Antibodies to pneumococcus are thought to represent the primary mechanism of naturally acquired resistance to colonization. Here, however, we show that, in patients with chronic obstructive pulmonary disease (COPD), resistance to pneumococcal colonization is not associated with higher concentrations of serum anti-capsular or non-capsular antibodies. We compared preacquisition serum antibody concentrations to capsular antigens 6B, 7F, 14, 19F, and 23F from patients with COPD who did and did not acquire pneumococcal respiratory tree colonization over the course of 2 years. Colonized patients did not have lower anti-capsular antibody concentrations than control subjects who did not acquire pneumococcus. We found no difference in functional antibody concentrations between colonized patients and control subjects. Furthermore, colonized patients had significantly higher preacquisition concentration of antibody directed against the whole cell and pneumococcal surface protein A than control subjects. We thus conclude that, in adult patients with COPD, resistance to pneumococcal colonization is unlikely to be determined by higher serum antibody concentrations to pneumococcal antigens.

*Streptococcus pneumoniae* is an important human pathogen causing asymptomatic carriage as well as important mucosal and systemic infections. Anti-capsular antibodies are thought to represent the single most important protective mechanism against invasive disease [1]. Antibodies to pneumococcal capsular polysaccharides were the basis of serum therapy in which passively transferred, serotype-specific antipneumococcal serum was shown to reduce mortality from pneumococcal pneumonia by half [2]. The development of pneumococcal polysaccharide vaccines for adults [3] and the efficacy of pneumococcal polysaccharide–protein conjugate vaccines in infants and children [4, 5] have confirmed that antibodies to polysaccharide antigens can provide excellent protection against invasive disease caused by pneumococci of the same serotype as well as that caused by cross-reacting serotypes.

Whether these anti-capsular antibodies represent the mechanism whereby unimmunized individuals develop resistance to pneumococcal infection is less clear. We recently presented seroepidemiologic data that suggest otherwise: the risk of invasive pneumococcal disease in childhood decreases in parallel for many different serotypes (which suggests a serotype-transcending mechanism) and before naturally acquired anti-capsular antibodies are detectable [6]. Antibodies to other, non-capsular antigens have also been suggested to mediate acquired resistance to pneumococcal infection [7, 8]. It has also generally been assumed that resistance...
to pneumococcal colonization is determined by antibodies to the pathogen. Pneumococcal conjugate vaccines, which stimulate the production of anti-capsular antibodies in immunized infants or toddlers, have been shown to reduce colonization with vaccine-type and cross-reactive pneumococci, demonstrating the sufficiency of anti-capsular antibodies for protection against nasopharyngeal carriage [9–12]. Similar to the case with protection from invasive disease, it remains unclear whether naturally acquired anti-capsular antibodies are the mechanism of protection from pneumococcal colonization. Definitive data to support any association between resistance to colonization with S. pneumoniae and higher levels of capsular or noncapsular antibodies are lacking.

Thus, we wished to examine whether any relationship could be demonstrated between antipneumococcal antibodies and the risk of acquisition of a pneumococcal strain in the respiratory tree. To this end, we studied serum samples collected from patients with chronic obstructive pulmonary disease (COPD) in the context of a study that examined the role of bacteria in pulmonary exacerbations [13, 14]. COPD is a major cause of morbidity and mortality in adults that affects >24 million people in the United States [15]. Patients with COPD are at high risk for respiratory exacerbations, characterized by increased shortness of breath, sputum production, and cough. In contrast to healthy adults, patients with COPD have frequent colonization with bacteria. A significant role of bacteria in exacerbations of COPD was confirmed by 2 studies [13, 14]. Here, we show that patients with COPD who acquire a new strain of pneumococcus in their sputum have, in general, higher preacquisition anti-capsular and noncapsular serum antibody concentrations than do patients with COPD who do not acquire a new pneumococcal strain over the course of a 2-year period.

PATIENTS, MATERIALS, AND METHODS

Patient population: Buffalo Veterans Affairs COPD study clinic. All bacterial isolates and serum samples were recovered from subjects enrolled in a prospective study from April 1994 through December 2002 at the COPD Study Clinic at the Buffalo Veterans Affairs Medical Center, as described elsewhere [13]. In brief, patients were included in the study if all of the following criteria were met: (1) the presence of chronic bronchitis; (2) the absence of asthma or bronchiectasis by clinical assessment; (3) the ability to comply with monthly study clinic visits; and (4) the absence of immunosuppression, malignancies, or other life-threatening illnesses. Patients were seen every month and at the time of suspected exacerbations. At each visit, they brought a spontaneously expectorated morning sputum sample, and blood samples were obtained. Although the timing of immunization is unknown, all subjects in the study had previously received 1 dose of pneumococcal polysaccharide vaccine.

