Amelioration of Experimental Autoimmune Encephalomyelitis in Lewis Rats by FTY720 Treatment

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Abbreviations:

CNS, central nervous system; EAE, Experimental autoimmune encephalomyelitis; RT-PCR, Reverse-transcription polymerase chain reaction; PBL, peripheral blood lymphocyte

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ABSTRACT

Experimental autoimmune encephalomyelitis (EAE) is a T cell-dependent autoimmune disease that reproduces the inflammatory demyelinating pathology of multiple sclerosis (MS). We investigated the efficacy and mechanism of immunosuppression against EAE by administering FTY720 in Lewis rats immunized with myelin basic protein (MBP) together with complete Freund’s adjuvant. FTY720 treatment almost completely protected the rats against disease. The protection by FTY720 was associated with a dramatic reduction in the number of lymphocytes staining for T-cell receptors in the spinal cord as examined by immunohistochemistry. The mRNA expression of Th1 cytokines IL-2, IL-6 and INF-γ in the spinal cord was also reduced dramatically as assessed by reverse-transcription polymerase chain reaction (RT-PCR). Furthermore, lymphocytes isolated from the spleen of FTY720-treated rats were transferred into naive recipient rats against EAE manifestation by reducing both disease incidence and clinical score. These results suggested that the protective anti-inflammatory effect of treatment with FTY720 was, to a large extent, due to the inhibition of encephalitogenic T-cell responses and/or their migration into the central nervous system (CNS), and may be a potential candidate for use in treating patients with MS.
Multiple sclerosis (MS) is a common and often disabling disease of the central nervous system (CNS). The early active MS lesions are characterized by the presence of mononuclear cell infiltrates around venules and small veins, followed by myelin breakdown and astrogliosis, resulting in irreversible disability. The etiology of the disease remains uncertain but is widely considered to involve organ-specific autoimmune destruction of CNS myelin.

Acute experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of the CNS, has been widely used as an animal model for testing novel therapeutic approaches for MS. The disease can be induced in different species of laboratory animals by injecting central nervous tissue antigens emulsified in an appropriate adjuvant, e.g. complete Freund’s adjuvant (CFA). In Lewis rats, a susceptible strain, EAE is manifested by a paralytic attack that affects the tail and hind limbs 11 to 14 days after injection of guinea pig myelin basic protein (MBP) as an encephalitogenic antigen. Consistent with this, EAE can also be induced in naive animals by transferring MBP-activated T cells. The initial observation by Paterson (Paterson, 1960) that the autoimmune disease EAE could be induced by transferring lymphocytes from activate-sensitized rats to naive histocompatible recipients confirmed the condition to be principally an immune cell-mediated phenomenon. Others also reported that relatively small numbers of spleen cells have transferred full clinical signs of EAE if cultured with mitogen concanavalin A (Con A) (Panitch and McFarlin, 1977) or with the antigen MBP, prior to transfer (Richert et al., 1979).

Clinically, the disease follows an acute and monophasic course. The main pathological event is the appearance of inflammatory cell infiltrates forming perivascular cuffs. The inflammatory infiltrates in acute EAE and MS contain
predominantly a diverse accumulation of T cells, macrophages and some B cells.

Pharmacological studies utilizing both the active and adoptive models of EAE have provided useful information on the mechanisms by which steroid and non-steroid immunomodulatory drugs may act and be of potential value in treating MS.

A potent immunosuppressive compound, ISP-1, and its derivatives, mycestericins, were isolated from the culture broth of *Isaria sinclairii*, a species of vegetative wasp (Fujita et al., 1994a). Chemical modification of ISP-1 led to a novel synthetic immunosuppressant, 2-amino-[2-(4-octylphenyl)ethyl]-1,3-propanediol hydrochloride (FTY720), which has more potent immunosuppressive activity and less toxicity than ISP-1 (Fujita et al., 1994b). FTY720 administered at 0.1mg/kg or more significantly prolonged the survival of skin, cardiac, liver, renal, pancreas, lung and small bowel allografts in rats (Brinkmann et al., 2001). Furthermore, FTY720 combined with cyclosporin A (CsA) or tacrolimus (FK506) produced synergistic immunosuppressive effects (Yanagawa et al., 1998).

