Airway Inflammation and Etiology of Acute Exacerbations of Chronic Bronchitis*

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Study objectives: **The etiologic role of bacterial pathogens isolated from sputum culture in 40 to 50% of acute exacerbations of chronic bronchitis (AECB) is controversial. If bacterial pathogens cause these AECB, they should be associated with greater neutrophilic airway inflammation than pathogen-negative exacerbations.**

Design: **This hypothesis was tested by comparing levels of interleukin (IL)-8, tumor necrosis factor (TNF)-α, and neutrophil elastase (NE) in 81 sputum samples obtained from 45 patients with AECB. Four groups were compared. In the first three groups, nontypeable *Haemophilus influenzae* (n = 20), *Haemophilus parainfluenzae* (n = 27), and *Moraxella catarrhalis* (n = 14) were isolated as sole pathogens, respectively. In the fourth group, only normal flora was isolated (n = 20). Paired samples, obtained from individual patients at different times, that differed in their culture results were also compared.**

Setting: **An outpatient research clinic at a Veterans Affairs Medical Center.**

Patients: **These patients were participating in a prospective, longitudinal study of the dynamics of bacterial infection in chronic bronchitis, for which they were seen in the study clinic on a monthly basis as well as when they were experiencing symptoms suggestive of AECB.**

Interventions: **None.**

Measurements and results: H influenzae **exacerbations were associated with significantly higher sputum IL-8, TNF-α, and NE. *M catarrhalis* exacerbations demonstrated significantly higher sputum TNF-α and NE when compared to pathogen-negative exacerbations. *H parainfluenzae*-associated exacerbations had an inflammatory profile similar to pathogen-negative exacerbations. Sputum elastase level distinguished bacterial from nonbacterial AECB and correlated with clinical severity of the AECB.**

Conclusions: **Increased airway inflammation associated with isolation of *H influenzae* and *M catarrhalis* supports an etiologic role of these pathogens in AECB.**

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Key words: airway inflammation; chronic bronchitis; exacerbation; *Haemophilus moraxella*

Abbreviations: AECB = acute exacerbations of chronic bronchitis; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; NE = neutrophil elastase; PWB = plate wash buffer; TNF = tumor necrosis factor

Chronic bronchitis is associated with intermittent exacerbations (acute exacerbations of chronic bronchitis [AECB]) that present with worsening of the chronic symptoms of productive cough and dyspnea. These exacerbations cause considerable morbidity and in patients with concomitant airway obstruction (COPD) are a major cause of mortality.[1] AECB can have one or more of several different etiologies.[2] Virus infection, identified by a fourfold rise in antibody titer or by viral isolation, causes a third of exacerbations.[3–5] Serologic evidence of atypical bacterial infection, mostly by *Chlamydia pneumoniae*, is seen in 5 to 10% of

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exacerbations.[6,7] Bacterial pathogens, especially nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella (Branhamella) catarrhalis*, are isolated from sputum in about 50% of exacerbations.[2]

The role of bacterial pathogens as a cause of AECB is controversial for several reasons.[8–11] Bacterial pathogens can be isolated from sputum during stable chronic bronchitis at the same frequency as during exacerbations. Serologic studies examining antibodies to common bacterial pathogens and placebo-controlled antibiotic trials in AECB have yielded confusing and contradictory results. Though alternative explanations exist for these observations, many authors have interpreted them to show that bacterial pathogens play no role in AECB.[8,11] This view holds that isolation of bacteria during AECB represents chronic colonization and is a mere epiphenomenon.

If bacterial pathogens were playing a role in AECB, one would expect a neutrophilic inflammatory response in the airways to the acute infection. Therefore, one would expect that airway inflammation during AECB should be related to sputum culture results. We therefore hypothesized that sputum collected during bacterial AECB exhibits neutrophilic inflammation to a greater degree than sputum collected during pathogen-negative AECB, which are of a viral or other etiology.

We tested this hypothesis by measuring markers of inflammation in a collection of sputum samples obtained during AECB observed during a prospective, longitudinal study of bacterial infection in chronic bronchitis. Pathogen-negative AECB were those in which only normal flora was isolated from sputum obtained at the time of diagnosis. Normal flora was defined as the absence of the following pathogens in sputum culture: Haemophilus spp., *M catarrhalis*, *S pneumoniae*, *Staphylococcus aureus*, Pseudomonas spp., and Gram-negative bacilli. Bacterial AECB were those in which any of these pathogens were isolated from sputum culture. The bacterial AECB included in this study were specifically those in which either *H influenzae* or *M catarrhalis* was the sole pathogen isolated from sputum obtained at the time of diagnosis. To examine the role of *Haemophilus parainfluenzae* as a pathogen in AECB, we included a group of exacerbations in which *H parainfluenzae* was the sole "pathogen" isolated. We measured neutrophilic inflammation by quantifying interleukin (IL)-8, tumor necrosis factor (TNF)-α, and neutrophil elastase (NE) in the sputum supernatants.[12–14] Pathogen-positive sputum samples were compared with pathogen-negative samples.

