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**STUDIES ON INFLAMMATION IN ATOPIC  
KERATOCONJUNCTIVITIS**

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To all patients who have shed their tears for me



## ABSTRACT

Atopic keratoconjunctivitis (AKC) is an ocular inflammatory condition associated with atopic dermatitis. AKC is classified as ocular allergy but with features quite different from common seasonal allergic conjunctivitis. The clinical picture includes eyelid eczema, blepharitis, conjunctivitis, and some degree of keratitis. The condition is chronic and normally starts off in young adulthood with periods of exacerbations during the following decades. Due to corneal affection, it is a potentially blinding disease. Little is known about the factors determining the development of AKC, the ongoing inflammation, and the severity and frequency of exacerbations.

The aim of the present studies was to characterize inflammation and to describe corneal complications in AKC. The possible influence of ocular and periocular microcolonization on the degree of inflammation in AKC was addressed in study I. In study II, treatment of eyelid eczema with either tacrolimus ointment or potent steroid ointment was analysed, with the objective to evaluate possible ocular effects. In study III, the ocular surface response to conjunctival provocation with airborne allergen was explored. In all three studies, tear cytokines were analysed as an objective parameter of inflammation. Two retrospective case series studies (IV and V) were performed presenting AKC patients with corneal emergencies.

No association was found between microcolonization and the degree of inflammation in AKC. However, a relationship between Staphylococcal enterotoxin B antibodies and disease severity was found. Significant differences in tear fluid cytokines comparing AKC subjects and healthy controls were shown in this study and the cytokine levels correlated well with conjunctival signs. Both tacrolimus ointment and steroid ointment had excellent and comparable effect on eyelid eczema. There were no change in ocular surface signs or cytokines following treatment, neither were there any ocular adverse events. In the provocation experiment, an immediate conjunctivitis was elicited and a significant increase in tear cytokines was documented 48 hours after allergen challenge in AKC subjects. The retrospective studies indicated AKC patients to be at risk for *Candida albicans* keratitis and spontaneous corneal perforation.

In conclusion, AKC presents with a spectrum of mild to severe inflammatory manifestations. Our studies indicate that bacterial colonization is unrelated to the inflammatory activity in moderate AKC, but antibodies to *Staphylococcus aureus* antigen could possibly be a marker of disease severity. Tear cytokines may further be markers of conjunctivitis and the provocation study indicates that allergen exposure may fuel the inflammation. Tacrolimus ointment appears to be a viable treatment option for eyelid eczema in AKC, but no evident improvement on the ocular surface was found after 3 weeks of medication. Further research is needed to unravel the exact causes of ocular surface inflammation and particularly of the debilitating keratopathy in AKC.

Keywords: allergy, atopic keratoconjunctivitis, atopy, chronic conjunctivitis, cytokine, keratitis, tear fluid

## LIST OF PUBLICATIONS

- I. Nivenius E, Montan PG, Chryssanthou E, Jung K, van Hage-Hamsten M, van der Ploeg I. No apparent association between periocular and ocular microcolonization and the degree of inflammation in patients with atopic keratoconjunctivitis. *Clin Exp Allergy*. 2004; 34: 725-30.
- II. Nivenius E, van der Ploeg I, Jung K, Chryssanthou E, van Hage M, Montan PG. Tacrolimus ointment vs. steroid ointment for eyelid dermatitis in patients with atopic keratoconjunctivitis. *Eye (Lond)*. 2007; 21: 968-75.
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- IV. Nivenius E, Montan PG. Atopic keratoconjunctivitis patients at risk for *Candida albicans* keratitis? A case study. Submitted.
- V. Nivenius E, Montan PG. Spontaneous corneal perforation associated with atopic keratoconjunctivitis? A case study. Submitted.

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## LIST OF ABBREVIATIONS

AE	Atopic dermatitis
AKC	Atopic keratoconjunctivitis
ABC	Atopic blepharoconjunctivitis
ANOVA	Analysis of variance
APC	Antigen presenting cell
CBA	Cytometric bead array
CCR-3	Chemokine receptor 3
CPT	Conjunctival provocation test
ECP	Eosinophil cationic protein
GCP	Giant capillary conjunctivitis
INF- $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IOP	Intraocular pressure
KC	Keratoconus
MBP	Major basic protein
MMP	Matrix metalloproteinase
MUC	Mucin
OCP	Ocular cicatricial pemphigoid
PAC	Perennial allergic conjunctivitis
S. aureus	Staphylococcus aureus
SAC	Seasonal allergic conjunctivitis
SEA	Staphylococcal enterotoxin A
SEB	Staphylococcal enterotoxin B
TNF- $\alpha$	Tumor necrosis factor alpha
VKC	Vernal keratoconjunctivitis

# 1 CONCEPTS IN IMMUNOLOGY AND ALLERGY

The immune system, required for defending the host against infection, is very complex. The defence mechanisms consist of the innate immunity that blocks the entry and achieves initial elimination of microbes, and the adaptive immunity, which generates a delayed but more targeted and effective defence.

## 1.1 SOME WORDS ABOUT CELLS INVOLVED IN OCULAR ALLERGY

When the host encounters microbes or in the case of allergic disease allergens, these are detected by antigen presenting cells (APCs) like **macrophages** and **dendritic cells**, which patrol the skin, mucosal sites, and the spleen. The APCs process and display the antigens to immune reactive cells, which initiate differentiation and activation.

The principle immune cells are the lymphocytes. All lymphocytes arise from stem cells in the bone marrow; B-lymphocytes (**B-cells**) mature in the bone marrow and T-lymphocytes (**T-cells**) in the thymus. B-cells produce antibodies against extracellular antigens while T-cells recognize and eradicate intracellular antigens. There are several types of T-cells. T-helper cells or CD4 + cells are particularly instrumental in allergy. Subsets of T-helper cells with different functions have been identified. The subsets that were first defined were called “type 1 helper T cells” (**Th1 cells**) and “type 2 helper T cells” (**Th2 cells**). Th1 cells typically produce the cytokine IFN- $\gamma$ , which is important in host defence against intracellular microbes. Th2 cells produce, among other cytokines, IL-4, IL-5 and IL-13, which stimulate the production of IgE and activates **eosinophils**. Mast cell activation through IgE binding of antigen is an important event in the defence against helminthic parasites but also in immediate hypersensitivity reactions.

## 1.2 ALLERGY AND ATOPY

At times the immune response is misdirected or uncontrolled and pathological immune reactions, called hypersensitivity reactions, develop. The word allergy originates from Greek, allos meaning "other" and ergon meaning "reaction", and was coined in the early 1900s by an Austrian scientist and paediatrician, Clemens von Pirquet, to describe altered reactions in the human body.

Allergy is the result of hypersensitivity reactions involving two principal immunological mechanisms.

**Immediate hypersensitivity reactions** are caused by the encounter of allergens with IgE antibodies bound to the mast cell surface. The binding of allergen causes a degranulation of the mast cell and release of vasoactive mediators.

**Delayed type hypersensitivity reactions** are T-cell mediated and antibodies do not play a direct role in this reaction.

Classically, allergy denotes IgE dependent reactions including asthma, allergic rhinitis and conjunctivitis, atopic eczema, urticaria, and anaphylaxis. The propensity of an

individual to produce IgE antibodies in response to various environmental antigens and to develop immediate hypersensitivity responses is called atopy and affected individuals are said to be atopic (Pepys 1994). An antigen that elicits an immediate hypersensitivity reaction is called an allergen. Most allergens are glucoproteins normally existing in our environment that are innocuous to normal cells and to non-sensitized individuals. Common allergen sources are pollen, mites, furred animals, moulds and foodstuffs.

