Minocycline prevents neurotoxicity induced by cerebrospinal fluid from patients with motor neurone disease

Tiina M. Tikka,1 Nina E. Vartiainen,1 Gundars Goldsteins,1 Simo S. Oja,3 Peter M. Andersen,4 Stefan L. Marklund5 and Jari Koistinaho1,2

1A.I. Virtanen Institute for Molecular Sciences, 2Department of Clinical Pathology, Kuopio University Hospital, University of Kuopio, Finland, 3Brain Research Center, University of Tampere Medical School and Department of Clinical Physiology, Tampere University Hospital, Tampere, Finland, 4Department of Pharmacology and Clinical Neurosciences, Umeå University Hospital and 5Department of Medical Biosciences, Clinical Chemistry, Umeå University Hospital, Umeå, Sweden

Correspondence to: J. Koistinaho, A. I. Virtanen Institute for Molecular Sciences, University of Kuopio, PO Box 1627, 70211 Kuopio, Finland
E-mail: jari.koistinaho@uku.fi

Summary
CSF from patients with motor neurone disease (MND) has been reported to be toxic to cultured primary neurones. We found that CSF from MND patients homozygous for the D90A CuZn-superoxide dismutase (CuZn-SOD) mutation, patients with sporadic MND and patients with familial MND without CuZn-SOD mutations significantly increased apoptosis and reduced phosphorylation of neurofilaments in cultured spinal cord neurones when compared with the effects of CSF from patients with other neurological diseases. Exposure of spinal cord cultures to MND CSF also triggered microglial activation. The toxicity of MND CSF was independent of the presence of the CuZn-SOD mutation, and it did not correlate with gelatinase activity or the presence of immunoglobulin G autoantibodies in the CSF. The concentrations of glutamate, aspartate and glycine in MND CSF were not elevated. Antagonists of N-methyl-D-aspartate (NMDA) and z-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate receptors prevented the toxic CSF-induced neuronal death but not microglial activation, whereas minocycline, a tetracycline derivative with anti-inflammatory potential independent of antimicrobial activity, reduced both the apoptotic neuronal death and microglial activation. We conclude that the cytotoxic action of CSF is prevalent in all MND cases and that microglia may mediate the toxicity of CSF by releasing excitotoxicity-enhancing factors.

Keywords: amyotrophic lateral sclerosis; cerebrospinal fluid, primary cell culture; inflammation; excitotoxicity

Abbreviations: AMPA = z-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; DMEM = Dulbecco’s modified Eagle medium; D90A = aspartic acid → alanine mutation at codon 90 in the CuZn-superoxide dismutase gene; HS-HI = heat-inactivated horse serum; IgG = immunoglobulin G; MND = motor neurone disease; SOD = superoxide dismutase

Introduction
Amyotrophic lateral sclerosis or motor neurone disease (MND) is a devastating fatal syndrome characterized by the progressive neurodegeneration of primarily motor neurones, although other types of neurones may also be affected (Smith and Appel, 1995; Shaw, 1999). About 10% of cases of MND are familial and in 20% of these patients the presence of mutations in the CuZn-superoxide dismutase (CuZn-SOD) gene has been associated with the disease (Andersen et al., 1997; Robberecht, 2000). Mutations in some patients with apparently sporadic MND have been found, but the significance of the mutations is uncertain (Jackson et al., 1997). Overall, the disease mechanism in cases with and without CuZn-SOD mutations remains elusive (Shaw, 1999).

Evidence is accumulating that disturbed glutamate homeostasis plays a role in MND. Glutamate is an essential amino acid neurotransmitter in the mammalian CNS, but when its extracellular concentration increases above the normal level, overstimulation of receptors for N-methyl-D-aspartate
Minocycline and CSF neurotoxicity

All MND cases fulfilled the E1 Escorial criteria for probable or definite MND (Brooks, 1994) and all patients were diagnosed by the same physician. Consent was obtained from the patients according to the Declaration of Helsinki. The study was approved by the Medical Research Ethics Committee and the Animal Care and Use Committee of Kuopio University and followed the NIH guidelines for the humane care of animals. Briefly, CSF was collected during 1995–1997 by lumbar thecal puncture into sterile tubes made of polystyrene (Cerbo, Trollhättan, Sweden) without additives. The tubes were immediately stored at –80°C until they were sent to the University of Kuopio for cell culture experiments, which were carried out during 1997–1998. The CSF samples were thawed and quickly divided into aliquots of 100 μl (without additives) in sterile Eppendorf tubes on ice and stored at –80°C until analysis.

