Characterization of Cytokine mRNA Transcripts in Conjunctival Cells in Patients With Allergic Conjunctivitis

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Purpose. The host response to allergens appears to be regulated by specific patterns of local cytokine production. More than 20,000 conjunctival superficial cells were collected with a special brush, a smaller version of the Cytobrush used in cervical cytology, from the upper palpebral conjunctiva.

Methods. Samples were obtained by cytology brush from seven patients with allergic conjunctivitis and from seven healthy volunteers. Giemsa staining, immunocytochemistry, and flow cytometric analysis were performed. Cytokine gene expression was assayed by the reverse-transcription–polymerase chain reaction method.

Results. Giemsa staining of cytocentrifuged preparations from patients with allergic conjunctivitis showed conjunctival epithelial cells with lymphocytes, mast cells, and eosinophils. In an immunohistochemical study, a few CD3- and CD4-bearing cells, but not CD20- and CD14-bearing cells, were seen in patients. In 82.6 ± 17% of the samples obtained from allergic patients, HLA-DR was present, but it was present in only 34.2 ± 17.8% of samples from control subjects (P = 0.0001) using flow cytometric analysis. Steady state transcripts of mRNA for cytokines were analyzed with RT-PCR in conjunctival cell samples, and results showed that samples from allergic conjunctivitis expressed increased transcripts of interleukin 4 and interleukin 13 but virtually no interleukin 2 or interferon-γ; six samples from seven healthy subjects expressed no interleukin 2, interleukin 4, interleukin 13, or interferon-γ transcripts.

Conclusions. These results suggest that the clinical features of allergic conjunctivitis in humans are associated with a specific local pattern of proinflammatory cytokine expression. Invest Ophthalmol Vis Sci. 1997;38:1350-1357.

Cytokines have been detected in conjunctival cells by immunohistochemical analysis, and Th2-like T helper cells are present in the conjunctiva.6 Mast cell lines produce lymphokines in response to cross-linkage of FceRI.7 Interleukin 4 (IL-4) stimulates B cells to class-switching plasma cells that produce immunoglobulin E (IgE).8-10 Allergen and IgE combine to stimulate mast cells to secrete histamine and other inflammatory mediators.11 Results reported in previous studies have shown the presence of mast cells in conjunctival samples12 and IgE in tears,13-17 and we have demonstrated increased levels of IL-4 in tears by enzyme-linked immunosorbent assay (ELISA).18 Histamine and IL-4 are increased in cultured supernatants of samples in allergic conjunctivitis obtained by brush cytology.19 Studies of lymphocyte signaling in mice indicate a complex series of regulatory interactions between lymphocyte subsets. In mice, clones of CD4-bearing T cells show divergence into Th1- and Th2-cell subpopulations.1 The Th1 cells produce the

Allergic conjunctivitis is one of the most common ocular surface diseases, but the pathogenesis is unknown. Different patterns of cytokine production are characteristic of certain subgroups of T helper cells, termed Th1 and Th2, the former mediating delayed-type hypersensitivity and latter mediating IgE synthesis and immediate-type hypersensitivity reactions.1 Two Th2 cells have been studied in other allergic diseases but have not been reported in allergic conjunctivitis.3,4
cytokines IL-2 and interferon (IFN)-γ that are associated with delayed-type hypersensitivity reactions. The Th2 cells produce the interleukins IL-4, IL-5, and IL-13 that stimulate humoral responses. In humans, the divergence of CD4-bearing T cells into Th1 and Th2 subsets has been less clear. However, certain infections and allergic diseases have shown a predominance of Th1- or Th2-like activity, respectively.2 However, relatively little is known about the functional activity of CD4-bearing T cells at the actual site of allergic conjunctivitis. Therefore, to establish the involvement of these cytokines in allergic disease, we conducted the present study to further examine expression of Th2-like cytokines in conjunctival cytologic samples from patients with allergic conjunctivitis. In addition, we assayed the T-cell infiltration within these samples, and their HLA-DR (an activation marker) expression, which is also thought to be of importance in allergic tissue responses.

