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Immunoglobulins from amyotrophic lateral sclerosis patients enhance spontaneous transmitter release from motor-nerve terminals

(neuromuscular junction/phrenic nerve–diaphragm preparation)

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ABSTRACT **Amyotrophic lateral sclerosis (ALS) is an incapacitating neuromuscular disease of unknown etiology. Although laboratory evidence is lacking, circumstantial evidence supports the importance of immune factors in the pathogenesis of ALS. In the present study immunoglobulins from 4 of 8 ALS patients induced a significant increase in spontaneous quantal transmitter release as monitored by miniature end-plate potential (MEPP) frequency in mouse phrenic nerve–diaphragm preparations at 23°C, whereas immunoglobulins from normal individuals and from patients with other neuromuscular diseases had no effect. At 32°C neither normal nor disease control immunoglobulins influenced MEPP frequency, but 8 of 11 ALS immunoglobulin samples produced a significant increase in spontaneous quantal transmitter release. The enhancing effect could be prevented by 10 mM Mg^{2+}. No effects were noted on MEPP amplitude and muscle resting potential. These findings suggest that the presynaptic terminal of the motor neuron may be an early target and that immunological factors may play an important role in the disease process.**

Amyotrophic lateral sclerosis (ALS) is a relentless incapacitating neuromuscular disease characterized by selective degeneration of lower and upper motor neurons. Despite intensive investigation, the etiology is unknown and effective therapy is lacking. Viruses (1–3), toxins (4), impaired aging (5), and altered tropic-factor function (6) have been implicated but not proven. There are no convincing laboratory demonstrations of specific antibodies in ALS. Recent attempts with cultured spinal neurons have been unsuccessful in documenting that ALS serum affects either cholinergic activity (7) or neurofilament protein expression (8). Furthermore, efforts to replicate binding of ALS serum globulins to a muscle protein have been unsuccessful using the recombinantly synthesized protein or the original extraction process (9–11).

However, autoimmunity still remains likely because of the increased incidence of associated autoimmune disorders (12) and the increased frequency of paraproteinemias (13) in ALS patients. Because of such circumstantial clinical evidence, we have continued our search for antibodies in ALS. We have investigated the effects of ALS immunoglobulins on spontaneous transmitter release in mouse phrenic nerve–diaphragm preparations. The present report provides evidence that immunoglobulins from ALS patients can alter spontaneous transmitter release from motor neurons in this *in vitro* assay system.

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MATERIALS AND METHODS

The experiments were performed with hemidiaphragms dissected after cervical dislocation from male BALB/c or Swiss mice weighing 25–30 g. The muscle was pinned to a small disc of Sylgard, which then was placed in an incubation chamber containing 5–10 ml of Krebs–Ringer solution (137 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgSO_4$/12 mM $NaHCO_3$/1 mM NaH_2PO_4/11 mM glucose, pH 7.4). The solution was oxygenated by continuous bubbling with a mixture of 5% CO_2 and 95% O_2. After the muscle was rinsed several times with Krebs–Ringer solution at room temperature, the final incubation solution containing ALS, control, or no globulins was added. After 4–5 hr at room temperature or 2 hr at 32°C the muscle and the incubation solution were transferred to the recording chamber. Control muscles incubated without immunoglobulins were always studied with an immunoglobulin-treated preparation. For that purpose two strips of muscles were obtained from the same hemidiaphragm. One was immediately placed in the 32°C bath while the other was maintained for 1 hr at room temperature before being placed in the 32°C bath. One of the two strips was incubated with immunoglobulins and the other without. An alternate choice was taken by one member of the technical staff. The bath temperature was continuously monitored with a thermistor placed immediately adjacent to the muscle and kept at the desired temperature within the range of ±0.5°C.

Intracellular recordings were made with glass microelectrodes filled with 3 M KCl of 5- to 15-MΩ resistance. The electrode was inserted into fibers near end-plate regions, which were located visually by the ends of intramuscular branches of the phrenic nerve. The electrode was connected to a high-input impedance amplifier and the output was fed to an oscilloscope (Tektronix 5113) and stored on a PCM videorecorder system (Medical Systems, Greenville, NY). Spontaneous miniature end-plate potentials (MEPPs) were analyzed by a computer (IBM PC, Tecmar Labmaster A/D converter).

Control experiments were carried out to assure the stability of the nerve terminal properties during the incubation and recording time. No differences were observed in MEPP frequency after 4–8 hr at 23°C or 4 hr at 32°C. The recording time was about 1 hr, during which 14–30 muscle fibers were studied in each muscle to obtain the mean MEPP frequency. The amplitude and frequency of MEPPs in each fiber were obtained during 60- to 180-sec recording periods. Slowly rising MEPPs like those described in botulinum-poisoned muscles (14) were infrequently observed in control as well as ALS-treated muscle fibers. Sub-MEPPs (15) were recognized in some recordings but the effect of ALS immunoglobulins on their characteristics was not studied systematically. Slowly

Abbreviations: ALS, amyotrophic lateral sclerosis; DC, disease control; MEPP, miniature end-plate potential.

rising MEPPs and sub-MEPPs were not included in the analysis.

