Haemophilus influenzae and airway inflammation in chronic bronchitis
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Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic *Haemophilus influenzae* airway infection

Comparison with noninfected patients with Chronic Obstructive Pulmonary Disease

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Abstract

Nonencapsulated *Haemophilus influenzae* often causes chronic infections of the lower respiratory tract in both nonobstructive and obstructive chronic bronchitis. We assessed airway inflammation in clinically stable, chronically *H. influenzae*-infected patients with nonobstructive (CB-HI, n = 10) and in patients with obstructive chronic bronchitis (COPD-HI, n = 10) by analyses of the sol phase of spontaneously expectorated sputum (SSP). As compared with the CB-HI group, the COPD-HI group had significantly higher (p < 0.05) levels of myeloperoxidase (MPO) and tumor necrosis factor (TNF)-α in their SSP, whereas the degree of plasma protein leakage (SSP-to-serum ratio of plasma proteins) and the levels of interleukin (IL)-8, secretory IgA, and lactoferrin were similar in the two groups. These findings point to differences in pathophysiology in CB-HI and COPD-HI patients. The high level of TNF-α in the SSP of COPD-HI patients is in accord with the proposed role of TNF-α in the development of airway obstruction in COPD patients. In apparent contradiction, low levels of TNF-α were found in the SSP of noninfected but otherwise similar COPD patients (n = 9). This finding, however, does not exclude an exaggerated TNF-α response to infection or another stimulus in the airways of COPD patients. The SSP levels of MPO and IL-8, and the degree of plasma protein leakage in the COPD-HI group, were retrospectively compared with and found significantly higher than those of noninfected COPD patients, suggesting a more marked inflammatory response in COPD-HI. Whether this reflects a direct cause-and-effect relationship should be addressed in a future long-term prospective study involving repeated measurements in the same patients.

Introduction

Chronic bronchitis (CB) refers to a condition of chronic or recurrent increase in the volume of bronchial secretions sufficient to cause expectoration [1,2]. CB can be associated with chronic airway obstruction (i.e., chronic obstructive pulmonary disease [COPD]) [1,2]. Both nonobstructive and obstructive CB are characterized by chronic inflammation of the airway wall, increased permeability of the airway mucosal microvasculature and epithelium, and hypertrophy of airway submucosal glands [3-5]. In both patients with nonobstructive and those with obstructive CB, nonencapsulated *Haemophilus influenzae* in particular frequently causes recurrent or persistent infections
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of the lower respiratory tract [6-8]. *In vitro* studies and studies employing animal models have indicated that *H. influenzae* may stimulate local inflammatory processes by inducing inflammatory mediators [9,10] and by causing epithelial damage [11,12]. In the present study, we sought to assess airway inflammation in patients with nonobstructive and obstructive CB who were chronically infected with *H. influenzae*. Because other bacterial species frequently occur in the airways of CB patients [6], which may induce a different inflammatory response [13], we studied patients in whom there was no indication of an infection with other bacterial species than *H. influenzae*.

Airway inflammation in nonobstructive and obstructive CB without an evident bacterial airway infection is characterized by airway neutrophilia [4,14,15], which has been shown to be correlated with airflow obstruction in COPD patients [14,15]. Additionally, tumor necrosis factor (TNF)-α has been implicated in the pathogenesis of chronic airway obstruction [16], since relatively high levels of this cytokine were detected in induced sputum samples from smokers with chronic airway obstruction as compared with smokers without obstruction. Moreover, the TNF2 allele, which has been implicated in greater TNF-α production, has been found at a higher frequency in smokers with obstructive chronic bronchitis [17].

In previous studies, we have shown that analysis of the sputum sol phase (SSP) of spontaneously expectorated sputum can be used to assess parameters of airway inflammation [18,19]. Here we report a study comparing airway inflammation as evaluated by analysis of spontaneously expectorated sputum from chronically infected CB patients with and without airway obstruction. Myeloperoxidase (MPO), interleukin (IL)-8, TNF-α, plasma protein leakage, and secretion of products from epithelial cells were measured as markers of inflammation in the SSP. We also studied whether levels of inflammatory indices in the SSP correlated with those in the sputum gel phase (SGP), as had previously been found in analyses of sputum from noninfected COPD patients [18]. Furthermore, we determined TNF-α levels in the SSP of noninfected COPD patients [19,20] in order to compare them with those in the SSP of infected but otherwise similar COPD patients.
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Materials and Methods

