L-type VGCCs or to a closely coupled antigen. Using a solubilized and highly purified preparation of L-type VGCCs obtained from rabbit skeletal muscle and an enzyme-linked immunosorbent assay (ELISA), we examined how serum and immunoglobulins from patients with amyotrophic lateral sclerosis react with L-type VGCC protein.

### METHODS

#### Study Subjects

Forty-eight patients given a diagnosis of amyotrophic lateral sclerosis were randomly selected and evaluated on the basis of their medical history, physical examination, electromyographic studies, muscle biopsy, bulbar-function tests, and exclusionary clinical biochemical studies.<sup>9</sup> A second group of patients served as controls and received diagnoses on the basis of clinical history, physical findings, and tests appropriate to each disease. This group of patients included 9 patients with Lambert-Eaton syndrome, 15 patients with Guillain-Barré syndrome, 12 patients with other types of motor neuron disease, 17 control patients with autoimmune neurologic disease, and 18 control patients with non-

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autoimmune neurologic disease. In addition, 25 healthy subjects were recruited from among department staff members and their families. The characteristics of the study subjects are shown in Table 1.

### Preparation of Serum and Immunoglobulin

Randomly selected serum samples were collected during patient admissions and clinic visits at Baylor College of Medicine-Methodist Hospital in 1990 through 1992. Patients with recent symptoms of acute infectious illness were excluded. Blood was drawn from the patients and normal controls after an overnight fast. The samples were centrifuged for 10 minutes at  $1500\times g$  and for 20 minutes at  $10,000\times g$ . The supernatants were mixed with sodium citrate buffer (pH 5; final concentration, 0.1 mol per liter) for 24 hours, centrifuged at  $10,000\times g$  for 30 minutes, equilibrated to pH 7.4, and stored at  $-80^{\circ}\text{C}$  until needed.

Immunoglobulins were purified from previously untested samples of citrate-treated serum or plasma from patients with a combination of precipitation with 45 percent ammonium sulfate and ion-exchange chromatography with a high flow rate (AMF Cuno, Meriden, Conn.).<sup>29</sup> In this technique, the samples underwent fractionation and dialysis with ammonium sulfate and were individually applied to cation-exchange cartridges; bound IgG was then eluted through anion-exchange cartridges before being concentrated by pressure dialysis (Amicon, Lexington, Mass.). The purity of the IgG samples was 90 percent. Some IgG samples were further purified by protein A-agarose affinity chromatography, though the cross-contamination of samples resulting from this method necessitated the use of fresh affinity matrix for each sample of IgG purified.

The total IgG concentration in the serum was measured with a Technicon RA-1000 system analyzer (Tarrytown, N.Y.), in which

immune-complex turbidity occurs at 340 nm, according to tests with serum IgG and human IgG antiserum standards.

### Purification of Antigens

Because complexes of L-type VGCCs from skeletal muscle were prepared in several laboratories, the ultimate degree of purification differed. Partially purified complexes were prepared in two laboratories. Before ELISA the samples underwent tissue homogenization, differential centrifugation, pressure fractionation of microsomes and sucrose-gradient fractionation, solubilization of L-type VGCCs with digitonin, wheat-germ agglutinin affinity chromatography, and low-pressure anion-exchange chromatography.<sup>30</sup> The typical concentrations obtained with these methods ranged from 0.02 to 0.04 nmol of L-type VGCCs per milligram of protein. More highly purified L-type VGCCs from skeletal muscle (containing 1.7 nmol of L-type VGCCs per milligram of protein) were prepared in a third laboratory.<sup>31</sup> Product purity was determined electrophoretically with the use of subunit-specific antibody labels produced against L-type VGCCs from rabbit skeletal muscle.<sup>30,32</sup>

Cytosolic proteins from rabbit skeletal muscle and microsomes depleted of L-type VGCCs were produced by collecting supernatant fractions of muscle homogenates treated with 0.6 mol of potassium chloride per liter and pellets of microsomal membrane extracted with 4 percent digitonin, respectively.<sup>30</sup> The samples underwent dialysis against 50 mmol of TRIS buffer (pH 7.4) per liter, which contained 0.1 mol of sodium chloride per liter (for cytosolic proteins), or against 10 mmol of 3-(*N*-morpholino)-2-hydroxypropane-sulfonic acid buffer (pH 7.4) per liter, which contained 0.1 mol of sodium chloride per liter and 0.05 percent Tween 20 detergent (for microsomes), and a brief period of sonication (microsomes only) before undergoing ELISA. The cytoskeletal proteins derived from the human brain<sup>33</sup> were a gift of Dr. K. Angelides. Characterization of cytosolic and cytoskeletal protein fractions showed that there was essentially no binding of dihydropyridine antagonist specific for L-type VGCCs and no immunoreactive L-type VGCC protein on immunoblotting of polyacrylamide electrophoretic gels. The digitonin-treated microsome pellets retained approximately 10 percent of the total bound radiolabeled dihydropyridine ligand. Fractions enriched for other skeletal-muscle proteins, including ryanodine-binding calcium-release channels, calcium ATPase, and calsequestrin, were prepared as previously described.<sup>30,34,35</sup>

