Original Article

Cytokines in nasal lavages and plasma and their correlation with clinical parameters in cystic fibrosis

Marthe S. Paats, Ingrid M. Bergen, Marleen Bakker, Rogier A.S. Hoek, Karin J. Nietzman-Lammering, Henk C. Hoogsteden, Rudi W. Hendriks 1, Menno M. van der Eerden *1

Department of Pulmonary Medicine, Erasmus MC Rotterdam, ’s-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

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Abstract

Background: Because persistent inflammation plays a dominant role in cystic fibrosis (CF), we assessed systemic and local upper airway responses during and after pulmonary exacerbation.

Methods: We followed a cohort of Pseudomonas aeruginosa-infected adult CF patients (n = 16) over time in pulmonary exacerbation and in stable disease. Interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-17A, IL-22, interferon-γ and TNFα levels were measured in sputum, nasal lavages and plasma.

Results: In CF patients IL-6 and IL-10 levels in nasal lavages were significantly increased in exacerbation compared with stable disease. Systemic IL-6 significantly correlated with CRP levels and FEV1 (%predicted), independently of disease status. Systemic IL-10 also correlated significantly with CRP and FEV1 (%predicted), but only in exacerbation. Other cytokines tested did not discriminate between exacerbation and stable disease.

Conclusions: Determination of IL-6 and IL-10 in nasal lavages may provide a minimally invasive tool in the assessment of an exacerbation in CF.

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Keywords: Cystic fibrosis; Cytokines; Inflammation; Nasal lavages; Interleukin

1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) defect predisposes CF patients to chronic respiratory infections, resulting in progressive tissue damage due to airway inflammation [1,2]. Airway inflammation in CF, however, begins already in early infancy [3,4] and, although clearly associated with infection, there is still some uncertainty about whether CF lungs are innately primed for a pro-inflammatory response. Recurrent and persistent lung infections with bacteria such as Staphylococcus aureus (S. aureus) and Pseudomonas aeruginosa (P. aeruginosa) are common from infancy and the incidence of infection increases with age [5]. In adulthood, most CF patients are chronically infected with P. aeruginosa [6], and this chronic infection results in a neutrophil-dominated lower airway inflammation and progression of obstructive lung disease and bronchiectasis [1,2].

Similar to the lower airways, mucociliary clearance of the upper airways is impaired by the causative CFTR defect [7–9]. Sinonasal involvement in CF has been proposed as a major source for chronic bronchopulmonary infection with opportunistic bacteria [10]. S. aureus and P. aeruginosa are known to colonise the upper airways in CF patients [11] and therefore this site may function as a gateway and reservoir for subsequent pulmonary infection [12].

The inflammatory activity reported in CF is not restricted to acute pulmonary exacerbations of CF, but has also been demonstrated during phases of clinical stability [13–15]. Inflammatory mediators as markers of the host response to infection may reflect the intensity of the lung injury and may...
of great assistance [16,17]. Nasal lavage is one of these unpleasant for patients. Surrogate markers, collected by accurately, but bronchoscopy with BAL is invasive and unpleasant for patients. Bronchoalveolar lavage (BAL) probably reflects the airway inflammation most crucial role in progressive lung damage in CF, it is important to find reliable markers of its severity. Bronchoalveolar lavage cytokine levels in exacerbation to those in stable disease. Furthermore, since systemic inflammation has also been reported in CF [15,21], collection of peripheral blood may also serve as a minimally invasive tool in the assessment of pulmonary inflammation.

We hypothesized that the inflammatory response in CF is increased in patients in acute exacerbations compared with those in stable disease and that the intensity of this inflammatory response – as reflected by the cytokine production – correlates with disease severity as defined by pulmonary function testing (FEV1 %predicted) and C-reactive protein (CRP) levels. We especially assessed whether local nasal or sputum inflammatory responses are indicative for acute pulmonary exacerbations in CF. To test this hypothesis, we followed a cohort of P. aeruginosa-infected adult CF patients over time and measured cytokine levels in stable disease as well as in acute pulmonary exacerbation. Cytokines reflecting innate and adaptive immune activity were determined both locally, in sputum and nasal lavages, and systemically in plasma.

