博 士 学 位 論 文

アマンタジンは実験的自己免疫性脳脊髄炎の重症度を調整する

近 畿 大 学 大 学 院
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福　本　雄　太

Doctoral Dissertation

Amantadine regulates the severity of experimental autoimmune encephalomyelitis

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課博 ・ 論博

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論文題目

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Amantadine regulates the severity of experimental autoimmune encephalomyelitis

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Abstract

Background：

Amantadine has been reported to have a neuroprotective effect, and it is therefore expected to have further clinical application.

Aim：

In this study, we examined the therapeutic effect of the N-methyl-D-aspartate (NMDA) receptor antagonist amantadine on experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis (MS).

Methods：

EAE was induced in C57BL/6 mice. Amantadine was administered at doses of 40 mg/kg dissolved in phosphate buffer saline (PBS) by feeding cannula every other day. The control group received PBS only. The immunized mice were examined and scored daily until day35. T-cell proliferation assay, pathological analysis and analysis of regulatory cells were performed.

Results：

Although amantadine did not significantly suppress the incidence or severity of EAE, it significantly reduced clinical symptoms in the recovery phase. There was also a significant increase in $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in response to amantadine treatment. These results suggest that amantadine promotes symptom recovery in EAE by acting in an immunosuppressive manner.

Conclusion：

Amantadine may be an effective therapy for inflammatory neurological diseases such as MS.

Keywords：experimental autoimmune encephalomyelitis, amantadine, foxp3, regulatory cell, multiple sclerosis

Abbreviations

BrdU, bromodeoxyuridine; CBA, cytometric bead array; EAE, experimental autoimmune encephalomyelitis; ELISA, Enzyme-linked immunosorbent assay; HE, hematoxylin and eosin stain; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; p.i., post immunization; PBS, phosphate buffered saline; PT, pertussis toxin

1. Introduction

The drug amantadine acts in an inhibitory manner on the N-methyl-D-aspartate (NMDA) receptor and is widely used as a therapeutic agent for depression, Parkinson's syndrome,[1] and infection with influenza A.[2] Amantadine modulates dopamine and serotonin in the brain and is therefore effective in improving ambition and the spontaneous decline that can be seen in cerebral infarction sequelae. Memantine, an NMDAR inhibitor that is similar to amantadine, improves stroke outcomes in an apparently non-neuroprotective manner by increasing brain-derived neurotrophic factor signaling, reducing reactive astrogliosis, and improving vascularization, which leads to improved recovery of sensory and motor cortical function.[3] Amantadine has also been reported to have a neuroprotective effect, and it is therefore expected to have further clinical application. Attempts have been made to use it to improve cognitive impairment and fatigue in multiple sclerosis (MS).[4-6]

MS is an inflammatory demyelination disease of the central nervous system (CNS), and while a pathogenic immune mechanism has been suggested, the details of its etiology are unknown. One potential mechanism that may be involved in the disease, however, is excitotoxicity.[7] An elevation in glutamate in the cerebrospinal fluid has been observed in the brains of MS patients and in experimental autoimmune encephalomyelitis (EAE).[8] Glutamate that is released by macrophages may be involved in axonal damage and oligodendrocyte pathology in MS lesions.[9]

EAE is considered to be an animal model of MS because it causes inflammatory demyelination in the CNS by immunizing the myelin antigens of the CNS.[10] Here, we examine the therapeutic effect of amantadine on EAE, and consider its therapeutic application in central nervous system inflammatory diseases such as MS.

2. Materials and Methods

2.1. Induction and evaluation of active immunization EAE

EAE was induced in C57BL6 mice by subcutaneous injection of a 0.2 ml emulsion containing 100 μg myelin oligodendrocyte glycoprotein (MOG)$_{35-55}$ in complete Freund's adjuvant with 5 mg/ml mycobacterium tuberculosis H37RA (Difco Laboratories, Detroit, MI, USA) and 300 ng pertussis toxin (Sigma-Aldrich, St. Louis, MO, USA). The emulsion was injected intraperitoneally on days 0 and 2 post-immunization (p.i.). Amantadine (Tanabe-Mitsubishi, Osaka, Japan) was administered at doses of 40 mg/kg dissolved in phosphate buffer saline (PBS) by feeding a cannula every other day beginning at day 0. The control group received PBS only. The dosage of amantadine was determined in preliminary experiments. The dosage

was distributed on a log scale that was used to find the maximum dosage without adverse effects and the minimum dosage with efficacy. The dosage of amantadine for this experiment was calculated to be 10-fold higher than the human dosage.

