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Bacterial Infection in Chronic Obstructive Pulmonary Disease in 2000: a State-of-the-Art Review

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INTRODUCTION

It is estimated that in 1995, 16.4 million people in the United States suffered from chronic obstructive pulmonary disease (COPD) (226). COPD is also the fourth most common cause of death in the United States (14). Both the prevalence of and mortality from this disease have been increasing worldwide (118, 226, 330). COPD is defined physiologically by the presence of irreversible or partially reversible airway obstruction in patients with chronic bronchitis and/or emphysema (14). Chronic bronchitis is defined clinically by the presence of cough with sputum production for most days of at least 3 months a year for 2 consecutive years (200). Other causes of chronic cough need to be excluded. Emphysema is defined pathologically as permanent dilation of the airspaces distal to the terminal bronchioles, accompanied by destruction of the alveolar septa in the absence of fibrosis (292). More than 80% of COPD cases encountered in the Western world are related to tobacco smoke exposure. Occupational exposures and alpha-1 antitrypsin deficiency are uncommon precedents for the development of COPD (177, 234).

Several potential contributions of bacterial infection to the etiology, pathogenesis, and clinical course of COPD can be identified (219). However, the precise role of bacterial infection in COPD has been a source of controversy for several decades (175, 296, 307). Opinion regarding the contribution of bacteria to the pathogenesis of COPD has ranged from the idea that it has a preeminent role (along with mucus hypersecretion) as embodied in the British hypothesis in the 1950s and 1960s, to the idea that it is a mere epiphenomenon in the 1970s and 1980s (200, 296, 307). In the last decade, new research techniques have become available, and traditionally noninfectious diseases such as peptic ulcer have been shown to be of infectious origin (240). This has renewed interest in the area of bacteria and COPD, and these new research methodologies should lead to a precise delineation of the contribution of bacterial infection to this disease.

Five potential pathways by which bacteria could contribute to the course and pathogenesis of COPD can be identified. (i) Childhood lower respiratory tract infection impairs lung growth, reflected in smaller lung volumes in adulthood. (ii) Bacteria cause a substantial proportion of acute exacerbations of chronic bronchitis which cause considerable morbidity and mortality. (iii) Chronic colonization of the lower respiratory tract by bacterial pathogens amplifies the chronic inflammatory response present in COPD and leads to progressive airway obstruction (vicious circle hypothesis). (iv) Bacterial pathogens invade and persist in respiratory tissues, alter the host response to cigarette smoke, or induce a chronic inflammatory response and thus contribute to the pathogenesis of COPD. (v) Bacterial antigens in the lower airway induce hypersensitivity that enhances airway hyperreactivity and induces eosinophilic inflammation. Evidence supporting these roles will be discussed in this review, with an emphasis on information gained from newer research techniques in the last decade. The second part of this review will discuss each of the major pathogens, with emphasis on recent developments related specifically to infections in COPD.

POTENTIAL ROLES OF BACTERIAL INFECTION IN COPD

Childhood Lower Respiratory Tract Infection and Adult Lung Function

Four recent studies have reported lung function (measured by spirometry) in cohorts of adult patients for whom reliable information was available regarding the incidence of lower respiratory tract infection (bronchitis, pneumonia, or whooping cough) in childhood (<14 years of age) (Table 1) (26, 152, 279, 280). These studies have consistently shown a lower forced expiratory volume in 1 s (FEV_1) and often a lower forced vital capacity among adults who experienced childhood lower respiratory tract infection compared to others in the cohort who did not experience such infection (26, 152, 279, 280). FEV_1 and forced vital capacity are widely used tests of pulmonary function. This association is seen after controlling for confounding factors such as tobacco exposure. The magnitude of this defect in FEV_1 has varied among the studies but tends to be greater in older cohorts. The extent of decrease in FEV_1 is unlikely to cause symptomatic pulmonary disease per se but could make the individual susceptible to the effects of additional injurious agents, such as tobacco smoke, and environmental or occupational exposure to air-borne pollutants. The defect in lung function is not airway obstruction, as the FEV_1/FVC ratio is

TABLE 1. Association of childhood lower respiratory tract infection with lung function in adults

Study	No. in study	Lower respiratory tract infection history	Age at followup (yr)	Effect on FEV_1
Barker et al. (26)	639 (all male)	Bronchitis or pneumonia in first yr	59–67	Lowered by 200 ml
Shaheen et al. (279)	618	Pneumonia or bronchitis in first 2 yr	67–74	Lowered by 650 ml in males with pneumonia
Johnston et al. (152)	1,392	Pneumonia or whooping cough in first 7 yr	34–35	Lowered by 102 ml with pneumonia
Shaheen et al. (280)	239	Pneumonia in first 14 yr	57.6 ± 4.3	Lowered by 390 ml
		Bronchitis in first 14 yr		Lowered by 130 ml

preserved. Instead, it is consistent with "smaller lungs", suggesting impaired lung growth.

Although the association between childhood lower respiratory tract infection and impaired lung function in adulthood is now well established, there is ongoing debate whether this association reflects a cause-effect relationship. Such a relationship could be explained by damage caused to a vulnerable lung undergoing rapid postnatal growth and maturation by the infectious process. If this were the case, then the effect of the infection on lung function should be seen only in the first 2 years of life, the major period of postnatal lung growth, but not in later childhood (3 to 14 years). However, this has not been a consistent observation in the studies to date (26, 152, 279, 280). An alternative explanation for the observed association between childhood lower respiratory tract infection and impaired lung function in adulthood is that an undetermined genetic factor predisposes these individuals to lower respiratory tract infections in childhood as well as a lower FEV_1 in adulthood. This explanation implies that impaired lung growth antedates the respiratory tract infection, with the infectious episode a result of the vulnerability of smaller lungs to infection in childhood.

The etiology of childhood lower respiratory tract infection was not established in these studies, and therefore whether the impact of viral infection differs from that of bacterial infection is not known. Though it is likely that a substantial proportion of these childhood infections were viral, bacterial infection, especially with *Streptococcus pneumoniae* and *Haemophilus influenzae*, is a common cause of severe pneumonia in children (333). The impact of childhood bacterial lower respiratory tract infection on the prevalence of COPD is likely to be greater in developing countries, where these infections are common and are often inadequately treated.

Bacterial Pathogens as a Cause of Acute Exacerbations of COPD

Bacteria are isolated from sputum in 40 to 60% of acute exacerbations of COPD (274). Table 2 shows the sputum bacteriology obtained in 14 clinical trials of antibiotics in acute exacerbation published in the last 4 years (12, 19, 53–55, 71, 74, 127, 170–172, 251, 278, 348). Variation in the relative incidence of specific pathogens is seen and may relate to patient inclusion criteria and sputum culture techniques. The three predominant bacterial species isolated are nontypeable *Haemophilus influenzae, Moraxella catarrhalis*, and *Streptococcus pneumoniae*. Other infrequently isolated potential pathogens are *Haemophilus parainfluenzae, Staphylococcus aureus, Pseudomonas aeruginosa*, and members of the family *Enterobacteriaceae*.

Whether isolation from sputum of a potential pathogen represents infection of the lower airway causing the exacerbation episode has been a controversial issue for several decades. In the 1950s and 1960s, the British hypothesis of the pathogenesis

TABLE 2. Bacterial pathogens isolated from sputum in recent studies of acute exacerbation of chronic bronchitis

Study	No. of patients	No. of culture positive	No. of bacterial isolates	% of bacterial isolates[a]						
				Haemophilus influenzae	*Moraxella catarrhalis*	*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Haemophilus parainfluenzae*	*Enterobacteriaceae*
Allegra et al. (12)	728	298	375	28	11	26	5	11	—[b]	15
Anzueto et al. (19)	2,180	673	777	13	18	7	17[c]	4	15	18
Chodosh et al. (55)	376	234	274	36	20	14	1	5	4	7
Chodosh et al. (54)	307	208	253	25	21	10	4	3	8	15
Chodosh et al. (53)	624	290	379	18	21	7	20[c]	DN[d]	6	DN
Davies et al. (71)	140	124	146	50	17	21	1	8	—	3
DeAbate et al. (74)	798	647	835	18	9	8	5	4	32	8
Habib et al. (127)	373	192	181	25	14	8	7	13	12	19
Langan et al. (170)	684	192	211	34	4	12	9	5	11	5
Langan et al. (172)	802	400	513	36	12	11	3	DN	27	DN
Langan et al. (171)	656	478	542	41	19	23	1	3	6	DN
Read et al. (251)	364	103	128	46	9	9	8	5	3	15
Shah et al. (278)	832	547	577	36	16	18	3	8	2	5
Wilson et al. (348)	750	287	342	31	15	25	5	1	5	5

[a] The values given for each bacterial pathogen represent the percentage of total isolates in each study.
[b] —, not reported.
[c] Increased incidence probably related to sputum processing in centralized laboratories.
[d] DN, details not available in the article to calculate percentage of isolates.

of COPD included as major contributors recurrent bacterial infection and mucus hypersecretion (200). With the realization of tobacco smoke exposure as the primary pathogenic mechanism of COPD and the emergence of evidence that mucus hypersecretion and worsening airway obstruction were independent, the British hypothesis fell into disfavor (28, 102, 145). Several longitudinal cohort studies in the 1960s and 1970s demonstrated that the incidence of bacterial isolation from sputum during exacerbations of COPD was not different from the incidence during stable COPD (123, 196). These studies also failed to demonstrate a higher bacterial titer in sputum during acute exacerbation than during stable COPD (123). Serological studies conducted in the same time period that compared serum antibody titers to airway bacterial pathogens such as nontypeable *H. influenzae* in COPD patients with those in controls arrived at confusing and contradictory conclusions (reviewed in reference 219). Results of placebo-controlled antibiotic trials in acute exacerbations have also been inconsistent, demonstrating either small or no benefit with antibiotic therapy (17, 269).

A common interpretation of these observations has been that isolation of bacteria from sputum during exacerbations represents chronic colonization, an innocent bystander role for the bacteria (95, 228, 296, 307). Alternative explanations for the contradictory observations from serological studies and antibiotic therapy should be considered (148, 219). Serological studies of antibodies to bacterial pathogens in exacerbations yielded confusing and contradictory results for the following reasons. (i) Laboratory strains were often used as the antigen instead of homologous patient strains. Thus, the strain variation in the surface antigens of the bacterial pathogens that has only recently been understood was not taken into account in these studies. (ii) The immunological methods employed in these studies often were not specific for antibodies to surface-exposed epitopes on the bacteria. A bacterial pathogen presents several hundred antigenic epitopes to a host, many of which are non-surface exposed and therefore potentially irrelevant to host defense. Furthermore, several of these epitopes are cross-reactive among bacterial species. A protective immune response that develops after an infection may be limited to a few epitopes on the bacterial surface. Detecting this response among the multitude of irrelevant, non-surface-exposed antigen-antibody interactions requires immunological assays that are specific for antibodies that bind to surface-exposed epitopes on the bacteria. Such assays include radioimmunoprecipitation assays, flow cytometry assays with whole bacterial cells as an antigen, and assays that measure functional (bactericidal or opsonophagocytic) antibodies. Immunoblots and whole bacterial immunodots that were used in previous serological studies do not have such specificity (115). (iii) Not all studies used true preinfection sera for comparison with the convalescent-phase serum. Instead, acute-phase serum taken at the time that the patient presents with symptoms was used. The symptoms of an acute exacerbation have often been present for several days, and therefore a serological response to the strain may be missed if an acute-phase serum is substituted for a preinfection serum.

Trials showing no benefit with antibiotics in acute exacerbations also have several potential explanations. (i) An exacerbation is a mucosal infection, and the use of antibiotics in other mucosal infections such as otitis media and sinusitis is also not associated with dramatic efficacy over placebo (345). This does not imply that mucosal infections are nonbacterial. (ii) The expected benefits from antibiotics in a mucosal infection are primarily a more rapid resolution of symptoms and prevention of complications. Unfortunately, most studies of antibiotics in acute exacerbations have not measured the speed of resolution of symptoms. Instead, the endpoint has been whether the treatment was successful at 3 weeks after the onset of the exacerbation. The systemic immune-inflammatory response would be expected to resolve a large proportion of bacterial exacerbations in this time period, disguising any potential effect of antibiotics (17). (iii) Many studies include patients with mild impairment of lung function who are likely to have a low rate of complications, making a difference from the placebo group prone to type 2 error. In other words, the study populations could have contained too few individuals with potential for benefit for a benefit to be observed. (iv) Exacerbations are nonbacterial in 50% of patients, with no expected benefit from antibiotics, again predisposing studies to a type 2 error. (v) Antibiotic resistance in some of these pathogens that may be compounded by lack of penetration into the bronchial tissues and fluids of some of the antibiotics is likely to diminish the effect of antibiotics in exacerbations.