Serum fractions were prepared from 17 ALS patients, 14 men (ages 39–72) and 3 women (ages 62, 64, and 72). The men had upper and lower motor-neuron disease and to a variable extent bulbar involvement of 9- to 43-month duration. All patients were evaluated at our center at Baylor and had undergone detailed clinical examination including history and physical examination and laboratory studies including electromyogram and muscle biopsy.

The disease control (DC) group consisted of 12 patients, 9 men (ages 40–64) and 3 women (ages 58, 60, and 63). Three patients had the post-polio syndrome with mild weakness, 2 patients had Eaton–Lambert syndrome with significant extremity and respiratory weakness, 2 patients had myasthenia gravis (1 of these with severe respiratory compromise), 2 patients had chronic inflammatory polyneuropathy with severe muscle weakness, 1 patient had diabetic motor and sensory neuropathy, 1 patient had Alzheimer disease, and 1 patient had motor and sensory neuropathy of unknown etiology.

The sera were heated at 56°C for 30 min, and the immunoglobulins were enriched by Rivanol (ethacridine lactate) precipitation followed by lyophilization followed by dialysis against Krebs–Ringer solution with no Ca (M_r 12,000 cutoff) and was subsequently stored after lyophilization (16). IgG was purified by protein A-Sephadex chromatography. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis demonstrated the presence of IgA, IgG, and IgM in the Rivanol-derived fractions and of IgG in protein A-derived fractions.

RESULTS

Transmitter release was studied after incubating the muscles for 4–5 hr at 23°C with or without immunoglobulins at 2 mg/ml. No differences were observed in MEPP amplitude in the presence or absence of globulins. ALS, normal, and DC samples all yielded comparable results with respect to MEPP amplitude (Table 1). Furthermore, none of the serum globulin samples affected the resting potential of muscle fibers.

The mean MEPP frequency of muscles in the absence of immunoglobulins was relatively constant among different preparations, with values ranging from 23–33 MEPPs per min at 23°C. The MEPP frequencies of muscles incubated with normal or DC immunoglobulins were similar to those incubated without immunoglobulins (Table 1). In contrast, a higher MEPP frequency was found in the group of muscles treated with ALS immunoglobulins. After removal of the ALS immunoglobulins, the enhanced MEPP frequency was reversible in three of the four cases, with values returning to the normal range.

Fig. 1*A* shows the average MEPP frequency recorded in muscles incubated at 23°C with normal, DC, and ALS immunoglobulins. Of the eight ALS immunoglobulins tested, four induced a significant increase of (50–120%) in MEPP frequency [$P < 0.005$, calculated by the nonparametric Mann–Whitney U test with the use of Tadpole software (17)].

Table 1. Resting potential and MEPP amplitude and frequency in untreated and immunoglobulin-treated muscle studied at 23°C

Immunoglobulins	Resting potential, mV	MEPP amplitude, mV	MEPP frequency, min^{-1}	n*
None	77.7 ± 0.5	1.93 ± 0.05	30 ± 1	149/9
Normal	80 ± 1	1.8 ± 0.1	31 ± 2	35/1
DC	78 ± 1	1.7 ± 0.1	29 ± 1	191/7
ALS	79.4 ± 0.5	2.0 ± 0.1	40 ± 4	324/8

Values (means ± SEM) were obtained after 4–5 hr of incubation at 23°C.
*No. of muscle fibers/no. of immunoglobulins.

The mean frequency of the four positive immunoglobulins was 49 ± 2 MEPPs per min (n = 205/4). Incubation of muscles with positive ALS immunoglobulins for longer periods (up to 7 hr) did not further increase the value of resting release. Furthermore, negative ALS immunoglobulins could not be made positive by prolonged incubation. However, dose–response curves were not carried out with all the negative ALS immunoglobulins and we cannot rule out the presence of enhancing effects at significantly higher doses of either ALS or control immunoglobulins.

The IgG fraction prepared from two of the positive ALS immunoglobulins was as effective in increasing the MEPP frequency as was the total immunoglobulin fraction (data not shown).

