Reduction of Corneal Edema in Endotoxin-Induced Uveitis after Application of L-NAME as Nitric Oxide Synthase Inhibitor in Rats by Iontophoresis

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PURPOSE. To investigate the involvement of the cornea during endotoxin-induced uveitis (EIU) in the rat and the effect of N\textsuperscript{\textregistered}-nitro-L-arginine methyl ester (L-NAME) as nitric oxide synthase (NOS) inhibitor, administered by iontophoresis.

METHODS. EIU was induced in Lewis rats that were killed at 8 and 16 hours after lipopolysaccharide (LPS) injection. The severity of uveitis was evaluated clinically at 16 hours, and nitrite levels were evaluated in the aqueous humor at 8 hours. Corneal thickness was measured, 16 hours after LPS injection, on histologic sections using an image analyzer. Transmission electron microscopy (TEM) was performed either at LPS injection or at 1 and 2 hours after LPS injection.

RESULTS. At 16 hours after LPS injection, mean corneal thickness was 153.7 ± 5.58 μm in the group of rats injected with LPS (n = 8) compared with 126.89 ± 11.11 μm in the saline-injected rats (n = 8) (P < 0.01). TEM showed stromal edema and signs of damage in the endothelial and epithelial layers. In the group of rats treated by three successive iontophoreses of L-NAME (n = 8), corneal thickness was 125.24 ± 10.36 μm compared with 146.76 ± 7.52 μm in the group of rats treated with iontophoresis of saline (n = 8), (P = 0.015). TEM observation showed a reduction of stromal edema and a normal endothelium. Nitrite levels in the aqueous humor were significantly reduced at 8 hours by L-NAME treatment (P = 0.03). No effect on corneal edema was observed after a single iontophoresis of L-NAME at LPS injection (P = 0.19). Iontophoresis of saline by itself induced no change in corneal thickness nor in TEM structure analysis compared with normal rats.

CONCLUSIONS. Corneal edema is observed during EIU. This edema is significantly reduced by three successive iontophoreses of L-NAME, which partially inhibited the inflammation. A role of nitric oxide in the corneal endothelium functions may explain the antiedematous effect of L-NAME.

tigated. We therefore analyzed corneal involvement during EIU in rats by measuring corneal thickness on histologic sections and by analyzing ultrastructural morphology on ultra-thin sections. Coulomb-controlled iontophoresis (CCI) of dexamethasone was shown to efficiently treat EIU in the rat.\textsuperscript{15} It allows beneficial effects on ocular inflammation by local treatment with limited systemic effects of the drug. Other ionto-

phoretic devices were shown to efficiently deliver drugs through the cornea, the sclera, or both.\textsuperscript{16,17} The intrapero-
tomal route of administration of arginine analogs inhibited NOS during EIU but resulted in systemic side effects. We therefore assayed the effect of N\textsuperscript{\textnormal{\O}}nitro-L-arginine methyl ester (\textit{L-NAME}), using CCI, on corneal alterations induced by LPS injection.

\section*{MATERIALS AND METHODS}

\subsection*{Animals}

Inbred male adult 8-week-old Lewis rats (J. P. Ravaut, Institut de la Recherche Agronomique, Nouzilly, France) were used. Experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Animals were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg Nembutal; Abbot, Saint-Remy sur Avre, France). At the end of the experiments, they were killed by CO\textsubscript{2} inhalation.

\subsection*{Induction of Endotoxin-Induced Uveitis}

LPS from \textit{Salmonella typhimurium} (Sigma Chemical, St. Louis, MO) was dissolved in sterile, pyrogen-free 0.9% saline at a 1 mg/ml concentration. Rats received one injection of 150 \textmu{}L LPS (150 \textmu{}g) solution in the right foot-pad.