In the last decade, several investigators have reexamined the issue of whether bacteria cause acute exacerbations of COPD using either new diagnostic modalities or new research techniques, including bronchoscopic sampling of the lower respiratory tract (96, 208, 239, 294), immune response to bacterial pathogens in exacerbations (49, 222, 355), molecular epidemiology of bacterial pathogens (116, 220, 270, 271, 288, 289, 290), and airway inflammation measurement and correlation with bacteriology (161, 293). These methods provide new data which contribute to a more rigorous evaluation of the etiology of exacerbations.

Bronchoscopic sampling of lower respiratory tract in exacerbations of COPD. An attractive approach to understanding the role of bacterial infection in exacerbated COPD is sampling of distal airway secretions for quantitative culture by protected specimen brush or by bronchoalveolar lavage (BAL) to determine bacterial concentrations in the distal airways. Such an approach has contributed tremendously to our understanding of nosocomial pneumonia (50). Samples obtained from the distal airways with these techniques have low levels of contamination by upper respiratory tract secretions. Bacterial concentrations above certain thresholds on quantitative culture have been found to correlate with tissue infection in patients with pneumonia (50). Four studies that have used this method in acute exacerbations have been published, and all have consistently shown significant bacterial infection of the distal airways in approximately 50% of patients experiencing an exacerbation (Table 3) (96, 208, 239, 294). The bacterial species isolated in these studies represent the same spectrum of pathogens commonly isolated from sputum cultures of patients with acute exacerbation.

The study done by Monso et al. is especially informative, as it included a control group of 29 patients with stable COPD (208). They demonstrated that exacerbation was associated twice as often with distal airway infection at $\geq 10^3$ CFU of pathogenic bacteria per ml and four times as often with $\geq 10^4$

TABLE 3. Bronchoscopic studies in acute exacerbations of COPD

Study	Subjects	Diagnostic methods	% of subjects with bacterial pathogen present	No. of isolates						
				H. influenzae	*M. catarrhalis*	*S. pneumoniae*	*H. parainfluenzae*	*P. aeruginosa*	Other	
									Gram negative	Gram positive
Fagon et al. (96)	50 ICU patients on ventilator	Protected specimen brush	50[a]	6	3	7	11	3	5	9
Monso et al. (208)	29 outpatients	Protected specimen brush	51.7	10	2	3		2		
Soler et al. (294)	50 ICU patients on ventilator[b]	Protected specimen brush, BAL, endotracheal aspirate	52	11	4	4		9	6	
Pela et al. (239)	40 outpatients	Protected specimen brush	52.5	1	2	10	1		1	7

[a] A positive culture was defined as $\geq 10^2$ CFU/ml instead of the usual $\geq 10^3$ CFU/ml.
[b] Twenty-one patients had antimicrobial therapy in the 24 h prior to admission to the intensive care unit (ICU).

CFU/ml ($P < 0.05$ for both comparisons) (Fig. 1). Soler et al. examined a more severely ill population of 50 patients who were placed on mechanical ventilation for an acute exacerbation and obtained lower airway secretions for culture by bronchoscopy with a protected specimen brush and BAL and tracheobronchial aspirates (294). Although 21 of their 50 patients had received antibiotics before the samples were obtained, bacterial infection was demonstrated in 21 of 50 (42%) patients and infection with a virus or atypical pathogen was found in 5 (10%) patients. The distribution of specific bacterial pathogens isolated in their study is remarkable for a large proportion of *Pseudomonas aeruginosa* and other gram-negative bacilli (14 of 50, 28%). Recently, two studies using sputum cultures have also demonstrated an increasing frequency of isolation of these groups of pathogens in exacerbations of severe COPD (90, 201). Whether this is due to environmental factors (such as antibiotic selection pressure or exposure to hospital flora from frequent exacerbations) or is related to a greater degree of host immune compromise is not clear.

The consistent results of these four studies and the greater rate of isolation of pathogenic bacteria in exacerbated than in stable COPD in the Monso study supports the pathogenic role of bacteria in a proportion of acute exacerbations of this chronic disease.

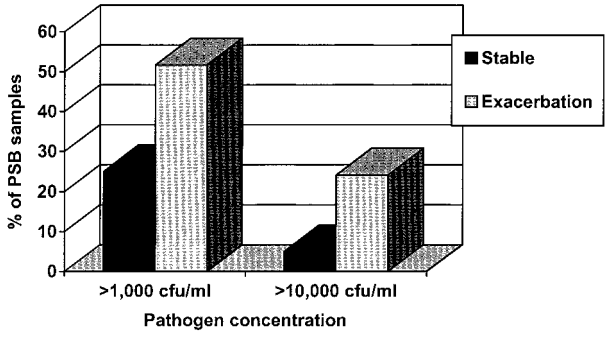

FIG. 1. Culture results of bronchoscopic samples obtained from patients with stable COPD and those with acute exacerbation. The number of positive samples at both pathogen concentrations was significantly greater ($P < 0.05$) in the exacerbation group. Data taken from the work of Monso et al. (208). PSB, protected specimen brush; CFU/ml, CFU of pathogenic bacteria per milliliter of epithelial lining fluid.

Immune responses to bacterial pathogens. Older studies of immune response to nontypeable *H. influenzae* in COPD had several limitations, as discussed above (219). Recently, we and other investigators have explored the immune response to bacterial pathogens in acute exacerbations of COPD with methods that avoid the pitfalls of earlier studies. These studies are discussed in detail later. These studies have demonstrated the development of specific immune response to infecting strains of nontypeable *H. influenzae* and *M. catarrhalis* and support the role of bacterial infection in acute exacerbations of COPD. Similar evidence with other bacterial species (see Table 2) would help us better define their role in acute exacerbations.

Molecular epidemiology of bacterial pathogens. Strains of a bacterial species can differ considerably in their surface antigenic structure. The "bacterial load" model of bacterial infection in COPD assumes that an increase in the titer of a bacterial species in the airway is responsible for the transition from stable COPD to exacerbation of COPD (346). Studies of bacterial titers in the sputum of patients with COPD have not supported this model (123). This model does not take into account the genetic diversity within the bacterial species, including alterations in surface antigenic structure. An alternative model is that infection with a bacterial strain with an antigenic structure new to the host leads to an immune and inflammatory response that presents clinically as an acute exacerbation. Longitudinal studies of patients with COPD in combination with molecular typing of the strains will allow investigators to test this model.

Airway inflammation and correlation with bacteriology. Bacterial infection of the lower airways during an acute exacerbation should be associated with neutrophilic inflammation as is seen in other mucosal sites such as the middle ear and sinuses. One would therefore expect airway inflammation in bacterial exacerbations to be associated with significantly greater neutrophilic inflammation than a nonbacterial exacerbation. Therefore, sputum culture results should correlate with measures of airway inflammation in acute exacerbations. Data from our laboratory support this hypothesis, with pathogen-positive acute exacerbations having substantially increased measures of airway inflammation in expectorated sputum compared to pathogen-negative exacerbations (276). Furthermore, Stickley et al. showed that increased purulence is associated with recovery of a bacterial pathogen at the time of exacerba-

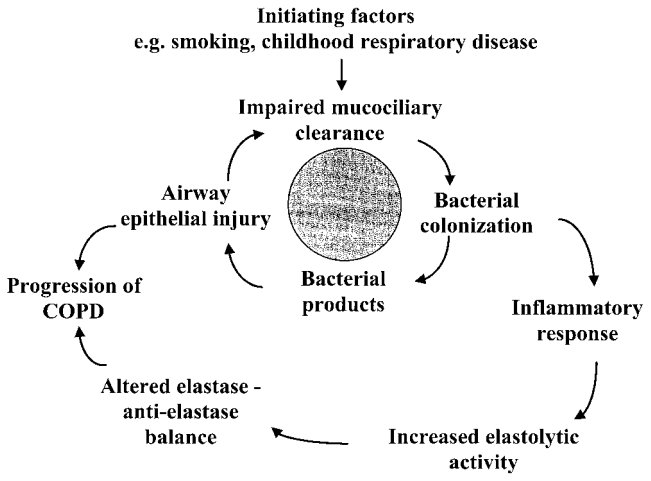

FIG. 2. Diagrammatic representation of the vicious circle hypothesis.

tion, suggesting that purulence is a marker for bacterial exacerbation (305a).

Overall, the weight of evidence indicates that bacterial pathogens cause approximately 40 to 50% of acute exacerbations of COPD. Further studies with sophisticated immunological assays, molecular epidemiology, and measurement of airway inflammation should refine our understanding of the pathogenesis of bacterial exacerbation and the mechanisms of protection and recurrence.

Vicious Circle Hypothesis

Tobacco smoking cannot be the sole factor responsible for the pathogenesis of COPD, as only a small proportion (15%) of smokers develop chronic bronchitis and an even smaller proportion go on to develop obstructive airway disease (COPD). In the absence of underlying lung disease, the tracheobronchial tree is sterile. In patients with COPD, the tracheobronchial tree is chronically colonized with potential respiratory pathogens, predominantly nontypeable *H. influenzae, S. pneumoniae*, and *M. catarrhalis* (124, 173, 208, 358). Several years ago, we proposed a vicious circle hypothesis to explain how chronic bacterial colonization of the lower airways in patients with COPD can perpetuate inflammation and contribute to progression of the disease (Fig. 2) (63, 219). A similar mechanism is believed to contribute to the pathogenesis of lung disease in individuals with cystic fibrosis. Substantial supporting evidence for this hypothesis in COPD, both in vitro and in vivo, has now accumulated and is discussed below.

Central to the vicious circle hypothesis is the notion that once bacterial pathogens have gained a foothold in the lower respiratory tract from impaired mucociliary clearance due to tobacco smoking, the bacteria persist by further impairing mucociliary clearance (Fig. 2). This impairment of mucociliary clearance can be due to enhanced mucus secretion, disruption of normal ciliary activity, and airway epithelial injury. Experimental evidence demonstrates that respiratory tract pathogens and their products can cause all of these effects in vitro.

Bacterial infection and chronic mucus hypersecretion. Adler et al. examined the effect of cell-free filtrates of broth cultures of nontypeable *H. influenzae, S. pneumoniae*, and *P. aeruginosa* on the secretion of mucous glycoproteins by explanted guinea pig airway tissue (1). Seven of 28 (25%) strains of nontypeable *H. influenzae*, 10 to 26 (34%) strains of *S. pneumoniae*, and 12 of 18 (66%) strains of *P. aeruginosa* stimulated mucin secretion. This stimulation was a true secretory effect and not passive release of preformed intracellular macromolecules due to cellular damage, as ultrastructural assessment (by light, transmission, and scanning electron microscopy) demonstrated an absence of cytotoxicity. The *Pseudomonas* stimulatory products were proteases of 60 to 100 kDa. The *Haemophilus* and pneumococcal stimulatory exoproducts were 50 to 300 kDa in size and did not possess proteolytic activity.

Bacterial infection and mucociliary clearance. The tracheobronchial ciliary escalator is of paramount importance in maintaining sterility of the lower respiratory tract by transporting bacteria trapped in mucus towards the pharynx (347). Disruption of this ciliary activity is therefore likely to be important in the establishment of chronic colonization in the tracheobronchial tree. Wilson et al. measured by photometry the effect of cell-free supernatants of nontypeable *H. influenzae, P. aeruginosa*, and *S. aureus* on ciliary beat frequency of strips of human nasal ciliary epithelium (349). Rapid inhibition of ciliary beat frequency was seen with nontypeable *H. influenzae* and *P. aeruginosa* but not with *S. aureus*. On direct examination, ciliary dyskinesia and ciliostasis were seen. Human neutrophil elastase inhibits ciliary activity and damages respiratory epithelium (15). Bacterial products in the airways may be a potent stimulus for neutrophil migration into the airways, and elastase released from these neutrophils can act synergistically with bacterial products and cause further inhibition of tracheobronchial ciliary function.

Bacterial infection and airway epithelial injury. An important component of the vicious circle hypothesis is the potentially damaging effects of bacteria and bacterial products on airway epithelial lining cells. Such epithelial injury in the large airways would contribute to bacterial persistence and in the small airways could contribute to the respiratory bronchiolitis that causes progressive airway obstruction (67, 107). In an in vitro tissue culture model of nasal turbinate epithelium, Read et al. have demonstrated that nontypeable *H. influenzae* causes airway epithelial injury (252). They studied these epithelia after 30 min, 14 h, and 24 h of incubation with a nontypeable *H. influenzae* strain. At 30 min, the airway epithelium and cilia were intact and the bacteria were associated with the overlying mucus layer. At 14 h, patchy injury developed to the airway epithelium, with bacterial cells now associating with these damaged epithelial cells but not with intact epithelium. At 24 h, detached epithelial cells with adherent bacteria were seen.

