Rhinovirus Infection Induces Degradation of Antimicrobial Peptides and Secondary Bacterial Infection in Chronic Obstructive Pulmonary Disease

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***Rationale*: Chronic obstructive pulmonary disease (COPD) exacerbations are associated with virus (mostly rhinovirus) and bacterial infections, but it is not known whether rhinovirus infections precipitate secondary bacterial infections.**

***Objectives*: To investigate relationships between rhinovirus infection and bacterial infection and the role of antimicrobial peptides in COPD exacerbations.**

***Methods*: We infected subjects with moderate COPD and smokers and nonsmokers with normal lung function with rhinovirus. Induced sputum was collected before and repeatedly after rhinovirus infection and virus and bacterial loads measured with quantitative polymerase chain reaction and culture. The antimicrobial peptides secretory leukoprotease inhibitor (SLPI), elafin, pentraxin, LL-37, α-defensins and β-defensin-2, and the protease neutrophil elastase were measured in sputum supernatants.**

***Measurements and Main Results*: After rhinovirus infection, secondary bacterial infection was detected in 60% of subjects with COPD, 9.5% of smokers, and 10% of nonsmokers ($P < 0.001$). Sputum virus load peaked on Days 5–9 and bacterial load on Day 15. Sputum neutrophil elastase was significantly increased and SLPI and elafin significantly reduced after rhinovirus infection exclusively in subjects with COPD with secondary bacterial infections, and SLPI and elafin levels correlated inversely with bacterial load.**

***Conclusions*: Rhinovirus infections are frequently followed by secondary bacterial infections in COPD and cleavage of the antimicrobial peptides SLPI and elafin by virus-induced neutrophil elastase may precipitate these secondary bacterial infections. Therapy targeting neutrophil elastase or enhancing innate immunity may be useful novel therapies for prevention of secondary bacterial infections in virus-induced COPD exacerbations.**

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Secondary bacterial infections are reported with influenza infection but it is not known whether bacterial infection is associated with other respiratory viruses, such as rhinoviruses, which are the most common viral cause of COPD exacerbations.

What This Study Adds to the Field

We report that experimental rhinovirus infection is followed by secondary bacterial infections in subjects with COPD but not in smokers or nonsmokers without COPD. Bacterial infections were associated with reduced levels of antimicrobial peptides suggesting that rhinovirus infection leads to impaired innate immune responses that predispose to bacterial infection.

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Chronic obstructive pulmonary disease (COPD) is a growing global epidemic and its prevalence is expected to increase markedly (1). Acute exacerbations are the major cause of morbidity and mortality in COPD and are associated with impaired quality of life (2), accelerated loss of lung function (3), and enormous healthcare costs (4). Respiratory infections cause most exacerbations (5, 6) with the relative contributions of viruses and bacteria still debated (7), and it is not known whether bacterial infections in COPD exacerbations occur *de novo* or secondary to an initial virus infection. Dual virus-bacterial infection has only been reported in a minority of COPD exacerbations (5, 6, 8, 9), consistent with the hypothesis that one may follow the other and therefore detection is frequently separated in time rather than simultaneous. Most viruses detected are rhinoviruses. Rhinovirus infection increases bacterial adherence to respiratory epithelial cells (10, 11), and impairs

macrophage responses to bacterial stimuli (12) *in vitro*. A temporal association between rhinovirus infection and invasive pneumococcal disease in children has been reported (13). These data suggest that rhinovirus infections may precipitate secondary bacterial infection but no *in vivo* data investigating this hypothesis exist. We have developed a unique human model of COPD exacerbation using experimental rhinovirus infection that induces the clinical features of an exacerbation and permits intensive repeated sampling of the lower airways (14, 15). The aims of this study were to determine whether rhinovirus infection can precipitate secondary bacterial infection *in vivo* and to investigate temporal relationships between viral and bacterial infection. Because there is known to be a lower respiratory microbiome in health and disease (16, 17) and because antimicrobial peptides are known to be important in antibacterial host defense (18), we also hypothesized that secondary bacterial infections may be caused by perturbations in this host defense mechanism as a consequence of rhinovirus infection. We therefore assessed levels of the major antimicrobial peptides (pentraxin 3, LL-37, α- and β-defensins, secretory leukoprotease inhibitor [SLPI], and elafin) in sputum before and during rhinovirus infections to determine whether levels were altered from baseline and how these may relate to secondary bacterial infections. Some of the results of this study have been previously reported in abstract form (19–22).

METHODS

Study Subjects

Ethical approval was obtained from UK Research Ethics Committees (study numbers 00/BA/459E, 07/H0712/138, and 11/LO/0400) and informed consent obtained from all subjects. The participants were recruited for two studies. The first included 13 subjects with COPD and 13 smokers without airway obstruction, and initial findings relating to virus infection and clinical outcomes have been reported (15). The second study (so far unreported) included 18 subjects with COPD and 15 smokers with identical inclusion criteria as the first study, and an additional control group of 19 nonsmokers. Further details regarding the study participants and inclusion criteria are in the online supplement.

