Production of Chemokines in the Lungs of Infants with Severe Respiratory Syncytial Virus Bronchiolitis

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Background. Respiratory syncytial virus (RSV) bronchiolitis in infants is characterized by a massive neutrophilic infiltrate into the airways. Chemokines direct migration of leukocytes and contribute to the pathogenesis of RSV disease. However, little is known about pulmonary chemokine responses to RSV in humans. Our aim was to characterize the production of chemokines in the lungs of infants with RSV bronchiolitis and how this production changes over time.

Methods. Chemokine mRNA and the concentration of chemokines were measured in nonbronchoscopic bronchoalveolar lavage (BAL) samples from infants with RSV bronchiolitis and from control infants. In infants with RSV bronchiolitis, changes in the concentrations of chemokines during the 7 days after intubation and between the days of intubation and extubation were examined.

Results. The production of chemokines within the lower respiratory tract was shown in all patients with RSV bronchiolitis. CXC chemokines (particularly CXCL10/interferon-inducible protein 10 and CXCL8/interleukin-8) were found to be the most abundant, but CC chemokines (CCL2/monocyte chemotactic protein 1 and CCL3/macrophage inflammatory protein–1α) were also present. Concentrations of some of these chemokines remained elevated over the course of the illness, whereas others decreased steadily. No differences in the concentrations were found between the days of intubation and extubation.

Conclusions. CXC chemokines predominate within the RSV-infected lung. Much of this response comes from inflammatory cells within the lower respiratory tract. Chemokine response patterns vary over time, possibly indicating different cellular sources for individual chemokines in the RSV-infected lung.
Table 1. Characteristics of infants and nonbronchoscopic bronchoalveolar lavage samples.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Infants with RSV bronchiolitis</th>
<th>Control infants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total patients/male, no.</td>
<td>47/21</td>
<td>10/2</td>
</tr>
<tr>
<td>Weight at admission, kg</td>
<td>4.0 (1.4)</td>
<td>3.3 (0.8)</td>
</tr>
<tr>
<td>Gestation at birth, weeks</td>
<td>34 (5.4)</td>
<td>38.4 (1.4)a</td>
</tr>
<tr>
<td>Age on admission, weeks</td>
<td>11.6 (9.8)</td>
<td>1.0 (2.5)a</td>
</tr>
<tr>
<td><strong>BAL fluid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples, no.</td>
<td>182</td>
<td>10</td>
</tr>
<tr>
<td>Total cell concentration (× 10⁶ cells/mL)</td>
<td>2.07 (3.71)</td>
<td>0.56 (0.36)a</td>
</tr>
<tr>
<td>Neutrophils, median (IQR), %</td>
<td>81.0 (17.5)</td>
<td>52.3 (27.7)a</td>
</tr>
<tr>
<td>Macrophages, median (IQR), %</td>
<td>10 (14.5)</td>
<td>32.7 (23.3)a</td>
</tr>
<tr>
<td>Lymphocytes, median (IQR), %</td>
<td>7.0 (6.0)</td>
<td>11.0 (13.0)</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD, unless indicated otherwise. IQR, interquartile range; RSV, respiratory syncytial virus.
a P < .01.

Chemokines play an important role in the recruitment and activation of leukocytes in a variety of inflammatory conditions in the lung. In vitro studies have shown that RSV stimulates the release of a number of chemokines from human respiratory epithelial cells, alveolar macrophages, and peripheral blood neutrophils [12–15] and that this release is cell-type specific and dependent on infection with replication-competent virus [16]. In mice, experimental infection with RSV results in the production of a range of chemokines, in amounts that parallel the intensity of inflammation in the lung [6, 16]. More-precise elucidation of the role of chemokines in the pathogenesis of RSV bronchiolitis has potential therapeutic implications, because a number of chemokine receptor antagonists are in development [17].

Studies of children with viral respiratory tract infections and children with RSV bronchiolitis have shown that they have increased production of chemokines, including CXCL8/interleukin (IL)–8, CCL5/RANTES, CCL3/macrophage inflammatory protein (MIP)–1α, and CCL2/monocyte chemotactic protein 1 (MCP-1) in the upper respiratory tract [18–23]. However, there is very little information about production of chemokines in the lower respiratory tract during acute RSV bronchiolitis in infants. We have undertaken a large study to investigate the production of several chemokines in the lower respiratory tracts of infants receiving mechanical ventilation for RSV bronchiolitis and to compare this production with that in a control group of infants receiving mechanical ventilation for nonrespiratory causes. We have also studied the patterns in this production throughout the duration of ventilation to assess how they change as the infants recover from their illnesses.