Bacteriological methods. Sputum samples were collected as described elsewhere [13, 14]. Cultures for isolation of S. pneumoniae were performed by plating serial dilutions of the sputum samples onto blood agar plates. S. pneumoniae was identified by colony morphology and optochin sensitivity. No patient who was found to be colonized with S. pneumoniae within 2 months of enrollment in the study was included in the present article.

Serum samples. Blood samples collected during clinic visits were allowed to clot. Serum was obtained by centrifugation and were stored at −80°C. We chose to analyze the serum sample 2 or 4 months before a visit at which a new strain of S. pneumoniae was isolated (referred to as the 2 month or 4 month preacquisition serum), to reduce the possibility that a patient preacquisition sample may in fact represent a sample obtained after colonization had occurred but had not yet been detected. Patients followed up in the same study who did not have any S. pneumoniae isolated for >24 months were defined as control subjects, and their serum samples were used for comparison. In our study, each serum assay represents a unique patient.

Measurements of serum antipneumococcal antibody concentration. All antibody measurements were performed by ELISA. The concentration of serum IgG to capsular polysaccharides type 6B, 7F, 14, 19F, and 23F was determined by ELISA according to published methods, using both C polysaccharide (CPS) and 22F absorption to remove nonspecific, cross-reactive antibodies [16]. The standard used in these assays was serum G141A-3 (a gift from Dr. George Siber, Wyeth-Lederle Vaccine, Pearl River, NY), which was assigned values of 6.64, 20.1, 31.5, 16.4, and 11.4 μg/mL for concentrations of IgG against types 6B, 7F, 14, 19F, and 23F, respectively.

A whole-cell pneumococcal IgG ELISA was developed by modifying a previously established assay in mice [17]. The whole-cell ELISA used pneumococcal strain Rx1AL−, an unencapsulated derivative of a serotype 2 strain. Rx1AL− is also lytA deficient; this mutation allows growth to high density and impairs the release of pneumolysin, which is thus concentrated in the bacterial pellet. Rx1AL− was grown to the late logarithmic phase in Todd-Hewitt broth supplemented with 0.5% yeast extract, harvested by centrifugation, killed by the addition of 70% ethanol, washed repeatedly, and resuspended in PBS. This ethanol-inactivated whole-cell bacterial suspension was used to coat the wells of a microtiter plate (Immulon II; Thermo Lab-systems)—100 μL of the suspension were used in each well, corresponding to ~10⁷ cells/well. The plate was incubated overnight at 4°C. The next day, wells were washed with 0.05% Tween 20 in PBS (PBST), and unbound sites in the wells were blocked by the addition of 5% fetal calf serum in PBST for 1 h at room temperature. After wells were washed, 100 μL of 8 serial dilutions of serum in PBST were added to the wells, and the plate was incubated at 37°C for 2 h. After the wells were washed
3 times with PBST, 100 μL of dilution of peroxidase-labeled goat anti–human IgG or IgM (Sigma-Aldrich) in PBST were added to each well, and the plate was incubated at room temperature for 1 h. After another wash, 100 μL of Sureblue peroxidase substrate (Kirkegaard & Perry Laboratories) were added to each well. After 15 min of color development in the dark, the reaction was stopped by the addition of 50 μL of 2 N sulfuric acid to each well, and the optical density at 450 nm was read. Controls included wells in which PBS was used instead of serum and wells in which PBS was used instead of the bacterial suspension. These controls consistently showed negligible color development. A standard was also used on all plates that consisted of serum from a healthy adult volunteer selected by virtue of high concentration of anti-pneumococcal antibodies.

A very similar protocol was developed for the determination of antibodies to the CPS (plates coated with 5 μg/mL CPS were obtained from Statens Serum Institut), pneumococcal surface protein A (PspA; plates coated with 1 μg/mL, provided by Dr. David Briles, University of Alabama, Birmingham) and pneumolysin (Ply; coated with 1.7 μg/mL, obtained as described elsewhere [18]).

