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Serum from Patients with Amyotrophic Lateral Sclerosis Induces the Expression of B-50/GAP-43 and Neurofilament in Cultured Rat Fetal Spinal Neurons

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease affecting motor neurons in the spinal cord, brain stem, and cortex. Cultures of fetal rat spinal cord cells were used to test sera from ALS patients (ALS sera) on their ability to influence the expression of the neuron-specific phosphoprotein B-50/GAP-43. Neurons were treated with ALS sera, sera of age-matched controls (CON sera), or sera of patients with autonomic neuropathy (AUTO sera) and fixed after 24 or 96 h. The levels of B-50 and neurofilament (NF) protein were assayed with an enzyme-linked immunoadsorbent assay (ELISA). No toxic effects of the ALS sera were observed. It appeared that after 24 h, both B-50 and NF levels were elevated in the ALS sera-treated cells by 12 and 11%, respectively. After 96 h, the B-50 level was 19% higher than in CON sera-treated neurons, and the NF level was 29% higher. AUTO sera did not differ from CON sera. The stimulating effect of ALS sera was absent if the sera were heated at 56°C for 30 min. We conclude that ALS serum induces the expression of B-50 and the subsequent axonal outgrowth and maturation in vitro. This induction might be a reflection in vitro of the processes underlying the collateral sprouting responses observed in ALS patients.

Index Entries: amyotrophic lateral sclerosis; B-50/GAP-43; tissue culture; fetal spinal neuron; B-50 ELISA; neurofilament ELISA.

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INTRODUCTION

The etiology of amyotrophic lateral sclerosis (ALS), which is characterized by motor neuron death in the spinal cord, brain stem, and cortex, is still unknown. Many hypotheses concerning the cause of motor neuron death have emerged and many model systems have been used to test them. A wide variety of defects or deficiencies have been postulated as causes for ALS: immunological disturbances (Drachman and Kuncl, 1989), systemic (neuro)toxicity (Roisen et al., 1982), (chronic) intoxications, in particular with heavy metals (Garruto et al., 1989), motor nerve growth factor deficiency (Appel, 1981), neuronal enzymatic/metabolic disorders (Beach et al., 1986; Bradley et al., 1987), alterations in glutamate neurotransmission (Nunn et al., 1987), viral infections (Provinciali et al., 1988) and hormonal dysfunctions (Patten and Pages, 1984). None of these putative causes has been unequivocally linked to the etiology of ALS, and with model systems of cultured neurons contradictory results have been found. This holds especially for the model system of (spinal) neuronal cultures to which sera of ALS patients (ALS sera) were added. Some authors found neurotoxic effects of (some of) these sera (Digby et al., 1985; Doherty et al., 1986; Maher et al., 1987), whereas others were not able to detect any effect on a variety of neuronal properties, such as survival (Touzeau and Kato, 1983), neurotransmitter metabolism (Touzeau and Kato, 1986), electrophysiological membrane properties (Erkman et al., 1989), and fiber outgrowth (Ebendal et al., 1989).

So far, in vitro neuronal reactions to ALS sera have been monitored by assaying survival or fiber outgrowth. In order to assess the influence of ALS sera on spinal neurons with respect to their ability to form sprouts—a stage that precedes outgrowth—we tested whether these sera could induce the regeneration- and development-linked neuron-specific phosphoprotein B-50/GAP-43 (Skene and Willard, 1981; Andreasen et al., 1983; for reviews *see* Benowitz and Routtenberg, 1987; Skene, 1989; Gispen et al., 1990; Liu and Storm, 1990). B-50 is a prerequisite for sprouting and can be used as a sensitive marker for regenerative responses (Van der Neut et al., 1990). As controls we used sera of age-matched subjects (CON sera), not afflicted with neuropathies of any kind, and sera of patients with autonomic neuropathy (AUTO sera).

