Nonspecificity of Assaying for IgG Antibody to Pneumolysin in Circulating Immune Complexes as a Means to Diagnose Pneumococcal Pneumonia

Daniel M. Musher,1,2 Rahul Mediwalav,1 Hoang M. Phan,1 George Chen,1,2 and Robert E. Baughn1,2,4

1Medical Service (Infectious Disease Section), Houston Veterans Affairs Medical Center, and Departments of 2Medicine, 3Microbiology/Immunology, and 4Dermatology, Baylor College of Medicine, Houston, Texas

Detection of immunoglobulin G (IgG) antibody to pneumolysin (PLY) in precipitated circulating immune complexes (CICs) has been used to diagnose pneumococcal pneumonia. With care to include appropriate controls, we precipitated and dissociated CICs and then assayed for IgG antibody to PLY. We detected IgG antibody to PLY in CICs that were precipitated from serum samples that were obtained at the time of admission to the hospital from 5 (23%) of 22 healthy adults, 7 (44%) of 16 subjects with stable chronic obstructive pulmonary disease, 10 (63%) of 16 subjects colonized with Streptococcus pneumoniae, and 9 (60%) of 15 patients with nonbacteremic pneumococcal pneumonia. Of the 16 patients with bacteremic pneumococcal pneumonia, 4 (25%) had IgG antibody to PLY at the time of admission, and 8 (50%) had IgG antibody to PLY in convalescence. Levels of IgG antibody in CICs closely correlated with serum levels of IgG antibody to PLY, implicating precipitation of free serum antibody in tests with false-positive results. Detection of IgG antibody to PLY in precipitated CICs is not a reliable method for diagnosing pneumococcal pneumonia.

In recent years, a surprising amount of controversy has surrounded the diagnosis of pneumococcal pneumonia. In the preantibiotic era, this diagnosis was not regarded as problematic. Lobar pneumonia was a common community-acquired infection, and 95% of cases were caused by Streptococcus pneumoniae [1]. In nonbacteremic cases, the causative organism was detected by means of sputum gram staining, culture, and, sometimes, mouse injection; the medical literature does not reflect much debate about the difficulty of establishing an etiologic diagnosis [1–4]. In contrast, despite the perception that most cases of community-acquired pneumonia are still caused by pneumococcus [5], some recent studies have successfully documented that this organism is the causative agent in relatively few cases (10%–30% of cases in some reports [5–9]).

The failure to diagnose pneumococcal pneumonia in the absence of positive blood culture results has motivated investigators to seek indirect ways of establishing the diagnosis, among which are the detection of rising titers of antibody to pneumolysin (PLY) [10–13], and, more recently, the detection of IgG antibody to PLY in circulating immune complexes (CICs) precipitated from serum [14–16]. Whereas rising titers of antibody to PLY were documented in a sequential study of individual subjects with bacteremic pneumococcal pneumonia by using baseline samples as controls [10], levels
of IgG antibody to PLY in CICs is not readily quantitated, and sequential studies of individual subjects have not been reported. To our knowledge, assaying for antibody to PLY in CICs has not been shown to be specific with use of age-matched controls, and the impact of pneumococcal colonization has not been studied. Notwithstanding, this technique has been the basis for diagnosis in recent epidemiological studies [17, 18] that found that pneumococcal vaccine was lacking in protective efficacy. In the present study, we precipitated CICs from serum samples that were obtained from patients with pneumonia and from appropriate control subjects, and we used accepted methods [14] to detect IgG antibody to PLY to gain insight into the diagnostic validity of this technique.

SUBJECTS AND METHODS

Subjects. The study included healthy young adults, healthy middle-aged adults, subjects with stable chronic obstructive pulmonary disease (COPD), subjects colonized with pneumococci, patients with bacteremic pneumococcal pneumonia, and patients with nonbacteremic pneumococcal pneumonia. The group of healthy, young adults (age range, 22–30 years) was comprised of medical students, interns, residents, or laboratory technicians who worked at or were rotating through the Veterans Affairs Medical Center in Houston. The healthy, middle-aged adults (age range, 50–64 years) were voluntary participants in a study of pneumococcal vaccination. Subjects in these 2 groups stated that they were in good health; no detailed medical history was obtained, and no physical examination was done. Subjects with stable COPD had medical histories and physical examinations that were consistent with this diagnosis, which was confirmed by the performance of pulmonary function studies. Colonized subjects were persons from whose sputum S. pneumoniae was isolated, but who had no symptoms, signs, or radiographic changes that suggested acute bacterial infection, who had received no treatment for pneumococcal infection, and who had no deterioration in their condition that was consistent with untreated bacterial disease. We usually obtained sputum samples for culture from such persons during evaluation of a relatively stable lung lesion, such as pulmonary fibrosis, emphysema, or malignancy.

