Local Interferon-\(\gamma\) Levels during Respiratory Syncytial Virus Lower Respiratory Tract Infection Are Associated with Disease Severity

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To investigate the role of cell-mediated immunity during respiratory syncytial virus (RSV) infection, interferon (IFN)-\(\gamma\) and interleukin (IL)-10 levels in nasopharyngeal secretions were measured in infants with lower respiratory tract infection (LRTI) caused by RSV. A novel technique was used to measure in vivo cytokine levels in nasopharyngeal aspirates (NPAs). Cytokine levels in the NPAs of 17 mechanically ventilated infants and 43 nonventilated hospitalized infants were compared. As expected, mechanically ventilated infants were significantly younger than nonventilated infants (7 vs. 14 weeks). IFN-\(\gamma\) levels were above the limit of detection in the NPAs of 3 (18%) mechanically ventilated infants and in the NPAs of 26 (60%) nonventilated infants. IL-10 levels in the NPAs of mechanically ventilated and nonventilated infants were comparable. It is hypothesized that maturation-related mechanisms have a key role in the development of RSV LRTI that results in mechanical ventilation.

Interferon (IFN)-\(\gamma\), a type II IFN, is produced by T cells and NK cells and has pleiotropic biological effects [1]. The properties of IFN-\(\gamma\) include direct antiviral activity, help in the generation and activation of cytotoxic T cells, stimulation of antigen presentation through induction of expression of major histocompatibility class I and II molecules, and activation of NK cells [2]. In addition, IFN-\(\gamma\) has a role in the regulation of the switch of antibody isotypes, including the switch in expression by B cells from IgM to IgG2a [2]. Taken together with these properties, IFN-\(\gamma\) is considered to be a key cytokine in inducing protective responses against viral pathogens.

A role for IFN-\(\gamma\) has been mentioned in the pathogenesis of respiratory syncytial virus (RSV) infections. We have shown that severe RSV lower respiratory tract infection (LRTI) that results in the need for mechanical ventilation is associated with low systemic proliferative responses and IFN-\(\gamma\) production [3]. In addition, the degree of oxygen saturation in the blood during RSV bronchiolitis was shown to be associated with decreased mRNA IFN-\(\gamma\) expression in the blood [4]. However, it is not certain how accurately these measurements in the blood reflect in vivo IFN-\(\gamma\) levels in the respiratory tract during RSV infection.

A few studies have investigated cytokine profiles in nasopharyngeal lavages (NPLs) during RSV LRTI [5–7]. However, one of the difficulties of studying cytokine levels in NPLs is the unpredictable recovery of volumes of secretions [8]. Consequently, the precise dilution factor in NPLs cannot be known. In addition, NPLs may lead to dilution of secretions to an extent that does not allow for the detection of immune mediators that are present in low concentrations in NPLs, including IFN-\(\gamma\). To overcome these disadvantages of NPLs, in the present study, we developed a novel method to measure cytokine levels in the respiratory tract. This technique was used to investigate IFN-\(\gamma\) levels in the nasopharynx during RSV LRTI. We tested the hypothesis that IFN-\(\gamma\) levels in the respiratory tract during RSV LRTI are associated with disease severity.

Methods

Study population. Children were included during one winter epidemic in 5 hospitals in the Netherlands. Inclusion criteria were as follows: hospital admission, symptoms of LRTI, age <13 months, and findings via immunofluorescence for RSV infection of epithelial cells in nasopharyngeal secretions. Symptoms of LRTI were severe chest cough, wheezing, hoarseness, stridor, shortness of breath [9], cyanosis, and apnea. Prematurely born infants with congenital heart disease or chronic lung disease and infants with wheezing illness before RSV bronchiolitis was diagnosed were not...
Nasopharyngeal aspirates (NPAs). Within 24 h after admission, nasopharyngeal secretions were aspirated with a 3.3-mm suction catheter (Vygon). Aspirates were placed on ice immediately and were stored at −80°C. If NPAs could not be obtained because of the small amount of secretion present in the nasopharynx, no second attempt was performed later in the course of disease. Before cytokine measurement, the NPAs were weighed, were diluted in dilution buffer (4°C) of the ELISA kits that were used for the cytokine assays (CLB), were sonicated on ice twice for 6 s (amplitude 6 μm), and were centrifuged at 10,000 g for 10 min at 4°C. For IL-8 measurement, NPAs were diluted 1:15,000; for measurement of other cytokines, NPAs were diluted 1:15.