Study Protocol

Subjects underwent clinical assessment including symptom diaries, lung function, and sputum induction before experimental infection with human rhinovirus 16, performed on Day 0 as previously reported (15). Subjects kept daily diary cards of symptoms and sputum sampling was repeated on Days 5, 9, 12, 15, 21, and 42 after virus inoculation. The protocols for the two studies were identical other than three time points for sputum sampling (Days 3, 28, and 35 postinoculation) that were discordant between the two studies, so these were not included in the present analysis. The study timeline is outlined in Table E2 in the online supplement.

Clinical Procedures

Virus inoculation and detection. Details regarding the preparation and safety testing of the rhinovirus 16 inoculum have been published (23). Ten tissue culture infective doses 50% of the virus were diluted in a total volume of 1 ml of 0.9% saline and inoculated in both nostrils using an atomizer (No. 286; DeVilbiss Co., Heston, UK). Rhinovirus infection was confirmed with a combination of virus culture, serology, and polymerase chain reaction according to previously established protocols (24). The sensitivity of this assay was 10^4 copies per milliliter. The criteria for successful infection are provided in the online supplement. Infection with viruses other than rhinovirus was excluded by testing nasal lavage samples at baseline and at the peak of upper respiratory symptoms with polymerase chain reaction (*see* online supplement).

Sputum induction. Sputum was induced by inhalations of hypertonic saline according to European Respiratory Society guidelines (25) and processed according to standard protocols (15). Details are provided in the online supplement.

Bacterial culture. Quantitative bacterial culture was performed on induced sputum samples on blood agar, chocolate agar, CLED agar, and Sabouraud agar in the Microbiology Laboratory at Imperial College Healthcare NHS Trust (5). Bacterial infection was defined as a colony count of greater than or equal to 10^5 cfu/ml of a potentially pathogenic microorganism (PPM) (*Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Staphylococcus aureus*, and *Haemophilus parainfluenzae*). Because there is debate as to whether *S. aureus* and *H. parainfluenzae* are causative organisms in COPD exacerbations (5, 7, 26), we also analyzed the data not counting these organisms as PPMs; these results are presented in the online supplement. All the sputum cultures were reviewed and there were no potentially pathogenic organisms detected at a load lower than 10^6 cfu/ml. The total bacterial load for each subject was defined as the sum of Log_{10} cfu/ml counts of all individual bacterial-positive cultures detected for that subject.

Antimicrobial peptides and inflammatory mediators. The antimicrobial peptides SLPI, elafin, pentraxin 3, human β-defensin-2 (HBD-2), α-defensins, and the cathelicidin LL-37 and the protease neutrophil elastase were measured in sputum supernatants using commercially available ELISA (*see* online supplement).

Statistical Analysis

Data are presented as mean (± SEM) values for normally distributed data or median (interquartile range) for nonparametric data. Changes from baseline were analyzed with repeated measures analysis of variance (Friedman test for nonparametric data) and, if significant, paired *t* tests or Wilcoxon matched pairs test. Differences between groups were analyzed using unpaired *t* tests or Mann-Whitney tests. Correlations between data sets were examined using Pearson correlation or Spearman rank correlation coefficient. Differences were considered significant for all statistical tests at *P* values of less than 0.05. All reported *P* values are two-sided. Analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Frequencies of Successful Rhinovirus Infection

A total of 31 subjects with COPD, 28 smokers, and 19 nonsmokers, were inoculated with low-dose rhinovirus 16 and 77 of 78 subjects completed the study through Day 42; one subject with COPD withdrew because of ill health believed unconnected to the study. No subjects were treated with corticosteroids (inhaled or oral) or antibiotics. Rhinovirus infection was confirmed in 20 of 30 subjects with COPD (66.7%); 22 of 28 smokers (78.6%); and 11 of 19 nonsmokers (58%). There were no significant differences in frequencies of successful virus infection between groups ($P = 0.53$).

Bacterial Infection during Experimental Rhinovirus Infection

Of the remaining 77 subjects, one smoker had a positive bacterial culture in the baseline sputum sample and one nonsmoker was unable to provide sputum samples; these subjects were excluded from further analyses. The clinical characteristics of the subjects infected with rhinovirus included in the final analysis are shown in Table 1. After successful rhinovirus infection a positive bacterial culture was detected in 12 (60%) of 20 of the subjects with COPD, 2 (9.5%) of 21 of the smokers, and 1 (10%) of 10 of the nonsmokers ($P < 0.001$). The time course of bacterial infection is shown in Figure 1A. Only subjects with COPD had significant increases in sputum bacterial load between baseline and postvirus infection samples (on Days 9, 12, and 15; $P < 0.05$ in each case) and bacterial load in subjects with COPD was significantly greater than smoking and nonsmoking groups on Day 9 ($P < 0.05$) than the nonsmoking group on Day 12 ($P < 0.05$). The individual bacterial species detected are listed in Table E4 and the bacterial loads in Table E5 in the online supplement.