The studies discussed above demonstrate that bacteria that colonize and infect the lower respiratory tract in COPD are capable of fostering an environment in the tracheobronchial tree in which they can persist, supporting the central tenet of the vicious circle hypothesis (Fig. 2). Recently, more attention is being directed towards another portion of the vicious circle, the effects of the chronic inflammatory response occasioned by bacterial products on the elastase-antielastase balance in the lung. If bacterial products in the tracheobronchial tree cause neutrophil influx and degranulation in the airways and lung parenchyma, they could contribute to the chronic inflamma-

tion, parenchymal lung damage, and progressive small airway obstruction seen in COPD (15, 140, 235).

Bacterial infection and airway inflammation. The presence of bacteria in the lower airways in patients with stable COPD has been labeled colonization. However, this bacterial presence is definitely abnormal and is not confined to the large airways. Bacteria have been shown to extend to the peripheral airways by cultures of bronchoscopic protected specimen brushings and BAL (208, 358). Even during colonization, bacteria in these airways are in a constant state of turnover, releasing extracellular products, undergoing lysis with release of a variety of proteins, lipooligosaccharide (LOS), and peptidoglycan (121). LOS is a potent inflammatory stimulus; in fact, repeated instillation of LOS can lead to the development of emphysema in hamsters (306). It is therefore quite likely that this colonization is actually a low-grade smoldering infection that induces chronic airway inflammation. In the large airways such inflammation would contribute to mucus production, and in the small airways it could contribute to respiratory bronchiolitis and progressive airway obstruction (79, 310). Recent data that support this hypothesis include in vitro experiments with LOS of nontypeable *H. influenzae* and a bronchoscopic study that demonstrates that bacterial colonization may be an independent stimulus to airway inflammation in patients with stable COPD, as described below. Furthermore, Hill et al. recently demonstrated an association between bacterial numbers and markers of airway inflammation in stable chronic bronchitis (141a).

Khair et al. incubated explant cultures of human bronchial epithelium with LOS from nontypeable *H. influenzae* at 10 and 100 μg/ml (161). Epithelial cell permeability, intracellular adhesion molecule-1 (ICAM-1) expression, and release of interleukin-6 (IL-6), IL-8, and tumor necrosis factor alpha (TNF-α) into the culture medium were measured. IL-6 and TNF-α secretion and ICAM-1 expression by the bronchial epithelial cells were significantly increased only by the higher concentration of LOS (100 μg/ml), while IL-8 expression was stimulated by LOS at both 10 and 100 μg/ml. The levels of inflammatory mediators attained in the culture medium were adequate to increase neutrophil chemotaxis and adherence in vitro. There was no increase in epithelial cell permeability.

In a recent study, Soler et al. compared the levels of several cytokines, including IL-1β, IL-6, IL-8, IL-10, and TNF-α, in BAL fluid obtained from 52 patients with stable COPD, 18 smokers, and 8 nonsmoking healthy controls (293). Among the smokers said to be without COPD, nine (50%) actually had chronic bronchitis, which confounds some of their findings, as discussed below. Bacterial colonization of the distal airways was determined by quantitative cultures of BAL fluid ($\geq 10^3$ CFU/ml was significant) and of protected specimen brush ($\geq 10^2$ CFU/ml was significant) samples from the distal airways. Pathogens isolated were classified into potential pathogenic microbes (PPM) and non-PPM. The PPM included nontypeable *H. influenzae, S. pneumoniae, M. catarrhalis, S. aureus, P. aeruginosa*, and gram-negative enteric bacteria. None of the healthy controls had PPM isolated in significant concentrations, compared to 42% of the smokers and 32% of the patients with COPD. Isolation of a significant number of PPM in the BAL was associated with significantly more polymorphonuclear cells and TNF-α compared to BAL which did not have PPM. A trend to higher IL-8 levels in the BAL was also seen. Isolation of non-PPM in significant amounts was not associated with an increase in BAL cytokines or airway neutrophilia. This study demonstrates that bacterial colonization of the lower respiratory tract with PPM occurs not only in patients with established COPD, but also in smokers who may have chronic bronchitis but do not have significant airway obstruction. Therefore, bacterial colonization of the lower airways appears to be an early phenomenon in the course of the disease. Furthermore, bacterial colonization is an independent stimulus for inflammation in the distal airways and therefore may contribute to the progression of COPD. This is analogous to young patients with cystic fibrosis in remission, who are chronically colonized in the distal airways mostly with *P. aeruginosa*, but also with nontypeable *H. influenzae* and *S. aureus*. This colonization is also associated with an active inflammatory process in the distal airways (164).

It is becoming increasingly apparent that the presence of bacteria in the lower airways in patients with COPD, even when they are clinically stable, is not innocuous. Nontypeable *H. influenzae* has the ability in vitro to disrupt ciliary motility, induce mucus hypersecretion, and damage the airway epithelium. These effects parallel the effects of tobacco smoke and contribute to the persistence of nontypeable *H. influenzae* in the lower airways of smokers. Nontypeable *H. influenzae* persistence in the lower airways appears to stimulate airway epithelium to produce proinflammatory cytokines, especially those that promote neutrophil chemotaxis, and therefore leads to additional airway inflammation. Whether this additional airway inflammation contributes to symptoms and progression of airway obstruction in COPD is unknown but should be a fertile area of investigation.

Chronic Bacterial Infection of Respiratory Tissues

Nontypeable *H. influenzae* has always been regarded as an extracellular pathogen that infects the airway lumen in COPD. Recently, invasion of the upper and lower respiratory tract tissues by this pathogen has been demonstrated. Whether COPD is associated with chronic *Chlamydia pneumoniae* infection of the respiratory tract has also recently been investigated. These studies used newer detection techniques with greater sensitivity than bacterial culture for determining the presence of bacterial organisms in tissue and made interesting and somewhat surprising observations.

Intracellular and intercellular invasion of *H. influenzae*. Nontypeable *H. influenzae* is present in the lumen of the respiratory tract, binds with specificity to mucin, and adheres to the surface of respiratory epithelial cells (see below). More recently, research from several groups has shown that the organism's niche in the human respiratory tract is not limited to adherence to the surface of epithelial cells. Several lines of investigation involving in vitro and in vivo studies have established that nontypeable *H. influenzae* invades beyond the surface of the respiratory epithelium.

Studies utilizing cultures of human epithelial cells have revealed that a small percentage of adherent nontypeable *H. influenzae* enter epithelial cells in a process that involves actin filaments and microtubules (303). Organ culture studies utilizing lung epithelial cells on permeable supports revealed clus-

ters of *H. influenzae* bacterial cells between cells, indicating that bacteria penetrated by paracytosis or passage between cells (326). Bacteria passed through confluent layers of epithelial cells without affecting the permeability or viability of the cell layer. Nontypeable *H. influenzae* which penetrate the epithelial cell layer in this model system are protected from the bactericidal activity of several antibiotics and antibody-mediated bactericidal activity (325). In assays employing primary human airway cultures. Ketterer et al. (160) showed that nontypeable *H. influenzae* adhered to and entered exclusively nonciliated cells in the population. The surface of infected cells showed evidence of cytoskeletal rearrangements, manifested by microvilli and lamellipodia extending toward bacteria, indicating that bacteria were entering epithelial cells by the process of macropinocytosis (160).

In addition to the these elegant in vitro studies, investigators from two centers have performed in vivo studies which confirm that nontypeable *H. influenzae* penetrate the mucosal surface during colonization of the human respiratory tract. In situ hybridization and selective cultures revealed that viable nontypeable *H. influenzae* are present in macrophagelike cells in the adenoids of children (104, 105). In a second approach to investigating whether nontypeable *H. influenzae* is present in intracellular or intercellular locations in the human respiratory tract, Moller et al. (207) obtained lung explants from patients undergoing lung transplant. *H. influenzae* was diffusely present in the epithelium, the submucosa of the bronchi, the bronchioles, the interstitium, and the alveolar epithelium, as determined by in situ hybridization and PCR.

In summary, these observations indicate that when nontypeable *H. influenzae* colonizes the human respiratory tract, the bacterium is present in several locations, including in the lumen of the respiratory tract, adhering to mucosal epithelial cells, in the interstitium of the submucosa, and within cells of the respiratory tract. Bacteria in tissues are protected from antibiotics and bactericidal antibodies and may act as reservoirs of infection (325). Tissue infection by nontypeable *H. influenzae* could also contribute to the pathogenesis of COPD directly or indirectly. Chronic low-grade infection could directly induce a chronic inflammatory response in the parenchyma and the airways of the lung that could be additive or synergistic to the inflammatory effects of tobacco smoke. Indirectly, such an infection could enhance the damaging effects of tobacco smoke on respiratory tissues. On the other hand, it is possible that this tissue infection is simply a marker of compromised local immunity. Whether tissue infection by nontypeable *H. influenzae* is seen in early COPD and the effect of this infection in tissue models need to be investigated.

Chronic *Chlamydia pneumoniae* infection in COPD. *C. pneumoniae* is an obligate intracellular atypical bacterial pathogen. Acute *C. pneumoniae* infection can cause bronchitis, pneumonia, and acute exacerbations of COPD (see below). Chronic infection with *C. pneumoniae* is being actively investigated as a cause of several systemic diseases, especially coronary artery disease (178). Von Hertzen et al. studied whether the incidence of chronic *C. pneumoniae* infection is increased in COPD (332). The presence of chronic *C. pneumoniae* infection was determined by three different methods: serum antibodies to *C. pneumoniae* (immunoglobulin G [IgG] and IgA and circulating immune complexes), sputum IgA antibodies to *C. pneumoniae*, and PCR of sputum for *C. pneumoniae* DNA. Two of the three methods had to yield positive results for the same patient to demonstrate a chronic *C. pneumoniae* infection. The incidence of chronic *C. pneumoniae* infection (as defined above) was 71% in patients with severe COPD, 46% in mild to moderate COPD, and 0% in the control group. Whether this chronic infection contributes to the pathogenesis of COPD as discussed above or is a reflection of compromised local immunity warrants further investigation.

Hypersensitivity to Bacterial Antigens

Allergic bronchopulmonary aspergillosis is an infectious disease with predominantly allergic manifestations mediated by a Th2-type immune response and characterized by IgE and eosinophil predominance (158). Inefficient removal of bacteria from the lower respiratory tract is characteristic of chronic bronchitis, resulting in prolonged contact between the airway lymphoid tissue and bacterial antigens. This could lead to the emergence of IgE antibodies to bacterial antigens, which could induce eosinophil infiltration and mast cell degranulation on repeated exposures to the bacterial antigens. An increased number of eosinophils is characteristic of airway inflammation in most patients with COPD, and tissue and airway lumen eosinophilia becomes more prominent during exacerbations (268). Furthermore, a small subgroup of patients with COPD have an eosinophilic bronchitis that is responsive to steroids (132).

The ability of bacterial pathogens to induce histamine release, hypersensitivity, and IgE-mediated inflammation has been investigated sporadically. Mast cells release histamine by non-IgE-mediated and IgE-mediated mechanisms. Clementsen et al. exposed mast cells obtained by BAL from the airways of patients with chronic bronchitis and normal individuals by BAL to Formalin-killed suspensions of nontypeable *H. influenzae, S. pneumoniae, M. catarrhalis*, and *S. aureus*. Nontypeable *H. influenzae* and *S. aureus* induced non-IgE-mediated and enhanced IgE-mediated histamine release (61). The enhancement of IgE-mediated histamine release appears to be mediated by the endotoxin of nontypeable *H. influenzae* (62). Histamine increases bronchial epithelium permeability, stimulates mucus secretion, and induces bronchoconstriction.

Patients with acute exacerbations of chronic bronchitis have had basophil-bound IgE and serum IgE to homologous strains of nontypeable *H. influenzae* and *S. pneumoniae* isolated from sputum with the acute exacerbation (162). In another study in asthmatics, 29% of patients had serum IgE antibodies to nontypeable *H. influenzae* and/or *S. pneumoniae* (238). This sensitization to bacterial antigens may contribute to the bronchoconstriction and airway inflammation seen with acute exacerbations of COPD.

These observations regarding histamine release and IgE to bacterial antigens suggest that bacterial pathogens, either directly or indirectly via a Th2-type immune response, could contribute to the eosinophilia, airway hyperreactivity, and bronchoconstriction seen in patients with COPD. Further investigation in this area is warranted, especially in the group of COPD patients with eosinophilic bronchitis (132).

