

Short communication

Eosinophil cationic protein in tears of normal subjects and patients affected by vernal keratoconjunctivitis

Leonardi A, Borghesan F, Faggian D, Secchi A, Plebani M. Eosinophil cationic protein in tears of normal subjects and patients affected by vernal keratoconjunctivitis.
Allergy 1995; 50: 610-613. © Munksgaard 1995.

The objectives of this study were to determine: 1) levels of tear eosinophil cationic protein (ECP) in patients with vernal keratoconjunctivitis (VKC); 2) the effect of pharmacologic therapy on ECP release; and 3) the correlation of this mediator with the severity of the disease. Tears were collected from 10 controls and 20 VKC patients before and after therapy for cytologic analysis and ECP measurement by radioimmunoassay. Ocular signs and symptoms were evaluated before tear collection. Mean ECP levels in controls were $7.5 \pm 0.4 \mu\text{g l}^{-1}$, and in VKC patients, $988.3 \pm 128 \mu\text{g l}^{-1}$ before therapy ($P < 0.001$) and $566.3 \pm 121 \mu\text{g l}^{-1}$ after therapy ($P < 0.005$). In dexamethasone (Dex) 0.1% or cyclosporin A (CsA) 2% patients (five per group), tear ECP decreased significantly after 7-14 days of treatment. Disodium cromoglycate (DSCG) 4% (five patients) for 14 days did not significantly affect ECP levels. ECP levels were significantly correlated with allergic signs ($P < 0.001$), symptoms ($P < 0.001$), and the number of eosinophils in tears ($P < 0.005$). The results of this study suggest that tear ECP levels accurately reflect the clinical status of VKC patients. The measurement of ECP may prove useful not only in the diagnosis and monitoring of allergic disease, but also as an objective parameter for the evaluation of new antiallergic therapies.

A. Leonardi¹, F. Borghesan²,
D. Faggian², A. Secchi¹, M. Plebani²

¹ Institute of Ophthalmology, Department of
Physiopathological Optics and ² Department of
Laboratory Medicine, University of Padua, Padua,
Italy

Key words: eosinophil cationic protein (ECP);
tears; vernal keratoconjunctivitis.

Mario Plebani, MD
Dipartimento di Medicina di Laboratorio
Laboratorio Centrale
Complesso Ospedale Università
via Giustiniani 2
Padova 35128
Italy

Accepted for publication 3 April 1995

Eosinophils are known to play a major role in the development of allergic diseases. Eosinophil cationic protein (ECP), originally purified by Olsson & Venge in 1974 (10), comprises 30% of the eosinophil granule matrix. Its toxic effect on parasites and epithelial cells has been demonstrated *in vitro* (18). ECP concentrations have proven to be correlated with the severity of allergic disease, and the presence of ECP in biologic fluids and tissues may now be considered a marker of eosinophil activation (3, 4, 9). An improved radioimmunoassay (RIA) for ECP has allowed the identification of this protein to a much higher degree of specificity and sensitivity (11). This has led to an increasing interest in the role of ECP in allergic disease: from its identification in biologic fluids as a means of identifying eosinophil-mediated disease processes, to inhibiting its release with pharmacologic or immunotherapeutic agents.

Vernal keratoconjunctivitis (VKC) is a relatively rare ocular allergic disease predominantly observed in children and young adults living in warm, southern climates and, less frequently, in northern regions (1). It is characterized histologically by marked mast-cell degranulation and eosinophil infiltration (1, 5). Clinically, the characteristic features include the presence of giant papillae on the tarsal conjunctiva, limbal infiltrates, and corneal involvement such as epithelial punctate defects or corneal ulcers. Eosinophil major basic protein (EMBP) has already been shown to play a role in the pathogenesis of epithelial erosions observed in VKC, indicating the importance of eosinophil activation in severe forms of this disease (15-17). Conjunctival eosinophil activation and degranulation, accompanied by high levels of tear ECP, were also previously reported in seven VKC patients (12). A toxic effect of ECP on human

corneal epithelial cells has recently been demonstrated *in vitro* (8).

The objectives of this study were threefold: 1) to measure tear ECP levels in normal subjects and in patients with active VKC; 2) to evaluate the effect of various topical, ocular drug therapies on tear ECP levels in VKC patients; and 3) to assess the correlation of this mediator with the severity of the disease.

Material and methods

Patients

Written informed consent was obtained from all patients, or, in the case of minors, from their parents. Bilateral tear samples were not successfully collected from all patients since a considerable amount of tears was required to perform the assay. With a capillary micropipette, 15 tear samples from 10 normal volunteers (mean age: 16 ± 2.1 years; five males, five females) were collected. A 5- μ l aliquot was used for tear cytologic analysis, the remainder being stored at -20° for subsequent determination of ECP.

