

Trypsin Inhibitory Capacity in Vernal Keratoconjunctivitis

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PURPOSE. To study tear trypsin inhibitory capacity (T-TIC), serum trypsin inhibitory capacity (S-TIC) and their relationship to matrix metalloproteinases (MMP)-1 and -9 in patients with vernal keratoconjunctivitis (VKC).

METHODS. In the first phase of the study, inactivation of α -1 antitrypsin (AAT) by MMP-1 and -9 was investigated in vitro. Subsequently, tear samples were collected after clinical evaluation from 14 patients with active VKC and 15 normal control subjects. Tear cytology was performed on all samples. Levels of T-TIC and S-TIC were determined by spectrophotometry, whereas levels of tear pro-MMP-1, pro-MMP-9, and active MMP-1 and -9 were determined by enzyme-linked immunosorbent assay (ELISA).

RESULTS. MMP-1 and -9 inactivated AAT in vitro. S-TIC was significantly higher ($P < 0.0001$), and T-TIC ($P < 0.0001$) significantly lower in VKC samples. Tear levels of pro-MMP-1 and pro-MMP-9, and the activity of MMP-1 and -9 was significantly greater in patients with VKC than in healthy subjects ($P < 0.0001$). There was no significant correlation between T-TIC, MMP-1, and MMP-9 activity. In addition, T-TIC was not correlated to the total clinical score or to single clinical sign scores.

CONCLUSIONS. In this study, tear trypsin inhibitory capacity was shown to be reduced and tear MMP-1 and -9 activity increased in patients with VKC. Although T-TIC did not correlate with VKC severity, a local reduced inhibitory capacity of AAT may facilitate or prolong conjunctival inflammation in VKC. (*Invest Ophthalmol Vis Sci.* 2007;48:264–269) DOI:10.1167/iovs.06-0758

The etiology and immunopathogenesis of vernal keratoconjunctivitis (VKC) remain unclear. A large percentage of the children and adolescents who have this severe, bilateral, ocular

inflammatory disease have no familial or personal history of atopic disease and have negative results on standard allergic diagnostic tests. This ambiguity makes the disease a challenge both for researchers seeking new answers to its immunopathology and treatment and for ophthalmologists who manage young patients who experience its grave inflammatory consequences.¹ The disease in most of the cases is seasonal, nevertheless, perennial cases are not rare, especially in patients living in warm climates. Its predominance during high pollen seasons lends credence to the widely accepted hypothesis that VKC is an immunologically mediated hypersensitivity reaction to environmental antigens.²

VKC is characterized by an intense inflammation of eosinophils and Th2-type lymphocytes.¹ Many cytokines, chemokines, mediators, and proteases, such as tryptase, chymase, and metalloproteinases (MMPs) have been found to be over-expressed in tears and tissues of patients affected by VKC, confirming the complexity of mechanisms involved in the pathogenesis of inflammatory cell infiltration.¹

Matrix metalloproteinases (MMPs) degrade all components of the extracellular matrix.³ These enzymes are implicated in many critical physiological and pathologic processes, including development, wound healing, angiogenesis, cancer, and inflammation.⁴ MMPs are inhibited by tissue inhibitors of MMPs (TIMP), and by the broad-spectrum inhibitor α_2 -macroglobulin. The functional activity of MMPs is controlled by the equilibrium between levels of activated MMPs and free TIMPs. MMP-1 typically degrades collagens I, III, and V, which are the main conjunctival and corneal collagens, whereas gelatinase B (MMP-9) typically degrades the basement membrane constituent, collagen IV, and other matrix proteins. MMP-9 has also been reported to play a crucial role in the migration of inflammatory cells through basement membranes and thus may be involved in eosinophil migration during allergic inflammation.⁵ MMP-9 is the major known proteinase expressed by eosinophils,⁶ and its increased immunostaining has been recently identified in eosinophils of patients with VKC.⁷

α -1 Antitrypsin (AAT) is the archetype of the serine protease inhibitor (Serpin) supergene family. This glycoprotein secreted by liver cells is considered an acute-phase reactant, since its plasma levels increase during host response to inflammation/tissue injury.^{8–10} After reaching peak concentrations in plasma, AAT diffuses into tissue spaces to protect against enzymes released from injured or inflamed cells.¹¹ A severe deficiency of AAT creates an imbalance between proteinases and inhibitors, clinically manifested in the lungs and vasculature by pathologic events such as cervical artery dissection and aneurysm.^{12,13}

