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Journal of Neuroimmunology 153 (2004) 108–121

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

Sphingosine 1-phosphate receptor agonists attenuate relapsing–remitting experimental autoimmune encephalitis in SJL mice

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Received 3 March 2004; received in revised form 23 April 2004; accepted 26 April 2004

Abstract

FTY720 is a prodrug for FTY-phosphate, an agonist at four of the five known receptors for sphingosine-1-phosphate (S1P). We show that administration of either FTY720 or FTY-P to SJL mice with established relapsing–remitting experimental autoimmune encephalitis (EAE) results in a rapid and sustained improvement in their clinical status, and a reversal of changes in expression of mRNAs encoding some myelin proteins and inflammatory mediators. EAE produced by adoptively transferring lymph node cells from immunized mice to naïve hosts is similarly ameliorated by FTY-P. Treatment with FTY-P is accompanied by a dose-responsive peripheral lymphopoenia.

Keywords: Relapsing–remitting EAE; FTY720; Lymphopoenia; Gene expression

1. Introduction

FTY720 is a structural analog of myriocin, a metabolite of the ascomycete fungus *Isaria sinclairia*, with some structural resemblance to sphingosine, an endogenous lysolipid. Sphingosine undergoes phosphorylation via sphingosine kinase leading to the formation of sphingosine-1-phosphate (S1P), the cognate ligand for the family of S1P receptors (S1PR). Activation of S1P receptors results in a plethora of physiological actions such as chemotaxis, cellular differentiation, survival and growth, and regulation of actin-based cytoskeletal reorganization which leads to cell adherence and cell shape changes (Goetzl and An, 1998; Chun, 1999; Fukushima et al., 2001).

FTY720 is a novel immunosuppressive agent which is active in various animal models of graft rejection and autoimmune disease, including graft versus host disease, type 1 diabetes, and rheumatoid arthritis. (Chiba et al., 1996; Suzuki et al., 1996a,b, 1998; Masubuchi et al., 1996; Matsuura et al., 2000). It is currently under development as an immunosuppressive agent for transplantation. It was thought initially that the mechanism of action was through induction of apoptosis in T lymphocytes (Nagahara et al., 2000; Suzuki et al., 1996a,b, 1997), and one effect of in vivo treatment with FTY720 is a profound lymphopoenia in the peripheral blood, with lymphocyte cell counts falling to as low as 5–10% of control levels at therapeutic doses of the compound. However, therapeutic effects are achieved in rats at doses of <1 mg/kg, at which the plasma concentrations are about two orders of magnitude lower than those required to drive T cell apoptosis in vitro (Yanagawa et al., 1998). A further argument against a role of apoptosis in most in vivo situations is provided by the observation that adoptively transferred fluorescently labeled lymphocytes disappear from the peripheral circulation on FTY720 treatment but reappear when drug treatment is discontinued (Pinschewer et al., 2000). It now appears that at least one of the mechanisms by which FTY720 achieves its effects in vivo is by a sequestration of circulating lymphocytes in peripheral lymph nodes (Pinschewer et al., 2000; Brinkmann et al., 2000, 2001a,b; Mandala et al., 2002, Xie et al., 2003).

FTY720 is a substrate for sphingosine kinase-2 (Sanchez et al., 2003) and phosphorylation in vivo (Mandala et al., 2002) has been demonstrated. The resultant ester (FTY-P) has a structure similar to sphingosine-1-phosphate, which is the preferred ligand at a group of G protein coupled