Patients
Patients were selected from the outpatient clinic of our hospital. Chronic bronchitis was defined as the production of sputum on most days for at least 3 months of the year during the previous 2 years [1]. COPD was defined according to the American Thoracic Society (ATS) guidelines [2]. Ten patients with nonobstructive CB and 10 patients with CB and irreversible airway obstruction, all chronically infected with *H. influenzae*, participated in the study. The patients had spontaneous sputum production of at least 1 g/24 h. The patients with obstructive CB had airway obstruction (FEV₁<80% predicted and/or a ratio of FEV₁ to VC<75% predicted) that was not reversible by inhalation of a bronchodilator [2]. Spirometry was performed on all patients, using a water-sealed spirometer according to standardized guidelines [21]. All patients had a history of chronic or recurrent infections of the lower respiratory tract with *H. influenzae*, with at least three positive sputum cultures during the year preceding the study, and monthly positive sputum cultures in the 3 months prior to the study. Although these patients had also had sputum cultures positive for other respiratory pathogens over the course of time, patients with sputum cultures positive for other pathogens in the 3 months before the present study were excluded. Patients with radiographic evidence of bronchiectasis or a subnormal level of any IgA, IgM, or IgG subclass were also excluded. All patients were clinically stable. Patients who needed antibiotic treatment within 6 weeks before the study were excluded. Systemic and/or inhaled corticosteroids were stopped at least 6 weeks before the collection of sputum. Patients were allowed to use inhaled β₂-agonists and anticholinergic drugs, and theophylline and/or N-acetylcysteine during the study.

The noninfected COPD patients had originally been recruited for another study [19,20]. Apart from the absence of infection of the lower respiratory tract, the selection criteria for the noninfected COPD patients were identical to those for the infected COPD patients. Lower respiratory tract infections were excluded in the noninfected patients through use of the same techniques used for confirming infection in the infected patients. The noninfected patients had no history of chronic or recurrent infections of the lower respiratory tract, and had had four consecutive sputum cultures negative over a period of at least 10 weeks before sputum was collected for the study.

The study was approved by the Medical Ethics Committee of the Academic Medical Center, Amsterdam, and informed consent was obtained from all of the subjects.
**Sputum collection and processing**

Patients collected sputum at home over a 24-hour period directly before visiting our hospital for lung function tests, as previously described [18,19]. They were instructed to minimize the collection of saliva (i.e., to rinse the mouth with water before expectorating sputum). The sputum was stored immediately by the patient at -20°C in the freezing compartment of the patient's home refrigerator, and was brought to the laboratory in a Perspex canister with ice to keep it cold [18,19]. A venous blood sample was taken at the same visit. A separate, fresh sputum sample was also obtained at the visit, and was used for semiquantitative bacteriologic culture according to the guidelines of the American Society for Microbiology (ASM) [22]. Sputum samples that showed significant upper respiratory tract contamination were excluded. To that end, two smears from each sputum sample were reviewed according to ASM guidelines [22], and samples that contained a mean of ≥10 squamous cells per low-power field (x10 objective) were excluded. The presence of *H. influenzae* was assessed by culturing a sputum sample on chocolate agar and by immunostaining a smear of sputum with the specific monoclonal antibody 8BD9 [23]. *H. influenzae* was further identified by its dependence on growth factors X and V and its inability to convert δ-aminolevulanic acid to porphyrins [24].

The 24-h sputum samples were thawed and the SSP was separated from the SGP by centrifugation at 50,000 x g at 4°C for 90 min [18,19]. The SSP and SGP were stored in aliquots at -80°C until analysis. The SGP was solubilized with dithiothreitol (DTT; Sigma Chemical Company, St. Louis, MO, USA) and deoxyribonuclease (DNAse; Sigma) [18,19].