Twenty patients affected by bilateral active VKC (mean age: 14 ± 6.1 years; 14 males, six females) were enrolled in the study. After clinical evaluation of ocular signs and symptoms, tear samples were collected, as above, for tear cytologic analysis and ECP determination.

Patients were treated with the following drug therapies: dexamethasone (Dex) 0.1% bilaterally four times daily (group 1; $n = 5$); cyclosporin A (CsA) 2% in castor oil (group 2; $n = 5$); and disodium cromoglycate (DSCG) 4% (group 3; $n = 5$). Clinical evaluation and tear collection were repeated after 5–7 days of steroid therapy and after 10–14 days of DSCG or CsA therapy. Tear ECP was determined in an additional five patients with a mild form of VKC who had received DSCG for 1–4 months (group 4). Treatments were allocated by the evaluation of each patient's clinical history from previous years. Patients whose allergic signs or symptoms worsened significantly during the DSCG treatment period were administered adjunctive steroid therapy. Table 1 specifies the number of tear samples available from each group before and after therapy.

A clinical score (0–4; 0 = absent; 4 = severe) was determined at each visit and for each eye for the four major symptoms: itching, tearing, photophobia, and foreign-body sensation, and for the five major signs: conjunctival erythema, eye swelling, discharge, papillae/limbus, and epithelial disease.

Methods

Tear cytologic analysis was done with precolored slides (Testsimplet[®], Boehringer, Mannheim, Ger-

many), the eosinophils present being counted in five microscopic fields of 0.15 mm^2 each.

ECP was measured by a commercially available RIA technique (Kabi Pharmacia, Uppsala, Sweden). The ECP standard was calibrated against pure ECP prepared according to Peterson et al. (11). To assess any deterioration in stored tear samples, ECP concentrations in the tears of five subjects were measured immediately after collection and 30 days after storage at -20° C. Storage time did not modify the mean values in tears. ECP determination in tears was precise and consistent, the intra-assay coefficient of variation ($\text{CV}\%$) varying from 4.03% to 6.97%, and the between-assay $\text{CV}\%$ from 5.1% to 8.1%. Assaying ECP in samples diluted to various strengths demonstrated a good correlation between obtained and expected values (mean recovery: 97%; range: 94–102%).

Statistics

Statistical analyses were performed with Student's *t*-test, the Mann-Whitney U-test, or the Wilcoxon signed-rank test, where appropriate. Results are listed as the mean $\pm$ standard error of the mean (SEM). Correlation coefficients were obtained by linear correlation analysis. For statistical significance, the assigned *P* value was ≤ 0.05 .

Results

The mean ECP level measured in control tears was $7.5 \pm 0.4 \text{ }\mu\text{g/l}$ (range: 5–11 $\mu\text{g/l}$). No subject had any signs or symptoms of external eye disease. Tear cytologic analysis demonstrated no eosinophils.

In pretherapy tear samples from VKC patients, mean ECP levels were $988.3 \pm 128.3 \text{ }\mu\text{g/l}$. After drug therapy, mean ECP levels were significantly decreased ($P = 0.05$) to $566.1 \pm 121.04 \text{ }\mu\text{g/l}$. Both pre- and posttherapy ECP levels were significantly greater in VKC patients than in controls ($P < 0.001$ and $P < 0.005$, respectively).

In group 1 (Dex), baseline mean tear ECP was $1346.8 \pm 300 \text{ }\mu\text{g/l}$ and, after 7 days of treatment, $573.4 \pm 170.1 \text{ }\mu\text{g/l}$ (Table 1). This difference was statistically significant ($P < 0.05$). The clinical scores of signs and symptoms were both significantly decreased after therapy ($P < 0.001$).

In group 2 (CsA), the mean ECP level was $1023.2 \pm 173.4 \text{ }\mu\text{g/l}$ at baseline, before therapy, and $161.6 \pm 62.9 \text{ }\mu\text{g/l}$ after therapy (Table 1). This difference was statistically significant ($P < 0.001$). The clinical scores of both signs and symptoms decreased significantly after treatment ($P < 0.001$).

In group 3 (DSCG), ECP levels in tears were $631.8 \pm 139.1 \text{ }\mu\text{g/l}$ at baseline and $961.6 \pm 262.6 \text{ }\mu\text{g/l}$ after treatment (Table 1). This difference was not

Table 1. ECP levels in tears ($\mu\text{g/l}$) before and after topical treatments

	Group 1 Dex	Group 2 CsA	Group 3 DSCG	Group 4 DSCG
Before	1346 $\pm$ 300 (24–2530) (n=9)	1023.2 $\pm$ 173.4 (258–2075) (n=10)	631.8 $\pm$ 139.1 (220–1690) (n=10)	
After	573.4 $\pm$ 170.1 (9–1300) (n=9)	161.6 $\pm$ 62.9 (6–612) (n=10)	961.6 $\pm$ 262.6 (43–2387) (n=10)	22.5 $\pm$ 4.7 (6–83) (n=9)
P value	0.05	0.001	NS	

n=number of samples available for ECP measurement.

statistically significant. The mean clinical score in the DSCG group was increased after treatment, with a worsening of corneal involvement and conjunctival inflammation.

