Infection Rate and Virus-Induced Cytokine Secretion in Experimental Rhinovirus Infection in Mucosal Organ Culture

Comparison Between Specimens From Patients With Chronic Rhinosinusitis With Nasal Polyps and Those From Normal Subjects

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Objective: To investigate the difference in susceptibility to rhinovirus (RV) infection and RV-induced inflammatory response between the nasal mucosae from patients with chronic rhinosinusitis with nasal polyps (CRS/NP) and subjects without CRS/NP (hereinafter, normal subjects).

Design: In vitro study.

Setting: Tertiary care rhinology clinic.

Patients: We conducted RV infection experiments on the organ cultures of NP s and inferior turbinate mucosae from 16 patients with CRS/NP and sphenoid sinus and inferior turbinate mucosae from 19 patients who underwent transsphenoidal pituitary surgery.

Main Outcome Measures: Successful RV-16 infection was determined by positive identification of RV on the surface fluid of organ culture using seminested reverse transcriptase–polymerase chain reaction. Effects of RV on interleukin 6 (IL-6) and IL-8 secretion were measured by enzyme-linked immunosorbent assay.

Results: The successful RV infection was achievable in 9 of 16 NP samples (56.3%) and 9 of 16 turbinate samples (56.3%) from patients with CRS/NP compared with 11 of 19 sphenoid sinus samples (57.9%) and 15 of 19 turbinate samples (78.9%) from normal subjects. The RV infection increased IL-6 and IL-8 secretion 236% and 173%, respectively, in NP samples, and 218% and 178%, respectively, in turbinate samples from patients with CRS/NP; compared with 231% and 145%, respectively, in sphenoid mucosa samples, and 181% and 148%, respectively, in turbinate samples from normal subjects. However, there were no statistical differences among the 4 groups.

Conclusion: These in vitro findings suggest that subjects with CRS/NP mucosa might not be more susceptible to RV infection, and did not secrete more cytokines in response to rhinovirus infection, than those with normal mucosa.

Thus, in our study, we hypothesized that the patients with CRS/NP might be more susceptible to RV infection and might show more intense RV-induced inflammatory response compared with normal subjects. To test this hypothesis in vitro, we conducted an RV infection experiment on the organ cultures of nasal mucosae, which we recently showed to be suitable for studying pathogenesis of RV infection. In this in vitro experimental infection, we compared the difference in the susceptibility to RV infection by assessing the rate of successful infection in different tissues. We also compared the virus-induced cytokine secretion, as a mirror of the severity of RV-induced inflammation, in NPs and inferior turbinate mucosae from patients with CRS/NP with inferior turbinate and sinus mucosae from normal individuals.

**METHODS**

**MUCOSAL SPECIMENS FOR ORGAN CULTURE**

Nasal polyps and inferior turbinate mucosae were harvested from 16 patients with CRS/NP (8 males and 8 females), ranging in age from 12 to 76 years (mean age, 29 years), admitted from July 2005 to January 2006 for surgical management of CRS/NP that had not been controlled with at least 1 month of treatment with oral antibiotics. Sphenoid sinus and inferior turbinate mucosae were also harvested from 19 individuals (9 males and 10 females), ranging in age from 13 to 62 years (mean age, 43 years), who underwent transsphenoidal surgery for pituitary adenoma during the same time period. Individuals with viral URIs or having family members with viral URIs in the preceding 2 months, or who had been treated with antihistamines or antibiotics during the previous month, were excluded from the study. In addition, individuals in the control group were routinely examined by plain sinus radiography to confirm the absence of sinus diseases. This study was approved by the institutional review board of Asan Medical Center, Seoul, South Korea, and all study subjects gave written informed consent.

**VIRAL STOCK PREPARATION**

Rhinovirus-16 was purchased from the American Type Culture Collection (ATCC) (Manassas, Virginia). Additional viral stocks were prepared by infection at 33°C of HeLa cells (ATCC), which have a low-input multiplicity of infection. When the infection was notably advanced, as evidenced by cytopathic effects, the cell supernatants were harvested, the cells were disrupted by freezing and thawing, and the debris was pelleted by low-speed centrifugation. Aliquots of clarified supernatants were frozen at -70°C after determining their viral titers.