Measurement of serum opsonophagocytic activity (OPA) directed against S. pneumoniae type 6B. Because the most common serotype isolated was serotype 6B, OPA to this serotype was measured using methods established elsewhere [19]. Briefly, human polymorphonuclear leukocytes (PMNs) from healthy adult donors were freshly isolated by ficoll-histopaque density-gradient centrifugation. Heat-inactivated human serum specimens were serially diluted in eight 3-fold steps in a 96-well microtiter plate with Hanks’ balanced salt solution/0.2% bovine serum albumin and were then incubated with 1000 cfu of a type 6 strain (0603, a 6B invasive isolate [20]) and complement (baby rabbit serum; final concentration, 12.5%) for 30 min at 37°C (final volume, 40 μL/well) on an orbital shaker. PMNs were then added at a 200:1 ratio (cells:bacteria; final volume, 80 μL/well), and the mixture was incubated for 45 min at 37°C with shaking. After incubation, samples from each well were diluted and plated onto blood agar plates overnight at 37°C and 5% CO₂. The OPA titer was calculated as the reciprocal of the serum dilution that caused a 50% reduction in colony-forming units (killing), compared with colony-forming units from control wells that contained all reagents except human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum. The lowest opsonophagocytic titer that could be measured by our method was 24, the final dilution of human serum.

Statistical analyses. Differences in clinical characteristics of patients were evaluated by Student’s t test or Wilcoxon rank sum test, depending on whether the data were normally distributed. Differences in antibody concentrations between colonized and uncolonized patients were evaluated by Student’s t test after the log transformation of data. Comparison of log-transformed serum antibody concentrations 2 and 4 months before the acquisition of pneumococcal colonization was performed using a paired t test. Logistic regression analysis was performed to determine which variables were independently and significantly associated with the acquisition of pneumococcus. For all comparisons, P < .05 was considered to be significant. Statistical analyses were performed using SPSS 11.0 for Macintosh (release 11.0.4; SPSS) and PRISM for Macintosh (version 4.0a; Prism).

RESULTS

Patient characteristics. Table 1 lists characteristics of the 29 patients who were colonized by S. pneumoniae at some time during follow-up, compared with 22 patients who remained free of S. pneumoniae colonization during follow-up as based on monthly sputum cultures. The 2 groups of patients were quite similar in clinical characteristics, including severity of underlying lung disease and duration in the study.

Serum anti-capsular pneumococcal antibodies. We determined serum anti-capsular antibody concentrations to serotype 6B, 7F, 14, 19F, and 23F in a subset of patients who did (n = 16) or did not (n = 13) develop respiratory tract colonization during the study period. As shown in figure 1A, for serotypes 6B, 7F, and 19F, the serum antibody concentration was significantly higher in the colonized group than in the uncolonized group (P < .001 for 6B and 7F; P < .05 for 19F). Tended to be higher for serotype 23F (P = 0.066), and was not significantly different for serotype 14 (P = 0.13). Furthermore, preacquisition serum samples from patients who subsequently became colonized with pneumococcus did not have, on average, lower antibodies to the specific colonizing serotype than did control subjects. As shown in figure 1B, 6 of 7 patients who became colonized with, for example, a type 6B strain had higher preacquisition anti–type 6B antibody concentrations than the average concentration in control subjects. Thus, preacquisition anti-capsular antibody concentrations in colonized patients were not lower than those of control patients.

Anti-type 6B opsonophagocytic antibodies. Next, we determined whether differences in opsonophagocytic antibodies could be appreciated between colonized and control subjects. Therefore, we measured OPA directed against the most commonly acquired serotype in our data set. As shown in figure 1C, among the colonized group, 7 patients were colonized at one point with a type 6B strain of pneumococcus. OPA against type 6B was determined in the 2-month preacquisition serum samples for these 7 colonized patients and compared with 12 serum samples from control patients. Overall, 2 of 7 colonized and 7 of 12 control patients had no detectable OPA against
type 6B in our assay (assigned an OPA titer of 12). Overall, the colonized group had a trend toward higher OPA titers than the uncolonized group (mean OPA titer 1515 vs. 565; \( P = .068 \), Student’s \( t \) test of log-transformed data).

**Serum antibodies to noncapsular antigens.** Having found no evidence of higher antibody concentrations or OPA among patients who escaped detectable colonization, we considered the hypothesis that the control group may have been protected by higher concentrations of antibody to noncapsular antigens. To evaluate this hypothesis, we measured antibodies to several noncapsular antigens using ELISA: CPS, PspA, Ply, and the whole unencapsulated organism (after the absorption of antibodies directed against CPS, as described in Patients, Materials, and Methods). As shown in figure 2, antibodies to these noncapsular antigens were either no different (as in the case of CPS or Ply) or were significantly higher (for the whole organism and PspA) in the colonized group, compared with the control group. There was significant correlation between IgG antibodies to the whole cell and PspA (Pearson’s \( R = 0.572; P < .001 \)) but little to no correlation between these antibodies and antibodies to Ply or CPS (\( P > .05 \)). By logistic regression analysis, a higher concentration of antibodies to the whole cell (after absorption with CPS) was significantly \( (P = .005) \) associated with pneumococcal colonization; antibodies to CPS, Ply, or PspA were not independent predictors of colonization \( (P > .05) \).

