Effects of Cloricromene, a Coumarin Derivative, on Endotoxin-Induced Uveitis in Lewis Rats

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PURPOSE. To investigate the effects of cloricromene, a coumarin derivative, on rats subjected to endotoxin-induced uveitis (EIU).

METHODS. Endotoxin uveitis was induced in male Lewis rats by a single footpad injection of 200 μg lipopolysaccharide (LPS). Cloricromene was topically applied to the rat eye twice at 1 hour before and 7 hours after injection of LPS. A separate group of animals was treated with vehicle. Rats were killed 16 hours after injection and the eyes enucleated for histologic examination and immunohistochemical analysis. The effect of treatment was also evaluated by slit lamp examination, by the number of intraocular inflammatory cells on histologic sections, and by measuring the protein and TNFα levels in the aqueous humor. Nitrite and nitrate production was also measured in the aqueous humor.

RESULTS. The histopathology of the iris-ciliary body included inflammatory cell infiltration and nuclear modification of vessel endothelial cells. Cloricromene treatment reduced the inflammatory cell infiltration and improved histologic status of the ocular tissue. Immunohistochemical analysis for P-selectin, intracellular adhesion molecule (ICAM)-1, nitroytrosine, and poly(ADP-ribose) synthetase (PARS) revealed a positive staining in inflammatory cell infiltration from LPS-treated rats. The degree of staining for P-selectin, ICAM-1, nitroytrosine, and PARS was markedly reduced in tissue sections obtained from LPS-recipient rats that had received cloricromene. Cloricromene strongly inhibited cell infiltration, protein exudation, TNFα production, and nitrite-nitrate formation.

CONCLUSIONS. This study provides the first evidence that cloricromene, a coumarin derivative, attenuates the degree of inflammation and tissue damage associated with EIU in rats. (Invest Ophthalmol Vis Sci. 2003;44:1178–1184) DOI: 10.1167/iovs.02-0559

Acute anterior uveitis is an inflammatory disorder that involves the iris and parts of the ciliary body. Anterior uveitis with marked cellular infiltration is often associated with Behçet’s disease, ankylosing spondylitis, Reiter’s syndrome, and HLA B27-associated uveitis in patients who have acute anterior uveitis report photophobia. Other symptoms may include redness, tearing, and reduced vision. Findings on examination are characteristic and include ciliary injection, keratic precipitates, cells, and protein flare in the aqueous and miosis with posterior synechiae. Although corticosteroids remain the mainstay of therapy for uveitis some patients are resistant to steroids. Furthermore, corticosteroids have a wide range of significant side effects, such as cataract, increase of intraocular pressure, increased susceptibility to microbial infection. The cascade of biological events leading to endotoxin-induced uveitis (EIU) initiated by lipopolysaccharide (LPS) injection is not clearly understood. Adhesion molecules and cytokines play a crucial role in the pathogenesis of uveitis. ICAM-1, P-selectin, TNFα, IL-1, and IL-6 are involved in the inflammatory process that occurs in uveitis.1–5 Cellular infiltration involves sequential steps of rolling, adhesion, and migration of leukocytes. The selectin family is a group of molecules involved in the first rolling phase, and their function precedes the roles of the other cell-adhesion molecules, such as ICAM-1 and leukocyte integrins. P-selectin is expressed in the vessels of the rat iris after LPS induction of EIU.1 An increased expression of ICAM-1 occurs in the iris of patients with uveitis, indicating an immunoregulatory function for adhesion molecules in the pathogenesis of uveitis.6 Expression of ICAM-1 has also been observed on the vascular endothelium of the rat ciliary body during EIU.7–8 Cytokines have been implicated as important mediators in the pathogenesis of EIU in the rat. Injection of TNFα, IL-1, IL-6, IL-8, IL-13, or IFN-γ into rat eyes causes acute uveitis that resembles the response to LPS.9–12 Moreover, increased levels of TNFα and other cytokines are found in the aqueous humor after injection of LPS.13 The inducible nitric oxide synthase-nitric oxide (iNOS-NO) pathway is also involved in the pathogenesis of EIU in rats. Epithelial cells of the iris-ciliary body and cells infiltrating the anterior segment of the eye are the major source of NO13–16. Evidence is accumulating that much of the NO-related injury may be due to the generation of peroxynitrite.17 NO and peroxynitrite cause apoptosis in a variety of cell types through the activation of nuclear enzyme poly(ADP-ribose) synthetase (PARS).18,19

