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# Alpha-Tocopherol Ameliorates Experimental Autoimmune Neuritis by Exerting Antioxidant Effects and Suppressing Cytokine Production

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## ABSTRACT

**Introduction:** Guillain-Barré syndrome (GBS) is an autoimmune neuropathy mediated by insult of anti-ganglioside antibodies and subsequent complement activation. Oxidative toxicity, resulting from insufficiently maintained cellular redox levels, has also been contributed to GBS pathogenesis; however, antioxidant therapy for GBS patients has not yet been established. The effectiveness of  $\alpha$ -tocopherol ( $\alpha$ T), a natural antioxidant, was evaluated as a treatment for experimental autoimmune neuritis (EAN), using an animal model of human GBS.

**Methods:** Female Lewis rats, immunized with 125  $\mu$ g of synthetic peptide from bovine P2 protein, were injected with 100 mg/kg of intraperitoneal  $\alpha$ T on only day 6 or on both days 6 and 13 post-immunization (p.i.). Lipid peroxidation products, histological alterations, and cytokine expression in the cauda equina and/or popliteal lymph nodes were sequentially evaluated.

**Results:** Flaccid paralysis developed from the tail tip at days 11-13 p.i. in both  $\alpha$ T-treated rats and non-treated control rats. The latter gradually progressed to flaccid paraplegia, and reached a peak of motor impairment on day 16 p.i., followed by spontaneous recovery. Double  $\alpha$ T administration, but not single administration, significantly improved the clinical course of EAN, decreased the level of the lipid peroxidic marker N epsilon-(hexanoyl) lysine, and suppressed mRNA expression of interferon-gamma and interleukin-10 in the popliteal lymph nodes and cauda equina. Histologically,  $\alpha$ T treatment showed reduced demyelination in the cauda equina compared to control rats.

**Conclusions:** Alpha-T ameliorated EAN by suppressing the production of pro-inflammatory cytokines and preventing oxidative damage. This demonstrates the potential of natural antioxidant treatment for GBS.

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**KEYWORDS:** Guillain-Barré syndrome, experimental autoimmune neuritis, alpha-tocopherol, antioxidant effect, immunoregulatory effect

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## Introduction

Guillain-Barré syndrome (GBS), an acute and monophasic polyneuropathy, develops as a result of immunological cross-reaction between the components of pathogenic microorganisms and human peripheral nerves. In many cases, respiratory or gastrointestinal infections precede the development of neurological symptoms by 1-2 weeks. Antibodies against glucolipids, especially gangliosides, which are essential for the maintenance of the cell membrane structure of peripheral nerves, are detected in the serum of about 50-60% of patients with GBS.<sup>1,2)</sup> These pathognomonic antibodies against gangliosides play a pivotal role in the development of GBS; however, numerous other factors, such as complement, cytokines, chemokines, lymphocytes, and macrophages, are additionally involved. In the last few decades, the importance of reactive oxygen species (ROS)<sup>3)</sup> and nitric oxide<sup>4)</sup> in the pathogenesis of GBS and immune-mediated peripheral neuropathy have been reported.

Treatments using plasmapheresis or intravenous immunoglobulins during the acute phase, together with supportive care, such as mechanical ventilation and management in an intensive care unit, have reduced mortality from cardiac arrest caused by autonomic failure in patients with GBS.<sup>5,6)</sup> Furthermore, it has been reported that eculizumab, a humanized antibody against the complement protein C5, combined with intravenous immunoglobulins for added potency, restored running ability in patients with severe GBS earlier than in placebo-treated patients.<sup>7)</sup> However, this treatment did not restore the ability to walk independently at four weeks in the expected proportion of patients. In practice, despite this advance in treatment, many patients with GBS suffer an incomplete recovery. Therefore, the development of more effective therapy for patients with GBS is necessary.

Experimental autoimmune neuritis (EAN) in rabbits, induced by immunization with homogenate solution of peripheral nerve tissue by Waksman et al. in 1955, enabled the development of animal models of GBS.<sup>8)</sup> Recently, EAN induced in the Lewis rat has been widely used to analyze pathophysiology and evaluate drug efficacy because of its high disease susceptibility.<sup>9)</sup> Both GBS and EAN are histologically characterized by infiltration of mononuclear cells into the perivascular cavities in the peripheral nerves, these cells are mostly macrophages, with some CD4<sup>+</sup> cells and CD8<sup>+</sup> cells present.<sup>10,11)</sup>

Numerous studies focusing on the development of experimental therapeutics for EAN have been reported to date. Immuno-suppressants,<sup>12,13)</sup> drugs used as disease-modifying therapy for multiple sclerosis,<sup>14,15)</sup> and matrix metalloproteinase inhibitors<sup>16,17)</sup> have been reported as efficacious treatments for EAN. Oxidative stress and peroxide-induced products have been suggested to be involved in disease pathogenesis and thus represent promising therapeutic targets for EAN and GBS.<sup>18,19)</sup> We have also shown that a hydroxyl radical scavenger edaravone ameliorates EAN by reducing the accumulation of hydroxyl radicals in the cauda equina.<sup>20)</sup>