Materials and Methods

Chronic Bronchitis Study Clinic

The Human Studies Subcommittee of the Department of Veterans Affairs Western New York Healthcare system approved our study protocol. All participants gave written informed consent. A total of 74 patients were enrolled in a prospective, longitudinal study of the dynamics and immune response to bacterial infection in chronic bronchitis from March 1994 to December 1997. Inclusion criteria were as follows: (1) the presence of chronic bronchitis as defined by the American Thoracic Society, [15] (2) the absence of asthma or bronchiectasis by clinical assessment, (3) the ability to comply with monthly clinic visits, and (4) the absence of immunosuppression (< 20 mg/d of prednisone was allowed) or other life-threatening illness. The subjects were seen monthly and whenever they were experiencing symptoms suggestive of AECB in an outpatient study clinic at the Buffalo Veteran Affairs Medical Center.

At each visit, clinical information, sputum, and serum samples were collected. Of a total of 1,503 clinic visits, the subject's COPD was clinically stable in 1,227 of these visits (81.6%), while in 276 visits (17.4%), the subject was experiencing an AECB. Whether a patient was stable or experiencing an exacerbation was determined as follows. At each visit, the subject was questioned about the status of his chronic respiratory symptoms (dyspnea; cough; sputum production, viscosity, and purulence), and the responses were graded as same as baseline or worse than baseline. If the symptom was reported to be worse than baseline, the subject was asked to grade it as somewhat worse or much worse than baseline. If there was minor (somewhat worse) worsening of two or more symptoms or a major (much worse) worsening of one or more symptoms, a clinical assessment was made as to the cause. If necessary, a chest radiograph was obtained. If no other cause, such as pneumonia, upper-respiratory infection, or congestive heart failure, was identified, the patient was determined to be experiencing an exacerbation.

We also calculated an overall clinical score at each visit, which served as a measure of clinical severity of the AECB episode. At each clinic visit in our longitudinal study, we assessed 10 clinical parameters (overall well-being; dyspnea; cough; sputum production, viscosity, and purulence; overall appearance, respiratory rate, wheezing, and rales) and graded each of these from 1 to 3 as described above. A score of 10 represented baseline, and a score of 30 represented the sickest patient.

Sputa collected were spontaneous morning samples. The whole sputum sample was weighed, graded macroscopically for purulence and viscosity, and homogenized by incubation at 37°C for 15 min with an equal volume of 0.1% dithiothreitol (Sputolysin; Calbiochem; San Diego, CA). Serial dilutions of homogenized sputum were prepared in phosphate-buffered saline solution and plated on blood, chocolate, and MacConkey agar plates. Bacterial identification was performed by standard techniques. If *H influenzae*, *M catarrhalis*, or *S pneumoniae* was present, we attempted to isolate and characterize 10 individual colonies of each bacterial species. Single colonies of other potential pathogens, including *H parainfluenzae*, *S aureus*, Pseudomonas spp., and Gram-negative bacilli were also isolated and characterized. Bacterial titers were measured by counting the number of colonies in the dilution plates and multiplying the count by the appropriate dilution factor. The remainder of the sputum sample was centrifuged at 25,000*g* for 45 min at 4°C, and the resultant supernatant was stored at − 70°C.

Of the 276 episodes of AECB, sputum samples were obtained for culture in 267 episodes. Normal flora was isolated in 83 of these samples (pathogen-negative AECB), while one or more

potential pathogens (Haemophilus spp., *M catarrhalis*, *S pneumoniae*, *S aureus*, Pseudomonas spp., and Gram-negative bacilli) were isolated in 184 episodes (bacterial AECB). Sputum supernatant obtained in 81 exacerbations in 45 patients was selected to be included in this study, as shown in Figure 1. These selection criteria allowed us to avoid overrepresentation by patients who had frequent exacerbations.

Comparison of Expectorated to Induced Sputum

Sputum induction with hypertonic saline solution has become a widely accepted research technique to obtain lower respiratory tract secretions in airway diseases such as asthma and COPD.[14,16,17] To assess the adequacy of our expectorated sputum samples as representative of the lower-airway milieu, 52 consecutive induced sputum samples from 31 patients were compared with spontaneously expectorated sputum collected by the patient on the morning of the induction. A volume of 30 mL of hypertonic (3%) saline solution was nebulized over 15 to 20 min with an ultrasonic nebulizer (model 099HD; DeVilbiss; Carlsbad, CA) to induce sputum production. The paired sputum samples were graded for purulence and viscosity, and subjected to Gram's staining, microscopic examination, and measurement of fibrinogen level (a marker for lower respiratory tract secretions). Table 1 shows the scoring system used to categorize the gross and microscopic features of the sputum specimens.