TABLE 1. CLINICAL CHARACTERISTICS OF STUDY SUBJECTS INCLUDED IN THE FINAL ANALYSIS

	Nonsmokers (*N* = *10*)	Smokers (*N* = *21*)	COPD (*N* = *20*)	*P* Value COPD vs. Smokers	*P* Value COPD vs. Nonsmokers
Age, yr	62.2 (53–71)	50.81 (40–66)	59.74 (44–72)	<0.01	NS
Sex, M:F	4:6	10:11	13:7	NS	NS
Smoking history, pack-years	0	33.86 (20–60)	44.15 (20–109)	<0.001	<0.001
Current smokers, current/ex	0	16/5	16/4	NS	N/A
Chronic bronchitis	N/A	N/A	17/20	N/A	N/A
FEV_1% predicted, mean	100.3 ± 3.36	96.20 ± 3.45	68.11 ± 1.58	<0.001	<0.001
FEV_1, L, mean	2.7 ± 0.18	3.26 ± 0.16	1.93 ± 0.09	<0.001	<0.01
FEV_1/FVC, mean	77.98 ± 1.09	78.05 ± 1.34	58.60 ± 1.87	<0.001	<0.001
Influenza vaccination	3/10	4/21	9/20	NS	NS
Antibiotics in the previous year	2/10	2/21	7/20	NS	NS
Exacerbations in the previous year	N/A	N/A	4/20 (20%)	N/A	N/A
>2 exacerbations in the previous year	N/A	N/A	0	N/A	N/A
Hospitalizations in the previous year	0/10	0/21	1/20	NS	NS

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; N/A = not applicable; NS = not significant.
Plus-or-minus values are mean ± SE; values in parentheses are ranges.

Frequency of Bacterial Infections in the Absence of Rhinovirus Infection

A number of subjects were inoculated with rhinovirus and went through the entire study protocol but were subsequently found to have no evidence of successful rhinovirus infection. We investigated bacterial detection in these subjects also to determine whether the increased bacteria detected in the rhinovirus-infected subjects with COPD were possibly related to sputum induction or bronchoscopy. In the nonrhinovirus-infected subjects bacterial infection was detected in 2 (20%) of 10 subjects with COPD, 1 (16.7%) of 6 smoking control subjects, and 1 (12.5%) of 8 nonsmoking control subjects. Only in the COPD group was the incidence of bacterial infection significantly higher in subjects infected with rhinovirus compared with subjects not infected (60% vs. 20%; $P = 0.038$) (*see* Table E6). The bacterial loads in the subjects not infected with rhinovirus are depicted in Figure 1B; there were no significant increases in bacterial load above baseline at any time point.

Temporal and Severity Relationships between Rhinovirus Infection and Bacterial Infection

There was no clear pattern in the temporal distribution of bacterial detections in the smokers infected with rhinovirus and nonsmokers (Figure 1A), nor in the subjects not infected with rhinovirus (Figure 1B). In the subjects infected with rhinovirus with COPD bacterial infection first appeared on Day 9 and bacterial load peaked on Day 15 (Figure 1A). In contrast, sputum virus load was maximal on Days 5 and 9 (Figure 1C), consistent with bacterial infection occurring secondary to virus infection. Peak rhinovirus loads in sputum were positively correlated with sputum bacterial loads ($r = 0.47$; $P = 0.039$), providing support for a causal relationship between the two.

Secondary Bacterial Infections and Underlying COPD Severity

Mean baseline FEV_1 and % predicted FEV_1 were significantly lower in the subjects with COPD with secondary bacterial infection, compared with those without bacterial infection (1.76 ± 0.12 L vs. 2.18 ± 0.10 L, $P = 0.026$; and 63.17 ± 1.45% vs. 68.75 ± 2.2%, $P = 0.04$, respectively). There were no significant differences in baseline FEV_1/FVC ratio, age, sex, or smoking history between bacteria-positive and -negative subjects with COPD (*see* Table E7).

Bacterial Infections and Clinical and Inflammatory Outcomes

All subjects with COPD had similar increases in lower respiratory symptoms during the viral infection. According to our previously established criteria (15), all subjects in the bacteria-positive group developed a COPD exacerbation as did seven of eight in the bacteria-negative group. In the bacteria-positive subjects there was an excess of total lower respiratory symptom scores and breathlessness on Days 20–25, but this was not statistically significant (Figures 2A and 2B). PEF fell significantly from baseline on Days 5–21 (Figure 2C), and FEV_1 on Days 9 and 21 (data not shown) in the bacteria-positive subjects but there was no significant change in either measure in the bacteria-negative subjects. Total sputum inflammatory cells (Days 9 and 15), sputum neutrophils (Days 9 and 12), and sputum neutrophil elastase (Day 9) increased significantly from baseline in the bacteria-positive subjects with COPD (Figures 2D–2F) but not in the bacteria-negative subjects. Supporting a relationship between severity of bacterial infection and pathologic responses, sputum bacterial load correlated significantly with peak inflammatory cell numbers and peak neutrophil numbers in sputum ($r = 0.75$, $P = 0.0001$ and $r = 0.68$, $P = 0.0014$, respectively).

Antimicrobial Peptides in Rhinovirus Infections

After rhinovirus infection sputum levels of pentraxin 3 increased significantly over baseline in the subjects with COPD on Days 5–21 (Figure 3A). In the smoking subject group levels were significantly increased on Day 9 and in the nonsmokers there was no significant induction (*see* Figure E1A). Pentraxin 3 levels were significantly higher in the COPD group compared with the nonsmokers on Days 5–21 and compared with the smokers on Day 15 (Figure 3A). Peak sputum pentraxin 3 levels in the subjects with COPD correlated with peak sputum virus load ($r = 0.45$; $P = 0.046$); peak sputum inflammatory cells ($r = 0.63$; $P = 0.0029$); peak sputum neutrophils ($r = 0.66$; $P = 0.0022$); and sputum bacterial load ($r = 0.52$; $P = 0.018$).

