Full Length Research Paper

Attenuated HtrA-mutant of *Streptococcus pneumoniae* induces protection in murine models of pneumococcal pneumonia and bacteremia

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*Streptococcus pneumoniae* (the pneumococcus) is a Gram-positive human pathogen that causes a number of diseases such as pneumonia, otitis media, bacteremia, and meningitis. The increasing rate by which the pneumococcus acquires resistance to the widely used antibiotics and the limitations of currently licensed vaccines have made pneumococcal diseases a major health problem. Search for more effective vaccines is ongoing and many pneumococcal proteins associated with virulence are now considered good candidates for new vaccine formulas. This study reports that intranasal immunization of mice with a live attenuated strain of the pneumococcus containing a deletion in the gene encoding for the High Temperature Requirement A (HtrA) protein, one of the major virulence factors in the pneumococcus, induces a cross-protection from pneumococcal infection. Mucosal and serum antibody levels were significantly increased in mice immunized with the HtrA mutant. Data presented here, together with other published reports, suggest that nasal immunization with live attenuated pneumococcal strains is a safe and effective strategy for the prevention of pneumococcal infections.

Key words: *S. pneumoniae*, HtrA protein, live attenuated vaccines, pneumococcal infection.

INTRODUCTION

The pneumococcus (*Streptococcus pneumoniae*) is a leading cause of many human diseases in both adults and children worldwide. These diseases include otitis media, pneumonia, bacteremia, and meningitis that result in millions of deaths every year (Klein, 1981; Gray and Dillon, 1986; Levine et al., 2006). The pneumococcus is carried in the upper respiratory tract by many healthy individuals. However, most infections do not occur following extended carriage but after the acquisition of recently acquired serotype (Gray et al., 1980; Johnson et al., 1991). The immune status and the virulence factors of pneumococcal strain therefore determine whether the pneumococcus will be confined to the nasopharynx or becomes invasive.

The continuous emergence of antibiotic-resistant strains of pneumococci (Butler et al., 1998) urges the need for developing effective vaccines against this life-threatening bacterium. Currently, there are four commercially available vaccines for the prevention of pneumococcal diseases; Pneumovax<sup>®</sup> 23, the 7-valent Pneumococcal Conjugate Vaccine (PCV7), PCV10, and PCV13. The first is a 23-valent polysaccharide vaccine containing the polysaccharide capsules of *S. pneumoniae* strains commonly involved in pneumococcal infection. Despite being effective in adults (Shapiro et al., 1991; Butler et al., 1993), this polysaccharide vaccine is unable to produce protective antibodies in children under two years of age (Butler et al., 1993). The reason for this is that polysaccharide antigens are T-cell independent, which stimulate mature B cells without the help of T cells. The B cells of young children do not respond to most polysaccharide antigens. The other problem with polysaccharide vaccine is the ability of pneumococcal strains to switch capsular type *in vivo* (Nesin et al., 1998).
Table 1. List of bacterial strains used in this study.

<table>
<thead>
<tr>
<th>S. pneumoniae strain</th>
<th>Details</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>Serotype 2, NCTC 7466</td>
<td>(Avery et al., 1944)</td>
</tr>
<tr>
<td>TIGR4</td>
<td>Serotype 4, clinical isolate</td>
<td>(Tettelin et al., 2001)</td>
</tr>
<tr>
<td>D39 ΔhtrA</td>
<td>D39 with replacement of htrA with spectinomycin cassette</td>
<td>(Ibrahim et al., 2004)</td>
</tr>
<tr>
<td>D39 ΔhtrA /htrA+</td>
<td>D39,ΔhtrA complemented with pAL2HtrA plasmid</td>
<td>(Ibrahim et al., 2004)</td>
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</table>

PCV7 contains polysaccharides from seven pneumococcal serotypes, which are most commonly associated with invasive and non-invasive infection in children, conjugated with T-cell dependent immunogenic proteins. This vaccine was introduced to overcome the limitation of polysaccharide vaccine and proved effective in infants and young children (Kayhty and Eskola, 1996; Klein, 1999). However, it has been shown to induce the emergence of nonvaccine pneumococcal serotypes (Toltzis and Jacobs, 2005) besides the limitation of its cost. Recently, two new pneumococcal conjugate vaccines were approved; the 10-valent vaccine (PCV10, Synflorix™, GlaxoSmithKline Inc., Belgium) and the 13-valent vaccine (PCV13, Prevenar®, Pfizer Inc., USA). These two vaccines contain three and six additional pneumococcal serotypes to PCV7, respectively and expected to prevent against more infections caused by S. pneumoniae. Several studies have also investigated live attenuated mutants or killed whole bacteria as potential candidates for pneumococcal vaccines (Talkington et al., 1996; Malley et al., 2001; Roche et al., 2007; Kim et al., 2012).

