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Plasma Endothelin-1 in Infants and Young Children with Acute Bronchiolitis and Viral Pneumonia

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SUMMARY **Respiratory syncytial virus (RSV) infections that occur during the first three years of life have been demonstrated to be associated with the development of childhood asthma. The mechanism of virus-triggered airway inflammation is not fully understood. Endothelin-1 is a potent bronchoconstrictor involved in many diseases including respiratory tract infections. Infants and young children diagnosed with either viral pneumonia or acute bronchiolitis, their age ranging between 2 months and 3 years, were recruited into this study. Nasopharyngeal aspirates were taken for detection of respiratory virus by antigen immunofluorescence stain, RT-PCR analysis and viral culture. Plasma endothelin-1 (ET-1) was measured by using a commercially available enzyme-linked immunosorbent assay (ELISA). Ten of the nineteen infants and children (52%) were positive for RSV infection, one co-infected with influenza A. Nine infants (90%) were positive for RSV subtype A. There was only one infant with subtype B. One of the RSV negative individuals was positive for influenza A. In addition, we recruited 10 patients without chronic underlying or respiratory tract illness as controls. ET-1 levels were significantly increased in RSV infection compared to the controls (3.6 ± 1.2 and 1.2 ± 1 pg/ml, respectively ($p < 0.05$). In conclusion, infants and young children who are infected with RSV have an increase in circulating plasma endothelin-1. This in turn may contribute to the subsequent development of childhood asthma.**

Acute bronchiolitis and viral pneumonia consume a substantial amount of resources of primary healthcare systems throughout the world.[1-3] Respiratory syncytial virus (RSV), a major pathogen within the paramyxovirus family, causes severe lung disease in young children as well as immunocompromised individuals. It is the most frequent cause of acute bronchiolitis and pneumonia in infants and young children requiring hospitalization.[4-5]

Studies done during the past few decades have expanded our knowledge extensively regarding the specific mechanisms involved in the pathogenesis of RSV bronchiolitis and subsequent chronic obstructive airway disease. It is known that RSV bronchiolitis and subsequent development of asthma may be triggered by Th2-type cytokines.[6] The airway's epithelial cells are the primary target cells for RSV infection. A growing body of evidence suggests that the epithelium is not only a physical barrier, but also has the potential to synthesize a variety of cytokines, e.g. interleukin-8 (IL-8), granulocyte macrophage-colony stimulating factor (GM-CSF) and transforming growth factor (TGF). During acute RSV infection the immune response may induce long-lasting detrimental effects, thereby contributing to post bronchiolitis wheezing.[7-9]

Endothelin-1 (ET-1) is another important cytokine with a major impact on asthmatic patients. It is an endothelial regulatory peptide present in pulmonary tissue where it exerts several biological effects both on bronchial and vascular smooth

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muscle cells.[10-11] Recent reports suggest that airway epithelial cells are capable to release this active peptide. It acts as a potent bronchoconstrictor in isolated human airways. In several studies, it has been shown to be increased in bronchoalveolar lavage fluid of asthmatic patients. The level of ET-1 was correlated with the severity of airway obstruction.[12-13] Our recent publication also demonstrated the relation between RSV infection and ET-1 release in pulmonary epithelial cell cultures.[14] The expression of endothelin-1 after RSV infection may be an important step in understanding the relation between viral infection, especially RSV and the development of asthma. Thus, this study attempted to validate our previous experiment which was undertaken in an *in vitro* setting.

MATERIALS AND METHODS

Design

We used a prospective trial to identify the relation between plasma ET-1 and acute bronchiolitis or viral pneumonia in infants and young children. The protocol of the study was approved by the Ethics Committee, Faculty of Medicine, Chulalongkorn University and informed consent was obtained from the parents or legal guadians.

Setting

Tertiary Care Referral Center (Chulalongkorn University Hospital, Bangkok, Thailand).

Population study

Patients and controls

Nineteen infants and children aged between 1 month and 3 years admitted with either acute bronchiolitis or viral pneumonia and another ten children without underlying chronic or other respiratory tract illnesses serving as controls were recruited into our study.

Definitions, inclusion and exclusion criteria

Diagnostic criteria for acute bronchiolitis or viral pneumonia were based on clinical grounds combined with chest radiographs.[15-16] Acute bronchiolitis was diagnosed if the following criteria were met: Patients aged between 1 month and 2 years presenting with a history of upper respiratory tract symptoms with or without fever; chest auscultation was positive for rhonchi or wheezing, and the chest radiograph showed hyperinflation.

