Protective Effect of Intravitreal Administration of Tresperimus, an Immunosuppressive Drug, on Experimental Autoimmune Uveoretinitis

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PURPOSE. To test the efficiency of locally administered tresperimus in experimental autoimmune uveoretinitis (EAU).

METHODS. EAU was induced in Lewis rats by S-antigen (S-Ag) immunization. Three intravitreal injections of tresperimus (prevention or prevention/treatment protocols) were performed at different time points after immunization. The pharmacokinetics of tresperimus was evaluated in the ocular tissues and plasma. The in vitro effect of tresperimus was evaluated on macrophages. EAU was graded clinically and histologically. Blood ocular barrier permeability was evaluated by protein concentration in ocular fluids. Immune response to S-Ag was examined by delayed type hypersensitivity, the expression of inflammatory cytokines in lymph nodes, ocular fluids and serum by multiplex ELISA, and in ocular cells by RT-PCR.

RESULTS. In vitro, tresperimus significantly reduced the production of inflammatory cytokines by lipopolysaccharide-stimulated macrophages. In vivo, in the treatment protocol, efficient tresperimus levels were measured in the eye but not in the plasma up to 8 days after the last injection. Tresperimus efficiently reduced inflammation, retinal damage, and blood ocular barrier permeability breakdown. It inhibited nitric oxide synthase-2 and nuclear factor κBp65 expression in ocular macrophages. IL-2 and IL-17 were decreased in ocular media, while IL-18 was increased. By contrast, IL-2 and IL-17 levels were not modified in inguinal lymph nodes draining the immunization site. Moreover, cytokine levels in serum and delayed type hypersensitivity to S-Ag were not different in control and treated rats. In the prevention/treatment protocol, ocular immunosuppressive effects were also observed.

CONCLUSIONS. Locally administered tresperimus appears to be a potential immunosuppressive agent in the management of intraocular inflammation. (Invest Ophthalmol Vis Sci. 2011;52:5414–5423) DOI:10.1167/iows.1046740

Tresperimus is an immunosuppressive drug structurally related to deoxyspergualin (DSG) that was initially developed as an antitumor agent. Tresperimus has been designed to be chemically stable in aqueous solution and was found to have favorable effects on transplant rejection. It has been shown to suppress graft rejection as efficiently as cyclosporine A. Indeed, a short course of tresperimus has similar or better effects compared to the effects of cyclosporine in bone marrow, cardiac, and skin transplant models. It is more effective than cyclosporine in a rat model of major histocompatibility complex (MHC)-mismatched cardiac transplantation. However, qualitatively, the effects are different. Prevention of rejection is related to the induction of donor-specific tolerance without affecting immunity to third party antigens. In addition, CD4+ T-cells from tresperimus-treated animals can transfer donor-specific tolerance to naïve animals, an effect not seen with cyclosporine or other traditional immunosuppressive drugs. The mechanism by which tolerance is induced is not clear. Tresperimus (like DSG) binds to Hsc70, a heat shock protein-chaperoned peptide that, among other effects, inhibits nuclear localization of nuclear factor (NF)-κB, which is required for CD40 and CD28 ligation signaling in antigen-presenting cells, an important early step in T-cell costimulation.

Many stimuli induce a cellular stress that results in the activation of signaling pathways involving the transcription factor NF-κB. NF-κB is present in the cytoplasm of all cells in a resting state sequestered by inhibitors of κB (IκB). After degradation of IκB, the p50/p65 component is released and translocated into the nucleus where it activates the transcription of genes involved in innate immunity, inflammation, or cell survival. NF-κB plays an important role in the activation of inflammatory molecules, including TNF-α and nitric oxide synthase-2 (NOS-2). Interestingly, we have previously shown that ocular injection of IL-13 reduced ocular inflammation with low NF-κB/p65 expression, promoted macrophage survival with a change of their phenotype, and reduced vascular leakage with low NOS-2 expression in vascular endothelial cells.

Tresperimus is studied here for its effect on experimental autoimmune uveoretinitis (EAU). EAU is an inflammatory disease model that shares many clinical and histopathologic features with human uveitis, such as sympathetic ophthalmia, birdshot retinochoroidopathy, Vogt-Koyanagi-Harada syndrome, Behçet’s disease, and sarcoidosis. EAU is a relevant model for the study of human posterior uveitis and the development of new therapeutic strategies. Indeed, biologic agents targeting cytokines and different soluble mediators of inflammation have shown their effects for specific ocular inflammatory diseases.
EAU in rats is induced by immunization with the purified retinal autoantigen S-antigen (S-Ag)15–20 that is also recognized by patients with uveitis. EAU is dependent on either CD4+ Th1 and Th17 effector cells.17 Indeed, both Th1 and Th17 can mediate EAU pathology.21,22 It has been shown that IFN-γ inhibits Th17 cell differentiation and that IL-17 inhibits Th1 induction.23–24 EAU mediated by Th1 or Th17 are clinically different.25–27 Th17 cells play a dominant role in EAU induced by interphotoreceptor retinoid-binding protein in the presence of pertussis toxin in mice.28 However, the role of IL-17 varies on species and stimulation conditions and extrapolation can hardly be made to human ocular pathology. Locally, macrophages and microglial cells29 amplify the reaction and induce the destruction of photoreceptors and retinal tissue.17–19 Monocytes/macrophages together with neutrophils12,19,30 are important effector cells in EAU, whereas T cells are acting more to initiate and maintain the response.31 Macrophages cross the blood-retinal barrier and infiltrate the retina, where their release of mediators such as NO2- and TNF-α can cause severe retinal damage and consequent loss of vision in humans.32

New therapeutic strategies for the treatment of uveitis are needed. Uveitis is an important cause of blindness worldwide. We investigated the effect of local administration of tresperimus (9 mM to achieve a 1-mM solution final in the vitreous; n = 6), and a control group received vehicle (saline; n = 6 rats). Clinical and histologic examinations were performed.

