Inflammation in *Achromobacter xylosoxidans* infected cystic fibrosis patients

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Abstract

*Background:* *Achromobacter xylosoxidans* infection may cause conspicuous chronic pulmonary inflammation in cystic fibrosis (CF) patients similar to *Pseudomonas aeruginosa* and the *Burkholderia cepacia* complex (*Bcc*). Evolution in lung function was compared in chronically infected patients. Cytokine concentrations in CF patients with and without chronic infection were compared to healthy controls.

*Methods:* Cytokines in serum and sputum were measured using multiplex bead based immunoassay.

*Results:* Sixty CF patients, 11 with *A. xylosoxidans*, 11 with *Bcc*, 21 with *P. aeruginosa* and 17 non-infected CF patients were compared to 11 healthy controls. *A. xylosoxidans* patients were younger, but had a FEV₁ decline similar to *P. aeruginosa* patients. *Bcc* patients had the steepest decline in FEV₁.

Serum levels of G-CSF, IL-6 and TNF-α were significantly higher in CF patients compared to healthy controls. Chronically infected CF patients had significantly higher serum levels of IFN-γ and IL-6 compared to non-infected CF patients. *Bcc* patients had significantly lower serum G-CSF and *A. xylosoxidans* patients had significantly higher sputum TNF-α compared to the other groups of chronically infected patients.

*Conclusion:* *A. xylosoxidans* can cause a level of inflammation similar to *P. aeruginosa* in chronically infected CF patients. *A. xylosoxidans* is a clinically important pathogen in CF and should be treated accordingly.

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1. Background

Recurrent and ultimately chronic pulmonary infection is a predominant cause of increased morbidity and mortality in cystic fibrosis (CF) patients [1–3]. Up to 80% of adult CF patients suffer from chronic, pulmonary infection with *Pseudomonas aeruginosa*, but an increasing number of other Gram-negative microorganisms are capable of causing chronic infections in CF patients [4,5].

In CF, the inflammatory response to pulmonary infections is exaggerated [6,7]. Increased levels of inflammatory cytokines have been found in sputum [8], BAL-fluid [9–11] and Exhaled Breath Condensate (EBC) [12] not only in chronically infected patients but even in CF infants without established infections [10,13,14]. Levels of cytokines in BAL-fluid are increased in individuals with airway infection compared to individuals without infection [7,9,15].

Inflammation caused by *P. aeruginosa* has been described through the studies of mouse models of chronic infection; by measurements of cytokines in BAL-fluid, sputum and serum [8,16]; by stimulation of peripheral blood mononuclear cells (PBMC) from patients with chronic *P. aeruginosa* infection [17] and measurements of specific antibody levels [18]. The increased morbidity and mortality in CF patients with this infection is well...
known [19]. A rapid increase in specific antibodies is associated to a poor prognosis in these patients [20].

The Burkholderia cepacia complex (Bcc) affects 3–4% of CF patients [4,21–26]. The prevalence varies greatly in individual centres since some Bcc genomovars have shown a high ability to cause cross infections within centres [27–29]. Bcc infection is feared due to the ability to cross infect [29,30] and to cause the cepacia syndrome — characterized by a sudden decline in clinical status and a high mortality [26,30–32]. B. cepacia lipopolysaccharide (LPS) can strongly stimulate the inflammatory response [33], but a study revealed no difference in levels of inflammatory markers between CF patients with B. cepacia and patients with chronic P. aeruginosa infection [34].

Another new cause of chronic pulmonary infection is Achromobacter xylosoxidans found in around 7% of the CF population [35]. The clinical significance of this infection is still not clearly established. A few studies have shown a negative impact on CF pulmonary disease, especially in patients with a rapid increase in specific antibodies [36], whereas other studies failed to demonstrate any correlation [37–39]. A recent study has shown an overrepresentation of A. xylosoxidans infected patients in the group of CF patients in need of lung transplantation compared to prevalence of A. xylosoxidans in the general CF population (presented as abstract 359 by Brown et al. at the 22nd NACFC in 2008, Orlando, Florida).

