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Immunization with LytB protein of *Streptococcus pneumoniae* activates complement-mediated phagocytosis and induces protection against pneumonia and sepsis

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ABSTRACT

The cell wall glucosaminidase LytB of *Streptococcus pneumoniae* is a surface exposed protein involved in daughter cell separation, biofilm formation and contributes to different aspects of the pathogenesis process. In this study we have characterized the antibody responses after immunization of mice with LytB in the presence of alhydrogel as an adjuvant. Enzyme-linked immunosorbent assays measuring different subclasses of immunoglobulin G, demonstrated that the antibody responses to LytB were predominantly IgG1 and IgG2b, followed by IgG3 and IgG2a subclasses. Complement-mediated immunity against two different pneumococcal serotypes was investigated using sera from immunized mice. Immunization with LytB increased the recognition of *S. pneumoniae* by complement components C1q and C3b demonstrating that anti-LytB antibodies trigger activation of the classical pathway. Phagocytosis assays showed that serum containing antibodies to LytB stimulates neutrophil-mediated phagocytosis against *S. pneumoniae*. Animal models of infection including invasive pneumonia and sepsis were performed with two different clinical isolates. Vaccination with LytB increased bacterial clearance and induced protection demonstrating that LytB might be a good candidate to be considered in a future protein-based vaccine against *S. pneumoniae*.

1. Introduction

Streptococcus pneumoniae is one of the leading etiologic agents of community-acquired pneumonia, sepsis and bacterial meningitis particularly affecting children and elderly adults [1,2]. Prevention of invasive pneumococcal disease (IPD) is one of the leading priorities in public health due to the high morbidity and mortality rates worldwide, especially in children in developing countries [3]. Current prophylactic measures against pneumococcal infection are based on polysaccharide-containing vaccines that may be conjugated to a carrier protein in order to elicit protection in children [4]. However, one of the major disadvantages of these polysaccharide vaccines is the extensive variability among the pneumococcal population with up to 96 different capsular polysaccharides (CPS) described to date [5]. An additional limitation is that capsular switching appeared after the introduction of pneumococcal conjugate vaccines (PCVs), which results in a serious concern because these strains may emerge by avoiding vaccine-induced immunity due to acquisition of capsular genes from non-vaccine serotypes [6–8]. To circumvent these problems, efforts are being made to investigate *S. pneumoniae* protein-based candidate vaccines that may protect against different serotypes. In this study we investigated the cell wall hydrolase LytB of *S. pneumoniae* as a potential vaccine candidate. LytB is located at sites close to the polar ends of the cell and is surface exposed. This enzyme has

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CFU, colony-forming units; CPS, capsular polysaccharide(s); ELISA, enzyme-linked immunosorbent assay(s); FAM-SE, 5,6-carboxyfluorescein succinimidyl ester; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced salt solution; HRP, horseradish peroxidase; IP, intraperitoneal; IPD, invasive pneumococcal disease; ISCIII, Instituto de Salud Carlos III; NMS, normal mouse serum; OD, optical density; OP, opsonophagocytosis; PCV, pneumococcal conjugate vaccine; PPSV, pneumococcal polysaccharide vaccine; RFI, relative percent fluorescence index; SD, standard deviation.

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N-acetylglucosaminidase activity and plays an essential role in daughter cell separation [9,10].

In terms of pathogenesis, LytB participates in biofilm formation, attachment to human epithelial cells and contributes to sepsis and pneumonia by increasing the ability of LytC to avoid complement-mediated immunity and phagocytosis [11–13]. From the prophylactic perspective, LytB might be a promising target for the development of a universal pneumococcal vaccine because an anti-LytB antiserum significantly protected mice from a lethal challenge with different pneumococcal strains [14]. In this study we show that immunization with LytB is immunogenic and enhances complement-mediated immunity and phagocytosis of different serotypes of *S. pneumoniae*. As a consequence, vaccination using LytB increases bacterial clearance and induces protection against pneumococcal sepsis and invasive pneumonia.

2. Materials and methods

2.1. Bacterial strains

S. pneumoniae clinical isolates used in this study included strain 957 (serotype 3; amoxicillin MIC = 0.015 μg ml^{-1}; erythromycin MIC = 0.25 μg ml^{-1}, levofloxacin MIC = 1 μg ml^{-1}, tetracycline MIC = 0.5 μg ml^{-1}, chloramphenicol MIC = 4 μg ml^{-1}) and strain 48 (serotype 23F; amoxicillin MIC = 16 μg ml^{-1}; erythromycin MIC > 128 μg ml^{-1}, levofloxacin MIC = 1 μg ml^{-1}, tetracycline MIC = 64 μg ml^{-1}, chloramphenicol MIC = 4 μg ml^{-1}). Pneumococcal isolates were cultured on blood agar plates at 37 °C in 5% CO_2 or in Todd-Hewitt broth supplemented with 0.5% yeast extract to an optical density at 580 nm (OD_{580}) of 0.4 (approximately 10^8 colony-forming units [CFU] ml^{-1}) and stored at −70 °C in 10% glycerol as single-use aliquots.