A group of rats received one intravitreal injection of tresperimus (n = 6) or saline (n = 6) at day 11 after immunization. Rats were examined by clinical and histopathologic analysis and no effect on EAU scores was detected (data not shown).

Different Analyses Were Performed

Ocular Examinations. Ocular examinations included the counting of inflammatory cells on paraffin eye sections, cytokine/chemokine analysis in ocular media by multiplex ELISA assay, NOS-2, and IL-10 expression in the cell pellet of ocular fluids by RT-PCR, and a protein evaluation using the Bradford assay.

Systemic Examinations. Inguinal lymph nodes were taken for RT-PCR analysis of cytokines (TNF-α, IFN-γ, IL-2, and IL-17). Delayed-type hypersensitivity (DTH) was estimated by ear assay, and serum cytokines were evaluated by multiplex ELISA assay.

In a third protocol, tresperimus levels in plasma and ocular tissues were measured 1 hour, 3 days, and then 8 days after the third injection (n = 6 at each time point; prevention protocol). Blood was collected, and then plasma samples were acidified for tresperimus stabilization with 4% (v/v) acid citric 0.5 M/disodium hydrogen phosphate 0.5 M (70:30; v/v). Pooled structures, aqueous humor/vitreous body (right eye), and retina/choroid (left eye) were collected and weighed (6 animals per sampling time). In addition, a group of nonimmunized Lewis rats received one intravitreal injection of tresperimus and plasma and ocular tissue levels were measured 1 hour and 3 days postinjection (n = 6 at each time point).

All samples were stored at ~20°C before analysis by liquid chromatography–mass spectrometry/mass spectrometry.

Clinical Examination

Animals were examined with a slit lamp (biomicroscope) at day 7 (i.e., one day after the first intravitreal injection, to evaluate the drug tolerance. Then rats were examined each day from day 11 up to the time of euthanatization to evaluate disease onset time and severity. The intensity of the clinical ocular inflammation was scored on a scale from 0 to 7 as described below.19 Pupils were dilated by instillation of one drop of mydriaticum: (0) no sign of inflammation, normal iris dilatation; (1) discrete inflammation of iris and conjunctiva, incomplete dilatation of iris; (2) dilatation of iris and conjunctival vessels, moderate blockade of iris dilatation; (3) hyperemia in iris associated with the Tyndall effect in the anterior chamber, nearly complete blockade of iris; (4–7) same signs as (3), but one point was added if synechia, fibrin, hypopcell (cell deposit in the inferior anterior chamber), or corneal edema are observed.

Histopathology

At the time of euthanatization (days 19 to 20 after immunization), enucleated rat eyes were processed, paraffin sections were cut through the papillary–optic nerve plane, and stained with hematoxylin and eosin–safran for histologic evaluation. Sections were examined by a masked investigator who scored the severity of EAU on a semiquantitative scale from 0 to 7 as follows16: (0) no tissue destruction and (1) through (7) limited to total destruction of the various layers of the retina, as follows: (1–2) destruction of outer segments of rods and cones; (3–4) destruction of the outer nuclear layer; (5–6) destruction of the inner nuclear layer; and (7) destruction of the ganglion cell layer.

Inflammatory Cell Counting

To quantify the effect of tresperimus on ocular inflammation, cells were counted on paraffin sections in the anterior segment of the eye at

### Methods

**Induction of EAU in Lewis rats**

Eight-week-old female Lewis rats (R. Janvier, Le Genest saint Isle, France) were immunized subcutaneously into both hind legs with 40 μg of S-Ag purified from bovine retinae emulsified (1:1) in complete Freund’s adjuvant (Difco, Detroit, MI) supplemented with 250 μg of Mycobacterium tuberculosis H37Ra (Difco) as previously described.19 The care and use of the animals was in compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

**EAU Treatment**

Drug Information and Administration. Tresperimus has a molecular weight of 390.41 g/mol (base), has a high solubility in NaCl or PBS (up to 5 mM base), and is stable at 67 μM for at least 24 hours in NaCl at room temperature and for 15 days at –20°C. Intravitreal injection was the chosen administration route to allow for better ocular tissue exposure and to limit systemic exposure to the compound. Five μL of a saline solution of tresperimus at 9 mM were injected intravitreally for a target vitreous concentration of approximately 1 mM.

Protocols of EAU Treatment. The administration of tresperimus was performed by intravitreal injections in both eyes using a 30-gauge disposable needle (BD-microfine syringe, Medical, Asnière, France). At the end of the experiments (19 to 20 days after immunization), blood collection was performed, the rats were killed, and the eyes and samples were collected for analysis.