During the last decades the incidence of atopic disease has been on the rise worldwide. Lately the incidence has reached a plateau in urbanized countries while an increase still is seen in developing countries (Williams H 2008, Asher 2006). Much effort has been invested to clarify the relationship with hereditary and environmental factors in the development of atopy (Blumenthal 2005, van den Oord 2009, Flohr 2011). There is, however, a discrepancy in that most, and not all, atopic individuals experience allergic disorders and conversely, some apparently allergic patients do not exhibit an atopic phenotype (Mentz G 1998, Flohr 2004).

## 2 OCULAR ALLERGY

Allergic conjunctivitis is one of the most common ocular surface disorders. Five subtypes are often described. The most frequent and also the mildest forms are seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) in which the allergic reaction is a typical immediate hypersensitivity reaction involving IgE response to antigen. In giant papillary conjunctivitis (GPC), the reaction is due to persistent contact of the conjunctiva with a foreign material and no certain IgE-mediated mechanism has been identified. For atopic keratoconjunctivitis (AKC) and vernal keratoconjunctivitis (VKC), the more chronic and severe subtypes of ocular allergy, the role of allergic sensitization is unclear.

**Table 1. Characteristic symptoms and treatment for allergic conjunctivitis**

Allergic condition	Clinical symptoms	Typical age	Immunopathology	Cells mainly involved	Treatment
SAC	Itching, redness, micropapillae	Teens, young adults	IgE mediated	Mast cells, Eosinophils	Antihistamines Mast cell stabilizers
PAC	Itching, redness, micropapillae	Adults	IgE mediated	Mast cells, Eosinophils	Antihistamines Mast cell stabilizers
GPC	Ocular irritation, giant papillae	Adults Related to contact lenses and iterative micro-trauma	Non-IgE mediated	Mast cells, T-cells	Antihistamines Mast cell stabilizers Corticosteroids
VKC	Persistent itch, cobblestone papillae, corneal ulceration	Children	IgE and non-IgE mediated	Mast cells, Eosinophils, T-cells	Antihistamines Mast cell stabilizers Corticosteroids Cyclosporine A
AKC	Atopic dermatitis, persistent itch, conjunctival and corneal inflammation	Adults	IgE and non-IgE mediated	Mast cells, Eosinophils, T-cells	Antihistamines Mast cell stabilizers Corticosteroids Cyclosporine A Tacrolimus

SAC= seasonal allergic conjunctivitis, PAC= perennial allergic conjunctivitis, GPC= giant papillary conjunctivitis, VKC=vernal keratoconjunctivitis, AKC= atopic keratoconjunctivitis

### 2.1 SEASONAL ALLERGIC CONJUNCTIVITIS (SAC)

SAC is very common and affects 15-20% of teenagers worldwide (Asher 2006). It is often associated with rhinitis and commonly referred to as hay fever or rhinoconjunctivitis. The ocular allergic response results from airborne, seasonally present allergens that dissolve in the tear film, bind to IgE on conjunctival mast cells and trigger the degranulation of inflammatory mediators. The symptoms include itch, swelling, redness, and watery discharge, but the cornea is never affected. Symptoms are seasonal, depending on type of allergy but typically last for 1-3 months. Treatment

consists of topical or oral antihistamines and mast cell stabilizers and is often self-managed. Ophthalmic corticosteroids are not indicated in SAC but intranasal steroids can have a beneficial effect (Bielory 2008). SAC can greatly affect quality of life. In severe cases, immunotherapy could be considered (Nelson 2011). Lately, also oral formulas have become available for some allergens (Durham 2010).

## **2.2 PERENNIAL ALLERGIC CONJUNCTIVITIS (PAC)**

PAC is considered a variant of SAC that persists throughout the year but with additional seasonal exacerbations (Dart 1986). In PAC, the most common allergens are dust mites and animal dander. The prevalence is reported to be lower than for SAC. The immunological mechanism, just as in SAC, involves allergen binding to IgE and mast cell degranulation. Compared to SAC, lower numbers of mast cells have been demonstrated along with few T-cells (Baddeley 1995). Symptoms are less prominent than in SAC but on the other hand the disease is chronic and might therefore be more disturbing. Treatment is not different from that in SAC.

## **2.3 GIANT PAPILLARY CONJUNCTIVITIS (GPC)**

GPC is primarily associated with contact lens wear, but exposed sutures or ocular prostheses could also be the cause. Early symptoms are discomfort, foreign body sensation and itch. Typical signs are subtarsal giant papillae formation and excessive mucus production. GPC is determined by two factors: repeated mechanical trauma and abnormal conjunctival immune response. Histological findings include cellular infiltrates typically found in allergic diseases; mast cells and eosinophils (Allansmith 1978) but the initiating mechanism remains unclear. High levels of neutrophil chemotactic factor, released by injured conjunctival cells have been proposed to induce inflammation (Elgebaly 1991). An increase in eotaxin has been discussed in contact lens GPC (Moschos, 2004, Tran 2011) but was not found in GPC caused by ocular prostheses (Sarac 2003). Recently, membranous epithelial cells, M-cells, have been suggested as an entry route for macromolecular material reaching conjunctival B-cells, which mediate the immune process (Zhong 2007). Treatment through elimination of the pathogenic factor is effective, change of contact lens type or cleaning regimens can help. Relief of symptoms may be achieved with cautious use of topical steroids.

## **2.4 VERNAL KERATOCONJUNCTIVITIS (VKC)**

VKC, along with AKC, is characterized by involvement of the cornea and together they constitute the most severe forms of ocular allergy. The word “vernal” means spring and flare-ups of the disease are common in early springtime although symptoms may occur all around the year. VKC is also referred to as spring catarrh. VKC typically affects young boys and the disease is more common in countries with sub-tropical or tropical climate but also in patients with an ethnic origin from such countries (Montan 1999). Symptoms include intensive itch, tearing and photophobia. The clinical signs are characterized by upper subtarsal flat-topped macropapillae or limbal papillae. Corneal involvement increases during periods of exacerbation, commonly in the form of superficial punctuate keratitis and occasionally with the development of shield ulcers

and corneal plaques (Cameron 1995, Iqbal 2003). The corneal affection sometimes causes pseudo-ptosis. The aetiology of VKC is unknown. Atopy is verified in 60% of VKC patients and about 50 % report other atopic disorders as asthma or rhinitis (Montan 1999), but atopic and non-atopic individuals follow a similar course. Histological findings include conjunctival eosinophils, mast cells and T-cells and cytokine studies have revealed an influence mainly of Th2 and less so Th1 cells (Leonardi 2006a, Leonardi 2006b). Eosinophils and eosinophilic toxic proteins have been related to the corneal affection in VKC (Montan 1996a, Leonardi 2003, Shoji 2009a). Recently, overexpression of histamine receptors was demonstrated possibly providing clues to the triggering mechanisms of disease (Leonardi 2011). Treatment aims at reducing symptoms and corneal involvement. Corticosteroids are needed in periods and for long-term treatment, Cyclosporine A might be a good alternative. VKC is most often self-limiting with spontaneous recovery after puberty (Leonardi 2002)

## **2.5 ATOPIC KERATOCONJUNCTIVITIS (AKC)**

In 1953, Hogan observed a relation between atopic dermatitis, eyelid eczema and ocular surface affection. He proposed the term Atopic keratoconjunctivitis (AKC) to include eyelid eczema, conjunctivitis and keratitis (Hogan 1953). Five years later one additional case was reported (Bartlett 1957) but not until 30 years later further reports emerged and interest among researchers arouse. To this day, the actual cause of the condition is not fully understood.

An association with atopic dermatitis (AD) is almost always found, but in a few AKC cases, AD occurred in childhood only which might have been over looked (Akova 1993). AD is a recurrent, chronic skin disease, with hereditary influence, first described in the 1920s (Coca 1929). It is clinically characterized by the presence of inflammatory lesions in typical locations according to the patient's age (Hanifin and Rajka 1980). AD often evolves in early childhood followed by other atopic manifestations, such as hay fever and asthma, called the atopic march (Ker 2009). Most individuals outgrow the disease but in some, the dermatitis is lifelong and AD is reported to affect 1-3% of adults worldwide (Katsaru 2011). AD may also rarely develop in adulthood (Katsaru 2011). Up to 80% of AD cases are found to be associated with atopy (Schmid-Grendelmeier 2001), but reports of IgE sensitization in AD varies widely (Flohr 2004).

