INTRODUCTION

Multiple sclerosis (MS) is a T-lymphocyte-mediated autoimmune disease that is characterized by inflammation in the central nervous system (CNS). Although many disease-modifying therapies (DMTs) are presumed effective in patients with MS, studies on the efficacy and safety of DMTs for preventing MS relapse are limited. Therefore, we tested the immunosuppressive anti-inflammatory effects of oral-formulated tacrolimus (FK506) on MS in a mouse model of experimental autoimmune encephalomyelitis (EAE). The mice were randomly divided into 3 experimental groups: an untreated EAE group, a low-dose tacrolimus-treated EAE group, and a high-dose tacrolimus-treated EAE group. After autoimmuneimmunization of the EAE mice with myelin oligodendrocyte glycoprotein, symptom severity scores, immunohistochemistry of the myelination of the spinal cord, and western blotting were used to evaluate the EAE mice. After the autoimmunization, the symptom scores of each EAE group significantly differed at times. The group treated with the larger tacrolimus dose had the lowest symptom scores. The tacrolimus-treated EAE groups exhibited less demyelination and inflammation and weak immunoreactivity for all of the immunization biomarkers. Our results revealed that oral-formulated tacrolimus inhibited the autoimmunization in MS pathogenesis by inactivating inflammatory cells.

Keywords: Multiple Sclerosis; Neuromyelitis Optica; Experimental Autoimmune Encephalomyelitis; EAE; Tacrolimus; FK506

The Anti-Inflammatory Effects of Oral-Formulated Tacrolimus in Mice with Experimental Autoimmune Encephalomyelitis

Myung-Jin Kim,1 Jung-Joon Sung,2 Seung Hyeon Kim,3 Jeong-Min Kim,1 Gye Sun Jeon,2 Seog-Kyun Mun,4 and Suk-Won Ahn1

1Department of Neurology, Chung-Ang University Hospital, Chung-Ang University College of Medicine, Seoul, Korea; 2Department of Neurology, Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea; 3Department of Neurology, Hanyang University Hospital, Hanyang University College of Medicine, Seoul, Korea; 4Department of Otorhinolaryngology, Head and Neck Surgery, Chung-Ang University College of Medicine, Seoul, Korea

Received: 22 December 2016  
Accepted: 28 May 2017

Address for Correspondence:  
Suk-Won Ahn, MD, PhD  
Department of Neurology, Chung-Ang University Hospital, Chung-Ang University College of Medicine, 102 Heukseok-ro, Dongjak-gu, Seoul 06973, Korea  
E-mail: icandr@hanmail.net

Funding: This research was supported by the Chung-Ang University Graduate Research Scholarship (2016), and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1D1A1B03036273).

Multiple sclerosis (MS) is a T-lymphocyte-mediated autoimmune disease that is characterized by inflammation in the central nervous system (CNS). Although many disease-modifying therapies (DMTs) are presumed effective in patients with MS, studies on the efficacy of DMTs for preventing the relapse of patients with MS are limited. In addition, DMTs can induce serious adverse effects (9,10).

We tested the anti-inflammatory effects of an oral-formulated immunosuppressant with a better side effect profile in a mouse model of experimental autoimmune encephalomyelitis (EAE) (11). Tacrolimus (FK506; macrolide lactone immunosuppressant) acts as a calcineurin inhibitor that blocks interleukin-2 production, which results in decreased T-cell proliferation (12,13). Tacrolimus is therefore used in the treatment of T-cell-mediated autoimmune diseases and prevention of organ transplant rejection (12,13). The EAE animal model is used most in studies of MS in laboratory animals. Both MS and EAE are characterized by perivascular inflammation and demyelination in the spinal cord and brain. EAE is a CD4+ T-cell-mediated autoimmune disease in which CNS inflammation is induced after the animals are immunized against a myelin-specific antigen that induces the migration of activated autoreactive T-cells across the blood-brain barrier and into the CNS (14,15).

In this study, we used C57BL/6 mice that had been immu-
Kim M-J, et al. • Tacrolimus in EAE Mice

nized against the myelin oligodendrocyte glycoprotein (MOG35–55) peptide, which is a method that is widely applied to induce EAE in animals. Because of the promising beneficial effects and safety of tacrolimus, the present study aimed to assess the therapeutic effects of oral tacrolimus in MS.