In search of a more sensitive assay, incubation and MEPP-frequency and -amplitude analysis were carried out at 32°C. MEPP frequency is clearly temperature-dependent, and the classical work on spontaneous acetylcholine release was carried out at 32°C (18). At this temperature MEPP frequencies are higher and more variable even in the absence of immunoglobulins (46–95 MEPPs per min). As a result, control muscles incubated without immunoglobulins were always studied with an experimental preparation. Immunoglobulins (2 mg/ml) from four normal individuals and five DC patients had no significant effect on MEPP frequency at 32°C (Table 2). In contrast, immunoglobulins from ALS patients increased MEPP frequency without affecting the muscle resting potential or the MEPP amplitude. Eight of the 11 ALS immunoglobulins tested produced a significant increase in MEPP frequency ($P < 0.005$) compared to normal and DC immunoglobulins (Fig. 1*B*). The mean MEPP frequency recorded from the muscles treated with those 8 positive immunoglobulin samples was 123 ± 10 MEPPs per min (n = 397/8) compared to 75 ± 5 per min (n = 223/8) recorded in the control untreated muscles. The distribution of MEPP frequency in untreated and in immunoglobulin-treated muscle fibers, and in particular positive ALS immunoglobulin-treated muscle fibers, could be fit to a normal Gaussian distribution. Dose–response curves were carried out with two positive ALS globulins. In one, a maximal effect was observed at 1–2 mg/ml with decreased effect at lower and higher concentrations. In a second case the effect was proportional to the concentration of globulins between 1 and 6 mg/ml.

The ratio between the MEPP frequencies recorded during the first half and the second half of the recording time of the experiments with positive ALS immunoglobulins was 0.99 ± 0.05 (n = 8), indicating that the stimulating effect had already developed within the first 2 hr of incubation. Most immunoglobulins were tested in two or more experiments, with equivalent values for MEPP frequencies and amplitude and muscle resting potential. Only two of the ALS immunoglobulins were tested at both 32°C and 23°C and at each temperature they increased the frequency of spontaneous transmitter release by 70–85%.

The frequency of the spontaneous release of transmitter is largely determined by presynaptic intracellular Ca^{2+} concentration (19–23). If extracellular Ca^{2+} did play a role in our experiments, increased Mg^{2+} would be expected to block the ALS immunoglobulin enhancement of transmitter release (24, 25). Four ALS immunoglobulins known to increase spontaneous resting in normal Krebs–Ringer solution were tested in Krebs–Ringer solution with 10 mM Mg^{2+} incubated at 32°C. The mean frequency of MEPPs of the four muscles treated with different ALS globulins in high Mg^{2+} was 54 ± 6, which is not different from a frequency of 60 ± 6 observed in four muscles incubated without immunoglobulins. Thus,