In AKC, a variety of ocular surface involvement is seen. Sub-classifications have been attempted to address these differences. The term atopic blepharoconjunctivitis (ABC) refers to patients with eyelid eczema and conjunctivitis but no corneal involvement. Sub-grouping in atopic versus non-atopic disease depending on IgE testing could be considered. In AKC, about 80% of the patients present with an atopic phenotype but symptoms and objective parameters of allergic inflammation do not differ between the atopic and non-atopic subgroups (Tuft 1991). A clinical grading depending on severity is relevant. Four subgroups have been proposed and could be of use in clinical studies (Calonge 2007), but the classification of mild, moderate, and severe disease is in line with most reports and easily applicable. Complex grading including all subtypes of ocular allergy are sometimes suggested (Shoji 2009b, Uchio 2008) but are of little use in severe chronic allergy.

### 2.5.1 Prevalence of AKC

In the entire population suffering from allergic conjunctivitis, AKC and VKC together are said to represent 2-8% but the figures are uncertain (Ono 2005, Uchio 2008). Mild cases of AKC may be misdiagnosed as dry eye or PAC and reports from ophthalmic centres probably represent only the more severe spectrum of disease. Analysing studies on AD patients with ocular findings could be one indirect method to estimate the spread of disease, see Table 2.

**Table 2. Reported ocular affection in atopic dermatitis patients**

Reference	AD patients	Ocular affection	Percentage
Kornerup 1959	100	3	3%
Karel 1965	128	41	32%
Amemiya 1980	44	14	32%
Garrity 1984	200	85	43%
Uchio 1998	216	70	32%
Dogru 1999	362	245	68%

AD=atopic dermatitis

AKC affects men and women equally (Power 1998) and no ethnic differences are reported. The disorder commonly presents in the early twenties and persists into the fifth or, in our experience, even the sixth decade of life. Exacerbations normally cease at 50- to 60-years of age, but visual impairment or irreversible visual loss may greatly affect daily life (Foster 1990, Power 1998).

AKC may affect children, although uncommonly, and only a few cases are identified in the literature (Hogan 1953, Onguchi 2006) and not more than five cases are managed at St Erik's Eye Hospital. Studies in children with AD verify that ocular complications are rare (Carmi E 2006, Kaujalgi 2009). In contrast, it is noted that AKC with early onset is associated with greater ocular surface damage in adult age and an extended duration of disease could be an explanation (Onguchi 2006). In children, there might be difficulties differentiating AKC from VKC due to similar features. Patients with a typical VKC picture might also have atopic dermatitis. While the treatment regimen is the same there are evident differences in prognosis. VKC typically disappears during puberty while AKC continues in adult life.

## 2.5.2 Clinical characteristics in AKC



Figure 1. Eyes of a woman with AKC (with permission given by the patient)

### 2.5.2.1 *Skin and eyelid affection in AKC*

The eyelid skin is thin and elastic to cover and protect the ocular surface. In AKC, a darkening and thickening of the eyelid skin with indurations and lichenification are common and during exacerbations, increased oedema, redness, and excoriations occur. Typically, some degree of blepharitis is present with thickened and inflamed lid margins. There can be maceration of the inner and outer cantus from constant wetting by tears and the eyelids are often glued together on awakening. The chronic inflammation may also distort the eyelid and ectropion is not uncommon. On top of itch and discomfort, the eyelid appearance is a major cosmetic concern for many patients.

### 2.5.2.2 *The conjunctiva in AKC*

Conjunctival involvement is part of all forms of AKC and may vary from mild to more severe inflammation. Subtarsal micropapillae are seen both in upper and lower tarsal conjunctiva. Papillary hypertrophy may rarely occur but the hallmark sign after years of disease is subtarsal fibrosis. In severe cases, fornix shortening, conjunctival fibrosis, and symblepharon formation are seen.

### 2.5.2.3 *The cornea in AKC*

All patients with AKC exhibit corneal affection at some point and most patients have lifelong corneal signs. Usually, superficial punctate keratitis is first seen in the lower part of the cornea. Larger erosions, sterile infiltrates, corneal plaque and scarring may follow. Neovascularization from limbus is seen in severe and long-standing disease. AKC patients are also prone to develop infectious keratitis with *Staphylococcus aureus* (*S. aureus*) (Hogan 1953) and herpes simplex (Easty 1975, Garrity 1984, Tuft 1991). The corneal affection also includes changes in corneal curvature. High-grade astigmatism and keratoconus are more common than in the general population (Tuft 1991, Power 1998). With confocal imaging, decreased density in corneal long nerve fibres and decreased nerve branching have been shown in AKC subjects, correlating with decreased corneal sensitivity and epithelial damage (Hu 2008).



Figure 2. Persistent epithelial defect in AKC (with permission given by the patient)

#### 2.5.2.4 *Intraocular changes in AKC*

Atopic cataract is reported as an entity by its own and was described before AKC was recognized (Milner 1941), but may be part of the AKC disease (Garrity 1984). Presenile cataract development with an anterior subcapsular cataract is typically described in atopic patients but also posterior subcapsular cataract is seen which could be related to atopy or to steroid treatment. Precautions before cataract surgery in AKC patients could be considered (Guglielmetti 2010). In cases of ectropion, eyelid surgery should be targeted prior to cataract extraction. A higher risk for retinal detachment has been reported in studies from Japan (Takahashi 1996, Yoneda 1995), but this is not commonly observed. Lastly, there are no epidemiological studies published proving an increased risk for glaucoma in AKC but fairly young subjects described in cohorts with severe disease suffered from glaucoma (Foster 1990). The intraocular pressure (IOP) should be checked regularly during topical steroid treatment.

#### 2.5.3 Differential diagnoses to AKC

In mild cases, AKC might be misdiagnosed as dry eyes or possibly PAC. Contact dermatitis with keratoconjunctivitis could resemble AKC. In patients presenting with severe inflammation, diagnoses such as rosacea, ocular cicatricial pemphigoid (OCP), Stevens-Johnson syndrome or chemical burns could be considered.

#### 2.5.4 Pathophysiological features of AKC

##### 2.5.4.1 *Cells and cytokines in AKC*

In SAC and PAC, the inciting allergens can be identified. The cascade of events with allergen recognition, stimulation of B-cells to IgE class switching and IgE production, binding of IgE to mast cells, and mast cell activation following exposure to allergen are well known. The mast cell release of mediators including histamine, prostaglandins and leukotrienes that cause itch, chemosis, and vessel dilatation are analysed in detail following conjunctival allergen challenge (CPT) (Proud 1990). Activated mast cells also release cytokines like IL-4, IL-13, TNF- $\alpha$  and chemokines that promote recruitment of eosinophils, neutrophils and Th2 cells (Macleod 1997, Anderson 2001). The majority of patients with AKC are atopic with increased IgE antibodies to a number of allergens. The IgE levels do not differ significantly when compared to other

forms of allergic conjunctivitis and do not correlate with disease severity (Tuft 1991). Further, the clinical symptoms and histological findings are comparable in atopic and non-atopic subjects (Tuft 1991). Clearly, other mechanisms than IgE sensitization play a role in the pathogenesis and T-cell infiltration has since long been implicated in the chronic inflammation in AKC. In conjunctival biopsies, T-cells, mast cells, and macrophages have been demonstrated (Foster 1991, Morgan 1991, Baddeley 1995, Metz 1996) as well as eosinophils and neutrophils (Bacon 1993) and basophils (Matsuda 2010). In tear fluid, high numbers of T-helper cells and activated B-cells (Aunduk 1998) and neutrophils and eosinophils (Leonardi 2000, Trocmé 2003) are found, reflecting the same pattern of T-cell mediated response. The cytokine spectrum, as analysed in biopsies (Power 1998), cell lines (Metz 1997, Calder 1999), and in tear fluid (Uchio 2000, Leonardi 2006a, Leonardi 2006b) demonstrate both typical Th1 and Th2 cytokines but with a Th1-dominant profile, which is not seen in VKC or other allergic conjunctivitis subgroups. The influence of the Th1 cytokines IL-2 and INF- $\gamma$  is seemingly of importance in the perpetuation of AKC.