Viral pneumonia was diagnosed if the following criteria were met: Upper respiratory tract symptoms with fever, tachypnea, upon chest auscultation rales, rhonchi or wheezing and interstitial infiltration in the chest radiograph.

Patients with the following criteria were excluded from our study: Previous hospital admissions due to respiratory tract illness, chronic respiratory tract illness, congenital heart disease, history of prematurity and pre-existing immunodeficiencies.

The diagnosis was confirmed by a pediatrician before deciding whether the subjects were eligible for the study. The chest radiographs were interpreted by radiologists who were not aware of the admission diagnosis.

Demographic and clinical data

Information on demographic and potentially confounding variables collected for infants included sex, race, age at admission, prior wheezing history, socioeconomic status and smoking by the mother or other members of the household.

Specimen collection

Within 48 hours after admission, specimens were collected by pharyngeal washing and divided into aliquots for different analyses. Venous blood samples were obtained and collected in ethylenediamine tetraacetic acid (EDTA) coated tubes. Tubes were immediately placed on ice. Blood samples were then centrifuged at 1,500 x *g* for 10 minutes and plasma was separated and stored at -70°C until testing.

RSV detection

RSV antigen detection

Diagnosis of RSV infection was done by antigen detection using a commercially available direct immunofluorescence monoclonal antibody test (Dakopatt, Glostrup, Denmark), reverse transcription-polymerase chain reaction (RT-PCR) analysis and confirmed by viral culture. Nasal wash specimens were used. RSV suptypes A or B were detected and differentiated by competitive RT-PCR.

Detection of RSV by RNA and subtyping

RNA was extracted from a 150 µl sample volume by the guandinium isothiocyanate-phenol-chloroform method described by Gilbert *et al.*[17] After isopropanol precipitation and washing with 75% ethanol, RNA samples were dried under vacuum and dissolved in 10 µl of 0.1% diethylpyrocarbonate in distilled water. To prepare cDNA, samples were heated to 65°C for 5 minutes and quick-chilled on ice for 2 minutes. Then 20 µl of cDNA mixture containing the RNA sample, 250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$, 50 mM

DTT, 20 U of RNase inhibitor and 200 U of multireverse transcriptase (Promega, Wi, USA) were incubated at 37°C for 1 hour.

The following primary primers were used in the study: RSV AB F, 5'-GTCTTACAGCCGTGATTAGG-3'; RSV AB R, 5'- GGGCTTTCTTTGGTTACTTC-3'. The secondary primers used were: RSV A F, 5'-GATGTTACGGTGGGGAGTCT-3'; RSV A R, 5'- GTACACTGTAGTTAATCACA-3'; RSV B F, 5'-AATGCTAAGATGGGGAGTTC-3'; RSV B R, 5'- GAAATTGAGTTAATGACAGC-3'.[18] Each primer pair was used at a concentration of 10 pmol. For the primary PCR, 5 µl of cDNA were added to 45 µl of a reaction mixture containing 10 x buffer, 25 mM $MgCl_2$ and 5 U of *Taq* DNA polymerase (Qiagen, CA, USA). The amplification reaction consisted of 1 cycle at 94°C for 2 minutes followed by 35 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 2 minutes. Five microliters of primary product was then transferred to 45 µl of the secondary amplification mixture as described above. The samples were then incubated for 1 cycle at 94°C for 2 minutes followed by 35 cycles at 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute. Amplicons were visualized by ethidium bromide staining following electrophoresis on 2% agarose gels.

Beta actin mRNA was used as an internal control. The primers were used as follows: forward, 5'-CCTTCCTGGGCATGGAGTCCT-3'; reverse, 3'-GGAGCAATGATC-TTGATCTTC-5'.[19]

Plasma endothelin-1 measurement

ET-1 levels were determined by a commercially available ELISA (R&D Systems, MN, USA) according to the manufacturer's recommendations.

Statistical analysis

All data are presented as means ± SD, where applicable. Data were analyzed by non-parametric Mann-Whitney U test to compare the mean values between two groups. A $p < 0.05$ was accepted as statistically significant.

