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-bearin 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-y; six samples from seven healthy subjects expressed no interleukin 2, interleukin 4, interleukin 13, or interferon-y 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.

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.1,2 Th2 cells have been studied in other allergic diseases but have not been reported in allergic conjunctivitis.3,4 Cytokines have been detected in conjunctival cells5 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 immunoabsorbant 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
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. 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), 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 EP antibody by the multiple antigen simultaneous test 16 (MAST 16) (SRL, Tokyo, Japan). Seven conjunctival cell samples were obtained from seven age- and sex-matched healthy subjects (aged 22 to 32; 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 EP antibody, according to results of the MAST 16 test. Informed consent for participation in the study was obtained from all participants. No corticosteroids, antihistamines, or mast cell–stabilizing agents were used for at least a 2-day period before samples were obtained.

Cell Sampling

 Conjunctival epithelial cell specimens were collected by cytology brush from upper palpebral conjunctiva after topical anesthesia in a daily outpatient clinic. More than 20,000 conjunctival superficial cells were collected with a special brush (Cytobrush Small, Meds-
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.

Samples from patients and healthy subjects were incubated with mouse anti–HLA–DR mAb (Becton-Dickinson) at 4°C for 30 minutes. After washing three times with phosphate-buffered saline containing 2% fetal bovine serum, the samples were incubated with FITC-labeled goat antimouse IgG (Fab Fractions, Bio Source, remodeling of superficial cells and HLA-DR were performed for RT-PCR, with RNA reverse-transcribed into 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 Biochemica) and FITC-labeled goat antimouse IgG as described above.

**Isolation and Amplification of RNA**

Cytokine mRNA expression in the conjunctival preparations was assayed by the reverse-transcription–polymerase chain reaction (RT–PCR) method. With phenol from these mixtures, RNA was isolated, and a 20-ng 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 Biochemica), 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, and then 72°C for 10 minutes. The reverse-transcription reaction was 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.

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.

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 × 10^7 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, IL-4, IL-13, and IFN-γ specific primers were obtained (Clontech Laboratories, Palo Alto, CA) and IL-15, 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. 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 cystein content (Table 1).
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 six samples, and IL-13 was observed in seven samples from seven patients with allergic conjunctivitis. In contrast, IL-2 and IFN-γ were not found in the samples from allergic patients (Fig. 4A). Transcripts of IL-4 and IL-13 were weakly visualized in one healthy subject. No cytokine expression was detected in the other samples from healthy subjects (Fig. 4B). There was a significant difference in the expression of IL-4 ($P = 0.005$; Fisher’s exact test) and IL-13 ($P = 0.029$) between patients and healthy subjects.

**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 immunocytology. 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.

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
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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.

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,36 CD54 adhesion molecules in allergic conjunctivitis,37 cytokines in ocular pemphigoid and Th2-like cytokines in vernal conjunctivitis.6 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 Th2.38,39 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,40 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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References

