LIPID MEDIATORS IN OCULAR INFLAMMATORY MODELS

(Van vetten afgeleide mediatoren in ontstekingsmodellen van het oog)

PROEFSCHRIFT

terverkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
opgezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het college van dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 24 oktober 1990 om 13.45 uur

door

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geboren te Gouda

1990
Offsetdrukkerij Haveka B.V.,
Alblasserdam

Promotiecommissie

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Publication of this thesis was made possible by grants of the Prof.Dr. H.J. Flieringa Stichting and Chibret Nederland.

Now there are four chief obstacles in grasping truth, which hinder every man, however learned, and scarcely allow any one to win a clear title to learning, namely, submission to faulty and unworthy authority, influence of custom popular prejudice, and concealment of our own ignorance accompanied by an ostentatious display of our knowledge.

Roger Bacon, Opus Maius 1268.
(Tr. R.B. Burke, 1928).

VOOR MIJN OUDERS

Contents

1. General introduction and objectives of the study

1.1 Aim of thesis

Uveitis, an acute or chronic inflammation of the uveal tissue of the eye, and keratitis, an inflammation of corneal tissue, may have their cause in bacterial, protozoal or viral infection. No etiological agent can be found in some patients. In these cases therapy has to be of symptomatic nature. One of the major causes of destruction of ocular tissues in uveitis and keratitis is the production of lysosomal enzymes by infiltrating white blood cells. Inhibition of either cellular infiltration of tissue or the prevention of lysosomal enzyme release by these cells will lead to less destruction of ocular tissue.

In this study we investigated the prevention of cellular infiltration of ocular tissue by topical or systemic administration of agents interfering with lipid mediators.

An Arthus reaction (delayed hypersensitivity reaction) of the cornea or uvea of the rabbit eye can be initiated by injection of antigen in respectively the corneal stroma or vitreous body. The clinical symptoms of the subsequent antigen-antibody reaction in the cornea are conjunctival hyperemia, an annular infiltration with leukocytes (ring of Wessely), stromal edema, and neovascularization of the cornea.[1]

Symptoms of the Arthus reaction elicited by intravitreal injection of an antigen are blood-aqueous barrier breakdown (visible with a slitlamp as a flare) and the presence of infiltrated leukocytes in the anterior chamber and vitreous

body.[2,3]

Phospholipid derivatives may act as mediators in human and animal inflammatory disease. These derivatives of cell membrane phospholipids are platelet-activating factor (PAF) and arachidonic acid. Arachidonic acid can be further metabolized to prostaglandins (PGs) and leukotrienes (LTs).

Platelet-activating factor can be produced by embryo chick retina upon stimulation with neurotransmitters such as acetylcholine and dopamine.[4] Systemic administration of PAF induces in the rabbit sludging in the retinal circulation and intense extravasation of plasma proteins while on fluoroangiographic pictures severe retinopathy is visible.[5] In addition (R)-PAF and lyso-platelet-activating factor (lyso-PAF) significantly impair the b-wave of the electroretinogram of the isolated rat retina, and these effects are dose-dependently inhibited by specific PAF-antagonists.[6] A PAF-antagonist also inhibits the transient increase in intra-ocular pressure induced by laser iridial burns; such a phenomenon is not inhibited by indomethacin.[7] Specific receptors for PAF in iris and ciliary body have been determined.[8,9]

Arachidonic acid can be metabolized by ocular tissues into PGs by the enzyme cyclooxygenase and into LTs by the enzyme 5-lipoxygenase.[10-13] The role of PGs and LTs as mediator in the inflammatory response of the eye in general[14-16] and of the Arthus phenomenon in particular is well known.[17-19] The PG level may be lowered by inhibition of cyclooxygenase. Cyclooxygenase inhibitors, however, do not decrease the severity of an Arthus reaction of cornea or uvea but even stimulate the leukocyte infiltration of the eye in these inflammatory diseases.[3,20-23]

The enzyme 5-lipoxygenase produces among others LTB_4, a very potent chemotactic substance.[24] In the eye its chemotactic potency has also been described.[25,26]

The main theme of this thesis is about the role of lipid derived mediators in the Arthus phenomenon in the eye and the effect of pharmacological inhibition of these mediators.

The role of a PAF-antagonist on corneal inflammation and the role of the second messenger cyclic adenosine monophosphate (cAMP) was investigated as well as the relative importance of lipoxygenase versus cyclooxygenase products in ocular inflammation. Phospolipase action in plasma membrane can make arachidonic acid available for conversion into cyclooxygenase or lipoxygenase products. Inhibition of arachidonic acid conversion to PGs may increase LTB production, which in part could be responsible for the aggravation of the Arthus reaction of the cornea or uvea. However, arachidonic acid can also be oxidized in mitochondria or used in esterification reactions, which at least in other tissues are of another order of magnitude. Inhibition of the ocular Arthus reaction by 5-lipoxygenase inhibitors is therefore of importance.

The following possibilities for modulation of the production of PGs and LTs from arachidonic acid products that have been used are:

- Replacement of arachidonic acid by a false substrate - columbinic acid. Columbinic acid replaces arachidonic acid in the cell membrane, but cannot be converted into prostaglandins or leukotrienes.[27]
- Replacement of arachidonic acid by a competitive substrate like eicosapentaenoic acid. Formation of prostaglandins and leukotri-

enes from eicosapentaenoic acid is possible, but these substances have a different biological effect compared to arachidonic acid derived products.[28-31]

- Specific pharmacological inhibition of either cyclooxygenase or 5-lipoxygenase.[32-34]
- Combined inhibition of the cyclooxygenase and 5-lipoxygenase enzymes or prevention of arachidonic acid release from cell membrane phospholipids.[35,36]

1.2 Historical introduction

1.2.1 Prostaglandins

A series of elegant experiments led Sir Thomas Lewis in 1927 to suggest that events in the acute inflammatory response were controlled by the local generation of chemical substances.[37] In 1930 Kurzak and Lieb, two New York gynecologists, discovered the smooth muscle-stimulating actions of seminal fluid on the human uterus.[38]

The actions of seminal plasma on isolated smooth muscle were first reported by von Euler[39-41] and independently by Goldblatt.[42] The chemistry of the active substances was not known by these early workers and they were called prostaglandins (PGs) by von Euler simply as a reflection of the site of production, the prostate.

In the mid 1960s, PGs were found to be oxidative metabolites of essential fatty acids, constituents of membrane phospholipids.[43,44]

1.2.2 Leukotrienes

In 1938 Feldberg and Kellaway showed that cobra venom injected into perfused lungs caused the release of a substance which slowly induced sustained contractions of guinea pig jejunem. The contractions were different from those induced by histamine and the substance was referred to as slow reacting substance.[45] A few years later it was demonstrated that a similar substance was released from the guinea pig pulmonary system upon immunological challenge.[46] Brocklehurst named the substance "slow reacting substance in anaphylaxis" (SRS-A).[47]

It is now known that these substances are identical. Despite many attempts SRS and SRS-A resisted chemical identification until the end of the 1970s, although it was known to be a sulphur containing lipid. The breakthrough came in 1979 when Samuelson's research group discovered new metabolites of arachidonic acid through the lipoxygenase pathway with biological and chemical properties of SRS and SRS-A.[48,49] These metabolites were named leukotrienes because they are produced by leukocytes and are characterized by the presence of three conjugated double bonds in the molecule. SRS and SRS-A have been shown to consist of a mixture of leukotrienes.

1.2.3 Platelet-activating factor

A reaction involving leukocytes and requiring antigen to trigger the release of histamine from rabbit platelets was reported in the sixties[50-52] and attributed to a factor actively

released from the leukocytes by a calcium- and temperature dependent process.[53,54] In 1972 and later, Benveniste et al.[55-57] described this substance and proposed the term platelet-activating factor, initiated its characterization, and showed that it was released from rabbit basophils by an IgE-dependent process.

This product has since been characterized as a phospolipase A_2 (PLA_2)-sensitive phospholipid[58] and further identified as 1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (Fig. 1)[59,60] The ether-lipid was finally synthesized and became available for pharmacological studies.[61]

Both PAF-acether (ace standing for acetate and ether for the ether bond) and AGEPC (acetyl glyceryl ether phosphoryl choline) are now used in the literature as synonyms for PAF.

2. Fatty acids as precursors of lipid mediators of inflammation

2.1 Dietary fatty acids

Fats form an important constituent of our diet. Dietary fats are mainly composed of triglycerides: a glycerol moiety with three fatty acids. There are many types of fatty acids, differing in chain length (number of carbon atoms) and in desaturation (number location and configuration of double bonds).

Fatty acids also are constituents of phospholipids, a class of cell membrane structural lipids. Generally a phospolipid is composed of a glycerol molecule which is linked at two of its hydroxyl groups to a long-chain fatty acid. The third hydroxyl group is esterified to a phosphate group which in turn is linked to either choline, ethanolamine, serine or inositol.

Recognition that some unsaturated fatty acids could not be synthetized by mammals led to the designation of essential and nonessential unsaturated fatty acids.

"Properly, the term essential fatty acids should include only those substances which are active both for growth and for maintenance of dermal integrity, limiting the term to linoleic and arachidonic acids and to such other acids as may be derived metabolically from them".[62]

Since the essential fatty acids are present in animal cell membranes any food containing such membranes will contain at least small amounts of most of the essential fatty acids. The main dietary sources of various essential fatty acids are as follows:

- Linoleic acid. This is present in substantial amounts in dairy products, organ meats such as liver, human milk and in many vegetable seed oils such as sunflower, safflower, corn and soy beans.
- Gamma-linolenic acid. This is present in small amounts in human milk and fresh cow milk; larger amounts are found in the seed oil of the evening primrose.
- Dihomo-gamma-linolenic acid. Moderate amounts are found in human milk.
- Arachidonic acid. Human milk contains moderate amounts and cow milk small amounts. Meat, egg yolks, some seaweeds and some shrimps contain substantial amounts.
- Eicosapentaenoic acid and docosahexaenoic acid are abundant in oily fish and shell fish, particularly from cold ocean areas.[63]

2.2 Metabolism of fatty acids

Incorporation of fatty acids in cell membrane phospolipids is accomplished either by replacement of de novo synthetized phospholipids or by replacement of the fatty acid part of a phospholipid by another fatty acid with the help of acyltransferase(s) after phospholipase action. There are at least two different acyltransferases. Both types are widely but not equally distributed within the cells. One specifically attaches a saturated fatty acid to the 1-position of a phospholipid molecule, the other acting at the 2-position of the molecule prefers unsaturated fatty acids.[64] This may be one of the mechanisms responsible for the predominant occupation of the 2-position of

the glycerol moiety of phospholipids by unsaturated fatty acids.

The biosynthesis of polyunsaturated fatty acids involves alternating desaturation and elongation reactions in which two hydrogen atoms are removed to create a new double bond and then two carbon atoms are added to lengthen the chain. Specific enzymes are required for each desaturation and elongation step, desaturases and elongases.

The consequence is that polyunsaturated fatty acids can be grouped in families determined by the place of their first double bond. The most important families in mammalian physiology are the n-9, n-6 and n-3 families with the first double bond on the 9th, 6th and 3rd carbon atom from the methyl end, respectively. Independent of any further desaturations or elongations unsaturated fatty acids will remain within their family.[65] The three series appear to compete with one another in a sense that for instance n-3 fatty acids can inhibit eicosanoid metabolism from n-6.[67]

In this manner the desaturation and elongation processes can be influenced by dietary factors. In addition hormonal factors are known to influence these processes.[66,67]

3. Biosynthesis and action of lipid mediators

3.1 Phospholipids

3.1.1 Phospholipids as precursors of lipid mediators

Eicosanoids is a general term used for the metabolites which are derived via oxidative metabolism of the fatty acid arachidonic acid (= eicosatetraenoic acid), eicosatrienoic acid (dihomo-gamma-linolenic acid) and eicosapentaenoic acid. They include prostaglandins, thromboxane, leukotrienes and other oxygenated fatty acid derivatives. Eicosanoids are generated in response to a wide variety of physiological and pharmacological stimuli and, since eicosanoids are not stored within cells, biosynthesis must immediately precede release. The precursor fatty acids can arise from the unesterified fatty acid pool but also by hydrolytic cleavage of intracellular lipid precursors: cholesteryl esters, mono- di- or tri-glycerides, and phosphatides. The phospholipid fraction constitutes the major intracellular source of the eicosanoid precursors. Therefore our attention wil be focussed on metabolism as eicosanoid precursors of these lipids.[68,69]

Platelet-activating factor is a phosphorus containing derivative of cell membrane phospholipids. Its metabolism will also be considered.

3.1.2 Phospholipases

Phospholipases are a class of enzymes that catalyze the hydrolysis of membrane phospholipids to release free fatty acids or one of the four phosphorylated bases.

In so far as they are relevant to the generation of the eicosanoids, phospholipids may be thought of as consisting of a three carbon glyceryl backbone to which fatty acids and phosphorylated bases are attached to the the glyceryl alchol by an ester linkage. The three positions on the glyceryl backbone are referred to as α, ß and α^1. Fatty acids are esterified on the α and ß position, whilst the α^1 position is esterified either to phosphoric acid or to a phosphorylated base; phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine or phosphatidylinositol.

A fatty acid that is esterified in position α can be liberated by phospholipase A_1, one that is esterified in position ß by phospholipase A_2 and phospholipase C hydrolyses the glyceryl-phosphate bond to liberate a phosphorylated base. Because unsaturated fatty acids (this includes all eicosanoid precursors) are generally attached in ß position to the glyceryl backbone phospholipase A_2 becomes potentially the most important regulatory enzyme for the entire eicosanoid cascade.[70,71] In addition to eicosanoids formed from arachidonic acid other phospholipase-derived products also have biological actions. Lysophospholipids, the co-products of phospholipase action are cytotoxic substances[72] and have been implicated in several human inflammatory conditions.[73,74] Acetylation of lysophospholipid at

the 2 hydroxy group gives rise to another lipid mediator, PAF-acether, a potent platelet aggregating substance and inducer of various inflammatory reactions. Other important products of phospholipid breakdown involved in transmembrane signal transduction (for instance from phosphoinositol) will not be discussed here.

3.1.3 Phospholipase inhibitors

Compounds that inhibit phopholipase A_2 may be subdivided into agents that inhibit the enzyme: a) by interacting directly with the enzyme; b) by interfering with the binding of the substrate; or c) by interacting or interfering with the binding of calcium ions.

a) Enzyme inhibitors.

Glucocorticoids inhibit phospholipase A_2 activity as measured by the release of arachidonic acid. This correlates well with their anti-inflammatory activity. The steroids induce biosynthesis of the phospholipase A_2 inhibitory protein[35,75] now called lipocortin.[76,77] Some compounds like para-bromophenacyl-bromide and several natural products including glycerrhizin alpha tocopherol, polymycin B and manoalide, a sesquiterterpenoid isolated from a sponge, may inhibit phospholipase A_2 directly.[78-81]

b) Inhibitors interacting with the substrate.