SUBJECTS AND METHODS
Patients and Healthy Subjects
Seven conjunctival cytology brush samples were obtained from seven patients with allergic conjunctivitis (seasonal allergic conjunctivitis),29 three men and four women (aged 14 to 42; mean age ± SD of 28.9 ± 7.1 years), who visited the Department of Ophthalmology, Tokyo Dental College of Chiba, Japan, in March and April 1995. Allergic conjunctivitis was diagnosed based on history including symptoms of ocular itching, tearing, redness, or ocular pain with supportive slit-lamp examinations showing hyperemia or papilla formation of the palpebral conjunctiva and filamentous discharge, or the finding of cedar pollen—(Cryptomeria japonica) specific serum IgE antibody by the multiple antigen simultaneous test 16 (MAST 16) (SRL, Tokyo, Japan).21 Seven conjunctival cell samples were obtained from seven age- and sex-matched healthy subjects (aged 22 to 52; mean age 28.2 ± 3.3 years) to serve as control samples. Healthy subjects had no symptoms or signs of allergic conjunctivitis and were negative for serum antigen–specific IgE antibody, according to results of the MAST 16 test.19,20

Conjugated sheep antihuman IgG, IgM, and IgA were obtained from Becton-Dickinson (Mountain View, CA) and anticytokeratin AE-3 was obtained from Boehringer Mannheim Biochemica (Indianapolis, IN) and was used as a conjunctival epithelial cell marker. Biotin–avidin immunoperoxidase staining was performed on acetone-fixed slides of samples. They were first stained with mAb, then with biotin-labeled goat antimouse Ig (Tago, Burlingame, CA), and finally with avidin–horseradish peroxidase (Vector, Burlingame, CA) and 3,3′-diaminobenzadine (Sigma, St. Louis, MO). All antibodies were diluted to give optimal staining results. Slides routinely were counterstained with hematoxylin.

Flow Cytometric Analysis
Flow cytometry (FACScan; Becton-Dickinson Immunocytometry System, Mountain View, CA) was performed on fixed laser power (600 mW at 488 nm) throughout the study. Standardization of the flow cytometer was achieved by measuring 4.05-μm fluorescent beads (Becton-Dickinson Immunocytometry System) that exhibited a constant light scatter and fluorescence. Background fluorescence reactivity was determined with IgG from control mice IgG. For the determination of HLA-DR expression, we used T cells from peripheral blood as a negative control and B cells from peripheral blood as a positive control. In this setting, expression of the antigen on positive controls was 100% and was < 5% on negative controls. Samples obtained by brush cytology from the patients and healthy subjects were stained with fluorescein isothiocyanate (FITC) and photomultiplied to a voltage high enough that the fluorescence histogram derived from the forward angle versus the side scatter-gated cell population, avoiding the lymphocytes and yielding a 1% to 5% background intensity of autofluorescence in the range.
TABLE 1. Nucleotide Sequences Used to Detect Cytokine Expression

<table>
<thead>
<tr>
<th>Sequence</th>
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<tr>
<td>IL-2</td>
</tr>
<tr>
<td>IL-4</td>
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<tr>
<td>IL-15</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>β-Actin</td>
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<td>102 bp</td>
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reserved for positively FITC-stained cells. For each sample, 10,000 cells were analyzed on a log fluorescence scale on the flow cytometer. To determine the keratin antigen, the mean log FITC-fluorescence channel of the positively stained cells was determined using a single-parameter histogram.

Isolation and Amplification of RNA

Cytokine mRNA expression in the conjunctival preparations was assayed by the reverse-transcription–polymerase chain reaction (RT–PCR) method.10 With phenol from these mixtures, RNA was isolated, and a 20-μg sample was used for RT–PCR, with RNA reverse-transcribed into complementary DNA (cDNA). For cDNA synthesis, 20 ng of the sample RNA solution was heated at 65°C for 5 minutes. After the addition of 20 units of ribonuclease inhibitor (Takara, Kyoto, Japan), 1 μl of 10-fold-concentrated PCR buffer (500 mM KCl; 200 mM Tris–HCl, pH 8.4; 25 mM MgCl2; 1 mg/ml bovine serum albumin), 1 μl of 1.25 mM dNTPs (dATP, dCTP, dGTP, dTTP from Pharmacia LKB Biotechnology, Uppsala, Sweden), 10 parts hexanucleotide mixture (Boehringer Mannheim, Germany), 0.1% dithiothreitol (Aldrich Chemical, Milwaukee, WI), and 20 units of reverse transcriptase (200 U/ml, Perkin Elmer-Cetus, Norwalk, CT) were added to the RNA solution. The reverse-transcription reaction was carried out at 42°C for 60 minutes, 94°C for 5 minutes.

