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NEW STRAINS OF BACTERIA AND EXACERBATIONS OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE

SANJAY SETHI, M.D., NANCY EVANS, R.N., BRYDON J.B. GRANT, M.D., AND TIMOTHY F. MURPHY, M.D.

ABSTRACT

Background The role of bacterial pathogens in acute exacerbations of chronic obstructive pulmonary disease is controversial. In older studies, the rates of isolation of bacterial pathogens from sputum were the same during acute exacerbations and during stable disease. However, these studies did not differentiate among strains within a bacterial species and therefore could not detect changes in strains over time. We hypothesized that the acquisition of a new strain of a pathogenic bacterial species is associated with exacerbation of chronic obstructive pulmonary disease.

Methods We conducted a prospective study in which clinical information and sputum samples for culture were collected monthly and during exacerbations from 81 outpatients with chronic obstructive pulmonary disease. Molecular typing of sputum isolates of nonencapsulated *Haemophilus influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* was performed.

Results Over a period of 56 months, the 81 patients made a total of 1975 clinic visits, 374 of which were made during exacerbations (mean, 2.1 per patient per year). On the basis of molecular typing, an exacerbation was diagnosed at 33.0 percent of the clinic visits that involved isolation of a new strain of a bacterial pathogen, as compared with 15.4 percent of visits at which no new strain was isolated ($P < 0.001$; relative risk of an exacerbation, 2.15; 95 percent confidence interval, 1.83 to 2.53). Isolation of a new strain of *H. influenzae*, *M. catarrhalis*, or *S. pneumoniae* was associated with a significantly increased risk of an exacerbation.

Conclusions The association between an exacerbation and the isolation of a new strain of a bacterial pathogen supports the causative role of bacteria in exacerbations of chronic obstructive pulmonary disease. (N Engl J Med 2002;347:465-71.)

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MORBIDITY and mortality among patients with chronic obstructive pulmonary disease are related in large part to acute exacerbations, which occur one to three times per year.¹⁻⁶ Our understanding of the cause and pathogenesis of these exacerbations is incomplete, and the role of bacterial pathogens is controversial.⁷⁻¹⁰

In studies performed decades ago, investigators followed patients with chronic obstructive pulmonary disease longitudinally, with periodic collection of sputum samples for culture, to determine whether there was an association between the isolation of bacterial pathogens in sputum and the occurrence of exacerbations.^{5,6,11} In these studies, the rate of isolation of potential bacterial pathogens from sputum samples during stable disease was identical to the rate during acute exacerbations. This finding led to the conclusion that bacterial pathogens do not cause exacerbations and that their presence in sputum is due to chronic colonization.^{7,12}

An increased understanding of the genetic heterogeneity among strains of a bacterial species exposes a major limitation of the older cohort studies.¹³ At the time of these studies, it was not possible to differentiate among strains of a pathogenic bacterial species. Therefore, all strains isolated from sputum over the course of the study were regarded as identical if they belonged to the same species. This approach did not allow for the detection of changes in strains over time. More recent studies have shown that the immune response to bacterial pathogens after exacerbations of

From the Divisions of Pulmonary and Critical Care Medicine (S.S., B.J.B.G.) and Infectious Diseases (T.F.M.), Department of Medicine, the Department of Microbiology (T.F.M.), and the Departments of Physiology and Biophysics and Social and Preventive Medicine (B.J.B.G.), State University of New York; and the Veterans Affairs Western New York Healthcare System (S.S., N.E., B.J.B.G., T.F.M.) — both in Buffalo, N.Y. Address reprint requests to Dr. Sethi at the Veterans Affairs Western New York Healthcare System (151), 3495 Bailey Ave., Buffalo, NY 14215, or at ssethi@buffalo.edu.

chronic obstructive pulmonary disease is characterized by considerable strain specificity, suggesting the importance of differentiation among strains of bacterial pathogens isolated over time from patients with chronic obstructive pulmonary disease.¹⁴⁻¹⁶

We hypothesized that the acquisition of a new strain of pathogenic bacterial species in a patient with chronic obstructive pulmonary disease who has no preexisting immunity to the strain leads to an exacerbation. To test this hypothesis, we conducted a study in which we obtained sputum samples monthly and during exacerbations in a cohort of patients with chronic obstructive pulmonary disease. Bacterial strains isolated from sputum obtained during periods of stable disease and during exacerbations were subjected to molecular typing. This report represents the results from the first 56 months of this study.