Sputum Fibrinogen Measurement

Fibrinogen is absent in saliva and is present in measurable amounts in lower respiratory tract secretions.[17] To assess the quality of the expectorated sputum samples, paired induced and expectorated sputa were subject to measurement of fibrinogen levels by using a competitive enzyme-linked immunosorbent assay (ELISA) as described previously.[17] Undiluted and tenfold dilutions of the samples were tested. The minimum fibrinogen level detectable was 30 ng/mL, and the linear range of this assay was 60 to 2,000 ng/mL.

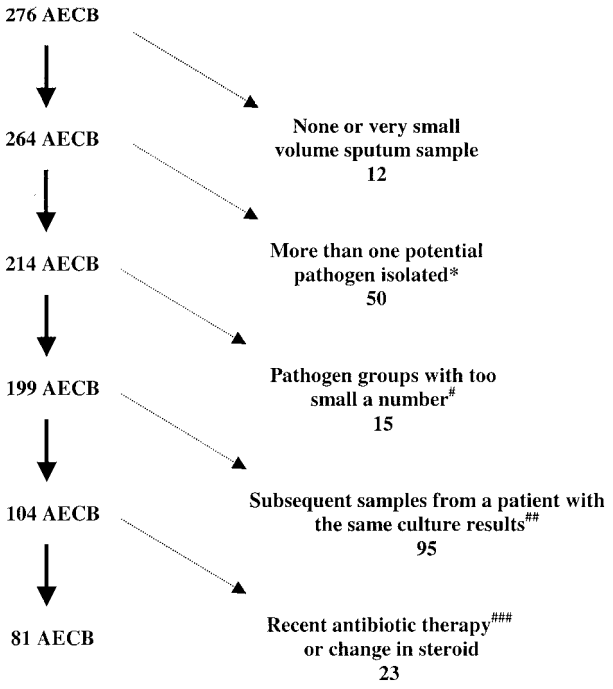

FIGURE 1. Sputum-sample selection flow sheet.

Table 1—*Scoring System for Comparison of Induced and Spontaneous Samples

Characteristics	Score 1	Score 2	Score 3
Purulence	Mucoid	Mucopurulent	Purulent
Viscosity	Thin, watery	Thick	Extremely thick
Nonsquamous cells	< 10/LPF	10–25/LPF	> 25/LPF
Squamous cells	< 10/LPF	10–25/LPF	> 25/LPF

*LPF = low power field of microscope (×10).

Sputum Inflammation Measurement

In all of the assays described below, Immulon-4 microtiter plates (Dynatech; Chantilly, VA) were used. All sputum supernatants and standards were tested in duplicate, and mean values were used. Intra-assay coefficient of variation of < 10% and interassay coefficient of variation of < 15% were accepted. Because of limited volume of sputum supernatant available for a small number of samples, all measurements were not performed on all samples (see "Results" section).

IL-8 Assay

A sandwich ELISA was developed. All cytokine reagents were obtained from R&D Systems, Minneapolis, MN. All intermediate washes were done with plate wash buffer ([PWB], phosphate-buffered saline solution with 0.05% Tween-20). Microtiter wells were coated with 50 μL of goat antihuman IL-8 antibody diluted to 4 μg/mL in carbonate-bicarbonate buffer (0.1M sodium carbonate, 0.1M sodium bicarbonate, pH 9.6) overnight at room temperature. The coating antibody was aspirated, and the wells were blocked with 300 μL of 3% nonfat dried milk in PWB for 1 h. The wells were washed, dried, and then coated with 50 μL of immunoassay stabilizer (Stabilcoat; Surmodics; Eden Prairie, MN) for 45 min. After removing the Stabilcoat, the plates were dried overnight at room temperature and stored at 4°C.

Serial dilutions of recombinant IL-8 and 1:10 dilution of sputum supernatants in 1% nonfat dried milk in PWB were prepared. Fifty microliters of the standard or sample was added to the wells and incubated at 37°C for 2 h. After washing, 50 μL of biotinylated antihuman IL-8 diluted to 200 ng/mL in 1% nonfat dried milk in PWB was added to the wells for 1 h at 37°C. After washing, bound biotinylated antibody was detected with streptavidin-horseradish-peroxidase, and the optical density of the wells was read at 450 nm. A standard curve was constructed from the wells containing recombinant IL-8, and the amount of IL-8 in the samples was read from this curve. The lower limit of detection of IL-8 in this assay was 5 pg/mL, and the linear range was 20 to 12,500 pg/mL.

TNF-α Assay

A sandwich ELISA similar to the IL-8 ELISA was developed with the appropriate reagents obtained from R&D Systems. Homogenized sputum supernatants were tested without further dilution. The lower limit of detection of TNF-α was 10 pg/mL, and the linear range was 80 to 5,000 pg/mL.

NE Assay

Free elastase activity in undiluted sputum supernatants was determined with a colorimetric assay with a synthetic substrate, N-methoxysuccinyl-ala-ala-pro-val p-nitroanilide, as described previously.[12] Standard elastase was site-titrated enzyme purified from sputum obtained from Elastin Productions (Owensville, MO). The minimum elastase activity detectable was 41 MU/mL, and the linear range was 94 to 3,200 MU/mL.