MATERIALS AND METHODS

Cell Culture

Dissociated fetal spinal neurons were cultured as described before (Van der Neut et al., 1990). In brief, spinal cords from 15-d-old rat fetuses were removed, freed from meninges and dorsal root ganglia, and dissociated enzymatically and mechanically. Cells were cultured in poly-(L-

lysine) coated 96-well microtiter plates (40,000 cells/well) in a medium consisting of Dulbecco's modification of Eagle's medium, supplemented with 5% horse serum (HS), 100 μg/mL streptomycin and 10 U/mL penicillin in a humidified atmosphere at 37°C. After 24 or 96 h the cultures were fixed.

Subjects

Sera of 16 ALS patients, 19 controls, and 5 patients with autonomic neuropathy were collected. The mean age of the ALS patients (2 female/14 male) was 52.1 y (range 31–77). As controls we used patients with migraine, disk prolapse (slipped disk), transient ischemic attack, or stenosis of the lateral recess. The mean age of the controls (8 female/11 male) was 56.3 y (range 38–71). Relevant details on the ALS patients are given in Table 1.

Sera

Venous blood samples from ALS patients or controls were taken at 8 AM. They were collected aseptically, allowed to clot at ambient temperature and spun down at 1000*g* for 15 min. The sera were collected, sterilized by filtration, aliquotted, and frozen at −26°C. Sera were added to the cultures 1 h after plating at a concentration of 5%. As a control condition we used cultures to which no human serum was added. In some cases the sera were decomplemented directly before use by heating them at 56°C for 30 min. Unless stated otherwise, the experiments were carried out with nondecomplemented sera.

Neuronal Survival

The neurons' viability was assayed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay described by Mosmann (1983).

B-50 ELISA

The B-50 ELISA was carried out as described before (Van der Neut et al., 1990), except that the cultures were fixed with 2% glutar-dialdehyde in PBS, instead of with methanol. Affinity-purified rabbit-anti-B-50 IgGs were produced as described elsewhere (Oestreicher et al., 1983). Cultured rat fibroblasts (B-50 negative cells), treated exactly as the neurons, were used as controls to account for nonspecific binding of antibodies to membrane constituents. Conjugate controls were determined by leaving out the first antibody in the ELISA procedure. The absorbance due to nonspecific antibody binding never exceeded 5% of the sample absorbance. B-50 levels were expressed as the percentage of the B-50 level in a control culture without human serum present.

Table 1
Characteristics of ALS Patients Participating in Study

ALS patient	Sex	Age (y)	Type[a]	Duration[b] (mo)	Survival[c] (mo)
1	female	60	U + L	12	>26
2	male	67	U + L	18	>32
3	male	63	U + L	11	12
4	male	31	U + L	24	>38
5	male	39	U + L	10	>24
6	male	68	U + L	33	>47
7	male	42	U + L	26	>40
8	male	45	U + L	72	73
9	female	35	U + L	36	>50
10	male	49	U + L	12	>26
11	male	54	U + L	25	>39
12	male	32	U + L	14	28
13	male	51	L	7	>21
14	male	77	U + L	31	>45
15	male	67	L	30	>44
16	male	54	L	7	>21

[a]U + L: upper and lower motor neurons affected at sample time; L: only lower motor neurons affected at sample time.
[b]Duration (in months) between first signs of ALS and sample time.
[c]Survival time (in months) as from first signs of ALS.

Neurofilament (NF) ELISA

The NF ELISA was carried out according to Doherty et al. (1984), with the monoclonal anti-NF antibody RT97 in a 1:2,000 dilution of ascites. The RT97 antibody recognizes antigenic determinants on the 200 and 155 kDa subunits of NF (Wood and Anderton, 1981). Controls and relative NF level calculations were as for the B-50 ELISA.

Statistical Analysis

For the statistical analysis of the data we used Mann Whitney's rank sum test.