The immunized mice were scored daily using the following scale: 0, no clinical signs; 1, limp tail; 2, partial hind leg paralysis; 3, total hind leg or partial hind and front leg paralysis; 4, total hind leg and partial front leg paralysis; and 5, moribund or dead. All mice used for the experiments were aged 8-16 weeks. All experiments were performed in accordance with the guidelines of the institutional ethics committee.

2.2. Peptides

MOG_{35-55} was purchased from Medical and Biological Laboratories CO., Ltd. (Nagano, Japan) for the induction of EAE. Peptides were > 90% pure, as determined by high performance liquid chromatography.

2.3. Pathological analysis

Spinal cords were removed from mice under anesthesia with isoflurane on day 35 after immunization. Spinal cords were fixed in 10% neutral buffered formalin and embedded in paraffin. Paraffin blocks were cut into 10 μm thick sections, and stained with hematoxylin and eosin (HE).

2.4. T cell proliferation assay

The T cell proliferative response with MOG_{35-55} was measured with bromodeoxyuridine (BrdU) cell proliferation and cytokine production assays. Mice were immunized with MOG_{35-55}, and were fed amantadine (treatment group) or PBS only (control group).

A single-cell suspension was prepared from the bilateral inguinal lymph nodes (LNs) of mice on day 14 after immunization. Cells were cultured in RPMI GlutaMAX (Gibco, Grand Island, NY, USA) with 10% heat-inactivated fetal bovine serum and were re-stimulated with MOG_{35-55} for 48 hours. During the final 4 hours of culture, BrdU was added to the wells. At the end of the culture, cells were fixed and anti-BrdU antibody was added to each well. After a one hour incubation, the proliferative reaction was analyzed with a spectrophotometer.

The cytokines in the culture supernatant were measured with a cytometric bead array (CBA Th1/Th2/Th17 kit; BD Bioscience, San Jose, CA). The concentrations of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17a, interferon (IFN)-γ, and tumor necrosis factor (TNF) were measured according to the manufacturer's guideline.

2.5. Analysis of regulatory cells

Spleen cells from immunized mice were re-stimulated with MOG35-55 and cultured with 10 μg/ml amantadine for 48 hours. The concentration of amantadine was determined to be the maximum concentration

at which cells in culture did not die.

Cultured cells were evaluated with a flow cytometer. The cells were stained with anti-mouse CD4 (FITC), CD25 (APC), and Foxp3 (PE) using a Mouse Regulatory T Cell Staining Kit (Thermo Fisher Scientific). The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ cells or $CD4^+$ $CD25^-$ $Foxp3^+$ cells were then calculated and compared between treatment groups.

2.6. Statistical analysis

Statistical analysis was performed using Excel and R software. The differences in EAE clinical scores, proliferative response, cytokine production, and Foxp3 expression were analyzed with the Mann-Whitney U test. A P value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Amantadine impacted the recovery phase of EAE.

Figure 1 shows the disease course of EAE in the amantadine-treated group (n=13) and the control group (n=12). There was a trend but no significant differences in the EAE cumulative score and the maximum EAE score between the amantadine-treated group and the control group. Amantadine-treated mice, however, had significantly lower EAE scores in the recovery phase when compared with the control group on day 27, day 28, day 32, day 33, day 34 and day 35 after immunization ($P < 0.05$).

Pathological changes in spinal cord sections were evaluated 35 days after immunization. Although there was no significant difference in pathological severity between the two groups, there was a trend toward a reduction in the pathological changes of EAE in the amantadine-treated group (Figure 2).

3.2. Amantadine did not influence the primary response.

The proliferative responses of MOG_{35-55}-specific T cells, as measured by uptake of BrdU, were not significantly different between the amantadine-treated and control groups (Figure 3A). The production of Th1/Th17-associated cytokines (IFN-γ and IL-17a) were also not significantly different between the amantadine-treated and control groups, but there was a trend toward a reduction in IFN-γ (Figure 3B and C). The other cytokines (IL-2, IL-4, IL-6, IL-10 and TNF) were not significantly different (data not shown).