**Analysis of inflammatory parameters in sputum**

Interleukin (IL)-8 was quantitated with an enzyme-linked immunosorbent assay (ELISA) [20]. TNF-α in the SSP was determined with the Medgenix TNF-α enzyme-amplified sensitivity immunoassay kit (Biosource, Fleurus, Belgium). As a standard, we used recombinant human (rh)TNF-α. The recovery of rhTNF-α in SSP was 102±3 % (mean±SEM). The peroxidase activity of MPO in the SSP was determined as a marker of neutrophilic inflammation [20,25]. The concentrations of albumin (Alb; molecular mass = 67 kD), and α2-macroglobulin (A2M; molecular mass = 725 kD) were determined for the study of plasma protein exudation [18,19,26]. The SSP-to-serum ratio (Q-protein) was calculated for each protein, thereby correcting for the variation in protein concentrations in the blood [18,19]. We also determined the relative coefficient of
excretion (RCE) (= QA2M/QAlb) of proteins from serum into the SSP, with an increase in RCE reflecting the loss of size selectivity of the respiratory membrane [18,27]. Lactoferrin [28], and secretory IgA (sIgA) [29,30] were used as markers for activation of epithelial cells. Both of these latter substances were measured with ELISAs [31]. All assays used in the study had previously been validated for analysis of sputum [18-20]. Reference samples were used in all assays to allow comparison of current with previous measurements.

TNF-α, MPO, Alb, and A2M were also assayed in DTT-treated SSP and SGP [18,19], and the protein levels in total sputum were calculated as follows: (SSP fraction x [protein]ssp) + (SGP fraction x [protein]scp). In these assays, MPO was determined as antigen. ELISA plates were coated in the same way as for the MPO activity assay [20,25], and standards and SSP samples were incubated for 2 h. After a wash, bound MPO was detected with biotinylated rabbit anti-human MPO in combination with streptavidin-poly-horseradish peroxidase (CLB, Amsterdam, The Netherlands). To this end, rabbit antihuman MPO (A 398; DAKO, Glostrup, Denmark) was biotinylated with N-succinimidyl-long-chain biotin (Pierce Chemical Company, Rockford, Illinois) according to the manufacturer’s instructions. As shown previously, DTT did not affect these assays [18,19]. The measurement of IL-8 was adversely affected by DTT treatment in our assay, and the SGP could therefore not be analyzed for IL-8. For the assay of TNF-α in SSP-DTT and SGP-DTT, we applied the Cyto-Sets antibody pairs (Biosource, Etten-Leur, The Netherlands). This TNF-α assay was not affected by DTT.

**Statistical analysis**

Differences between the patient groups were analyzed with a parametric one-way analysis of variance (ANOVA) or with the nonparametric Kruskall-Wallis one-way ANOVA test (KW test), as appropriate. In cases of an overall statistical difference, the differences between two groups were further analyzed, using either Student’s t test or the Mann-Whitney U test (MWU test). Values of p for pairwise comparisons were corrected for multiple comparisons through the use of Bonferroni’s or Dunn’s multiple comparisons test, as appropriate. Differences between parameters within a group of patients were analyzed with Wilcoxon’s signed-ranks test (WSR test). Spearman’s rank correlation test was used to assess correlations between parameters. A value of p<0.05 was considered significant.
Results

Analyses of the SSP of H. influenzae-infected CB patients
Sputum from 10 chronically H. influenzae-infected patients with nonobstructive CB (CB-HI) and 10 chronically H. influenzae-infected patients with obstructive chronic bronchitis (COPD-HI) was analyzed. The characteristics of the patients are listed in Table 1. The nonobstructive and the obstructive CB patients did not differ with respect to age, amount of sputum expectorated in 24 h, or relative amount of their SSP. The CB-HI patients were selected on the basis of normal results of spirometry. These patient's FEV₁ (% predicted) differed significantly from that of the COPD-HI group (p<0.001, Student's t test). The bacterial load, based on semiquantitative sputum cultures, was similar in both groups of patients, and was estimated as moderate to abundant, or about $10^4$ to $10^5$ cfu/ml.

Table 1. Patient characteristics of the study groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CB-HI</th>
<th>COPD-HI</th>
<th>COPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>Sex, F/M</td>
<td>6/4</td>
<td>3/7</td>
<td>3/6</td>
</tr>
<tr>
<td>Age, yr</td>
<td>52.4 (5.0)</td>
<td>62.8 (2.5)</td>
<td>58.9 (2.0)</td>
</tr>
<tr>
<td>FEV₁, % predicted*</td>
<td>99.2 (4.4)</td>
<td>65.0 (7.1)</td>
<td>57.6 (2.9)</td>
</tr>
<tr>
<td>Current/ex-smoker</td>
<td>3/4</td>
<td>5/4</td>
<td>7/2</td>
</tr>
<tr>
<td>24-h sputum weight, g</td>
<td>8.3 (3.0)</td>
<td>10.7 (2.2)</td>
<td>11.7 (3.0)</td>
</tr>
<tr>
<td>SSF</td>
<td>0.58 (0.07)</td>
<td>0.48 (0.06)</td>
<td>0.55 (0.04)</td>
</tr>
</tbody>
</table>

