Differences in Clinical Manifestation of *Streptococcus pneumoniae* Infection Are Not Correlated with In Vitro Production and Release of the Virulence Factors Pneumolysin and Lipoteichoic and Teichoic Acids

Annette Spreer, Astrid Lis, Joachim Gerber, Ralf René Reinert, Helmut Eiffert, and Roland Nau

Departments of Neurology and Bacteriology, University of Göttingen, Göttingen, and Institute of Medical Microbiology, National Reference Center for Streptococci, University of Aachen, Aachen, Germany

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Production and release of the pneumococcal virulence factors pneumolysin and lipoteichoic and teichoic acid in 75 clinical isolates were investigated. No difference was found between strains causing systemic infection or localized respiratory infection and isolates from asymptomatic carriers. This suggests that the presence of pneumolysin and lipoteichoic and teichoic acid is a necessary but not a sufficient condition for pneumococcal infection and development of invasive disease.

*Streptococcus pneumoniae* strains are the most common worldwide cause of respiratory infections and are responsible for a wide range of invasive diseases such as sepsis and meningitis (9, 16).

*S. pneumoniae* often colonizes the nasopharynx of asymptomatic carriers. Appropriate culturing of throat swabs yields pneumococci from 5 to 10% of healthy adults and 20 to 40% of healthy children on a single occasion of examination (16). Spreading from the nasopharynx-endogenous infections is common. The mechanisms of invasion and the conditions necessary for a local or systemic infection are not fully understood. Unlike certain other organisms, such as *Streptococcus pyogenes*, which produces a variety of tissue-damaging substances, *S. pneumoniae* produces few toxins and largely causes disease via replication in host tissues and generation of an intense inflammatory response (16).

Among the proinflammatory virulence factors of pneumococci, the membrane-damaging cytosolic protein pneumolysin and bacterial capsule components lipoteichoic acid (LTA) and teichoic acid (TA) are of particular importance. Pneumolysin is released from the cytoplasm during bacterial growth and lysis (1, 14) and acts in a proinflammatory manner by stimulating several effector cells of the acquired and innate immune systems (6). As a pore-forming toxin, pneumolysin is directly cytotoxic to a wide spectrum of mammalian cells, including neuronal cells (3, 19). It damages respiratory and ependymal epithelial cell layers (8, 12) and facilitates penetration and systemic dissemination of bacteria from alveoli during experimental pneumonia (17). Even though differences in virulence among pneumococcal strains for mice were not attributable to differences in pneumolysin production (2), the role of pneumolysin production and release in human disease remains uncertain. As principal constituents of the pneumococcal cell wall, LTA and TA strongly stimulate the generation and release of proinflammatory agonists by leukocytes and act as adhesion molecules for many gram-positive species (10).

In the present study we investigated whether the production and liberation of the virulence factors pneumolysin and LTA and TA are associated with the clinical manifestation of pneumococcal infection and the ability of different strains to cause localized or systemic infection. For this purpose, we examined 75 clinical isolates of *S. pneumoniae* classified in three groups as described below.

**Bacterial strains and growth.** The pneumococcal strains used for this study were clinical isolates of the strain collection of the National Reference Laboratory for Streptococci, Aachen, Germany, and three isolates from patients treated at the University Hospital of Göttingen. With regard to the clinical manifestation caused by each strain, the isolates were grouped as follows: group A contained 39 isolates from patients with pneumococcal sepsis or meningitis, group B contained 21 pneumococcal strains from patients with localized respiratory infection, and group C contained 15 strains of *S. pneumoniae* isolated from patients without clinical symptoms related to pneumococcal colonization. All strains were grown in tryptic soy broth for 14 h at 37°C. Bacterial counts were determined every 2 h by plating 10-μl samples of 10-fold dilutions of bacteria on blood agar plates, and log-linear regression analysis (Δlog CFU/milliliter/hour) was performed for all pneumococcal strains. The amounts of pneumolysin and LTA and TA were measured in samples drawn during mid-logarithmic growth (bacterial density, 7 × 10^5 to 3 × 10^8 CFU/ml). All measurements were normalized to a bacterial number of 10^8 CFU.