### ELISA

Detergent-free antigens were diluted in 0.1 mol of bicarbonate buffer (pH 8.4) per liter to a final concentration of 1 to 3  $\mu\text{g}$  of protein per milliliter before they were added to 96-well polystyrene ELISA plates (each microtiter well holds 100  $\mu\text{l}$ ; Corning, Corning, N.Y.) for overnight incubation at  $4^{\circ}\text{C}$ . Because the presence of detergents such as digitonin and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate greatly retarded the binding of protein antigens to plastic, samples containing detergent were incubated on ELISA plates for 48 hours at  $4^{\circ}\text{C}$ . The plating densities for L-type VGCC antigen, as determined by the binding of the dihydropyridine radioligand, were typically 2 to 4 fmol (in 0.125  $\mu\text{g}$  of protein) per microtiter well for partially purified preparations and up to 20 fmol (in 0.013  $\mu\text{g}$  of protein) per microtiter well for highly purified material. Unbound antigen was removed by rinsing the well with a solution of 0.9 percent sodium chloride and 0.05 percent Tween 20 (saline-Tween solution). Remaining protein-binding sites in the wells were subsequently blocked by the addition of 50 mmol of TRIS buffer at a pH of 7.4 per liter (blocking buffer; 250  $\mu\text{l}$  per well), which contained 1 percent fraction V bovine serum albumin (Sigma Chemical, St. Louis), 0.9 percent sodium chloride, and 0.05 percent Tween 20, for 2 hours at  $37^{\circ}\text{C}$  (for antigens that were not bound by detergent) or for 48 hours at  $4^{\circ}\text{C}$  (for samples containing detergent). Plating of immunoreactive L-type VGCCs on ELISA plates was confirmed by the addition of serially diluted mouse anti-L-type VGCC  $\gamma$ -subunit-specific monoclonal antibody to several microtiter wells in each plate and visualized with a goat antimouse alkaline phosphatase-conjugated second antibody at a dilution of 1:1000 (Promega Biotech, Madison, Wis.). Measurements of nonspecific antibody binding were made with antigen-free

Table 1. Clinical and Demographic Characteristics of the Study Subjects.

GROUP	No.	MEAN ( $\pm$ SE) AGE*	MALE/FEMALE RATIO
		$\bar{y}$	%
Patients with amyotrophic lateral sclerosis	48	51.8 $\pm$ 1.8	67/33
Patients with Lambert-Eaton syndrome	9	53.2 $\pm$ 4.4	89/11
Patients with Guillain-Barré syndrome	15	55.2 $\pm$ 6.0	60/40
Patients with other motor neuron diseases	12	53.2 $\pm$ 2.1	67/33
Familial amyotrophic lateral sclerosis	7		
Spinal muscular atrophy	3		
Post-polio syndrome	1		
Unidentified motor neuron disease	1		
Normal controls	25	48.8 $\pm$ 2.7	60/40
Controls with non-autoimmune neurologic disease	18	57.0 $\pm$ 4.0	67/33
Peripheral neuropathy	4		
Cerebrovascular accident or multi-infarct state	4		
Myelopathy	2		
Radiculopathy	1		
Charcot-Marie-Tooth syndrome	1		
Vertigo	2		
Arnold-Chiari deformity	1		
Hereditary spastic paraparesis	1		
Bulbar neuropathy	1		
Tourette's syndrome	1		
Controls with autoimmune neurologic disease	17	52.4 $\pm$ 5.0	47/53
Multiple sclerosis	5		
Myasthenia gravis	5		
Polymyositis	3		
Rheumatoid-arthritis vasculitis	2		
Systemic lupus erythematosus	1		
Paraneoplastic neuropathy	1		

\* $P>0.1$  for the comparison of ages between groups.

plates, treated with blocking buffer alone or in combination with digitonin, before the assay.