CB-HI=chronic bronchitis with chronic Haemophilus influenzae infection; COPD=chronic obstructive pulmonary disease; COPD-HI=chronic obstructive pulmonary disease with chronic Haemophilus influenzae infection; F=female; M= male; SSF=sputum sol fraction. Data are expressed as mean (SEM). *FEV₁ after-bronchodilation. †p<0.001 compared with COPD-HI.

The MPO levels in the SSP, assessed as a parameter of neutrophilic inflammation, were significantly higher in the COPD-HI than in the CB-HI group (Figure 1A; p<0.05, MWU test). High levels of IL-8 were detected in the SSP of both groups (Figure 1B). The levels of TNF-α in the SSP of the COPD-HI group were significantly higher than in that of the CB-HI group (Figure 2; p<0.05, MWU test). For comparison, TNF-α levels were determined in the SSP of nine noninfected COPD patients [19,20]. The patient characteristics of these noninfected (indicated as COPD) patients were similar to those
of the infected COPD-HI patients (Table 1). TNF-α levels were significantly lower in the SSP of the noninfected COPD patients than in that of either group of infected patients (Figure 2; COPD-HI: p < 0.001; CB-HI: p < 0.05, MWU test).

Figure 1. (A) MPO (nM) and (B) IL-8 (ng/ml) levels in the SSP of CB-HI (n = 10) and COPD-HI (n = 10) patients. Horizontal bars indicate median values.

Figure 2. TNF-α (pg/ml) in the SSP of CB-HI (n = 10), COPD-HI (n = 10), and COPD (n = 9) patients. Horizontal bars indicate median values.
The Q protein values for the COPD-HI and CB-HI groups are presented in Table 2. The QA2M (67 kD) values were significantly higher than the QA2M (725 kD) values (both p<0.01, WSR test), which is in accord with the greater permeation across the airway mucosa of molecules with a small molecular mass (Alb) than of molecules with a high molecular mass (A2M) [32]. Neither the Q proteins values nor the RCE values (Table 2) differed between the two groups of patients.

Table 2. Plasma protein exudation and levels of sIgA and lactoferrin in the sputum sol phase

<table>
<thead>
<tr>
<th></th>
<th>CB-HI</th>
<th>COPD-HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>QA2M</td>
<td>3.4 (0.5-20.7)</td>
<td>4.3 (0.5-12.6)</td>
</tr>
<tr>
<td>RCE (QA2M/QA2M)</td>
<td>0.27 (0.06-1.23)</td>
<td>0.49 (0.13-0.92)</td>
</tr>
<tr>
<td>sIgA, mg/L</td>
<td>144 (60-359)</td>
<td>108 (21-1017)</td>
</tr>
<tr>
<td>Lactoferrin, mg/L</td>
<td>152 (49-289)</td>
<td>119 (24-263)</td>
</tr>
</tbody>
</table>

CB-HI = chronic bronchitis with chronic Haemophilus influenzae infection; COPD-HI = chronic obstructive pulmonary disease with chronic Haemophilus influenzae infection; QA2M = ratio of α2-macroglobulin in sputum sol phase to α2-macroglobulin in serum; QA2M = ratio of albumin in sputum sol phase to albumin in serum; RCE = relative coefficient of excretion of proteins from serum into the sputum sol phase (QA2M/QA2M); sIgA = secretory immunoglobulin A. Values are presented as median (range).

The levels of secretory IgA (sIgA) and lactoferrin, assessed as markers of secretion by airway epithelial cells, did not differ in the SSP of the COPD-HI and CB-HI groups (Table 2). The levels of sIgA and lactoferrin were significantly correlated with each other within each group (CB-HI: r = 0.71; COPD-HI: r = 0.65, both p < 0.05 by Spearman’s rank correlation test).