Compounds which form complexes with phospholipids can influence phospholipase action because the complex formation

prevents catalytic action by phospholipase A_2. Examples of these compounds are chlorpromazine, mepacrine and volatile anesthetics like halothane.[82,83] Many amphiphilic compounds, as well as cold, may decrease membrane fluidity and prevent interaction of phospholipase(s) intercalated in the phospholipid bilayers of the biomembranes with their substates.

3.2 Platelet-activating factor

3.2.1 Pathophysiology of platelet-activating factor

Over the last few years, several publications have indicated that platelet-activating factor (PAF = PAF-acether), is a major mediator of inflammation and anaphylaxis.[84-86] PAF is produced by many types of stimulated cells including mononuclear phagocytes, basophils, platelets, endothelial cells and human polymorphonuclear neutrophils (PMN).[87-91] Its action was first thought to be restricted to platelet activation, but now it also seems to stimulate PMN function. Indeed, intravenous injection of purified PAF-acether into rabbits led both to the development of an acute neutropenia and to the formation of intravascular PMN aggregates.[92] It induces smooth muscle contraction,[93] enhances vascular permeability,[94,95] and leads to bronchoconstriction, hypotension and renal failure.[84-87,96]

Numerous interactions between PAF and other lipid mediators have been described. For instance, PAF-acether induces the release of leukotriene C_4 (LTC_4) from rat lung and, when platelets are present, the release of prostaglandins.

3.2.2 Inhibition of platelet-activating factor action

The discovery of potent and specific antagonists of PAF-acether has recently allowed a more detailed analysis of the role of this phospholipid in health and disease.

Three types of compounds of different origin and structure have been reported as PAF antagonists: PAF analogues (e.g. CV-3988), natural products (e.g. kadsurenone, BN 52021) and synthetic compounds (e.g. the hetrazepines brotizolam and WEB 2086, and 48740-RP).[99] The pharmacology of these antagonists is basically determined by the pharmacology of PAF.[98] It is to be expected that all the effects induced by exogenous PAF can be inhibited by such compounds. In addition, PAF involvement in animal models of diseases, like endotoxin shock and anaphylaxis, can be used for pharmacological characterization of these PAF antagonists, which appear to act by occupying PAF binding sites (or receptors) on the effector cells. These PAF antagonists displace the PAF molecules from receptor-binding sites, in most cases without themselves initiating any response. Their action is therefore a receptor-mediated process, competitive and reversible.[99,100]

3.3 Prostaglandins

3.3.1 Biosynthesis and action of prostaglandins

The PGs and related compounds are derived from 20-carbon essential fatty acids that contain three-, four or five double bonds; 8,11,14-eicosatrienoic acid (dihomo-gamma-linolenic acid),

5,8,11,14-eicosatetraenoic acid (arachidonic acid) and 5,8,11,14,17-eicosapentaenoic acid.

The chemical structure of PG consists of a cyclopentane ring and two alphatic side chains. The synthesis of PGs from fatty acids is preceded by the oxygenation and cyclization of the pentane ring by cyclooxygenase, leading to an unstable C15-hydroperoxy-C9-C11-endoperoxide prostaglandin G. Endoperoxides which are chemically unstable form the intermediate substrates for tissue-specific synthesis of a variety of biologically active PGs.[101] They fall into several main classes, designated by letters and distinguished by substitutions on the cyclopentane ring. The main classes are subdivided in accord with the number of double bonds in the side chains. This is indicated by subscript 1, 2 or 3 and reflects the fatty acid precursor. Thus, PGs derived from 8,11,14-eicosatrienoic acid carry the subscript 1; those derived from arachidonic acid carry the subscript 2; and those derived from 5,8,11,14,17-eicosapentaenoic acid carry the subscript 3. In man arachidonic acid is the most abundant precursor and there is little evidence that PGs of the 1 or 3 series are important. However, PGs of the 3 series have greater significance in fish and marine animals where 5,8,11,14,17-eicosapentaenoic acid is the precursor.

More than 100 natural PGs, isomers and their metabolites are now known. The E- and F-type PGs have been termed primary PGs.[102,103] They, as well as PGs of the A-, B-, C- and D-types, are relatively stable compounds while PGs G, H and I are unstable in aqueous media at physiological pH and temperature.

In inflammation most PGs are powerful vasodilators. By promoting blood flow in the inflamed region they enhance edema

formation and leukocyte infiltration.

The pain producing activity of bradykinin and of histamine is potentiated by PGs.[104]

Nevertheless, the view that the inflammatory process is exclusively promoted by prostaglandins is oversimplified. Evidence is accumulating that, depending on the experimental situation that is studied, PGE_2 displays either pro- or anti-inflammatory effects.[105-107] While such a dual function has not been convincingly shown for other products of AA-bioconversion, the di-homo-gamma-linoleic acid derived PGE_1 does share this property with PGE_2.[106-108] AA and di-homo-gamma-linoleic acid are essential fatty acids (EFA) and EFAs per se are reportedly either pro- or anti-inflammatory.[106,107,109]

While under some conditions PGE can counteract non-immune plasma exudation, its anti-inflammatory effects with model conditions in which immunecompetent lymphocytes and/or macrophages are involved deserve more attention. Besides their inflammatory effects,[105-107] the E-type prostaglandins have, in fact, been proposed as regulators of the immune response.[110,111] In addition, a modulatory involvement in immune reactions has also been ascribed to essential fatty acids, albeit the immunomodulatory effects of EFA are partially mediated through conversion to PGE.[109] Lymphocytes and macrophages, as pivotal targets for both the anti-inflammatory and immunomodulatory effects of PGE, form the obvious link between the two functions.[107]

3.3.2 Inhibition of prostaglandin action

3.3.2.1 Cyclooxygenase inhibitors

In 1971 Vane reported that aspirin and indomethacin could inhibit prostaglandin biosynthesis. He showed that this effect was exerted directly on the enzymatic conversion of arachidonic acid to prostaglandins. These drugs prevent production of the prostaglandin endoperoxides by the cyclooxygenase enzyme and as a result inhibit the synthesis of all of the products beyond this step in the metabolic pathway.[112,113] Cyclooxygenase inhibitors however promote the secretion of lysosomal enzymes and LTB_4.[114,115]

3.3.2.2 Prostaglandin antagonists

Most or all actions of the various prostaglandins are exerted through activation of specific receptors.[116,117] Polyphloretin-phosphate possesses prostaglandin-antagonist effects and can inhibit signs of ocular inflammation.[118]

3.4 Leukotrienes

3.4.1 Biosynthesis and action of leukotrienes

The leukotrienes constitute a group of acyclic 20-carbon essential fatty acid derivatives. Their name is derived from the fact that they were first isolated from leukocytes, and that they contain a triene, i.e. three conjugated double bonds. Their

numerical subscripts refer to the total number of double bonds, whereas the letters refer to the substituents in alphabetic order. Thus, all the LTs with the numerical subscript 3 are derived from eicosatrienoic acid, those with subscript 4 from arachidonic acid and those with subscript 5 from eicosapentaenoic acid.[119,120]

The LTs are formed primarily from arachidonic acid. The leukotrienes LTA_4, LTB_4, LTC_4, LTD_4, LTE_4 and LTF_4 arise through the 5-lipoxygenase pathway from arachidonic acid with as intermediate a 5-hydroperoxy-eicosatetraenoic acid (5-HPETE). Reaction of LTA_4 with gluthathione, a tripeptide, yields LTC_4 which can be further metabolized to respectively LTD_4, LTE_4 and LTF_4. Metabolism of LTA_4 with a hydrolase yields LTB_4.

Leukotrienes are formed analogously from 5,8,11-eicosatrienoic acid (LTB_3, LTC_3, LTD_3 and LTE_3) and from 5,8,11,14,17-eicosapentaenoic acid (LTB_5, LTC_5, LTD_5 and LTE_5). Leukotrienes may also arise through the 12- and 15-lipoxygenase pathways.[121]

The LTs exert marked effects in many biological systems.

Leukotriene B_4 is one of the strongest chemotactic agents known for leukocytes. It causes aggregation of cells, produces superoxide and mobilizes the calcium pool of the cells. LTB_5 is considerably less chemotactic.[121-124] LTC_4, LTD_4 and LTE_4 have little if any effect on leukocyte chemotaxis.

LTC_4, LTD_4 and LTE_4 give marked constriction of coronary arteries in a number of species and have a negative inotropic effect.[125-128] LTC_4 and LTD_4 contract human broncheal and tracheal muscles[129,130] and stimulate pulmonary mucus secretion.[131,132] In the skin erythema and whealing is caused by LTC_4, LTD_4 and LTE_4 and induration by LTB_4.[133,134]

3.4.2 Inhibition of leukotriene action

3.4.2.1 Enzyme inhibitors

There are many sites throughout the path of the synthesis of LTs where interruption can take place. Of course prevention of the release of unsaturated fatty acids will deprive the lipoxygenase as well as the cyclooxygenase pathway of substrate. This can be done effectively by the use of corticosteroids as has been discussed before.

Many cyclooxygenase inhibitors also inhibit to some extent 5-lipoxygenase. Examples are acetylsalicylic acid, indomethacin and phenylbutazone.[135]

Some selective 5-lipoxygenase inhibiting drugs have been developed[34,136] though none of these have been accepted at this stage as a safe and useful clinical drug.

3.4.2.2 Leukotriene antagonists

Receptor sites for LTC_4-LTD_4 have been demonstrated on the cell surface of leukocytes. LTC_4 and LTD_4 receptor antagonists have been developed, showing also in vivo activity in various animal models.[136,137]

3.5 Cyclic nucleotides

3.5.1 Cyclic nucleotides as second messengers in an eicosanoid induced inflammatory response

When a chemical mediator initiates a metabolic response in the cell this is effected through a membrane-linked specific receptor causing the formation of an intracellular messenger molecule. The intracellular messenger is often called the second messenger, which on its turn then stimulates or depresses some characteristic biochemical activity in the target cell. The intracellular activities of PGs and LTs are dependent on such receptor mediated processes.[116,136-138] The second messengers in this case are cyclic nucleotides, such as cyclic adenosine monophosphate (cAMP) or cyclic guanidine monophosphate (cGMP).

Adenosine triphosphate can be metabolized by the enzyme adenylcyclase to 3.5 cAMP and free pyrophosphate. The enzyme responsible for the destruction of cAMP is phosphodiesterase, which catalyzes the hydrolytic reaction by which cAMP is converted to adenosine 5'phosphate.[139]

Prostaglandin E and prostacyclin are among the most potent stimulators of cAMP generation. Prostaglandin E_2 gives rise to an activation of adenylcyclase and thereby an increase in the intracellular cAMP level.[140] A rise in cAMP is associated with a reduction of lymphocyte stimulation, cell mediated cytotoxicity, antibody- and lymphokine production, in polymorphonuclear leukocytes it accompanies lysosomal-enzyme release.[141]

A reduced capacity of the cyclooxygenase pathway in elicited macrophages is accompanied by low intracellular cAMP

levels. While products of the cyclooxygenase pathway of arachidonic acid metabolism inhibit lymphocyte functions by increasing cAMP, the same processes may be enhanced by products of the lipoxygenase pathway, possibly by increasing intracellular cGMP.[142,143] Leukotriene C_4 induces a rapid transient cAMP generation which is PG mediated in isolated macrophages.[142]

3.5.2 Pharmacological manipulation of the cyclic nucleotide level

The level of cAMP or cGMP may be influenced by inhibition or stimulation of the action of the regulating enzymes adenylcyclase or phosphodiesterase.

Cyclic nucleotide phosphodiesterase activity can be inhibited by caffeine and other methylxanthines, cromoglycate and many other agents. Activation of cyclic nucleotide phosphodiesterase is possible by insuline, imidazole and some other agents.[144]

Guanyl cyclase can be activated by α_2 adrenergic agonists, muscarinic agonists and opiates.[145] It can be inhibited by guanidine-triphosphate, other guanine nucleotides and fluoride.[146]

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Volume 7 number 6 1988

Current Eye Research

The effects of 3-isobutyl-methyl-xanthine on experimentally induced ocular inflammation in the rabbit

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Received on July 13, 1987; accepted on April 8, 1988

ABSTRACT

The effect of topical administration of 3-isobutyl-methyl-xanthine (IBMX), a potent phosphodiesterase inhibitor, was studied on an experimentally provoked uveitis in rabbits.

After presensitization with an intravitreal injection of human serum albumin (HSA), intravenous antigenic challenge induces blood-aqueous barrier breakdown and leukocyte infiltration. The effect of IBMX on the blood-aqueous barrier was determined by scoring the severity of the flare in the anterior chamber and by determination of the levels of ascorbic acid and protein in the aqueous. Treatment with IBMX 1% two times daily, significantly inhibited the breakdown of the blood-aqueous barrier and the increase in PGE_2 level of the aqueous humor. There was no effect on leukocyte infiltration. The therapeutic effect of IBMX in blood-aqueous barrier protection is comparable with the effect of topical treatment with the corticosteroid medrysone.

INTRODUCTION

Immunogenic uveitis in rabbits can be used as a model for the evaluation of ocular anti-inflammatory agents (1,2). HSA administered intravitreally to the rabbit eye produces ocular inflammatory symptoms which are similar to those observed during uveitis (3).

Immunogenic uveitis is caused by an immediate hypersensitivity reaction of the rabbit eye (4). This type of uveitis is caracterized by conjunctival hyperemia, cellular infiltration and a breakdown of the blood-aqueous barrier (1,2,5,6). Characteristics of the breakdown of the blood-aqueous barrier are increased protein and decreased ascorbic acid level in the aqueous.

Phosphodiesterase inhibitors such as theophylline, caffeine and IBMX, raise the level of intracellular cyclic adenosine monophosphate (cAMP) by preventing the chemical conversion of cAMP to AMP by phosphodiesterase (7).

The beneficial effects of an elevated intra-cellular cAMP level on an immediate hypersensitivity reaction in vitro and in vivo have been described (8,9,10,11). Systemically applied theophylline showed, for example, a marked reduction in exudate formation and leukocyte migration in a pleural immediate hypersensitivity reaction (9).

On the contrary disruption of the blood-aqueous barrier in the eye, induced by paracentesis or intravitreal injection of bacterial endotoxins can be antagonized by systemic treatment with imidazole, an activator of cAMP-phosphodiesterase (12,13,14). Systemically administered theophylline potentiates the blood-aqueous barrier disruption caused by topical application of prostaglandin E_2 (PGE_2) to the eye (15).