MATERIALS AND METHODS

Induction of EAE and animal care
All of the procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use. Animals exhibiting paralysis were kept on soft bed of each cage and fed and watered through animal feeding tube. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. If any mouse came to the moribund stage, it was decapitated after anesthesia with sodium pentobarbital. Sixty-four mice that were approximately 80 days old were randomly divided into 3 experimental groups: an untreated EAE group, a 5-mg/kg tacrolimus-treated EAE group, and a 10-mg/kg tacrolimus-treated EAE group. During the week before the start of and during the experiments, the animals were housed in individual cages with an ambient temperature of 23°C ± 3°C, stable air humidity, and a natural day-and-night cycle. The adoptive transfer of EAE was performed as described previously. Briefly, 11-week-old female C57BL/6 mice (Central Lab. Animal Inc., Seoul, Korea) were immunized against the MOG peptide, which is a method that is widely applied to induce EAE in animals. Because of the promising beneficial effects and safety of tacrolimus, the present study aimed to assess the therapeutic effects of oral tacrolimus in MS.

Immunoblotting and antibodies
Fourteen days after immunization, each mouse was perfused with phosphate-buffered saline (PBS) while they were deeply anesthetized, and the CNS was removed. The spinal cords were homogenized in ice-cold radio immunoprecipitation assay buffer containing protease inhibitor and phosphatase inhibitor cocktails (Catalog No. 01906845001 and 11697498001; Roche Diagnostics Corporation, Indianapolis, IN, USA). The samples were centrifuged at 14,000 rpm for 25 minutes at 4°C, and the supernatants were collected. The protein concentrations of the samples were measured with Thermo Scientific Pierce Micro BCA Protein Assay Kits (Thermo Fisher Scientific Inc., Waltham, MA, USA). The samples were denatured in loading buffer for 10 minutes at 95°C, loaded into 15% gel for separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then blotted onto nitrocellulose membranes with a Trans-Blot Cell system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). After the transfer, the membranes were blocked for 1 hour at room temperature with 5% skim milk (Duchefa Biochemie BV, Haarlem, The Netherlands) in Tris-buffered saline (20 mM Tris, 150 mM NaCl [pH 7.5]) with 0.1% Tween 20 (TBST). The membranes were then incubated overnight at 4°C with one of the following primary antibodies: MOG (1:1,000, Catalog No. ab109746; Abcam plc, Cambridge, UK), myelin basic protein (1:1,000, Catalog No. ab40390; Abcam plc), glial fibrillary acidic protein (GFAP; 1:4,000, Catalog No. ab7260; Abcam plc), ionized calcium binding adaptor 1 (Iba1, 1:250, Catalog No. ab1758; Abcam plc), or CD4 (1:200, Catalog No. sc-1140; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were then washed in TBST buffer and incubated for 1 hour with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (1:5,000, Catalog No. ADI-SAB-300; Enzo Life Sciences, Inc., Farmingdale, NY, USA) or HRP-conjugated donkey anti-goat IgG (1:2,000, Catalog No. A50-101P; Bethyl Laboratories, Inc., Montgomery, TX, USA) that was diluted in 5% (w/v) skim milk in TBST buffer. The membranes were then washed in TBST and developed with a Chemiluminescent Sensitive Plus HRP Microwell and/or Membrane Substrate (Surmodics, Inc., Eden Prairie, MN, USA). The membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) at room temperature for 20 minutes and then incubated with an antibody to β-actin (1:1,000, Catalog No. 4970; Cell Signaling Technology, Inc., Danvers, MA, USA), which was used as a loading control.