RESULTS

Twenty nine infants and children were enrolled in the study. Ten of 19 patients were positive for RSV (52.5%) and nine patients were negative for RSV (47.5%); one with a co-infection of influenza A. Ten patients without chronic underlying diseases of the respiratory tract were recruited as controls. Among the RSV negative individuals, one of 19 patients (5%) was positive for influenza A by viral culture and developed respiratory failure. Eight out of ten (80%) RSV positive patients were detected by immunofluorescence staining (IFA) and nine out of ten RSV positive patients (90%) were detected by RT-PCR technique. Table 1 shows the demographics of patients enrolled in this study. Among those patients negative for RSV, there were 8 infants (42%) diagnosed with acute bronchiolitis and the remaining ones with viral pneumonia. A surprisingly high number of initial antibiotics were used in these children (32%).

The level of plasma ET-1 was significantly increased in infants positive for RSV compared to the controls (3.6 ± 1.2 pg/ml *vs* 1.2 ± 1.0, $p = 0.002$). Plasma ET-1 was also significantly increased in RSV-negative infants with acute bronchiolitis or viral pneumonia (2.38 ± 1.4 pg/ml, $p < 0.03$) compared to the controls. In addition, the RSV-positive infants had significantly higher ET-1 levels compared to the RSV-negative ones ($p < 0.02$). The level of ET-1 in the controls was measured at 1.2 ± 1 pg/ml (Table 1, Fig. 1). This was comparable with previously published data.[20,26-27] Two infants (10.5%) subsequently developed respiratory failure and required mechanical ventilation. One

Table 1 Comparison of the study population, demographics and plasma endothelin-1 levels (pg/ml) of patients and control

	RSV positive	RSV negative	Control	*p-value* compared to control
Number	10	9	10	-
Sex M:F	4:6	7:2	5:5	-
Age (months, mean ± SD)	10.4 ± 7.6	11.5 ± 4.9	84 ± 8.5	< 0.05
Plasma ET-1 (pg/ml)	3.6 ± 1.2	2.38 ± 1.4	1.2 ± 1.0	< 0.05

of them was positive for RSV and another was positive for influenza A. Almost all RSV-positive infants (9/10) displayed subtype A. Only one of ten showed RSV subtype B. Fig. 2 demonstrates PCR bands of RSV subtype A presented at 343 bp and RSV subtype B presented at 183 bp.

DISCUSSION

RSV is the main cause of hospitalization for respiratory tract infection in young children worldwide.[21-22] It was noted that recurrent episodes of wheezing followed an episode of bronchiolitis in infancy. Rooney and Williams[23] monitored 62 children for 2 to 7 years after hospitalization with RSV proven bronchiolitis. They previously reported that 56% of children had further episodes of wheezing and 43% had multiple episodes. Subsequently, a number of controlled studies were carried out.[24] However, the mechanism of viral infection especially RSV and the development of asthma is not clearly understood. This study was undertaken to evaluate the relationship of RSV infection and the production of ET-1. Behera *et al.* showed that RSV can induce the expression of 5-LO and endothelin-1 in the epithelial cells leading to the production of leukotrienes.[25] We observed a significant increase in production of ET-1 comparing the RSV positive group, the RSV negative group and the control. Our study demonstrated the relationship of RSV infection and ET-1 production in infants and children with acute bronchiolitis and viral pneumonia, although we did not follow ET-1 plasma levels during their hospitalization. This may further substantiate our data. There were some discrepancies in ages between the control and the target group. However, previous data have shown that the level of plasma ET-1 could be physio-

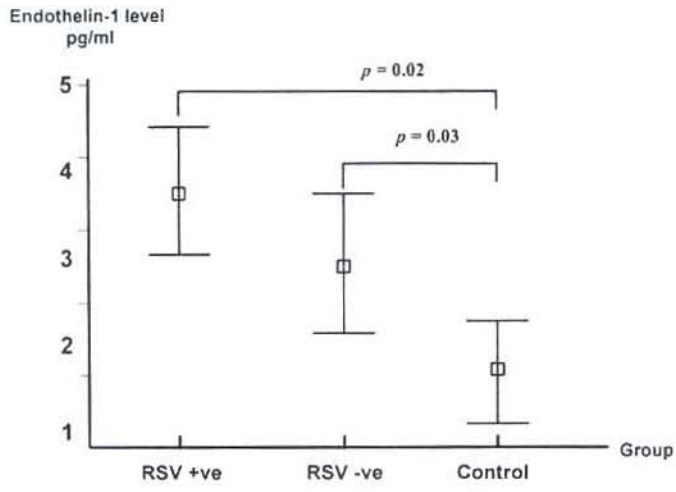

Fig. 1 Comparison of endothelin-1 levels among RSV positive patients, RSV negative patients and controls