These observations in the eye suggest that accumulation of intraocular cAMP promotes the barrier damage and that cAMP might be the common effector of the barrier breakdown, caused by prostaglandin as well as by non prostaglandin agents.

The object of this study is to evaluate the action of IBMX as topically given phosphodiesterase inhibitor, on immunogenic uveitis. We also compared its action with that of the corticosteroid medrysone.

MATERIALS AND METHODS

Animals

Chinchilla rabbits (2,0 to 3,0 kg, aged 6 to 8 months) were kept in our animal facilities with free access to water and standard food.

HSA-induced uveitis

Ocular sensitization was induced by an intravitreal injection of 30 µl of HSA (20% solution, CLB, Amsterdam, The Netherlands) in the right eye. The left eye was injected with 30 µl sterile

saline. The injection was performed with a 30-gauge needle through the pars plana, after local anaesthesia with tetracaine 2% eye drops.

The eyes were daily examined with a slitlamp for signs of an inflammatory response. The response usually took place within 8 to 20 days in the HSA injected eyes. Animals that did not respond with this initial inflammatory reaction were excluded from further experiments (about 10% of the animals). The primary inflammatory response was then allowed to subside until the eyes appeared to be normal on biomicroscopy. This took approximately 3-6 weeks from the time of injection.

After six weeks the animals were challenged intravenously with HSA (1 mg/kg). Within two hours sensitized eyes developed an inflammatory response which reached a maximum at 24 hours and remained constant till 72 hours after the challenge dose. This second inflammatory reaction was used for our pharmacological experiments.

Slitlamp examination

Before commencement of the experiment all animals were checked for the signs of any ocular inflammation by slitlamp examination. The second inflammatory response was observed and graded according to Hogan (16) for hyperemia, flare and cells at zero, six, 24 and 72 hours after the intravenous challenge with HSA. The observer did not know which treatment the animals received.

Sampling of aqueous humor

At 72 hours paracentesis was performed under general anaesthesia with hypnorm^{R} (fluanison 10 mg/ml and phentanyl citrate 0,2 mg/ml) 0,75 ml/kg body weight. With a 27 gauge needle approximately 200 µl aqueous was aspirated.

The aqueous was analyzed for white cell count, protein, ascorbic acid and PGE_2.

Assays

- The total protein concentration of the aqueous was determined according to Bradford (17) using HSA as standard.

- Ascorbate was measured in aqueous within 1 hr. after aspiration, using a colorimetric test set (Boehringer-Mannheim, Mannheim, West Germany).

- PGE_2 was determined by a Radio Immuno Assay kit (New England Nuclear, Boston, Mass., USA).

- The cell counts of the aqueous humor were performed with a haemocytometer.

Drug treatment

A 1% suspension of IBMX (Fluka AG, Buchs, Switzerland) was prepared using 0,5% methylcellulose as the vehicle.

A 1% suspension of medrysone in polyvinylalcohol 1,4% (HMS liquifilm) was obtained from Allergan (Irvine, CA, USA). The control group was treated with methylcellulose 0,5%.

At 16 hours prior to challenge and twice daily after challenge, the inflamed eyes were treated topically with 30 µl of either IBMX, medrysone or the vehicle (0,5% methylcellulose).

Statistics

For protein, ascorbate, PGE_2 and cell count the values of the uninflamed left eye were subtracted from the values of the inflamed right eye. On the mean differences of these values statistical analysis was performed.

For the Hogan score, analysis was performed on the mean values observed in the inflamed eyes.

Differences between mean values were tested for significance with the Student t-test for unpaired data.

RESULTS

HSA induced anterior uveitis

Intravenous administration of HSA to animals induced a mild anterior uveitis within two hours, that lasted for approximately 14 days in the HSA sensitized eye. No posterior synechiae were seen. The uveitis was characterized by hyperemia which was maximal at six hours and by aqueous flare and aqueous cellular infiltration with a maximum after 24 hours (fig. 1, a,b,c).

In the nonsensitized contralateral eyes no hyperemia, aqueous flare or aqueous cellular response were noted.

At 72 hours protein and PGE_2 levels in the aqueous humor were significantly increased and

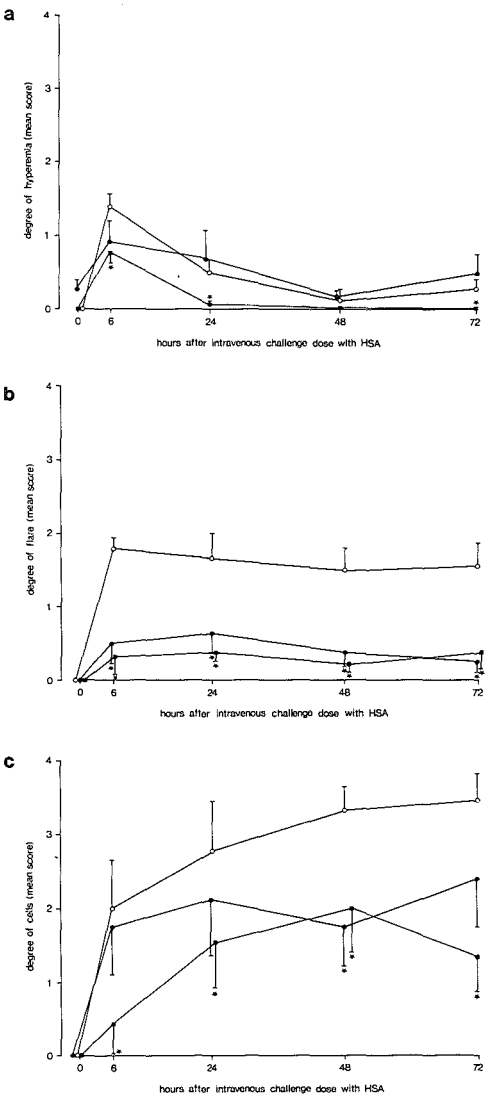

Fig. 1
Hogan's score (mean ± SEM) for:
a) conjunctival hyperemia
b) flare
c) cells
in untreated HSA-induced uveitis, n=9 (O), treated with IBMX 1%, n=8 (●) or medrysone 1%, n=9 (■).
* $P < 0.05$, significance of treated v.s. untreated group.

ascorbate was decreased in the HSA-sensitized eyes in comparison with the non-sensitized contralateral eyes (table 1).

Slitlamp examination

- Conjunctival hyperemia (fig. 1a)

Pretreatment with IBMX induced some conjunctival hyperemia before the uveitis was provoked by the intravenous challenge dose. After medrysone pretreatment no hyperemia was observed.

Conjunctival hyperemia in the medrysone treated group was significantly decreased at 6, 24 and 72 hours compared to the treated group.

IBMX had no effect on conjunctival hyperemia compared to the placebo group.

- Aqueous flare (fig. 1b)

Both IBMX and medrysone significantly reduced the aqueous flare during the course of the uveitis.

- Aqueous white blood cells (fig.1c, table 1)

Hogan's score for white cells was significantly diminished at 6, 48 and 72 hours in the medrysone treated group and in the IBMX treated group at 48 hours.

The cell count at 72 hours was significantly reduced in the medrysone treated group but not in the IBMX treated group (table 1).

Composition of aqueous humor

- Protein

IBMX and medrysone both significantly inhibited the increase in protein content of the aqueous at 72 hours.

- Ascorbate

IBMX and medrysone both significantly inhibited a decrease in ascorbic acid content of the aqueous at 72 hours.

- PGE_2

IBMX but not medrysone significantly inhibited the production of PGE_2 in the aqueous at 72 hours.

DISCUSSION

The results indicate that IBMX and the steroid medrysone are effective inhibitors of several inflammatory responses in the HSA-induced immune complex uveitis.

Medrysone reduced Hogan's score for conjunctival hyperemia, flare and aqueous cells. In the

TABLE 1

MEAN DIFFERENCES FOR PROTEIN, ASCORBATE, PGE_2 AND CELL COUNT IN THE AQUEOUS BETWEEN HSA-INDUCED UVEITIS EYES WITH AND WITHOUT TREATMENT AND THE UNINFLAMED CONTRALATERAL EYES AT 72 HOURS.

	Protein (g/l) OD-OS	Ascorbate (mg/l) OD-OS	PGE_2 (pg/ml) OD-OS	Cell count (10^4/ml) OD-OS
Non treated HSA eyes (n=9)	3.3 ± 0.7	-56.0 ± 13.7	146 ± 61	16.4 ± 5.7
IBMX treated HSA eyes (n=8)	1.6 ± 0.6*	-4.1 ± 2.7***	11 ± 6***	10.6 ± 6.4
Medrysone treated HSA eyes (n=9)	1.5 ± 0.4*	-14.0 ± 5.5**	130 ± 16	2.1 ± 1.5*

Values represent the differences between the levels of inflamed eyes (OD) and uninflamed eyes (OS).
All values are mean ± SEM.
Mean protein and ascorbate values in uninflamed eyes (n=26), were 0,5 g/l and 181 mg/l. In the uninflamed eyes PGE_2 was below detection limit (< 25 pg/ml), and cells were not present.
Significance of difference v.s. control: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

aqueous of medrysone treated rabbits obtained 72 hours after the challenge dose, there was a decreased level of protein, an increased level of ascorbic acid, and a reduced cell count in comparison to vehicle treated animals. No change was observed in the levels of PGE_2. IBMX reduced Hogan's score for aqueous cells at some time points and reduced flare during the whole course of the inflammation. There was no effect on hyperemia, but this observation was hindered by the fact that in a normal non-inflamed rabbit eye some (1+ in Hogan's score) hyperemia was produced by topical treatment with IBMX.

At 72 hours IBMX reduced the increase of protein, the decrease of ascorbic acid, and the increase of PGE_2, but not the aqueous cell count as compared to the non-treated inflamed eyes of the controls.

Medrysone protects the eye against blood-aqueous barrier disruption as well as cellular infiltration in immunogenic uveitis. IBMX only protects the blood-aqueous barrier but has no significant effect on cellular infiltration.

The mechanism of action of corticosteroids is explained by the induced synthesis of lipocortins specific proteins, that are inhibitors of phospholipase A_2 (18). In this way the amount of arachidonic acid available as a substrate for the cyclooxygenase and lipoxygenase pathway is diminished, which may reduce the production of prostaglandins respectively leukotrienes. The mechanism of phosphodiesterase inhibitors in inflammation is explained by the intracellular elevation of cAMP, which reduces the production of PGE_2 in an immediate hypersensitive reaction (9) and the release of lysosomal enzymes in leukocytes at the site of the inflammation (8,10,19). Topical treatment with theophylline reportedly had no effect on blood-aqueous barrier breakdown caused by PGE_2 and systemic treatment increases the blood-aqueous barrier disruption in an inflammatory response which was not accompanied by cellular infiltration (15). These observations of the above study were not confirmed in our experiments using IBMX as inhibitor of phosphodiesterase. However, IBMX is 15 times more potent than theophylline as inhibitor of phosphodiesterase (7) and our model is different in respect of infiltration with leukocytes. It is conceivable that IBMX decreases the production of prostaglandins in these leukocytes and that by this way the blood-aqueous barrier is protected in our model.

Therefore our conclusion is that although intraocular cAMP may cause blood-aqueous barrier

damage, in HSA-induced uveitis in rabbits topical IBMX, probably by elevation of intracellular cAMP levels, markedly supresses a leukocyte-mediated damage of the barrier.

ACKNOWLEDGEMENTS

The authors wish to thank prof. I.L. Bonta, for the suggestion to use IBMX in our inflammatory model and for his critical review of our manuscript, and Mrs. M. Wissing for secretarial assistance.

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Volume 7 number 4 1988

Current Eye Research

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

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Received on July 13, 1987; accepted on February 29, 1988

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 A_2 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 C_4 (LTC_4) and leukotriene D_4 (LTD_4) may cause vascoconstriction followed by capillary leakage and edema (5), while leukotriene B_4 (LTB_4) 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 A_2-activity (7). In this way by depriving the cyclooxygenase as well as the lipoxygenase 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 cycloplasmic receptor and translocation of this complex to the nucleus where it induces the synthesis of a protein whith anti-phospholipase properties. Several of these proteins gave 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

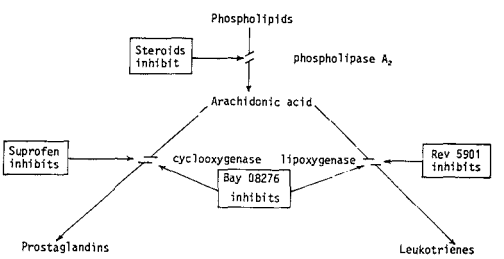

Fig. 1 Schematic representation of the arachidonic acid pathway to inflammatory mediators and interference with the used lipoxygenase and cyclooxygenase inhibitors in this pathway.

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 ulcus in the course of microbi-allergic keratitis, a corneal ulcus 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 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,

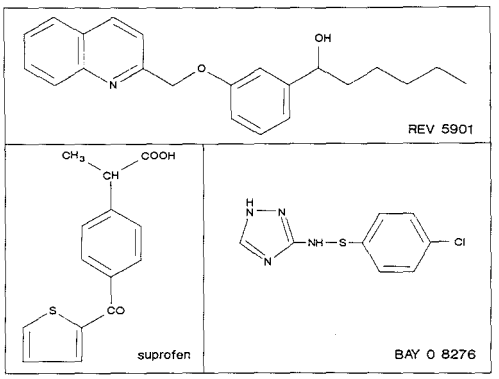

Fig. 2 Structural formulas of the lipoxygenase and cyclooxygenase inhibitors used in treating immunogenic keratitis.

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 Wesseley'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 trapezoideal 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 Wesseley'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

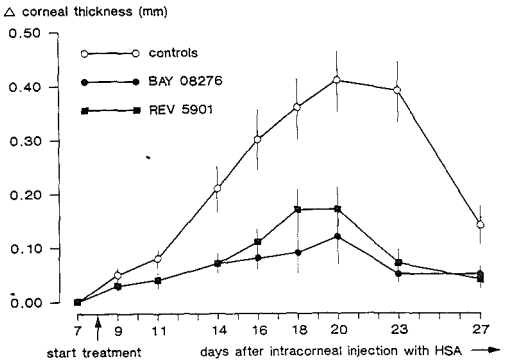

Fig. 3 Comparison of changes in corneal thickness during immunogenic keratitis treated with the lipoxygenase inhibitors Bay 08276 (n=8), Rev 5901 (n=8) and a vehicle treated control group (n=16). Mean Δ corneal thickness was calculated from the difference between the pachymetric measurements before intraocular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

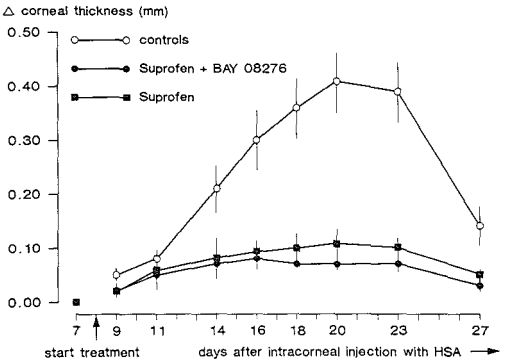

Fig. 4 Comparison of changes in corneal thickness during immunogenic keratitis treated with the cyclooxygenase inhibitor suprofen (n=8), combined treatment with Bay 08276 and suprofen (n=8) and a vehicle treated control group (n=16). Mean Δ corneal thickness was calculated from the difference between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

In the eight rabbits treated with Bay 08276 or Rev 5901 the period of corneal opacification was shorter in comparison with the controls. Wesseley'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 lipoxygenase 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 lipoxygenase 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 appearence 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 corneal neovascularization, but corneal edema was strongly inhibited by suprofen.