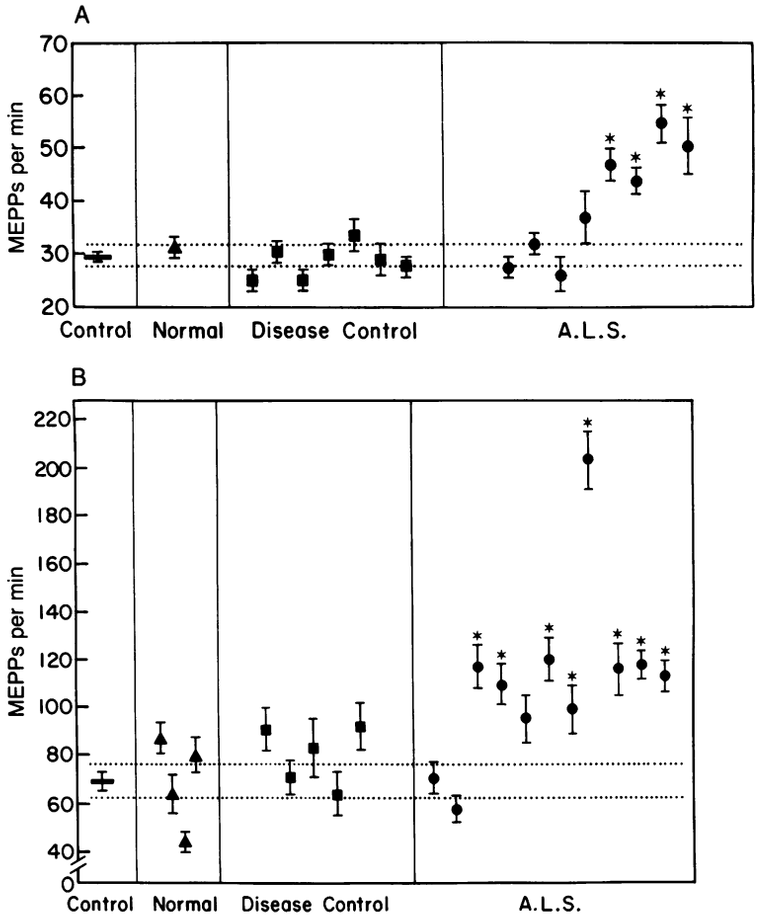

FIG. 1. Mean frequency of MEPPs recorded in muscle fibers incubated at 23°C (*A*) or 32°C (*B*) with no globulins (control; bar) or with normal (▲), DC (■), or ALS (●) globulins at 2 mg/ml. Area between the broken lines indicates the mean ± 2 times the SEM of the MEPP frequency of the muscle fibers incubated without globulins. Each point represents the mean MEPP frequency (± SEM) recorded in no less than 14 muscle fibers incubated in the presence of globulin from the same patient. DC globulins were obtained from patients with post-polio syndrome, chronic inflammatory neuropathy, myasthenia gravis, Eaton–Lambert syndrome, or diabetic neuropathy. Significant differences ($P < 0.005$) compared with control muscle fibers (incubated without globulins) are denoted by asterisks. The analysis of the variance of the ALS group compared with the normal plus DC group is significantly different, with a $P < 0.05$.

the transmitter release increased by ALS immunoglobulins can be prevented by increasing extracellular Mg^{2+}.

DISCUSSION

We have shown in two separate series of experiments that a number of ALS immunoglobulins enhance the spontaneous release of transmitter from the nerve terminals without affecting the postsynaptic receptors or the muscle membrane potential. The high ratio of positive ALS immunoglobulins found in the experiments performed at 32°C suggests that temperature increases the sensitivity of the assay. However, the effect of two positive ALS immunoglobulins studied at 23°C was not potentiated at a higher temperature. It remains to be shown whether ALS immunoglobulins that are negative at 23°C will become positive when tested at 32°C.

It is known that high external Mg^{2+} concentration reduces the frequency of MEPPs and the quantal content of the evoked potential by diminishing the influx of Ca^{2+} into the nerve terminal (24, 25). The blocking effect of Mg^{2+} on the enhancement of transmitter release produced by ALS immunoglobulins suggests that Ca^{2+} entry may play a role in this phenomenon. However, a nonspecific action of Mg^{2+} on the membrane or on the immunoglobulin receptor site could also explain these results. Unfortunately, agents like Mn^{2+}, Co^{2+}, or verapamil, which are capable of blocking evoked release, are not suitable for testing this hypothesis because they induce large increases in MEPP frequency (26–28).

Table 2. Resting potential and MEPP amplitude and frequency in untreated and immunoglobulin-treated muscle studied at 32°C

Immuno-globulins	Resting potential, mV	MEPP amplitude, mV	MEPP frequency, min^{-1}	*n**
None	67 ± 2	1.5 ± 0.2	71 ± 7	80/4
Normal	66 ± 2	1.4 ± 0.2	69 ± 4	69/4
None	61 ± 2	1.7 ± 0.2	74 ± 5	77/5
DC	60 ± 2	1.6 ± 0.1	80 ± 5	82/5
None	63 ± 1	1.45 ± 0.09	70 ± 4	237/11
ALS	62.5 ± 0.9	1.5 ± 1	110 ± 10	438/11

Values were obtained after 2 hr of incubation at 32°C.
*No. of muscle fibers/no. of immunoglobulins.

With the two series of experiments combined, we have found a statistically positive result in 10 of the 17 ALS immunoglobulins. These samples were obtained from patients of different ages and at different times after the onset of the disease. One of the ALS patients with negative reactivity had a family history of ALS. Thus, 10 of 16 sporadic ALS patients, or 62%, were positive in our assay. Only 3 of the 16 patients were female, and 2 of these women gave negative results. Thus, 9 of 13 male sporadic ALS cases gave positive results. In comparing patients whose immunoglobulins were positive with those whose immunoglobulins were negative, there was a suggestive correlation with the degree of respiratory deficit as an index of the extent of motor-neuron disease. The 6 ALS patients with the most significantly positive immunoglobulin effects had a greater impairment of respiratory function than 5 ALS patients with negative immunoglobulins. In the former, vital capacity was 40 ± 7% of predicted (mean ± SEM), and in the latter, it was 68 ± 10% ($P < 0.025$).

The key question is whether our results with ALS immunoglobulins can shed light on the degeneration of motor neurons that is the hallmark of ALS. The significance of enhanced resting MEPP frequency *per se* is unclear and our data do not address the mechanism of upper motor-neuron compromise. It is possible that the increased MEPP fre-

quency is not the relevant variable but is an epiphenomenon that has allowed us to detect the antibodies. It is also possible that sustained levels of high intracellular Ca^{2+} leading to enhanced MEPP frequency could also be responsible for deleterious effects on motor neurons (29). However, since dying-back of the motor axon is not the main pathology in ALS (30), either the altered signal in the axon terminal is communicated to the soma or the immunoglobulins themselves could be transported back to the cell body. Either process could lead to overall cell dysfunction. Regardless of the specific mechanism, the demonstration that a high percentage of our ALS patients possess immunoglobulins that alter MEPP frequency suggests that the presynaptic terminal of the motor neuron may be an early target and that immunological factors may play an important role in the disease process.

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