**PATIENTS AND METHODS**

**Patients.** During the 2000–2001 and 2001–2002 RSV seasons, 182 nonbronchoscopic bronchoalveolar lavage (BAL) samples were collected from 47 infants receiving mechanical ventilation for RSV bronchiolitis. From these infants, BAL samples were collected during the first 7 days of mechanical ventilation or until the infant was extubated, as described elsewhere [24]. BAL samples were processed in accordance with the recent guidelines of the European Respiratory Society [25]. The characteristics of the patients and the BAL samples are presented in table 1. Twenty-three of the 47 infants were born preterm (at <37 weeks of gestation), and, of these, 14 required mechanical ventilation during the neonatal period. None of the infants in the study had received passive immunoprophylaxis for RSV bronchiolitis.

The control group was composed of 10 full-term (birth at ≥38 weeks of gestation) infants receiving mechanical ventilation for noninfective and nonrespiratory causes. BAL was performed on the day of intubation (day 1) on 5 infants before they underwent cardiac surgery and on 5 infants before they underwent abdominal surgery. The pediatric research ethics committee of Alder Hey Children’s Hospital approved the present study, and informed consent was obtained from the parents of all infants.

**Sample preparation and RNA isolation.** Standard techniques were used to assess cell concentrations and differential cell counts. BAL fluid was centrifuged, and the supernatant was removed and stored at −80°C. The cell pellet was resuspended in Trizol (Gibco), and RNA was isolated in accordance with the manufacturer’s instructions. The RNA was dried, resuspended in hybridization buffer (BD Biosciences), and stored at −20°C until analysis.

**Ribonuclease protection assay.** The expression of chemokine mRNA was measured using an RNase protection assay. The RNase protection assay was performed in accordance with the manufacturer’s instructions (BD Biosciences), as described elsewhere [26], using a customized probe set that contained the CXC chemokines CXCL8 and CXCL10/interferon (IFN)–inducible protein 10 (IP-10) and the CC chemokines CCL2, CCL3,
CCL4/MIP-1β, CCL5, and CCL11/eotaxin. The level of chemokine transcript was normalized to that of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In brief, probe solutions were hybridized to samples of patient RNA over-night. After RNase digestion, samples underwent phenol extraction before precipitation in ethanol. Each sample was loaded on a polyacrylamide gel. The gels were exposed to autoradiographic film, and densitometric analysis was performed with the Image program (National Institutes of Health). The level of chemokine mRNA expressed relative to that of GAPDH control transcript present was determined in a large selection of samples.

**ELISA.** Concentrations of CXCL8, CXCL10, CCL2, CCL3, CCL5, and CCL11 in BAL samples from infants with RSV bronchiolitis and from control infants were quantified using commercially available ELISAs (R&D Systems). The volume of BAL fluid recovered from the lungs of infants varied depending on the volume of saline instilled, which generally depended on the weight of the infant. This variation in the volume of BAL fluid meant that it was sometimes not possible to perform a complete analysis of the chemokine profile for every sample. All samples were measured in duplicate. Changes in concentrations of chemokines were examined over time both graphically and by comparing concentrations on day 1 and on the day of extubation (day X), as described elsewhere [27].

**Statistical analyses.** Characteristics of the BAL fluid and clinical characteristics were expressed as the mean ± SD or median (interquartile range), and differences were examined using the Student’s t test or the Mann-Whitney rank sum test, depending on the distribution of the data. Chemokine/GAPDH mRNA data were normally distributed and were displayed graphically as the mean ± SE. Concentrations of chemokines, which were not normally distributed, were expressed as the median (interquartile range) when tabulated. When displayed graphically, these data were plotted as the mean ± SE, for reasons of consistency. Differences between infants with RSV bronchiolitis and control infants were examined using an independent 2-sample t test, whereas differences in levels between day 1 and day X were examined using the paired-samples t test. All statistical tests were 2 tailed, and P = .05 was considered to be statistically significant. Statistical calculations were performed using SPSS software (version 10.0.5; SPSS).