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0165-5728/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneuroim.2004.04.015

receptors with five known members (S1P receptors, S1PR). Mandala et al. (2002) showed that FTY720 itself has weak or no activity at any of these receptors but that the phosphate ester is an agonist with low nanomolar potency at four of the five receptors, and these observations were further substantiated by Brinkmann et al. (2002). A nonhydrolysable phosphonate analogue of FTY-P retained sufficient potency at the four S1P receptors to have in vivo efficacy in a lymphopoenia assay. FTY720, sphingosine-1-phosphate, and the phosphonate analogue of FTY-P all caused a rapid and reversible peripheral lymphopoenia in rats and mice, reaching a nadir at 4 h postinjection. The potency of compounds in this assay reflected their intrinsic affinities at the four receptors, indicating that one or more of these receptors is indeed the molecular target of these compounds (Mandala et al., 2002). Recent reports using selective agonists demonstrate that S1P1 is the target for lymphopenia (Forrest et al., 2004; Sanna et al., 2004). Moreover, lymphocytes genetically deleted for S1P1 have thymic emigration and recirculation defects similar to that achieved with the receptor agonists, suggesting that S1P1 is required for egress (Matloubian et al., 2004) and that the agonists induce lymphopenia by downregulating S1P1 on lymphocytes (Graler and Goetzl, 2004).

Two papers have appeared indicating that FTY720 is active in rodent models of experimental autoimmune encephalitis (EAE), an animal model of multiple sclerosis. Brinkmann et al. (2002) treated Wistar rats with FTY720 (0.3 mg/kg/day) from the day of induction of EAE (day 0) in Wistar rats. They showed that rats thus treated did not develop EAE in this monophasic model. In a different acute monophasic rat model, in which myelin basic protein is used as the immunogen in Lewis rats, Fujino et al. (2003) showed once again that dosing rats orally from day 0 almost completely suppressed the development of disease. This was associated with a marked reduction in the number of T lymphocytes infiltrating the spinal cord and a reduction in the levels of the TH1 cytokines IL-2, IL-6, and interferon gamma. While these data are impressive, the efficacy of FTY720 when given at the onset of clinical symptoms or on established EAE is unknown. In the present investigation, we have examined the efficacy of FTY720 and its phosphate ester on an established disease state in the relapsing–remitting EAE model in SJL mouse (SJL rr-EAE), a model which mimics several features of human MS.

EAE can be induced in SJL strain mice by immunizing them with a peptide fragment of the myelin protein, proteolipid protein (PLP), together with pertussis toxin (PTX) treatment (McRae et al., 1992). The resulting EAE shares many features with human multiple sclerosis. It is a chronic disease from which the mice never recover and has a relapsing–remitting pattern similar to that of the major form of human multiple sclerosis (McRae et al., 1992, 1995). In addition to the infiltration of T lymphocytes into the brain and the spinal cord (and unlike the common rat models), these mice also exhibit both demyelination and also axonal damage (Sobel et al., 1990; Marracci et al., 2002). Demyelination has for many decades been regarded as the hallmark of multiple sclerosis, but in recent years, the importance of the axonal damage and eventual neuron loss as the key processes underlying the relentless progression of the disease has been recognised (Trapp et al., 1998, 1999; Wujek et al., 2002). In addition to confirming these features of the SJL-rr model, we also show that in this EAE model, significant changes in the quantitative expression of mRNAs encoding both myelin-related proteins and mediators such as granulocyte-macrophage colony stimulating factor (GM-CSF) and inducible nitric oxide synthase occur.

In this study, we characterise the effects of FTY720 and its phosphorylated derivative on the clinical state, and levels of circulating lymphocytes in the SJL mouse rr-EAE model. Initiating dosing just prior to the onset of clinical signs delays and blunts the first phase of the disease as previously reported in the rat models. More significantly for the clinical setting, if treatment with these compounds is delayed until the peak of the first phase of disease, there is an immediate and rapid improvement in the clinical status of the animals which is maintained for as long as dosing is continued. Treatment with FTY-P is associated with a partial reversal in the gene expression changes seen in untreated animals with the disease. We also show that there is a correlation between the magnitude of clinical improvement and the levels of lymphopoenia achieved at any given dose of FTY-P, but this correlation is incomplete, indicating that nonselective S1P receptor agonism may exert its effects in this model by additional mechanisms. FTY-P is also effective in improving clinical status and preventing mortality in an adoptive transfer version of the disease.

