Modulation of immunogenic keratitis in rabbits by topical administration of inhibitors of lipoxygenase and cyclooxygenase

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ABSTRACT

Intrastromal injection with human serum albumin (HSA) in the rabbit cornea induced edema and a ring-shaped leukocyte infiltrate followed by neovascularization.

The effect of topically administered lipoxygenase and cyclooxygenase inhibitors on this inflammatory keratitis was studied.

The lipoxygenase inhibitors Bay 08276 and Rev 5901 and the cyclooxygenase inhibitor suprofen were given as 1% eye drops three times daily during the experiment. In eyes treated with lipoxygenase inhibitors leukocyte infiltration, neovascularization and edema formation decreased. In eyes treated with a cyclooxygenase inhibitor the period of neovascularization was slightly shortened and corneal edema decreased. No influence on leukocyte infiltration was seen.

INTRODUCTION

Prostaglandins and leukotrienes play an important role in inflammation (1,2).

These inflammatory mediators are derived mainly from the fatty acid arachidonic acid. This precursor fatty acid is stored as component of the phospholipids in cell membranes and is released enzymatically by phospholipase A₂ following a variety of stimuli (3). Arachidonic acid is rapidly oxygenated either by a cyclooxygenase to prostaglandins (PGs) or by a lipoxygenase to leukotrienes (LTs). In general prostaglandins contribute to the formation of edema and erythema (4). Leukotriene C₄ (LTC₄) and leukotriene D₄ (LTD₄) may cause vasoconstriction followed by capillary leakage and edema (5), while leukotriene B₄ (LTB₄) is a potent chemotactic agent which plays a role in the recruitment of leukocytes into the site of inflammation (6).

Steroidal anti-inflammatory agents inhibit the release of arachidonic acid by indirect inhibition of phospholipase A₂-activity (7). In this way by depriving the cyclooxygenase as well as the lip-

oxygenase pathway of their substrate these agents are effective as anti-inflammatory drugs (fig. 1). The mechanism of action depends on binding of the steroid to a cytoplasmic receptor and translocation of this complex to the nucleus where it induces the synthesis of a protein with anti-phospholipase properties. Several of these proteins have been described and they have been named lipocortins (8). Current non-steroidal anti-inflammatory drugs like indomethacin or suprofen are inhibitors of cyclooxygenase preventing the formation of prostaglandins (1,9). Recently compounds have been developed which selectively inhibit lipoxygenase, preventing formation of leukotrienes (2,10,11). These lipoxygenase inhibitors could have, either alone or in combination with a cyclooxygenase inhibitor, a significant effect in treatment of non-infective inflammatory disorders (fig. 1).

The role of prostaglandins and leukotrienes as mediators in the ocular inflammatory response in general and of the cornea in particular has been

Fig. 1 Schematic representation of the arachidonic acid pathway to inflammatory mediators and interference with the used lipoxygenase and cyclooxygenase inhibitors in this pathway.

described (12-15). Arachidonic acid can be metabolized by the cornea to products of cyclooxygenase and lipoxygenase (16-18). The stimulatory effect of LTB_4 on leukocyte infiltration in the eye has been observed (14,15,19). Prostaglandins are known as potent mediators in the development of corneal neovascularization (16,20).

Topically administered corticosteroids are the most potent drugs for suppression of corneal inflammatory symptoms. Corticosteroids however have an array of side effects on the eye. Their ability to elevate intraocular pressure, inhibit wound healing, facilitate the growth of fungi and enhance herpes simplex virus replication has been reported (21-25). The reports on the effect of cyclooxygenase inhibiting drugs on corneal inflammation are contradictory. Stimulation as well as suppression of corneal inflammatory symptoms have been reported (26-32). Lipoxygenase inhibiting compounds have not been tested on this model although reports on the use of lipoxygenase inhibiting drugs on corneal transplantation, epithelial wound healing and immune complex uveitis have appeared (16,33,34).

A delayed hypersensitivity reaction of the rabbit cornea can be used as a model for testing anti-inflammatory properties of drugs (26-31). In this animal model the intrastromal injection of antigen induces an annular zone of antigen-antibody precipitate (35,36-39,40) which provokes an opaque ring in the cornea consisting mainly of infiltrate of polymorphonuclear leukocytes. Hereafter neovascularization starts from the limbus (3,35,36). Manifestations of an immediate hypersensitivity reaction in the cornea in man are among others; a marginal ulcer in the course of microbi-allergic keratitis, a corneal ulcer in the course of periarteritis nodosa and herpes simplex accompanied disciform keratitis (37). Because leukocyte infiltration plays a major role in this type of corneal disease the treatment with lipoxygenase inhibitors is of interest. These inhibitors are supposed to prevent LTB_4 formation and thereby chemotaxis of polymorphonuclear leukocytes in the cornea.

