

IL-10 and RANTES are Elevated in Nasopharyngeal Secretions of Children with Respiratory Syncytial Virus Infection

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ABSTRACT

Background: Respiratory syncytial virus (RSV) infection causes asthma-like symptoms in infants and young children. Although an increase in several mediators in the airway during RSV infection has been reported, the mechanisms involved in airway inflammation are not fully understood. The aim of this study was to investigate the immunological deviation associated with airway inflammation by measuring cytokine and chemokine levels in the airway during RSV infection.

Methods: One hundred and ten children under 3 years of age with respiratory symptoms were enrolled in this study from November 2004 through January 2005. Nasopharyngeal secretions (NPAs) were gently aspirated and analyzed with RSV antigen, thereafter the concentrations of IL-4, IL-10, IFN- γ , and RANTES were measured using an ELISA kit. We also investigated the prognosis of each child after 1 year by reference to clinical records or by interviews and re-evaluated the cytokine and chemokine levels.

Results: Of the subjects, 70 children were RSV positive and 40 were negative. Only 4 children were given a diagnosis of asthma by the pediatrician when NPAs were collected. The levels of IL-4, IL-10, and RANTES were significantly higher in the RSV-positive patients than RSV-negative patients with *P* values at 0.0362, 0.0007, and 0.0047, respectively. In contrast, there was no significant difference in the levels of IFN- γ . Furthermore, there was a significant positive correlation between IL-10 and RANTES.

Conclusions: The increased production of IL-4, IL-10, and RANTES in the airway may play an important role in the pathophysiological mechanisms of RSV infection.

KEY WORDS

Asthma, interleukin-10, RANTES, respiratory syncytial virus, wheeze

INTRODUCTION

Respiratory syncytial virus (RSV) is a non-segmented, single-stranded RNA virus belonging to the *Paramixoviridae* family. Most young children within their first few years of life are susceptible to infection with this toxic virus.¹ RSV infection produces respiratory symptoms and 30% of patients develop lower res-

piratory tract illness, including bronchiolitis, or respiratory failure.² In Japan, RSV infection was diagnosed in 31.4% of hospitalized patients under 3 years of age with lower respiratory infection.³ The development of asthma following RSV infection remains unclear from previous clinical studies.⁴ Sigurs *et al.* reported in their elegant study that infants with severe RSV infection developed a higher risk of sensitization to aller-

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gens and allergic asthma.^{5,6} In contrast, Stein *et al.* reported that RSV infection in the first year of life was a risk factor for the development of recurrent wheeze before 11 years of age, however this association did not persist to 13 years of age.⁷ Taussig *et al.* reported 3 phenotypes of wheeze in early childhood through adolescence in the Tucson Children's Respiratory Study.⁸ Some patients with RSV infection-induced wheeze under 3 years of age subsequently developed non-atopic recurrent wheeze. Although they suffered recurrent wheeze until 7 years of age, they finally outgrew the condition and exhibited no more signs of bronchial hyperresponsiveness. Therefore the condition of these patients was not considered to be associated with atopic asthma. These observations indicated that not only RSV infection, but also differences in the self-immune response to RSV, might contribute to the asthma-like symptoms in children.

Recently, the level of several inflammatory cytokines in the airway, including interleukin (IL)-2,⁹ IL-4,¹⁰ IL-5,¹¹ IL-6,¹² IL-9,¹³ IL-10,¹⁰ IL-12,¹⁰ IL-18,^{10,14} tumor necrotic factor (TNF)- α ,¹⁵ and interferon (IFN)- γ ^{10,16} have been reported to be associated with RSV infection. However, the mechanisms that are involved in airway inflammation in response to viral-induced bronchiolitis are not fully understood. Based on the current understanding of allergic diseases including asthma, these conditions are considered to arise from an irregular balance among T-cell phenotypes. This entails not only a shift in the T-helper 1/T-helper 2 (Th1/Th2) balance, but also includes the action of the regulatory T cells that play an important role in the pathophysiological response against various stimuli such as respiratory viruses and airborne allergens. Indeed IL-10, which is produced by regulatory T cells, is characterized by the abolition of allergen-induced specific T-cell proliferation and the suppression of Th1-type cytokine (IFN- γ) or Th2-type cytokine (IL-4) production.¹⁷ Hence, we investigated the cytokine profile in the airways of young children with RSV infection measuring the cytokine level in nasopharyngeal aspirates (NPAs). Moreover, we measured chemokine [regulated on activation normal T-cell expressed and secreted (RANTES)] production, which may be associated with the attraction of inflammatory cells such as Th2-type lymphocytes or eosinophils.