#### 2.5.4.2 *Eosinophils in AKC*

Eosinophils are believed to be critically involved in the keratopathy in the chronic ocular allergy conditions AKC and VKC. In the activated state, eosinophils release preformed basic proteins of which the most important are the eosinophil cationic protein (ECP) and major basic protein (MBP). These proteins have toxic properties affecting epithelial cell viability, morphology and migration (Trocmé 1997). Increased numbers of eosinophils are found in AKC (Foster 1991, Bacon 1993) and eosinophil activation has been shown to correlate with disease (Hingorani 1998). In AKC and VKC, higher ECP values in tear fluid compared to GPC and SAC subjects as well as a significant correlation between ECP values and severity of disease have been demonstrated (Montan 1996a). Participation of eosinophils in corneal ulceration in AKC has further been highlighted with the finding of depositions of ECP and MBP in corneal buttons (Messmer 2002). Eosinophil recruitment and activation is at least in part dependent on eotaxin (Fukagawa 1999) and conjunctival fibroblasts have been proposed to be one source of eotaxin in severe allergic tissues (Leonardi 2003). Eotaxin binds to chemokine receptor 3 (CCR-3) and treatment with anti-CCR-3 antibodies for corneal ulcers in ocular allergy has been suggested (Fukagawa 2002). Recently, experimental mice studies demonstrated that blockade of CCR-3 suppressed not only eosinophilic inflammation but also mast cell degranulation (Komatsu 2008, Miyazaki 2009) and this might be a future therapeutic approach in ocular allergy.

#### 2.5.4.3 *Mucins in AKC*

In AKC patients, abnormal mucin tear levels have been found associated with corneal damage (Dogru 2005). The ocular surface epithelium mainly expresses the cell surface-associated mucins MUC-1, MUC -4 and MUC -16, which are involved in surface protection and lubrication, and the gel-forming mucin MUC5AC that promotes lubrication as well as clearance of allergens, pathogens, and debris (Gipson 2004). In AKC, alterations in both cell surface-associated and secreted mucins have recently been described. Squamous metaplasia of the conjunctival epithelium and goblet cell loss, accompanied by decreased levels of the goblet cell-specific mucin MUC5AC, have been found suggesting a link between epithelial damage and loss of lubrication (Dogru

2005). Along with the decrease in MUC5AC, an increase in the expression of MUC-1, MUC-2, and MUC-4, has been demonstrated as a possible compensating mechanism (Hu 2007).

#### 2.5.4.4 *S. aureus* in AKC

More than 80% of AD patients are colonized with *S. aureus* as compared with only 10% of healthy individuals (Higaki 1999, Breuer 2002). Similar proportions are found in AKC (Tuft 1991, Tabuchi 2004). Deficiencies in the innate immunity, with decreased secretion of antimicrobial peptides (Ong 2002) and reduced levels of skin surface IgA (Imayama 1994) and of tear IgA (Toshitani 1999), has been related to this altered colonization. The presence of *S. aureus*, seems to be both a consequence and a cause of disease and numerous pathogenic roles have been suggested in AD, including production of superantigens, super antigen-specific IgE and stimulation of Toll-like receptors (Lin 2007). Some of these phenomena could possibly be important in AKC. Superantigens are a class of antigens that cause non-specific activation of T-cells and massive cytokine release. A relation between corneal disease, increased cytokine production, and exposure to *S. aureus* enterotoxin B has been experimentally verified (Thakur 1997).

*S. aureus*-derived superantigens can also function as classic allergens, inducing production of specific IgE antibodies. Several studies have found correlations between disease severity in AD and IgE antibodies to *S. aureus* superantigens (Leung 1993, Nomura 1999, Breuer 2000). While *S. aureus* superantigens are found also in healthy skin, induction of IgE production is rarely found in healthy subjects (Leung 1993). Presence of *S. aureus* A antigen (SEA) and *S. aureus* B antigen (SEB) antibodies in tears have been found in VKC and in AKC but not in PAC or controls, possibly indicating a relation to disease (Shoji 2003). Another potential role of *S. aureus* in chronic ocular inflammation in AKC has been demonstrated through activation of epithelial cells via Toll-like receptors (Cook 2005). Similarly, aggravation of allergic conjunctivitis after *S. aureus* exposure involving Toll-like receptors has been demonstrated in animal studies (Chung 2009).

#### 2.5.5 Treatment in AKC

Already in 1953, Hogan found that corticosteroid treatment, topically and at times orally, was essential for AKC patients. Steroid treatment given locally as drops or ointment or systemically as tablets has been the backbone of treatment since then but warrants regular IOP control and cautious use. Although treatment with sodium cromoglycate showed some relief in a small study of AKC patients (Jay 1981), the standard antiallergic strategy with allergen avoidance, moistening eye drops, mast cell stabilizers, and antihistamines has a questionable place in moderate to severe cases.

Cyclosporine A is a calcineurin inhibitor and has been used as eye drops in ophthalmology since the 1980s. In a study using 2 % cyclosporine drops, improvement and a marked down regulation of T-cell activity was demonstrated in AKC patients (Hingorani 1999). Further trials have evaluated the efficacy of 0.05-0.1% cyclosporine drops with relief of symptoms and decreased need for steroid treatment (Apek 2004, Ebihara 2009), but in our experience a concentration of at least 1% is preferable.

Systemic treatment with Cyclosporine A has been beneficial in severe cases (Hoang-Xuan 1997, Anzaar 2008, Cornish 2010) but careful monitoring of side effects is needed for.

Tacrolimus (FK-506), also a calcineurin inhibitor, has emerged as an important contribution in anti-inflammatory treatment since about 15 years. Tacrolimus ointment has been effective in eyelid eczema treatment (Rikkers 2003, Zirbi 2009) and ophthalmic solutions show promising results but are only available in Japan (Ohashi 2010, Wakamatsu 2011). Topical treatment has not been associated with serious side effects. Tacrolimus does not seem to induce increased IOP (Remitz 2011) and does not cause atrophy of connective tissue (Kyllönen 2004). Systemic tacrolimus can also be considered in severe disease (Stumpf 2006, Anzaar 2008) but careful observation is required to avoid adverse effects.

Antibiotic drops are not indicated in the chronic phase of AKC but should be prescribed preventively in corneal ulcers. For suspected infectious keratitis topical broad spectrum antibiotic treatment is immediately indicated.

### **3 AIMS OF THE STUDIES**

The overall aim was to evaluate and further characterize the chronic inflammation in AKC in relation to possibly aggravating factors but also in response to periorcular eczema treatment.

The following specific aspects were studied

- The presence of microcolonization of the ocular and periorcular surface and its relation to
  - o Symptoms and clinical findings of the ocular surface
  - o Tear cytology and cytokines
  - o Conjunctival histology
- The effect of eyelid treatment with tacrolimus or group II steroid ointment on
  - o Symptoms and clinical findings of the eyelid skin and ocular surface
  - o Intraocular pressure (IOP)
  - o Microcolonization
  - o Tear cytology and cytokines
- The response to conjunctival allergen challenge in terms of
  - o Symptoms and clinical findings in the conjunctiva
  - o Tear cytokines

Two case studies addressing rare corneal disorders in AKC patients were included with the aim to highlight corneal complications and to suggest possible causative factors.