Leukocyte infiltration has been regarded by some authors as a prerequisite of corneal edema and neovascularization (36,41,42). Therefore these symptoms might also be influenced by the use of lipoxygenase inhibitors.

The purpose of our present report was to compare the anti-inflammatory effects of two selected lipoxygenase inhibitors, a corticosteroid and a cyclooxygenase inhibitor.

MATERIALS AND METHODS

Animals

The experiments were performed in male pigmented chinchilla rabbits weighing 2.0-2.5 kg. All eyes were initially examined with a slit lamp. Only animals without any sign of ocular inflammation were included in the study. Each pharmacological trial consisted of 8 animals. The vehicle treated control group consisted of 16 animals.

Immunization

Immunization of the pigmented rabbits was performed by injection of 20 μ l pyrogen free human serum albumin (HSA) (20% solution, C.L.B., Amsterdam, The Netherlands) into the cornea of both eyes, according to Morawiecki (43), after corneal anaesthesia with 0.4% oxybuprocaine and sedation by intramuscular injection (0,75 ml/kg body weight) of Hypnorm^R, containing fluanison 10 mg and phentanyl citrate 0.2 mg per ml.

Drug treatment

Rabbit eyes were treated with Bay 08276 (N-1,2,4 triazol-3-1-p-chlorophenylsulfamide, Bayer AG, Wuppertal, Germany), Rev 5901: (a-pentyl-3-2-quinolinyl-methoxy)-benzenemethanol, (Revlon Health Care, Tuckahoe, N.Y., U.S.A.) and Suprofen (Alcon, Fort Worth, Texas, U.S.A.) (fig. 2). Because all drugs used in this study were badly solvable in water they were applied as suspension in hydroxypropylmethylcellulose eye drops as vehicle, composed of 0,5% hydroxypropylmethylcellulose and 0,9% NaCl in water, freshly prepared every four days. To have some idea about the relative potency of these experimental drugs all were prepared as 1% suspensions. Fluorometholone 0,1% in 1,4% polyvinylalcohol (Allergan, Irvine,

Fig. 2 Structural formulas of the lipooxygenase and cyclooxygenase inhibitors used in treating immunogenic keratitis.

CA, U.S.A.) was used as a positive control. Controls were treated with 0,5% hydroxypropylmethylcellulose in 0,9% NaCl only.

Treatment with the above mentioned preparations, one drop three times a day instilled into the conjunctival sac, was started eight days after immunization and continued for the duration of the experiments.

Parameters of ocular inflammation

The keratitis of the rabbit eye was evaluated by measuring corneal edema formation, neovascularization and the occurrence of Wessely's phenomenon in the cornea. This white corneal ring is composed of precipitated antigen-antibody complexes and inflammatory cells (35,38,41,43). These three parameters of corneal inflammation can be well observed in vivo. The clinical observation was organized in a masked fashion and for each animal the values of both eyes were averaged.

Corneal aspect:

We counted the number of days during which opaque rings or a diffuse completely opaque cornea was visible as well as the number of days on which vessels were present in the cornea.

Pachymetry:

A Haag-Streit slit lamp with a pachymeter fitted with central fixation lights according to Mishima

and Hedbys (44) was used for measurements of corneal thickness (36). From each eye the mean of three measurements was taken.

Central corneal thickness was measured before and at the 7, 9, 11, 14, 16, 18, 20, 23 and 27th day after intrastromal injection with HSA.

For each animal the differences between the pachymetry measurements before and after intraocular injection with HSA were recorded as Δ corneal thickness or edema formation.

For each animal separately Δ corneal thickness was plotted in time and from these graphs the area under the curve was measured. Area under the curve was calculated using the trapezoidal rule between zero time and 27 days.

Statistical analysis

Data were analyzed by non-parametric methods to avoid assumptions about the distribution of the variables involved.

Wilcoxon's signed rank test was applied for the mean pachymetry data obtained at several time points in the treated and untreated groups during the period of inflammation and the Mann-Whitney U-test served for analysis of the duration of neovascularization and corneal opacification in the treated and the untreated eyes at any given time. Significance of difference is given for two-tailed observations, P values < 0,05 were regarded as significant.

RESULTS

Non-treated eyes

The corneas treated only with hydroxypropylmethylcellulose showed, seven to ten days after injection of HSA, clouding starting at the limbus as well as formation of Wessely's ring on about day 14-17.