2. Methods

2.1. Study design

A prospective study was performed in CF patients with chronic P. aeruginosa infection between January and December 2010. Patients admitted for intravenous antibiotic therapy during acute respiratory exacerbation to the Erasmus MC, a CF centre with a total adult population of 120 patients, were enrolled in the study. The medical ethics committee of the Erasmus MC approved of the study. Written informed consent was obtained from each patient.

An airway exacerbation was defined as previously described by Fuchs et al. [22], and was said to have occurred when a patient was treated with parenteral antibiotics for any 4 of the following 12 signs or symptoms: change in sputum; new or increased hemoptysis; increased cough; increased dyspnea; malaise, fatigue, or lethargy; temperature above 38 °C; anorexia or weight loss; sinus pain or tenderness; change in sinus discharge; change in physical examination of the chest; decrease in pulmonary function by 10% or more from a previously recorded value; or radiographic changes indicative of pulmonary infection. Exclusion criteria were known immunodeficiency or autoimmune disease, the use of systemic corticosteroids, and the presence of allergic bronchopulmonary aspergillosis (ABPA) or allergic asthma. In all patients intravenous antibiotics were administered for 21 days. Selection of antibiotics was based on the sensitivity of the cultured bacteria and consisted in all patients of two antibiotics with different mechanisms of action.

Upon admission, we collected venous blood and sputum samples and performed nasal lavages in all patients. Additionally, spirometry and routine biochemical analysis (including CRP) were done. Measurements were repeated approximately 3 months after hospital admission when patients were clinically stable, which was defined as no need for intravenous antibiotics for at least 6 weeks prior to measurements. Chronic P. aeruginosa infection was diagnosed if the organism was isolated in at least 3 consecutive sputum samples within a 6-month period.

2.2. Collection of sputa, nasal lavages and plasma

Sputum samples were collected by spontaneous coughing, and were stored at 4 °C for a maximum of 2 h until processing. Samples were processed using Sputolysin (Calbiochem). Briefly, 1 ml of 10% Sputolysin was added per 1 mg of sputum, and incubated for 15 min at 37 °C while vigorously shaking. Subsequently, samples were centrifuged at 600 × g for 10 min at 4 °C, and supernatants were aliquoted.

Nasal lavage was performed essentially as previously described [12]. However, instead of using 10 ml of sterile isotonic saline, we inserted 5 ml into each nostril with a 10 ml syringe with a slightly reclined position of the head during occlusion of the soft palate. Specimens were stored at 4 °C for a maximum of 2 h until processed. Nasal lavages were centrifuged at 1200 × g for 10 min at 4 °C and supernatants were aliquoted. To obtain plasma, venous blood was collected into EDTA containing vials and centrifuged at 1200 × g for 10 min at 4 °C. All samples were stored at −80 °C until cytokine analysis.

2.3. Cytokine level measurements

Levels of IL-6, IL-10, IL-8, IL-1β, TNFα, IL-17A, IL-22, IFNγ, IL-4, IL-5 and IL-2 in sputum samples and nasal lavages were determined using Multiplex assays (FlowCytomix, eBioscience) according to manufacturer’s instructions. Levels of IL-6, IL-10, IL-8, IL-1β, TNFα, IL-17A, IL-22, and IL-4 in plasma were assessed by enzyme-linked immunosorbent assay (ELISA) using commercially available assays (IL-8 OptEIA Set, BD Biosciences; all other cytokines Ready-Set-Go kits, eBioscience). Specific sensitivity levels can be found in the manufacturers’ manuals.

2.4. Statistical analysis

Data are shown as mean values (±SD) in cases of normally distributed data or median values with interquartile ranges (IQRs) when not normally distributed. Cytokine levels were not normally distributed and therefore nonparametric tests were used to make comparisons between groups (Kruskal–Wallis test for across group comparison of three or more groups, Mann–Whitney U-test for pair-wise analyses). Paired data were tested using the paired Wilcoxon rank test. Correlations were calculated by using Spearman’s Rank correlation coefficient.
Data analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 and Prism 5.01 (GraphPad). Statistical significance was taken as a p-value < 0.05.

3. Results

3.1. Clinical characteristics of the study population

Sixteen CF patients with chronic *P. aeruginosa* infection were included in this study. No patients who met the inclusion criteria refused participation. All patients were studied at admission for an acute pulmonary exacerbation and three months later (range 74–122 days) when clinically stable. Clinical characteristics of the study population are shown in Table 1.

