Prophylactic Treatment with Intravenous Immunoglobulin Attenuates Experimental Optic Neuritis in Mice

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Optic neuritis is characterized by optic nerve inflammation, demyelination and axonal loss. Intravenous immunoglobulin (IVIg) has been reported to be effective for steroid-resistant patients. However, there is no report investigating the histopathological efficacy of IVIg in optic neuritis models. In this study, we examined the effects of IVIg on optic neuritis of experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune optic neuritis (EAON). Inflammation, demyelination and axonal loss were assessed in the optic nerve sections. IVIg showed dose-dependent prevention of clinical symptoms in EAON. IVIg provided an anti-inflammatory effect in both EAE and EAON, associated with improved demyelination. Axonal loss in EAE was also significantly attenuated. These results suggest that IVIg has neuroprotective properties in experimental optic neuritis, and is a promising new treatment for optic neuritis.

Key words intravenous immunoglobulin; optic neuritis; neuroprotection; experimental autoimmune optic neuritis; experimental autoimmune encephalomyelitis

INTRODUCTION

Optic neuritis is an inflammation of the optic nerve, and is often observed in patients with multiple sclerosis (MS) and neuromyelitis optica (NMO).1,2 This is an acute and self-limited episode, resulting in temporary or permanent loss of vision via demyelination and axonal loss.3,4 For the treatment of optic neuritis, corticosteroids are used to hasten visual recovery.5 However, alternative options for the treatment are needed because of the existence of the patients with steroid-resistance.

Intravenous administration of immunoglobulin such as sulfonated immunoglobulin G (IgG) exerts anti-inflammatory and neuroprotective effects. It has been shown that intravenous immunoglobulin (IVIg) has pleiotropic actions on the immune system, including microbial and toxin inhibition, complement deactivation, receptor blockade, anti-idiotypic binding and modulating cytokine production.6,7 Other possible actions of IVIg are reported, including inhibition of antibody production, inhibition of B cell differentiation, restoring the balance between T-helper 1 (Th1) and T-helper 2 (Th2) cells and modulating cell migration.8 Clinically, IVIg has been an established treatment in neurological diseases such as Guillain–Barré syndrome (GBS), chronic inflammatory demyelinating polyneuropathy (CIDP), multifocal motor neuropathy (MMN) and myasthenia gravis (MG) in addition to Kawasaki disease (KD).9 Recent reports demonstrate that IVIg achieved visual improvement in the corticosteroid-refractory optic neuritis in MS.10 Given these reports, it is possible that IVIg will become an alternative option for the treatment of optic neuritis.

A Clinical trial investigating the effect of IVIg in optic neuritis patients is ongoing in Japan. However, there is little literature reporting efficacy of IVIg in optic neuritis models.11 In this study, to determine whether IVIg attenuates optic neuritis in mice, we performed histopathological analysis using experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune optic neuritis (EAON) models.

MATERIALS AND METHODS

Induction and Scoring of EAE and EAON Eight-week-old female C57BL/6J mice were purchased from Charles River (Yokohama, Japan). The method of EAE induction has been described previously.12 Briefly, at 9 weeks of age, mice were anesthetized with pentobarbital, and a total of 200 µg myelin oligodendrocyte glycoprotein peptide (MOG35–55; Qiagen, Tokyo, Japan) emulsified in Adjuvant Complete Freund (FCA; Becton Dickinson, GA, U.S.A.), containing 1000 µg Mycobacterium tuberculosis (MT; Becton Dickinson), was injected subcutaneously into the flank for immunization. In addition, 500ng pertussis toxin (PT; List Biological Laboratories, CA, U.S.A.) was injected by intraperitoneally on days 0 and 2 after immunization.

EAON was induced according to the method by Matsunaga et al.13 Briefly, mice were subcutaneously immunized with MOG35–55 emulsified in FCA containing MT and dimethyl
sulfoxide (DMSO) in the neck region, following the injection of PT. Each dose of MOG35-55, MT and PT injected in EAON mice was the same as in EAE mice. Severity of clinical symptoms was scored using a 9-point scale as follows: 0, no signs; 1, tail paralysis; 2, waddling gait; 3, partial limb paralysis; 4, paralysis of one limb; 5, paralysis of one limb and partial paralysis of another; 6, paralysis of two limbs; 7, moribund; 8, death; 9.

The experiment was conducted in a blind fashion. The total scores from days 12 to 45 were calculated.

All procedures were conducted according to protocols approved by the Chemo-Sero-Therapeutic Research Institute (currently KM Biologics Co., Ltd.) under the approval of the Animal Experiment Committee.

IV Ig Treatment Experiment 1: clinical symptoms in EAON were evaluated. To evaluate time course and dose-dependency of clinical symptoms in EAON, mice were administered with 100, 200, 400 and 800 mg/kg of IV Ig or an equal volume of saline for 5 d from days 0 to 4 after immunization.

Experiment 2: histopathological evaluation in both EAE and EAON was performed. Both EAE and EAON mice were administered with 800 mg/kg of IV Ig or an equal volume of saline for 5 d from days 0 to 4 after immunization. These doses were found previously to be effective against paralysis in EAE mice.