However, we have not identified whether the tissue peroxidic process is subsequently induced by ROS, including hydroxyl radicals. It is necessary to measure a lipid peroxidic marker in the cauda equine, such as malondialdehyde or N epsilon-(hexanoyl) lysine (HEL), to estimate the severity of impairment severity in lipid-rich myelin and evaluate treatment effects. Vitamin E is a natural antioxidant produced by plants alone.<sup>21)</sup> Among eight different forms with similar chromanol structures, alpha-, beta-, gamma-, and delta-tocopherol and alpha-, beta-, gamma-, and delta-tocotrienol, only alpha-tocopherol ( $\alpha$ T) meets the criteria for fulfilling the human vitamin E requirement. The main function of  $\alpha$ T in the human body is a fat-soluble antioxidant. Fats, which are an integral part of all cell membranes, are vulnerable to damage through lipid peroxidation by ROS. Anti-inflammatory effects of  $\alpha$ T, such as allergy airway inflammation, i.e., allergic asthma<sup>22,23)</sup> and multiple sclerosis, which is an inflammatory disease of the central nervous system,<sup>24)</sup> have been observed in some preclinical animal models. However,  $\alpha$ T has no proven efficacy against carrageenan-induced air pouch inflammation<sup>25)</sup> and vascular injury with high-fructose-induced insulin resistance.<sup>26)</sup>

This study was conducted to evaluate the ability of  $\alpha$ T to improve the clinical course of EAN and to explore its underlying mechanisms of action associated with regulation of lipid peroxidic production and cellular immune response in this disease.

## Materials and Methods

### Rats

Female Lewis rats aged 6-7 weeks were purchased from the Charles River Japan (Yokohama, Japan). Rats were housed at the Toho University Ohashi Experimental Animal Laboratory according to the regulations and guide-

lines of Toho University's Animal Experiment Committee. All efforts were made to minimize the suffering of rats and to reduce the number of rats used. This research was approved by the Toho University Animal Experiment Committee (Approval Number 17-53-298 and 18-54-298).

#### **Induction and evaluation of EAN**

Rats anesthetized with sevoflurane (Mylan, Osaka, Japan) were subcutaneously injected with 125 µg of synthetic peptide corresponding to the amino acid residues 53-78 of bovine P2 protein (TESPFGKNTSEISFKLGQFEETTADNR, Operon, Tokyo, Japan), emulsified in an equal volume of Complete Freund's adjuvant (Sigma-Aldrich, MO, USA), in the right footpad. Motor function was observed daily and scored according to the following scales: Tail; 0 = no clinical signs; 1 = paralysis of the tail tip; 2 = incomplete paralysis of the entire tail; 3 = complete paralysis of the entire tail, forelimbs; 0 = no clinical signs; 1 = unable to climb fence by forelimbs; 2 = unable to walk; 3 = complete paralysis, left hind limb; 0 = no clinical signs; 1 = paralysis of the toe only; 2 = incomplete dorsiflexion of the foot joint while walking; 3 = complete paralysis (drags legs when walking). A total clinical score as disease severity, 0-9 points, was calculated as the sum of daily all parts scores of rats in each group.

The number of rats was  $n = 4$  in each group in the therapeutic regimen 1, and  $n = 25$ , day 0-7 post-immunization (p.i.);  $n = 20$ , day 8-10 p.i.;  $n = 15$ , day 11-14 p.i.;  $n = 10$ , day 15-20 p.i.;  $n = 5$ , day 21-28 p.i. in each group in regimen 2.

#### **Alpha-tocopherol treatment**

D-alpha-tocopherol (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) was dissolved in 1% ethanol diluted in phosphate-buffered saline. Rats in the treatment group were intraperitoneally injected with  $\alpha$ T solution equivalent to 100 mg/kg on day 6 only under therapeutic regimen 1 and both days 6 and 13 p.i. under therapeutic regimen 2. Rats in the non-treated control group were injected with vehicle only.

#### **Histopathology**

For histological assessment, additional rats were immunized and treated with  $\alpha$ T solution or vehicle, as performed under regimen 2 ( $n = 5$  for non-treated control rats,  $n = 6$  for  $\alpha$ T treated rats). At day 16 p.i., the rats were perfused with ice-cold phosphate-buffered saline, and the cauda equina at the levels of L 2-3 were collected. The cauda equina were fixed with 10% buffered formalin, embedded in paraffin, and cut into 5 µm-thick serial sections for staining of the infiltrating cells with hematoxylin & eo-

sin (H&E) and of myelin with Luxol fast blue (LFB).