The aim of the present study was to evaluate the inflammatory response to A. xylosoxidans by comparing concentrations of inflammatory markers in serum, EBC and sputum from patients chronically infected with P. aeruginosa, Bcc and A. xylosoxidans, respectively. CF patients without chronic, pulmonary infections caused by Gram-negative microorganisms and healthy controls.

2. Methods

2.1. Study design

Cross sectional and retrospective study of patients followed at the Copenhagen CF centre. Patients were invited to participate at outpatient visits and samples for cytokine measurements were collected during a 2 year period.

2.2. Treatment strategy at Copenhagen CF centre

Patients are seen on a regular, monthly basis. At all visits, the clinical status of the patients is assessed by weight, height and lung function parameters, forced vital capacity (FVC) and forced expiratory volume in 1 s (FEV1) (Masterscreen Pneumo, Jaeger) are obtained according to the ATS guidelines [40], using reference values from Polgar [41]. Lower respiratory tract secretions for microbiological investigation are obtained by coughing or by endo-laryngeal suctioning. Specific, precipitating antibodies are obtained at least once every year, and chronic infection is defined as 6 consecutive positive sputum samples and/or elevated levels of specific, precipitating antibodies [20]. The specificity of the antibody response is determined by absorption experiments [20]. CF patients with chronic, Gram-negative, pulmonary infections are treated with 3-monthly elective courses of 2 weeks of i.v. antibiotics and with continuous inhaled antibiotics.

2.3. Patients

All patients treated at CF centre Copenhagen were eligible for the study. Patients were characterized as non-infected or chronically infected with A. xylosoxidans, Bcc or P. aeruginosa. Preferably, subjects without chronic infections were chosen among patients ≥15 years securing their own agreement to give informed consent and better age-match to chronically infected patients. Patients chronically infected with more than one Gram-negative microorganism were not included in analyses to avoid contributing inflammatory stimulation from more than one infectious agent. All patients had a confirmed diagnosis of CF based on abnormal sweat electrolytes, characteristic clinical features and genotype.

Healthy controls were recruited from the staff at the centre.

2.4. Lung function parameters and specific antibodies

Highest value of FEV1 and highest level of specific antibodies were collected retrospectively for each year of observation, starting the year prior to development of chronic infection, up to 12 years after development of chronic infection. Slope of decline of FEV1 was calculated as change in % of predicted/year, including all years of chronic infection.

Highest value of FEV1 in the year prior to development of chronic infection in the A. xylosoxidans group was compared to a group of controls without chronic, pulmonary infections. Two CF controls, matched on age (±1 year) and gender, were found for each A. xylosoxidans infected patient.

2.5. Biofilms

Gram-stained smears of sputum were searched for biofilms of Bcc and A. xylosoxidans by microscopy as previously reported in relation to chronic infection with mucoid P. aeruginosa [42].

2.6. Samples for cytokine measurements

Samples were taken at least 2 months after a course of i.v. antibiotics to avoid any antibiotic suppression of inflammation [43–45]. The following samples were collected from each participant: serum sample, EBC and, if possible, sputum sample. Age, duration of chronic infection, FEV1 and level of specific antibodies, were noted at the time of the sample.

Serum: blood samples were allowed to clot. Serum was collected after centrifugation for 10 min at 3000 rpm.

Exhaled Breath Condensate: samples were collected using Ecoscreen® (Jaeger, Hoechberg, Germany). Patients were allowed to breathe normally for 15 min, wearing a nose-clip. After collection, EBC was frozen immediately.

Sputum: Fresh, spontaneously expectorated sputum samples were frozen and stored at −80 °C until preparation. Samples were ultra-centrifuged for 4 h at 38,000 rpm. Non-infected CF
patients were not able to produce sputum, why only sputum samples from the chronically infected groups were analyzed.