We have previously reported that MMP-1 and -9 tear levels were significantly increased in subjects with VKC.^{1,6} It has also been reported that AAT may be cleaved by MMPs¹⁴ and may play an important role in the immunoregulation of some diseases.^{15,16} The purpose of the present study was to evaluate a possible relationship between AAT and MMP. Trypsin inhibitory capacity (TIC), a marker of AAT activity, was measured in serum and tears of normal subjects and patients with VKC, and

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these levels were then correlated with the activity of MMP-1 and -9.

MATERIALS AND METHODS

The chromogenic substrates for trypsin BAPNA (H-D-pip-Phe-Arg-*p*-nitroanilide), 1,10-phenanthroline, N- α -*p*-tosyl-L-lysyl chloromethylketone and heparin (210-6) were obtained from Sigma-Aldrich (St. Louis, MO). The human active MMP-1 (F1M00) and human active MMP-9 fluorescence assays (F9M00) and the human pro-MMP-1 immunoassay (DMP100) were all obtained from R&D Systems (Hornby, ON, Canada). The human pro-MMP-9 immunoassay was obtained from Chemicon International (Temecula, CA).

Calculation of the Residual Inhibitory Capacity of MMP-Treated α -1 Antitrypsin

α -1 Antitrypsin was allowed to react with MMP-1 and -9 at 37°C in 50 mM HEPES buffer [pH 7.4] containing 100 mM NaCl and 5 mM CaCl₂, to approximate physiologic conditions. After various intervals, reactions were stopped by diluting 100-fold with 0.1 mM 1,10-phenanthroline and 0.01% Nonidet P-40 in 50 mM HEPES buffer (pH 7.4; MMP inhibition buffer). Nonidet P-40 was included to reduce protein binding to the test tubes. The amount of α -1 antitrypsin inactivated at each time point was determined by titration with trypsin as follows: portions (0–100 μ L) of the stopped reaction mixtures were added to 20 μ L of trypsin solution in wells of a flat-bottomed 96-well microtiter plate. The volume in each well was adjusted to a total of 120 μ L with MMP inhibition buffer. After incubation for 60 minutes at 23°C, to allow sufficient time for reaction of α -1 antitrypsin with enzyme, 40 μ L of BAPNA substrate was added to each well, and continuous rates of substrate hydrolysis were measured over 10 to 60 minutes with a plate reader. The rate of substrate hydrolysis was proportional to the fraction of residual α -1 antitrypsin activity.

Patients and Samples

After clinical evaluation, tear samples and peripheral blood were obtained from 14 active patients with VKC (mean age, 14.8 \pm 6.4 years; range, 7–24; 12 males and 2 females) and 15 normal, age-matched subjects (mean age 16.3 \pm 6.37 years; range, 7–30; 13 males and 2 females) as a control group. None of the subjects in the control group used contact lenses or had any ocular inflammatory signs and symptoms. The research adhered to the tenets of the Declaration of Helsinki and was approved by the Padova University Ethic Committee and Review Board, and by Zahedan University Medical Sciences Research Committee. Before samples were collected, written informed consent was obtained from all subjects or, in the case of minors, from their parents after explanation of the nature and the possible consequences of the study. All samples were collected from patients and normal subjects who presented to the Ophthalmology Department of Padua University.

Diagnosis of VKC was based on clinical history and evaluation of signs and symptoms. Clinical scores (0–3) for each ocular symptom (itching, tearing, photophobia, and foreign body sensation) and each sign (conjunctival erythema, mucous discharge, papillae, limbal infiltrates, and corneal epithelial disease) were assigned at the time of the visit. Patients included in the study were free of topical antihistamines and mast cell stabilizers for at least 3 days, topical corticosteroids for at least 7 days, and systemic antiallergic treatment for at least 2 weeks. All patients were in an active inflammatory phase of the disease with active limbal infiltrates (limbal VKC) or tarsal giant papillae (tarsal VKC) and with at least three positive symptoms. Patients with VKC were tested for skin test reactivity and the presence of specific IgE in serum for common environmental allergens (CAP-System; Pharmacia & Upjohn, Uppsala, Sweden).

