NOTES

Activation of Human Complement by the Pneumococcal Toxin Pneumolysin

JAMES C. PATON,¹* BRENTON ROWAN-KELLY,² AND ANTONIO FERRANTE²

Department of Microbiology¹ and University Department of Paediatrics² Adelaide Children’s Hospital, North Adelaide, South Australia, 5006 Australia

Received 7 November 1983/Accepted 6 December 1983

Highly purified pneumolysin (at a concentration of 10 μg/ml) caused significant activation of human complement, as measured by conversion of C3. Complement activation in the presence of pneumolysin was not observed in sera chelated with a combination of Mg²⁺ and ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid, and activation was only slight in C2-deficient sera. This suggests that the toxin is capable of activating the classical complement pathway. Treatment of normal human serum with pneumolysin also significantly reduced its opsonic activity for Streptococcus pneumoniae.

There is substantial evidence that serum complement plays an important role in host resistance to pneumococcal infections (4, 6, 16). Maximal opsonization of pneumococci requires an intact classical and alternate complement pathway, and thus complement deficiency can result in an inability to clear these organisms (6, 8, 16). Several workers (3, 12) have reported diminished serum complement levels in some individuals with pneumococcal infections and have suggested that during an infection, pneumococcal antigens may activate complement to such an extent that serum opsonic activity is compromised.

A number of serotypes of pneumococci have been shown to be capable of activating both complement pathways (2, 11). Cell wall teichoic acid is known to be an important activator of the alternate pathway (17), but the pneumococcal antigen(s) responsible for mediating classical pathway activation has not yet been identified. Interestingly, purified capsular polysaccharides have been shown to be only weak activators of the classical complement pathway and are effective only at high concentrations (500 to 1,000 μg/ml) (2).

We have been examining the role of pneumolysin, a sulphydryl-activated cytolytic toxin produced by Streptococcus pneumoniae, in this organism’s pathogenicity. Previous studies have shown that highly purified pneumolysin inhibits the antimicrobial activity of human polymorphonuclear leukocytes (PMNLs) (9) and also that immunization of mice with the toxin increased their survival time after challenge with virulent pneumococci (10). In the present study, we examined the ability of highly purified pneumolysin to activate human complement in vitro. The effect of pneumolysin treatment on the opsonic activity of serum was also investigated.

Pneumolysin was purified from a type 1 strain of S. pneumoniae and assayed as previously described (10). The final preparation migrated as a single major protein species (which accounted for greater than 97% of total protein) when analyzed by polyacrylamide gel electrophoresis in either the presence or absence of sodium dodecyl sulfate. The purified material had a specific activity of at least 500,000 hemolytic units per mg of protein (10) (1 hemolytic unit/ml will lyse 50% of a 1% suspension of human erythrocytes in 30 min at 37°C). Total protein was measured by the method of Bradford (1).

Complement activation was assessed by measuring conversion of C3 by two-dimensional crossed immunoelectrophoresis with goat anti-human C3 (Cappel Laboratories, Cochranville, Pa.). The electrophoresis was carried out in 1% agarose with 75 mM barbitone–2 mM calcium lactate buffer, pH 8.6. The first dimension was electrophoresed at 5 V/cm for 4 h, and the second dimension was run at 2 V/cm overnight. After washing, the plates were dried and stained with Coomassie brilliant blue R-250. C3 conversion was quantitated by measurement of the area under the peaks.

When samples of normal (pooled) human serum were incubated at 37°C for 60 min in the presence of pneumolysin (10 μg/ml), 17.8% C3 conversion occurred (Table 1). Conversion was reduced proportionally when lower toxin concentrations were used (result not shown). The conversion observed at 10 μg of pneumolysin per ml was not due to the hemolytic activity of the toxin, because even greater C3 conversion (26.6%) was observed in serum treated with pneumolysin that had been Inactivated by prior exposure to cholesterol (1 μg of cholesterol per μg of toxin) (Table 1). This enhancement was statistically significant (P < 0.02, by paired t test).

No C3 conversion was observed when sera were treated with 10 μg of pneumolysin per ml in the presence of either 5 mM EDTA or a combination of 0.35 mM MgCl₂ and 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (EGTA) (Table 1). This suggests that pneumolysin causes complement activation via the classical pathway. This is supported by the finding that incubation of serum from a patient genetically deficient in C2 (15) with pneumolysin resulted in very little (only 3%) C3 conversion (Table 1).

