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Immunological Characteristics of Patients with Vernal Keratoconjunctivitis

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Purpose: Previously, we have reported that local excision and immunosuppressive treatment are useful in treating patients with very severe vernal keratoconjunctivitis (VKC).

Methods: We measured serum levels of immunoglobulins, cytokines, and interleukin-2 receptors (IL-2R) and the concentration of IL-4 in tears of 10 patients with severe VKC, and made a comparison with data from 10 healthy controls. Brush cytology specimens were examined to determine the number of inflammatory cells and the human lymphocyte antigen (HLA)-DR expression in conjunctiva.

Results: The mean serum level of total immunoglobulin (Ig)E, but not IgG, was higher in severe VKC patients compared with healthy controls. Specific IgE positivity to housedust, dust mites, and cat antigens was observed in 60–80% of patients. The level of IL-4 and IL-2R in serum and IL-4 in tears before treatment was higher in patients with VKC compared with controls. Serum levels of IL-2 and interferon-γ did not increase. Brush cytology specimens from patients expressed HLA-DR.

Conclusions: These results suggest that VKC could be a combination of type I allergic disease and an inflammatory cell disease including activated T cells, especially Th2 cells. Therefore, the surgical removal of inflammatory cells combined with immunosuppressive therapy could be advocated as a method of treatment. **Jpn J Ophthalmol 2002;46:244–248**

Key Words: Antibodies, cytokines, human lymphocyte antigen-DR, inflammatory cells, interleukin-2 receptors.

Introduction

Vernal keratoconjunctivitis (VKC) is thought to be a severe type of allergic disease that interferes with the patient's normal lifestyle. We have previously reported that combined excision of giant papillary formations and supratarsal injection of corticosteroid with long-term use of cyclosporine A (CsA) eyedrops are useful in treating patients with very severe VKC.[1] CsA is a potent immunomodulator that inhibits the clonal expansion of interleukin (IL)-2 mediated lymphocytes.[2,3] Recent studies have shown that cytokines are involved in the regulation and stimulation of cells, especially T cells, that are important in the pathogenesis of allergic disease.[4–9]

Maggi et al[10] detected Th2-like helper T cells in the conjunctiva of patients with VKC. IgE and IgG production by human lymphocytes is induced by interleukin (IL)-4 and suppressed by interferon (IFN)-γ.[11] The IL-2 receptor (IL-2R), a subunit of the IL-2 cell-surface receptor, is secreted from cell membranes during T cell activation and is thought to be an important marker of T cell activation.[12] We evaluated the levels of these cytokines in serum and tears before treatment, and measured serum levels of total and antigen-specific immunoglobulins. We also determined the number of inflammatory cells in brush cytology samples, and evaluated human lymphocyte antigen (HLA)-DR expression in conjuncti-

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val epithelial cells, which are thought to be indirect indicators of the activation of the Th2 and Th1 subsets of T cells, to study the immunomodulating effects of surgical excision and CsA on VKC.

Materials and Methods

Ten Japanese patients (6 men and 4 women, aged 13 to 29 years; mean age = 18.3 ± 5.1 years) who had suffered from severe bilateral chronic VKC for more than 1 year were evaluated in this study. Their stage of disease was thought to be refractory. All patients had been treated with eyedrops containing 0.1% betamethasone sodium phosphate (Sanbetasone®; Santen, Osaka), 0.1% fluorometholone (0.1% Flumetholone®; Santen), or cromolyn sodium (Intal®; Fujisawa, Osaka). One patient also had received short-term therapy with systemic corticosteroids (maximal dose of betamethasone: 3 mg/day). All patients had the palpebral type of VKC with giant papillary proliferations, but they did not have atopic dermatitis or systemic diseases. At least 2 days before sampling, they received only cromolyn sodium eyedrops (Intal) four times/day and no systemic treatment such as corticosteroids. Then, they underwent 0.05% CsA eyedrop[1] treatment for 1 month and received surgical treatment 1 month later at our hospital. Two patients refused blood examinations. They were included in this study because they had typical giant papillary proliferations and corneal erosion, and because of their history. All subjects provided written informed consent before participation.

The control group consisted of 10 age-matched healthy volunteers, who had no ocular allergic symptoms and/or history, and who tested negative for the multiple antigen simultaneous test (MAST) 16 (SRL Company, Tokyo).[13,14]

The serum level of total IgE was determined by fluoroenzyme immunoassay (Pharmacia, Tokyo). The serum level of total IgG was assayed by the latex agglutination method (SRL), and serum levels of antigen-specific IgE were determined by the MAST-16 (SRL). Serum levels of IL-2 and of human IFN-γ were determined by radioimmunoassay (Medgenix Diagnostics, Fleurs, Belgium). The sensitivities of the assays were 0.8 U/mL for IL-2 and 1.0 U/mL for IFN-γ. The levels of IL-4 in serum and tears were determined by a sandwich enzyme-linked immunoassay (ELISA) with a minimum sensitivity of 0.01 pg/mL (SRL) according to previously described methods.[13] Tear samples of >30 mL were collected in a way to prevent local reflex tearing with a micropipette without any added solution. Tears were stored at −20°C until assayed. IL-2R was measured by an immunoradiometric assay (Medgenix) with a minimum sensitivity of 90 U/mL.