The serial sections were assessed in a blind fashion for EAN, characterized by a LFB-negative area, which is occupied by H&E positive cells. The total areas corresponding to EAN foci and that corresponding to the nerve roots were measured using Image J (National Institutes of Health, Bethesda, MD) after conversion to binary images (7-32 nerve roots for each rat; five non-treated control rats and six treated rats). The ratio of EAN foci to the entire nerve root cross section area was compared in both groups of rats.

#### **Enzyme-linked immunosorbent assay (ELISA) of N epsilon-(hexanoyl) lysine (HEL)**

The cauda equina tissues were homogenized in a solution of radioimmunoprecipitation assay lysis buffer system (Santa Cruz Biotechnology, CA, USA). The samples were centrifuged at  $900 \times g$  for 10 min at 4°C in an Eppendorf 5424 Microcentrifuge (Eppendorf AG, Hamburg, Germany). The supernatants were stored at -80°C for later use to evaluate HEL levels as the lipid peroxidic marker in the cauda equina. The HEL was quantified using a HEL ELISA Kit (Nikken Seil Co. Ltd., Shizuoka, Japan). All procedures were performed according to the instructions of the manufacturer. The absorbance was measured at 450 nm with an iMark™ Microplate Absorbance Reader (BIORAD, Tokyo, Japan). HEL concentrations were expressed as nmol/L.

#### **Sequential gene expression analysis by real-time polymerase chain reaction (RT-PCR)**

Rats anesthetized using sevoflurane were thoroughly perfused with ice-cold phosphate-buffered saline, and the popliteal lymph nodes and cauda equina were collected. The popliteal lymph nodes were mechanically dissociated by passing through a 100-µm strainer, and the resulting isolates were washed with Hanks' Balanced Salt Solution. This was followed by centrifugation at  $300 \times g$  for 5 min by Tabletop Centrifuge Model 5400 (Kubota Corp., Osaka, Japan); then, the supernatant was decanted and the pellet resuspended to a concentration of approximately  $1 \times 10^6$  cells/mL of medium. The lymphocytes were dissolved by RLT buffer (Qiagen, KK, Tokyo, Japan) and the cauda equina tissue was preserved in RNAlater (Qiagen, KK, Tokyo, Japan) and stored at -20°C for later use to analyze gene expression. Before extracting RNA, the cauda equina was crushed using a tissue grinder (Shakeman™, Biomedical Science, Tokyo, Japan). RNA extraction and cDNA synthesis were conducted using commercially available kits

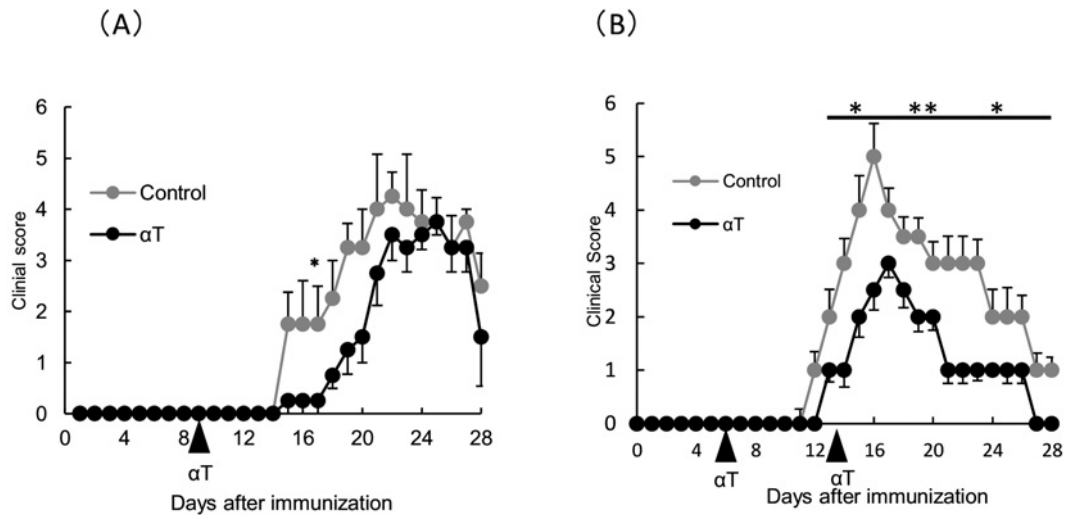


Fig. 1 Alpha-tocopherol effectively ameliorates the clinical course of experimental autoimmune neuritis (EAN)

(A) Sequential profile of motor impairment of rats in therapeutic regimen 1

(B) Sequential profile of motor impairment of rats in therapeutic regimen 2

Rats were immunized with SP-26 to induce EAN by injecting with 100 mg/kg of  $\alpha$ T dissolved in 1% ethanol on day 6 p.i. in regimen 1 and on days 6 and 13 p.i. in regimen 2. The rats that received vehicle served as controls. The severity of EAN was scored on a 0-9 points. Data show median  $\pm$  SEM. Fig. 1B was based on one representative experiment out of two independent experiments, with five samples per experiment with similar results.  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*) were considered to represent statistical significance according to the Mann-Whitney U test.