All patients used azithromycin as maintenance therapy. In all CF patients CRP levels were raised when in acute pulmonary exacerbation compared with stable disease, but no statistical significance was reached. FEV1 values decreased when in pulmonary exacerbation but this was also not a significant difference. Nine patients had a history of nasal polyps.

3.2. Local cytokine levels

In sputa of CF patients, levels of IL-10, IL-8, IL-1β, TNFα, IL-22 and IL-4 were detectable but no significant differences were found between clinically stable patients and patients in exacerbation (Table 2). In nasal lavages, only IL-6, IL-10, IL-8 and IL-1β levels could be detected. IL-6 and IL-10 levels in nasal lavages were significantly higher in patients in acute pulmonary exacerbation compared with the same patients in stable disease (Table 2). A pair-wise analysis of CF patients in our study population without nasal polyps (n = 7) also revealed a significant raise in IL-6 and IL-10 levels in exacerbation compared with stable disease (Fig. 1). Levels of IFNγ, IL-5, IL-2 and IL-17A were not detectable in sputa or nasal lavages.

For all cytokines except for IL-6, sputum levels were higher than levels found in nasal lavages of CF patients (Table 2).

3.3. Systemic cytokine levels

The concentrations of IL-6, IL-10, IL-8, IL-1β and IL-22 in plasma of CF patients were above detection levels but no significant differences were found between CF patients in stable disease and in exacerbation (Table 2).

IFNγ, IL-17A, IL-5 and IL-2 were not detectable in sputa and nasal lavages of CF patients and were therefore not determined in plasma. We did measure IL-17A in plasma of CF patients but concentrations were below detection levels.

3.4. Local versus systemic cytokine levels

Levels of most cytokines were higher in local compartments than systemically. IL-10, IL-8, IL-1β, TNFα and IL-4 levels were significantly higher in sputum compared with plasma, both in clinically stable patients and in patients in exacerbation (Table 2). In contrast, IL-6 was not detectable in sputum of CF patients regardless of clinical status, but could be detected in the plasma of all patients (Table 2).

Levels of IL-8 and IL-1β in nasal lavages exceeded systemic levels (Table 2). IL-22 was below detection in nasal lavages but notable levels were found in both sputum and plasma of all patients.

3.5. Correlations between cytokine levels and clinical parameters

When correlating plasma cytokine levels of CF patients with markers of disease severity, we found significant associations with systemic levels of IL-6 and IL-10. FEV1 (%predicted) showed a strong inverse correlation with plasma IL-6 levels, independently of clinical status (Fig. 2A). For plasma IL-10 levels a significant inverse correlation with FEV1 (%predicted) was also found, but only in CF patients in pulmonary exacerbation (Fig. 2B).

For CRP levels, we also found significant associations with plasma IL-6 and IL-10 levels: plasma IL-6 significantly correlated with CRP in both stable disease and exacerbation (Fig. 3A), whereas plasma IL-10 levels only correlated significantly with CRP in patients when in exacerbation (Fig. 3B). All other cytokines that were detectable in plasma did not correlate with either FEV1 or CRP (data not shown).

Thus, in contrast to IL-10, plasma IL-6 levels significantly correlated with markers of disease severity independently of clinical status. Cytokine levels in sputa and nasal lavages did not show any significant correlations with either FEV1 or CRP levels (data not shown).
Table 2
Cytokine levels in sputa, nasal lavages and plasma of CF patients when clinically stable and in acute pulmonary exacerbation.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Stable (pg/ml)</th>
<th>Exacerbation (pg/ml)</th>
<th>Plasma (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>b.d.</td>
<td>5.0 (1–10)</td>
<td>4.6 (2.4–7.5)</td>
</tr>
<tr>
<td>IL-10</td>
<td>401 (218–1542)</td>
<td>6.0 (3–62)*</td>
<td>3.6 (1.6–13.8)</td>
</tr>
<tr>
<td>IL-1</td>
<td>1685 (1127–2541)</td>
<td>2.5 (0–4)</td>
<td>1.7 (0.5–2.7)</td>
</tr>
<tr>
<td>IL-8</td>
<td>6025 (2221–9375)</td>
<td>712 (351–1474)</td>
<td>0 (0–2.0)</td>
</tr>
<tr>
<td>IL-10</td>
<td>408 (105–895)</td>
<td>120 (10–298)</td>
<td>3.6 (0–6.8)</td>
</tr>
<tr>
<td>INFα</td>
<td>133 (0–235)</td>
<td>b.d.</td>
<td>b.d.</td>
</tr>
<tr>
<td>TNFα</td>
<td>175 (0–212)</td>
<td>b.d.</td>
<td>b.d.</td>
</tr>
<tr>
<td>IL-4</td>
<td>b.d.</td>
<td>b.d.</td>
<td>b.d.</td>
</tr>
</tbody>
</table>