3.3. Amantadine increased the proportion of regulatory T-cells.

In the primary response to MOG_{35-55}, the cells from the amantadine-treated group increased $CD4^+$ $CD25^+$ $Foxp3^+$ T cells, but not $CD4^+$ $CD25^-$ $Foxp3^+$ T cells (Figure 4). The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in the group treated with 10 μg/ml amantadine was significantly higher than that of the control group (1.70 ± 0.59 vs. 2.44 ± 1.41, $p < 0.05$).

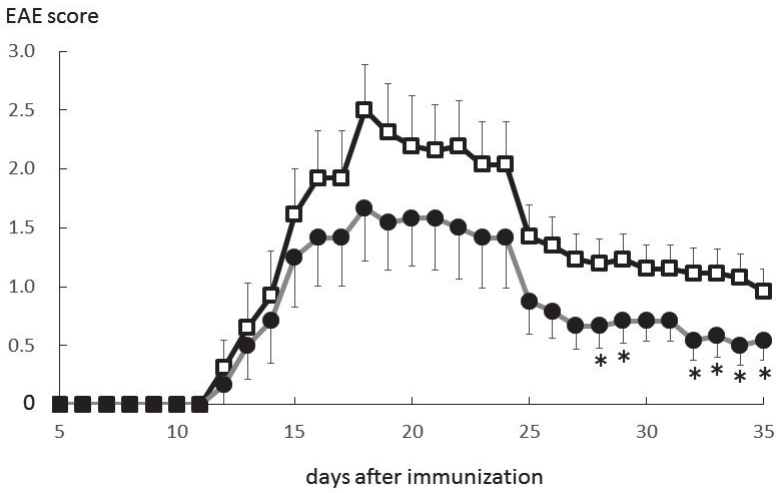

Figure 1. Disease course of EAE

There was a trend but no significant difference between the amantadine-treated group and the control group in regards to EAE cumulative score (control group : 35.77 ± 5.38 ; treated group : 23.46 ± 6.17) and maximum EAE score (control group : 3.00 ± 0.41 ; treated group : 2.17 ± 0.50). However, amantadine-treated mice had significantly lower EAE scores in the recovery phase when compared with the control group (control group : treated group ; day 27, 1.35 ± 0.24 : 0.67 ± 0.20 ; day 28, 1.31 ± 0.23 : 0.67 ± 0.19 ; day 32, 1.12 ± 0.21 : 0.54 ± 0.17 ; day 33, 1.19 ± 0.22 : 0.58 ± 0.18 ; day 34, 1.08 ± 0.20 : 0.50 ± 0.16 ; day 35, 0.96 ± 0.22 : 0.54 ± 0.17).

A representative experiment of two independent experiments is expressed as mean ± S.E.M. Amantadine was administrated to mice starting from the day of immunization (●). Control mice were administrated vehicle alone (□). *represents a P value of < 0.05 using the Mann-Whitney U test.