(RNeasy Mini kit, Qiagen, Tokyo, Japan; and iScript RT Supermix for RT-qPCR, Bio-Rad, Tokyo, Japan). Resulting cDNA was used as a template for real-time PCR using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Tokyo, Japan) and gene-specific primers (Perfect Real Time Primer, TAKARA BIO, Otsu, Japan and TaqMan Gene Expression assays, Applied Biosystems, Foster City, CA, USA). Gene expression of *interferon-gamma* (*IFN- $\gamma$* ) (RA 056832), *interleukin (IL)-10* (RA008616), *interleukin (IL)-17a* (Rn01757168\_m1), and *forkhead box protein 3* (*Foxp3*) (RA 031375) were analyzed in comparison to that of *Gapdh* (RA 015380, used as an endogenous control) by CFX96 Touch™ Real-Time PCR Detection System (BIO-RAD, Tokyo, Japan). Gene expression levels are shown as a relative copy number determined using the method of delta threshold ( $2^{-\Delta\Delta Ct}$ ).

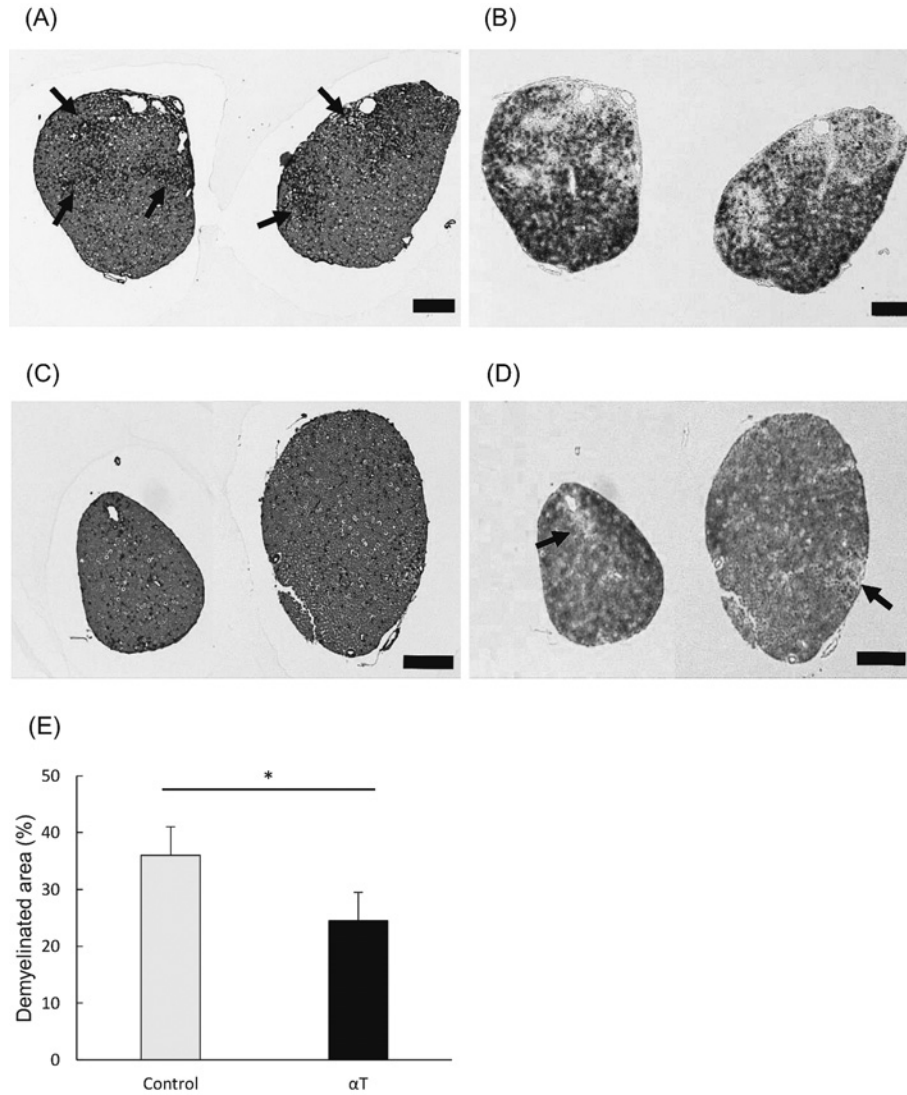
#### Statistical analysis

Statcel 3 software (OMS Publishing Inc., Saitama, Japan) was used for determining statistical significance. For clinical scores, data obtained by ELISA and RT-PCR between the two groups were analyzed by nonparametric Mann-Whitney U test. Data were considered statistically significant where  $P < 0.05$ .