**EXPERIMENTAL RV INFECTION IN THE ORGAN CULTURE**

Organ cultures of the mucosal specimens were grown as described in our previous study. In brief, mucosal tissues were washed 3 times in HEPES buffer to remove blood cells and mucus, divided into 4 small pieces with a 4-mm punch biopsy specimens (Stiefel, Offenbach, Germany), and placed in minimal essential medium (GIBCO, Grand Island, New York), supplemented with 2 mM of L-glutamine, 20 mM of HEPES, penicillin G (100 U/mL), streptomycin sulfate (100 µg/mL), and amphotericin B (0.25 µg/mL) for 4 hours at 4°C. Each tissue sample was placed at the air-liquid interface, with the epithelium up and the submucosa down, on a 5 × 5-mm support of gelfoam (Johnson & Johnson, Gargrave, England), prehydrated for at least 24 hours with culture medium (Figure 1). Each fragment was placed in a well of a 24-well plate (Nunc; Nunc, Roskilde, Denmark), along with 1.5 mL of culture medium, so that the epithelium was above the liquid phase. To the top of each fragment was applied 30 µL of RV-16 (50% tissue culture infection dose, in units per milliliter) for 4 hours, after which the samples were washed 4 times with phosphate-buffered saline to remove previously applied RVs. The RV-treated mucosae were transferred to a second set of identical plates and incubated for an additional 48 hours at 33°C.

**DETERMINATION OF SUCCESSFUL RV INFECTION**

In RV-infected tissues, RVs were detectable from the surface fluid after a certain interval from the virus application, indicating that RVs were actively replicating in the epithelial cells. Because not all the tissues on which RVs were applied became infected, the success of RV infection that can be translated into susceptibility of a specific mucosal specimen to RV was determined by confirming positive detection of RV from the surface wash fluid of the organ culture 48 hours after infection using a 2-step reverse transcriptase–polymerase chain reaction (RT-PCR), as described in the next subsection.

**PREPARATION OF RNA AND PICORNAVIRUS RT-PCR**

RNA was extracted from mucosal surface fluid using Trizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer’s protocol. Picornavirus RNA was reverse transcribed and amplified using the primers OL26 (5'-GCACCTCTGTTCCTCCC-3') and OL27 (5'-CGGACACCCAAATAG-3'), which amplified a 388-bp fragment from the 3'-noncoding region of the picornavirus genome, and the Superscript One-Step RTPCR with Platinum Taq kit (Invitrogen, Gaithersburg, Maryland). Each PCR mixture contained 2 X reaction buffer, 0.4 mM of each deoxyribonucleotide triphosphate (dNTP), 2.4 mM of magnesium sulfate, 0.5 µL of each primer, 0.4 µL of Platinum Taq mix, and 8.8 µL of the RNA template in a final volume of 20 µL. Amplification was performed in a thermal cycler (Perkins Elmer 9600, Minneapolis, Minnesota) programmed for initial cDNA synthesis and predenaturation (30°C for 1 hour and 94°C for 2 minutes), followed by 36 cycles of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 55°C, and extension for 30 seconds at 68°C.
Culture media after RV infection were stored at −70°C until assayed. The interleukin 6 (IL-6) and IL-8 concentrations were assayed using enzyme-linked immunosorbent assay kits (Biotechnology Co, Nivelles, Belgium), according to the manufacturer's protocol.

**STATISTICAL ANALYSIS**

Differences in RV-16 infection rate between groups were determined by the McNemar test, and differences in cytokine secretion between groups were analyzed using the Mann-Whitney U test. All statistical analyses were performed using the PC-SAS statistical package (version 6.04; SAS Institute, Cary, North Carolina), and P < .05 was considered statistically significant.

**RESULTS**

**SEMINESTED RT-PCR FOR RV-16 DETECTION FROM THE MUCOSAL SURFACE FLUID**

After an experimental RV infection, among the surface wash fluids of the 16 NP and inferior turbinate mucosa from patients with CRS/NP, 9 nasal polyps (56.3%) and 9 turbinate mucosa (56.3%) showed the 292-bp RV-specific band, indicating a successful RV infection. Of the 19 sphenoid and inferior turbinate mucosa from the control group, 11 sphenoid mucosa (57.9%) and 15 turbinate mucosa (78.9%) were successfully infected with RV by showing positive identification of the 292-bp RV-specific band (Figure 2), but the differences were not statistically significant (P > .05 for all comparisons). These results suggest a similar degree of susceptibility to RV infection among the different mucosal organ cultures studied.