2. Materials and methods

2.1. Experimental autoimmune encephalitis

2.1.1. SJL mouse

Female SJL mice (6–9 weeks old) were obtained from the Jackson laboratory (Bar Harbor, ME). They were housed in a 12-h light / dark cycle with access to food and water ad lib. All animal procedures were conducted in accordance with protocols approved by the local animal care committee.

2.1.2. Active immunization

EAE was induced following the methods of McRae et al. (1992). A 20-mer peptide based on the mouse proteolipid protein (PLP) sequence 139–151 (His–Ser–Leu–Gly–Lys–Trp–Leu–Gly–His–Pro–Asp–Lys–Phe (custom synthesized by American Peptide, Sunnyvale, CA, 98% purity) was used to induce EAE. Peptide was dissolved at 5 mg/ml in distilled water and emulsified with an equal

volume of Freund's adjuvant containing 5 mg/ml of H37Ra *M. tuberculosis* (Difco) following the procedure of Stevens et al. (1999). On the day of initiation of EAE, referred to as day 1 of the experiment, mice received a single injection of 100 ul of emulsion subcutaneously in a skin fold at the back of the neck. On day 1, they were also given 250 ng of pertussis toxin (List Biological Labs, Campbell, USA) from a solution of 50 ug/ml in 0.01 M sodium phosphate buffer pH7.0 with 0.9% NaCl intraperitoneally. This injection was repeated on day 3. Control animals received an injection of water/Freund's emulsion and both pertussis injections.

Mice were weighed daily and assessed using a clinical scale as follows: 0 = healthy mouse, 1 = flaccid tail, 2 = hind limb weakness, 3 = paralysis of one or both hind limbs, 4 = forelimb paralysis, and 5 = death. Animals were euthanased if they reached a score of 4. Mice which appeared to be dehydrated were given 1 ml of saline ip per day, and cages containing mice with clinical scores of above 2 were provided with wet chow on the cage base to ensure that animals could reach their food.

Drugs and experimental compounds were given by intraperitoneal injection or by oral gavage as appropriate. FTY-P was synthesized at the Merck Research Laboratories, Rahway. Mitoxantrone was purchased from Calbiochem as a 10-mg/ml solution and was diluted for use in saline.

In some experiments, animals were bled retroorbitally at a frequency not greater than once per week to provide blood samples for lymphocyte counting. At the termination of experiments, tissues (brain, spinal cord, liver) were taken and snap frozen on dry ice for biochemical analysis. In some cases, tissues to be used for RNA preparation were collected and stored overnight at 4 °C in an RNA preservative solution (RNAlater, Qiagen), and then placed at −70 °C until RNA preparation.

2.1.3. EAE induction in SJL/J mice via adoptive transfer of PLP-reactive lymphocytes

EAE was passively induced by adoptive transfer of PLP-reactive lymphocytes. Thoracic and popliteal lymph nodes were collected 11–12 days after immunization of donor mice with PLP in CFA. Free lymph node cells were obtained by mechanically dissociating the nodes, and red blood cells were hypotonically lysed by a 2-min treatment in 0.144 M (0.95%) ammonium chloride, 17 mM Tris–HCl, pH7.4. These cells were cultured in Click's medium (EHAA) supplemented with 10% foetal calf serum and 50 uM beta mercaptoethanol. Cells were stimulated for 48–72 h in the presence of 25 ug/ml $PLP_{139-151}$ or BSA as a negative control. Proliferation of the stimulated cells was confirmed by [^{3}H]- thymidine incorporation and microscopically by the appearance of blasts. Recipient SJL mice were injected intraperitoneally with PLP-reactive lymphocytes (6–12.5 million cells/mouse in a volume of 0.5 ml phosphate buffered saline per mouse). Clinical evaluation of EAE was conducted using the methods described above.

2.2. Lymphopoenia assays

The lymphopoenia assay was performed using an in vitro microprocessor controlled automatic blood analyzer instrument (H-2000 Hematology Analyzer (Hospitex Diagnostics LP, Webster, TX) using whole blood samples.