Human serum or IgG, which had been diluted in blocking buffer, was added to antigen-coated wells for two hours at 37°C. Human IgG was assayed at concentrations ranging from 0.05 to 200 µg per milliliter, whereas serum was added in a dilution of 1:50 to 1:156,250. The unbound reagents were removed by multiple rinses with saline-Tween solution, and alkaline phosphatase-conjugated goat antihuman Fc-specific IgG in a dilution of 1:2000 in 100 µl of blocking buffer per microtiter well (Tago, Burlingame, Calif.) was added to wells for one hour at 37°C. After the wells were rinsed with saline-Tween solution, para-nitrophenyl phosphate (0.4 mg per milliliter) in a buffer consisting of 0.1 mol of sodium carbonate and 1 mmol of magnesium chloride per liter at a pH of 9.5 (Sigma Chemical) was introduced into the wells (95 µl per microtiter well), and the alkaline phosphatase activity from bound antibody complexes was assayed spectrophotometrically on a Bio-Tek 8000 microplate reader (Bio-Tek, Winooski, Vt.) at 37°C through a 405-nm filter (with simultaneous automatic subtraction of background optical-density readings at 490 nm).

#### Assessment of Disease Progression

Patients followed in the Baylor Amyotrophic Lateral Sclerosis Clinic underwent monthly or bimonthly tests of clinical function.<sup>36</sup> The scores of tests assessing the degree of motor disability for each patient were plotted as a function of time in months. Patients were included in an assessment of disease progression when the rate of change in their disability score (disease progression) could be modeled by linear regression ( $r^2 > 0.9$ ) and when 12 months of data were available near the time of phlebotomy. Patients taking immunosuppressants were excluded from this analysis.

#### Statistical Analysis

All statistical evaluations were performed with an IBM-compatible 386/33 computer, with a STATA statistical computer-program package (Computing Resource Center, Los Angeles).

### RESULTS

#### Reactivity of L-Type VGCCs with Patient Serum on ELISA

Serum IgG from patients with amyotrophic lateral sclerosis demonstrated selective and sensitive binding of L-type VGCCs on ELISA. Serum samples from 48 patients with amyotrophic lateral sclerosis, 25 normal subjects, 17 control patients with autoimmune neurologic diseases, 18 control patients with non-autoimmune neurologic diseases, 9 patients with Lambert-Eaton syndrome, 15 patients with Guillain-Barré syndrome, and 12 patients with other types of motor neuron disease were assayed to determine their reactivity to highly purified L-type VGCC antigen from rabbit skeletal muscle (plating concentration, 20 fmol per microtiter well). Most serum samples from the control groups reacted minimally with plastic-bound L-type VGCCs (Fig. 1). These values had normal distributions, with almost identical means and standard errors (Table 2). Because the ELISA results for patients with amyotrophic lateral sclerosis, Lambert-Eaton syndrome, or Guillain-Barré syndrome were not distributed normally, we used nonparametric statistical tests for other statistical analyses. When significant antigen binding was defined as an optical density of more than 2 SD above the mean values for the control population, serum from 36 of the 48 patients with amyotrophic lateral sclerosis (75 percent) had significant binding to L-type VGCCs, whereas less

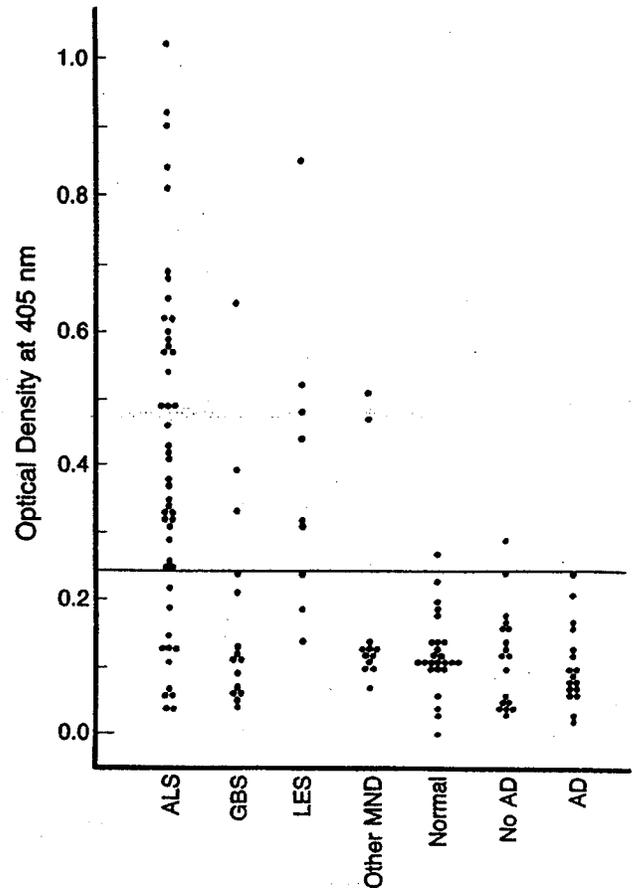


Figure 1. Quantitative Comparison of Serum Binding to L-Type VGCCs from Skeletal Muscle.

