Original article

Allergic mediators in tear from children with seasonal and perennial allergic conjunctivitis

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ABSTRACT

Purpose: To evaluate the concentration of allergic mediators in tears of children with seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) compared with controls.

Methods: Twenty children with allergic conjunctivitis (17 SAC and 3 PAC) and sixteen healthy children were included in this study. Tear samples were collected using a Merocel sponge (Oasis, 0525), and immediately eluted by incubation in elution buffer and subsequent centrifugation at 20,000 rpm for 30 min at 4 °C. Concentrations of histamine (HIS), tryptase (TPS), eosinophil chemotactic factor (ECF), major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN), IgE and E-selectin were measured using enzyme linked immunosorbent assays (ELISA). Data were compared with the Mann–Whitney U test (P < 0.05), and multivariate analyses were also performed.

Results: Tear levels of TPS (P = 0.014), MBP (P = 0.032), ECP (P = 0.0041), IgE (P = 0.014) and EDN (P = 0.00077) showed significant differences in children with SAC and PAC compared to controls.

Conclusion: The simultaneous analyses of allergic mediators in the tears of children with SAC and PAC showed a significant elevated concentration in EDN, ECP and MBP in allergic group and decreased levels in IgE and TPS. Statistical analyses showed a diagnostic accuracy of 94.4% using the eight molecules panel.

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Mediadores alérgicos en lágrimas de niños con conjuntivitis alérgica estacional y perenne

RESUMEN

Objetivo: Evaluar la concentración de diferentes moléculas mediadoras de alergia en lágrima de niños con conjuntivitis alérgica estacional (CAE) y conjuntivitis alérgica perenne (CAP) frente a controles.

Métodos: Se incluyeron 20 niños con conjuntivitis alérgica (17 CAE y 3 CAP), y 16 niños sanos utilizados como controles. Las muestras de lágrima se obtuvieron mediante una hemosteta
Introduction

The eye and its structures are involved in the immunological hypersensitivity reactions of the body. Allergic conjunctivitis is made up of a group of ocular surface inflammations that have exhibited a significant increase in recent years. About 25% of the general population exhibit some type of allergic disease, and the majority of allergic patients exhibit expressions in the various target organs, with frequent allergic involvement at the ocular level.\(^1\)\(^2\)

During childhood, 3 basic expressions can be found: seasonal allergic conjunctivitis (SAC) which usually arises due to hyperreactivity to pollen, perennial allergic conjunctivitis (PAC) against mites and epithelium, and the severest form of allergic conjunctivitis which is vernal keratoconjunctivitis (VKC). The latter is a form of ocular allergic reaction specific to childhood which in most cases recedes in adolescence but courses chronically with very severe episodes which could produce severe corneal involvement.

SAC is a form of acute conjunctivitis which expresses in outbreaks linked to the pollinization season. Frequently, the ocular clinic involves nasal and pharyngeal symptoms, notably irritation, burning feeling and photophobia. It can also involve mucin secretion, conjunctival chemosis, palpebral edema and, less frequently, corneal involvement.\(^3\)

In what concerns PAC, this group comprises allergic conjunctivitis exceeding 3 weeks of evolution.\(^4\) It arises from continuous and not intensive antigenic exposure, possibly involving seasonal exacerbation generally due to pollen allergy, even though it generally is of annual duration due to allergy to dust, mites, fungi, animal hair and other allergens. Its onset is progressive and could become acute or subacute after bacterial and viral infections or due to chronic trauma such as the use of contact lenses. Its initial evolution is intermittent, subsequently becoming chronic. It courses with irritation, itching, foreign body feeling and moderate intensity tearing which becomes worse with humidity, light, contaminated atmosphere and air-conditioning. Occasionally, its diagnostic becomes difficult due to its similarity with other chronic conjunctivitis causes with which it can also be associated. It is also more susceptible to secondary infections in atopic patients. Tests are positive for pneumoallergens (with immediate reaction), dust and mites (producing a double reaction) in 80% of the cases. In this type of condition, IgE is raised in 60% of the cases.\(^4\)

In all its forms there is an initial stage of enhanced sensitivity and, after successive exposures to the allergens, the allergic reaction sets in, with an early stage involving mastocytes and histamine and a later stage involving eosinophils and neutrophils. Virtually all the proteins and cells involved in the inflammation are present in the ocular allergy.\(^5\)

When an allergic entity is suspected, the patient must be referred for skin tests followed by specific and overall IgE serum levels and a hemogram with eosinophil count. Ocular diagnostic tools include the conjunctival provocation test (CPT), involving the administration of one drop of allergen on the conjunctiva to observe the local and specific conjunctival response.\(^6\) The limitation of this provocation test is related to the low availability and high costs of water-soluble, preservative-free allergens.

