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Combined effects of lactoferrin and lysozyme on *Streptococcus pneumoniae* killing

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ABSTRACT

Streptococcus pneumoniae is a common colonizer of the human nasopharynx, which can occasionally spread to sterile sites, causing diseases such as otitis media, sinusitis, pneumonia, meningitis and bacteremia. Human apolactoferrin (ALF) and lysozyme (LZ) are two important components of the mucosal innate immune system, exhibiting lytic effects against a wide range of microorganisms. Since they are found in similar niches of the host, it has been proposed that ALF and LZ could act synergistically in controlling bacterial spread throughout the mucosa. The combination of ALF and LZ has been shown to enhance killing of different pathogens *in vitro*, with ALF facilitating the latter action of LZ. The aim of the present work was to investigate the combined effects of ALF and LZ on *S pneumoniae*. Concomitant addition of ALF and LZ had a synergistic killing effect on one of the pneumococci tested. Furthermore, the combination of ALF and ALZ was more bactericidal than lysozyme alone in all pneumococcal strains. Pneumococcal surface protein A (PspA), an important vaccine candidate, partially protects pneumococci from ALF mediated killing, while antibodies against one PspA enhance killing of the homologous strain by ALF. However, the serological variability of this molecule could limit the effect of anti-PspA antibodies on different pneumococci. Therefore, we investigated the ability of anti-PspA antibodies to increase ALF-mediated killing of strains that express different PspAs, and found that antisera to the N-terminal region of PspA were able to increase pneumococcal lysis by ALF, independently of the sequence similarities between the molecule expressed on the bacterial surface and that used to produce the antibodies. LF binding to the pneumococcal surface was confirmed by flow cytometry, and found to be inhibited in presence of anti-PspA antibodies. On a whole, the results suggest a contribution of ALF and LZ to pneumococcal clearance, and confirm PspA's ability to interact with ALF.

1. Introduction

Antimicrobial proteins are essential components of the innate immune system, exhibiting several mechanisms of defense against a wide spectrum of pathogens. These molecules are produced in large amounts in all secretory fluids, as the first line of protection from mucosal pathogens [1]. Two of the major antimicrobial proteins are lactoferrin and lysozyme [2].

Human lactoferrin (hLF) is a mucosal glycoprotein, present in high concentrations in milk (from 1 to 7 g/L) [3], and also found in secretions such as saliva, tear, semen and neutrophil granules. It is a multifunctional protein, displaying immunomodulatory effects [3,4], bacteriostatic and antimicrobial activities against a wide variety of pathogens, including fungi, bacteria, viruses and parasites [5]. The bacteriostatic effect of hLF is due to its ability to sequester iron, an essential component for bacterial growth. The lytic action of hLF, on the other hand, is related to the iron free (apolactoferrin-ALF) form of the molecule, which can undergo proteolysis and produce cationic peptides (lactoferricins-LFN) that destabilize microbial membranes [6,7]. The mechanism of action of lactoferricins in Gram-negative bacteria involves the release of lipopolysaccharide, leading to an increase in the permeability of the outer

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membrane [6,8]. In Gram-positive microorganisms, it has been proposed that lactoferrin can interact with lipoteichoic acid, reducing the negative charge of the bacterial membrane, and thus allowing for further interaction with other lytic proteins, as lysozyme [6,9].

Lysozyme (LZ) is a bactericidal and fungicidal polypeptide, abundant in the respiratory tract secretions of mammals (>500 μg/mL) [10], and also present at high levels in human milk, vacuoles of phagocytic cells, interstitial fluid and in saliva [11]. LZ acts as a muramidase on the cell wall, hydrolysing the β 1–4 glycosidic link between N-acetylmuramic acid and N-acetylglucosamine, the structural components of peptidoglycans [11]. A non-muramidase function has also been described for LZ, which involves the production of a cationic antimicrobial peptide that leads to membrane permeabilization [12]. The direct lytic effects of LZ have been widely demonstrated against several Gram-positive bacteria [1,13,14], while Gram-negative pathogens show an increased resistance to LZ action [8,11], and usually require the action of other host factors to become susceptible. It has also been suggested that lysozyme can potentiate the action of the complement system, as well as recognition of bacterial surface structures by immunoglobulins [8].

Several studies have indicated that LF and LZ can act in synergism. The combination of the two proteins has been shown to increase killing of many organisms [8,15]. In Gram-negative bacteria, it was suggested that the alteration of permeability of the external membrane caused by the action of LF facilitates access of LZ to the cell wall peptidoglycan [8]. A similar synergistic action was observed in *Staphylococcus epidermidis* – a Gram-positive bacterium – where binding of LF to lipoteichoic acid on the bacterial membrane leads to a decrease on the negative charge of that surface, thus facilitating LZ access to the underlying peptidoglycan [9].