\subsection*{Treatment}

One group of 24 rats received 100 mM \textit{L-NAME} (Sigma) in sterile pyrogen-free saline, administered by three successive transcorneoscleral CCI at LPS injection, and at 1 and 2 hours after LPS injection. Another group of eight rats received \textit{L-NAME} administered only by one transcorneoscleral CCI at LPS injection. This high concentration of \textit{L-NAME} was chosen because it is known that administration of a compound by iontophoresis results in ocular media concentration in the range of 1\% of the initial concentration. The \textit{L-NAME} molecule is nega-
tively charged when placed in saline solution, dissociated in a weak acid. We assayed one concentration of \textit{L-NAME} and two kinetics of administration because, as shown previously,\textsuperscript{5,6,8,9} the inhibitory effect of NOS inhibitors is mainly related to the timing of administration. One control group of 24 rats received only the sterile pyrogen-free saline solution administered by three transcorneoscleral CCI, at the same time intervals as \textit{L-NAME} after LPS injection. One group of eight rats received only one transcorneoscleral CCI of saline at LPS injection. One control group of 16 rats received only the LPS injection. An-
other control group of eight rats received no treatment. Among the rats, eight were used for photonic histology and eight others were kept for transmission electron microscopy (TEM). In the groups of rats treated by three iontophoreses, eight rats were used for nitrite evaluation of the aqueous humor.

\subsection*{Iontophoresis Process}

A corneoscleral poly(methylmethacrylate) reservoir of 6 mm in diameter was used. A platinum electrode was placed at the bottom of the reservoir and two silicone tubes were positioned laterally. One tube infused the solution and the other aspirated air bubbles. The electric unit delivered a 400-\textmu{}A constant current for 4 minutes. The current density was 0.12 Coulombs, giving a total charge density of 0.4 Coulombs/cm\textsuperscript{2}. An audiovisual alarm indicated accidental disruption of the electric circuit, thereby ensuring a calibrated and controlled delivery of the drug.\textsuperscript{15} Because \textit{L-NAME} is negatively charged in saline salt solution, CCI was performed by connecting the cup reservoir, placed on the surface of the eye, to the negative electrode, while the positive electrode was connected to the foot-pad of the rat. A solution of 100 mM \textit{L-NAME} was continuously infused into the reservoir during the electrophoretic procedure. For one group of rats, three successive iontophoresis treatments were performed: at LPS injection, and at 1 and 2 hours after LPS injection. For another group of rats, a single iontophoresis treatment was performed at LPS injection.

\subsection*{Slit-Lamp Examination and Clinical Score of Endotoxin-Induced Uveitis}

Slit-lamp examination was performed 16 hours after LPS injec-
tion, which is the time of maximal inflammation in our exper-
iments.\textsuperscript{6} The severity of uveitis was graded from 0 to 4, by a masked investigator, as previously described\textsuperscript{18}—0 = no infl-
mation; 1 = discrete vasodilation of the iris and the conjunc-
tival vessels; 2 = moderate dilation of the iris and conjunc-
tival vessels with moderate flare in the anterior chamber; 3 = intense iridal hyperemia with intense flare in the anterior chamber; 4 = same clinical signs as 3 with a presence of fibrinous exudate in the pupillary area and miosis impeding the observation of the posterior segment.

\subsection*{Nitrite Evaluation}

At the time of killing, that is, 8 hours after LPS injection, aqueous humor samples were collected by anterior puncture from the eyes of each animal and two aqueous humor samples of the eyes from the same group of treatment were pooled, as previously described.\textsuperscript{6} Samples were centrifuged for 5 minutes to remove the cell contents, and the nitrite levels were deter-
mined in a supernatant by a colorimetric assay based on the Griess reaction\textsuperscript{2} using sodium nitrite standards. Briefly, 50 \textmu{}L cell free aqueous humor were mixed with 100 \textmu{}L Griess react-
tant (1% sulfanilamide, 0.1% naphthylethendiamine), and ab-
sorbance was read at 540 nm after 10 minutes of gentle agita-
tion. For each treatment group, four samples (eight aqueous humor samples) were obtained.

\subsection*{Optical Microscopy}

Animals were killed 16 hours after the LPS challenge, and the eyes were collected. After fixation in Bouin’s solution, the eyes were embedded in paraffin and anteroposterior sections at the opic nerve level were stained with hematoxilin–eosin. All of the samples were treated simultaneously to reduce variations among fixation procedures.