Data Analysis

Nonparametric statistical methods were used for all data with Statview 5.0 software. For unpaired data, the Kruskall-Wallis and Mann-Whitney *U* rank tests were used; for paired data, the Wilcoxon signed-rank test was used. Correlation was tested with the Spearman coefficient. A $p < 0.05$ was considered significant.

Results

Patient Demographics

As shown in Table 2, our population was predominantly an elderly male population. On enrollment, 26 of the subjects (58%) had severe COPD (FEV_1 < 50% predicted), 12 subjects (27%) had moderate COPD (FEV_1 of 50 to 64% predicted), 2 subjects (4%) had mild COPD (FEV_1 of 65 to 80% predicted), and 5 subjects (11%) had chronic bronchitis only.

Comparison of Expectorated and Induced Sputa

The only significant differences between the induced and expectorated sputa were that the expectorated sputa were more purulent and viscous on gross examination than the induced sputa (Table 3). This could reflect a pooling of secretions overnight in the lower respiratory tract or a dilution of the induced sputa by inhaled saline solution. Microscopic purulence and salivary contamination were similar in the two groups, as well as the isolation rate for the major pathogens (*H influenzae*, *M catarrhalis*, *S pneumoniae*; Table 3). These results demonstrate that spontaneously expectorated sputa in patients with COPD are comparable to induced sputa and can be used to study the lower respiratory tract milieu.

Table 2—*Demographic Characteristics of Patients Whose Sputum Samples Were Included in This Study*

Characteristics	Data
Mean age (range), yr	65.47 (46–82)
Sex, No.	
Male	44
Female	1
Race, No.	
White	36
African American	9
Mean duration since diagnosis of COPD (range), yr	14.96 (2–58)
Smoking status on enrollment, No.	
Ex-smoker	26
Current smoker	19
Mean smoking pack-years (range)	92.07 (10–185)
Mean FEV_1 (range), L	1.68 (0.59–3.93)
Mean FEV_1 (range), % predicted	49.07 (17–99)

Comparison of Sputum Samples Grouped by Culture Results

The 81 sputum samples studied were classified into four groups: *H influenzae* isolated as sole pathogen on culture (n = 20), *H parainfluenzae* isolated as sole pathogen on culture (n = 27), *M catarrhalis* isolated as sole pathogen on culture (n = 14), and normal flora only isolated on culture (n = 20). If our hypothesis is correct, one or more inflammatory molecules in sputum should be significantly increased in the samples that contained bacterial pathogens (*H influenzae*, *H parainfluenzae*, or *M catarrhalis*) compared to samples in which these pathogens were absent (normal flora only).

IL-8 was detectable in all 81 sputa tested, TNF-α in 40 of the 79 sputa tested, and free NE activity in 66 of the 80 sputa tested. Figure 2 depicts the results obtained. *H influenzae* exacerbations were associated with significantly higher sputum IL-8, TNF-α, and NE when compared to pathogen-negative (normal flora only) exacerbations. *M catarrhalis* exacerbations were associated with significantly higher sputum TNF-α and NE when compared to pathogen-negative (normal flora only) exacerbations. Except for a significantly higher sputum NE, airway inflammation in *H parainfluenzae* exacerbations was similar to pathogen-negative (normal flora only) exacerbations. There were differences in the intensity and nature of inflammation among the different pathogens. *H influenzae* exacerbations were associated with significantly greater sputum IL-8, TNF-α, and NE, while *M catarrhalis* exacerbations were associated with significantly greater sputum TNF-α when compared to *H parainfluenzae* exacerbations. The *H influenzae* and *M catarrhalis* groups did not differ in any of the sputum inflammatory parameters.

These results demonstrate that isolation of *H influenzae* or *M catarrhalis* from sputum in an AECB is associated with an airway inflammatory profile characteristic of neutrophilic inflammation. In contrast, nonbacterial AECB are not associated with such an inflammatory profile. Airway inflammation in AECB associated with *H parainfluenzae* is heterogeneous; however, as a group, these AECB have an airway inflammatory profile very similar to nonbacterial exacerbations.

Table 3—*Comparison of Induced and Spontaneous Sputa

Characteristics	Spontaneous	Induced	p Value
Weight, mg	7.84 (1–35)	6.94 (1–28)	NS
Purulence score	2.33 (1–3)	2.00 (1–3)	0.016
Viscosity score	1.80 (1–3)	1.59 (1–3)	0.02
Nonsquamous cells/LPF score	2.77 (1–3)	2.69 (1–3)	NS
Squamous cells/LPF score	1.94 (1–3)	1.79 (1–3)	NS
Nontypeable *H influenzae* isolated, %	28.8	25	NS
M catarrhalis isolated, %	15.4	15.4	NS
S pneumoniae isolated, %	9.6	7.5	NS
Sputum fibrinogen level, ng/mL, mean (± 1 SD)	4,400 (± 4,100)	3,600 (± 4,000)	NS

*Values are presented as mean (range) unless otherwise indicated. NS = not significant; see Table 1 for other abbreviation.

