Chronic Inflammatory Demyelinating Polyneuropathy Sera Inhibit Axonal Growth of Mouse Dorsal Root Ganglion Neurons by Activation of Rho-Kinase

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Clinical course and prognosis are variable among patients with chronic inflammatory demyelinating polyneuropathy (CIDP), whereas the extent of axonal degeneration is the major prognostic factor. We studied the effects of sera from CIDP patients on axonal growth in cultured mouse dorsal root ganglion neurons. Compared with control sera, CIDP sera prominently suppressed axonal outgrowth of dorsal root ganglion neurons and shortened axonal length. The inhibitory activity was abolished by adding Y27632, a Rho-kinase inhibitor. These findings suggest that CIDP sera inhibit axonal elongation by Rho-kinase activation, and some serum factors may be responsible for development of axonal degeneration in CIDP.

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Chronic inflammatory demyelinating polyneuropathy (CIDP) is an immune-mediated neuropathy characterized by electrodiagnostic and pathological evidence of peripheral nerve demyelination.[1] It is likely that CIDP is a heterogeneous disorder, having a wide range of clinical expression ranging from subacute to chronic progression, and a monophasic disorder remitting to a chronic persistent or relapsing course. Therefore, outcomes are also variable among CIDP patients.[1–3] Whether specific clinical, electrodiagnostic, and laboratory features are associated with prognosis of CIDP is not sufficiently understood, but previous studies have suggested that the extent of secondary axonal degeneration is the major prognostic factor in CIDP.[4,5] Extensive axonal loss evidenced by prominent muscle atrophy leads to little or no responses to immunomodulating treatments, and thereby insufficient clinical recovery.[6]

Peripheral axonal degeneration is usually associated with collateral sprouting and axonal regeneration, but clinical recovery in CIDP patients with axonal degeneration appears to be slow and incomplete, suggesting that some factors could inhibit axonal regeneration. For example, some myelin components such as myelin-associated glycoprotein (MAG) trigger the inhibitory signal on neurite axonal growth via the Rho-kinase pathway, which is one of the major mechanisms for the lack of ability of axonal regeneration in the central nervous system, and this inhibitory pathway is also present in the peripheral nervous system.[7–9] It is possible that after extensive peripheral nerve demyelination, fractions of MAG with the inhibitory activity or some other active peptides contained in patients' sera could suppress axonal regeneration by activation of the Rho/Rho-kinase pathway. We therefore studied whether serum factors in CIDP patients affect axonal growth in mouse dorsal root ganglion (DRG) neurons and whether Rho-kinase inhibition reverses the effects.

Subjects and Methods

Serum Samples

Serum was collected from eight CIDP patients (7 men; age, 34–64 years) in their initial progressive phase or at relapse. The patients' condition fulfilled published criteria.[2] Disease duration ranged from 2 months to 19 years. Atrophy in the distal muscles was present in four patients, mild in three patients (Patients 1–3 in the Table), and severe in Patient 8. Control sera were obtained from 5 healthy subjects and from 14 patients with multiple sclerosis (n = 5), neuromyelitis optica (n = 5), or vasculitic neuropathy (n = 4; see the Table). Patients with vasculitic neuropathy caused by allergic granulomatous angiitis showed prominent muscular atrophy and served as neuropathy control patients. Serum was stored at −80°C and inactivated at 56°C for 30 minutes before use.

Dorsal Root Ganglion Cultures

DRGs were removed from adult C57BL/6J mice and dissociated into single cells by incubation with 0.25% trypsin for 30 minutes at 37°C. Dulbecco's modified eagle's medium/F12 medium (Invitrogen Corporation, Carlsbad, CA) containing 10% fetal bovine serum was added, and the cells were centrifuged at 1,000 rpm for 5 minutes. Neurons were plated on poly-L-lysine–coated chamber slides.