Patients with bacteremic pneumococcal pneumonia [19] had a clinical presentation that was consistent with pneumonia, had radiographic confirmation of a pulmonary infiltrate, and had ≥1 blood cultures that yielded S. pneumoniae. Patients with nonbacteremic pneumococcal pneumonia [19] had all or nearly all of the following: clinical presentation suggestive of pneumonia with cough, sputum production, subjective fever and/or chills, physical findings of pneumonia, and a distinct infiltrate that appeared on a plain chest radiograph. For every patient, microscopic examination of sputum showed ≥20 WBCs per epithelial cell with large numbers of gram-positive cocci in pairs and chains and no or rare other bacterial forms. Sputum culture yielded S. pneumoniae and no other likely bacterial pathogen, and the results of ≥1 cultures of blood specimens obtained before antibiotic therapy was begun were negative. This study utilized convenience samples from frozen stores (–70°C).

Isolation and dissociation of CICs. The method of Leinonen et al. [14] was followed with precise adherence to previously reported protocol. Equal volumes (100 μL) of 7% polyethylene glycol (PEG) in borate buffer (pH 8.2) and serum were incubated overnight at 4°C (final concentration of polyethylene glycol, 3.5%). The resulting precipitate was collected by centrifugation at 1500g at 4°C, washed twice with 3.5% polyethylene glycol in borate buffer (pH 8.2), and then dissociated with 100 μL of borate buffer (pH 12); this suspension was allowed to stand for 1 h after which time it was used in the ELISA.

ELISA. Wells of microtiter plates (Immuno II, Dynatech) were coated with 1 μg/mL of a mutant form of PLY (pneumolysoid B [obtained from James C. Paton, Women’s and Children’s Hospital, Adelaide, South Australia, Australia]) in PBS/mL [14, 20]. Pneumolysoid B is a recombinant protein toxoid derivative of PLY that is expressed in Escherichia coli [21]. Plates were incubated overnight at 4°C. Wells were washed after this step and all subsequent steps with PBS containing 0.2% polysorbate. Plates were blocked with PBS containing 2% bovine serum albumin for 1 h at 37°C. Duplicate samples of sera (in 3 dilutions [1:100, 1:300, and 1:900] in PBS [pH 8.5]) and resuspended dissociated immune complexes (at dilutions of 1:50, 1:100, and 1:300 in PBS [pH 7.2]) from each subject were studied on a single plate. Every plate contained 6 3-fold dilutions of a laboratory reference standard serum with a known concentration of antibody to PLY (obtained from David Briles, University of Alabama, Birmingham).

Alkaline phosphatase–conjugated goat antibody (suspended at a dilution of 1:6000 in PBS; Sigma Chemical) was used to detect human IgG antibody. The reaction was developed in the dark for 20 min at 37°C, with p-nitrophenol phosphate in 0.2 M Tris buffer (pH 9.7). Absorbance was determined by use of an ELISA reader (Dynatech) at 405 nm; the absorbance of wells coated with PBS to which all reagents had been sequentially added was subtracted from every value. Some control wells on each plate had all reagents added except diluted serum; antibody to PLY was judged to be present only if the final absorbance exceeded that for these control wells.

