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SHORT COMMUNICATION

Nonencapsulated *Streptococcus pneumoniae* resists extracellular human neutrophil elastase- and cathepsin G-mediated killing

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Abstract

Although the *Streptococcus pneumoniae* polysaccharide capsule is an important virulence factor, ~ 15% of carriage isolates are nonencapsulated. Nonencapsulated *S. pneumoniae* are a cause of mucosal infections. Recent studies have shown that neutrophils kill *S. pneumoniae* predominately through neutrophil proteases, such as elastase and cathepsin G. Another recent finding is that nonencapsulated pneumococci have greater resistance to resist cationic antimicrobial peptides that are important in mucosal immunity. We here show that nonencapsulated pneumococci have greater resistance to extracellular human neutrophil elastase- and cathepsin G-mediated killing than isogenic encapsulated pneumococci. Resistance to extracellular neutrophil protease-mediated killing is likely to be of greater relative importance on mucosal surfaces compared to other body sites.

Streptococcus pneumoniae is a major human pathogen. The contribution of *S. pneumoniae* virulence factors in host respiratory colonization and disease varies according to the *in vivo* location of the bacterium (Kadioglu *et al.*, 2008). The presence of pneumococcal polysaccharide capsule, which inhibits opsonophagocytosis, is an important virulence factor. There are currently 93 known capsular serotypes of *S. pneumoniae*. Invasive *S. pneumoniae* infections are caused virtually exclusive by encapsulated strains. The majority of pneumococcal nasopharygeal isolates are also encapsulated. However, pneumococci colonizing the nasopharynx phenotypically show reduced polysaccharide capsule expression compared to pneumococci causing invasive disease (Kim & Weiser, 1998). Moreover, up to 18% of pneumococcal nasopharygeal isolates are nonserotypeable, and up to 15% of pneumococcal nasopharygeal isolates are truly nonencapsulated and lack the genes encoding the enzymes required for capsule synthesis. Other nonserotypeable strains express yet unknown capsule serotypes or display extreme down-regulation of capsule expression(Marsh *et al.*, 2010).

Truly nonencapsulated pneumococci may be a cause of outbreaks of mucosal disease particularly conjunctivitis and have been related to acute otitis media (Martin *et al.*, 2003; Hanage *et al.*, 2006). Thus, nonencapsulated pneumococci may be highly contagious and cause mucosal disease (Martin *et al.*, 2003).

The microbial and host factors that determine carriage are still incompletely characterized. Neutrophils recruited by IL-17 expressing CD4$^+$ T cells seem to contribute to mucosal clearance of pneumococci (Malley *et al.*, 2005; Zhang *et al.*, 2009). Neutrophils kill and degrade bacteria by a range of mechanisms including reactive oxygen species and antimicrobial peptides. The concept has emerged that neutrophil proteases such as neutrophil elastase and cathepsin G also contribute significantly to intracellular and extracellular killing of bacteria (Reeves *et al.*, 2002; Pham, 2006). Thus, neutrophil proteases may

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be effective in killing bacteria even in the absence of effective phagocytosis. Patients with deficiency of neutrophil serine protease activity due to Papillon–Lefevre syndrome suffer impaired host defence clinically evident as severe periodontitis and pyogenic liver and renal abscesses (Van Dyke *et al.*, 1984; Almuneef *et al.*, 2003).

The importance of neutrophil elastase and cathepsin G for intracellular and extracellular killing of *S. pneumoniae* by neutrophils was demonstrated recently and may be relevant for colonization (Standish & Weiser, 2009). Extracellular neutrophil protease is present on the conjunctival and nasal mucosa as it can be demonstrated in tear fluid and nasal secretions (Sakata *et al.*, 1997; Innes *et al.*, 2009).

The prevalence of nonencapsulated pneumococci on mucosal surfaces compared to the almost complete absence of nonencapsulated pneumococci in invasive disease suggests nonencapsulated pneumococci possess resistance to important mucosal defences. Indeed, nonencapsulated pneumococci possess greater resistance to cationic antimicrobial peptides (the α-defensin human neutrophil protein 1–3) (Peschel, 2002; Beiter *et al.*, 2008).