Sputum LL-37 levels were increased significantly from baseline in the COPD group on Day 12 (Figure 3B) but there was no change from baseline in either control group (*see* Figure E1B). Peak sputum LL-37 levels in subjects with COPD correlated with peak sputum virus load ($r = 0.53$; $P = 0.017$); peak sputum inflammatory cells ($r = 0.85$; $P < 0.0001$); peak sputum neutrophils ($r = 0.85$; $P < 0.0001$); sputum bacterial load ($r = 0.49$; $P = 0.03$); and peak sputum pentraxin 3 levels ($r = 0.59$; $P = 0.0061$).

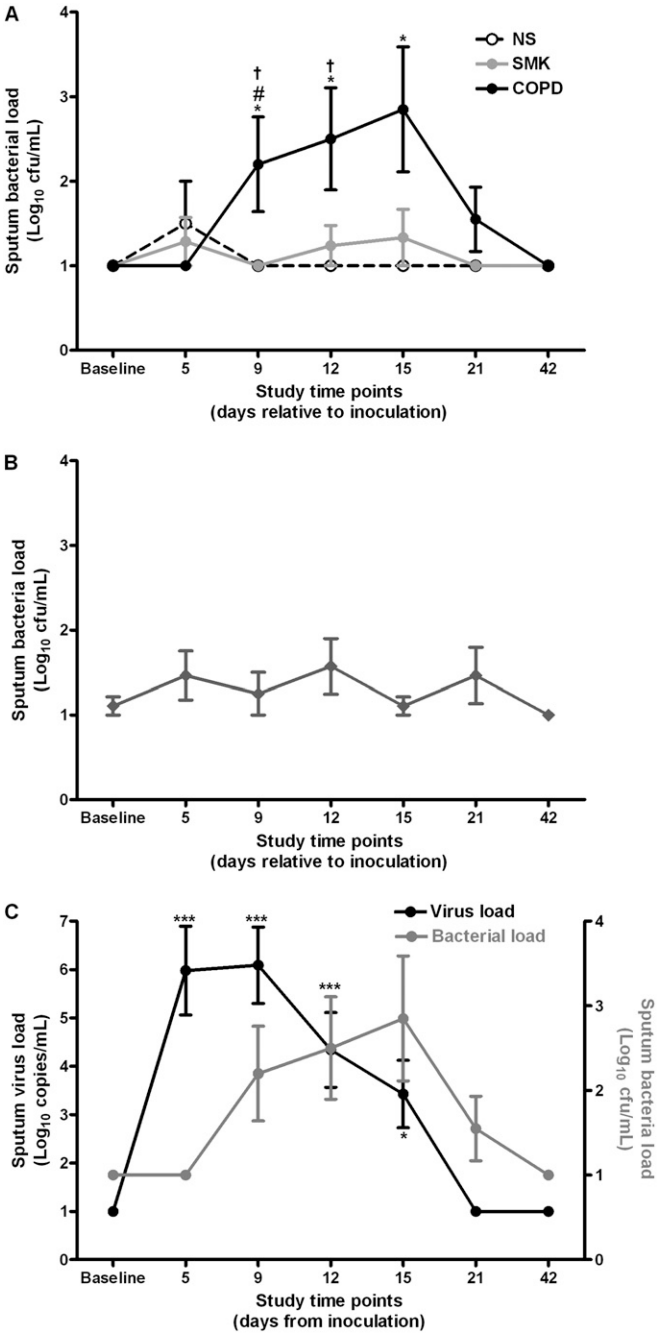

Figure 1. Time course of bacterial load. (*A*) Time course of bacterial load in subjects successfully infected with rhinovirus. (*B*) Time course of bacterial load in all subjects in whom rhinovirus infection was not confirmed but who underwent the same study procedures as the infected subjects. (*C*) Time course of sputum virus load and bacterial load in subjects with COPD. All data mean ± SEM. $^{*}P < 0.05$ compared with baseline. $^{***}P < 0.001$ compared with baseline. $^{\dagger}P < 0.05$ compared with NS. $^{\#}P < 0.05$ compared with SMK. COPD = chronic obstructive pulmonary disease; NS = nonsmokers; SMK = smokers.

Sputum α-defensin increased above baseline in the COPD group on Days 9–21 (Figure 3C), but there were no significant changes from baseline in the control groups (*see* Figure E1C). There were no correlations between α-defensin levels and clinical, virologic, or bacterial parameters.

Sputum levels of HBD-2 were very low and there were no significant changes from baseline the COPD group (Figure 3D) or the smoking control subjects (*see* Figure E1D). In the nonsmokers levels were significantly increased on Day 21 compared with baseline (*see* Figure E1D). There were no correlations between HBD-2 levels and clinical, virologic, or bacterial parameters.

There were no increases above baseline in SLPI or elafin levels at any time point in the COPD group (Figures 3E and 3F), nor in either control group (*see* Figures E1E and E1F), and there were no significant differences between the groups.