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 IC_{50} (μM). (n.d. = not determined).

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 cornal 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 lipoxygenase 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 lipoxygenase 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 lipoxygenase 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.

ACKNOWLEDGEMENT

We thank Mr. J. Verkerk for assistance with animal experiments and Mrs. M. Wissing for secretarial assistance.

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Volume 7 number 6 1988

Current Eye Research

Modulation of immunogenic keratitis in rabbits by topical administration of poly-unsaturated fatty acids

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Received on July 13, 1987; accepted on April 22, 1988

ABSTRACT

Several unsaturated fatty acids are precursors of prostaglandins and leukotrienes. Depending on their precursor, these prostaglandins and leukotrienes have different biological characteristics. The effects of topically administered fatty acids on an experimentally provoked inflammatory keratitis were studied in rabbits . Intrastromal injection with human serum albumin induced in the cornea a ring-shaped infiltration with leukocytes, corneal edema and neovascularization.
Arachidonic, γ-linolenic, dihomo-γ-linolenic (DHGL), eicosapentaenoic (EPA) and columbinic acid were given as eye drops in a suspension in hydroxypropylmethylcellulose 0.5% three times daily during the experiment. EPA, DHGL, columbinic, and γ-linolenic, but not arachidonic acid, showed a significant inhibition of either leukocyte infiltration, edema or neovascularization. The inhibitory effects of these fatty acids may be caused by topical inhibition of the formation of prostaglandins and leukotrienes in the arachidonic acid cascade in the rabbit cornea.

INTRODUCTION

The arachidonic acid cascade is the sequence of events taking place in a tissue when cell membranes, triggered by mechanical, physical or chemical trauma or immune complex deposition, produce the fatty acid arachidonic acid in a kind of autolytic process where the phospholipids of cell membranes are attacked by the enzyme phospholipase A_2.

Arachidonic acid on its turn can be converted by two different ways to prostaglandins or leukotrienes. In ocular inflammation prostaglandins are produced by the enzyme cyclooxygenase and leukotrienes by the enzyme lipoxygenase (1-6).
The prostaglandins are responsible for inflammatory signs such as vasodilatation and tissue swelling by edema formation (7,8). The leukotrienes are involved in leukocyte infiltration by chemotaxis (9-12).

The aim of our study was to intervene in the inflammatory response of the rabbit cornea through possible inhibition of the production of prostaglandins and leukotrienes by topical application of various unsaturated fatty acids.

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 µl pyrogen free human serum albumin (HSA, 20% solution, C.L.B., Amsterdam, The Netherlands) into the cornea of both eyes, according to Morawiecki (13), 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

EPA, DHGL, γ-linolenic acid and arachidonic acid were obtained from Sigma (St. Louis, MO, USA). Columbinic acid was a gift from Dr. Houtsmuller (Unilever Research Laboratories, Vlaardingen, The Netherlands). The fatty acids were stored in the dark under nitrogen to prevent oxidation.

Fatty acid suspensions in concentrations of 3% for columbinic acid and 1 % for EPA, DHGL, γ-linolenic and arachidonic acid, were prepared with

0,5% hydroxypropylmethylcellulose in phosphate buffered saline (pH 7.4) as vehicle.

Both eyes of rabbits were treated with a fatty acid preparation, one drop of 30 µl three times daily instilled unto the conjunctival sac. Treatment was started eight days after immunization and continued for the duration of the experiment. Controls were treated with the vehicle only.

Parameters of ocular inflammation

Keratitis of the rabbit eye was evaluated by measuring corneal edema formation, neovascularization and the occurrance of the annular leukocyte infiltrate in the cornea (Wesseley's ring) (14). 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:

The number of days were recorded during which an opaque corneal ring or a completely opaque cornea was visible as well as the number of days on which growth of vessels into the cornea was visible.

Pachymetry:

A Haag-Streit slit lamp with a pachymeter fitted with central fixation lights according to Mishima and Hedbys (15) was used for measurements of corneal thickness. From each eye the mean of three measurements was taken. Central corneal thickness was measured before and at regular time intervals 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 corneal edema. For each animal separately Δ corneal thickness was plotted against time and from these graphs the area under the curve was calculated using the trapezoideal rule between zero time and 27 days.

Determination of fatty acids in lipids of corneal tissue after topical treatment with eicosapentaenoic acid or columbinic acid

Three groups of four rabbits with normal eyes received during four days a treatment with either the vehicle or a suspension of 3% columbinic acid or 1% EPA. Rabbits were sacrificied using an overdose of penthothal on the fifth day four hours after they received the last dose of the eye drops. Using a 14 mm trephane the corneas were dissected from the intact enucleated eye. The corneas were washed four times in saline to prevent contamination in the analytical procedure with topically applied fatty acids. In each of the three groups of animals right and left corneas were pooled separately for fatty acid analysis.

One volume of methanol was added to the pooled samples and they were stored at -60°C till biochemical analysis. Lipids were extracted from the corneal tissues with a mixture of chloroform: methanol (2:1). The chloroform layer was concentrated with a stream of nitrogen and the residue was transesterified with methanolic hydrochloric acid (2h, 65°C). After extraction with a mixture of hexane/diethyl ether 50/50 and evaporation of the solvent with a stream of nitrogen, the fatty acid esters were chromatographed over silica columns with hexane/diethyl ether 90/10.

The fatty acid methyl esters were analyzed with gas liquid chromatography after removal of the solvent with a stream of nitrogen. A HP 5880 Gas Chromatograph equipped with an automatic sampler (7672 A, Hewlett-Packard) and a FID detector employing a WCOT glass capillary column (CP SIL 88, l = 25 cm, i.d. = 0.22) were used; injection temperature 225°C, detection at 350°C, programmed from 110°C to 186°C with 2°C/min. and 10 min. hold at the final temperature.

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 untreated eyes at any given time.

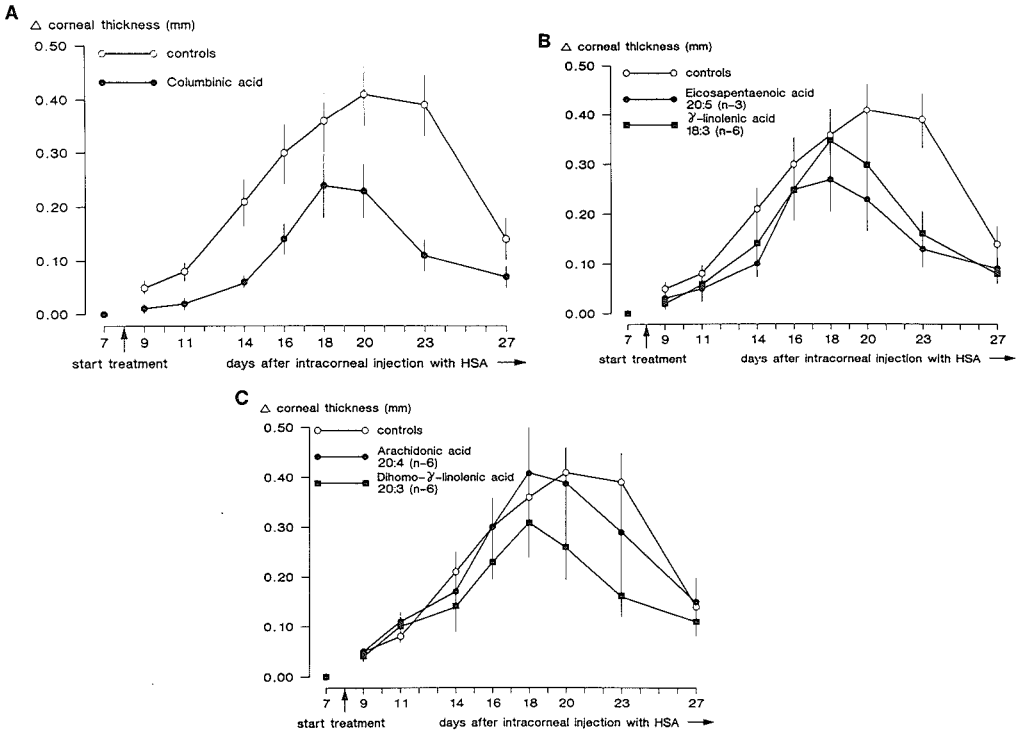

Fig. 1
Changes in corneal thickness during immunogenic keratitis in rabbits (controls; n=16). Eyes were treated topically with suspensions of
(A) columbinic acid 3% (n=8; P < 0.01)
(B) EPA 1% (n=8; P < 0.01); γ-linolenic acid (n=8; P < 0.01)
(C) DHGL 1% (n=8; P < 0.01); arachidonic acid (n=8; n.s.).
P denotes significance of difference vs. controls; n = number of animals; n.s. = not significant.

Significance of difference is given for two tailed observations, P values < 0.05 were regarded as significant.

On the data of the fatty acid analysis no statistical analysis was performed.

RESULTS

Non-treated eyes

The appearence of keratitis in vehicle treated eyes was as follows. One week to ten days after intracorneal injection of HSA, clouding of the cornea started at the limbus and on about day 14-17 a white ring of opacification known as Wesseley's ring (13) appeared.

The ring was present for one to eight days. Within an interval of two to four days vascularization of the cornea started from the limbus, 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 white ring formation and neovascularization. Corneal edema formation recorded with pachymetry

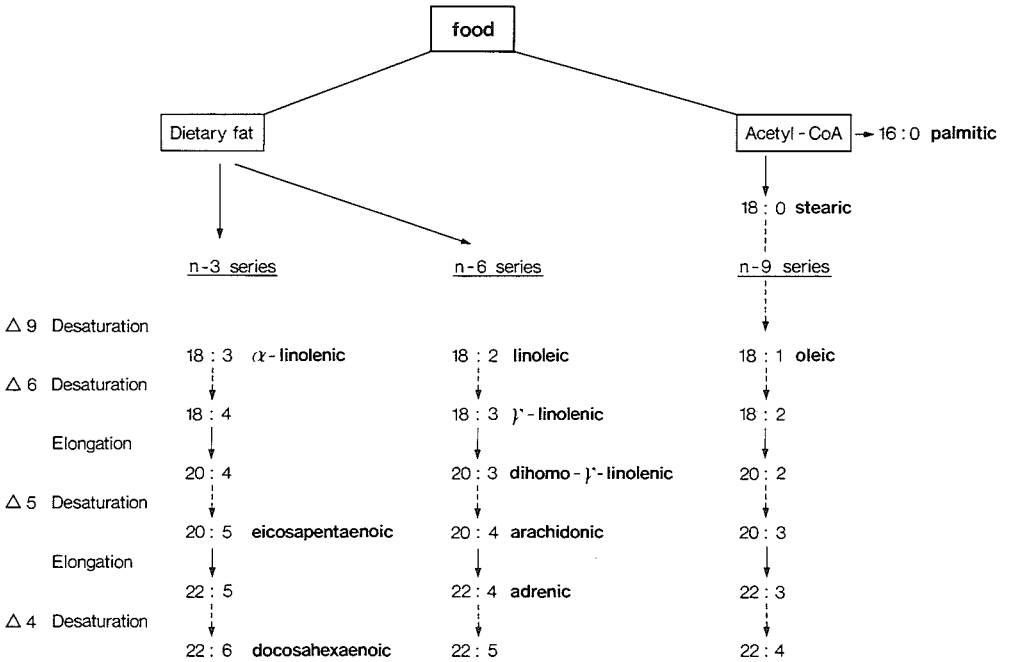

Fig. 2
Schematic representation of three families of unsaturated fatty acids. The place of the first double bound is designated by n. First number of the fatty acid designates chain length, second number after : the number of double bonds of the fatty acid. The sequential reactions are indicated with bold arrows for the elongation reactions and dashed arrows for the desaturation reactions catalyzed by the Δ 9,6,5 and 4 desaturase enzymes.

started around day seven and lasted till day 30 (fig. 1).

Treatment with fatty acids

In rabbits treated with EPA, columbinic acid, DHGL and γ-linolenic acid the period of corneal opacification was significantly shorter in comparison with the controls. Vessel growth was significantly diminished after treatment with EPA, columbinic acid and γ-linolenic acid (table I). These substances and DHGL also significantly inhibited corneal edema formation (Fig. 1, table I).

Topical application of arachidonic acid neither increased nor decreased the inflammatory response (table I, fig. 1c).

Fatty acids in lipids of corneal tissue after topical treatment with eicosapentaenoic acid or columbinic acid (Table II)

After four days of topical treatment, EPA treated animals showed the occurrence of 1,8% EPA (20:5 n-3) and 2.5% of its metabolite (22:5 n-3) in the corneal phospholipids. Columbinic acid treated animals showed the occurrence of 5,6% of this fatty acid in the corneal phospholipids.

The level of arachidonic acid (20:4, n-6) after treatment with both EPA and columbinic acid, decreased to 5,9% and 4,9% respectively in comparison to the level of 7,5% of arachidonic acid in the controls. Also the level of its metabolite

TABLE I

CORNEAL OPACITY, GROWTH OF VESSELS 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	7,2 ± 0,8	100 ± 13 (2-183)
Columbinic acid 3% (18:2 n-6trans) (n=8)	3,7 ± 0,7***(2-5,5)	3,7 ± 0,7** (0-5,5)	47 ± 10^{++}(3-85)
Eicosapentaenoic acid 1% (20:5 n-3) (n=8)	3,3 ± 0,5***(2-5,5)	4,3 ± 0,8* (1,5-7)	60 ± 11^{++}(22-118)
Dihomo-γ-linolenic acid 1% (20:3 n-6) (n=8)	3,9 ± 0,5** (2-5,5)	4,9 ± 0,9 (1,5-9)	70 ± 15^{+} (18-152)
γ-linolenic acid 1% (18:3 n-6) (n=8)	4,6 ± 0,5* (2,5-6)	4,8 ± 0,6** (2-7)	71 ± 12^{++}(24-100)
Arachidonic acid 1% (20:4 n-6) (n=8)	5,3 ± 1,0 (2-9,5)	6,4 ± 1,0 (0-8)	97 ± 21 (16-201)

AUC: area under the curve
° : mean ± SEM (range)
Significance of difference vs controls for duration of corneal opacity and vessel growth was calculated with the Mann-Whitney U test.
* P < 0,05; ** P < 0,01; *** P < 0,002
Mean pachymetry values of controls and treated animals at various points during the inflammation were tested for significance with Wilcoxon's signed rank test
$^{+}$ P < 0,05; $^{++}$ P < 0,01

(22:4 n=6) was decreased in the same ratio as in the controls to 2,5% and 1,9% respectively. The oleic acid (18:1) level decreased and the palmitic acid (16:0) level increased in the corneal phospholipids of EPA and columbinic acid treated animals.