Neurite Outgrowth Assays

For outgrowth assays, plated cells were stimulated 30 minutes in the presence or absence of Y27632, a specific inhib-

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Table. **Measurements of Neurite Length in the Absence or Presence of Rho-Kinase Inhibitor, Y27632**

Samples	Neurite Length (μm)		p^a
	Untreated	Y27632 Treated	
Control (no serum) (n = 3), mean (SEM)	101 (6)	96 (8)	NS
MAG-Fc (n = 3), mean (SEM)	46 (2)[b]	110 (10)	0.0001
Normal (n = 5), mean (SEM)	96 (4)	104 (2)	NS
CIDP (n = 8), mean (SEM)	49 (7)[b]	103 (10)	0.001
Patient no.[c]			
1 (2 mo)	50	140	
2 (4 mo)	24	77	
3 (5 mo)	48	72	
4 (8 mo)	40	99	
5 (14 mo)	30	78	
6 (14 mo)	56	137	
7 (15 mo)	59	134	
8 (228 mo)	90	94	
Vasculitic neuropathy (n = 4) Mean (SEM)	92 (7)	94 (12)	NS
Multiple sclerosis (n = 5), mean (SEM)	109 (9)	101 (10)	NS
Neuromyelitis optica (n = 5), mean (SEM)	92 (7)	102 (10)	NS

[a]Untreated versus treated. [b]$p < 0.01$ compared with normal serum. [c]Disease duration (months).
SEM = standard error of the mean; NS = not significant; MAG = myelin-associated glycoprotein; CIDP = chronic inflammatory demyelinating polyneuropathy.

itor of Rho-kinase (20μM; EMD Chemicals, San Diego, CA), and incubated for 24 hours. Then the cells were fixed in 4% paraformaldehyde in 0.1M phosphate-buffered saline and immunostained with Tuj-1 (1:1,000; Covance, Princeton, NJ) antibody recognizing the neuron-specific β-tubulin III protein. After staining, the length of the longest neurite for each β-tubulin III–positive neuron was determined. Where indicated, recombinant rat MAG-Fc chimera (25μg/ml; R&D systems, Minneapolis, MN) was added to the medium after plating.[10,11] Samples were added to the medium at the concentration of 10% (vol/vol) to examine the effect of CIDP serum. Photomicrographs of β-tubulin III–immunostained DRG neurons were captured using Image-pro software (Media Cybernetics, Bethesda, MD) from randomly selected fields. Neurite lengths were measured using the Scion image software (Scion Corporation, Frederick, MD). Then to examine whether CIDP sera cause axonal degeneration (shortening of axonal growth), we added the sera 24 hours after culture.

Statistical Analysis

Data are represented as the mean ± standard error of the mean of three independent experiments. Statistical analyses were performed using Student's *t* test for paired data. Median values among groups were compared with one-way analysis of variance followed by Tukey's multiple-comparison test.

Results

The neurite outgrowth assay allowed detailed morphometric analysis of individual neurons. DRG neurons derived from adult mice were cultured in chemically defined medium at low density, resulting in individual neurons without neuron's contacts (Fig 1). In a few hours, the neurons showed outgrowth of neurites. Neurite length in CIDP serum-containing cell cultures was clearly shorter than in normal serum-containing cell culture. Neurite outgrowth inhibition was also ob-