Tear samples were collected from the outer canthus with a microcapillary tube, and centrifuged for 10 minutes at 1000 rpm to separate

the cells from the tear fluid. Tear volume ranged from 50 to 200 μ L. Supernatants were stored at -80°C until assaying.

Peripheral whole-blood samples (5.4 mL) were collected in tubes (Vacutainers; BD Biosciences-Falcon Labware, Bedford, MA) containing 0.6 mL of trisodium citrate (0.13 M) as anticoagulant and immediately put on ice. After centrifugation at 3000g for 15 minutes, plasma aliquots were stored at -40°C and tested within 1 month.

Determination of Serum- and Tear-TIC

TIC was measured in all serum samples as previously described.^{9,10} Briefly, each serum sample was diluted 100 times with Tris-base buffer (pH 8.2; 100 μ M, and at 37°C) and then mixed with diluted trypsin solution. These solutions were then mixed with trypsin synthetic substrate (BAPNA) and incubated at 37°C for 5 minutes (bovine serum albumin was used as the control). The absorbance value for each sample was read by a spectrophotometer at 400 nm, and serum TIC (S-TIC) was calculated. For determination of TIC in tears (T-TIC), samples were diluted five times only. The experimental procedure was the same as that for serum.

MMP-1 and -9 Activity in Tears

A fluorometric assay designed to measure enzyme activity quantitatively was performed. Monoclonal antibodies specific for MMP-1 and -9 (R&D Systems) were precoated onto black microplates. Standards and samples were pipetted into wells. Free MMP-1 and -9 in standards or samples bound to the immobilized antibody. After any unbound material was washed away, a fluorogenic substrate linked to a quencher was added and active enzyme present in samples cleaved the peptide linker between the fluorophore and the quencher, allowing a fluorescent signal to be read that was proportional to the amount of enzyme activity in the sample.

Statistics

Statistical analysis was performed with commercial software (SPSS ver. 11.0; SPSS, Chicago, IL). Data are expressed as the mean \pm SD. Analyses were calculated with ANOVA, unpaired *t*-test, and Spearman's rank correlation. *P* < 0.05 was considered significant.

RESULTS

ATT Inactivation by MMP-1 and -9

MMP-1 and -9 were shown to inactivate AAT in vitro (Table 1). Inactivation rates were expressed as moles of AAT inactivated by 1 mole of MMP-1 or -9 in 1 second. MMP-9 had a greater effect on AAT than did MMP-1. Because AAT is a major source of antiprotease activity^{9,17,18} including trypsin,^{15,16} we determined whether the trypsin inhibitory capacity in VKC tears was modulated by MMP-1 and -9 activity.

Patients

The mean age of patients with VKC and normal control subjects was similar. Of the 14 patients with VKC, six had the tarsal form of the disease and eight the limbal form. Two of the patients with tarsal disease and one with limbal disease had corneal complications. Table 2 summarizes the demographic and clinical data of patients with VKC. Tarsal VKC had a

TABLE 1. Comparison of the Rates of Inactivation of ATT by MMP-1 and -9

	MMP-1	MMP-9
ATT	0.192 \pm 0.06	0.324 \pm 0.421

Rates of inactivation are expressed as $\text{M}^{-1} \text{s}^{-1} \times 10^{-4}$.

TABLE 2. Clinical and Laboratory Data from Patients with VKC

Pt. No.	Gender/Age	VKC Type	Serum Specific IgE/Prick	Total IgE	Eosinophil Count $\times 10^9/L$	Total Clinical Score (0–3)	Corneal Clinical Score (0–3)	Papillae/Limbus Score (0–3)
1	M/12	T	G, D, P, C, T	569	1.0	14	0	2
2	F/7	L	Negative	37.4	0.1	10	0	1
3	M/10	T	D, F	279	1.0	12	1	2
4	M/21	L	Cat	120	0.3	14	0	2
5	M/10	T	Negative	45	0.1	9	0	1
6	M/12	T	Negative	46	0.1	12	0	2
7	M/7	L	G, P	122	0.2	16	0	2
8	F/24	L	Negative	23.6	0.0	11	0	2
9	M/6	T	D, G	172	0.3	12	1	2
10	F/13	T	D, C, T, F	505	1.0	30	3	3
11	M/5	L	G	186	0.2	20	2	3
12	M/16	L	G, D, P, T, F	204	0.1	17	1	3
13	M/12	L	Negative	95	0.3	8	0	1
14	M/9	L	Negative	174	0.5	12	0	1