Primary spinal cord cultures

The neurotoxicity of CSF samples from five MND patients homozygous for the D90A CuZn-SOD mutation, five patients with familial MND, 16 patients with sporadic MND (all without CuZn-SOD mutations) and 24 control patients with other neurological diseases (Table 1) was screened in rat spinal cord cultures. These cultures were prepared from 14-day-old embryos. The spinal cord tissues were minced, trypsinized [0.25% trypsin-EDTA (ethylene diaminetetraacetic acid)] and resuspended in high-glucose Dulbecco’s modified Eagle medium (DMEM) (Life Technologies, Roskilde, Denmark) containing 10% FBS (foetal bovine serum) and 10% heat-inactivated horse serum (HS-HI). A single-cell suspension was prepared and the cells were cultured on poly-L-lysine-coated 96-well plates (1 × 10^5 cells per well) maintained at 37°C in a 7.5% CO_2 incubator. The medium was changed on the following day to DMEM containing 5% FBS and 5% HS-HI. After 4 days in vitro, 5 μM cytosine β-D-arabinofuranoside (Sigma, St Louis, Mo., USA) was added for 24 h to inhibit the growth of non-neuronal cells. This procedure results in mixed spinal cord cultures consisting of neurones (70%), astrocytes (25%) and a few other non-neuronal cell types, including microglia (5%). These neurones express functional glutamate receptors of different types (Vartiainen et al., 1999).

Initial screening of CSF samples

At day 7, cultures were exposed to each individual CSF sample for 24 h. The exposure medium contained 25% CSF in DMEM + 1% HS-HI. After the exposure, the cultures were fixed with 4% paraformaldehyde, rinsed in 0.1 M phosphate-buffered saline and incubated with the nuclear binding dye bis-benzimide (5 μg/ml, Hoechst 33342; Sigma) for 5 min to detect fragmented and condensed nuclei, markers of apoptotic cell death. The apoptotic (fragmented chromatins) and preapoptotic (condensed nuclei) neurones were counted from 10–15 random fields of 4 × 10^3 mm² per well and from four to six wells per treatment using an inverted fluorescence microscope (Nikon Diaphot 300; Nikon, Tokyo, Japan).

Subjects and methods

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equipped with appropriate filter sets; the numbers were expressed as a ratio with respect to total cell number. The bisbenzimide-stained cultures were also processed for immunohistochemical analysis of neurofilament phosphorylation using monoclonal antibodies specific for phosphorylated and non-phosphorylated medium- and high-molecular weight neurofilaments (SMI-31 and SMI-32, mouse monoclonal; dilution 1 : 5000; Sternberger Monoclonals, Baltimore, Md., USA). After 48 h of incubation with the primary antibodies at 4°C, antibody binding was visualized using fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G (IgG) (dilution 1 : 70; Jackson ImmunoResearch, West Grove, Pa., USA) as secondary antibody. We counted the immunoreactive cells from six to 10 random fields of 1.5 × 1.2 mm² area per well from four to six wells per treatment using a Nikon Diaphot 300 inverted fluorescence microscope equipped with appropriate filter sets.