**Detection and quantification of virulence factors.** Pneumolysin was detected using quantitative immunoblotting (18). We measured both extracellular (culture supernatant) and cytoplasmic (pellet after centrifugation) pneumolysin during mid-logarithmic growth. Cell disintegration in the pellets was achieved by adding sodium dodecyl sulfate-containing reducing sample buffer and boiling for 5 min. The interday variability of pneumolysin quantification was
evaluated by repeated blotting of a defined sample (n = 13; 85 ± 31 pg [mean ± standard deviation]). Furthermore, we investigated the reproducibility of the cytoplasmic and extracellular pneumolysin production by a laboratory strain (D39) by analyzing samples of strain D39 grown repeatedly as described above (n = 6; cytoplasmic pneumolysin, 1,300 ± 518 pg/10⁶ CFU [mean ± standard deviation]; extracellular pneumolysin levels for all cultures were below the detection limit of 10 pg/10⁶ CFU). To evaluate a possible influence of the culture medium, four pneumococcal strains were comparatively grown in tryptic soy broth and Todd-Hewitt broth: no substantial difference was identifiable.

LTA and TA levels were quantified in culture supernatants by an enzyme immunoassay with monoclonal antiphosphorylcholine antibody TEPC-15 as a capture antibody and polyclonal rabbit-anti-LTA and -TA antibodies as detector antibodies (20). For a laboratory serotype 3 strain grown repeatedly on six different days for 6 h in tryptic soy broth (log CFU/ml, 8.11 ± 0.50), LTA and TA concentrations in culture supernatants amounted to 1,746 ± 1,211 ng/ml (mean ± standard deviation).

Statistics. Since normal distribution was not always present, groups were compared by the nonparametric Kruskall-Wallis test, using the software InStat 3 (GraphPad, San Diego, Calif.).

Comparison of genomic sequences of pneumolysin. We compared a part of the genomic sequence of pneumolysin, which appears to be present in nearly all pneumococcal strains (11), for 7 strains classified as apathogenic (group C) and 10 highly pathogenic strains (group A). On the basis of the comparative alignment of a group of cholesterol-dependent cytolysins (pneumolysin of S. pneumoniae, perfringolysin of Clostridium perfringens, aerolysin of Aeromonas hydrophila, and hemolysin of Staphylococcus aureus) (13), a 284-bp nucleotide sequence containing the code for six single fully conserved amino acids was chosen, supposing that exchanges in those highly conserved regions would alter the pathophysiological function of the toxin. The amplification was performed according to standard methods using the primers 1113gtaacagg caag gtggat1120 and 1402gaaatgggc aggatttgac1420 (pneumolysin gene: National Center for Biotechnology Information nucleotide database M17717 [21]).

In vitro growth kinetics (Δlog CFU/milliliter/hour) of S. pneumoniae isolates did not differ significantly between the groups A, B, and C (P = 0.22) (Fig. 1a). When normalized to a bacterial number of 10⁶ CFU, the amount of extracellular pneumolysin in the culture supernatants (Fig. 1b) ranged from 18 to 17,300 pg/10⁶ CFU (median = 330 pg/10⁶ CFU) and the amount of intracellular pneumolysin (Fig. 1c) ranged from 54 to 75,000 pg/10⁶ CFU (median = 1,500 pg/10⁶ CFU; P = 0.13).
In comparing groups A to C, no significant difference was found with respect to levels of extracellular pneumolysin ($P = 0.84$) or cytoplasmic pneumolysin ($P = 0.92$). The pneumolysin production or release did not depend on the serotype investigated (data not shown). The release was not correlated with the growth rate of the strain (Spearman’s rank correlation coefficient $r_s = 0.05; P = 0.66$). The extracellular and intracellular amounts of pneumolysin were correlated ($r_s = 0.53; P < 0.0001$). The ratio between extracellular and cytoplasmic pneumolysin levels was calculated for each strain (Fig. 1d). During logarithmic growth, the culture supernatant of most strains contained less pneumolysin than the pellet. No significant difference was found among groups A to C with respect to the extracellular pneumolysin/intracellular pneumolysin ratio ($P = 0.76$).

LTA and TA in a range from 0.2 to 603 ng/10^9 CFU (Fig. 1c) were detected in culture supernatants of pneumococcal strains. No significant difference was found among groups A to C ($P = 0.91$), and no correlation was found between LTA release and bacterial growth rate ($r_s = -0.12; P = 0.33$).