## **4 STUDY DESIGN**

### **Paper I**

In a cross-sectional study, microcolonization of the eyelid, cilae, and conjunctiva was analysed in AKC patients and healthy controls, using cultures for aerobic bacteria and fungi and serum analyses for SEB and *Malassezia* antibodies. The correlation with clinical signs of the disease, inflammatory cells in conjunctival biopsy and in tear fluid, and cytokines in tear fluid was investigated.

### **Paper II**

In a double-blind cross-over study, three weeks of eyelid treatment with tacrolimus ointment, Protopic® 0.1%, and a group II steroid, clobetasone butyrate, Emovat®, was evaluated in AKC patients with reference to clinically observed and subjectively reported efficacy, effect on IOP and microcolonization, and influence on tear cytokines.

### **Paper III**

The conjunctival response to local provocation with aeroallergen in AKC patients was studied. A single high dose of relevant antigen was given in one eye in sensitized AKC patients. Clinical signs and symptoms were documented and tear fluid cytokines were analysed at baseline, after 8 hours and after 48 hours.

### **Paper IV and V (case studies)**

AKC patients with severe corneal affection treated at St Erik's Eye Hospital since 1990 were reviewed, revealing two rare complications earlier not associated AKC: *Candida albicans* keratitis and spontaneous corneal perforation. These cases are presented and discussed.

### **Ethics (I-V)**

Approval was obtained for each study from the local ethics committee.

## **5 MATERIAL AND METHODS**

### **5.1 PATIENTS AND CONTROLS (I-V)**

St. Erik's Eye Hospital, inaugurated in 1990, is the only referral centre for severe ocular surface disease in the Stockholm County with approximately 2 million inhabitants. Presumably all patients with severe AKC are referred to the corneal department. During the time of the studies, these patients have mainly been managed by Dr. Per Montan and in later years also by myself. A group of 30-40 severe cases are followed on a regular basis while about 100 patients with mild to moderate disease are seen occasionally. Patients known to the investigators were recruited to the studies. Besides AKC diagnosis, a 2-week washout of anti-allergic, anti-inflammatory and anti-microbial treatment was required in studies I-III. The demand to halt anti-inflammatory treatment was particularly challenging to candidate participants with severe disease, which lead to study cohorts with moderate disease. In all, 35 patients were included; three patients participated in all three studies and seven patients in two of them. In study I, twelve healthy controls and in study III, five healthy controls were recruited from hospital staff. Inclusion criteria for controls were no history of allergy or ocular disease and negative IgE serology. In study III, five SAC subjects were recruited via advertisements, inclusion criteria were history of SAC, positive skin prick test, absence of any other eye affection and no anti-allergic or anti-inflammatory medication 2 weeks prior to the study. The patients reviewed in the case studies were managed at St Erik's Eye Hospital in a period of 20 years and known to the authors. All participants received written information prior to the trials and case studies, and a written informed consent was obtained.

### **5.2 ASSESSMENTS OF SYMPTOMS AND SIGNS (I-III)**

Grading of the clinical signs was slightly modified across the studies to allow for a more detailed assessment of the parameters of interest. In study I, a 3-grade score system (0 corresponding to no signs at all, 1 to mild and 2 to severe signs) determined the degree of lid eczema, blepharitis, conjunctivitis and keratitis. In study II, where response to eyelid treatment was sought, a detailed scoring was designed. Signs of eyelid eczema were categorized as: oedema, redness, excoriations, crusts/oozing, and lichenification. Signs of blepharitis were categorized as: oedema, redness, and crusts/oozing. All signs were scored 0-3: 0: no, 1: mild, 2: moderate, and 3: severe signs. Concerning conjunctival and corneal signs a less detailed scoring was used (0 = no signs at all; 1 = mild; 2 = moderate and 3 = severe signs). In the provocation study, only conjunctival signs of redness and chemosis were assessed using a 4-grade score system as above.

For subjective signs in study II and III, a VAS scale 1-10 was used, and in study II, a symptom diary was kept.

### **5.3 ATOPY AND ANTIBODY TESTING (I-III)**

Venous blood samples were obtained in all three studies. The blood was left in room temperature until centrifuged for 10 minutes at 1500g to separate sera, and thereafter frozen at -20°C pending further analysis.

In study I, IgE-mediated allergy was screened with Phadiatop® Pharmacia CAP System Specific IgE FEIA (Pharmacia Diagnostics, Uppsala, Sweden) and specific serum IgE against SEB and *M. sympodialis* was assessed with Pharmacia CAP System (Pharmacia Diagnostics AB, Uppsala, Sweden).

In study II, serum IgE against SEB was again analysed with the same system.

In study III, a skin prick test was performed with Soluprick SQ *Betula verrucosa* (silver birch), 10 HEP and Soluprick SQ Grass Pollen *Phleum pratense* (timothy grass), 10 HEP (ALK-Abello´ Nordic), to give immediate information about sensitization. Serum specific IgE to birch and timothy was analysed with the ImmunoCAP® System (Phadia AB, Uppsala, Sweden).

### **5.4 CULTURES (I, II)**

Presence of bacteria and *Malassezia* and *Candida* species was investigated in culture sampled from lashes, lid margin and inferior palpebral conjunctiva in study I. For isolation of bacteria, hematin agar plates and unmoistened swabs (Copan, Brescia, Italy) were used. For *Malassezia* species, Leeming & Notman agar (LNA) plates and for *Candida*, CHROMagar *Candida* plates (CHROMagar Co., Paris, France) were used. In study II, eyelids, eyelid margins, and conjunctiva were cultured as above with the exceptions that, in view of negative results in the study I, *Malassezia* was not sampled from the conjunctiva and that *Candida* was not cultured at all.

### **5.5 TEAR COLLECTION (I-III)**

Tear fluid was collected from the external canthus with a glass capillary tube and transferred to Eppendorff tubes and immediately frozen to -80°C pending further analysis. The most severely affected eye was sampled in study I, in study II, pooled samples were obtained and in study III, tears from both eyes were simultaneously sampled by two investigators.

### **5.6 TEAR CYTOLOGY (I- II)**

Two µl of tear fluid was immediately fixed with 2 µl of 4% formaldehyde for cytology analysis. This sample was placed as a drop on a glass microscope slide and dried prior to staining with May Grünwald (BDH, VWR, Stockholm, Sweden)/Giemsa (BDH). Eosinophil, neutrophil, macrophage and lymphocyte numbers were determined in the first study and eosinophil and neutrophil numbers in the second study by blinded observers.

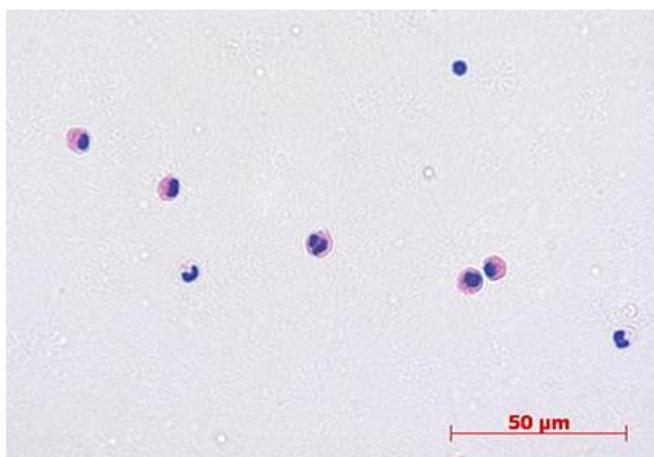


Figure 3. Eosinophils in tear fluid from AKC patient.

