Pneumolysin is an important virulence factor of Streptococcus pneumoniae. This study examined the hypothesis that human antibody to pneumolysin provides protection against pneumococcal infection. At the time of hospital admission, patients with nonbacteremic pneumococcal pneumonia had higher levels of serum anti-pneumolysin IgG than did patients with bacteremic pneumococcal pneumonia or uninfected control subjects. IgG levels rose significantly during convalescence in patients with bacteremic pneumonia, reaching levels observed in nonbacteremic patients. Purified human anti-pneumolysin IgG protected mice against intraperitoneal challenge with S. pneumoniae types 1 or 4 in a dose-related fashion; mice that received anti-pneumolysin IgG had a greater likelihood of surviving challenge and had negative blood cultures. Pneumolysin damages epithelial cells and inhibits phagocytic function of polymorphonuclear leukocytes. One hypothesis that might explain the study results is that, early in infection, IgG to pneumolysin blocks these effects in the alveoli, thereby protecting the host against bacteremic pneumococcal disease.

Strong evidence implicates pneumolysin, a thiol-dependent toxin that is nearly uniformly present in Streptococcus pneumoniae, as an important contributor to pneumococcal virulence. Instillation of pneumolysin into the bronchi of rodents causes acute inflammatory changes of bacterial pneumonia [1], genetically engineered pneumococcal mutants that lack pneumolysin have greatly diminished capacity to cause disease [2], and immunization with pneumolysin enhances survival of mice after pneumococcal challenge [3].

Historically, studies of immunity to S. pneumoniae have focused on antibody to pneumococcal capsular polysaccharide (CPS). Some persons, however, fail to make IgG to most CPS [4], but they remain free of pneumococcal infection. Others may be at risk of pneumococcal infection because they are colonized with S. pneumoniae and generate no anti-CPS IgG or generate IgG that is poorly functional [5], yet resist infection. It seems reasonable to postulate that naturally acquired antibody to a noncapsular constituent of pneumococcus may contribute to this resistance. Here, we describe clinical and laboratory observations that provide further evidence that IgG to pneumolysin may protect humans against pneumococcal infection.

Subjects and Methods

Subjects. Healthy middle-aged adults (50–64 years old) were volunteers in a study of pneumococcal vaccination. They had no recognized medical condition and took no medications that might interfere with normal immune responses. Subjects with stable chronic obstructive pulmonary disease had medical histories consistent with this diagnosis and confirmation by pulmonary function studies. Some were receiving treatment or were previously treated with glucocorticosteroids. Colonized subjects were persons from whose respiratory secretions S. pneumoniae was grown but who had no symptoms, signs, or radiographic changes that suggested an acute bacterial infection and who showed no deterioration, even though they were not treated with antibiotics. Such persons usually had a sputum culture during evaluation of a relatively stable lung lesion, such as pulmonary fibrosis, emphysema, or malignancy.

Patients with bacteremic pneumococcal pneumonia [6] had a clinical presentation consistent with pneumonia, radiographic confirmation of a pulmonary infiltrate, and ≥1 blood culture that yielded S. pneumoniae; patients who did not survive ≥10 days were not included. Patients with nonbacteremic pneumococcal pneumonia [6] had many or all of the following signs or symptoms: a clinical presentation suggestive of pneumonia with cough, sputum production, subjective fever, and/or chills and physical findings of pneumonia. All had a distinct infiltrate on plain chest radiography. In every case, microscopic examination of sputum showed ≥20 white blood cells


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CONCISE COMMUNICATION

Protection against Bacteremic Pneumococcal Infection by Antibody to Pneumolysin

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per epithelial cell with numerous gram-positive coci in pairs and chains and no, or few, other bacterial forms. Sputum culture yielded \textit{S. pneumoniae} and no other likely bacterial pathogen, and \textgeq{}1 blood culture obtained before antibiotic therapy was negative. Genetic non-responders to CPS were healthy adults who failed to make IgG to CPS of \textit{S. pneumoniae} types 1 and 4, as well as most other CPS after vaccination with 23-valent capsular polysaccharide pneumococcal vaccine [4].

**ELISA.** Microtiter plates were coated overnight at 4°C with 1 \(\mu\)g/mL pneumolysoid B (provided by J. Paton, Adelaide, Australia). For wash steps, we used PBS containing 0.2\% Tween. For blocking steps, we used 2\% bovine serum albumin (Sigma) in PBS. Three dilutions of duplicate serum samples from 1 subject in each group were studied on a single plate. All plates included 6 three-fold dilutions of a human serum sample, in which the concentration of anti-pneumolysin antibody had been quantitated (provided by D. Briles, University of Alabama, Birmingham). Alkaline phosphatase-conjugated goat antibody diluted 1:6000 in PBS (Sigma Chemical) was used to detect human IgG.