Results of ELISA for individual citrate-treated serum samples (dilution, 1:1250) assayed against purified L-type VGCC antigen are shown. Mean values for individual serum samples tested in duplicate wells in three experiments are depicted after a 3-hour incubation at 37°C, followed by 15 hours of incubation at 25°C. The horizontal line represents the value 2 SD above the pooled mean control values. Most of the reactions in serum samples from patients with amyotrophic lateral sclerosis (ALS) were detectable at dilutions of 1:6250 to 1:25,000. GBS denotes Guillain-Barré syndrome, LES Lambert-Eaton syndrome, Other MND other types of motor neuron disease, Normal normal controls, No AD non-autoimmune disease, and AD autoimmune disease.

than 5 percent of the samples from control patients with no motor neuron disease had significant binding to L-type VGCCs (Fig. 1).

Other strongly reactive serum samples included those from 6 of 9 patients with Lambert-Eaton syndrome; 3 of 15 patients with Guillain-Barré syndrome; 1 patient with motor neuron disease of the upper and lower extremities, serum monoclonal gammopathy, and a protracted clinical course; and 1 patient with clinical amyotrophic lateral sclerosis, whose brother had identical symptoms and multiple inflammatory foci in the ventral horn of the spinal cord at autopsy. However, serum samples from patients with multigenerational familial amyotrophic lateral sclerosis, familial spinal muscular atrophy, post-polio syn-

drome, recent central nervous system injury, or chronic autoimmune disease of the nervous system were all nonreactive on ELISA.

No antibody-binding specificity to other tested antigens, including cytosolic and cytoskeletal proteins (Fig. 2), and GM<sub>1</sub> gangliosides was found. Antibody titers among patients with amyotrophic lateral sclerosis were reduced almost to control levels when muscle microsomes depleted of L-type VGCCs were tested.

The differences in the level of serum IgG binding to L-type VGCC antigen were not explained by differences in the total serum IgG concentration. Total serum IgG levels in patients with amyotrophic lateral sclerosis ranged from 5 to 18 mg per milliliter and were not significantly different from the levels in patients with autoimmune diseases. When ELISAs were performed that tested constant serum concentrations of IgG, the results were equally sensitive and selective.

#### Reactivity of L-Type VGCCs with Purified Patient IgG

IgG behaved similarly to serum fractions when IgG isolated from similar patient populations was added at equal protein concentrations to ELISA plates pretreated with either partially purified (plating concentration, 4 fmol per microtiter well) or highly purified (plating concentration, 20 fmol per microtiter well) L-type VGCCs. IgG from patients with amyotrophic lateral sclerosis bound to partially purified VGCC antigen, differentiating their action from that of most control patients with neurologic disease ( $P < 0.02$  by the Wilcoxon rank-sum test) (Fig.

Table 2. Binding of Serum Antibody from the Study Subjects to L-Type VGCCs, as Measured by ELISA.

GROUP	No.	OPTICAL DENSITY AT 405 NM	
		MEAN $\pm$ SE	MEDIAN
Patients with amyotrophic lateral sclerosis	48	0.43 $\pm$ 0.04	0.38
Patients with Lambert-Eaton syndrome	9	0.39 $\pm$ 0.07	0.32
Patients with Guillain-Barré syndrome	15	0.18 $\pm$ 0.04	0.11
Patients with other motor neuron diseases	12	0.18 $\pm$ 0.04	0.13
Normal controls	25	0.12 $\pm$ 0.01	0.11
Controls with non-autoimmune disease	18	0.12 $\pm$ 0.02	0.12
Controls with autoimmune disease	17	0.11 $\pm$ 0.02	0.09

