

Review

Allergy and allergic mediators in tears



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ABSTRACT

The identification of inflammatory mediators in the tear fluid have been extensively used in ocular allergy to find either a 'disease marker', to better understand the immune mechanisms involved in the ocular surface inflammation, or to identify potential targets for therapeutic interventions. While the clinical characteristics allow a relatively convincing diagnosis of ocular allergic diseases, in the initial, non active phases, or in the chronic stages, the diagnosis may not be clear. Although not highly specific, total tear IgE can be measured with local tests by inserting a paper strip in the lower meniscus. The measurement of tear specific inflammatory markers, such as histamine, tryptase, ECP, IL-4, IL-5 and eotaxin, may be useful for the diagnosis or monitoring ocular allergy. New technologies such as multiplex bead assays, membrane-bound antibody array and proteomic techniques can characterize the distribution of a wide range of bioactive trace proteins in tears. Dozens of mediators, cytokines, chemokines, growth factors, angiogenic modulators, enzymes and inhibitors were thus identified in small tear samples using these techniques, providing the possible identification of specific biomarker for either specific disease or disease activity. However, to date, there is no a single specific laboratory test suitable for the diagnosis and monitoring of allergic conjunctivitis.

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1. Introduction

Approximately one third of the world population is affected by some form of allergic disease and ocular involvement is estimated to be present in 40–60% of this population. Allergic conjunctivitis is a localized allergic condition frequently associated with rhinitis but often observed as the only or prevalent allergic sensitization. This disease ranges in severity from mild forms, which can still interfere significantly with quality of life, to severe cases characterized by potential impairment of visual function.

The term allergic conjunctivitis refers to a collection of hypersensitivity disorders that affects the lid and conjunctiva. Various clinical forms are included in the classification of ocular allergy each of them requires a differential diagnosis that is usually clinical, yet can be substantiated by objective laboratory parameters (Leonardi et al., 2012). While their clinical characteristics allow a relatively convincing diagnosis of ocular allergy both in the initial or chronic stages, there can be some confusion as to what form of allergy is present. At times, pseudo-allergic forms, with clinical manifestations similar to allergy but with a non-allergic equivocal pathogenesis, are difficult to distinguish from allergic forms, with their

precisely defined pathogenic mechanisms. In fact, several ocular surface diseases, including tear film dysfunction, blepharitis, sub-acute and chronic infections, toxic and mechanical conjunctivitis may mimic the clinical pictures of ocular allergy. To date, there is no specific laboratory test suitable for the diagnosis and monitoring of allergic conjunctivitis. Ancillary tests, such as skin prick and the identification of serum specific IgE, can be useful for diagnosis and treatment, however it is well known that the results are often not correlated with the ocular disease. The identification of inflammatory mediators in the tear fluid have been extensively used in ocular allergy to find either a 'disease marker', to better understand the immune mechanisms involved in the ocular surface inflammation, or to identify potential targets for therapeutic interventions.

1.1. Tear collection

Tear samples can be easily obtained from the ocular surface using different methods and techniques making mediator search an attractive tool in ocular allergy. There different methods for sampling of tears: the microcapillary tube method, the filter paper and ophthalmic sponges. It is preferable to collect tears from both eyes since in many cases of allergic conjunctivitis there is a unilateral predominance.

Aspiration of tears by glass capillary tubes or pipettes can yield volumes of 20–50 µl, but collecting is tedious, time-consuming,

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sometimes uncomfortable for patients and children, and may provoke the production of reflex tearing by touching the conjunctiva with the tube. Tears can be recovered from a Schirmer strip but tear reflection is very common due to strong irritation by the strip. Various sponges and extraction buffers can be used, making it difficult to assess the feasibility of the protocols and to compare the results. Moreover, some cytokines bind tightly to the sponges, and diffusing cytokines out of the sponges during the extraction procedure can be difficult. With all these methods, tear reflection may occur easily diluting factors that are going to be evaluated. Thus the ultimate outcome could be also affected by the tear collection method chosen and the consistency of the extraction protocol (Inic-Kanada et al., 2012). In our experience, the best method for avoiding reflex tearing is the capillary tube.

2. Ocular allergy: classification and clinical phenotypes

The current allergic nomenclature is based on pathophysiology according to the different hypersensitivity mechanisms still commonly used in the clinical practice. Ten years ago the European Academy of Allergy and Clinical Immunology and the Nomenclature Review Committee of the World Allergy Organization proposed to distinguish allergic from non allergic hypersensitivity reactions dividing them into IgE- and non-IgE-mediated hypersensitivities (Johansson et al., 2004, 2001). According to this view and to better understand ocular pathology in the future, a new classification of ocular allergy has been recently proposed dividing ocular surface allergic- from non-allergic hypersensitivity disorders (Leonardi et al., 2012). However, single clinical entities maintain the traditional names.

2.1. Seasonal/intermittent (SAC) and perennial/persistent allergic conjunctivitis (PAC)

Seasonal allergic conjunctivitis, or hay fever, one of the most common allergic diseases, results in significant morbidity and presents an increasing economic burden because of direct health expenditures, as well as less evident cost factors, such as lost work time. SAC and PAC are IgE-mediated allergic disease usually associated with exposure to airborne allergens. SAC is characterized by seasonal/intermittent ocular itching, commonly associated to rhinitis, and non specific signs such as conjunctival redness and eyelid edema, which arise and subside with the patient's exposure to the offending allergen(s). PAC is related to allergens that are present year round, such as dust mites, animal dander and molds or to multiple allergen sensitizations. It is a chronic condition, with persistent, frequently mild symptoms, enhanced by higher or longer exposure to allergen and exacerbated by non-specific irritating substances. It is characterized by ocular itching, burning, eye watering and conjunctival redness, conjunctival and eyelid edema and small tarsal conjunctival papillae. In both forms, the only pathognomic symptom is itching.

2.2. Vernal keratoconjunctivitis (VKC)

VKC is a persistent and severe form of ocular allergy affecting children and young adults, particularly in warm climate. VKC usually appears between age 4 and 12 and usually heals after puberty. The disease is more frequent in boys (sex ratio, 3/1) before puberty. The IgE-mediated mechanism found in approximately 50% of the patients does not explain completely the severity and the clinical course of this disease, which is probably related also to T cell-mediated responses, massive eosinophil attraction and activation, and non-specific hyper-reactivity.

Intense itching, tearing and photophobia are the classical symptoms of VKC patients. Disease exacerbation can be triggered by either allergen re-exposure or, more frequently, by non-specific stimuli such as sun light, wind, and dust. The tarsal form of the disease is characterized by irregularly sized hypertrophic papillae, leading to a cobblestone appearance of the upper tarsal plate. The limbal form is characterized by transient, multiple limbal or conjunctival gelatinous yellow-gray infiltrates with white points on the top, known as Horner-Trantas's dots, and papillae at the limbus. Punctate keratitis, epithelial macroerosions or ulcers and plaques are signs of corneal involvement, which resolves with different levels of subepithelial scarring.