**Evaluation of serum anti-pneumococcal antibody 4 months before the acquisition of pneumococcus.** A potential explanation for the paradoxical finding that patients who develop pneumococcal colonization have higher concentrations of preacquisition antibody to pneumococcal noncapsular antigens is that acquisition had in fact already occurred but had not been detected by the 2 previous sputum cultures. To examine this possibility, we analyzed serum antibody titers from colonized patients 4 months before the acquisition of the pneumococcal strain. Because we found that antibodies to the whole cell were significantly associated with pneumococcal colonization, we compared these serum antibody concentrations at 2 and 4 months before the detection of pneumococcus by paired-samples \( t \) test; there was no difference in antibody concentrations at the 2 time points (mean \( \pm SE \) antibody concentrations at 2 vs. 4 months, 18,036 \( \pm 5653 \) vs. 15,943 \( \pm 4057 \); \( P = .45 \)), arguing against a recent boosting event. Furthermore, even 4 months before acquisition, colonized patients had significantly higher antibody concentrations against the whole cell than did uncolonized patients \( (P < .001, t \) test).

**DISCUSSION**

Several vaccines based on purified capsular polysaccharide have provided protection against invasive *S. pneumoniae* disease in adults via an antibody-dependent mechanism. Moreover, capsular polysaccharide–protein conjugate vaccines used in infants and toddlers have been shown to reduce colonization by vaccine-type pneumococcal strains. This reduction in nasopharyngeal colonization has had a dramatic impact on the epidemiology of pneumococcal disease: the conjugate vaccine in the United States has prevented more than twice as many cases of invasive pneumococcal disease through indirect effects on pneumococcal transmission (i.e., herd immunity) as through its direct effect of protecting vaccinated children [21]. Although highly effective against vaccine serotypes, this vaccine strategy may eventually be limited by the phenomenon of serotype replacement [10, 22–24], limited serotype coverage, high cost, and difficulties in production that have led to periods of shortage since licensure. A better understanding of the mechanisms that underlie immunity to pneumococcal colonization could
Figure 1. Serum anti-capsular antibody concentrations in patients with chronic obstructive pulmonary disease (COPD) with and without pneumococcal colonization. A, Serum anti-capsular IgG directed against capsular serotypes 6B, 7F, 14, 19F, and 23F compared between patients who did not (white columns) and did (black columns) acquire pneumococcus during the study period. Serum samples from the colonized patients were obtained 2 months before the acquisition of pneumococcal colonization. Patients who acquired pneumococcus had significantly higher concentrations of antibodies to types 6B, 7F, and 19F than patients who did not acquire a pneumococcal strain. There was a trend toward higher antibody concentrations to serotype 23F ($P = .066$) and no difference in type 14 antibody concentrations ($P = .16$). *$P < .05$; **$P < .001$. B, Ratio of specific anti-capsular antibody concentration of colonized patients to mean anti-capsular antibody of control subjects. For patients colonized with 1 of the 5 tested capsular types (6B, 7F, 14, 19F, and 23F), we compared antibody concentrations of the specific serotype with the mean concentration of antibody against that serotype in the control population. The ratio of these 2 values was plotted. A value of 1 (dotted line) signifies that the individual’s anti-capsular antibody concentration is equal to the mean of the control population. In 11 of 15 cases, this ratio was $>1$. C, Anti–type 6B opsonophagocytic titers measured in selected patients who did not acquire pneumococcus ($n = 12$; white squares) and patients who developed respiratory tree colonization with a type 6B strain ($n = 7$; black triangles). The preacquisition opsonophagocytic activity (OPA) titers tended to be higher in the colonized group than in the noncolonized group ($P = .068$). The dashed line represents the lower limit of detection of this assay (OPA titer of 24; patients with undetectable OPA were assigned a titer of 12).

pave the way for the development of alternative, or complementary, species-specific pneumococcal vaccines.

