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Identification of Local Th2 and Th0 Lymphocytes in Vernal Conjunctivitis by Cytokine Flow Cytometry

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PURPOSE. Th2 lymphocytes may play a key role in the development of allergic diseases such as vernal keratoconjunctivitis (VKC). Cytokine flow cytometry of tear samples was used to identify the phenotypical and functional properties of lymphocytes at the actual site of the allergic reaction.

METHODS. Tear and blood samples were obtained from patients affected by active VKC ($n = 12$) and from normal control subjects ($n = 10$). Tears were obtained after gentle scraping of the tarsal and bulbar conjunctiva. Tear and blood samples were placed in a solution of brefeldin-A, phorbol myristate acetate (PMA), ionomycin, and RPMI for 4 hours and then processed for flow cytometry. Lymphocytes were marked with the monoclonal antibodies, anti-IFN- γ and anti-interleukin (IL)-4. Levels of IL-4, IL-2, IFN- γ , IL-2R, total IgE, eosinophil cationic protein (ECP), eosinophil protein X/neurotoxin (EPX), and myeloperoxidase (MPO) were also evaluated in serum.

RESULTS. Expression of IL-4 was observed in $9.2\% \pm 9.5\%$ of lymphocytes in tears of patients with VKC. Of the 12 patients with VKC, 8 (67%) had tear lymphocytes positive for IL-4 (Th2). Two patients (17%) had a double population of lymphocytes: One was positive for Th2, and the other was positive for both IL-4 and IFN- γ (Th0). One patient (8%) was positive for IFN- γ (Th1) only, and one patient was negative for both ILs. No differences in the

percentage of Th2 lymphocytes were found between tarsal and limbal patients. The percentage of Th2 lymphocytes was significantly correlated with the severity of the disease. No positive lymphocytes were found in tears of control subjects. Eosinophils, serum IgE, ECP, and EPX were all significantly higher in VKC than in control subjects.

CONCLUSIONS. In ocular allergic diseases, local lymphocytes expressed the Th2 phenotype and, to a lesser degree, the Th0 phenotype. Although results of systemic allergic markers can be inconclusive in patients with VKC, flow cytometry demonstrated a local lymphocyte phenotype that can account for the clinical and histologic abnormalities of VKC. (*Invest Ophthalmol Vis Sci*. 1999;40:3036-3040)

Vernal keratoconjunctivitis (VKC) is a severe, bilateral, recurrent inflammatory eye disease occurring mostly in children and young adults and histologically characterized by increased numbers of eosinophils, mast cells, and mononuclear cells, the latter mainly consisting of CD4⁺ T-helper (Th) lymphocytes.¹ CD4⁺ Th cells can be classified into distinct types by their cytokine profile.² Type 1 (Th1) cells, which produce interleukin (IL)-2, interferon gamma (IFN- γ) and tumor necrosis factor-beta (TNF- β), are known to promote delayed-type hypersensitivity. Type 2 (Th2) cells, which produce IL-3, IL-4, IL-5, IL-10, and IL-13, are thought to aid in humoral responses such as IgE isotype switching and mast cell and eosinophil growth and differentiation. In the absence of clear polarizing signals, CD4⁺ T cell subsets with a more heterogeneous profile of cytokine production than Th1 or Th2 are designated Th0. These Th0 subsets mediate intermediate effects, depending on the cytokines produced and the nature of responding cells. To date, the few studies of cytokine production by conjunctival Th cells have used immunoassays or mRNA analyses, neither of which provided consistent information about the production of different cytokines from individual cells. A prevalence of Th2 type clones has been shown in lymphocyte culture derived from the conjunctival biopsy specimens of a small group of patients with VKC.^{3,4}

In the present study, the expression of IFN- γ and IL-4 cytokines in fresh lymphocytes obtained from conjunctival scrapings and peripheral blood of patients with VKC was investigated using flow cytometry. Cytokine flow cytometry of conjunctival lymphocytes may closely reflect the actual cytokine production and thus the functional properties of these cells at the site of the inflammation, minimizing artifacts due to long-term culture. In addition to flow cytometry, serum cyto-

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kines and other systemic markers of allergic inflammation were considered and correlated with the clinical condition.