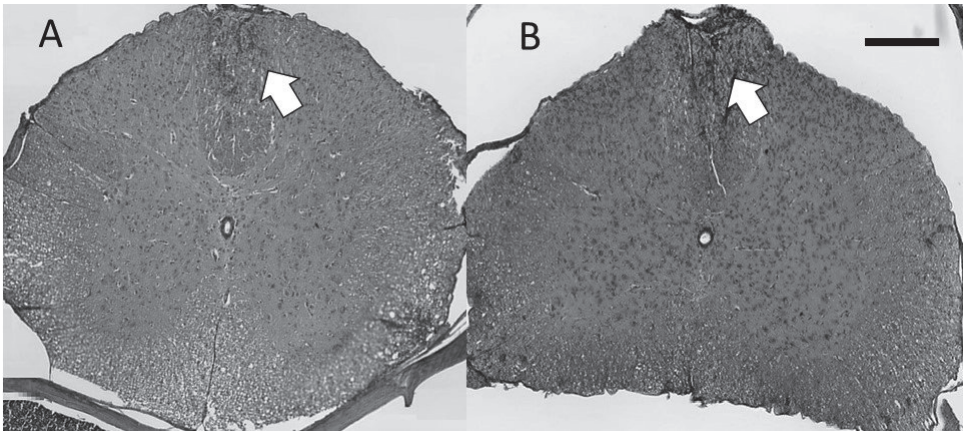

Figure 2. Pathological findings

Spinal cord sections from EAE day 35 after immunization are shown. Arrows show cellular infiltrations in the HE stained section. Both amantadine treated mice (A) and control mice (B) showed mild cellular infiltration. Although there was no significant difference in pathological severity between the two groups, the amantadine group trended toward a reduction in pathological changes in EAE. Scale bar = 100 μm.

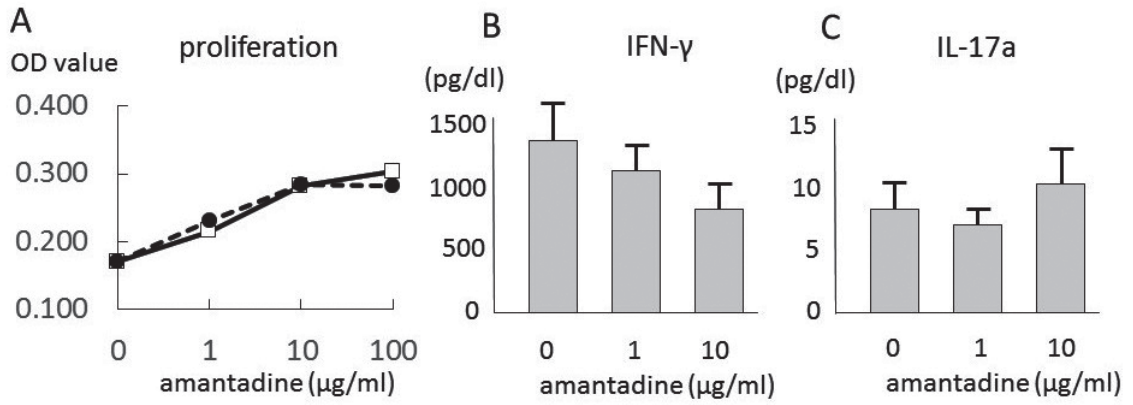

Figure 3. The proliferative responses of MOG_{35-55}-specific T cells
The proliferative responses of MOG_{35-55}-specific T cells, as measured by a BrdU assay, showed no significant differences in the uptake OD value between the amantadine-treated group (●, 0.28 ± 0.12, n=5) and the control group (□, 0.30 ± 0.12, n=5). The value represents the mean ± SD (A). Although there was a trend toward a reduction in IFN-γ, the production of Th1/Th17-associated cytokines (IFN-γ and IL-17a) were also not significantly different between the amantadine-treated and control groups (B, C).

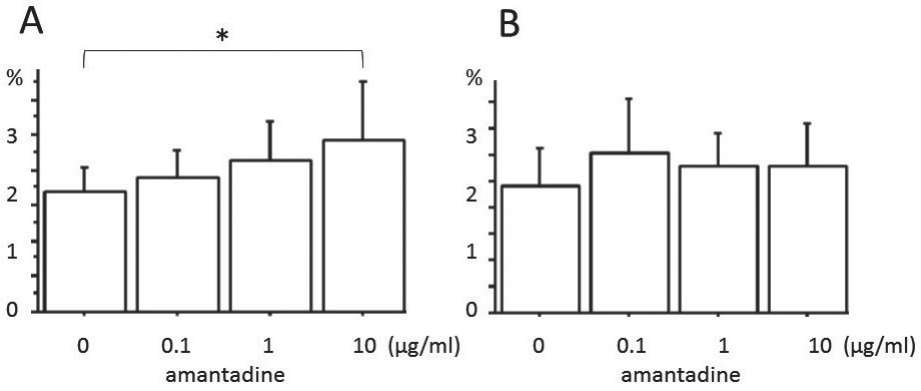

Figure 4. Amantadine induced $CD4^+$ $CD25^+$ $Foxp3^+$ T cells
Spleen cells from immunized mice were re-stimulated with MOG_{35-55} and cultured with 10 μg/ml amantadine for 48 hours. The cells were stained with anti-mouse CD4 (FITC), CD25 (APC) and Foxp3 (PE), and were analyzed with a flow cytometer. The cells from the amantadine treated group significantly increased the number of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (A), but not $CD25^-$ T cells (B). The values represent the average percentage ± SD, n=4. * represents a p value of < 0.05 using the Mann-Whitney U test.