Increases in Neutrophil Elastase and Reductions in Elafin and SLPI after Rhinovirus Infection Were Exclusive to Subjects with COPD with Secondary Bacterial Infections

Because neutrophil elastase has been reported to degrade SLPI and elafin (27, 28) and strong induction of neutrophil elastase on Day 9 was limited to the subjects with COPD who developed secondary bacterial infections (Figure 2D), we hypothesized that high levels of rhinovirus-induced neutrophil elastase may degrade these two antimicrobial peptides. Consistent with our hypothesis, in the bacteria-positive subjects with COPD sputum elafin levels were significantly reduced compared with baseline levels on Day 9 (−68.47 ng/ml; $P = 0.042$). In contrast, in the bacteria-negative subjects with COPD there was a nonsignificant increase from baseline in sputum elafin on Days 9 and 12 (39.17 ng/ml, $P = 0.28$; 159.2 ng/ml, $P = 0.078$) (Figure 4A). Sputum elafin levels were significantly lower in the bacteria-positive compared with the bacteria-negative subjects with COPD on Days 9 (−2013 ng/ml vs. 775.2 ng/ml; $P = 0.015$) and 12 (−46.93 vs. 159.2 ng/ml; $P = 0.023$) (Figure 4C) and on Day 9 correlated inversely with bacterial load ($r = -0.71$; $P = 0.004$) and peak sputum neutrophils ($r = -0.55$; $P = 0.016$).

Similar nonsignificant trends were observed for sputum SLPI (Figure 4B). Sputum SLPI levels were lower in the bacteria-positive compared with the bacteria-negative subjects with COPD on Day 9 (−1542 ng/ml vs. 383.4 ng/ml; $P = 0.07$) and on Day 12 (−68.47 ng/ml vs. 39.17 ng/ml; $P = 0.023$) (Figure 4D) and on Day 12 correlated inversely with bacterial load ($r = -0.51$; $P = 0.023$). There were no significant differences between bacteria-positive and bacteria-negative subjects with COPD in pentraxin 3, LL-37, α-defensin, or HBD-2 levels (data not shown).

DISCUSSION

We have used our human experimental rhinovirus infection model to demonstrate that secondary bacterial infections occur in 60% of subjects with COPD after a rhinovirus infection and that this occurs significantly more frequently than in smoking and nonsmoking subjects and in subjects with COPD who underwent the same sampling protocol but did not develop rhinovirus infections. We report relationships between virus load and secondary bacterial infection, and that breathlessness, airflow obstruction, and airway inflammation were more severe or more prolonged in subjects with COPD with secondary bacterial infection. Secondary bacterial infection in subjects with COPD was associated with high levels of rhinovirus-induced neutrophil elastase and with reductions in the antimicrobial molecules SLPI and elafin.

Respiratory infections are the commonest causes of COPD exacerbations with viruses and bacteria frequently detected (5, 9), but it is not known whether bacterial infections occur *de novo* or follow an initial virus infection. Patients frequently report colds before exacerbations (6, 29) and *in vitro* mechanisms linking rhinovirus infections to increased susceptibility to bacterial infection have been reported (10–12). However, rates of dual virus-bacterial infection in COPD exacerbations are

