Correlation between inflammatory mediators in the nasopharyngeal secretion and in the serum of children with lower respiratory tract infection caused by respiratory syncytial virus and disease severity*

Correlação entre mediadores inflamatórios na secreção nasofaríngea e no soro de crianças com infecção do trato respiratório inferior por vírus sincicial respiratório e a gravidade da doença

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Abstract

Objective: To determine whether the concentrations of inflammatory mediators (CCL5, soluble intercellular adhesion molecule type 1 [sICAM-1], TNF-α, IL-6 and IL-10) in the nasopharyngeal secretion and in the serum of children with lower respiratory tract infection (LRTI) caused by respiratory syncytial virus (RSV) correlate with the clinical markers of disease severity. **Methods:** Between July of 2004 and December of 2005, 30 children less than three months of age, diagnosed with LRTI caused by RSV and admitted to a neonatal ICU, were included in this study. **Results:** The severity of disease at hospital admission, as determined with a modified clinical scoring system, presented a significant positive correlation with sICAM-1 and IL-10 concentrations in the nasopharyngeal secretion, as well as with IL-6 concentrations in the serum, of the patients. In addition, serum IL-6 concentrations presented a significant positive correlation with the duration of oxygen therapy and with the length of hospital stay. **Conclusions:** At hospital admission, the concentrations of sICAM-1 and IL-10 in the nasopharyngeal secretion, as well as the concentration of IL-6 in the serum, could be used as markers of severity in patients with LRTI caused by RSV. The serum levels of IL-6 determined at admission could also be used to predict prolonged oxygen supplementation and hospital stay.

Keywords: Respiratory syncytial virus, human; Chemokine CCL5; Intercellular adhesion molecule-1; Interleukin-6; Interleukin-10; Tumor necrosis factor-alpha.

Resumo

Objetivo: Avaliar se as concentrações dos mediadores inflamatórios (CCL5, *soluble intercellular adhesion molecule type* 1 [sICAM-1], TNF-α, IL-6 e IL-10) na secreção nasofaríngea e no soro de crianças com infecção do trato respiratório inferior (ITRI) por vírus sincicial respiratório (VSR) apresentam correlação com os marcadores clínicos de gravidade da doença. **Métodos:** Entre julho de 2004 e dezembro de 2005, 30 crianças com idade inferior a três meses, diagnosticadas com ITRI por VSR e admitidas em uma UTI neonatal foram incluídas neste estudo. **Resultados:** Houve uma correlação positiva significante entre a gravidade da doença na admissão hospitalar, determinada por um sistema de escore clínico modificado, e as concentrações de sICAM-1 e de IL-10 na secreção nasofaríngea e de IL-6 no soro dos pacientes. Houve também uma correlação positiva significante entre a concentração de IL-6 no soro e o tempo de oxigenoterapia e a duração da internação. **Conclusões:** As concentrações de sICAM-1 e IL-10 na secreção nasofaríngea e de IL-6 no soro determinadas na admissão poderiam ser usadas como marcadores de gravidade da ITRI por VSR. Os níveis de IL-6 determinados no soro na admissão também poderiam ser usados para predizer o prolongamento da oxigenoterapia e da duração da internação.

Descritores: Vírus sincicial respiratório humano; Quimiocina CCL5; Molécula 1 de adesão intercelular; Interleucina-6; Interleucina-10; Fator de necrose tumoral alfa.

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Introduction

Lower respiratory tract infections (LRTIs) caused by respiratory syncytial virus (RSV) constitute one of the most common and most severe types of diseases presenting in the first months of life,[1] especially in children less than six weeks of age and in those who are born prematurely.[2] The RSV accounts for 50-90% of the cases of bronchiolitis and for approximately 50% of all childhood pneumonias, principally in the autumn and winter months.[3-5]

In vitro studies and studies of children with RSV infection have demonstrated that the epithelial cells of the airways and the alveolar macrophages produce various inflammatory mediators, such as prostaglandins, leukotrienes, cytokines (TNF-α, IFN-, IL-1, IL-2, IL-6 and IL-10), chemokines (IL-8 and CCL5), soluble intercellular adhesion molecule type 1 (sICAM-1) and growth factors.[6,7] Lower concentrations of Th1 cytokines, such as TNF-α, and higher concentrations of Th2 cytokines, such as IL-6, have been described in the acute phase of severe disease caused by RSV.[8]

The cytokines TNF-α and IL-1, synthesized by respiratory epithelial cells infected with RSV, activate the cascade of pro-inflammatory mediators, as well as promoting recruitment, migration and adhesion of specific types of leukocytes (monocytes, neutrophils and T lymphocytes) to the tissues affected by the viral activity, with subsequent degranulation and an increase in tissue damage. Inflammatory cell activation initiates the production of new pro-inflammatory and anti-inflammatory mediators.[9] The increase in inflammatory mediators has a significant effect on the initial inflammatory response and on late immunological events.