Data are represented as median values (IQR). IL: interleukin. TNF: tumour necrosis factor.
*p = 0.04; **p = 0.02, §p = 0.08 for clinically stable compared with exacerbation CF (Mann–Whitney U-test). b.d.: below detection. All n = 16 except for sputum (n = 14), nasal lavages (n = 13) and plasma (n = 15) in stable disease. When stated ‘b.d.’, cytokine levels were below detection limits in all patients, except for sputum IL-6 in exacerbation, where only one patient had levels above detection limits. In the other samples, cytokine levels were generally above detection limits, except for IL-10 in nasal lavages in stable disease (4/13 b.d.), IL-8 in plasma in stable disease (6/15 b.d.) and in exacerbation (5/15 b.d.), IL-22 in sputum in stable disease (4/14 b.d.) and in exacerbation (3/14 b.d.), and IL-4 in sputum in stable disease (4/14 b.d.).

4. Discussion

Previous studies have shown that inflammatory activity in CF is present during acute pulmonary exacerbations, as well as in phases of clinical stability [13–15]. To our knowledge, this is the first study investigating inflammatory responses simultaneously in sputa, nasal lavages and plasma of P. aeruginosa-infected adult CF patients both when in exacerbation and when clinically stable. We studied a large panel of cytokines which are of potential interest for CF. Our most important findings are that IL-6 and IL-10 levels in nasal lavages of CF patients were significantly higher in exacerbation compared with stable disease. Moreover, although cytokine levels in sputum and plasma of CF patients were unable to discriminate between exacerbations and stable phases of disease, systemic IL-6 levels did significantly correlate with CRP levels and FEV1 (%predicted), independently of disease status. Systemic IL-10 levels also correlated with CRP and FEV1 (%predicted), but only in exacerbation. Additionally, we found that cytokine levels were generally higher in sputum and nasal lavages than in plasma. The physiological differences might even be more substantial, as nasal lavage and sputum processing procedures introduce dilution of cytokine levels. Our findings therefore support the concept of an inflammatory response that is compartmentalized to the airways.

The cytokines IL-6 and IL-10 are major regulators of the host inflammatory response. There are only few reports about cytokine levels in nasal lavages of CF patients. It has been demonstrated that the neutrophilic inflammation which is prominent in CF lower airways, is also present in the nasal airways of CF patients [18,19]. Noah et al. measured IL-6 and IL-10 levels in nasal lavages of very young children with CF and found similar levels in these patients when compared with controls [19]. However, in this study no distinction was made between CF patients in exacerbation or newly diagnosed infants, and control subjects were all children with an indication for bronchoscopy [19]. Our findings in nasal lavages are in agreement with reported analyses in exhaled breath condensates of CF patients which contained increased IL-6 levels during exacerbation and decreased levels after antibiotic treatment [23]. Contradictory results have been published on the influence of nasal polyps on cytokine levels [20]. We performed a subanalysis in CF patients without nasal polyps. Also in this paired analysis, patients in exacerbation had increased levels of IL-6 and IL-10 compared with stable clinical conditions.

In line with previous reports [24,25] we were unable to detect IL-6 in sputum of CF patients, irrespective of clinical status. Some groups have been able to detect very low concentrations of IL-6 in sputum samples of CF patients, but findings are contradictory. Eickmeier et al. found higher IL-6 levels in induced sputum samples of clinically stable CF patients when compared with healthy individuals [14], whereas another group reported lower levels of IL-6 in sputa of children with CF compared with healthy adult individuals [26]. Nixon et al. did not detect differences in IL-6 levels in patients in exacerbation versus stable disease but did find a relationship between sputum IL-6 levels and FEV1 (%predicted) which was present during acute exacerbations only [15].