2.2. Vaccination experiments in mice

BALB/c mice were bred by the Instituto de Salud Carlos III (ISCIII) animal facility. All mice used were 8–16 weeks old and, within each experiment, groups of mice were matched for age and sex. Animal experiments were performed at ISCIII in accordance with Spanish legislation (RD 1201/2005) and EU regulations (218/63/EU). The animal experiments performed in this work were approved by the Animal Care and Use Committee of ISCIII (CBA PA 52_2011-v2 and PROEX 218/15).

The pneumococcal LytB protein used for immunization studies was purified as previously described [9,10] and was prepared for animal inoculations at 20–40 μg in Alum (Alhydrogel; aluminum hydroxide, InvivoGen) as the adjuvant in a 1:1 proportion. Groups of 5 mice were immunized by intraperitoneal (IP) inoculation of 200 μl of Alum alone or 200 μl of LytB protein preparation in Alum adjuvant on days 0, 7, and 14 as previously described [15]. Animals were euthanized on day 21 and blood was collected from cardiac puncture and conserved as pooled for further *in vitro* assays. For protection experiments against sepsis, groups of 10 mice were immunized as previously described, followed by IP challenge on day 21 with 10^3 CFU/per mouse of serotype 3 strain or 10^7 CFU per mouse of serotype 23F strain representing at least the lethal dose 50 (LD_{50}) of each strain for sepsis infection. The immunization schedule for protection against pneumonia was the same as before although the bacterial challenge was performed by the intranasal route with 50 μl of 10^4 CFU/mouse of serotype 3 strain or 5×10^7 CFU/mouse of serotype 23F strain representing at least the LD_{50} of each strain for pneumonia. Bacterial counts were determined during the first 24–48 h from blood samples (6 μl per mouse) obtained from the tail vein of infected animals as previously described [16]. The development of disease was monitored daily, and mice were sacrificed when they exhibited severe signs of disease.

2.3. Enzyme linked immunosorbent assays to detect Ig subclasses

Specific antibody titers in pooled sera from five mice of each group were measured by enzyme-linked immunosorbent assays (ELISA) using 96-well polystyrene Maxisorp plates (Nunc) coated with 0.5 μg of purified LytB protein for 2 h at 37 °C and blocked with a PBS-2% bovine serum albumin (BSA) solution as previously described [17]. Bound antibodies were detected by using horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 and IgA; Santa Cruz) for 30 min and developed using *o*-phenylenediamine (Sigma-Aldrich) before determining the OD_{492} using a microtiter plate reader (Anthos 2020).

2.4. Activation of complement immunity

Complement activation was assessed using flow cytometry assays, as described before [17–19]. Briefly, C3b deposition was analyzed by incubating 5×10^6 CFU of *S. pneumoniae* in 10 μl of serum (diluted to 50% in PBS) for 30 min at 37 °C using pooled sera from mice immunized with Alum alone or immunized with LytB−Alum. Bacteria were then incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse C3b antibody (ICN-Cappel) diluted 1/300 in PBS. Then, bacteria were fixed in 3% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD Biosciences) using forward and side scatter parameters to gate on at least 25,000 cells. This assay was adapted for C1q binding incubating for 1 h with rabbit anti-mouse C1q antibody (Abcam) followed by an additional incubation with FITC-conjugated polyclonal goat anti-rabbit IgG. The results were expressed as a relative percent fluorescence index (RFI) that measures not only the proportion of fluorescent bacteria positive for the host serum component investigated but also the intensity of fluorescence that quantify the immune component bound [19,20].

2.5. Opsonophagocytosis

Phagocytosis was evaluated using a flow cytometry assay including *S. pneumoniae* strains described above labeled with 5,6-carboxyfluorescein succinimidyl ester (FAM-SE; Molecular Probes) and human HL-60 cells (CCL-240; ATCC) differentiated to granulocytes. The general conditions of the assay were based on those described previously except that clinical isolates of serotype 3 and 23F were incubated with pooled sera from mice immunized with Alum alone or immunized with the mixture LytB-Alum [16,21]. Infection assays were performed with a ratio of 10 bacteria per cell. A minimum of 6000 cells were analyzed using a FACS Calibur flow cytometer. Results were expressed as a RFI defined as the proportion of positive cells for fluorescent bacteria multiplied by the geometric mean of fluorescence intensity, which correlates with the amount of bacteria phagocytosed per cell.

2.6. T cell response

Experiments investigating cellular populations of lymphocytes were performed in non-immunized mice and in mice immunized with Alum alone or vaccinated with LytB mixed with Alum as explained above. Briefly, single-cell suspensions of the spleens of each group, were prepared in staining buffer (2% fetal calf serum in PBS). Detection of CD3, CD4 and CD8 was performed using standard protocols with the following antibodies diluted 1/300 in staining buffer (Armenian hamster anti-mouse CD3-phycoerythrin (PE), Tonbo; rat anti-mouse CD4-FITC, Biolegend;

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