**RESULTS**

**Expression of chemokine mRNA relative to that of GAPDH.** The expression of chemokine mRNA relative to that of GAPDH mRNA was measured in BAL samples from 23 infants with RSV bronchiolitis on a total of 73 occasions. The pattern of expression of chemokine mRNA was similar in all infants with RSV bronchiolitis in all samples. Strong expression of CXCL8, CXCL10, CCL2, CCL3, and CCL4 mRNA relative to that of GAPDH mRNA was found in most samples (figure 1). Weak expression of CCL5 mRNA was detected in some samples, and no expression of CCL11 mRNA was detected in any sample. When full-term infants and preterm infants with RSV bronchiolitis were analyzed separately, no significant differences in expression of chemokine mRNA were found (data not shown).

**Concentrations of chemokines.** In infants with RSV bronchiolitis and in control infants, concentrations of CXCL10, CXCL8, CCL2, CCL3, CCL5, and CCL11 were measured in 80, 141, 167, 179, 182, and 157 BAL samples, respectively. There were significant differences between infants with RSV bronchiolitis and control infants on day 1 in the concentrations of CXCL10, CXCL8, CCL2, CCL3, and CCL5 (figure 2A and 2B). When full-term infants and preterm infants with RSV bronchiolitis were analyzed separately, no significant differences in the concentrations of chemokines were found (data not shown).

Mean concentrations of chemokines were plotted over the course of the 7 days after intubation (figure 3A–3F). Similar concentrations of CXCL8, CCL2, and CCL11 were found on most days during this period. Concentrations of CCL3, CCL5, and CXCL10 appeared to decrease between day 1 and day 7. When mean concentrations of chemokines on day 1 and day X were compared (table 2), no significant differences were found.

**DISCUSSION**

This is the first large study, of which we are aware, that has investigated the production of chemokines in the lower respiratory tract of infants with RSV bronchiolitis and carefully doc-
Figure 2. Concentrations of chemokines in infants with respiratory syncytial virus (RSV) bronchiolitis relative to those in control infants, on the day of intubation. Differences in mean ± SE concentrations of CXC (A) and CC chemokines (B) in nonbronchoscopic bronchoalveolar lavage samples from infants with RSV bronchiolitis and control infants are shown for CXCL10/interferon-inducible protein 10 (IP-10), CXCL8/interleukin-8 (IL-8), CCL2/monocyte chemotactic protein 1 (MCP-1), CCL3/macrophage inflammatory protein–1α (MIP-1α), CCL5/RANTES, and CCL11/eotaxin. The most abundant chemokine measured was CXCL10, for which the concentration on day 1 was ~4 times that of CXCL8 and ~140 times that of CCL3. * \( P \leq .01 \) ** \( P \leq .05 \).

umented how this production changes over the course of the illness. Strong up-regulation of the expression of CXC (CXCL8/IL-8 and CXCL10/IP-10) and CC (CCL2/MCP-1, CCL3/MIP-1α, and CCL4/MIP-1β) mRNA in particular was shown in inflammatory cells in BAL samples from all infants with RSV bronchiolitis. On day 1, concentrations of most chemokines were greater in BAL samples from infants with RSV bronchiolitis than in BAL samples from control infants. Although the concentrations of some chemokines did not change over time, the concentrations of others declined steadily. The time course of clinical illness varied in infants, so we compared concentrations of chemokines at 2 critical time points (day 1 and day X), and no differences in concentrations were observed for any of the chemokines studied.

A number of studies have examined the production of chemokines in the upper respiratory tracts of RSV-infected individuals. Noah and Becker studied chemokines in the nasal secretions of adults experimentally infected with RSV [28]. They found that production of CXCL8, CCL2, CCL3, and CCL5, but not CCL11, increased at the time of virus shedding and symptomatic illness. Hornsleth et al. found that the CXCL8 to CCL5 ratio in nasopharyngeal secretions was directly related to the severity of RSV disease [22]. We have shown elsewhere that the amount of CXCL8 mRNA in nasopharyngeal aspirate is strongly associated with the risk of severe disease as determined by a requirement for supplemental oxygen [29]. Garofalo et al. studied levels of CCL2, CCL3, and CCL5 in nasopharyngeal secretions of infants with RSV infection [21]. Concentrations of CCL3 were significantly greater in hypoxic infants with RSV bronchiolitis than in either nonhypoxic infants with RSV bronchiolitis or infants with RSV upper respiratory tract infection alone. Concentrations of CCL2 and CCL3 were also found to be inversely correlate with oxygen saturation levels.