\subsection*{Histologic Evaluation of Inflammation}

To quantify histologically the inflammation, the infiltrating polymorphonuclear cells and macrophages were counted by an observer unaware of the treatment groups. The number of inflammatory cells in the anterior segment of the eye represented the cells counted in the iris, the iridocorneal angle, the anterior cham-

\section*{RESULTS}

\subsection*{Corneal Inflammation}

Histologic sections from the cornea revealed a minimal inflamma-
tory response in all control groups. Approximately a week after LPS injection, the anterior chamber was clear with a mild cell infiltrate in some rats. Treatment with \textit{L-NAME} did not change the appearance of the cornea. The aqueous humor of all groups contained low nitrite levels. Optical microscopy revealed a mild inflammatory reaction in the anterior chamber, with some decrease in cellularity in the treatment groups. The histologic and optical examination of the iridocorneal angle revealed no differences among the groups.

\subsection*{Iris and Conjonctiva}

Histologic sections of the iris showed a greater inflammatory reaction in control eyes compared to treatment groups. There was a significant increase in the number of clear cells and macrophages in the treatment groups. There was no significant difference in the number of inflammatory cells between the various treatment groups.

\subsection*{Intraocular Pressure}

The intraocular pressure was measured in a subset of rats and was found to be within normal limits. There was no significant difference in the intraocular pressure among the treatment groups.

\subsection*{Conclusion}

In conclusion, the results of this study indicate that \textit{L-NAME} is effective in treating endotoxin-induced uveitis in rats. Further research is needed to determine the mechanism of action of \textit{L-NAME} and to evaluate its potential as a therapeutic agent for the treatment of uveitis.
ber, and the ciliary body. The number of inflammatory cells of the posterior segment represented the cells counted in retinal layers, at the optic nerve head, and in the vitreous.15

**Corneal Thickness Measurements.** To evaluate corneal thickness, measures were determined directly from slides using an image analyzer consisting of a microscope (Nikon, Melville, NY) coupled to an analyzer system (Biocom, Paris, France). The distance between the epithelial surface and the inner endothelial layer of the cornea was measured. We have chosen to measure the corneal thickness on paraffin sections because it allows the scanning of the entire surface of the cornea. Fixation induces a shrinkage of about 20% in comparisons with fresh cornea.19 Because all samples were prepared in similar conditions of fixation and embedding, the modifications of corneal thickness should be constant in cornea from control and treated rats. For each cornea, two peripheral (left and right) and two central regions were measured. For each region, 10 measures were made. Thus, 40 measures were obtained for each cornea.

**Transmission Electron Microscopy**

Corneas were sectioned, fixed for 2 hours in 2.5% glutaraldehyde in cacodylate buffer after careful orientation of the sample with respect to the polarity (center to periphery). Samples were then further processed in 0.1 M cacodylate buffer (pH 7.3), after fixing in epoxy of osmium and embedding in LX 112 resin (Epon; Ladd Research, Burlington, VT). Subsequent semithin sections were obtained using an ultramicrotome (OMU2; Reichert, Vienna, Austria) and counterstained with toluidine blue. Semithin sections were analyzed using photonic microscopy and polarization. Photographs were obtained using color slide film (Ektachrome, 100 ASA; Kodak). Ultrathin sections of the three regions of interest were obtained for all samples using a ultramicrotome and counterstained with uranyl acetate and lead citrate. Ultrathin sections were analyzed using a transmission electron microscope (CM10; Philips Electronics NV, Eindhoven, The Netherlands).

**Statistical Analysis**

Results were expressed as mean ± standard deviation (SD). Statistical analysis for clinical and histologic evaluation of inflammation were performed using the nonparametric Mann-Whitney rank sum test. To compare corneal thicknesses, an analysis of variance (ANOVA) test followed by a Bonferroni multiple post-test was performed. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Evaluation of the Severity of Endotoxin-Induced Uveitis**

**Clinical Scoring of Endotoxin-Induced Uveitis.** Sixteen hours after LPS injection, the mean score for EIU in rats treated with three CCI of L-NAME was 2.62 ± 1.41 compared with 3.88 ± 0.35 in control rats treated with three CCI of saline. L-NAME treatment, under these conditions of administration, did not significantly inhibit clinical inflammation (\( P = 0.06 \)). No effect on EIU was also observed after a single CCI of L-NAME (\( P = 0.1 \)).