A total of four separate experiments were performed on a total of 81 rats. Thirty rats were used for the evaluation of tresperimus levels in plasma and ocular tissues (pharmacokinetics [PK] experiment).

In a first protocol, “prevention,” three intravitreal injections (5 μL) were performed at days 6, 9, and 12 after S-Ag immunization. Three groups of six rats were injected: one group received tresperimus (9 mM to achieve a 1-mM solution final in the vitreous; n = 6), a control group received vehicle (saline; n = 6 rats), and control uveitis rats were left untreated (n = 6). Animals were examined clinically with a slit lamp from day 9 after S-Ag immunization up to the time of euthanatization. Histopathology of the eyes was performed and immunostaining was processed on cryostat sections.
the optic nerve level after staining with hematoxylin and eosin-safran (at least five sections per eye). The cell number was expressed as the mean ± SEM of cell number counted in both eyes per animal. This counting was performed in the protocols of “prevention” and “prevention/treatment.”

**Immunohistochemistry**

Eyes (two eyes per group) were collected and fixed, and cryostat sections were cut and stained for immunohistochemistry at day 19 to 20 after immunization. Samples were fixed in 4% paraformaldehyde, embedded in ornithine carbamoyltransferase (OCT); Tissue-Tek, Miles Inc., Elkhart, IN) and 10-µm anteroposterior cryostat ocular sections were cut at the level of the optic nerve and stained for cell surface markers as previously described. Sections were incubated with rabbit polyclonal antibody against NOS-2 (1:100; Beckton Dickinson Biosciences, San Jose, CA), rabbit polyclonal antibodies directed against NF-κB/p65 (1:100), followed by Alexa 488-conjugated goat anti-rabbit antibody (green; Molecular Probes, Eugene, OR). Macrophages were detected by incubation with purified mouse monoclonal antibody (mAb) anti-macrosialin CD68 (clone ED1; macrophages and dendritic cells; 1/50; Serotec, Oxford, UK) followed by goat polyclonal antibody (red; 1:250). Sections were mounted with mounting medium (DAPI Vectashield, Vector Laboratories, Burlingame, CA) and observed by fluorescence photomicroscopy (FXa; Microphot, Nikon, Melville, NY). Digitized micrographs are obtained with a digital camera (Spot; BFI Optilas, Evry, France).

**Immune Response Evaluation**

Delayed Type Hypersensitivity (DTH) was estimated by ear assay measuring the specific anti-S-Ag response. Eighteen days after S-Ag immunization and the intravitreal injection of tresperimus (n = 12) or saline (n = 11), rats were tested for cutaneous response to S-Ag in the ear.

RNA Isolation and RT-PCR in the Lymph Nodes and in Cells Infiltrating the Eye. Total RNA was isolated from lymph nodes draining the immunizing site 19 to 20 days after immunization and from cells collected after centrifugation (2000 g/5 minutes) of aqueous humor/vitreous body from eyes of each group by Trizol reagent (Invitrogen, Carlsbad, CA). Inflammatory cytokines TNF-α, IFN-γ, IL-2, IL-17, and NOS-2 and IL-10 and sense primers and antisense primers were obtained from Genosys (Paris, France) and the PCR amplification was performed according to the manufacturer’s instructions.

**Cytokine and Chemokine Concentrations in Ocular Fluids (Pooled Aqueous Humor and Vitreous Body).** Cytokine and chemokine concentrations in ocular fluids were measured at 19 to 20 days after immunization by multiplex ELISA assay (xMAP technology assay) as described in the manufacturer’s protocol: cytokines MCP-1/CCL2, MIP1-α/CCL3, RANTES/CCL5, IP10/CXCL10 (IFN-inducible protein-10), and GRO/KC; inflammatory mediators IL-1β, IL-18 and TNF-α; Th1/Th2/Th17 cytokines IL-2 and IFN-γ/IL-4, IL-5, IL-6, IL-10, IL-13/IL-17, and VEGF (Clinsciences, San Diego, CA). The nonparametric Mann–Whitney U test was used for two groups, and the one-way ANOVA test followed by the Bonferroni multiple comparison tests was used for multiple groups. P < 0.05 was considered statistically significant.

**RESULTS**

**Pharmacokinetics of Tresperimus in Ocular Tissues and Plasma after Intravitreal Injections**

<table>
<thead>
<tr>
<th>Tresperimus Concentrations (n = 6)</th>
<th>Immunized Rats (3 Intravitreal Injections, Prevention Protocol)</th>
<th>Nonimmunized Rats (1 Intravitreal Injection)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 Hour</td>
<td>3 Days</td>
</tr>
<tr>
<td>Aqueous humor/vitreous, µM</td>
<td>270 ± 61</td>
<td>2.2 ± 1</td>
</tr>
<tr>
<td>Retina/choroid, µM</td>
<td>155 ± 25</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Plasma, µM</td>
<td>0.11 ± 0.03</td>
<td>BLQ</td>
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Values are Mean ± SEM. BLQ, below the lower limit of quantification (6 ng/mL).

Detection thresholds for all the analytes were estimated around 1 to 10 pg/mL.