METHODS

STUDY DESIGN

One hundred and ten children under 3 years of age were enrolled in this study from November 2004 through January 2005 at the Daido Hospital in Aichi. The inclusion criteria were those individuals who had at least one respiratory symptom, such as cough, wheeze, running nose, sneeze, or snore, and who were suspected by professional pediatricians to be infected with RSV. We excluded children who had car-

diac disease, immunodeficiency, chronic inflammatory disease, congenital respiratory disorder, chronic lung disease after infantile respiratory distress syndrome, and immature infants of less than 2,500 g weight at birth. No children were treated with antibiotics or steroid when NPAs were collected. Four children were given a diagnosis of asthma by the pediatrician and 18 children had atopic status when NPAs were collected. After obtaining written informed consent from the parents, within a few days with symptoms NPAs were gently aspirated by well-trained nurses, using a sterilized suction catheter of 10 Fr diameter connected to a graduated trap-tube. After the collection of 1 ml NPA samples, 2 ml of sterilized saline was immediately added, and the resulting suspension was well mixed at the hospital laboratory. These samples were analyzed by using a Directigen EZ RSV™ Test kit (BD, NJ, USA) to detect the RSV antigen. The samples were centrifuged at 3000 rpm for 15 minutes at 4°C, and thereafter the supernatants were collected and stored at -80°C until assay. We excluded any samples with visual blood contamination. We analyzed the concentrations of IL-4, IL-10, IFN- γ , and RANTES using an ELISA kit (R&D systems, Minneapolis, MN, USA) following the protocol recommended by the manufacturer.

A physical examination of each patient was undertaken by pediatricians before the NPA collection. Using auscultation, we confirmed the presence of wheeze in those who had apparent wheeze on expiration. The diagnosis of asthma was defined as having a history of wheeze of more than three times. Atopy was defined as a positive level of more than one specific IgE antibody against house dust mite, grass pollen, cow's milk, or hen's egg white.

We followed the prognosis of patients by reference to patient records 1 year from the date on which the initial diagnosis had been made. This study was approved by the Ethics Committee of Daido Hospital and carried out at a single hospital setting for both inpatients and outpatients.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Mann-Whitney *U* test and Spearman rank correlation to test for similarities and differences between the RSV-positive group [RSV (+)] and the RSV-negative group [RSV (-)]. The association between RSV and atopy, atopy and wheeze, atopy and developing asthma were analyzed by using Fisher's exact test. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

SUBJECTS

The backgrounds of RSV (+) patients (*n* = 70) and RSV (-) patients (*n* = 40) are shown in Table 1. There was no significant difference in the mean ages of RSV

Table 1 Subject background and prognosis: Atopy/non-atopy was defined by a specific IgE antibody to common allergens. Transient wheeze was wheeze experienced less than 3 times post-infection over a period of 1 year. Asthma was diagnosed by a pediatrician.

	RSV (+)		RSV (-)	
n (female)	70 (33)		40 (20)	
Atopy	13 (18%)		5 (13%)	
Average age	1.22 ± 0.86		1.11 ± 0.78	
Wheezing	+	-	+	-
N (admission)	54 (34)	16 (7)	20 (11)	20 (4)
Prognosis				
Atopy/non-atopy	13/34	5/7	5/10	1/18
Transient wheeze	14 (30%)	0	7 (47%)	0
Asthma	7 (15%)	2 (17%)	8 (50%)	4 (21%)