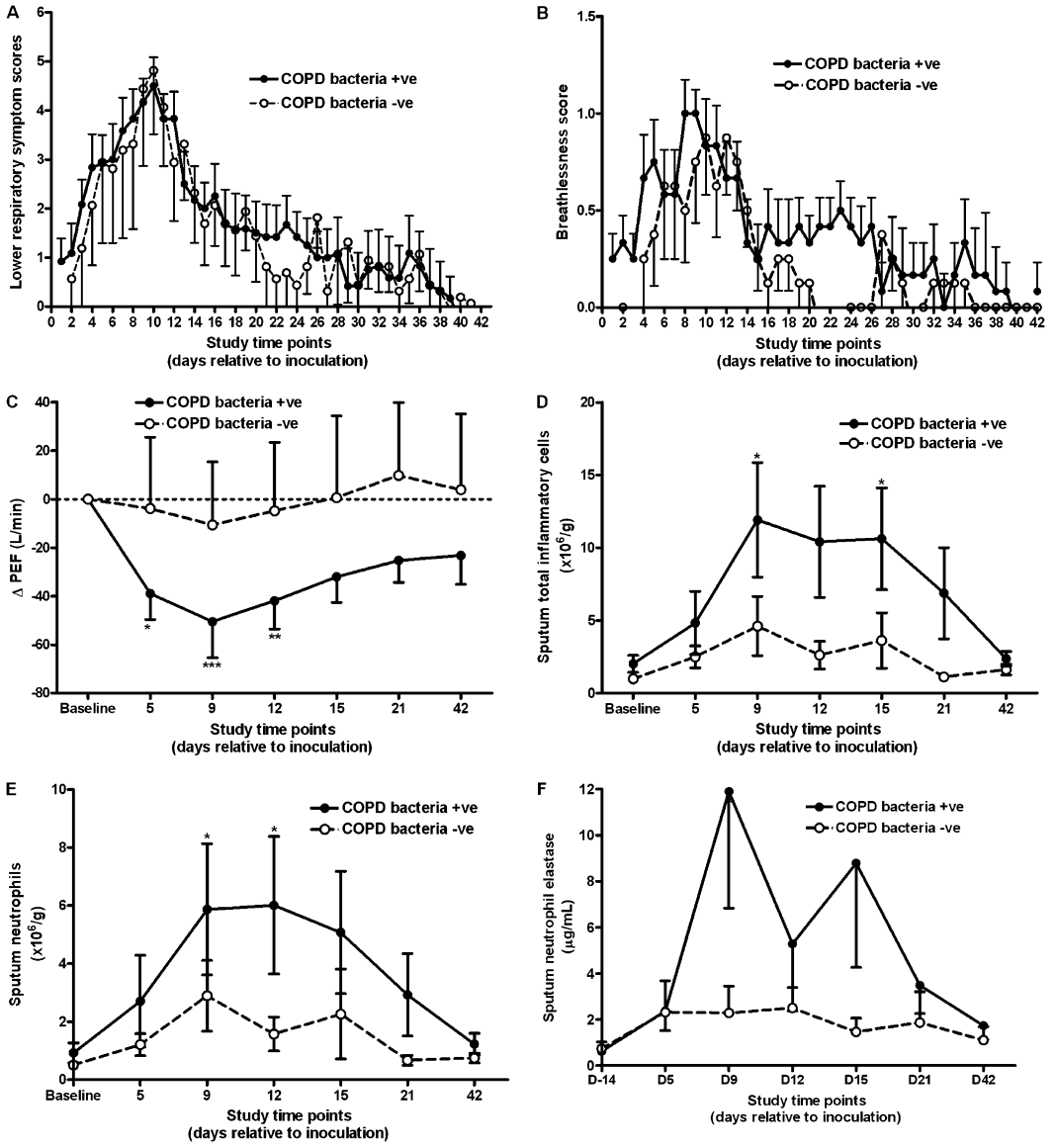

Figure 2. Clinical and inflammatory parameters in subjects with chronic obstructive pulmonary disease (COPD) with and without bacterial infection. (*A*) Total lower respiratory symptom scores. (*B*) Breathlessness scores. (*C*) Peak expiratory flow. (*D*) Total sputum inflammatory cells. (*E*) Sputum neutrophil numbers. (*F*) Sputum neutrophil elastase levels. All data mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with baseline.

relatively low (5, 6, 8, 9), consistent with one infection following the other, so that only relatively infrequently are the two detected together. When patients are sampled at the onset of exacerbation and again 5–7 days later 36% of exacerbations in which a virus was detected at onset developed secondary bacterial infection (6), but 71% of bacterial exacerbations had reported symptoms of a viral upper respiratory tract infection before onset, so the true association may be even higher (6).

Experimental infection studies uniquely allow examination of temporal relationships between viral and bacterial infection in a manner difficult to achieve in naturally occurring exacerbations. We report that 60% of subjects with COPD developed bacterial infection after rhinovirus infection and virus load in sputum peaked on Days 5–9 postinoculation, whereas bacterial load peaked on Day 15. Further evidence providing support for a causal link was the positive correlation between virus and bacterial loads. Therefore, these are the first *in vivo* data directly linking rhinovirus infection to secondary bacterial infections in COPD and suggest that studies of naturally occurring exacerbations have underestimated the rates of dual infection caused by virus and bacterial infections occurring at different times (5, 30).

No bacteria were present at baseline before rhinovirus infection suggesting either that the bacterial infections were *de novo* acquisition of new organisms, or that bacteria were present at levels below the sensitivity of culture at baseline, and that immune suppression consequent on virus infection resulted in overgrowth of these organisms to detectable levels. Determining which of these is the predominant mechanism requires further studies using more sophisticated bacterial detection methods, such as quantitative polymerase chain reaction, bacterial sequencing methodologies, and experimental rhinovirus infection studies that include subjects with COPD with bacterial colonization.

Bacteria cultures should be accompanied by appropriate clinical symptoms and physiologic and pathologic changes to fulfill accepted definitions of infection. We therefore examined the effect of bacterial infection on clinical and inflammatory outcomes and report that the peak of symptoms, airflow obstruction, and airways inflammation occurred on Day 9 postinoculation, coinciding with peak virus load. However, in the bacteria-positive subjects breathlessness, airflow obstruction, and airways inflammation persisted on Day 15, when virus load was falling, and Day 21 when virus load was undetectable, suggesting that secondary