A striking feature of FTY720 is that it induces a marked decrease in the number of peripheral blood lymphocytes (PBL), especially T cells, at doses that prolong allograft survival (Hoshino et al., 1996). A recent report showed that FTY720 selectively induces cell death in mature T-lymphocyte, especially CD4-positive cells, in peripheral blood without depressing bone marrow (Enosawa et al., 1996). It has been hypothesized that apoptotic cell death of lymphocytes and acceleration of lymphocyte homing decrease the number of lymphocytes (Suzuki et al., 1996b; Chiba et al., 1998; Yanagawa et al., 1998).

Recently, Brinkmann et al. reported that FTY720 prevented development of EAE in Wister rats (Brinkmann et al., 2002). We attempted to confirm and extend their findings
by evaluating the suppressive effects of FTY720 on EAE in Lewis rats, which have presented evidence that the immune and neuroendocrine system can contribute to susceptibility to inflammatory autoimmune disease (MacPhee and Mason, 1988). In the present study, we show that oral administration of FTY720 almost completely protected rats immunized with MBP/CFA against EAE, resulting in a dramatic reduction of leukocyte infiltration into the CNS and decreased expression of IL-2, IL-6 and INF-γ in the CNS. Furthermore, the capacity to generate disease could be inhibited when isolated spleen cells were transferred from FTY720-treated rats to naive Lewis rats.
MATERIALS AND METHODS

Animals

We purchased 250 to 280g, ten-week-old male inbred Lewis rats from Shizuoka Laboratory Animal Center (Shizuoka, Japan). All animals were provided water and food ad libitum, and were housed in accordance with institutional animal care policies.

Induction of acute EAE

The methods of acute EAE induction were similar to those published previously (Schmitz et al., 1991). We emulsified MBP (kindly provided by Dr. W. F. Hickey) in 0.9% saline in an equal volume of complete Freund’s adjuvant (ICN Biomedicals, Inc., Aurora, OH) containing 4mg/ml of heat-inactivated *Mycobacterium butyricum* (Difco, Detroit, MI) then immunized male Lewis rats with 0.1 ml of emulsion subcutaneously on the dorsum of two sides of the tail. The total dose of MBP was 50 to 75 µg/rat.

Induction of adoptive transferred EAE

For adoptive transferred EAE, we immunized rats with MBP as described above. Fourteen days later, we prepared spleen cell suspensions from the actively EAE-induced Lewis rats with FTY720-treated and saline-treated control, by Ficoll® Isopaque (Lymphocyte Rat, Cedar Lane, Ontario) density-gradient centrifugation. We harvested interface layer cells and washed them twice in PBS then used these cells, consisting of lymphocytes, for the following procedure. We cultured isolated erythrocyte-free lymphocyte suspensions from the immunized rats for two days with 50µg/ml ConA (Wako, Osaka, Japan). After washing them with RPMI1640 (Sigma Ltd., St. Louis, MO), we injected 6 x 10⁶ cells into naive Lewis rats.

Chemical compound

FTY720, a gift from Yoshitomi Pharmaceutical Industries (Osaka, Japan), was
dissolved in physiological saline.

**Treatment schedule of rats**

The rats were treated with either FTY720 (1mg/kg/day) or saline. The drug was given orally once a day on days 0-14 after immunization with MBP.

**Specimens**

Three animals from each group were sacrificed under ether anesthesia on days 7, 14, 21, and 28 after sensitization. The spinal cord and spleen were removed quickly. Blocks up to 1 cm³ were embedded in OCT compound (Tissue-Tek, Elkhart, IN), and snap frozen in isopentane, which was pre-cooled in acetone and dry ice, and 6µm frozen sections were cut in a cryostat for DNA fragmentation analysis and immunohistology. A second portion of the spinal cord and spleen was immediately snap-frozen for subsequent molecular analyses, and a third portion of the samples was fixed in 10% neutral buffered formalin for neuropathology.

**Clinical grading of EAE**

Rats were evaluated daily and graded by a blinded investigator according to the following scale: grade 0= no signs; grade 1= limp tail; grade 2= hind limb weakness sufficient to impair righting; grade 3= paraplegia; grade 4= paraplegia with forelimb weakness, moribund condition.

