Natural killer cells in vernal keratoconjunctivitis

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Purpose: Recent studies suggest that natural killer (NK) cells exert effector/regulatory properties on both innate and adaptive responses via release of different cytokines. While some information indicates NK cells in allergic asthma and atopic dermatitis, no data are available for allergic conjunctivitis. The aim of this study was to evaluate NK in the blood and the conjunctiva of patients with vernal keratoconjunctivitis (VKC).

Methods: Six patients with active VKC and six healthy subjects were included in the study. Blood samples and conjunctival biopsies were taken from each patient. NK cells in blood and conjunctiva were quantified by flow cytometry and immunohistochemistry, respectively. Clinical findings of the patients were recorded, conjunctival immune infiltrates were characterized, and both parameters were correlated to NK cell number.

Results: Compared to healthy subjects, NK cells were significantly decreased in the blood and increased in the conjunctiva of patients with VKC.

Conclusions: Together with lymphocytes, eosinophils, and mast cells, NK cells constitute a significant proportion of the immune cells infiltrating VKC conjunctiva. This finding indicates a potential role of NK and innate immunity in the regulation of allergic reactions and in diseases such as VKC. New therapeutic alternatives for modulating allergic inflammation might target NK cells.

Vernal keratoconjunctivitis (VKC) is a chronic bilateral inflammation of the conjunctiva characterized by hyperemia, chemosis, photophobia, and mucous discharge. The hallmark of the disease is the presence of giant papillae on the upper tarsal conjunctiva or limbus [1]. Originally classified as an allergic conjunctivitis-type disease, VKC was considered for many years to be the expression of a classical type I immunoglobulin-E (IgE)-mediated hypersensitivity reaction of the conjunctiva, according to the Coombs and Gell definition [2]. Recent studies have demonstrated that only 50% of patients with VKC are sensitive to the skin/Radio-Allergo-Sorbent Test (skin/RAST) [3], and that a more complex IgE-independent pathogenic mechanism is involved with the contribution of many cells and mediators detected in the serum, tears, and conjunctiva of patients with VKC [4]. Conjunctival allergen challenge and biochemical functional studies indicate a Th2-driven mechanism [5,6] for VKC, and a definition, similar to that of asthma, of “an allergic inflammatory disease with mast cells, eosinophils and lymphocytes” [7]. This traditional view of the allergic reaction has been recently revisited, and an adjuvant role of innate immunity in allergic inflammation has been proposed. Epithelial cells, dendritic cells (prototypical antigen presenting cells), natural killer (NK) cells, as well as mast cells/basophils, through expression of various membrane receptors, particularly toll-like receptors (TLR), might also participate in the innate immunity response by playing a regulatory role in allergy [8-10]. In line with this hypothesis, TLRs have been shown to be differentially expressed in the conjunctiva of patients with VKC when compared to healthy subjects [11].

NK cells are granular lymphocytes that play a crucial role in innate immunity due to their cytotoxic ability to destroy virus-infected cells and certain tumor cell targets without prior stimulation [12]. Recent data suggest that a subpopulation of NK cells (NK-T) are important regulators of both innate and acquired response through the release of several immunoregulatory mediators (including TNFα, TNFβ, TGFβ, GM-CSF, MIP-1α, IL-1, IL-2, IL-3, IL-4, IL-5, IL-8, IL-10 and IL-13) [13-16]. Indeed, NK cells can be triggered by specific T-cell receptor activation to secrete cytokines that promote Th2 responses instead of Th1 responses, suggesting that NK cells might play a role in regulating the balance between Th1/Th2 lymphocytes [15,16].

In the present study, we sought to compare NK cells in the peripheral blood and in the conjunctiva of VKC patients with those of healthy subjects.