Whole blood from EAE animals was collected by retroorbital bleeding, using EDTA as an anticoagulant. The blood was used within 4 h at room temperature for cell counting. Lymphopoenia was defined in our assay as the reduction of lymphocyte cell density (cells/μl) as compared to PLP control vehicle. Data were expressed by plotting cell density as a function of time (days in vivo) and analyzed using software Prism™ 3.0 (San Diego, CA) All values are expressed as mean ± standard error of mean (S.E.M.).

2.3. Molecular biology

2.3.1. Isolation of total RNA and reverse transcription

Total RNA from mouse spinal cord, brain, lung, and heart (LPS-treated) was isolated using an RNeasy® Protect Midi Kit (Qiagen). Total RNA of PLP-treated mouse lymphocytes was isolated using an RNeasy® Protect Mini Kit (Qiagen). RNA was treated with RNase-free DNase I to remove genomic DNA contamination. Total RNA (1.2 μg) from each was reverse transcribed using a RETROscript™ Kit (Ambion). In each reverse transcription reaction, a reaction-omitting reverse transcriptase was included for the assessment of genomic DNA contamination.

2.3.2. Cloning, construction, and DNA sequencing of mini genes

The cDNA encoding myelin components (MAG, PLP, MBP, MOG, and CNPase) and β actin was amplified from mouse brain with polymerase chain reaction (PCR) techniques. The cDNA encoding GM-CSF and iNOS was amplified from mouse lung and LPS-induced heart, respectively. For cloning of mini genes for myelin components, iNOS and β actin, 3 μl of cDNA was amplified with 2.5 U of *PfuTurbo*® DNA polymerase (Stratagene), 200 nM dNTP, and 10 pmol of each primer in a total volume of 50 μl for 30 cycles in GeneAmp® 9700 thermocycler (ABI). Each cycle consisted of 30 s at 95 °C, 30 s at 55 °C, and 45 s at 75 °C. GM-CSF mini gene was amplified from 3 μl of cDNA using 2.5 U of Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen), 200 nM dNTP, 2 mM $MgSO_4$, 1 × High Fidelity PCR buffer, and 10 pmol of each primer in a total volume of 50 μl. PCR was performed using a GeneAmp® 9700 thermocycler (ABI) with incubation at 94 °C for 30 s, 55 °C for 30 s, and 74 °C for 45 s for 30 cycles. The PCR-amplified fragments were subcloned into PCR cloning vectors, pCR®–BluntII-TOPO®, or pCR®II–TOPO® (Invitrogen) using a PCR Cloning Kit (Invitrogen). The integrity of these genes was confirmed by sequencing using an ABI 3100 automated fluorescence

sequencer (ABI). The sequence was analyzed using software Sequencher.

2.3.3. TaqMan® PCR

Quantitative real-time PCR was carried out using an ABI Prism 7900 sequence detector on 1 μl of cDNA samples using 900 nM each primer, 250 nM TaqMan probe, and 25 μl of TaqMan® Universal PCR Master Mix, in a total volume of 50 μl. PCR was carried out with incubation at 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Primers used for these genes were designed from sequences in the Genebank database (Table 1). Additional reactions were performed on each 96-well plate using a known dilution of DNA from mini genes or cDNA as PCR template for constructing a standard curve relating threshold cycle to cDNA concentration. Data were analyzed using software SDS2.0. All data were normalized to β actin and expressed as %control.

2.4. Statistics

All values are expressed as mean ± standard error of mean (S.E.M., $n=4$ individual animals each with 2–3 replicates). Data were analyzed by analysis of variance (ANOVA) followed by post hoc analysis (Neuman–Keul's test; Prizm 3.0, GraphPad, San Diego, CA) and statistical significance inferred at a $p \leq 0.05$.