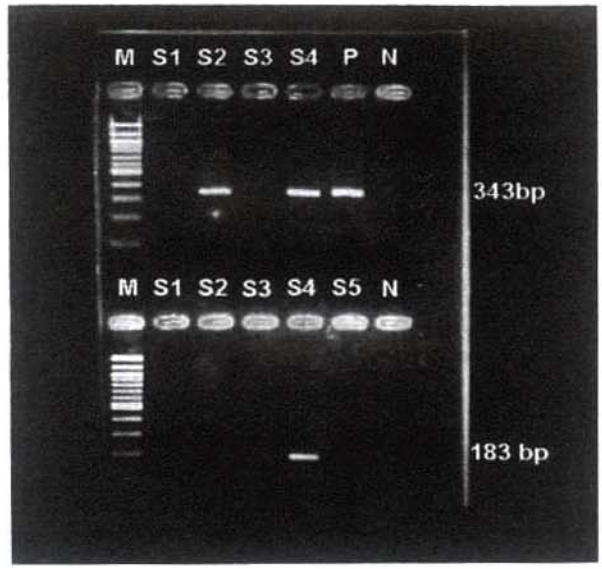

Fig. 2 RSV subtype determination from nasal wash specimens in patients with acute bronchiolitis or viral pneumonia by using RT-PCR technique. Amplified PCR products were analyzed by 2% agarose gel electrophoresis. Upper panel from left to right: lane M, a 100 bp DNA ladder marker; lanes S2, S4 and P (positive controls), positive bands for RSV subtype A present at 343 bp; lanes S1, S3 and N (negative control), negative for RSV type A. Lower panel from left to right: lane M, a 100 bp DNA ladder marker; lanes S1-3, S5 and N (negative controls), negative for RSV subtype B; S4, positive for RSV subtype B. All Beta actin bands were present in each PCR product (data not shown).

ologically elevated during the neonatal period and subsequently decreased to very low level overtime (~1 pg/ml).[26-27] Therefore, the difference in ages between the two groups should have no adverse effect on overall results.

RSV infection has been associated with a T-cell response characterized primarily by the production of cytokines by type 2 helper T cells. The same response was observed during episodes of asthma.[28] Both are characterized by the recruitment of T-cells and eosinophils and the release of soluble mediators, such as histamine, kinin and other leukotrienes. Endothelin-1 is a newly isolated vasoactive peptide. Recently, it was shown that the lung is also the main source of ET-1 production. ET-1 can also stimulate fibroblast proliferation, collagen deposition and contribute to airway wall thickening which underlies bronchial hyperresponsiveness.[29-30] Little is known about the control of ET-1 expression by airway epithelial cells. However, potent proinflammatory cytokines such as interleukin-1, TNF-α or LPS can stimulate the release of ET-1.[31-32] Our previous data suggested that different RSV infectivity may change the production of ET-1.[14] Furthermore, our results demonstrated the predominance of RSV subtype A over subtype B in infants with bronchiolitis or viral pneumonia in Thailand. The RSV subtype A infection was reported to be more prevalent and clinically severe than subtype B.[33-34] In Thailand, Tantivanich *et al.* previously demonstrated RSV subgroup determination by RT-PCR in 50 infants and children with suspected respiratory infections,[34] although they did not differentiate their patients into acute bronchiolitis or viral pneumonia which might be important. Thus our report determined RSV subgroups by using RT-PCR technique in these young children in relation to the production of ET-1. However, there were some limitations in our study. The RSV culture was positive in only 50% (5/10) of the cases. This could be explained from the difficulty of handling and the long delay in transport of nasopharyngeal specimens to virology laboratory which is located in another institution. Besides, RSV is very hard to isolate. Thus our RSV detection is based primarily on either positive by the RT-PCR technique or immunofluorescence staining.

In conclusion, this study suggests that RSV infection has a relationship with the production of ET-1. These findings may be beneficial in future vaccine development.[35-36] Even though our data cover a small group of infants but the findings may have major clinical implications. The presence of this important cytokine from epithelial cells may imply subsequent development of asthma. Its regulation might serve as a potential novel treatment in asthmatic patient. Further large clinical trials together with applied basic science research seem warranted.

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