METHODS

Patients and tissue handling: The study, performed in spring, involved six patients with active VKC (males between 7-35 years old) and six healthy subjects with no history of atopic...
ocular/systemic diseases (males between 24-26 years old). VKC diagnosis was based on the presence of recurrent symptoms of itching, photophobia, and tearing in early spring. The symptoms were associated with a mild to severe cobblestone-like appearance in the upper tarsal conjunctiva, mucous discharge, epithelial keratopathy, and at least two eosinophils per optic field (x100/oil immersion) in the conjunctival scraping. Two out of six patients showed sensitization to Dermatophagoides pteronissinus with a skin prick test. Symptoms (itching, tearing, and photophobia) and signs (tarsal and bulbar papillae, hyperemia, secretion, and corneal epithelial involvement) were recorded and graded on a scale of 0-3 (0, absent; 1, mild; 2, mild; and 3, severe). A total symptom score (from 0-9) and a total sign score (from 0-15) were calculated. Table 1 summarizes the characteristics of the patients included in the study. One physician (A.L.) carried out all ocular examinations by slit lamp biomicroscopy. All patients were without treatment for at least one week prior to the study. Blood samples and conjunctival biopsies were collected from both VKC and healthy subjects and flow cytometry and immunohistochemistry were performed to identify circulating and conjunctival immune cells. Conjunctival biopsy from healthy subjects was performed at the time of surgery, for strabismus.

The study adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from both VKC and healthy subjects, and institutional human experimentation committee approval was granted.

**Flow cytometry:** For specific staining, 150 µl of peripheral blood were pretreated with NH4CL-EDTA for 10 min at room temperature to lyse erythrocytes It was then incubated for 30 min at 4°C with CD3-FITC, CD16-PE, or CD56-PerCP conjugated monoclonal antibodies, according to the manufacturer’s instructions (Simultest CD3/CD16+CD56; Code 340042; Becton Dickinson Biosciences, San Jose, CA).

All samples were analyzed through a FACScan flow cytometer (Becton Dickinson) equipped with an argon laser emitting at 488 nm. Forward and side scatter signals were collected as linear signals, and all emissions were collected on a four-decade logarithmic scale. FITC, PE, and PerCP signals were measured, respectively at 530 nm, 575 nm and 670 nm. Spectral overlap was minimized by electronic compensation with Calibrite beads (Becton Dickinson) before each series. CellQuest® software (Becton Dickinson) was used to acquire and evaluate 5,000 events with preserved side scatter signals and high membrane staining for specific antibodies.

**Histological and immunohistochemical evaluations:** Conjunctival specimens were post-fixed in 4% p-formaldehyde-100 mM phosphate-buffered saline (PBS) and cut in 3 µm sections (HM 325 Microm; BioOptica, Milan, Italy). Sections were either processed for basic histology (hematoxylin and eosin; BioOptica), immunohistochemistry, or immunofluorescence. Briefly, sections were stained with monoclonal antihuman CD4 (1/50; Dako Corp, Carpenteria, CA), EG2 (1/50; Peninsula, Upsala, Sweden), AA1 (1/200; R&D Systems, Minneapolis, MN), and CD56 (undiluted clone T199; BD) antibodies and developed using ABC-peroxidase (Vectastain Elite ABC, Vector Laboratories, Inc., Burlingame, CA) or with the appropriate fluorescent technique. As a negative control, sections were stained with a nonspecific isotype antibody, T-helper lymphocytes (CD4+), activated eosinophils (EG2+), mast cells (AA1+), and NK cells (CD56+) were counted under light microscopy, in a blind-fashion, in three optic fields (40X magnification) for each of three randomly selected areas. Arko32 software (version 10.06.06) was used for image analysis. Data are shown as means±SEM.

For fluorescent analysis, specific binding of the primary antibody was detected using a cy2-conjugated donkey antimouse-antirabbit-antigot antibody (Jackson Laboratory, West Grove, PA), and evaluated at 60X magnification in an oil immersion (E2000U confocal microscope; Nikon, Tokyo, Japan). Brightness and contrast level were evaluated using C1 software (Nikon), and the images were transferred to the Adobe Photoshop 7.0 program (Adobe Systems Inc., San Jose, CA). To detect nonspecific binding, we performed control immunofluorescent staining by substituting the primary antibody with control isotype IgG (data not shown).