**In Situ Assay for DNA Fragmentation**

As previously described (Li et al., 2001), we used Apop Tag™ Plus Kit (Oncor, Gaithersburg, MD), which utilizes certain reagents for non-isotopic DNA end-extension in situ and other reagents for immunohistochemical staining of the extended DNA technique, to detect DNA fragmentation. Briefly, we cut cryosections (6µm), fixed them in 10% neutral buffered formalin in a coplin jar, and quenched them in 0.5 to 1%
hydrogen peroxide in PBS for 5 minutes at room temperature. We then incubated each section with a working strength terminal deoxynucleotidyl transferase (TdT) reaction mixture consisting of 38µl reaction buffer and 16µl TdT enzyme in a humidified chamber at 37°C for one hour and terminated the reaction with a pre-warmed working strength stop/wash buffer for 30 minutes at 37°C. To visualize incorporated TdT, we incubated sections with peroxidase-conjugated anti-Digoxigenin antibody for 30 minutes at room temperature, washed them three times in a coplin jar, and incubated them with DAB substrate working solution for 3 to 6 minutes at room temperature. The reaction was terminated by washing with H2O and sections were counterstained with methyl green and mounted. Negative controls were prepared by substituting PBS for the TdT enzyme in the reaction mixture.

**Reverse-transcription polymerase chain reaction (RT-PCR)**

We extracted total cellular RNA from frozen spinal cord and spleen tissue by using ISOGEN (Nippon gene, Tokyo, Japan) as described previously (Li et al., 2001) and confirmed the RNA quality on formaldehyde-agarose gels. One microgram total RNA was used for first-strand cDNA synthesis in 20 µl of 100 mM Tris-HCl, 500 mM KCl, 5 mM MgCl2, 1mM dNTP, 1 U/µl RNase inhibitor, random 9 mers primer, and 0.25 U/µl avian myeloblastosis virus reverse transcriptase (Takara, Shiga, Japan). We performed PCR amplification in a 100µl reaction mixture containing 200 µM of each of the regular dNTPs, 10 pmol of each primer, and 2.5U Taq DNA polymerase (TaKaRa) using primers IL-2 (300 base pair, bp), 5'-CAGCTGTGGCT GGACTTACAGG-3' and 5'-CACAGTTGATGGCTCATCATCG-3'; IL-6 (294 bp), 5'-GACTTCACAGAGGATACCC-3' and 5'-TAAGTTGTTCACAAACTCC-3'; INF-γ (310 bp), 5'-GGATATCTGGAGAATTGCAAGAAG-3' and 5'-GCTAGATT
CTGGTGACAGCTGGTG-3'; β-actin (461 bp)

5'-CATCGTGGGCCGCTCTAGGCA-3' and

5'-CCGGCCAGCCAAGTCCAGACGC-3'. We used the TaKaRa Thermal Cycler 480 PCR system and the "hot start" technique to increase specificity. The thermal cycling parameters were denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension for 90 sec at 72 °C (40 cycles). PCR products (10 µl) were analyzed on 1 to 1.8% agarose gels. We visualized prominent bands of the correct size with ethidium bromide staining and measured the intensity of each band by a compact digital camera (DC40) with analysis software (BioMax 1D image analysis software; Eastman Kodak Co., Rochester, NY) as described in a previous paper (Li et al., 2001). The relative quantities of genes are presented as the ratio between the intensity of IL-2, IL-6 or INF-γ band and that of the house keeping gene β-actin.

**Immunohistology and neuropathology**

We characterized the cells with monoclonal antibodies R73 (α/β T-cell receptor) to evaluate T-cell infiltrates (Serotic, Oxford, UK), air-dried the slides, fixed them in acetone at −20°C overnight, and then air-dried them for one hour. The primary antibody consisted of mouse IgG1 isotype diluted to 1:50 in a solution containing 2% bovine serum albumin and 0.1 % sodium azide in PBS. The second antibody consisted of goat antibody to mouse IgG conjugated to alkaline phosphatase (Santa Cruz Biochemicals, Santa Cruz, CA) diluted at 1:100 in the above working solution. Color development was performed with an alkaline phosphatase substrate kit (Vector Laboratories Inc, Burlingame, CA). We obtained an optimum section morphology when the sections were air dried for one hour before counterstaining in hematoxylin (Sigma).