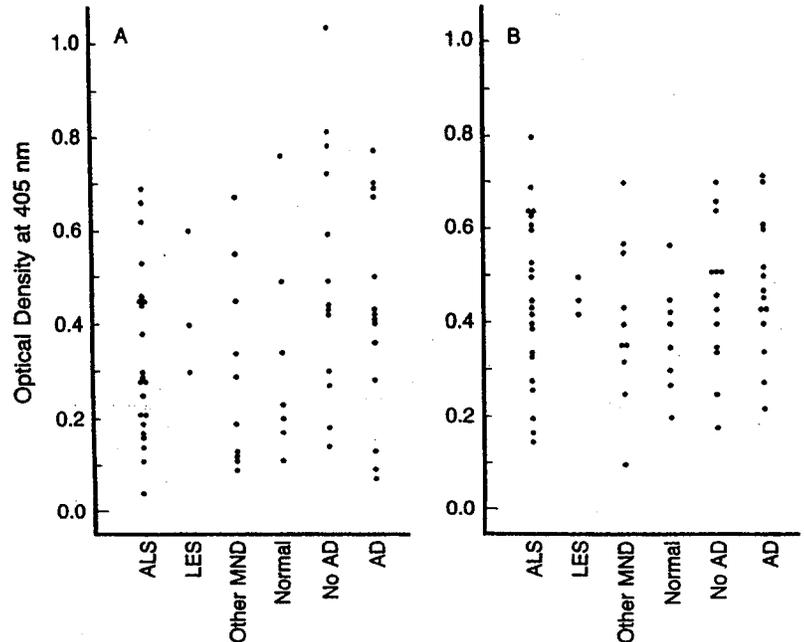


Figure 2. Quantitative Comparison of Serum Binding to Cytoskeletal and Cytoplasmic Proteins.

Shown are the results of ELISA for some of the serum samples depicted in Figure 1 assayed against either cytoskeletal proteins derived from human brain or cytoplasmic proteins from rabbit skeletal muscle. In Panel A, a partially purified fraction containing human neurofilament protein was precipitated onto microtiter plates at a final concentration of 10  $\mu$ g per milliliter. The presence of plated neurofilaments was confirmed by an assay with monoclonal antibodies directed against the 160-kd and 200-kd species. In Panel B, 0.6 mol of potassium chloride-soluble cytoplasmic proteins per liter were plated at a final concentration of 10  $\mu$ g per milliliter. For both assays, serum was diluted 1:1250 before testing. Each point represents the mean value of optical-density readings of product formation from the reaction of *N*-*P*-nitrophenyl alkaline phosphate with alkaline phosphatase, measured in triplicate wells after two hours of incubation at 37°C. Greater dilutions of serum (up to 1:31,250) provided qualitatively similar results (data not shown) when tested against cytoskeletal or cytoplasmic proteins, and serum-titer curves against these antigens (data not shown) did not reveal greater binding by serum from the patients with amyotrophic lateral sclerosis than by serum from other groups of subjects at any tested dilution. The abbreviations are defined in the legend to Figure 1.

3A). IgG fractions from two patients with the Lambert-Eaton syndrome also reacted strongly in this assay. The differences in the IgG responses of the various groups of subjects were more apparent when highly purified L-type VGCCs were used as antigens (two-sample Wilcoxon *z* score, 3.97;  $P < 0.001$ ) (Fig. 3B). The IgG from patients with amyotrophic lateral sclerosis did not react specifically with cytoskeletal proteins, cytosolic proteins, GM<sub>1</sub> gangliosides, or various purified muscle-membrane proteins, including calcium ATPase, calsequestrin, and the ryanodine receptor.

Such binding was not qualitatively affected by the method of VGCC purification or by the laboratory in which it was prepared. IgG from individual patients with amyotrophic lateral sclerosis had quantitatively similar levels of reactivity and qualitatively similar rank-ordered responses when tested against L-type VGCCs prepared in a similar manner by two laboratories, and qualitatively similar rank-ordered re-

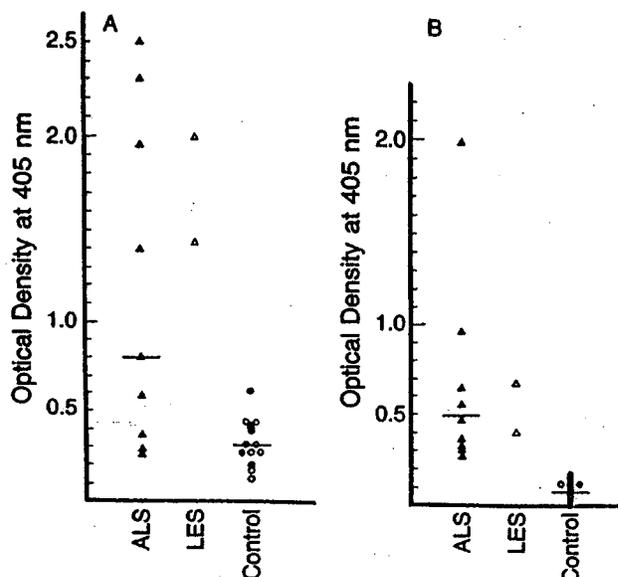


Figure 3. Quantitative Comparison of IgG Binding to L-Type VGCCs from Skeletal Muscle.