TABLE 4. Typing systems for nontypeable *Haemophilus influenzae*

Typing system	Basis of strain differentiation	References
OMP subtyping	Molecular mass differences of OMPs	23, 116, 206, 216
Restriction endonuclease analysis	Molecular size of small fragments of genomic DNA restricted with frequently cutting restriction enzymes	43, 93, 116, 271
Electrophoretic typing	Electrophoretic mobility of isoforms of metabolic enzymes	224, 243, 244
RAPD and REP-PCR[a]	DNA fingerprints of PCR-amplified genomic DNA using various primers	32, 153, 154, 206, 289, 319
Ribotyping and long PCR ribotyping	Restriction enzyme patterns of ribosomal DNA	288–290
Pulsed field gel electrophoresis	Molecular size of large fragments of genomic DNA restricted with infrequently cutting restriction enzymes	270

[a] RAPD, randomly amplified polymorphic DNA; REP, repetitive extragenic palindrome.

BACTERIAL PATHOGENS

Nontypeable *Haemophilus influenzae*

Dynamics of colonization and molecular epidemiology. Nontypeable *H. influenzae* strains are common inhabitants of the human upper respiratory tract, being present in up to three-fourths of healthy adults. When serial cultures are performed, the organism can be recovered from the sputum of virtually all patients with chronic bronchitis. Adults with chronic bronchitis are colonized in the lower airways with nontypeable *H. influenzae* and other bacteria (45, 173). Colonization with nontypeable *H. influenzae* is a dynamic process, with new strains being acquired and replacing old strains periodically (271). Multiple strains frequently colonize the respiratory tract simultaneously in the setting of chronic bronchitis (116, 220).

The development of typing systems for nontypeable *H. influenzae* has led to important information about the epidemiology of respiratory tract colonization and infection. Earlier studies with outer membrane protein (OMP) subtyping and restriction endonuclease analysis were important in beginning to understand epidemiology and pathogenesis (23, 116, 216). The development and application of more powerful typing systems have further characterized the epidemiology of respiratory tract colonization and also elucidated genetic relationships among nontypeable *H. influenzae* strains (224, 243, 270, 289). Typing systems for nontypeable *H. influenzae* and the basis of strain differentiation for each are listed in Table 4.

Studies in which prospectively collected strains are subjected to genomic typing will reveal important data about colonization patterns. For example, it will be important to know how long individual strains of nontypeable *H. influenzae* colonize the respiratory tract of adults with COPD. Such studies will reveal whether acquisition of a new strain predicts the occurrence of an exacerbation. Application of strain typing to analysis of colonization and exacerbation patterns will begin to reveal whether protective immune responses to individual strains occur. Such information will be important in more precisely defining the role of nontypeable *H. influenzae* in causing exacerbations and designing immunization strategies as vaccines are developed.

Mechanisms of adherence. (i) Mucin binding. The first step in the pathogenesis of infection by nontypeable *H. influenzae* is colonization of the respiratory tract. Since the human respiratory mucosa is covered with mucus, bacteria initially encounter mucus in the respiratory tract. The mucus gel is a complex mixture of secreted molecules, cells, and debris, including mucins, which are high-molecular-weight glycoproteins with O-glycoside-linked carbohydrate side chains. Mucins bind bacteria and therefore likely influence bacterial adhesion to the epithelium. Mucin-bacterium interactions may serve as a host defense mechanism facilitating removal of bacteria from the respiratory tract by the mucociliary elevator. Alternatively, binding of bacteria to mucin may represent the initial step in bacterial adherence to the epithelium and colonization of the respiratory tract.

Analysis of the interaction of nontypeable *H. influenzae* with purified human nasopharyngeal mucin reveals a specific interaction between mucin and the bacterium (72, 165). Binding of mucin is mediated by OMPs P2, P5, and a third as yet unidentified OMP (253, 254). Furthermore, it appears that a protein-oligosaccharide interaction is responsible for binding, because asialo-mucin does not bind to nontypeable *H. influenzae* OMPs (254). Elucidating the molecular interaction of mucin with nontypeable *H. influenzae* will be important in understanding mechanisms of pathogenesis and may lead to the development of strategies to prevent colonization and infection.

(ii) Adherence to respiratory mucosa. Research in the past decade has witnessed the identification and characterization of multiple adhesins expressed by nontypeable *H. influenzae* (300, 301) (Table 5). Teleologically, the expression of multiple adhesin molecules and the ability to modulate expression of these adhesins support the notion that adherence to the respiratory tract is critical for survival of the bacterium.

Like many gram-negative bacteria, some strains of nontypeable *H. influenzae* express pili, which mediate adherence to mucosal cells (110, 156). The pili of *H. influenzae* are hairlike projections composed of polymeric helical structures with a

TABLE 5. Adhesins of nontypeable *Haemophilus influenzae*

Adhesin	Approx. molecular mass (kDa)	% of strains[a]	References
Pili	27	~33	109, 110, 195, 322, 323
HMW1 and HMW2	125	70–80	20, 21, 24, 230, 304
Hia (*H. influenzae adhesin*)	115	20–30	25
Hap (*Haemophilus* adhesin and penetration)	155 (110)[b]	100	302
OMP P5 (fimbrin)	35	100	231, 284
OapA (opacity-associated protein A)	47	100	246, 341

[a] Percentage of strains which are capable of expressing the adhesin.
[b] The gene product is 155 kDa, and the processed protein is 110 kDa.

distal-tip adhesin. The gene cluster responsible for the biogenesis of pili contains five genes: *hifA*, encodes the major structural protein, *hifB* encodes a periplasmic chaperone, *hifC* encodes an outer membrane usher, and *hifD* and *hifE* encode minor protein subunits and participate in the biogenesis of pili (323). Phase variation of pilus expression is mediated by slipped-strand mispairing in the promoter site of *hifA* and *hifB* (322). Examination of clinical isolates from children has revealed that only one third of such isolates contain the pilus gene cluster and are capable of expressing pili (300). The proportion of piliated strains of nontypeable *H. influenzae* recovered from adults with chronic bronchitis has not been rigorously studied.

The observation that nonpiliated strains of nontypeable *H. influenzae* are capable of adhering to cultured human epithelial cells suggested the presence of nonpilus adhesins. Barenkamp and coworkers identified the high-molecular-weight surface proteins HMW1 and HMW2 and isolated the genes which encode the proteins (22). Analysis of the sequences revealed similarity with *Bordetella pertussis* filamentous hemagglutinin, a known adhesin molecule. Construction of isogenic mutants which lack HMW1 and HMW2 and expression of the recombinant proteins in *Escherichia coli* clearly established these proteins as adhesins of *H. influenzae* (304). Approximately 70 to 80% of nontypeable *H. influenzae* strains express HMW1 and HMW2 (305).

Strains which lack HMW1 and HMW2 are capable of adherence to epithelial cells in vitro, suggesting the presence of additional adhesin molecules. A gene which encodes another adhesin was identified in and isolated from a nontypeable strain which lacked the genes which encode HMW1 and HMW2 (25). The gene has been named *hia* and encodes a protein of ~115 kDa. Analysis of an isogenic mutant and expression of recombinant Hia in *E. coli* have established that Hia is an adhesin for nontypeable *H influenzae*. Hia has sequence homology with Hsf of *H. influenzae* type b strains and also demonstrates binding characteristics similar to Hsf (300).

Another gene which encodes a 155-kDa nonpilus adhesin was described by St. Geme et al. (139, 302). Hap is present in all strains and is involved in adherence and invasion of cultured human epithelial cells. Hap shows significant homology to serine-type IgA1 proteases of *H. influenzae* and *Neisseria* species but is distinct from IgA1 protease. Like IgA1 protease, Hap is synthesized as a larger preprotein that contains a prokaryotic signal sequence which facilitates transport to the periplasm. The carboxy terminus then inserts into the outer membrane and forms a pore through which the remainder of the protein passes. The Hap protein cleaves a 110-kDa fragment, which is released, and the remaining 45-kDa fragment remains associated with the membrane (138, 247). The precise role of the Hap protein in pathogenesis is still unclear, but one intriguing possibility is that the proteolytic activity is important once the bacterium is intracellular (300).

Strains of nontypeable *H. influenzae* recovered from children with otitis media express a nonhemagglutinating surface appendage which has been called a fimbria by Sirakova et al. (231, 284). This ~36-kDa protein is OMP P5, which is an OMP A-like protein (210). Disruption of the gene results in reduced adherence to human oropharyngeal cells and alteration in the ability to cause otitis media in chinchillas. Immunization of chinchillas with OMP P5 is protective in animals challenged with the homologous strain (284).

OapA (opacity-associated protein A) is responsible for the transparent-colony phenotype of *H. influenzae* and is required for efficient colonization of the nasopharynx in an infant rat model of *H. influenzae* carriage (341). More recently, OapA has been identified as an adhesin which mediates adherence of nontypeable *H. influenzae* to Chang epithelial cells (246). The protein is present in all strains of *H. influenzae* examined thus far.

The adherence of nontypeable *H. influenzae* to the human respiratory tract mucosal surface is the result of a complex interaction of bacterial adhesins and host molecules. Several adhesins have been identified, and their precise roles in the pathogenesis of infection of humans remain to be defined. Elucidating the mechanisms of the interactions between adhesins and host, the antigenic structure of the molecules involved, the relative importance of the various adhesins, the ability of the bacterium to modulate expression of adhesins, and the conditions under which specific adhesins are expressed will be important in understanding the molecular mechanisms of pathogenesis. Such observations may lead directly to developing novel strategies to prevent colonization or infection by nontypeable *H. influenzae* in the setting of chronic bronchitis.

(iii) Intracellular and intercellular invasion. Nontypeable *H. influenzae* is present in the lumen of the respiratory tract, binds with specificity to mucin, and adheres to the surface of respiratory epithelial cells. More recently, research from several groups has shown that the organism's niche in the human respiratory tract is not limited to adherence to the surface of epithelial cells. Several lines of investigation involving in vitro and in vivo studies have established that nontypeable *H. influenzae* invades beyond the surface of the respiratory epithelium. These studies were discussed previously.

Iron uptake. Bacteria require a source of iron for several metabolic processes. In the human host, most iron is present intracellularly in heme-containing compounds or bound to ferritin. Most extracellular iron is bound to transferrin or lactoferrin. The level of free iron is below the level necessary to support bacterial growth. Bacteria have developed mechanisms to acquire iron for growth in the human host.

Iron acquisition by *H. influenzae* is a complex process which involves several components. Iron is acquired from transferrin by transferrin-binding proteins in the outer membrane, and subsequent transport of iron from the periplasmic space to the cytoplasm is dependent on the *hitA, hitB*, and *hitC* genes. Molecules which are involved in iron uptake are summarized in Table 6.

Since *H. influenzae* lacks the enzymes necessary to convert δ-aminolevulinic acid to protoporphyrin IX, the organism requires heme for growth. Indeed, the requirement for heme is used in the clinical microbiology laboratory to confirm the identity of a clinical isolate as *H. influenzae*. Several molecules are involved in the uptake of heme, and these are summarized in Table 6.

A comprehensive discussion of the mechanisms of iron and heme uptake is beyond the scope of this review. Suffice it to say that these mechanisms are the focus of intense investigation and are important from the perspective of understanding the pathogenesis of *H. influenzae* infection. Furthermore, proteins

TABLE 6. Proteins involved in uptake of iron and heme by *Haemophilus influenzae*[a]

Protein	Gene	Molecular mass (kDa)	Location	Function	References
Tbp1	*tbpA*	95	Outer membrane	Transferrin transport	111, 112, 179
Tbp2	*tbpB*	68–85	Outer membrane	Transferrin binding	111, 112, 179
FbpA	*hitA*	36	Periplasmic space	Iron binding	272
HitB	*hitB*	51	Cytoplasmic membrane	Permease	272
HitC	*hitC*	40	Cytoplasmic membrane	Energy transduction	272
HxuA	*hxuA*	100	Secreted	Heme/hemopexin binding	65, 66
HxuB	*hxuB*	60	Outer membrane	Release of HxuA	66
HxuC	*hxuC*	78	Outer membrane	Heme transport	66
57-kDa protein	?	57	Outer membrane	Hemopexin binding	350, 351
HbpA	*hbpA*	61	?	Heme binding	131
HgpA	*hgpA*	120	Outer membrane	Hemoglobin/haptoglobin binding	150
HgpB	*hgpB*	115	?	Hemoglobin/haptoglobin binding	257
HhuA	*hhuA*	115	Outer membrane	Hemoglobin/haptoglobin binding	185
P4	*hel*	30	Outer membrane	Heme transport	256

[a] Adapted from reference 249 with permission of the publisher.

involved in iron uptake are the subject of study as potential vaccine antigens, and the observation that these proteins are transcribed and expressed in vivo further supports their potential as vaccines (142, 344).

