Downregulation of Endotoxin-Induced Uveitis by Intravitreal Injection of Vasoactive Intestinal Peptide Encapsulated in Liposomes

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PURPOSE. To reestablish the immunosuppressive microenvironment of the eye, disrupted by ocular inflammation during endotoxin-induced uveitis (EIU), by means of intravitreal injection of vasoactive intestinal peptide (VIP) in saline or encapsulated in liposomes, to increase its bioavailability and efficiency.

METHODS. EIU was induced in Lewis rats by subcutaneous injection of lipopolysaccharide (LPS). Simultaneously, animals were intravitreally injected with saline, saline/VIP, VIP-loaded liposomes (VIP-Lip), or unloaded liposomes. EIU severity and cellular infiltration were assessed by clinical examination and specific immunostaining. VIP concentration was determined in ocular fluids by ELISA. Ocular expression of inflammatory cytokine and chemokine mRNAs was detected by semiquantitative RT-PCR. Biodistribution of rhodamine-conjugated liposomes (Rh-Lip) was analyzed by immunohistochemistry in eyes and regional cervical lymph nodes (LNs).

RESULTS. Twenty-four hours after intravitreal injection of VIP-Lip, VIP concentration in ocular fluids was 15 times higher than after saline/VIP injection. At that time, EIU clinical severity, ocular infiltrating polymorphonuclear leukocytes (PMNs), and, to a lesser extent, ED1+ macrophages, as well as inflammatory cytokine and chemokine mRNA expression, were significantly reduced in VIP-Lip-injected rats compared with rats injected with saline/VIP, unloaded liposomes, or saline. Rh-Lip was distributed in vitreous, ciliary body, conjunctiva, retina, and sclera. It was internalized by macrophages and PMNs, and VIP colocalized with liposomes at least up to 14 days after injection. In cervical LNs, resident macrophages internalized VIP-Rh-Lip, and some adjacent lymphocytes showed VIP expression.

CONCLUSIONS. VIP was efficient at reducing EIU only when formulated in liposomes, which enhanced its immunosuppressive effect and controlled its delivery to all tissues affected by or involved in ocular inflammation. (Invest Ophthalmol Vis Sci. 2007;48:3230–3238) DOI:10.1167/iovs.06-1305

Uveitis is the inflammation of the tissues forming the uveal tract, namely iris, ciliary body, choroid, and contiguous structures. In humans, Gram-negative bacterial infection is considered a major factor in acute anterior uveitis. In rats, a single systemic injection of endotoxin, the lipopolysaccharide (LPS), induces acute bilateral anterior uveitis with posterior involvement. Endotoxin-induced uveitis (EIU) is characterized by the breakdown of the blood-ocular barrier, the ocular infiltration of inflammatory cells, and the production of inflammatory cytokines and chemokines. EIU is a useful model for human acute anterior uveitis, particularly that associated with Crohn’s disease, ankylosing spondylitis, and arthritis, leading to severe visual impairment or blindness.

The mainstay of human uveitis treatment tends to inhibit the immune response by systemic agents and, more recently, anti–interleukin (IL)-2 receptor antibody, alpha interferon, or drugs inhibiting the binding of tumor necrosis factor alpha (TNF)-alpha to its receptors. These strategies are successfully used in patients with recalcitrant uveitis; however, because of their systemic administration, all these drugs induce potentially severe adverse effects. Moreover, in certain ocular tissues, tight junctions reduce the passage of drugs from the blood to the eye, thus limiting the bioavailability of immunosuppressors in ocular tissues.

To overcome these limitations, local administration of drugs directly into the eye is particularly interesting for treating ocular diseases. In experimental models, local delivery of immunomodulatory molecules has shown its efficacy in limiting ocular inflammation. In humans, injection into the vitreous cavity (i.e., intravitreal injection) is routinely used for the intraocular delivery of corticosteroids, antimetabolites, and, more recently, anti–VEGF therapies. However, to reach and maintain efficient drug concentration within the eye, repeated intraocular injections may be necessary, leading to adverse effects and complications. In addition, high acute intraocular concentrations of drugs may induce severe local toxicity and intraocular media variations. To avoid these problems, it is important to define a controlled drug release system and to determine which therapeutic molecules are best tolerated by the intraocular milieu.