MATERIALS AND METHODS

Patients and Healthy Subjects

Tear and blood samples were obtained from patients affected by active VKC ($n = 12$; mean age, 12.6 ± 6 years), and normal healthy control subjects ($n = 10$; mean age, 9.7 ± 3.3 years). A group of 20 adult healthy subjects (mean age, 38.3 ± 10 years) was included as an additional control, from whom only blood samples were obtained and analyzed. Of the 12 patients with VKC (2 girls and 10 boys), 6 had the tarsal form of the disease and 6 the limbal form. All the patients were instructed to discontinue therapy for at least 5 days before the visit. For each patient, a clinical score (range, 0–4; 0, absent; 4, severe) was assigned to the four major symptoms (itching, tearing, photophobia, and foreign body sensation) and to the six major signs (conjunctival erythema and chemosis, discharge, papillae, limbal infiltrates, and corneal epithelial disease). The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained from all subjects for participation in the study.

Tears were collected after gentle scraping of the tarsal and bulbar conjunctiva. Two hundred to 350 μ l of tear fluid was collected using a capillary tube and placed in vials (Eppendorf, Fremont, CA) with 20 μ l RPMI 1640 (Sigma, St. Louis, MO). Tear cytology on precolored slides (Testsimplet; Boehringer Mannheim, Mannheim, Germany) and cell counting in the Burkert chamber were performed before flow cytometry.

Flow Cytometric Analysis

Samples of heparinized peripheral blood were processed according to the standard procedure of double or triple direct immunofluorescence. The following: fluorescein-isothiocyanate (FITC)-, phycoerythrin (Pe)- and peridinin-chlorophyll-protein (PerCP)- conjugated monoclonal antibodies (mAbs) to human lymphocyte cell-surface antigens were used: Leu-4-FITC (anti-CD3), Leu-3a-Pe/PerCP (anti-CD4), Leu-2a-Pe (anti-CD8), Leu-11c-Pe (anti-CD16), Leu-12-FITC (anti-CD19), Leu-19-Pe (anti-CD56), and anti-HLA-DR-Pe (all purchased from Becton Dickinson, Mountain View, CA) and T4/4B4-FITC/Pe (anti-CD4/CD29) and T4/2H4-FITC/Pe (anti-CD4/CD45RA; both from Coulter, Miami, FL). Data analysis was performed on a flow cytometer (FACSSca Immunocytometry System; Becton Dickinson) equipped with an argon laser emitting at 488 nm. Correlate analysis of forward scatter and right-angle scatter was used to establish a lymphocyte gate.

Intracellular cytokine staining (cytokine flow cytometry) was performed according to Prussin.⁵ Peripheral blood and tear samples were processed in the same manner. Each blood and tear sample of patients with VKC and normal subjects was divided into two aliquots. The activated aliquot was processed after stimulation with ionomycin and phorbol myristate acetate (PMA) in the presence of brefeldin-A (BFA), and the nonactivated aliquot was processed without this stimulus in the presence of BFA. Briefly, tears (100 μ l) were diluted with 400 μ l RPMI to obtain the same volume (500 μ l) for each sample. To 500 μ l of blood- and tear-activated samples was added 50 μ l BFA (final concentration, 10 μ g/ml; Sigma), 130 μ l PMA (final concentration, 25 ng/ml; Sigma), 100 μ l ionomycin (final con-

centration, 1 μ g/ml; Sigma), and 220 μ l RPMI. All samples were then incubated for 4 hours in CO₂ at 37°C. Blood samples from the additional control group were similarly processed but incubated for 4 and 8 hours. Isotype-matched control subjects were prepared with 450 μ l RPMI and 50 μ l BFA. Ten microliters of mAb anti-CD4 conjugated with PerCP was added to all samples and incubated for an additional 20 minutes. After washing, samples were fixed (100 μ l of component A, Fix and Perm; Caltag, South San Francisco, CA) and incubated for 15 minutes at room temperature. After two washings, 100 μ l of fixative (component B; Fix and Perm; Caltag), 20 μ l of mAb anti-Hu-IFN- γ -FITC (IgG_{2b}), and 20 μ l of anti-Hu-IL-4-Pe (IgG₁) were added. Isotype-matched control subjects were prepared with IgG₁-Pe and IgG_{2b}-FITC at the same concentration as the anti-cytokine mAb. After a 30-minute incubation in the dark, samples were washed and then fixed with 500 μ l of 1% paraformaldehyde.