Total clinical score: sum of scores of 10 signs and symptoms (0 to 3 for each of them; 0, absent; 1, mild; 2, moderate; 3, severe), itching, tearing, photophobia, mucous discharge, foreign body sensation, hyperemia, keratitis, trantas dors, limbal infiltration, and giant papillae. L, limbal VKC; T, tarsal VKC; D, *Dermatophagoides*; G, *Graminaceae*; P, *Parietariae*; C, *Compositae*; Alt, *Alternaria alternata*; Tr, tree pollen; Cat, cat dander.

significantly higher mean clinical score of signs and symptoms compared with limbal disease ($P < 0.01$).

S- and T-TIC in Normal Subjects and Patients with VKC

S- and T-TIC were calculated to identify the serum ATT activity. S-TIC in healthy individuals (2.895 ± 0.0402 micromoles/min per milliliter) was significantly lower than in patients with VKC (3.334 ± 0.222 micromoles/min per milliliter; $P < 0.0001$; Fig. 1A). In contrast, T-TIC of healthy individuals (0.1847 ± 0.004 micromoles/min per milliliter) was significantly higher ($P < 0.0001$) than in patients with VKC (0.0760 ± 0.016 micromoles/min per milliliter; Fig. 1B).

Tear Pro-MMP-1 and -9 Levels

Tear levels of pro-MMP-1 were significantly higher in patients with VKC (52.12 ± 11.80 ng/mL, $P < 0.0001$; Fig. 2A) compared with the low levels found in normal subjects (5.213 ± 0.5637 ng/mL). Concentrations were similar in tarsal and limbal VKC. Tear levels of pro-MMP-9 (Fig. 2B) were also significantly increased in VKC (269.3 ± 71.0 ng/mL) compared to normal subjects (10.41 ± 1.667 , $P < 0.0001$), but were similar in tarsal and limbal VKC.

Tear MMP-1 and -9 Activities

Mean tear MMP-1 activity in patients with VKC (63.76 ± 14.53 fluorescence units [FU]) was significantly higher than in nor-

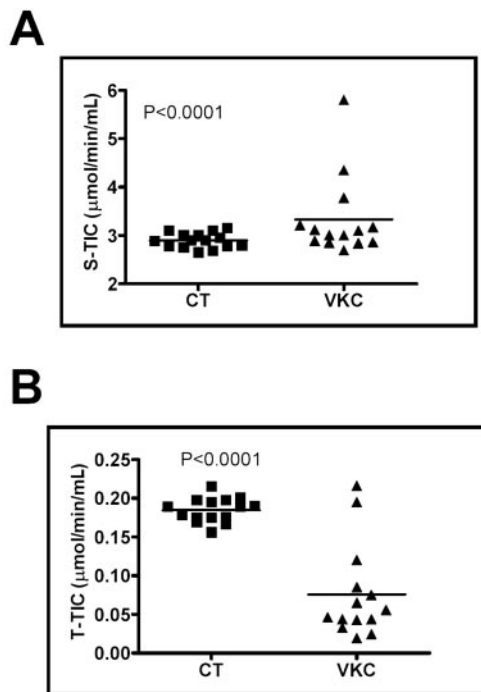


FIGURE 1. (A) S- and (B) T-TIC in VKC and normal subjects (CT). S-TIC was significantly higher in VKC individuals ($P < 0.0001$) and T-TIC was significantly lower in patients with VKC ($P < 0.0001$).

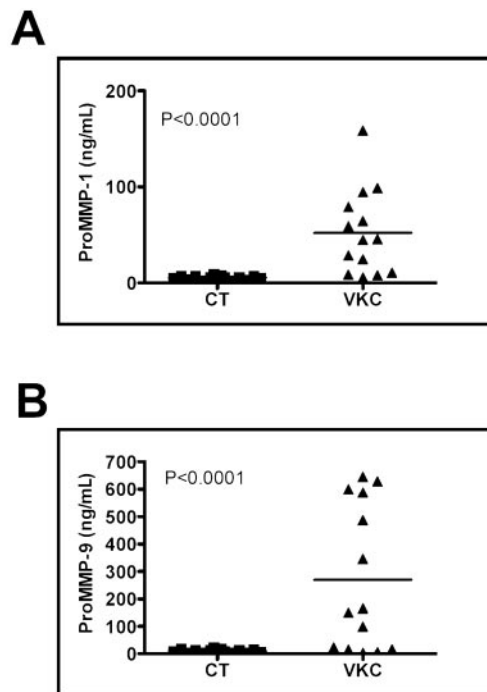


FIGURE 2. Concentrations of pro-MMP-1 (A) and pro-MMP-9 (B) in tears of patients with VKC and normal subjects. Pro-MMP-1 and -9 were significantly higher in patients with VKC ($P < 0.0001$).

