

A flow cytometric study about the immunopathology of vernal keratoconjunctivitis

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Background: Vernal keratoconjunctivitis (VKC) is a bilateral seasonal conjunctival inflammation. Exact pathogenesis of the disease is unknown, but some evidences suggest T_H lymphocyte-mediated immune reactions.

Objective: The aim of this study was to investigate the role of the T lymphocyte and its subsets in the pathogenesis of VKC. **Methods:** We obtained tear samples from patients with VKC and normal volunteers during active (spring) and quiescent (winter) periods. The patients' records were also obtained for assessment of symptom scores. The percentages of CD4/29+, CD4/45RA+, CD4+, and CD8+ in tear samples were established by using flow cytometry, and the results of all three groups were compared with each other by using the Wilcoxon matched-pair signed-rank test and the Mann-Whitney U test. **Results:** The percentages of CD4/29+ and CD4+ cells in tears of patients with VKC increased significantly in the active period and decreased to normal levels in the quiescent stage. In contrast, the percentages of CD4/45RA+ and CD8+ cells in tears of patients with VKC did not show any significant change between spring and winter. The patients' symptoms were significantly lower in the quiescent period (winter) compared with the active stage (spring).

Conclusion: We propose that increased numbers of CD4+ and CD4/29+ cells in tears may be exacerbating the disease during the spring season. (*J Allergy Clin Immunol* 1998;101:821-4.)

Key words: Vernal keratoconjunctivitis, CD4+ lymphocyte, CD4/29+ lymphocyte, CD4/45RA+ lymphocyte, immunopathology

Vernal keratoconjunctivitis (VKC) is a bilateral, seasonal inflammation caused by allergy, which is usually seen in young patients.^{1,2} It is encountered most frequently between childhood and puberty.³ The disease is more common in dry, warm climates such as the Middle East and Mediterranean basin.⁴ VKC tends to be seasonal, with symptoms exacerbating with the onset of spring and declining during the fall. The exact pathogenesis of the disease is unknown. Although strong evidence points to the role of type-1 hypersensitivity, type-4 hypersensitivity may also be involved. Evidence for the role of type-1 hypersensitivity includes high concentra-

Abbreviations used

FCM: Flow cytometry

VKC: Vernal keratoconjunctivitis

tions of eosinophils in the conjunctiva, relatively high incidence of systemic eosinophilia, elevated tear IgE levels and IgG antibodies (including specific antibodies to allergens such as grass and ragweed), and response to agents that inhibit mast cell degranulation.^{2,4-7} Several other findings, however, could not be explained by this mechanism. Immunohistochemical studies of VKC biopsy specimens showed a multicellular inflammatory infiltrate with prevalence of activated eosinophils, mast cells, and T_H lymphocytes in both the epithelium and subepithelium.^{8,9} These findings suggest that a type-4 hypersensitivity reaction may play a role in the pathogenesis of VKC. This study was designed to investigate the role of T cells in the pathogenesis of the disease. For this purpose, we compared the percentages of the CD4+, CD8+, CD4/29+, and CD4/45RA+ cells from the tear samples of patients with active and nonactive VKC with those of normal volunteers.

METHODS

After obtaining approval from the medical ethics committee of our hospital (Eskisehir Air Force Hospital), the study was commenced. The subjects of this study (group A) were 14 men and six women between the ages of 11 and 22 years who had a typical history of bilateral ocular inflammation that began in the early spring, abated late summer, and was present for at least the last 2 years. All had large papillae (>1 mm) on the upper tarsal conjunctiva that covered more than two thirds of the tarsal area, limbal hypertrophy, Trantas' dots, and typical mucoid discharge. Corneal involvement was observed in eleven patients, among whom five had a micropannus of the peripheral cornea that was mostly situated on the upper region, four had farinaceous epitheliopathy (Keratitis vernalis of Tobgy), and two had characteristic shield-shaped central corneal ulcers. All patients had received topical cromolyn sodium or steroid drops at some time, but none had been prescribed topical ocular or systemic medication for at least 1 month before the flow cytometric analysis.