To ensure the specificity of the staining procedure, each sample had a control in which the specific binding of the anti-IL4 and anti-IFN- γ mAbs was blocked with a molar excess of recombinant cytokine (IL-4 and IFN- γ ; PharMingen, San Diego, CA). Samples were analyzed on the flow cytometer. Because lymphocytes had not been separated from other leukocytes and epithelial cells in the tear samples, 30,000 events were acquired, reflecting the total number of nonspecific cells in the sample and not the number of CD4 cells. The gating, however, was on only CD4-positive lymphocytes. Three-color dot plots were generated by plotting IL-4 versus IFN- γ fluorescence after gating to exclude dead and/or contaminating non-CD4⁺ lymphocytes from the analysis. Results are expressed as the percentage of cytokine-producing cells within the CD4⁺ population. One-parameter histograms demonstrating cytokine staining were created by commercial software (Lysis II; Becton Dickinson, San José, CA), and set markers statistics were performed on the basis of the staining of isotype-matched control subjects.

Cytokine and Mediator Assay

In patients with VKC and control subjects, serum and tear levels of IL-4 (by enzyme-linked immunosorbent assay [ELISA]; Endogen, Woburn, MA), serum levels of IFN- γ (Immuno Radio-metric Assay [IRMA]; Biosource-Europe, Fleurus, Belgium), serum levels of IL-2 (ELISA), and IL-2R (by chemiluminescence; Immunolite-ILR2; Euro/DPC, Llanberis, UK) were measured according to their respective standard protocols. The lower detection assay limit for IFN- γ was 1 U/ml; for IL-4, 2 pg/ml; for IL-2, 6 pg/ml; and for IL-2R, 50 U/ml. In serum, the following tests were also performed: total IgE (fluoroenzyme immunoassay [FEIA]; Pharmacia, Uppsala, Sweden) eosinophil cationic protein (ECP), eosinophil protein X/neurotoxin (EPX), and myeloperoxidase (MPO; by radioimmunoassay; Pharmacia).

Statistics

Data from the VKC and control groups were compared using the Mann-Whitney test. The Spearman correlation was used to correlate different parameters with the severity of the clinical disease. $P < 0.05$ was considered significant. All data are expressed as mean $\pm$ SD.

RESULTS

Mean total cell number was 88,500 (range, 15,500–180,000) in VKC tear samples and 18,800 (range, 7,500–26,500) in normal samples. The percentage of CD4⁺ T cells was $2.9\% \pm 1.6\%$ in VKC tear samples and $2.5\% \pm 1.0\%$ in normal samples. In VKC tears, the expression of IL-4 within the CD4⁺ T-cell population was identified by flow cytometry in $9.2\% \pm 9.5\%$ of lymphocytes (Table 1). Only activated aliquots were positive for intracellular cytokine staining (Fig. 1). Of the 12 patients with VKC, 8 (67%) had tear lymphocytes positive for only IL-4 (Th2); two (17%) had a double population of lymphocytes (one positive to IL-4 and the other to both IL-4 and IFN- γ [Th0]); one (8%) was positive only to IFN- γ (Th1); and one was negative to both ILs. In one tear sample from the normal subject group, a small percentage of Th1 lymphocytes was found. The percentage of IL-4-positive lymphocytes was significantly increased in patients with VKC compared with normal samples (9.2% versus 0% ; $P = 0.001$).