Systemic levels of IL-6 in our study were low but detectable and although no significant differences were found between patients in exacerbation and patients in stable disease, we did find a significant inverse correlation with FEV1 (%predicted) and a significant positive correlation with CRP. This suggests that systemic inflammation is an independent risk factor for disease progression in CF. Furthermore, the close correlations of systemic IL-6 and IL-10 with CRP levels, support the use of these cytokines as circulating markers of inflammation in CF. Colombo et al. also measured systemic IL-6 levels in CF children but could not detect biological activity [24]. Our results are supported by several previous reports, including Nixon et al. who observed that systemic IL-6 levels were similar in exacerbation versus stable disease and significantly correlated with CRP levels [15]. Gifford et al. reported a reduction of serum IL-6 in CF patients showing improvement of FEV1 upon antibiotic treatment [27]. Finally, in a larger cohort of adult CF patients in stable clinical conditions, Ngan et al. found that plasma IL-6 levels correlated significantly with FEV1 (%predicted) [28]. This supports the use of IL-6 as circulating marker of inflammation in CF.

Data on IL-10 levels in sputum or serum and clinical status of CF patients are limited and contradictory. Two groups have previously studied sputum IL-10 levels but no comparisons between exacerbation and stable disease have been made [23,26].
Serum IL-10 levels were only reported in one study in which they were below detection level [24]. One group reported data on sputum IL-10 levels analysed by a multi-parametric biochip array [29]. They found that levels of IL-10 in induced sputum decreased after 15 days of exacerbation treatment.

Our finding that IL-10 levels in nasal lavages of CF patients were increased during exacerbation may seem inconsistent with the supposed role of IL-10 as an anti-inflammatory mediator. Especially, since it has been reported that IL-10 production by airway epithelial cells or T cell clones from CF patients is reduced [13,30,31]. However, it cannot be excluded that IL-10 has immunostimulatory properties in this context, because (i) under certain conditions IL-10 enhances proliferation or cytokine production by T cells or NK cells [31,32] and (ii) when overexpressed in the lungs of transgenic mice, IL-10 causes an inflammatory response and subepithelial fibrosis [33]. Further research will be required to identify pro- or anti-inflammatory roles of IL-10 during exacerbations in CF patients.

The main limitation of this study, although comparable to other studies investigating cytokines in CF [13–15], is the small number of patients included. Therefore, our observations need to be interpreted with caution and verified in a larger group of patients. Furthermore, the results presented in this study were obtained with spontaneously expectorated sputum. The collection of spontaneous sputum is non-invasive, but not all CF patients produce sputum on a consistent basis. In contrast, nasal lavages are minimally invasive and could therefore be performed in most CF patients regardless of age or clinical status, although incidentally some patients have difficulties to perform a nasal lavage. Direct comparison of cytokine concentrations in plasma and nasal lavages, however, is complicated because of fluid dilution that is associated with the nasal lavage procedure. Azithromycin and corticosteroids have known immunomodulatory effects [34,35]. In our study, all patients used azithromycin as maintenance therapy and continued the usage when in exacerbation. The conclusions of our study are therefore not likely to be influenced by this. In addition, we verified that our results were not influenced by inhaled corticosteroid (ICS) or nasal corticosteroid (NCS) usage: no correlations were found between ICS use or NCS use and cytokine levels (data not shown). Furthermore, cytokine profiles of the two patients in our cohort with a history of non-allergic asthma, did not significantly differ from those without non-allergic asthma. Our patient cohort was not...
homogenous as three patients were co-infected with *S. aureus*, but this would make our study group more representative of the CF patients in clinical practice.

In conclusion, our study provides a comprehensive analysis of cytokine profiles reflecting innate and adaptive immune activity in local and systemic compartments in adult *P. aeruginosa*-infected CF patients. We showed that levels of IL-6 and IL-10 in nasal lavages may provide a minimally invasive tool in the assessment of an exacerbation in CF patients. Furthermore, because of the relationship between plasma IL-6 concentrations and FEV1 and CRP levels, independent of clinical status, systemic IL-6 might be a valuable marker of systemic inflammation in CF. Future studies could investigate whether measurements of other inflammatory markers in nasal lavage than those detected within the presented study are useful to define pro-inflammatory status and potentially identify a subpopulation of CF patients that might benefit from systemic anti-inflammatory therapy.

References