All samples were stored at −80 °C prior to analysis.

2.7. Cytokines

Cytokines in serum, EBC and sputum were measured by a multiplexed flow cytometric assay using a human cytokine LINCoplex kit (MPXHYCTO-60 K, Linco Research, St. Charles, MO, USA) and a human high sensitivity LINCoplex kit (HSCYTO-60SK, Linco Research, St. Charles, MO, USA) on a Luminex® 100™ system (Ramcon, Birkerød, Denmark). In serum and sputum, measurements of G-CSF, IFN-γ, TNF-α, IL-6, IL-8 and IL-10 were done. In EBC measurements of IFN-γ, TNF-α, IL-1β, IL-6 and IL-10 were done. Analysis was done according to the instructions of the manufacturer. Samples were measured in duplicates. The range of the standard curves for all cytokines measured was 3.2–10.000 pg/ml. To minimize variation, the same person ran each entire set of samples on the same day. Based on the standard curves, the coefficient of variation (CV) was calculated and did not exceed 20%.

2.8. Microbiology

Bacterial resistance against commonly used antibiotics was tested in isolates from included subjects. A strain was defined as multiresistant if simultaneous resistances to at least 3 different groups of antibiotics were found (aminoglycosides, 3rd generation cephalosporins, colistin, aztreonam, meropenem, ciprofloxacin, tetracycline, chloramphenicol, sulfamethoxazole with trimethoprim and penicillin with β-lactamase inhibitor).

2.9. Statistics

Data and measurements with a normal distribution were compared using one-way ANOVA. More than two groups of data that were not normally distributed were compared using Kruskal–Wallis test. Mann–Whitney Rank Sum test was used for comparison of two groups of data without normal distribution. Correlation of duration of chronic infection to level of FEV₁ and specific antibodies, respectively, was done in the individual groups of chronically infected patients using Spearman’s correlation. Level of significance was defined as 0.05 (two-tailed).

3. Results

Sixty-seven CF patients and 11 healthy controls were included. Fifty CF patients had chronic pulmonary infections: 14 patients with *A. xylosoxidans*, 21 with *P. aeruginosa*, 13 with *B. multivorans* and 2 with *B. cepacia*. The last 15 patients were grouped together as the *Bcc* group. Of the chronically infected CF patients, 3 patients in the *A. xylosoxidans* group and 4 patients in the *Bcc* group were co-infected with other Gram-negative microorganisms. Six patients were co-infected with *P. aeruginosa* (2 in the *A. xylosoxidans* group, 4 in the *Bcc* group) and one patient initially having chronic *A. xylosoxidans* infection developed chronic *B. multivorans* infection and continued to have sputum cultures positive for both microorganisms. All 7 patients with co-infections were excluded from further analyses, leaving 11 patients in the *A. xylosoxidans* group and 11 patients in the *Bcc* group. Seventeen CF patients did not have chronic, Gram-negative, pulmonary infection. Demographic data are shown in Table 1. CF patients without chronic infections were younger compared to the *P. aeruginosa* group, the *Bcc* group and the healthy controls, but not when compared to the *A. xylosoxidans* group. Non-infected CF patients had the highest FEV₁ when comparing all groups of CF patients (Table 1). Within the group of chronically infected patients, the *A. xylosoxidans* group was younger (*p*<0.003). The *P. aeruginosa* group had the longest duration of chronic infection (*p*<0.002) (Table 1).