No difference in the percentage of Th2 lymphocytes was found between those with the tarsal and those with the limbal form of the disease. The percentage of Th2 lymphocytes was correlated with the total clinical score of the disease ($P < 0.001$) and with the degree of corneal involvement ($P < 0.05$). In five of the six patients with VKC who were negative for specific serum IgE, local Th2 cells were identified. Cytokine flow cytometry in peripheral blood samples was negative except in one of the normal subjects, in whom 3.9% of lymphocytes were positive for IFN- γ . In the additional control group, in which blood samples were activated for 4 and 8 hours, a lower expression of IL-4 and more IFN- γ -positive cells were detected at 8 hours' than at 4 hours' incubation time (IL-4, $0.7\% \pm 0\%$ versus 0% ; IFN- γ , $24.7\% \pm 6.6\%$ versus $2.1\% \pm 3\%$, respectively).

Because of the limited quantity of tear samples, it was possible to measure levels of tear IL-4 only in six patients with VKC and in five normal subjects. Results showed that this cytokine was found only in one patient with VKC (5 pg/ml).

The number of eosinophils and the levels of serum IgE, ECP, and EPX were all significantly increased in patients with VKC compared with control subjects (Table 2). However,

these values were not correlated with the severity of the ocular allergic disease expressed by the total score of signs and symptoms. No differences between VKC and control subjects were found in the percentage of peripheral blood lymphocytes CD3⁺, CD4⁺, CD8⁺, and CD16⁺/CD56⁺ (natural killer cells). A significantly increased percentage of HLA-DR⁺ lymphocytes and CD19⁺ lymphocytes was observed in VKC samples (Table 2). CD4⁺/CD29⁺ lymphocytes were also significantly increased in VKC compared with control subjects ($15.6\% \pm 4.5\%$ versus $10.6\% \pm 2.8\%$; $P = 0.002$), whereas CD4⁺/CD45RA⁺ cells were reduced ($14.5\% \pm 5\%$ versus $20.5\% \pm 4.1\%$; $P = 0.01$). None of these values was correlated with the severity of the disease. Only serum levels of sIL-2R were increased in patients with VKC compared with control subjects (Table 2).

After differentiation of tarsal versus limbal VKC, the only statistically significant systemic parameter was the higher number of peripheral blood eosinophils in tarsal ($731 \pm 277 \times 10^6/l$) than in limbal VKC ($316 \pm 234 \times 10^6/l$; $P = 0.02$).

DISCUSSION

A prevalent Th2 response seems to be involved in the pathophysiology of atopic diseases. Among the first descriptions of the cloning of CD4⁺ cells from allergic tissues, Maggi et al.⁵ obtained from conjunctival specimens of three patients with VKC T-cell clones that produced mostly IL-4 and little IFN- γ and that supported IgE synthesis in vitro. IL-4 has also been found to be increased in tears from patients with VKC—however, with no evidence of the cell source.⁶ An increased expression of IL-3, IL-4, and IL-5 mRNA has also been shown in VKC conjunctival tissues using in situ hybridization histochemistry.⁷

Cytokine flow cytometry is a single-cell technique in which individual cells are analyzed as they pass through the laser in single file.⁸ In the present study, this technique was applied to conjunctival-derived lymphocytes freshly collected from a relatively diverse group of patients with VKC. The goal was to identify the functional properties of effector Th cells in vivo by evaluating fresh ex vivo cytokine production at the single-cell level, without the interference of possible in vitro

TABLE 1. Characteristics of Patients with VKC: Tear Cytology (Light Microscopy), Percentage of CD4⁺, Th1, Th2, and Th0 in Tears (Flow Cytometry)

Patient	Sex/ Age	Clinical Form	Previous/ Associated Atopic Diseases	Ocular Clinical Score	Total Tear Cell Number $\times 10^5$	Eosinophil Tear Cytology (%)	Lymphocyte Tear Cytology (%)	Tear CD4 ⁺ (%)	Tear Th1 (% CD4)	Tear Th2 (% CD4)	Tear Th0 (% CD4)
1	F/8	T	E	35	50	34	25	3.8	0	5.7	0
2	M/7	T	—	17	80	62	10	1.7	0	0	0
3	M/20	T	E	21	15.5	5	37	5.5	0	25	0
4	M/10	T	As	18	40	25	12	2	0	8	2
5	M/20	T	E	25	178	50	5	1.9	0	12.3	1.7
6	M/7	T	—	38	19	55	16	2.2	0	16.7	3.8
7	M/7	L	—	24	139	80	9	1.5	0	8.5	0
8	M/7	L	—	8	100	57	7	1.8	2.3	0	0
9	M/24	L	E, R	21	120	80	8	2.5	0	3.6	0
10	M/7	L	—	14	63	8	24	3	0	1.4	0
11	M/6	L	—	17	180	20	7	2.4	0	2.1	0
12	F/7	L	—	26	78	55	17	7	0	28	0

T, tarsal; L, limbal; E, eczema; As, asthma; R, rhinitis.