The apparent activation of the classical complement pathway may have been due to the presence of specific antibody in the sera, although only very low levels of antibody to the purified pneumolysin could be detected with an enzyme-linked immunosorbent assay (results not shown).

To examine the potential biological significance of activation of complement by pneumolysin, we tested the opsonic activity of the sera for S. pneumoniae. A type 1 strain of S. pneumoniae was suspended to a concentration of 3.0 × 10⁹/ml in Hanks balanced salt solution supplemented with

* Corresponding author.
TABLE 1. C3 Conversion in serum treated with pneumolysin

<table>
<thead>
<tr>
<th>Serum treatment</th>
<th>C3 conversion (%) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum + 10 µg of pneumolysin per ml</td>
<td>17.8 ± 1.5</td>
</tr>
<tr>
<td>Normal serum + 10 µg of pneumolysin pretreated with 26.6 ± 1.4 cholesterol per ml</td>
<td></td>
</tr>
<tr>
<td>Normal serum + 10 µg of pneumolysin per ml + 5 mM Mg++</td>
<td>0</td>
</tr>
<tr>
<td>EGTA + 0.35 mM Mg++</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0</td>
</tr>
<tr>
<td>C2-deficient serum + 10 µg of pneumolysin per ml</td>
<td>3.0 ± 0.8</td>
</tr>
</tbody>
</table>

*Normal human serum or C2-deficient serum was incubated at 37°C for 60 min with the indicated additions. Conversion of C3 was measured by crossed immunoelectrophoresis as described in the text and expressed as a percentage, after correction for background C3 conversion present in the appropriate control (serum which was incubated without additions). The results shown represent the means of three experiments ± standard error of the mean.

33% normal human serum that had been pretreated with or without 10 µg of pneumolysin or cholesterol-inactivated pneumolysin per ml. After incubation at 37°C for 30 min, the *S. pneumoniae* cells were harvested by centrifugation and resuspended in Hanks balanced salt solution without added serum. Portions of these suspensions containing 10⁶ *S. pneumoniae* cells were added to 10⁵ human polymorphonuclear leukocytes (PMNLs) (prepared as described previously [9]) in a final volume of 0.5 ml of Hanks balanced salt solution. The mixtures were incubated at 37°C with end-to-end shaking in an atmosphere of 95% air–5% CO₂, and at various times samples were withdrawn, diluted in water, and plated on blood agar to determine the number of viable *S. pneumoniae*. The rate at which the *S. pneumoniae* were engulfed and killed by the PMNLs was used as a measure of the opsonic activity of the respective serum (Fig. 1). When *S. pneumoniae* cells were preopsonized with control serum, they could be readily engulfed and killed by the PMNLs, with only 22% of the original number of bacteria remaining viable after 60 min. However, when the bacteria were preopsonized with sera treated with either pneumolysin or cholesterol-inactivated pneumolysin, very little decrease in the number of viable *S. pneumoniae* occurred during the subsequent incubation with the PMNLs.

Thus, pneumolysin-induced C3 conversion could be correlated with a significant inhibition of serum opsonic activity—an event likely to be of considerable biological significance. The consequences of complement activation by a free circulating toxin during a pneumococcal infection could also include generalized inflammation, resulting from generation of the anaphylatoxic peptides C3a and C5a. This may be of particular importance in pneumococcal pneumonia, since administration of C3a and C5a has been shown to induce lethal pulmonary injury in guinea pigs (13).

Johnson et al. (7) have shown that very high concentrations of pneumolysin (ca. 500 µg/ml) activated human serum to become chemotactic for neutrophils. This result could perhaps be explained by our present findings, since C5a generated by complement activation is also chemotactic. Interestingly, classical complement pathway activation has also been reported for another (unrelated) membrane-active toxin, the staphylococcal alpha-toxin (5). This activation occurred at toxin concentrations similar to those used in the present study and also was not dependent on the hemolytic activity of the toxin.

Although a rise in antibody to pneumolysin has been reported in a proportion of patients with pneumococcal pneumonia (14), the actual amount of the toxin produced during an infection is not known. Measurement of circulating pneumolysin levels is complicated by inhibition of the hemolytic activity of the toxin by serum cholesterol. A highly significant finding of the present study, however, is that the activation of complement by pneumolysin was actually enhanced by cholesterol treatment.

This work was supported by grants from the National Health and Medical Research Council and the Adelaide Children’s Hospital Research Trust.

**LITERATURE CITED**