2.3. Atopic keratoconjunctivitis (AKC)

AKC is a persistent inflammatory condition involving the eyelid skin, the conjunctiva and possibly the cornea, and that can be defined as the ocular manifestation of atopic dermatitis. The hallmark sign of AKC is an eczematous, erythematous, exudative lesion of the lids often associated with chronic blepharitis and meibomian gland dysfunction. The conjunctival hyperaemia and chemosis affect predominantly the inferior fornix and palpebral conjunctiva. The limbus may also be involved. The disease often leads to cornea lesions and can be complicated by conjunctival fibrosis, *Staphylococcus aureus* colonization of the eyelid, herpes simplex keratitis, keratoconus, retina detachment, cataract with consequence sustained vision deterioration.

2.4. Giant papillary conjunctivitis (GPC)

GPC is an adverse ocular reaction to contact lenses, ocular prostheses, post-operative sutures or some irregularities of the eyeball surface. GPC is a non-allergic hypersensitivity inflammation of the external ocular surface that may overlap with other forms of ocular allergy. The early stages of GPC may be asymptomatic but the initial signs can be observed by slit lamp examination. Increasing intolerance to contact lenses may lead to their discontinuation. Clinically it is characterized by small to giant papillae of the upper tarsal conjunctiva, redness and discharge.

2.5. Contact blepharoconjunctivitis (CBC)

CBC is a result of allergic/irritant reactions after contact with different substances usually applied to the eyelid skin or the conjunctival sac. It is characterized by edema, eyelid skin redness, eczema or lichenification, conjunctival redness and papillae.

3. Allergic inflammation

In sensitized patients, the immediate hypersensitivity associated with ocular allergy is characterized by allergen-mediated cross-linking of IgE on mast cells, leading to degranulation and release of mediators localized in specialized granules, including histamine, tryptase, leukotrienes, cytokines, and platelet-activating factors and the *de novo* synthesis and secretion of cytokines, chemokines and eicosanoids. These mediators stimulate nerve endings, dilate blood vessels, and recruit inflammatory cells to the reaction site, causing immediate clinical symptoms such as itching, redness, and lid and conjunctival edema (early phase) (Fig. 1). The late phase reaction is associated with an accumulation of inflammatory cells in the conjunctiva. This immediate or early response lasts clinically 20–30 min as demonstrated by the specific conjunctival provocation test (CPT). In fact, CPT reproduces signs and symptoms of the allergic reaction and induces enhanced tear levels of histamine, tryptase, prostaglandins, leukotrienes, and the

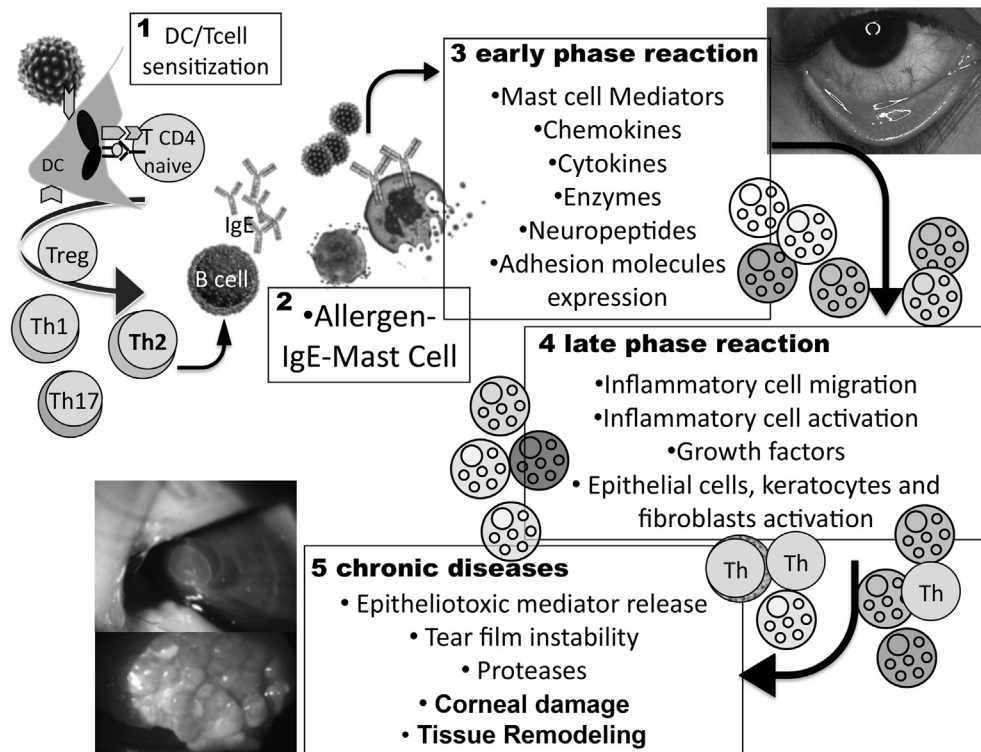


Fig. 1. Ocular allergic inflammation. In predisposed patients, dendritic cells (DC) trigger naïve T cells to differentiate into Th2 lymphocyte. These cells stimulate B-cell to produce allergen specific IgE (1). After the natural exposure to the sensitizing allergen (2), specific IgE on mast cells cause the release of inflammatory mediators and the immediate clinical manifestation of the disease (3). Mast cell degranulation induces the expression of chemokines and adhesion molecules promoting the recruitment of inflammatory cells in the conjunctival mucosa and the induction of the late-phase reaction (4). Chronic allergic diseases are characterized by corneal damage due to epitheliotoxic factors released by activated eosinophils a giant papillae formation (5).

recruitment of inflammatory cells (Leonardi et al., 2007b). Conjunctival mast cells are 100% tryptase and chymase positive (connective type mast cells) and express IL-4 and other cytokines. IL-4 plays a key role in allergy promoting T cell growth, inducing IgE production from B cells, up-regulating the adhesion molecules and regulating T helper type 2 (Th2) lymphocytes differentiation (Anderson et al., 2001). Together with tumor necrosis factor (TNF)- α , IL4 is a promoter of adhesion molecules expression and chemokine secretion from corneal keratocytes, conjunctival fibroblasts and epithelial cells, all contributing to inflammatory cells recruitment and the induction of the allergic inflammatory late phase reaction (Fig. 1).

The presence of Th2 cells and Th2-type cytokines has been proven to have a pathogenetic role in ocular allergic diseases. However, during the active inflammatory phase of these diseases, multiple cytokines are over-expressed and produced (Leonardi et al., 2006a) including the typical Th1-type cytokine, interferon (IFN)- γ , which may contribute to increasing the ocular inflammation similarly to what has been shown in animal models. During the ocular inflammatory process, several inflammatory mediators, cytokines and free oxygen radicals are released onto the ocular surface and into the tear film, causing a wide range of corneal clinical manifestations. For example, inflammatory cells, cytokines and chemokines liberated from eosinophils, Th2 cells, and tear film instability may act concomitantly in the pathogenesis of shield ulcer (Fig. 1). Eosinophils and eosinophil-derived proteins and collagenases, in particular metalloprotease (MMP)-9, have been shown to damage the corneal epithelium and basement membrane (Leonardi et al., 2003a).

Multiple mediators, cytokines, chemokines, receptors, proteases, growth factors, intracellular signals, regulatory and inhibitory

pathways, and other unknown factors and pathways are differently expressed, ultimately resulting in the many clinical manifestations of ocular allergic disease. A better understanding of the mechanisms involved in ocular surface immunity is necessary for identifying biological disease markers, new classification criteria and new therapeutic strategies.