**Statistical analyses**
Recipient survival times were compared among the groups by Gehan’s generalized Wilcoxon test. Comparisons of the mean day of onset of disease and mean peak disease severity between any two groups of rats were analyzed by the Student’s t test; P values less than 0.05 were considered significant.
RESULTS

Effect of FTY720 on Lewis EAE rats

A total of 51 rats (22 FTY720-treated and 29 saline-treated) were used in these studies. The treated and control groups were compared with regard to maximal clinical score and time to clinical onset of EAE. The results showed that 40% of the rats died after EAE induction. However, FTY720 administration almost completely prevented EAE-induced rat death (Fig. 1A). The difference in maximum clinical score between FTY720 and control groups was significant with P<0.0001 using the Student’s t test. FTY720-treated rats were less subject to EAE induction than saline-treated rats (Figs. 1B and C). Furthermore, FTY720 treatment also prevented the decrease of body weight in EAE rats (Fig. 1D), in addition to reducing the clinical score.

Effect of FTY720 on the formation of inflammatory lesions in the CNS

We performed histological studies of spinal cords to investigate the effect of FTY720 blockade on the formation of inflammatory lesions in the CNS. As shown in Fig. 2A-D, inflammatory lesions were readily detectable in control rats, whereas the spinal cords from rats administered FTY720 exhibited a complete absence of inflammatory-cell infiltrates.

Effect of FTY720 on the infiltration of T lymphocytes

In EAE, MBP-specific T lymphocytes attack the myelinated tissue of the CNS. EAE in Lewis rats generally has an acute, monophasic course. We identified the expression of T-cell receptors in CNS to investigate T lymphocyte infiltration and the effect of FTY720 on those cells. As shown in Figs. 2J-L, infiltration of T lymphocytes was found in the spinal cords of saline-treated rats. However, administration of FTY720 dramatically decreased infiltration of T lymphocytes (Figs. 2N-P). By day 14, this
difference was more marked; there were also more T cells in the portal tracts of the control group than in that of the FTY720-treated group.

**Effect of FTY720 on the induction of apoptosis in the CNS**

Apoptosis related to EAE is well known in Lewis rats (Pender et al., 1991). To identify apoptosis in the CNS, we performed TUNEL staining of the spinal cords of Lewis rats with EAE. We observed many apoptotic cells in the spinal cords of control saline-treated rats on days 21 and 28 with the TUNEL method but none in FTY720-treated rats (Fig. 2S and T vs. W and X).

**Activation of infiltrating cells and suppression of cytokine production**

The development of clinical EAE has been associated with the production of various inflammatory cytokines associated with the Th1 phenotype, including IL-2, IL-6 and IFN-γ (Ando et al., 1989; Samoilova et al., 1998). The mRNA levels of these inflammatory products have been identified and quantified in the spinal cords and spleen both of FTY720-administered and control saline-administered rats by the RT-PCR method. Expression of these cytokines was dramatically reduced in the spinal cords in FTY720-treated rats (Figs. 3A and B), whereas very little reduction was seen in spleens of the FTY720- and control saline-administered rats (Figs. 4A and B).

**Adoptive transfer of protection against EAE**

To understand the mechanism involved in suppressing EAE by administering FTY720, we tested whether this lack of response could be adoptively transferred by spleen cells from the FTY720-treated donors. As shown in Fig. 5, the results demonstrated that Con A-activated splenocytes from rats administered saline successfully transferred EAE to naive recipient rats. In contrast, Con A-stimulated spleen cells from FTY720-treated donors transferred into naive recipient rats against
EAE manifestation by reducing both disease incidence and clinical score (Fig. 5).
DISCUSSION

Despite numerous advances in the past decade, the cause and pathogenesis of inflammatory CNS demyelinating disorder, MS, remain unknown. EAE, an inflammatory CNS demyelinating disorder that serves as the prime animal model for MS, can be induced in a number of species by immunization with myelin components or injection of autoimmune T lymphocytes and has been used to study immune tolerance (Zamvil and Steinman, 1990). Recently, EAE research has reached a stage where a considerable range of new therapeutic strategies has emerged, and some of them may be very close to clinical application. A common thread in these strategies is that they could become useful for treating many different cell-mediated autoimmune diseases.