Streptococcus pneumoniae is a common colonizer of the nasopharynx of healthy children and adults, from where it can, under certain circumstances, spread to sterile sites such as the lungs, meninges and blood, causing intense inflammatory responses and leading to diseases like pneumonia, meningitis and septicemia [16]. Its incidence and mortality are higher among the elderly and young children, being responsible for 1.1 million deaths of children under five years of age [17].

The currently available pneumococcal vaccines are based on capsular polysaccharides, alone or in conjugation with carrier proteins. While free polysaccharides fail to protect the major risk groups, conjugate vaccines have limited coverage, are associated with serotype replacement [18] and are too expensive for use in the parts of the world with the greatest need without heavy subsidies [19]. Furthermore, an increase in antibiotic resistance has been observed in the last decades [20,21], reinforcing the need for broad ranging, cost effective vaccines.

Pneumococcal colonization is a necessary step for all pneumococcal diseases [22], and the bacterium has developed many strategies to evade host immune responses at the mucosal level. It has been demonstrated that pneumococci can bind to lactoferrin, increasing bacterial resistance to lactoferrin-mediated killing [23]. The bacterial receptor for LF has been identified as being Pneumococcal surface protein A (PspA) [23–25], a surface exposed coiled-coil protein also recognized as an inhibitor of C3 deposition on the bacterium surface [26].

PspA is an important candidate for inclusion in a protein-based pneumococcal vaccine, inducing robust immune responses in different animal models, which correlate with protection against sepsis, pneumonia, and colonization [27–32]. Particularly, the ability of PspA to reduce pneumococcal colonization in mice – either as a DNA vaccine [30] or in presence of mucosal adjuvants [33,34] – suggests an important role for PspA during the initial stages of pneumococcal infection. The N-terminal region of the molecule (which protrudes from outside the capsule) is responsible for interaction with complement and lactoferrin, and confers protection against local and systemic infection [29,31,32,35]. Pneumococcal incubation with a recombinant N-terminal PspA fragment has been shown to prevent pneumococcal lysis by hLF *in vitro*, while antibodies against PspA were able to increase killing by hLF [36]. However, the N-terminal region of PspA exhibits structural and serological variability [31,37], which can limit vaccine coverage. Based on the sequence variations of a region within the N-terminus of the protein known as the clade defining region, PspAs have been divided into six clades, grouped in three families [38]. Several studies have shown that mouse immunization with PspA fragments from different clades induced antibodies with variable degrees of cross-recognition and cross-protection [31,35,37]. Therefore, it is important to determine the width of anti-PspA-mediated pneumococcal lysis by ALF, in order to establish the efficacy of PspA-based formulations against different pneumococcal strains.

Since lysozyme is another protein present in great amounts at mucosal sites, the present study also investigated the lytic effects of this molecule on pneumococci, as well as the contribution of the polysaccharide capsule in preventing bacterial death. The combined effects of ALF and LZ on pneumococcal killing were examined, and the results provide an insight on the interactions between pneumococci and the immune system during the initial stages of infection.

2. Material and methods

2.1. Bacterial strains and growth conditions

Frozen pneumococcal isolates of different serotypes and expressing different PspAs (Table 1) were thawed, plated on blood agar (Neoprov) and incubated at 37 °C under anaerobic conditions for 16 h. Colonies were transferred to 5 mL of Todd Hewitt medium (Gibco) supplemented with 0.5% yeast extract (THY) and grown up to an $O.D._{600\ nm} = 0.4–0.5$; the samples were diluted with THY to an $O.D._{600\ nm} < 0.05$ and regrown up to a final $O.D._{600\ nm} = 0.1–0.2$ (corresponding to approximately 10^7 CFU/mL). This protocol has been described by *Shaper* et al. [36] as necessary for limiting the amount of capsule deposited on the bacterial surface, therefore allowing for a better exposure of PspA, and to better mimic the bacterial morphology during colonization [39].

2.2. Immunization of BALB/c mice with recombinant PspAs

Gene fragments encoding the N-terminal region of PspA from

Table 1
Pneumococcal strains used in this study.

Strain	PspA clade	PspA family	Capsule type	Reference
245/00	1	1	14	[37]
94/01	2	1	18A	[37]
A66.1[b]	2	1	3	[26]
D39[b]	2	1	2	[40]
RM200[a]	2	1	–	[41]
679/99	3	2	6B	[29]
259/98	3	2	14	[35]
3JYP 2670[b]	4	2	3	[29]
P101	4	2	19F	IAL[c]

[a] Non encapsulated, autolysin-negative strain, with the *ply* gene substituted by *pdT*. Kindly provided by Dr. Richard Malley (Children's Hospital).
[b] Kindly provided by Dr. David Briles (AUB).
[c] P101 is a clinical isolate from Instituto Adolpho Lutz (IAL), São Paulo, Brazil.