Clinical characteristics of EAE
All of the animals were checked daily after tacrolimus treatment, and their symptoms were recorded. EAE mice show flaccid paralysis, which is characterized by decreased muscle tone that progresses from the tail upward through the body (16,19). A 6-point scale (0–5) was used to rate the severity of the symptoms, with a score of 1 denoting tail paralysis, a score of 4 indicating quadriplegia, and a score of 5 signifying death. An investigator who was blind to the groups rated the experimental animals as follows: 0, no clinical disease; 0.5, piloerection; 1, tail weakness; 1.5, tail paralysis; 2, hind limb weakness; 3, hind limb paralysis; 3.5, forelimb weakness; 4, forelimb paralysis; or 5, moribund or death. The symptom scores of the mice are expressed in mean ± standard error. The data were analyzed with analysis of variance tests and Dunnett’s post-hoc tests, if necessary (SPSS, version 19.0; IBM Corp., Armonk, NY, USA). P values less than 0.05 were considered statistically significant.
Immunohistochemistry
After the mice were killed and the spinal cord removed, the spinal cord was fixed with 4% (w/v) paraformaldehyde in PBS buffer overnight at 4°C. The spinal cords were dehydrated in 30% sucrose at 4°C until the tissue sank. The tissue was then embedded in O.C.T. compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen with dry ice (21,22). The samples were cut into 10-μm coronal sections from the cervical spine to thoracic spine. The sections were stained with Luxol fast blue (LFB) stain or hematoxylin & eosin (22). For the LFB stain, which was performed to stain myelin, the sections were incubated overnight at 60°C in 0.1% LFB (Solvent Blue 38; Sigma-Aldrich Co., LLC, St. Louis, MO, USA). The sections were rinsed in distilled water (DW) twice and then differentiated by dipping them in 0.01% lithium carbonate (Cat#L0224; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). The sections were rinse twice in DW and then Nissl stained by incubating them in 0.1% cresyl violet (Sigma-Aldrich Co., LLC) for 5 minutes (22,23). The sections were then rinsed quickly in fresh DW. The sections were dehydrated by dipping them in 3 changes of absolute ethanol (Merck Chemicals GmbH, Darmstadt, Germany). For the hematoxylin & eosin, which was performed to analyze inflammation, the tissue sections were hydrated in DW for 10 minutes, stained with Mayer’s Hematoxylin solution (Sigma-Aldrich Co., LLC) for 10 minutes, washed in DW for 5 minutes, and then stained with Eosin Y solution, alcoholic (Sigma-Aldrich Co., LLC) for 3 minutes (22,23). The sections were rinsed quickly in 70% Ethanol and dehydrated in 3 changes of absolute ethanol (Merck Chemicals GmbH). All of the sections were mounted on slides and cover slipped with Permount (Thermo Fisher Scientific Inc.). Light microscopy was used to examine the slides.

Ethics statement
All of the procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines and were approved by IACUC (Permit No. 2015-00026) of the Chung-Ang University for the care and use.

RESULTS
In this study, we evaluated the clinical EAE symptom scores in each EAE group every day during the 14-day study in order to examine the functional loss and alterations of the mice in this EAE model. In addition, we examined inflammatory cell infiltration and demyelination in the spinal cord specimens with immunohistochemistry to determine if the severity of the spinal cord histopathology correlated with tacrolimus administration. Furthermore, we investigated autoimmunization and CNS inflammation by evaluating the presence of activated microglial cells, interleukin-2, macrophages, and T-cell activation in the EAE animals.

After autoimmunization with MOG35–55, the EAE symptom scores increased gradually in each group over time, and significant differences were found among the groups beginning the 10th day after immunization (Fig. 1; P < 0.05). The tacrolimus-treated mice had lower symptom scores compared with the untreated mice. The 10-mg/kg tacrolimus-treated group had lower EAE symptom scores compared with the 5-mg/kg tacrolimus-treated group. These results demonstrated that the oral form of tacrolimus improved the clinical symptoms and disease progress of the EAE mice model.

We stained the spinal cord sections with LFB to visualize myelin and the demyelination zones in the white matter (Fig. 2). Tacrolimus treatment remarkably maintained the levels of myelination in the spinal cords of the EAE mice at levels similar to those in control mice. The untreated EAE mice showed marked demyelination in the white matter of the spinal cord and inflammatory cell infiltration in the perivascular area. The tacrolimus-treated EAE groups exhibited higher myelination levels and decreased inflammation compared with the control EAE mice. In addition, compared with the 5-mg/kg tacrolimus-treated EAE mice, the 10-mg/kg tacrolimus-treated EAE mice had higher levels of myelination and less inflammatory infiltration, and cuffed vessels were observed in the spinal cord. Tacrolimus treatment markedly inhibited autoimmunization and the infiltration of inflammatory cells into the spinal cords of the EAE mice. These results demonstrated that tacrolimus treatment inhibited the immune cascade abnormal infiltration of inflammatory cells, and spinal cord demyelination of the EAE mice.