Eye symptom severity scores were obtained from each patient at the active (spring) and quiescent (winter) stages. The severity of the patients' symptoms of itching, photophobia, watering, and mucoidal discharge were examined. For this purpose, each patient was instructed to grade his or her symptoms of itching, photophobia, watering, and mucoidal

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TABLE I. The results of FCM tear analyses and statistical analyses

	CD4+ cells			CD8+ cells			CD4/45RA+ cells			CD4/29+ cells		
	Range	Mean ± SD	<i>p</i> Value	Range	Mean ± SD	<i>p</i> Value	Range	Mean ± SD	<i>p</i> Value	Range	Mean ± SD	<i>p</i> Value
FCM analyses												
Group A1	8.4-17 (37-85)*	12.3 ± 1.2	—	0.6-2.7 (3-14)	1.4 ± 0.28	—	0.6-1.1 (4-6)	0.87 ± 0.11	—	7.4-14.5 (33-78)	12.1 ± 1.2	—
Group A2	0.75-5.4 (4-28)	2.8 ± 0.98	—	0.7-2.3 (4-12)	1.42 ± 0.3	—	0.7-1.2 (4-6)	0.89 ± 0.09	—	0.6-2.1 (3-11)	1.3 ± 0.17	—
Group B	0.8-5.1 (4-27)	2.7 ± 1.1	—	0.6-2.2 (3-11)	1.39 ± 0.24	—	0.4-1.4 (3-7)	0.90 ± 0.23	—	0.3-2.2 (2-12)	1.2 ± 0.32	—
Statistical analyses												
Between groups A1 and A2†	—	—	<0.05	—	—	>0.5	—	—	>0.5	—	—	<0.05
Between groups A1 and B‡	—	—	<0.05	—	—	>0.5	—	—	>0.5	—	—	<0.05
Between groups A2 and B‡	—	—	>0.5	—	—	>0.5	—	—	>0.5	—	—	>0.5

Group A1, Patients with VKC in the active period (tears obtained during spring); Group A2, patients with VKC in the quiescent period (tears obtained during winter); Group B, normal volunteers.

*Absolute number of cells.

†Analyses performed with Wilcoxon matched-pair signed-rank test.

‡Analyses performed with Mann-Whitney U test.

discharge on a scale from 0 to 10. Symptom scores from each patient were recorded on separate paper. We obtained tear samples from each patient in the active group (A1) and the quiescent group (A2). Symptom score analyses of the patients were repeated in the quiescent stage and compared with the active stage by using Wilcoxon matched-pair signed-rank test. Shield ulcer and farinaceous keratopathy were not observed in any patient when examined during the quiescent stage, but micropannus persisted in patients when already present. The conjunctival signs (upper tarsal papillae) changed little for the better between the active and quiescent stages.

The control subjects (group B) were 18 volunteers who were ophthalmologically normal except for refractive errors, with no systemic or ocular allergic symptoms. The 11 men and seven women ranged in age from 9 to 21 years (mean ± SD, 15.2 ± 2.0 years).

Tear samples (minimum, 100 µl; mean ± SD, 114 µl ± 11 µl) were obtained from patients and control subjects. For this purpose, microhematocrit tubes (100 pcs NRIS microhematocrit tubes; Modulohm A/S 6-8 Vasekaer DK-2730, Herlev, Denmark) were introduced into the inferior tear meniscus. Tears were expelled into a plastic tube by using a bulb attached to one end of the tube. This process was repeated three to four times at 5- or 6-minute intervals. Separate plastic tubes were used for each patient or volunteer. Each specimen was dispensed equally into four glass tubes of 25 µl each, and 20 µl of isotopic antibody (Cytostat Coulter Clone; Coulter Immunology, a division of Coulter Corp., Hialeah, Fla.; cat. no.: 6603796) conjugated with both fluorescein isothiocyanate and rhodamine (RD1) was added to the control tubes. These tubes were used as internal controls for each specimen. Twenty microliters of CD4 RD1/CD8 fluorescein isothiocyanate antibody (cat. no.: 603821, Coulter) for T_H and suppressor T lymphocytes, respectively; 20 µl of CD4/29 RD1 antibody (cat. no.: 6603799, Coulter) for memory T_H lymphocytes; and 20 µl CD4/45 RD1 antibody (cat. no.: 6003800) for naive T_H lymphocytes were added separately to three tubes of tears from each patient or volunteer. After incubation of all tubes at room

temperature for 5 minutes, the cells in the tear samples were fixed with 1% sodium azide and 1% paraformaldehyde.