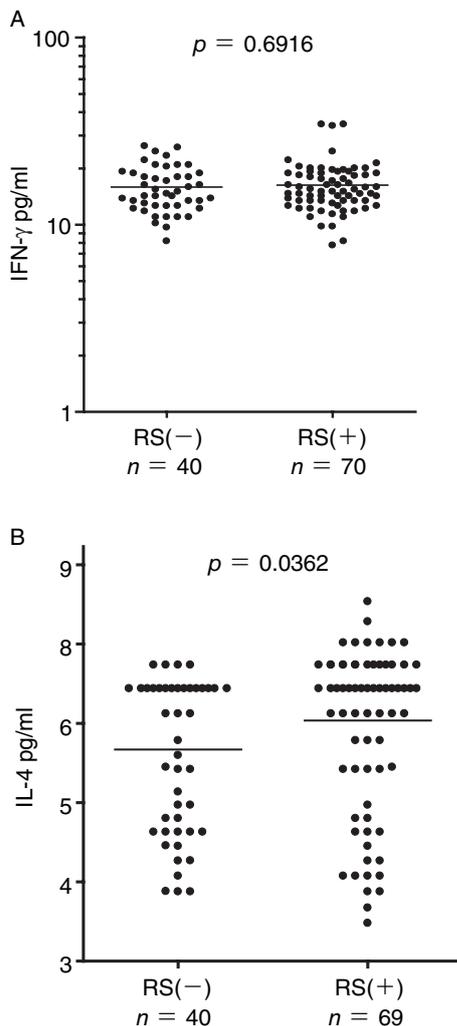


Fig. 1 IFN- γ and IL-4 levels in RSV (+) and RSV (-) patients: **(A)** For IFN- γ , there was no significant difference between the groups ($p = 0.6916$). **(B)** IL-4 was increased to a greater extent in RSV (+) than in RSV (-) patients ($p = 0.0362$) (Mann-Whitney U test).

(+) and RSV (-) patients, (1.22 ± 0.86 years and 1.11 ± 0.78 years, respectively). Four children with RSV (+) were given a diagnosis of asthma by a pediatrician. Thirteen children (18%) in the RSV (+) group and 5 (13%) in the RSV (-) group were atopic when NPAs were collected. There was no significant association between RSV and atopy ($p = 0.5004$). Furthermore there was no significant association between atopy and wheeze when the NPAs were collected ($p = 0.7636$). The presence of wheeze was significantly higher in RSV (+) children ($n = 54$, 77%) than in RSV (-) children ($n = 20$, 50%). There was no patient in this study who needed tracheal intubation or mechanical ventilation. Among the RSV (+) patients, 41 children (59%) were admitted, whereas among the RSV (-) patients 15 children (38%) were admitted to the hospital.

MEASUREMENT OF CYTOKINES AND CHEMOKINES

IFN- γ is the representative Th1-type cytokine which suppresses the production of Th2-type cytokines and enhances cellular immunity. Unexpectedly, we observed no significant difference in the levels of IFN- γ between the two groups ($p = 0.6916$) (Fig. 1A). IL-4, which is one of the Th2-type cytokines, induces the proliferation of Th2-type cells and suppresses the development of Th1-type cells. Moreover IL-4 induces IgE production and is strongly associated with allergies. We excluded one patient because the IL-4 level was below the detection limit. We found no background differences in this patient. Although the concentration range of IL-4 was very narrow, we observed a small but significant increase in the level of IL-4 in RSV (+) patients as compared with RSV (-) patients ($p = 0.0362$) (Fig. 1B).

IL-10 is one of the inhibitory cytokines produced by regulatory T cells. IL-10 was shown to down-regulate production of not only Th2-type cytokine such as IL-4 but also Th1-type cytokine such as IFN- γ .^{17,18} Although we excluded 38 patients, 21 in the