4. Discussion

In this study, we showed that amantadine has a therapeutic effect on EAE. Although the therapeutic effect of amantadine was only seen in the recovery period, it also tended to suppress the EAE maximum score. It can thus be presumed that it also has an effect on the acute phase of EAE. Amantadine did not, however, affect the primary response of pathogenic T cells. Thus, amantadine has no strong therapeutic effect in modifying the acute condition of EAE. On the other hand, in the recovery phase of EAE, amantadine significantly ameliorated the clinical symptoms, which thus leads to the conclusion that it has an effect on neural repair and neuroprotection.

In a rat model of EAE with optic neuritis, administration of another NMDA receptor antagonist, memantine, resulted in the protection of retinal ganglion cells and axons, and reduced demyelination of the optic nerve.[11] Another report showed that memantine suppressed the development of EAE in a dose-dependent manner in rats.[12] This therapeutic effect was not due to dampened CNS inflammation and the number of IFN-γ mRNA-expressing cells were not reduced. It has thus been hypothesized that the mechanisms responsible for reversible neurological deficits in EAE may involve NMDA receptors.[13]

In a rat EAE model, glutamate receptors were shown to be associated with neurodegenerative and neurotoxic processes,[14] and amantadine was shown to have a neuroprotective effect. Another study of EAE using Lewis rats demonstrated that amantadine and memantine suppressed neurological symptoms of EAE and reduced the expression of pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the brain.[15] An in vitro study of amantadine revealed that amantadine reduced the production of the pro-inflammatory cytokines IFN-γ and TNF-alpha and increased the production of the negative immunoregulator IL-10.[16] Another in vitro experiment with Con A stimulation demonstrated that amantadine has a direct, inhibitory, and dose-dependent effect on T lymphocytes.[17] In our experiments, amantadine did not suppress the primary response of pathogenic T cells, but there was a trend in suppression of the Th1 cytokine IFN-γ.

In an investigation using the collagen-induced arthritis model (CIA), memantine treatment significantly improved the course of CIA and up-regulated the expression of Foxp3 in spleen $CD4^+$ T cells, followed by an increase in $CD4^+$ $CD25^+$ regulatory T cells.[18] Our experiment showed that amantadine significantly increases regulatory $CD4^+$ $CD25^+$ $Fop3^+$ T cells. The mechanism up-regulating regulatory T cell is unclear. NMDA receptor subunits are present in rodent and human T lymphocytes. Stimulation of NMDA receptors modulates immune responses.[9] Thus, amantadine may have been involved in up-regulating regulatory T cells. In a murine model of respiratory infection with Bordetella pertussis, $CD25^-$ $CD4^+$ $Foxp3^+$ cells are the dominant population in the lung, gut, and liver.[19] In our experiments, these cells were not increased by amantadine. These results align with the hypothesis that nerve repair in EAE is due to the immunoregulatory action of amantadine via $CD4^+$ $CD25^+$ $Foxp3^+$ T cells.

In some reports using the rat EAE model, memantine modified blood-brain barrier (BBB) dysfunction and neurological deficits during the acute phase of EAE.[20, 21] The pathological findings of our study showed,

however, that amantadine did not suppress cell infiltration into the CNS. Since the effect of amantadine is dose-dependent, the dosage of amantadine in our study may not have been sufficient to yield an effect. Further studies are needed in order to elucidate effects of amantadine for human and understand the mechanism of immune effects and other neuroprotective effects.

As amantadine has been applied to clinical care for quite some time, its long-term safety has been confirmed and it is also inexpensive. Further explorations into its application in other neurological diseases are therefore warranted. Given that the therapeutic effect of amantadine alone is presumed to be weak, it may be considered as an add-on to other therapeutic agents, but further experimental studies are necessary to confirm this hypothesis.

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Conflict of Interest

The authors report no conflicts of interest in this work.

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