Strain-specific Immune Response to *Haemophilus influenzae* in Chronic Obstructive Pulmonary Disease

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Previous studies of immune response to *Haemophilus influenzae* after exacerbations of chronic obstructive pulmonary disease (COPD) have yielded contradictory results. Using homologous (infecting) strains and immunoassays to surface-exposed epitopes, we tested the hypothesis that adults with COPD make new antibodies to strain-specific, surface-exposed epitopes on *H. influenzae* after exacerbations. We collected clinical information, sputum, and serum monthly and during exacerbations from 81 patients with COPD over 56 months. Serum antibodies to *H. influenzae* after exacerbations associated with *H. influenzae* in sputum were detected with whole bacterial cell ELISA and bactericidal assays. An immune response to homologous *H. influenzae* occurred after 22 of 36 (61.1%) exacerbations with newly acquired strains compared with 7 of 33 (21.2%) exacerbations with preexisting strains (odds ratio [OR] = 4.4; 95%, 1.8 to 10.8; p = 0.001). An absence of an immune response was strongly associated with complement sensitivity (OR = 0.03; 95% confidence interval, 0.003 to 0.22; p = 0.001). New bactericidal antibodies developed after exacerbations were highly strain specific, showing bactericidal activity for only 11 of 90 (12.2%) heterologous strains. Development of an immune response to *H. influenzae* supports its role in causing exacerbations. The strain specificity of the immune response likely represents a mechanism of recurrent exacerbations.

Keywords: chronic obstructive pulmonary disease; *Haemophilus influenzae*; humoral immunity; exacerbation

Exacerbations contribute significantly to the morbidity and mortality associated with chronic obstructive pulmonary disease (COPD) (1–4). Nontypeable *Haemophilus influenzae* is the bacterial pathogen most commonly isolated from sputum during exacerbations (5, 6). However, the role of *H. influenzae* in causing acute exacerbations is controversial, in part because of inconsistent results in studies of immune responses to *H. influenzae* (7–15). Several limitations of these serologic studies have likely contributed to contradictory results (7–15). Many previous studies compared antibodies in single sera obtained from patients with sera obtained from healthy control subjects rather than between paired sera obtained from individual patients before and after the exacerbation, potentially failing to detect significant immune responses (7, 8, 10, 11). Several studies used a single or a small panel of laboratory strains of *H. influenzae* as an antigen instead of the strains recovered from sputum during the exacerbations (7–11, 14, 15). In view of the considerable variation in the surface antigenic structure among *H. influenzae* strains, such a method fails to detect strain-specific immune responses (6, 12). Furthermore, immunoassays used in several studies were not specific for antibodies to epitopes exposed on the bacterial surface, an approach that will not detect potentially protective antibodies in the background of antibodies to non–surface-exposed or cross-reactive epitopes (7–11, 14, 15). In this study, we used homologous (infecting) strains, immunoassays to detect antibodies to surface-exposed epitopes, and paired patient serum samples to test the hypothesis that adults with COPD make new antibodies to surface-exposed epitopes on *H. influenzae* after exacerbations.

Strains of *H. influenzae* that are new to a patient are associated with the development of an exacerbation (5). Consistent with that observation, we further hypothesized that the immune response that develops after exacerbation to the homologous (infecting) strain will have limited ability to kill other (heterologous) *H. influenzae* strains (16–20). Such a strain-specific immune response will protect against recurrent exacerbation due to the homologous strain but will leave the patient susceptible to exacerbations caused by antigenically unrelated heterologous strains.

To test these hypotheses, we conducted a study in which we obtained clinical information and serum and sputum samples monthly and during exacerbations from a cohort of patients with COPD. Serum antibodies to homologous and heterologous strains of *H. influenzae* were measured with whole bacterial cell ELISA and bactericidal assay. Some of the results of these studies have been previously reported in the form of an abstract (21, 22).