There is overlap between groups in the values obtained of the various inflammatory molecules measured; however, 16 of 19 pathogen-negative (normal flora only) sputa (84.2%) have a NE level < 350 MU/mL, while only 10 of 34 *H influenzae* and *M catarrhalis* sputa (29.4%) have a level below this value. In the *H parainfluenzae* group, 12 of 27 sputa (44.4%) have sputum NE < 350 MU/mL.

Comparison of Sputum Inflammation Within Patients

Sputum samples used in this study were collected on a longitudinal basis, with individual patients contributing from one to four samples. Comparison of sputum inflammation in pairs of samples obtained from individual patients during two exacerbations, which differ only in the presence or absence of pulmonary pathogens, is a more rigorous test of our hypothesis. Such an analysis accounts for the variability among patients in the baseline level of airway inflammation, and the differences seen within a patient should reflect the presence of pathogens in the airways.

All possible pairs of samples available were included in this analysis. In order to obtain at least 10 pairs for analysis, we combined *H influenzae* and *M catarrhalis* into a single pathogen-positive group. To further elucidate the role of *H parainfluenzae* as a pathogen, we treated it in this analysis both as a nonpathogen and as a pathogen by comparing it with pathogen-positive exacerbations and with pathogen-negative exacerbations (Table 4).

Pathogen-positive AECB were associated with significantly higher sputum IL-8, TNF-α, and NE than pathogen-negative AECB (Table 4, Fig 3). When compared to *H parainfluenzae* exacerbations, sputum IL-8, TNF-α, and NE were significantly increased in pathogen-positive AECB. *H parainfluenzae* exacerbations did not differ in any of the sputum inflammatory parameters from the pathogen-negative (normal flora only) AECB (Table 4).

These results confirm those obtained with the sputum samples grouped by culture results. They show that within a patient, a bacterial (*H influenzae* and *M catarrhalis*) AECB is associated with significantly greater neutrophilic airway inflammation than a nonbacterial AECB. Airway inflammation in AECB associated with *H parainfluenzae* is heterogeneous; however, within a patient, they resemble pathogen-negative AECB.

Correlation Between Symptoms and Sputum Inflammation

Clinical scores for the exacerbations included in this study ranged from 12 to 26, with a mean score of 17.5. There was no difference in clinical score among the groups of exacerbations defined by culture results (Kruskal-Wallis, p = not significant). There was a significant correlation between clinical score with sputum NE ($\rho = 0.449$; $p < 0.001$; Fig 4). This correlation was unaffected by exclusion of the four outliers with clinical scores > 24. However, none of the other inflammatory markers measured showed such a relationship. This observation demonstrates that sputum elastase is a marker of the clinical severity of an AECB and could reflect a cause-effect relationship between the two parameters.

Correlation Among the Inflammatory Markers and With Bacterial Titers in Sputum

All the three inflammatory markers measured in the sputum demonstrated significant correlation with each other with ρ values of 0.504 for IL-8 and TNF-α ($p < 0.001$), 0.396 for IL-8 and NE ($p < 0.001$), and 0.571 for TNF-α and NE ($p < 0.001$).

Bacterial titers per gram of sputum (mean ± 1 SEM) of *H influenzae* ($4.89 \pm 1.03 \times 10^8$) and *M catarrhalis* ($4.71 \pm 1.13 \times 10^8$) did not differ from each other but were significantly greater than *H parainfluenzae* ($4.35 \pm 3.69 \times 10^7$) with a p value