Statistical analysis. The Student’s t test was performed. Correlation between sets of continuous variables was assessed by use of Excel 5.0 (Microsoft).
RESULTS

IgG antibody in dissociated CICs. In every group of subjects that we studied, $\geq 2$ individuals (\$\geq 11\%\$) had detectable IgG antibody to PLY in precipitated and dissociated CICs (table 1). Such IgG antibody was detected in serum samples from 2 (11\%) of 18 healthy young adults, 5 (23\%) of 22 healthy middle-aged adults, 7 (44\%) of 16 persons with stable COPD, and 10 (63\%) of 16 uninfected patients who were colonized with S. pneumoniae. Of the 15 patients with nonbacteremic pneumococcal pneumonia, 9 patients (60\%) who were in the acute phase of infection had IgG antibody to PLY, and 10 patients (67\%) who were in convalescence had IgG antibody to PLY. It is interesting that of 16 patients with bacteremic pneumococcal pneumonia, 4 patients (25\%) who were in the acute phase of infection had IgG antibody to PLY, and 8 patients (50\%) who were in convalescence had IgG antibody to PLY.

The mean levels of IgG antibody to PLY for those subjects who had measurable levels of antibody in CICs followed a similar pattern (table 1). Most important was the finding that levels in colonized persons were identical with those for patients who had recovered from pneumococcal pneumonia, whether bacteremic or nonbacteremic. The mean level of IgG antibody to PLY was somewhat lower in the samples obtained from patients with bacteremic pneumococcal disease at the time of admission than in those from age-matched controls (\(P > .05\)).

In precipitated CICs from serum samples from 22 healthy middle-aged adults who were controls, the level of IgG antibody was $>2$ SDs above the mean level for that group in 1 adult (5\%), compared with 3 patients (19\%) in the group with COPD, 5 patients (31\%) in the group with pneumococcal colonization, and 5 patients (33\%) in the group with nonbacteremic pneumococcal pneumonia. Of those patients with bacteremic pneumococcal pneumonia, none in the acute phase of infection had levels of IgG antibody to PLY in CICs $>2$ SDs above the mean level, although 6 patients (38\%) who were in convalescence did.

Association between IgG antibody to PLY in CICs and serum. To examine the hypothesis that free serum IgG antibody precipitates along with CICs, we sought to relate the concentrations of antibody to PLY in serum to those in preparations of CICs. There was a significant correlation between the level of IgG antibody detected in serum and that in dissociated CICs when analyzed for individual subjects (figure 1A; \(r = .44\); \(P < .001\)) or for the mean levels for each group of subjects (figure 1B; \(r = .78\); \(P < .05\)).

DISCUSSION

By documenting the presence of IgG antibody to PLY in preparations of dissociated CICs precipitated from serum samples from persons without pneumococcal pneumonia, the present study shows that this technique cannot be used as a means

<table>
<thead>
<tr>
<th>Subject group</th>
<th>No. of subjects</th>
<th>No. (%) of subjects with IgG antibody in CICs</th>
<th>Mean level of IgG antibody in CICs, $\mu$g/mL</th>
<th>No. (%) of subjects with level of IgG antibody $&gt;2$ SD above mean level for control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Young (age range, 22–30 y)</td>
<td>18</td>
<td>2 (11)</td>
<td>0.20</td>
<td>—</td>
</tr>
<tr>
<td>Middle-aged (age range, 50–64 y)</td>
<td>22</td>
<td>5 (23)</td>
<td>0.42</td>
<td>1 (5)</td>
</tr>
<tr>
<td>Colonized or infected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD</td>
<td>16</td>
<td>7 (44)</td>
<td>0.45</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Pneumococcal colonization</td>
<td>16</td>
<td>10 (63)</td>
<td>0.73</td>
<td>5 (31)</td>
</tr>
<tr>
<td>Pneumococcal pneumonia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonbacteremic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute infection</td>
<td>15</td>
<td>9 (60)</td>
<td>0.54</td>
<td>5 (33)</td>
</tr>
<tr>
<td>Convalescence</td>
<td>15</td>
<td>10 (67)</td>
<td>0.74</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Bacteremic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acute infection</td>
<td>16</td>
<td>4 (25)</td>
<td>0.28</td>
<td>0</td>
</tr>
<tr>
<td>Convalescent</td>
<td>16</td>
<td>8 (50)</td>
<td>0.71</td>
<td>6 (38)</td>
</tr>
</tbody>
</table>

NOTE. COPD, chronic obstructive pulmonary disease.