**Mechanism of CSF toxicity**

After the initial screening for CSF toxicity, we carried out another set of experiments in which the spinal cord cultures were exposed for 24 h to 25% of control CSF and CSF pooled from the MND CSF samples. Some of these cultures had been pretreated with 10 μM MK-801, a non-competitive NMDA receptor antagonist (Tocris Cookson, Bristol, UK), 50 μM CNQX (6-cyano-7 nitroquinoxaline-2,3-dione), an AMPA/kainate receptor antagonist (Sigma) or 20 nM minocycline, a semisynthetic tetracycline derivative (Sigma). All compounds were dissolved in DMEM containing 5% HI-HS, which was also used as a zero control. After fixing the cultures with 4% paraformaldehyde, the proportion of apoptotic cells was determined using bis-benzimide staining as described above. Subsequently, the cultures were subjected to immunohistochemical analysis of neuronal survival using a neuron-specific NeuN antibody (mouse monoclonal, 1 : 100; Chemicon, Temecula, Calif., USA) and microglial proliferation using a CD11b antibody (mouse monoclonal antibody OX-42, 1 : 1500; Serotec, Oxford, UK). After 24 h of incubation with these primary antibodies at 4°C, the cultures were reacted with biotinylated anti-mouse IgG (1 : 200; Amersham International, Amersham, UK) for 2 h, rinsed in phosphate buffer for 30 min, then incubated with avidin–biotin complex (1 : 200; Vector Laboratories, Burlingame, Calif., USA) for 2 h. After careful washing in phosphate buffer, diaminobenzidine (0.2 mg/ml; Sigma) was used as a substrate for the colour reaction, which was visualized with 0.3% H₂O₂. The number of immunoreactive cells was quantified as described above for neurofilament immunoreactivity.

**Table 1  Characteristics of test subjects**

<table>
<thead>
<tr>
<th>MND patients</th>
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<th>Control subjects</th>
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<tr>
<td>Sex</td>
<td>Age (years)</td>
<td>Diagnosis</td>
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<tr>
<td>M</td>
<td>48</td>
<td>D90A</td>
</tr>
<tr>
<td>M</td>
<td>62</td>
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<tr>
<td>F</td>
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<tr>
<td>F</td>
<td>80</td>
<td>sMND</td>
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</tbody>
</table>
| M | 59 | sMND | 18 | + = alive; F = female; M = male; fMND = familial MND; sMND = sporadic MND; MS = multiple sclerosis.
Analysis of CSF

The autoreactivity of CSF samples against neural proteins was screened by western blotting using human spinal cord homogenate as antigen. A protein sample of 50 mg was separated by 10% SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) and electrophoresis was performed for 80 min at a constant voltage of 150 V. The separated protein bands were blotted onto polyvinylidene difluoride membrane that had been pretreated with 5% skimmed milk in Tris-buffered saline for 30 min, and the separated protein bands were blotted onto polyvinylidine membrane was cut into slices 3 mm wide. The membrane slices were incubated with 20% CSF in Tris-buffered saline containing 0.2% Tween for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase-labelled anti-IgG (Amersham International) for 2 h. Diaminobenzidine (Sigma) was used as the substrate for the colour reaction.

The amino acid concentrations of freshly thawed CSF samples were measured by ion-exchange chromatography using o-phthalaldehyde derivatization and an automated amino acid analyser (Pharmacia Biotech; Biochrom, Cambridge, UK) equipped with fluorescence detection at the highest sensitivity. (Each CSF sample contained 1-2,4-diaminobutyric acid as internal standard.)

The activities of matrix metalloproteases 2 (gelatinase A) and 9 (gelatinase B) were measured from 10 μl samples of CSF using gelatin [0.15% porcine skin gelatin (Sigma) in 8% sodium dodecyl sulphate] zymography. After electrophoresis, the gels were washed, renatured and incubated in collagenase buffer (21 mM Tris–HCl, pH 7.6, 10 mM CaCl₂, and 0.04% NaN₃) for 20 h at 37°C before single-step staining with Phast-Gel Blue R (Pharmacia Biotech).

Statistical analysis

Statistical significance was evaluated by analysis of variance and the two-tailed t-test. P < 0.05 was considered significant.