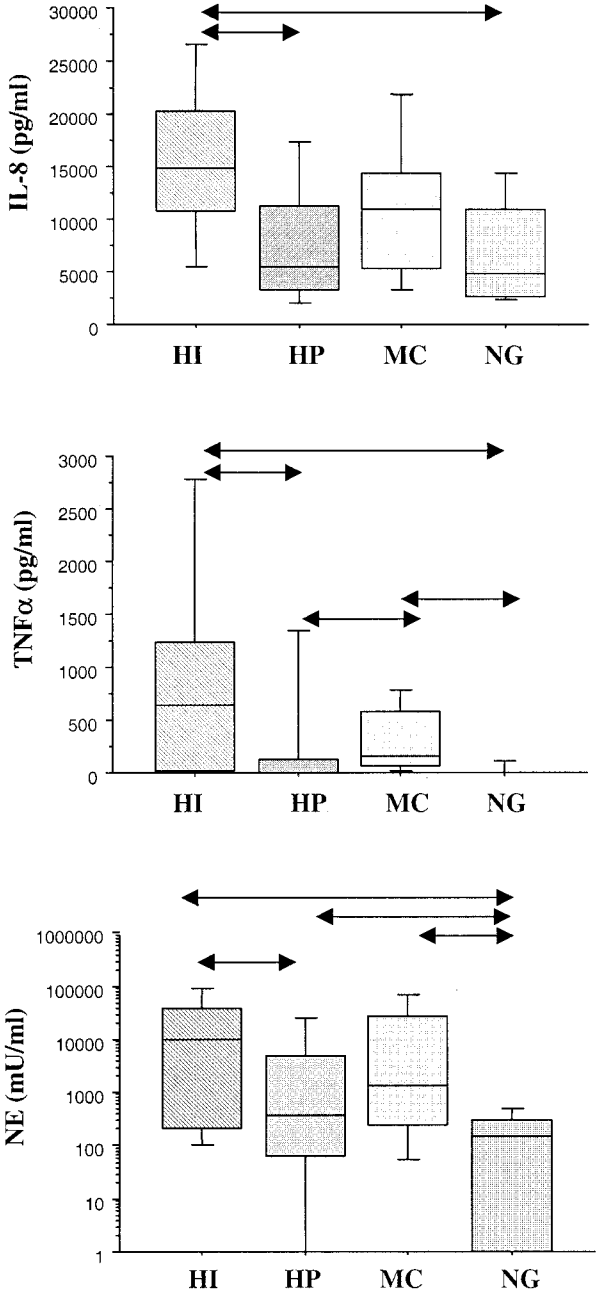

FIGURE 2. Box plots of sputum inflammation in the unpaired groups. The vertical bar represents 10th and 90th percentile values, the box encompasses the 25th to 75th interquartile range, and the horizontal line in the box represent median values. The arrows represent significant ($p < 0.05$) differences by the Mann-Whitney U rank test. HI = *H influenzae* isolated as the sole pathogen on culture; HP = *H parainfluenzae* isolated as the sole pathogen on culture; MC = *M catarrhalis* isolated as the sole pathogen on culture; NG = only normal flora isolated on culture.

< 0.001 for both comparisons. There was significant correlation between bacterial titers and sputum IL-8 ($\rho = 0.382$; $p = 0.003$), TNF-α ($\rho = 0.437$; $p < 0.001$), and NE ($\rho = 0.381$; $p = 0.003$). These results suggest that bacterial infection is acting as an inflammatory stimulus, and as the stimulus increases, there is a corresponding increase in the airway inflammatory response.

DISCUSSION

Several new research approaches are being used to investigate the role of bacteria in the etiology of AECB. Bronchoscopy with culture of distal airway secretions during AECB has demonstrated that pathogenic bacteria are present in about 50% and are likely to be the etiologic agents.[18–20] We have shown development of strain-specific bactericidal antibodies to infecting strains of *H influenzae* after AECB, further supporting the role of bacteria in causing AECB.[21] Molecular typing of sputum isolates of *H influenzae* from patients with COPD has demonstrated a dynamic turnover of bacterial strains in these patients, which could contribute to recurrent exacerbations (unpublished data). The present study provides another line of evidence supporting an etiologic role of *H influenzae* and *M catarrhalis* in AECB. Extending these observations to other potential pathogens in AECB, specifically *S pneumoniae* and *P aeruginosa*, will be important.

S pneumoniae was isolated in 10 of the 276 episodes of AECB observed in our longitudinal study. In only four of these episodes (in three subjects), *S pneumoniae* was a sole pathogen (Fig 1). This low rate of infection by *S pneumoniae* could be related to almost all our subjects having received the pneumococcal vaccine. In addition, the pneumococcus appears to be more prevalent in the earlier stages of COPD, while 85% of our subjects had moderate or severe obstructive disease.[22,23]

The present study demonstrates a strong association between recovering *H influenzae* and *M catarrhalis* in sputa during exacerbations and neutrophilic airway inflammation. We did not enumerate neutrophils in sputa; however, previous studies have shown excellent correlation between measured levels of IL-8 and NE levels and sputum or BAL neutrophil counts.[12,24] Both *H influenzae* and *M catarrhalis* are clearly associated with mucosal infections at other airway sites, such as the middle ear and sinuses. *H influenzae* and its products reduce mucociliary clearance, increase mucus secretion, and cause bronchial epithelial damage *in vitro*.[25,26] Lipooligosaccharide of *H influenzae* induces IL-8, IL-6, and TNF-α secretion from bronchial epithelial cells *in vitro*.[27] It is therefore likely that a similar inflammatory process is being engendered by these mucosal pathogens during AECB. An alternative explanation of our findings is that the pathogens recovered are actually oropharyngeal isolates and were isolated

Table 4—*Comparison of Sputum Inflammation in Paired Samples

Sputum Culture Pairs	Pairs, No.	IL-8 Median Difference, pg/mL†	TNF-α Median Difference, pg/mL	NE Median Difference, mU/mL
PP–NG	15	4,760 (p = 0.005)	24 (p = 0.043)	5,628 (p = 0.017)
PP–HP	20	7,650 (p = 0.049)	57 (p = 0.021)	454 (p = 0.041)
HP–NG	10	−916	0	58

*PP = *H influenzae* or *M catarrhalis* isolated as the sole pathogen on culture; see Figure 2 legend for other abbreviations.