## 5.7 CONJUNCTIVAL CYTOLOGY (I)

To analyse inflammatory conjunctival cells, a biopsy specimen was excised from the lower, mid-tarsal conjunctival area with scissors under local anaesthesia. This was done in patients only and not in the controls. The tissue was paraffin embedded, sliced to 4 μm thickness, deparaffinized, rehydrated, and stained with the avidin–biotin complex (ABC) technique. The primary antibodies were EG2 (1: 20; specific for eosinophil cationic protein and secreted forms of eosinophil protein X; Pharmacia Diagnostics), elastase (1: 50; specific for neutrophils, Dako, Copenhagen, Denmark) and AA1 (1: 50; which stains mast cell tryptase, Dako). With the AA1 antibody, pre-treatment with pronase 0.05% was performed. Seven-μm-thick acetone fixed cryostat sections were stained with the primary antibodies anti-Leu-4 (1: 32, reacting with CD3, ‘panTcells’, Becton-Dickinson, SanJose´, CA, USA) and anti-IgE (clone B3102E8, 1: 1000 ImmunKemi, Järfälla, Sweden/Southern Biotechnology Associates, Birmingham, AL, USA). The ABC method was used and counterstaining was performed with Mayer’s hematoxylin. Cell numbers were counted/mm length of the conjunctival surface by one masked observer.

## 5.8 CYTOKINE ANALYSES (I-III)

Flow cytometric methods were used for cytokine analysis in all studies. The basic principles of flow cytometry include labelling of the sample with fluorescent dye molecules binding specifically to components of interest. The samples, containing cells or synthetic beads, are then loaded on the flow cytometer. The instrument shines light of a given wavelength on a thin stream where single cells (or beads) pass. Detectors record the reactions of the light when hitting the molecules or beads and thus the different molecules can be identified. With different fluorescent probes and multiple laser as well as multiple detectors, a huge number of signals can be recorded.

In study I, the concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5 and IL-10 were investigated. Cytometric bead array (CBA) (Becton Dickinson, Stockholm, Sweden) with beads coated with antibodies to the different cytokines was used. Depending on different fluorescence intensity of the different beads, the concentrations of multiple cytokines were analysed simultaneously by flow cytometry.

In study II, the concentrations of IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, GM-CSF, IFN- $\gamma$  and TNF- $\alpha$  were detected with the Luminex method. This method also uses beads but the beads carry variable quantities of two different fluorescent dyes that produce up to 100 different shades of colour and allows simultaneous analysis of an even larger number of cytokines in preformed kits. In study III, the concentrations of IFN- $\gamma$ , TNF- $\alpha$ , IL-5, IL-6, IL-10 and IL-13 were again analysed with the CBA method (BD Biosciences, San Jose, CA, USA), using a different laboratory from that used in the study I.

The choice and number of cytokines analysed was to some extent dependent on the method and kits available at that time. Our aim was, according to findings in previous reports (Metz 1997, Calder 1999), to analyse the balance between Th1 and Th2 subsets of cytokines, and in study I and III we could select accordingly. In study II, the multiplex method offered a broader cytokine spectrum including the cytokines we were interested in.

## **5.9 EYELID TREATMENT (II)**

Tacrolimus ointment was chosen being an interesting substance with little previous documentation for eyelid eczema treatment. The 0.1% concentration was favoured over the 0.03% given its higher potency and lack of an increased risk profile (Hanafin 2001, Soter 2001). As an active comparator, the steroid ointment clobetasone butyrate was selected on account of evidence of an equal anti-inflammatory action to that of tacrolimus 0.1% (Reitamo 2002). In addition, the ointment base with White Vaseline was the same for both products, which facilitated blinding. The treatment period of three weeks was designed from a safety point-of-view as we hypothesized that the steroid ointment could induce IOP elevations. The onset of the anti-inflammatory effect of both treatments was previously reported within two weeks (Ruzicka 1997, Reitamo 2002). A research nurse managed randomization and drug distribution.

## **5.10 CONJUNCTIVAL ALLERGEN CHALLENGE (III)**

Single, high dose conjunctival challenge, had been performed earlier by our group with good results and was previously proven to induce late phase changes in SAC (Montan 1996b, Bonini 1990). In study III, the allergen to which the patient exhibited the greatest reaction in skin prick testing was chosen for conjunctival challenge. A 40  $\mu$ l drop of highly concentrated birch or grass allergen, 100 000 SQ-E of Aquagen (ALK-Abello', Nordic; Kungsbacka, Sweden), was given in the lower fornix of the right eye. A 40  $\mu$ l drop of the albumin containing diluent (ALK-Abello', Nordic; Kungsbacka, Sweden) was given in the other eye.

## **5.11 STATISTICS I-III**

In study I, the Mann-Whitney U-test was used to analyse variable differences between AKC subjects and controls.

In study II, significant carryover or rebound phenomena of the treatments were evaluated with analysis of variance (ANOVA). All post-treatment values were compared with the baseline values. For parametric variables, paired t-test and for nonparametric variables, Wilcoxon paired test was used. Categorical data were analysed with the Sign test. Post hoc analyses of baseline global objective scores in

relation to treatment preference and to the presence of SEB were performed with the Mann-Whitney test.

In study III, Wilcoxon matched pair test was used to analyse variable differences before and after provocation and between the provoked and unprovoked eye. Differences between the groups were analysed with Mann-Whitney U-test.

The Spearman rank correlation coefficient was used to explore various associations in all three studies.

## 6 RESULTS AND DISCUSSION

### 6.1 MICROCOLONIZATION IN AKC (STUDY I)

In study I, the hypothesis was tested that bacteria and fungi would be linked to inflammatory changes in AKC, since such an association was previously found in patients with AD (Higaki 1999, Breuer 2002). Despite the fact that *S. aureus* was frequently isolated (73% showed growth on the lid margins vs. 0% of controls), no relation was found between the severity of AKC (as assessed clinically or objectively in biopsies or tear samples) and the presence of microorganisms. No growth and scant growth were shown for *Candida* and *M. sympodialis*, respectively, in the study group as well as in controls. In terms of sensitization to microorganism antigens, patients showed a high frequency of IgE antibodies to *M. sympodialis* (80%) while only 2 exhibited IgE to SEB. In contrast, control sera were all negative. Interestingly, the two patients with positive IgE to SEB presented the highest scores for clinical signs. As a means to confirm this finding, four additional AKC subjects with severe keratopathy were sampled and two of these evidenced IgE to SEB. Significantly higher levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-5 and IL-10 were shown in AKC patients compared to those in controls. The highest cytokine concentrations were observed for IFN- $\gamma$  supporting the notion of a Th1 profile in AKC and an association was found between conjunctival signs and the levels of all cytokines except IL-5.

In this cross-sectional investigation we found no evidence to suggest that periocular or ocular microcolonization is related to inflammatory parameters in AKC. Our results are in line with a previous study demonstrating comparable isolates of *S. aureus* on the lids of AD patients with and without ocular affection (Tuft 1992). It is supported also by the findings in study II, where colonizing patterns were unchanged following treatment with almost total clearing of eczema. However, it is far from certain that on-going inflammation in AKC would be synchronous with the local presence of bacteria and a longitudinal study with repeated sampling would be necessary to rule out a pathogenic role of microbes in AKC. Even though it may be suspected that altered skin colonization is a consequence rather than a cause in AD, it does provoke an immune response. In AD, hypersensitivity to *Malassezia* is discussed as a pathogenic factor (Bäck 2001, Svejgaard 2004, Bayrou 2005) but no association with other atopic manifestations, like asthma was previously found (Waerestad 1985) and no association with severity of AKC was found in our study. *S. aureus* on the other hand, may still be indirectly instrumental in the triggering or perpetuation of inflammation in AKC and merits further investigation since positive SEB IgE was found in the more severe cases participating in our study.