4. IgE in tears

The immunoglobulin IgE plays a pivotal role in the pathogenesis of type I allergic reaction. Measurement of IgE protein levels in serum reflects systemic atopic diathesis independently of a specific sensitization. The measurement of allergen-specific IgE is a marker of sensitization to a specific antigen. Tear IgE concentrations increase in allergic conjunctivitis patients, suggesting that measuring tear IgE concentrations can help to diagnose IgE-mediated allergic conjunctivitis (Leonardi et al., 1993).

4.1. Measurement of total IgE in tears

Total tear IgE analysis has been used for differential diagnosis of IgE mediated diseases, or when a lack of sample makes it impossible to perform specific tear IgE analyses. Different methods can be used: the traditional paper radio immunosorbent test (PRIST) and immunochromatographic methods. Total tear IgE evaluation has come back into fashion after the commercialization of simple and rapid diagnostic tests for the semi-quantitative determination.

IgE in tears are normally absent or very low due to the blood–tear barrier. Tear samples taken from healthy individuals or from non-allergic conjunctivitis patients give negative results. Using an immunosorbent method, tear IgE concentrations showed

significant increases in VKC, SAC and PAC when compared with normal subjects (Nomura and Takamura, 1998).

Lacrytest® (ADIATEC S.A, Nantes, France) is a rapid immunoassay for a qualitative total IgE determination in tears with levels above the normal value (less than 2.5 kU/l or 3 ng/ml). The test utilizes paper strips that are applied directly to the lower fornix of the conjunctiva in a manner similar to the Schirmer's test. Anti-IgE antibodies inside the strip reveal, with a colorimetric system, the presence of total tear IgE in a matter of minutes: the absence of a red line indicates a negative finding; the appearance of two red lines indicates a positive finding and functioning of the assay. A semiquantitative result is possible by reading the color intensity. This test has a sensitivity and specificity of 91.7% and 98.5%, respectively (Monzon et al., 2009; Sirbikaite and Ehlers, 2007). In our experience, the test is well correlated with results of conjunctival provocation (CPT) results but poorly correlated with skin test results, confirming that local sensitization may occur. This test is currently out of market.

Similar to the previous test, ENTEBE IgE IC® (Hepatica, Mataram, Indonesia) uses tear samples collected by Schirmer filter paper immersed in a reaction buffer solution and eluted at room temperature. The immunochromatography test strip, is then included in the same reaction buffer solution for reactive labeling. Results are determined by visual observation, indicated by a red colored line. Tear IgE indices for VKC, AKC and SAC patients were significantly correlation with both total IgE levels in serum and clinical scores (Inada et al., 2009).

Allerwatch® test is an other new quick diagnostic test for measuring total IgE in tears using immunochromatography method. Results can be promptly obtained during a consultation (within 10 min). Using this method, the total tear IgE score (0, 1, 2) was correlated with the scores for objective signs of allergic conjunctivitis (Mimura et al., 2012).

These immunological assays have interesting potential applications as a local ocular 'marker' of allergy that presently does not exist. Given the scarce quantity of tear samples, the limited correlation between ocular symptomatology and systemic allergic tests and the rapid and simple execution of these tests, it would be auspicious to have available to the eye specialist, allergist, or even general practitioner, an easily measurable marker for ocular allergic disease.

4.2. Tear specific IgE

Detection of allergen-specific IgE in serum is normally performed with an enzyme-linked immunosorbent assay (ELISA) and CAP radioallergosorbent test as standard methods. Levels of specific IgE are undetectable in tears of non-allergic subjects. For each assay, at least 50 µl of tears are required, thus it is not always possible to obtain a sufficient quantity of sample. The assay is identical to that used for serum IgE measurement, even if there are no standardized reference parameters for the eye. We have in the past observed little correlation between tear and serum IgE and between tear IgE and skin tests: 30% of SAC or PAC patients and 50% of VKC patients were positive only to local tear tests (Leonardi et al., 1993). Tear specific IgE levels are, however, well correlated with CPT results. Similarly, the highly significant correlation between allergic conjunctivitis and the presence of specific tear IgE emphasized the diagnostic value of immunoblots with tear IgE, especially in cases in which serum provides inconclusive results (Hoffmann-Sommergruber et al., 1996).

Immfast Check J1, a new commercial immunochromatographic test, has been used to measure specific tear IgE in moderate to severe cases of allergic conjunctivitis. This kit is specifically designed for direct measurement of IgE against cedar pollen, cat dander, and

house dust mites. Tear samples are obtained by Schirmer strips and eluted in a buffer than processed. Results are classified into 4 categories according to a semiquantitative scale determined by visual observation and confirmed using an image software. Specific IgE scores were significantly higher in the allergic group with a very high sensitivity (98.4%) and specificity (97.7%), suggesting this test to be performed in an outpatient basis (Mimura et al., 2011).

We recently used in tear samples of VKC patients, a multiple specific microarray technique (ImmunoCAP ISAC, Phadia, Milan, Italy) for direct measurement of IgE directed against 103 components derived from 47 allergens. Specific IgE were detected in 6 of 10 patients and in 3 of them, allergen sensitization was detected only by tear IgE. This method detects allergen sensitization at component level and may further add important information by defining both cross and co-sensitization to a large variety of allergen molecules in small tear samples. The presence of specific IgE only in tears of VKC patients reinforce the concept of possible local sensitization.

In summary, patients with high total IgE in tears have a high probability of allergic sensitization, however measurement of tear allergen-specific IgE antibodies is a more specific diagnostic marker of allergic sensitization.

5. Tear cytology

It is particularly useful to qualitatively define conjunctival inflammation in the active phase of allergic disease with cytological analysis. A cytological test can be performed using tears, conjunctival scrapings, brush cytology, impression cytology or conjunctival biopsy. The presence of even one eosinophil is highly indicative of an allergic pathology, while their absence does not exclude an allergic diagnosis. In particular, tear cytology is rapid and easy to perform: a few microliters of tears collected from the external canthus with a glass capillary are immediately placed on a slide. Precolored slides, such as those treated with May Grunwald-Giemsa, or rapid dyes can be used for a response in several minutes. It is less traumatic than conjunctival scrapings or brush cytology.

In patients with allergic eye disease the cellular constitution of the tear film is characterized by the presence of neutrophils, eosinophils and lymphocytes as proved by the response to the CPT (Bonini et al., 1990). A secondary immediate, late and delayed conjunctival responses, induced by the nasal provocation test with allergen, were associated with different cellular profiles in the tears (Pelikan, 2012). Increased number of eosinophils, neutrophils, basophils and epithelial cells found in tears after nasal specific provocation confirms that nasal and conjunctival allergic reactions are related each other. Collected tear cells can be also evaluated by flow cytometry: the percentages of T cells, activated B cells, and T-helper/T-suppressor cell ratios were found to be higher in tears of patients with AKC than in controls (Avunduk et al., 1998). The intracytoplasmic cytokine expression with flow cytometry provides information not only on the phenotype of the inflammatory cells involved but also on their state of activation. With this techniques, the presence of Th2 lymphocytes in tear fluid of patients with VKC have been demonstrated (Leonardi et al., 1999). Recently, variable levels of Th17 cells were detected in tears-derived cells in clinical subtypes of conjunctivitis. Intracellular co-expression of T cell subset-specific transcription factors has identified a predominance of Th2 T cells in VKC, which is inversely correlated with Th17 cells, suggesting VKC is mainly a Th2-driven response. The lack of correlation with IL-17 levels in tears fluids suggests these cells were not the source of this cytokine (Galatowicz et al., 2010).