CsA and FK506 are well known immunosuppressants and have contributed to preventing EAE. For instance, actively induced EAE can be inhibited by administering CsA orally at 1 mg/kg/day (Bolton et al., 1982b; Deguchi et al., 1991); adoptive transfer-induced EAE can also be inhibited (Bolton et al., 1982a). Inamura et al. demonstrated that FK506, like CsA also inhibited actively induced EAE (Inamura et al., 1988). Bolton et al. showed that inhibition of adoptive transfer-induce EAE using the drug (Bolton, 1992). These immunosuppressants are known to exert their immunosuppressive activity by inhibiting the production of Th1-associated cytokines in Ag-stimulated T cells (Borel, 1990). Although CsA binds to cyclophilin and FK506 to FK506-binding protein (FKBP), both cyclophilin/CsA and FKBP/FK506 complexes inhibit the phosphatase activity of calcineurin that activates the Nuclear factor of activated T cell (NF-AT) involved in promoting IL-2 gene transcription (Liu et al., 1991). Because CsA and FK506 affect the same process of T cell activation, they
exhibit quite similar side effects, such as renal and liver toxicities (Platz et al., 1994). Thus, CsA- or FK506-based multiple drug therapy with steroids or other immunosuppressants has been widely used to reduce the side effects of individual immunosuppressants in clinical situations (McWhinnie and Morris, 1991). These immunosuppressants also cause metabolic derangements and organ toxicities at therapeutic doses. Therefore, drug therapies to disable or eliminate only T cells that are involved in a particular disease would potentially be very useful. The latest progress of immunosuppressive therapy has brought enormous advantages not only in the field of organ transplantation but also in the treatment of allergic and autoimmune diseases.

There is thus great interest in the recently characterized and potent immunosuppressant FTY720. FTY720 is a synthetic drug produced by modifying ISP-I purified from culture filtrates of *Isaria sircrailii*, an Ascomycete. FTY720 has demonstrated a unique mechanism to trigger rat spleen cells and several cell lines undergoing apoptosis in in vitro systems and in animal organ transplantation models, and has an effective immunosuppressive activity for preventing allograft rejection without toxic side effects (Suzuki et al., 1996a; Suzuki et al., 1996b). Through a mechanism completely different from CsA, FK506, DSG and other conventional immunosuppressants, FTY720 prevents allograft rejection by inducing apoptosis cell death in peripheral lymphocytes (Suzuki et al., 1996b) and accelerating lymphocyte homing (Chiba et al., 1998; Yanagawa et al., 1998).

The present study was undertaken to investigate effects of FTY720 upon the course and pathology of EAE, a T-cell mediated demyelinating disease of the central nervous system. Kitabayashi et al. (2000) demonstrated that FTY720 prevents development of experimental autoimmune myocarditis. Furthermore, similar autoimmune diseases such
as experimental autoimmune thyroiditis (Hozumi et al., 1999), experimental autoimmun e uveoretinitis (Kurose et al., 2000), autoimmune type I diabetes (Yan et al., 1998), and systemic lupus erythematosus (Okazaki et al., 2002) were prevented in FTY720-treated animals. Consistent with the above studies, administration of FTY720 improved clinical scores dramatically in EAE in Lewis rats (Fig. 1). As demonstrated in a previous study, the expression of adhesion molecules related to T cell trafficking is enhanced in spinal cords, and monoclonal antibodies of these molecules inhibit EAE disease (Lee and Benveniste, 1999). Furthermore, increased T cell infiltration of spinal cords has, in fact, been described in various reports (Sun et al., 2000). Therefore, infiltrated T cells were thought to be closely involved in the development of EAE (Hickey et al., 1991). The present study also confirmed T cell infiltration by immunohistochemical staining anti-T cell receptor monoclonal antibody (Fig. 2). Other studies using FTY720 and autoimmune disease models found T cell elimination in the inflammation lesion (Yan et al., 1998; Hozumi et al., 1999; Kitabayashi et al., 2000; Kurose et al., 2000; Okazaki et al., 2002). Consistent with the above studies, we demonstrated a marked reduction in central nervous system damage and infiltrating cells in FTY720-treated rats compared with control rats (Fig. 2). Therefore, FTY720 administration might inhibit EAE development might by inhibiting encephalitogenic T cell responses and/or their migration into the CNS. These findings have identified FTY720 as a possible therapeutic agent for human MS.

