Fingolimod (FTY720) as an Acute Rescue Therapy for Intraocular Inflammatory Disease

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Objective: To examine the efficacy of the immunomodulatory drug fingolimod (FTY720) as a rescue therapy for noninfectious intraocular inflammation.

Methods: Experimental autoimmune uveoretinitis, the murine correlate of human uveitis, was induced in B10.RIII mice. The mice were treated with 2 oral doses of fingolimod daily, either during early ocular infiltration or following clinical onset of the disease. At subsequent times, retinal infiltrates were examined and enumerated using flow cytometry, and structural disease was assessed and scored using histology.

Results: Fingolimod treatment, administered 2 days before disease onset, prevented inflammatory cells from infiltrating the retina, with corroborative suppression of histologic disease. A single dose of fingolimod was sufficient in clearing infiltrating leukocytes from the retina within 2 hours of treatment. Furthermore, a single dose of fingolimod administered after disease onset not only abolished retinal infiltrates but also prevented disease relapse for at least 3 weeks.

Conclusions: A short-term, high-dose treatment with fingolimod rapidly reduces ocular infiltrates in experimental autoimmune uveoretinitis, leading to a normal myeloid cell count within the retina. When given at the early stages of intraocular inflammation, fingolimod resolves disease.

Clinical Relevance: This study directly demonstrates the therapeutic potential of fingolimod and an acute rescue intervention for human noninfectious posterior-segment intraocular inflammatory disease.

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Experimental autoimmune uveoretinitis (EAU) serves as a preclinical model of human uveitis, permitting assessment of immunotherapeutic efficacy, with proven successful translation into clinical practice. Moreover, the close clinicopathologic correlation between EAU and human uveitis allows us to dissect immunopathologic mechanisms of autoimmune inflammation and tissue damage, identifying novel pathways to facilitate the development of immunotherapies. Experimental autoimmune uveoretinitis in mice is initiated by activation of CD4+ T cells specific for ocular antigens, which are most frequently located within or around photoreceptor segments. We use a model system in which EAU is induced by administration of dominant peptides from interphotoreceptor retinoid-binding protein in an appropriate adjuvant. Infiltration by ocular antigen–specific T cells recruits macrophages into the eye and activates them, generating structural damage via mechanisms that include the secretion of nitric oxide.

Fingolimod (FTY720) is a potent immunomodulator that generates lymphopenia in circulating blood coupled with an increase in T cells in the secondary lymphoid tissue. Fingolimod mediates this effect by binding to and subsequently downregulating expression of sphingosine-1 phosphate receptor 1. Fingolimod suppresses experimental allergic encephalomyelitis in both mice and rats. Preliminary reports of clinical trials in multiple sclerosis have shown reduced lesion burden and symptoms. For ocular inflammation, continual daily fingolimod treatment showed partial suppression of disease.
severity in rat S-antigen–induced EAU,\textsuperscript{24} including successful daily therapy given either at the time of immunization or at disease onset. However, this study only examined the clinical disease.

Considering the mechanisms of fingolimod action and current preclinical efficacy and clinical trial data, we wished to extend these observations and examine the potential therapeutic use of fingolimod as a rescue therapy, given at the peak of acute retinal infiltration. Herein, we demonstrate that a single dose of the drug rapidly reduces infiltration and prevents subsequent retinal damage. We suggest that fingolimod may be a highly effective nonsteroidal option for rescue intervention in sight-threatening uveitis—during acute presentation or for relapses of chronic disease.

### METHODS

#### MICE, EAU INDUCTION, AND SCORING

Female B10.RIII mice were originally obtained from Harlan UK Limited (Oxford, England), and a breeding colony was established within the Animal Services Unit at the University of Bristol. All mice were housed under specific pathogen-free conditions, with food and water continuously available. The mice were aged between 6 and 8 weeks at the time of disease induction. Treatment of the animals conformed to the United Kingdom Home Office’s regulations for animal research and to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The mice were immunized subcutaneously with 50 μg of mouse interphotoreceptor retinoid-binding protein (IPRBP)\textsubscript{161-180},\textsubscript{184,185} SGPYIYSLHPGNTILHVD peptide (synthesized by Sigma Genosys, Poole, England, to 95% purity) in phosphate-buffered saline (PBS) (2% vol/vol dimethyl sulfoxide) in an emulsion with complete Freund adjuvant (1 mg/mL; 1:1 vol/vol) supplemented with 1.5-mg/mL Mycobacterium tuberculosis complete H37 Ra (BD Biosciences, Oxford). Mice also received 1 μg of Bordetella pertussis toxin intraperitoneally at the time of immunization. The onset of clinical disease was determined by using topical endoscopic fundus imaging. Some mice received 10-mg/kg fingolimod (Cayman Chemicals, Ann Arbor, Michigan) administered in 200 μL of PBS by oral gavage, according to the treatment regimens indicated. Similarly, control mice were given 200 μL of PBS in accordance with previously published protocols.\textsuperscript{25}