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. A total of 81 patients were enrolled between March 1994 and December 1998. After initial recruitment, additional patients were recruited as needed to maintain active follow-up of 50 patients. Inclusion criteria were the presence of chronic bronchitis¹⁷; the absence of asthma and bronchiectasis on the basis of a clinical assessment; an ability to comply with a schedule of monthly clinical visits; and the absence of immunosuppressive or other life-threatening disorders. The patients were seen monthly, as well as whenever they had symptoms suggestive of an exacerbation, at an outpatient clinic in the Buffalo Veterans Affairs Medical Center.

At each visit, clinical information and sputum and serum samples were obtained. The patients were questioned about the status of their chronic respiratory symptoms (dyspnea, cough, sputum production, viscosity, and purulence), and the responses were graded as 1 (at the usual level), 2 (somewhat worse than usual), or 3 (much worse than usual). A minor worsening of two or more symptoms or a major worsening of one or more symptoms prompted a clinical assessment of the cause. If the patient had fever (a temperature that exceeded 38.3°C), appeared ill, or had signs of consolidation on examination of the lungs, a chest film was obtained to rule out pneumonia. If other causes of the worsening of symptoms, such as pneumonia, upper respiratory infection, and congestive heart failure, were ruled out, the patient was considered to be having an exacerbation of chronic obstructive pulmonary disease. The determination of whether the patient had stable disease or an exacerbation was made before the results of sputum cultures were available.

Sputum Samples

The study personnel who processed the sputum samples were unaware of the clinical status of the patients. Samples of sputum that had been spontaneously expectorated in the morning were homogenized by incubation at 37°C for 15 minutes with an equal volume of 0.1 percent dithiothreitol (Sputolysin, Calbiochem). Serial dilutions of homogenized sputum in phosphate-buffered saline were placed on blood, chocolate, and MacConkey agar plates. Bacterial identification was performed with the use of standard techniques. If *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* was present, up to 10 individual colonies of each bacterial species were isolated and saved as frozen stocks at -70°C.

Sputum isolates were classified as potential pathogens or as normal flora. Potential pathogens were *H. influenzae*, *M. catarrhalis*,

S. pneumoniae, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and other gram-negative rods.^{18,19} Other bacterial species were classified as normal flora.

Molecular Typing

Isolates of *H. influenzae* were typed by polyacrylamide-gel electrophoresis of cell lysates, as previously described.²⁰ This method is based largely on the mobility of major outer-membrane proteins that vary among strains.²¹ Because of the occurrence of multiple strains in a single sputum sample, every available isolate (a total of 2159 isolates from 375 sputum samples) of *H. influenzae* were typed. To determine whether more than one strain was present in a sputum sample, 10 isolates from 25 sputum samples with *M. catarrhalis* and the same number with *S. pneumoniae* were individually typed. A single strain was present in every instance. Therefore, single isolates of *M. catarrhalis*, *S. pneumoniae*, and *P. aeruginosa* strains were subsequently typed by pulsed-field gel electrophoresis, as previously described.²²⁻²⁵ A single isolate of *P. aeruginosa* had been saved from each sputum sample as indicated by the study protocol, and additional isolates were therefore not available for typing.

Each strain was categorized as old or new on the basis of molecular typing. A new strain was one that had not been isolated from sputum samples obtained previously from an individual patient. An old strain was one that had been isolated from sputum obtained from a previous visit. In six instances, a strain isolated at the time of an exacerbation had been identified as a new strain at a visit less than four weeks earlier, when the patient had had stable disease. These strains were classified as new strains for the exacerbation-related visit.