The total amount of the free fatty acids was 4% of the amount of phospholipid bound fatty acids. Columbinic acid and EPA occurred in the free fatty acid fraction, however the level of these fatty acids was low compared to the phospholipid bound fraction.

DISCUSSION

Treatment with the fatty acids columbinic acid, EPA and γ-linolenic acid was effective in the inhibition of leukocyte infiltration, neovascularization and corneal edema formation. Regarding neovascularization and corneal edema, columbinic acid showed the most effective inhibition. EPA was the most effective inhibitor of leukocyte infiltration. DHGL showed a significant inhibition of leukocyte infiltration and edema formation but not of neovascularization. Arachidonic acid treatment had neither an inhibitory nor a stimulating effect on the parameters of the immune-complex keratitis. 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 the fatty acids used in this model of corneal disease. In our impression the number of days on which corneal opacification occurred were directly correlated with the area of corneal

TABLE II

THE EFFECT OF TOPICAL ADMINISTRATION OF COLUMBINIC ACID (18:3 5,9,12) OR EICOSAPENTAENOIC ACID (20:5 n-3) ON THE COMPOSITION OF RABBIT CORNAL TISSUE PHOSPHOLIPID BOUND AND FREE FATTY ACIDS.

Fatty acid		Fatty acid composition (weight %)					
Numerical symbol	Trivial name of of fatty acid	Controls PL	Controls FFA	Columbinic acid PL	Columbinic acid FFA	Eicosapentaenoic acid PL	Eicosapentaenoic acid FFA
16:0	palmitic	10.9	0.7	13.8	0.4	15.8	0.7
18:0	stearic	8.9	1.4	10.6	0.9	9.0	1.1
18:1 n-9	oleic	42.5	0.8	37.1	1.4	35.7	1.1
18:2 n-6	linoleic	0.9	0.0	0.7	0.1	0.5	0.0
18:3 n-6 (trans)	columbinic	0.0	0.0	5.6	0.4	0.0	0.0
20:4 n-6	arachidonic	7.5	0.0	5.9	0.2	4.9	0.0
20:5 n-3	eicosapentaenoic	0.0	0.0	0.0	0.0	1.8	0.1
22:4 n-6		3.0	0.0	2.5	0.1	1.9	0.0
22:5 n-3		0.7	0.0	0.5	0.0	2.5	0.1
22:6 n-3	docosahexanoic	0.3	0.0	0.0	0.0	0.3	0.0

The numerical symbol designates the chain length and the number of double bounds of fatty acid, n designates the place of the first double bound.
Values are the mean of two pooled corneal tissue preparations obtained from four different animals, extracted and analyzed as described in the Methods section.
(PL = phospholipid bound fatty acids, FFA = free fatty acids).

opacification. The same was observed for corneal neovascularization.

All animals, treated with vehicle only, responded with corneal opacification, neovascularization and edema. The appearance of opaque rings and neovascularization in the cornea is in accordance with previous observations using this model of corneal anaphylaxis (16-18).

Histology has shown that the opaque ring consisted mainly of a polymorphonuclear leukocyte infiltrate (24). Chemotactic factors produced at the site of the antigen-antibody precipitate may attract leukocytes. The invaded leukocytes will produce prostaglandins and chemotactic leukotrienes (5,6). Prostaglandins, especially PGE_1 and PGE_2, have been determined as the most important stimuli for neovascularization (19). The leukocyte products will result in even more leukocyte infiltrate, neovascularization and edema. Agents that can prevent the formation of prostaglandins and chemotactic leukotrienes in the cornea will eventually lead to a decrease of the inflammatory symptoms of immune complex keratitis (18).

In vitro cyclooxygenase inhibition can be achieved in different ways; by enzyme inhibition with non-steroidal anti-inflammatory drugs (19) or by competition with structurally related fatty acids of arachidonic acid (20). Many different fatty acids can interact with the active site of cyclooxygenase, but the number and position of the

double bonds determine how well inhibitors can prevent synthesis of prostaglandin (20). Inhibition of lipoxygenase is possible by some experimental compounds (21-23). Little is known about the competitive inhibitory effects of fatty acids on the activity of lipoxygenase.

Figure 2 shows a classification of the fatty acids we used in our experiments. We selected these fatty acids because of their different possible inhibitory effects on the formation of prostaglandins and leukotrienes from arachidonic acid. The γ-linolenic acid and DHGL can be converted by desaturase enzymes to arachidonic acid (24). DHGL can also be converted directly to prostaglandins of the ^{1}-series for example PGE_1, by cyclooxygenase and to 15 hydroxyeicosatetraenoic acid by lipoxygenase (25,26). EPA, a fatty acid found in fish oil prepared from various kinds of marine fishes, can be metabolized to prostaglandins of the 3-series and leukotrienes of the 5-series (27-30). Columbinic acid is a newly discovered type of unsaturated fatty acid of botanical origin (31) and differs in its structure from γ-linolenic acid by cis-trans isomerie at one of the double bonds. Formation of the arachidonic acid by desaturation can not take place from this fatty acid (31,32). DHGL and arachidonic acid possess the same substrate specificity for the production of prostaglandins by cyclooxygenase (33), but biological activity of PGE_1 (DHGL-derived) is less compared to PGE_2 (arachidonic acid-derived) (33). Substrate specificity of EPA for cyclooxygenase is much less in comparison with arachidonic acid and DHGL (33). EPA-derived PGE_3-formation hardly takes place in vivo in the rabbit eye (34).

We determined the incorporation of the two most efficient inhibitors of immunogenic keratitis, EPA and columbinic acid in the cornea after four days of topical application. Both EPA and columbinic acid showed an efficient incorporation into the phospholipids of the cornea, with concomittant lower levels of arachidonic acid, free fatty acid level was low. Phospholipase A_2-activation by an inflammatory stimulus therefore may lead to a release of these poly-unsaturated fatty acids besides lower levels of the naturally occurring ararchidonic adcid from the cell membranes of corneal tissue.

In conclusion local application of unsaturated fatty acids opens new perspectives in the treatment of corneal inflammatory diseases through modulation of the inflammatory response resulting in less leukocyte infiltration, neovascularization and corneal edema formation.

ACKNOWLEDGEMENTS

We thank Mr. J. Verkerk and Mr. W. Heerooms for assistence with animal experiments, Mr. P. Moret (Unilever Research Laboratory, Vlaardingen, The Netherlands) for the fatty acid analysis, Dr. U.T.M. Houtsmuller for his gift of columbinic acid and Mrs. M. Wissing for secretarial assistance.

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Ginkgolides - Chemistry, Biology, Pharmacology and Clinical Perspectives. P. Braquet (Ed.)
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INTERFERENCE OF A GINKGOLIDE WITH MODELS OF CORNEAL DISEASES

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INTRODUCTION

Platelet-activating factor (1-O-alkyl-2-acetyl-glycero-3-phosphocholine, PAF-acether) is one of the most potent inducers of platelet aggregation and has a wide spectrum of biological activities as a very potent mediator of inflammation, increasing vascular permeability.

A specific PAF-acether antagonist BN 52021, a ginkgolide isolated from *Ginkgo biloba* extract which interferes with receptors for PAF-acether (3), is now available.

Various cell types are potential sources for the formation of PAF-acether in pathophysiological conditions; PAF-acether is released during chemical or immune stimulation of neutrophils, vascular endothelium, thrombocytes and macrophages (2, 12).

In the eye, PAF-acether decrease the biwave response of the rat electroretinogram *in vitro* (6), and when systemically administered PAF-acether gives rise to edema of the rabbit retina *in vivo* (4). Treatment with the *Ginkgo biloba* extract resulted in protection of the retinal tissue against argon laser damage (5).

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In the cornea, implantation of polymere pellets impregnated with PAF-acether causes formation of newly formed vessels in this normally non-vascular tissue (9).

Immune keratitis can be used as a model to evaluate the effect of drugs on leukocyte infiltration, neovascularization and edema in corneal tissue.

The participation of mediators in this pathology is beginning to be demonstrated but the results obtained on animal models with specific inhibitors of cyclooxygenase are contradictory. Corticosteroids prevent the occurrence of keratitis completely and nonsteroid antiinflammatory drugs such as indomethacin demonstrated inhibition of leukocyte infiltration at high concentrations (1). However, at lower concentrations an increase of leukocyte infiltration was observed (11), indicating that inhibition of the formation of prostaglandins in the cyclooxygenase pathway may facilitate the generation of leukotrienes in the lipoxygenase pathway and that at high concentrations inhibition of both pathways takes place.

In order to clarify the possible role for PAF-acether in the model of corneal inflammation and in corneal wound healing we investigated the effect of topical application with the PAF-acether receptor antagonist BN 52021.

MATERIALS AND METHODS

Animals

Male pigmented Chinchilla rabbits (2-2.5 kg) were sedated with Hypnorm (fluanison 10 mg/ml and phentanyl citrate 0.2 mg/ml) 0.75 ml/kg body weight and oxybuprocain (novesin) was applied on the cornea for topical anesthesia.

Corneal Denudation

The entire corneal epithelium was removed from both eyes by scraping with a perpendicular blade from limbus to limbus. The removal of the corneal epithelium was performed carefully in order to preserve as far as possible the integrity of the basement membrane (Fig. 1).

Immune Keratitis

Immunization was performed by injection of 20 μl of pyrogen-free human serum albumin (20% solution) using a 27 gauge needle in the corneal stroma of both eyes according to Morawiecki (8) (Fig. 2).

Wound Healing

Wounds after corneal scraping were stained with fluorescein (0.25% solution of fluorescein sodium), the eye was washed with NaCl 0.9% in order

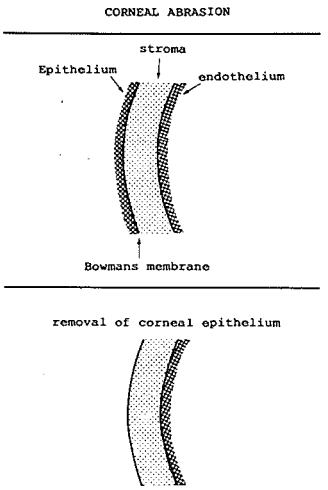

Figure 1 Schematic representation of corneal denudation.

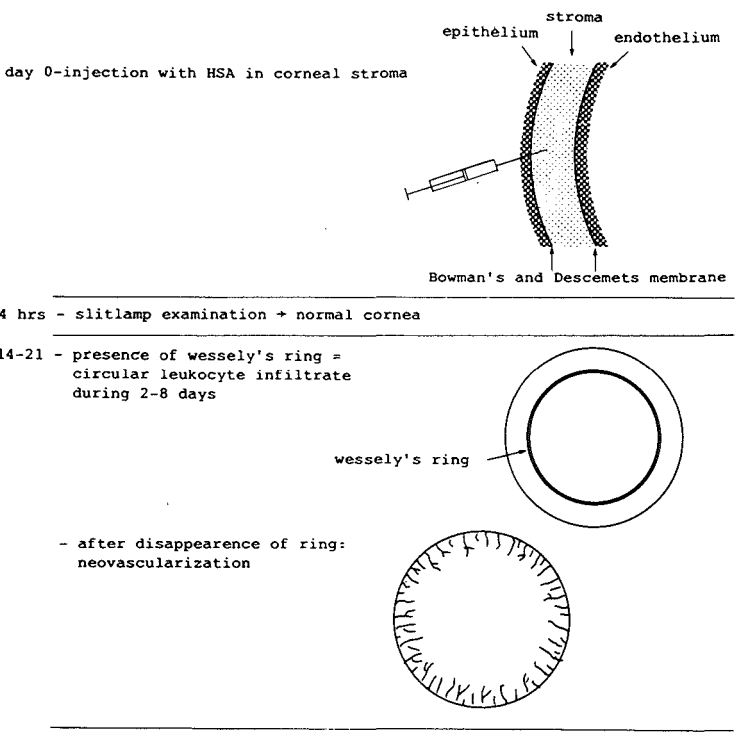

Figure 2 Schematic representation of the model of immunogenic keratitis.

to remove excess fluorescein and photographed at regular time intervals until healed. Photographs were projected and wounded areas were determined by planimetry. Results were calculated in percent re-epithealization of the cornea.

Corneal Thickness

Corneal thickness was measured with a Haag-Streit slit lamp and pachometer, fitted with central fixation lights according to Mishima and Hedbys (7). The use of the fixation lights is essential in order to obtain consistent measurements of central corneal thickness. From each eye the mean of three measurements was taken. The difference between the corneal thickness after and before abrasion or immunization is presented as corneal thickness.

In the immune keratitis experiment for each animal the values of both eyes were averaged.

The observations were organized in a masked fashion, where the observer was not aware of the schedule of drug treatment.

Corneal Aspect

The number of days was recorded during which a white ring or a diffuse, completely opaque cornea was present and during which neovascularization was visible.

Drug Treatment

BN 52021 was freshly prepared every day as a 1% suspension in viscous ophthalmic solvent containing 0.5% hydroxypropylmethylcellulose and 0.9% NaCl without addition of preservatives.

In the corneal abrasion experiment in eight rabbits at random one eye was treated with 30 μl of a 1% suspension of BN 52021 and the other eye with the vehicle, three times daily by instillation into the conjunctival sac. Treatment started one day before abrasion and continued for the duration of the experiment.

In the immune keratitis experiment in eight rabbits both eyes were treated with 30 μl of BN 52021 and in eight rabbits both eyes received the vehicle three times daily by instillation into the conjunctival sac. Treatment started eight days after immunization and continued for the duration of the experiments.

RESULTS

Immune Keratitis

Seven to ten days after intracorneal injection of HSA the appearance of keratitis in the eyes of the vehicle-treated rabbits started with clouding of the cornea at the limbus and between day 14 and 17 an opaque ring was formed, known as Wessely's ring (13), remaining for one to eight days. Sometimes the whole cornea became completely opaque in this period. Two to four days after the appearance of the white ring vascularization of the cornea started from the limbus and progressed until about day 24 and then regressed quickly, resulting in all cases in a clear cornea 30 days after immunization. Swelling of the cornea as recorded by pachometry started at about eight days after immunization and lasted until about day 30.