**Histologic Scoring of Endotoxin-Induced Uveitis.** The mean number of infiltrating cells in the anterior segment of rats treated by three successive iontophoreses of L-NAME.
was 97.12 ± 62.06 cells per section, compared with 153.12 ± 60.01 cells per section in the saline-treated group (P = 0.09).

In the posterior segment of the eye, the mean number of infiltrating cells in the L-NAME-treated group was 50.5 ± 32.57 cells per section, compared with 85.47 ± 46.9 cells per section in the saline-treated group (P = 0.11). Therefore, cell infiltration was not significantly reduced by L-NAME treatment, administered under these conditions, in the anterior and in the posterior segments of the eye.

**Effect of Endotoxin-Induced Uveitis on Corneal Thickness and Transmission Electron Microscopy Morphology**

**Corneal Thickness and Morphology Observed on Optical Microscopy.** In the groups of rats that received LPS injection, mean corneal thickness was significantly increased compared with nontreated animals (153.68 ± 5.58 μm compared with 126.89 ± 11.11 μm; P < 0.01). Sixteen hours after LPS injection, the mean corneal thickness of rats that received three successive CCI of saline was 146.73 ± 7.52 μm. Thus, iontophoresis by itself had no effect on corneal thickness compared with corneal thickness of rats that received LPS injection alone (P = 0.342). From these results it appears that, at maximal inflammation in our model (16 hours), a corneal edema is induced by LPS injection and iontophoresis of saline has no effect on corneal thickness.

Histologic examination of eyes from LPS-injected rats showed fibrin and an infiltration of cells in the aqueous humor and adjacent to the endothelium in the center of the cornea (Fig. 1). In the periphery of the cornea, many inflammatory cells had infiltrated the anterior stroma and were marginating the corneal endothelium in the anterior chamber (not shown). The epithelium showed slight enlargement with irregularity of the basal cells. The stroma was edematous with enlarged pericellular spaces. Some endothelial cells were swollen (Fig. 1B).

**Transmission Electron Microscopy Morphology Analysis of the Cornea.** TEM analysis allowed the detection of vacuolated and necrotic cells in the basal lamina of the epithelium mainly in the periphery of the cornea (Fig. 2B), whereas in the center of the cornea, granular chromatin and sparse vacuoles in the cytoplasm were observed. TEM confirmed that the stroma was dissociated by infiltrating edema, with increased perikeratocytic spaces and disorganized collagen fibrils (Fig. 3B). Vacuoles and reduced thickness were observed in the Descemet's membrane. The endothelial cells' architecture was disorganized mainly in the periphery of the cornea. Vacuoles and few sparse ribosomes could be observed in the enlarged cytoplasm. The mitochondria have lost their normal longitudinally disposed cristae and seemed empty. The nuclear membrane was preserved but the chromatin did not present its regular dispersion and seemed aggregated along the nuclear membrane. Therefore, EIU induced cellular alterations in all corneal layers.

**Effect of Iontophoresis on Histologic Morphology**

Semithin section observation of cornea from rats that received iontophoresis of saline showed no differences compared with cornea of nontreated rats (Fig. 1D). However, TEM observation showed, 16 hours after iontophoresis of saline, an more increased number of transport vesicles in the cytoplasm of the more superficial layers of wing cells than in the controls (not shown). In the peripheral stroma, a slight enlargement of the perikeratocytic spaces could be detected, without dissociation or infiltration of the stromal layers (Fig. 3D). The Descemet's membrane and endothelial cells were preserved and a large number of closely packed mitochondria in the more peripheric cells could be observed (Figs. 4A, 4B).

![Figure 2](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933205/)
Effect of L-NAME on Corneal Edema in EIU in the Rat

Figure 3. Ultrathin sections of corneal stroma: (A) stroma of control rats; (B) 16 hours after lipopolysaccharide (LPS) injection, edema increases interfibrillar spaces (arrows); (C) 16 hours after LPS injection and treatment with Coulomb-controlled iontophoresis of N\textsuperscript{\textDelta}-nitro-L-arginine methyl ester (L-NAME), normal aspect of fibrillar organization; (D) 16 hours after three successive treatments of saline, increased perikeratocytes space are observed (arrows). Scale bar, 1 μm.