The High Temperature Requirement A protein (HtrA) is a stress-induced serine protease that has a major role in helping organisms to survive environmental stresses such as oxidative stress and elevated temperatures (Zumbrunn and Trueb, 1996; Ponting, 1997; Gray et al., 2000). This protease is required for virulence in many Gram-negative bacteria (Baumler et al., 1994; Elzer et al., 1996; Li et al., 1996) and is also a virulence factor in Gram-positive bacteria such as Streptococcus pyogenes (Jones et al., 2001). HtrA was identified as a virulence factor of S. pneumoniae in a screen using signature-tagged mutagenesis (Hava and Camilli, 2002) and was also found to play a role in nasopharyngeal colonization of the pneumococcus (Sebert et al., 2002). The author of this study has recently reported that HtrA is a major virulence factor of S. pneumoniae. HtrA-deficient mutants of both serotype 2 (D39) and serotype 4 (TIGR4) pneumococci were attenuated in mouse models of pneumococcal pneumonia and bacteremia (Ibrahim et al., 2004). However, our mutant strains colonized the nasopharynx in the same way as the wild type strains contrary to a published report (Sebert et al., 2002). The main focus of current study aimed at investigating the possibility of using HtrA-attenuated strains as live vaccines to elicit an immune response and to provide protection against wild type lethal strains of S. pneumoniae.

MATERIALS AND METHODS

Bacterial strains and challenge dose preparation

S. pneumoniae strains used in this study are listed in Table 1. Bacteria were grown on blood agar base number 2 (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (BAB) and on Brain Heart Infusion (BHI). The challenge dose was prepared by passing S. pneumoniae strains through mice as described in a published report (Alexander et al., 1994), and aliquots were stored at -80°C. Pneumococci could be stored for at least 3 months at -80°C with no significant loss of viability. Strain validation was carried out by checking bacterial susceptibility to the antibiotic optochin (Difco) and by serotyping via the Quellung reaction.

When required, a sample was thawed rapidly and bacteria were harvested by centrifugation before re-suspension in an appropriate volume of sterile phosphate-buffered saline (PBS) (Oxoid).

Murine model of nasopharyngeal colonization

Female outbred MF1 mice (25-30 g) were used in this study when they were 9 weeks old as standard models of pneumococcal pneumonia and bacteremia. During all experiments, animals were supplied with balanced rodent food and water ad libitum. Pneumococci colonizing the nasopharynx of the test animals were collected for counting by doing nasopharyngeal lavage at two time points; 12 h-post-infection and 48 h-post-infection. Groups of mice (six mice each) were intranasally infected with 1 × 10^4 CFU/mouse as follows: Mice were lightly anaesthetized with 1.5% (vol/vol) halothane (Zeneca Pharmaceuticals, Macclesfield, UK) over oxygen (1.5 L/min) using a calibrated vaporizer. Anaesthesia was confirmed when mice failed to produce a reflex reaction when gently pinched. The infectious dose (1 × 10^4 CFU/mouse) was administered in 50 µl of bacterial suspension to the nostrils of mice held vertical (Kadioglu et al., 2000) using a Proline® pipette (Biohit) and mice were laid on their backs until they recovered. At the indicated time points after intranasal infection, mice were sacrificed and the nasopharynx was washed by injecting 1 ml of sterile PBS into the trachea and collecting the fluid coming from the naris. The nasopharyngeal washes were then plated onto BAB with 20 µg/ml of neomycin to prevent the growth of other contaminants.

Immunization of mice and infectious challenges

Groups of ten mice were immunized with the live attenuated HtrA-mutant of the type 2 strain D39 (D39 ΔhtrA) to assess its ability to protect against pneumococcal infection. Fifty microliter (50µl) of PBS containing 1×10^6 CFU of the mutant strain were administered
intranasally as described above. Control mice were inoculated with PBS alone.