Numerous immunosuppressive factors, such as transforming growth factor-beta, alpha-melanocyte-stimulating hormone, and vasoactive intestinal peptide (VIP), are produced locally within the eye and participate in the immunologic homeostasis of the ocular microenvironment. These endogenous molecules may therefore be used to treat ocular inflammation in the event of disruption of the immune system. Vasoactive intestinal peptide, a 28-aa neuropeptide, exhibits many interesting immunosuppressive properties in vitro and in vivo. Previous studies have shown that VIP interacts with many cell types involved in inflammatory reaction, including endothelial cells,
macrophages, and lymphocytes, through its receptors PAC1, VPAC1, and VPAC2.

In vitro, VIP inhibits the secretion of chemokines, nitric oxide, and inflammatory cytokines by LPS-stimulated macrophages and enhances the production of the anti-inflammatory cytokine IL-10. In vivo, treatment with VIP reduces the severity of inflammation in experimental models of autoimmune encephalomyelitis, arthritis, and Crohn’s disease. Intraperitoneally administered VIP also protects mice against the lethal effects of high doses of LPS.

Nevertheless, when tested in experimental models of ocular inflammation (EU) and experimental autoimmune uveoretinitis (EAU), VIP exacerbated or inhibited the ocular disease or showed no therapeutic effect. These conflicting results could be attributed to the differences in injection protocols or to the poor stability of VIP in the biological environment, compromising the bioavailability of VIP in the ocular media. Interestingly, VIP encapsulation in sterically stabilized liposomes was reported to prevent its degradation in vitro and in vivo, to preserve its activity by maintaining its α-helix conformation, and to prolong its release. Liposomal formulation of VIP may be promising for the treatment of ocular inflammation.

In the present study, we evaluated the effect of intravitreal injection in rats of saline/VIP or VIP encapsulated in liposomes (VIP-Lip) on the severity of LPS-induced ocular inflammation (EU) at cellular and molecular levels. The fate of liposomes loaded with VIP in the eye and in regional draining lymph nodes (LNs) was also analyzed.

**Materials and Methods**

**Materials**

Egg yolk phosphatidylcholine (PC) was a gift from Lipoid GmbH (Ludwigshafen, Germany). Egg yolk phosphatidylglycerol (PG), cholesterol (CHOL), trehalose, and lipopolysaccharide (LPS) from Salmonella typhimurium were purchased from Sigma-Aldrich Co. (St. Louis, MO). VIP was obtained from Bachem AG (Bubendorf, Switzerland). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(poly(ethylene glycol))-2000] (PEG-DSPE) and phosphatidylethanolamine-N-[lysamine rhodamine B sulfonyl] (PE-Rhod) were purchased from Avanti Polar Lipids (Alabaster, AL). Sodium dodecyl sulfate (SDS) was obtained from Bio-Rad Laboratories (Hercules, CA). Tropicamide 5% and tetracaine 1% were provided by CibaVision Ophthalmics (Toulouse, France). Sodium pentobarbital was provided by Ceva sante animale (Libourne, France).

Purified mouse monoclonal antibody (mAb) anti-macrosialin CD68, a cyttoplasmic antigen in rat monocytes, macrophages, and dendritic cells (clone ED1), and purified mAb anti-sialoadhesin CD169 (clone ED3) were purchased from Serotec Ltd. (Oxford, UK). Alexa Fluor 647-conjugated goat anti-mouse IgG (H+L) and Alexa 488-conjugated goat anti-mouse IgG (H+L) were obtained from Molecular Probes (Eugene, OR). Rabbit mAb anti-VIP was provided by Bachem UK (Merseyside, UK).

Fluoroprobe Alexa 488-conjugated goat anti-rabbit IgG (H+L) was provided by Interchim (Montluçon, France). Mounting medium (DAPI Vectashield) was purchased from Vector Laboratories Inc. (Burlingame, CA).

**Animals**

Eight-week-old male Lewis rats (Charles River, Saint Aubin les Elbeuf, France) weighing approximately 280 g each were used. Animals were maintained on a 12-hour-light/12-hour-dark cycle. Food and water were supplied ad libitum. Animals were handled and cared for in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research.

**Liposome Preparation**

Pegylated liposomes used to encapsulate VIP were composed of PC, PG, CHOL, and PEG-DSPE (68 mM; molar ratio, 50:10:35:5). Liposomes were first prepared by the thin-film hydration method, and VIP was encapsulated by the lyophilization–rehydration method, as described by Séjourné et al. VIP-Lip was separated from free VIP by ultracentrifugation for 1 hour at 150,000g and 4°C (7.155; rotor 70.1 TI; Beckman, Palo Alto, CA) after dilution of 250 μL liposomes in saline to obtain a minimal volume of 1 mL. The supernatant was removed, and the resultant pellet was resuspended in a required amount of saline to obtain 250 mg final suspension. Unloaded liposomes were prepared as described except that VIP was not added. Rhodamine-conjugated liposomes (unloaded Rh-Lip and VIP-Rh-Lip) were also prepared as described. In this formulation, 2.04 mM of PC was replaced by 2.04 mM PE-Rhod. The final molar ratio of PC/PG/CHOL/PEG-DSPE/PE-Rhod was 47:10:35:5:3.