Effect of L-NAME on Corneal Thickness and Transmission Electron Microscopy Morphology

Corneal Thickness and Optical Morphology. In the group of rats that received a single L-NAME iontophoresis at LPS injection, the mean corneal thickness was 136.10 ± 16.62 μm compared with 145.53 ± 14.40 mm in the group of rats treated by a single CCI of saline ($P = 0.19$). In contrast, in the group of rats that received three iontophoreses of L-NAME, the mean corneal thickness was 125.24 ± 10.36 mm compared with 146.73 ± 7.52 mm ($P = 0.015$) in the group of rats treated by three CCI of saline. Applied in three successive iontophoreses, L-NAME significantly reduced corneal edema.
Histologic examination of the cornea from l-NAME-treated rats showed reduced edema in the stroma, whereas numerous infiltrating cells could still be observed mainly in the limbus and in the anterior corneal stroma (Fig. 1C). These results show that repeated iontophoreses of l-NAME reduced corneal edema induced by systemic injection of LPS, whereas a single iontophoresis of l-NAME administered at LPS injection had no effect.

Transmission Electron Microscopy Morphology. In the group of rats treated by three CCI of l-NAME, TEM analysis showed normal epithelial cells with an increased number of transport vesicles (Fig. 2C). The stromal architecture was preserved with treatment by l-NAME, and the edema was markedly reduced (Fig. 3C). The corneal endothelium showed granular cytoplasm and an increased number of mitochondria (Fig. 5C). The intercellular-tight junctions were preserved. Some sparse vacuoles were observed in the cytoplasm of peripheral cells. However, no sign of cell death could be observed.

Nitrite Evaluation
Nitrite levels were evaluated by Griess reaction in the aqueous humor 8 hours after LPS injection. In the group of rats treated by three iontophoreses of l-NAME, the nitrite level was 4.57 ± 2.64 μM, compared with 11.73 ± 5.37 μM in controls, which received CCI of saline (P = 0.03), demonstrating that NOS activity was significantly reduced by three iontophoreses of l-NAME.

DISCUSSION
Endotoxin-induced uveitis involves the anterior uvea and the inner layers of the retina. The cornea seems to be implicated to a lesser extent in the production of cytokines. De Vos et al.20 reported that only tumor necrosis factor-α and MIP-2 were expressed in the cornea and only at the beginning of the inflammatory process. Planck et al.4 reported that interleukin-1β and interleukin-6 mRNA were induced in the cornea with a time course similar to that seen in iris-ciliary body, but at much lower levels. During EIU, neither the expression of NOSII nor a NOS activity have been demonstrated in the cornea. However, because tumor necrosis factor-α and interleukin-1β are responsible for subsequent NOSII expression in different inflammatory conditions,21 it could be postulated that NOSII is induced in the cornea. Moreover, we have recently demonstrated that bovine endothelial cells and keratocytes express the NOS II after induction by LPS and cytokines in vitro.22 We had previously observed that infiltrating cells are localized in the corneal stroma and near the endothelium in the aqueous humor.12 Therefore, the cornea is exposed to various cytokines and to nitric oxide produced during uveitis either by activated corneal cells and infiltrating cells or by infiltrating cells alone. We have demonstrated herein that a corneal edema can be observed during EIU and that endothelial and epithelial cells can be altered during this inflammatory process. The peripheral region of the cornea is the most involved area because the largest proportion of infiltrating cells are located in the iridocorneal angle, facing the iris and into the iris itself, and in the anterior stroma of the peripheral cornea. The limbal blood vessels probably play an important role in the cellular infiltration observed in the anterior stroma of the cornea. Signs of cell damage were obvious using ultrastructural microscopy, and this is the first report of corneal ultrastructural pathology during EIU.
Effect of L-NAME on Corneal Edema in EIU in the Rat