Purified IgG (5  $\mu\text{g}$  per 100  $\mu\text{l}$ ) was added to microtiter wells pretreated with either partially purified (Panel A) or highly purified (Panel B) L-type VGCCs from skeletal muscle. Each point represents the mean values of optical-density readings (as explained in the legend for Figure 2), measured in triplicate samples in two experiments after a 3-hour incubation at 37°C (Panel A) or a 3-hour incubation at 37°C followed by 15 hours of incubation at 25°C (Panel B). The bars represent the median values. Solid triangles depict values for patients with amyotrophic lateral sclerosis (ALS), open triangles values for patients with Lambert-Eaton syndrome (LES), solid circles values for control patients with neurologic disease, and open circles values for normal controls.

sponses (though quantitatively reduced values) when tested against more highly purified L-type VGCCs produced in a third laboratory. These quantitative reductions in binding after further antigen purification might be explained by the removal of contaminating proteins with which other immunoglobulins react, increasing specificity. Since there were quantitative differences in binding on ELISA of both monoclonal antibodies and patient IgG in some preparations of L-type VGCCs in the absence of changes in subunit electrophoretic mobilities on denaturing gels, secondary, tertiary, or quaternary structures of L-type VGCCs may also be important to antibody-antigen interactions and may vary between preparations.

The reaction rates on ELISA were much slower for IgG from patients with amyotrophic lateral sclerosis and Lambert-Eaton syndrome than for subunit-specific monoclonal antibodies (a response time of several hours as compared with one of 10 to 15 minutes) when tested against partially purified L-type VGCCs. Longer reaction times were required when serum samples were tested. The reaction rates increased with increasing IgG concentrations (>10  $\mu\text{g}$  per assay) or high serum titers (dilution, <1:250) but were associated with simultaneous reductions in the specificity of the

reaction. The greatest degree of IgG binding specificity was noted with lower IgG concentrations (<5  $\mu\text{g}$  per assay) or greater serum dilutions (>1:1250). The specificity of the reaction also increased when highly purified antigen was used, although the reaction rates decreased for both patient IgG and monoclonal antibody.

Nonspecifically bound second antibody did not contribute to the reaction rate, as assayed with primary antibodies from which Fc regions had been removed. Large background reactions were observed with 3 percent of the serum samples and 10 percent of the IgG samples added to blocked, antigen-free ELISA plates, and these results were subtracted from the binding values for antigen-coated plates to provide specific data on VGCC binding.

#### Relation of the Response to ELISA and Disease Progression

The ranked disease-progression rates for 38 patients with amyotrophic lateral sclerosis directly correlated with their serum reactivities on ELISA (Spearman rank-correlation coefficient, 0.62) (Fig. 4A). When these patients were grouped according to their serum reactions on ELISA, two rates of disease progression were evident (two-sample Wilcoxon rank-sum  $z$  statistic, -4.17;  $P < 0.001$ ) (Fig. 4B); strong statistical correlations were found between patients with a slow clinical course and weak serum reactivity on ELISA and between patients with more rapid disease progression and strong serum reactivity on ELISA. However, the degree of serum reactivity on ELISA did not correlate with disease severity at the time of phlebotomy (Spearman rank-correlation coefficient, 0.16).

Serum reactivity on ELISA appeared to be constant over time, as tested three times over a 20-month period in samples from three patients with amyotrophic lateral sclerosis (variation between sample titers, <20 percent for each patient), despite obvious functional deterioration in these patients during the same period. The age of the patients did not correlate with the rate of disease progression or the response to ELISA (Spearman rank-correlation coefficients, 0.01 and -0.19, respectively), nor did the sex of the patients affect these two variables (sex-specific Spearman rank-correlation coefficients, 0.73 for women and 0.54 for men).