mal subjects (14.63 ± 1.046 FU; $P < 0.0001$, Fig. 3A). Similarly, mean tear MMP-9 activity in patients with VKC (21.53 ± 4.544 FU) was significantly higher than that in normal subjects (10.79 ± 1.001 FU; $P < 0.0001$, Fig. 3B). No differences in MMP activities were found between the two subgroups: tarsal and limbal VKC.

Correlation between T-TIC and MMP-1 or MMP-9 Activity

Since MMP-1 and -9 were shown to inactivate AAT in vitro (Table 1), and since their activity was increased in VKC tears (Figs. 3A, 3B), we investigated the relationship between T-TIC and MMP-1 or -9 activity in VKC tears. As shown in Figures 4A and 4B, T-TIC was not related to MMP-1 ($P < 0.1910$) or MMP-9 ($P < 0.1448$) in patients with VKC. This lack of relationship was also observed when the patients were subclassified by limbal or tarsal VKC (data not shown).

Correlation between T-TIC and Clinical Scores in VKC

To explore the possibility that proteases play a role in VKC development,^{2,10-23} we investigated whether there is any relationship between T-TIC and total or single sign and symptom clinical scores in patients with VKC. The results showed that neither the total clinical score ($P < 0.3412$; Fig. 5A) nor the papilla limbus score ($P < 0.4950$; Fig. 5B), nor the number of days the subject was steroid-free before the samples were collected was related to T-TIC in all patients with VKC, as well as in the subgroups of limbal and tarsal VKC (data not shown).

Of note, S-TIC correlated significantly with total serum IgE levels ($P < 0.0126$; data not shown).

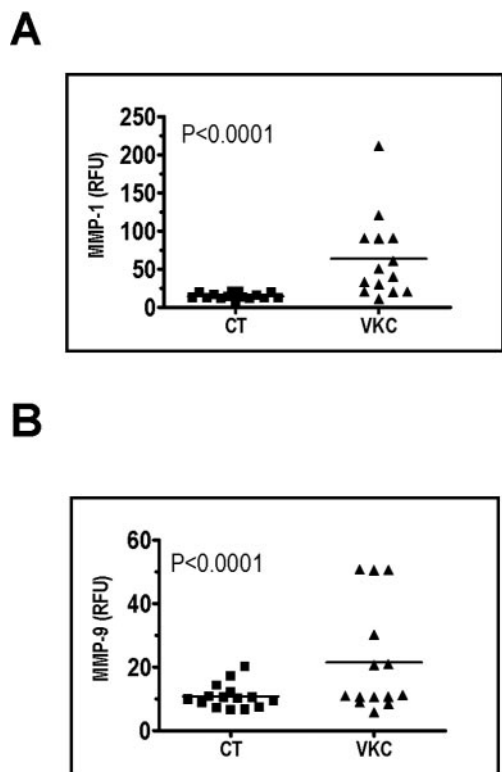


FIGURE 3. Comparison of tear MMP-1 (A) and -9 (B) activities in VKC and normal subjects. Active MMP-1 and -9 were significantly higher in VKC ($P < 0.0001$).