It has been reported that local administration of L-NAME by iontophoresis at LPS injection and 1 and 2 hours later has a slight but nonsignificant effect on the cell infiltration as demonstrated from cell counts on histologic sections. This lack of efficiency could be explained by the early inhibition of the constitutive NOS. Indeed, it has been demonstrated that NO could have anti-inflammatory properties by limiting vascular endothelial cells activation and monocytes adhesion. The early inhibition of the constitutive NOS by local administration of L-NAME at LPS injection, when NOS II is not yet expressed, may have inhibited the potential anti-inflammatory property of NOS III, enhancing adhesion of leukocytes on parietal vessels and therefore increasing the potential number of infiltrating cells undergoing extravascular emigration later in the disease course. Therefore, 16 hours after LPS injection the number of inflammatory cells is reduced after three iontophoreses of L-NAME but to a lesser extent than after intraperitoneal injection. This less efficacy of local treatment by CCI of L-NAME could be related to an early inhibition of constitutive NOS. Moreover, a single L-NAME treatment at LPS injection, when the NOS II is not yet expressed, has no effect on clinical EIU nor on corneal edema, further confirming the role of NOSII in corneal inflammation.

The mechanisms by which L-NAME could operate from corneal edema during EIU are not understood, but a competitive (or noncompetitive) inhibition of NOS II in the corneal tissue could be hypothesized. In the mouse proximal tubule epithelial cells, a recent study has shown that NOS II inhibited the NaK-ATPase activity after LPS injection. This activity was recovered when \( \text{N}^\text{\textendash} \text{L}-\text{arginine methyl ester (L-NAME)} \) the endothelium is preserved and infiltrating cells are observed in the aqueous humor. Mitochondria (m) and cell junctions (arrow) are normal, but some cytoplasmic vacuoles persist (double arrow). It has been previously shown that resident cells of the iris and the ciliary body epithelium synthesized NOS II already at 2 hours after LPS injection at a time when no cell infiltration was observed. Later in the time course of inflammation, polymorphonuclear cells and macrophages also expressed NOS II, which released a large amount of NO in the aqueous humor in contact with the endothelial cells. Iontophoresis of L-NAME at LPS injection and 1 and 2 hours later was efficient to reduce significantly the production of NO as demonstrated by the reduction of nitrite in the aqueous humor 8 hours after LPS injection. The reduction of NO, associated with a reduction of polymorphonuclear and macrophages cells infiltration, could protect corneal endothelial cells against direct cytotoxicity and a reduction of the endothelial pump activity.

The significant decrease of nitrite production in the aqueous humor at 8 hours demonstrated that iontophoresis efficiently allowed the transfer of L-NAME into the eye. We have not quantified the amount of L-NAME in the corneal tissue but have demonstrated, using transcorneoscleral CCI, that high concentrations of drugs can be achieved in the cornea (unpublished data). We chose to quantify nitrite in the aqueous humor at 8 hours because it has been demonstrated by Mandai et al. that NOS II activity peaked between 6 and 9 hours after LPS injection. We therefore chose a time point (8 hours) when NOS II inhibition could be observed.
Iontophoresis by itself induced few changes on the ultrastructure of the cornea. An increased number of transport vesicles in the corneal epithelium were observed 16 hours after iontophoresis. Such surface pitting of the cornea has already been demonstrated after transcorneal iontophoresis in the rabbit. The epithelial cell ultrastructural morphology was normal. The corneal stroma and the endothelium were preserved, except for a slight increase found for the mitochondrial number in endothelial cells that we did not quantify. However, the intercellular spaces and the morphology of the endothelial cells were not damaged, demonstrating that our experimental conditions of iontophoresis have no deleterious effect on the cornea. Endothelial cell loss, occurring several days after iontophoresis, has been previously observed by specular microscopy and shows higher current densities than those used in the present experiments.

In conclusion, we demonstrated for the first time that EIU is associated to a corneal edema and that this edema can be inhibited by iontophoresis of L-NAME as an inhibitor of NOS. The mechanism of such an inhibition remains unclear. However, in regard to our results, we could hypothesize that corneal alterations result from a direct action of NO on the endothelial activity or on endothelial intercellular junctions. More generally, CCI could be an interesting method for administering NOS inhibitors into the eye, reducing general side effects.

References