In summary, evaluation of the number and percentage of leukocytes on the ocular surface can be essential to the decision of how to proceed with further diagnostic tests.

6. Mast cell mediators

The number of mast cells in the human conjunctiva has been calculated to be between 5000 and 6000 cell/mm³. This number is increased in all ocular allergic diseases even in the absence of a substantial leukocyte infiltration (Anderson et al., 2001). During pollen season, the median mast cell number in the lamina propria was increased by 61% in SAC patients compared to normal subjects and remained increased in allergic patients out of season (Anderson et al., 1997). Increased expression of stem cell factor and several other cytokines and chemokines in the allergic conjunctiva may explain mast cell migration, proliferation, survival and activation typical of ocular allergy (see also Section 8). Besides the gamut of preformed and newly formed mediators, activated conjunctival mast cells release cytokines such as IL-4, IL-6, IL-8, IL-13, TNF- α and transforming growth factor (TGF)- β , all of which have profound effects on the mucosa, including chemokines and adhesion molecules expression that contribute to the recruitment of inflammatory cells.

6.1. Histamine

Histamine exerts its activities by binding to H₁, H₂, H₃ and H₄ receptors (R), which are important therapeutic targets for various allergic and non allergic diseases (Dunford et al., 2006). All four histamine receptor subtypes are important for conjunctival goblet cell secretion (Hayashi et al., 2012) and are present in the human normal conjunctiva, while H₁R, H₂R and H₄R are over expressed in VKC (Leonardi et al., 2011b). Rapidly released upon mast cell activation, the interaction with H₁R is responsible for most of the allergic signs and symptoms: redness, swelling and itching. It has been calculated that a single conjunctival mast cell contains 4.6 pg of histamine (Miller et al., 1996), signifying that the total potential amount of histamine that can be released with massive mast cell degranulation is 23 ng/mm³. The measurement of tear histamine, carried out with RIA or ELISA (Table 1), is a relatively simple assay to perform given the commercially available kits. In the tear film of normal subjects, histamine was found at concentrations of 5–10 ng/ml, whereas tear samples of patients with active VKC contain significantly higher levels (Abelson et al., 1995) but not in SAC (Martinez et al., 2011). Histamine is rapidly degraded in tears by histaminase enzymes, thus high values are observed in tears only immediately after massive mast cell degranulation, such as after a provocation test, or, as we have demonstrated, in conditions of enzyme deficit such as in VKC (Abelson et al., 1995; Bacon et al., 2000; Leonardi, 2000). Although histamine measurement cannot be considered the ideal marker of allergic inflammation, tear levels measured immediately after the CPT may mirror the level of mast cell activation (Leonardi and Abelson, 2003). On the contrary, no differences in histamine tear levels were found between different time points of analysis after nasal grass pollen provocation (Callebaut et al., 2010). Using a novel high performance liquid chromatography method, the tear histamine content was low in physiological conditions, and did not vary in relation to age and sex. Histamine levels were significantly higher in patients affected by allergic or *Haemophilus influenzae*-associated conjunctivitis confirming that histamine tear levels are not specifically increased exclusively in allergic conditions (Venza et al., 2004).

6.2. Tryptase and chymase

Tear tryptase is a marker for conjunctival mast cell activation and, as such, its measurement might be useful in the diagnosis of allergic disease (Butrus et al., 1990; Magrini et al., 1996). However, it is also possible that in other forms of non-allergic conjunctivitis,

non-specific activation of mast cells may occur. Tear tryptase can be assayed using the UniCAP method (sensitivity 1 mg/L). After CPT, mean tryptase concentration significantly increased at 20 min and then decreased to normal at 40 min (Bacon et al., 2000; Jedrzejczak-Czechowicz et al., 2011) (Table 1). Tryptase tear levels were found to be markedly increased in patients with severe VKC, significantly decreased following treatment and correlated with the improvement in the clinical signs and symptoms suggesting that tryptase measurement in tears may serve as a good diagnostic tool for ocular allergy and for monitoring of activity of the disease (Tabbara, 2001) and to evaluate and compare different agents (Bonini et al., 1997; Leonardi et al., 2000b).

Mast cell chymase levels and activity in tears were also increased in VKC patients and correlated significantly with disease severity suggesting that it may be a sensitive marker for determining the severity of VKC (Ebihara et al., 2004).

6.3. Prostaglandins and leukotrienes

High performance liquid chromatography and immunoassays have demonstrated the presence of increased levels of PGD₂, LTC₄, LTD₄, and LTE₄ in tears after conjunctival challenge (Proud et al., 1990). Increased levels of LTB₄ and LTC₄ were also found in tears of contact lens associated inflammation and ocular prosthesis associated GPC (Irkec et al., 1999). Proinflammatory leukotrienes play a pivotal role in allergic reaction and goblet cell mucous production in the conjunctiva, which can be actively terminated by treatment with resolvins (Dartt et al., 2011).

In summary, the increased concentrations of mast cell derived mediators in tears, in particular tryptase and histamine, have been considered biomarkers of allergic IgE-mediated conjunctival response.

7. Eosinophils derived mediators

Eosinophils are known to play a major role in the development of all allergic diseases. Eosinophil major basic protein (MBP), eosinophil cationic protein (ECP), and eosinophil neurotoxin (EPX/EDN) are released by activated eosinophils during allergic reactions. The presence of these proteins in biologic fluids and tissues is now considered a specific marker for eosinophil activation (Bonini et al., 1993).

7.1. Eosinophil cationic protein (ECP)

ECP is thought to play a major role in the pathogenesis of VKC and AKC having a toxic effect on human corneal epithelium and potentially leading to vernal corneal shield ulcer. ECP levels in biological fluids correlate with the severity of some allergic diseases, thus it can be considered a marker of eosinophil activation. Tear ECP tear levels were increased only in the late-phase reaction after CPT, partially correlating with clinical symptoms but not with tear cytology. These values were similar to values found in an active seasonal reaction (Table 1) (Jedrzejczak-Czechowicz et al., 2011; Leonardi et al., 2000a; Montan et al., 1996; Muromoto et al., 2006; Oh et al., 1999). Patients with SAC, VKC and AKC, but not GPC, rosacea, and bacterial conjunctivitis, had significantly increased tear and serum levels of ECP and EPX, confirming that ocular allergic diseases are essentially systemic disorders with an increased activation of local and peripheral eosinophils (Leonardi et al., 2000a). Increased tear concentration of EDN, ECP and MBP were also found in children with active SAC and PAC (Martinez et al., 2011; Oh et al., 1999). Elevated ECP tear levels have been detected in patients with active VKC and AKC and correlated with the severity of the disease (Leonardi et al., 1995; Montan and van

Table 1

Tear mediators in ocular allergic diseases.

