Original Article

Effect of Phosphodiesterase-3 Inhibition on Experimental Autoimmune Neuritis

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ABSTRACT

Background: The phosphodiesterase-3 inhibitor cilostazol (CLZ) is widely prescribed as an antiplatelet agent in treatment for peripheral arterial disease. The immune modulating effect of CLZ has been unveiled in experimental autoimmune encephalomyelitis, the animal model for human multiple sclerosis; however, the effect of CLZ on immune-mediated peripheral nerve disease is unclear.

Methods: Female Lewis rats were immunized with synthetic peptide from bovine P2 protein to induce experimental autoimmune neuritis (EAN). Ten or 30 mg/kg/day of CLZ was administered daily from 1 day post immunization (dpi) or after onset of paralysis. Immunohistochemistry and real-time PCR were studied on sequentially removed cauda equina (CE).

Results: Motor paralysis developed at 11 dpi in all rats; however, subsequent paralysis was suppressed by 30 mg/kg/day of CLZ. CLZ administered after onset of paralysis suppressed motor disturbance compared with sham-treated EAN rats. Improvement of motor function roughly correlated with shrinkage of demyelination foci and inflammatory cell accumulation. Real-time PCR analysis for proinflammatory cytokine interferon (IFN)-gamma, anti-inflammatory cytokine interleukin (IL)-10, or cell adhesion molecule E-selectin (E-sel) mRNA expression revealed an upregulation of IL-10 message before motor paralysis onset and suppression of IFN and E-sel messages at the paralysis onset phase.

Conclusions: CLZ treatment ameliorates EAN via IL-10 upregulation with reciprocal suppression of IFN and E-sel expression in peripheral nervous system.

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KEYWORDS: experimental autoimmune neuritis, phosphodiesterase-3 inhibitor, interferon-gamma, interleukin-10, E-selectin

Introduction

Experimental autoimmune neuritis (EAN), which is induced by immunizing model animals with various antigens,[1] serves as an animal model of human Guillain-Barré syndrome, the most common acquired demyelinating inflammatory peripheral neuritis in humans.[2] The immunological and pathological backgrounds of EAN have been well characterized in the context of elucidating those of experimental autoimmune encephalomyelitis (EAE), an

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animal model of human multiple sclerosis (MS) and a counterpart of EAN in the central nervous system. It has been shown that Th1 and Th17 cytokines play a crucial role in the development of EAN and that Th2 cytokine interleukin (IL)-10 is crucial for amelioration of EAN.[3, 4] Briefly, in disease developing stage, activated memory T cells infiltrate into the peripheral nervous system (PNS) prior to development of motor paralysis. These cells produce proinflammatory cytokines (interferon (IFN)-gamma, IL-1, tumor necrosis factor (TNF)-alpha) and chemokines in the PNS to attract and activate mononuclear phagocytes that finally cause demyelination of peripheral nerves, resulting in motor paralysis. At peak stage of motor paralysis, anti-inflammatory cytokine IL-10 is produced, which reciprocally inhibits proinflammatory cytokines, leading to spontaneous recovery.[3]

Chemokines and cell adhesion molecules collaborate with Th1 and Th17 cytokines,[5] and help many cell lineages to infiltrate from the blood stream to peripheral nerves during the development of, or recovery from EAN.[6] During this cell trafficking process, various cell lineages play their own roles, such as destruction of the myelin sheath, removing myelin debris, or facilitating regeneration. Thus, different cell lineages must cross the blood-nerve-barrier (BNB) at different disease stages depending on their specific roles.