**Isolation of IgG to pneumolysin.** IgG was purified from pre-vaccination serum of genetic nonresponders to pneumococcal vaccine, who lacked antibody to CPS 1 or 4 after vaccination, by fast performance liquid chromatography with the use of HiTrap protein A affinity columns (Amersham-Pharmacia Biotech), as recommended by the manufacturer. Pooled IgG fractions were subjected to affinity chromatography with a HiTrap NHS column (Amersham-Pharmacia Biotech). Pneumolysoid was dissolved to yield 10 mg in 1 mL of coupling buffer (pneumolysoid B in initial experiments; pneumolysoid PdD in final experiments; provided by J. Paton). Acid-eluted IgG fractions were immediately neutralized to pH 7.4 with 1 M NaOH. The pooled fractions contained 1.93 mg/mL IgG to pneumolysin and no detectable IgG to CPS of serotypes 1 or 4. IgG was concentrated with a Centricon-30 system (Amicon) to obtain desired final concentrations for injection into mice.

**Passive immunization.** Purified IgG to pneumolysin was diluted in saline, and 0.1-mL volumes were injected intraperitoneally (ip) into groups of 6–10 outbred Swiss white mice (weights, 17–18 g). Controls received 0.1 mL of saline and, in 1 experiment, additional controls received an unrelated antibody preparation. The IgG dose was selected to yield plasma IgG levels similar to those observed in humans, calculated by approximating blood volume at 7\% of body weight and plasma volume at 60\% of blood volume. Forty minutes later, mice were injected ip with 10 LD_{50} \textit{S. pneumoniae} type 1 or type 4. Colony-forming units were verified by dilution and plating at the end of the injection period. Mice were observed every 6 h for 7 days; no deaths occurred after that time. In one experiment, anti-	extit{Pseudomonas} serum (Sigma) was used as an additional, unrelated control.

**Statistics.** IgG concentrations were converted to log_{10}. After verifying that log-transformed data were normally distributed, we averaged the transformed data and carried out Student’s \textit{t} test. We report results as the anti-log of means.

**Results**

**Antibody to pneumolysin in human subjects.** Mean IgG to pneumolysin in the serum of healthy middle-aged adults was 2.24 \(\mu\)g/mL (figure 1). Antibody levels were similar in adults with stable chronic obstructive pulmonary disease (2.78 \(\mu\)g/mL; \(P = .2\)) but were higher in those colonized with \textit{S. pneumoniae} (4.22 \(\mu\)g/mL; \(P = .007\)). Serum IgG at the time of admission for nonbacteremic pneumococcal pneumonia was 5.75 \(\mu\)g/mL (\(P = .003\) vs. control subjects), and this level rose in convalescence to 6.72 \(\mu\)g/mL. Most striking was the finding that patients with bacteremic pneumococcal pneumonia had mean antibody to pneumolysin IgG of 1.93 \(\mu\)g/mL at admission, a value similar to that of uninfected control subjects (\(P = .82\)) but significantly lower than that for patients with nonbacteremic pneumococcal pneumonia (\(P = .04\)). In bacteremic patients, after \textgeq{}10 days, mean anti-pneumolysin IgG increased significantly to 6.14 \(\mu\)g/mL (\(P = .01\)).

**Mouse protection by IgG to pneumolysin.** In initial experiments, IgG to pneumolysin was isolated from high-titered pooled human serum by pneumolysoid B column. When tiny doses of the eluted IgG were found to protect mice (data not shown), we subjected the preparation to ELISA for IgG to CPS types 1 and 4. Sufficient concentrations of anticapsular antibody were deleted to account for the observed protective effect. This contamination with anti-CPS IgG was attributed to the capacity of pneumolysoid B to bind Fc of human IgG nonspecifically. Accordingly, we used 2 approaches to obtain IgG free of antibody to CPS and with the least possible contamination by IgG to other putative virulence factors. First, we coated the column used to extract anti-pneumolysin with pneumolysoid PdD that binds Fe only minimally; second, serum used to extract anti-pneumolysin IgG was devoid of antibody.
to the CPS under study. The extracted IgG did not react in an ELISA for IgG to CPS 1 or 4.