Histopathological Evaluation of Optic Nerves In experiment 2, on days 14 and 21 after immunization, optic nerves of both sides (five mice per group at each time point) were isolated, fixed in 10% formalin neutral buffer, embedded in paraffin, and cut in a coronal section. For histological analysis, sections were stained with hematoxylin and eosin (H&E) to detect inflammation, Klüver–Barrera (KB) for demyelination, and Bodian for axonal damage. Presence of inflammatory cell infiltration in the H&E specimens was assessed in a blind fashion according to previously published criteria: no lesion; 0, moderate cell infiltration; 1, strong cell infiltration; 2, massive cell infiltration; 3, Specimens of the three nonimmunized mice in the other experiment were also evaluated as the intact control. Immunohistochemical studies were performed using the antibodies against cell-specific markers: Iba1 (microglia), myelin basic protein (MBP; oligodendrocyte) and Glial Fibrillary Acidic Protein (GFAP; astrocyte). Sections were deparaffinized and rehydrated. Endogenous peroxidases were quenched with 10% H2O2 for 10 min. For Iba1 staining, antigen retrieval was performed using Target Retrieval Solution (Dako, Tokyo, Japan) at 100°C (microwave) for 15 min. For MBP and GFAP staining, sections were incubated with Protease (Nichirei Biosciences, Tokyo, Japan) at room temperature for 10 min. Nonspecific binding was blocked with normal goat serum (Nichirei Biosciences). The following primary antibodies were used: rabbit anti-Iba1 antibody (1:500; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), rabbit anti-MBP antibody (1:500; Nichirei Biosciences) and rabbit anti-GFAP antibody (1:500; Dako). Primary antibodies were applied overnight at 4°C. For secondary antibody, biotin-conjugated goat polyclonal anti-rabbit IgG (Nichirei Biosciences) was used. Peroxidase-conjugated streptavidin (Nichirei Biosciences) and 3–3′-diaminobenzidine tetrahydrochloride (DAB; Nichirei Biosciences) were used for visualization. Cell nuclei were stained with hematoxylin. After staining, one image containing whole bilateral optic nerves was acquired by a digital camera (DFC480; Leica, Tokyo, Japan). The whole cell surface area and the stained cell surface area were analyzed using Win ROOF 2013 (Mitani, Fukui, Japan). Data were expressed as the stained cell surface area per unit by dividing the stained cell surface area by the whole cell surface area.

Statistics SAS 9.2 (SAS Institute, Tokyo, Japan) was used for statistical analysis, Excel 2010 (Microsoft, Redmond, OR, U.S.A.) for graph generation. For the total scores of clinical symptoms from days 12 to 45 in EAON mice, Shirley–Williams multiple comparison test was used. For histopathological evaluation, Mann–Whitney U test was used. The Spearman rank order correlation was used to determine the association among the clinical symptoms and the histological scores. Differences were considered statistically significant at p < 0.05.
IVIg Treatment Prevents Clinical Symptoms in EAON

In experiment 1, time course and dose-dependency of clinical symptoms in EAON were evaluated. Mice treated with saline showed paralysis from approximately day 12 after immunization, peaking several days later, maintaining the high score until day 45 after immunization (Fig. 1). The time course of clinical symptoms in EAON was almost the same way as that in EAE. Treatment with IVIg significantly showed dose-dependent prevention of total scores of clinical symptoms compared to the control (Fig. 1). IVIg Treatment Reduces Optic Nerve Inflammation

In experiment 2, optic nerve sections stained with HE were examined for areas of inflammatory cell infiltration, and inflammation was graded on 0- to 3-point scale. Inflammatory cell infiltration in EAE on day 14 after immunization. Attenuated inflammation in IVIg-treated EAE on day 14 after immunization.
infiltration was detected in most optic nerves of both EAE and EAON mice, confirming the presence of optic neuritis (Fig. 2). Treatment with 800 mg/kg of IVIg in EAE and EAON significantly reduced inflammation compared to in saline-treated EAE and EAON mice, respectively, on both days 14 and 21 after immunization (Fig. 2). To further confirm cell inflammation, optic nerve sections were stained with Iba1 antibody, a microglia-specific marker. Treatment with 800 mg/kg of IVIg in EAE and EAON significantly decreased the Iba1-positive area per unit compared to in saline-treated EAE and EAON mice respectively, on day 14 after immunization (Fig. 3).

**IVIg Treatment Reduces Optic Nerve Demyelination**

Optic nerve demyelination begins after the onset of inflammation and can be detected by KB staining of myelin in optic nerves. Treatment with 800 mg/kg of IVIg in EAE significantly increased the KB-positive area per unit compared to in saline-treated EAE mice, on both days 14 and 21 after immunization (Fig. 4). To further confirm demyelination, optic nerve sections were stained with MBP antibody, an oligodendrocyte-specific marker. Treatment with 800 mg/kg of IVIg in EAON significantly increased the MBP-positive area per unit compared to in saline-treated EAON mice, on day 21 after immunization (Fig. 5).