Cyclic-AMP (cAMP) is an intracellular mediator with multifaceted roles, which is degraded by phosphodiesterase (PDE). Among the many roles of cAMP, suppressive roles for inflammatory cytokines are of special interest.[7] PDEs act as proinflammatory agents through degradation of anti-inflammatory cAMP in many tissues and increase during autoimmune inflammatory processes.[8] Thus, a number of experimental therapeutics based on PDE inhibition have been attempted in a variety of models.[9–12] There have also been many reports on PDE inhibition in EAE;[13–22] however, only a small number of experimental therapeutics regarding PDE inhibition for EAN have been reported.[23, 24]

PDEs comprise several subtypes. The pattern of activation of PDE subtypes differs depending on the immune-mediated neurological disorder, e.g., MS patients have a high serum concentration of PDE-3 compared with other subtypes of PDE.[8] It is expected that inhibiting a specific PDE subtype would have a unique immunomodulatory effect on various autoimmune diseases.

The effect of CLZ on EAE is already known, i.e., that CLZ suppresses EAE via cell adhesion molecule (CAM) downregulation;[19, 22] however, the effect of CLZ on EAN has not been reported. Thus, we attempted to investigate whether CLZ can suppress EAN as well, and, if so, to characterize cytokine and CAM expression in PNS sequentially.

Materials and Methods

Animals and induction of EAN

Female Lewis rats, 7 weeks old; (Charles River Japan, Yokohama), were kept in Toho University Ohashi Experimental Animal Laboratory according to the regulations and guidelines of Toho University Animal Experiment Committee. 150 μg of synthetic peptide (SP26: Operon, Tokyo) corresponding to the 53-78 amino acid sequence of the bovine myelin P2 protein was emulsified in complete Freund's adjuvant (CFA: Sigma-Aldrich, St. Louis, MO) and injected intradermally to the right footpads of the rats to induce EAN[25] under sevoflurane inhalation.

Cilostazol (CLZ) treatment

Experiment 1

We investigated the effects of different doses and timings of CLZ in experiment 1. CLZ was suspended in 0.5% carboxylmethyl cellulose (CMC) aqueous solution, administered daily via a feeding tube. Following immunization, CLZ 30 mg/kg/day (high dose group, n = 6) or CLZ 10 mg/kg/day (low dose group, n = 6) as well as CMC only (vehicle group, n = 11) was administered. Immediately after the onset of motor paralysis, several rats in the vehicle group were randomly selected and started receiving high dose CLZ administration (30 mg/kg/day : delay group, n = 5).

Experiment 2

The sequential histopathological and mRNA expression studies were investigated in experiment 2. CLZ was administered daily using a feeding tube, as described above. Following immunization, CLZ 30 mg/kg/day (high dose group) or CMC only (vehicle group, n = 25) was administered. Number of rats were, n = 25, 0-7 day post immunization (dpi), n = 20, 8-11 dpi, n = 15; 12-14 dpi, n = 10; 15-21 dpi, n = 5; 22-28 dpi, in both groups.

Severity of paralysis

From the day of immunization (= 0 dpi.), motor impairment in three regions-the tail, forelimbs, and left hind limb-was evaluated daily. The scores for the tail were: 0 = as-

ymptomatic, 1 = paralysis of the tail tip, 2 = incomplete paralysis of the entire tail, 3 = complete paralysis of the whole tail. Scores for the forelimbs were: 0 = asymptomatic, 1 = unable to climb fence using forelimbs, 2 = unable to walk, 3 = complete paralysis. Scores for the left hind limb were: 0 = asymptomatic, 1 = paralysis of the toes only, 2 = incomplete dorsiflexion of the foot joints while walking, 3 = complete paralysis (drags legs when walking). Finally, the scores of these three sites were added together for the EAN score (0-9).