This ring remained present for one to eight days. Two to four days after the ring became visible, vascularization of the cornea started over 360° from the limbus towards the center, progressed till about day 22-25 and then regressed quickly resulting in all cases in a clear cornea 30 days after the injection of the HSA.

All animals injected with HSA responded with this

Fig. 3 Comparison of changes in corneal thickness during immunogenic keratitis treated with the lipoxxygenase inhibitors Bay 08276 (n=8), Rev 5901 (n=8) and a vehicle treated control group (n=16). Mean Δ corneal thickness was calculated from the difference between the pachymetric measurements before intraocular injection with HSA and any given time thereafter (mean $\pm$ SEM).

Fig. 4 Comparison of changes in corneal thickness during immunogenic keratitis treated with the cyclooxygenase inhibitor suprofen (n=8), combined treatment with Bay 08276 and suprofen (n=8) and a vehicle treated control group (n=16). Mean Δ corneal thickness was calculated from the difference between the pachymetric measurements before intraocular injection with HSA and any given time thereafter (mean $\pm$ SEM).

ring formation and neovascularization. Corneal edema formation recorded with pachymetry started around day seven and lasted till day 30 (fig. 3, 4).

Treatment with corticosteroid

None of the six rabbits treated with fluorometholone 0.1% eye drops developed any visible sign of infiltration or neovascularization. There was no difference in corneal thickness of these eyes compared to non-inflamed eyes (not shown).

Treatment with lipoxxygenase inhibitors

In the eight rabbits treated with Bay 08276 or Rev 5901 the period of corneal opacification was shorter in comparison with the controls. Wessley's ring in the treated group was frequently incomplete, and corneal opacification was less intense. Vessel growth was significantly diminished (table 1). We noted a marked inhibition of corneal edema with Bay 08276 treatment, and a less strong inhibition in Rev 5901 treated animals (table 1, fig. 3).

Treatment with a cyclooxygenase inhibitor

In eight rabbits treated with suprofen the period

of corneal opacification was not significantly shortened. The period of vessel growth was significantly shortened. Also a marked inhibition of corneal edema in suprofen treated animals was noted (table 1, fig. 4).

Combined treatment with a lipoxxygenase and a cyclooxygenase inhibitor

The eight rabbits treated with a combination of Bay 08276 and suprofen showed a shorter period of corneal opacification and vessel growth than the controls (table 1).

Also a strong inhibition of corneal edema with combined treatment was noted (table 1, fig. 4). There was no significant difference between the effect of combined treatment with Bay 08276 and suprofen as compared to treatment with the lipoxxygenase inhibitor Bay 08276 alone.

DISCUSSION

Animals treated only with the hydroxypropylmethylcellulose vehicle in this study responded for 100% with corneal opacification, neovascularization and

TABLE 1
CORNEAL OPACITY, GROWTH AND EDEMA FORMATION DURING IMMUNOGENIC KERATITIS

	Duration of corneal opacity (days) [°]	Duration of corneal neovascularization (days) [°]	Pachymetry AUC (% compared to controls) [°]
Controls (n=16)	6,7 ± 0,5 (3-12)	7,2 ± 0,8 (1-12)	100 ± 13 (2-183)
Bay 08276 1% (n=8)	1,3 ± 0,5** (0-5,5)	2,1 ± 0,7** (0-3)	27 ± 8* (2-77)
Rev 5901 1% (n=8)	3,4 ± 0,6** (1-6)	2,4 ± 0,8** (0-6,5)	36 ± 8* (6-71)
Suprofen 1% (n=8)	4,8 ± 1,3 (0-8)	4,0 ± 0,9* (0-7,5)	32 ± 9* (11-93)
Bay 08276 1% + Suprofen 1% (n=8)	1,1 ± 0,3** (0-2,5)	1,9 ± 0,7** (0-5,5)	23 ± 6* (0-58)

[°] : mean ± SEM (range); n : number of animals; AUC: area under the curve.
Significance of difference vs controls for duration of corneal opacity and vessel growth was calculated with the Mann-Whitney U test.
Mean pachymetry values of controls and treated animals at various time points during the inflammation were tested for significance of difference with Wilcoxon's signed rank test.
* p < 0,01
** p < 0,002

edema. The appearance of opaque rings and neovascularization in the cornea is in accordance with previous observations using this model of corneal anaphylaxis (27,31,35,45). Histology has shown that the opaque ring consisted mainly of a polymorphonuclear leukocyte infiltrate (31,36). The difference in response rate to the antigen of the present experiments with previous work on this model must be due to the greater sensitivity of chinchilla rabbits compared to Dutch blue belts (31). This is in accordance with observations made by Sery (45).