From 17 different strains of S. pneumoniae, a 284-bp nucleotide sequence containing the code for six single fully conserved residues in the group of cholesterol-dependent cytolysins was determined. A limited number of base exchanges was observed, with two resulting amino acid exchanges involving the highly conserved amino acids in pneumococcal isolates from healthy carriers (strain CB 01, Trp379 → Arg; strain CB 15, Gly386 → Ala) (21).

Data about outcome for the patients were only available for 13 clinical isolates (Table 1). Neither the amount of extracellular pneumolysin nor the amount of intracellular pneumolysin was correlated with outcome ($r_s = 0.22, P = 0.47$, and $r_s = 0.18, P = 0.56$, respectively).

Carriage of S. pneumoniae is common among children and adults, and most acquisitions of a pneumococcus will not lead to disease. Nevertheless, spreading of pneumococci causes localized or disseminated diseases.

In search of factors determining whether a pneumococcal strain colonizes the nasopharynx of a healthy carrier or causes local or invasive disease, we studied 75 clinical isolates of S. pneumoniae from patients with sepsis or meningitis (group A) or localized respiratory disease (group B) and from healthy carriers (group C). The in vitro growth characteristics of the strains did not differ significantly among these groups. Therefore, we investigated a possible influence of the production and release of the virulence factors pneumolysin and LTA and TA on the clinical presentation of pneumococcal infection. Determining a relation between pneumolysin release and the invasiveness of a pneumococcal strain would provide a tool to facilitate the decision for a prophylactic treatment of asymptomatic carriers.

On the basis of the spectrum of pathogenic functions of pneumolysin (disruption of the epithelial cell layer, cytotoxicity, and proinflammatory effects), the role of pneumolysin during pneumococcal invasion has been discussed extensively. Although pneumolysin is known to be produced by virtually all clinical isolates of pneumococci (11, 15), the concentrations of cytoplasmic and extracellular pneumolysin were found to differ between different strains over 3 orders of magnitude (2, 5). Therefore, we hypothesized that the difference in pneumolysin production and release among pneumococcal strains colonizing the nasopharynx of healthy carriers and those causing invasive diseases could be a quantitative one. Yet no correlation was found between the origin of the isolates and the production and release of pneumolysin during the logarithmic growth phase in vitro.

Although a large variation range has been reported for pneumolysin production by different strains (2, 5), the variation range of extracellular amounts of pneumolysin observed in this study during logarithmic growth remains remarkable. Quantitation of the protein pneumolysin by immunoblotting and determination of the bacterial titer by 10-fold dilution as performed here are certainly of limited accuracy, but this imprecision could not be the causative factor for differences of nearly 3 log.

The ratio of extracellular pneumolysin levels to intracellular pneumolysin levels during the logarithmic growth phase differed between the strains in a range from 0.04 to 4.16 (median = 0.27). This observation compares well with the report of Benton and coworkers (2), who described two principal types

### TABLE 1. Outcome for 13 patients with pneumococcal respiratory and invasive infection in comparison to pneumolysin production and release of the isolated pneumococcal strain in vitro

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clinical presentation</th>
<th>Clinical classified group</th>
<th>Cytoplasmatic pneumolysin (pg/10^9 CFU)</th>
<th>Extracellular pneumolysin (pg/10^9 CFU)</th>
<th>Outcome</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKI 540</td>
<td>Meningitis</td>
<td>A</td>
<td>3,420</td>
<td>158</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>RKI 567</td>
<td>Meningitis</td>
<td>A</td>
<td>5,500</td>
<td>9,867</td>
<td>Deafness</td>
<td>2</td>
</tr>
<tr>
<td>RKI 1041</td>
<td>Meningitis</td>
<td>A</td>
<td>17,250</td>
<td>1,667</td>
<td>Death</td>
<td>3</td>
</tr>
<tr>
<td>RKI 1064</td>
<td>Meningitis</td>
<td>A</td>
<td>900</td>
<td>387</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>RKI 1112</td>
<td>Sepsis</td>
<td>A</td>
<td>417</td>
<td>21</td>
<td>Death</td>
<td>3</td>
</tr>
<tr>
<td>Gø 1</td>
<td>Meningitis</td>
<td>A</td>
<td>675</td>
<td>300</td>
<td>Neurological sequelae</td>
<td>2</td>
</tr>
<tr>
<td>Gø 2</td>
<td>Meningitis</td>
<td>A</td>
<td>65</td>
<td>48</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>Gø 3</td>
<td>Meningitis</td>
<td>A</td>
<td>1,086</td>
<td>2,714</td>
<td>Death</td>
<td>3</td>
</tr>
<tr>
<td>RKI 228</td>
<td>Respiratory infection</td>
<td>B</td>
<td>550</td>
<td>2,290</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>RKI 421</td>
<td>Respiratory infection</td>
<td>B</td>
<td>1,075</td>
<td>150</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>RKI 484</td>
<td>Respiratory infection</td>
<td>B</td>
<td>7,500</td>
<td>5,700</td>
<td>No sequelae</td>
<td>1</td>
</tr>
<tr>
<td>RKI 662</td>
<td>Respiratory infection</td>
<td>B</td>
<td>1,003</td>
<td>170</td>
<td>Sequelae</td>
<td>2</td>
</tr>
<tr>
<td>RKI 1027</td>
<td>Respiratory infection</td>
<td>B</td>
<td>5,000</td>
<td>1,600</td>
<td>Sequelae</td>
<td>2</td>
</tr>
</tbody>
</table>