†Median difference is the median of the differences between the first and second values of each pair of samples. Only significant p values obtained with the Wilcoxon signed rank test are shown.

in the sputum from salivary contamination. Several bronchoscopic studies of distal airway bacterial flora in AECB have shown that *H influenzae* and *M catarrhalis* are often present in concentrations of $> 10^3$/mL in the distal airways during AECB.[18–20] Laurenzi et al[28] and Haas et al[29] have shown that in patients with COPD, *H influenzae* is simultaneously recovered in the upper and the lower airways. These studies would support the notion that most, if not all, of the sputum isolates of *H influenzae* and *M catarrhalis* in the present study reflect bronchial infection rather than oropharyngeal colonization.

Measuring inflammatory molecules in sputum as done in this study is now a widely accepted method of studying airway inflammation, but is associated with significant limitations. Contamination with oropharyngeal flora has been discussed above. Contamination with saliva could variably dilute the inflammatory markers present in sputum and could have influenced our results. Microscopic evaluation was not performed in the 81 samples included in this study; however, macroscopic evaluation revealed that only four of these samples were mucoid (three in the *H parainfluenzae* group and one in the normal flora-only group). Seventy-seven of the 81 samples were either entirely purulent or contained multiple flecks of pus, indicating their predominantly lower-respiratory origin. Another major limitation of sputum is that it represents secretions from all levels of the bronchial tree. Therefore, it is not possible to determine from this study if the neutrophilic inflammation in bacterial AECB is confined to the large airways or involves the small airways also. Future studies examining distal airway inflammation in bacterial AECB are needed to extend our observations.

Though *H parainfluenzae* is frequently isolated in sputum obtained during stable and unstable COPD, its role of as an airway pathogen in COPD is ill defined.[30,31] Whether this sputum isolation is from oropharyngeal contamination or from the lower respiratory tract is unclear because *H parainfluenzae* is a common constituent of normal oropharyngeal flora.[32] Furthermore, little evidence exists to suggest that *H parainfluenzae* is a mucosal pathogen in other respiratory tract sites, most notably the middle ear and the sinus cavity. Bronchoscopic studies of AECB have added to the confusion. In the three published studies, *H parainfluenzae* was the most common pathogen isolated in the study by Fagon et al,[18] while it was completely absent in the other two studies.[19,20] In this study, sputa that contained *H parainfluenzae* resembled pathogen-negative sputa. However, unlike the pathogen-negative sputa, the *H parainfluenzae* sputa are a heterogeneous group. For example, while only 3 of 19 normal flora-only sputa (15.8%) have free neutrophil elastase activity of > 350 MU/mL, 15 of 27 *H parainfluenzae* sputa (55.6%) have elastase levels exceeding this value. Some of these sputa had elastase values resembling pathogen-positive exacerbations.

This heterogeneity among the *H parainfluenzae* sputa can be explained in two possible ways. It is possible that *H parainfluenzae* sputa with an inflammatory profile that resembles pathogen-positive samples are actually harboring *H influenzae* that was missed because of sampling error, in spite of our efforts to isolate and characterize multiple Haemophilus colonies. Another potential explanation is that a proportion of *H parainfluenzae* strains are more virulent and cause mucosal infections associated with significant neutrophilic airway inflammation. Further study of the virulence of *H parainfluenzae* strains and the immune response to these strains is needed to arrive at definitive conclusions regarding their role in AECB.

The abundant NE activity found in our sputa during bacterial AECB suggests that these exacerbations could increase the airway elastase burden and cause airway epithelial injury and remodeling. Such a process in the distal small airways could contribute to progressive airway obstruction.[33,34] How does one reconcile our data that bacterial AECB are associated with free elastase and could therefore contribute to progression of COPD with epidemiologic evidence that the number of exacerbations is not predictive of decline in lung function over time?[35–37] There are several reasons to question the conclusions of these epidemiologic studies. These studies were