## Results

First, to determine the anti-inflammatory properties of  $\alpha$ T, we examined its effects in EAN. All rats in both  $\alpha$ T-treated and non-treated control groups developed EAN. The administration of 100 mg/kg of  $\alpha$ T on day 6 p.i. alone resulted in decreased severity of the clinical course at the acute phase compared to that of the non-treated control rats ( $P < 0.05$  on day 17 i.p.; Fig. 1A). However, disease severity was similar to that in the non-treated controls by the middle of the recovery phase. Upon testing another therapeutic regimen, we found that administration of the same dose of  $\alpha$ T on days 6 and 13 p.i. was optimal for suppressing EAN. In this regimen, disease severity was significantly lower on day 14-28 p.i. in the  $\alpha$ T-treated rats than the non-treated control rats ( $P < 0.05$  and  $P < 0.01$ , respectively; Fig. 1B). These results suggest that multiple-dose administration of  $\alpha$ T has a significant therapeutic effect in EAN.

Second, histological examination of the cauda equina of the non-treated control group revealed clear multifocal foci of mononuclear cell infiltration on day 16 p.i. (Fig. 2A). In serial sections, these foci coincided with patchy non-



**Fig. 2** Alpha-tocopherol treatment reduces inflammation of the cauda equina. On day 16 post injection, the cauda equina were collected, and 5  $\mu\text{m}$ -thick sections from the levels of L2-3 were stained with hematoxylin & eosin and Luxol fast blue. Photomicrographs of the cauda equina in EAN rats treated with vehicle (non-treated control groups). Arrows in (A) (hematoxylin & eosin staining) indicate inflammatory cellular infiltration. The same foci in the serial section shown in (B) (Luxol fast blue staining) are not stained, indicating demyelination with cell infiltration. Conversely, photomicrographs of the cauda equina of  $\alpha\text{T}$ -treated EAN rats indicate less inflammatory cellular infiltration as shown in (C) (hematoxylin & eosin staining) and less demyelinating foci indicated by arrows in (D) (Luxol fast blue staining). Bars indicate 100  $\mu\text{m}$ . The rate of demyelinated areas in the whole cauda equina on day 16 p.i. (E) were significantly smaller in the  $\alpha\text{T}$ -treated rats than those in the non-treated rats. Data show mean  $\pm$  SEM. Values of  $P < 0.05$  (\*) were considered to represent statistical significance.

stained foci by LFB staining, indicating demyelination (Fig. 2B). Thus, we defined such lesions as EAN foci. The proportion of total EAN foci area to the whole transverse area in  $\alpha\text{T}$ -treated rats was significantly smaller than in the non-control rats ( $P < 0.05$ , Fig. 2C).

Third, we found that the HEL level was elevated in the cauda equine of EAN rats, from the induction phase to the acute phase. In particular, significant elevation was observed in non-treated control rats compared to the normal rats ( $P < 0.05$  on days 7, 10, and 14 i.p., respectively, Fig. 3).

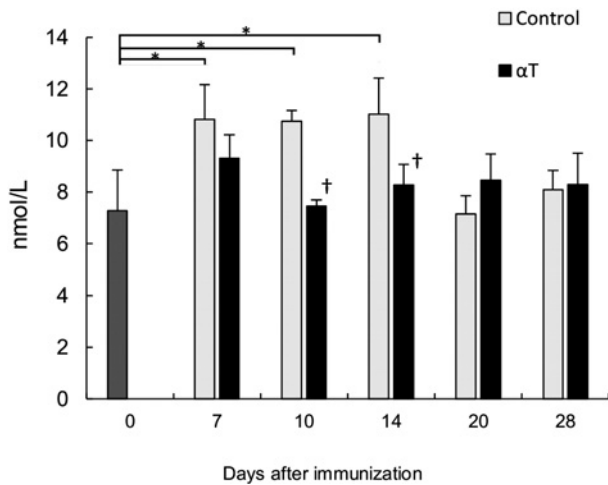


Fig. 3 Alpha-tocopherol reduces formation of N epsilon-(hexanonyl) lysine (HEL) in the cauda equina. Rats were dissected on days 7, 10, 14, 20, and 28 post injection. The formation of HEL in the cauda equina was analyzed using ELISA. Data show mean  $\pm$  SEM. Values of  $P < 0.05$  (\*) obtained on comparison with the normal rats were considered to represent statistical significance.  $P < 0.05$  ( $\dagger$ ),  $P < 0.01$  ( $\dagger\dagger$ ) compared with the non-treated control rats.

However, this was decreased to a significantly low level in  $\alpha$ T-treated rats compared to the non-treated rats ( $P < 0.05$  on days 10 and 14 i.p., respectively, Fig. 3).