Antigenic variation of surface proteins. (i) Antigenic heterogeneity. Nontypeable *H. influenzae* expresses six to eight major proteins in its outer membrane. Studies in the 1980s demonstrated that a high degree of variability in the molecular weights of these outer membrane proteins existed among strains of nontypeable *H. influenzae* (23, 216). OMP P2, which coustitutes approximately half of the protein content of the outer membrane, shows a particularly high degree of size variability among strains (216). P2 is the major porin protein of *H. influenzae*, allowing small hydrophilic molecules to pass through the outer membrane (318). Analysis of the sequence of the gene which encodes P2 revealed that portions of the protein which are buried within the outer membrane are relatively conserved among strains but that several of the eight loops which are exposed on the bacterial surface show a high degree of sequence variability among strains (31, 84, 283). Since antibodies to P2 elicit strain-specific protection (117, 157, 312), these observations suggested that antigenic heterogeneity of the major surface protein plays a role in the ability of nontypeable *H. influenzae* to cause recurrent respiratory tract infections in humans.

(ii) Point mutations under immune selective pressure. Analysis of OMP patterns from strains of nontypeable *H. influenzae* recovered prospectively from patients with chronic bronchitis reveals a high degree of turnover of strains, with frequent infection by new strains in some patients and persistent infection by the same strain in other patients (116). Among the strains which show persistence in the respiratory tract, variants with changes in the molecular weight of P2 but identical DNA fingerprints have been observed (116, 117). To determine the mechanism of this antigenic drift, Duim et al. (85) studied the sequences of the genes encoding P2 in these variants. The antigenic drift resulted from single-base changes in the P2 gene, all generating amino acid changes in surface-exposed loops of the P2 protein (85). Similar single-base changes were observed in the P2 gene from variants selected in subcutaneous cages implanted in rabbits and from a variant which survived antibody-mediated killing in vitro (85, 86, 329). All of the point mutations in the P2 gene were nonsynonymous, since they resulted in amino acid changes. Since all of the substitutions resulted in amino acid changes, these mutations produced a selective advantage for the bacterium. These observations strongly suggested that the accumulation of point mutations under immune selective pressure resulted in antigenic drift of surface-exposed regions of a major OMP. This mechanism of evading an immune response by the host could allow persistent *H. influenzae* infection in COPD.

(iii) Horizontal transfer of genes. Recent studies in an Aboriginal community in the Northern Territory of Australia reveal another mechanism by which nontypeable *H. influenzae* alters its P2 molecule to evade host defenses. Rural Aboriginal children are heavily colonized by nontypeable *H. influenzae* in the nasopharynx at an early age (174). Ribotyping of prospectively recovered isolates has revealed that the children are colonized by multiple strains of *H. influenzae* simultaneously and that strains are acquired and cleared frequently, resulting in a high rate of turnover (288). By determining the sequences of P2 genes from selected strains, Smith-Vaughn et al. (291) demonstrated the presence of identical P2 genes in strains with different genetic backgrounds. In view of the wide diversity of P2 gene sequences, the authors concluded that horizontal transfer of the P2 gene occurred among strains. The presence in the human respiratory tract of simultaneous, multiple strains of a bacterium which is competent for DNA uptake provides a powerful mechanism for the bacterium to alter expression of surface molecules. This phenomenon is likely to occur in other settings in which multiple strains of nontypeable *H. influenzae* colonize the respiratory tract, such as cystic fibrosis (206) and chronic bronchitis (220).

Antigenic variation of LOS. (i) Structure. Endotoxin, or lipopolysaccharide, is the major glycolipid in the outer membrane of gram-negative bacteria. Endotoxin is essential to the integrity and functioning of the bacterial cell wall. Nonenteric gram-negative mucosal pathogens, including *H. influenzae*, express an endotoxin molecule which lacks the long, repeating polysaccharide side chains which are typical of lipopolysaccharide of enteric gram-negative bacteria such as *E. coli* and *Sal-*

monella spp. Therefore, the endotoxin of *H. influenzae* is more accurately called LOS.

LOS is involved in several stages in the pathogenesis of infection, including colonization of the respiratory tract and cytotoxic injury to target tissues. The importance of LOS in pathogenesis has generated considerable interest in studies of the biosynthesis and structure of the molecule. Such studies are complicated because it is necessary to study tertiary gene products of genes which are turned on and off at high frequencies. Nevertheless, considerable new information about LOS biosynthesis and structure has been obtained in the past decade.

LOS contains a membrane-anchoring lipid A portion. This part of the molecule is responsible for its endotoxin like properties, including mitogenicity, pyrogenicity, platelet aggregation, cytokine activation, and adjuvant activity. Lipid A is linked by a single 2-keto-3-deoxyoctulosonic acid molecule to a heterogeneous oligosaccharide composed of glucose, galactose, and heptose. Marked intrastrain and interstrain variation in the size of LOS is observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This variation is a result of differences in the quantity and assembly of the neutral sugars, particularly galactose (340).

Surface determinants which are essential for the organism at one stage of colonization or infection may be unnecessary or even detrimental at a later stage of infection. Bacteria have evolved adaptive mechanisms for phenotypic variation of surface molecules, including LOS (262).

The LOS of *H. influenzae* shows considerable structural heterogeneity both between strains and within a clonal population derived from a single strain. This heterogeneity occurs as a result of several mechanisms. LOS is the end product of a complex biosynthetic process, and some variation occurs as a result of factors which influence the interaction of enzymes, regulatory proteins, and substrates (262). Such factors may determine the number of phosphate substitutions, anomeric linkages, saccharide branching chains, and other structural modifications, so that LOS is expressed as a family of molecules on the bacterial surface. A variety of environmental factors influence LOS structure as well. Such factors include exposure to serum; exposure to mixtures of glucose, lactate, urea, and bicarbonate in vitro; alteration of growth rate; and cystine limitation.

(ii) Phase variation. Another mechanism by which *H. influenzae* alters its LOS is phase variation, which is the ability to regulate the expression of molecules by turning on and off the expression of selected genes. The LOS of *H. influenzae* demonstrates phase variation, which occurs through a mechanism known as slipped-strand mispairing (141). The *lic* locus, which is responsible for synthesis of oligosaccharide structures, contains open reading frames which are preceded by multiple tandem repeats of the tetramer 5′-CAAT-3′. Alterations in the number of repeats through the nonrecombinational mechanism of slipped-strand mispairing shift upstream initiation codons into or out of frame, creating a translational switch and resulting in phase variation (141). Multiple oligosaccharide structures undergo phase variation in a complex pattern. Some genes vary independently, and some vary in a coordinate fashion with other genes. As a result, the bacterium has the ability to display a varied array of LOS structures on its surface. This ability enables *H. influenzae* to adapt to its environment in the various stages of colonization and infection.

(iii) Molecular mimicry of host tissue. The LOS of many strains of *H. influenzae* contain a terminal digalactoside, Gal-α-(1-4)-β-Gal, which is also present in human glycosphingolipids in the urinary tract, intestinal epithelium, and erythrocytes (187). The mimicry of host tissue may be an adaptive mechanism which promotes bacterial survival in the respiratory tract of the host.

The LOS components which resemble moieties in human tissue can be altered by the addition of sialic acid both in vitro and in vivo (187). Indeed, many strains of *H. influenzae* contain sialylated LOS (188). The oligosaccharide portion of sialylated LOS may also resemble sialylated oligosaccharides present in human glycosphingolipids. Sialylated LOS may play a variety of potential roles in the pathogenesis of colonization and infection by *H. influenzae* (187). These include antirecognition of bacterial surface antigens by the host, downregulation of opsonophagocytosis by bacteria, since bacteria with sialylated LOS are more resistant to phagocytosis, decreased adherence of bacteria to host cells or to other bacteria, intracellular survival of bacteria, and alteration of bacterial or host cell signaling pathways.

In summary, *H. influenzae* has an enormous capacity to alter the expression of its LOS by a variety of mechanisms. The mechanisms which have evolved illustrate some of the adaptive potential of surface bacterial determinants and their role in colonization and infection of the human respiratory tract.

Immune response. (i) Interpreting the literature. Human antibody responses to nontypeable *H. influenzae* in patients with COPD have been studied for decades. Two elements of the experimental design of such studies are critical in interpreting this literature: (i) the importance of using the homologous infecting isolate as the source of antigen in the immunoassays, and (ii) the importance of using immunoassays which detect antibodies to epitopes which are exposed on the surface of the intact bacterium.

Evidence is mounting that most immune responses following infection by nontypeable *H. influenzae* are strain specific (see below). Therefore, studies with laboratory isolates rather than homologous clinical isolates need to be reinterpreted in this context.

(ii) Strain-specific immune responses. OMP P2 is strongly immunogenic in experimental animals and humans (117, 213, 298, 354). Analysis of monoclonal antibodies to P2 which were generated by immunizing mice with whole bacterial cells revealed that most antibodies were directed toward a single surface-exposed loop on the P2 protein (125, 126). All of these antibodies were highly specific for the immunizing strain. This observation suggested that nontypeable *H. influenzae* expresses an immunodominant, strain-specific epitope on the bacterial surface.

To test the hypothesis that the expression of strain-specific and immunodominant epitopes on the bacterial surface induces a strain-specific immune response, mice and rabbits were challenged with whole cells of a strain of nontypeable *H. influenzae* (354). Analysis of the antibody response with immunoblot, bactericidal, and immunoprecipitation assays revealed a prominent antibody response almost exclusively to a single surface-exposed loop of the P2 molecule (354). These obser-

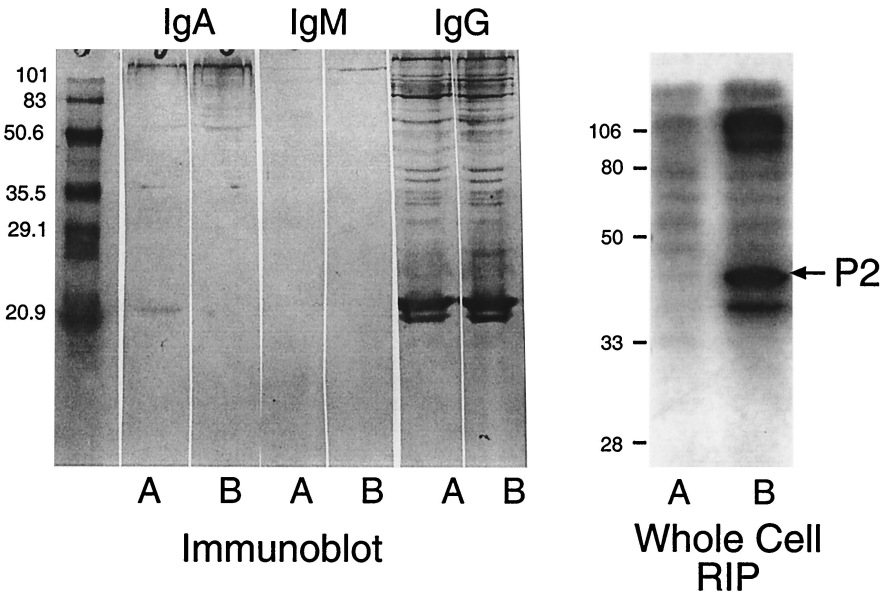

FIG. 3. Immunoblot assay (left panel) and whole-cell radioimmunoprecipitation (RIP) assay (right panel) with preexacerbation serum (lanes A) and postexacerbation serum (lanes B) from an adult with COPD who experienced an exacerbation due to nontypeable *H. influenzae*. Assays were performed with the homologous infecting strain. The positions of molecular mass standards are noted (in kilodaltons) to the left of each panel. Note that immunoblot assays detect antibodies to many bands with minimal difference between pre- and postexacerbation sera. By contrast, whole-cell radioimmunoprecipitation assays with the same sera show the development of new antibodies to P2 (arrow) and higher-molecular-mass proteins following the exacerbation.

vations, along with studies involving P2 in longitudinally collected isolates from adults with COPD (84–86, 117), support the notion that the surface-exposed loops of the P2 protein are under intense immune selective pressure. The expression of strain-specific, immunodominant epitopes represents a mechanism by which the bacterium induces antibodies which will protect against recurrent infection by the homologous strain but will not protect against infection by heterologous strains.

To determine whether a similar phenomenon occurs in humans, serum from two adults with exacerbations of COPD due to nontypeable *H. influenzae* were characterized (355). Both patients developed new bactericidal antibodies to their infecting strain. Immunoblot assays with homologous strains revealed antibodies to many antigens, with minimal differences between pre-and postexacerbation sera (Fig. 3). This observation illustrates the second critical element in the design of experiments to characterize the immune response to nontypeable *H. influenzae*. Immunoassays which detect antibodies to epitopes which are present on the bacterial surface will measure the potentially relevant antibody responses.