In view of the high frequency, morbidity and mortality of childhood pulmonary infections caused by RSV, together with the importance of the inflammatory mediators CCL5, sICAM-1, TNF-α, IL-6 and IL-10 in the genesis of inflammatory and immunological processes, as well as the absence of studies conducted in Brazil, correlating the inflammatory response in the respiratory epithelium and in the serum of children less than three months of age with the severity of the respiratory disease caused by RSV, we decided to conduct a study to determine the role of these inflammatory mediators in the pathogenesis of LRTI caused by RSV in the first three months of age.

The objective of this study was to determine whether the concentrations of inflammatory mediators (CCL5, sICAM-1, TNF-α, IL-6 and IL-10) in the nasopharyngeal secretion and in the serum of children less than three months of age and diagnosed with LRTI caused by RSV correlate with disease severity.

Methods

In the period between July of 2004 and December of 2005, 30 children less than three months of age, diagnosed with LRTI caused by RSV, without comorbidities and admitted to the *Unidade de Cuidados Intensivos Neonatal* (UCINE, Neonatal Intensive Care Unit) of the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (HC-FMUSP, University of São Paulo School of Medicine *Hospital das Clínicas*) Institute for Children, were included in this cross-sectional study. Those children presented with bronchiolitis or pneumonia, or a combination of the two. During the study period, 39 children were admitted to the UCINE for LRTI. In our study, there was no control group of healthy children in the same age bracket due to ethical implications, such as the question of whether samples should be collected from suckling infants without respiratory symptoms. All parents or legal guardians agreed to their children's participation in the study.

Nine patients with LRTI and negative or inconclusive tests for RSV in the airways were excluded, as were those with RSV infection associated with cyanotic heart disease, bronchopulmonary dysplasia, gastroesophageal reflux disease, bacterial sepsis or a family history of atopy, as well as those with RSV infection receiving bronchodilators or corticosteroids, either alone or in combination. The diseases mentioned above, as well as the use of bronchodilators or anti-inflammatory agents, can alter the respiratory pattern and the levels of inflammatory mediators.

Clinical and radiological criteria were used in order to define LRTI. The clinical criteria included findings of tachypnea, cyanosis, chest retractions, rhonchi or diffuse or localized wheezing or crackles on physical examination of the chest, which was performed by one of the authors. The radiological criteria included diffuse lung hyper-

Table 1 - Modified clinical scoring system.

Score	Respiratory rate, breaths/min	Wheezing	Oxygen saturation	Accessory muscle recruitment
0	≤ 30	None	≥ 95%	None
1	31-45	End of exhalation (stethoscope auscultation)	90-94%	Minimal
2	46-60	Total exhalation and inhalation (stethoscope auscultation)	85-89%	Moderate
3	> 60	Exhalation and inhalation (no stethoscope required)	< 85%	Maximal

Score values and classification of severity: lower than or equal to 3: normal; from 4 to 6: mild; from 7 to 9: moderate; and from 10 to 12: severe. Adapted from De Boeck et al.[10]

inflation or interstitial/alveolar opacification, or a combination of the two.

The following clinical markers were used in order to evaluate the severity of the respiratory disease: a clinical scoring system (Table 1), adapted from De Boeck et al.[10]; duration of oxygen therapy; duration of mechanical ventilation; and length of hospital stay.

For each participant, we used specific protocols for pulmonary diseases, including evaluation of comorbidities, testing for respiratory viruses (RSV, influenza, parainfluenza, metapneumovirus and rhinovirus) and blood culture. Patients who had conjunctivitis prior to or concomitantly with the respiratory condition, as well as those with LRTI and whose mothers had leukorrhea at the time of delivery, were submitted to *Chlamydia trachomatis* serology. The study was approved by the Research Ethics Committee of the Department of Pediatrics and by the HC-FMUSP Research Ethics Committee. The parents or legal guardians of the children gave written informed consent after receiving information regarding the objectives of the study and the procedures to which their children would be submitted.

In order to measure the concentrations of the inflammatory mediators CCL5; sICAM-1; TNF-α; IL-6; and IL-10, nasopharyngeal aspirates and peripheral blood were collected in the first 24 h of the hospital stay, during routine respiratory therapy. From the children involved in the study, even from those who were intubated, we did not collect any secretions other than nasopharyngeal secretions. We did so in order to standardize the method for collecting respiratory secretion, since the collection of nasopharyngeal aspiration is a low-risk procedure, as well as being a procedure in which it is easy to obtain specimens that are immunologically relevant to the entire respiratory tract.