The purpose of the present study was to investigate the effects of cloricromene (AD6) on EIU induced by injection of LPS in the rat. We investigated the effects of AD6 on the iris-ciliary body (histology), cellular infiltration, protein extravasation, cytokine production, formation of adhesion molecules, and nitroytrosine and PARS activity caused by LPS. In addition, we investigated the effects of AD6 on nitrite-nitrate production.

METHODS

Animals

Male Lewis rats weighing 160 to 180 g were obtained from Charles River (Calco, Italy). All the animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Drugs and Chemicals

AD6 (8-monochloro-3-β-diethylaminoethyl-4-methyl-7-ethoxy-carbonyl-methoxy coumarin; cloricromene) was obtained from Fidia Laboratories (Abano Terme, Italy). Primary anti-P-selectin, anti-ICAM-1, anti-nitroytrosine, and anti-poly(ADP-ribose) synthetase antibodies were...
from DBA (Milan, Italy). All other reagents were purchased from Sigma Chemical Co. (Milan, Italy).

AD6 ocular tolerability was evaluated, and no signs of ocular inflammation or discomfort were observed (data not shown).

Induction of EIU, Protein, and TNFα Detection

LPS (Salmonella minnesota) was dissolved in sterile, pyrogen-free saline. Uveitis was induced in rats by injection of 200 μL of LPS into the foot. An ointment (40 μg) containing 1% AD6 was topically applied to both eyes twice, 1 hour before and 7 hours after injection. A control group received injection of LPS and a topical ointment containing only the excipients (vehicle), 1 hour before and 7 hours after injection of LPS. Sixteen hours after the induction of uveitis, the eyes were examined by slit lamp by an observer unaware of the treatment. Uveitis in each eye was graded according to a previously reported scoring system20, 0, no inflammatory reaction; 1, discrete dilatation of the iris and conjunctival vessels; 2, moderate dilatation of the iris and conjunctival vessels; 3, intense iridal hyperemia with flare in anterior chamber; and 4, same clinical signs as 3, plus the presence of fibrinous exudate in the pupillary area with intense flare in the anterior chamber. No signs of uveitis were observed in the animals at the beginning of each experiment. Immediately after the slit lamp examination, the rats were killed, and the aqueous humor and iris-ciliary body (see Myeloperoxidase Measurement section) of both eyes were collected. Aqueous humor samples were pooled, and the protein concentration was determined by the Bradford assay. In a separate set of animals treated in the same way as just described the TNFα level was evaluated in the aqueous humor at 16 hours after injection of LPS. The assay was performed with commercial ELISA kit (Calbiochem-Novabiochem Corp., La Jolla, CA).

Histologic Examination

A separate set of rats, treated in the same way as described earlier, were used for the histologic study. For microscopic histologic evaluation, rat eyes were collected and fixed in a solution 0.1% glutaraldehyde (25%) and 4% paraformaldehyde in phosphate buffer. Indirect immunofluorescence staining was performed on 7-μm-thick sections. After deparaffinization, the sections were hydrated (three times for 15 minutes in PBS) permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 minutes. Non-specific adsorption was minimized by incubating the sections in 2% normal goat serum in phosphate-buffered saline for 20 minutes. An ointment containing 1% AD6 was topically applied to both eyes twice, 1 hour before and 7 hours after injection of LPS. Three groups of animals (sham-treated, control, and AD6 groups) were used for the histologic study. Iris-ciliary bodies were collected and studied by light microscopy (Dialux 22; Leitz, Wetzlar, Germany).