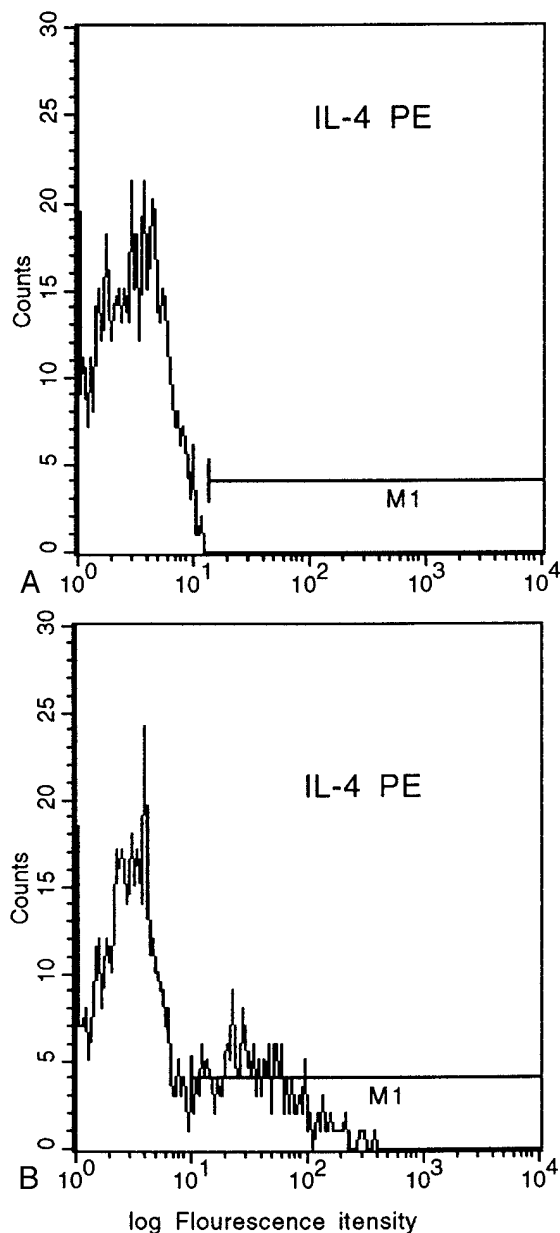


FIGURE 1. Intracellular cytokine-specific staining in VKC tears. Tear samples were stimulated for 4 hours with PMA and BFA. PE-labeled anti-IL-4 antibody and anti-CD4 PerCp were then incubated with either molar excess of the corresponding recombinant cytokine (IL-4) (A) or an equivalent volume of phosphate-buffered saline (B) before staining. Single-color histograms were generated by gating on CD4-positive cells.

artifacts of a cloning procedure. Only after a short polyclonal stimulation, 0% to 28% of the tear CD4⁺ lymphocytes of patients with VKC were capable of producing IL-4 and/or IFN- γ . The specificity of staining was demonstrated by the positive intracellular staining in aliquots activated with PMA and BFA compared with those to which a molar excess of recombinant cytokine was added. Conversely, this method did not show cytokine expression in T cells from the peripheral blood of young patients with VKC and age-matched control subjects. However, a higher expression of intracellular cytokines was

TABLE 2. Cell Counts, Mediators, and Cytokines in Peripheral Blood of Patients with VKC and Controls