In order to determine the clinical significance of the tacrolimus-mediated decreases in CD4 T-cell activity, we investigated
Fig. 2. Histopathology of EAE mice. The spinal cord specimens of the untreated EAE mice exhibited marked demyelination (A) and inflammatory cell infiltration in the perivascular area (D). However, the tacrolimus-treated EAE mice exhibited preserved myelination and decreased inflammation compared with the untreated group. Additionally, the 10-mg/kg tacrolimus-treated EAE mice exhibited more myelination (C) and decreased inflammation (F) compared with the 5-mg/kg tacrolimus-treated EAE mice (B, E). EAE = experimental autoimmune encephalomyelitis.

Fig. 3. Western blots of EAE mice. Fourteen days after the immunization, specimens from the untreated EAE group and tacrolimus-treated groups were investigated with western blots. The blots of the samples from the tacrolimus-treated EAE mice contained bands with strong immunoreactivity to MOG and weak immunoreactivity to CD4 compared with those in the untreated mice (A). In addition, the blots of the samples from the tacrolimus-treated EAE mice had weak immunoreactivity to Iba1 and GFAP compared with those in the untreated EAE mice (B). EAE = experimental autoimmune encephalomyelitis, MOG = myelin oligodendrocyte glycoprotein, Iba1 = ionized calcium binding adaptor 1, GFAP = glial fibrillary acidic protein.
whether tacrolimus prevented the clinical symptoms of EAE in the mice. During tacrolimus treatment, the in vivo functions of the CD4 cells were blocked in the EAE mice. Taken together, these results suggested that CD4 T-cell activity plays an important role in the tacrolimus-mediated decrease in inflammatory reaction by autoimmunity in EAE model.

Western blotting was used to compare the levels of immunization biomarkers in the untreated and tacrolimus-treated EAE groups (Fig. 3). Compared with the untreated EAE mice, the tacrolimus-treated EAE mice exhibited stronger immunoreactivity to MOG and weaker immunoreactivity to CD4, Iba1, and GFAP. Collectively, these results suggested that tacrolimus suppressed the EAE in the mice by inhibiting T-cell activation.

**DISCUSSION**

In this study, we evaluated the anti-inflammatory effects of tacrolimus in an EAE mouse model and found that tacrolimus decreased the severity of the clinical symptoms of EAE and inhibited autoimmunization and inflammation in the EAE model. Thus, these results suggested that tacrolimus might be effective in the treatment of MS. EAE in mice and MS are thought to have similar causes: the infiltration of autoreactive T-cells and associated inflammatory cells into the CNS, which results in the immune-associated CNS demyelination that is observed in patients with MS and EAE animals (24,25).

MS is a devastating demyelinating disease of the human CNS, and expensive DMTs are currently used to treat patients with MS (26). However, therapies that effectively prevent the relapse of MS are not yet available. In particular, the limited efficacy and severe side effects, as well as treatment costs, of these therapies often limit their availability. The side effects include flu-like symptoms, menstrual disorders in women, decreased neutrophil and white blood cell counts, progressive multifocal encephalopathy, cardiac problems, increased aspartate transaminase and alanine transaminase levels, and the development of neutralizing antibodies (9,10,27,28). Therefore, it is necessary to investigate safe, effective, and less expensive therapeutic options for patients with MS.

The results of the present study suggested that tacrolimus might be useful for treating patients with MS because of its neuroprotective properties, safety, and reasonable cost. The oral form of tacrolimus, which has been clinically approved for other diseases (12,13), has recently been shown to have significant immunosuppressive properties that result in the inhibition of CNS demyelination and axonal injury in EAE mice and patients with MS. Furthermore, these results suggest that the anti-inflammatory properties of immunosuppression are responsible for the decreased clinical symptoms in the EAE mice, and these properties might be critical for the effective inhibition of relapse in patients with MS.

Relatively few studies have been conducted on the use of tacrolimus as a treatment for immune-associated CNS diseases, and, to the best of our knowledge, no studies have investigated the use of the approved oral form of tacrolimus in EAE model. Of course, a few studies revealed therapeutic pathomechanisms of tacrolimus on EAE model by the peritoneal injections of FK506, however our study identified therapeutic effectiveness of oral formulated tacrolimus which would be much feasible as strategic therapeutics compared with injection (29,30). Also, a clinical study on the combination therapy of tacrolimus and interferon beta was reported, however it did not identify pure therapeutic effects of oral formulated tacrolimus in MS because interferon therapy has been established DMT for MS (31).