Immunohistochemical Localization of P-Selectin, ICAM-1, Nitrotyrosine, and PARS

Three groups of animals (sham-treated, control, and AD6 groups) were used for the immunohistochemical study. Iris-ciliary bodies were fixed in an immunofixation solution of 0.1% glutaraldehyde (25%) and 4% paraformaldehyde in phosphate buffer. Indirect immunofluorescence staining was performed on 7-μm-thick sections. After deparaffinization, the sections were hydrated (three times for 15 minutes in PBS) permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 20 minutes. Non-specific adsorption was minimized by incubating the sections in 2% normal goat serum in phosphate-buffered saline for 20 minutes. Sections were incubated overnight with (1) a rabbit anti-human polyclonal antibody directed at P-selectin21,22 (CD62P), which reacts with rat and mouse anti-rat antibody directed at ICAM-121-23 (CD54); 1:500 in PBS vol/vol; DBA) and (2) an anti-nitrotyrosine rabbit polyclonal antibody21-23 (1:500 in PBS vol/vol; DBA) or with anti-poly(ADP-ribose) goat polyclonal rabbit antibody21-23 (1:500 in PBS vol/vol). Sections were washed with PBS, and incubated with the secondary antibody (tetramethylrhodamine isothiocyanate [TRITC]–conjugated anti-rabbit and FITC–conjugated anti-mouse; Jackson ImmunoResearch, West Grove, PA, or TRITC-conjugated anti-goat antibody; 1:100 in PBS vol/vol) for 2 hours at room temperature. Sections were washed as before, mounted with 90% glycerol in PBS, and observed with a confocal microscope (LSN 510; Carl Zeiss, Oberkochen, Germany) equipped with a 40× oil objective. To verify the binding specificity for ICAM-1, PARS, or P-selectin, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). To confirm that the immunoreaction for the nitrotyrosine was specific, some sections were also incubated with the primary antibody (anti-nitrotyrosine) in the presence of excess nitrotyrosine (10 mM) to verify the binding specificity. In these situations, no positive staining was found in the sections (data not shown), indicating that the immunoreaction was positive in all the experiments performed. Micrographs (n = 5) were assessed by densitometry using image-analysis software (Optilab; Grafter, Mirmande; France) software on a computer (CPU G3-266; Macintosh; Apple Computer, Cupertino, CA).

Evaluation of Cellular Infiltration and MPO Measurement

The number of infiltrating cells was counted on histologic sections of the iris-ciliary body. Neutrophils were counted by an investigator unaware of the treatment groups. For each animal, the number of cells represents the mean count in three serial sections. The activity of MPO, a hemoprotein located in azurophilic granules of neutrophils, was used as a biochemical marker for infiltration of neutrophil into tissues. In the present study, MPO was measured as previously described.24 Briefly, 16 hours after injection of LPS, the iris-ciliary bodies were collected and weighed (see Induction of EIU section). Tissue samples were homogenized in a solution containing 0.5% hexa-decyltrimethylammonium bromide dissolved in potassium phosphate buffer (50 mM; pH 6). The homogenate was sonicated for 10 seconds, frozen thawed three times, and centrifuged at 40,000 g for 15 minutes at 4°C. An aliquot of the supernatant was then allowed to react with a solution of o-dianisidine hydrochloride (0.167 mg/mL) and 0.0005% hydrogen peroxide. The rate of change in absorbance was measured spectrophotometrically at 460 nm. Concentrations of MPO are expressed as units per milligram of wet tissue.

Nitrite Detection

In a separate set of animals treated in the same way as described earlier (see Induction of EIU section) the nitrite levels were evaluated. At 16 hours after injection of LPS, aqueous humor samples were collected from the eyes and pooled. Nitrite-nitrate production, an indicator of NO synthesis, was measured by a colorimetric assay. Briefly, the nitrate in the aqueous was first reduced to nitrite by incubation with nitrate reductase (670 μM/mL) and reduced nicotinamide adenine dinucleotide phosphate (NADPH; 160 μM) at room temperature for 3 hours. The nitrite concentration in the sample was then measured by the Griess reaction, by adding 100 μL of Griess reagent (0.1% naphthylethylene diamide dihydrochloride in H2O, 1% sodium nitrite in 5% ethylenediamine dihydrochloride H2O; 1:1) to 50 μL samples and the absorbance was read at 540 nm.