With the patient in the supine position, head on the midline, a disposable silicone tube (number six or eight) was introduced up to the posterior nasopharynx. In order to prevent any dilution-related differences among the specimens studied, we did not employ the previous administration of saline solution. This tube was connected to a plastic vial, and this vial was attached to a plastic extension tube connected to a vacuum pump. The entire procedure was performed in a sterile way, and we collected a quantity of nasopharyngeal secretion that was sufficient for processing. The vials containing the specimens collected were labeled, stored on ice and immediately taken to the laboratory.

A vial containing approximately 1 mL of nasopharyngeal aspirate was used in the testing for respiratory viruses by direct immunofluorescence with specific monoclonal antibodies (Respiratory Viruses Panel 1 Viral Screening & Identification Kit; Chemicon International Inc., Temecula, CA, USA) for rapid viral antigen detection (sensitivity of 80-90% and specificity > 95%) and viral isolation in culture of HEp-2 (human epidermoid carcinoma) cells, NCI-H292 (human lung mucoepidermoid carcinoma) cells, MDCK (Madin-Darby canine kidney) cells and Vero (African green monkey kidney) cells, with a sensitivity of 60-90% and a specificity of 100%, when the rapid test result was negative for respiratory viruses.[11] Therefore, we attempted to reduce the number of false-negative results to a minimum. Real-time RT-PCR was used in order to identify metapneumovirus and rhinovirus, as well as to confirm the presence of RSV.

Another vial, this one containing 2 mL of nasopharyngeal aspirate, and a tube with silicone gel containing 2 mL of peripheral blood were

sent to the laboratory for immediate centrifugation at 3,000 rpm for 5 min. The supernatant of nasopharyngeal secretion and the serum were fractioned into five plastic Eppendorf tubes, each containing 200 µL, and stored at −70°C for later determination of the concentration of inflammatory mediators.

The supernatant of nasopharyngeal secretion and the serum were defrosted, and the concentrations of CCL5, sICAM-1, TNF-α, IL-6 and IL-10 were determined by enzyme-linked immunosorbent assay using Quantikine kits (R&D Systems, Minneapolis, MN, USA), in accordance with the manufacturer instructions. The analysis of the levels of inflammatory mediators was performed in duplicate. The reading was performed by spectrophotometry with a 450-nm filter, and the lower limits of detection were as follows: CCL5, 8 pg/mL; sICAM-1, 15.6 pg/mL; TNF-α, 4.4 pg/mL; IL-6, 4.7 pg/mL; and IL-10, 3.9 pg/mL. The immunoenzymatic assays used presented intra-assay precision of 1-4% and inter-assay precision of 2-7%.

The concentrations of inflammatory mediators in the nasopharyngeal secretion and in the serum were compared using the nonparametric Mann-Whitney test. The presence of a correlation between the concentrations of inflammatory mediators (nasopharyngeal secretion and serum) and the clinical markers of the severity of the respiratory disease caused by RSV was investigated using Spearman's correlation coefficient.[12] The study was designed to identify clinically significant associations. The sample size (n = 30) was calculated based on the values obtained for the concentrations of CCL5, TNF-α and IL-6 (mean and standard deviation) by Chung & Kim[13] and by Wang et al.[14] The level of significance was set at $p < 0.05$. The database was created using Excel 2007, and the statistical program used was the Statistical Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Of the 30 patients with LRTI caused by RSV included in the study, 20 (67%) were male and 22 (73%) were born at term. The mean weight at hospital admission was 3,593 g (range, 2,000-4,820 g). The mean age at the onset of signs and symptoms was 24.3 days (range, 11-49 days), and the mean age at admission was 27.7 days (range, 12-50 days). Regarding diagnosis, 9 children (30%) had bronchiolitis, 3 (10%) had pneumonia and 18 (60%) had bronchiolitis accompanied by pneumonia. At hospital admission, as determined with the modified clinical scoring system, 16 patients (53%) had a normal to mild respiratory condition and 14 (47%) had a moderate to severe respiratory condition. The mean score was 6.4 (range, 1-12). During the UCINE stay, 26 patients (87%) required oxygen therapy, and the mean duration of oxygen supplementation was 8.5 days (range, 1-43 days). Mechanical ventilation was required in 10 children (33%), and the mean duration was 9.7 days (range, 3-32 days). The mean length of hospital stay was 11.9 days (range, 4-50 days). At hospital admission, 2 children (7%) with RSV infection had concomitant infection with the following etiologic agents: *Chlamydia trachomatis* (one case) and human metapneumovirus (one case). Two children (7%) developed sepsis by oxacillin-resistant, coagulase-negative *Staphylococcus* sp. during the hospital stay, and