**Cytokine and Chemokine Concentrations in Peritoneal Macrophages.** Macrophages were collected by washing the peritoneal cavity with a saline solution (NaCl 0.9%) containing heparin (0.4%) and polymyxine B (1 mg × L⁻¹). The collected peritoneal cells (in suspensions) were centrifuged at 1000 g (22°C, 5 minutes), and placed in MEM containing 2% fetal calf serum and 1% penicillin-streptomycin. Afterward, cells (1 × 10⁶/2/mL/well) were layered on six-well plates and maintained at 37°C under 5% CO₂ atmosphere during 2 hours. Nonadherent cells were removed by three successive washings with phosphate buffer saline (PBS, pH 7.4) and adherent cells (macrophages) were used for in vitro LPS stimulation with or without tresperimus treatment. Macrophages were pretreated overnight with tresperimus (1 mM) and then stimulated, in presence or not with tresperimus, with 100 ng/mL of LPS for 6 hours. Supernatants were collected and chemokines and cytokines concentrations were analyzed by multiplex ELISA (xMAP technology assay) following the manufacturer’s protocol (Clinsciences).

**Protein Determination in Aqueous Humor/Vitreous Body**

Aqueous humor/vitreous body from rat eyes injected with Tresperimus (n = 12) and from control rats (n = 12) injected with saline (prevention protocol) and from rat eyes injected with Tresperimus (n = 4) and from control rats (n = 5) injected with saline (prevention/treatment protocol) were collected from both eyes of each animal and pooled. After centrifugation protein concentration was determined in 1 µL of each supernatant using a Bradford assay with γ globulin as the standard (Biorad, Les Ulis, France).

**Statistical Analysis**

Data are presented as mean ± SEM. Statistical analysis was performed with commercially available software (Prism5; Graphpad Software, San Diego, CA). The nonparametric Mann–Whitney U test was used for two groups, and the one-way ANOVA test followed by the Bonferroni multiple comparison tests was used for multiple groups. P < 0.05 was considered statistically significant.
Ocular tissues were highly exposed to tresperimus, with significant contents (>10 μM) in retina/choroid 8 days postinjection. Moreover, the elimination rate of tresperimus was slower in retina/choroid than in aqueous humor/vitreous body.

An impact of the EAU pathology on the tresperimus ocular pharmacokinetics was observed, with a faster elimination from ocular tissues of immunized animals. This observation was linked with slightly higher plasma concentration (range, 19–89 ng/mL).

**FIGURE 1.** In vitro effect of tresperimus on the production of cytokines and chemokines by rat peritoneal macrophages stimulated with LPS. After overnight pretreatment with tresperimus (1 mM), macrophages were stimulated with 100 ng/mL of LPS for 6 hours, in presence or not with tresperimus. Chemokine and cytokine concentrations were analyzed in supernatants by multiplex ELISA (xMAP technology assay) following the manufacturer’s protocol (Clinisciences, Montrouge, France). Data are mean ± SEM. Significantly reduced concentrations of IL-1β (P < 0.04), IL-6 (P < 0.001), TNF-α (P < 0.001), CCL2/MCP1 (P < 0.001), CCL3/MIP1-α (P < 0.01), CCL5/RANTES (P < 0.01), CXCL10/IP-10 (P < 0.01), and a significant increase of IL-18 (P < 0.001) were detected in the presence of tresperimus compared to saline.

**FIGURE 2.** Effect of tresperimus injection on clinical EAU. (A) Intravitreal injections (5 μL/eye) at days 6, 9, and 12 after S-Ag immunization of tresperimus, saline, or no intraocular treatment (prevention protocol; 6 rats/group). Data are mean ± SEM (day 13, *P < 0.02; days 14–19, ***P < .0006 compared to saline-injected rats, or compared to rats that did not receive any intraocular treatment; day 13, *P < .02; day 19, ***P < .0006). (B) Intravitreal injections (5 μL/eye) at days 9, 12, and 15 after S-Ag immunization of tresperimus (5 rats/group) or saline (6 rats/group; prevention/treatment protocol). Data are mean ± SEM (days 13–19, ***P < .0006 compared to saline-injected rats). Data represent two separate experiments.
In Vitro Effect of Tresperimus on Production of Cytokines and Chemokines by Rat Peritoneal Macrophages Stimulated with LPS

Treatment by tresperimus of peritoneal macrophages stimulated with LPS reduced the concentration of the inflammatory cytokines IL-1β, IL-6, and TNF-α (Fig. 1). LPS alone did not increase the production of IL-18 by macrophages, but in conjunction with tresperimus, macrophages produced enhanced levels of IL-18 (Fig. 1). Other cytokines were detected in the supernatants of macrophages stimulated with LPS, but tresperimus treatment did not modify their concentration (data not shown). This was true particularly for anti-inflammatory/Th2 cytokines (IL-4, IL-5, IL-10, and IL-13). Tresperimus treatment had no effect on the production by macrophages of IL-2, IL-17, and IFN-γ, which are mainly produced by T lymphocytes in vivo. In addition, tresperimus treatment of macrophages had no effect on the production of CXCL10 (IP-10) and VEGF in vitro.