The cells were examined with a flow cytometer. PMT-log2, PMT-log3 parameters with forward scatters and side scatters were used. Cells restricted in a fixed area by the forward scatters and side scatters were then studied (500 to 550 cells; mean ± SD, 521 ± 9.5 cells). The results were expressed in terms of percentages of CD4+, CD8+, CD4/29+, and CD4/45RA+. FCM tear analyses were performed in both the active (group A1) and quiescent stages (group A2). Wilcoxon matched-pair signed-rank test was used for statistical analysis between groups A1 and A2, whereas the Mann-Whitney U test was used for statistical analyses between groups A1 and B and A2 and B. Statistical significance was accepted if *p* values were less than 0.05.

RESULTS

The percentages of CD4+ cells in the tear samples of groups A1, A2, and B ranged from 8.4% to 17% (mean ± SD, 12.3% ± 1.2%), 0.75% to 5.4% (mean ± SD, 2.81% ± 0.98%), and 0.8% to 5.1% (mean ± SD, 2.7% ± 1.1%), respectively. There was a statistically significant difference in CD4+ cells when group A1 was compared with both groups A2 and B (both *p* < 0.05); however, no significant difference was observed between groups A2 and B (*p* > 0.5). A similar trend was observed with CD4/29+ cells for which percentage ranges for groups A1, A2, and B were 7.4% to 14.5% (mean ± SD, 12.1% ± 1.2%), 0.6% to 2.1%, (mean ± SD, 1.3% ± 0.17%), and 0.3% to 2.2% (mean ± SD, 1.2% ± 0.32%), respectively. The other cells in the FCM window were B lymphocytes, conjunctival epithelial cells, and polymorphonuclear leukocytes. Polymorphonuclear leukocytes were more granular (y-axis side scatter) and had a larger cell size (x-axis forward scatter). Epithelial cells were also larger than lymphocytes. Monoclonal antibodies

TABLE II. The results of symptom score analyses

	Itching	Photophobia	Mucoid discharge	Watering	Total
Group A1 (n = 20)	6.3 ± 1.1*	6.2 ± 1.4	8.5 ± 1.7	5.4 ± 1.8	26.8 ± 3.1
Group A2 (n = 20)	2.8 ± 0.7	4.2 ± 1.3	2.8 ± 1.0	3.9 ± 0.7	16.8 ± 2.8

Statistical analysis was performed with Wilcoxon matched-pair signed-rank test; $p < 0.05$ for all symptoms. *Group A1*, patients with VKC in the active period (tears obtained during spring); *Group A2*, patients with VKC in the quiescent period (tears obtained during winter).

*Mean ± SD (patients scoring of his or her symptoms using numbers from 0 to 10).

known to be specific for T lymphocytes were not present on the cell surfaces of B lymphocytes. There was no significant difference when percentages of CD8+ and CD4/45RA+ cells were compared among all groups (Table I).

Patient symptoms were at a significantly lower level during the winter season compared with the spring season ($p < 0.05$ for itching, watering, mucoidal discharge, photophobia, and total symptom score) (Table II).

DISCUSSION

FCM measures the physical and chemical properties of individual cells or particles moving single file in a fluid stream.¹⁰ The cell suspension passes through the flow cytometer in a single file and the individual cells are sequentially illuminated by a light source. As the exited fluorochrome returns to resting energy level, light of a specific wavelength is emitted and detected by a photo-receptor that converts this energy into electric impulses analyzed by a computer software program.¹¹

Tears contain many cells originating from corneal-conjunctival epithelial cells, subepithelial lymphoid tissue, and conjunctival vessels.¹² Lymphocytes are the most abundant, but large numbers of conjunctival epithelial cells and polymorphonuclear leukocytes are also found.¹² Thus it is possible to say that tears may represent cellular components of conjunctiva and subepithelial tissue. Although the use of FCM currently involves measurement of at least 10,000 cells,^{10, 11} the technique is also suitable for detecting surface receptors on a small number of tear cells.¹³