**Physicochemical Characterization of Liposomes**

Liposome mean size was determined before and after encapsulation by quasi-elastic light scattering (NanoSizer N4 Plus; Beckman Coulter, Margency, France) after suspension dilution 150 times in a saline solution. Measurement conditions were: 20°C; 1 mPa s; refractory index, 1.33; 1-minute data acquisition; detection angle, 90°. Measurements, made in triplicate, were expressed as mean diameter ± SD.

The PC concentration in purified liposomes was evaluated by enzymatic phospholipid assay (Phospholipides enzymatiques PAP 150; BioMérieux, Lyon, France). Because rhodamine interferes with absorbance measurements, the phospholipid concentration of Rh-Lip suspensions was determined by Bartlett inorganic phosphate assay.

The concentration of 28-aa VIP in liposomes was determined by VIP ELISA (VIP EIA kit extraction free; Peninsula Laboratories Inc., San Carlos, CA). The principle of the assay is based on the competition between biotinylated VIP and nonbiotinylated peptide (either standard or unknown) to bind on anti–VIP antibody that recognizes 28-aa VIP. Modifications were performed in the protocol to quantify VIP in liposome suspensions instead of in serum or plasma. Liposomes were solubilized by the addition of SDS 1% (1.06 mmol lipid/g SDS). Samples were then diluted in pH 7.7 buffer to reach VIP concentrations ranging from 0.1 to 10 ng/mL. The calibration curve was also prepared with a pH 7.7 buffer instead of standard diluent provided by the kit. Results were expressed as mean ± SD.

**Endotoxin-Induced Uveitis Induction and Clinical Examination**

EU was induced by subcutaneous injection of 0.1 mL sterile pyrogen-free saline containing 350 μg/kg LPS. Animals were examined with a slit lamp biomicroscope 24 hours later, corresponding to the time of maximal severity of EU. The intensity of clinical ocular inflammation was scored on a scale from 0 to 4 for each eye, derived from a scale described previously as follows: 0, no sign of inflammation; 1, discrete inflammation in iris and conjunctiva; 2, dilatation of iris and conjunctiva vessels; 3, hyperemia in iris associated with Tyndall effect in anterior chamber; 4, same signs as in 3, but a point was added if synechia or fibrin was observed.

**Intravitreal Injection Protocols**

Lewis rats were anesthetized by intraperitoneal injection of 0.15 mL pentobarbital (5.47 g/100 mL saline). Pups were diluted by instillation of one drop of tropicamide 5%. One drop of tetracaine 1% was administered for local anesthesia. Intravitreal injections (10 μL) were performed in both eyes using sterile syringes fitted with a 30-gauge needle (Microfine; Becton Dickinson AG, Meylan, France), as previously described. Intravitreal injections were performed simultaneously with EU induction, as described. To detect any proinflammatory effect of VIP in the eye, four normal rats received intravitreal injection of unloaded liposomes (50 mM lipids).

The therapeutic effect of VIP on EU was tested in 36 rats. Four groups of rats received intravitreal injections of VIP-Lip (0.55 mg/mL VIP, 50 mM lipids; n = 9), saline/VIP (0.55 mg/mL VIP; n = 9),...
unloaded Lip (50 mM lipids, n = 9), or saline alone (n = 9). Eyes were used for immunohistochemistry and RT-PCR analysis.

To quantify VIP concentration in vitreous body and aqueous humor, 18 EIU rats received saline/VIP (n = 9) or VIP-Rh-Lip (n = 9). Rats were killed at 2, 6, and 24 hours (n = 3 per time point and per group). Secondary LNs were collected for biodistribution study.

Twenty-six EIU rats were used for the biodistribution study in ocular tissues and secondary lymphoid organs of unloaded Rh-Lip (n = 7) and VIP-Rh-Lip (n = 10) at 6 hours, 24 hours, 7 days, and 14 days. Biodistribution of VIP after intravitreal injection of saline/VIP (n = 7) was analyzed in ocular tissues. Control rats received intravitreal injections of saline (n = 2).