RESULTS

Fetal rat spinal neurons were cultured, treated with ALS sera and assayed for NF and B-50 protein. Although negative effects of complement on neuronal survival have been recorded, we observed no light-microscopical signs of cytotoxicity (swelling, vacuolization, detachment). Moreover, the absence of cytotoxic constituents was supported by the MTT assay, which showed an increasing metabolic activity with increasing CON or ALS serum concentration (Fig. 1). In Fig. 2, the effect of the

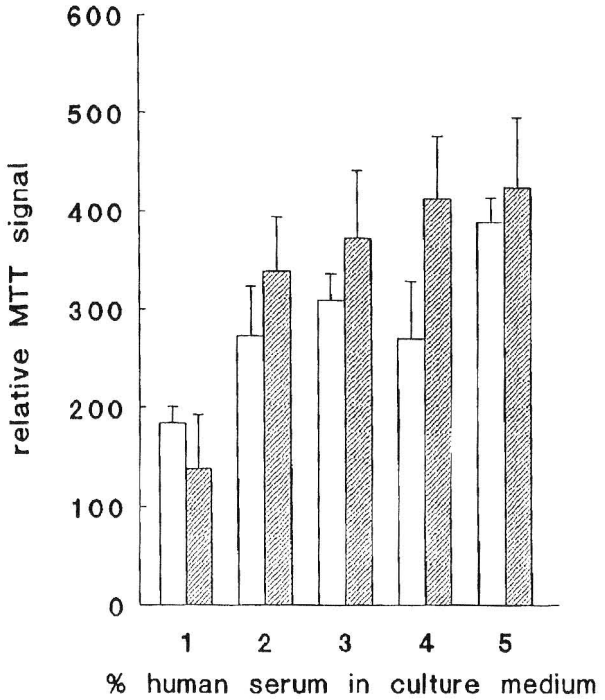

Fig. 1. The absence of neurotoxic effects of CON (open bars) and ALS sera (hatched bars) was demonstrated with the MTT assay described by Mosmann (1983). Dilutions of sera (1–5%) were applied to the cultures and after 96 h the mitochondrial activity was assayed.

sera on the B-50 content of the cultures is shown. It appears that the B-50 level is increased in both CON and ALS sera-treated cultures. As the neurons are cultured in a relatively poor medium (5% HS), this effect may be ascribed to a general suppletion with nutritional constituents. However, in cultures treated with ALS sera, the mean B-50 content is 12% higher after 24 h than those treated with CON sera ($P = 0.118$), and 19% higher ($P = 0.028$) after 96 h. AUTO sera do not differ from CON sera. In Fig. 3 the NF content of the spinal neurons is shown. As for B-50, the mean NF content is elevated in CON and ALS serum-treated cultures. After 24 h, NF levels are 11% higher in ALS sera-treated cultures than in CON sera-treated cultures ($P = 0.047$), and after 96 h this stimulating effect has increased to 29% ($P = 0.025$). The increase in stimulation is absent if AUTO sera are added to the cultures.

If the sera were exposed to heat treatment (56°C for 30 min), the stimulating effect of the ALS sera over the CON sera on B-50 and NF formation was abolished (Fig. 4).

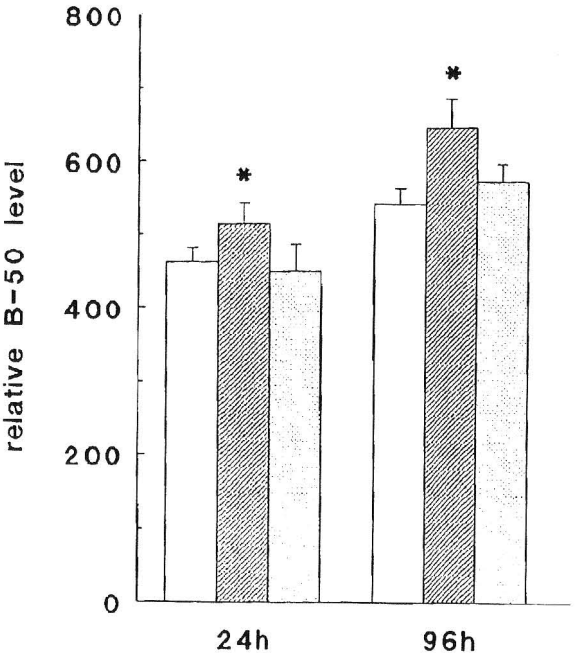

Fig. 2. Effect of CON (open bars), ALS (hatched bars), or AUTO sera (stippled bars) on the B-50 level in spinal neurons after 24 and 96 h in culture. B-50 was determined with an ELISA as described under Methods. Measurements were carried out in quadruplicate wells. Values given are means ± s.e.m. and are expressed as a percentage of the absorbance in cultures without human serum.