Mediator	Tear collection	Method	Unit	Normal levels	CPT	SAC/PAC	VKC	AKC	Author
IgE	Sponge	ELISA	ng/ml	22.1 ± 9.9		8.97 ± 12.04			Martinez et al., 2011
IgE	Capillary tube	PRIST	ng/ml	52.1 ± 9.7		194.7 ± 21/134.8 ± 23	322.2 ± 45.7		Nomura and Takamura 1998
IgE	Filter paper	ICR	IgE index	0		4.0 ± 3.1	27.5 ± 15	19.8 ± 15	Inada et al., 2009
Histamine	Capillary tube	ELISA	ng/ml	0.85 ± 0.2			11.1 ± 2		Abelson et al., 1995
Histamine	Capillary tube	ELISA	ng/ml	10 ± 4	15 ± 8				Leonardi et al., 2000a,b
Histamine	Capillary tube	ELISA	nM/L	5 ± 5	30 ± 27				Leonardi et al., 2003a,b,c
Histamine	Sponge	ELISA	ng/ml	4 ± 11		56 ± 130			Martinez et al., 2011
Histamine	Sponge	RIA	nmol/L	1 ± 1	7 ± 1				Bacon et al., 2000
Histamine	Filter paper	ELISA	ng/ml	0.75 ± 0.15	0.69 ± 0.15				Callebaut et al., 2010
Histamine		HPLC	ng/ml	2.26		23.61			Venza et al., 2004
Tryptase	Sponge	RIA		3. ± 3.2		0.6 ± 0.1	14.5 ± 13		Magrini et al., 1996
Tryptase	Sponge	RIA	μg/L	1 ± 1	23 ± 4				Bacon et al., 2000
Tryptase	Capillary tube	RIA	mg/L	0	16 (0–65)				Leonardi, 2000
Tryptase	Filter paper	UniCap		0	5.8 ± 1.9				Jedrzejczak-Czechowicz et al., 2011
Tryptase	Sponge	ELISA	ng/ml	32 ± 15		26.6 ± 13.7			Martinez et al., 2011
Tryptase	Capillary tube	UniCap	ng/ml				16 ± 27		Tabbara, 2001
Chymase		ELISA	mU	0.11 ± 0.05			1.91 ± 0.71		Ebihara et al., 2004
ECP	Capillary tube	RIA	μg/L	7.5 ± 0.4			988.3 ± 128		Leonardi et al., 1995
ECP	Capillary tube	RIA	μg/L	<20 (0–30)		70 (4–540)	470 (19–6000)	215 (36–1900)	Montan et al., 1996
ECP	Capillary tube	RIA	μg/L				560 ± 756.7		Leonardi et al., 1997
ECP	Capillary tube	UniCap	μg/L	3.9 ± 3.8		54.9 ± 117.7			Oh et al., 1999
ECP	Sponge	RIA	μg/L	0.5 ± 0.05	1.5 ± 0.5 (EPR)	5 ± 1 (LPR)			Bacon et al., 2000
ECP	Filter paper	ELISA	μg/L	5.06 ± 3.3		54.9 ± 117.7			Muromoto et al., 2006
ECP	Filter paper	CL	ng/ml				3092 ± 1658		Hori et al., 2011
ECP	Capillary tube	UniCap	μg/ml				1152 ± 654.0		Leonardi et al., 2007a,b
ECP	Sponge	UniCap	μg/L	1.7 ± 1		10.6 ± 7.7 (LPR)			Jedrzejczak-Czechowicz 2011
ECP	Capillary tube	ELISA	ng/ml					2680 ± 2342	Wakamatsu et al., 2011
ECP	Sponge	ELISA	ng/ml	5.5 ± 11.6		143.8 ± 209			Martinez et al., 2011
IL-4	Micro Pipette	ELISA	pg/ml	0.9 0.5		3.5 ± 2.5	23.9 ± 22.5		Fujishima et al., 1995
IL-4	Capillary tube	ELISA	pg/ml	0					Leonardi, 2006a,b
IL-4	Capillary tube	ELISA	Unit	60 ± 160		350 ± 450			Uchio et al., 2000

CPT = conjunctival provocation test; SAC = seasonal allergic conjunctivitis; PAC = perennial allergic conjunctivitis; VKC = vernal keratoconjunctivitis; AKC = atopic keratoconjunctivitis; ICR = immunochromatography; CL = chemiluminescence; ECP = eosinophil cationic protein.

Hage-Hamsten, 1996; Wakamatsu et al., 2012). Thus, the measurement of ECP in tears is a valid tool for use not only in the monitoring of ocular allergic diseases, but also in the evaluation of topical therapies, for which an objective factor is needed to complement clinical assessments (Hori et al., 2011; Leonardi et al., 1997, 1995, 2007a; Wakamatsu et al., 2011).

In summary, ECP tear levels may be considered a specific biomarker of both allergic IgE- and non-IgE-mediated allergic conjunctival inflammations.

8. Cytokines and chemokines

Determination of tear cytokine and chemokine levels and their receptors expression are not yet used for diagnosis, but only for the study of allergic physiopathology or for the evaluation of efficacy of anti-allergic agents. For example, a recent study in the animal model suggest that interfering with CCR7 function therapeutically may inhibit the progression of allergic conjunctivitis (Schlereth et al., 2012). Several studies have revealed distinct differences in the cytokine/chemokine concentrations in tears between the various manifestations of ocular allergy using various methods. SAC and GPC are characterized by an overall lack of significant cytokine changes in tears, whereas VKC and AKC are characterized by increased concentrations of Th1, Th2, pro-inflammatory cytokines and chemokines (Cook, 2004; Leonardi et al., 2006a). Data from individual studies have yielded surprisingly wide ranges of values for the concentration of various cytokines in normal a pathological tear fluid. For example, the concentration of IL-8 has been reported to range from non-detectable to as much of 800 pg/ml. Yet relatively few cytokines have been measured simultaneously in the same tear sample using the traditional ELISA techniques. Recently, measuring simultaneously various mediators in one sample of 10–20 μ L of tear fluid allow to describe a better picture of the local allergic inflammatory cascade.

8.1. Cytokines and chemokines measurement by ELISA

This technique has been carried out mostly to detect Th2-type cytokines in different phenotypes of allergic conjunctivitis. In the first tear cytokine studies, tear IL-4 levels were significantly higher in VKC, AKC and GPC patients than in SAC and controls raising the possibility that the increased level of IL-4 in tears could play a role in allergic disease and its severity in patients (Fujishima et al., 1995; Uchio et al., 2000). Tear IL-5 levels in VKC and AKC patients with giant papillae, were higher than those in SAC, AKC without giant papillae and normal controls (Uchio et al., 2000). Similarly, IL-4, IL-5, and TNF- α concentrations were significantly higher in the eyes of patients with AKC compared with the eyes of healthy control subjects (Wakamatsu et al., 2010).

In a different study, IL-4 tear levels were increased in VKC and AKC compared with normal subjects, but only IFN- γ significantly correlated with corneal involvement. The IL-4/13-dominant profile was found in 50% of VKC and in 17% of AKC patients, while a IFN- γ -dominant profile was found in 18% of VKC and in 17% of AKC patients suggesting that different cytokine profiles may sustain different allergic phenotypes (Leonardi et al., 2006b).

Among the cellular mediators and cytokines implicated in the development of allergic inflammation, TNF- α is one powerful pro-inflammatory cytokine thought to be involved in the pathogenesis of allergic diseases. Tear TNF- α levels were increased in 6 of 12 patients with VKC compared to undetectable levels in tear samples from patients with active SAC and in the control group. However, after CPT, only in one patient TNF- α levels were detectable. It has been suggested that blocking this key inflammatory mediator may

down regulate the entire immunologic reaction in severe ocular allergies (Leonardi et al., 2003b).