In the additional five mild VKC patients (group 4) for whom tear samples were available only after DSCG therapy, mean ECP levels were $22.5 \pm 4.7 \mu\text{g/l}$. This value was significantly higher than in controls ($P < 0.05$) but significantly lower than in pretreatment values of severe VKC ($P < 0.05$).

A significant correlation was observed between ECP levels in all samples and both allergic signs ($r = 0.485$, $P < 0.001$) and allergic symptoms ($r = 0.582$, $P < 0.001$). In evaluation of the tear samples in which both ECP measurements and cell counts were performed, ECP levels and number of eosinophils were significantly correlated ($r = 0.403$, $P < 0.005$). The number of eosinophils in tears was also significantly correlated with allergic signs ($r = 0.524$, $P < 0.001$) and symptoms ($r = 0.572$, $P < 0.01$).

After both Dex and CsA therapy, the ECP levels in tears significantly decreased, yet the number of eosinophils in tears did not significantly change.

Discussion

VKC is an often severe, ocular allergic inflammation in which both immediate and delayed hypersensitivity appears to be involved. The conjunctival infiltration of eosinophils, basophils, and mononuclear cells, in addition to a constantly increased number of mast cells and an increased deposition of collagen, are the typical histologic features of VKC (1). The measurement of an eosinophil-derived mediator in tears of VKC patients is especially relevant since these cells may comprise 20–50% of inflammatory cells present in the tear fluid during the active phases of the disease.

Activated eosinophils which release toxic factors such as the presently studied ECP (7), in addition to EMBP, eosinophil peroxidase, eosinophil-derived neurotoxin (EDN), and eosinophil protein-X (EPX),

are known to play a pivotal role in allergic eye inflammations (2). Eosinophil-derived proteins have been shown to be toxic to epithelial cells *in vitro*, to increase nonspecific hyperreactivity, and to alter *in vitro* reactivity to histamine (6, 19).

Monitoring of VKC patients is usually performed by clinical evaluation of signs and symptoms (13). Tear cytologic analysis and the measurement of ECP and EDN/EPX, recently found increased in the serum of patients with active VKC, have also been considered useful markers of ocular allergic inflammation (14).

The results of this study have shown that tear ECP levels were 130 times higher in samples collected from VKC patients than in control, nonallergic volunteers. Wide variations in ECP levels found in baseline tears of VKC patients (24–2530 $\mu\text{g/l}$) may reflect a varying range of activation of eosinophils. Additionally, local drug therapy (DSCG, Dex, and CsA analyzed as one "treatment group") was shown significantly to decrease tear ECP levels to almost half the pretreatment levels.

The anti-inflammatory/immunosuppressive agents dramatically decreased levels of tear ECP: Dex by 58%, and CsA by 84%. Signs and symptoms of the disease were also significantly ameliorated by both treatments. In contrast, in severe VKC, DSCG not only failed to alter significantly tear ECP levels, but also failed to improve the clinical aspect of the disease. In the five mild VKC patients treated with DSCG for several months, and for whom only during-treatment tear samples were available, the levels of ECP were comparatively low; significantly higher (3X) than normal volunteers, yet still significantly lower than those with severe VKC. Since these levels were so much lower than even the poststeroid and post-CsA values, they may reflect the mildness of the disease in these patients. Nevertheless, in all patients, regardless of therapy, ECP levels in tears were significantly correlated with signs and symptoms, thus accurately reflecting the clinical progression and status of VKC patients. The number of eosinophils in tears was also correlated with the clinical score and with the levels of ECP, but did not decrease significantly, as did the ECP levels after therapy. This suggests that eosinophils can be present but not activated in the conjunctiva.

In conclusion, ECP is a protein easily detectable and measurable in tears with a high degree of sensitivity. Tear ECP, as a useful, noninvasive marker of eosinophil activation, was also shown to be increased in VKC patients, to be dramatically reduced by pharmacologic intervention, and to correlate significantly with allergic signs and symptoms. The great variability in tear ECP levels among VKC patients, probably due to varying degrees of eosinophil activation, suggests that ECP measurement is best

used as a marker of disease progression in individual patients over time. While the total number of VKC patients in this study was adequate considering the rarity of the disease, larger groups need to be studied to confirm the specific drug effects on tear ECP suggested by the present study. The measurement of ECP may prove useful not only in the diagnosis and monitoring of ocular allergic diseases, but also in the evaluation of new anti-allergic therapies for which an objective parameter is needed to complement subjective clinical assessments.