Statistical Analysis

The unit of analysis was the clinic visit. The relative risk of an exacerbation when a pathogen or a new strain was present was calculated with the use of generalized estimating equations to take into account the patients' repeated visits (S Plus 6.0, Insightful).²⁶ An unstructured correlation matrix was used. The absence of an exacerbation, a pathogen, or a new strain was coded as 0, and the presence as 1. The coefficient of the relation between the risk of an exacerbation due to a pathogen or a new strain was the log of the relative risk when a quasilielihood approach was used with a logarithmic-link function.²⁷ An alternative approach is to estimate the odds ratio with the use of conditional logistic regression. With this alternative approach, the results were similar (not shown) and did not affect our overall conclusions.

To determine the effect of the influenza season, the months of December, January, February, and March were designated as the influenza season and coded as 1, and the other months were coded as 0. The coding for influenza season was added to the pathogen or new strain as another predictor variable for the risk of an exacerbation, together with a term for the interaction between the two predictor variables.

RESULTS

Characteristics of the Patients and the Visits

The majority of the patients were elderly men with moderate-to-severe chronic obstructive pulmonary disease (Table 1). At all but one visit, the assessment of the patient was completed. The characteristics of these 1975 completed visits are shown in Table 2. Sputum was not available at a total of 148 clinic visits (11 visits during exacerbations and 137 at the time of stable disease). Absence of sputum was significantly less frequent during exacerbations than during stable disease (2.9 percent vs. 8.6 percent, $P < 0.001$).

TABLE 1. BASE-LINE CHARACTERISTICS OF 81 PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE.*

CHARACTERISTIC	VALUE
Age — yr	
Mean	66.5±9.4
Range	45–84
Sex — no.	
Male	79
Female	2
Race — no.	
White	66
Black	15
Interval since diagnosis — yr	
Mean	11.7±11.6
Range	0–54
Smoking status — no.	
Former smoker	52
Current smoker	29
Pack-years of smoking	
Mean	84.2±38.3
Range	10–185
FEV ₁ — liters	
Mean	1.62±0.75
Range	0.47–4.07
FEV ₁ — % of predicted	
Mean	47.3±19.5
Range	15–99
Airway obstruction — no. (%)	
Severe (FEV ₁ <50 percent of predicted)	47 (58)
Moderate (FEV ₁ 50–69 percent of predicted)	25 (31)
Mild (FEV ₁ ≥70 percent of predicted)	4 (5)
None (chronic bronchitis only)	5 (6)

*Plus-minus values are means ±SD. FEV₁ denotes forced expiratory volume in one second.

For 26 exacerbations, the patient had received at least one dose of an antibiotic within 48 hours before the clinic visit. Pathogens were isolated in sputum samples obtained at 8 (30.8 percent) of these 26 visits, as compared with 134 (39.8 percent) of 337 visits during exacerbations for which there had been no antibiotic treatment within the previous 48 hours, but the effect of antibiotic use on the rate of isolation of pathogens was not statistically significant ($P=0.43$). Visits during exacerbations preceded by antibiotic treatment were therefore included in the data analysis. The influenza season did not significantly affect the incidence of exacerbations, the rate of isolation of pathogens, or the rate of isolation of new strains.

Excluding the 1 incomplete visit and the 148 visits at which sputum samples were not obtained, 1827 visits were analyzed for the association between bacterial isolation and exacerbation. Pathogenic bacteria were isolated from sputum samples obtained at 601 of the 1827 visits (32.9 percent). Isolation of a bacterial pathogen was associated with a significant increase in the incidence of exacerbations. An exacerbation was present in 142 of the 601 visits at which pathogens

TABLE 2. CHARACTERISTICS OF 1975 COMPLETED CLINIC VISITS.