The contribution of stem cell factor (SCF) to the pathogenesis of VKC has been suggested since SCF promotes mast cell adhesion, migration, proliferation, survival and release of histamine and tryptase. Tear SCF levels in patients with moderate or severe VKC were significantly increased compared with the level in healthy controls (Nakatani et al., 2007). Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine associated with the generation of cell-mediated immune responses, was elevated in tears as well as serum in patients of severe atopic dermatitis suggesting that MIF may play an important role in the induction or enhancement of ophthalmic features related to severe atopic dermatitis (Kitaichi et al., 2006). The inflammatory/fibrogenic cytokines, TGF- β 1, IL-1 β , IL-6 and TNF- α , and procollagens I and III were found significantly increased in tears of active VKC patients but not in non-active patients suggesting that multiple pro-inflammatory mediators are involved in maintaining the inflammatory and remodeling processes (Leonardi et al., 1998). Osteopontin (OPN), a non collagenous adhesive matrix protein that is expressed by activated macrophages, was found significantly increased in tears of AKC and VKC patients, again suggesting its pathophysiological role in severe ocular allergic conditions associated to tissue remodeling (Uchio et al., 2002).

Several chemokines and their receptors involved in the recruitment and activation of inflammatory cells in allergic diseases have been found by tissue expression without quantitative analysis. The factors mostly studied in tears are the members of eotaxin family, the potent eosinophil chemotactic and activating peptides, eotaxin-1 (CCL11) and eotaxin-2 (CCL24). Both chemokines were found significantly increased in tears of VKC and AKC patients compared with those of normal patients (Fukagawa et al., 1999; Leonardi et al., 2003c; Shoji et al., 2009). Interestingly, mucus contained higher levels of both chemokines than did the tears. While eotaxin-1 is produced by conjunctival fibroblasts and not by conjunctival epithelial cells, when stimulated by IL-4 and TNF- α , eotaxin-2 is produced primarily by monocytes and macrophages (Leonardi et al., 2003c). In these different studies, tear eotaxin was always correlated with either the percent of eosinophils in tear fluid, the clinical score and/or the corneal involvement in VKC patients.

Tear eotaxin-1 was also found increased in active SAC suggesting that it may be used to confirm a diagnosis of seasonal ocular allergy (Eperon et al., 2004).

The chemokine profile, growth related oncogene (GRO)- α , eotaxin-2 and thymus and activation-regulated chemokine (TARC) in the VKC were elevated compared with those in the controls, but they decreased significantly after the treatment with tacrolimus (Hori et al., 2011). Thus, measurement of selected cytokines and chemokines by ELISA techniques may be considered a local marker of allergic inflammation.

8.2. Cytokines and chemokines measurement by stationary phase protein array technology

Membrane arrays, are relatively non expensive and can be processed by laboratories with minimal equipment and facilities and may have widespread clinical diagnostic applicability (Sack et al., 2007). Dozens of cytokines, chemokines, growth factors, angiogenic modulators, enzymes and inhibitors can be identified in small tear samples using these techniques. Membrane array characterization allowed for the identification of up to 80 chemokines, cytokines and growth factors in one tear sample giving a more global picture of immunoregulation of the ocular surface (Sack et al., 2005).

In a study with methodological details on these techniques, data revealed strong signals for the pro-inflammatory mediators IL-1 α and TNF- α especially in the ‘closed eye’ tear samples from allergic patients (Sack et al., 2007). These two pro-inflammatory mediators play initiating roles in ocular surface diseases, including allergy. Moreover, the accumulation after overnight eye closure of ocular surface tissue and inflammatory cell products arising from the sub-clinical state of inflammation typical of the closed eye microenvironment and the ongoing allergic inflammation, explain the occurrence overnight of corneal ulcers typical of severe VKC patients. In the same study, enhanced signals for IL-2, IL-4, IL-5 and IFN- γ in allergic patients confirms the contribution of both Th1 and Th2 processes in a variety of chronic ocular surface allergic diseases (Sack et al., 2007).

Using a similar array for 40 inflammatory mediators and 25 μ L tear sample from either VKC, GPC patients or normal subjects, strong signals for many inflammatory mediators have been reported with dramatic differences observed in the patterns of distribution in tear samples from the three populations and further changes associated with therapeutic intervention (Shoji et al., 2006). In VKC patients, eotaxin, eotaxin-2, IL-4, IL-6, IL-6 soluble receptor (IL-6sR), IL-7, IL-11, macrophage inflammatory protein (MIP)-1delta, tissue inhibitor of metalloproteinases (TIMP)-2, monocyte chemoattractant protein (MCP)-1, and macrophage-colony stimulating factor (M-CSF) were increased 4–8 times the values in the control group. In the patients with GPC, IL-6, M-CSF, monokine-induced gamma interferon (MIG), eotaxin-2, IL-6sR, IL-11, MIP-1delta, and TIMP-2, were also increased over the control values. However, only eotaxin-2 and IL-6sR relative increases were statistically significant in the VKC group. The strong signals for IL-8, but only trace to negligible signals for many Th1/Th2 cytokines may suggest that the low sensitivity of some of the assays coupled with a very high level of cross talk between capture and probe antibodies probably made impossible the identification of these proteins such as many Th1/Th2-like cytokines.

An extensive study using three microwell plate antibody arrays specific for 26 compounds (Th1/Th2 cytokines, growth factors and MMPs) revealed that the frequencies of positive signal for IL-4, IL-5 and IL-10 were significantly greater in tears of VKC patients than in control samples (Leonardi et al., 2009). Similarly, the frequency of positive signals for several growth factors, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10 were significantly greater in VKC than in normal tears.

The main problem with these techniques is that tears contain several factors that can complex with micro-well bound Ig decreasing its binding capacity in ELISA assay-like conditions and a proteases that can selectively clip micro-well bound Ig reducing the efficiency of ELISA assays in general (Sack et al., 2007). Given all these factors and the variable nature of tear fluid, it is not surprising that the results of different studies are highly dependent upon the nature of the assay, the volume, type of the assayed tear sample, as well as the individual donor, making extremely difficult to obtain quantitative data. Conversely, these techniques are relatively easy to perform and non expensive allowing to screen several mediators in small size samples.

8.3. Cytokines and chemokines measurement by multiplexed bead analysis

The multiplexed bead based flow cytometry opens up new opportunities for the study and diagnosis of ocular allergy. The technique combines the principles of a sandwich ELISA with the potential of flow cytofluorimetry and allows the simultaneous measurement of various mediators in one sample of 10–20 μ L of tear fluid. More than just the assaying of a single cytokine, it is possible to identify relationships between cytokines or groups of cytokines with opposing or complementary functions.

In the first study using this technology, a quantitative method for simultaneously assaying six cytokines in tears has been described in allergic and non-allergic patients, showing significant differences in the ratios of pro-inflammatory cytokines between the two groups (Cook et al., 2001) (Table 2).

In a further study, 14 different cytokines and chemokines were simultaneously measured in tears of SAC, VKC, AKC and GPC patients using the Luminex technology (Leonardi et al., 2006a). Type Th2 cytokines, IL-4, IL-5 and IL-13, were significantly increased in all forms of allergic disease, particularly in VKC, compared to normal tears. IFN- γ and other pro-inflammatory cytokines such as IL-1, IL-6 and TNF- α were increased especially in the more severe forms of VKC and AKC. Among the chemokines, eotaxin was elevated in VKC and was correlated with IL-5 and IL-4 levels. IL-8 was exceptionally high in GPC tears. In GPC, the microtrauma induced by the contact lens edge is responsible for the inflammatory reaction probably through IL-8 and other cytokine/chemokine production. The cytokine tear profile was similar in adults and children with VKC, however, the limbal disease in adults had a more Th1-type cytokine profile compared to the tarsal form (Leonardi et al., 2013).