(iii) Antibodies to surface-exposed epitopes. Figure 3 shows that serum from a patient who experienced an exacerbation due to nontypeable *H. influenzae* had antibodies to many antigens before the exacerbation and in spite of developing new bactericidal antibodies, had no new detectable antibodies by immunoblot assay. This observation is consistent with that of Groeneveld et al. (115), who showed that patients with abundant antibodies to *H. influenzae* in serum and sputum still experienced infection. Immunoblot assays detect antibodies to many epitopes on OMPs, including those which are buried within the outer membrane and not available for binding on the intact bacterium. Antibodies which bind to epitopes which are not on the bacterial surface are not likely to be protective. The OMPs of *H. influenzae* share cross-reactive epitopes with the OMPs of many gram-negative bacteria. Most of the cross-reactive epitopes are buried in the membrane and not on the bacterial surface. In order to detect the meaningful and potentially protective immune response, immunoassays which specifically detect antibodies to epitopes on the bacterial surface should be used. Such assays include whole-cell radioimmunoprecipitation assays, flow cytometry, and functional assays such bactericidal and opsonophagocytosis assays. Subjecting the serum in Fig. 3 to whole-cell radioimmunoprecipitation revealed that the patient developed new antibodies to P2 and to higher-molecular-mass proteins, an observation which was missed by immunoblot assay. Adsorption studies further established that new bactericidal antibodies were directed at strain-specific epitopes on the P2 protein (355).

In summary, the literature on the human immune response to nontypeable *H. influenzae* must be interpreted with caution. Recent research with improved study design reveals that adults with COPD make strain-specific antibody responses to surface-exposed epitopes following infection with nontypeable *H. influenzae* (222, 355).

Prospects for vaccines. In view of the morbidity, mortality, and health care costs associated with bacterial infection in chronic bronchitis, there is interest in developing vaccines to prevent bacterial infections in this population. A large number of investigators in academia and industry are conducting research in pursuit of vaccines to prevent infections caused by nontypeable *H. influenzae*.

(i) Correlates of protection. Identifying a protective immune response is a critical step in developing a vaccine to prevent any infection. It may be possible to generate immune responses

to a variety of bacterial antigens, but the key question is whether that immune response will be effective in preventing infection in the target population. Clinical trials are necessary to establish the efficacy of a vaccine. However, in characterizing and evaluating potential vaccine antigens prior to clinical trials, in vitro and in vivo assays are useful in predicting which antigens are most likely to generate protective immune responses.

Animal models of infection have been widely used in testing vaccine antigens for a variety of infectious agents. Several models have been useful in evaluating vaccine antigens for nontypeable *H. influenzae*. These include the chinchilla model of otitis media (21, 76, 114) and various pulmonary and nasopharyngeal clearance models in the mouse and rat (130, 144, 166, 167, 204, 335, 352). The ability of an antigen to generate a protective immune response in an animal model is used as a rationale to proceed with further testing of a potential vaccine antigen. However, it is worth noting that the correlation between protection in animal models and protection in humans for nontypeable *H. influenzae* has not been established in any animal model to date.

The best correlate of protection for infection by nontypeable *H. influenzae* appears to be a bactericidal antibody response. The presence of serum bactericidal antibody is associated with protection from otitis media due to nontypeable *H. influenzae* in children (91, 282). In view of this observation, the ability of an antigen to generate bactericidal antibodies is used as a second strategy for identifying potential vaccine antigens.

(ii) Vaccine strategies. Nontypeable *H. influenzae* causes mucosal infections. Therefore, a mucosal immune response may be the most effective in preventing infections (106). One avenue of investigation is the development of technologies for the mucosal delivery of vaccine antigens to generate a mucosal immune response (167, 169, 335, 336). This exciting and potentially fruitful approach is being pursued by several groups of investigators.

While the induction of mucosal immune responses to prevent infections caused by nontypeable *H. influenzae* is rational, whether such an immune response will be protective remains to be seen. As noted above, the best correlate of protection from otitis media (also a mucosal infection) is the presence of serum bactericidal antibody to the infecting strain. Furthermore, conjugate vaccines for *H. influenzae* type b infections are administered systemically and are also effective in reducing or eliminating colonization of the respiratory tract by type b strains. Therefore, systemically administered vaccines for preventing infections due to nontypeable *H. influenzae* are also a viable approach. Whether systemic vaccines, mucosal vaccines, or a combination will be most effective awaits further study.

Vaccines to prevent infections caused by nontypeable *H. influenzae* in patients with chronic bronchitis will also have application in preventing otitis media in infants and children. The three most common bacterial causes of otitis media and exacerbations of chronic bronchitis are *S. pneumoniae*, nontypeable *H. influenzae*, and *M. catarrhalis*. As a result, vaccine formulations to be tested in humans may include combinations of vaccine antigens from these three organisms.

(iii) Vaccine antigens. A vaccine should be capable of generating an immune response which is effective in preventing infection by all or most strains of a species in the target population. A vaccine antigen should have several characteristics. (i) It should be conserved among strains so that the immune response is effective against many strains. This characteristic is especially important for nontypeable *H influenzae* in view of the extensive antigenic heterogeneity observed in surface antigens. (ii) The vaccine antigen should be located on the bacterial cell surface so that protective antibodies can bind to the intact bacterium. (iii) The vaccine should generate an immune response which is protective from infection in the target population. (iv) The vaccine antigen must be immunogenic in the target population.

Several surface proteins of nontypeable *H. influenzae* have the characteristics of potential vaccine antigens and are therefore generating substantial interest as vaccines. Several integral OMPs are being investigated, including P4, P5, P6, protein D, OMP26, D15, iron-regulated proteins, and others (11, 101, 113, 144, 150, 168, 179, 180, 185, 214, 215, 284, 295, 344). In addition, the adhesin molecules discussed earlier in this review are also under consideration as potential vaccine antigens (Table 5). Antibodies (particularly mucosal antibodies) to adhesins may be capable of blocking adherence of the organism to the respiratory mucosa. Finally, there is interest in using detoxified LOS as a vaccine antigen (120, 122). Research in the next decade promises substantial progress in the challenge of developing vaccines for nontypeable *H. influenzae*.

(iv) Clinical trials with killed whole-cell oral vaccine. Placebo-controlled clinical trials with a vaccine formulation consisting of Formalin-killed whole bacteria of a nontypeable *H. influenzae* strain have been conducted in Australia and Papua New Guinea (58, 59, 176, 308). The vaccine produced a statistically significant reduction in the frequency of exacerbations compared to placebo-treated controls. The apparent protective effect was present for 1 year. Although a specific mucosal antibody response is observed following vaccination (60), no clear correlation between clinical protection and either salivary antibody to *H. influenzae* or colonization with *H. influenzae* was observed (58). The mechanism of the protective effect is not known at this time. These provocative studies may provide important information about mechanisms of protection in the human respiratory tract and support the concept that vaccines offer potential in reducing bacterial infections in COPD.

Moraxella catarrhalis

***M. catarrhalis* as a cause of exacerbations of COPD.** Nontypeable *H. influenzae* and *S. pneumoniae* have long been recognized as causes of purulent exacerbations of COPD. More recently, *M. catarrhalis* has been implicated as an etiologic agent in such exacerbations. The recognition of *M. catarrhalis* as a human lower respiratory tract pathogen has been delayed for several reasons. The organism colonizes the upper respiratory tract of children and adults in the absence of clinical signs of infection. Another factor accounting for the difficulty in recognizing the role of this organism as a human pathogen is the observation that the colony morphology of *M. catarrhalis* is difficult to distinguish from that of commensal *Neisseria* species, which are part of the normal upper respiratory tract flora. Finally, *M. catarrhalis* causes noninvasive infections, so is rarely recovered in blood or pleural fluid cultures.

Four principal lines of evidence implicate *M. catarrhalis* in

TABLE 7. Typing methods for *Moraxella catarrhalis*

Category and methods	Assay	Basis of strain differentiation	References
Phenotypic			
SDS-PAGE and immunoblot assay	Immunoblot of bacterial lysates with normal human serum	Antigenic and molecular weight differences of cellular proteins	197, 209, 259
Esterase electrophoretic polymorphism	Detection of specific esterases by electrophoresis	Relative electrophoretic mobility of esterases	77, 241
LOS typing	Inhibition ELISA with typing antisera	Antigenic differences in LOS	320
Genotypic			
Restriction enzyme analysis of genomic DNA			
Agarose gel electrophoresis	Electrophoretic separation of small fragments of DNA	DNA sequence differences detected by restriction enzymes	77, 79, 94, 147, 197, 209, 236, 237, 259
Pulsed-field gel electrophoresis	Electrophoretic separation of large fragments of DNA	DNA sequence differences detected by restriction enzymes	159, 163, 190, 261, 334
DNA probe	Southern blot probed with labeled random genomic fragments	DNA sequence differences in selected regions	30
Ribotyping	Southern blot probed with labeled rRNA	DNA sequence differences detected by restriction enzymes and hybridization with rRNA	44, 77
Randomly amplified polymorphic DNA analysis	Electrophoretic separation of DNA fragments amplified by PCR from genomic DNA	DNA sequence differences detected by PCR amplification with random primers	334

this setting, and these have been reviewed recently (56, 211, 212). (i) Using strict criteria to evaluate the quality of sputum samples, a subset of patients with exacerbations of COPD have sputum smears which show a predominance of gram-negative diplococci on Gram strain and nearly pure cultures of *M. catarrhalis* (49, 198, 227, 299, 331). (ii) Pure cultures of *M. catarrhalis* have been obtained in transtracheal aspirates from patients experiencing exacerbations and pneumonia (10, 81, 128, 229, 343). (iii) Clinical improvement is seen in patients with *M. catarrhalis* infections following specific antibiotic therapy. Many penicillins are not active against *M. catarrhalis* because most strains produce β-lactamase. Patients with β-lactamase-positive strains who fail therapy with β-lactam antibiotics show clinical improvement following administration of an antibiotic active against *M. catarrhalis* (198, 227). (iv) Patients with chronic bronchitis who experience exacerbations associated with clinical and laboratory evidence of *M. catarrhalis* infection develop a new bactericidal antibody response to the homologous strain (49). The observation of a specific immune response to the organism following clinical infection provided evidence that the bacterium caused the infection.

Taken together, these lines of evidence indicate that *M. catarrhalis* causes exacerbations of COPD. It is difficult to estimate the proportion of exacerbations which are due to *M. catarrhalis*. However, one study performed in a Veterans Administration facility found that 30% of exacerbations were caused by *M. catarrhalis* (328). In our prospective study of patients with COPD, *M. catarrhalis* is the second most common cause of exacerbations after nontypeable *H. influenzae* (unpublished observations).

Typing systems. Initial studies involving the analysis of banding patterns of OMPs by SDS-PAGE revealed that the molecular masses of OMPs were quite similar among strains of *M. catarrhalis* (27). This observation precluded the use of OMP patterns for strain differentiation of *M. catarrhalis*. The sole currently available serotyping system for *M. catarrhalis* is based on antigenic differences in LOS among strains (248, 320). This system distinguishes three LOS types. The method is limited by the small number of serotypes and the observation that 60% of clinical isolates belong to a single serotype. Additional work on the antigenic structure of LOS will be important to determine whether serotyping based on LOS structure will be feasible.

The application of DNA-based molecular typing systems to epidemiologically well-defined strains of *M. catarrhalis* has been useful in studying nosocomial outbreaks and is providing important observations about the epidemiology of colonization and infection by *M. catarrhalis*. Table 7 summarizes typing methods for *M. catarrhalis*.

Epidemiology of colonization. *M. catarrhalis* is recovered exclusively from humans. No animal or environmental reservoirs for the organism have been identified. The bacterium colonizes mainly the respiratory tract, although it is rarely recovered from the genitourinary tract (80, 286). A strong relationship exists between rate of colonization and age in a population. Upper respiratory tract colonization with *M. catarrhalis* is common throughout infancy (16, 94, 184, 321, 324). For example, in one study involving monthly nasopharyngeal cultures taken from healthy infants from birth, 78% of infants were colonized at some time during the first 2 years of life (94). The prevalence of colonization with *M. catarrhalis* decreases with age, so that approximately 1 to 5% of healthy adults are colonized with *M. catarrhalis* (88, 155, 242, 321).