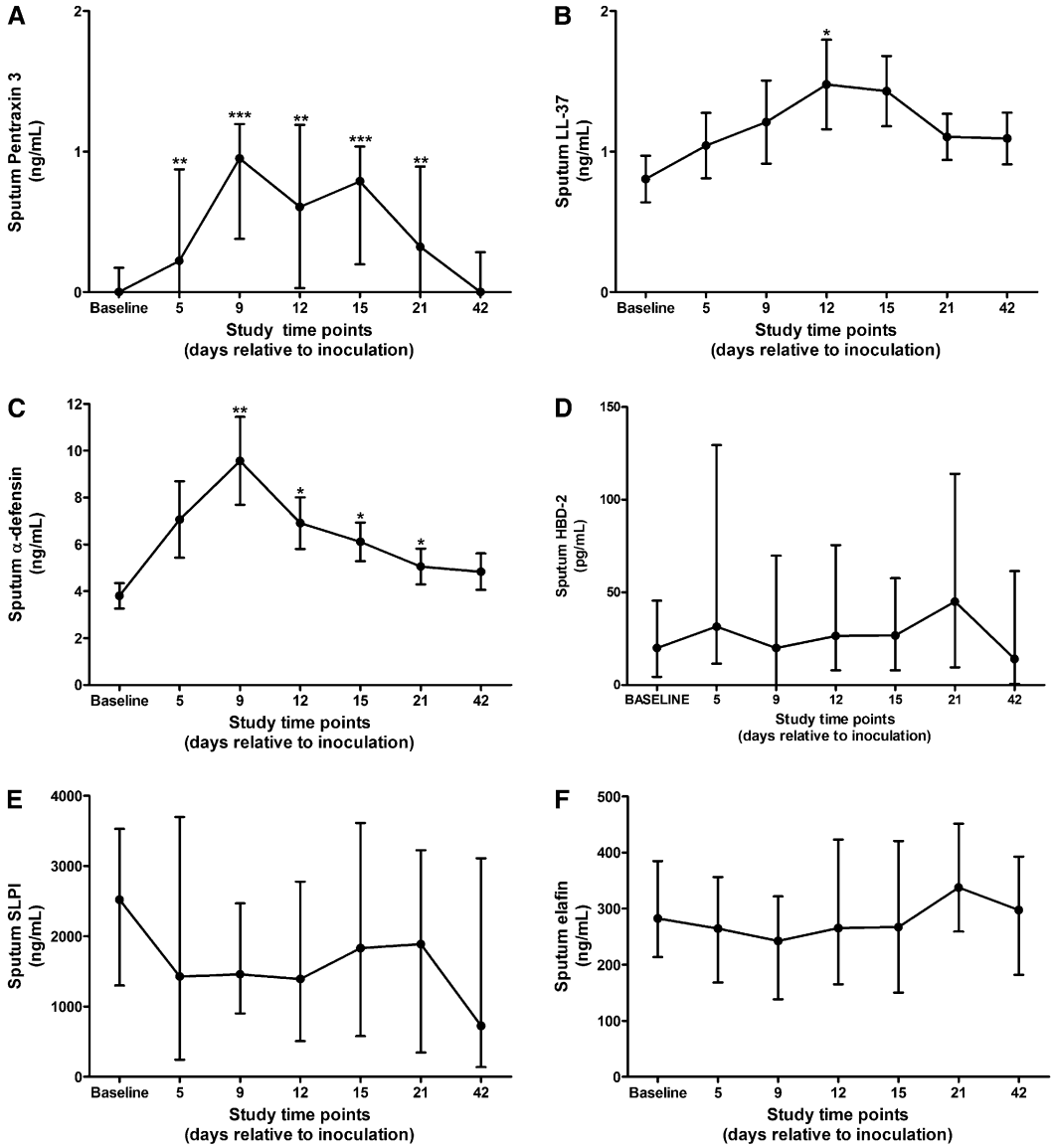

Figure 3. Levels of antimicrobial peptides in sputum in all subject groups. (*A*) Pentraxin 3. (*B*) LL-37. (*C*) α-defensins. (*D*) human β-defensin-2 (HBD-2). (*E*) Secretory leukoprotease inhibitor (SLPI). (*F*) Elafin. *A*, *B*, and *C* are mean ± SEM; *D*, *E*, and *F* are median ± IQR. $*P < 0.05$, $**P < 0.01$ and, $***P < 0.001$ compared with baseline.

bacterial infections are likely to prolong the duration of initially virus-induced COPD exacerbations. After rhinovirus infection sputum levels of neutrophil elastase were higher and levels of the antimicrobial peptides SLPI and elafin lower in bacteria-positive subjects with COPD compared with those in whom no bacteria were detected. Low levels of SLPI in bacterial infections in COPD have been reported (31–33), but these studies did not establish whether these were a cause or consequence of infection. We report that neutrophil elastase was elevated on Day 9 and SLPI and elafin fell on Days 9 and 12, before the peak in bacterial load on Day 15, and SLPI and elafin correlated inversely with bacterial load, suggesting that their deficiency increases susceptibility to secondary bacterial infection. However, we cannot definitively exclude that the reduced levels of SLPI and elafin are secondary to bacterial infection. SLPI and elafin are cleaved by neutrophil elastase (27, 28) and an inverse relationship between neutrophil elastase and SLPI has been reported in cystic fibrosis (27) and COPD (31). Neutrophil elastase levels were higher in bacteria-positive subjects with COPD, so low levels of SLPI and elafin may be caused by their cleavage by rhinovirus-induced neutrophil elastase. The actions of these molecules are complex and include antimicrobial, immunomodulatory and antiprotease effects. Although it is likely that the antimicrobial actions of SLPI and elafin are a key to their role in secondary bacterial infections, we cannot exclude that other mechanisms may be relevant. In contrast, rhinovirus infection in COPD was associated with consistently high sputum levels of pentraxin 3, LL-37, and α-defensins, suggesting that these molecules may have less potent antimicrobial activities than SLPI and elafin. HBD-2 levels in sputum were very low and increased after rhinovirus infection in the nonsmokers only, suggesting that this defensin is unlikely to be important in this context and consistent with a report that smoking suppresses HBD-2 levels in pneumonia (34).

