Abstract. 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. Brush cytology technique was used to obtain conjunctival cells from 30 patients with allergic conjunctivitis and 20 controls. Samples were assayed for the presence of RSV sequences with the reverse transcription polymerase chain reaction (RT-PCR) and the nested polymerase chain reaction (nested PCR). In order to further investigate the relation of RSV and allergic conjunctivitis, we performed an animal study. BALB/c mice were either infected with live RSV or sham infected intranasally. The mice were exposed to ovalbumin (OVA) with alum adjuvant. At differing intervals, levels of OVA concentration in serum and IgG-anti-OVA antibody were measured by ELISA and IgE-anti OVA antibody in serum was assessed by passive cutaneous anaphylaxis. RSV sequences were detected in 7 (23%) of 30 patient samples and 1 (5%) of 20 control samples. IgG and IgE antibody responses to OVA in serum were demonstrable only in RSV-infected mice immunized with OVA and alum. Our results proved that PCR could detect RSV sequences in conjunctival samples. RSV may be a significant pathogenic factor in allergic conjunctivitis. (Keio J Med 43 (4): 201 -205, December 1994)

Key words: RSV, allergic conjunctivitis, RT-PCR, brush cytology, IgE-anti-OVA antibody activity

Introduction

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 people are exposed to antigen developed allergic conjunctivitis. Its pathogenesis remains unclear. Respiratory syncytial virus (RSV) is a major pathogen in severe respiratory tract infections in newborns and infants. It is associated with significant morbidity, including bronchiolitis, pneumonia and refractive airway disease. Epidemiologic and immunologic studies indicate that the incidence of RSV involvement in respiratory pathology in adults and in otitis media in children is increasing. Infection with RSV has been reported to be associated with Th2 CD4 T-cell infiltrate (mouse lymphocyte), elevated virus-specific IgE and IgG, production of cytokines, release of histamine or leukotrienes, and suppression of T-cell function. In addition, it facilitates the access of antigens to the antibody-forming cells in the airway and develops the virus-specific IgE activity and virus-induced suppression of regulatory T-cell subpopulations.

We investigated the possible involvement of RSV in allergic conjunctivitis, using a newly developed technique, brush cytology of the conjunctiva, to collect samples, and the polymerase chain reaction (PCR) assay. We then decided to investigate the effects of RSV infection on the kinetics of antigen uptake and serum antibody responses during acute infection with RSV in BALB/c mice.

Methods

Patients

We examined 30 symptomatic patients with allergic conjunctivitis (13 males and 17 females) aged 17 to 76 years old (mean age of 25.4 ± 11.3) 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, redness, tearing and mucous discharge and the results of slit lamp examinations that revealed papillae, chemosis,
and hyperemia of the palpebral conjunctiva, and positive results for antigen specific serum IgE antibodies to 16 antigens (Table 1). We also examined 20 normal controls (11 males and 9 females) aged 18 to 33 years old (mean age of 25.6 ± 8.1). The control subjects had no symptoms or signs of allergic conjunctivitis and were negative for IgE antibodies to 16 antigens. Informed consents for participation in the study were 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 (Fig 1). Its nylon bristles repel the negative charge of the epithelial cell membrane and increase the number of collected epithelial cells.15 With this method, it is possible to collect about 8,000 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 cytology15 were kept in 2 ml of isotonic sodium chloride solution, and stored at −80°C until assayed.

Detection of RSV by PCR

RSV sequences in the conjunctival preparations were assayed by reverse transcription polymerase chain reaction (RT-PCR) and nested polymerase chain reaction (nested PCR). RNA was isolated from the collected cells by the method of Chomczynski and Sacchi17 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 min for flattening of template RNA and then cooled rapidly. After the addition of 20 units of ribonuclease inhibitor (Takara, Kyoto, Japan), 1 μl of 10X PCR buffer [500 mM KCl, 200 mM Tris-HCl buffer (pH 8.4), 25 mM MgCl2, 1 mg/ml BSA], 1 μl of 1.25 mM dNTP's (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 min, then at 94°C for 5 min, and then cooled rapidly.

Specific primers (Table 2) were synthesized on a model 391 PCR-Mate (Applied Biosystems, Inc, Tokyo, Japan), based on the RNA sequences previously identified by Collins et al18 RNA sequences that expressed the fusion glycoprotein of RSV were chosen as the target sequences, because of the similarities among subtypes of RSV.19 The primer and probe sequences for RSV showed no significant homology, as Okamoto et al had previously reported.20

The PCR reaction mixture contained 10 μl of cDNA, 10 μl of 10X PCR buffer, 11 μl of 20 mM MgCl2, 16 μl of 1.25 M dNTP's, 42.5 μl of DEPC-water, 100 mM 5' 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 min, the reaction mixture was amplified for 35 cycles at 94°C for 30 sec, at 55°C for 30 sec, and at 72°C for 60 sec, followed by re-extension for 10 min at 72°C.

