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The effect of FTY720 in the Theiler's virus model of multiple sclerosis

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ABSTRACT

FTY720 (fingolimod) has demonstrated efficacy in multiple sclerosis (MS). We evaluated the effects of FTY720 on progressive disability, viral load, and antibody responses in mice infected with Theiler's murine encephalomyocarditis virus (TMEV). FTY720 and phosphorylated FTY720 (FTY720-P) were detected in the brain after intraperitoneal injection of the drug. Bioactivity of FTY720 was confirmed by reduced numbers of mononuclear cells in the spleen and blood after treatment. No significant differences were found in disability progression, viral load, and serum antibody responses between the FTY720-treated versus the PBS-treated mice. There was less production of IgG within the CNS in the FTY-treated group on some measures.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) in humans that causes demyelination and progressive neurological disability. There is no cure for MS, and more effective therapies are needed. FTY720 (also called fingolimod or Gilenya) is an oral therapy recently approved for use in relapsing MS by the FDA, that has demonstrated efficacy in MS superior to placebo as well as to interferon beta, the most commonly used drug for MS treatment [1,2].

FTY720 is effectively phosphorylated by sphingosine kinases in vivo, and the active metabolite FTY720-P targets a new class of G-protein coupled receptors, termed S1P receptors [3]. FTY720-P likely acts as a functional antagonist, down-modulating lymphocytic S1P1 receptors [4], and this is associated with retention of central- but not effector memory T cells in lymphoid organs [5]. FTY720 crosses the blood–brain barrier (BBB) and attains substantial concentrations in the CNS [6]. Thus, the drug may also directly affect neural cells [7,8].

In order to learn more about mechanisms of action of FTY720, we analyzed its effects in the Theiler's murine encephalomyocarditis virus (TMEV)-induced, immune-mediated demyelinating disease (TIDD) model, which is characterized by progressive weakness and robust systemic and CNS antiviral antibody and cellular responses [9–11]. Our goal was to test the effect of FTY720 on the progressive neurological disability, CNS histology, the viral load, and the antiviral immune response in TIDD.

2. Materials and methods

2.1. FTY720

FTY720 was obtained from Novartis Institutes for Biomedical Research (Basel, Switzerland) and was dissolved in distilled water. A dose of 0.3 mg/kg/day, called "low dose" (FTY-L) or 3.0 mg/kg/day, called "standard dose" (FTY-S), was given daily by intraperitoneal (IP) injection in a volume of 0.1 ml until necropsy. The selection of doses of FTY720 was based on earlier studies in murine viral infection [12] or experimental autoimmune encephalomyelitis (EAE) in which 3 mg/kg/day was effective in decreasing disease [13,14]. There was no peritoneal serositis induced by the IP injections, based on examination at necropsy.

2.2. Mice and virus

All animal work utilized protocols reviewed and approved by the University Animal Care and Use Committee at UMDNJ-New Jersey Medical School. SJL mice purchased from Harlan Laboratories (Indianapolis, IN) were 4–8-week old females, and were housed in isolator cages in the Research Resource Facility at New Jersey Medical School. The virus used was the BeAn strain of TMEV, obtained originally

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from Steven Miller (Northwestern University), and passaged in a hamster fibroblast line, BHK, as previously described [15]. PFU were determined by a cytopathic effect (CPE) assay. 25–30 μl of a PBS solution containing 3×10^8 PFU/ml BeAn (i.e., 7–9 million PFU per mouse) was injected into the right frontal lobe of mouse brain. Injections of FTY720 were begun at 3 different time points prior to or after the development of weakness, at 30(n = 81), 60(n = 30), or 85(n = 30) days post-infection (DPI); the results did not substantively vary according to time post-infection (p.i.). I.e., for all 141 infected mice, comparisons between FTY720- and PBS-treated mice showed no differences, and there were no significant differences in outcome of any measures according to when treatment with FTY720 or PBS was initiated. Injections of therapy, FTY720 or PBS, were continued in each animal until the animal was necropsied. Mice were necropsied at any average of 150 days p.i., or earlier if their severe neurological disability endangered their life, but never prior to 100 days p.i. Techniques used in the necropsies were performed as previously described [16–18], including anesthesia, intracerebral (IC) injection, perfusion with PBS, CSF collection by cisternal tap, and the collection of blood and other tissues.

2.3. Isolation of mononuclear cells (MNCs)

MNCs were obtained from spleen and spinal cord (SC) as previously described [16,19]. In brief, spleen was minced on frosted glass slides to obtain single cell suspensions; a Percoll gradient was used to isolate cerebral MNCs. Ammonium chloride was then used to lyse red blood cells.

2.4. Quantification of FTY720 and FTY720-P in the blood and brain

Whole blood was collected by cardiac puncture. Brains were collected after cardiac perfusion with 5 ml PBS. Blood and brains were snap-frozen and kept in −80 °C until analysis. Concentrations of FTY720 and FTY720-P were determined by high-pressure liquid chromatography (Agilent 1100; Agilent, Waldbronn, Germany) with mass spectrometric detection as described previously [6].