In both groups, the levels of TNF-α were correlated with the RCE, with the correlation being statistically significant in the COPD-HI group (CB-HI: r = 0.61, p = 0.07; COPD-HI: r = 0.75, p < 0.05; Spearman’s rank correlation test). The levels of MPO in the SSP also showed a correlation with the RCE, which tended to be significant in the CB-HI group (CB-HI: r = 0.58, p = 0.08; COPD-HI: r = 0.49, p = 0.16; Spearman’s rank correlation test).

**Distribution of proteins between the SSP and the SGP**

The distribution of MPO, Alb, and A2M between the SSP and the SGP has been studied previously in sputum from noninfected patients [18,19], but not in sputum from infected
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patients. In the present study, sufficient sputum was available from 16 patients chronically infected with *H. influenzae* to permit these additional analyses (CB-HI: n = 8; COPD-HI: n = 8) (Table 3).

Table 3. Proteins* in dithiothreitol-treated sputum sol and gel phases and in sputum

<table>
<thead>
<tr>
<th></th>
<th>CB-HI (n = 8)</th>
<th>COPD-HI (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP-DTT</td>
<td>7.7 (2.2-152)</td>
<td>19.7 (2.4-263)</td>
</tr>
<tr>
<td>SGP-DTT</td>
<td>174 (68-260)*</td>
<td>333 (19-620)**</td>
</tr>
<tr>
<td>Sputum</td>
<td>54 (2.0-25.3)*</td>
<td>242 (9-535)†</td>
</tr>
<tr>
<td>Alb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP-DTT</td>
<td>421 (322-2330)</td>
<td>410 (315-1040)</td>
</tr>
<tr>
<td>SGP-DTT</td>
<td>502 (292-1774)</td>
<td>618 (330-1045)</td>
</tr>
<tr>
<td>Sputum</td>
<td>632 (290-2010)</td>
<td>540 (330-1045)</td>
</tr>
<tr>
<td>A2M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP-DTT</td>
<td>14.2 (4.9-129)</td>
<td>16.0 (2.0-27.5)</td>
</tr>
<tr>
<td>SGP-DTT</td>
<td>49.3 (11.4-144)</td>
<td>32.7 (3.5-50.5)‡</td>
</tr>
<tr>
<td>Sputum</td>
<td>33.9 (9.1-104)</td>
<td>28.1 (2.6-44.3)†</td>
</tr>
<tr>
<td>RCE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSP-DTT</td>
<td>0.39 (0.26-2.04)</td>
<td>0.50 (0.14-1.06)</td>
</tr>
<tr>
<td>Sputum</td>
<td>0.78 (0.39-3.96)‡</td>
<td>0.95 (0.29-1.57)†</td>
</tr>
</tbody>
</table>

A2M = α2-macroglobulin; Alb = albumin; CB-HI = chronic bronchitis with chronic *Haemophilus influenzae* infection; COPD-HI = chronic obstructive pulmonary disease with chronic *Haemophilus influenzae* infection; MPO = myeloperoxidase; RCE = relative coefficient of excretion of proteins from serum into the sputum sol phase; SGP-DTT = dithiothreitol-treated sputum gel phase; SSP-DTT = dithiothreitol-treated sputum sol phase.

Values are presented as median (range). * in mg/L; †p<0.05, WSR test, compared with levels in SSP-DTT; ‡ p<0.01, WSR test, compared with levels in SSP-DTT; § MPO in CB-HI group, analyzed in seven patients.

The levels of MPO in DTT-treated SSP (SSP-DTT) and DTT-treated SGP (SGP-DTT) were significantly correlated with one another (r = 0.51, p < 0.05, n = 15; Spearman’s rank correlation test). In both groups, the MPO levels in SGP-DTT were significantly higher than those in SSP-DTT (Table 3). The average sputum-to-SSP ratios of MPO in the nonobstructive and obstructive CB patients were similar (CB-HI = 9.2 and COPD-HI = 9.7).

The levels of Alb and A2M in SSP-DTT and SGP-DTT were also significantly correlated (Alb: r = 0.71, p < 0.005; A2M: r = 0.74, p < 0.001; n = 16, Spearman’s rank correlation test). The levels of Alb in SSP-DTT and SGP-DTT were similar, whereas the
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Levels of A2M in SGP-DTT were slightly higher than those in SSP-DTT (Table 3). This difference was statistically significant only in the COPD-HI group (Table 3). As a consequence, total sputum levels of Alb did not differ from those in SSP-DTT, whereas the total sputum levels of A2M were higher than those in SSP-DTT. In both groups, the RCE in total sputum was significantly higher than the RCE in SSP-DTT (Table 3), whereas neither RCEPUTUM, nor RCESSPDTT differed in the two groups.

