Respiratory syncytial virus and allergic conjunctivitis

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The pathogenesis of allergic conjunctivitis is largely conjectural. We investigated the possible involvement of respiratory syncytial virus (RSV), a ubiquitous respiratory pathogen, in the development of allergic conjunctivitis through immune mechanisms. A new technique of brush cytology was used to obtain conjunctival cells from 30 patients with allergic conjunctivitis and 20 control subjects. Samples were assayed for the presence of RSV sequences with the reverse-transcription polymerase chain reaction and the nested polymerase chain reaction. Specific primers and inner primers were synthesized on the basis of RNA sequences previously identified. RSV sequences were detected in 7 of 30 (23%) patient samples and 1 of 20 (5%) control samples. Our results proved that polymerase chain reaction could detect RSV sequences in conjunctival samples. RSV may be a significant pathogenic factor in allergic conjunctivitis. (J ALLERGY CLIN IMMUNOL 1995;95:663-7.)

Key words: *allergic conjunctivitis, reverse-transcription polymerase chain reaction (RT-PCR), nested PCR, brush cytology*

Allergic conjunctivitis is one of the most common ocular surface diseases that occurs in all age groups and is associated with seasonal factors. Although antigen is a key factor, not all persons who are exposed to antigen have allergic conjunctivitis. Its pathogenesis remains unclear.[1] Respiratory syncytial virus (RSV) is a major pathogen in severe respiratory tract infections in newborns and infants.[2] It is associated with significant morbidity, including bronchiolitis, pneumonia, and refractive airway disease.[3] Epidemiologic and immunologic studies indicate that the incidence of RSV involvement in respiratory disease in adults[4] and in otitis media in children[5, 6] is increasing. Infection with RSV has been reported to be associated with T_{H2} CD4 T-cell infiltrate (mouse lymphocyte), elevated virus-specific IgE[7] and IgG,[8] production of cytokines,[9] release of histamine[10] or leukotrienes,[11] and suppression of T-cell function.[12] In addition, it facilitates the access of antigens to the antibody-forming cells in the airway and increases the virus-specific IgE activity and virus-induced suppression of regulatory T-cell subpopulations.[13, 14]

Abbreviations used
PCR: Polymerase chain reaction
RSV: Respiratory syncytial virus
RT-PCR: Reverse transcription polymerase chain reaction

We investigated the possible involvement of RSV in allergic conjunctivitis, using a newly developed technique, brush cytology of the conjunctiva,[15, 16] to collect samples and the polymerase chain reaction (PCR) assay.[17]

METHODS

Patients

We examined 30 patients with symptoms of allergic conjunctivitis (13 men and 17 women) aged 17 to 76 years (mean age, 25.4 ± 11.3 years) who visited Tokyo Dental College in Chiba, Japan, between March and May of 1991. A diagnosis of allergic conjunctivitis was based on reported symptoms, including ocular itching,

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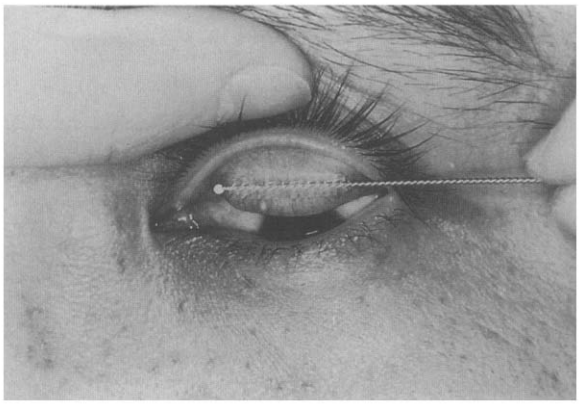

FIG. 1. Brush cytology technique. Brush cytology technique was used to collect conjunctival epithelial cells. Both the upper and lower tarsal conjunctivae were scraped several times.

TABLE I. Multiple antigen simultaneous test of 16 antigens: Positive results in 30 patients

Antigen	Positive results (%)
House dust	63
Ragweed mix I	10
Japanese cedar	83
Alternaria spp.	0
Mite	73
Mugwort	10
Penicillium spp.	7
Aspergillus spp.	7
Egg white	20
Sweet vernal grass	17
Cladosperium spp.	0
Cat	17
Soybean	10
Timothy grass	7
Candida spp.	7
Dog	7

redness, tearing, and mucous discharge; the results of slit lamp examinations that revealed papillae, chemosis, and hyperemia of the palpebral conjunctiva[18, 19]; and positive results for antigen-specific serum IgE antibodies to 16 antigens (Table I). We also examined 20 normal control subjects (11 men and 9 women) aged 18 to 33 years (mean age, 25.6 ± 8.1 years). The control subjects had no symptoms or signs of allergic conjunctivitis and had negative results for IgE antibodies to 16 antigens (Table I).[19] Informed consent for participation in the study was obtained from all patients and normal volunteers.