3.1. Biofilms

Seven of eight and 6/6 of the CF patients with chronic *A. xylosoxidans* and *Bcc*, respectively, had detectable biofilm-like clusters of Gram-negative rods surrounded by a matrix in sputum (Fig. 1). The remaining patients in these two groups either harboured other Gram-negative rods or only produced pharyngeal

<table>
<thead>
<tr>
<th>CF patients according to infectious status</th>
<th>Healthy controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No chronic infection</td>
<td><em>A. xylosoxidans</em></td>
</tr>
<tr>
<td>Number (males)</td>
<td>17 (6)</td>
<td>11 (6)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>17.8 (8.6–25.7)*</td>
<td>20.4 (16.1–26.9)*</td>
</tr>
<tr>
<td>Number of specific, precipitating antibodies</td>
<td>19 (1–43)</td>
<td>23 (3–48)</td>
</tr>
<tr>
<td>FEV₁% predicted</td>
<td>83.2 (51.8–97.4)*</td>
<td>83 (30.9–104.2)</td>
</tr>
<tr>
<td>Chronic infection duration (years)</td>
<td>4.6 (2.8–10.4)</td>
<td>16.9 (0.1–30.7)*</td>
</tr>
<tr>
<td>AF508 homozygous</td>
<td>10 (7 F508/other)</td>
<td>7 (4 F508/other)</td>
</tr>
<tr>
<td>CF related diabetes</td>
<td>4/17</td>
<td>2/11</td>
</tr>
</tbody>
</table>

All data were assessed at the time of sample collection. All groups were compared using Kruskal–Wallis test (*p*-values given in table). When Kruskal–Wallis test showed a significant result, the groups were compared to each other, two at a time, using Mann–Whitney test.

* p<0.01 using Mann–Whitney test.
secretions dominated by squamous epithelial cells. The CF patients with chronic mucoid P. aeruginosa lung infections have previously been shown to have biofilm in sputum [42]. Compared to P. aeruginosa biofilms in sputum, the biofilms of A. xylosoxidans and Bcc looked similar with a dark, condensed appearance where the bacterial cells were located very close to each other in contrast to the biofilms of mucoid P. aeruginosa where the abundant light alginate matrix leads to separation of the individual bacterial cells.

3.2. Specific antibodies and change in lung function

In all groups of chronically infected patients, a significant positive correlation between duration of chronic infection and levels of specific antibodies was found (Spearman’s rho = 0.587, p < 0.001).

When comparing level of FEV$_1$ in the year prior to development of chronic infection in the 3 groups of chronically infected patients, no difference was found. Patients in the A. xylosoxidans, Bcc and P. aeruginosa groups had a median (range) FEV$_1$, % of predicted, of 94.7 (55.7 to 118.9), 103.5 (54.9 to 110.8) and 83.5 (51.7 to 116.5), respectively (n.s.). The A. xylosoxidans group was compared to a group of controls matched on age and gender. In the year prior to development of chronic infection in the A. xylosoxidans group, the CF controls without chronic infections had a median (range) FEV$_1$, % of predicted, of 94.4 (74.2–126.4) (n.s.).

In all groups of chronically infected patients, a small, but significant negative correlation between duration of chronic infection and levels of FEV$_1$ decline, % of predicted/year was found (Spearman’s rho = -0.287, p < 0.001). The A. xylosoxidans, Bcc and P. aeruginosa group had a median (range) change in FEV$_1$ of $-1.7 (-7.5$ to $+2.3)$, $-3.7 (-7.6$ to $-0.2)$ and $-0.9 (-9.1$ to $+2.1)$, respectively. The Bcc group had a greater lung function decline compared to the other groups (p < 0.005) (Fig. 2).

3.3. Serum levels of cytokines

CF patients had higher concentrations of TNFα in serum compared to healthy controls (Fig. 3) (p < 0.003). No difference was found when comparing CF patients with and without chronic, Gram-negative infections. Concentrations of G-CSF were highest in the A. xylosoxidans and the P. aeruginosa groups compared to all other groups (Fig. 4) (p < 0.001). Within the groups of chronically infected patients, the Bcc group had lower levels of G-CSF compared to the A. xylosoxidans group (p < 0.02) and to the P. aeruginosa group (p < 0.01).
Concentrations of IL-6 were higher in chronically infected patients when compared to non-infected patients and healthy controls ($p<0.003$) (Fig. 5). We found no difference in IL-6 when comparing non-infected CF patients to healthy controls.

