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Research paper

Pneumococcal DNA-binding proteins released through autolysis induce the production of proinflammatory cytokines via toll-like receptor 4

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ABSTRACT

Streptococcus pneumoniae is a leading cause of bacterial pneumonia. Our previous study suggested that *S. pneumoniae* autolysis-dependently releases intracellular pneumolysin, which subsequently leads to lung injury. In this study, we hypothesized that pneumococcal autolysis induces the leakage of additional intracellular molecules that could increase the pathogenicity of *S. pneumoniae*. Liquid chromatography tandem-mass spectrometry analysis identified that chaperone protein DnaK, elongation factor Tu (EF-Tu), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were released with pneumococcal DNA by autolysis. We demonstrated that recombinant (r) DnaK, rEF-Tu, and rGAPDH induced significantly higher levels of interleukin-6 and tumor necrosis factor production in peritoneal macrophages and THP-1-derived macrophage-like cells via toll-like receptor 4. Furthermore, the DNA-binding activity of these proteins was confirmed by surface plasmon resonance assay. We demonstrated that pneumococcal DnaK, EF-Tu, and GAPDH induced the production of proinflammatory cytokines in macrophages, and might cause host tissue damage and affect the development of pneumococcal diseases.

1. Introduction

Streptococcus pneumoniae, also known as pneumococcus, is a Gram-positive diplococcus and major human pathogen. This bacterium asymptomatically colonizes the upper respiratory airway and causes common clinical syndromes, such as otitis media, sinusitis, bronchitis, and empyema, or even severe life-threatening diseases, including pneumonia, meningitis, and septicemia [1,2]. Pneumococcal infections have led to significant morbidity and mortality worldwide especially in children under 5 years old and adults over 65 years old in developing countries [3].

A variety of pneumococcal virulence factors, including the autolytic enzyme LytA, contribute to the development of pneumococcal diseases [4]. LytA is responsible for the characteristic autolytic behavior associated with pneumococcus. It has been reported that LytA potentially contributes to pneumococcal pathogenesis by catalyzing the release of intracellular toxins and generating proinflammatory cell wall fragments [5]. Our previous study suggested that *S. pneumoniae* autolysis-dependently releases pneumolysin (PLY), which is a cholesterol-dependent cytolytic pore-forming toxin, that induces the disruption of pulmonary immune defenses [6]. Therefore, autolysis plays a central role in the pathogenesis of pneumococcal diseases.

The innate immune response is the first line of defense against any bacterial infection. In this regard, macrophages respond immediately to diverse microbial pathogens and control the replication of invading pathogens [7,8]. Macrophages express various pattern recognition receptors, such as toll-like receptors (TLRs), which activate downstream signaling and induce the production of proinflammatory cytokines.

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TLRs play a crucial role against microbial infections, initiating and activating both host innate and adaptive immunity [9]. They recognize the presence of microbial pathogens via detection of conserved structures. It has been shown that the cell wall components of pneumococci, such as lipoteichoic acid and peptidoglycan are recognized by TLR2 [10]. TLR4 is a key component of the innate response to Gram-negative infections through the recognition of lipopolysaccharide (LPS). In addition, PLY is also considered to be a TLR4 ligand. TLR4-mutant mice are more susceptible to lethal infection after intranasal infection with pneumococcus [11]. It has also been reported that TLR9 plays an important role in the recognition of pneumococcus [12]. However, the possibility that other pneumococcal virulent factors interact with the host innate immune system and participate in pneumococcal pathogenesis remains to be explored.

In this study, we hypothesized that pneumococcal autolysis induces the leakage of additional intracellular virulent factors, which increase the pathogenicity of *S. pneumoniae*. Here, we demonstrated that chaperone protein DnaK, elongation factor Tu (EF-Tu), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were released into the pneumococcal culture supernatant by autolysis. In addition, we examined their activity of inducing the production of proinflammatory cytokines in mouse peritoneal macrophages and THP-1-derived macrophage-like cells.