To perform the PCR assay, 10 μl of the cDNA reaction mixture was diluted with 90 μl of PCR buffer, followed by the addition of 20 pmol of 5′ and 3′ primers, 1.25 mM dNTPs, 20 mM MgCl2, and 2 units of thermostable Taq polymerase (Perkin Elmer-Cetus) and was overlaid with 150 μl of mineral oil. The reaction was started by denaturing the RNA–cDNA hybrid by heating at 94°C for 45 seconds, annealing the primers at 60°C for 45 seconds, and extending the primers at 72°C for 2 minutes. Amplification was performed with a DNA thermal cycler (Perkin Elmer-Cetus). After being denatured at 94°C for 10 minutes, the reaction mixture was passed through 35 cycles consisting of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 2 minutes, with a final extension for 7 minutes at 72°C.24

Then, 10-μl samples of the PCR products were analyzed by electrophoresis on a 1.7% agarose gel containing ethidium bromide. The specificity of the amplified products was validated by their predicted size on the agarose gel. To confirm the PCR reaction, the amplified products were purified by electrophoresis on a 4% Nusieve (FMC, Rockland, ME) agarose gel. The predominant band was cut out of the gel and placed in 0.1 ml of TE (0.01M Tris–HCl, pH 8; 0.1 mM EDTA). Gel-purified PCR products were ligated into P17 blue vector. Dideoxynucleotide chain-termination sequencing was performed according to the manufacturer’s protocol. Three independent clones were sequenced.

Peripheral blood mononuclear cells (2 × 107 cells) were stimulated for 24 hours with 50 U/ml recombinant IL-2 and 1 μg/ml phytohemagglutinin for use as positive controls for all cytokines.

Synthesis of Primers and Probes

Interleukins IL-2,25,26 IL-4,27 and IFN-γ28 and β-actin29 specific primers were obtained (Clontech Laboratories, Palo Alto, CA) and IL-13, was synthesized on model 391 PCR-Mate (Applied Biosystems, Foster City, CA) and purified on Sephadex G50 columns (Pharmacia LKB Biotechnology) and by high-performance liquid chromatography.30 This primer sequence was specific and showed no significant homology, as confirmed by a computer-assisted search of updated versions of GenBank, and were chosen with a balanced nucleotide composition ranging from 40% to 60% glycine content (Table 1).

Statistical Analyses

Data are shown as the mean ±SD. Statistical analysis of superficial cells and HLA–DR were performed using the unpaired, two-tailed Student’s t-test, and analysis of expression of cytokines was performed using Fisher’s exact test, with a level of P < 0.05 accepted as statistically significant.
RESULTS

Giemsa and Immunohistochemical Staining

The mean cells collected from 14 samples was 26,400 ± 10,700. May-Grunwald-Giemsa staining of cytocentrifuged preparations from patients with allergic conjunctivitis showed conjunctival epithelial cells with lymphocytes, mast cells, and eosinophils. Higher numbers of lymphocytes (mean, 3.6 ± 1.5%) were obtained in samples from allergic patients than in sam-
A marked expression of IL-4 transcript was observed in cell samples from patients with allergic conjunctivitis, but less than that from healthy subjects (samples from healthy subjects = normal; samples from patients with allergic conjunctivitis = allergy).

In contrast, IL-2 and IFN-γ were not found in the cell samples from seven patients with allergic conjunctivitis. Recently, the cDNA encoding IL-13, a T-cell-derived cytokine, was cloned and expressed. Interleukin-13 induced IgG4 and IgE synthesis by human B cells IL-4 independently. Because IL-15 is produced for a much longer period after T-cell activation than IL-4, it has been suggested that IL-13 has an important role in the regulation of prolonged IgE synthesis in allergic disease. Transcript for this cytokine was demonstrated in cell samples from patients with allergic conjunctivitis and in one healthy subject in the current study.