Immunohistological Examination

Experiment 1

Rats were deeply anesthetized by sevoflurane inhalation, perfused thoroughly via the left ventricle using ice-cold 0.1 M phosphate buffered saline (PBS) at 14 dpi (high dose, low dose, and vehicle groups, n = 3, respectively) and 21 dpi (high dose, low dose, and vehicle groups, n = 3, respectively, and delay group, n = 5). Cauda equina (CE) was removed, fixed in 10% neutral buffered formalin solution, embedded in paraffin. 7-8 μm thick serial sections at the levels of L2-3 were prepared. Immunohistological staining (Avidin-Biotin Complex (ABC) method with 3,3'-diaminobenzidine (DAB) as chromogen, (VECTASTAIN ABC kit; Vector, Burlingame, CA) using anti-CD68 monoclonal antibodies (Ab31630: Abcam, Tokyo) and anti-S-100 monoclonal antibodies (Ab4066, Clone 4C4.9: Abcam) was performed on serial sections following the standard antigen retrieval method. The S-100 negative area, which is occupied by CD68 positive cells in the serial section, was considered as an EAN focus. In S-100 stained slices, all nerve roots in a single section were examined. The area of total EAN foci and of the whole transverse area of corresponding nerve roots was measured using Image J (National Institutes of Health (NIH), Bethesda, MD) after conversion to binary images (5-15 nerve roots for each rat, 3-6 rats for each time points). The proportion of EAN foci to the whole nerve root cross section area in both groups was compared sequentially.

Experiment 2

On 7, 11, 14, 21, and 28 dpi, rats in the high dose group and vehicle group were deeply anesthetized (n = 5 for each time point). CE were then removed and fixed, stained, and morphometrically analyzed for experiment 1. 5-15 nerve roots per each rat, 5 rats for each time point were analyzed.

Analysis of mRNA in the peripheral nerves

Rats in experiment 2 as described above were used. After 7, 11, 14, 21, and 28 dpi, rats in the high dose group and vehicle group were deeply anesthetized (n = 5 for each time point); after thorough perfusion using ice-cold 0.1 M PBS, the cauda equina were removed and then preserved in RNAlater (QIAGEN, Tokyo). After crushing the tissue using a tissue grinder (Shakeman™, Biomedical Science, Tokyo) , total RNA was extracted using an RNeasy mini kit (QIAGEN). Resulted RNA was reverse transcribed using iScript™ cDNA synthesis kit with oligo-dT primer (BIO-RAD, Tokyo) following the manufacturer's instructions. Resulted cDNA was then used as a template for real-time PCR using iTaq™ Universal SYBR® Green Supermix™ and gene-specific primers (Perfect Real Time Primer (TAKARA BIO, Otsu)). CFX96TM Real-Time System C1000 Touch™ Thermal Cycler (BIO-RAD) was used. Firstly, the gene expression levels of IFN-gamma, IL-10, E-sel, and housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined. The expression levels of target genes were expressed as a ratio to those of housekeeping gene expression level in the same sample. These ratios were then compared with those at 0 dpi (ΔΔ Ct method). These ratios were compared between the high dose group and vehicle group. Data from samples showing inconsistent melting curves were excluded.

Statistical Analysis

Mann-Whitney U test was used for analysis and a value of $p < 0.05$ was considered to have significant difference. For the analysis of time course of motor impairment, Friedman test was employed and a value of $p < 0.05$ was considered to have significance.

Approval for Animal Experiments

This research was approved by the Toho University Animal Experiment Committee (Approval Number 13-51-215, 14-52-215, 15-53-215 16-54-215).

Results

Severity of paralysis (Fig. 1)

All rats developed the first sign of motor paralysis, i.e., flaccid paralysis of the tail tip, at 11 dpi. Motor paralysis reached a peak at approximately 15 dpi, then gradually improved. In the experiment 1, there was a significant symptomatic reduction in the delay group compared with the