Intravitreal Injection of Tresperimus Significantly Reduced the Clinical Scoring of EAU

In the prevention protocol, 6, 9, and 12 days after immunization, rats received an intravitreal injection of 5 µL/eye of saline, tresperimus, or no intraocular treatment (6 rats/group). The tresperimus treatment led to a significant reduction of the clinical severity of EAU from day 13 after immunization compared to rats that received injections of saline (day 13, *P < 0.02; days 14–19, ***P < .0006) or compared to rats that did not receive any intravitreal treatment (day 12, *P < 0.02; day 19, ***P < .0006; Fig. 2A). Disease severity was significantly reduced by the treatment up to 19 days after immunization, indicating that intravitreal therapy was very effective.

In the prevention/treatment protocol, the intravitreal injection of tresperimus at days 9, 12, and 15 after S-Ag immunization also led to a significant reduction of clinical EAU (Fig. 2B).

Intraocular Injection of Tresperimus Protects Retina from Destruction and Limits Ocular Inflammatory Cell Infiltration

In the prevention protocol, rats presented very low EAU at histologic grading level (mean EAU severity grade, 1.45 ± 0.26; n = 10; P = 0.007) compared to control rats injected with saline (mean EAU severity grade, 3.25 ± 0.5; n = 10; Fig. 3A) and compared to rats that did not receive any intraocular treatment (mean EAU severity grade, 3.15 ± 0.6; n = 10; P = 0.08).

In the prevention/treatment protocol, consistent with clinical results, rats treated with tresperimus showed a significant reduction of EAU at the histologic level (mean EAU severity grade, 2.67 ± 0.33; n = 3; P = 0.05) compared to EAU in control rats injected with saline (mean EAU severity grade, 4.87 ± 0.13; n = 4; Fig. 3A).

The histopathologic examination of retinas from control rats injected with saline in the prevention protocol (Fig. 3B) revealed severe posterior uveitis with extensive destruction of the photoreceptor cell layer (Figs. 3Ba and 3Bb; white asterisks), inflammatory cell infiltration in the subretinal space (arrow), and fibrin exudates in the vitreous body (arrowhead). Numerous inflammatory cells were present in the vitreous body at the optic nerve head level (Fig. 3Bc; arrow). In contrast, in rats treated with tresperimus (Fig. 3C), the photoreceptor cell layer was largely spared from destruction (Fig. 3Cc; white asterisks) or showed only partial loss of the outer segments (Fig. 3Cd; arrow) with infiltration by inflammatory cells limited to the choroid (Fig. 3Cd; arrowhead). No inflammation was visible at the optic nerve head level (Fig. 3CF; arrow). In the prevention/treatment protocol, retinas from control rats injected with saline (Fig. 3D) showed extensive posterior uveitis with large destruction of the photoreceptor cell layer (Fig. 3Dg; white asterisk), whereas in rats treated with tresperimus (Fig. 3E) the photoreceptor cell layer showed no alteration nor inflammation (Fig. 3EH; white asterisk).

Quantification of Infiltrating Cells Present in the Anterior Chamber of the Eye

To confirm the inhibitory effect of tresperimus treatment on EAU, the number of cells present in the anterior segment of the eye was counted on paraffin sections at the optic nerve level after staining with hematoxylin and eosin–safran. Rats treated with three intravitreal injections of tresperimus (prevention protocol) presented very low inflammatory cell infiltration in the anterior segment of the eye (mean number of cells/eye,
significantly lower amount of proteins (prevention protocol showed, at the day 20 collection of samples, a
amount of proteins in aqueous humor/vitreous body from rats injected into the vitreous body with tresperimus in the preven-
tion protocol had a significant reduction of inflammatory cells in the anterior segment of the eye (n = 3; P = 0.05) compared to the intense ocular inflammation in saline injected controls (n = 4).

68.8 ± 17.3; n = 9; P = 0.0006) compared to control rats injected with saline (mean number of cells/eye, 254.8 ± 44.3; n = 10; Fig. 4).

Effect of Intravitreal Injection of Tresperimus on Local Ocular Immune Response

Blood–Ocular Barrier Breakdown. Examination of the amount of proteins in aqueous humor/vitreous body from rats injected into the vitreous body with tresperimus in the prevention protocol showed, at the day 20 collection of samples, a significantly lower amount of proteins (P = 0.05) compared to samples from rats injected with saline (Table 2A), which means that the treatment induced a diminution of protein leakage and a protection of blood–ocular barrier in treated rats.

A significant reduction of the amount of proteins was also observed in aqueous humor/vitreous body from rats treated with tresperimus in the prevention/treatment protocol compared to samples from rats injected with saline (Table 2).

Intraocular Injection of Tresperimus Modulates Macrophage Activity. As shown by immunostaining in control rats injected with saline (Figs. 5F–5H), numerous ED1-positive macrophages and lymphocytes expressed cytoplasmic and nuclear expression of NF-κBp65, principally in the vitreous body where infiltration by numerous inflammatory cells is visible. In contrast, in tresperimus-treated rats in the “prevention” protocol, low inflammatory cell infiltration is visible in ocular tissues (Figs. 5C–5E), with a reduced number of infiltrating cells in ocular tissues and media. Ocular infiltrating cells show only a cytoplasmic expression of NF-κBp65 without any nuclear translocation suggesting that these cells are deactivated.