Four weeks after immunization, mice were challenged intranasally with 1x10^6 CFU of the wild type strains D39 and TIGR4 and also with the complemented strain D39 ΔhtrA /htrA+. Survival of mice was observed over a period of nine days. When showing signs of lethargy or upon becoming moribund, mice were humanely euthanized and were considered to reach the endpoint of the experiment (Toth, 1997). Mice that survived the course of infection were assigned an arbitrary survival time of 216 h for statistical analysis.

For intravenous infection, Bacterial suspensions were prepared from standard inocula to give 2x 10^6 CFU/ml. Mice were placed in a ventilated, heated Perspex box for 5 min in order to expose veins and were restrained in appropriate apparatus. One hundred microliter (100 µl) of bacterial suspension was administered directly into the bloodstream via the lateral vein using 1-ml insulin syringe (Microfine, 12.7 mm, Becton Dickinson). A bleed was taken from a separate vein immediately following injection to ensure successful infection by bacterial enumeration (Kerr et al., 2002). Survival of mice was observed over a period of nine days.

Measurement of bacterial load and antibody titers

Four days post-infection, viable count of bacteria in the nasal lavage fluids collected from the nasopharynx of challenged mice was determined in serial dilutions plated on BAB selective media containing 20 µg/ml of neomycin. Dead mice were given arbitrary values just above the maximum values for the statistical analysis.

Samples of sera and lavage fluids were stored at -20°C to measure antibody concentrations. The antibody titer to S. pneumoniae was determined by enzyme-linked immunosorbent assay (ELISA) according to published protocols (Medina et al., 2008; Vintini et al., 2010). PBS-washed whole bacteria diluted with coating buffer (0.015 M Na2CO3, 0.035 M NaHCO3) to an optical density of 0.1, were fixed by overnight incubation at 4°C on 96-well plates. Non-specific protein binding sites were blocked with PBS containing 5% non-fat milk. Samples were diluted (Serum 1:100 and Nasal Lavage 1:4) with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Peroxidase-conjugated goat anti-mouse IgA and IgG (Fc specific; Sigma Chemical, St Louis, USA) were diluted (1:500) in PBS-T. Antibodies were revealed with a substrate solution [o-phenylenediamine (Sigma)] in citrate-phosphate buffer (pH 5, containing 0.05% H2O2) and the reaction was stopped by the addition of 1 M H2SO4. Readings were carried out at 493 nm and samples were considered negative for the presence of specific antibodies when OD493 < 0.1.

Statistical analysis

Statistical analyses were carried out using StatView 5. Comparison of colonization among groups, survival times, and ELISA data were analyzed by using nonparametric Mann-Whitney U analysis. In all analyses, a p-value less than 0.05 was considered statistically significant.

RESULTS

HtrA-mutant displayed similar colonization densities compared to the wild type strains.

Since the ability of vaccine strain to induce protection is dependent on its persistence on mucosal surface, this study tested the effect of HtrA mutation on the degree of nasopharyngeal colonization by the pneumococcus after intranasal infection. 12 and 48 h post-infection, colonization densities by the HtrA mutant of type 2 strain was comparable to those of the wild types of both type 2 and type 4 parent strains, and also to that of the complemented strain of type 2, D39 ΔhtrA /htrA+ (Table 2). This suggests that HtrA mutation did not affect the colonization fitness of the mutant strain and raised the expectations that this live attenuated mutant could be able to induce immunity against virulent wild type strains.

Ability of HtrA mutant to induce protection

To test the ability of HtrA mutant to induce protection against pneumococci, two pneumococcal infection models were used; a pneumonia model and a bacteraemia model. In the pneumonia model, mice were immunized with the HtrA mutant and were challenged intranasally with the infectious dose of the tested strains four weeks after immunization. Following challenge with parent strains D39 and TIGR4 as well as the complemented strain D39 ΔhtrA /htrA+, survival of mice in all immunized groups was significantly longer than that of the non-immunized control group of either D39 (Figure 1A) or TIGR4 (Figure 1B). These data suggest that colonization by the live attenuated HtrA mutant can induce mucosal immunity.

In the systemic bacteraemia model, survival of mice after intravenous infection with the tested strains was also significantly longer than that of the control groups (Figure 2 A and B) suggesting that HtrA mutant can also

Table 2. Pneumococcal colonization of the nasopharynx of mice after intranasal infection.