In addition, we analyzed the distribution of TNF-α in sputum from nine patients (CB-HI: n = 4; COPD-HI: n = 5). The levels of TNF-α in the SGP were lower than in the SSP, and the average sputum-to-SSP ratios were similar in both subgroups of patients (CB-HI: 0.7; COPD-HI: 0.6).

Discussion

The results of our study indicate that patients with CB and irreversible airway obstruction who have chronic H. influenzae airway infection have higher levels of MPO and TNF-α in their sputum than do chronically H. influenzae-infected CB patients without airway obstruction. In contrast, other parameters of local airway inflammation and epithelial cell activation (i.e., the degree of plasma protein exudation and the levels of IL-8, sIgA, and lactoferrin) were similar in these two groups of patients.

Our observations of the levels of TNF-α are of particular interest. TNF-α was implicated in the pathogenesis of COPD by Keatings and coworkers [16], who detected higher levels of TNF-α in induced sputum samples from COPD patients than in sputum samples from healthy smokers, patients with asthma, and nonsmoking healthy controls. The high levels TNF-α in the COPD-HI patients as compared with those of the CB-HI patients in our study would appear to support this proposal. In this respect, it is of interest whether the two CB-HI patients in our study who had high levels of TNF-α in the SSP, both of whom were relatively young smokers (39 and 36 years), will develop airway obstruction over time. On the other hand, although the TNF-α levels in the SSP were much higher in the chronically infected COPD than in the noninfected COPD patients in our study, the degree of airway obstruction was similar in the two groups [19,20]. This finding indicates that TNF-α is present in low concentrations in the SSP of clinically stable noninfected COPD patients, but does not exclude an exaggerated TNF-α response to infection or another stimulus in these patients.
As in the case of the TNF-α data for the CB-HI group, there were two outliers for the MPO data. The patient with the highest TNF-α level also had the highest MPO level, but the two other outliers occurred in different patients. These outliers did not cause the observed differences between the CB-HI and COPD-HI patients.

We found that in both subgroups of infected CB patients, levels of TNF-α and MPO were correlated with the RCE, a parameter considered to reflect the loss of size selectivity of the respiratory membrane [18,19]. However, neither plasma protein exudation, nor the RCE differed between the two groups of chronically infected patients. Since the subject's sputum had been stored at -20°C before separation of the sol from the gel phase, the increased levels of TNF-α in the SSP of the COPD-HI patients may reflect increments in the number of TNF-α-containing cells or higher intracellular levels of TNF-α, rather than reflecting the release of TNF-α by these cells. Likewise, the increased levels of MPO may not reflect activation of neutrophils, but only the presence of these cells. Therefore, TNF-α and MPO may not have contributed fully to the extent of inflammation and epithelial-cell activation in the COPD-HI patients. Analysis of induced sputum may allow differentiation of released from intracellular TNF-α and MPO. However, direct comparison of induced and spontaneously expectorated sputum cannot be made, because induced sputum appears to contain a higher proportion of viable cells than does spontaneously expectorated sputum [33]. Although this is an important issue for resolution in future studies, our results indicate that COPD-HI patients have a local increase in the levels of TNF-α and MPO in their SSP, and/or a local increase in the numbers of cells containing TNF-α and MPO, which points to differences in the pathophysiology of CB-HI and COPD-HI.

Analysis of inflammatory parameters in the SSP of spontaneously expectorated 24-h sputum has been previously validated for sputum from noninfected COPD patients [18]. In our study, analysis of sputum collected from clinically stable COPD patients at two subsequent visits with a 2-week interval yielded highly reproducible findings despite possible variations in collection and storage of sputum by the patients. Also, analysis of the SSP was found to adequately reflect that of total sputum (i.e., SSP+SGP) from noninfected COPD patients [18]. We analyzed sputum from chronically H. influenzae-infected CB patients with and without chronic airway obstruction. In both groups of infected CB patients, the SGP contained significantly higher levels of MPO and A2M than did the SSP, whereas the levels of Alb in the SGP and SSP were similar. Levels of TNF-α were higher in the SSP than in the SGP. The distribution of the foregoing proteins between the SGP and the SSP did not differ in the CB-HI and COPD-HI groups.
However, the distribution of proteins between the SGP and SSP of infected COPD patients was clearly different from that observed for noninfected COPD patients [18,19]. MPO and A2M were retained in greater quantity in the SGP of infected than of noninfected COPD patients [18,19]. Considered collectively, this shows that analysis of the SSP from chronically H. influenzae-infected patients with both nonobstructive and obstructive CB adequately reflects the levels of proteins in total sputum. Furthermore, because of the observed differences in distribution of proteins in the SGP and SSP of infected as opposed to noninfected patients, our findings also indicate the need for caution in direct comparisons of levels of proteins in the SSP of different groups of patients.