3. Results

3.1. EAE in SJL mice

Mice injected subcutaneously with a single dose of PLP emulsified in CFA, together with intraperitoneal doses of PTX on the first and third days, began to lose body weight and manifest clinical signs at about day 7. The decline in body weight and increase in clinical scores occurred rapidly, reaching their maxima at about day 14. At this point, mice had typically lost about 30–35% of their body weight, and mean clinical scores were usually about 3. In our hands, the subsequent evolution of the disease showed some interexperiment variations. In some experiments, the first phase of disease would remit to a low clinical score by about day 25, with a slow gain of body weight to about 90% of control values. In those cases where such a clear remission from first phase was observed, mice spontaneously relapsed with subsequent bouts of severe disease, separated by intervening remissions (Fig. 1A). In other experiments, the relapsing–remitting pattern was less obvious, and after a clear first phase with a lower degree of remission, the mean clinical scores showed a chronic secondary type of progression (Fig. 1B).

In both of these patterns, the clinical scores (during remission in the case of clearly rr-EAE) became progressively higher as the disease advanced. After the first phase,

Table 1
Primer and probe sequences used for mouse Taq-Man experiments

Gene	Accession #	Forward primer (F)/TaqMan® probe (TM)/Reverse primer (R)
PLP	M15442	F: AGCGGGTGTGTCATTGTTTG TM: 5′AAACTTGTCGGGATGTCCTAGCCA R: 5′ACAACAGTCAGGGCATAGGTGAT
MBP	M11291; XM_129053; M15062	F: 5′GACCCAAGATGAAAACCCAGTAGT TM: 5′CATTTCTTCAAGAACATTGTGACACCT R: 5′TTGGGATGGAGGTGGTGTTC
MAG	NM_010758	F: 5′CGCACGGTGGAGCTGAGT TM: 5′TCATGTATGCACCTTGGAAGCCC R: 5′CCACCACCGTCCCATTCA
MOG	U64572	F: 5′TGTAGGCCTTGTATTCCTCTTCCT TM: 5′ACGAAGTTTTCCTCTCAGTCTGTGCTG R: 5′GTCCGATGGAGATTCTCTACTTCTG
CNPase	M31810	F: 5′TGTGCTGCACTGTACAACCAAAT TM: 5′CACCACCTCCTGCTGGGCGTATTCT R: 5′AGGCCTTGCCATACGATCTCT
iNOS	U43428	F: 5′AAATCCCTCCTGATCTTGTGTTG TM: 5′ACTCGTACTTGGGATGCTCCATGGTCA R: 5′CAACCCGAGCTCCTGGAA
GM-CSF	X02333	F: 5′AGAAGTCGTCTCTAACGAGTTCTCCTT TM: 5′CGGGTCTGCACACATGTTAGCTTCTT R: 5′GTAGACCCTGCTCGAATATCTTCAG
β actin	X03672	F: 5′CGATGCCCTGAGGCTCTTT TM: 5′CCAGCCTTCCTTCTTGGGTATG R: 5′TTTCATGGATGCCACAGGATT
INFγ	K00083	F: 5′GCATAGATGTGGAAGAAAAGAGTCTC TM: 5′CATCCTTTTGCCAGTTCCTCCAGA R: 5′GCTCTGCAGGATTTTCATGTCA