**VIP Concentration in Aqueous Humor and Vitreous Body after Injection of VIP-Rh-Lip and Saline/VIP in EIU Rats**

At different time points after injection, aqueous humor and vitreous body from both eyes of each animal were collected, pooled, and immediately frozen. To determine 28-aa VIP concentration in aqueous humor and vitreous body (i.e., free VIP and VIP contained in liposomes), liposomes were solubilized by dilution of each sample with SDS 1% (final SDS concentration, 0.15%). Total VIP concentration was then determined with a VIP ELISA assay kit according to the manufacturer’s instructions (Bachem UK), after dilution with the standard diluent provided by the kit. Concentrations of VIP in ocular fluids of untreated EIU rats were under the detection limit of the kit (0.1 ng/mL).

**Tissue Collection and Processing for Immunohistochemistry**

Immediately after the rats were killed, eyes and right and left subman- dibular and superficial cervical LNs (collectively named cervical LNs) were collected and processed as previously described.54 All tissues were postfixed in 4% paraformaldehyde containing 5% sucrose for 2 hours before immersion for an additional 2 hours in phosphate buffer containing 5% sucrose and overnight in phosphate buffer containing 15% sucrose. The next day, samples were embedded and frozen in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura Finetek, Zoeterwoude, Netherlands) and were stored at −80°C. Frozen sections (10-μm thick) of LNs and anteroposterior sections of eyes at the optic nerve level were cut using a cryostat (CM 3050S; Leica, Oberkochen, Germany) and were mounted on gelatin-coated slides for immunohistochemical analysis.

**Immunohistochemistry**

Immunostaining was performed on frozen eye and LN sections, as previously described.55 To count the number of inflammatory cells on eye sections, the slides were incubated with mouse monoclonal antibody ED1 at a dilution of 1:50. Secondary Alexa 488 (green)-conjugated goat anti–mouse antibody was then applied. Different controls were included in every staining run: (1) negative controls without primary mAbs; (2) isotype controls with the addition of normal serum immunoglobulin in place of goat polyclonal antibodies; (3) normal mouse immunoglobulin of the same isotype for monoclonal mouse antibodies. No rhodamine fluorescence was observed on eye sections after injections of saline/VIP or saline alone. Sections were mounted with an anti–fade medium with mounting medium (DAPI Vectashield; Vector Laboratories) and were observed by fluorescence photomicroscopy (FXA; Microphot; Nikon, Melville, NY). Digitized micrographs were obtained with a digital camera (Spot; BFI Optilas, Evry, France).

**Epifluorescence and Confocal Microscopy and Image Analysis**

Epifluorescence microscopy of eye and LN sections was performed using ×25 (PL Fluorat; Leitz), oil immersion ×25 (NPL Fluorat; Leitz), and ×50 (PL Fluorat; Leitz) objectives mounted on a fluorescence microscope (Leitz, Aristoplan, Rueil-Malmaison, France) with the appropriate excitation filters. Images were collected with a camera (SPOT; BFI Optilas) and the appropriate software (SPOT Advanced 3.1 for Windows 95/98/NT; Diagnostic Instruments). Confocal microscopy with a laser scanning confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) was performed to further characterize the distribution of unloaded Rh-Lip and VIP-Rh-Lip in LNs. The microscope was equipped with an argon laser giving 488 nm (blue excitation, green emission) and a helium/neon laser giving 543 nm (green excitation, red emission), which allowed two fluorochromes to be observed. Sections were analyzed at ×63 oil immersion objective (plan-APOCHROMAT NA 1.4; Carl Zeiss); sequential images were merged and false colored using image browser software (LSM 510; Carl Zeiss) to produce a composite multicolor image. Final image processing was performed with image editing software (Adobe Photoshop CS2; Mountain View, CA).

**Cell Counting**

To quantify EIU and the effect of VIP-Lip treatment, all ED1+ cells were counted across the entire ocular cross-sections (ciliary body, iris, aqueous humor, vitreous body, retina, and choroid) obtained from animals injected with VIP-Lip (n = 7 eyes), saline/VIP (n = 5 eyes), unloaded Lip (n = 6 eyes), and saline (n = 4 eyes). Cells showing the characteristic morphologic appearance of PMNs, with trilobed nuclei after DAPI staining, were also counted on the same sections, in aqueous humor and vitreous body. Cell number was expressed as the mean ± SE of total cell number per animal, as previously described.55