Sample preparation

Conjunctival samples were collected with a special brush, a smaller version of the Cytobrush used in cervical cytology (Cytobrush Small; Medscand, Malmö, Sweden) (Fig. 1). Its nylon bristles repel the negative charge of the epithelial cell membrane and increase the number of collected epithelial cells.[15, 16] With this method, it is possible to collect about 8000 superficial cells from the conjunctiva.[15] However, it is still necessary to use PCR analysis because of the relatively small number of cells. The epithelial cells obtained by brush cytology[15] were kept in 2 ml of isotonic sodium chloride solution and stored at −80° C until assayed.

TABLE II. Nucleotides in detection of RSV

RT-PCR (first PCR; product size, 391 bp)
5′:GGTGTTGGATCTGCAATCGCCA
3′:AACTTTTTCTGATCATTTGT
Nested PCR (second PCR; product size, 207 bp)
5′:AAGTGCTCTACTATCCACA
3′:CACTAAATTCCCTGGTAATC
β-Actin (product size, 202 bp)
5′:CCTTCCTGGGCATGGAGTCCTG
3′:GGAGCAATGATCTTGATCTTC

Detection of RSV by PCR

RSV sequences in the conjunctival preparations were assayed by reverse transcription PCR (RT-PCR) and nested PCR. RNA was isolated from the collected cells by the method of Chomczynski and Sacchi,[20] and a 10 ng sample was used for RT-PCR and nested PCR. For cDNA synthesis, 10 ng of the sample RNA solution was heated at 65° C for 5 minutes for flattering template RNA and then cooled rapidly. After the addition of 20 units of ribonuclease inhibitor (Takara, Kyoto, Japan), 1 μl of 10× PCR buffer (500 mmol/L KCl, 200 mmol/L Tris-HCl buffer [pH 8.4], 25 mmol/L $MgCl_2$, 1 mg/ml bovine serum albumin), 1 μl of 1.25 mmol/L deoxyribonuclease triphosphates (dATP, dCTP, dGTP, dTTP [Pharmacia Co., Tokyo, Japan]) and 20 units of Rous-associated virus 2 reverse transcriptase (RTase; Takara, Kyoto, Japan) were added to the RNA solution. The mixture was heated at 42° C for 60 minutes, then at 94° C for 5 minutes, and then cooled rapidly.

Specific primers (Table II) were synthesized on a model 391 PCR-Mate (Applied Biosystems, Inc., Tokyo, Japan), based on the RNA sequences previously identified by Collins et al.[21] RNA sequences that expressed the fusion glycoprotein of RSV were chosen as the target sequences because of the similarities among subtypes of RSV.[22] The primer and probe sequences for RSV showed no significant homology, as previously reported by Okamoto et al.[23]

The PCR reaction mixture contained 10 μl of cDNA, 10 μl of 10× PCR buffer, 11 μl of 20 mmol/L $MgCl_2$, 16 μl of 1.25 mmol/L deoxyribonucleoside triphosphates, 42.5 μl of diethyl pyrocarbonate in water, 100 pmol/L 5′