Table 2 - Comparison of the concentrations of inflammatory mediators in the nasopharyngeal secretion and in the serum of children less than three months of age determined at admission and lower respiratory tract infection caused by respiratory syncytial virus.[a]

Inflammatory mediator	Concentrations, pg/mL		p
	Nasopharyngeal secretion[a]	Serum	
	(n = 30)	(n = 30)	
CCL5	240.8 (98.8-554.4)	842.7 (764.8-878.4)	< 0.001
sICAM-1	774.7 (529.0-1.062.0)	1.573.3 (1.513.0-1.631.0)	< 0.001
TNF-α	41.1 (12.5-258.1)	4.4 (4.4-4.4)	< 0.001
IL-6	209.4 (138.9-325.0)	11.6 (7.5-41.4)	< 0.001
IL-10	11.9 (11.9-57.4)	53 (53-53)	< 0.001

sICAM-1: soluble intercellular adhesion molecule type 1. [a]Results expressed as median (interquartile range).

Table 3 - Correlations of high concentrations of inflammatory mediators in the nasopharyngeal secretion and in the serum with clinical markers of the severity of the disease caused by respiratory syncytial virus.[a]

Inflammatory mediator	Type of sample	Modified clinical scoring system (used at admission)	Duration of oxygen therapy, days	Duration of mechanical ventilation, days	Length of hospital stay, days
		(n = 30)	(n = 26)	(n = 10)	(n = 30)
CCL5	Nasopharyngeal secretion	0.325 (0.079)	0.307 (0.128)	−0.253 (0.470)	0.096 (0.614)
	Serum	0.057 (0.765)	0.077 (0.708)	−0.025 (0.946)	0.113 (0.553)
sICAM-1	Nasopharyngeal secretion	0.401 (0.028)	0.224 (0.271)	−0.018 (0.973)	0.360 (0.051)
	Serum	−0.031 (0.871)	−0.173 (0.397)	−0.605 (0.067)	−0.042 (0.827)
TNF-α	Nasopharyngeal secretion	0.120 (0.527)	0.168 (0.412)	0.108 (0.759)	0.268 (0.152)
	Serum	−0.113 (0.552)	−0.148 (0.472)	−0.118 (0.733)	0.036 (0.848)
IL-6	Nasopharyngeal secretion	0.317 (0.088)	0.162 (0.430)	−0.408 (0.248)	0.143 (0.451)
	Serum	0.469 (0.009)	0.445 (0.023)	−0.222 (0.537)	0.572 (0.001)
IL-10	Nasopharyngeal secretion	0.412 (0.024)	0.271 (0.181)	−0.231 (0.514)	0.181 (0.337)
	Serum	−0.099 (0.604)	−0.122 (0.551)	−0.118 (0.733)	0.064 (0.735)

sICAM-1: soluble intercellular adhesion molecule type 1. r: Spearman's correlation coefficient. [a]Results expressed as r (p value).

3 (10%) developed urinary tract infection, two of which were cases of infection with *Escherichia coli* and one of which was a case of infection with *Enterococcus faecalis*. There were no deaths among the patients involved in the study. None of the children received ribavirin, bronchodilators or corticosteroids during the UCINE stay.

At hospital admission, the median concentrations of CCL5, sICAM-1 and IL-1 in the serum of the children with RSV infection were higher than were those in their nasopharyngeal secretion, in a statistically significant manner, whereas the median concentrations of IL-6 and TNF-α in their nasopharyngeal secretion were significantly higher (Table 2).

The analysis of the correlations between disease severity at hospital admission, as determined with the modified clinical scoring system, and the concentrations of inflammatory mediators in the nasopharyngeal secretion and in the serum (first specimens) of the patients with LRTI caused by RSV revealed that disease severity presented a significant positive correlation with the concentrations of sICAM-1 (r = 0.401; p = 0.028) and IL-10 (r = 0.412; p = 0.024) in the nasopharyngeal secretion, as well as with the concentrations of IL-6 (r = 0.469; p = 0.009) in the serum (Table 3).

Discussion

The prevalence of RSV bronchiolitis and RSV pneumonia is high in the pediatric population in all continents, there being a broad spectrum of clinical manifestations and pulmonary involvement of varying severity.[15-18] Studies of the response of inflammatory mediators during RSV infection have contributed to furthering the understanding of the pathogenesis of the disease caused by the virus and the understanding of the immune response.