* When results were analyzed to determine whether an outcome was related to pneumolysin production, no sequelae was scored 1, any sequelae was scored 2, and death was scored 3. Then, Spearman’s rank correlation was performed. Neither the amount of extracellular pneumolysin nor the amount of intracellular pneumolysin was correlated with outcome ($r_s = 0.22, P = 0.47$, and $r_s = 0.18, P = 0.56$, respectively).
of pneumolysin production during in vitro growth. As in the present study, for strain D39 they found a rise of cytoplasmic pneumolysin but no pneumolysin release until the late log phase. In contrast, for another strain (WU2) a measurable extracellular pneumolysin titer detectable prior to cytoplasmic titers was found, possibly due to small amounts of autolysis that can begin in the early- to mid-log phase. Alternatively, a nonautolytic release of pneumolysin prior to generalized lysis appears more probable, since Balachandran and coworkers describe a non-autolysin-dependent lysis-independent pneumolysin release (1). When comparing these studies with the present data, it remains to be discussed that in most cases (except that of Cima-Cabal et al. [5], who used an enzyme-linked immunosorbent assay with monoclonal antibodies to detect pneumolysin) a hemolysis assay was used to detect pneumolysin. This assay can only detect functional active pneumolysin, whereas in Western blot analysis pneumolysin with preserved primary structure is detected.

LTA and TA are surface-associated adhesion amphiphiles of gram-positive bacteria, including pneumococci (7), probably facilitating colonization and invasion of bacteria into deep tissue. Owing to the capacity to stimulate the generation and release of proinflammatory mediators (10), LTA and TA can contribute to cell damage in infectious sites. In a setting of asymptomatic colonization of the nasopharynx, differences in spontaneous release of LTA and TA could affect the ability of pneumococcal strains to cause invasive disease. Yet no difference in levels of LTA and TA release was found among the three groups with different pathogenicity characteristics in vitro.

In conclusion, with respect to the in vitro production and release of pneumolysin and LTA and TA during logarithmic growth no difference was found between pneumococcal strains isolated from patients with invasive disease or localized respiratory infection and strains from carriers without clinical signs of pneumococcal disease. Although the conditions in vitro might not necessarily reflect expression of virulence factors in vivo, our data suggest that the presence of virulence factors pneumolysin, LTA, and TA is a necessary factor for virulence but is not sufficient to explain the differences in invasiveness and pathogenicity between pneumococcal strains.

In large clinical studies a number of predisposing factors for invasive pneumococcal infection were identified, such as factors disturbing the local immune defense mechanisms, previous viral infections, or cigarette smoking as well as factors compromising the systemic immune response such as alcoholism, splenectomy, immunosuppression, metabolic disorder, advanced age, or immature immune response in young children. At least one of these factors was present in the majority of adults with invasive disease (4, 16). It is possible that host factors identified as predisposing factors may influence in vivo production and release of virulence factors. Although we studied only two of a wide spectrum of potential virulence factors in vitro, our results support the concept that for acquisition of an invasive pneumococcal infection after colonization, the host conditions are of predominant importance.

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REFERENCES