VARIABLE	VALUE
No. of visits during stable disease (%)	1601 (81.1)
No. of visits during exacerbations (%)	374 (18.9)
Total no. of visits/patient	
Mean	24.4
Range	2–65
No. of visits during exacerbations/patient	
Mean	4.6
Range	0–22
Mean no. of visits during exacerbations/patient/yr	2.1
Mean no. of days between visits	33.6

were isolated from sputum (23.6 percent), as compared with 221 of 1226 visits at which no pathogens were isolated from sputum (18.0 percent; $P<0.001$); the relative risk of an exacerbation for patients with pathogens was 1.44 (95 percent confidence interval, 1.24 to 1.68) (Table 3). An analysis of individual bacterial species showed a significant increase in the frequency of exacerbations with isolation of *M. catarrhalis* and *S. pneumoniae*. Isolation of *H. influenzae*, *P. aeruginosa*, and gram-negative bacilli was not associated with an increased frequency of exacerbations. Isolation of *S. aureus* was associated with a decreased frequency of exacerbation ($P=0.007$; relative risk, 0.15; 95 percent confidence interval, 0.04 to 0.60); however, this finding requires confirmation with a larger number of isolates.

Acute Exacerbations and Acquisition of New Strains

Two examples of molecular typing and time lines are shown in Figure 1. Data were analyzed to test the hypothesis that acquisition of a new bacterial strain was associated with an exacerbation. The 148 visits at which sputum was not available were excluded because of the absence of bacteriologic data. The 80 initial clinic visits at which sputum was obtained were excluded because of our inability to determine whether the strain isolated from sputum was new or old. In addition, visits associated with an ongoing exacerbation that had been present at the time of the previous visit (a total of 39 visits) were excluded. *S. aureus* and gram-negative bacilli other than *P. aeruginosa* were not subjected to molecular typing because of the small number of strains available. Therefore, the 47 visits at which these species were the only pathogens isolated were excluded. For six visits during stable disease, acquisition of a new strain was followed by an exacerbation within four weeks with the same strain of bacteria isolated from sputum; these six visits were also excluded from the analysis. Therefore, a total of 1353

TABLE 3. RELATIVE RISK OF AN EXACERBATION ACCORDING TO WHETHER A BACTERIAL PATHOGEN WAS ISOLATED.

PATHOGEN	FREQUENCY OF EXACERBATION		P VALUE	RELATIVE RISK (95% CI)*
	PATHOGEN	NO PATHOGEN		
	no. of exacerbations/ total no. of visits (%)			
Any pathogen	142/601 (23.6)	221/1226 (18.0)	<0.001	1.44 (1.24–1.68)
<i>Haemophilus influenzae</i>	77/375 (20.5)	286/1452 (19.7)	0.18	1.14 (0.94–1.38)
<i>Moraxella catarrhalis</i>	46/133 (34.6)	317/1694 (18.7)	<0.001	1.99 (1.52–2.62)
<i>Streptococcus pneumoniae</i>	13/52 (25.0)	350/1775 (19.7)	0.02	1.40 (1.05–1.87)
<i>Pseudomonas aeruginosa</i>	14/65 (21.5)	349/1762 (19.8)	0.66	1.09 (0.74–1.60)
<i>Staphylococcus aureus</i>	0/19 (0)	363/1808 (20.1)	0.007	0.15 (0.04–0.60)
Other gram-negative rods	6/56 (10.7)	357/1771 (20.2)	0.20	0.76 (0.49–1.16)

*The relative risk of an exacerbation was for the presence of a pathogen in sputum, as compared with its absence. Relative risks were calculated with the use of generalized estimating equations. CI denotes confidence interval.

visits during stable disease and 302 visits during exacerbations were included in the analysis of the relation between strain acquisition and exacerbation.

Isolation of a new strain of a pathogen was associated with a significant increase in the frequency of exacerbation. Eighty-nine of 270 visits at which new strains were isolated (33.0 percent) were associated with exacerbations, as compared with 213 of 1385 visits at which no new strains were isolated (15.4 percent; $P<0.001$; relative risk, 2.15; 95 percent confidence interval, 1.83 to 2.53) (Table 4). The relative risk of an exacerbation in association with the isolation of a new strain of *H. influenzae* was 1.69 (95 percent confidence interval, 1.37 to 2.09; $P<0.001$). This finding contrasts with the absence of an association between the isolation of *H. influenzae* and an exacerbation (Table 3), demonstrating the importance of strain analysis. The relative risk of an exacerbation in association with the isolation of a new strain was 2.96 for *M. catarrhalis* (95 percent confidence interval, 2.39 to 3.67; $P<0.001$) and 1.77 for *S. pneumoniae* (95 percent