As previously reported (Bonetti et al., 1997), a number of apoptotic cells were invariably associated with clinical disease in saline-treated control EAE rats. Our study found apoptotic cell death in the spinal cords of EAE rats, but not in rats treated with FTY720. This observation was correlated with the lack of infiltration cells in the spinal
Patterns of cytokine expression in spinal cords of EAE Lewis rats have been reported previously, and elevation of cytokine expressions in such tissues is believed to contribute to pathology (Sun et al., 2000). These reports suggested that autoreactive T cells in spinal cords were activated by Th1-associated cytokines (IL-2, IL-6 and IFN-γ) but not Th2-associated cytokines (IL-4 and IL-10). Therefore, elevation of cytokine expressions was thought to be an important component of EAE disease in addition to the T cell infiltration into the spinal cord. In our present EAE model, mRNA expressions of Th1-associated cytokines (IL-2, IL-6 and IFN-γ) in the spinal cords were markedly decreased in rats that had been administered FTY720 compared to control saline-treated rats (Fig. 3). It seems that a lack of infiltration in the spinal cord in EAE rats treated with FTY720 resulted from a lack of inflammation, so cytokines were not upregulated in the spinal cord. Therefore, these data indicated that FTY720 inhibits the induction of at least three inflammatory cytokines in vivo by preventing T cells from infiltrating spinal cords. In contrast to spinal cords, the expression of cytokines was not inhibited in spleen with FTY720 treatment (Fig. 4). This is consistent with a previous report by Yanagawa et al. (Yanagawa et al., 1998). In that study, FTY720 significantly reduced the number of peripheral blood T cells in skin-allografted rats. Furthermore, FTY720 markedly decreased T cell infiltration into allografts while, in contrast to CsA, had little effect on IL-2 and IFN-γ mRNA in expression in allografts.

Pinschewer et al. reported that FTY720 impairs the circulation and homing of effect T cells to peripheral lesions without affecting the induction and expansion of immune responses in secondary lymphoid organs (Pinschewer et al., 2000). To verify whether the above mechanism is involved in our model, we attempted to adoptively transfer to
naive Lewis rat using spleen cells isolated from actively EAE-induced Lewis rats with FTY720 treatment. Clinical EAE was adoptively transferred to cell recipients using the lymphocytes with saline-treated control rats, while EAE was not induced in spleen cells isolated from rats treated with FTY720 (Fig. 5). We therefore speculated that the MBP-specific spleen cells might not be included in cells transferred from FTY720-treated rats, although there is some possibility of inhibiting the induction of encephalitogenic T cells, due to its inhibition of the encephalitogenic T cell migration and homing to peripheral organs including the spleen. Further studies are needed to clarify this.

More recent reports found that FTY720 targets sphingosine 1-phosphate (S1P) receptors (Brinkmann et al., 2002; Mandala et al., 2002). Those studies demonstrated that FTY720 was phosphorylated by sphingosine kinase and that the phosphorylated compound is a potent agonist at four sphingosine 1-phosphate receptors, and the effects of FTY720 are actively induced. Furthermore, the studies speculated that EAE might relate to a direct effect on neuronal cells and/or oligodendrocytes expressing S1P receptors. Because activation of S1P receptors can antagonize apoptotic processes, which are associated with early stages of progressive neurodegenerative and demielinating diseases (Brinkmann et al., 2002). Consistent with the above reports, FTY720 both prevented active induction of EAE and adoptively transferred EAE, a principally immune cell-mediated phenomenon. These data indicated that FTY720 administration might inhibit EAE development by inhibiting encephalitogenic T cell responses and/or their migration into the CNS.