As shown in figure 2, prior injection of anti-pneumolysin IgG calculated to yield serum levels of 10 or 2.5 μg/mL protected mice against challenge with 40 cfu (10 LD₅₀) of S. pneumoniae type 4; mortality rate was 0% and 20%, respectively, versus 100% in recipients of saline or anti-Pseudomonas serum. A broader range of concentrations of anti-pneumolysin IgG was studied in mice that then were challenged with 40 cfu (10 LD₅₀) of S. pneumoniae type 4; some degree of protection was observed with IgG levels as low as 0.75 μg/mL (figure 2B).

Mechanism for the observed protection. Mice that were pretreated with anti-pneumolysin IgG to achieve 10 μg/mL and control mice were challenged ip with 10 LD₅₀ and were killed 12 h (3 each, treated and control mice) and 18 h (2 each, treated and control mice) after challenge. Heart blood of the anti-pneumolysin–treated mice contained no detectable pneumococci (<100 cfu/mL) vs. >10³–2×10⁷ cfu/mL in the blood of control mice.

Discussion

In recent years, the capacity of pneumolysin to initiate a broad array of pathologic changes has become increasingly apparent [7]. Pneumolysin inhibits ciliary action of respiratory epithelial [8] and ependymal [9] cells, damages the integrity of alveolar lining cells [10], and reduces migration and phagocytic function of polymorphonuclear leukocytes [11]. Pneumolysin also induces an acute inflammatory reaction [1]. Pneumococcal mutants that lack the capacity to make pneumolysin have greatly reduced virulence for mice, although careful studies have reached somewhat different conclusions regarding the relative contributions of cytotoxic and inflammatory capacities of pneumolysin to the final outcome of infection [2, 12]. In addition, mutant bacteria that lack autolysin are not as pathogenic, presumably because pneumolysin is not released to the external milieu. It seems reasonable to postulate that pneumolysin–induced damage to alveolar lining cells and/or arriving phagocytes might facilitate pneumococcal invasion, and antibody to pneumolysin might reduce the damage, thereby inhibiting invasion.

We found that nearly all adults have measurable levels of IgG to pneumolysin (authors’ unpublished data). In the present study, pneumococcal colonization was associated with higher antibody levels, which is consistent with the recent demonstration that colonization of children leads to emergence of anti-pneumolysin antibody [13]. Our finding that colonized subjects and patients with nonbacteremic pneumococcal pneumonia have substantially higher levels of IgG to pneumolysin than do patients with bacteremic pneumococcal pneumonia suggests that this antibody may play some role in protection. Although the possibility remains that anti-pneumolysin IgG is present in bacteremic patients but bound to pneumolysin that is present in the lung, it is opposed by the important study of Amdahl et al. [14], who showed an inverse association between the level of antibody to pneumolysin in banked preinfection serum samples and susceptibility to pneumococcal pneumonia among human immunodeficiency virus–infected patients.

On the basis of these findings, we studied the capacity of human IgG to protect mice against challenge with 2 representative mouse-virulent serotypes of S. pneumoniae. Alexander et al. [3] showed that immunization of mice with pneumolysin toxoid stimulates mean IgG levels of ~4.9 μg/mL and that these levels are associated with protection against challenge by ≥9 pneumococcal serotypes. In our study, this concentration of human IgG fully protected mice against ip challenge with 10

![Figure 2](image-url)
LD₉₀ S. pneumoniae type 1 or type 4, and levels as low as 0.75 μg/mL provided some protection. The finding that mice given IgG to pneumolysin did not have bacteremia is consistent with a contribution of IgG to local defenses against invasiveness in the peritoneal cavity or with enhanced capacity to clear organisms from the bloodstream [15]. Because pneumolysin is not expressed on bacterial surfaces and because the mechanisms cited above would explain the contribution of antibody to diminished invasiveness, this latter concept appears to provide the better explanation. Pneumonia is preceded by colonization, an event that sensitizes humans to CPS. Because there is no anamnestic response to polysaccharides, antibody to pneumolysin is likely to be present or to appear more rapidly than antibody to CPS. If aspiration or inhalation of pneumococci occurs soon after colonization, IgG to pneumolysin may provide partial protection until anti-CPS antibody appears. In persons who cannot generate anti-CPS IgG or whose IgG is poorly functional, antibody to pneumolysin may protect against pneumonia or at least reduce the likelihood of bacteremic disease. Despite these data, which appear to show the importance of pneumolysin as a virulence factor for S. pneumoniae, it is worth noting that naturally occurring pneumococcal disease of horses in the United Kingdom is caused by a clone that does not produce pneumolysin.

References