Table 2. Proteins (μg/mL) in Aqueous Humor/Vitreous Body of Rats Injected Intravenously with Saline Compared to Rats Injected with Tresperimus

<table>
<thead>
<tr>
<th>Group</th>
<th>Saline</th>
<th>Tresperimus</th>
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<tbody>
<tr>
<td>Protocol prevention*</td>
<td>45.7 ± 5.4</td>
<td>34.2 ± 6.4†</td>
</tr>
<tr>
<td>Protocol prevention/treatment†</td>
<td>41.3 ± 13.8</td>
<td>13.9 ± 3.3</td>
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* Data are mean ± SEM of 12 rats/group (P = 0.05).
† Data are mean ± SEM of six rats in the saline group and four rats in the tresperimus group (P = 0.1).

where infiltration by numerous inflammatory cells is visible. In contrast, in tresperimus-treated rats in the “prevention” protocol, low inflammatory cell infiltration is visible in ocular tissues.

NOS-2 and IL-10 Expression in Infiltrating Inflammatory Cells Present in Aqueous Humor/Vitreous Body (Semiquantitative RT-PCR). In agreement with NF-κBp65 expression and localization, a reduction of NOS-2 expression was observed in ED1-positive cells in rat eyes treated with three injections of tresperimus compared to controls injected with saline (Figs. 5A and 5B).

Using semiquantitative RT-PCR, we evaluated NOS-2 mRNA expression in infiltrating ocular cells obtained after centrifugation of ocular fluids of saline injected rats (n = 5) compared to tresperimus-treated rats (n = 6; prevention protocol). We show that NOS-2 mRNA expression in infiltrating intraocular cells contained in aqueous humor/vitreous from tresperimus-treated rats was significantly reduced (P = 0.004) compared to cells from saline-injected rats (Fig. 5I). In tresperimus-treated rats (n = 4) in the prevention/treatment protocol, a significant reduction (P = 0.03) of the expression of NOS-2 was detected in infiltrating intraocular cells compared to cells from saline-injected rats (n = 4; Fig. 5I). Only in the prevention/treatment protocol, IL-10 expression was significantly increased (data not shown).

Cytokines in Aqueous Humor/Vitreous Body (Multiplex ELISA Assay, xMAP Technology Assay). Cytokine concentration was measured in aqueous humor/vitreous body at the time of death (i.e., at day 20 after immunization). A significant reduction of IL-2 and IL-17 concentrations (P ≤ 0.02 and P ≤ 0.03, respectively), no significant effect on IFN-γ (not shown) but a significant increase of IL-18 (P ≤ 0.008) was detected in aqueous humor/vitreous body from tresperimus-injected rats (prevention protocol) compared to rats injected with saline (Fig. 6). Lower levels of IL-6, IL-4, and VEGF were found in tresperimus-treated rats compared to controls (not significant; data not shown). Amounts of TNF-α, MIP-1α, IL-13, IL-5, and IP10 were undetectable in treated and control samples.
Intravitreal Injection of Tresperimus Has No Effect on Systemic Immune Response In Vivo Using the Prevention-Protocol Treatment

Cytokines in Inguinal Lymph Nodes (RT-PCR). No difference in levels of TNF-α, IL-2, IFN-γ, and IL-17 was detected in inguinal lymph nodes from treated and control rats, indicating that the treatment has no systemic effect (Fig. 7A).

DTH. DTH was estimated by ear assay measuring the specific anti-S-Ag response. Rats injected intravitreally with tresperimus at 6, 9, and 12 days after S-Ag immunization were tested on day 18 postimmunization with S-Ag by subcutaneous injection into one ear. Rats treated with tresperimus did not exhibit a significant reduction of ear swelling at 24 or 48 hours compared to control rats that received intravitreal injection of saline, showing that T-cell reactivity toward S-Ag in vivo is not reduced by tresperimus treatment and confirming that the treatment has no systemic effect (Fig. 7B).

No significant changes were observed in any measured cytokines using multiplex ELISA in the serum of rats treated with tresperimus, suggesting again no systemic effect of tresperimus using this treatment regimen.

Effect of Tresperimus on Cytokines in the Serum in the Prevention/Treatment Protocol

When tresperimus was administered at 9, 12, and 15 days after immunization, contrary to what was observed in the previous protocol, IL-4, IL-2, IL-6, and IFN-γ levels were significantly reduced without IL-10 increase compared with control rats (not shown). This serum cytokine profile suggested an effect on the systemic response.

DISCUSSION

Tresperimus is an agent that has shown its effects in tolerance induction, but the mechanisms of action of tresperimus are unclear. In the present study, we showed for the first time that intravitreal administration of tresperimus is of great benefit in the treatment of an autoimmune ocular disease, EAU, a model of ocular inflammation leading to retinal destruction. It is a clinically relevant model for human ocular inflammation that allows for the testing of new therapeutic strategies.

Interestingly, tresperimus is a very soluble molecule in saline, and its injection in the posterior pole of the rat eye at the level of the pars plana allowed tresperimus to diffuse into the anterior and posterior segments of the eye as shown by its effectiveness on anterior and posterior ocular inflammation in EAU. Indeed, after the intravitreal injection in S-Ag-immunized animals, tresperimus diffused rapidly (in 1 hour) to the retina/choroid tissues (155 mM) and decreased until 8 days to reach a level (11 mM) still effective in transplantation rat models (> 10 mM). In addition, very low levels of tresperimus were found in the plasma with no effect on the systemic immune response, as tested in the prevention protocol.
In the present study, we show that three intravitreal injections of tresperimus performed after S-Ag immunization were necessary to reduce clinical ocular inflammation and preserve retinal photoreceptors. Tresperimus treatment was also performed in a prevention/treatment protocol (i.e., at days 9, 12, and 15 after S-Ag immunization). Tresperimus treatment also led to a significant reduction of the clinical and pathologic manifestations of EAU, revealing that tresperimus could be used for both the prevention and treatment of intraocular inflammation.