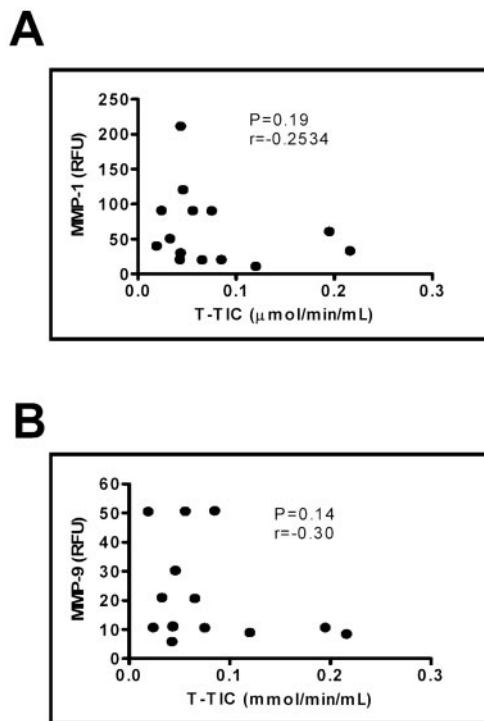


FIGURE 4. Correlation of tear MMP-1 (A) and MMP-9 (B) activities and tear trypsin inhibitory capacity (T-TIC) in VKC. T-TIC did not correlate with MMP-1 ($P < 0.1910$) or -9 ($P < 0.1448$).

DISCUSSION

AAT is a 52-kDa glycoprotein secreted by hepatocytes and, to a lesser extent, by lung epithelial cells and phagocytes. AAT

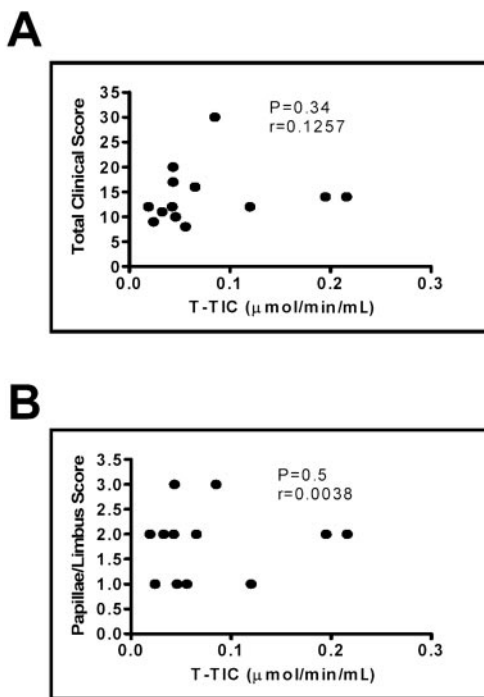


FIGURE 5. Correlation between tear trypsin inhibitory capacity (T-TIC) and total clinical score in VKC (A), and correlation between T-TIC and giant papillae/limbal infiltrate score (B). Neither total clinical score ($P = 0.34$) nor papillae/limbus score ($P = 0.50$) correlated with T-TIC.

expression in conjunctival epithelium is regulated by Sp1 transcription factor.²⁴ This protein belongs to the serine protease inhibitor (SERPINS) superfamily.²⁵ Many biological processes require a balance between proteases that initiate essential proteolytic pathways and the inhibitors that limit excessive protease activity. Of the many families of protease inhibitors, serpins appear to control key intracellular and extracellular pathways²⁶ and are unique in that they are activated only by drastically changing shape.²⁷

AAT is sensitive to inactivation by nonsubstrate proteinases. In vitro, human neutrophil elastase is known to inactivate several serpins, including AAT, in a substratelike manner by cleaving them near the inhibitory site.²⁸ Some MMPs also inactivate AAT,^{14,29} downregulating its inhibitory capacity at the site of inflammation, and thus allowing for greater neutrophil elastase activity. In fact, neutrophil elastase has been shown to be highly expressed in conjunctival specimens from patients with VKC.³⁰

Since MMP-1 and -9, shown to inactivate AAT in vitro (Table 1), have previously been shown to increase in tears of patients with VKC,^{1,6} we investigated whether T-TIC, a marker of AAT activity, is modified in this disease.

S-TIC was in fact significantly higher in the VKC group than in normal subjects (Fig. 1A; $P < 0.0001$). AAT, an acute phase reactant, is upregulated during the acute-phase response to tissue necrosis and inflammation. Serum levels and activity increase in rheumatoid arthritis, bacterial infections, viral hepatitis, uveitis, vasculitis, carcinomatosis and after estrogen rises after puberty, during pregnancy, or in conjunction with contraceptive medication use.^{9,10,31–33} Because VKC is a severe chronic ocular allergic disease characterized by an intense conjunctival inflammation with predominance of eosinophils and Th2-type lymphocytes and increased leukocyte activation markers in serum even in the absence of systemic signs and symptoms,¹ an increase in S-TIC was not unexpected in individuals with VKC.