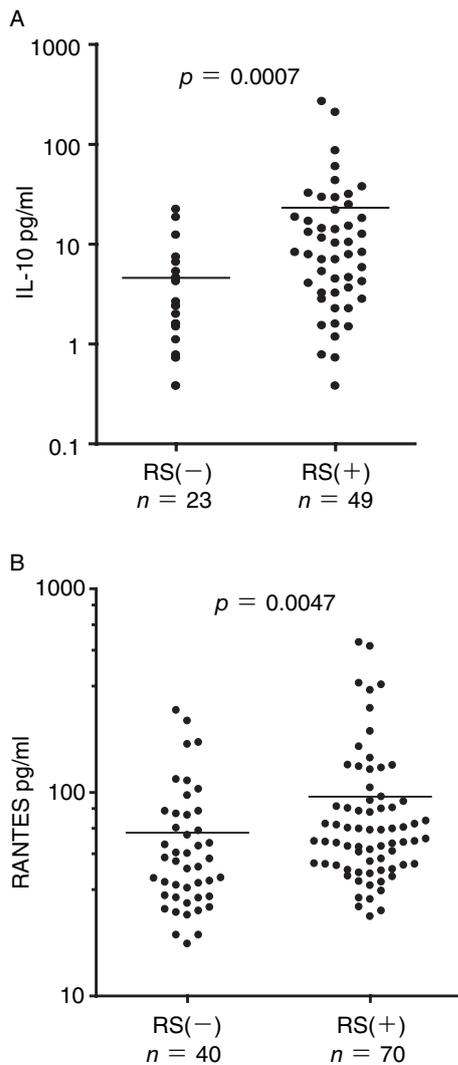


Fig. 2 IL-10 and RANTES levels in RSV (+) and RSV (-) patients: **(A)** IL-10 and **(B)** RANTES were increased to a greater extent in RSV (+) than in RSV (-) patients ($p = 0.0007$ and $p = 0.0047$, respectively; determined by the Mann-Whitney U test).

RSV (+) group and 17 in the RSV (-) group, because their levels were below the limit of detection, we found no differences in the backgrounds of these patients. The levels of IL-10 in the RSV (+) patients were significantly higher than in the RSV (-) patients ($p = 0.0007$) (Fig. 2A).

RANTES is one of the inflammatory chemokines which is thought to be secreted by airway epithelial cells or by Th2-type cells. RANTES is a potential chemoattractant for Th2-type cells and eosinophils. In our study, the levels of RANTES were significantly increased in the RSV (+) patients compared with the RSV (-) patients ($p = 0.0047$) (Fig. 2B).

We compared cytokine levels between patients with wheeze and without wheeze when NPAs were

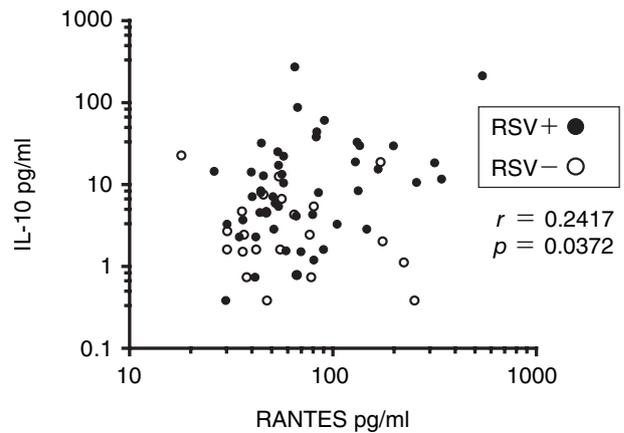


Fig. 3 Correlation between IL-10 and RANTES (1): In all subjects, IL-10 and RANTES were correlated ($r = 0.2417$, $p = 0.0372$; Spearman rank correlation).

collected. In the level of IL-4, IL-10 and RANTES there was no difference between the groups ($p = 0.5724$, $p = 0.7474$, and $p = 0.6672$, respectively). We also compared cytokine levels between atopic and non-atopic patients. There was no difference between the groups (IL-4; $p = 0.9948$, IL-10; $p = 0.3743$ and RANTES; $p = 0.0645$, respectively).

CORRELATION BETWEEN IL-10 AND RANTES

Both IL-10 and RANTES were elevated in the NPAs of RSV (+) children. We analyzed the relationship between IL-10 and RANTES. Expectedly, we found a positive correlation between RANTES and IL-10 in all patients. ($r = 0.2417$ $p = 0.0372$) (Fig. 3). Interestingly the positive correlation between IL-10 and RANTES was significant in the RSV (+) patients ($r = 0.3662$, $p = 0.0097$) (Fig. 4A), but not significant in the RSV (-) patients ($p = 0.3579$) (Fig. 4B).

RELATIONSHIP BETWEEN CYTOKINE AND CHEMOKINE PROFILES, AND PROGNOSIS

After 1 year, 93 patients: 59 RSV (+) and 34 RSV (-), were reevaluated by reference to hospital records or by interview. The prognosis of each case was determined as follows: 1) patients who had wheeze less than 2 times in the past year (transient wheeze), 2) patients who had wheeze 2 times (recurrent wheeze) or were given a diagnosis by a physician of asthma in the past year, and 3) patients who did not have wheeze. Fourteen RSV (+) patients with wheeze (30%) and 7 RSV (-) patients with wheeze (47%) experienced transient wheeze (Table 1). In RSV (+) subjects 15% of patients with wheeze and 17% without wheeze were given a diagnosis of asthma. There was no significant association between atopy and the development of asthma ($p = 0.4951$). Among RSV (-) subjects 50% of patients with wheeze were given a diagnosis of asthma. However, only 21% of patients