Other allergic tests measure the presence of various allergy mediators in tears such as IgE, histamine, trypsin, the eosinophil cationic protein and various cytokines.\(^7\) Most tests are determined in vitro at specialized labs which require expensive or sophisticated equipment to analyze the concentration. In vivo ocular allergy diagnostic methods based on the presence of IgE in tears utilize paper strips that are placed directly upon the inferior fornix of the conjunctiva. The strip contains anti-IgE antibodies and the presence of IgE is chromatically determined in just a few minutes. However, this test is not very sensitive for allergic conjunctivitis (20%) and is not very commonly used in clinical practice.\(^8\)

Although there are many ocular inflammation diagnostic methods, some are very expensive and difficult to carry out while others lack sensitivity. Accordingly, we consider it necessary to develop new ocular allergy diagnostic tests that should be differential, simple and economic and which could be utilized directly by specialists in their practices.
The objective of this study was to assess the concentration of various allergy mediator molecules in children tears with seasonal allergy and perennial allergy against tears of control children to design an ocular allergy diagnostic tear system based on biomarkers.

**Subjects, material and methods**

Tear samples of 20 children with SAC and PAC and 16 healthy children utilized as controls were analyzed at the Ophthalmology Service of the Cruces Hospital in Baracaldo. The tutors of all the children involved in the study signed an informed consent authorizing the collection of tear samples.

The allergic subjects were included in the study under the following criteria: diagnosed allergy clinic history with subclinical symptomatic outbreak; slight irritation and tearing, scratching and/or blinking and conjunctival hyperemia. Allergologists referred the suspected cases of SAC (outbreaks linked to the pollenization season) or PAC (evolution exceeding 3 weeks) to the Ophthalmology Service which confirmed the disease with slit lamp. The age of the children ranged between 5 and 14 years. The exclusion criteria were the existence of other ocular conditions, joint administration of topical drugs, ophthalmological surgery within the last 6 months and the existence of systemic diseases with repercussion on the ocular surface. VKC was excluded from the study because it is much more severe and the presence of papillae precluded the existence of problems in the differential diagnostic.

The control group was made up by children between 5 and 14 years of age without any ocular clinic or condition on the ocular surface and without any diagnosed systemic allergy. The samples were taken by qualified staff in strict compliance with the Helsinki declaration principles and with the approval of the Ethical Research Committee of our hospital.

Tear samples were taken of both eyes without anesthetics utilizing polyvinyl acetate swabs (PVA ref. 0525, OASIS, USA). To this end, the swab was placed on the edge of the lower eyelid without touching the ocular surface to avoid irritation. The tip of the swab was cut and imibed to obtain all the proteins it contained. Subsequently it was centrifuged at 20,000 rpm for 30 min. The obtained tear was quantified with the EZQ method (Protein Quantification Kit, BioRad) to determine the overall protein concentration in each tear sample. Subsequently, it was frozen at −80°C for subsequent analysis. The analyzed molecules were histamine (HIS), triptase (TPS), eosinophil cationic protein (ECP), basic protein (MBP), eosinophil cationic protein (ECP), eosinophil neurotoxin (EDN), IgE and E-selectin. The analysis was made utilizing the ELISA quantitative technique (enzyme-linked immunosorbent assay) of the sandwich type. In all cases, the color reactant was TMB and therefore the wavelength utilized for determining the concentrations in all the essays was of 450 nm. The statistical study was carried out utilizing the nonparametric U Mann–Whitney test with a significance level of P < 0.05. The critical mediator values were found with graphical representations based on point diagrams, and the sensitivity and specificity levels were determined with the multivariate logistic regression analysis (PEJ SPSS 18.0, Chicago, IL USA).

**Results**

Twenty child tear samples were analyzed (17 had SAC and 3 PAC) and compared with 16 control tear samples of healthy children. The allergy group tear samples were collected between March and June 2009 while the control group tear samples were collected in November 2009, the season with the lowest allergic impact.

The mean age of the allergy group was of 9 ± 3.21 (85% boys, 15% girls), and the mean age of the control group was of 10.25 ± 3.02 (81% boys, 19% girls). No statistically significant differences were found between the age of the ocular allergy group and the control group (P = 0.275).

The TOS tear levels TPS (P = 0.014), MBP (P = 0.032), ECP (P = 0.0041), IgE (P = 0.014) and EDN (P < 0.001) exhibited statistically significant differences in the allergy group when compared to the control group (Table 1).