2. Pené J, Rouxet F, Briere F, et al. IgE production by normal human lymphocytes is induced by interleukin...
and suppressed by interferons γ and δ and prosta-
3. Herrrood HG. Interleukins in immunologic and aller-
S. Cytokines in the conjunctiva of acute and chronic 
mucous membrane pemphigoid: An immunohisto-
1993;231:563–570.
Th-2–like helper T cells in the conjunctiva of patients 
1174.
Mast cell lines produce lymphokines in response to 
cross-linkage of FcεRI or to calcium ionophores. 
encoding the murine IgGl induction factor by a novel 
646.
9. Prete GD, Maggi E, Farronchi P, et al. IL-4 is an essen-
tial factor for the IgE synthesis induced in vitro by human T 
10. Tepper RL, Levinson DA, Stanger BZ, et al. IL-4 in-
duces allergen-like inflammatory disease and alters T 
cell development in transgenic mice. Cell. 1990; 
11. Ishizaka K. Basic mechanisms of IgE-mediated hyper-
12. Tsubota K, Takamura E, Hasegawa T, Kobayashi T. 
Expression of interleukin 4 (IL-4) in tears of patients 
13. Matsuki S, Ando F. Aging alteration of immunoglobu-
14. Allansmith MR, Hahn GS, Simon MA. Tissue, tear and 
serum IgE concentrations in vernal conjunctivitis. Curr Opin 
15. Fujishima H, Saito I, Takeuchi T, Shinozaki N, Tsubota 
K. Respiratory syncytial virus and allergic conjunctivitis. J Allergy 
cell adhesion molecules in the salivary and lacrimal glands of 
17. Taniguchi T, Matsui H, Fujita T, et al. Structure and 
expression of a cloned cDNA for human interleukin-
of the human interleukin 2 gene. Proc Natl Acad Sci 
19. Yokota T, Otsuka T, Mosmann T, et al. Isolation and charac-
terization of a human interleukin cDNA clone, 
homologous to mouse B-cell stimulatory factor 1, that 
expresses B-cell- and T-cell-stimulating activities. 
immune interferon cDNA in E. coli and monkey cells. 
21. Ponte P, Ng S, Engel J, Gunning P, Kedes L. Evolution-
ary conservation in the untranslated regions of act in 
mRNAs: DNA sequence of a human beta-actin cDNA. 
22. Tsubota K, Kajiwara K, Ugajin S, Hasegawa T. Con-
23. Fujishima H, Okamoto Y, Saito I, Tsubota K. Respira-
tory syncytial virus and allergic conjunctivitis. J Allergy 
24. Bottomly K. A functional dichotomy in CD4+ T lym-
25. Mosmann TR, Coffman RL. Two types of mouse 
helper T-cell clone: implications for immune regula-
human IL-2 induces IgE and IgG synthesis by normal 
1990;144:2123–2130.
27. Prete GD, Farronchi P, et al. IL-4 is an essen-
tial factor for the IgE synthesis induced in vitro by human T 
28. Tepper RL, Levinson DA, Stanger BZ, et al. IL-4 in-
duces allergen-like inflammatory disease and alters T 
cell development in transgenic mice. Cell. 1990; 
29. Ishizaka K. Basic mechanisms of IgE-mediated hyper-
30. Tsubota K, Takamura E, Hasegawa T, Kobayashi T. 
Detection by brush cytology of mast cells and eosino-
phils in allergic and vernal conjunctivitis. Cornea. 
31. Matsuki S, Ando F. Aging alteration of immunoglobul-
in E level in human tear fluid. Folia Ophtalmol Jpn. 
32. Allansmith MR, Hahn GS, Simon MA. Tissue, tear and 
serum IgE concentrations in vernal conjunctivitis. Am J 
33. Tuft SJ, Kemeny DM, Dart JK, Buckley RJ. Clinical 
features of atopic keratoconjunctivitis. Ophthalmology. 
34. Inselr MS, Lim JM, Queng JT, Wanisison C, McGovern 
JP. Tear and serum IgE concentrations by Tandem-
R IgE immunoradiometric assay in allergic patients. 
Ophthalmology. 1987;94:945–948.
35. Kari O, Salo OP, Bjorksten F, Backman A. Allergic 
conjunctivitis, total and specific IgE in the tear fluid. 
36. Fujishima H, Takeuchi T, Shimazaki N, Saito I, Tsubota 
K. Measurement of interleukin 4 (IL-4) in tears of 
patients with seasonal allergic conjunctivitis and vernal 
37. Fujishima H, Takeuchi T, Shinozaki N, Tsubota 
K. Measurement of interleukin-4 and histamine 
in superficial cells of conjunctiva in patients with aller-
38. Friedlaender MH. Conjunctivitis of allergic origin: 
Clinical presentation and differential diagnosis. Surv 
conjunctivitis caused by pollen (Cryptomeria 
Japanica D. Don) out of season. Asian Pac J Allergy 
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