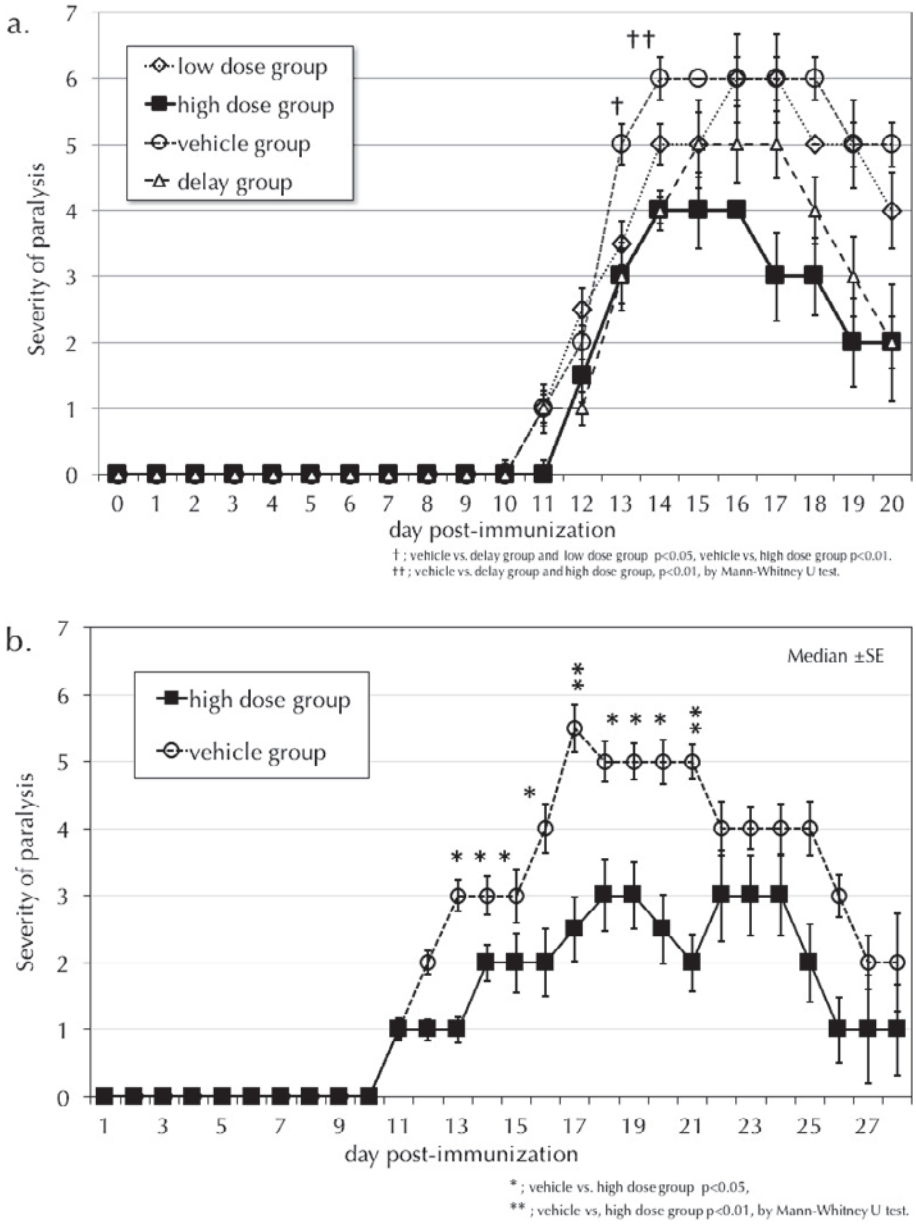

Fig. 1 The effect of cilostazol on the severity of paralysis

a) Temporal profile of motor impairment of rats in experiment 1.

b) Temporal profile of motor impairment of rats in experiment 2.

In experiment 1 (a), there was a significant symptomatic reduction on 14 day post immunization (dpi) in the delay group compared with the vehicle group (†; $p<0.05$, by Mann-Whitney U test).