Statistical Analysis

All values are expressed as mean ± SD. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A Mann-Whitney test was used for ordinal variables. P < 0.05 was considered significant. The figures from histologic and immunohistochemical experiments are representative of at least three experiments (five slides for each eye from different animals) performed on different experimental days.

RESULTS

EIU and Clinical Score

The clinical inflammation score, which was determined 16 hours after injection of LPS, was significantly (P < 0.001) lower in the AD6 group than in the control (treated with topical application of the vehicle and injected with LPS). Animals treated with AD6 showed a inflammatory response of lower intensity than the control. Figure 1 shows that uveitis developed in AD6-treated rats with a mean intensity of 1.5 ± 0.5 vs. 3.4 ± 0.5 in the control rats.
Increased amounts of proteins were detected in the aqueous humor 16 hours after injection of LPS. AD6 significantly reduced the leakage of proteins in the anterior segment of the eye (Fig. 2A; \(P < 0.001\)). The levels of TNF-\(\alpha\) in the aqueous humor were significantly lower in AD6-treated rats than in the control group (treated with topical application of the vehicle and injected with LPS; Fig. 2B; \(P < 0.001\)).

### Histopathology of EIU

Histologic evaluation of iris-ciliary body tissues from LPS-injected rats revealed signs of severe uveitis with massive neutrophil infiltration (Figs. 3B, 3D). Epithelial cells from LPS-injected rats showed markedly reduced cytoplasm and scarcely visible nuclei (Fig. 3D, inset). In AD6-treated animals, the severity of uveitis was significantly reduced (Fig. 3C), and the shape of the epithelium (Fig. 3C, inset) was similar to the epithelium of the sham-treated rats (Fig. 3A, inset). The number of inflammatory cells was significantly \((P < 0.001)\) reduced in the group treated with AD6 (Fig. 4A). These data compare well with the measurement of MPO activity in iris-ciliary body samples. Treatment with AD6 significantly reduced the MPO activity elicited by LPS (Fig 4B; \(P < 0.001\)).

### P-Selectin and ICAM-1 Immunostaining

The results of immunohistochemical staining for adhesion molecules correlated with the histologic results and the intensity of the infiltrating cells. Immunohistochemical analysis of iris-ciliary body sections obtained from rats injected with LPS revealed positive staining for P-selectin (Fig. 5B). In contrast, no positive P-selectin staining was found in the iris-ciliary body of AD6-treated rats (Fig. 5C). Eye tissue sections from sham-treated rats (receiving the saline injection and topical application of the vehicle) showed no positive staining for P-selectin (Fig. 5A) and ICAM-1 (Fig. 5D). Tissue sections obtained from rats injected with LPS revealed intense positive staining for ICAM-1 (Fig. 5E). In contrast, no positive ICAM-1 staining was found in the iris-ciliary body samples obtained from AD6-treated rats (Fig. 5F).

### Nitrotyrosine and PARS Immunostaining

Immunohistochemical analysis of iris-ciliary body sections obtained from rats injected with LPS revealed positive staining for nitrotyrosine (Fig. 5K). In contrast, no positive nitrotyrosine staining was found in the iris-ciliary body of AD6-treated rats (Fig. 5L). Immunohistochemical analysis of iris-ciliary body sections obtained from rats injected with LPS also revealed intense positive staining for PARS (Fig. 5N). In contrast, no
specific positive staining for PARS was found in the iris-ciliary body of AD6-treated rats (Fig. 5G). No staining was observed for nitrotyrosine or PARS in the iris-ciliary body obtained from sham-treated rats (Figs. 5J, 5M).

Nitrite Levels

Nitrite levels were evaluated by the Griess reaction in the aqueous humor 16 hours after LPS injection. In the group of rats treated with AD6, the nitrite levels were significantly lower (P < 0.001; n = 10–12 eyes) compared with the control (treated with topical application of the vehicle and injected with LPS) 8.2 ± 3.1 and 19.5 ± 4.7 μM respectively, demonstrating that NOS activity was reduced.