More than 20,000 conjunctival superficial cells were collected from all the subjects with a special brush (Cytobrush, small; Medscand, Malmo, Sweden) from the upper palpebral conjunctiva. They were kept in 1 mL of phosphate-buffered saline (PBS; pH 7.4). These cells were counted by hemocytometer. The 20,000 cells were washed once with medium. Ten thousand cells were examined by flow cytometer (FACScan®; Becton Dickinson, Rutherford, NJ, USA). Ten thousand cells were spread on glass slides and fixed with acetone. These cells were treated with May-Grünwald stain (Muto Pure Chemicals, Tokyo) and Giemsa stain (Daiichi Pure Chemical, Tokyo), and compared with samples from the controls.

Flow cytometry with FACScan® (Becton-Dickinson) was carried out at a fixed laser power (600 mW at 488 nm). Standardization of the flow cytometer conditions, background fluorescence reactivity, and control conjunctival epithelial cells were previously reported.[6] Briefly, conjunctival epithelial cells (1×10^4) were first incubated with mouse monoclonal anti-HLA-DR antibody (Becton-Dickinson, Mountain View, CA, USA) at 4°C for 30 minutes. After washing with PBS containing 2% fetal bovine serum, the samples were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Fab Fractions, Bio Source, Camarillo, CA, USA) at 4°C for 30 minutes. The samples were washed and then run on the flow cytometer. For analyzing the expression of cytokeratin, the samples were fixed with 0.1% formaldehyde in PBS, and made permeable with digitonin (Sigma, St. Louis, MO, USA) (10 mg/mL) at 4°C for 5 minutes. These samples were stained with anti-keratin AE-3 (Boehringer Mannheim Biochemica, Indianapolis, IN, USA) and FITC-labeled goat anti-mouse IgG.

Data are reported as absolute values and as mean ± SD, and were analyzed by the unpaired two-tailed Student *t*-test. A probability value smaller than .05 was considered statistically significant.

Results

The mean serum concentration of IL-4 was significantly higher in the 10 patients than in the controls ($P = .01$) (Table 1). Serum levels of IL-2 and IFN-γ were undetectable. The mean tear concentration of IL-4 was 26.1 ± 23.8 pg/mL (Table 1). The mean level of IL-2R was also significantly higher in the patients (470.2 ± 174.1 U/mL) than in the controls (320.2 ± 136.8 U/mL) ($P = .05$) (Table 1). The mean

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Table 1. Total IgE, IgG, and Cytokines in Serum and Tears*

Patient	Sex	Age	Total IgE (U/mL)	Total IgG (pg/dL)	IL-2 (U/mL)	IL-2R (U/mL)	IFN-γ (U/mL)	IL-4 (pg/mL)	Tear IL-4 (pg/mL)
1	M	21	79	1450	1.4	300	<0.1	2.8	5.3
2	F	29	5300	1920	<0.8	262	<0.1	1.1	3.7
3	M	13	540	1230	<0.8	411	<0.1	3.3	31.1
4	M	13	660	1430	<0.8	541	<0.1	2.1	14.6
5	F	13	71	1420	<0.8	826	<0.1	3.0	42.7
6	F	19	700	1460	<0.8	452	<0.1	2.0	11.2
7	M	16	11000	1060	<0.8	636	<0.1	3.3	13.6
8	M	22	7800	1600	<0.8	406	<0.1	3.0	10.5
9	F	16	63	928	<0.8	310	<0.1	1.1	52.6
10	M	21	1100	1570	<0.8	558	<0.1	1.7	75.2
Mean		18.3	2731.3	1406.8		470.2		2.3	26.1
SD		5.1	3912.6	281.6		171.4		0.9	23.8
P-value			.05	.08		.05		.01	.01

*IL: interleukin, IFN: interferon.