In experiment 2 (b), severity of paralysis was significantly low on 13, 14, 15, and 18 dpi in the high dose group compared with the vehicle group (*; $p<0.05$, **; $p<0.01$, by Mann-Whitney U test).

vehicle group (Mann--Whitney U test, $p < 0.05$; 14 dpi), although significant differences were not observed between the low dose group and vehicle group. In experiment 2, the EAN score was significantly low at 13, 14, 15, and 18 dpi in the high dose group compared with the vehicle group ($p < 0.01$ at 12, 13, 14, 21 dpi; and $p < 0.05$ at 15-20 dpi; by Friedman test : $p < 0.05$ at 13, 14, 18 dpi; and $p < 0.01$ at 15 dpi by Mann-Whitney U test).

Histological analysis (Fig. 2, 3)

Patchy S-100 negative foci were observed in the cauda equina after 11 dpi in all EAN rats in both experiment 1 and experiment 2. These foci were occupied by CD68 posi-

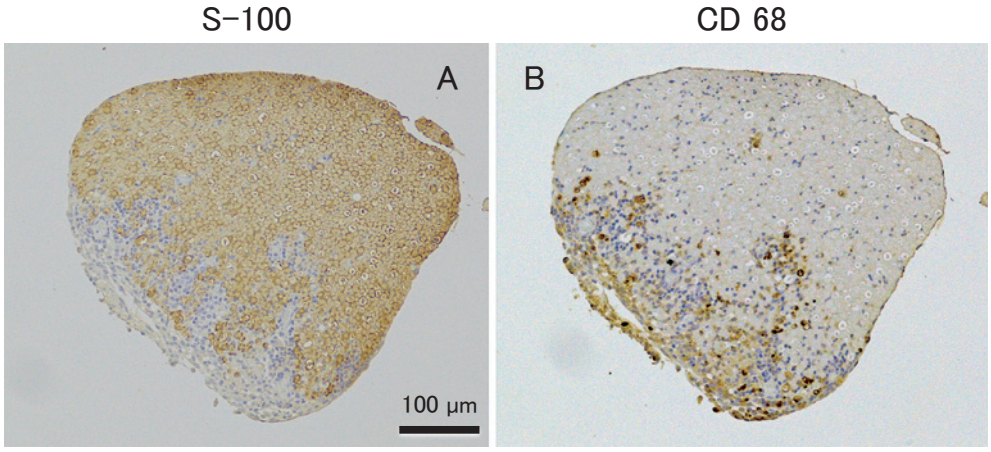

Fig. 2 Histological examination of the cauda equina
a) S-100 immunostaining in cauda equina from vehicle-treated EAN rat at 11 dpi.
b) CD68 immunostaining in cauda equina from vehicle-treated EAN rat at 11 dpi.
Patchy S-100 negative foci were observed in the cauda equina in all EAN rats (a). These foci were occupied by CD68 positive mononuclear cells in the serial section (b).

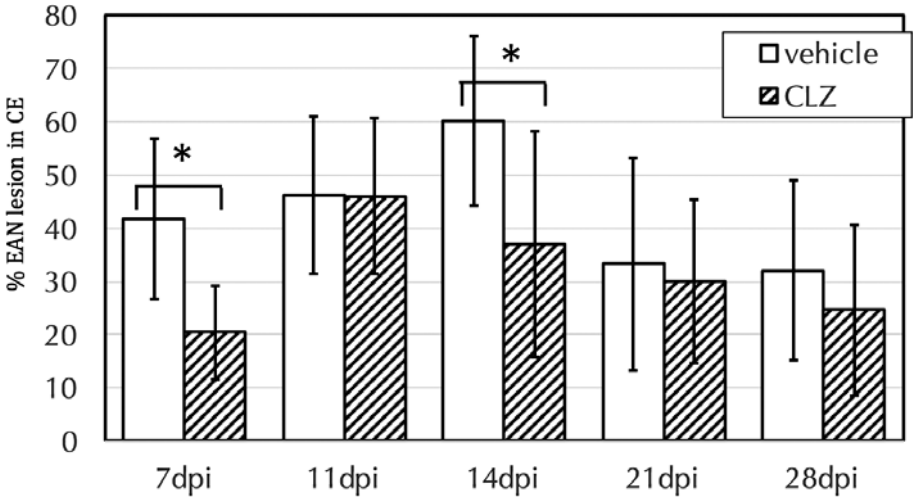

Fig. 3 Sequential observation for the proportions of S-100 negative foci with infiltrated CD68 positive cells in cauda equina
The proportions of demyelinated areas in the whole cauda equina were significantly smaller in the high dose group compared with the vehicle group on 7 and 14 dpi (*; $p < 0.05$, by Mann-Whitney U test. Bars indicate standard error).