**RNA Isolation and Semiquantitative RT-PCR of Intraocular Cytokines and Chemokines**

Total ocular RNA was isolated from freshly enucleated eyes of each group (4 eyes/group) 24 hours after LPS injection by the acid guani- dinium thiocyanate-phenol-chloroform method, as previously described.56 In each sample, the concentration was readjusted according to the RNA optic density at 260 nm. PCR fragments were analyzed by 3% agarose gel electrophoresis and visualized by ethidium bromide staining under UV light. To verify that equal amounts of RNA were added in each PCR reaction within an experiment and to verify a uniform amplification process, GAPDH mRNA was also transcribed and amplified for each sample. Relative band intensity was calculated in comparison with that for GAPDH. TNF-α, IL-1β, MCP-1, MIP-2, IFN-γ, CINC-1, and GAPDH sense and antisense primers were obtained from αGenosys (Paris, France), and PCR amplification was performed according to the manufacturer’s instructions. These primers were designed to amplify specifically the cDNA fragments representing mature mRNA transcripts of 162 bp for GAPDH, 294 bp for TNF-α, 257 bp for MCP-1, 334 bp for MIP-2, 287 bp for IFN-γ, and 71 bp for CINC-1.

**Statistical Analysis**

EIU clinical scores and cell counting were presented as mean ± SEM. Severity of EIU was compared using the nonparametric Mann–Whitney U test followed by the Bonferroni multiple comparison test. A P value adjusted by the multiple comparison test was calculated in each experiment. P < 0.05 was considered statistically significant. VIP concentrations were presented as mean ± SD. Measurements were compared using the Student’s t-test. P < 0.05 was considered statistically significant.
RESULTS

Physicochemical Characterization of Liposomes

Liposomes were between 300 and 600 nm for VIP-Lip and between 250 and 400 nm for unloaded Lip. VIP encapsulation yield was 37% ± 7%, corresponding to an encapsulation efficiency of 3.0 ± 0.4 mmol VIP/mol lipids.

Therapeutic Effect of Saline/VIP and VIP-Lip in EIU Rats

Clinical ocular inflammation was evaluated at slit lamp examination 24 hours after EIU induction. As shown in Figure 1, intravitreal injection of unloaded Lip (50 mM lipids) and saline/VIP (0.55 mg/mL VIP, 50 mM lipids) had no effect on EIU compared with animals receiving saline intravitreally (mean clinical scores: 2.8 ± 0.19, 3.1 ± 0.21, and 3.2 ± 0.19, respectively). By contrast, intravitreal injection of VIP-Lip (0.55 mg/mL VIP, 50 mM lipids) markedly reduced the clinical score of uveitis from 3.2 ± 0.19 to 1.5 ± 0.21 (P = 0.0002).

No inflammatory sign was detected in 7 of 8 eyes in normal rats injected with unloaded Lip (50 mM lipids; Fig. 1). One synchia was observed in 1 of 8 eyes.

VIP Concentration in Aqueous Humor and Vitreous Body

VIP concentration in pooled aqueous humor and vitreous body decreased as a function of time for saline/VIP and VIP-Lip (Fig. 2). Concentrations were not significantly different 2 and 6 hours after injection. However, at 24 hours, VIP concentration was 15 times higher after VIP-Lip injection compared with saline/VIP (155 ng/mL vs. 10 ± 4 ng/mL; P = 0.05).

Effect of Saline/VIP and VIP-Lip on Ocular Inflammatory Response

Inflammatory cells infiltrating the eye were counted 24 hours after EIU induction. Compared with unloaded Lip and saline intravitreal injections, saline/VIP injection had no effect on the number of inflammatory cells (ED1+ cells and PMNs) in the anterior and posterior segments in EIU rats (Fig. 3A). By contrast, inflammatory cell infiltration was significantly reduced within the eyes of EIU rats receiving VIP-Lip injection (P ≤ 0.04) compared with saline/VIP, unloaded Lip, and saline. Interestingly, VIP-Lip treatment did not affect the number of resident ED1+ macrophages in the ciliary body (Fig. 3B). VIP-Lip and saline/VIP induced a significant decrease (P ≤ 0.05) in the number of infiltrating ED1+ macrophages in aqueous humor and iris only when compared with saline-injected rats (Fig. 3C).

In the posterior segment, the number of ED1+ cells was significantly decreased in VIP-Lip treated rats (P ≤ 0.05) compared with rats injected with saline/VIP, unloaded Lip, or saline (Fig. 3D).