Concentrations of IFN-γ were higher in chronically infected patients compared to CF patients without chronic infections ($p<0.04$) (Fig. 6). Chronically infected CF patients had concentrations similar to healthy controls.

3.4. Exhaled Breath Condensate

No difference in pH between the groups was found (data not shown). Levels of cytokines in the EBC were very low. All samples had TNF-α, IL-1β, IL-6 and IL-10 below detection limit and 14 of 33 samples had IFN-γ below detection limit.

3.5. Sputum levels of cytokines

The A. xylosoxidans group had higher concentrations of TNF-α compared to the other chronically infected groups ($p<0.03$) (Fig. 7). G-CSF, IFN-γ, IL-6, IL-8 and IL-10 were measurable in sputum, but no difference in concentrations was found when comparing the 3 groups.

3.6. Antimicrobial susceptibility

In the A. xylosoxidans group, 8 of 11 strains were resistant to 3 or more groups of antibiotics. In the Bcc group, 7 of 11 patients harboured multiresistant strains. In the P. aeruginosa group the number was 11 of 21 patients (n.s.).

4. Discussion

Our results show significantly enhanced inflammatory parameters in combination with progressed lung disease in CF patients with chronic A. xylosoxidans lung infection. The ability of this microorganism to cause chronic infection or chronic colonization is known, but it has been difficult to establish the clinical significance of this infection. Recent studies have indicated that patients chronically infected with A. xylosoxidans may have a more rapid decrease in lung function compared to CF patients without this infection [36] and a higher percentage of A. xylosoxidans infected CF patients has been observed among CF patients awaiting lung transplantation compared to the general CF community (Brown et al., abstract 359, NACFC 2008). Furthermore, patients with a rapid increase in specific,
precipitating antibodies to *A. xylosoxidans* had a more rapid decline in lung function [36].

It has been suggested that acquisition of chronic *A. xylosoxidans* infection is associated to a poor pulmonary status measured by level of FEV<sub>1</sub>, % of predicted, at time of establishment of chronic infection [37]. In the present study, patients with chronic *A. xylosoxidans* had levels of FEV<sub>1</sub> similar to controls without chronic infection at time of establishment of chronic infection. We found that although patients chronically infected with *A. xylosoxidans* were younger compared to the other groups of chronically infected patients and with duration of chronic infection shorter than the *P. aeruginosa* group, the decline in FEV<sub>1</sub> was as high as in the *P. aeruginosa* group, although lower than in the *Bcc* group.

Biofilms are consortia of bacteria grown in clusters surrounded by a self produced polymer matrix (polysaccharides, proteins, DNA). Both *A. xylosoxidans* and *Bcc* had detectable biofilm-like structures in sputum and this was also the case with mucoid *P. aeruginosa* as reported previously [42]. The biofilms of *A. xylosoxidans* and *Bcc* appeared more dark and condensed, maybe due to the presence of extracellular DNA in the biofilm matrix, and this can maybe explain the reduction of the incidence of such bacteria in sputum of CF patients treated with nebulized DNase [46]. The presence of biofilms in sputum from CF patients infected with *A. xylosoxidans* and *Bcc* could explain the unsuccessful eradication of these chronic infections by antibiotic therapy. Furthermore, similar to chronic *P. aeruginosa* infection, the presence of biofilms can worsen the chronic, probably immune complex-mediated, inflammation and tissue damage in these patients [42], since the production of antibodies against biofilm components such as alginate, LPS, elastase and alkaline protease can lead to formation of immune complexes [47,48].