tive mononuclear cells in the serial section (Fig. 2) and were thus considered demyelinated lesions.

In experiment 1, these foci tended to be suppressed in the high dose group at 14 and 21 dpi compared with other groups; however, delay group on 21 dpi did not show any remarkable difference.

In experiment 2, these foci increased in number as paralysis became exacerbated in both the high dose group and vehicle group, finally developed into large confluent lesions. The proportions of these demyelinated areas in the whole cauda equina were significantly smaller in the high dose group compared with the vehicle group at 7 and 14 dpi (Fig. 3).

Expression of mRNA in the cauda equina (Fig. 4)

IFN message (Fig. 4a)

In the vehicle group, the increase in IFN message started at 7 dpi, maintained a high level through 14 dpi, then decreased afterward. In the CLZ group, the increase in IFN message started at 11 dpi. At 7 dpi, IFN message in the CLZ group tended to be decreased compared with the

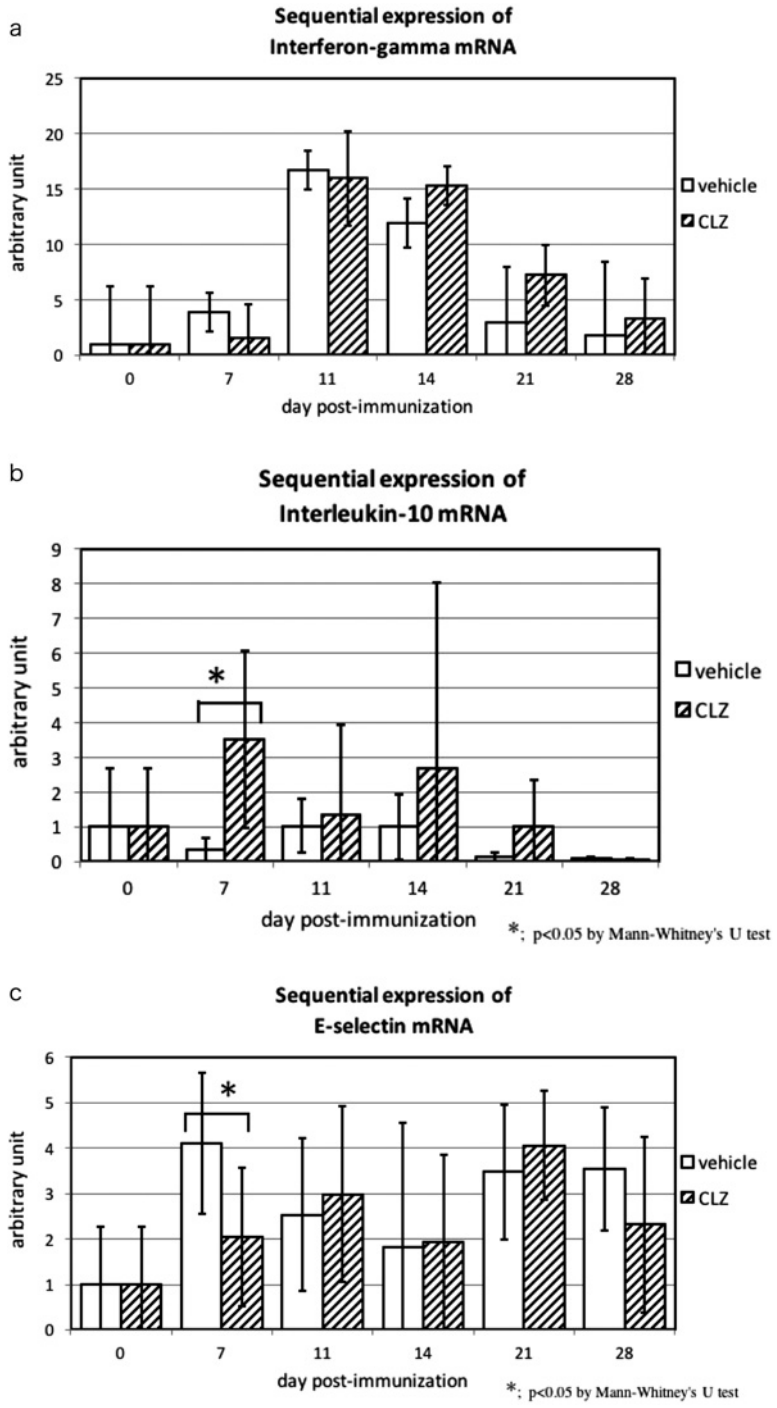

Fig. 4 Sequential expression of mRNA in the cauda equina

a. Interferon (IFN)-gamma message in vehicle group started upregulation at 7 dpi, peaked at 11 dpi, then declined. IFN message in CLZ group tended to be decreased compared with vehicle group although not significant statistically.

b. Interleukin-10 (IL-10) message expression surged at 7 dpi in CLZ group. This difference was statistically significant; however, at the other time points, changes in IL-10 message expressions were not significant.

c. E-selectin expression shows two peaks, at 7 dpi and 21 dpi in vehicle group. E-selectin expression in CLZ group was suppressed significantly compared with vehicle group at 7 dpi.