The aim of this study was to investigate the effect of the presence of capsule on the *in vitro* pneumococcal resistance to extracellular human neutrophil elastase and cathepsin G.

The *in vitro* bactericidal activities of elastase and cathepsin G were determined as described previously (Standish & Weiser, 2009). In brief, original cultures of pneumococcal wild-type strains and nonencapsulated derivatives (wild-type strain D39 (serotype 2), TIGR4 (serotype 4) and G54 (serotype 19F) and isogenic nonencapsulated derivatives) (Bootsma *et al.*, 2007), were grown to mid-log in tryptic soy broth (TSB) at 37 °C, 5% CO_2 without agitation, washed twice in PBS, and then ~ 10^7 CFU/mL *S. pneumoniae* were incubated in the presence or absence (control) of purified human 3.39 μM neutrophil elastase (NE; Calbiochem Cat. No. 324681) and 2.1 μM neutrophil cathepsin G (CG; Calbiochem Cat. No. 219373) in a total volume of 100 μL of 10 mM sodium phosphate, 1% tryptic soy, at 37 °C, 5% CO_2. The bactericidal reaction was terminated after 2 h by 1 : 10 dilution in 10 mM sodium phosphate. Viable counts of colony forming units were determined by plating serial dilutions of the pneumococcal culture on tryptic soy agar (TSA) plates supplemented with 250 U/mL bovine liver catalase (Sigma). All assays were performed in duplicate on at least three different days, at 37 °C, 5% CO_2 without agitation.

Following 2 h incubation with human neutrophil elastase wild-type encapsulated serotypes 2, 4 and 19F pneumococcal strains showed significantly less resistance to killing than the isogenic nonencapsulated derivatives (Fig. 1a). Differences between encapsulated and nonencapsulated strains were analysed by Student's *t*-test. A *P* value < 0.05 was considered statistically significant. Similarly following 2 h incubation with human neutrophil cathepsin G wild-type encapsulated serotypes 2, 4 and 19F pneumococcal strains showed significantly less resistance to killing than the isogenic nonencapsulated derivatives (Fig. 1b). We observed an especially strong effect for the nonencapsulated serotype 2 strain (D39), for which we do not have a good explanation.

The main finding of our study is that the absence of the pneumococcal polysaccharide capsule increases the resistance of pneumococci to extracellular human neutrophil elastase- and cathepsin G-mediated killing.