Results

Initial screening

The proportion of neurones with DNA fragmentation typical of apoptosis was dramatically increased in the presence of CSF from the D90A MND (9.5 ± 6.7%), familial MND (11.3 ± 5.8%) and sporadic MND (9.8 ± 5.6%) patients compared with the presence of CSF from control patients (1.8 ± 0.6%) (Figs 1 and 2). The neurotoxicity of the CSF was associated with dephosphorylation of neurofilaments in neuronal cell bodies, as the neurofilament phosphorylation index for control CSF-treated cultures was 4.4 ± 2.1; it was 1.3 ± 0.5 for D90A MND CSF-treated cultures, 1.2 ± 0.4 for familial MND CSF-treated cultures and 1.7 ± 0.6 for sporadic MND CSF-treated cultures (Figs 1 and 2). The results from these screening experiments indicate that CSFs from patients with the D90A CuZn-SOD mutation, sporadic MND or familial MND without CuZn-SOD mutations were equally toxic. The toxicity of CSF did not correlate with patient survival time, even though in two cases of D90A CuZn-SOD with survival times of 144 and 264 months the apoptosis percentages were 2.28 and 6.33%, respectively, considerably below the mean value of this patient group (9.5%).

Mechanism of CSF toxicity

When the CSF pooled from all MND patients was analysed, neuronal cell counts revealed a 34% reduction in surviving neurones and a clear increase in the percentage of apoptotic neurones (Fig. 3A), as expected after the initial screening studies. The neurotoxicity of the CSF was associated with proliferation of microglial cells (Fig. 3B), which increased in number (cells per 1.5 × 1² mm² area) from 22 ± 1.2 in control CSF-treated cultures to 32.1 ± 2.1 in MND CSF-treated cultures. Because our previous studies had shown that excitotoxicity in the same cell culture model was preceded by neurofilament dephosphorylation and increased by preceding activation of microglia (Vartiainen et al., 1999; Tikka et al., 2001), we tested the effect of glutamate receptor antagonists on CSF toxicity. Administration of 10 μM MK-801, an antagonist of NMDA glutamate receptors, and 50 μM CNQX, an antagonist of AMPA/kainate glutamate receptors, prevented the neurotoxicity of MND CSF (Fig. 3A) but not microglial proliferation (Fig. 3B), suggesting that ionotropic glutamate receptors mediate the neuronal death downstream or independently of microgliosis.
CSF analysis
The concentrations of glutamate (1.88 ± 0.24 μM in MND and 2.44 ± 0.70 μM in controls), aspartate (3.16 ± 0.26 μM in MND and 3.58 ± 2.50 μM in controls) and glycine (490 ± 211 μM in MND and 475 ± 137 μM in controls) were not significantly different in MND and control CSF samples.

Gelatin zymography showed a high level of variation of matrix metalloprotease 2 (gelatinase A) activity in CSF among the patients and no significant difference between MND and control patients was seen (arbitrary scanning units in a 10 μl sample: 66.5 ± 37.7 in controls and 46.1 ± 16.7 in MND; P > 0.05, two-tailed t-test). Matrix metalloprotease 9 (gelatinase B) was detected in one MND patient.

Western blotting using human spinal cord tissue as the antigen source detected IgG autoantibodies in 23% of all CSFs without correlation with toxicity.

Together, these studies ruled out the direct role of endogenous glutamate, aspartate and glycine, as well as gelatinases and IgG autoantibodies, in CSF neurotoxicity. Because minocycline reduces excitotoxicity by inhibiting microglial activation (Tikka et al., 2001; Tikka and Koistinaho, 2001), we studied the effect of minocycline on the neurotoxicity of CSF. We found that administration of 20 nM minocycline completely inhibited microglial proliferation and apoptotic neuronal death induced by toxic CSF (Fig. 3).

Discussion
These results extend previously published studies by showing that CSF from MND patients with and without CuZn-SOD mutations is toxic to spinal cord neurones and that this toxicity of MND CSF is associated with microglial proliferation, neurofilament dephosphorylation and the apoptotic death of neurones. Activation of both NMDA and AMPA/kainate receptors is required for toxicity, even though the concentrations of the endogenous excitatory amino acids glutamate, aspartate and glycine are low in MND CSF.