Although the ability of conjugate vaccine–induced anti-capsular antibodies to protect against pneumococcal colonization is clear, less is known about the natural development of immunity to pneumococcal colonization. Previously, several investigators have evaluated the relationship between naturally acquired antibodies to pneumococcus and colonization. The prevalence and duration of colonization decrease with age [25], and many antibody responses increase with age [26, 27]. Although several studies have documented a homotypic anti-capsular serum antibody response to colonizing pneumococcal serotypes [28–31], there is little direct evidence that such naturally acquired antibody represents the mechanism whereby humans become resistant to pneumococcal colonization. In fact, in the sole published example of experimental pneumococcal colonization of humans, antibodies to the capsular polysaccharide did not predict protection against colonization [32]. In a longitudinal study examining the relationship between antibodies and carriage in adults, Goldblatt et al. [31] showed that, among 6 serotypes tested, only anti-capsular antibody concentration to serotype 14 was significantly associated with reduced odds of carriage.

In the present study, we sought to evaluate whether any association could be demonstrated between serum anti-pneumococcal antibodies and lower respiratory tract colonization in an adult patient population at risk for pneumococcal infection. At the start of the present study, we had hypothesized that patients with higher serum antibodies to capsular and/or noncapsular pneumococcal antigens would be less likely to acquire a new strain of pneumococcus over the course of the defined study period. However, no such association could be demonstrated. To the contrary, patients in the colonized group had, on average, higher antipneumococcal antibody concentrations than control subjects in the uncolonized group, even when individuals’ antibodies to the colonizing capsular type were examined. Patients in the colonized group had significantly higher serum anti-capsular antibody concentrations to
several of the tested serotypes; in no case was their antibody concentration lower than that in the control group. Furthermore, the higher OPA titer in patients subsequently colonized with serotype 6B suggests that colonization is not the result of lower functional serum anti-capsular antibody concentrations. Overall, our results do not support the hypothesis that higher levels of serum antipneumococcal antibodies protect patients with COPD from the acquisition of pneumococcus in the respiratory tree; instead, the higher antipneumococcal (capsular and noncapsular) antibody concentrations may simply reflect more-frequent pneumococcal colonization, as has been suggested by others [33–35]. Our data thus suggest that factors other than serum antibodies contribute to resistance to pneumococcal colonization in this study population.

What might some of these factors be? Our study was limited by the absence of any mucosal antibody data, which may be conferring protection against colonization. As vaccine studies have suggested, however, salivary antibody concentrations generally correlate with serum antibody concentrations [36], making it seem unlikely that resistance to colonization in these patients with COPD is mediated by mucosal antibodies. Using animal models, our group and that of J. Weiser (University of Pennsylvania, Philadelphia) have shown that, independently of antibody, CD4+ T cells contribute to resistance to pneumococcal colonization [17, 37, 38] or clearance of carriage [39, 40]. Whether this phenomenon is also occurring in humans is unclear at present, but it is tempting to speculate that acquired cellular immune responses to pneumococcus may be contributing to naturally acquired resistance to colonization. Such a possibility has been recently supported by a study that found that peripheral blood mononuclear cells (PBMCs) from children colonized with pneumococcus had significantly lower CD4+ T cell proliferative responses to pneumolysin than PBMCs obtained from noncolonized children [41]. Other factors, such as differences in exposure to colonized children, innate immune responses, and interspecies bacterial competition [42], may also be responsible.

Our study has some limitations. First, although all patients in the study received the pneumococcal polysaccharide vaccine, the timing of this immunization (as well as number of previous doses) is unknown. Although the timing of previous immunization could certainly affect the antibody concentration to capsular (as well as noncapsular [43, 44]) antigens, this would not invalidate the finding that higher levels of antibody did not appear to correlate with enhanced protection against pneumococcal colonization. Second, no information was available regarding the prior colonization status of these patients with COPD. Patients in the uncolonized group may have been colonized with pneumococcus just before or after the study had been completed. However, such a possibility would not explain the higher antibody concentrations found in colonized patients. Third, we did not evaluate IgA antibodies to pneumococcus, which could play a role in the prevention of pneumococcal colonization [45]. Finally, because our study population included only patients with COPD in whom we evaluated the presence of pneumococcus in expectorated sputum, our findings may not be generalizable to other populations, including healthy younger adults or children.

In summary, we found that higher levels of antipneumococcal antibodies do not correlate with protection from pneumococcal colonization. Factors other than serum antibodies are therefore likely to contribute to protection against pneu-
coccal respiratory tree colonization in this high-risk population. Further elucidation of the mechanisms of protection against pneumococcal colonization in humans may lead to the development of novel strategies for the prevention of pneumococcal disease in high-risk groups.

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References