METHODS

Study Design

The Human Studies Subcommittee of the Veterans Affairs Western New York Healthcare System approved the study protocol. All participants gave written informed consent. After initial recruitment, additional patients were recruited as needed to maintain active follow-up of 50 patients. To achieve this goal, a total of 81 patients with chronic bronchitis were enrolled between March 1994 and December 1998. Inclusion criteria have been described previously (5). The patients were seen at the Buffalo Veterans Affairs Medical Center monthly and whenever there were symptoms suggestive of an exacerbation.

At each visit, clinical information and sputum and serum samples were collected. The criteria for defining an exacerbation have been described previously (5).

Patient Samples and Bacterial Strains

The study personnel who processed the sputum samples were unaware of the clinical status of the patients. Sputum samples were spontaneously expectorated morning samples. Sputum samples were homogenized by incubation at 37°C for 15 minutes with an equal volume of 0.1% dithiothreitol (Sputolysin; Calbiochem, La Jolla, CA). Serial dilutions of homogenized sputum in phosphate-buffered saline (PBS) were plated on blood, chocolate, and MacConkey agar plates. Bacterial identification was performed by standard techniques. If *H. influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* were present, up to 10 individual colonies of each bacterial species were isolated and saved as frozen stocks at −70°C. Serum was separated from 10 ml of blood collected at each clinic visit and stored in aliquots at −70°C.

Bacterial isolates from sputum were classified as potential pathogens or as normal flora. Potential pathogens included nontypeable *H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, *Pseudomonas aeruginosa*, *Staphylo-*

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coccus aureus, and other gram-negative enteric rods. Other bacterial species were classified as normal flora. Sputum isolates of *H. influenzae* were typed as described previously, and each strain was categorized as preexisting or new (5). A new strain was one that had not been isolated from sputum samples previously obtained from an individual patient. A preexisting strain was one that had been isolated from sputum obtained from a previous clinic visit.

Each *H. influenzae* strain was tested with two homologous patient sera to determine whether serum antibodies to the infecting strain develop after an exacerbation. The preexacerbation serum was obtained at a clinic visit before the exacerbation visit and the postexacerbation serum was obtained at least 4 weeks after the exacerbation visit.

Whole Bacterial Cell ELISA

The level of serum IgG to whole bacterial cell preparations of homologous isolates of *H. influenzae* was determined by ELISA as described previously for *M. catarrhalis* (23). Preexacerbation and postexacerbation sera were tested simultaneously. The percentage change in optical density at 450 nm (OD_{450}) between postexacerbation and preexacerbation sera was calculated. To determine the level of a significant percentage change between postexacerbation and preexacerbation serum, 15 paired samples of sera obtained 2 months apart from subjects in the study clinic who had not experienced *H. influenzae* infection by sputum culture during that period were tested in ELISA with heterologous *H. influenzae* strains.

Bactericidal Assays

The presence of serum antibodies capable of antibody-mediated complement-dependent killing of strains of *H. influenzae* was determined by bactericidal assays. Bacteria grown to the midlogarithmic phase were diluted in PBS containing calcium and magnesium (PCGM) and volumes of 20 μl added to wells of a 96-well plate. Ten-microliter aliquots of heat-inactivated (56°C for 30 minutes) serum were added to the wells (final serum concentration of 20%). An exogenous source of complement was prepared by depleting a healthy volunteer's serum of IgG by protein G chromatography (20). After incubating the bacteria with the serum for 30 minutes, 5 μl of complement was added (a final complement concentration of 10%), and the incubation continued for an additional 30 minutes at 37°C. Duplicate aliquots of the well contents were plated on chocolate agar and colony counts of surviving bacteria performed after overnight incubation. Control wells were performed simultaneously in each assay in which either complement or serum were omitted. A percentage kill was determined by calculating the colony count obtained after incubation with serum and complement as a percentage of the colony count obtained after incubation with serum alone and subtracting the value obtained from 100. A 50% kill was regarded as significant. Sera that demonstrated significant bacterial killing at 20% concentration were titrated with serial twofold dilution to determine the minimum bactericidal concentration.