Several studies have surveyed the results of cultures of sputum samples and examined the clinical status of the patients from whom *M. catarrhalis* was recovered (41, 242, 287). These studies show that sputum samples which grow *M. catarrhalis* are more likely to be recovered from patients with chronic lung diseases than from healthy adults. These and other studies suggest that adults with chronic lung diseases are colonized

TABLE 8. Proteins involved in uptake of iron by *Moraxella catarrhalis*

Protein	Gene	Molecular mass (kDa)	Function	References
TbpA	*tbpA*	115–120	Transferrin transport	183
TbpB (OMP B1)	*tbpB*	80–84	Binds transferrin	183, 192, 225, 258, 275, 356
CopB (OMP B2)	*copB*	80	Under study	2, 5, 134, 136, 277
LbpA	*lbpA*	110	Binds lactoferrin	39, 40, 83
LbpB	*lbpB*	98	Binds lactoferrin	39, 40, 83, 356

with *M. catarrhalis* at a higher rate than are healthy adults. However, this observation has not been studied rigorously.

Two studies have examined the dynamics of respiratory tract colonization by subjecting isolates of *M. catarrhalis* recovered prospectively to molecular typing (94, 163). One study involved healthy children, while the other involved adults with bronchiectasis. Both studies showed that strains of *M. catarrhalis* are eliminated and acquired frequently, indicating that colonization with *M. catarrhalis* is a dynamic process. The dynamics of colonization of the respiratory tract in COPD is an important area of investigation. Such information is important in elucidating the precise role of *M. catarrhalis* in causing exacerbations, understanding the role of the immune response in clearing strains from the respiratory tract, devising a rational approach to antibiotic therapy, and designing an effective vaccine strategy.

Surface antigens. (i) Iron-regulated proteins. *M. catarrhalis* can utilize human transferrin and lactoferrin as sources of iron in the absence of siderophore production (46). Work in the last few years has led to the identification and characterization of transferrin- and lactoferrrin-binding proteins (Table 8). The arrangement of the genes encoding transferrin-binding proteins in *M. catarrhalis* differs from the arrangement in other organisms. However, overall, *M. catarrhalis* iron transport appears to function similarly to that of other mucosal pathogens, such as *H. influenzae* and the pathogenic *Neisseria* species.

(ii) OMPs. OMP patterns observed by SDS-PAGE show a high degree of similarity among strains from diverse clinical and geographic sources (27). Over the past decade, several OMPs have been characterized in some detail (Table 9).

UspA (ubiquitous surface protein A, also called HMW-OMP) migrates as an oligomer in SDS-PAGE. UspA is encoded by two genes, *uspA1* and *uspA2*, which encode proteins with deduced molecular masses of 88 and 62 kDa, respectively. Sequence analysis reveals that UspA1 and UspA2 both have an internal segment of 140 amino acids with 93% identity. Furthermore, several different and repetitive amino acid motifs are present in the two proteins (64). UspA1 is an adhesin, and UspA2 is involved in serum resistance (3, 199). The UspA proteins have generated considerable interest as vaccine antigens.

M. catarrhalis expresses a trypsin-sensitive, heat-modifiable hemagglutinin (100). Fitzgerald et al. (98) report that strains of *M. catarrhalis* which contain a 200-kDa protein agglutinate human erythrocytes, whereas strains which lack the 200-kDa protein do not. The authors further report that strains of *M. catarrhalis* recovered from adults with signs of clinical infection are more likely to hemagglutinate than strains recovered from patients who are colonized and lack clinical signs of infection (100). They suggest that hemagglutination may be a marker of pathogenicity.

OMP CD is a heat-modifiable protein which migrates aberrantly in SDS-PAGE (217, 273). The deduced amino acid sequence reveals a protein of 46 kDa, whereas the protein migrates at an apparent molecular mass of 60 kDa (218). This aberrant migration is due to a proline-rich region in the central part of the protein. OMP CD is highly conserved among strains and shows homology with OprF, a porin in *Pseudomonas* species (218). OMP CD is abundantly expressed on the bacterial surface (273). The nucleotide sequence encoding OMP CD in strains of *M. catarrhalis* which persist in the human respiratory tract is stable, indicating that OMP CD does not appear to change under immune selective pressure in vivo (146). OMP CD binds specifically to mucin purified from the human respiratory tract (254).

OMP E is another heat-modifiable protein which is abundantly expressed on the bacterial surface (36, 37). OMP E is a 50-kDa protein which is highly conserved among strains of *M. catarrhalis*. The function of OMP E is not yet known; it has borderline homology with FadL of *E. coli*, a protein which is involved in fatty acid transport.

(iii) LOS. The outer membrane of *M. catarrhalis* contains LOS which lacks the long O-polysaccharide side chains present in enteric lipopolysaccharide and is thus similar in structure to the LOS of other nonenteric gram-negative bacteria such as *Haemophilus* and *Neisseria* spp. (143). In addition, the LOS of *M. catarrhalis* has at least one epitope in common with the LOS of other nonenteric gram-negative bacteria (47).

TABLE 9. OMPs of *Moraxella catarrhalis*

OMP	Molecular mass (kDa)	Function	References
UspA1 (HMW OMP)	88 (oligomer)[a]	Putative adhesin	3, 4, 51, 52, 64, 135, 199
UspA2 (HMW OMP)	62 (oligomer)[a]	Involved in serum resistance	3, 4, 51, 52, 64, 135, 199
200-kDa protein	200	Hemagglutination?	98, 99
OMP CD	46[b]	Porin	146, 217, 218, 273, 353
OMP E	50	Unknown	36, 37

[a] Molecular mass varies among strains.
[b] OMP CD runs aberrantly (apparent molecular mass, ~60 kDa) in SDS-PAGE.

TABLE 10. Animal models of *Moraxella catarrhalis* infection

Model	Animal	Route and inoculum (CFU)	Outcome measurement	Observations	References
Pulmonary clearance	Mouse	Lung (1×10^5–5×10^5)	Rate of clearance of bacteria from lungs	*M. catarrhalis* cleared from lungs by 24 h	134, 136, 186, 232, 317, 327
Otitis media	Chinchilla	Intrabulbar (3×10^8)	Signs of otitis media and clearance of bacteria from middle ear	*M. catarrhalis* cleared from middle ear by 5 days	57, 82
Systemic infection	SCID mouse	Intranasal, intraperitoneal, or intravenous (10^2–10^7)	Clinical and postmortem findings	*M. catarrhalis* not recovered from blood	133

The lipid A portion of the LOS molecule of *M. catarrhalis* is similar in structure to the lipid A of other gram-negative bacteria and is responsible for the profound biological effects of LOS (143, 151, 191). Antigenic differences in the LOS among strains of *M. catarrhalis* reside in the oligosaccharide portion of the molecule (87, 103). The observation that 95% of strains belong to just three LOS serotypes indicates that less antigenic heterogeneity is present in the LOS of *M. catarrhalis* than in that of other nonenteric gram-negative bacteria such as *Haemophilus* and *Neisseria* spp., whose LOS show enormous antigenic heterogeneity among strains.

(iv) Pili. Many gram-negative species express pili (also called fimbriae), which mediate adherence to host cells. Strains of *M. catarrhalis* recovered from the human respiratory tract express pili, as observed by electron microscopy (6–9, 189, 260). Several lines of evidence indicate that some strains express type 4 pili (189). Little else is known about the role of pili in colonization, and this is an area which deserves study.

Immune response. A large number of studies have been performed to characterize the serological response to *M. catarrhalis*, and these have been reviewed recently by Christensen (56). This review will consider observations and issues related specifically to the human immune response to *M. catarrhalis* in COPD.

Chapman et al. (49) demonstrated that adults with COPD develop new bactericidal antibodies to their homologous isolates of *M. catarrhalis* following lower respiratory tract infection. This observation is important in defining *M. catarrhalis* as a pathogen and also emphasizes the importance of using immunoassays which detect antibodies to surface-exposed epitopes to elucidate a potentially protective human immune response to *M. catarrhalis*. Whether this immune response following infection with *M. catarrhalis* is strain- specific, as observed with nontypeable *H. influenzae*, remains to be determined.

Little information is available regarding antibody responses to individual antigens of *M. catarrhalis* in patients with COPD. The human antibody response in patients with COPD to OMP CD and OMP E, which are highly conserved surface proteins, is quite variable among individuals (37, 217). The majority of patients who experienced exacerbations due to *M. catarrhalis* had detectable serum IgG to the antigens, but none developed new antibodies to OMPs CD and E following infection. Analysis of the mucosal antibody response revealed that IgA was the predominant immunoglobulin to OMPs CD and E in sputum supernatants (37, 217). Antibodies to surface-exposed epitopes on OMP B1 are observed in the serum of patients with bronchiectasis (275). The antibody response to OMP B1 in patients with COPD has not yet been rigorously evaluated.

The immune response to *M. catarrhalis* in COPD deserves further study. Since *M. catarrhalis* causes predominantly mucosal infections, characterization of the mucosal immune response will be important. Identifying the nature of a protective immune response to *M. catarrhalis* will be important as well. As discussed in regard to studies of the immune response to nontypeable *H. influenzae*, attention should be paid to determining whether infection with *M. catarrhalis* induces immune responses to strain-specific or antigenically conserved antigens in patients with COPD. In addition, careful attention should be paid to using immunoassays which are capable of detecting antibodies to epitopes which are available on the bacterial surface, because such antibodies are most likely to be associated with a protective immune response.

Prospects for vaccines. The recognition of the importance of *M. catarrhalis* as a cause of lower respiratory tract infection in patients with COPD and the role of the bacterium as a cause of otitis media in children has stimulated research which has led to much progress in identifying potential vaccine antigens. Several OMPs have been the focus of study as potential vaccine antigens. The ideal OMP would contain abundantly expressed surface-exposed epitopes in all phases of growth, be highly conserved among strains, be expressed in vivo, and generate a protective immune response in the population that is susceptible to infection with *M. catarrhalis*. The OMPs listed in Tables 8 and 9 have several of these characteristics to a greater or lesser extent and are the focus of intense investigation. In addition, a detoxified LOS molecule has been studied as a potential vaccine antigen (119).

In spite of substantial progress in identifying antigens with vaccine potential, an important factor which is limiting the development of vaccines for *M. catarrhalis* is the lack of information about what constitutes a protective immune response to *M. catarrhalis*. A number of studies have demonstrated immune responses to *M. catarrhalis* in humans and animal models; however, none of the immune responses identified thus far are clearly associated with protection from infection.

Considerable effort has been devoted to developing a useful animal model for *M. catarrhalis*. Identifying such an animal model has been difficult. Table 10 lists three animal model systems which have been used to study *M. catarrhalis*. While animal models have yielded important information, particularly in assessing potential vaccine antigens, each of the proposed models has significant limitations. *M. catarrhalis* is an exclusively human pathogen and does not cause natural disease in animals. The immune response to *M. catarrhalis* in humans may differ from that observed in animals. Furthermore, none of the animal models developed thus far is a true model of infection. Rather, they measure the rate of clearance

of the bacterium from the animal. The mouse pulmonary clearance model has been the most widely used model to assess potential vaccine antigens.

Another approach to identifying a correlate of protection is in vitro assays of functional immune responses. For example, the presence of serum bactericidal antibodies is associated with protection from otitis media due to nontypeable *H. influenzae* (91, 282). The identification of such a correlate of protection has guided vaccine development. UspA, OMP CD, and OMP B1 of *M. catarrhalis* are targets of bactericidal antibodies (51, 225, 353). Whether bactericidal antibodies to *M. catarrhalis* are associated with protection awaits further study.

Streptococcus pneumoniae

Streptococcus pneumoniae has been studied extensively since its first isolation in 1881. Indeed, some of the most important discoveries in microbiology and infectious diseases resulted from research on the pneumococcus. These landmark discoveries include identification of DNA as genetic material, the association of capsular polysaccharide with bacterial virulence, the role of bacterial capsule in resistance to phagocytosis, the concept of type-specific immunity to bacterial infection, and the use of polysaccharide antigens as vaccines (13, 219). *S. pneumoniae* is the most common cause of community-acquired pneumonia, and the organism is an important cause of invasive infections in adults. The frequency of invasive human infection by the pneumococcus and the serious nature of many of these infections have demanded the attention of investigators, who have studied the epidemiology and pathogenesis of and immunity to pneumococcal infection.

In contrast to invasive pneumococcal infections, the role of *S. pneumoniae* in respiratory tract infections in COPD has received far less attention for several reasons. As discussed previously in this review, a reliable method to distinguish between colonization and clinical infection in individual patients does not currently exist. Most pneumococcal infections in the setting of COPD are not associated with bacteremia, so the presence of the bacterium in a normally sterile body fluid cannot be used as a diagnostic tool. Furthermore, the pneumoccus is frequently recovered from the sputum of patients with chronic bronchitis even in the absence of clinical infection; therefore, sputum cultures will not reveal the etiology of an exacerbation. These difficulties in defining infection, along with the association of *S. pneumoniae* with less fulminant infections in COPD, account for the relative lack of information on the role of the pneumococcus in this clinical setting.