Although one might have expected a local increase in tear ATT due to increased conjunctival blood vessel permeability in active patients with VKC, T-TIC was significantly lower in patients with VKC than in normal individuals (Fig. 1B), indicating a reduced local production or inhibition of its activation in this disease. This finding may have been due to greater MMP-1- and -9-mediated inactivation of AAT. In fact, pro-MMP-1, pro-MMP-9, and active MMP-1 and -9 were significantly higher in patients with VKC than in normal individuals (Figs. 2A, 2B, 3A, 3B). However, there was no significant correlation between active MMP-1, MMP-9, and T-TIC (Fig. 4A, 4B), and thus multiple factors must be responsible for the reduction of T-TIC in patients with VKC. Although the severity of the disease varied among the patients, TIC did not correlate with either signs and symptoms or previous steroid treatment, suggesting that the low levels of ATT activity found in tears may be a peculiar characteristic of VKC rather than a marker of disease activity. In fact, tear AAT levels have been shown to increase in diseases of ocular inflammation such as allergic conjunctivitis, bacterial conjunctival and corneal infections, and acute adenoviral keratoconjunctivitis.^{34,35} Active transport, transudation from serum, local production, or a combination of these factors may give origin to this protein in tears.

Eosinophils are the major effector cells in allergic inflammatory response. They are increased and activated in tears and tissues of all allergic eye diseases^{1,36,37} and produce reactive oxygen species (ROS). It is well-known that AAT is oxidized and thus inactivated by free radicals released from activated neutrophils, mast cells, and eosinophils.^{14,38} It has also been demonstrated that the major cause of inactivation is oxidation of the methionine residue to methionine sulfoxide, located at its active center.^{38,39} Methionine residues in proteins

are oxidized by various molecules,⁴⁰ and once oxidized are found in the sera of patients with diseases that are associated with the presence of free radicals.⁴¹ Considering the high number of activated leukocytes in the inflamed VKC conjunctiva¹ ROS produced by these cells may be involved in the decrease of T-TIC observed in patients with VKC.

Several proteases, such as tryptase, chymase, urokinase-type (uPA), tissue type (tPA) plasminogen activators, and metalloproteases (MMPs) have been found to be overexpressed in tears and tissues of patients affected by VKC, yet without changes in inhibitors PAI-1 and TIMP-1.^{1,2,6} AAT does not directly inhibit tryptase⁴² but does effectively inhibit chymase.^{43,44} 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.^{45,46} Chymase also triggers the activation or degradation of endogenous bioactive peptides such as interleukin-1 β , stem cell factor, and endothelin,^{47–49} and participates in inflammatory reactions related to the degranulation of mast cells. In fact, chymase tear levels have been found to be increased in patients with VKC,¹⁹ in whom the number of mast cells is increased 60% to 200%.¹ Conjunctival mast cells are mostly of the connective tissue type (MC_{CT}), which contain tryptase, chymase, cathepsin-G, and carboxypeptidase-A, whereas mucosal mast cells contain only tryptase.^{22,50} The role of chymase in conjunctival allergic inflammation is still unclear, but the present findings suggest that decreased activity of AAT in tears may potentiate the activity of mast cell-derived proteases in VKC.

It has been suggested that a reduced level or activity of AAT may increase the propensity to develop asthma,⁵¹ or cold urticaria⁴⁴ and might enhance the severity of airway hyperresponsiveness.⁵² This is very interesting, because nonspecific ocular hyperreactivity, one of the least defined features of VKC, may be related to the present findings.

In conclusion, we found that (1) MMP-1 and -9 inactivated the protease inhibitor AAT in vitro; (2) TIC (as expression of AAT activity) was significantly lower in tears of the patients with VKC and higher in serum compared with normal subjects; (3) tear levels and activity of MMP-1 and -9 were increased in VKC; (4) tear MMP-1 and -9 activities did not correlate significantly with AAT activity. Thus, other factors in addition to these MMPs must contribute to the reduction of this major protease inhibitor in tears. We hypothesize that several pathways that limit local inflammation are downregulated in VKC. The present results suggest that, in conjunction with judicious anti-inflammatory coverage, additional local antioxidant and antiprotease treatment or possible local AAT replacement therapy may be useful in the treatment of VKC.

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