DISCUSSION

In the present study, topical administration of AD6 inhibited ocular inflammation in EIU at clinical and histologic levels. AD6 is a nonanticoagulant coumarin derivative with anti thrombotic, antiplatelet actions and with beneficial effects in various models of ischemia and shock. This report highlights the effect of AD6 on EIU. EIU is an experimental model of acute ocular inflammation characterized by a breakdown of the blood-aqueous barrier. LPS activates a variety of inflammatory signals, and many factors may contribute to the pathogenesis of EIU. Despite this, it has been demonstrated that many factors are involved in the pathogenesis of uveitis, the contribution of many others remains to be elucidated. In this study, we investigated the biological events leading to EIU after injection of LPS and whether AD6, a coumarin derivative, can reverse these events. Histopathologic and immunohistochemical studies showed that the neutrophil represents a key cell in the pathogenesis of EIU. It has been demonstrated that cytokines modulate the adhesion molecule expression and that they are important for selective lymphocyte trafficking into the eye during uveitis.

P-selectin and ICAM-1 are expressed on endothelial cells in response to specific stimuli and both proteins are upregulated by proinflammatory cytokines such as TNFα. Altavilla et al. demonstrated that TNFα induces production of E-selectin in a splanchnic artery occlusion (SAO) shock rat model. They showed that AD6 decreases serum levels of TNFα and E-selectin, reduces leukopenia and MPO activity on the ileum and lung, blunts the reduction of arterial blood pressure, and reduces the damage in the ileum and lung tissues. Recently, the ability of AD6 to inhibit LPS-induced production of TNFα in a dose-dependent manner has been demonstrated, as was previously demonstrated both in vitro in peritoneal macrophages and in vivo in myocardial ischemia-reperfusion injury. A reduction by AD6 of production of free radicals has been reported in vivo after myocardial ischemia and reperfusion. More recently, Zanna and Bevilacqua showed the scavenger effect of AD6 for neutrophil generation of free radicals, which further supports the quencher effect of this drug.

Corsini et al. showed that AD6 inhibits LPS-induced transcription of TNFα and activation of nuclear factor (NF)-κB by interfering with LPS-induced cellular oxidative activity. These results demonstrate that AD6 interferes with the early signal transduction pathway triggered by LPS. The mechanism by which AD6 inhibits activation of NF-κB and subsequent neo synthesis of TNFα could be related to the scavenger effect against ROS. The data obtained on TNFα in the present study are in accordance with findings in these studies. TNFα represents a pivotal mediator in the development of EIU. Topical treatment with AD6 reduced by 2.6-fold (P < 0.001) the levels of TNFα in the aqueous humor of rats with EIU. This result is probably crucial to block the cascade of events that cause the inflammatory response. This hypothesis was strengthened by findings in immunohistochemical analysis. In fact, in our study, no positive staining for P-selectin and ICAM-1 was found in the iris-ciliary body samples obtained from rats treated with AD6. This latter result is extremely important, because the rolling phase of leukocytes and the subsequent tight adherence with endothelial cells are inhibited.

TNFα is implicated in the NO synthesis pathway; in fact, the induction of iNOS is mediated by the release of endogenous TNFα. The iNOS-NO pathway is involved in the pathogenesis of EIU. It has been demonstrated by direct evidence of the expression of iNOS mRNA in the iris-ciliary body that NO is one of the proinflammatory mediators of EIU, which is implicated in the inflammatory cascade of events taking place after injection of LPS. Simultaneous generation of NO and superoxide favors the production of a toxic reaction product such as peroxynitrite anion (ONOO−). Peroxynitrite with lipids cause lipid peroxidation with malondialdehyde and formation of conjugated diene. Furthermore, NO and peroxynitrite cause apoptosis in a variety of cell types.