**Statistical evaluation:** The nonparametric Mann-Whitney test (StatView II for PC; Abacus Concepts Inc., Barkley, CA) was used for statistical comparison of the number of circulating and conjunctival immune cells in patients with VKC versus healthy subjects. The Spearman rho test was employed to correlate the number of circulating and conjunctival NK cells to signs, symptoms, and IgE serum levels and to the number of

| Table 1. Characteristics of patients with vernal keratoconjunctivitis |
|-------------------------|------------------|
| **Age (yr)** | **VKC patients** |
| Range | 7-35 |
| Mean | 18±11 |
| **Gender (n)** | Male |
| | 6 |
| **Atopic associated condition (n)** | Asthma | 1 |
| | Rhinitis | 1 |
| **VKC course (n)** | Seasonal | 3 |
| | Perennial | 3 |
| **VKC form (n)** | Limbal | 0 |
| | Tarsal | 5 |
| | Mixed | 1 |
| **Total symptoms score (mean±SD)** | 3.5±1.4 |
| **Total signs score (mean±SD)** | 6.1±1.5 |

This table summarizes the characteristics of the patients included in the study.
of circulating and conjunctival eosinophils. Data are represented in graphs as mean±SEM, and p values less than 0.05 were considered statistically significant.

RESULTS
Circulating NK cells (CD56+ and CD16+) were decreased in patients with VKC compared to healthy subjects. The decrease in NK cells was significant when considering NK cell number (Figure 1A; VKC: 744±401 versus healthy subjects: 1554±649; p<0.05) as well as percent NK cells (CD56+ and CD16+ cells/CD3+ cells; VKC: 5.6%±1.9% versus healthy subjects: 10.9%±5%; Figure 1B; p<0.05).

Immunohistochemistry of the conjunctiva showed a significant increase of CD4+ T-helper lymphocytes (VKC: 56.1±17.5/optic field versus control: 5.6±2.6/optic field; p<0.01), activated eosinophils (VKC: 30.9±8.3/optic field versus control: 0±0/optic field; p<0.01) and AA1+ mast cells (VKC: 41.3±11.6/optic field versus control: 2.2±2.5/optic field; p<0.01) in patients with VKC compared to healthy subjects (Figure 2).

CD56+-bearing NK cells were detected in all conjunctiva of patients with VKC and in 75% (4/6) of healthy subjects. Statistical analysis showed a significant (p<0.01) increase in the number of CD56+ cells infiltrating the conjunctiva of patients with VKC (24±10.5 cells/optic field) when compared to healthy subjects (5.9±2.6 cells/optic field; Figure 3).

No significant correlations were observed between the number of circulating or conjunctival NK cells and the signs and symptoms scores or plasma IgE levels.

DISCUSSION
This report demonstrates that, relative to healthy normal subjects, patients with VKC have a decreased number of circulating NK cells and an increased number of NK cells together with Th lymphocytes, activated eosinophils, and mast cells in the conjunctiva.