2.5. ELISA for albumin, total IgG, anti-TMEV binding antibody (Bab)

CSF and serum albumin, total IgG, as well as anti-TMEV IgG (TMEV-IgG) were assayed by ELISA as previously described [16]. The indices were calculated according to the following formulas: Quotient Albumin (Q_{alb}) = $albumin_{CSF}/albumin_{serum}\times1000$; Quotient IgG ($Q_{IgG}$) = $IgG_{CSF}/IgG_{serum}\times1000$ IgG Index = Q_{IgG}/Q_{alb}.

TMEV-specific binding antibody (TMEV-Bab) was determined as previously described. In brief, total IgG concentrations in the serum and CSF were diluted to the same level. Each sample was then assigned an arbitrary unit (AU) of anti-TMEV binding activity according to the standard curve in the anti-TMEV ELISA. The Bab index was calculated as the following ratio: TMEV-Bab Index = AU_{CSF}/AU_{serum}.

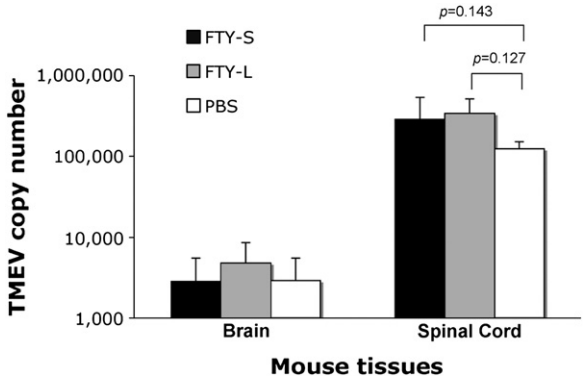

Fig. 1. Viral load in mouse tissues. Brain and spinal cord (SC) were collected at necropsy. Viral load was expressed as mean copy number of virus in each 0.5 ug RNA from tissues as calculated from a standard of known virus copy number. Error bars represented standard error. n = 15 in FTY-S, n = 30 in FTY-L, n = 36 in PBS.

2.6. Neutralizing antibody (Nab) assay

TMEV-specific neutralizing antibody (TMEV-Nab) was measured using the modified cytopathic effect assay (CPE) [20,21]. In brief, 1500 PFU of TMEV was incubated with serial three-fold dilutions of mouse serum and CSF for 2 h, followed by incubation with BHK cells for 48 h in a 96-well microplate format. Live BHK cells were then exposed to naphthol blue-black dye, and absorbance read on an ELISA reader at 620 nm. Neutralizing titers were expressed as the inverse of that dilution of serum able to block 90% of the cytopathic effect of the virus; i.e. to lower the cytopathic effect of 1500 PFU of virus to 150 PFU of virus under conditions in which the change from 1500 PFU to 150 PFU is in the linear part of the curve of the cytopathic effect dilution curve. The Nab index was calculated as the ratio: Quotient TMEV-Nab ($Q_{TMEV\text{-}Nab}$) = $TMEV\text{-}Nab_{CSF}/TMEV\text{-}Nab_{serum}\times1000$; then the TMEV-NAb index was calculated as: TMEV-Nab Index = $Q_{TMEV\text{-}Nab}/Q_{IgG}$.

2.7. Real-time RT-PCR for in situ IgG production and TMEV load

Expression of IgG and TMEV RNA was performed essentially as previously described with specific primers and probes for murine IgG1 and TMEV [18,22]. Total RNA was isolated from fresh, homogenized tissue samples using the Trizol One-Step Isolation method; reverse transcription (RT) was performed in a Gene Amp PCR system 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA). Taqman RT-PCR was performed in an ABI 7000 Sequence Detection System (PE Applied Biosystems). To determine the viral load in tissue, a standard curve was obtained when the threshold cycle (C_T) values of the samples were plotted against known TMEV concentration. Viral load in tissue was determined by referring the C_T values of experimental samples to the standard curve. Results were expressed as estimated TMEV numbers in each 0.5 μg RNA from tissues. Mouse GAPDH mRNA levels were used as a measure of RNA quality. The IgG1 mRNA level was expressed as the relative expression index of 2 to the power of $-\Delta C_T$, where $-\Delta C_T = -(C_{T\text{-}IgG} - C_{T\text{-}GAPDH})$.

2.8. Rotarod testing for progressive disability

Progressive disability in mice was assessed as previously described [17]. Rotarod data were expressed as a neurological function index (NFI). The NFI value at any time point was the mean of the last 3 time indices divided by the mean time indices from day 15 to day 45 after infection. Time indices were the time on the Rotarod for that day divided by the mean of the 2 maximum times for that mouse. Data shown are pooled data for the 3 different times post-infection in which FTY720 was administered. There were no significant differences between the FTY720- and PBS-treated groups at any of these time periods.

2.9. Histology

Sections of lumbar spinal cord were harvested and postfixed in 4% formaldehyde overnight. The tissues were then transferred to PBS until processing into paraffin blocks using standard techniques. Two separate lumbar areas were examined at least 2 mm apart. Sections of 10 μm were cut, placed on glass slides and histochemical staining performed using standard methods for overall morphology (Haematoxylin and Eosin, H&E), myelin (solochrome) and axonal density (Bielshowsky's silver stain). T cells were examined using anti-CD3 (AbD Serotec, Oxford, UK), microglia/macrophages by anti-ionized calcium binding adaptor molecule 1 (Iba-1, Wako Pure Chemical Industries, Osaka, JP) and astrocytes were examined using anti-glial fibrillary acidic protein