### 6.2 EYELID ECZEMA TREATMENT IN AKC (STUDY II)

The aim of study II was to evaluate the efficacy and safety of tacrolimus 0.1% relative to a mid potent corticosteroid ointment, clobetasone butyrate 0.05% ointment for treatment b.i.d. of eyelid eczema in AKC patients. Successful treatment of eyelid eczema with tacrolimus ointment in AKC patients had been reported in a few non-

controlled, open-label studies (Mayer 2001, Freeman 2004, Kawakita 2004). A double-masked explorative crossover study with 3-week treatment periods was designed. The main objective was to evaluate the safety with special reference to the intraocular pressure and to compare the effects of the treatments on eyelid eczema and any potential impact on ocular surface inflammation in patients with AKC.

Both treatments were effective in reducing signs and symptoms of eyelid eczema, with a near statistically proven benefit for tacrolimus with regard to total eyelid skin and margin eczema scores ( $P = 0.05$ ). Some patients experienced a transient burning feeling the first days of tacrolimus treatment but no serious adverse events occurred. Interestingly, intraocular pressure was not evidently affected by either treatment but three weeks of treatment might have been too short to rule out this risk. No alleviation of conjunctivitis or keratitis was noted in response to either medication, contrary to what has been described in reports on individual patients treated with tacrolimus ointment (Rikkerts 2003). The lack of effect was apparently confirmed in cytokine analyses showing no decrease after treatment periods but again the periods might have been too short for positive secondary effects to manifest.

Proportions of patients with presence of bacteria (95% had *S. aureus*) remained unchanged during the study regardless of type of treatment and degree of general symptoms, seemingly confirming the results of study I. As a follow-up investigation to study I, IgE to SEB was once again tested and a significant correlation was found between positive sera and higher clinical baseline scores for eczema and eye surface parameters. Thus, *S. aureus* may indirectly through an immune mechanism have a pathogenetic role in AKC.

There are now 2-year data confirming the safety of tacrolimus 0.03% in relation to IOP (Remitz 2010). Following our study, a great number of AKC patients reporting to our clinic have been put on tacrolimus 0.1% for eyelid dermatitis with a generally good response and with no evident rises in IOP. The initial burning sensation can be unbearable to some patients but with 1-2 weeks of steroid pre-treatment this can be overcome. Based on experimental studies (Niwa 2003), a black box warning for skin cancer following cutaneous tacrolimus was decided by the FDA 2004. No further reports of malignancy have emerged since then but physicians should be careful to inform patients to make a break in treatment over the sunnier periods of the year.

### **6.3 CONJUNCTIVAL CHALLENGE IN AKC (STUDY III)**

In study III, the role of atopy in AKC was explored by using an experimental allergen provocation model with either birch or grass pollen extracts. Previously, conjunctival challenge had not been performed in this group of patients. It was uncertain if the procedure would induce any reaction at all given the chronically inflamed status of the eyes with a possibly high threshold for increased symptoms and signs. Conversely, a provocation of a protracted inflammatory reaction could not be ruled out and it was evident that the fear of such an event made recruitment to the experiment difficult. The provocation did, however, evoke an immediate hypersensitivity reaction in the AKC subjects comparable to the reaction in SAC, as described in the literature (Möller 1984, Ableson 1990) and as evidenced in a few subjects included in the study for reference

purpose. Symptoms declined within 2 hours in all subjects and no patient experienced prolonged discomfort or exacerbation of disease following the provocation. In contrast, subclinical changes were found in tears of AKC patients after 48 hours. A significant increase in IFN- $\gamma$  and IL-6 was found compared to baseline values and significantly higher concentrations of IFN- $\gamma$ , IL-6, and IL-10 were demonstrated at 48 hours compared to those in the unprovoked eye. INF- $\gamma$  is known to have a pro-inflammatory role in chronic conjunctivitis stimulating production and expression of adhesion molecules, chemokines and co-stimulatory molecules by conjunctival (Stahl 2003, Zahn 2003, Leonardi 2006a) and corneal cells (Yannariello-Brown 1998). Increased levels of IFN- $\gamma$  in tears have also been shown to correlate with disease activity in AKC (Leonardi 2006b). In study I, both INF- $\gamma$  and IL-10 strongly correlated with conjunctival signs. Not previously reported in chronic allergy, in study III, the concentration of the pro-inflammatory INF- $\gamma$  and anti-inflammatory IL-10 correlated at all time points in the AKC patients and changes in clusters of cytokines may give further insights in the nature of inflammatory reactions. The serological allergen specific IgE-levels did not correlate with cytokine concentrations or clinical signs neither at baseline nor after provocation. Still, the experiment indicates that allergen exposure may affect the disease course in AKC. Our findings should stimulate further research with larger and matched groups of AKC and SAC patients to unravel possible differences between clearly allergen driven seasonal conjunctivitis and chronically manifest allergic conjunctivitis in which allergen exposure is but one of many pathogenic factors.

#### **6.4 CYTOKINE ANALYSES (STUDY I-III)**

Cytokine analyses have broadened our understanding of inflammatory disorders in recent decade, although study results have varied considerably (Uchio 2000, Cook 2001, Leonardi 2006a, Leonardi 2006b). In studies I-III, cytokine concentrations in tear fluid were analysed to seek objective indicators of ocular surface inflammation. A drawback was that during the progress of our work the laboratory, Capio Diagnostik, St. Göran's Hospital, Stockholm, Sweden, changed their method of analysis, so that cytokine analyses of studies I and II were performed differently. In contrast, the same method was used in studies I and III but the analysis in study III was done at Division of Cellular immunology, Karolinska University Laboratory, Huddinge, Sweden, since the Capio Diagnostik laboratory no longer conducted this analysis. Tear collection, however, was carried out by the same operators and by the same method across the studies including a standardized wash-out period of treatment for enrolled AKC subjects. In study I, all cytokines were detectable both in patients and in controls, but with significantly higher values in patients. A correlation with conjunctival signs was evident for most cytokines. These seemingly robust and significant results were not reproduced in studies II-III, view Table 3.

**Table 3. Median cytokine concentration in pg/ml in tear fluid at baseline in the three studies**

Cytokine	AKC Study I N=15	AKC Study II N=16	AKC Study III N=11	CT Study I N=12	CT Study III N=5	SAC Study III N=5
IFN- $\gamma$	800	98	63	562	46	44
TNF- $\alpha$	53	18	0	32	0	0
IL-2	101	27	-	64	-	-
IL-4	66	47	-	43	-	-
IL-5	36	1	0	21	0	0
IL-6	-	64	189	-	86	141
IL-10	66	45	54	47	31	24
IL-13	-	142	42	-	7	12

AKC= Atopic keratoconjunctivitis, CT= Healthy controls, SAC=Seasonal allergic conjunctivitis “-“= cytokine not analysed.

In study I, tear fluid was collected from the most affected eye. In study II, tear fluid was pooled from the right and the left eye. In study III, each eye was collected simultaneously, values reported above from the right eye.

Different methods of analysis might have affected the results and in study III, storage time at  $-80^{\circ}\text{C}$  was prolonged, which might have been unfavourable. Still, the cytokine results in studies II and III were of interest, since the main objective was to investigate changes following treatment and provocation interventions within the same individual. Large variations in tear cytokine concentrations are indeed observed between other studies performed by the same group (Leonardi 2006a, Leonardi 2006b). One may conclude that the concentrations of cytokines may not be comparable between different studies and methods, and that improved and standardized methods both for collection and for analyses of tear fluid cytokines are needed.

Studies IV and V are case series dealing with severe corneal manifestations in AKC aiming to highlight the risk for complications in this patient group.

Both the true prevalence of AKC and the risk to develop advanced and sight-threatening disease are unknown, as are the predisposing factors. In our experience, severe complications are seen primarily after 10-20 years of disease in middle aged patients. This age range is found also in case reports of patients requiring systemic treatment (Stumpf 2006, Anzaar 2007).