DISCUSSION

In the processes leading to reinnervation, three phases can be distinguished: (a) an early phase characterized by sprouting, (b) an intermediate phase in which axonal elongation and NF formation take place, and (c) a final phase characterized by the formation of synaptic contacts. We studied the effects of ALS sera on the early and intermediate phases in a quantitative way by measuring B-50 as a sensitive marker for sprouting, and NF as a measure for the subsequent neurite outgrowth. We have monitored the sprouting response and neurite elongation in cultured fetal spinal neurons under relatively poor culture conditions (DMEM supplemented with 5% HS), when neurite outgrowth is not maximal; thus it is still possible to detect the effect of outgrowth stimulating factors, that would, under optimal culture conditions, be masked by

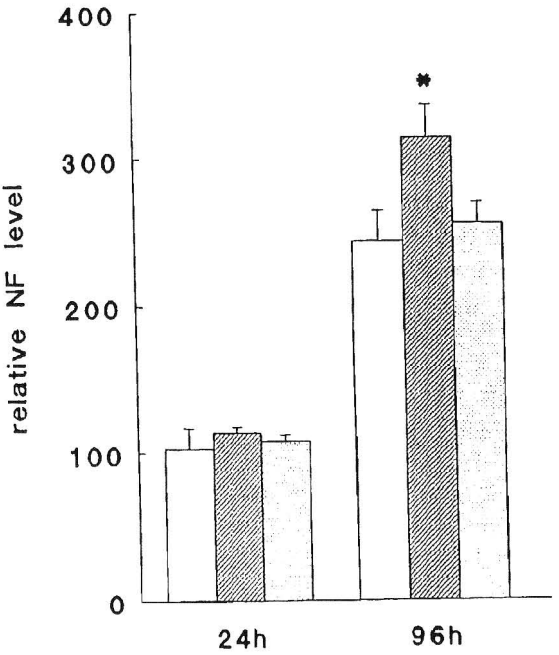

Fig. 3. NF in spinal neurons in culture, treated with CON (open bars), ALS (hatched bars), or AUTO sera (stippled bars) for 24 h or 96 h in culture as measured with an ELISA. *See* legend to Fig. 1 and text for more details.

other growth supporting constituents. Sera of ALS patients apparently contain a factor or factors, which, if present in cultures of fetal spinal neurons, induces the expression of B-50 as a first sign of sprouting (Fig. 2). This sprouting response is followed by neurite outgrowth and maturation, as demonstrated by the increased levels of the 155/200 kDa subunits of NF (Fig. 3).

The melanocortin peptides (αMSH, ACTH-fragments) exert a trophic effect on neurons both in a model system of peripheral nerve regeneration (Verhaagen et al., 1987) and in slice cultures of rat spinal cord (Van der Neut et al., 1988). Recently it was shown that the $ACTH_{4-9}$ analog, ORG2766, could induce collateral sprouting in partially denervated rat soleus muscle (De Koning et al., 1989). Although the extent of collateral sprouting in ALS is less than in some other motor neuron diseases (Stålberg et al., 1982), it is clear that collateral sprouting in ALS patients forms a prominent compensatory mechanism in the restoration of former contacts between nerve and muscle (Coërs et al., 1973). Electromyographic studies have shown that in ALS patients the fiber density, i.e., the number of innervated muscle fibers per axon, is increased during

