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Active release of pneumolysin prepores and pores by mammalian cells undergoing a *Streptococcus pneumoniae* attack

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ABSTRACT

Background: Streptococcus pneumoniae is a potent human pathogen. Its pore-forming exotoxin pneumolysin is instrumental for breaching the host's epithelial barrier and for the incapacitation of the immune system.
Methods and results: Using a combination of life imaging and cryo-electron microscopy we show that pneumolysin, released by cultured bacteria, is capable of permeabilizing the plasmalemma of host cells. However, such permeabilization does not lead to cell lysis since pneumolysin is actively removed by the host cells. The process of pore elimination starts with the formation of pore-bearing plasmalemmal nanotubes and proceeds by the shedding of pores that are embedded in the membrane of released microvesicles. Pneumolysin prepores are likewise removed. The protein composition of the toxin-induced microvesicles, assessed by mass spectrometry, is suggestive of a Ca^{2+}-triggered mechanism encompassing the proteins of the annexin family and members of the endosomal sorting complex required for transport (ESCRT) complex.
Conclusions: S. pneumoniae releases sufficient amounts of pneumolysin to perforate the plasmalemma of host cells, however, the immediate cell lysis, which is frequently reported as a result of treatment with purified and artificially concentrated toxin, appears to be an unlikely event *in vivo* since the toxin pores are efficiently eliminated by microvesicle shedding. Therefore the dysregulation of cellular homeostasis occurring as a result of transient pore formation/elimination should be held responsible for the damaging toxin action.
General significance: We have achieved a comprehensive view of a general plasma membrane repair mechanism after injury by a major bacterial toxin.

1. Introduction

Streptococcus pneumoniae is a potent human pathogen. Infection leads to common diseases such as otitis media, meningitis and pneumonia, which affect several million people and is responsible for significant infant death in developing countries [1]. The bacterial exotoxin pneumolysin (PLY) is instrumental for the breach of epithelial and endothelial barriers and the incapacitation of the host's immune system [2,3].

PLY belongs to the large family of cholesterol-dependent cytolysins (CDC), toxins that are structurally related and characterized by their large trans-membrane pore [4]. Other prevalent pathogens producing CDCs are *Streptococcus pyogenes* (streptolysin O, SLO) and numerous other Gram positive bacteria, such as *Clostridium*, *Listeria* and *Bacillus* [5].

During the progress of infection PLY is released by the bacteria as soluble monomers that bind to cholesterol-rich microdomains within the plasma membrane of the host cells [2,6]. After binding, PLY assembles in circular oligomeric prepores, undergoes a conformational change and perforates the plasmalemmal lipid bilayer. The formation of transmembrane pores leads to the loss of plasmalemmal integrity that

Abbreviations: AEC, primary human airway epithelial cells; ALG-2, apoptosis-linked gene 2; ALIX, ALG-2 interacting protein X; BCA, bicinchoninic acid; CCD, charge-coupled device; CDC, cholesterol-dependent cytolysins; Chmp4B, charged multivesicular body protein 4B; ESCRT, endosomal sorting complex required for transport; FACS, fluorescence activated cell sorting; FBS, Fetal Bovine Serum; FT, Fourier transform detector; HBE, human bronchial epithelial cells; HEK 293, human embryonic kidney cells; HRP, horseradish peroxidase; Hsp90, heat shock protein 90; NAD, nonlinear anisotropic diffusion; PBS, phosphate buffered saline; PLY, pneumolysin; PMSS, protein match score summation; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SLO, streptolysin O; Vps4, vacuolar protein sorting 4.

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might result in the lysis of targeted cells and ultimately in extended tissue damage at the site of infection and overwhelming immune responses [2].

However, plasmalemmal perforation by pore-forming toxins does not necessarily entail an unfavorable prognosis with respect to cellular survival. Largely depending on the concentration of a pore-forming toxin, the repertoire of cellular responses is considerable and ranges from the activation of intracellular and transmembrane signaling cascades *i.e.* for the initiation of the release of cytokines at non-lytic toxin concentrations, to imminent lytic cell death at lytic ones [2,7,8].

Lytic cell death has frequently been documented by using purified, concentrated exotoxins [2,7]. Recently, we have shown that even at non-lytic concentrations of purified PLY the majority of targeted host cells are being perforated; however, these injuries are rapidly and efficiently resealed [9]. Ca^{2+}-dependent recruitment of the repair machinery leads to plugging of toxin pores that are either expelled into the extracellular space or internalized into the cellular interior [7, 10–12].

Here we show that during their growth bacteria produce enough PLY to perforate the plasma membrane of human cells. However, the host cells are capable of efficiently eliminating the active toxin pores and thus do not succumb to lytic death. Evaluating the mechanisms that are responsible for the elimination of toxin pores, we show that they are actively ejected and that both prepores and functional toxin pores are expelled in a Ca^{2+}-triggered mechanism. Individual toxin pores and prepores have been identified by cryo-electron microscopy. Mass spectrometry results demonstrate that microvesicles are highly enriched in PLY, annexins, actin-binding and Ca^{2+}-regulated proteins and that they also contain components of the ESCRT machinery.