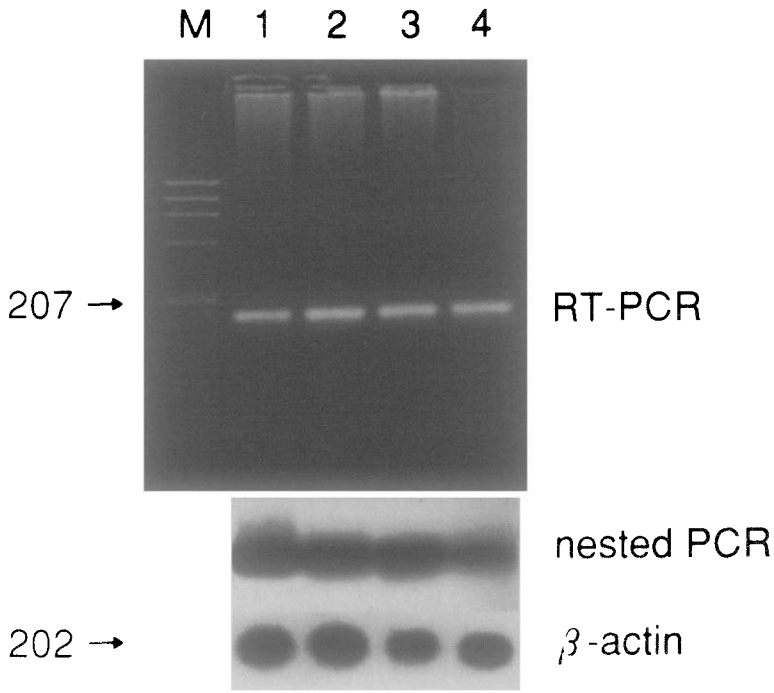

FIG. 2. Representative RSV-positive samples obtained by brush cytology from patients with allergic conjunctivitis. RT-PCR amplified products for RSV were analyzed by electrophoresis on a 1.7% agarose gel and stained with ethidium bromide. Southern blot analysis was performed on the amplified product of clinical samples obtained by nested PCR. Amplified products were denatured by alkali, neutralized, and applied to a nylon membrane. Each of these samples was hybridized with phosphorous 32–labeled RSV-specific internal probe (Table II). *Lane M,* marker (X174/Hae III digest).

and 3′ primers and 2.5 units of thermostable Taq polymerase (Perkin Elmer Cetus, Yokohama, Japan). Amplification was performed with a DNA thermal cycler (MJ Research Inc., Tokyo, Japan). After being denatured at 94° C for 10 minutes, the reaction mixture was amplified for 35 cycles at 94° C for 30 seconds, at 55° C for 30 seconds, and at 72° C for 60 seconds, followed by reextension for 10 minutes at 72° C.

The second PCR was performed in the same manner with inner primers. Then, 10 μl samples of the first and second PCR products were analyzed by electrophoresis on a 1.7% agarose gel. Southern blotting, with one of the inner primers as an internal probe, was used to confirm the specificity of the positive PCR results.

RSV-infected Hep-2 cells, but not mumps virus–infected Vero cells or parainfluenza type II virus–infected GM-K cells, were used as controls.[23] The addition of 1 μg of yeast RNA (Torula yeast RNA: 5′Prime→3′Prime) to various dilutions of RNA from RSV-infected Hep-2 cells did not affect the results of RT-PCR.

RESULTS

By using brush cytology, we obtained superficial cells from patients with allergic conjunctivitis, including samples from the conjunctival epithelium, lymphocytes, eosinophils, mast cells, and some tears.

RT-PCR detected a discrete band in seven samples from patients with allergic conjunctivitis, but not in samples from control subjects. Southern blotting confirmed the specificity of the positive PCR results. After nested PCR, RSV sequences were clearly detected in the same samples (Fig. 2). By our technique, 7 of 30 samples (23%) from patients with allergic conjunctivitis and 1 of the 20 control samples (5%) were positive for the presence of RSV.

DISCUSSION

Our study is the first investigation on the role of RSV in allergic conjunctivitis. Genomic sequences of RSV were identified in superficial cells and tears collected from the conjunctivae of patients with allergic conjunctivitis. The depressive effects of RSV on the mucociliary system, neutrophils, and T-cell function may predispose these patients to the development of bacterial infection.[12, 13] These effects include possible alterations of mucosal transport mechanisms secondary to the release of

potent pharmacologic mediators from mucosal mast cells and polymorphonuclear leukocytes or other lymphokines released during acute RSV infection.[24] RSV-antibody immune complexes may also be involved in mucosal damage.

RSV may produce a number of immunologic effects that contribute to the pathogenesis of bronchiolitis in RSV-infected children.[10, 25, 26] In vitro studies show an increased lymphoproliferative response in peripheral smears obtained from patients with bronchiolitis caused by RSV.[25] It has been suggested that in vivo infections with viral agents such as RSV may function as adjuvants for other inhaled allergens and environmental antigens available in the mucosal lumen during acute viral infection.[27] Production of IgE in response to a variety of protein antigens and allergens appears to be regulated by T suppressor lymphocytes.[28-32] Welliver et al.[10] have shown that RSV-specific IgE antibodies and histamine are present in respiratory secretions and serum from RSV-infected infants with severe wheezing. Virus-induced wheezing is attributable to allergic reactivity and hyperreactive airway disease.[33, 34] Moreover, an IgE response initially induced by one antigen can be induced by the stimulation of an IgE response to another antigen.[35]

Allergic conjunctivitis is not the usual manifestation of a simple acute RSV infection. It is still unclear which is the initiated sequence, allergic conjunctivitis or RSV infection. Although RSV infection may easily occur in patients with allergic conjunctivitis and may simply be the bystander, the animal experiment supports our hypothesis that allergic conjunctivitis follows the RSV infection. The disturbed humoral and cell-mediated immune responses in the conjunctiva that are associated with RSV infection may lead to inflammation. Further study is necessary to compare the detection rate of RSV in patients with first-year allergic conjunctivitis and patients with allergic conjunctivitis of longer duration. Our findings underscore the importance of conducting further studies on the relationship between RSV and allergic conjunctivitis.

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