#### DISCUSSION

Our study shows that patients with amyotrophic lateral sclerosis have serum IgG that reacts with L-type VGCCs. Such antibodies are found selectively, but not solely, in these patients, and the titers of these antibodies appear to be associated with the rate of progression of amyotrophic lateral sclerosis. Samples of IgG from the same patients that were previously prepared and used to document electrophysiologic interactions with VGCCs<sup>26,27</sup> also bound purified protein from L-type VGCCs on ELISA.

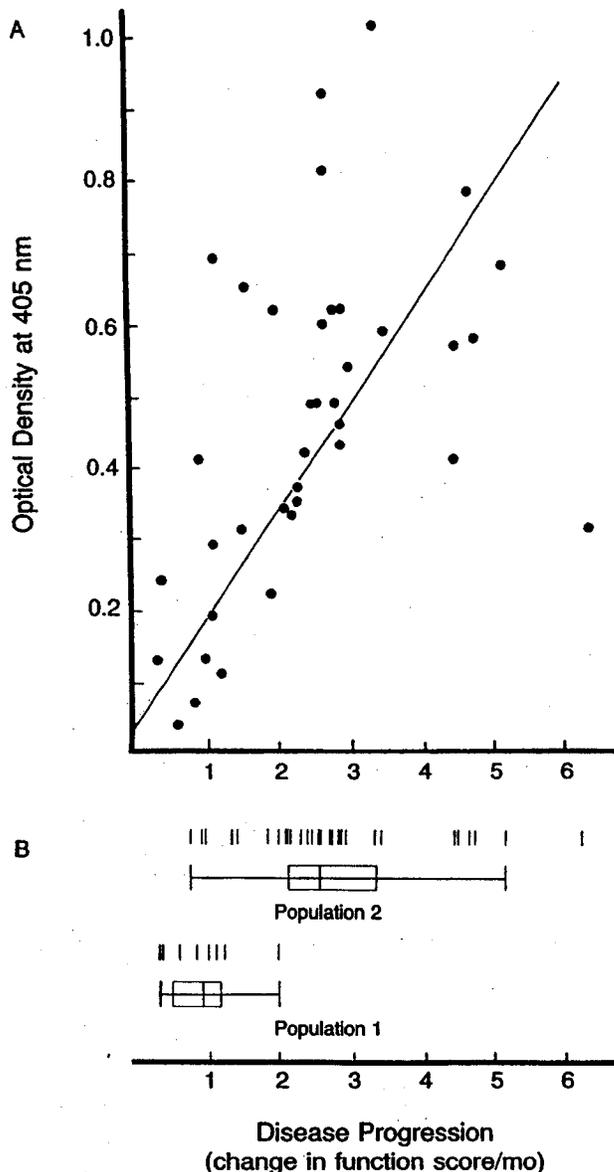


Figure 4. Serum ELISA Reactions as a Function of Individual Rates of Disease Progression.

In Panel A, rates of disease progression for 38 of the 48 patients with amyotrophic lateral sclerosis are plotted as a function of serum reactivity on ELISA. The line was obtained by least-squares regression analysis for these data (adjusted  $r^2$ , 0.8). The same data are presented in Panel B, with the patients divided into two populations on the basis of their reactions on ELISA: values for population 1 were less than 2 SD above the values for the control population, and values for population 2 were more than 2 SD above the control values. These populations are plotted as a function of the rate of disease progression. The enclosed boxes represent the 25th through 75th percentiles, the vertical line in each box represents the median, and the small vertical lines above the population-range markers indicate the individual rates in each population. The rates of disease progression were calculated from serial disease-progression scores and plotted as a function of the time since the first symptoms appeared. These scores, ranging from a low of 30 points for normal persons to a high of 164 points for patients with maximal motor dysfunction, were determined from monthly or bimonthly assessments of bulbar function, respiratory function, strength of muscles in the extremities, and skeletal-muscle function.

The presence of serum antibodies to VGCCs is not specific to patients with amyotrophic lateral sclerosis. Positive reactions were also seen with serum from patients with Lambert-Eaton syndrome, an autoimmune disease involving antibodies cross-reactive with N-type<sup>37</sup> and L-type<sup>38</sup> VGCCs and with synaptotagmin.<sup>39</sup> However, IgG from patients with Lambert-Eaton syndrome differs functionally from IgG from patients with amyotrophic lateral sclerosis in terms of having opposite effects on ion flux in pre-synaptic calcium channels and miniature end-plate potential frequency measured in the neuromuscular junctions of mice,<sup>25,40</sup> and in having apparent differences in the number of VGCCs observed morphologically or pharmacologically in various tissues from patients.<sup>41,42</sup> These differences may result from separable, disease-specific recognition sites on calcium channels that mediate opposite functional effects, just as different antibody-binding sites on the thyroid-stimulating-hormone receptor mediate hyperthyroidism or hypothyroidism.<sup>43</sup>