Levels of CCL5, sICAM-1, TNF-α, IL-6 and IL-10 were detected in all of the nasopharyngeal secretion and serum specimens from the children with LRTI caused by RSV admitted to the UCINE, confirming the role of these inflammatory mediators in the pathogenesis of the disease. Our results were similar to those reported by other authors[19,20] and suggest that the pro-inflammatory mediators CCL5, sICAM-1, TNF-α and IL-6, as well as the regulatory cytokine IL-10, play a fundamental role in the local inflammatory response and in the RSV-induced systemic response, although mucosal immunity can be independent from the systemic response. We chose to study the inflammatory mediators CCL5, sICAM-1, TNF-α, IL-6 and IL-10 due to the importance of these cytokines in the

genesis of the inflammatory and immune processes caused by RSV and the absence of studies correlating the levels of these mediators with the severity of the respiratory disease caused by RSV in children less than three months of age.

The importance of cytokines and chemokines in the severity of LRTIs caused by RSV has yet to be fully elucidated. Previous studies have demonstrated that specific inflammatory mediators and their gene polymorphisms, as well as the imbalance in the immune response,[21] can contribute to the severity of the viral disease.[22] The proper production of pro-inflammatory and anti-inflammatory mediators promotes a potent antiviral activity, reducing the pathogenesis, the morbidity and the mortality of the respiratory disease caused by RSV. Younger age translates to a greater difficulty in regulating the production of pro-inflammatory and anti-inflammatory mediators.[23,24]

In Brazil, this is the first study to evaluate whether the concentrations of inflammatory mediators (CCL5, sICAM-1, TNF-α, IL-6 and IL-10) in the nasopharyngeal secretion and in the serum correlate with the clinical markers of the severity of LRTI caused by RSV in patients less than three months of age. In our study, the patients with higher concentrations of sICAM-1 and IL-10 in the nasopharyngeal secretion and higher concentrations of IL-6 in the serum (Table 3) presented with a more severe respiratory condition at hospital admission, as determined with the clinical scoring system adapted from De Boeck et al.[10] The patients with higher concentrations of IL-6 at hospital admission required prolonged oxygen therapy and hospital stays.

Our results suggest that the increased local and systemic production of certain pro-inflammatory and anti-inflammatory mediators (sICAM-1 and IL-10 by the respiratory epithelium and IL-6 by the mononuclear cells and neutrophils in the peripheral blood) in the acute phase of LRTI caused by RSV contributed to the more severe and prolonged clinical evolution of some patients. At hospital admission, the concentrations of sICAM-1 and IL-10 in the nasopharyngeal secretion, as well as the concentrations of IL-6 in the serum, proved to be appropriate markers of the severity of LRTI caused by RSV. The concentrations of IL-6 determined at admission were able to predict which patients would require prolonged oxygen therapy and hospital stay. Some authors[25-27] have also demonstrated that the increase in the concentrations of pro-inflammatory and anti-inflammatory mediators at the onset of the infection is related to the greater severity of the disease caused by RSV.

The association between high IL-10 levels in the nasopharyngeal secretion and the severity of the respiratory condition possibly occurred due to the immunosuppressive behavior of this cytokine.[28,29] The higher morbidity of the children with a moderate to severe respiratory condition can be partially explained by the more intense activity of the inflammatory cascade in some individuals, with an increase in the damage to the respiratory epithelium already damaged by RSV activity, and by the presence of coinfection with other etiologic agents at hospital admission. Despite this, there were no deaths in our sample, partly due to the absence of specific risk factors for severe disease caused by RSV, such as bronchopulmonary dysplasia and congenital heart disease, and also due to the fact that most of our patients were born at term.

One of the limitations of the present study was the fact that we did not evaluate the association between the severity of the respiratory condition caused by RSV and the genetic polymorphisms of the cytokines. This will be the object of new studies involving larger samples. Real-time RT-PCR was not performed for all of the most common respiratory viruses due to the difficulty in collecting nasopharyngeal samples from suckling infants and also due to the attempt at reducing the risk of hypoxia and trauma caused by prolonged aspirations.

In conclusion, we believe that this study serves as a basis to suggest that, at hospital admission, the concentrations of the pro-inflammatory mediators sICAM-1 in the nasopharyngeal secretion and IL-6 in the serum, as well as the concentration of the regulatory cytokine IL-10 in the nasopharyngeal secretion, constitute good parameters for evaluating the inflammatory and immune response in LRTI caused by RSV. These concentrations can be used as markers of disease severity. These observations could contribute to the development of new therapeutic strategies aimed at the immunomodulation of the disease caused by RSV.

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