Statistical Analysis

Patients who experienced at least one *H. influenzae* exacerbation were compared with patients who experienced no such exacerbations by unpaired *t* test for continuous data and by chi-square analysis for nominal data.

The unit of analysis was an exacerbation strain. The relationship of antibody response and whether the strain of *H. influenzae* was new to the patient was determined with generalized estimating equations to take into account that patients contributed more than one exacerbation strain (24). An exchangeable correlation matrix was used with a binomial distribution and a logit link function. The coefficients of this regression are the logarithm of the odds ratio (OR). ORs of having an antibody response to *H. influenzae* when a new strain was present compared with when a preexisting strain was present were calculated. Similar analysis was performed to calculate the OR of having an ELISA response to *H. influenzae* when the strain was complement sensitive compared with when it was complement resistant.

The OR of finding a concomitant new pathogen when a new *H. influenzae* strain was present in the sputum compared with when a preexisting strain was present in the sputum was calculated, as described previously here, except the unit of analysis was an exacerbation.

RESULTS

Patient Demographics

Of the 81 patients, 35 had at least one exacerbation of COPD associated with *H. influenzae* isolation from sputum over 56 months for a total of 77 exacerbations. Table 1 compares these 35 patients with the 46 patients who did not experience a *H. influenzae* exacerbation. No differences were observed in the two groups in age, sex, racial distribution, tobacco consumption, and severity of airway obstruction. Patients with *H. influenzae* exacerbations had been observed for a significantly longer period in the study clinic and had had more exacerbations observed. Ten exacerbations were excluded from further study, three that were seen on the first clinic visit and seven that represented repeat visits for an exacerbation with the same strain isolated from sputum less than 4 weeks earlier. Therefore, 67 exacerbations in 33 patients (range of 1–7 per patient) were included in this study.

In four exacerbations, two strains of *H. influenzae* were isolated simultaneously. Two of these strains could not be studied further. One was not recoverable from frozen stock, and another demonstrated autoaggregation (clumping) in growth media. Therefore, 69 strains of *H. influenzae* were available for analysis. Molecular typing demonstrated that 36 strains were new to the patient, and 33 were preexisting strains.

Development of Serum Antibodies by Whole Bacterial Cell ELISA After Exacerbation

The 15 paired control samples from patients without *H. influenzae* infection demonstrated 0.4 ± 8.6% (mean ± 1 SD) change in OD_{450}, with a 22.1% change representing the upper limit of the 99% confidence interval (CI). Therefore, an increase in OD_{450} of more than 22.1% between the postexacerbation and preexacerbation sera was regarded as a significant serum IgG immune response to the infecting strain after an exacerbation.

ELISA assays detected a significant IgG response to 26 of the 69 (37.7%) strains (Figure 1). Exacerbation strains of *H. influenzae* new to the patient were significantly more likely to be associated with an immune response by ELISA, which was seen with 21 of 36 (58.3%) new strains as compared with 5 of 33 (15.2%) preexisting strains (OR = 7.7; 95% CI, 2.5 to 24.1; $p < 0.001$).

Development of Bactericidal Antibodies After Exacerbation

Bactericidal assays were attempted with all 69 strains and were successfully performed with 50 strains. One strain demonstrated autoaggregation in PBS. Eighteen strains (10 new and 8 preexisting) were killed by 10% complement in the absence of serum antibodies. This complement sensitivity was seen with three complement sources: two IgG-depleted normal human sera and one hypogammaglobulinemic serum.

Development of bactericidal antibodies to the homologous strain after exacerbation was demonstrated if the postexacerbation serum was bactericidal and the corresponding preexacerbation serum was not bactericidal at 20% concentration or if there was a two-dilution (fourfold) increase in titer between the preexacerbation and the postexacerbation serum. A bactericidal antibody response after exacerbation was seen with 23 of 50 strains (46%) (Figures 2A and 2B). Exacerbation strains of *H. influenzae* new to the patient were significantly more likely to be associated with development of bactericidal antibodies, which was seen with 18 of 26 (69.2%) new strains as compared with 5 of 24 (20.8%) preexisting strains (OR = 8.7; 95% CI, 2.7 to 28.0; $p < 0.001$).