As expected, when tresperimus was administered later in the time course of the disease, it induced the modification of cytokines in the serum. At this time point, tresperimus incompletely protected the blood–ocular barriers, even though at the end of the follow-up period it significantly reduced inflammatory cells and protein in the eye. This could be explained by a limited systemic passage of intravitreally injected trespermus, which in this protocol was not quantified. Whether such systemic diffusion could occur in larger animals—and, more importantly, in humans—will have to be explored.

To examine the level of action of trespermus, we analyzed systemic and local immune responses in the prevention protocol. We show here that when trespermus was administered in the eye early after immunization (first injection at 6 days), it did not affect the systemic immune response. Indeed, in inguinal lymph nodes draining the immunizing site, the level of inflammatory cytokines, such as TNF-α, and cytokines produced by T lymphocytes, such as IL-2, IFN-γ, and IL-17 were not modified by trespermus treatment. In addition, DTH to S-Ag was not different in control and treated rats, further suggesting that the treatment did not modify the systemic T-cell reactivity to S-Ag. Moreover, under this treatment regimen, serum cytokines were not significantly modified. In contrast, trespermus treatment was very effective on ocular pathology and local immune response, which is consistent with the presence of trespermus in the eye but below therapeutic levels in the serum. This could be explained by a systemic diffusion could occur in larger animals—and, more importantly, in humans—will have to be explored.

Macrophages have been reported to play an important role in tissue destruction.33–35 Our data suggest that trespermus treatment regulates both macrophage activation and T-cell-mediated response in the eye. This was confirmed by the in vitro analysis of cytokine production by rat peritoneal macrophages after LPS stimulation. Trespermus induced a reduction of IL-1, IL-6, and TNF-α and an increase of IL-18, which correlates well with the results observed in vivo in EAU.

To further determine whether the reduction of EAU scores in trespermus-treated rats is related to a regulation of macrophage activation, we examined by semiquantitative RT-PCR the expression of Nos-2 in infiltrating cells present in aqueous humor/vitreous body from treated and control rats in prevention and prevention/treatment protocols. In both protocols of treatment, expression of Nos-2 was detected by RT-PCR in ocular inflammatory cells collected from control rats, whereas the trespermus treatment suppressed Nos-2 expression in infiltrating macrophages. This result was confirmed by immunohistochemistry; ED1-positive macrophages present in the aqueous humor from trespermus-treated rats did not express Nos-2 compared to control rats. In addition, treatment allowed for the reduction of NF-κBp65 nuclear expression in ocular infiltrating macrophages. These results suggest that the reduction of Nos-2 expression in the cells of aqueous humor/vitreous in treated rats could be related to a diminution of the number of ocular infiltrating cells and/or to a less expression of Nos-2 in these cells. Interestingly, only in the prevention/treatment protocol, in addition to the downregulation of Nos-2, ocular infiltrating cells showed an increased expression of IL-10, suggesting that in addition to the possible systemic effect, a local immunomodulation is taking place.

**FIGURE 6.** Effect of three trespermus intravitreal injections on intraocular cytokines. Cytokine concentration was measured by multiplex ELISA at the time of death (i.e., day 20 after immunization) in aqueous humor/vitreous body from rats that received three intravitreal injections of trespermus (5 μL/eye/injection; black bars; n = 9 rats) at days 6, 9, and 12 after S-Ag immunization compared to controls that received three intravitreal saline injections (hashed bars; n = 12 rats). Significantly reduced concentrations of IL-2 and IL-17 (P ≤ 0.02 and P ≤ 0.03 respectively) and a significant increase of IL-18 (P ≤ 0.008) were detected in aqueous humor/vitreous body from trespermus-injected rats compared to rats injected with saline.

**FIGURE 7.** Effect of intravitreal injections of trespermus on systemic immune response. (A) Cytokine mRNA expression in inguinal lymph nodes. mRNA expression was evaluated by semiquantitative PCR. Total mRNA was isolated from inguinal lymph nodes at day 19 after immunization and reverse-transcribed into cDNA. The PCR fragments were analyzed by agarose gel electrophoresis. Results are obtained in six rats injected into the eye with trespermus and five rats injected with saline (prevention protocol). Agarose gel electrophoresis of the PCR product is presented below. Primers were designed to amplify specifically the cDNA fragments mature mRNA transcripts of 70 bp for 18S, 397 bp for IL-2, 287 bp for IFN-γ, 295 bp for TNF-α, and 64 bp for IL-17. (B) Specific DTH (mm) to S-Ag. DTH measured by ear assay showed that trespermus treatment did not modify ear swelling compared to saline injection as measured at 24 and 48 hours (P = 0.8 and P = 0.4, respectively). Data are mean ± SEM of 12 rats injected with saline and 11 rats treated with trespermus. **Data are mean ± SEM of 12 rats injected with saline and 11 rats treated with trespermus.**
Altogether, our results suggest that the reduction of EAU in tresperimus-treated rats (with both protocols) is related to a reduced activation of intraocular macrophages or to a change of their phenotype that contributes to reduced tissue damage.\textsuperscript{30} The present results are consistent with previous reports\textsuperscript{30} on the generation of “alternatively activated” macrophages\textsuperscript{7} during PKC\textsubscript{\textalpha} and IL-13-treatment of ocular inflammation. Tresperimus has been reported to inhibit nuclear localization of NF-kB\textsuperscript{B} in antigen-presenting cells, which is required for T cell costimulation.\textsuperscript{4} This suggests that local treatment with tresperimus could have an effect on the macrophage ocular presentation of S-Ag to T lymphocytes infiltrating the ocular tissue, thereby reducing ocular amplification of the immune response.