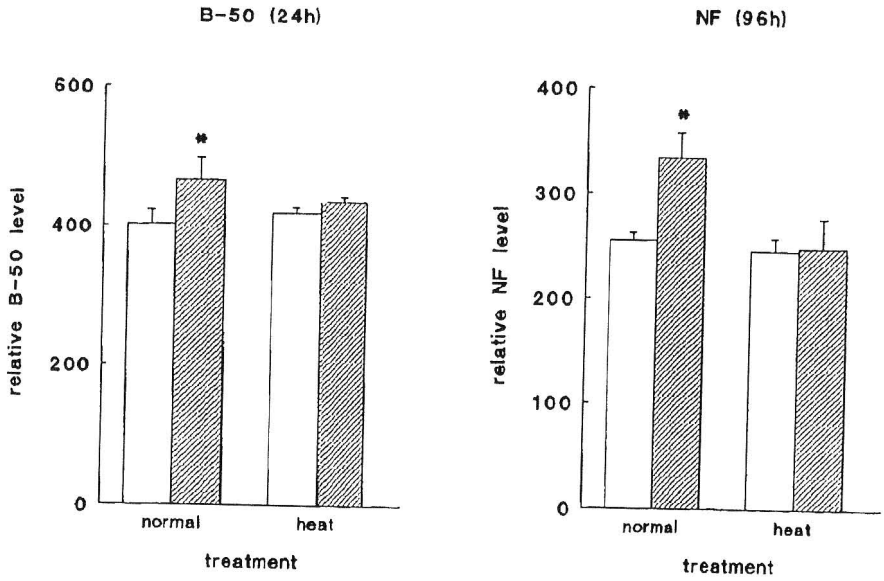

Fig. 4. Heat treatment (56°C, 30 min) of the sera abolishes the differences between CON (open bars) and ALS sera (hatched bars): The stimulating effect of ALS sera can no longer be observed. *See* legend to Fig. 1 and text for more details.

the course of the disease, which points to reinnervation processes (Carleton and Brown, 1979; Stålberg, 1982; Dengler et al., 1990). Furthermore, collateral sprouting has been shown to be present in muscle biopsies of ALS patients by histological techniques (Bjornskov et al., 1984). It is well established that denervated fibers in a muscle (Slack and Pockett, 1982; Nurcombe et al., 1984), or extracts of muscle (Appel et al., 1989; Iwasaki et al., 1989) contain soluble factors that can induce sprouting and nerve outgrowth in vitro, and it can therefore be inferred that factors produced by denervated muscle fibers in ALS patients account for the neurotrophic effect on our spinal cord cells. Although it was proposed that sprouting stimuli are restricted within a denervated muscle or even within a small portion of the muscle (Brown, 1984), the poor membrane integrity, as indicated by high carbonic anhydrase III levels in ALS patients (Väänänen et al., 1988), may allow factors from the muscle to enter the circulation. Another source of endogenous neurotrophic factors may be the degenerating nerve itself: it was shown that extracts of damaged nerves contain trophic activity (Politis and Spencer, 1983). Factors like those mentioned above could account for the trophic effect which we observed on rat spinal cord cells in vitro and might be responsible for the collateral sprouting processes in vivo. However, the observation that AUTO sera are not able to stimulate B-50- and NF-formation does not rule out the possibility that sera of patients with other diseases or trauma

affecting motor neuron-muscle functioning, which also leads to reinnervation in vivo, would have the same stimulating effect in vitro as have ALS sera.

The magnitude of the stimulating effect (20–30%) of ALS sera resembles the magnitude of other findings in regeneration: In the peripheral nerve regeneration system of crushed rat sciatic nerves mentioned above, Verhaagen et al. (1987) showed that αMSH treatment stimulated the number of sprouts to the same extent (about 30%), whereas the recovery of sensorimotor function by this peptide is greatly enhanced (Van der Zee et al., 1988). Also, Van der Neut et al. (1988) reported an effect of 30–40% in an in vitro study; this number apparently represents a biological maximum of outgrowth and NF formation under these conditions.

In conclusion, a possible consequence of motor neuron death in ALS patients may be the induction of target-derived (motor) nerve growth stimulating factors. Although the observation that heat treatment abolishes the stimulating effect of the ALS sera suggests that it is produced by a heat-labile polypeptide factor, the exact nature of these factors remains to be characterized.

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