All animals injected with HSA responded with white ring formation and neovascularization. Control rabbits with normal cornea treated with BN 52021 showed no changes in the aspect of the cornea.

In rabbits treated with BN 52021 the period of corneal opacification was significantly shorter but not the period of neovascularization, as compared to that in non-treated animals (Table 1). The extent of swelling, determined as mean area under the curve (Fig. 3), was significantly different from that in untreated controls.

Table 1 Immunogenic keratitis in rabbits treated with topical BN 52021

	Period of corneal opacity (days)	Period of corneal neovascularization (days)	Pachometry AUC (% of control)
Controls (n = 8)	5.5 + 0.7	6.8 + 1.6	100 + 23
BN 52021 1% (n = 8)	2.5* + 1.6	7.0 + 1.6	59* + 21

AUC: area under the curve.
All values are mean + SEM.
*Mann-Whitney U test ($P < 0.05$ for BN 52021 *vs.* control).
*Wilcoxon's signed rank test ($P < 0.01$ for BN 52021 *vs.* control).

Corneal Abrasion

In the vehicle-treated eyes complete restoration of the corneal surface was observed by 168 hours after scraping of the epithelium.

In all animals improvement was observed in the treated eye at 6 hours with complete restoration at 96 hours (Fig. 4).

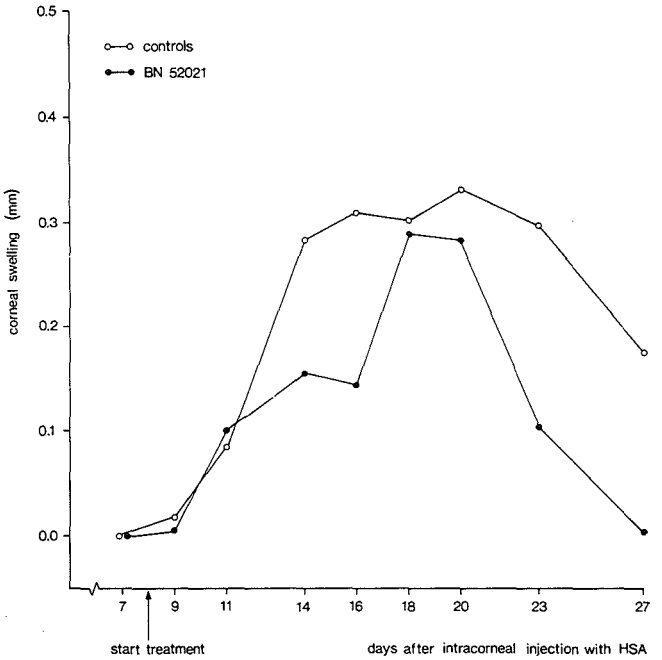

Figure 3 Effect of treatment with eye drops containing 1% BN 52021 (●) on corneal thickness as compared to vehicle-treated controls (○) in rabbits during immune keratitis provoked by injection with human serum albumin (HSA).

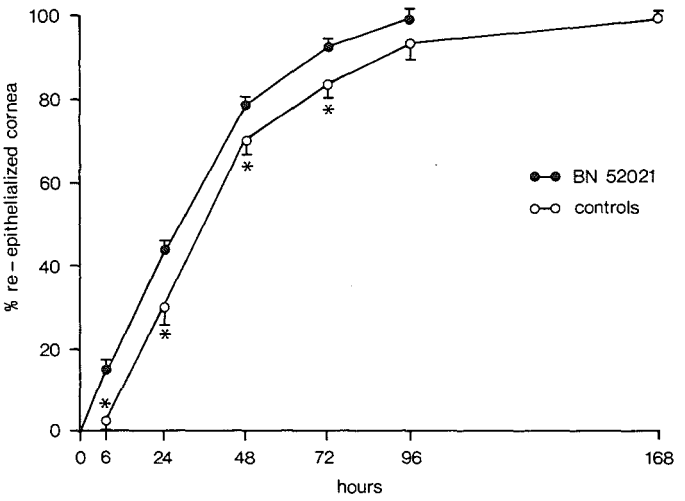

Figure 4 Effect of treatment with eye drops containing 1% BN 52021 (●) on corneal re-epithelialization as compared to vehicle-treated controls (○) in rabbits after corneal denudation.

Corneal swelling in vehicle-treated eyes was observed until 168 hours after scraping of the epithelium. In the eyes treated with BN 52021, after 6 hours less swelling was recorded as compared to the vehicle-treated contralateral eye. This difference in swelling persisted until at 168 hours after scraping the corneal swelling in the treated eyes had disappeared and the corneal thickness was the same as before the experiment (Fig. 5).

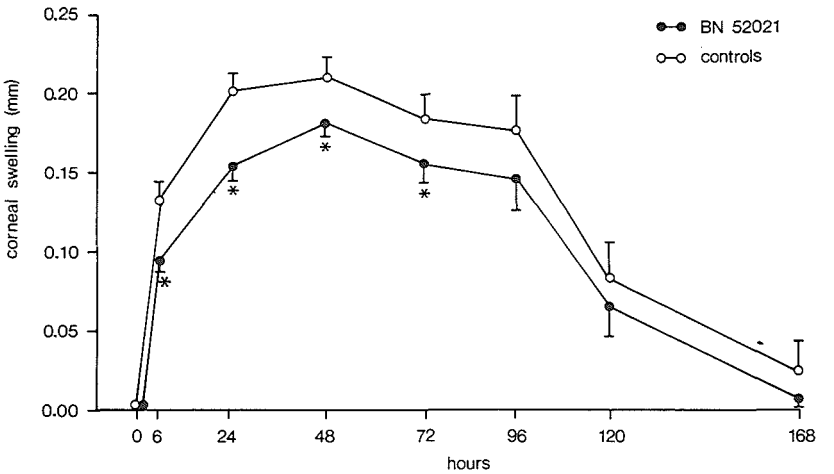

Figure 5 Effect of treatment with eye drops containing 1% BN 52021 (●) on corneal swelling as compared to vehicle-treated controls (○) in rabbits after corneal denudation.

DISCUSSION

From a therapeutic point of view topical treatment of affections of the eye has the advantage of a direct accessibility to the surface of the eye with medications and the possibility of rapid resorption through the cornea into the inner eye. In our experiments a viscous solvent was used, giving a longer cornea-contact time, in order to promote local drug resorption into the cornea.

In preliminary experiments with the model of immune keratitis we tried to ameliorate drug resorption in the cornea by removal of the corneal epithelium, which might be a barrier against drug penetration. There was no difference in the observed effect of BN 52021 on immune keratitis; however, it was striking that the epithelial defect healed earlier in the eye treated with BN 52021 as compared to the vehicle-treated contralateral eye. This prompted us to investigate in a separate experiment the effect of BN 52021 after corneal denudation in a normal eye.

The effect of the specific PAF-acether antagonist BN 52021 on leukocyte infiltration and corneal swelling in immune keratitis and on wound healing and corneal swelling after corneal denudation reveal a role for PAF-acether in these signs of ocular inflammation. Rochels (9) proved that PAF-acether, implanted in polymere pellets in the rabbit cornea, gives rise to neovascularization. This process of PAF-acether-induced corneal neovascularization can be fully inhibited by topical treatment with indomethacin, suggesting the formation of prostaglandins as a necessary step in the growth of new vessels. If there exists a positive feedback system between PAF and prostaglandins in the inflamed cornea this system is not inhibited efficiently enough by BN 52021 to prevent to some extent neovascularization.

In both models BN 52021 was given prophylactically before signs of inflammation started to occur. Corneal thickness in immune keratitis was only influenced before and after maximal swelling in contrast to the swelling after denudation, which was directly less intensive from the beginning and throughout the experiment.

From studies with nonsteroidal antiinflammatory compounds such as indomethacin and flurbiprofen it is known that prostaglandins probably do not play a role in conjunctival epithelial cell migration after corneal denudation (10). The time-related effect of BN 52021 on corneal swelling and reepithelialization already within six hours after corneal denudation and the parallel in the course of these parameters until complete healing of the cornea suggest a common cause mediated by PAF-acether. From the experiments it cannot be determined if improved healing of corneal wounds is the cause or the effect of decreased corneal swelling.

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Investigative Ophthalmology & Visual Science, Vol. 31, No. 8, August 1990

The Influence of a Fish Oil Dietary Supplement on Immunogenic Keratitis

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Fish lipids contain large amounts of eicosapentaenoic acid (EPA) and docosahexaenoic acid. These fatty acids are known to have an influence on prostaglandin (PG) and leukotriene (LT) synthesis. We studied the effect of a fish oil dietary supplement on an immune-complex-induced keratitis of the rabbit eye and compared it with the effect of a sunflower seed oil dietary supplement, rich in linoleic acid. Immune complex keratitis induced by intrastromal injection of human serum albumin (HSA) was characterized by leukocyte infiltrate, neovascularization, and corneal edema. Animals given a fish oil diet showed significantly less leukocyte infiltrate, neovascularization, and corneal edema, compared to those given a sunflower seed oil diet. Invest Ophthalmol Vis Sci 31:000–000, 1990

Prostaglandins (PGs) and leukotrienes (LTs) play an important role in ocular inflammation.[1–8] PGs contribute to the formation of edema and erythema.[9,10] LTB_4 is a potent chemotactic agent that plays a role in the migration of leukocytes into sites of inflammation.[11–13] These inflammatory mediators are derived from the fatty acid arachidonic acid (AA) by metabolic conversion catalyzed by the enzymes cyclooxygenase and lipoxygenase (Fig. 1). AA is released from the phospholipids of the cell membrane in response to injury or inflammation by phospholipase A_2.[14] In addition to AA, some other fatty acids, such as eicosapentaenoic acid (EPA) and dihomo-γ-linolenic acid (DHGL), may also give rise to PG and LT formation. If PGs and LTs are synthesized from a fatty acid, each fatty acid has its own PG and LT counterparts with different chemical structures and biologic behaviors.[15]

The fatty acid composition of the diet determines the ratio in which fatty acids are incorporated into the phospholipids of the cell membrane.[16] There is considerable evidence that human populations whose dietary protein is mainly derived from fish are at low risk for cardiovascular disease.[17–22] A starch- and casein-derived diet containing 6.7% fish oil has shown a beneficial influence on edema formation, systemic lupus erythematosus, immunologically induced arthritis, and glomerulonephritis in experimental animals.[23–26] Fish lipid contains large amounts of EPA and docosahexaenoic acid (DCHA), which are only very minor components of food derived from terrestrial plants and animals. EPA and DCHA as dietary supplements lead to diminished synthesis of PGE_2 and LTB_4[16,27–30] as a result of competitive inhibition of metabolism of AA in the cyclooxygenase and lipoxygenase pathways by EPA and the formation of PGE_3 and LTB_5 from EPA (Fig. 1).[15,28–33] Rabbit, monkey, and human uvea and conjunctiva are able to metabolize EPA to PGE_3 and LTB_5 in vitro.[34,35]

A type III immune complex reaction of the rabbit cornea can be used as a model for testing anti-inflammatory properties of drugs. In this animal model, after intrastromal injection of antigen, the eye first remains quiet for 10–14 days. During this time, specific antibody production is generated in the local draining lymph nodes and spleen. An abrupt onset of inflammation occurs when sufficient specific antibody complexes with local antigen. These antibody–antigen complexes activate the complement cascade with the production of chemotactic factors that are responsible for polymorphonuclear cell infiltration.[36] These polymorphonuclear leukocytes (PMLs) produce, among others, AA products. PGs and LTs are major factors in neovascularization and edema formation of the cornea as well as in the positive feedback mechanism of PML infiltrate formation.[37]

We compared the influence of a diet containing 6.7% fish oil and 2.7% sunflower seed oil with a diet containing 9.4% sunflower seed oil, rich in linoleic acid (LA), on the clinical appearance of human serum albumin (HSA)-induced immunogenic keratitis in rabbits.

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Submitted for publication: June 7, 1989; accepted January 9, 1990.

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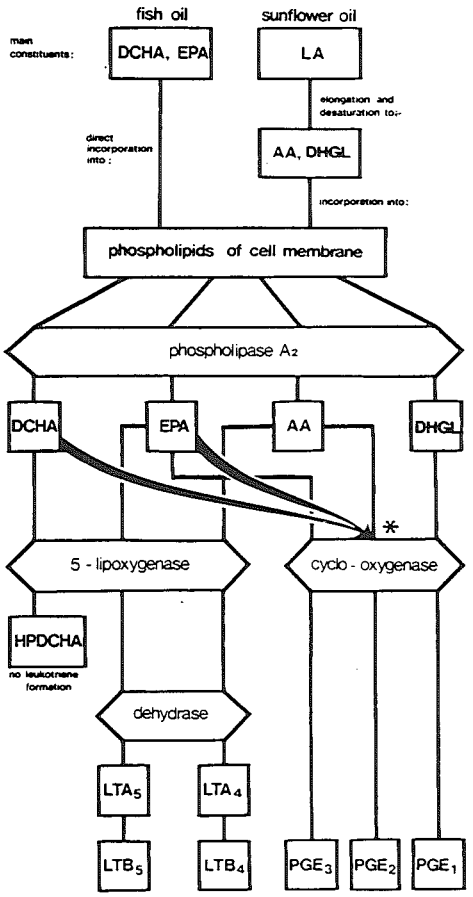

Fig. 1. PGE and LTB formation from their precursor fatty acids. DCHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; DHGL, dihomo-γ-linolenic acid. *, Competitive inhibition.

Materials and Methods

Animals

The experiments were performed in male pigmented chinchilla rabbits weighing at least 2.0 kg. All eyes were initially examined with a slit lamp. Only animals without any sign of ocular inflammation were included in the study. All procedures in this study adhered to the ARVO Resolution of the Use of Animals in Research.

Diet and Feeding Procedures

The rabbits were maintained on standard rabbit diet (Hope Farms, Woerden, The Netherlands) prior to commencing the experimental diet. Because sufficient control of the exact fatty acid composition of a commercial rabbit diet is not possible, we chose a semi-synthetic experimental diet in which the composition of nutrients can be controlled exactly. Both diets contained 2.7% sunflower seed oil, and the total fat was increased to 9.4% by adding either 6.7% more sunflower seed oil or 6.7% fish oil. These two diets will be referred to as "the sunflower seed oil diet" and "the fish oil diet". The composition of the experimental diets is given in Tables 1 and 2.

Diet and water were given ad libitum to both groups. Body weight was measured twice a week.