Increased inflammation in CF patients is well known and high levels of cytokines in BAL-fluid have been shown even in CF infants [7,9,10,14]. Not only in BAL-fluid, but also in sputum, very high levels of IL-1β, IL-6, IL-8 and TNF-α can be found in CF patients [7,8,15,43–45,49–53]. These cytokines increase neutrophilic inflammation in CF airways. Lower than normal levels of IL-10 have been found in CF epithelial cells and airway secretions [49,54], leading to inhibited down-regulation of neutrophilic inflammation. Concentrations of cytokines in serum are low and have been difficult to measure [8,43,53], but some correlation to clinical condition has been found in earlier studies [52,55–57].

The clinical course of the CF lung disease probably depends on the nature and degree of the inflammatory response. Chronic *P. aeruginosa* infection in CF patients is characterized by a Th2 response with higher serum levels of G-CSF and IL-4 [58], and *P. aeruginosa* infected CF patients have a higher proportion of Th2 lymphocytes in BAL-fluid [59]. Th1 response, characterized by higher serum levels of IFN-γ and GM-CSF, is dominant in CF patients with chronic *P. aeruginosa* infection and milder disease compared to CF patients with chronic *P. aeruginosa* and more severe disease [60,61]. Finally, increasing serum levels of G-CSF are correlated to decreasing levels of FEV<sub>1</sub> in CF patients with chronic *P. aeruginosa* infection [57].

In the present study we have shown that patients chronically infected with *A. xylosoxidans* had levels of G-CSF in serum (Fig. 4) comparable to patients with *P. aeruginosa* and a similar rate of decline of lung function after development of chronic infection (Fig. 2). Patients in the *Bcc* group had lower levels of G-CSF in serum, yet this group had the fastest decline in lung function after development of chronic infection. A possible explanation could be a different inflammatory response in the *Bcc* group.

Furthermore we found higher levels of TNF-α in serum from all CF patients compared to healthy controls. This might be a result of other, intermittent, infections, such as *Staphylococcus aureus* or other microorganisms that all CF patients suffer from and confirms a report of signs of “background” CF inflammation found even in newly diagnosed CF infants or maybe shows the presence of another focus outside the lung, e.g. the paranasal sinuses [62,63].

IL-6 levels were significantly higher in chronically infected CF patients when compared to CF patients without chronic, Gram-negative infections. IL-6 is a pro-inflammatory cytokine that has not been very extensively studied in CF patients. IL-6 gene expression can be induced by *P. aeruginosa* infection in a CF bronchial epithelial cell line [64], and IL-6 levels in BAL-fluid were increased in CF children with increasing number of CF pathogens cultured in BAL-fluid [15]. Finally, IL-6 has been measured in serum in CF patients chronically infected with *P. aeruginosa*. These patients had higher levels of IL-6 compared to healthy controls [8], and even slightly higher levels than the levels found in the present study. Our study confirms that increased concentrations of IL-6 seem to be related to the occurrence of infections.

High cytokine concentrations were found in sputum samples in all chronically infected patients. Concentrations of TNF-α in sputum were higher in the *A. xylosoxidans* group when compared to other infected groups (Fig. 7). The clinical relevance of the augmented TNF-α in sputum of the *A. xylosoxidans* group is uncertain, but may be particularly important at the site of the chronic pulmonary infection.

Intrinsic resistance to many antibiotics characterizes the *A. xylosoxidans* and *Bcc* strains. We found similar incidences of
multiresistant strains in all 3 groups of chronically infected patients but different clinical courses in the groups. A direct connection between occurrence of multiresistance and a worse clinical course could not be found in the patients included for this study.

The inflammatory response in Bcc infected patients appears to differ from the response found in CF patients chronically infected with P. aeruginosa. This might partly explain the different and more aggressive nature of disease related to Bcc infection and should be further investigated.

5. Conclusion

A. xylosoxidans is a clinically important pathogen in CF patients causing inflammation and clinical deterioration similar to the changes found in P. aeruginosa infected CF patients.

References