To determine the mechanisms by which  $\alpha$ T improves the clinical course and inhibits inflammatory cell infiltration in the cauda equina, we assayed gene expression of three cytokines and one transcription factor using lymphocytes of the popliteal lymph nodes and the cauda equina from  $\alpha$ T-treated or non-treated control rats. As shown in Fig. 4, *IFN- $\gamma$*  expression and the level of representative pro-inflammatory cytokines was substantially reduced in both the popliteal lymph nodes and the cauda equina of  $\alpha$ T-treated rats ( $P < 0.05$  on days 10, 14 and 20 i.p. and  $P < 0.05$  on days 10 and 14 i.p., respectively, Fig. 4A and E). Alpha-T treatment also decreased the expression of *IL-10* ( $P < 0.05$  on day 10 and 20 i.p. and  $P < 0.05$  on days 10, 14 and 20 i.p. respectively, Fig. 4B and F), a cytokine that reciprocally inhibits pro-inflammatory cytokines, resulting in the spontaneous recovery of EAN. We also tested the expression of *IL-17* and transcription factor *FoxP3*; however, no significant differences were found between the two groups (Fig. 4C, D, G, and H).

## Discussion

The main results of this study can be summarized as follows: (1)  $\alpha$ T treatment ameliorated the clinical course of

EAN. (2) The levels of the lipid peroxidic marker, HEL, increased in the cauda equina from the induction phase to the acute phase of EAN; however, (3) this increase in the levels of HEL and the gene expression of cytokines *IFN- $\gamma$*  and *IL-10* were suppressed in the cauda equina and the popliteal lymph nodes of  $\alpha$ T-treated rats.

To improve the clinical course of EAN, double  $\alpha$ T injection during the induction and acute phases was required. However, in a previous study, a single  $\alpha$ T injection on day 3 p.i. alone, with the same dose as in the present study, was reportedly sufficient to improve the symptoms of experimental autoimmune encephalomyelitis (EAE) in mice as an animal model of multiple sclerosis.<sup>24)</sup> This difference may be attributable to variations in disease susceptibility and severity,  $\alpha$ T pharmacokinetics, and immunization protocols between the species studied.

The initiation of lipid peroxidation in normal tissue is a step in the production of fatty acid radicals. The most well-known initiators in living cells are ROS, hydroxyl radicals ( $\text{OH}^\bullet$ ), and hydroperoxyl radical ( $\text{HOO}^\bullet$ ), which combine with a hydrogen atom to form water and a fatty acid radical. Phagocytes, such as macrophages and neutrophils, act as ROS production sources in inflamed tissue. In addition to their direct phagocytic effects, the activated nicotinamide adenine dinucleotide phosphate oxidase present in the cell membranes of these cells generates superoxide ( $\text{O}^\bullet$ ); further, spontaneously produced hydrogen peroxide undergoes further reactions to generate other ROS.<sup>27)</sup> We previously reported that the levels of 2, 3-dihydroxybenzoic acid, a reliable indicator of  $\text{OH}^\bullet$  damage,<sup>28)</sup> started increasing from day 7 p.i. in EAN cauda equina tissue and showed a significant increase on days 11 and 14 p.i. compared with level in normal rats.<sup>20)</sup> Such increased  $\text{OH}^\bullet$  should cause lipid peroxidation in peripheral nerve myelin, a lipid-rich substance formed by Schwann cells. In fact, deposit of the lipid peroxidic marker, malondialdehyde, were found to be consistent with infiltrating  $\text{CD45}^+\text{CD3}^-$  cells around myelin of the cauda equina.<sup>20)</sup> A previous clinical report noted that oxidative stress markers were deposited on marginal perineurial cells in cases of chronic inflammatory demyelinating polyneuropathy.<sup>29)</sup>

HEL is the initial product derived from lipid peroxide during the process of lipid peroxidation by ROS.<sup>30)</sup> Unlike the conventional aldehyde-based lipid peroxidic marker, malondialdehyde, it can capture the initial stage of lipid peroxidation as a stable marker of oxidative stress. HEL levels in EAN cauda equina increased during the same