References

- ALLANSMITH MR. The eye and immunology. St Louis, MO: CV Mosby, 1982.
- BONINI S, TOMASSINI M, BONINI S, CAPRON M, BALSANO F. The eosinophil has a pivotal role in allergic inflammation of the eye. *Int Arch Allergy Immunol* 1992; 99: 354–8.
- BONINI S, TOMASSINI M, ADRIANI E, et al. Markers of eosinophilic inflammation in allergic diseases. *Allergy* 1993; 48 (Suppl. 17): 133–7.
- BOUSQUET J, CHANEZ P, LACOSTE JY, et al. Eosinophilic inflammation in asthma. *N Engl J Med* 1990; 323: 1033–9.
- EASTY DL, BIRKENSHAW M, MERRETT T, MERRETT J, ENTWHISTLE C, AMER B. Immunological investigations in vernal eye disease. *Trans Ophthalmol Soc U K* 1980; 100: 98–107.
- FLAVAHAN NA, SLIFMAN NR, GLEICH GJ, VANHOUTTE PM. Human eosinophil major basic protein causes hyper-reactivity of respiratory smooth muscle: role of the epithelium. *Am Rev Respir Dis* 1988; 138: 685–8.
- FRIGAS E, GLEICH J. The eosinophil and the pathophysiology of asthma. *J Allergy Clin Immunol* 1986; 77: 527–37.
- HALLBERG CK, BRYSK MM, TYRING SK, GLEICH GJ, TROCME SD. Toxic effects of eosinophil cationic protein on cultured human corneal epithelium [Abstract]. *Invest Ophthalmol Vis Sci* 1994; 35 (Suppl.): 1943.
- HEDIN G, AHLSTEDT S, ENANDER I, HAKANSSON L, VENGE P. Eosinophil cationic protein (ECP), eosinophil chemotactic activity (ECA), neutrophil chemotactic activity (NCA), and tryptase in serum before and during bronchial challenge in cat-allergic children with asthma. *Pediatr Allergy Immunol* 1992; 3: 144–9.
- OLSSON I, VENGE P. Cationic proteins of human granulocytes. II. Separation of the cationic proteins of the granules of leukemic myeloid cells. *Blood* 1974; 44: 235–46.
- PETERSON CG, ENANDER I, NYSTRAND J, ANDERSON AS, NILSSON L, VENGE P. Radioimmunoassay of human eosinophil cationic protein (ECP) by an improved method. Establishment of normal levels in serum and turnover *in vivo*. *Clin Exp Allergy* 1991; 21: 561–7.
- SAIGA T, UENO N, SHIMIZU Y. Deposition of eosinophil cationic protein in conjunctival tissues and tears in vernal keratoconjunctivitis [Abstract]. *Invest Ophthalmol Vis Sci* 1991; 32 (Suppl.): 682.
- SECCHI AG, TOGNON MS, LEONARDI A. Topical use of cyclosporine in the treatment of vernal keratoconjunctivitis. *Am J Ophthalmol* 1990; 110: 137–42.
- TOMASSINI M, MAGRINI L, BONINI S, LAMBIASE A, BONINI S. Increased serum levels of eosinophil cationic protein and eosinophil-derived neurotoxin (protein X0) in vernal keratoconjunctivitis. *Ophthalmology* 1994; 101: 1808–11.
- TROCME SD, KEPHART GM, ALLANSMITH MR, BURNE WM, GLEICH GJ. Conjunctival deposition of eosinophil granule major basic protein in vernal keratoconjunctivitis and contact lens-associated giant papillary conjunctivitis. *Am J Ophthalmol* 1989; 108: 57–63.
- TROCME SD, KEPHART GM, BURNE WM, BUCKLEY RJ, GLEICH GJ. Eosinophil granule major basic protein deposition in corneal ulcers associated with vernal keratoconjunctivitis. *Am J Ophthalmol* 1993; 115: 640–3.
- UDELL IJ, GLEICH GJ, ALLANSMITH MR, ACKERMAN MR, ABELSON MB. Eosinophil granule major basic protein and Charcot-Leyden crystal protein in human tears. *Am J Ophthalmol* 1981; 92: 824–8.
- VENGE P, PETERSON CGB. Eosinophil biochemistry and killing mechanisms. In: MORLEY J, ed. *Eosinophils and asthma*. London: Academic Press, 1989: 163–77.
- VENGE P, DAHL R, FREDENS K, PETERSON CG. Epithelial injury by human eosinophils. *Am Rev Respir Dis* 1988; 138: 54–7.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.