## **6.5 CANDIDA ALBICANS KERATITIS IN AKC (STUDY IV)**

Four AKC patients with *Candida albicans* (*C. albicans*) keratitis were diagnosed during a period of 20 years at St Eriks Eye Hospital. They are presented in case study IV. During the same period of time, a total of 14 cases of fungal keratitis were managed

in the hospital. Nine were caused by *C. albicans* and four of these patients had AKC. In temperate and urbanized areas, fungus is a very rare cause of keratitis and the main risk factor at our latitude is ocular surface disease (Tanure 2000, Galarreta 2007, Tuft 2009). Previously, AKC has only once been discussed as a particular predisposing disorder for fungal keratitis (Galarreta 2007).

The patients described in this study were all men, 37-44 years old and in good general health except for atopic disease and AKC. Three of them had received antibiotic and steroid treatment for the keratitis before the *Candida* diagnosis was made, highlighting the difficulty to clinically distinguish keratitis origin.

Besides chronic ocular inflammation, longstanding epithelial defects, and possible steroid use AKC patients might be at higher risk for *Candida* infection through altered skin barrier and colonization. In AKC patients with ulcerative blepharitis, *Candida* species have been found to be important colonizers (Huber-Spitzky 1992). In our study I, however, *Candida* was not recovered from any location in patients with moderate AKC. The only report found on skin colonization with *Candida* in AD, indicates a high prevalence but the relation to severity was not analysed (Arzumanyan 2000). On the other hand, an association between IgE antibodies to *C. albicans* and severity of AD severity has been demonstrated (Savolainen 1993). We propose that *C. albicans* should be considered as a possible causative organism when managing keratitis in this patient group. Cultures are essential and if *Candida* is found, treatment should be prompt and continued for a long time. The outcome of the four patients was depressingly poor.

## 6.6 SPONTANEOUS CORNEAL PERFORATION IN AKC (STUDY V)

In study V, we reported on six incidents of spontaneous corneal perforations in three AKC patients. All of them had previous corneal affection. Two patients had keratoconus (KC), one of whom had also a history of keratitis, healed since 1.5 years. Both were using rigid contact lenses. The third patient had corneal scarring with advanced astigmatism. In reports on spontaneous perforations in KC, these were always preceded by corneal hydrops (Rubsamen 1991, Nicoli 1999, Lam 2011) and in systemic disorders, such as rheumatoid arthritis (Malik 2006) and Sjögrens syndrome (Brejchova 2009) perforations are characterized by initial epithelial defects followed by stromal thinning and subsequent perforation. In the perforations presented by us, there was no documentation of hydrops or history of preceding corneal ulceration. The perforations were sudden and without premonition.

Corneal remodelling and breakdown is at least in part related to metalloproteinase (MMP) activity (Page-Masow 2007). One could speculate that an imbalance in these mediators led to perforation in our cases. An overexpression of MMPs or decrease in MMP inhibitors has been proposed in discussions of KC pathogenesis (Kenney 1994, Mackiewicz 2006) and increased levels of several MMPs have been demonstrated in corneal melting in systemic disorders (Brejchova 2009, 2010). In allergic conjunctivitis, little is known of the role of MMPs. MMP-2 and -9 have been demonstrated in tear fluid in VKC but also in some SAC subjects (Kumagai 2002). The activity of MMP-9 has further been correlated with corneal involvement in VKC (Leonardi 2003b). In patients with atopic blepharoconjunctivitis, increased levels of

MMP-8 in tear fluid have been demonstrated compared to healthy controls (Määttä 2008) but there are no studies on MMP activity in AKC.

The combination of corneal ectasia and inflammation probably led to perforation in these AKC patients. For initial management, patching with amniotic membrane transplant or corneal lamellar transplant, were successful. In two of the patients, perforation eventually occurred bilaterally and one of them experienced repeated perforations, underscoring the importance of observation also of the contra lateral eye in this group of patients. Systemic treatment could be indicated and cross-linking may possibly be considered for the fellow eye in similar situations. The role of MMP activity in AKC might be of further interest to explore with special attention to corneal thinning and presence of KC in AKC

## 7 SUMMARY AND PERSPECTIVES

AKC is a complex inflammatory disease, where atopy, imbalanced T-cell activity and an altered skin flora are prominent features. Whether these features are secondary phenomena or actual causes of the condition is not clear. The factors determining why some AD patients develop AKC and others do not, the mechanisms triggering exacerbations of disease, and the chain of events leading to severe corneal damage in some AKC patients are unknown. We aimed to characterize the inflammatory response to microbes, to eyelid treatment and to pollen exposure and to gain knowledge of severe corneal complications in patients suffering from AKC. We did not identify an association between ocular microcolonization and ocular surface inflammation although IgE against SEB may be a valid indicator of disease activity. We found no effect on ocular inflammation with effective eyelid eczema treatment leaving the issue of a link between adjacent eczema and conjunctivitis unresolved. We observed increased ocular surface inflammation with relevant allergen provocation and finally, we found AKC to be overrepresented in the rare corneal conditions *Candida* keratitis and spontaneous corneal perforation. Our results and conclusions should be viewed in light of the rather limited number of participants and that mainly moderate disease was studied.

What might be the objectives in future research on inflammation in AKC?

Investigation of cells and mediators in tears is a valuable tool in studies of ocular allergy. Tear collection offers the possibility of repeated sampling given its non-invasive nature but the lack of standard collection and analysis techniques is, however, a limitation and our results showed large variations across the studies. Tear collection with the capillary tube technique as used by us is the most common one, but absorption with Schirmer paper is another option (Wakamatsu 2010). Development of a quick, easily repeatable, non-irritative method along with the design of standard analysis kits of markers and mediators would definitely imply a leap forward. In odontology research, small volumes of gingival fluid are absorbed with a predesigned paper strip and the technique is successful for detection of a variety of mediators. (Tsilingaridis 2003, Grant 2010). A similar predesigned strip could possibly be adequate for tear fluid collection.

Although much is still to be learned about the relevance of the Th1/Th2 paradigm in allergy, another subset of T-helper cells called Th17 was identified a few years ago. Th17 activity has been implicated in a number of inflammatory disorders including atopy (Milner 2011). For instance, secretion of the Th17 cytokines, IL-22 and IL-17A has been shown to increase in response to staphylococcal toxins in the skin of AD patients (Niebuhr 2010). This may further indicate how colonization with *S. aureus* can contribute to inflammation in AD and possibly also in AKC. In this context, a possible correlation with serum IgE to SEB merits further investigation.

Further studies of the allergen-induced conjunctivitis using CPT should be carried out comparing AKC with SAC. With an extended investigation of a wide array of tear inflammatory markers (including those derived from the Th17 subset) a broader understanding of the differences between chronic and seasonal allergy could be gained.

In many investigations of allergic ocular disorders, a key role of eosinophil inflammation and an association between eosinophil products and corneal affection is suggested (Leonardi 1995, Montan 1996a). This was not analysed in our studies but could be of value to further explore since blocking of eosinophil recruitment could be a possible future therapeutic target (Komatsu 2008, Miyazki 2009).

Corneal thinning, manifesting as frank KC, is seen in AKC and even corneal perforation may rarely occur as observed by us. Possibly an imbalance in MMP activation and inhibition could be a cause. The levels of MMPs as influencing factors are previously not analysed in AKC but in VKC an association between MMP-9 and corneal affection has been observed (Leonardi 2003b). Indication of collagen degradation, its possible association with topographic corneal changes and the relation with e.g. eosinophil involvement, would be of interest to evaluate in patients with AKC. Possibly the classical definition of KC being a non-inflammatory disorder can be challenged when seen as a feature in AKC.

The AKC patient suffers from a multitude of inflammatory manifestations of which the more severe have a profound impact on cosmetics and visual function. This makes AKC one of the more challenging ocular surface diseases to manage. Further investigations, with the ultimate aim of finding means to improve the quality of life in AKC patients, should be given priority.

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