P-value: compared to normal controls (n:10). IgE and IgG in control: 71.2 ± 67.6 U/mL and 1222.2 ± 150.6 pg/dL. IL-2R, IL-4 in serum, and IL-4 in tears in control: 320 ± 136.8 u/mL, 0.8 ± 0.4 pg/mL, and 0.9 ± 0.6 pg/mL (data of IL-4 in tears in control is from Fujishima et al.[13] The mean serum level of total IgE was elevated in 7 (70%) of the 10 patients. The IgG level was not elevated. Specific IgE antigens were present for housedust in 6 patients (60%), dust mites in 8 patients (80%), and cat in 6 patients (60%) (See Table 2). In the 10 controls, serum IgE and IgG were not present.

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May-Grünwald-Giemsa staining of the specimens obtained from the 10 controls showed very few inflammatory cells, and more than 99% were conjunctival epithelial cells. Patient samples showed lymphocytes, neutrophils, eosinophils, and basophils (Figure 1). HLA-DR expression was observed in 84.5 ± 15.9% of cells obtained from patients, compared with 45.3 ± 13.4% of cells obtained from controls ($P = .009$) (Figure 2).

Discussion

Cromolyn sodium was being used by patients at the time of sample collection. This drug can stabilize mast cells and may alter cytokine release, but the severity of the disease in these patients did not permit us to schedule a washout period. Although we do not know how this drug affected the results, we suppose that inflammation or cytokine release might have occurred to a greater extent if no drug treatment had been given.

The mean serum level of total IgE, but not IgG, was elevated, and 60–80% of patients were positive for dust mites and housedust antigen-specific IgE in the present study, suggesting that systemic allergic reaction in these patients was mediated by IgE. The level of total IgE was not elevated in 3 patients. Vir-

Table 2. Antigen-specific IgE (U/mL) Present in Vernal Keratoconjunctivitis Patients*

Patient	Antigen-specific IgE (U/mL)
1	HD, dust mites, egg white, soybean, RAG, sweet vernal, timothy, sugi pollen, Candida, Alternaria, cat, dog
2	Egg white, soybean, sweet vernal
3	HD, dust mites, RAG, Candida, cat, dog
4	HD, dust mites, egg white, soybean, RAG, mugwort, timothy, sugi pollen, Penicillium, Cladosporium, cat
5	HD, dust mites, soybean
6	Dust mites, egg white, Alternaria, dog
7	Mugwort, sweet vernal, timothy, sugi pollen, cat
8	HD, dust mites, cat
9	HD, dust mites, egg white, soybean, mugwort, cat
10	Dust mites, sweet vernal, Penicillium, Cladosporium, Aspergillus, cat, dog

*VKC: vernal keratoconjunctivitis, HD: house dust, RAG: ragweed mix 1, sugipollen: Japanese cryptomeria pollen.

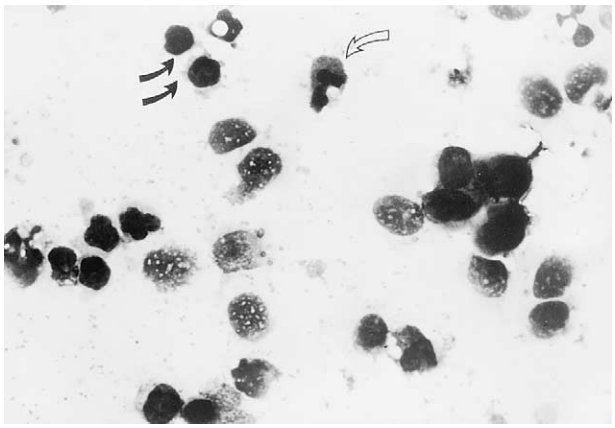

Figure 1. Brush cytology samples from superior palpebral conjunctiva of vernal keratoconjunctivitis patient; stained with May-Grünwald stain and Giemsa stain. Lymphocytes, neutrophils, and eosinophils were observed before treatment. The number of epithelial cells in patient samples was less than in control samples. Black arrows indicate lymphocytes. The white arrow indicates eosinophils.