In both anterior and posterior segments, the number of PMNs was significantly reduced by VIP-Lip injection (P = 0.001) but not by saline/VIP (Figs. 3E, 3F) compared with unloaded Lip-injected rats. Compared with saline-injected controls, the number of PMNs in the VIP-Lip–treated group was significantly decreased in the anterior segment (P = 0.02) but not in the posterior segment because of high SEM values in this group.

Thus, intravitreal injection of VIP-Lip led to a diminished infiltration of inflammatory cells (ED1+ cells and PMNs) in anterior and posterior segments of the eye but did not modify the number of ED1+ resident macrophages in the ciliary body. An effect of saline/VIP injection on the number of ED1+ macrophages was only observed in aqueous humor and iris.

To better understand the molecular mechanisms at the origin of the reduction of EIU severity, the expression of cytokine mRNAs was investigated by semiquantitative RT-PCR analysis of four eyes in each group taken 24 hours after EIU induction. Reduced expression of TNF-α, IL-1β, MCP-1, MIP-2, IFN-γ, and CINC-1 mRNAs was detected in VIP-Lip–injected eyes compared with saline and unloaded Lip. An effect of saline/VIP was evidenced on TNF-α, IL-1β, and MIP-2 mRNA expression, but not on MCP-1, IFN-γ, and CINC-1 mRNA expression (data not shown).

Intraocular Biodistribution of VIP-Rh-Lip and Unloaded Rh-Lip in EIU Rats

Intraocular biodistribution of VIP-Rh-Lip or unloaded Rh-Lip at various time points after injection into the vitreous is summarized in Table 1. From 6 to 24 hours after intravitreal injection,
VIP-Rh-Lip was seen in the vitreous body (Fig. 4A), accumulating along the internal limiting membrane of the retina (Fig. 4B), and in the anterior segment close to the ciliary body (Fig. 4C).

Liposomes were also found within some ocular tissues, such as the ciliary muscles (Fig. 4C), the conjunctiva (Fig. 4D), and the sclera (Table 1). In all these tissues, large numbers of Rh-Lip (red) containing VIP (green) were detected in the conjunctiva (Fig. 4J). CB, ciliary body; CM, ciliary muscles; CONJ, conjunctiva; I, iris; R, retina; V, vitreous body. Photographs are representative of similar sections stained for 5 rats/group. Scale bars, 20 μm.

FIGURE 4. Ocular biodistribution of rhodamine-conjugated liposomes containing VIP (VIP-Rh-Lip) in EU rats. At 6 and 24 hours after LPS injection into the footpad and intravitreal injection of VIP-Rh-Lip, liposomes (red) and VIP (green) are observed in the vitreous body (A, E, F), ciliary body and muscles (C), and conjunctiva (D). In the vitreous body, liposomes (red) accumulate along the internal limiting membrane of the retina with evidence of internalization by the colocalization (yellow) of liposomes (red) with ED1+ macrophages (green; B, arrow). At 24 hours, liposomes (red) and VIP (green) are internalized in substantial numbers of (F) PMN cells, and liposomes (red) are internalized in (G) ED1+ cells (green). Diffusion of free VIP (green) is observed within the retina (E). ED1+ cells (green) located along the iris also internalize liposomes (red) (H, arrow). At 14 days, liposomes (red) are observed next to the ciliary body (I) and VIP (green)-loaded liposomes (red) are detectable within the conjunctiva (J).

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<th>Intravitreal VIP-Rh-LIP and Subcutaneous LPS</th>
<th>Intravitreal Unloaded Rh-Lip and Subcutaneous LPS</th>
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<tr>
<td><strong>Time after Injection</strong></td>
<td><strong>6 h and 1 d</strong> (n = 5)</td>
<td><strong>7 d and 14 d</strong> (n = 5)</td>
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<tr>
<td><strong>Vitreous</strong></td>
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<td><strong>Retina</strong></td>
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Relative intensity of liposome dispersion scored arbitrarily on the whole section as follows: no liposome, −; low, +; medium, ++; high, +++.
At days 7 and 14, the number of liposomes had decreased in ocular tissues; most of the detected liposomes were localized in the vitreous body, close to the ciliary body (Fig. 4l), and within the conjunctiva (Fig. 4j) and were still loaded with VIP. The presence of liposomes was not observed within the aqueous humor, iris, or retina (Table 1). At day 7, VIP-Rh-Lip that had been internalized by resident ED1⁺ cells was detected at the ciliary body base (not shown).