At various times after immunization, eyes were snap-frozen in optimal cutting temperature compound. Serial 12-μm cryosections were cut and fixed in acetone for 10 minutes. They were stained with rat antimouse monoclonal anti-CD45 antibody (Serotec, Oxford) and counterstained with hematoxylin before being scored for inflammatory infiltrates (presence of CD45\textsuperscript{+} cells) and structural disease (disruption of morphology) as previously described.\textsuperscript{26-28} Briefly, structural disease was scored in 3 areas (rod outer segments, neuronal layers, and retinal morphology) to give a total possible structural score of 12 points; the number of CD45\textsuperscript{+} cells was qualitatively scored in 6 areas (ciliary body, vitreous, vasculitis [mural or extravascular cells], rod outer segment, and choroid) to yield a total possible score of 30.

#### ISOLATION OF SINGLE-CELL SUSPENSIONS

Retinal-infiltrating cells were isolated by dissecting retinas and digesting them in complete RPMI supplemented with 10% vol/vol fetal calf serum, 1 mM 4- (2-Hydroxyethyl)-1-piperazineneethanesulfonic acid (Invitrogen, Paisley, Scotland), 0.5-mg/mL collagenase D, and 750-μ/mL deoxyribonuclease I. After 20 minutes at 37°C, an additional 0.5-mg/mL collagenase D and 750-μ/mL deoxyribonuclease were added. The mixture was then incubated for an additional 10 minutes at 37°C. Cell suspensions were then forced through a 40-μm cell strainer using a syringe plunger. Cell suspensions were incubated with 24G2 cell supernatant for 5 minutes at 4°C, before incubation with fluorochrome-conjugated antimouse monoclonal antibodies against CD4, CD11b, and CD45 at 4°C for 20 minutes. Cell suspensions were acquired using a 3-laser LSR-II flow cytometer (BD Cytometry Systems, Oxford). Analysis was carried out using FlowJo software (TreeStar, San Carlos, California). Cell numbers were calculated by reference to a known-cells standard as previously reported.\textsuperscript{29} Briefly, splenocytes, at a range of known cell concentrations, were acquired using a fixed and stable flow rate for 1 minute. Based on total cell number acquired during this time, a standard curve was generated and used to interpolate cell concentrations of ocular infiltrating cells acquired at the same flow rate for the same time. Using Prism 4 software (GraphPad Software Inc, San Diego, California), comparisons of statistical significance between groups were assessed using the Mann-Whitney U test.

Fingolimod has been successful in preventing inflammation in numerous systems,\textsuperscript{22-24,30} including relapsing-remitting multiple sclerosis,\textsuperscript{23} ocular inflammation,\textsuperscript{24} limiting inflammation, and infiltration following transplantation,\textsuperscript{31-33} and as a rescue therapy following cardiac transfer, dramatically extending allograft survival\textsuperscript{34} (and as a single dose, limiting adverse events that may accrue with chronic therapy\textsuperscript{35}). We wished to test the efficacy of short-term treatment with fingolimod in EAU. Retinal inflammatory cell infiltration during EAU can be observed several days before clinical disease (from day 11 in B10.RIII mice\textsuperscript{36}); thus, we tested whether or not fingolimod treatment at this time could reverse early infiltration. We administered fingolimod orally on days 11 and 12 following EAU induction and enumerated cellular infiltrates in retinas on day 13. In addition, we ascertained whether such treatment was sufficient to prevent onset by examining eyes histologically on day 13 for signs of disease. Fingolimod treatment significantly reduced the number of retinal-infiltrating macrophages and T cells at day 13, a time when we routinely observe the initial influx of large numbers of immune cells (Figure 1). The number of cells was lower or equivalent to that observed at day 11. Importantly, this reduction in infiltrates was also accompanied by a reduction in early structural damage normally observed in EAU (Figure 1).