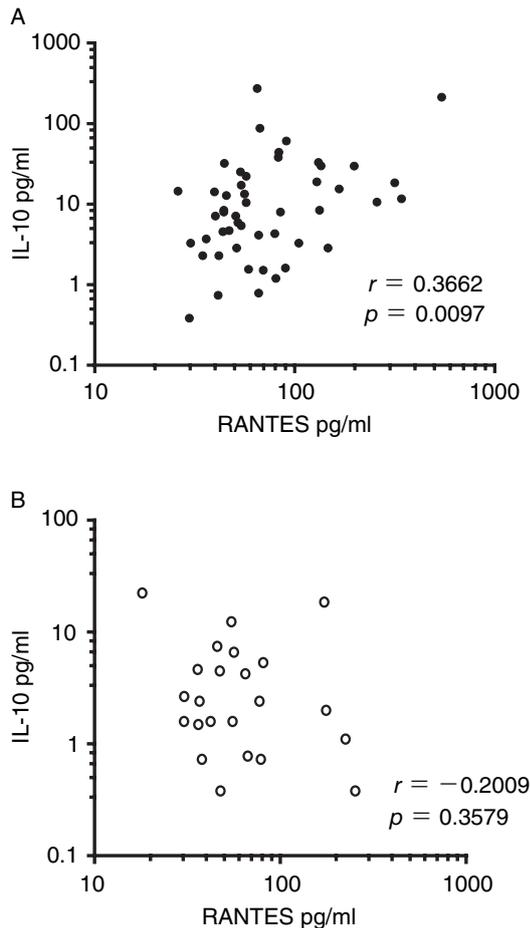


Fig. 4 Correlation between IL-10 and RANTES (2): (A) In RSV (+) patients, there was a significant positive correlation between IL-10 and RANTES ($r = 0.3662$, $p = 0.0097$). (B) In RSV (-) patients, IL-10 was not significantly correlated with RANTES.

without wheeze were given a diagnosis of asthma. In both the RSV (+) and RSV (-) groups, patients without wheeze did not experience wheeze. We re-evaluated the level of IL-4, IL-10, INF- γ , and RANTES in NPAs with asthma or recurrent wheeze. There were no significant differences between patients with asthma and recurrent wheeze or without. Moreover we found no significant correlation between atopic status and RSV infection.

DISCUSSION

In this study we demonstrated the shift of pro-inflammatory cytokine and chemokine production with RSV infection, based on the following observations. Firstly, the levels of IL-4, IL-10, and RANTES in the NPAs were all elevated in patients with RSV infection. Secondly, there was no significant difference between the RSV (+) and (-) patients with respect to the levels of INF- γ in the NPAs. Thirdly, IL-10 was significantly correlated with RANTES in RSV (+) patients,

but not in RSV (-) patients.

Recurrent wheeze following RSV-induced bronchiolitis is thought to be one of the predisposing factors in the development of asthma. Approximately 30% of children with RSV infection develop bronchiolitis. The impact of RSV infection on the health-related quality of life is critical.¹⁹ The mechanisms of post-bronchiolitis wheezing induced by RSV infection have been reported in many studies. Copenhagen *et al.* reported that cord blood INF- γ responses were inversely related to the frequency of viral respiratory infections.²⁰ Severe respiratory failure was observed in RSV-infected infants with lower production of INF- γ , indicating that immunological clearance of the virus could be affected in these patients.²¹ In contrast to previous findings, we observed no difference in the INF- γ levels between RSV (+) and RSV (-) children (Fig. 1A). Our patients had only mild to moderate respiratory problems, thus this discrepancy might be attributable to the difference in clinical severity compared with the previous studies.

On the other hand, we observed a small but significant elevation of IL-4 in patients with RSV infection (Fig. 1B). Kristjansson *et al.* reported that the level of IL-4 was significantly higher in RSV-infected infants of less than 3 months of age compared with those above 3 months.²² Since we analyzed patients of all ages together in this study, it may explain the observed small difference in the IL-4 levels. IL-4 is an important Th2 cytokine and is associated with airway hyperresponsiveness.²³ The increased production of IL-4 can affect local responses to allergens.²⁴ As a consequence, asthma-like symptoms can be enhanced by a shift in the immune system from Th1 to Th2. However, the elevation of IL-8 production in blood rather than a skewing of the Th1/Th2 cytokine balance, such as the switch from INF- γ to IL-4 production from peripheral lymphocytes, was reported to be a marker for severity in RSV infection.²⁵ This might be due to the difference in the local and systemic production of cytokines in response to viral infections.²⁶ Further investigations will be needed to clarify these mechanisms.