**IL-6 AND IL-8 QUANTIFICATION**

Compared with the uninfected control samples, the level of secretion of IL-6 and IL-8 increased to 236% and 173%, respectively, in RV-infected NP samples from patients with CRS/NP, and to 218% and 178%, respectively, in infected turbinate samples from patients with CRS/NP (Figure 3). However, there were no statistical differences among the 4 groups (P > .05 for all comparisons).

**COMMENT**

Using the organ culture model and RT-PCR, we recently showed that only 50% of normal nasal mucosa could be infected with RV. Similarly, during the in vivo RV challenge of volunteers, not every volunteer was actually infected, and only a small fraction of cells were infected. When epithelial cells are infected with RV, viral replication takes place, and RVs are released to the cell surface, allowing their detection in the surface fluid.
of epithelial cells. Thus, the success of experimental RV infection can be determined by positive identification of RV from the surface wash fluid of epithelial cells using cell culture or PCR methods. Therefore, in our study, with an assumption that the in vitro success rate of RV infection in a certain mucosal organ culture may reflect the in vivo susceptibility to RV of the original host tissue, we have used this organ culture model for experimental RV infection to compare the susceptibility to RV infection and virus-induced cytokine secretion in NPs and inferior turbinate mucosae from patients with CRS/NP and in normal inferior turbinate and sinus mucosae.

We found that 56.3% of NPs and 56.3% of turbinate mucosae samples from patients with CRS/NP were successfully infected by RV, compared with 57.9% of sphenoid and 78.9% of turbinate mucosae samples from normal subjects. Although the highest RV infection rate was observed in normal turbinate mucosae, none of the differences was statistically significant (P>0.05). These results suggest that, under the same environmental conditions, patients with CRS/NP and normal subjects may have a similar probability of catching colds when exposed to RV.

Rhinovirus infection stimulates the secretion of IL-6 and IL-8, and there is substantive evidence for direct correlations between the levels of IL-6 and IL-8 secretion and the severity of RV cold symptoms. Interleukin 6 causes cold symptoms by inducing pyrexia, and IL-8 plays an important role in the recruitment and activation of neutrophils, resulting in the release of the contents of their cytotoxic granules. Therefore, to determine the physiologic changes in the organ culture model induced by experimental RV infection, we assayed the increases in IL-6 and IL-8 secretion in samples from patients with CRS/NP and normal subjects. We found that RV infection enhanced the secretion of both cytokines in all organ culture samples tested. Although the increases in both cytokines differed slightly among the groups, there were no significant differences (P>0.05) in RV-induced cytokine secretion, suggesting that, during RV colds, both patients with CRS/NP and normal subjects may undergo similar degree of inflammatory processes, which are critical in the development of cold symptoms.

In this study, the in vitro susceptibility to RV infection and RV-induced inflammatory responses did not differ significantly (P>0.05) between patients with CRS/NP and normal subjects. The results were not only in disagreement with those suggested in a previous report, but they also could not prove our working hypothesis that patients with CRS/NP might be more susceptible to RV infection and might show more intense RV-induced inflammatory response compared with the normal subjects. However, to better understand the real susceptibility to RV infection and the severity of RV-induced inflammation in patients with CRS/NP, further epidemiological studies or in vivo experimental RV infection study in volunteers are highly required.

Nonetheless, in conclusion, our in vitro results suggest that CRS/NP mucosa might not have increased susceptibility to RV infection or more intense cytokine response by RV infection when compared with normal mucosa.

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Author Contributions: Drs Wang, Chung, Lee, and Jang and Ms Kwon had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Wang and Jang. Acquisition of data: Kwon, Chung, and Lee. Analysis and interpretation of data: Wang and Jang. Drafting of the manuscript: Chung. Critical revision of the manuscript for important intellectual content: Wang, Kwon, Lee, and Jang. Administrative, technical, and material support: Kwon, Chung, Lee, and Jang. Study supervision: Jang.

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REFERENCES