Our findings indicate that toxins other than glutamate, aspartate and glycine are present in MND CSF and that these toxins are able to activate ionotropic glutamate receptors directly or indirectly, leading to neuronal death. Because antagonists of NMDA or AMPA/kainate receptors did not inhibit microglial proliferation but minocycline blocked
microglial proliferation and provided neuroprotection. Microglial proliferation is not secondary to neuronal death. Our previous studies have shown that microglial proliferation precedes and contributes to excitotoxic neuronal death in spinal cord cultures, and that minocycline, which is not known to interfere with glutamate receptors, provides neuroprotection by inhibiting microglial proliferation and activation (Tikka et al., 2001; Tikka and Koistinaho, 2001). Together, our present and previous experiments suggest that MND CSF includes molecules that trigger microglial proliferation and the release of microglial toxins that bind and activate ionotropic glutamate receptors. Alternatively, toxic molecules in MND CSF may act simultaneously on microglia and ionotropic glutamate receptors on neurones and inhibition of either effect reduces the neurotoxicity of CSF. This toxicity of MND CSF was not correlated with the presence of IgG autoantibodies to human spinal cord proteins, excluding the direct contributory role of autoimmunity (La Bella et al., 1997). Neither did extracellular matrix metalloproteinases, which have been shown to be increased in MND tissues (Lim et al., 1996), explain the toxicity of MND CSF in the present study.

In agreement with our findings, previous studies have demonstrated that exposure to MND CSF induces neurodegenerative changes in chick spinal cord cultures (Nagaraja et al., 1994) and reduces the number of surviving neurones in cultures of rat cortical neurones (Couratier, 1993). In the cortical culture model, administration of NMDA receptor antagonists had no effect, whereas treatment with AMPA/kainate receptor antagonists blocked the toxicity (Couratier et al., 1993), which conflicts with our finding that receptors of both AMPA/kainate and NMDA are involved in the MND CSF-induced death of spinal cord neurones. In general, the role of different glutamate receptors in the death of motor neurones is controversial. Selective sensitivity of spinal motor neurones to the activation of AMPA/kainate receptors has been demonstrated in slice culture models (Kaal et al., 2000; Rothstein et al., 1993) and in mixed spinal cord neuronal cultures (Carriedo et al., 2000), whereas numerous other studies suggest that NMDA receptors mediate glutamate-induced motor neurone death in the spinal cord and that NMDA receptors are also involved in the excitotoxic death of cortical motor neurones (Choi et al., 1988; Regan and Choi, 1991; Fryer et al., 1999; Van Den Bosch and Robberecht, 2000; Van Westerlaak et al., 2001; Tikka and Koistinaho, 2001; Urushitani et al., 2001). A recent study by Van Den Bosch and Robberecht (2000) demonstrated that NMDA receptors are responsible for spinal motor neurone death induced by both short-term and long-term exposure to glutamate. In the same study, motor neurone death mediated by AMPA receptors became apparent only when desensitization of these receptors was prevented. Thus, the roles of different glutamate receptors in neuronal death seem to depend on the experimental protocol and the cell culture model used. For example, it should be noted that, whereas Couratier et al. (1993) used cortical cultures with a neurone : glia ratio of 95 : 5, our cultures were prepared from the spinal cord and had a neurone : glia ratio of 70 : 30. Considering that Shahani et al. (1998) reported reactive astrogliosis in neonatal rat spinal cord after exposure to MND CSF and that we found some evidence for a putative role of microglia in MND CSF toxicity, the different percentages of glial cells present in cultures may well contribute to the discrepancy in NMDA receptor-mediated neurotoxicity (Vandenbergh et al., 1998; Tikka and Koistinaho, 2001).

Neural tissues develop microglial activation and proliferation as a typical response to almost any kind of insult (Kreutzberg, 1996). An increased number of microglial cells and their transformation into phagocytic macrophage-like cells have been reported in all acute brain injuries and more...
chronic neurodegenerative diseases, including MND (McGeer and McGeer, 1995; Hirano, 1996; Yrjänheikki et al., 1998, 1999). In transgenic mouse models of familial MND, the time course of microglial activation parallels that of motor neurone loss (Hall et al., 1998; Almer et al., 1999). As microglia are known to release various neurotoxic molecules, such as excitatory amino acids, extracellular proteases, cytokines and free radicals such as nitric oxide (NO) (Kreutzberg, 1996), it is possible that these cells contribute to motor neurone death in MND. We have also shown previously that microglia significantly increase the excitotoxic death of cultured spinal cord neurones and, most importantly, that treatment with minocycline protects these cells from excitotoxicity by inhibiting microglial activation and proliferation (Tikka et al., 2001; Tikka and Koistinaho, 2001).