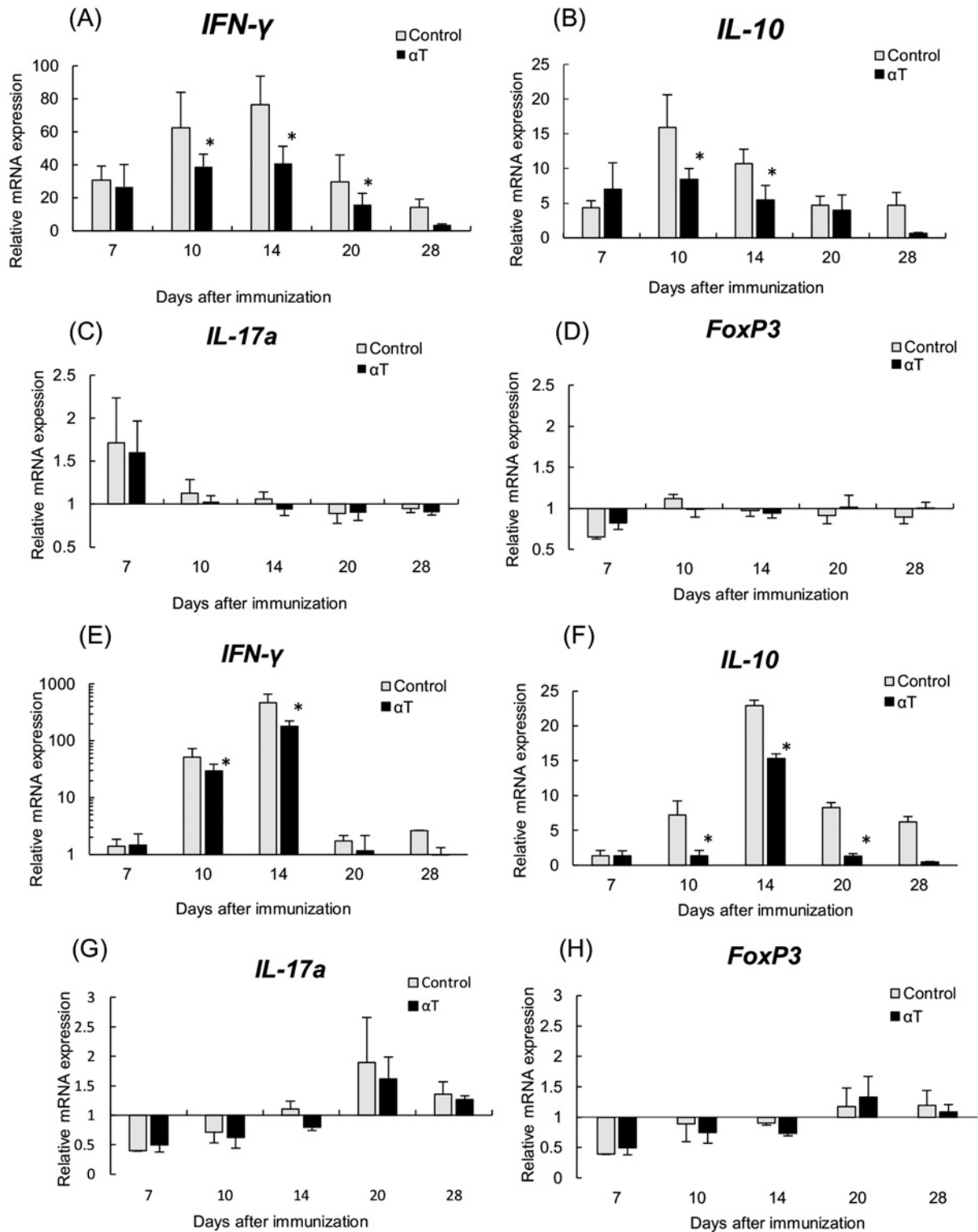


Fig. 4 Alpha-tocopherol regulates sequential cytokine gene expression in the popliteal lymph nodes and the cauda equina

Rats were dissected on days 7, 10, 14, 20, and 28 post injection. Gene expression in the popliteal lymph nodes is shown in (A), (B), (C), and (D), and in the cauda equina in (E), (F), (G), and (H). Messenger RNAs were isolated from the popliteal lymph nodes and the cauda equina and analyzed for the expression of *IFN- $\gamma$*  (A, E), *IL-10* (B, F), *IL-17a* (C, G), and *Foxp3* (D, H). Data show mean  $\pm$  SEM. Values of  $P < 0.05$  (\*) were considered to represent statistical significance.



phase in which the hydroxyl radicals increased; however, this increase was modest in  $\alpha$ T-treated rats. To our knowledge, this is the first report on sequential lipid peroxidation in the peripheral nerve that preceded the development of the clinical symptoms of EAN.

Oxidative damage, which is considered an important component of the stress response, plays a fundamental role in the disruption of biological function, gene regulation, and structure of cell components in myelin. Parallel model-membrane studies have shown that  $\alpha$ T is capable of adopting two different locations in the bilayer; this property endows it with the ability to scavenge both cytosolic ROS and lipid radicals.<sup>31</sup> Therefore,  $\alpha$ T undoubtedly prevents a chain reaction of lipid oxidation in the cauda equina tissue of EAN.