The diet for the two groups of rabbits contained 30.1 g fat per 1.000 kcal (energy = 28%, weight = 9.4%). The fish oil was extracted from mackerel and contained 13.3% EPA (20:5) and 7.1% DCHA (22:6).

The animals were gradually acclimated to their experimental dietary supplements in five steps of 2 weeks each. At each step a higher percentage of the standard diet was replaced by the experimental diets.

The animals were kept for at least 6 months on a 100% semi-synthetic diet prior to the experiments. (Materials for the diets were provided by Unilever, Vlaardingen, The Netherlands.) Weights were similar in rabbits fed with fish-oil-enriched or sunflower seed oil enriched food at the completion of this feeding period. Before the start of adjusting the animals to the experimental diets, weights were 1450 ± 36 g and 1514 ± 44 g, and at the time of the keratitis experiments were 2751 ± 101 g and 2625 ± 115 g (mean ± SEM), respectively, for the animals that received the sunflower seed oil diet or fish oil diet.

Immunization

Immunization of the pigmented rabbits was performed by injection of 20 μl pyrogen-free HSA (20% solution; CLB, Amsterdam, The Netherlands) into the cornea of both eyes according to Morawiecki,[38] after corneal anesthesia with 0.4% Oxybuprocaine and sedation by Hypnorm™ (fluanison 10 mg/ml and phentanyl citrate 0.2 mg/ml), 0.75 ml/kg body weight.

Parameters of Ocular Inflammation

The effect of the diet on the clinical appearance of this HSA-induced immunogenic keratitis of the rabbit eye was evaluated by measuring corneal edema

Table 1. Composition of semi-synthetic rabbit diets

Ingredient	Diet: Fish oil (g/1000 kcal)	Diet: Sunflower seed oil (g/1000 kcal)	Weight (%)		Energy (%)	
Corn starch*	140	140	43.7		49	
Casein†	62	62	19.3		23	
Crude fiber‡	60	60	18.7		—	
Salts mixture§	25	25	7.8		—	
Vitamin mixture‖	2	2	0.6		—	
Grass meal¶	1.5	1.5	0.5		—	
Sulfa quinoxaline	0.04	0.04	0.0		—	
Fish oil	21.5	—	6.7	—	20	—
Sunflower seed oil	8.6	30.1	2.7	9.4	8	28
	320.6	320.6	100	100	100	100

* 4.1 kcal/g (N.V. Honig's Artikelen, Koog a/d Zaan, The Netherlands).
† 4.1 kcal/g (D.M.V., Veghel, The Netherlands).
‡ Sawdust (sterilized) (Broekman Instituut, Someren, The Netherlands).
§ Composition in milligrams: $CaCO_3$ 7250; $CoSO_4$ 0.3; Cu citrate 12; Fe(III) citrate 290; KCl 5970; KH_2PO_4 1930; KIO_3 0.3; $MgHPO_4 \cdot 3H_2O$ 6320; $MnSO_4 \cdot 4H_2O$ 72; Na acetate 3100; Zn citrate 35.
‖ Composition in milligrams: vitamin A acetate 12; p-amino-benzoic acid 147; biotin 0.5; cholecalciferol 5; choline 587; all-rac-α-tocopherol-acetate 60; folic acid 3; *myo*-inositol 294; vitamin K 5; nicotinamide 45; pantothenic acid (Ca salt) 17, pyridoxine 7; riboflavin 7; thiamin (monocitrate) 18.
¶ Coöperative Landbouwvereniging "De Samenwerking", Maasland, The Netherlands.

formation, neovascularization, and the occurrence of an annular leukocyte infiltrate in the cornea (Wessely's ring).[39] These three parameters of corneal inflammation can be well observed in vivo. The clinical observation was organized in a masked fashion.

Table 2. The fatty acid composition of sunflower seed oil and fish oil used in the experimental diets

Fatty acid: Numerical symbol	Fatty acid: Trivial name	Fatty acid composition (Wt/Wt %): Sunflower seed oil*	Fatty acid composition (Wt/Wt %): Fish oil†
14:0	myristic	0.1	11.5
15:0	pentedecanoic	0.1	—
16:0	palmitic	7.1	24.1
16:1 (n = 7)	hexadecanoic	0.1	12.4
17:0	heptadecanoic	0.1	—
18:0	stearic	4.3	3.7
18:1 (n = 9)	oleic	18.8	14.9
18:2 (n = 6)	linoleic (LA)	68.3	1.4
18:4 (n = 3)	octadecatetranoic	—	4.0
20:0	arachidic	0.3	—
20:1 (n = 6)	eicosenoic	0.2	1.5
20:4 (n = 6)	arachidonic (AA)	—	0.7
20:5 (n = 3)	eicosapentaenoic (EPA)	—	12.3
22:0	behenic	0.6	—
22:1 (n = 3)	erucic	—	1.0
22:5 (n = 3)	docosapentaenoic	—	2.1
22:6 (n = 3)	docosahexaenoic (DCHA)	—	9.0
Total		100.0	100.0

The numbers are the percentages of total fatty acids. The numerical symbol designates the chain length and the number of double bonds in the fatty acid, and n designates the place of the first double bond, counting from the methyl end.
* Union N.V., Antwerp, Belgium.
† Unimills B.V., Zwijndrecht, The Netherlands.

Corneal aspect: The number of days during which opaque rings or a diffuse completely opaque cornea were visible were recorded from color slides taken daily (flash intensity 50 mW, distance 5 cm). For each animal the values of both eyes were averaged.

Neovascularization: The extent of vessel growth into the cornea was determined every other day from the color slides. Diapositives were projected and enlarged 12 times. By planimetry the area with new vessels was determined as a percentage of the total corneal surface. The percentage of vessel growth was plotted against time in days, and from these graphs the area under the curve was measured by planimetry. For each animal the values of both eyes were averaged.

Pachymetry: A Haag-Streit slit lamp with a pachymeter fitted with central fixation lights according to Mishima and Hedbys[40] was used for these measurements. The use of the fixation lights is essential in order to obtain consistent measurements of central thickness. In inflamed edematous corneas, pachymetry measurements were subject to greater inaccuracy, which, however, did not exceed a relative measurement error of 5%. From each eye the mean of three measurements was taken. For each animal the values of both eyes were averaged.

Central corneal thickness was measured before intracorneal injection with HSA and every other day from day 7 until day 29. For each animal the difference between pachymetry measurement before intra-

ocular injection with HSA and the measurement at any time thereafter was recorded as the change in corneal thickness. For each animal the corneal thickness was plotted in time, and from this graph the area under the curve was measured.

Determination of Anti-HSA Immunoglobulins by Enzyme-Linked Immunosorbent Assay (ELISA)

Serum was obtained from the rabbits of the two dietary regimens on day 29 after the first intracorneal injection with HSA. Microcuvettes (Gilford, Cleveland, OH) were coated with coating buffer containing 10 μg/ml of the HSA, used as antigen for the intracorneal injections on day 0. After a coating period of 1 hr at room temperature the cuvettes were washed three times with phosphate-buffered saline (PBS) containing 0.1% Tween 80. Subsequently 250-μl serum samples, diluted 1/10.000 in 0.1% Tween in PBS were added to the cuvettes. After an incubation period of 1 hr at room temperature the cuvettes were washed three times with 0.1% Tween in PBS. Subsequently the cuvettes were incubated with a 1/500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin for 1 hr at room temperature (Nordic, Tilburg, The Netherlands). After being washed three times with 0.1% Tween in PBS, bound peroxidase-labeled antibody was developed at room temperature by adding 350 μl ABTS solution (0.16 mM 2,2-azino-di-3-ethyl-benzthiazoline-6-sulphonate [Boehringer, Mannheim, West Germany] + 0.15% H_2O_2 in 0.05 M citric acid, pH 4.0). The green reaction product was measured after 20 min in a spectrophotometer (EIA; Gilford) at 405 mm.

As a standard, a sample of purified rabbit anti-HSA immunoglobulin was used. This standard contained 10.000 ELISA units per 0.37 μg rabbit anti-HSA immunoglobulin.

Statistical Analysis

Data were analyzed by nonparametric methods to avoid assumptions about the distribution of the variables involved.

Wilcoxon's signed-rank test was applied for pachymetry data obtained in the treated and untreated groups during the period of inflammation. The Mann-Whitney U-test was applied for the difference in the area under the curve of neovascularization and for the difference in the duration of corneal opacification between dietary groups. Significance of difference was obtained from a table for two-tailed observations, and P values < 0.05 were regarded as significant. All values are given in mean ± SEM.

Results

Clinical Observations

The appearance of keratitis in rabbits fed a sunflower seed oil diet was as follows. One week to 10 days after intracorneal injection of HSA, clouding of the cornea started at the limbus, and on approximately days 14–17 a white ring of opacification, also called Wessely's ring, formed.[39] This ring was present for 1–8 days. Two to 4 days after Wessely's ring was first noted, neovascularization of the cornea started from the limbus. The neovascularization progressed until approximately days 22–25 and then regressed quickly. In all cases we observed a clear cornea 30 days after the injection of the HSA.

All animals injected with HSA responded with white ring formation and neovascularization. Corneal edema formation, recorded with pachymetry, started around day 7 and lasted until day 30 (Fig. 2, Table 3).

In the rabbits fed a fish oil diet, the period of corneal opacification was significantly shorter compared to that of animals fed a sunflower seed oil diet (Fig. 3, Table 3). Moreover, the white ring frequently appeared incomplete, and corneal opacification was less intense. Vessel growth measured as area under the

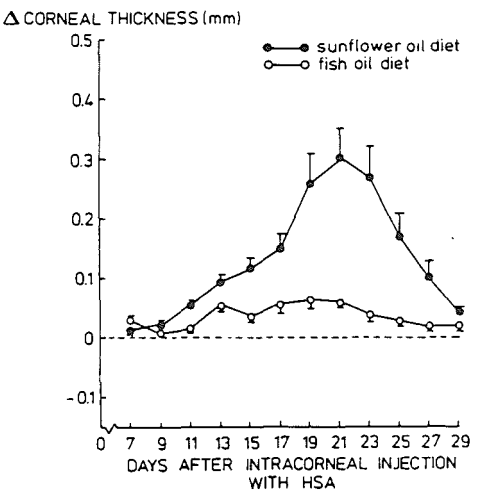

Fig. 2. Comparison of changes in mean corneal thickness during immunogenic keratitis treated with two different dietary regimens (fish oil diet, n = 8 or sunflower seed oil diet, n = 10). Δ corneal thickness was calculated from the difference between the pachymetry measurement before intraocular injection with HSA and at any given time thereafter.

Table 3. Immunogenic keratitis in rabbits on a supplemented diet

Diet	*Duration of corneal opacity** (days)	*Vessel growth AUC** (%)	*Corneal thickness AUC†* (%)
Sunflower seed oil (n = 10)	4.8 ± 0.5	100 ± 15	100 ± 15
Fish oil (n = 8)	2.6 ± 0.6‡	16 ± 8§	27 ± 6‖

All values are mean ± SEM.
AUC, area under the curve (100% in sunflower seed oil diet).
* Significance of difference for duration of corneal opacity and vessel growth was calculated with the Mann-Whitney U-test.
† Mean corneal thickness values of the animals on the two dietary regimens at various time points during the inflammation were tested for significance with Wilcoxon's signed rank test.
‡ $P < 0.01$.
§ $P < 0.001$.
‖ $P < 0.005$.

curve was significantly diminished (Fig. 4, Table 3). A significant inhibition of corneal edema was noted in rabbits given a fish oil diet as compared to those given a sunflower seed oil diet (Fig. 2, Table 3).

Systemic Antibody Response to Intracorneal Injected HSA

There was no significant difference in systemic response to HSA in the two different dietary regimens. Animals on a sunflower seed oil diet showed a serum level of 8.12 ± 2.17 μg/ml (mean ± SEM) of anti-HSA IgG immunoglobulins, and those on a fish oil diet 8.38 ± 3.26 μg/ml serum (mean ± SEM).

Discussion

The three parameters of corneal inflammation we used, Wessely's ring, swelling, and neovascularization, can be observed well in the model of immunogenic keratitis and are known to be at least partially

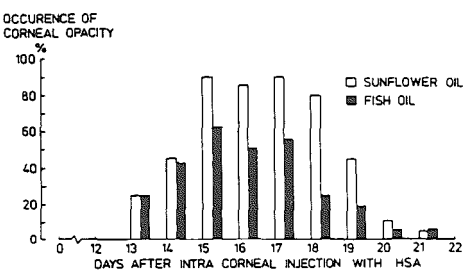

Fig. 3. Days on which opaque rings or a diffuse completely opaque cornea were visible during immunogenic keratitis treated with two different dietary regimens (fish oil diet, n = 8 or sunflower seed oil diet, n = 10). Corneal opacity was recorded from color slides taken daily.

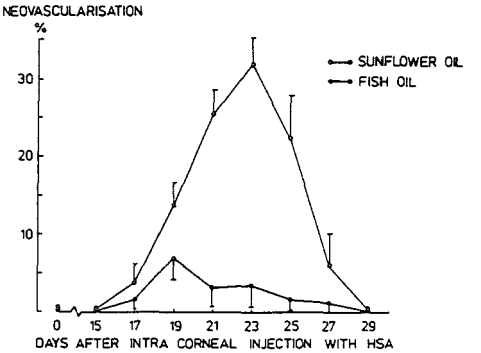

Fig. 4. Comparison of changes in mean corneal neovascularization during immunogenic keratitis treated with two different dietary regimens (fish oil diet, n = 8 or sunflower seed oil diet, n = 10). Mean corneal neovascularization was calculated as percentage of total corneal surface by planimetry from color slides taken of the inflamed corneas.

mediated by the formation of PGs and LTs.[41–44] Rabbits on a diet containing 9.4% sunflower seed oil were the controls in our study. For health reasons, in order to avoid deficiency of essential fatty acids the experimental group received 2.7% sunflower seed oil made up to 9.4% with fish oil. The values found for the parameters during immunogenic keratitis in the control group were not significantly different from those in rabbits maintained on standard rabbit diet.[37]

The suppressive effect of the fish oil dietary supplement was significant on all three clinical parameters. Apart from a shorter period of infiltration with leukocytes, the observed amelioration of the immunogenic keratitis by the diet may be due also to altered metabolic activity of the invaded leukocytes. Most authors consider the presence of PML in corneal inflammation responsible for the later development of neovascularization and the concomitant edema formation.[45–48] Others believe that the corneal tissues, especially the epithelium, can produce chemotactic and neovasculogenic substances without the infiltration of the PML.[49,50] In this case leukocytes are not a prerequisite of neovascularization, but rather by their presence merely potentiate the neovascular response.