Bactericidal antibodies to the homologous infecting strain were not detected in 25 of the 26 (96.2%) preexacerbation sera

TABLE 1. SUBJECTS WHO EXPERIENCED AT LEAST ONE *HAEMOPHILUS INFLUENZAE* EXACERBATION COMPARED WITH THOSE WHO HAD NONE OBSERVED DURING THE 56 MONTHS OF FOLLOW-UP IN THE CHRONIC OBSTRUCTIVE PULMONARY DISEASE STUDY CLINIC

Variable	Subjects with at Least One *H. Influenzae* Exacerbation (*n* = 35)	Subjects with No *H. Influenzae* Exacerbation (*n* = 46)	p Value
Age, mean ± SEM	64.7 ± 1.4	67.9 ± 1.5	0.13
Male sex	33	46	0.18
White	27	39	0.40
FEV_1, mean ± SEM	1.5 ± 0.1	1.7 ± 0.1	0.39
FEV_1 % predicted, mean ± SEM	45.4 ± 3.7	48.7 ± 2.7	0.46
Current smoker at enrollment	11	18	0.49
Pack years of smoking, mean ± SEM	85.2 ± 5.8	83.5 ± 6.2	0.85
Years of observation, mean ± SEM	2.9 ± 0.2	1.6 ± 0.2	< 0.001
Number of exacerbations, mean ± SEM	3.4 ± 0.6	2.8 ± 0.6	< 0.001

Definition of abbreviation: *H. Influenzae* = *Haemophilus influenzae*.

when the *H. influenzae* strain was new to the patient. However, preexacerbation sera were bactericidal for 18 of 24 (75%) preexisting *H. influenzae* strains.

Development of either ELISA or Bactericidal Immune Response

Concordant results were seen in 42 of 50 (84%) instances when both bactericidal and ELISA assays were completed with a strain and set of sera. An immune response was detected exclusively by ELISA with five and by the bactericidal assay with three strains. An immune response by either assay developed to 29 of 69 (42%) strains. Exacerbation strains of *H. influenzae* new to the patient were much more likely to be associated with an immune response by either assay, which was seen with 22 of 36 (61.1%) new strains as compared with 7 of 33 (21.2%) preexisting strains (OR = 4.4; 95% CI, 1.8 to 10.8; p = 0.001).

Complement Sensitivity and Development of Immune Response

Eighteen strains of *H. influenzae* were killed by 10% complement in the absence of antibodies *in vitro*. Complement sensitivity was strongly associated with absence of a serum IgG response by ELISA, which was found to only 1 of 18 (5.6%) complement-sensitive strains (0 of 10 new and 1 of 8 preexisting strains) compared with 28 of 50 (56%) complement-resistant strains (OR = 0.03; 95% CI, 0.003 to 0.22; p = 0.001).

Strain Specificity of the Immune Response

To determine the strain specificity of postexacerbation bactericidal antibodies, 10 postexacerbation sera from 10 patients that killed homologous strains were studied. Each serum was tested with nine heterologous *H. influenzae* strains in bactericidal assays at 20% concentration for a total of 90 assays. If a postexacerbation serum killed a heterologous strain, the corresponding preexacerbation serum was also tested with the heterologous strain. Postexacerbation sera were bactericidal in 17 of the 90 (18.9%) assays with heterologous strains. Six of the 17 corresponding preexacerbation sera were also bactericidal for the heterologous strain. Therefore, new bactericidal antibodies that developed after an exacerbation killed in 11 of 90 (12.2%) assays with heterologous strains. Of the 10 postexacerbation sera tested, 4 killed only the homologous strain, 4 killed one, 1 killed three,