This study has a number of important implications for understanding the pathogenesis and etiology of COPD exacerbations. These data suggest that a substantial proportion of exacerbations attributed to bacterial infection alone may have been preceded and precipitated by viral infection, but the virus are no longer detectable at the time of presentation. Secondary bacterial infections occurred in 60% of subjects with moderate COPD and no bacterial colonization, and in patients with more severe COPD in whom exacerbations are more frequent (35) and bacterial colonization more prevalent (36), secondary bacterial infections are likely to be even more frequent and of greater functional significance. Our finding of relationships between secondary

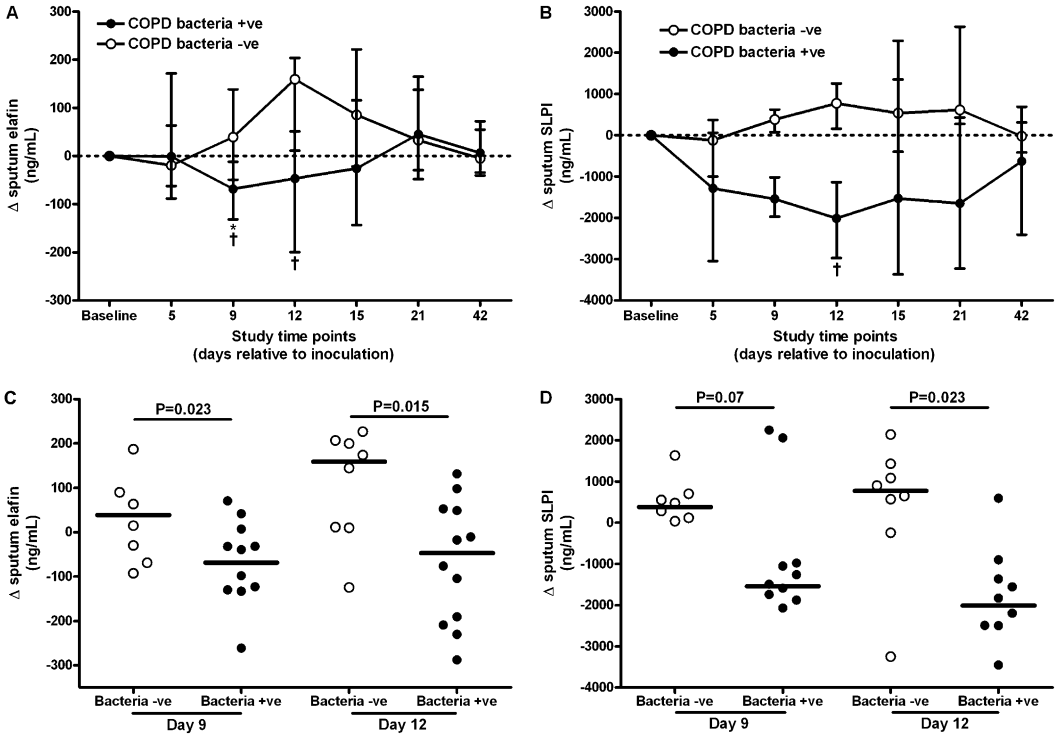

Figure 4. Change from baseline of levels of elafin and secretory leukoprotease inhibitor (SLPI) in sputum in subjects with chronic obstructive pulmonary disease (COPD) with and without bacterial infection. (*A*) Time course of sputum elafin. (*B*) Time course of sputum SLPI. (*C*) Change from baseline of sputum elafin levels on Days 9 and 12. (*D*) Change from baseline of sputum SLPI levels on Days 9 and 12. All data median ± IQR. $^{*}P < 0.05$ compared with baseline, $^{\dagger}P < 0.05$ compared with subjects without bacterial infection.

bacterial infections and the underlying severity of COPD within our Global Initiative for Chronic Obstructive Lung Disease stage II patients with COPD supports this hypothesis.

These findings strengthen the case for trials of antiviral therapies as early interventions at the onset of cold symptoms in subjects with COPD, because they raise the prospect that they may not only reduce severity of or prevent virus-induced COPD exacerbations, but they also have the potential to prevent secondary bacterial infections. In addition, administration of exogenous inhaled SLPI or elafin, or inhibitors of neutrophil elastase are now identified as potential novel therapeutic approaches to prevent secondary bacterial infections in COPD exacerbations.

The main limitation of our study is the small numbers of subjects and the limitation of experimental infection to subjects with moderate COPD. However, these results are only achievable in experimental infection studies that, because of the inherent difficulties of such an intensive study design, will always be small. We believe that such studies are a powerful and unique tool to investigate the role of infection in COPD and generate important data that complement equally important information gained from naturally occurring exacerbations. These data need replicating in future experimental studies and where possible in naturally occurring viral infections in patients with COPD.

In conclusion, secondary bacterial infection is common after rhinovirus infection in COPD and is associated with high levels of neutrophil elastase and with reduction in levels of the antimicrobial peptides elafin and SLPI. Treating respiratory virus infections in patients with COPD holds promise as a novel therapeutic approach for COPD exacerbations, as does administration of SLPI and elafin or elastase inhibitors.

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