Bars indicate standard error.

vehicle group, although not statistically significant.

IL-10 message (Fig. 4b)

In the vehicle group, IL-10 message levels were constantly low, contrary to the CLZ group where IL-10 message level increased at 7 dpi. This difference was statistically significant ($p < 0.05$ by Mann-Whitney U-test). IL-10 messages thereafter fluctuated without a significant trend.

E-sel message (Fig. 4c)

In the vehicle group, E-sel expression appeared to have biphasic upregulation (7-11 dpi and 21-28 dpi). On the contrary, E-sel expression in the CLZ group showed a high peak at 21-28 dpi, which was identical to the second peak seen in the vehicle group, and the only low peak was at 7-11 dpi. The expression level at 7 dpi was significantly lower in the CLZ group compared with the vehicle group ($p < 0.05$ by Mann-Whitney U test).

Discussion

In the present study, we investigated the effect of CLZ, a selective PDE-3 inhibitor, on EAN rats with regard to severity of paralysis, histological findings, and mRNA expression (Th1 and Th2 cytokines and E-selectin). EAN was suppressed by CLZ administration symptomatically and histologically.

In experiment 1, we compared two different doses of CLZ, i.e., 10 and 30 mg/kg/day. These doses were determined according to previous reports.[19, 26]

All groups developed EAN at 11 dpi and peaked at approximately 15 dpi, followed by gradual improvement. CLZ suppressed EAN severity dose-dependently. It suppressed EAN even when started after onset of paralysis although the effect was weaker than when rats received CLZ before EAN onset. From the point of view of real therapeutics for humans, this is a crucial finding.