NK cells play a crucial role in innate immunity, having the ability to directly lyse virus-infected cells [12]. Aside from their cytotoxic activity, NK cells can produce and release high amounts of both Th1 (IFNγ) and Th2 (IL4, IL5, and IL13) cytokines, enabling these cells to also regulate acquired immune response [13-16]. A new concept proposes classifying NK cells by their predominant Th1 (NK-1) or Th2 (NK-2) cytokine pattern [15]. Both subsets might inhibit or stimulate aspects of the allergic and normal immune response by specific patterns of cytokine release [17]. Recently, it was proposed that NK cells play a crucial role in the pathogenesis of allergic diseases by altering the balance between Th1/Th2 lymphocytes. NK cells might be triggered to secrete cytokines (IL-4, IL-5, and IL-13) that promote Th2 rather than Th1 responses [15,16]. In an animal model of allergic asthma, Schuster and coworkers detected increased numbers of NK cells along with CD4 and CD8 expressing T cells in both bronchoalveolar lavage fluid (BALF) and lung parenchyma [18]. In a murine model of allergic peritonitis, NK-derived IL-5 contributed to the observed eosinophil infiltration [19]. Furthermore, NK-specific depletion inhibited allergen-induced pulmonary eosinophil and CD3-T cell infiltration, and blunted the increase of IL-4, IL-5, and IL-12 in BALF [20]. Allergen-specific immunotherapy in humans was also shown to decrease NK cell activity [21]. All these data indicate a key role of NK cells in the control of allergic disease. The present findings of decreased circulating NK in VKC patients appear at first to

Figure 1. Circulating natural killer cells in patients with vernal keratoconjunctivitis compared to healthy subjects. A: Flow cytometry demonstrated a significant decrease of circulating natural killer cells (cells/µl) in vernal keratoconjunctivitis (VKC) patients compared to healthy subjects (p<0.05). B: The percent natural killer cell decrease was significant (CD56+ and CD16+ cells/CD3+ cells; p<0.05).
diverge from the hypothesis that NK is an important regulator of the allergic reaction. However, similar decreases in circulating NK cells have been described in atopic diseases such as asthma and atopic dermatitis [22-24]. In these prior studies, the decrease of circulating NK cells was shown to not be due to any intrinsic defect in the ability of NK cells to proliferate, but merely reflected accumulation of these cells in the site of inflammation [22]. In line with this hypothesis, we demonstrated a significant increase of NK cells infiltrating the conjunctiva in inflamed VKC tissues. The number of NK cells in VKC conjunctiva was similar to that of eosinophils—cells long considered to be crucial players in the pathogenesis of VKC. This finding indicates that NK cells may be involved in the pathophysiology of VKC, modulating allergic inflammation through the release of cytokines that influence the balance between Th1 and Th2 responses and the resulting conjunctival eosinophil infiltration.

Surprisingly, no significant correlation was observed between the number of circulating or conjunctival NK cells and the clinical signs and symptoms of VKC. This lack of correlation might indicate an all or none relationship with VKC that is unaffected by changes in the severity signs and symptoms. Conversely, it might simply reflect the low sample size of VKC.

Figure 2. Evaluation of inflammatory cells in the conjunctiva of patients with vernal keratoconjunctivitis. The graph (A) illustrates a significant increase of T-helper lymphocytes (CD4+/green; B), activated eosinophils (EG2+/green; C), and mast cells (AA1+/green; D) in vernal keratoconjunctivitis (VKC) conjunctiva compared to healthy subjects (E, F, G, respectively). No specific binding was detected when sections were incubated with isotype IgG (data not shown; 40X magnification).
Figure 3. Immunohistochemical evaluation of natural killer cells in vernal keratoconjunctivitis conjunctiva. Graph A illustrates the significant increase of natural killer cells in the conjunctiva of patients with vernal keratoconjunctivitis (VKC) compared to healthy subjects. Natural killer cells were identified by CD56 expression quantified by immunohistochemistry (B=VKC and C=healthy subjects) and confocal analysis (D=VKC and E=healthy subjects). No specific immune staining was observed in the presence of isotype IgG (F=control staining; 40X magnification).
patients. A more extensive study including a larger population is required to confirm these initial findings. Nonetheless, this study indicates a potential role of NK cells in VKC pathophysiology as well as a link between innate and specific immunity in allergic diseases. These novel findings appear of clinical relevance since a deeper understanding of the role of NK in VKC might lead to new pharmacological strategies focused on selective modulation of Th1 and Th2 polarization of NK cells, using factors such as IL-7, IL-15, and α-galactosylceramide [25,26].

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REFERENCES