$^*$ For persons with detectable IgG antibody to pneumolysin in preparations of CICs.
to diagnose pneumococcal pneumonia. By use of a technique identical to that previously described by Leinonen et al. [14], we found such antibody in dissociated CICs obtained from 23% of healthy middle-aged adults, 44% of patients with COPD, and 63% of persons colonized with S. pneumoniae, compared with 60% of patients who had tests performed at the time of admission for nonbacteremic pneumococcal pneumonia. Complexes extracted from serum samples obtained from patients at the time of admission for bacteremic pneumococcal pneumonia were less likely to contain IgG antibody to PLY than were those from patients with nonbacteremic infection, and when detected, such antibody was present at lower concentrations. Among patients who were convalescent, however, differences between those with nonbacteremic and those with bacteremic pneumococcal pneumonia were not detected. These findings support the observation, that in bacteremic patients, levels of IgG antibody to PLY are low or zero [22]; detection of higher levels of IgG antibody in CICs from patients who recovered from bacteremic infection paralleled higher serum IgG levels. In fact, the best explanation for the detection of IgG antibody to PLY in preparations of complexes seems to be that serum IgG antibody is precipitated along with CICs. Thus, for the groups that were studied, there was a good association between sera that contained higher concentrations of IgG antibody and those with such antibody detected in the precipitated CICs.

Well-controlled studies [10, 11, 13] have described a 2-fold increase in titers of antibody to PLY in 83% of patients with bacteremic pneumococcal pneumonia and in 45%–82% of those with nonbacteremic pneumococcal pneumonia. However, to our knowledge, none of these reports addressed the possibility that pneumococcal colonization might stimulate antibody to PLY. Detection of IgG antibody to PLY in dissociated CICs was subsequently proposed as a valid means to diagnose pneumococcal pneumonia. Leinonen et al. [14] detected PLY-specific IgG antibody in CICs from 11 of 11 patients with bacteremic pneumococcal pneumonia, and Porath et al. [23] demonstrated a sensitivity of 88% for this technique for the diagnosis of bacteremic disease in children. Leinonen et al. [14] did not present data for age-matched control subjects or persons with stable COPD, and neither study presented data for persons that were colonized with S. pneumoniae.

Diagnosis of pneumococcal infection by detection of IgG antibody to PLY in precipitated CICs has had a substantial impact on the interpretation of recent studies of pneumococcal vaccine. Ortqvist et al. [18] administered either pneumococcal vaccine or a placebo to nearly 700 Swedish adults who had been recently hospitalized for pneumonia; bacteremic pneumococcal pneumonia subsequently occurred in 1 vaccinated subject and 5 control subjects, which is consistent with a protective effect of vaccine against bacteremic disease. In large part by their use of serological techniques, however, these investigators identified a total of 35 cases of pneumococcal pneumonia (19 in vaccine recipients and 16 in control subjects) and concluded that the vaccine offered no benefit.

In a study of 2800 Finnish adults, Koivula et al. [17] found the same incidence of pneumococcal pneumonia among persons who received pneumococcal and influenza vaccines as among those who received influenza vaccine alone. In this study, all diagnoses were made serologically. Of those persons in whom pneumococcal pneumonia was diagnosed, only 18%
had 2-fold increases in titers of antibody to PLY; in 89% of the patients, CICs in acute-phase or convalescent-phase serum samples contained IgG antibody to PLY. These tests yielded concordant results in only 7% of cases, and most diagnoses (81%) were based on the detection of IgG antibody in precipitated CICs. If rising levels of antibody to PLY are 45%–83% sensitive in the diagnosis of pneumococcal pneumonia, another technique could not serve as the sole method for making the diagnosis in >80% of cases. Finally, if this serological technique were valid, the annual incidence of pneumococcal pneumonia among persons >60 years of age in Varkaus, Finland, would be 700–800 cases per 100,000 persons, which far exceeds the rate of infection reported elsewhere in Europe or Scandinavia.

In conclusion, by studying appropriate control subjects, we have found that assaying for IgG antibody to PLY in CICs, as it has been used to date, is not a valid method for diagnosing pneumococcal pneumonia. These findings raise substantial questions about recent reports that claim to show the lack of efficacy of pneumococcal vaccine. They should also encourage the use of more specific means to diagnose pneumococcal infection in future studies.

**References**


3. Finland M, Sutliff WD. Infections with pneumococcus type III and type VIII: characterization of pneumonia caused by pneumococcus type III and that associated with a biologically closely related organism, pneumococcus type VIII. Arch Intern Med 1934; 53:481–507.