A review of work on invasive infections caused by *S. pneumoniae* is beyond the scope of this review. Selected aspects of epidemiology, pathogenesis, and pneumococcal vaccines as they relate to infection in adults with COPD will be considered.

Epidemiology of colonization. The rate of respiratory tract colonization by *S. pneumoniae* varies with age. Prevalence studies reveal that 20 to 40% of healthy children and 10 to 20% of healthy adults are colonized at any one time (108, 137, 221). Several longitudinal studies have established that colonization of the upper airway with *S. pneumoniae* is common in infants and children (74, 89, 92, 181, 255, 285). Most children are colonized with the pneumococcus at some time during the first 2 years of life. Surprisingly little information is available from longitudinal studies of adults with COPD. Cultures of expectorated sputum from adults experiencing exacerbations of COPD reveal that *S. pneumoniae* is isolated from 7 to 26% of such samples (Table 2). More information is needed on the dynamics of colonization of patients with COPD, including the frequency of simultaneous colonization by multiple strains, the duration of colonization of individual strains, the rate of turnover of strains, and the distribution of capsular serotypes in patients with COPD. Such information will help to guide vaccination strategies.

Pathogenesis. (i) Virulence factors and surface antigens. The polysaccharide capsule of *S. pneumoniae* is the major virulence factor for the organism. Encapsulated strains are 10^5 times more virulent than isogenic mutants lacking capsule (316, 338, 339). Capsule accounts for resistance to phagocytosis and survival in the bloodstream, and antibodies to capsular polysaccharide are protective from infection. Ninety serotypes based on structural differences in capsular polysaccharide have been identified.

Cell wall polysaccharide is a major surface antigen which has a common structure among all serotypes. Its structure consists of teichoic acid which contains phosphoryl choline. Enzyme-linked immunosorbent assays (ELISAs) to measure antibodies to type-specific polysaccharide sometimes detect antibodies to cell wall polysaccharide, so caution must be used in interpreting the results of these assays (223).

Cell wall and cell wall polysaccharide induce inflammation. The cell wall consists of peptidoglycan, which is a single macromolecule consisting of a variety of distinct glycopeptides. These cell wall fragments have potent biological effects and play a major role in the inflammation observed in pneumococcal infection (48, 313, 315). The lower airways of adults with COPD are colonized by pneumococci, which are continuously dividing and shedding cell wall fragments into the airways. These bacterial antigens may contribute to the airway inflammation observed in COPD.

Pneumolysin is a thiol-activated toxin which has a variety of toxic effects on different cell types (250, 266). Pneumolysin-negative mutants are less virulent than their parental strains, and antibodies are protective in animal models (34, 35, 314). Autolysin lyses pneumococci upon release of pneumolysin by the bacterium (35). Neuraminidase may facilitate attachment to epithelial cells by cleaving sialic acid from the host glycolipids and gangliosides (311). PspA is a surface protein which is required for full virulence of the pneumococcus, and antibodies to PspA are protective in a mouse model (42). *S. pneumoniae* expresses IgA1 protease, which may facilitate colonization by cleaving secretory IgA in the respiratory tract (337). However, elucidation of the precise role of IgA1 protease in pathogenesis awaits further work.

(ii) Adherence. *S. pneumoniae* binds to respiratory tract epithelial cells to colonize the human respiratory tract. Work in the past decade has identified several adhesins and has begun to elucidate some of the molecular mechanisms of adherence of pneumococci to cells in the respiratory tract.

Pneumococcal isolates undergo spontaneous phase variation between opaque and transparent colony morphologies. These differences in colony morphology correlate with rates of autolysis and appear to be relevant to virulence, particularly in

adherence to host cells. Transparent variants adhere more readily to host cells and are able to colonize the nasopharynx of mice more efficiently than opaque variants (69, 342). The mechanism of enhanced colonization appears to involve increased adherence to GlcNAc and platelet-activating factor receptors on host cells (69).

Airway epithelial cells express receptors which show different specificities for the transparent and opaque variants. Activation of host cells by cytokines alters expression of receptors. For example, IL-1 activates epithelial cells, and TNF-α activates endothelial cells to express platelet-activating factor, which is a receptor for the pneumococcus (68). Such mechanisms likely contribute to the outcome of pneumococcal infections.

Several adhesins have been identified on the pneumococcal surface. PsaA is a 37-kDa protein which mediates attachment to type II pneumocytes and is involved in colonization of the respiratory tract in mice (33, 73). CbdA is a choline-binding protein of 75 kDa which is an adhesin and a determinant of virulence (264). A pyruvate oxidase, encoded by *spxB*, is important in the expression of adhesins, since a mutation in *spxB* leads to downregulation of multiple adhesive properties of *S. pneumoniae* (297). Although pneumolysin is a cytoplasmic protein released only after autolysis, it may play an indirect role in adherence by directly damaging epithelial surfaces (250). In vitro studies confirm reduced adherence to epithelial cells in pneumolysin-deficient mutants (266). However, pneumolysin does not appear to be a major determinant in nasopharyngeal colonization in mice (266).

The molecular mechanisms of adherence of *S. pneumoniae* and induction of inflammation by pneumococcal antigens are areas of research which will lead to novel methods of treatment and prevention of pneumococcal infection.

Pneumococcal vaccine. Anticapsular antibody provides the greatest degree of protection from invasive infection, and this observation has guided vaccine development for the pneumococcus. The Centers for Disease Control and the American College of Physicians recommend that all patients with COPD receive the 23-valent capsular polysaccharide vaccine. While this approach is rational, a critical review of the literature discloses no convincing evidence that immunization of adults with COPD reduces the incidence of pneumococcal infection in COPD (reviewed in reference 219). No new trials which would change that conclusion have been published (97, 233).

The capsular polysaccharide vaccine is not uniformly immunogenic in the elderly, and this observation likely accounts for the variable efficacy of the vaccine in this population (182, 263, 265, 267). Since COPD is a disease of the elderly, immune responses in this population are of particular relevance with regard to COPD. It has been estimated that the vaccine has an efficacy of 50 to 75% (149). Recently developed conjugate vaccines in which pneumococcal capsular polysaccharides are coupled to protein carriers hold promise as being more immunogenic. Some early trials in infants and children have revealed good immunogenicity and reduction in nasopharyngeal colonization by the pneumococcus (18, 70, 193, 194, 357). The ability of the vaccines to eradicate colonization may be especially useful in patients with COPD, since colonization likely contributes to airway inflammation and exacerbations are mucosal infections. Unfortunately, early studies with conjugate vaccines in the elderly suggest that they may not offer a major advantage in older populations (245, 281). Further study of pneumococcal conjugate vaccines in patients with COPD is clearly warranted.

Antibodies to capsular polysaccharide of *S. pneumoniae* are generally measured by ELISA. Recent efforts have been directed toward improving the specificity and standardizing the quantitation of capsule-specific antibodies by ELISA. Furthermore, since serum levels of antibodies to capsule do not always correlate with protection, recent work has focused on functional antibody responses, particularly in the elderly (149, 263). Romero-Steiner et al. (263) showed that functional immune responses, measured by in vitro killing assays, after pneumococcal vaccination were significantly lower in elderly adults than in young adults. Interestingly, functional antibody responses did not correlate closely with antibody levels measured by ELISA but did show correlation with antibody avidity. In view of these observations, it is important to critically assess indicators of vaccine protection by correlating clinical outcomes with antibody levels, functional activity, and antibody avidity to have an accurate surrogate for protection against pneumococcal infection (149). In addition, it will be important to assess the efficacy of pneumococcal vaccines specifically in patients with COPD, since protection against recurrent exacerbations and colonization by the pneumococcus may be different from protection from invasive infection, which has been the most common measure of efficacy of the vaccines in trials to date.

Haemophilus parainfluenzae

H. parainfluenzae is present as part of the normal upper respiratory tract flora in humans. Therefore, the bacterium is frequently recovered from expectorated sputum from adults with COPD (309). The presence of *H. parainfluenzae* in the sputum of 2 to 27% of patients experiencing exacerbations (Table 2) has raised the question of whether *H. parainfluenzae* causes exacerbations of COPD. The presence of the organism in sputum during an exacerbation does not establish etiology. In order to address this question, one must examine the type of evidence which has established that *H. influenzae* and *M. catarrhalis* cause exacerbations.

Bronchoscopy with the protected specimen brush performed during exacerbations has been used as a method to establish the etiology of some exacerbations in four recent studies (96, 208, 239, 294) (Table 3). Bacteria were present in 50 to 72% of patients with exacerbations. In one study (96), 25% of the samples contained *H. parainfluenzae*, while in the other three studies, *H. parainfluenzae* was not recovered at all (208, 239, 294). The discrepancy is difficult to explain, but these studies do not provide convincing evidence one way or the other for *H. parainfluenzae*'s being a common cause of exacerbations.

Documenting an immune response to a putative pathogen is another approach to establishing etiology. Mitchell and Hill (202) recently studied the immune response to *H. parainfluenzae* in three patients with chronic bronchitis and showed that these patients had higher titers of antibodies to *H. parainfluenzae* than healthy controls. Essentially no other work on the immune response to *H. parainfluenzae* in COPD has been published. This is an important area of research to pursue.

TABLE 11. *Chlamydia pneumoniae* as a cause of acute exacerbations of COPD

Reference	Country	No. of exacerbations studied	% caused by *C. pneumoniae*
Blasi et al. (38)	Italy	142	4
Mogulkac et al. (205)	Turkey	49	16 (sole etiology), 6 (other agents)
Miyashita et al. (203)	Japan	77	7.8
Beaty et al. (29)	United States	44	5

Overall, there is simply not enough evidence at this time to state with any degree of confidence the role, if any, that *H. parainfluenzae* plays as a pathogen in COPD.

Chlamydia pneumoniae

C. pneumoniae is an obligate intracellular human pathogen which causes acute infections of the upper and lower respiratory tract, including pharyngitis, sinusitis, bronchitis, and community-acquired pneumonia (129). Chronic infection with *C. pneumoniae* occurs in patients with COPD, as discussed above. Studies of *C. pneumoniae* in COPD are complicated by several observations. (i) The organism is difficult to cultivate and detect directly in the respiratory tract. (ii) Coinfection with other bacteria is common. (iii) Variability among authors exists in interpretation of the results of serological assays. (iv) By early adulthood, at least half of the population worldwide is serologically positive for *C. pneumoniae* (129). (v) Smoking is associated with increased levels of serum antibodies to *C. pneumoniae* in patients with and without COPD. (vi) Serological conversion occurs even in the absence of symptoms.

C. pneumoniae likely causes a small proportion of acute exacerbations of COPD, an observation based on serological evidence. Table 11 summarizes four studies which used serological criteria to establish the presence of acute *C. pneumoniae* infection in adults experiencing acute exacerbations of COPD. Although geographic variation may occur, the best estimates are that 5 to 10% of exacerbations of COPD are associated with *C. pneumoniae*.

Gram-Negative Bacilli

Samples collected from the lower airways with the protected specimen brush contain gram-negative bacilli, including *Pseudomonas aeruginosa, E. coli*, and *Proteus mirabilis*, in a small proportion of patients experiencing acute exacerbations of COPD (96, 208, 239, 294) (Table 3). These bacteria are isolated most often in the setting of severe exacerbations in patients with advanced COPD. The presence of these bacteria in the lower airways could reflect selective pressure by repeated exposure to antibiotics, a greater degree of compromise of lung defenses, or a combination of these factors. Defining the role of gram-negative bacilli in acute exacerbations is important in understanding the pathogenesis of infection and also in guiding a rational approach to antimicrobial therapy. Analysis of immune responses to these putative pathogens following exacerbations is a fruitful avenue of research to lead to a more precise definition of the role of these gram-negative bacilli in acute exacerbations of COPD.

CONCLUSIONS AND FUTURE DIRECTIONS

Research in the past decade has produced exciting new observations on the important role of bacteria in COPD. Better techniques to sample the lower airways, advances in techniques to study molecular mechanisms of microbial pathogenesis, and powerful methods to study host inflammatory responses provide opportunities to more precisely elucidate the role of bacteria in the pathogenesis and course of COPD. Emphasis should be placed on defining the contribution of bacteria in the lower airways to airway inflammation which is a hallmark of COPD. Identifying the microbial antigens responsible will allow investigators to design novel interventions to test the hypothesis that bacterial colonization of the lower airways accelerates progression of the disease. Developing reliable and widely applicable methods to define the etiology of exacerbations will be important in devising strategies to better treat and prevent exacerbations. Finally, the development of vaccines to prevent infections caused by nontypeable *H. influenzae, M. catarrhalis*, and *S. pneumoniae* would have a major impact on the course of COPD.

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