To evaluate whether the biodistribution of liposomes was or was not affected by the delivery of VIP in EIU rats, unloaded Rh-Lip was intravitreally injected simultaneously with subcutaneous administration of LPS. At 24 hours, the biodistribution profile of unloaded Rh-Lip was not different from the one observed after VIP-Rh-Lip injection (Table 1; vitreous body, Fig. 5a; anterior chamber, Fig. 5b; internal limiting membrane of retina, Figs. 5c, 5d; iris, Fig. 5e). Free liposomes were observed in the vitreous body, on the retinal surface, and in the internal layers of the retina (Fig. 5c; enlargement in Fig. 5d). At 24 hours, many inflammatory cells, PMNs (Figs. 5a, 5b), and ED1⁺ macrophages (Fig. 5e) containing or not containing liposomes infiltrated the ocular tissues and were still present in the eye after 14 days (Table 1). No difference in biodistribution between VIP-Rh-Lip and unloaded Rh-Lip was detected at 7 and 14 days (Table 1). VIP was not detectable by immunohistochemistry in any ocular tissue or at any time after intravitreal injection of unloaded Rh-Lip and saline/VIP.

**Biodistribution of VIP-Rh-Lip and Unloaded Rh-Lip in Draining Cervical Lymph Nodes**

Fluorescent liposomes were observed in cervical LNs as early as 2 hours (data not shown), at 24 hours (Fig. 6a), and up to 14 days (Fig. 6b) after intravitreal injection. Biodistribution of unloaded Rh-Lip and VIP-Rh-Lip in the cervical LNs was identical at 2, 6, and 24 hours and 14 days (data not shown). At the cellular level, VIP-Rh-Lip was detected within resident ED3⁺ (CD169) macrophages located in the subcapsular sinus of the LN (Figs. 6a, 6b). Free VIP (green) was apparent in macrophages. However, most VIP appeared to be encapsulated within Rh-Lip as yellow granules at 24 hours (Figs. 6c, 6d) and up to 14 days after injection (Fig. 6f). In addition, VIP expression not associated with liposomes was visualized in small, round cells close to large ED3⁺ macrophages in the T-cell area (Fig. 6e) and the germinal center (data not shown) of LNs of animals that received unloaded Rh-Lip or VIP-Rh-Lip.

**DISCUSSION**

The aim of this work was to evaluate the effect of intraocular injection of VIP, a peptide endogenously present in the normal eye, as a new immunomodulatory agent for the treatment of EIU. Because VIP is poorly stable in biological media, it was encapsulated in liposomes to improve its protection against degradation and to control its release.

First, the intraocular tolerance of unloaded liposomes was studied in normal rats. No inflammation was observed in the injected eyes. Indeed, the lipids present in liposomes are well tolerated, as previously reported in rabbits57 and in humans.58 These results prompted us to use, in our experimental protocols, the relatively high concentrations of lipids (50 mM) necessary to inject large amounts of VIP without any potential adverse effect.

In the present article, we demonstrate a strong protective effect of intravitreal injection of liposome-encapsulated VIP on EIU in the rat. In a previous study, however, Zhang et al.45 reported that saline/VIP injected intraperitoneally exacerbated EIU in mice. The reasons for this discrepancy are unknown. It can be hypothesized that variations in animal and mouse.

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**FIGURE 5.** Ocular biodistribution of rhodamine-conjugated unloaded liposomes in EIU rats. At 24 hours, free liposomes (red) are present in the vitreous (A, C) and aqueous humor (B) or are internalized within PMN cells (B, arrow) and internal layers of the retina (asterisk). Liposomes are observed in the limiting membrane of the retina (arrow) or internalized by PMNs (arrowhead) 24 hours after injection (C, D). (D) is an enlargement of the area selected in (C). Internalization of liposomes (red) in numerous ED1⁺ cells (green) adjacent to anterior layers of the iris at 24 hours (E, arrow). AC, anterior chamber; I, iris; R, retina; V, vitreous body. Photographs are representative of similar sections stained for minimum 5 rats/group. Scale bars, 20 µm.