S. aureus colonization is common in AKC, potentially activating epithelial cells via toll-like receptor 2 (TLR-2). Although there is no evidence that periocular and ocular bacterial micro-colonization are related to inflammatory parameters in AKC, these patients showed significantly higher levels of IFN- γ , TNF- α , IL-2, IL-4, IL-5 and IL-10 than controls (Nivenius et al., 2004). An association was found between conjunctival signs and the levels of all cytokines except IL-5. However, the contribution of *S. aureus* to on-going inflammation in AKC can not be dismissed. When challenged with conjunctival provocation with airborne allergens, AKC patients showed a significant increase of IFN- γ , IL-6 and IL-10 in the tear fluid at 48 h after provocation vs. the control eye confirming that even though many of these patients experience no obvious seasonal variation a typical IgE-mediated allergic reaction can be induced with evidence of an inflammatory late phase that explains the chronicity of this disease (Nivenius et al., 2012).

In summary, although several cytokines, chemokines and their receptors have been found over-expressed in allergic ocular inflammations using different methods, none of them seems to be ideal to be used as clinical biomarker. However, some key cytokine or receptor antagonist may be further used to down-regulate the conjunctival immune response.

Table 2

Tear cytokines detected by multiplexed bead cytokine assay based on reported studies (8.3).

	IL2	IL4	IL5	IL10	IL12	IL13	IFN γ	IL1 β	IL6	TNF α	IL8	MCP1	RANTES	Eotaxin
SAC	NS	↑	↑	NS	NS	↑	NS	↑	NS	NS	NS	NS	NS	NS
AKC	↑	↑	↑	↑	NS	NS	↑	↑	↑	↑	↑	↑	NS	NS
GPC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	↑	NS	NS	NS
VKC	NS	↑	↑	↑	↑	↑	↑	↑	↑	↑	NS	↑	NS	↑

NS = Non statistically significant increased compared to tears from non-allergic subjects; (↑) = significantly increased compared to tears from non-allergic subjects.

9. Growth factors and angiogenic factors

Several growth factors, collagens and other extracellular matrix proteins are over-expressed in VKC and AKC tissues and may be involved tissue growth and remodeling. Results from an angiogenic and growth factor arrays showed significantly increased expressions of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and thrombopoietin (TPO) in VKC compared to normal tears, with a relatively high number of VKC patients expressing basic fibroblast growth factor (bFGF) and heparin binding epithelial growth factor (HB-EGF) (Leonardi et al., 2009). Expression of VEGF, TGF- β 1, bFGF, and platelet-derived growth factor-BB (PDGF-BB), was previously shown in VKC conjunctival tissues by immunohistochemistry. In particular the TGF- β /Smad signaling pathway is over-expressed in VKC tissues (Leonardi et al., 2011a).

In summary, the increased production and activation of growth factors, together with an imbalance between MMPs, and their natural tissue inhibitors, TIMP, are all probably involved in the pathogenesis of conjunctival inflammation, remodeling and corneal changes typical of chronic severe allergies.

10. Proteases and their inhibitors

Several proteases, such as tryptase, chymase, urokinase-type, tissue type plasminogen activators and MMPs, have been found to be over expressed in tears and tissues of patients affected by VKC, yet without changes in the inhibitors, plasminogen activator inhibitor (PAI)-1 and TIMP-1 (Leonardi et al., 2003a, 2005). Tryptase has been demonstrated to have several biological activities: degradation of fibrinogen and fibronectin; hydrolysis of neuro-peptides; stimulation of fibroblast proliferation; production of C3a from C3; potentiation of histamine effects and activation of mast cells. In combination with the other mast cell serine protease, chymase, tryptase may be implicated in the activation of other proteases, such as MMPs, which are all involved in extracellular matrix degradation and inflammatory cell infiltration. Chymase is a chymotrypsin-like mast cell protease that plays a major role in the formation of angiotensin II by conversion of angiotensin I independent from its converting enzyme. However, the role of chymase in conjunctival allergic inflammation is still unclear.

Conjunctival and corneal remodeling are accompanied by degradation of ECM in addition to synthesis and deposition of new matrix. Tear levels of pro-MMP-1 and pro-MMP-9 were significantly increased in patients with VKC compared with control subjects (Leonardi et al., 2003a). MMP-1/TIMP-1, MMP-9/TIMP-1 molar ratios, and MMP-1 and MMP-9 activities were significantly increased in VKC tears compared with control samples. Interestingly, MMP-9 activity correlated significantly with corneal involvement and giant papillae formation. Greater levels and activity of MMP correlated with clinical findings in patients with VKC, suggesting that proteases are involved in allergic inflammation.

Alpha-1 antitrypsin (AAT) is the archetype of the serine protease inhibitor (Serpin) supergene family. Since AAT is a major source of antiprotease activity including trypsin, the trypsin inhibitory capacity, a marker of AAT activity, was evaluated in VKC tears and its modulation by MMP-1 and MMP-9 activity. Tear trypsin inhibitory capacity were measured in serum and tears of normal subjects and VKC patients by a fluorimetric assay (Ghavami et al., 2007). Tear trypsin inhibitory capacity of healthy individuals was significantly higher than VKC patients indicating a reduced local production or inhibition of its activation in this disease. This finding might have been due to greater MMP-1 and -9-mediated inactivation of AAT. Even though AAT does not directly inhibit mast cell tryptase but does effectively inhibit chymase, the decreased activity of AAT in

tears may potentiate the activity of mast cell derived proteases in VKC.

Plasminogen activators play a role not only in fibrinolysis but also in events such as chemotaxis, collagen degradation and cell spreading. Thus the serine protease, urokinase, a potent chemo-attractant for leukocytes, may be involved in the pathogenesis of the allergic inflammation. Tear levels of urokinase, tissue plasminogen activator and tear plasminogen activity levels were significantly greater in VKC patients whereas PAI-1 tear levels were not detectable, suggesting that an imbalance between urokinase activation and inhibition may be involved in the cell recruitment and tissue remodeling typical of VKC (Leonardi et al., 2005).

In summary, an imbalance between proteases and their inhibitors may facilitate inflammatory cell transmigration, tissue remodeling, and corneal damage in ocular allergies suggesting some of these factors as potential target for therapeutical interventions.

11. Neuromediators

Neurogenic inflammation may be involved in ocular allergic inflammation as an initiating mechanism after mast cell activation or in the axon reflex by sensitive conjunctival fibers. Mainly released at the sensory nerve endings or from immune and/or structural cells, neuropeptides and neurotrophins might either upregulate or downregulate inflammatory processes. These mediators contribute to ocular allergy by acting directly on cells responsible for both early and late phase reactions as well as on epithelial cells and fibroblasts, leading to either self-limiting or chronic states. Increased plasma levels of Substance P (SP) and nerve growth factor (NGF), and an obvious alteration in muscarinic and beta1-adrenergic receptor, vasoactive intestinal peptide (VIP), protein gene product 9.5, and NGF tissue expression have been reported in VKC (Lambiase et al., 1995; Motterle et al., 2006) confirming the involvement of an autonomic dysfunction in the pathogenesis of VKC. Tear levels of SP, calcitonine gene related peptide (CGRP), neuropeptide Y (NPY), and VIP were evaluated, by ELISA, in allergic individuals before and after conjunctival allergen challenge. After CPT, SP, CGRP, and VIP were significantly increased in allergic tears as compared to baseline (Sacchetti et al., 2011). Conversely, no differences in absolute SP tear levels were found between the different time points after nasal grass pollen provocation (Callebaut et al., 2010).