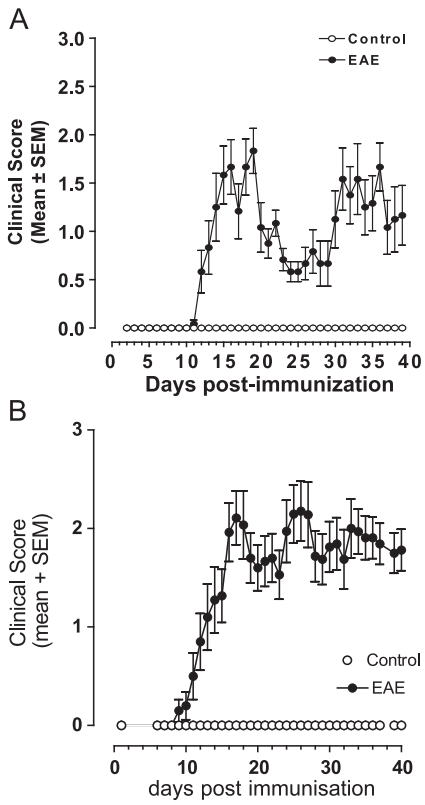

Fig. 1. Representative time course of clinical evolution of EAE in two separate experiments. (A) A typical relapsing–remitting time course is seen for animals induced for EAE by immunization with PLP and treatment with pertussis toxin. (B) A less clear remission is seen after phase 1, and the animals remain chronically sick at a clinical score of about 2.

body weight was little affected during subsequent relapses. Mortality was seen at levels which varied between experiments from between about 10% and 30%, but almost all mortality occurred in the first phase, and animals surviving this phase were unlikely to die subsequently.

3.2. Gene expression changes during EAE

We used the Taq-Man technique to examine quantitative changes in the expression of several myelin-related and inflammation-related genes during the course of EAE. In these studies, gene expression was initially expressed as a percentage of the expression of the β actin-encoding gene, whose expression was essentially constant between different samples. We then derived a final value for the expression in EAE samples by expressing the values as a percentage of the levels observed in disease-free CFA/PTX-treated controls taken at the same time. Fig. 2A represents the clinical evolution of the disease in the EAE experiment from which these time course samples were derived. There was, as was always observed in this model, a clear first phase, beginning at about day 7 and peaking with a mean clinical score of 2 at day 15, and this was followed by a relatively small remission to a mean score of about 1.25 by day 20, at which point the animals remained stable until day 30. At day 30, the clinical scores worsened, but in this experiment, a chronic secondary phase was seen without further evidence of clear relapses and remissions.

We studied the expression of the myelin-related proteins PLP, MBP (Fig. 2B and C), and also CNPase, MAG, and MOG (data not shown). The change in expression of the latter three mRNAs was very similar to that shown for MBP and PLP. The first phase of the disease was marked by a profound reduction in the level of these mRNAs, all of which fell to 26% or less than control levels at day 14. There was a subsequent recovery of expression at day 27, somewhat prior to the minor improvement in clinical score that began at day 30. Thereafter, expression levels again declined at day 44, at which time the clinical status was slowly worsening.

The expressions of the three inflammatory mediators IFNγ, iNOS, and GM-CSF were similarly markedly elevated during the first phase (Fig. 2D–F) and significantly reduced in the first small remission. In all three cases, they remained somewhat higher during the later phases of the disease, with GM-CSF showing the largest elevation (>1000-fold at all time points) over control levels at these later phases.

3.3. Clinical effect of mitoxantrone, FTY720, and FTY-P

We examined the effect in this model of mitoxantrone, a clinically used drug. Because of inherent toxicity, we were able to dose only for 6 days. When the drug was given for 6 days from day 6–12, it delayed the onset of disease and blunted the severity of the first phase, but at later time points, there appeared to be little difference in the condition of these animals from that of untreated controls (Fig. 3).

We initiated once-daily dosing of mice with FTY720 at 3 mpk, beginning at day 7, 14, or 25 after immunization. Animals dosed from day 7 showed a delay in onset of clinical signs of a few days, and the severity of the first phase was blunted compared with control mice (Fig. 4A). The degree of weight loss in this group was also reduced as compared with vehicle-treated mice. After resolution of this mild first phase, the mice remained at a very low and stable clinical score for the duration of the compound dosing. Much more dramatic effects were seen if doing was initiated at the peak of the first phase, when clinical scores were at their highest (Fig. 4B). A rapid improvement in the clinical score was initiated and maintained over several days, until by day 22, the mice had reached a similar low score as the mice treated from day 7. In this experiment, the remission at day 25 in the untreated control animals was relatively modest, and the clinical scores of mice which received FTY from this point declined to a level significantly below

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