Fish oil dietary supplementation may markedly alter the eicosanoid formation in endotoxin-induced anterior uveal inflammation; in contrast to our findings in immunogenic keratitis, however, it has no effect on cellular infiltration.[51] This difference in inflammatory response is due possibly to differences in tissue synthesis of LTB_4, a potent chemoattractant for leukocytes. The iris ciliary body of the rabbit has low levels of lipoxygenase activity, unlike the cornea, in

which lipoxygenase activity is more prominent.[7] In our study the effect of fish oil supplement on corneal inflammation may be ascribed to a diminished synthesis of PGs in the cornea by EPA- and DCHA-induced inhibition of cyclooxygenase and to the formation of the less active LTB_5 and PGE_3 from EPA (Fig. 1).[27–33]

In immunochemical keratitis the reaction in the cornea starts with the formation of antibody–antigen complexes.[46] Although an effect of the EPA-enriched diet on antibody formation has been observed,[52] in our experiments a diet containing EPA compared to one containing LA does affect the systemic antibody response to HSA.

Key words: fish oil, keratitis, eye, prostaglandin, leukotriene

Acknowledgments

The authors thank Mr. W. Heerooms for assistance with animal experiments; Unilever, Vlaardingen, for providing the material for the animal diets; and Mrs. M. Wissing and Mrs. C. H. M. Muijlwijk-Planting for secretarial assistance.

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10. Conclusions

The steroid medrysone and IBMX are effective inhibitors of several inflammatory responses in the human serum albumin induced immune complex uveitis. Medrysone protects the eye against blood-aqueous barrier disruption as well as against cellular infiltration in immunogenic uveitis. IBMX only protects the blood-aqueous barrier but has no significant effect on cellular infiltration.

The mechanism of action of corticosteroids is explained by the induced synthesis of lipocortins - specific proteins, that are inhibitors of phospholipase A_2. The mechanism of phosphodiesterase inhibitors in inflammation is explained by the intracellular elevation of cAMP, which reduces the production of prostaglandin E_2 in an immediate hypersensitive reaction. It is conceivable that IBMX decreases the production of PGs in the infiltrated leukocytes and that by this way the blood-aqueous barrier is protected in our model.

In HSA induced immunogenic keratitis the suppressive effect of a local corticosteroid used as a positive control confirms earlier observations using this model. The lipoxygenase inhibitors Bay 08276 and Rev 5901 were both effective in the inhibition of leukocyte infiltration, neovascularization and corneal edema.

Efficacy of inhibition of leukotriene B_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.

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

Treatment of immunogenic keratitis with the fatty acids columbinic acid, eicosapentaenoic acid and gamma-linolenic acid was effective in the inhibition of leukocyte infiltration, neovascularization and corneal edema formation. Columbinic acid showed the most effective inhibition with regard to neovascularization and corneal edema. Eicosapentaenoic was the most effective inhibitor of leukocyte infiltration. Dihomo-gamma-linolenic acid showed a significant inhibition of leukocyte infiltration and edema formation but not of neovascularization. Arachidonic acid treatment had neither an inhibitory nor a stimulating effect on the parameters of the immune-complex keratitis.

We determined the incorporation of the two most efficient inhibitors of immunogenic keratitis, EPA and columbinic acid, in the cornea after four days of topical application. Both EPA and columbinic acid showed an efficient incorporation into the phospholipids of the cornea, with concomitant lower levels of intracorneal arachidonic acid; free fatty acid was low. Phospholipase A_2-activation by an inflammatory stimulus therefore may lead to a release of these poly-unsaturated fatty acids besides

lower levels of the naturally occurring arachidonic acid from the cell membranes of corneal tissue.

In conclusion local application of unsaturated fatty acids opens new perspectives in the treatment of corneal inflammatory diseases through modulation of the inflammatory response resulting in less leukocyte infiltration, neovascularization and corneal edema formation.

The effect of the specific PAF-acether antagonist BN 52021 on leukocyte infiltration and corneal swelling in immune keratitis revealed a role for PAF-acether in these signs of ocular inflammation. The effect of BN 52021 after corneal denudation in a normal eye showed a significant therapeutic effect on wound healing and corneal swelling after corneal denudation.

A common cause mediated by PAF-acether was suggested by the time-related effect of BN 52021 on corneal swelling and re-epithelialization within six hours after corneal denudation as well as by the parallel course of these parameters until complete corneal healing. From the experiments it cannot be determined if improved healing of corneal wounds is the cause or the effect of decreased corneal swelling.

For the study on the influence of a fish oil diet on ocular inflammation, we used the model of immunogenic keratitis. The immunogenic keratitis could be significantly suppressed by an eicosapentaenoic acid containing fish oil diet. Significance was obtained on all three clinical parameters and was an indication for altered formation of PGs and LTs. Rabbits on linoleic acid enriched diet were the controls in our study. The values found for the parameters during immunogenic keratitis were not significantly different from those in rabbits maintained on

standard diet.

In immunochemic keratitis the reaction in the cornea started with the formation of antibody antigen complexes. Although an effect of the EPA enriched diet on antibody formation had been observed, in our experiments feeding of EPA did not have an effect on the systemic antibody response to HSA.

In conclusion EPA feeding had a significant inhibitive effect on immune complex inflammation of the rabbit cornea.

11. Summary

The effect of topical administration of 3-isobutyl-methyl-xanthine (IBMX), a potent phosphodiesterase inhibitor, was studied on an experimentally provoked uveitis in rabbits. After presensitization with an intravitreal injection of human serum albumin (HSA) intravenous antigenic challenge induced blood-aqueous barrier breakdown and leukocyte infiltration. Treatment with IBMX 1% two times daily, significantly inhibited the breakdown of the blood-aqueous barrier and the increase in PGE_2 level of the aqueous humor. There was no effect on leukocyte infiltration. The therapeutic effect of IBMX in blood-aqueous barrier protection was comparable with the effect of topical treatment with the corticosteroid medrysone.

Intrastromal injection with 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 and a PAF-acether antagonist on this inflammatory keratitis was studied. Also the topical and systemic application of several unsaturated fatty acids were evaluated.

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.

Several unsaturated fatty acids are precursors of prostaglandins and leukotrienes. Depending on their precursor, these prostaglandins and leukotrienes have different biological characteristics. Arachidonic, gamma-linolenic, dihomo-gamma-linolenic (DHGL), eicosapentaenoic (EPA) and columbinic acid were given as eye drops 0.5% three times daily during the experiment. Eicosapentaenoic acid, DHGL, columbinic, and gamma-linolenic, but not arachidonic acid, showed a significant inhibition of either leukocyte infiltration, edema or neovascularization. The inhibitory effects of these fatty acids may be caused by topical inhibition of the formation of prostaglandins and leukotrienes in the arachidonic acid cascade in the rabbit cornea.

Platelet activating factor (1-O-alkyl-2-acetyl-glycero-3-phosphocholine, PAF-acether) is one of the most potent inducers of platelet aggregation and has a wide spectrum of biological activities as a very potent mediator of inflammation, increasing vascular permeability.

We tested the PAF-acether antagonist BN 52021 in the models of corneal abrasion and immunogenic keratitis. It was applied three times daily as a 1% suspension. After corneal abrasion, in the vehicle-treated eyes complete restoration of the corneal surface was observed by 168 hours after scraping of the epithelium. In the eyes treated with BN 52021, after 6 hours less swelling was recorded as compared to the vehicle-treated contralateral eye. This difference in swelling persisted until at 168 hours after scraping the corneal swelling in the treated eyes had disappeared and the corneal thickness was the same as before the experiment.

During immunogenic keratitis rabbits treated with BN 52021 showed a significantly shorter period of corneal opacification. The period of neovascularization was not different from that in non-treated animals. The extent of swelling, determined as mean area under the curve, was significantly different from that in untreated controls.

Fish lipids contain large amounts of eicosapentaenoic acid and docosahexaenoic acid. These fatty acids are known to have an influence on prostaglandin and leukotriene synthesis. We studied the effect of a fish oil diet on an immune complex induced keratitis of the rabbit eye and compared the effects of this diet with the effects of a sunflower oil diet, rich in linoleic acid. Fish oil treated animals showed significantly less leukocyte infiltrate, neovascularization and corneal edema in comparison with sunflower oil fed rabbits.

12. Samenvatting

Het effect van locale toediening van de fosfodiesterase-remmer 3-isobutyl-methyl-xanthine (IBMX) werd bestudeerd bij een experimentele immuuncomplex uveitis in het konijnenoog. Deze ontsteking ontstaat na sensibilisatie met een intravitreale injectie van humaan-serum albumine (HSA) als antigeen. Intraveneuze toediening van dit antigeen daarna geeft aanleiding tot een bloed-kamerwater-doorbraak en leukocyteninfiltratie van het oog. Behandeling met IBMX 1% tweemaal daags gaf een significante bescherming van de bloed-kamerwater-barrière en een daling van het prostaglandine E_2-gehalte van het kamerwater. Er was geen effect op het aantal geinfiltreerde leukocyten te zien. Het therapeutisch effect van IBMX werd vergeleken met het effect van lokale behandeling met het corticosteroid medrysone.

Intrastromale injectie met HSA als antigeen in de cornea van het konijnenoog induceert corneaoedeem en een ringvormig leukocyteninfiltraat gevolgd door neovascularisatie in het hoornvlies. Het effect van lokaal toegediende lipoxygenase- en cyclo-oxygenaseremmers en een PAF-acether-antagonist op deze keratitis werd bestudeerd. Op ditzelfde ziektemodel werd het therapeutisch effect van lokale en systemisch toegediende onverzadigde vetzuren geëvalueerd.

De lipoxygenaseremmers BAY 08276 en Rev 5901 en de cyclo-oxygenaseremmer suprofen werden driemaal daags als 1% oogdruppels gegeven. Ogen behandeld met de lipoxygenaseremmers gaven een significante vermindering van het leukocyteninfiltraat, de neovascularisatie en het oedeem te zien. In ogen behandeld met

een cyclo-oxygenaseremmer verminderde het corneaoedeem en was de neovascularisatie minder lang aanwezig. Er werd geen invloed op de ernst van het leukocyteninfiltraat geconstateerd.

Uit een aantal onverzadigde vetzuren kunnen prostaglandinen en leukotrienen gevormd worden. Afhankelijk van het het vetzuur hebben de daaruit gevormde prostaglandinen en leukotrienen andere biologische eigenschappen. Bij konijnen met immunogene keratitis werden arachidonzuur, gamma-linoleenzuur, dihomo-gamma-linoleenzuur (DHGL), eicosapentaanzuur (EPA) en akeleizuur als 0,5% oogdruppels driemaal daags toegediend. EPA, DGHL, akeleizuur en gamma-linoleenzuur gaven een significante remming van hetzij leukocyteninfiltraat hetzij de oedeemvorming c.q. de neovascularisatie te zien. Arachidonzuur had noch een stimulerende, noch een remmende invloed op het ontstekingsproces. Het remmend effect van deze vetzuren lijkt te verklaren door lokale remming van de vorming van prostaglandinen en leukotrienen uit arachidonzuur in de cornea.

Thrombocyten activerende factor (platelet-activating factor; 1-O-alkyl-2-acetyl-glyceryl-3-phosphocholine, PAF-acether) is een van de meest krachtige bloedplaatjesaggregerende stoffen. Naast andere biologische eigenschappen is het een belangrijke ontstekingsmediator welke onder andere een verhoogde vasculaire permeabiliteit veroorzaakt. De PAF-acether-antagonist BN 52021 werd driemaal daags als 1% suspensie toegediend in een ziektemodel bestaande uit abrasio corneae en een immunogene keratitis. Na abrasio corneae was in de niet-behandelde ogen het corneaepitheel 168 uur na de abrasio gesloten. In ogen behandeld met BN 52021 was 6 uur na de abrasio minder corneaoedeem dan bij de niet-behandelde ogen te zien. Dit verschil in oedeemvorming bleef

aanwezig gedurende het hele proces van epitheelregeneratie. Na sluiting van het corneaepitheel was de corneadikte hetzelfde als voor het experiment. Tijdens immunogene keratitis was bij ogen behandeld met BN 52021 de duur van het leukocyteninfiltraat en de mate van corneaoedeem significant verminderd ten opzichte van onbehandelde ogen, de corneaneovascularisatie werd niet door BN 52021-behandeling beïnvloed.

Vetten van vis bevatten grote hoeveelheden eicosapentaanzuur en docosahexaanzuur. Deze vetzuren hebben een invloed op prostaglandinen- en leukotrienensynthese. We bestudeerden het effect van een visoliedieet op een immuuncomplex-geinduceerde keratitis van het konijnenoog. Dit dieet werd vergeleken met het effect van een zonnebloemoliedieet rijk aan linolzuur. Visolie-behandelde dieren lieten significant minder corneaoedeem, leukocyteninfiltraat en corneaneovascularisatie zien vergeleken met zonnebloemolie-gevoede konijnen.

13. Publications

N.L.J. Verbey is co-author of the following publications:

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Hoyng PhFJ, Verbey NLJ, Thörig L, Van Haeringen NJ.
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Verbey NLJ, Van Haeringen NJ, De Jong PTVM.
Modulation of immunogenic keratitis in rabbits by topical administration of inhibitors of lipoxygenase and cyclooxygenase.
Curr Eye Res 1988;7:361-368.
This thesis chapter 6.

Verbey NLJ, Van Haeringen NJ, De Jong PTVM.
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Verbey NLJ, Van Haeringen NJ.
The influence of a fish oil diet on immunogenic keratitis.
Invest Ophthalmol Vis Sci 1990;31: in press.
This thesis chapter 9.

14. Acknowledgements

I am greatly indebted to Prof.Dr. P.T.V.M. de Jong for his readiness to be promotor of this thesis and his critical review of the manuscripts.

I thank Dr. N.J. van Haeringen for his supervision, critical comments and stimulation during the entire study.

I wish to thank Drs. A.J. Otto for his help and enthousiasm.

I wish to thank Dr. U.T.M. Houtsmuller, Dr. D. Nugteren, G. Kivits and E. Haddeman of the Unilever Research Laboratory in Vlaardingen for their advice which proved to be invaluable in avoiding most of the pitfalls in nutritional research.

I wish to thank Prof.Dr. I.L. Bonta, Prof.Dr. W.C. Hülsmann, Prof. J.H.P. Wilson, Prof.Dr. J.A. Oosterhuis and Prof.Dr. T.E.W. Feltkamp for their critical review of this thesis.

I would like to thank J.G. Verkerk and W. Heerooms for technical assistance, especially in handling the animals.

I am grateful to mrs. C.H.M. Muijlwijk-Planting and mrs. M. Wissing for secretarial help, and to N. Bakker and C.B. Schotel for illustrative and photographic assistance.

I would like to express my gratitude to the scientific board and the management of the Netherlands Ophthalmic Research Institute for giving me the opportunity to do the research described in this thesis.

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