However, the results of our study provide evidence that the oral administration of tacrolimus to mice suppressed the disease process underlying EAE, inhibited the invasion of mononuclear cells into the spinal cord, and restored myelination in the CNS. Additionally, we did not observe any side effects in any of the mice treated with tacrolimus. Therefore, this study would be the cornerstone for further clinical researches on MS with using tacrolimus.

In summary, these results revealed that tacrolimus was therapeutic by inhibiting autoimmunization in EAE mice. These therapeutic effects of tacrolimus might result in the inactivation of the CD4 T-cell immune pathway and decreased inflammatory cells. In conclusion, the results of the present study suggested that the oral administration of tacrolimus might be an ideal alternative DMT for patients with MS because of its safety, anti-inflammatory effects, and affordability. Further studies are required to identify the therapeutic dose of tacrolimus in patients with MS.

**DISCLOSURE**

The authors have no potential conflicts of interest to disclose.

**AUTHOR CONTRIBUTION**

Conceptualization: Kim MJ, Sung JJ, Kim SH, Ahn SW. Data curation: Kim MJ, Kim JM, Mun SK, Ahn SW. Investigation: Kim MJ, Kim JM, Mun SK, Ahn SW. Writing - original draft: Kim MJ, Jeon GS, Mun SK, Ahn SW.

**ORCID**

Myung-Jin Kim https://orcid.org/0000-0001-9199-1724  
Jung-Joon Sung https://orcid.org/0000-0001-7525-5313  
Seung-Hyun Kim https://orcid.org/0000-0001-9644-9598  
Jeong-Min Kim https://orcid.org/0000-0001-7213-5527  
Gye Sun Jeon https://orcid.org/0000-0001-5090-4292  
Seog-Kyun Mun https://orcid.org/0000-0001-8624-2964
REFERENCES