The detection of high malondialdehyde levels and antioxidant activity in cerebrospinal fluid of patients with GBS<sup>32</sup> indirectly indicates an increase in lipid peroxidation and antioxidant demand at the site of inflammation. Kumar et al. reported that increased plasma vitamin E and serum total glutathione-S-transferase levels in patients with GBS may represent the compensatory effects of vitamin E due to the lack of availability of superoxide dismutase/vitamin C.<sup>33</sup> Low levels of serum uric acid, a naturally occurring antioxidant in human blood, were also observed in patients with GBS.<sup>34</sup> Furthermore, in patients with GBS and Hughes severity scores at month 3, there was a negative correlation between total thiol levels, a novel oxidative stress parameter; thus, increasing antioxidant activity has been thought to promote motor function recovery in GBS patients.<sup>35</sup> These findings support the finding that antioxidants represent a potential therapeutic approach to remedy the imbalance of the oxidative-reductive reaction in GBS.

In addition to its antioxidant activity,  $\alpha$ T contributes to the function of the immune system. Supplemental intake of vitamin E reported to increase the number of macrophages during heat stress<sup>36</sup> and enhances phagocytic capacity.<sup>37</sup> The immunological activities, in addition to the antioxidant effects of  $\alpha$ T, may be regulated by intracellular signal transduction via modification of the plasma membrane structure.<sup>38</sup> When  $\alpha$ T is distributed in lipid rafts consisting of sphingolipids and cholesterol-rich microstructures, the gaps between the microstructures are horizontally widened, followed by a decrease in the density of microstructures. It is believed that such structural changes in the cell membrane affect the transduction of

various intracellular signals. During cytokine production induced by lipopolysaccharide,  $\alpha$ T is incorporated at the boundaries of lipid rafts and in non-raft areas on the plasma membrane; this prevents the association of Toll-like receptor-4 receptor with CD14 located in the raft, thus attenuating pro-inflammatory activity.<sup>38</sup>

With respect to cellular immunity, T-helper (Th) cells are generally divided into two main types of cells according to their cytokine productivity: Th1 cells that produce IFN- $\gamma$  and IL-2; and Th2 cells, which produce IL-4 and IL-5.<sup>39</sup> EAN is primarily regulated by Th1 cells. IFN- $\gamma$  production from Th1 cells is increased in the cauda equina from the pre-symptomatic to the peak phase of EAN; reciprocally, IL-10 production from Th2 cells is increased from the peak through the recovery phase.<sup>40</sup> Among Th2 cytokines, only the level of IL-10 is increased, although another Th2 cytokine, IL-4, is not detected in the peripheral nerve system. In the present study,  $\alpha$ T treatment significantly suppressed production both of IFN- $\gamma$  and IL-10; however, IL-17 and transcription factor FoxP3 levels in the popliteal lymph nodes and the cauda equina were only slightly changed. Alpha-T treatment significantly decreased the production of IFN- $\gamma$  from splenocyte in EAE after stimulation with myelin oligodendrocyte glycoprotein, whereas that of IL-10 increased slightly, as revealed by an *ex vivo* assay.<sup>24</sup> In the present study,  $\alpha$ T-treated rats showed consistently milder clinical symptoms accompanied by lower IL-10 levels, from the acute to early recovery phase, compared to the non-treated control rats. This result suggests that the therapeutic effect of  $\alpha$ T on EAN, which reduces macrophage infiltration into the cauda equina, is mainly responsible for the suppression of IFN- $\gamma$  production from Th1 cell. As IL-10 production to counteract inflammatory effects is dependent on the level of IFN- $\gamma$ , it is reasonable to attribute the low IL-10 production to low IFN- $\gamma$  production. A known mechanism that accounts for decreased IFN- $\gamma$  production in the cauda equina in response to  $\alpha$ T treatment is the regulation of important chemotactic molecules such as monocyte chemoattractant protein-1.<sup>41</sup> The low IFN- $\gamma$  production in the popliteal lymph nodes suggests that  $\alpha$ T regulates the phase in which naïve T cells are functionally and phenotypically transformed into Th1 and/or Th2 cells in response to specific stimuli presented by the dendritic cells. In this regard, however, it is possible that additional unknown mechanisms are involved; further study is needed to understand  $\alpha$ T-induced IFN- $\gamma$  suppression in EAN.

This study has several limitations. First, the dose of drug in the present experimental therapeutic study is much higher than that used in human patients when converted to the amount per body weight. Therefore, the equivalent dose of  $\alpha$ T cannot be applied directly in human patients. Second, it is not known whether the effects seen in the rat tests can be achieved in humans, because the target antigens and detailed pathological mechanism underlying cellular immunity is yet to be clarified in human GBS. Despite these limitations, however, the findings represent important progress toward the development of novel treatment for GBS.

In conclusion,  $\alpha$ T ameliorates EAN through anti-oxidative effects and suppression of pro-inflammatory cytokine production, not through up-regulation of anti-inflammatory cytokine production.

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**Conflicts of interest:** None declared.

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