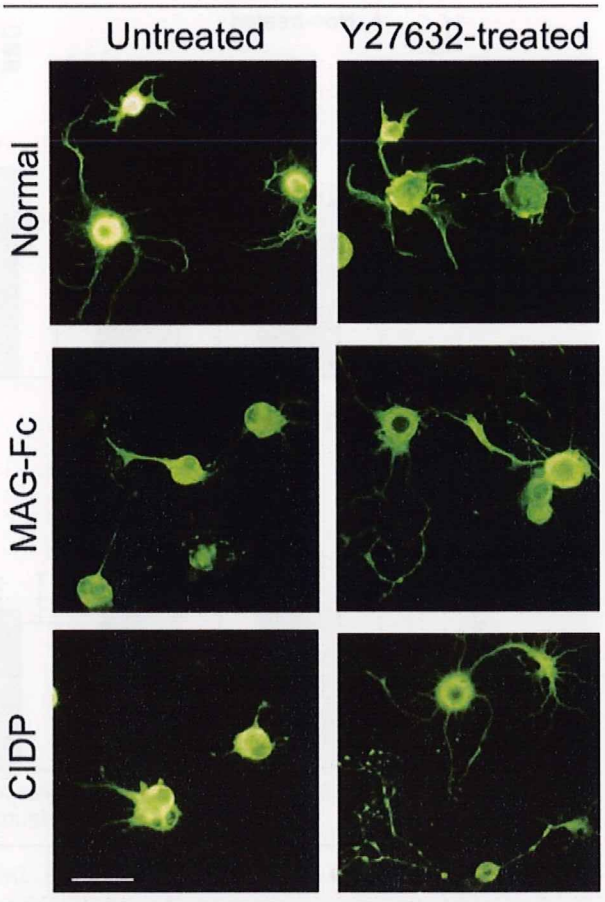

Fig 1. Effects of serum from chronic inflammatory demyelinating polyneuropathy (CIDP) patients on axonal growth of mouse dorsal root ganglion (DRG) neurons in the presence or absence of Y27632, a specific Rho-kinase inhibitor. Compared with normal serum, myelin-associated glycoprotein (MAG)-Fc (positive control) and CIDP serum suppress axonal elongation. The inhibitory effect is canceled by adding Y27632. Scale bar, 50 μm.

served in MAG-Fc treatment. Therefore, we tested whether the neuronal effects of CIDP serum are Rho-kinase–dependent using Y27632, a specific inhibitor of Rho-kinase. The inhibitory activity by MAG-Fc and CIDP sera was abolished by Y27632 (Fig 2), and the effects were similar at the concentration of 20 and 2μM.

The Table shows the effects of sera obtained from eight individual CIDP patients, compared with sera from normal subjects and disease control patients. Sera from all but one CIDP patient prominently suppressed axonal outgrowth. Patients 1 to 7 whose sera had the inhibitory effects had disease duration of 2 to 15 months. Patient 8 had long-standing CIDP (19 years) and severe muscle wasting in the four limbs; only this patient's serum did not affect the axonal growth. The CIDP group showed significantly shorter axonal length than the normal and disease control groups. The inhibitory effects of CIDP sera were canceled by adding Y27632, whereas normal human serum and inflammatory disease control sera from patients with multiple sclerosis, neuromyelitis optica, or allergic granulomatous angiitis had no significant effects on neurite outgrowth of DRG neurons, and they were not affected by Y27632.

To examine the effects of immunoglobulins contained in CIDP sera, we purified IgG and IgM from human sera using Melon Gel IgG Purification Kits (Pierce, Rockford, IL) and HiTrap IgM Purification HP (GE Healthcare, Little Chalfont, Bucks, UK), and depleted IgG from serum pools using protein G-agarose beads. Purified IgG and IgM, and IgG-depleted serum was used at a concentration of 10 and 20% in the culture medium for neurite outgrowth assay. The finding showed that IgG and IgM did not affect the neurite growth in our culture system.

Figure 2 shows the effects of sera added after 24-hour culture, when axonal growth developed. Sera from three CIDP patients (Patients 2, 4, and 5 in the Table) shortened axonal length prominently at 48 hours, whereas sera from patients with vasculitic neuropathy had no obvious effects on axonal growth.