Histological examination revealed that the size of the demyelinated area in the CE roughly correlated with the severity of paralysis in EAN rats (Fig. 3). CLZ treatment suppressed the area of demyelination in CE although it was statistically significant only at 7 and 14 dpi. The reason why histological findings were not as significant as motor signs remains unclear. We postulate that, at the peak of inflammation, several humoral factors such as TNF-alpha produced within the CE block nerve conduction of the remaining myelinated nerve fibers.[27]

To clarify the mechanism of CLZ in suppressing EAN symptomatically and histologically, mRNA expressions of Th1 and Th2 cytokines and cell adhesion molecule E-selectin in CE were studied in experiment 2. In general, Th1 cytokine IFN expression is upregulated in CE from the pre-symptomatic stage through the peak stage of untreated EAN rats and reciprocally Th2 cytokine IL-10 expression is upregulated in CE from the peak through recovery stage of untreated EAN rats.[3] It has been known that IL-10 treatment suppresses EAN via downregulation of Th1 cytokines.[28, 29] This indicates that IL-10 can be a candidate for therapeutic target of EAN. In the present study, given that our focus was on cytokine kinetics in CE during the evolution of EAN, CE from EAN rats after the disease peak stage were not examined. The results of cytokine profiles from the present study are therefore slightly different from those in our previous study,[3] i.e., peak of IL-10 expression at 16-18 dpi in CE from untreated rats (vehicle group) was not observed. However, a surge in IL-10 expression at 7 dpi was observed in CLZ-treated rats (Fig. 4b). At that time given that motor paralysis has not yet developed and in the vehicle group IFN expression has only just started to rise, IFN cannot stimulate IL-10 via TNF-alpha[30] at this point. The direct, non-specific effect of CLZ on IL-10 expression can be postulated. In fact, few reports show that IL-10 can be upregulated by cAMP-elevating drugs[30] or CLZ.[31] In this later report, IL-10 similarly showed upregulation although a different animal model was investigated (limb ischemia model in rat) and a different CLZ dose (12 mg/kg/day) was used. Therefore, the effect of CLZ on IL-10 upregulation as mentioned above[30] should be further investigated using in-vitro experiments.

The sequential expression of IFN messages in the CE from the vehicle group was identical to that in the previous report.[3] Moreover, IFN expression in the CE from the high dose group showed a trend of suppression at 7 dpi. Subsequently, it showed a tendency of delayed stimulation through 28 dpi, although there was no significant difference (Fig. 4a). At the least, a slight suppression of IFN expression at 7 dpi is agreement with the fact that antigen-specific T cell proliferation and IFN production were suppressed by CLZ treatment in EAE.[22] Taken together, our findings suggest that CLZ treatment contributes more to IL-10 stimulation than to IFN suppression in EAN. It is likely that proinflammatory cytokines other than IFN (TNF-alpha, IL-1, and IL-17) were suppressed to a greater extent, resulting in mitigation of EAN.

Additionally, suppression of the cell adhesion molecule E-sel expression within the CE was observed. Endothelial

cells express E-sel upon activation by cytokines TNF-alpha and IL-10.[32] Suppression of E-sel expression in CLZ-treated endothelial cells has been previously reported.[33] Although P-selectin suppression has already been reported in a CLZ-treated EAE model,[22] its effect on E-sel expression is unknown. Since, in our experiments, animals were thoroughly perfused to remove blood from PNS vasculature, P-selectin expression was not studied. E-sel expression was suppressed at 7 dpi, which is in agreement with a surge in IL-10 message. We hypothesized that upregulated IL-10 at 7 dpi might stimulate endothelial cells to suppress E-sel as it triggered suppression of cell trafficking into CE at 7-11 dpi. Meanwhile, a surge in IL-10 message also suppressed IFN expression within CE and cooperated in suppressing demyelination of CE, which resulted in mitigation of EAN.

In addition, few studies have investigated drug administration after disease onset for experimental treatment of EAN. Hence, our results of CLZ administration after disease onset (in experiment 1) might help in developing supportive therapeutics in the future.

In conclusion, EAN can be suppressed by CLZ administration even when treatment is started after disease onset via IL-10 upregulation in the PNS. Further study is needed to confirm the effect of CLZ on cytokine and cell adhesion molecule expression in PNS components.

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Conflicts of interest: Non declared.

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