confidence interval, 1.14 to 2.75; $P=0.01$). For these two pathogens, strain analysis magnified the association shown by analysis of the rate of isolation and the occurrence of an exacerbation (Table 3). Isolation of new strains of *P. aeruginosa* was not associated with exacerbations (relative risk, 0.61; 95 percent confidence interval, 0.21 to 1.82; $P=0.38$).

DISCUSSION

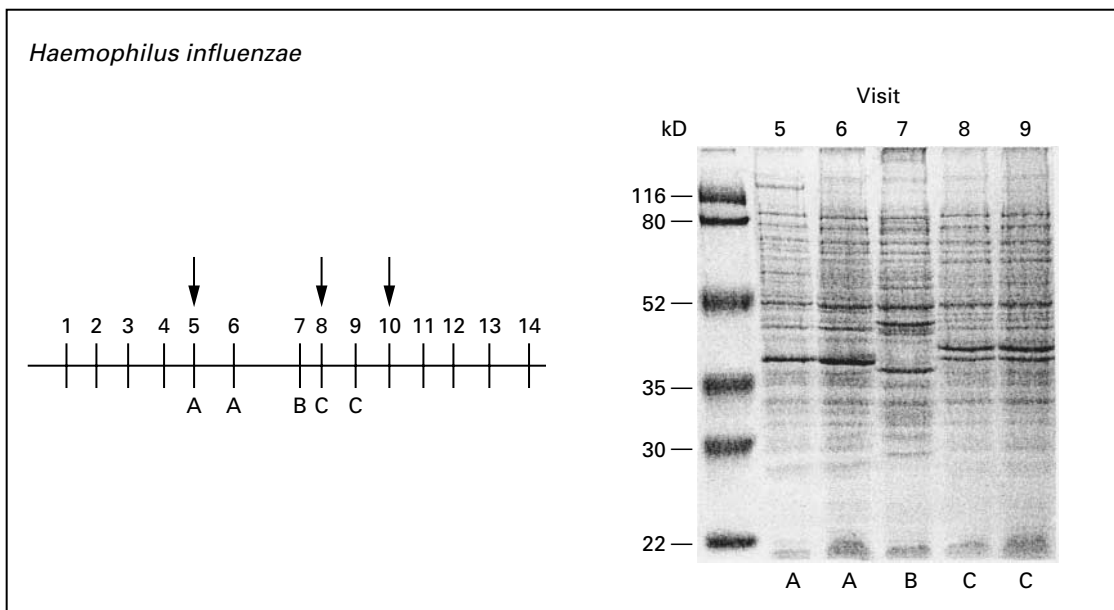
We used molecular typing of bacteria in a prospective study to test the hypothesis that the acquisition of new strains of bacterial pathogens is associated with exacerbations of chronic obstructive pulmonary disease. Such an association was demonstrated for *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* — the three major pathogens implicated in acute exacerbations of chronic obstructive pulmonary disease. The findings for *S. pneumoniae* need to be confirmed with a larger number of isolates. With *P. aeruginosa*, no association was observed; however, the number of isolates was small.

Figure 1 (facing page). Time Lines and Molecular Typing for Two Patients.

The horizontal lines are time lines, with each number indicating a clinic visit. The arrows indicate exacerbations. Isolates of each bacterial species were assigned types on the basis of banding patterns on gel electrophoresis. The first isolate from each patient was assigned the letter A, as were all subsequent isolates with an identical banding pattern. Subsequent isolates with different banding patterns were assigned consecutive letters (B, C, D, and so forth). The lettering system was applicable to the individual patient. An isolate labeled A from one patient, for example, was not the same strain as an isolate labeled A from another patient.