	Total IgE (kU/l)	Eosinophils ($\times 10^9/l$)	Lymphocytes ($\times 10^9/l$)	CD4 ⁺ (%)	CD19 ⁺ (%)	HLA-DR ⁺ (%)	ECP ($\mu g/l$)	EPX ($\mu g/l$)	MPO ($\mu g/l$)	IL-4 (pg/ml)	IFN- γ (U/ml)	IL-2 (pg/ml)	IL-2R (U/ml)
VKC (<i>n</i> = 12)	213 $\pm$ 176	0.54 $\pm$ 0.3	2.2 $\pm$ 0.5	38.6 $\pm$ 7	15.3 $\pm$ 3	20.4 $\pm$ 5	36 $\pm$ 27	76 $\pm$ 63	280 $\pm$ 151	<2	1.2 $\pm$ 0.4	2.5 $\pm$ 5	824 $\pm$ 325
Control (<i>n</i> = 10)	33 $\pm$ 22	0.07 $\pm$ 0.05	2.5 $\pm$ 1.2	43.3 $\pm$ 5	11.8 $\pm$ 2	14.6 $\pm$ 1	7.8 $\pm$ 3	16 $\pm$ 3	189 $\pm$ 130	<2	1.3 $\pm$ 0.4	<6	425 $\pm$ 154
<i>P</i>	0.0028	0.0002	NS	NS	0.01	0.003	0.002	0.003	NS	NS	NS	NS	0.003

Data are expressed as mean $\pm$ SD. NS, not significant.

detected in peripheral blood of adult control subjects when a longer incubation time was used. These latter findings are in agreement with previous studies of peripheral blood cells in which different cloning procedures were implemented, cultured lymphocytes were selected, or longer or different cell stimulations were used,⁸⁻¹⁰ showing that data are influenced by varying methods. However, the great difference between local findings in VKC and normal subjects, and between findings in tears and in peripheral blood in VKC after a short stimulation, may better reflect the physiologic situation of already-activated lymphocytes only at the target organ.

Although 68% of patients with VKC had local Th2 cells, only 17% had both Th2 and Th0 cells. It was notable that most of the patients negative to prick tests and/or serum-specific IgE had local IL-4-positive T-cells. The presence of Th2 cells correlated significantly with the clinical severity of the disease, demonstrating that actively producing effector T-cells play a proinflammatory role in VKC. Whether infiltrating T cells in the inflamed conjunctiva can recognize environmental allergens and thus contribute to the development of a clinical inflammation and to serum and local levels of IgE is still unknown. Several exogenous stimuli, such as aeroallergens and other specific or nonspecific stimuli, may act together in a genetically predisposed host, resulting in a preferential Th2 response. The Th0 cells identified in some patients with VKC may be either a precursor to polarized Th1 or Th2 phenotypes or a stable, differentiated population that is present in conditions in which environmental allergens and antigens induce both cell-mediated and humoral immunity.⁹ The presence of IFN- γ , although in a small percentage of cases, may explain why some patients are negative for local IgE, because this cytokine exerts a negative regulatory influence on IL-4-driven IgE synthesis. This finding supports the hypothesis that a delayed-type hypersensitivity reaction may coexist with immediate-type reactions in VKC.

Alterations of other systemic parameters considered in the present study involved a systemic B-cell activation and polyclonal IgE production, although no Th2 cells were found in peripheral blood. The observed increase in IL-2R serum levels may be considered as a nonspecific marker of T-cell activation. The numbers of peripheral blood eosinophils and eosinophil activation markers were higher in VKC than in control samples, confirming that VKC is a systemic disorder that has an almost exclusive target site in conjunctival tissue.

Th2-derived cytokines may be the effectors of many of the clinical and histologic aspects of VKC. IL-3 and IL-5 are mast cell and eosinophil differentiation factors and may be responsible for the high number of conjunctival mast cells and eosin-

ophils seen in VKC. An excess of either IL-4 or IL-13, which are essential for IgE production, may result in the high levels of IgE that can be found only in tears. However, other proinflammatory cytokines and abnormalities have been found in VKC¹¹, the pathogenesis of which remains far from clear.

Although results of the present study were limited by the small amount of samples, cytokine flow cytometry clearly demonstrated active Th cells from a mixed population of inflammatory cells freshly derived from the site of the reaction. With this technique, the cytokine production of a small T-cell subpopulation was identified without prior cell purification. These results provide further support that Th2- and Th0-type responses predominate in the conjunctival mucosa of patients with VKC.

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