**DISCUSSION**

To determine the T-cell-cytokine-mRNA profile in the cell samples, which were obtained by brush cytology and included various cell types, we have examined the population of eosinophils, CD3−, CD4−, CD14−, CD20−, and AE-3− expressing conjunctival epithelial cells by immunocytochemistry. Giemsa and immunohistochemical staining of cytocentrifuged preparations showed that most conjunctival cells obtained from healthy subjects were epithelial cells. Cell samples from patients with allergic conjunctivitis showed small numbers of inflammatory cells, including lymphocytes, mast cells, and eosinophils, but an increased percentage of lymphocytes. Lymphocytes are usually present in the substantia propria and may have been loosened by edema. We previously detected histamine and IL-4 in cell samples obtained from patients with seasonal allergic conjunctivitis. Expression of HLA-DR was also observed with greater frequency in cell samples from the allergic patients compared with that in samples from healthy subjects. The expression of HLA-DR antigen on conjunctival cells is regulated by T cells. Because HLA-DR is an activation marker, our flow cytometric data imply that activation of conjunctival epithelial cells is present in patients with allergic conjunctivitis. Our results suggest that activation of Th2 cytokine expression may lead to HLA-DR expression in conjunctival epithelial cells, implying a linkage with pathology of allergic conjunctivitis.

Two subsets of helper T cells, designated Th1 and Th2, have been characterized in mice and humans, and a linkage of the Th2 pattern to allergic disease has been demonstrated. We have detected IL-2, IFN-γ, IL-4, and IL-13 expression by RT-PCR to define the divergence into Th1 and Th2 subpopulations. The different spectra of cytokines produced by these cell types is critical to their definition. In our study's findings the pattern of steady state mRNA expression determined by RT-PCR of cells from patients with allergic conjunctivitis differs from that in cells from healthy subjects. The observation of T lymphocytes in cell samples from patients with allergic conjunctivitis and the demonstration of cells expressing mRNA for IL-4, IL-13, but not IFN-γ, suggest that Th2-like helper T cells may participate in allergic conjunctivitis.

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FIGURE 4. Expressions of cytokine transcripts in brush cytology samples from (A) patients with allergic conjunctivitis and in (B) those from one healthy subject. (A) Reverse-transcription–polymerase chain reaction-amplified products of interleukin 4 and interleukin 13 RNA underwent electrophoresis in a 1.7% agarose gel and stained with ethidium bromide. Interleukin 2 and interferon-γ were not observed in samples from patients with allergic conjunctivitis. (B) Interleukin 4 and interleukin 13 appeared at low levels in one healthy subject. These four cytokines were not seen in the other six healthy subjects. Lane P = positive control; lane M = marker; Hae III digest. Magnification, ×174.

study. The weak expression of IL-4 and IL-13 transcript in a single healthy subject may have been caused by other atopic or allergic disease that was not discovered on initial selection.

By immunocytologic analysis, only a small number of T cells and other inflammatory cells were stained, with the majority of cells obtained by brush cytology identified as epithelial cells. Conjunctival epithelial cells express HLA-DR in trachoma, CD54 adhesion molecules in allergic conjunctivitis, cytokines in ocular pemphigoid, and Th2-like cytokines in vernal conjunctivitis. Conjunctival cells may play a role in allergic disease, and it is possible that IL-4, produced by conjunctival epithelial cells, could promote development of Th2. The separation of conjunctival epithelial cells from lymphocytes by brush cytology will be necessary to study the source of IL-4 further. Accordingly, the large population of conjunctival epithelial cells must be studied more precisely.

Although we have previously described levels of IL-4 in antigen stimulate peripheral blood lymphocytes in patients with allergic conjunctivitis, results from this study demonstrated that the Th2-like cytokine is transcribed at the ocular surface where the allergic reaction is occurring. The local proinflammatory cytokine milieu may have played an important role in allergic conjunctivitis.

Keywords
allergic conjunctivitis, conjunctival cells, cytokine, reverse-transcription–polymerase chain reaction, Th2 cells

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