Although fingolimod prevented retinal infiltration, an effective rescue therapy must also be able to rapidly reconstitute normal myeloid cell numbers and function in the retina. Previous studies have demonstrated that a single oral dose of fingolimod generates maximal lymphopenia in circulating blood in as little as 4 hours.\textsuperscript{37} Whether a single dose of fingolimod can reduce inflammatory cell numbers within target organs has yet to be tested. We induced EAU in B10.RIII mice; 13 days later at the first

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In this study, we report for the first time that a short-term high dose of fingolimod given in the early stages of ocular autoimmunity rapidly prevents retinal damage. We clearly demonstrate that fingolimod not only prevents infiltration of target organs, but also reduces existing infiltration. Our data support the plausible translation of fingolimod into the management of noninfectious uveitis, as it may prove to be a highly effective rescue therapy for patients with acute or relapsing disease, precluding the need for high-dose corticosteroid therapy or prolonged biologic therapy as currently used. Previous studies of fingolimod have not examined this role; instead, therapeutic approaches have focused on the long-term use of fingolimod. In particular, a previous preclinical in vivo study of the use of fingolimod to treat EAU disease progression was monitored only for a short pe-
As fingolimod has been shown to act by reducing the number of circulating lymphocytes, during remitting stages of autoimmune disease such treatment is unnecessary, and with long-term use there is a significant chance of accruing detrimental effects. To circumvent these issues, we used short-term treatment with a high dose of fingolimod as a therapeutic strategy for acute disease. While the fingolimod dose chosen (10 mg/kg) is somewhat higher than that which is currently used in human multiple sclerosis trials (therefore having a potential for increased toxicity), it is in line with doses previously used in preclinical studies. We have shown that

Figure 3. Thirteen and 14 days after induction of experimental autoimmune uveoretinitis (EAU), mice were given either fingolimod (FTY720) (10 mg/kg) by oral gavage (treated mice) or phosphate-buffered saline (PBS) (control mice). On days 14, 15, 21, and 35, eyes from the treated and control mice were enucleated. A, Total number of immune cells per retina. B-D, Individual histologic disease scores. E, Histologic images from control and treated mice. These data are representative of 2 independent experiments.

treatment with fingolimod after retinal inflammatory infiltration is as effective in preventing ocular damage as treatment before disease onset. Reinitiation of EAU is brought about by infiltration of CD4+ T cells specific for ocular antigens, which recruit inflammatory macrophages to the target organ. Previously, fingolimod has been shown to exhibit its effects on lymphocyte homing, though there are some previous reports regarding an effect of fingolimod on myeloid cell trafficking.50,61 Fingolimod also reduces macrophage infiltrates in the eye. However, we do not yet fully understand if this is due to a direct effect on infiltrating macrophages or whether macrophage infiltration relies on the continued presence of CD4+ T cells. It is conceivable that myeloid ingress into inflammatory sites is such a dynamic process, with rapid influx and egress/turndown, that removal of T cells from this site rapidly suppresses the signal for further entrance of any new myeloid cells, leading to an apparent deletion of such cells from this site. However, because we propose this treatment as a short-term rescue therapy, any long-term detrimental effects on immunity should be obviated.

We timed treatment to coincide with the point when experimentally clinical signs as well as CD4+ infiltrates are more manifest; therefore, this treatment arguably has more direct clinical translational relevance. Previously, fingolimod has been successfully combined with immunosuppressive treatments, such as steroids, to prevent transplant rejection.12-13 The data support that fingolimod represents an excellent candidate for acute therapy of autoimmune disease. However, the effectiveness of our treatment protocol for patients with uveitis, or indeed other autoimmune diseases, remains to be tested with or without concomitant standard immunosuppressive therapy.

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Ophthalmic Images

Unusual Appearance of “Lepra Pearls”
Manohar Babu Balasundaram, MS

A 30-year-old Indian man had lepromatous leprosy with chronic granulomatous anterior uveitis. A, Multiple “lepra pearls” can be seen in his left eye, many with translucent envelopes and 1 appearing pearly white, which is typical of the classic pearl. B, Photograph after topical steroid use showing loss of the translucent inflammatory envelope.