We observed a significant increase in the IL-10 levels in RSV (+) patients (Fig. 2A). IL-10 is thought to be produced by Th2-type cells. However, recent investigations indicate that IL-10 is produced by T regulatory cells.²⁷ IL-10 has been demonstrated to suppress the production and response of INF- γ and IL-4.²⁸ Bont *et al.* reported that IL-10 produced by monocytes in RSV-infected infants was implicated in post-bronchiolitis wheeze.²⁹ Other viruses in addition to RSV, such as rhinovirus, also induced the expression of the IL-10 gene in sputum from patients with acute asthma attacks.³⁰ In an animal asthma model, IL-10 has been reported to be the essential cytokine contributing to airway hyperresponsiveness.^{31,32} Taken together these observations indicate that IL-10 changes

the immune response and thereby affects airway reactivity. This finding is in contrast to a previous study by Chung *et al.*³³ They investigated only RSV bronchiolitis and reported that the levels of IL-10 were significantly lower in patients with atopy than in those without atopy. In our patients with subsequently diagnosed asthma or recurrent wheezing, we observed no significant difference in the levels of IL-10 between RSV (+) and RSV (-) individuals. Thus, multiple factors may be involved in the development of asthma or recurrent wheeze post RSV infection.

The chemokine RANTES plays important roles in the pathogenesis of asthma.³⁴ In this study, RANTES was significantly higher in RSV (+) children than in RSV (-) children (Fig. 2B). Chung *et al.* reported that RANTES and eosinophil cationic protein in the NPAs of RSV-bronchiolitis patients were significantly higher than in controls.³⁵ They also indicated that RANTES was significantly increased in infants who had experienced subsequent wheezing. They concluded that RANTES could have predictive value with respect to the development of airway hyperactivity.³⁴ In our study we did not show any difference in RANTES levels of patients with subsequent wheezing with RSV infection. *In vivo* studies have demonstrated that different kinetics were involved in the production of RANTES post RSV infection, thus this may account for the comparable RANTES levels observed in the present study.

After 1 year, we re-evaluated the study subjects and found that IL-4, IL-10 and RANTES levels were not correlated to the atopic status or the development of asthma. In early childhood, virus infection was observed to be one of the risk factors for developing asthma at 7 years of age.⁴ Thus, we will need to undertake careful re-evaluation in the future.

We observed a significant positive correlation between IL-10 and RANTES in the RSV (+) patients, but not in the RSV (-) patients. There was no correlation between IL-10 and either IL-4 or IFN- γ , suggesting that at an early phase of RSV infection IL-10 may not affect Th1 or Th2 cell cytokine production. On the other hand, IL-10 regulates IL-4 and IFN- γ within normal levels, but does not affect RANTES in the early phase. Jafri *et al.* measured the cytokine levels after inoculation of RSV in BALB/c mice. They found time differences in the production of each cytokine.³⁶ We measured cytokine levels not in the recovery phase but in the acute phase. In contrast, Legg *et al.* reported that the ratio of IL-4 to IFN- γ increased in acute bronchiolitis to a greater extent than in RSV-induced upper respiratory infection. They also indicated that the ratio of IL-10 to IL-12 was significantly higher in patients with acute bronchiolitis than in those with upper respiratory infection.¹⁰ Recently, Heaton *et al.* studied the *in vitro* T-cell response patterns associated with different wheezing phenotypes in children. They reported that bronchial hyperre-

sponsiveness in non-atopic patients was associated with an elevation of antigen-specific and polyclonal IL-10 production.³⁷ Thereby the new findings of implication with IL-10 and RANTES in our study may play an important role in the pathophysiological mechanisms associated with RSV infection.

CONCLUSION

We conclude that IL-10 and RANTES increase in the NPAs of patients with RSV infection and that they act synergistically. Further study of the immunological mechanism between Th1/Th2-type cells and regulatory T cells will be needed to clarify the phenotype of wheeze in RSV infection.

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