**IVIg Treatment Reduces Axonal Loss in Optic Nerve**

To examine whether IVIg can protect from axonal loss, optic nerve sections were stained with Bodian. Treatment with 800 mg/kg of IVIg in EAE significantly increased the Bodian-positive area per unit compared to in saline-treated EAE mice, on both days 14 and 21 after immunization (Fig. 6).

**Effects of IVIg Treatment on Astrocytes**

To examine whether IVIg exerts an influence on astrocytes, optic nerve sections were stained with GFAP antibody, an astrocyte-specific marker. Treatment with 800 mg/kg of IVIg in EAE significantly decreased the GFAP-positive area per unit compared to in saline-treated EAE mice, on day 21 after immunization (Fig. 7).

**Correlation between Clinical Symptoms and Histopathological Grades**

We examined if the severity of clinical symptoms, optic nerve inflammation (H&E and Iba1), demyelination (KB and MBP) and axonal loss (Bodian) correlate with each other in EAE and EAON. Clinical symptoms were significantly correlated with H&E in EAE (r = 0.8537, p < 0.001) and EAON (r = 0.8239, p < 0.001), respectively. In EAE, Iba1 (r = 0.7611, p < 0.001), KB (r = −0.7672, p < 0.001), MBP (r = −0.5459, p < 0.05), and Bodian (r = −0.7739, p < 0.001) were significantly correlated with H&E, respectively. Meanwhile, in EAON, only Iba1 (r = 0.7328, p < 0.001) and MBP (r = −0.6361, p < 0.01) were significantly correlated with H&E, respectively.

**DISCUSSION**

Here, we show that IVIg attenuates experimental optic neuritis in EAE and EAON. This is supported by the findings that IVIg significantly attenuates the inflammation in the
optic nerve (Figs. 2, 3). In addition, demyelination and axon loss was also inhibited by IVIg (Figs. 4–6). The observed anti-inflammatory and neuroprotective effects of IVIg were consistent with findings in a variety of other diseases models. The protective effects of IVIg in the optic nerve are considered to result in the prevention of clinical symptoms because of the correlation between clinical symptoms and histopathological grades. This is the first report that IVIg attenuates inflammation, demyelination and axon loss in optic nerve in mouse models.

The neuroprotective effects of IVIg were slightly different between EAE and EAON. This may be due to the difference in histopathological time course. EAE is widely used as an animal model of MS. Although EAE is usually characterized by inflammation and neurodegeneration in the spinal cord or brain, optic neuritis also appeared. EAON is induced by inflammation and neurodegeneration in the spinal cord or brain, and Iba1 staining) was higher on day 14 than on day 21, the demyelination and axon loss were comparative between days 14 and 21. In EAN, the inflammation (Iba1 staining) was higher on day 21 than on day 14, the demyelination appeared on day 21 but not on day 14, and the axon loss was low on both days 14 and 21. These results suggest that the inflammation is followed by the demyelination and axon loss and that the histopathological changes in EAE appear faster than those in EAN. Thus, obvious neuroprotection of IVIg may be easily detectable in EAE.

The detailed mechanisms underlying the neuroprotection of IVIg remain unclear, but two possibilities can be considered. One is a direct neuroprotective effect of IVIg. The other is the anti-inflammatory effect of IVIg. Our data demonstrate that the inflammation, which is followed by the demyelination and axon loss, was suppressed by IVIg (Fig. 2). In early phase of EAE, microglia is known to induce the inflammatory response by secreting pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β, and nitric oxide. In this study, the Iba1-positive area on day 14 (early phase) was decreased by IVIg, suggesting that IVIg suppresses the migration of microglia (Fig. 3). In addition, IVIg is reported to decrease serum TNF-α level in EAE. On the other hand, microglia produces neurotrophic factor such as brain derived neurotrophic factor (BDNF), and acts as neuroprotective in the middle or late phase of EAE. No obvious suppression of IVIg on the Iba1-positive area on day 21 (late phase) was observed, suggesting the possibility that IVIg do not affect neuroprotective microglia. Collectively, neuroprotective effects of IVIg may be due to anti-inflammation including microglial suppression, and direct protection of the optic nerve. Further studies are needed to reveal the detailed mechanism of IVIg neuroprotection, and the microglia subtype-specific effects of IVIg.

IVIg decreased GFAP-positive area per unit, astrocyte-specific marker, on day 21 in EAE (Fig. 7). On day 21, the GFAP-positive area in the saline group was increased compared with that on day 14. Consistent with the previous reports, the inflammation was followed by increased GFAP expression in our EAE model, indicating that microglia induces the changes in astrocyte. Thus, the effect of IVIg on astrocyte may be due to the inhibition of microglia activation.

Prophylactic IVIg treatment is considered to be the limita-

tion in this study, and would let us investigate the therapeutic effect of IVIg in animal models. However, the findings that IVIg attenuates experimental optic neuritis in mice make it a promising option for the treatment of optic neuritis patients.

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