<table>
<thead>
<tr>
<th>Time post-infection (H)</th>
<th>Mean log CFU/ml ± SEM*</th>
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<tbody>
<tr>
<td></td>
<td>D39</td>
</tr>
<tr>
<td>12</td>
<td>4.62 ± 0.72</td>
</tr>
<tr>
<td>48</td>
<td>4.53 ± 1.12</td>
</tr>
</tbody>
</table>

Groups of mice were infected with 10^6 CFU/mouse of each strain. At the indicated time points, bacteria colonizing the nasopharynx were collected and counted on BAB plates. * Standard Error of the Mean. No significant differences were observed in the number of bacteria colonizing the nasopharynx in mutant strains compared to the wild type strain at both time points.
Figure 1. Survival of immunized MF1 mice after intranasal challenge. Groups of mice were immunized with the HtrA mutant and four weeks after immunization, they were challenged intranasally with $1 \times 10^6$ CFU/mouse of the indicated pneumococcal strains. The non-immunized group in (A) was challenged with D39 wild type strain, while the non-immunized group in (B) was challenged with TIGR4 wild type strain. *, $P=0.002$, **, $P=0.036$ longer survival compared to non-immunized group.

This study also tested the effect of immunization with the HtrA mutant on the colonization densities by wild type strains. Viable count of D39, TIGR4, and the complemented strain D39 $\Delta$htrA /htrA+ in the nasal lavage after intranasal immunization with the attenuated live vaccine strain was significantly reduced compared to the control groups (Figure 3). These data suggest that virulent wild type strains were unable to survive in the nasopharynx of immunized mice and therefore indicate a role of mucosal immunity conferred by the attenuated HtrA mutant.

Effect of immunization with HtrA mutant on antibody titers

This study explored the mechanism of protection of the live attenuated strain of the *S. pneumoniae* by investigating the role of humoral immunity. To evaluate the effect of HtrA mutation on the specific antibody responses in mucosal and systemic compartments, the IgG titers to whole pneumococci were measured in serum of mice groups in which there was protection from D39, TIGR4, and D39 $\Delta$htrA /htrA+ compared to that in the non-immunized control groups. Data showed that the levels of IgG in serum of immunized mice were...
Figure 2. Survival of immunized MF1 mice after intravenous challenge. Groups of mice were immunized with the HtrA mutant and four weeks after immunization they were challenged intravenously with $1 \times 10^5$ CFU/ mouse of the indicated strains. The non-immunized group in (A) was challenged with D39 wild type strain, while the non-immunized group in (B) was challenged with TIGR4 wild type strain. *, $P = 0.001$, **, $P = 0.021$, ***, $P = 0.015$ longer survival compared to non-immunized group.

Figure 2. Survival of immunized MF1 mice after intravenous challenge. Groups of mice were immunized with the HtrA mutant and four weeks after immunization they were challenged intravenously with $1 \times 10^5$ CFU/mouse of the indicated strains. The non-immunized group in (A) was challenged with D39 wild type strain, while the non-immunized group in (B) was challenged with TIGR4 wild type strain. *, $P = 0.001$, **, $P = 0.021$, ***, $P = 0.015$ longer survival compared to non-immunized group.

significantly higher than that in serum of the non-immunized mice (Figure 4A).

In the nasal lavage fluid, the levels of pneumococcus-specific IgA induced by the HtrA mutant on the mucosal surface were also increased in the immunized mice while animals that did not receive the live attenuated mutant showed very low mucosal antibody level (Figure 4B). Thus our data suggest that nasal immunization with the live attenuated pneumococcal HtrA mutant was able to enhance the specific humoral immune responses at the systemic and mucosal levels.