In Panel A, each letter under the time line represents a positive sputum culture for *H. influenzae* in one patient. Molecular typing was performed with sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and staining with Coomassie blue. Whole bacterial-cell lysates of isolates recovered at visits 5 through 9 are shown. Three molecular types were identified. In Panel B, each letter under the time line represents a positive culture for *M. catarrhalis* in another patient. Molecular typing was performed with the use of pulsed-field gel electrophoresis and ethidium bromide staining. *Sma*I-digested DNA from isolates recovered at visits 1, 3, 10, 29, and 33 are shown. Five molecular types were identified.

A



B

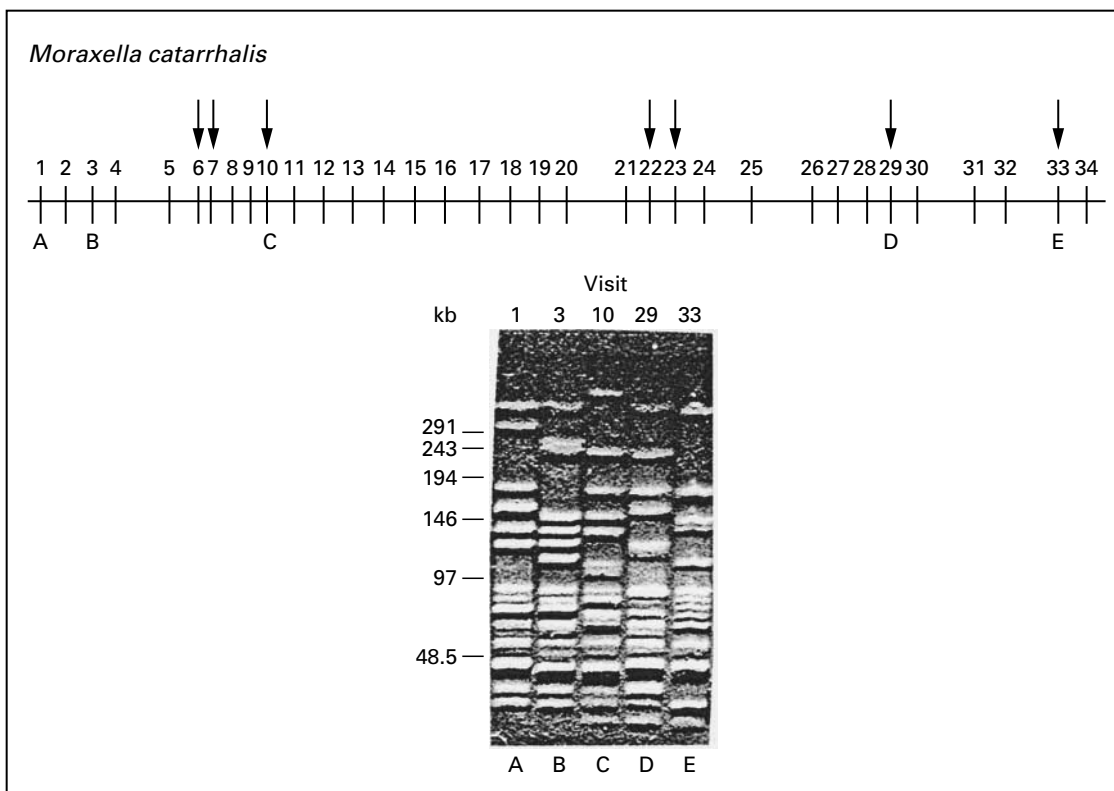


TABLE 4. RELATIVE RISK OF AN EXACERBATION ACCORDING TO WHETHER A NEW STRAIN OF BACTERIAL PATHOGEN WAS ISOLATED.