In addition, activated macrophages have been shown to synthesize IL-18,\textsuperscript{56} which is a pleiotropic cytokine identified as a Th1-type cytokine also involved in autoimmunity diabetes.\textsuperscript{35} Interestingly, IL-18 regulates pathogenic retinal neovascularization by promoting regression of abnormal neovascularization in an oxygen-induced retinopathy mouse model.\textsuperscript{58} Intraocular and subretinal neovascularization were observed during EAU,\textsuperscript{30,59} with an imbalance between VEGF and TGF-\beta.\textsuperscript{60} The role of IL-18 in EAU is not clearly identified. In EAU in mice, IL-18 was reported as a coinducer of both Th1 and Th2 cytokines.\textsuperscript{41} IL-18 is constitutively expressed in ocular tissues, but its role remains undetermined because the IL-18 gene is not necessary for the development of EAU in mice immunized by the IRBP peptide.\textsuperscript{41} Interestingly, IL-18 induced the Th2 cytokine IL-13\textsuperscript{42} that in our hands generated “alternatively activated” macrophages during ocular inflammation.\textsuperscript{7} In the present situation, rats treated with tresperimus and presenting with a reduced EAU at its resolution showed a significant increased expression of IL-18 in aqueous humor/vitreous fluids, suggesting that in our experimental conditions in Lewis rats, IL-18 was participating to the reduction of ocular inflammation and lesions. Interestingly, during Behçet’s disease, the detection of increased levels of IL-18 was reported in patients with inactive disease, implying that Th1 activation and subclinical inflammation persist during the inactive period of the disease.\textsuperscript{43}

After antigenic stimuli, different T-helper cells develop that are known as Th1 (IFN-\gamma-producing cells), Th17 (IL-17-producing cells), and Th2 (IL-4, IL-5, and IL-13-producing cells). Th1 and Th17 cells are involved in autoimmune disorders, while Th2 cells have a role in allergies and asthma.\textsuperscript{44} In the present study, we show that three intraocular injections of tresperimus performed after S-Ag immunization in the prevention protocol reduced the intraocular concentration of the T cell–specific cytokines IL-2 and IL-17. Cytokine concentration in pooled aqueous humor and vitreous body was determined by multiplex ELISA, 21 days after S-Ag immunization in rats treated with tresperimus or saline. It shows that a reduction of severity of EAU in tresperimus-treated rats is related to significant reduced levels of IL-2 and IL-17. It has been reported that IL-2 promotes Th17 expansion, providing explanations for the efficacy of IL-2R antibody therapy in uveitis, and suggests that antagonism of Th17 that contributes to ocular pathology could be used for the treatment of chronic ocular inflammation.\textsuperscript{21} Indeed, EAU in rats may be suppressed by anti-IL-17 antibody injection with suppression of antigen-specific DTH and lymphocyte proliferation assay.\textsuperscript{45} This confirms that IL-17 plays a crucial role in EAU and that tresperimus reduces EAU severity by reducing Th17 responses. Tresperimus appears to reduce autoimmunity by inhibiting proliferation as shown by significant reduced responses of IL-2 and Th17, and this effect appears to be comonitant with increased levels of Th1 (IL-18)-inducing molecules. Our results suggest that IL-18 inhibits Th17 induction during EAU, and this is in agreement with previously published observations of increased Th17 induction in the absence of IL-18 in atherosclerosis.\textsuperscript{46}

Conclusion
Tresperimus is a very effective drug by local administration on the experimental model of autoimmune uveoretinitis. Its solubility in water allows tresperimus to be injected directly into the vitreous body and to diffuse to the ocular tissues. It persists at a therapeutic level for at least 8 days in the eye. Systemic administration of tresperimus has shown its effectiveness in bone marrow, cardiac, and skin transplant models. However, secondary effects, such as neutropenia and hypotensive effects, were detected after systemic high dose of treatment that impeded its clinical use. Our present study shows that tresperimus is effective in an experimental model of autoimmune uveitis after intraocular administration in rats. The dose administered in the eye is so low compared with the higher dose of tresperimus that would be used systemically in humans that systemic side effects would not occur. Because tresperimus has already been used in humans, its clinical ocular application could be very fast. Tresperimus is a promising local therapy for ocular inflammation and other Th17-mediated autoimmune diseases, but additional studies are needed before its use in clinical practice.

References


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