Little is known about the effect of chronic airway infection on local airway inflammation in COPD. The characteristics of the noninfected COPD patients [19,20], whose SSP we analyzed for TNF-α were similar to those of the infected COPD patients in our study. Because we assessed the same parameters in the SSP of the noninfected and infected COPD patients, using the same methodology and the same standards [19,20], we were able to compare inflammatory parameters in the SSP of these two groups of patients. We found significantly lower levels of MPO (COPD: median = 12.7 nM; range = 5.6 to 36.6 nM [19] and IL-8 (COPD: median = 16.3 ng/ml; range = 9.3 to 56.7 ng/ml [20]), as well as of TNF-α, in the noninfected COPD group than in the COPD-HI group (both p < 0.001, MWU test). Moreover, the parameters of plasma protein leakage QAlb (COPD: median = 4.28; range = 3.39 to 10.90) and QA2M (COPD: median = 0.80; range = 0.40 to 5.66), as well as the RCE (COPD: median = 0.13; range = 0.07 to 0.52), were significantly lower in the noninfected COPD patients [19] than in the COPD-HI group (all p < 0.05, MWU test). This indicates that local airway inflammation and inflammatory damage are greater in chronically infected COPD patients than in noninfected COPD patients. Because of the different distribution of inflammatory markers between the SSP and SGP of sputum from infected and noninfected patients, caution is advised in reaching conclusions based on analyses only of the SSP. However, we found that levels of MPO and A2M in both the SSP and the SGP of infected patients were higher than those in the respective sputum phases of noninfected patients, indicating that differences in MPO and QA2M/QAlb in the total sputum of infected versus noninfected COPD patients are even greater.

Whether the more pronounced airway inflammation in chronically infected COPD patients results in a more rapid decline of lung function remains unknown [6,34]. In only one of four prospective studies was it concluded that more frequent episodes of
Acute infection were associated with a more rapid decline in lung function [6]. Furthermore, little is known about the impact of recurrent and persistent bacterial airway infections on the decline in lung function in COPD patients [6,34,35]. The differences between noninfected and infected COPD patients that we found in levels of the proinflammatory mediator TNF-α may also represent a primary difference between these two groups. It could be envisaged that COPD patients with an exaggerated production of TNF-α have greater airway inflammation, as a result of which *H. influenzae* can more easily colonize these patients’ airways [6,34,36]. Despite these clear differences between infected and noninfected COPD patients, the data in the present study are based on relatively small numbers of patients and, except for TNF-α, on comparison with historic data [19,20]. Our findings are an incentive for a larger, and prospective study on the role of bacterial infections in airway inflammation in COPD patients.

In summary, we have shown that local airway inflammation as assessed by the analysis of spontaneously expectorated sputum differs in chronically *H. influenzae*-infected patients with nonobstructive CB and those with obstructive CB. Specifically, higher levels of TNF-α and MPO in the airways of the patients with obstructive CB point to differences in the pathophysiology of CB-HI and COPD-HI. It remains to be determined whether similar differences are evident in bacterial infections other than with *H. influenzae*. This is of interest because patients with CB often experience airway infections with other bacterial pathogens [6]. Comparison with the noninfected COPD patient group suggests that airway inflammation is more pronounced in chronically infected COPD patients. Whether this reflects a direct cause-and-effect relationship for inflammation in the two groups should be addressed in a future, long-term prospective study involving repeated measurements in the same individual patient. Furthermore, in view of the observed differences in airway inflammation, further studies are warranted to determine whether recurrent and/or persistent bacterial infections are associated with a more rapid decline of lung function in COPD patients.

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