Cytokine levels in NPAs were used as an approximation for cytokine levels in fluids of the lower airways. The potential systematic error caused by sampling secretions from the upper airways was evaluated by correlating cytokine levels (IL-8 and IL-10) in NPAs and tracheobronchial aspirates (TBAs) [5]. In a subset of 10 mechanically ventilated patients with RSV bronchiolitis, NPAs and TBAs were collected simultaneously. Identical methods of aspiration and subsequent analysis were used for NPAs and TBAs.

Cytokine assays. Concentrations of IL-4, IL-8, IL-10, and IFN-γ were determined with ELISA kits supplied by the Dutch Laboratory for Blood Transfusion. The limit of detection of the assay was 2 pg/mL for IL-4, 2.5 pg/mL for IL-8, 2.5 pg/mL for IL-10, and 4 pg/mL for IFN-γ. IL-12 concentrations were determined with ELISA kits from R&D; the limit of detection for IL-12 was 7.8 pg/mL.

Statistical analysis. Cytokine levels in NPAs had nonparametric distributions. They are expressed as medians and ranges. Pearson’s correlation coefficient was used to analyze the relationship between cytokine levels in NPAs and TBAs. Spearman’s correlation coefficient was used to analyze the relationship between IFN-γ levels in NPAs and age. The Mann-Whitney U test was used to analyze differences in cytokine levels between ventilated and nonventilated infants. All tests of significance were 2-sided. P < .05 was considered to be statistically significant.

Results

Subject characteristics. The investigated population consisted of 75 patients. Forty-four patients (59%) were boys, with a median age at the time of RSV bronchiolitis of 9 weeks. Seventeen (23%) infants were born prematurely (median gestational age, 34 weeks; range, 27–36 weeks). Seventeen subjects (23%) required mechanical ventilation. As expected, median age in mechanically ventilated infants was lower than in nonventilated infants (7 vs. 14 weeks; P = .002), and a significantly higher percentage was born prematurely (47% vs. 14%; P = .006). None of the children was immunodeficient. None of the prematurely born infants had received RSV prophylaxis. None of the patients received ribavirin or systemic anti-inflammatory agents, including corticosteroids. All patients survived.

Cytokine levels in NPAs. NPAs were collected from 17 mechanically ventilated patients and from 43 nonventilated patients. No NPAs were obtained from 15 patients (20%). In these cases, an insufficient amount of secretion was present in the nasopharynx to be aspirated. These 15 infants were all nonventilated infants. IL-8 levels (range, 5–1300 ng/mL) and IL-10 levels (range, 40–3600 pg/mL) in NPAs were detectable in all patients. IFN-γ could be measured in the NPAs of 28 infants (53%). IL-4 and IL-12 levels in NPAs were below the limits of detection in all NPA samples.

In a subgroup of 10 mechanically ventilated infants with RSV infection, NPA and TBA samples were collected simultaneously. In 3 patients, the amount of TBA was not sufficient for IL-10 measurement. The correlation coefficient for IL-8 levels in NPAs and TBAs was .5 (P = .03; n = 10); for IL-10, the correlation coefficient was .91 (P < .01; n = 7; figure 1).

Differences in cytokine levels in NPAs between ventilated and nonventilated infants were not statistically significant. However, IL-8 levels tended to be slightly lower in ventilated infants (median, 95 ng/mL; range, 2–1300 ng/mL) than in nonventilated infants (median, 265 ng/mL; range, 2–1000 ng/mL; P = .08).

Figure 1. Correlation between cytokine levels in nasopharyngeal aspirates (NPAs) and tracheobronchial aspirates (TBAs) in patients with mechanically ventilated respiratory syncytial virus bronchiolitis. Pearson’s correlation coefficients are shown. A. Correlation for interleukin (IL)-8. B. Correlation for IL-10. Aspirates were taken simultaneously within 24 h after mechanical ventilation was begun.
and nonventilated patients were analyzed. The geometric mean IL-8 levels in ventilated and nonventilated patients were 214 ng/mL (95% confidence interval [CI], 148–309 ng/mL) and 166 ng/mL (95% CI, 129–209 ng/mL; not significant), respectively. The geometric mean IL-10 levels in ventilated and nonventilated patients were 347 pg/mL (95% CI, 159–794 pg/mL) and 447 pg/mL (95% CI, 309–660 pg/mL; not significant), respectively. IFN-γ levels in NPAs were below the limit of detection in 14 (82%) of 17 mechanically ventilated patients and in 17 (40%) of 43 nonventilated patients (P < .001; figure 2).