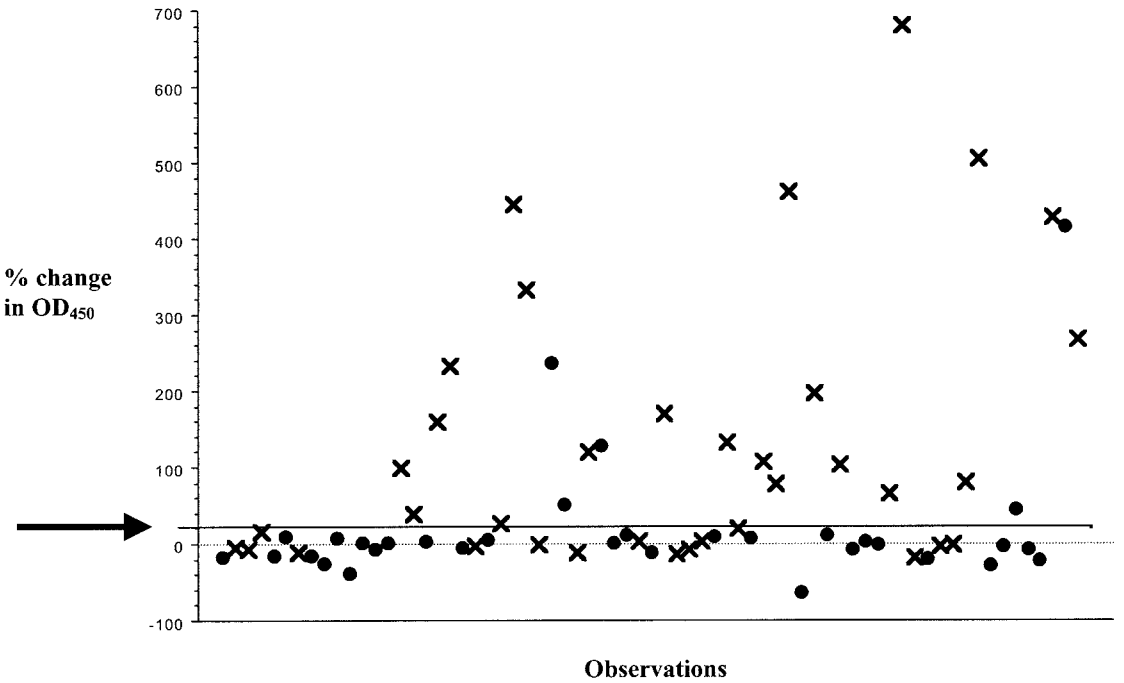

Figure 1. ELISA results obtained with all 69 strains. The percentage change in OD_{450} of serum IgG antibodies to the homologous strain of *Haemophilus influenzae* is shown. Observations with new strains are represented by an *X*, and observations with preexisting strains are represented by a *closed circle*. A *horizontal line* is at 22.1% and represents a significant change in optical density at 450 mn (OD_{450}).