Studies of the production of chemokines in the lower respiratory tracts of infants with RSV disease have been very limited. A study of 10 RSV-infected infants found detectable amounts of CXCL8, CCL3, and CCL5 in lower respiratory tract secretions, compared with little or none in lower respiratory tract secretions from 10 control infants [30]. Other studies have shown that concentrations of CCL3 and CCL5 in tracheal secretions from infants receiving mechanical ventilation for RSV bronchiolitis correlate with concentrations in nasopharyngeal secretions [31]. Some results of the present study are similar to those reported in a smaller study by Sheeran et al. [23]. This research group measured concentrations of CXCL8, CCL3, and CCL5 in tracheal aspirates on the first, third, and fifth days after hospitalization in 14 infants receiving mechanical ventilation for RSV bronchiolitis. Concentrations of all 3 chemokines were elevated on the first day after hospitalization and then declined over course of the 5-day study period. Concentrations of CXCL8 and CCL5 in tracheal aspirate correlated with markers of clinical severity of disease defined as days receiving mechanical ventilation and days receiving supplemental oxygen. These researchers suggested that strategies aimed at down-regulation of the expression/production of chemokines may be clinically worthwhile in RSV disease.

We were unable to detect viable epithelial cells in any of our samples [11]. Thus, the presence of chemokine mRNA in our samples suggests that at least some of the chemokine response in this group of patients is coming from inflammatory cells...
within the lower respiratory tract. It may be that release of chemokines by these cells increases severity of RSV disease by amplifying the immune response to RSV. However, no study to date has been able to investigate whether the chemokine responses in the lower respiratory tracts of infants with mild RSV disease differ from those in infants with severe RSV disease.

Interestingly, in the present study, no differences in the production of chemokines or the expression of chemokine mRNA were found between full-term and preterm infants with RSV bronchiolitis. This finding is in marked contrast to our findings that were published elsewhere regarding the striking differences found in concentrations of proinflammatory cytokines and IL-
To our knowledge, this is the first time that CXCL10 has been found in respiratory secretions from infants with RSV disease. CXCL10 is secreted in response to IFN-γ by a variety of cell types, including neutrophils, monocytes, and T lymphocytes [36]. It is a selective monocyte, a Th1 lymphocyte chemoattractant, and one of the few chemokines capable of binding receptors from different classes of chemokines (both CXCR3 and CCR3) [37]. Expression of CXCL10 has been found in studies of in vitro–infected respiratory epithelial cells [38] and in animal models of RSV infection [6, 39, 40], but not as yet in clinical studies of humans with RSV disease.

Of particular interest in the present study were the very large quantities of CXCL10 present in some BAL samples. Mean concentrations of CXCL10 were ∼4 times those of CXCL8 and ∼140 times those of CCL3. Although quantities of chemokines do not necessarily correlate with biological effect, these results suggest that CXCL10 plays an important role in the pathogenesis of RSV disease.

Given their predominance, neutrophils are the likely source of CXCL10 in our samples. Release of CXCL10 by these cells may recruit Th1 lymphocytes to the lower respiratory tract. Many neutrophils within the RSV-infected lower respiratory tract are also in the process of apoptosis. CXCL10 is a monocyte chemoattractant, and, once in the lung, phagocytosis of apoptotic neutrophils by monocytes could potentially protect the lung from the harmful effects of the disintegration of neutrophils.

In the present study, we aimed to describe the chemokine response in the lungs of infants with severe RSV bronchiolitis. An ultimate aim is to understand how the immunological mechanisms in infants with mild RSV disease differ from those in infants with severe RSV disease and whether an overexuberant immunological response contributes to pathogenesis. This will require further painstaking work in vitro and in animal models and further clinical studies that examine the relationship between chemokine responses and RSV load. Other studies are required to determine whether these responses are specific to bronchiolitis or whether they occur in other viral and bacterial lower respiratory tract infections in children. However, we believe that the present study, which has carefully documented the chemokine response in the lungs over time and has identified novel chemokines in this process, will help direct future studies in how to achieve this overall goal.

In summary, we have made a number of intriguing findings about chemokine production in the lower respiratory tract of infants with RSV bronchiolitis, including the identification and quantification of chemokines that have not previously been de-
scribed in this disease. Whether these responses are protective or contribute to the pathogenesis of the disease needs to be determined. These chemokines may also be important in the pathogenesis of other lower respiratory tract infections in children.

Acknowledgments

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References