Peroxynitrite activates the nuclear enzyme PARS, and the role of PARS in the apoptotic process has been demonstrated in several cell types. PARS plays an important role in the regulation of gene expression and cell differentiation, and the activation of PARS may play an important role in inflammation. It has been demonstrated that the inhibition of PARS attenuates the recruitment of neutrophils in rodent models of inflammation. In agreement with these data we demon-
strated a significant ($P < 0.001$) decrease in neutrophils in iris-ciliary body samples obtained from EIU-affected rats topically treated with AD6. This result was confirmed by the significant ($P < 0.001$) decrease in MPO activity observed in iris-ciliary body samples obtained from rats topically treated with AD6. The coumarin derivative AD6 attenuates the degree of chronic inflammation and tissue damage associated with collagen-induced arthritis in the rat. In particular, AD6 inhibits plasma levels of TNFα, IL-1β, nitrite, and malondialdehyde. Furthermore, the degree of staining for iNOS, cyclooxygenase (COX)-2, nitrotyrosine, and PARS was dramatically reduced in inflamed joints from collagen-treated rats that had been treated with AD6. In the present study, we observed that topical treatment with AD6 significantly ($P < 0.001$) re-

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**Figure 5.** Immunohistochemical localization of P-selectin (A–C), ICAM-1 (D–F), nitrotyrosine (J–L), and PARS (M–O) in the iris-ciliary body. Eye tissue sections obtained from sham-treated rats (A, D, J, M), control rats (B, E, K, N) and from AD6-treated rats (C, F, L, O). (G–I, P–R) Transmission light micrographs. Magnification, ×125.
duced the degree of ocular inflammation and tissue damage associated with EIU. Furthermore, we showed that topical treatment with AD6 reduced by 3.6-fold ($P < 0.001$) the levels of protein in the aqueous humor. We demonstrated by immunohistochemical analysis that EIU leads to a significant increase in the degree of nitrotyrosination of proteins in the iris-ciliary body as well as to a substantial increase in PARS activity. In contrast, no positive staining for nitrotyrosine and PARS was found in the iris-ciliary body samples obtained from rats topically treated with AD6.

Increased nitrotyrosine staining is considered as an indication of increased nitrosative stress rather than a specific marker of the generation of peroxynitrite.\textsuperscript{41–42} Reactive oxygen species (ROS) and peroxynitrite produce cellular injury and necrosis through several mechanisms including peroxidation of membrane lipids, protein denaturation, and DNA damage. ROS produce strand breaks in DNA which triggers energy-consuming DNA repair mechanisms and activates the nuclear enzyme PARS, resulting in the depletion of its substrate, nicotinamide adenine dinucleotide (NAD), in vitro and a reduction in the rate of glycolysis. Because NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, its depletion leads to a rapid decline in intracellular adenosine triphosphate (ATP). This process has been termed the “PARS suicide hypothesis.”\textsuperscript{57–60} In the present study we demonstrated, for the first time, that PARS activity increases in the EIU, but the most important fact is that we demonstrated that topical treatment with AD6 can inhibit the increase in PARS activity.

In conclusion, this study demonstrates for the first time that the coumarin derivative AD6 attenuates the uveitis induced by LPS in the rat at different levels. The antiinflammatory effects of AD6 may be dependent on a combination of pharmacologic properties of this drug that could be due to the block of some specific cytokines. First, AD6 exerts antiangiogenic effects by decreasing VEGF, which prevents the expression of INOS and the production of NO and, ultimately, the formation of peroxynitrite. Peroxynitrite induces the activation of PARS through an increase of DNA strand breakage and a decrease of intracellular levels of NAD and ATP. Second, AD6 prevented the activation of PARS, probably because of its effect on TNFa. Third, the block of P-selectin and ICAM-1 by AD6, could be related to the inhibition of TNFa. Although we cannot determine the exact mechanism of action of AD6, it is reasonable to hypothesize that the drug exerts its effects, both in blocking the PARS pathway and blocking the adhesion molecules, through an inhibition of TNFa probably mediated by the inhibition of NF-kB. However, we cannot rule out the possibility that AD6 exerts its pharmacologic effects by means of the other mechanisms that are at the moment unknown. Therefore, further studies are needed to explain fully the mechanism of action of this drug. The results of the present study suggest that AD6 could have an important role in the limitation of ocular inflammation and may be an effective agent in the treatment of human uveitis.

References