In summary, the increasing interest into neurogenic inflammation may explain some of the specific and non-specific conjunctival hyper-reactivity reactions. Locally released neuropeptides may act directly on the epithelia, blood vessels and immune cells, potentially modulating protective and inflammatory responses of the ocular surface.

12. Oxidative stress and reactive oxygen species

Increased oxidative stress status, specially elevation of reactive oxygen species (ROS), has been described in allergic patients. Inflammatory cells, environmental air pollutants and pollen grains generate oxidative stress in the conjunctiva and participate in the worsening of disease inflammation and symptoms (Bacsi et al., 2005). The early phase lipid peroxidation marker was significantly increased in tears of patients with AKC compared with controls together with higher percentages of cells positively stained for the same factor, and late phase lipid peroxidation marker in palpebral conjunctival samples suggesting an increased lipid peroxidation status in AKC (Wakamatsu et al., 2010). A close relationship between ROS production, peroxidative lipid membrane damage and the inflammatory pathological process in allergic

keratoconjunctivitis may be postulated, suggesting that antioxidants might serve as a possible therapeutic agents.

13. Mucous, mucins and tear film instability

Chronic ocular allergies are associated with tear film instability, increased goblet cell mucin secretion into tears and thus mucous discharge. Down regulation of goblet cell-derived mucins (MUC5AC) with up-regulation of MUCs 1, 2, 4 and 16 mRNA expression has been shown in atopic eyes with allergic shield corneal ulcers compared with eyes without ulcers and eyes of control subjects (Dogru et al., 2008, 2006). These findings suggest that in addition to the presence of ocular surface inflammation, ocular allergy may cause ocular damage through mechanical tear film instability. MUC16 up-regulation, which is involved in the protection of the ocular surface epithelia, could be a manifestation of an ocular surface defense response that compensates for the ailing ocular surface health resulting from the decrease in MUC5AC. Persistence of inflammation and further decline of expression of the major ocular surface mucin, MUC5AC, may stimulate up-regulation of other epithelial mucins such as MUC16 to protect the ocular surface.

14. Proteomics

Several methods of proteomic analyses have been explored to obtain differential data on a broad range of proteins detected in tears. These include use of two dimensional gel-electrophoresis, coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), MALDI analysis, trypsin digestion with differential labeling followed by tandem mass spectrometry, use of biochips coupled with surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, HPLC, and membrane bound antibody array (MA).

Using a two-dimensional polyacrylamide gel electrophoresis protein separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry peptide identification, six differentially expressed proteins, IL-4, phospholipase A2, albumin, lactoferrin, hemopexin, and lipocalin, were displayed in pooled tears of VKC patients (Pong et al., 2010). The increased hemopexin concentration in VKC tears was significantly associated with disease severity (Pong et al., 2011). The presence in tears of the inflammatory protein, hemopexin, may be attributed simply to increased vascular permeability from the circulation. However, tear hemopexin in VKC may function to modulate the local inflammatory response and be involved in tissue repair through fibrosis and angiogenesis. We recently performed a proteomic analysis with iTRAQ technology, a powerful tool for screening large numbers of proteins in tears (submitted). This analysis revealed previously undetected proteins that might participate in the pathogenesis of VKC, as well as demonstrating the profound changes in tear composition associated with prolonged allergic inflammation.

15. Conclusions

The concentration and distribution of inflammatory mediators or inhibitors in the tear fluid have been extensively used in ocular allergy to find a 'disease marker', to better understand the immune mechanisms involved, or to identify potential targets for therapeutic interventions (Enriquez-de-Salamanca et al., 2012). It is possible that patients with diseases can have a different protein profile and, therefore, protein or peptide analysis can be used as a possible fingerprint for disease biomarkers and pathological molecule identification. The critical limitation is the volume of the

tear sample, which can be collected without stimulating the reflex tearing.

Mediators, chemokines and cytokines are expressed and produced locally not only by inflammatory cells, but also by epithelial and connective tissue cells of the ocular surface and thus it is impossible at the moment to identify directly in patients the origin and state of activation of many of these inflammatory mechanisms. New technologies such as multiplex bead assays, membrane-bound antibody array and proteomic techniques can characterize the distribution of a wide range of bioactive trace proteins in tears. Dozens of mediators, cytokines, chemokines, growth factors, angiogenic modulators, enzymes and inhibitors have been identified in small tear samples using these techniques in addition to the traditional ELISA or RIA assays. In several studies, these analysis allowed the identification of previously undetected proteins that may participate in the host defense system or in different inflammatory processes. The ability to measure multiple cytokines in tears, combined with knowledge obtained from in-vitro analysis of the individual and combined effects of these factors on various conjunctival cells (i.e. mast cells, epithelial cells, fibroblasts) has facilitated further understanding of specific processes contributing to maintenance of inflammation and progression of vision-threatening disease and paved the way toward new therapeutic targets.

Although several mediators and factors can be detected in tears as a marker of ocular inflammation, none can be considered as a clear marker of ocular allergy. So far, none of the local tests here described have been standardized to reach the clinical use in patients, excluding one total IgE assays specifically designed for tears and probably none of the described factors or tests is suitable to be considered the single specific disease marker. More likely, a combination of several of them may be required to target either a single disease phenotype, activity phase, or therapeutic effect. One possibility is to design an assay kit to detect a panel of tear markers, such as total IgE, tryptase, eotaxin, ECP, IL-4, IL-5, MMP-9 which have been found constantly increased in ocular allergic diseases and validate it in the different ocular allergic phenotypes (Table 3).

Most of studies here reviewed were performed in relatively small cohorts of patients. A consensus in classification and phenotyping of patients among different studies is essential for proper tear biomarker designation. However, all this work has built up massive body of knowledge in the field of mediators and biomarkers in ocular allergy and will help us to develop new diagnostic tests and to find new therapeutic strategies, including the development of targeted or personalized therapies for ocular allergic diseases.

Table 3
Potential tear markers in ocular allergic inflammation.

Marker	Origin	Type of conjunctivitis	Specificity
IgE	B cells	Allergic	Low
Allergen-Specific IgE	B cells	Allergic	High
Histamine	Mast cell, basophils	Allergic, Non allergic	Low
Tryptase	Mast cell	Allergic	High
ECP	Eosinophils	Allergic	High
IL-4	Th2 cells, Mast cells	Allergic, immune	High
IL-5	Th2-cells, Eosinophils	Allergic, immune	High
Eotaxin	Fibroblasts	Allergic	High
MMP-9	Inflammatory and structural cells	Allergic dry eye	Low
Neuromediators	Nerve endings	Allergic, non allergic	Low
Hemopexin	Inflammatory and structural cells	Allergic, non allergic	Low

ECP = eosinophil cationic protein; MMP = metalloprotease.

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