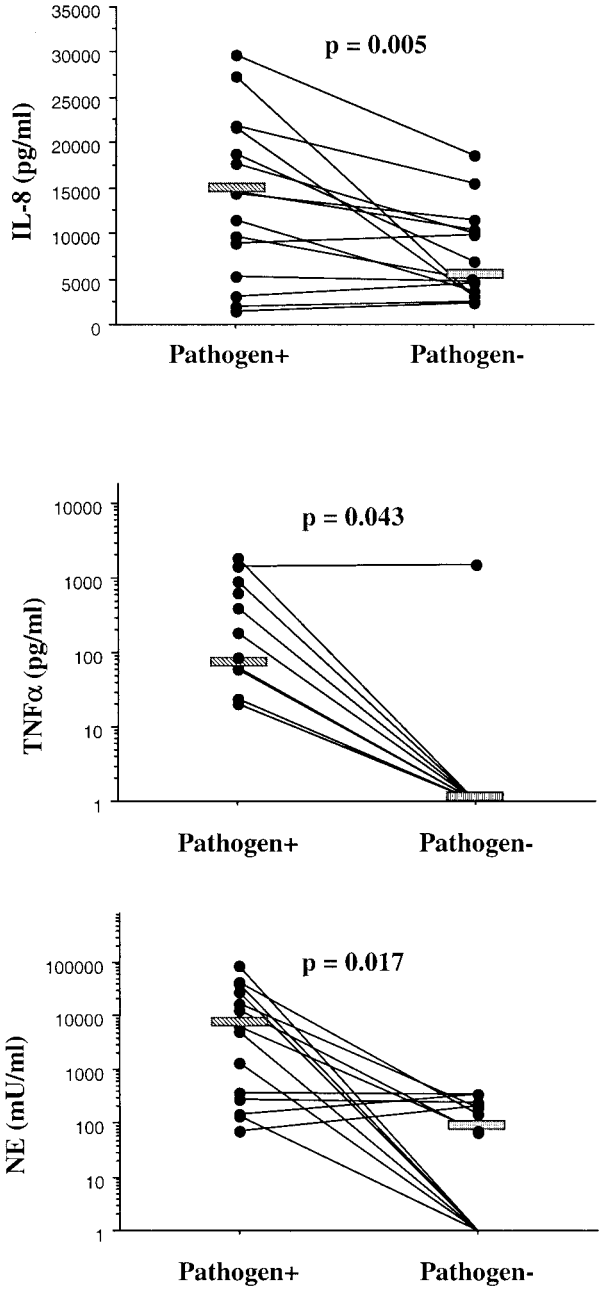

FIGURE 3. Paired comparison of airway inflammation in pathogen-positive (pathogen+) AECB with pathogen-negative (pathogen-) AECB (normal flora only). Lines connect the measured values from individual patients. Horizontal bars represent median values. The p values obtained with the Wilcoxon signed-rank test are shown.

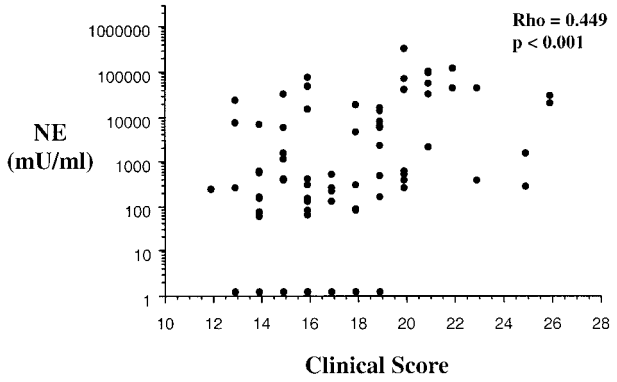

FIGURE 4. Correlation of free sputum elastase activity (NE) with the clinical score obtained during each exacerbation. Free sputum elastase activity is expressed on the y-axis, while the clinical score is expressed on the x-axis. The Spearman correlation coefficient (Rho [ρ]) and the corresponding p value are also shown.

generally conducted in cohorts of middle-aged men with either no lung disease or mild chronic bronchitis, rather than cohorts of patients with established COPD. The impact of AECB on lung function may be quite different in these two populations. These studies measured occurrence of AECB by patient recall in questionnaires administered every 6 months to 1 year. This can be a major potential source of error. These studies did not define the etiology of the recorded AECB; therefore, the actual number of bacterial AECB was not measured. If only bacterial AECB contribute to loss of lung function, definitive conclusions about their role in progression of COPD cannot be drawn from these studies. Finally, two epidemiologic studies[38,39] that studied patients with established COPD and had closer follow-up showed a significant relationship between the decline of lung function and the occurrence of AECB.

Clinical features cannot readily distinguish bacterial from nonbacterial AECB. Sputum purulence has often been described as a marker for bacterial exacerbations, but sputum was mucoid on gross examination in only 4 of the 81 exacerbations included in this study. On the other hand, a sputum elastase (NE) level of 350 MU/mL had a sensitivity of 70.6%, specificity of 84.2%, positive predictive value of 88.9%, and negative predictive value of 61.5% in distinguishing bacterial (*H influenzae* or *M catarrhalis*) from nonbacterial (normal flora-only) AECB. This suggests that a semiquantitative method of measuring free sputum elastase, possibly as a "dipstick," could be used in clinical practice to distinguish bacterial from nonbacterial AECB, which would be extremely useful in identifying patients who would benefit from antibiotics.

In summary, this study demonstrates that *H influenzae* and *Mcatarrhalis* are associated with a neutrophilic inflammatory response in the airways in AECB. Furthermore, it shows that when *H parainfluenzae* is isolated from sputum during AECB, in most instances it is not the etiologic agent. Whether free elastase in the sputum can be used as a clinical marker to distinguish bacterial from nonbacterial

AECB and to estimate the severity of an AECB is an interesting proposition that needs to be explored further. The extent to which bacterial AECB involves the small airways of the lung and contributes to progressive airway obstruction needs to be investigated.

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