**FIGURE 6.** Regional lymph node biodistribution of rhodamine-conjugated liposomes containing VIP (VIP-Rh-Lip) in EIU rats, observed by confocal microscopy. VIP-Rh-Lip (red) is located within subcapsular sinus macrophages stained with ED3⁺ (green) 24 hours after intravitreal injection. (A) After intravitreal injection. (B) After intravitreal injection. (C) and (D) are enlarged images of the area selected in (A). VIP colocalized with rhodamine-conjugated liposomes (red) within resident macrophages appears as yellow granules (C). (D) is an enlargement of the area selected in (C) showing the presence of VIP within Rh-Lip. (E) Enlargement of inset in (C) depicting the presence of small round cells expressing VIP (green) in proximity to large macrophages containing VIP-Rh-Lip. VIP-Rh-Lip was still detectable in subcapsular macrophages 14 days after intravitreal injection (F). VIP appears green, Rh-Lip appears red, and colocalization is indicated in yellow. Scale bars: (A–C, F) 20 µm.
strains, administration route, dose, and, above all, formulation of VIP injected may differentially affect the outcome of EIU. Furthermore, liposomal encapsulation of VIP leads to a reduced amount of free VIP in direct contact with the ocular tissues. Katayama et al. reported that intravitreal injection of large amounts of VIP (5 nmol or more) directly in contact with ocular tissues induced inflammatory reaction and ocular toxicity in normal rabbits. In the present study, we did not observe such effects, probably because the retention of high doses of VIP (15 nmol/eye) in liposomes and the slow release rate of VIP prevented toxic concentration in direct contact with ocular tissues.

A twofold reduction of EIU clinical score was observed when intravitreal injection of VIP encapsulated in liposomes (score, 1.5 ± 0.21) was performed, whereas saline/VIP was inefficient (score, 3.1 ± 0.21; similar to control score). This result can be explained by the high concentration of 28-aa VIP detected in pooled aqueous humor and vitreous body 24 hours after VIP-Rh-Lip injection. Indeed, VIP concentrations were 15 times higher in ocular fluids after VIP-Rh-Lip injection than after saline/VIP injection.

As shown by immunostaining, VIP could not be detected in saline/VIP-injected eye sections, whereas colocalization between Rh-liposomes and VIP was visible in ocular tissues and fluids of animals injected with VIP-Rh-Lip. Colocalization of VIP and liposomes was still observed after 2 weeks, indicating that liposomes remained stable during the experiments and that VIP was still encapsulated in liposomes and was progressively released from the formulation, as previously described for several drugs.

The stability of the vesicles in biological media can be attributed to CHOL, which reduces liposome fluidity after VIP-Rh-Lip injection. Indeed, VIP concentrations were 15 times higher in ocular fluids after VIP-Rh-Lip injection than after saline/VIP injection.

To detect a possible effect of intraocular injection of VIP-Lip on the systemic immune system, the fate of liposomes was also studied in draining LNs. Indeed, liposomes were observed within ED3+ subcapsular sinus macrophages in cervical LNs as early as 2 hours after intravitreal injection (data not shown), indicating that through local intravitreal injection, liposomes are able to reach draining LNs and induce a subsequent systemic effect on the immune response. We showed recently that intravitreal injection of tamoxifen-loaded pegylated nanoparticles inhibited EAU with a reduced systemic Th1-type response resulting from a form of immune deviation.

In the present study, detection of fluorescent liposomes in the conjunctiva, the sclera, and the cervical LNs suggests that VIP-Rh-Lip reaches the lymph through the conjunctival lymphatics and enters the LNs through the lymph, probably in a non-cell-associated form, as previously described after anterior chamber injection of soluble antigen. VIP-Rh-Lip was detectable up to at least 14 days, showing that liposomes internalized within subcapsular sinus macrophages are not degraded or are only slowly degraded. Similar to what occurs in the eye, the presence of free VIP in resident macrophages ED3+ suggests that these cells participate in the retention or the slow release of VIP in the LN. Free VIP not associated with Rh-Lip was also detected in small, round cells in the T-cell area and the germinal center of the LN. Although the nature of these cells was not determined in this study, their morphology and localization suggest that they are T and B lymphocytes, respectively. It is tempting to propose that the release of free VIP from macrophages that have internalized VIP-Rh-Lip will stimulate the production of VIP by T and B lymphocytes in the LNs up to 14 days after intravitreal injection. Our results sug-
gest that a local injection of VIP-Rh-Lip could have a systemic and persistent regulatory effect on immune cell populations involved in the development and severity of EAU, a T-cell-dependent model of ocular inflammation.

In conclusion, in the present study, we define a sustained drug release system able to maintain a stable and therapeutic VIP concentration within the eye during EIU. This work brings a proof of concept for such potential therapies in uveitis. Furthermore, we show that liposomes injected into the eye reach the cervical LN. This way of delivery may thus potentially act on immune cells in the periphery and could represent an advantage for the treatment of ocular inflammation involving cell-mediated immune responses such as observed in EAU. Encapsulation of VIP in liposomes, allowing its protection and slow release in ocular tissues and LNs, may open new perspectives for uveitis treatment.

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References