In conclusion, our findings suggest that administration of FTY720 effectively prevents development of EAE in rat models. Although this study did not precisely
examine the adverse effects of the drug, none of the FTY720-treated rats died during the therapy and the drug-treated rats gained body weight during therapy. The data suggested that FTY720 may be safety for the clinical situation. FTY720 might be a candidate for treating patients with MS because of its strong capacity to suppress EAE and because of its therapeutic effects.
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FTY720, a novel immunosuppressant, induces sequestration of circulating mature lymphocytes by acceleration of lymphocyte homing in rats. II. FTY720 prolongs skin allograft survival by decreasing T cell infiltration into grafts but not cytokine production in vivo. *J Immunol* **160**:5493-5499.

FOOTNOTES

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FIGURE LEGENDS

Fig.1 Suppression of acute EAE in the Lewis rat by FTY720.

Rats were immunized with MBP/CFA to induce EAE, as described in Materials and Methods. FTY720 (squares) or control (circles) saline was given daily by oral administration at 1mg/kg/day. (A) Survival (n = 6 / FTY720), (n = 6 / control); (B) Maximum Clinical Score (n=12 / FTY720), (n =19 / control); (C) Mean Clinical Score (n = 23 / FTY720), (n = 16 / control); (D) Body weight loss (n = 23 / FTY720), (n = 16 / control). Data complied from four separate experiments.

Fig.2 Histological findings of the spinal cord in the EAE Lewis rat.

Histological findings of the MBP/CFA-immunized rats treated with FTY720 or control saline. In HE staining in the spinal cords of rats, inflammatory lesions were readily detectable in control rats, whereas the spinal cords from rats administered FTY720 exhibited a complete absence of inflammatory-cell infiltrates (A – H). Influx of inflammatory T-cells in the spinal cords of rats was shown in the control rats, whereas administration of FTY720 dramatically decreased infiltration of T lymphocytes (I - P). Detection of apoptotic cells in the spinal cords of rats by the use of the TUNEL method was shown in control rats, whereas treatment of FTY720 decrease of TUNEL positive cells in the spinal cords of rats (Q – X). Original magnification x 100. Data are representative of three separate experiments.

Fig.3 Reverse transcriptase-polymerase chain reaction (RT-PCR) for IL-2, IL-6, INF-γ mRNAs in the spinal cords of the EAE Lewis rat.
(A) Representative data show the intensity of RT-PCR products obtained from spinal cord samples in each group. Compared with control rats, mRNA expression for IL-2, IL-6 and INF-γ decreased in the FTY720-treated rats. (B) The intensity of each band was calculated using Kodak Digital Science 1D Image Analysis Software. The relative quantities of the genes are presented as the ratio of intensities of IL-2, IL-6 and INF-γ bands against those of housekeeping gene, β-actin. Data are representative of three independent experiments and indicate the mean ratio of triplicate results in each experiment.

Fig. 4 Reverse transcriptase-polymerase chain reaction (RT-PCR) for IL-2, IL-6, INF-γ mRNAs in the spleen of the EAE Lewis rat.

(A) Representative data show the intensity of RT-PCR products obtained from spleen samples in each group. Compared with control rats, no decrease of mRNA expression for IL-2, IL-6 and INF-γ was shown in the FTY720-treated rats. (B) The relative quantities of the genes in spleen were plotted against those of control as described in Fig. 3

Fig. 5 Prevention of adoptively transferred EAE by treatment with FTY720

Rats were adoptively transferred spleen cells from MBP/CFA-immunized rats treated with FTY720 (squares) or control saline (circles). (A) Survival; (B) Maximum Clinical Score; (C) Mean Clinical Score; (D) Body weight loss. Data complied from two separate experiments.
Figure 1

A

% of Survival

--- FTY720
--- Control

Days Post Immunization

B

Maximum Clinical Score

***

Control  FTY720

C

Mean Clinical Score

Days Post Immunization

D

% of Body Weight

FTY720
Control
Figure 4

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A

Day 7 | Day 14 | Day 21 | Day 28

Control | FTY720 | Control | FTY720 | Control | FTY720 | Control | FTY720

IL-2  | INF-γ | β-Actin

B

Relative Intensity to β-Actin

Days Post Immunization

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Control | FTY720