The suppressive effect of a local corticosteroid used as a positive control on the model of immunogenic keratitis confirms earlier observations using this model (31,34). The lipoxygenase inhibitors Bay 08276 and Rev 5901 were both effective in the inhibition of leukocyte infiltration, neovascularization and corneal edema. Bay 08276 was the most potent one of the two. This drug is also effective in the prevention of neovascularization and leukocyte infiltration in experimental corneal transplants and alkaline burns (16). The cyclooxygenase inhibitor suprofen has a limited effect on

TABLE 2
SELECTIVITY OF LIPOXYGENASE AND CYCLOOXYGENASE INHIBITORS IN WHOLE CELL ASSAYS IN VITRO*

Compound	Eicosanoid generation	
	LTB ₄	PGE ₂
Bay 08276	10	100
Rev 5901	3	n.d.
Indomethacin	n.d.	0,3
Suprofen	n.d.	0,2

* Calculated after data given by Todd, Jardin et al. and McMillan et al. (9,10,49) Values represent IC₅₀ (μM). (n.d. = not determined).

corneal neovascularization, but corneal edema was strongly inhibited by suprofen. Because we counted the number of days corneal opacification was visible and not the number of leukocytes involved, we could have underestimated the effect of lipoxygenase and cyclooxygenase inhibitors in this model of corneal disease. Our impression was that the number of days on which corneal opacification occurred were directly proportional to the size of the area of opacifi-

cation. The same was observed for the duration of corneal neovascularization.

Efficacy of inhibition of LTB_4 by Bay 08276 and Rev 5901 appears to coincide with inhibition of corneal opacity and thus of leukocyte infiltration and neovascularization. Less inhibition of edema by Rev 5901 as compared to Bay 08276 may be related to absence of any effect on PG formation, whereas Bay 08276 is less specific and may also inhibit PG formation. Suprofen as inhibitor of PG and not of LTB_4 formation only effects edema formation efficiently and has little effect on neovascularization and no influence on corneal opacity (table 2).

Chemotactic factors produced at the site of the antigen-antibody precipitate attract leukocytes. The invaded leukocytes on their turn will produce prostaglandins and chemotactic leukotrienes and this will result in even more leukocytes, infiltration by leukocytes, neovascularization and edema.

A review of several experimental models of corneal neovascularization has revealed that a cellular inflammatory reaction occurs in most of them prior to neovascularization (36). This suggests that polymorphonuclear leukocytes directly or indirectly may provoke corneal vascularization. Eliason (46) however found that in the absence of invading leukocytes an injured cornea is also capable of producing an angiogenic factor from its own native elements. The most important stimuli for neovascularization have been determined to be PGE_1 and PGE_2 (16,10). The efficacy of specific lipoxigenase inhibitors in the prevention of neovascularization by blocking LT formation suggests that infiltrated leukocytes play a major part in the occurrence of this symptom. Perhaps polymorphonuclear leukocytes are not necessary for the process of neovascularization, but they seem either to produce prostaglandins or other angiogenic factors of their own or to enhance the production of these substances in corneal tissue.

Corneal edema is frequently observed in association with corneal inflammation. Chusid (42) demonstrated a significant increase in corneal

water content in normal animals compared to neutropenic animals after induction of corneal inflammation by intracorneal injection of chemotactic agents. He concluded that the edema formation is due to the elaboration and release of certain polymorphonuclear leukocyte products within the cornea.

Post surgical cornea edema has been reported to be reduced after systemic administration of the cyclooxygenase inhibitor naproxen^R (47).

In our experiments treatment with the cyclooxygenase inhibitor suprofen resulted in an almost complete absence of corneal edema, despite the presence of leukocyte infiltrate and neovascularization. This suggests, prostaglandin production must play a very important role in the development of corneal edema. The effect of lipoxigenase inhibitors on corneal edema is probably due to prevention of the positive feedback phenomenon in leukocyte infiltration as described above.

The effect of cyclooxygenase inhibiting drugs on corneal inflammatory symptoms is controversial. Indomethacin has been reported to have a suppressive effect on corneal neovascularization (28-30,48) and suprofen on polymorphonuclear leukocyte accumulation (32). Other investigators have found a stimulation of polymorphonuclear leukocyte accumulation (27,31).

Our conclusion is that the lipoxigenase inhibitors we used, have a strong inhibiting influence on symptoms of corneal inflammation. In view of the known undesirable side effects of steroid these drugs may be of interest as topical ophthalmic preparations.

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