In order to determine the critical concentration of each mediator, dot diagram illustrations were made (Interactive Dot Diagram). With this type of illustration it was possible to obtain the critical concentration value that provides maximum sensitivity and specificity (Fig. 1). The critical percentage values attained for each mediator with specificity and sensitivity are shown in Table 2. The analysis of the comparative ROC curves (Fig. 2) illustrates the relationship between sensitivity and specificity for a given mediator. When a comparative analysis between different mediators is made, it can be seen which would give the best results as a diagnostic tool, i.e., the one having the largest area below the curve. According to our results, the ocular allergy mediator in tear exhibiting the largest area under the curve is EDN (95% sensitivity and 68.7% specificity). However, the multivariate logistic regression analysis which also constitutes a predictive model indicates that the use of the 8 mediators as a diagnostic tool allow for correct diagnostic of 94.4% of patients, both healthy and allergic (Table 3).

**Discussion**

Ocular allergy is highly prevalent in the general population and is one of the most frequent ocular surface conditions in ophthalmological practice. The symptoms caused by the various types of ocular allergy are common to many other ocular surface and systemic conditions, with slight outbreaks in infants frequently being erroneously diagnosed as tics. For this reason, when the diagnostic is doubtful or difficult it is necessary to utilize alternative or supplementary methods, mainly for primary care pediatricians. Many inflammatory mediators can be measured in tears, both for diagnostic and prognostic purposes and/or with experimental ends. The majority of mediators indirectly determine the specific IgE concentration with in vivo tests on the patient, or directly in the lab.

Accordingly, the conjunctival provocation test analyzes the presence of specific IgE over the conjunctiva and the lacry test, which is a direct method, measures the amount of IgE in the tear. The in vitro methods comprise highly sophisticated tests that analyzed various allergic mediators in tears,
Table 1 – Allergic mediator concentration values in children tears with seasonal allergic conjunctivitis and perennial allergic conjunctivitis compared with tear values in control children.

<table>
<thead>
<tr>
<th>Allergic mediators</th>
<th>SAC/PAC allergy mediator concentration in tears (ng/mL)</th>
<th>Allergy mediator concentration in control tears (ng/mL)</th>
<th>P &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS</td>
<td>26.6 ± 13.79</td>
<td>32.9 ± 15.12</td>
<td>0.014</td>
</tr>
<tr>
<td>MBP</td>
<td>43.68 ± 37.2</td>
<td>24.92 ± 20.38</td>
<td>0.03</td>
</tr>
<tr>
<td>HIS</td>
<td>56 ± 130</td>
<td>4 ± 11</td>
<td>0.09</td>
</tr>
<tr>
<td>ECP</td>
<td>143.8 ± 209.9</td>
<td>5.5 ± 11.6</td>
<td>0.0041</td>
</tr>
<tr>
<td>EDN</td>
<td>322.5 ± 380.75</td>
<td>8.25 ± 12.25</td>
<td>0.0007</td>
</tr>
<tr>
<td>E-selectin</td>
<td>8.95 ± 4.46</td>
<td>9.16 ± 6.60</td>
<td>0.456</td>
</tr>
<tr>
<td>ECF</td>
<td>9.28 ± 5.91</td>
<td>5.74 ± 7.45</td>
<td>0.06</td>
</tr>
<tr>
<td>IgE</td>
<td>8.97 ± 12.04</td>
<td>22.12 ± 19.9</td>
<td>0.014</td>
</tr>
</tbody>
</table>

Significance value P < 0.05.

Fig. 1 – The critical concentration of each allergic mediator with interactive dot diagrams. With this type of illustration it is possible to obtain the critical concentration value providing maximum sensitivity and specificity. Group 0: control group; group 1: ocular allergy group.

Table 2 – Critical values obtained for each mediator with specificity and sensitivity indicated in percentages.

<table>
<thead>
<tr>
<th>Allergic mediators</th>
<th>Concentration in tears (ng/mL)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPS</td>
<td>≤23.8</td>
<td>75</td>
<td>68.7</td>
</tr>
<tr>
<td>MBP</td>
<td>&gt;12.55</td>
<td>85</td>
<td>50</td>
</tr>
<tr>
<td>HIS</td>
<td>&gt;5</td>
<td>50</td>
<td>87.5</td>
</tr>
<tr>
<td>ECP</td>
<td>&gt;3</td>
<td>80</td>
<td>81.2</td>
</tr>
<tr>
<td>EDN</td>
<td>&gt;3.5</td>
<td>95</td>
<td>68.7</td>
</tr>
<tr>
<td>E-selectin</td>
<td>&gt;9.7</td>
<td>40</td>
<td>75</td>
</tr>
<tr>
<td>ECF</td>
<td>&gt;6.8</td>
<td>70</td>
<td>87.5</td>
</tr>
<tr>
<td>IgE</td>
<td>≤13.45</td>
<td>90</td>
<td>56.2</td>
</tr>
</tbody>
</table>
such as the ELISA technique which utilizes specific antibodies against the mediators to be analyzed with an enzymatic marker, or the RAST test with basic principles identical to the ELISA technique but utilizing radioactive isotopes. Other indirect techniques are the histamine release test or the basophil release test, which do not provide greater usefulness for clinical diagnostic. However, they can be useful for experimental or research purposes. In many cases, these methods exhibit a low sensitivity and in many cases require a lab for analysis with highly expensive and sophisticated methods. All these reasons evidence the need of a test that can be carried out in the medical practice directly on the patient, which should be specific, sensitive and affordable.