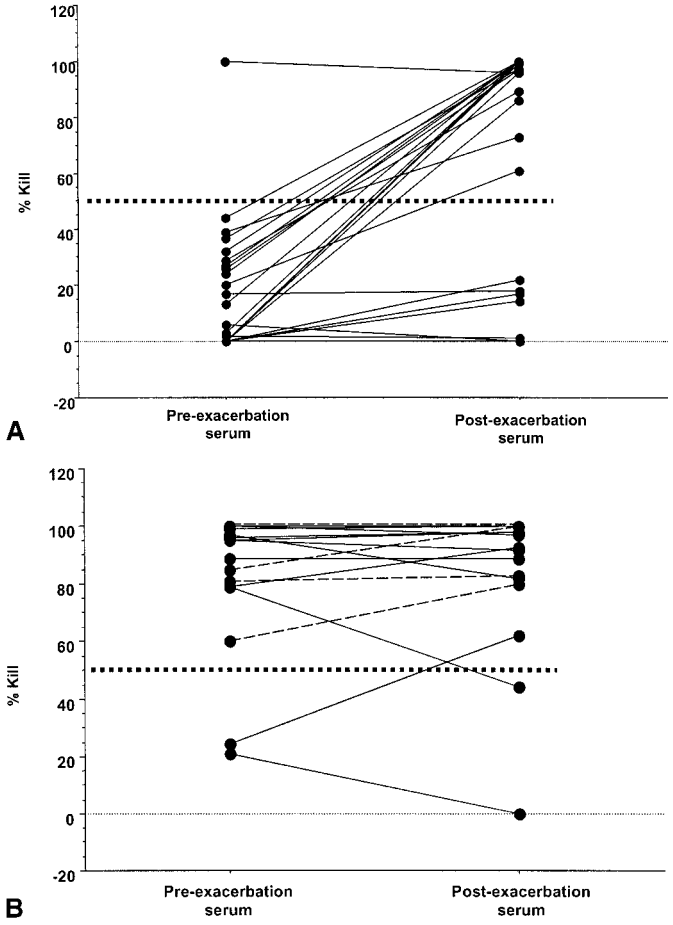

Figure 2. Bactericidal assay results with 50 complement-resistant strains. *Lines* connect the measured values with preexacerbation and postexacerbation sera with a homologous *H. influenzae* strain. The serum concentration is 20% in all assays. The *dotted lines* in *B* represent four pairs of sera in which titration by twofold dilution demonstrated that the postexacerbation serum were able to kill the homologous strain at a fourfold lower concentration. *A* represents results with new strains, and *B* represents results obtained with preexisting strains. The *dashed horizontal line* at 50% kill represents the threshold value above which sera were regarded as having bactericidal activity. Seven assay results in *A* overlap in the preexacerbation (less than 5%) and postexacerbation (more than 95%) values. Thirteen assay results in *B* overlap in the preexacerbation (more than 95%) and postexacerbation (more than 95%) values.

and 1 killed four heterologous strains. Therefore, 8 of the 10 postexacerbation sera were highly strain specific, whereas 2 demonstrated partial strain specificity.

Simultaneous Isolation of Other Bacterial Pathogens from Sputum

For this analysis, two exacerbations that had a new and a preexisting strain of *H. influenzae* simultaneously isolated were classified as exacerbations associated with new strains of *H. influenzae*. Other respiratory pathogens were simultaneously isolated from sputum in 1 of the 36 exacerbations with new strains and with 10 of the 31 exacerbations associated with preexisting strains of *H. influenzae* (Table 2). Furthermore, the other bacterial pathogen isolated was new to the patient, as determined by molecular typing in 9 of the 11 instances (Table 2). Exacerbations with a preexisting *H. influenzae* strain were significantly more frequently associated with a concomitant pathogen new to the patient than exacerbations with a new *H. influenzae* strain (OR = 14.9; 95% CI, 1.6 to 138.3; p = 0.018).

DISCUSSION

In this study, we have demonstrated that after an exacerbation of COPD associated with a *H. influenzae* strain isolated from sputum, there is development of serum antibodies to the infecting strain in the majority of instances when that strain was newly acquired by the patient. These antibodies are directed to antigens that are exposed on the bacterial surface and are therefore potentially protective. Indeed, serum bactericidal antibodies are associated with protection from otitis media due to *H. influenzae* (16). In addition, the immune response that develops is quite specific to the homologous strain and therefore likely will not protect against infectious exacerbations by other strains of *H. influenzae* with a different antigenic structure. Therefore, these observations substantiate the role of *H. influenzae* in a proportion of exacerbations and help explain a mechanism for recurrent exacerbations with *H. influenzae* in COPD.