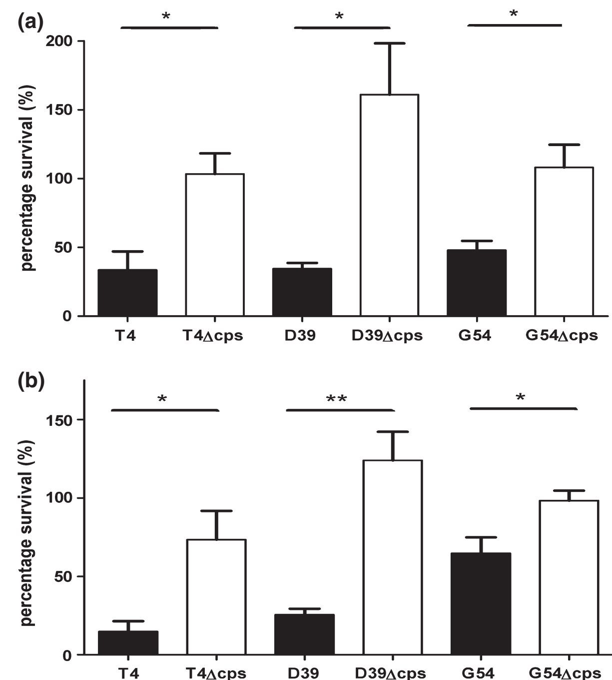

Fig. 1. (a) Survival rates of *S. pneumoniae* serotypes and nonencapsulated derivatives following exposition to human neutrophil elastase (concentration 3.39 μM) compared to control. Encapsulated *S. pneumoniae* wild-type strains (black bars) showed decreased survival rates compared to nonencapsulated derivatives (white bars). Values are mean percentage survival ± SEM: TIGR4 (33.45 ± 13.56) vs. TIGR4Δcps (103.3 ± 15.05); D39 (41.82 ± 5.309) vs. D39Δcps (161.0 ± 37.29; G54 wild type (48.05 ± 6.602) vs. G54Δcps (108.1 ± 16.58). (b) Survival rates of *S. pneumoniae* serotypes and nonencapsulated derivatives after 2 h exposition to human neutrophil cathepsin G (concentration 2.1 μM) compared to control (mean ± SEM). Encapsulated *S. pneumoniae* wild-type strains (black bars) showed decreased survival rates compared to nonencapsulated derivatives (white bars). Values are TIGR4 wild type (14.94 ± 6.503) vs. TIGR4Δcps (73.39 ± 18.35); D39 wild type (22.82 ± 1.700) vs. D39Δcps (124.0 ± 18.12); G54 wild type (64.71 ± 10.19) vs. G54Δcps (98.26 ± 6.422); (**P* < 0.05, ***P* < 0.01).

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The pneumococcal targets of neutrophil protease have not yet been identified, but it is likely that essential pneumococcal surface proteins are degraded by neutrophil proteases. How the absence of capsule increases resistance to human neutrophil elastase- and cathepsin G-mediated killing is unclear.

A potential explanation is that positive surface charges modifications, such as incorporation of positively charged D-alanine in lipoteichoic acids exposed on nonencapsulated pneumococci, repulses the positively charged proteases and thus increase resistance to degradation, whereas presence of pneumococcal polysaccharide capsule masks these positive charge modifications and increases susceptibility to the proteases. This mechanism is employed by different bacterial species including pneumococci to resist cationic antimicrobial peptides (Peschel, 2002; Beiter *et al.*, 2008).

An alternative explanation is the release of anionic bacterial decoys, specifically by nonencapsulated pneumococci, which may trap the positively charged (cationic) human neutrophil proteases. Before the role of neutrophil proteases in microbial killing was elucidated, it was shown that pneumococci release a highly charged polyanion that functions as a neutrophil elastase inhibitor during growth. This pneumococcal elastase inhibitor was identified as pneumococcal RNA released from autolysing pneumococci, whereas released pneumococcal polysaccharide capsule was only weakly inhibitory towards neutrophil elastase (Vered *et al.*, 1988). Notably, nonencapsulated pneumococci show more autolysis and release more pneumococcal RNA during growth than encapsulated pneumococci (Vered *et al.*, 1988; Fernebro *et al.*, 2004). Moreover, increased autolysis in nonencapsulated strains compared to encapsulated strains may even have underestimated the higher viable counts in our study. Secreted bacterial RNA fragments may impact pathogenesis (Obregon-Henao *et al.*, 2012).

The contribution of *S. pneumoniae* virulence factors in host respiratory colonization and disease varies according to the *in vivo* location of the bacterium. In line with our findings, others described previously that nonencapsulated pneumococci possess increased resistance against cationic antimicrobial peptides compared to encapsulated pneumococci (Beiter *et al.*, 2008). Pneumococcal resistance to extracellular neutrophil proteases may be of greater relative importance than inhibition of opsonophagocytosis on the mucosal surface in comparison with other body compartments such as the bloodstream or lung parenchyma. On the mucosal surface, phagocytosis may be ineffective, but neutrophil degranulation and release of toxic substances including neutrophil proteases may effectively kill pneumococci. However, definitive *in vivo* data demonstrating the contribution of extracellular killing of pneumococci are lacking (Coonrod *et al.*, 1987).

We conclude that human neutrophil proteases elastase and cathepsin G are active against pneumococci in general; however, nonencapsulated pneumococci show increased resistance to extracellular human neutrophil protease-mediated killing compared to encapsulated pneumococci. The mechanism of this increased resistance and the effect on human colonization and (mucosal) infection remain to be elucidated.

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The authors declare that no conflict of interest exists.

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