The second PCR was performed in the same manner using 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, using 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. 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. For the recovery of RNA and purity and integrity, we have checked the amount of RNA and we have also detected expression of β-actin for every single PCR.

Animals, virus, and antigen

Male BALB/c-Ros mice, 8 weeks old, raised on ovalbumin-free food (food excluded ovalbumin) were used. Ten mice were used for each group. Long strain of RSV was prepared as previously described and stored in aliquots at −70°C. The stock virus had a titer of 2 × 10^7 plaque-forming units/ml (PFU/ml). Aluminum hydroxide (Amphojel tablets, Wyeth Laboratories, Philadelphia, PA) was suspended in PBS at 5 mg/ml and homogenized by sonication. Ovalbumin, grade V (Sigma chemical Co, St. Louis, MO), was dissolved at a concentration of 400 μg/ml in the alum (aluminum hydroxide) suspension.

Protocols for animal study

Two groups of mice were either infected with RSV or sham infected on day 0. RSV was inoculated by intranasal administration of 50 μl (=10^6 PFU). Control animals were sham infected with a virus-free HEp-2 cell suspension. Animals were lightly anesthetized by inhalation of methoxyflurane. Four to eight days later, they received five doses of 5.0 ml/day (0.4 mg/ml) of OVA in alum every day by inhalation. Blood samples were obtained by retroorbital bleeding. Serum was stored in aliquots at −20°C. Serum IgG and IgE OVA-specific antibody activity were measured prior to and 17 and 32 days after the RSV inoculation.

Assays

Serum IgG anti-OVA activity was determined by a sandwich-type ELISA. Rabbit anti-OVA antiserum (IgG fraction) was coated to polyvinyl chloride microtiter plates, and peroxidase-conjugated IgG fraction was used for detection (both Cooper Biomedical). Results were expressed as the reciprocal of the minimum diluted concentration required to obtain optical density 0.5 (O.D. 0.5).

OVA-specific IgE antibody was detected by passive cutaneous anaphylaxis (PCA). PCA titers were expressed as the reciprocal of the highest dilution giving a positive reaction.

Statistical analysis of these results were evaluated by Fisher’s exact test.

Results

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

RT-PCR detected as discrete band in seven samples from patients with allergic conjunctivitis, but not from controls. 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 (23%) of 30 samples from patients with allergic conjunctivitis and 1 (5%) of the 20 control samples (Fig 3) were positive for the presence of RSV.

Serum IgG OVA-specific antibody activity is shown in Fig 2.
Fig 3. RSV positive late. 7 (23%) of 30 samples from patients with allergic conjunctivitis and 1 (5%) of 20 control samples contained RSV.

Fig 4. Serum IgG-anti-OVA antibody in mice infected with RSV or sham infected. Solid circles = RSV-infected mice; open circles = RSV sham-infected mice. Antibody activity was expressed as ELISA titer (reciprocal dilution) giving an O.D.0.5 (optical density of 0.5). Y-axis is the reciprocal of the minimum diluted concentration required to obtain an O.D.0.5.

Fig 5. Serum IgE-anti-OVA antibody in mice infected with RSV or sham infected. Solid circles = RSV-infected mice; open circles = RSV sham-infected mice. PCA (passive cutaneous anaphylaxis) titers were expressed as the reciprocal of the highest dilution giving a positive reaction where a blue lesion of a diameter greater than 5 mm. Vertical line: 2X, 3X---6X. The difference observed at day 32 was statistically significantly (P<0.05). Data means titers ± SE of ten mouse.

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 increased serum IgG- and IgE-anti-OVA in RSV infected mice indicates that RSV may play an important role in the pathogenesis of 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. These effects include possible alterations of mucosal transport mechanisms secondary to the release of potent pharmacological mediators from mucosal mast cells and polymorphonuclear leukocytes, or other lymphokines released during acute RSV infection. 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. In vitro studies show an increased lymphoproliferative response in peripheral smears obtained from patients with bronchiolitis due to RSV. 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. Production of IgE in response to a variety of protein antigens and allergens appears to be regulated by T suppressor lymphocytes. Welliver et al 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. Moreover an IgE response initially induced by one antigen can be induced by the stimulation of an IgE response to another antigen.

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 allergic conjunctivitis and it may simply the bystander, the animal experiment supports our hypothesis that allergic conjunctivitis follows the RSV infection. Only RSV infected mice developed serum IgG and IgE-anti-OVA supported the concept that RSV infection occurs first and encourages the development of allergic conjunctivitis. 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 so as to compare the detection rate of RSV in the first year allergic conjunctivitis patients and more established patients with allergic conjunctivitis, and set up the conjunctival cultivation to study the effects of RSV infection in vitro. Our findings underscore the importance of conducting further studies on the relation between RSV and allergic conjunctivitis.

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