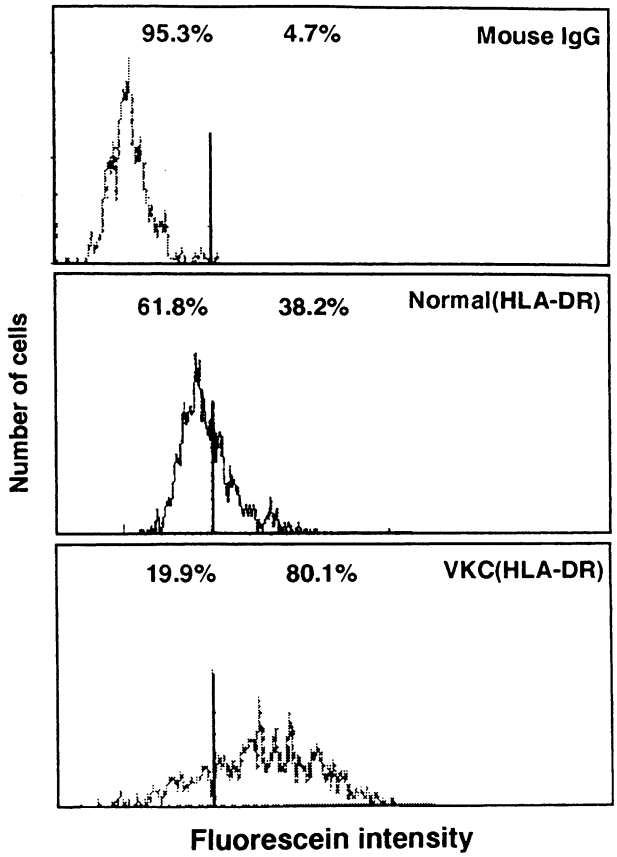

Figure 2. Analysis of human lymphocyte antigen (HLA)-DR molecule expression by FACScan® in cells obtained by brush cytology from a control subject and a representative patient. Upper panel: Indication of positive cells appears on the right-hand side of the vertical bar. Background control fluorescence reactivity was determined with only fluorescein isothiocyanate-labeled goat anti-mouse IgG. HLA-DR expression was less than 5%. Center panel: 38.4% of cells from control subject showed HLA-DR expression. Lower panel: 80.1% of cells from vernal keratoconjunctivitis patients showed HLA-DR expression.

tanen et al found no relationship between locally infiltrating Th-2-like cells and IgE response in patients with atopic dermatitis.[15] Serial studies in patients with VKC who are IgE-negative might clarify any relationship between Th-2-like cells and IgE.

Although serum levels of IL-2 and IFN-γ were undetectable in the present study, serum and tear concentrations of IL-4 were significantly higher in the patients than in the controls. Concentration of IL-4 in tears was significantly higher in the present 10 patients than in the healthy subjects observed in our previous study (0.9 ± 0.6 pg/mL) (P = .01).[13] The present study is the first to demonstrate the presence of IL-4 in the serum of patients with VKC. The recently developed sandwich ELISA is capable of detecting very low concentrations of protein.[13] Previous studies have shown that serum levels of IL-4 are elevated in patients with adult bronchial asthma[12] and in patients with atopic dermatitis, compared with values in controls. The present data in patients with VKC were consistent with the results of these previous studies. The elevated level of IL-4 suggests a preferential activation of Th-2 cells in the patients with VKC.

In the present study, patients with VKC exhibited an elevated serum IL-2R level as compared with the controls, indicating the presence of T- and B-cell activation. Matsumoto et al previously reported that the serum level of IL-2R was elevated in patients with bronchial asthma. The present findings suggest that VKC is a T-cell-activated systemic disease, not just an ocular disease. Also, these results imply that treatment with either cyclosporine or methylprednisolone may have reduced the inflammation, because the effect of treatment on the serum level of IL-2R may be variable.

Expression of HLA-DR was observed in brush cytology samples from both the patients and the controls. The mean expression of HLA-DR from controls was 45.3%. The slight DR expression on the conjunctival epithelial cells in controls may occur because the conjunctiva is always exposed to the environment and unknown antigens might stimulate the epithelial cells, or because the brush cytology process could affect the expression of HLA-DR. Although

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some of the factors were related to the HLA-DR expression in normal conjunctiva, the expression on the conjunctiva of patients in this study was significantly higher than normal. T cells regulate the expression of HLA-DR antigen on glandular epithelial cells.[16] The expression of HLA-DR is tightly regulated by such factors as IFN-γ, which up-regulates the antigen expression. Accordingly, the present findings suggest that the conjunctival cells have been stimulated by the cytokines that up-regulate the antigen. El-Asrar et al reported in an immunohistochemical study that the epithelial and stromal number of HLA-DR-positive cells in the conjunctival biopsy specimens obtained from patients with VKC decreased after topical CsA treatment.[17] This evidence might indicate that HLA-DR-positive cells are involved in the pathogenesis of VKC.

In our previous report, many inflammatory cells were detected in patient brush cytology samples that were not seen in control samples or which disappeared after therapy.[1] These inflammatory cells may be producing Th-2-like cytokines in the conjunctiva and serum, and also producing serum IgE and IL-2R in patients with VKC.[10] El-Asrar et al also reported that an immunohistochemical study showed that IgE-positive plasma cells and mast cells remained unaltered after topical CsA.[17] These results imply that VKC might not only be an IgE-mediated allergic disease but also a combination of local or systemic T-cell-related inflammatory diseases. The T-cell-related inflammation might be attacking patients with VKC. The suppression and/or removal of these activated cells by CsA or surgical treatment may thus be a useful treatment strategy in patients with VKC.

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