2. Materials and methods

2.1. Bacterial strains and reagents

S. pneumoniae D39 (NCTC 7466) strain was purchased from the National Collection of Type Cultures (Salisbury, UK). Inactivation of *lytA* gene in *S. pneumoniae* D39 strain was performed as described previously [13]. *S. pneumoniae* was grown in tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ, USA), with 100 μg/mL spectinomycin (Sigma-Aldrich, St. Louis, MO, USA) added to the medium to allow for *lytA*-negative mutant-strain (Δ*lytA*) selection. Recombinant (r) LytA protein was kindly provided by Dr. Yuuki Sakaue (Niigata University, Niigata, Japan). Polyinosinic-polycytidylic acid [TLR3 ligand; Poly (I:C)], LPS (TLR4 ligand) from *Escherichia coli*, resiquimod (TLR7 ligand; R848), and cytosine-phosphate-guanosine oligodeoxynucleotides 1668 (TLR9 ligand; CpG ODN 1668) were purchased from InvivoGen (Toulouse, France). Antibodies against pneumococcal DnaK, EF-Tu, and GAPDH were generated by Eurofins Genomics K.K. (Tokyo, Japan). Briefly, rabbits were immunized intracutaneously with 200 μg of rDnaK, rEF-Tu, or rGAPDH emulsified with an equal volume of Freund's complete adjuvant. Two weeks later, booster immunizations were done using the same amount of each recombinant protein emulsified with incomplete Freund's adjuvant. Second booster immunizations were done 2 weeks later. Ten days after the second booster immunizations, rabbits were sacrificed and sera were extracted from blood collected by cardiac puncture. Immunoglobulin G (IgG) antibodies were purified using protein A column.

2.2. Animals

In this study, male, 8–10 weeks old 3d mice (*Unc93b1* mutant; TLR3, TLR7, and TLR9 ligand-unresponsive), its wild-type littermate control mice, C3H/HeN, C3H/HeJ mice, and BALB/c mice were used. 3d mice were obtained as described previously [14], and all other mice were purchased from Nihon CLEA (Tokyo, Japan). Mice were maintained under standard conditions in accordance with our institutional guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of Niigata University.

2.3. Preparation of bacterial supernatants and purification of extracellular DNA

S. pneumoniae D39 and Δ*lytA* strains were grown statically in TSB at 37 °C under aerobic conditions. Overnight cultures were inoculated into fresh TSB to allow continued bacterial growth. Optical densities of 600 nm at each time points were analyzed using miniphoto518R (TAITEC, Saitama, Japan). After 6-, 12-, and 24-h incubation, bacterial supernatants were collected by centrifugation at 3000 × *g* for 10 min and cells were removed by filtration using a 0.22-μm pore size membrane filter (Merck Millipore, Billerica, MA, USA). Real-time polymerase chain reaction (PCR) was performed to quantify extracellular DNA (eDNA) in the bacterial supernatant, as described below. After 12-h incubation, eDNA was also purified from pneumococcal culture supernatants by phenol-chloroform purification and ethanol precipitation, and then stored in Tris-EDTA buffer (10 mM Tris; 0.1 mM EDTA; pH 8.0) at −20 °C until it was required for further assays.

2.4. Quantification of DNA by real-time PCR

To determine the concentration of eDNA in bacterial culture supernatants, absolute quantification in real-time PCR was performed with the StepOnePlus real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) using the SYBR Green detection protocol. Optimization of the real-time PCR reaction was performed according to the manufacturer's instructions. To prepare a standard curve, *S. pneumoniae* DNA was extracted and purified using GenElute™ bacterial genomic DNA kit (Sigma-Aldrich) according to the kit manual, and then the concentration was determined using a spectrophotometer (e-spect; Malcom, Tokyo, Japan). The primers used for real-time PCR were designed to target a fragment of PLY-encoding gene of *S. pneumoniae*, and were based on a published sequence [15]. The forward primer oligonucleotide sequence was 5′-AGCGATAGCTTTCTCCAAGTGG-3′, and the reverse primer sequence was 5′-CTTAGCCAACAAATCGTTTACCG-3′.

2.5. Cell preparation and culture

Mice were injected intraperitoneally with 4 mL of 4% thioglycolate medium (Becton Dickinson, Franklin Lakes, NJ, USA). Four days later, peritoneal macrophages were isolated by peritoneal lavage using 10 mL sterile phosphate buffered saline. The cells (1×10^5 cells/200 μL) were seeded onto a 96-well plate (Becton Dickinson) and cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640; Wako Pure Chemical Industries, Osaka, Japan) at 37 °C in 95% air and 5% CO_2. After 60 min, cells were washed with RPMI 1640 to remove non-adherent cells. The recovered adherent cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Japan Bio Serum, Hiroshima, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako Pure Chemical Industries).

Human monocytic cell line THP-1 (ATCC TIB-202) were obtained from RIKEN Cell Bank (Ibaraki, Japan). The cells (1×10^5 cells/200 μL) were incubated in RPMI 1640 supplemented with 200 nM phorbol 12-myristate 13-acetate (Cayman Chemical, Ann Arbor, MI, USA) to induce differentiation into macrophage-like cells at 37 °C in 95% air and 5% CO_2. After 48 h of incubation, the cells were washed with RPMI 1640 and cultured further in the medium without FBS for 24 h, and then the medium was changed to remove the cytokines induced by cell adherence. The recovered adherent cells were grown in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin.

2.6. Protein identification

Pneumococcal eDNA from the bacterial culture supernatant, purified DNA, or TSB as control was mixed with 2% sodium dodecyl sulfate (SDS) sample buffer, heated at 99 °C for 3 min, separated by SDS-