In this study it has been found that the simultaneous analysis of determined allergic mediators in child tears indicates altered concentrations in children with SAC and PAC compared to control children.

Mastocytes have always been considered as the main cell involved in allergic reactions. In addition to the well-known effect induced by the crossing of IgE-type antibodies with IgE receptors with high affinity for the antigen and due to the release of various mediators such as histamine which causes the immediate degranulation of mastocytes, it is known that these cells also produce and release other factors such as triptase. Similarly, eosinophils and their toxic proteins are increased and activated in tears, conjunctiva and serum in all ocular diseases and account for the corneal damage they cause. Other inflammatory cells such as neutrophils are also involved in the allergic response and contribute to the inflammation through the reclusion of other inflammatory cells and mediators. The analyzed molecules are derived from all these cells involved in the allergic response. Accordingly, histamine is the most important and abundant mediator found in the initial stage of an anaphylactic reaction. It is able to activate the T-cell suppressor and therefore to inhibit the production of IgE. This would explain the low IgE levels in pathologic individuals obtained in this study, as the histamine levels in the tears of children with SAC and PAC were high. This feedback process in which the effecting molecule, in this case HIS, causes the inhibition of the molecule in charge of its release could explain how the high levels of HIS in tears could be related with low levels of IgE and vice versa.

In addition, in contrast with what was expected, we have also found in this study lower TPS concentrations in the tears of children with ocular allergy than in control tears. Even so, it has been described that it is possible to have a nonspecific activation of mastocytes due to an increase in the levels of the molecules that produce their activation such as triptase and, in a second stage, after the mastocytes have been activated, the triptase levels diminish giving rise to the release of HIS. On the other hand, in this study the ECP, EDN and MBP values were increased in the tears of children with ocular allergies when compared with the tears of healthy control children. ECP, EDN and MBP are the main mediator proteins derived from activated eosinophils. ECP and EDN are found in the matrix of eosinophil granules, whereas MBP is found in the heart of the granules.

ECP is significantly increased in the tears of all allergic conjunctivitis forms, even in patients with negative tests for total and specific IgE. ECP correlates with the severity of the corneal compartments during the aggressive stages of the disease in which the protein is released of activated eosinophils and it is a strong indicator of the pathogenesis of the damage caused to the corneal epithelium in severe ocular allergy forms. High EDN levels have also been found in the tears of ocular allergy patients due to the activation of conjunctival eosinophils. Although EDN has a high homology in sequence with ECP, EDN has 100 times more ribonuclease activity than ECP. This neurotoxin could be a mediator in the activation of eosinophils and of degranulation.

Some molecules of all the analyzed mediators provide good sensitivity values (EDN, IgE, MBP) and others provide good specificity values (HIS and ECF), but there is no mediator that can act on its own as a good and effective diagnostic tool for ocular allergy due to the high standard deviations produced by the variations in the concentration of these mediators between individuals in the same group. However, after carrying out a multivariate analysis by means of multiple logistic regression it was seen that the joint study of several of these mediators in the form of ocular allergy mediator panels could lead to success percentages exceeding 94%.

In conclusion, the joint analysis of allergic mediators in child tears constitutes an important diagnostic tool to supplement clinical assessments, mainly in the cases exhibiting
similarity with other causes of chronic conjunctivitis and/or blinking tics. The diagnostic system which utilizes 8 allergy biomarkers discussed in this study is highly sensitive and specific, and we believe that, as it is a multivariate system, it would allow higher diagnostic success than existing tests which analyze only one marker. The success percentage in diagnostic is very high (94%), which makes it a more effective system than existing tests which exhibit considerably lower success percentages. At present, our group is researching a device for anchoring the 8 allergy markers that will provide a reading of the results in a few minutes, enabling its use in the medical practice without the need of external labs. This method must be simple, fast and affordable. Even so, clinical assessments must prevail and guide the execution of lab tests in order to confirm a presumed diagnostic or point towards tentative hypotheses.

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Conflict of interests

None of the authors have declared any conflict of interests.

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