After immunization with whole bacterial cells in animal models, a substantial proportion of bactericidal antibodies that develop to *H. influenzae* are to a major outer membrane protein P2 (19, 25). The same was observed for human bactericidal antibodies that developed after exacerbations of COPD in two patients from our study (20). Extracellular portions of outer membrane protein P2 show a high degree of sequence variability among strains of *H. influenzae* (26–29). We speculate that the strain-specific immune response observed in this study is directed at heterogeneous surface epitopes on outer membrane protein P2 and other surface antigens of *H. influenzae*.

Exacerbations of COPD associated with preexisting strains of *H. influenzae* were not associated with an immune response in the majority of instances, suggesting that other infectious or noninfectious factors caused these exacerbations. Other bacterial pathogens that were new to the patient were isolated in eight of these exacerbations and likely induced the increased symptoms. Viruses and atypical bacteria are associated with exacerbations of COPD and were not investigated in this study (30, 31). Changes in the surface antigenic structure *in vivo* in a preexisting strain of *H. influenzae* may allow it to escape the immune response and induce an exacerbation, another possible explanation for these exacerbations (25, 32–34).

The absence of a host immune response to complement sensitive strains of *H. influenzae* is an intriguing and novel observation. We speculate that either because of their sensitivity to complement or because of other virulence determinants associated with complement sensitivity, these strains colonize the mucosal surface rather than invade the bronchial mucosa. Therefore, they may fail to elicit a humoral immune response. This observation needs further investigation to provide valuable insight into the virulence mechanisms of nontypeable *H. influenzae*.

Limitations of our study include the reliance on sputum samples to define infection with their low sensitivity and specificity. This study did not assay the mucosal immune response, which may be important, especially with complement-sensitive strains of *H. influenzae* (9, 10, 35). Simultaneous study of viral and atypical bacterial infection would have provided a more comprehensive assessment of the infectious etiology of exacerbations.

We have recently demonstrated that acquisition of new strains of bacterial pathogens in COPD is associated with a substantial increase in risk of exacerbation (5). Bacterial pathogens are isolated in significant concentrations from bronchoscopic samples during acute exacerbations (36–39). Neutrophilic airway inflammation is associated with isolation of bacterial pathogens from sputum (40, 41). This study lends another line

TABLE 2. BACTERIAL PATHOGENS ISOLATED SIMULTANEOUSLY FROM SPUTUM IN 11 OF THE 67 EXACERBATIONS INCLUDED IN THIS STUDY

Patient Number	Visit Number	Pathogen	Concomitant Pathogen Strain New or Preexisting*	*H. Influenzae* Strain New or Preexisting*
1	18	*M. catarrhalis*	New	Preexisting
5	6	*M. catarrhalis*	New	Preexisting
5	19	*S. pneumoniae*	Preexisting	Preexisting
5	35	*M. catarrhalis*	Preexisting	Preexisting
5	37	*M. catarrhalis*	New	Preexisting
18	17	*P. aeruginosa*	New	Preexisting
22	3	*S. pneumoniae*	New	Preexisting
32	6	*M. catarrhalis*	New	Preexisting
39	29	*M. catarrhalis*	New	Preexisting
60	11	Enterobacter	New	New
74	11	*M. catarrhalis*	New	Preexisting
		S. pneumoniae	New	

Definition of abbreviations: *M. catarrhalis* = *Moraxella catarrhalis*; *S. pneumoniae* = *Streptococcus pneumoniae*; *P. aeruginosa* = *Pseudomonas aeruginosa*.

* A new strain was one that had not been previously isolated from sputum from that patient since enrollment in the study clinic, and a preexisting strain was one that had been previously isolated from sputum from that patient.

of evidence that bacteria indeed cause a substantial proportion of exacerbations of COPD. Recurrent exacerbations with *H. influenzae* in COPD have been ascribed previously to periodic increases in bacterial concentration in the airways. This study supports a new mechanistic explanation for recurrent exacerbations with *H. influenzae* in these patients. Specifically, patients make strain-specific antibodies after exacerbations, leaving the host susceptible to reinfection by other strains of *H. influenzae*.

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