DISCUSSION

*S. pneumoniae* is an important human pathogen that continues to cause life-threatening infections in both developing and developed countries (Mulolland, 2007; Scott, 2008). Despite the widespread use of antibiotics, the emergence of antibiotic resistant pneumococcal strains and some problems associated with vaccines have made pneumococcal diseases a major health problem. Since the pneumococcus enters the host primarily through the respiratory mucosa, vaccination strategies by the nasal route are of great interest and one approach for the prevention of pneumococcal infection is the use of live attenuated mutants which contain deletions in the genes encoding for major virulence factors. Most vaccines delivered parenterally are not completely effective to induce mucosal immunity (Walker, 1994). Moreover, the nasal route proved to be the most effective pathway to induce protective immunity against
Figure 3. Bacterial load in the nasopharynx of mice challenged intranasally with the indicated strains after immunization with the HtrA-mutant. D39 non-immunized group was used as a control group for type 2 strains while TIGR4 non-immunized group was used to control for type 4 strain. Viable count of bacteria in the nasopharynx of challenged mice was determined in serial dilutions plated on BAB selective media containing 20 µg/ml of neomycin. Values represent the means ± standard error of the means. The dotted line represents the limit of detection of the assay. *, $P < 0.006$ for type 2 strains and **, $P = 0.013$ for type 4 strain.

Figure 4. Antibody titers measured by ELISA. Groups of mice were immunized with the HtrA-mutant strain and challenged with the indicated strains. (A) Levels of IgG in serum against pneumococcal HtrA-mutant following immunization in the indicated group of mice. Values are expressed as geometric mean titers (GMT) ± standard errors of the means (SEM). (B) Levels of IgA against pneumococcal HtrA-mutant in nasal lavage of mice following immunization in the indicated groups. Values are expressed as GMT/µg protein ± SEM. *, $P < 0.001$. 
the pneumococcus (Wu et al., 1997; Daniels et al., 2010).

It has been previously reported that deletion in the gene for High Temperature Requirement A (HtrA) protein in the pneumococcus produced a highly attenuated mutant (Ibrahim et al., 2004). On this basis, it was hypothesized that, if able to colonize the nasopharynx, HtrA mutant could induce protective immunity against pneumococcal infection. This study, therefore, addressed whether mucosal and systemic immunity can be induced through nasal immunization with a live attenuated HtrA-mutant of the pneumococcus.

This study first confirmed that the colonization fitness of the mutant is not affected by deletion of the HtrA gene. Contrary to a published report (Sebert et al., 2002), our data suggested that HtrA mutant did not lose the capability to colonize the nasopharynx of mice due to deletion of HtrA. This could be due to differences between strains used. This study then tested the ability of our mutant to induce protection from virulent pneumococcal strains after intranasal immunization. Our findings demonstrated that intranasal immunization with HtrA mutant provided protection against the wild type strains of two different serotypes (type 2 and type 4) when studied in both pneumonia and bacteraemia models. Other studies have shown similar findings with other pneumococcal antigens after intranasal colonization with the vaccine strains either in the same genetic background (Trzcinski et al., 2005) or in different backgrounds (Roche et al., 2007). Protection induced by HtrA mutant was also confirmed by the inability of wild type strains to colonize the nasopharynx of immunized mice after intranasal infection. Failure of wild type strains to cause infection in immunized mice offered the possibility that the clearance of the virulent strains could be due to mucosal immunity.

It is known that nasopharyngeal colonization stimulates the production of secretory IgA and serum IgG antibodies. Antibodies against pneumococcal proteins in serum and saliva have been associated with increased protection against pneumococcal carriage (Zhang et al., 2006). This study investigated the role of mucosal and systemic immunity in the cross protection conferred by the HtrA mutant. Levels of pneumococcal specific antibodies IgG and IgA were significantly elevated in all groups of immunized mice; D39, TIGR4, and D39ΔhtrA compared to the control groups. IgG and IgA antibodies are significant for the defense against respiratory infections. IgG promotes phagocytosis and prevents local propagation of the pathogen in the alveoli as well as its passage into blood. IgA prevents pathogen adherence to epithelial cells, thus reducing colonization (Twigg, 2005). These antibodies were also shown to opsonize pneumococci, making it easier for phagocytes to recognize, ingest and clear bacteria from the respiratory tract (Gordon et al., 2000). In this study, failure of the wild type strains to colonize the nasopharynx could be attributed to the increased level of IgA induced by the live attenuated HtrA mutant in immunized mice. Moreover, the increase in serum IgG levels could account for the protection from sepsis observed after intravenous infection.

In conclusion, this study demonstrated that nasal immunization with the live attenuated HtrA strain produced cross-protection against pneumococcal infection in the two genetic backgrounds studied. This study also provided additional evidence that the nasal route is optimistic when considering vaccination strategies in human. However, effectiveness of immunization with this live attenuated strain is not guaranteed in human.

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REFERENCES