NO released by microglia could contribute to excitotoxic neuronal death in MND. Astrocytes and microglia both show induction of inducible NO synthase in transgenic mouse models of MND (Almer et al., 1999) and increased protein nitration has been found in both the sporadic and the familial form of MND as well as in a transgenic mouse model of the disease (Beal, 1997; Ferrante et al., 1997). Importantly, peroxynitrite, a reactive product of NO and superoxide, is able to induce tyrosine nitration of neurofilaments in motor neurones and glutamate transporter in astrocytes (Beckman et al., 1993; Trotti et al., 1996). Because minocycline inhibits the expression of inducible nitric oxide synthase at the mRNA and protein levels (Amin et al., 1996; Yrjänheikki et al., 1998), NO is a good candidate for mediating the neurotoxicity of MND CSF.

The cytotoxic factor in CSF is unknown and it remains to be shown that this factor is involved in the pathogenesis of MND in vivo. With this reservation in mind, it is interesting that CSF from sporadic and familial cases showed similar cytotoxic properties and that the effect was similar in the five MND cases homozygous for the D90A CuZn-SOD mutation. This suggests a common cytotoxic mechanism. The D90A CuZn-SOD mutation is, in the homozygous state, associated with very slowly progressive disease and a mean survival of 14 years, contrasting with the much faster disease progression rate reported for a few patients who were heterozygous for the D90A mutation (Andersen et al., 1996), most patients with other CuZn-SOD mutations and for MND patients without CuZn-SOD mutations (Haverkamp et al., 1995). To explain this paradox, we have proposed the existence of a modifying factor, which is co-inherited with the D90A CuZn-SOD allele and, through an unknown mechanism, reduces the cytotoxic effect of the D90A-mutated CuZn-SOD molecule (Andersen et al., 1996, 1997). A haplotype study of all known MND cases worldwide with the D90A allele supports the existence of such a factor (Al-Chalabi et al., 1998). The present results, showing an equal cytotoxic effect in all three patient groups, suggest that the putative modifying protective factor is not consistently present or active in the CSF.

MND CSF induced the accumulation of non-phosphorylated neurofilaments and a reduction in the amount of phosphorylated neurofilaments in neuronal cell bodies. This finding contrasts with reports of the accumulation of phosphorylated neurofilaments in MND motor neurones (Carpenter, 1968; Chou and Fakadej, 1971; Hirano, 1991; Figlewicz et al., 1994). Previous in vivo (Wang et al., 1992, 1994) and in vitro (Vartiainen et al., 1999) studies have demonstrated a similar dephosphorylation response to the administration of kainate, although an opposite response to kainate has also been reported (Hugon and Vallat, 1990; Terro et al., 1996). Because both in vivo and in vitro studies have indicated that dephosphorylation sensitizes neurofilaments to proteolysis by calpain, which is activated in neurones on stimulation of the NMDA receptor (Siman et al., 1989; del Cerro et al., 1994; Roberts-Lewis et al., 1994; Hell et al., 1996), and neurones resistant to kainate contain phosphorylated neurofilaments (Terro et al., 1996), it is possible that accumulation of phosphorylated neurofilaments is not an indication of severe injury of these particular cells, but rather an adaptation mechanism in surviving neurones (Vartiainen et al., 1999; Julien and Beaulieu, 2000).

Most importantly, our study shows that minocycline, an antibiotic that is widely used clinically, provides neuroprotection against MND CSF toxicity, which agrees with previous findings that minocycline reduces inflammation and neuronal death in ischaemic stroke in animals (Yrjänheikki et al., 1998, 1999), delays progression of Huntington’s disease in a transgenic mouse model (Chen et al., 2000) and is neuroprotective in animal models of brain trauma (Sanchez-Mejia et al., 2001) and Parkinson’s disease (He et al., 2001). Minocycline may be a new candidate for the treatment of ALS.

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