To investigate whether differences in IFN-γ in NPAs were explained by differences in age, we analyzed the correlation between age and IFN-γ in NPAs in nonventilated infants. No correlation was found (r = −.02; P = .87). Therefore, we consider it to be unlikely that differences in age explain differences in IFN-γ levels in NPAs.

**Discussion**

The main finding of this study was that in vivo IFN-γ levels in NPAs were severely decreased in mechanically ventilated infants with RSV LRTI, compared with hospitalized infants with RSV LRTI who did not require mechanical ventilation. To our knowledge, this is the first study to actually measure local IFN-γ levels during RSV LRTI.

The method to measure cytokine profiles in nasopharyngeal secretion that has been used most widely is NPL [10]. As a result of the unpredictable recovery of volumes of secretions, the dilution factor in NPL is not known. In a group of 52 infants with upper respiratory tract infections, estimated dilution factors in nasal lavage fluid varied widely, from 1.8 to 432 [8]. Correction for the dilution by normalizing cytokine levels to albumin concentrations in NPLs does not appear to be an option, because albumin levels also increase during upper respiratory tract illness as a result of increased vascular permeability [10]. This implies that only ratios between cytokines, but not cytokine concentrations, can be established accurately in NPLs. Moreover, large dilutions potentially result in undetectable levels of cytokines that are present in relatively low concentrations, including IL-10 and IFN-γ [7]. In the present study, this drawback of NPL was overcome, although IFN-γ levels in NPAs were still below the limits of detection in almost all mechanically ventilated infants.

Other disadvantages of analysis of NPAs can be considered. It is conceivable that NPLs contain cytokines from the epithelial fluid lining the nasopharynx, which may not be found in NPAs. These cytokines could be relevant. Another drawback of analysis of NPAs is that it can be performed only if nasal secretions are present in amounts that can be aspirated. Consequently, in the present study, NPAs could not be taken from infants with small amounts of nasal secretions. In addition, a pilot study of control infants showed that the amount of secretions in healthy infants was insufficient to be aspirated. The validity of analysis of NPAs was estimated by comparing cytokine levels in NPAs and TBAs [7]. A high correlation between cytokine levels in NPAs and TBAs was found, which indicates the similarity between cytokine profiles in NPAs and the lower respiratory tract. Taken into account its disadvantages, analysis of NPAs appears to be a suitable and easy method to study cytokine levels that are present at low levels in the respiratory tract.

Evidence exists that insufficient antiviral immunity has a role in the pathogenesis of severe RSV LRTI. It was shown that viral titers found in nasopharyngeal samples from RSV-infected infants are associated with disease severity and are highest in mechanically ventilated infants [11, 12]. Hall et al. [12] already speculated that direct antiviral activity of IFNs, as well as cell-mediated immunity, may be important in the pathogenesis of RSV LRTI. Indirect evidence for this hypothesis came from more recent studies that showed that RSV LRTI is associated with decreased IFN-γ production by peripheral blood mononuclear cells [3, 4]. The results of the present study directly confirm that severe RSV LRTI is associated with reduced local levels of IFN-γ.

Mechanically ventilated infants with RSV LRTI were significantly younger than nonventilated infants. Therefore, it is conceivable that maturation-related mechanisms may explain low systemic and local IFN-γ production in the respiratory tract of mechanically ventilated infants during RSV LRTI. Indeed, T cell–mediated responses are delayed during the first 4–8 weeks of postnatal age, accompanied by impaired capacity to produce IFN-γ [13]. In addition to maturation-related mechanisms, reduced IL-12 production by antigen-presenting cells may explain low IFN-γ levels in the NPAs of mechanically ventilated infants.
ventilated infants during RSV LRTI. IL-12 is a prerequisite for IFN-γ production by T cells and NK cells [1]. In line with this hypothesis, we have shown previously that monocyte IL-12 production is inversely related to duration of mechanical ventilation during RSV LRTI [14].

In summary, analysis of NPAs is a novel technique to detect in vivo cytokine levels in the respiratory tract during RSV LRTI. We have shown decreased IFN-γ levels in NPAs during RSV LRTI in infants with most-severe disease. This finding strongly suggests an important role for IFN-γ in the pathogenesis of RSV LRTI.

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References