One concern with regard to our data is whether antibodies from the serum of patients with amyotrophic lateral sclerosis that are cross-reactive with L-type VGCCs have a primary role in the pathogenesis of the disease or are due to the destruction of motor neurons or to denervation. Two observations favor a primary role: serum from patients with familial amyotrophic lateral sclerosis does not appear to possess such antibodies, even though the degree of denervation and motor-neuron destruction is comparable for both the familial and idiopathic forms of the disease; and antibody titers to L-type VGCCs correlate with the rate of disease progression but not with the stage of disease in patients with amyotrophic lateral sclerosis. In electrophysiologic experiments with L-type VGCCs isolated from skeletal muscle, antibodies from patients with amyotrophic lateral sclerosis reacted only with the extracellular portion of the calcium channel.<sup>44</sup> If the production of these antibodies was due to the destruction of motor neurons, one might anticipate the occurrence of reactivity to intracellular- and extracellular-channel epitopes. However, the presence of L-type VGCC antibody in 66 percent of the patients with Lambert-Eaton syndrome and 20 percent of the patients with Guillain-Barré syndrome suggests that serum antibodies in amyotrophic lateral sclerosis may not lead directly to the death of motor neurons.

Other problems in the attempt to implicate calcium-channel antibodies in the pathogenesis of motor neuron disease include the use of L-type VGCC protein isolated from rabbit muscle, because of limitations in existing purification techniques, when amyotrophic lateral sclerosis is not known to affect muscle directly. Neuronal N-type VGCCs have been localized to presynaptic terminals of motor neurons,<sup>45</sup> and electrophysiologic experiments in our laboratories using a neuronal cell line that expresses central N-type but not L-type VGCCs<sup>46</sup> indicate that IgG from patients with

amyotrophic lateral sclerosis can alter N-type calcium currents. Since structural<sup>47</sup> and pharmacologic<sup>48</sup> similarities and immunologic cross-reactivity<sup>49-53</sup> have been demonstrated between some epitopes on different VGCCs, antibodies from patients with amyotrophic lateral sclerosis may interact with both N-type and L-type VGCCs at epitopes common to both channels. IgG from patients with Lambert-Eaton syndrome has been shown to block both N-type and L-type VGCCs.<sup>54</sup>

An apparently unresolved paradox concerns the mechanism by which the short-term partial blockade of VGCCs in vitro can later translate into enhanced miniature end-plate potential frequency, which is observed after the transfer of IgG from patients with amyotrophic lateral sclerosis to mice.<sup>25</sup> The key questions are whether intracellular calcium is increased and whether IgG binding to VGCCs changes the number of VGCCs, leads to covalent alterations (e.g., phosphorylation) of existing channels, or enhances the release of calcium from internal stores. Any of these mechanisms could increase the release of acetylcholine. Alternatively, IgG binding to VGCCs may enhance the internalization of IgG, which in turn could enhance the release of acetylcholine and initiate a cascade eventuating in cell death. Furthermore, it is possible that the reactivity of IgG from patients with amyotrophic lateral sclerosis with neuronal N-type or T-type VGCCs may differ from that observed with peripheral L-type VGCCs; for example, it may enhance rather than inhibit calcium currents.

Finally, the hypothesis that amyotrophic lateral sclerosis is an autoimmune disease raises numerous clinical points, among the most important of which is that standard immunotherapies used to treat autoimmune conditions have no proved benefit in this disease.<sup>55,56</sup> One possible explanation for the general ineffectiveness of immunosuppression is that by the time symptoms appear, the motor-neuron pool may be greatly reduced. This situation is analogous to that in autoimmune insulin-dependent diabetes: standard immunotherapies are ineffective by the time clinical disease is manifest, since there has already been extensive destruction of beta cells.<sup>57</sup> Moreover, once the destructive processes have been initiated, the loss of remaining viable motor neurons may be independent of the initial antibody attack. For some cells, this may be caused by alterations in cellular calcium homeostasis or other reactions potentially triggered by the presence of IgG in motor neurons. Certainly, detailed studies of the interaction of such antibodies with central neurons are required to establish whether their role is of primary or secondary importance in the pathogenesis of amyotrophic lateral sclerosis.

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