1. Compston A, Coles A. Multiple sclerosis. Lancet 2008; 372: 1502-17.
2. Bar-Or A. Immunology of multiple sclerosis. Neurol Clin 2005; 23: 149-75.
3. Frohman EM, Racke MK, Raine CS. Multiple sclerosis—the plaque and its pathogenesis. N Engl J Med 2006; 354: 942-55.
4. Weinschenker BG, Blass B, Rice GP; Noseworthy J, Carriere W, Baskerville J, Ebers GC. The natural history of multiple sclerosis: a geographically based study. I. Clinical course and disability. Brain 1989; 112: 133-46.
5. Brinkmann V, Billich A, Baumrukter T, Heinig P, Schmouder R, Francis G, Aradhye S, Burtin P. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 2010; 9: 883-97.
6. Kappos L, Polman CH, Freedman MS, Edan G, Hartung HP, Miller DH, Montalban X, Barkhof F, Bauer L, Jakobs P, et al. Treatment with interferon-beta-1b delays conversion to clinically definite and McDonald MS in patients with clinically isolated syndromes. Neurology 2006; 67: 1342-9.
7. Neuhaus O, Farina C, Wekerle H, Hohlfeld R. Mechanisms of action of glatiramer acetate in multiple sclerosis. Neurology 2005; 65: 702-8.
8. Mikol DD, Barkhof F, Chang P, Coyle PK, Jeffery DR, Schwid SR, Stohbini B, Uitdehaag B. REGARD study group. Comparison of subcutaneous interferon-beta-1a with glatiramer acetate in patients with relapsing-multiple sclerosis (the REBIF vs Glatiramer Acetate in Relapsing MS Disease [REGARD] study): a multicentre, randomised, parallel, open-label trial. Lancet Neurol 2010; 9: 903-15.
9. Walther EU, Hohlfeld R. Multiple sclerosis: side effects of interferon beta therapy and their management. Neurology 1999; 53: 1622-7.
10. Herndon RM, Rudick RA, Munschauer ME, Friedmann MS, Edan G, Saidel A, Coats ME, Labutta R, Richert JR, Cohan SL, Genain C, et al. Eight-year immunogenicity and safety of interferon-beta-1a-Avonex treatment in patients with multiple sclerosis. Mult Scler 2005; 11: 409-19.
11. Pahan K. Neuroimmune pharmacological control of EAE. J Neuroimmune Pharmacol 2010; 5: 165-7.
12. Furukawa Y, Yoshikawa H, Iwasa K, Yamada M. Clinical efficacy and cytokine network-modulating effects of tacrolimus in myasthenia gravis. J Neuroimmunol 2008; 195: 109-14.
13. Friedrich RB, Coradini K, Fonseca FN, Gutierrez SS, Beck RC, Pohlmann AR. Lipid-core nanocapsules improved antiedematogenic activity of tacrolimus in adjuvant-induced arthritis model. J Nanosci Nanotechnol 2010; 16: 1265-74.
14. Mondal S, Roy A, Pahan K. Functional blocking monoclonal antibodies against IL-12p40 homodimer inhibit adoptive transfer of experimental allergic encephalomyelitis. J Immunol 2009; 182: 5013-23.
15. Kuerten S, Lehmann PV. The immune pathogenesis of experimental autoimmune encephalomyelitis: lessons learned for multiple sclerosis? J Interferon Cytokine Res 2011; 31: 907-16.
16. Miller SD, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol 2010; Chapter 15: Unit 15.1.
17. Mendel I, Kerlero de Rosbo N, Ben-Nun A. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. Eur J Immunol 1995; 25: 1551-9.
18. Pachner AR. Experimental models of multiple sclerosis. Curr Opin Neurol 2011; 24: 291-9.
19. van der Star BJ, Vogel DY, Kipp M, Puente F, Baker D, Amor S. In vitro and in vivo models of multiple sclerosis. CNS Neurool Disord Drug Targets 2012; 11: 570-88.
20. Hoefstetter IH, Shive CL, Forsthuber TG. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. J Immunol 2002; 169: 117-25.
21. Cinar O, Semiz O, Can A. A microscopic survey on the efficiency of well-known routine chemical fixatives on cryosections. Acta Histochem 2006; 108: 487-96.
22. Lamberts R, Goldsmith PC. Fixation, fine structure, and immunostaining for neuropeptides: perfusion versus immersion of the neuroendocrine hypothalamus. J Histochem Cytochem 1986; 34: 389-98.
23. Mondal S, Pahan K. Cinnamon ameliorates experimental allergic encephalomyelitis in mice via regulatory T cells: implications for multiple sclerosis therapy. PLoS One 2015; 10: e0116566.
24. Vighietta V, Baecker-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004; 199: 971-9.
25. Trapp BD, Peterson J, Ransohoff RM, Rudick R, Mork S, Bö L. Axonal transaction in the lesions of multiple sclerosis. N Engl J Med 1998; 338: 278-85.
26. Kobelt G, Berg J, Atherly D, Hadjimichael O. Costs and quality of life in multiple sclerosis: a cross-sectional study in the United States. Neurology 2006; 66: 1696-702.
27. Petersen B, Bendtzen K, Koch-Henriksen N, Ravnborg M, Ross C, Sorensen PS; Danish Multiple Sclerosis Group. Persistence of neutralizing antibodies after discontinuation of IFN-beta therapy in patients with relapsing-remitting multiple sclerosis. Mult Scler 2006; 12: 247-52.
28. Frohman EM, Brannon K, Alexander S, Sims D, Phillips JT, O’Leary S, Hawk AR. Lipid-core nanocapsules improved antiedematogenic activity of tacrolimus in adjuvant-induced arthritis model. J Nanosci Nanotechnol 2010; 16: 1265-74.
29. Mondal S, Roy A, Pahan K. Functional blocking monoclonal antibodies against IL-12p40 homodimer inhibit adoptive transfer of experimental allergic encephalomyelitis. J Immunol 2009; 182: 5013-23.
30. Kuerten S, Lehmann PV. The immune pathogenesis of experimental autoimmune encephalomyelitis: lessons learned for multiple sclerosis? J Interferon Cytokine Res 2011; 31: 907-16.
31. Miller SD, Karpus WJ, Davidson TS. Experimental autoimmune encephalomyelitis in the mouse. Curr Protoc Immunol 2010; Chapter 15: Unit 15.1.