2. Materials and methods

2.1. Mammalian cell culture and transfections

Human embryonic kidney cells (HEK 293) and neuroblastoma SH-SY5Y cells were cultured as described [13]. HeLa cells were maintained in DMEM (Dulbecco's modified Eagle's medium. Gibco, Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum. Gibco, Life Technologies, Paisley, UK) and 1% penicillin-streptomycin (Gibco, Life Technologies, Paisley, UK). Cell cultures were grown in 5% CO_2 at 37 °C. Transfections were performed as described in [14]. Cells were transiently transfected with the coding sequence of human annexin A2, A6 or porcine annexin A1 cloned into the Living Colors Fluorescent protein vector pEYFP-N1 (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) [14] and/or with a human charged multivesicular body protein 4B (Chmp4B)-mCherry construct [15]. Transfected cells were seeded on coverslips and were incubated for 48 h, reaching 80–90% confluence. The transfection rate for the fluorescently-labeled constructs ranged between 70 and 90%.

2.2. Bacterial cultures

The clinical pneumococcal isolates were collected in Switzerland from 1 to 10-year-old children of both sexes. Strains 103.57, 211.25, 109.74, 307.14, 207.06, 208.41, 106.66, 207.31 and 202.67 were from nasopharyngeal swabs (otitis), whereas strains B101.77, B103.21 were from blood cultures (septicemia). The clinical pneumococcal isolates as well as the pneumococcal strains D39 and PLN-A (D39 deficient in expressing PLY) were cultured in BHI (Brain Heart Infusion Broth, Sigma-Aldrich, Buchs, Switzerland) at 37 °C. The PLN-A strain carries an erythromycin resistance marker [16], therefore the medium was supplemented with 1 μg/ml erythromycin (Sigma-Aldrich). For the generation of bacterial supernatants, bacteria grown to their stationary phase ($OD_{500} = 1.0$) were pelleted (5000 ×*g*) for 15 min. The supernatants were filtered through a syringe filter with a pore size of 0.2 μm (VWR, Dietikon, Schweiz). Overnight cultures from D39 strain used for co-culturing experiments were diluted to $OD_{500} = 0.01$ in DMEM medium supplemented with 10% heat-inactivated FBS and 20 mM HEPES (Merck, Zug, Switzerland) and grown to early stationary phase. For the establishment of growth curves, the overnight cultures were diluted to $OD_{500} = 0.01$ and the OD_{500} was measured every 1.5 h.

2.3. Recombinant toxins

The nontoxic PLY mutant ΔA146R147 exhibits a double-amino acid deletion within the PLY sequence [17] and is N-terminally tagged with GFP. Recombinant PLY and EGFP-tagged PLY were cloned and purified as follows: all primers were synthesized by Microsynth (Balgach, Switzerland) and are detailed in the Supplementary File 1. Restriction enzymes were from Fermentas (ordering partner: Fisher Scientific, Wohlen, Switzerland) and all other reagents used in the cloning experiments were purchased from Promega (Dübendorf, Switzerland) if not otherwise stated. Isolation of the genomic DNA of the strain D39 was performed with the E.Z.N.A. ® Bacterial DNA Kit (VWR). The PLY gene and the EGFP gene (from pN1-EGFP vector; Clontech, Saint-Germain-en-Laye, France) were amplified. The PCR products as well as the pET28a vector were cut with the complementing restriction enzymes (BamHI, XhoI) and purified by the PCR Clean-up Kit (Qiagen, Hilden, Germany). First the PLY PCR product was ligated into the pET28a vector (Novagen, Madison, USA; digested with BamHI and XhoI). Subsequently, the EGFP PCR-product was ligated into the pET28a-PLY vector (digested with NheI and BamHI) by the T4-DNA ligase. Positive clones were sequenced. The pET28a-PLY and the pET28a-EGFP-PLY vector were transformed in BL21 (DE3) pLysS competent cells. Protein expression was induced with 1 mM IPTG (Sigma-Aldrich) at the bacterial culture $OD_{500} = 0.5–0.7$. Bacterial cells were incubated for 4 h at 37 °C and harvested by centrifugation (5000 ×*g*, 4 °C, 30 min). The recombinant proteins were purified with Protino® Ni-NTA 1 ml columns (MACHEREY-NAGEL, Oensingen, Switzerland) according to the manufacturer's instructions. The protein content of the eluates was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and high protein contents were pooled. Two dialysis steps to phosphate buffered saline (PBS) (1 mM DTT added) were performed with a cellulose membrane tube that retains proteins with ≥12 kDa (Sigma-Aldrich) according to the manufacturer's instructions. The protein concentration was determined with a bicinchoninic acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Reinach, Switzerland) and the hemolytic activity of PLY as well as EGFP-PLY was assessed by a hemolysis assay.

2.4. Hemolysis assay

Human red blood cells were isolated from whole blood of healthy volunteers and were stored in the Alsever's solution (Sigma-Aldrich). Before each experiment red blood cell suspension was centrifuged (3000 ×*g*, 4 °C, 10 min). Pelleted erythrocytes were washed twice with PBS (3000 ×*g*, 4 °C, 10 min) and used as the source (100%) in the hemolysis assay. Serial dilutions of bacterial culture ($OD_{500} = 1.0$) supernatants or recombinant toxins, pre-activated with 5 mM DTT, were incubated with 1% human red blood cells at 37 °C for 60 min in PBS. The absorbance of the supernatant was measured at 450 nm and the hemolytic activity was calculated according to the formula: $\text{hemolysis}\ (\%) = ((A_{\text{sample}} - A_{\text{negative control}})/(A_{\text{positive control}} - A_{\text{negative control}})) \times 100$. Negative control = 1% human red blood cells in PBS. Positive control = 1% human red blood cells in H_2O.

2.5. Confocal microscopy

If not otherwise stated Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES, pH = 7.4) containing 2.5 mM $CaCl_2$ (calcium Tyrode's buffer) or 100 μM EGTA was used in all experiments. Imaging was performed as described in [9]. In brief,