The exact pathogenic mechanism of VKC is not fully understood. However, immunohistochemical studies provide us with important clues about the processes involved in the disease. Immunohistochemical studies reveal a complex immunologic basis and chronic inflammatory component of VKC. Involvement of secondary inflammatory cells (particularly eosinophils and T_H cells) in addition to the mast cells resident in the conjunctival substantia propria can lead to severe inflammatory damage.¹⁴ Immunohistochemical studies of VKC biopsy specimens show multicellular inflammatory infiltrate with prevalence of activated eosinophils, mast cells, and T_H lymphocytes in the epithelium and substantia propria.^{8, 9} Mast cells and eosinophils are important inflammatory cells in all of the ocular allergies. However, infiltration of the conjunctiva with T_H cells was reported

to be important only for VKC, atopic keratoconjunctivitis, and giant papillary conjunctivitis.^{13, 15} In this study we have found an increased T_H lymphocyte concentration in the tears of patients with active VKC. This increase was due to CD4/29+ (memory T_H cells) subset predominance because no significant difference was observed between groups (patients with active VKC, patients with nonactive VKC, and control subjects) with respect to CD4/45RA+ (naive T_H) cell count.

The T_H cell role in the pathogenesis of VKC is supported by cloning T_{H2} -like T_H cells from the conjunctival epithelium of patients with VKC.¹⁶ Enhanced T_H cells may stimulate IgE production of B cells because IgE- and eosinophil-mediated immune reactions are dependent on the activation of the T_H cells.¹⁷ IL-4, a lymphokine produced by T_H cells, plays an important role in B-lymphocyte activation and is required for production of IgE. Other secretors of T_H cells (IL-3 and IL-5) activate eosinophils and modulate the function of mast cells.¹⁷⁻¹⁹ These features of T_H cells suggest that they may play an important role in allergic diseases of the conjunctiva. Indeed, concentration of IL-4 in the tears of patients with VKC was reported to be significantly higher than that found in control subjects.²⁰ It was postulated that VKC can represent a phenotypic model of up-regulation of the cytokine gene cluster on chromosome 5q, which, through its products (IL-3, IL-4, IL-5, and granulocyte colony-stimulating factor), regulates T_{H2} prevalence.⁸ We demonstrated the predominance of CD4/29+ (memory T_H) lymphocytes in the tears of patients with VKC in the active period, and this predominance was correlated with the enhanced disease symptoms. The number of CD4/29+ cells in tears decreased and reached the normal level as the disease went into its quiescent period during the winter. The decline of CD4/29+ cells in tears was accompanied by a decrease of the disease symptoms. The reason for the decrease in CD4/29+ cell concentration during the quiescent period is unknown. However, it was reported in a previous study²¹ that T_H cells in blood samples from patients allergic to pollen were higher in the peak ragweed and birch pollen season (early spring) than after spring, but the authors gave us no information about the T_H cell subgroups.

We found that concentration of CD8+ cells in tears did not differ among the groups of patients with active VKC, patients with nonactive VKC, and normal volunteers. Similarly, CD8+ cell count in blood samples from

patients allergic to pollen was reported not to change from early to late spring.²¹ This result may indicate that CD8+ cells have little, if any, role in the pathogenesis of VKC, in contrast to their role in atopic keratoconjunctivitis.^{22, 23}

In conclusion, we think that our study may indicate that enhanced numbers of tear CD4/29+ cells may exacerbate VKC during spring through an unknown mechanism or mechanisms. The study also gives us further evidence as to the role of type-4 hypersensitivity reactions in the pathogenesis of VKC. It is probable that this information may also explain the mechanism of the efficacy of topical cyclosporin-A treatment in VKC^{24, 25} because a recent immunohistopathologic study revealed that the clinical improvement in VKC after topical cyclosporin therapy may result from its immunomodulating effect on the components of cell-mediated and humoral immune response. In contrast, the drug has no immunomodulating effect on mast cells and IgE-mediated allergic response.²⁶ New treatment modalities in the exacerbating period of the disease may be established on the basis of the data obtained in this study. Clearly, additional studies are needed to explore the role of T_H cells and their subsets in the pathogenesis of VKC.

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