NEW STRAIN	FREQUENCY OF EXACERBATION		P VALUE	RELATIVE RISK (95% CI)*
	NEW STRAIN	NO NEW STRAIN		
	no. of exacerbations/ total no. of visits (%)			
Any strain	89/270 (33.0)	213/1385 (15.4)	<0.001	2.15 (1.83–2.53)
<i>Haemophilus influenzae</i>	38/145 (26.2)†	257/1503 (17.1)	<0.001	1.69 (1.37–2.09)
<i>Moraxella catarrhalis</i>	41/84 (48.8)	261/1571 (16.6)	<0.001	2.96 (2.39–3.67)
<i>Streptococcus pneumoniae</i>	8/25 (32.0)	294/1630 (18.0)	0.01	1.77 (1.14–2.75)
<i>Pseudomonas aeruginosa</i>	3/22 (13.6)‡	297/1631 (18.2)	0.38	0.61 (0.21–1.82)

*The relative risk of an exacerbation was for the presence of a new strain in sputum, as compared with its absence. Relative risks were calculated with the use of generalized estimating equations. CI denotes confidence interval.

†Seven visits were excluded because of simultaneous isolation of new strains of *M. catarrhalis* (six visits) and *P. aeruginosa* (one visit).

‡Two visits were excluded because of simultaneous isolation of new strains of *M. catarrhalis*.

An association between the acquisition of a new strain and an exacerbation of disease does not prove causation. However, this finding contributes to the growing body of evidence that bacteria cause a substantial proportion of exacerbations. This body of evidence includes data obtained during exacerbations that show an association between increased airway inflammation in sputum and the isolation of bacterial pathogens,^{28,29} the development of a strain-specific immune response to the infecting bacterial strains,¹⁴ and the isolation of bacteria in substantial numbers from specimens obtained from the distal airways by bronchoscopy.^{18,30}

Our observations suggest a mechanism that explains recurrent bacterial exacerbations of chronic obstructive pulmonary disease. We speculate that a strain-specific protective immune response develops after an exacerbation, leaving the patient susceptible to infection by other strains of the same bacterial species.^{8,14,31} Acquisition of a strain to which the patient is susceptible leads to an exacerbation.¹⁵

The limitations of our study include a reliance on analysis of sputum samples, which have low sensitivity and specificity. In a large prospective study, however, the use of more invasive methods for repeated sampling of lower-airway secretions is impractical. The frequency of isolation of *S. pneumoniae* was relatively low in this study. However, several other investigators have reported a low rate of isolation of this pathogen in association with acute exacerbations, particularly in patients with moderate-to-severe chronic obstructive pulmonary disease, which the majority of our patients had.^{32–34}

Some of our patients had new bacterial strains in the

absence of an exacerbation. One possible explanation is that these strains were less virulent than the strains associated with exacerbations and therefore induced a less intense host inflammatory response that did not cause symptoms. Indeed, strains of *H. influenzae* vary in their ability to induce inflammatory responses in tissue culture.³⁵ Another possible explanation is that these new strains did cause symptoms, but they were not severe enough to prompt the patient to seek medical help. Seemungal et al. studied daily symptoms and peak expiratory flows in patients with chronic obstructive pulmonary disease and found that as many as half the exacerbations were not reported by the patients.²

Some exacerbations occur in the absence of bacteria in sputum. In addition, in our study, the strain isolated was a preexisting strain in approximately a quarter of the visits during exacerbations when bacterial pathogens were present in sputum. There are several possible mechanisms for these exacerbations. Respiratory-virus infection is present in a third of exacerbations.³¹ There is serologic evidence of infection with *Mycoplasma pneumoniae* or *Chlamydia pneumoniae* in 5 to 10 percent of exacerbations.³¹ Our study design was limited by the fact that we did not identify infections with viruses and atypical bacteria. Another possible mechanism for exacerbations caused by preexisting strains is a modification of the antigenic structure that allows the strain to evade the host immune response and multiply in the airways, causing increased inflammation and therefore symptoms.³⁶ Such a modification, which occurs with *H. influenzae* in animal models and in patients with chronic obstructive pulmonary disease, requires further investigation.³⁶

In summary, the isolation of new strains of *H. influ-*

enzae, *M. catarrhalis*, and *S. pneumoniae* in patients with chronic obstructive pulmonary disease was associated with acute exacerbations. This finding provides evidence that many acute exacerbations of chronic obstructive pulmonary disease are due to bacterial infection.

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