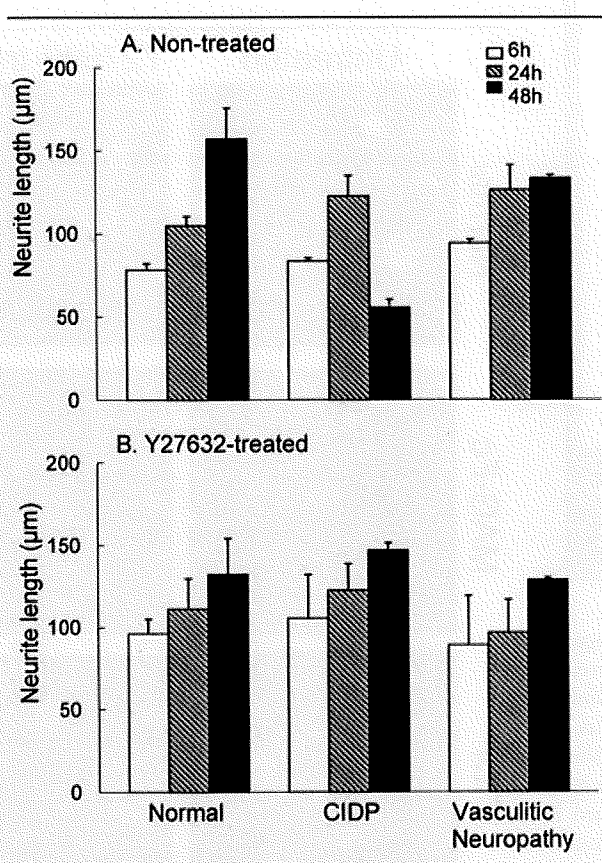

Fig 2. Measurements of the mean axonal length. Sera were added after culture for 24 hours. In contrast with sera from healthy control subjects (n = 3) and patients with vasculitic neuropathy (n = 3), adding sera from patients with chronic inflammatory demyelinating polyneuropathy (CIDP; n = 3) at 24 hours resulted in substantial shortening of axonal growth at 48 hours. Inhibition of neurite outgrowth by CIDP serum was blocked by adding Y27632 (Rho-kinase inhibitor). White bars *represent 6 hours;* hatched bars *represent 24 hours;* black bars *represent 48 hours.*

Discussion

Our results show that CIDP sera suppress axonal elongation of mouse DRG neurons, and this inhibitory effect was abolished by adding a Rho-kinase inhibitor, Y27632. Moreover, CIDP sera added after development of axonal elongation shortened the axonal length. Sera obtained from other inflammatory disease control subjects did not have such effects. These findings suggest that CIDP sera specifically inhibit axonal growth and degenerate axons, and the inhibitory activity is mediated by activation of the Rho-kinase pathway.

Rho-kinase is a serine/threonine protein kinase and one of the major downstream effectors of the small GTPase Rho. Several types of myelin-associated neurite outgrowth inhibitors trigger the inhibitory signals via the Rho/Rho-kinase pathway.[7–9] Rho activation by outgrowth inhibitors such as MAG and Nogo is mediated through $p75^{NTR}$ receptor, which enhances the dissociation of the Rho-guanine nucleotide dissociation inhibitor (Rho-GDI) from RhoA after stimulation by the neurite outgrowth inhibitors.[12] To elucidate the signal cascades of neurite outgrowth inhibition via the activation of Rho/Rho-kinase, the next step would be to test whether $p75^{NTR}$ is essential for neurite outgrowth inhibition induced by CIDP serum.[12]

Many studies have shown that Rho-kinase is involved in the pathogenesis of a variety of diseases. Therefore, the Rho/Rho-kinase pathway is considered to be a promising target for new pharmacological treatments.[13–16] Several types of Rho-kinase inhibitors have

been reported. Y27632 is in the category of 4-aminopyridine derivatives.[17] Y27632 inhibits both Rho-kinase I and II by competitively binding to the adenosine triphosphate binding pocket.[18–20] Another Rho-kinase inhibitor, fasudil, is safe and clinically available for treatment of cerebral vasospasm after subarachnoid hemorrhage and, therefore, is a candidate for treatment of axonal loss in CIDP patients.

Our results suggest that some substances in serum of CIDP patients inhibit axonal regeneration. Sera from seven of the eight CIDP patients, whose disease duration ranged from 2 to 15 months, clearly inhibited axonal growth. The inhibitory activity is possibly responsible for poor recovery from axonal loss in some CIDP patients. Serum from the remaining one patient with severe muscle atrophy and long-standing CIDP (19 years) did not show such inhibitory effects, but this patient's serum was tested after stabilization of symptoms for 10 years; therefore, disease activity itself could be low at the time of examination.

Our finding also showed that CIDP sera cause shortening of axonal length after development of axonal growth, and this cannot be explained by inhibitory effects of axonal growth mediated by MAG-triggered Rho-kinase activation. However, our results demonstrated that the shortening of axons was also reversed by adding Y27632, suggesting that the effects are also Rho-kinase dependent.

Molecules responsible for the inhibition are currently unknown, but candidates include inflammatory cytokines, and fragments of MAG or other myelin components that trigger inhibitory signals of axonal growth. Our findings could provide new insights into the pathogenesis of CIDP. Further studies will be required to identify the neurite outgrowth inhibitory factor in CIDP sera. Rho-kinase inhibition may be a new treatment option for CIDP patients with secondary axonal loss and resulting incomplete clinical recovery.

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