Enhancement of Clearance of Bacteria from Murine Lungs by Immunization with Detoxified Lipooligosaccharide from *Moraxella catarrhalis* Conjugated to Proteins

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Received 22 February 2000/Returned for modification 1 May 2000/Accepted 9 June 2000

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*M. catarrhalis* strain 25238 detoxified lipooligosaccharide (dLOS)-protein conjugates induced a significant rise of bactericidal anti-LOS antibodies in animals. This study reports the effect of active or passive immunization with the conjugates or their antisera on pulmonary clearance of *M. catarrhalis* in an aerosol challenge mouse model. Mice were injected subcutaneously with dLOS-tetanus toxoid (dLOS-TT), dLOS–high-molecular-weight proteins (dLOS-HMP) from nontypeable *Haemophilus influenzae* (NTHi), or nonconjugated materials in Ribi adjuvant and then challenged with *M. catarrhalis* strain 25238 or O35E or NTHi strain 12. Immunization with dLOS-TT or dLOS-HMP generated a significant rise of serum anti-LOS immunoglobulin G and 68% and 35 to 41% reductions of bacteria in lungs compared with the control (*P < 0.01*) following challenge with homologous strain 25238 and heterologous strain O35E, respectively. Serum anti-LOS antibody levels correlated with its bactericidal titers against *M. catarrhalis* and bacterial CFU in lungs. Additionally, immunization with dLOS-HMP generated a 54% reduction of NTHi strain 12 compared with the control (*P < 0.01*). Passive immunization with a rabbit antisera against dLOS-TT conferred a significant reduction of strain 25238 CFU in lungs in a dose- and time-dependent pattern compared with preimmune serum-treated mice. Kinetic examination of lung tissue sections demonstrated that antisera-treated mice initiated and offset inflammatory responses more rapidly than preimmune serum-treated mice. These data indicate that LOS antibodies (whether active or passive) play a major role in the enhancement of pulmonary clearance of different test strains of *M. catarrhalis* in mice. In addition, dLOS-HMP is a potential candidate for a bivalent vaccine against *M. catarrhalis* and NTHi infections.

*M. catarrhalis* has emerged as a significant human pathogen and may be the cause of more childhood infectious diseases than previously thought (8, 16, 33). The most *M. catarrhalis*-susceptible populations are very young children and the elderly. In young children, *M. catarrhalis* is the third-most-common cause of otitis media, associated with 15 to 20% of all cases reported, following *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) (6, 17). More than 70% of children are likely to experience at least one episode of otitis media by the age of 3 years (41). Recurrent or chronic otitis media can lead to hearing and/or speech impairment or to language delay. *M. catarrhalis* is also a significant cause of sinusitis and persistent cough in young children (2, 25). In the elderly, especially those with chronic obstructive pulmonary diseases or compromised immune systems, *M. catarrhalis* can account for lower respiratory tract infections such as bronchitis or pneumonia. Although invasive diseases caused by *M. catarrhalis* such as bacteremia, meningitis, and endocarditis are less common, they can be fatal (11, 30, 32). Currently, the rate of β-lactamase-producing strains in some areas of the United States has increased to 95% (12), and more clinical isolates are resistant to β-lactam antibiotics (24).

Active immunization with an effective vaccine would be an efficient approach to prevent *M. catarrhalis* infections. Much research has been performed on the outer membrane protein antigens of *M. catarrhalis* in an attempt to identify potential vaccination antigens (9, 28, 34, 35). This approach has led to the identification of several outer membrane proteins, such as the ubiquitous surface protein A (UspA), B1, B2, CD, and E. Some of these antigens have been reported to be protective in a murine model of human diseases (9, 34). Currently there is no vaccine available to prevent the diseases caused by *M. catarrhalis*, largely because the pathogenic mechanism and the host immune response to this pathogen have yet to be clarified.

The lipooligosaccharide (LOS) molecule is a prominent surface component of *M. catarrhalis* and has been implicated as a virulence factor important in the pathogenesis of this organism (13, 21). Less attention has been paid to *M. catarrhalis* LOS as a vaccine component due to its toxicity and weak immunogenicity in vivo. However, LOS has several characteristics that make it an attractive vaccine candidate. Serum antibodies to LOS developed in patients with *M. catarrhalis* infections (36) and the convalescent-phase anti-LOS immunoglobulin G (IgG) demonstrated bactericidal activity against *M. catarrhalis* (40). In addition, the serological properties of LOS in humans suggest a less variable structure of LOS (36). Only three major antigenic types of *M. catarrhalis* LOS can be distinguished, and more than 90% of 302 strains expressed one of three LOS serotypes (A, 61%; B, 29%; C, 5%) (44). It is possible that a vaccine candidate including two to three types of LOS would generate anti-LOS antibodies with bactericidal activity against majority of the pathogenic strains of *M. catarrhalis*.

The LOS molecule is too toxic to be administered to humans in its native form, and detoxified LOS (dLOS; hapten) does not elicit antibodies in vivo. In a previous study, we used *M. catarrhalis* strain 25238 as a source of LOS (serotype A) and covalently bound the dLOS to a carrier protein, tetanus toxoid (TT), or to a high-molecular-weight protein (HMP) from...
NTHi strain 12. Both proteins improved the immunogenicity of the dLOS. The conjugates were immunogenic and induced bactericidal antibodies against the homologous strain as well as some heterologous strains when these tested in animals (26). In this study we further evaluated the protective effect of these conjugates on the pulmonary clearance of M. catarrhalis homologous strain 25238, heterologous strain O35E, and NTHi strain 12, using an aerosol challenge mouse model.

**MATERIALS AND METHODS**

**Mice.** Female BALB/c mice (5 or 10 weeks of age) were obtained from Taconic Farms Inc. (Germantown, N.Y.). The mice were housed in an animal facility in accordance with National Institutes of Health guidelines under animal study protocol 850-98.

**Bacterial strains and culture conditions.** M. catarrhalis (43) and NTHi strain 12 (3) were provided by E. J. Hansen and S. J. Barenkamp, respectively. These strains were grown on chocolate agar at 37°C with 5% CO2 for 16 h; then three to five clones were transferred to new plates and incubated for 3.5 to 4 h, or until mid-logarithmic phase. Each bacterial suspension was prepared to a desired concentration with sterile phosphate-buffered saline (PBS, pH 7.0) containing 0.1% gelatin, 0.15 mM CaCl2, and 0.5 mM MgCl2, and stored on ice until use. The bacterial concentration was determined by a 65% transmission at 540 nm. The final bacterial number was confirmed by counting the CFU after overnight incubation of the plates at 37°C with 5% CO2.

**Conjugate vaccines.** Conjugates were obtained and prepared as described previously (26). The toxicity of dLOS before conjugation was only 1 endotoxin unit/ml, which was 20,000-fold lower than that of LOS as determined by the Limulus amebocyte lysate assay. The composition of dLOS-TT was 103 µg of dLOS and 266 µg of TT per ml with a molar ratio of dLOS to TT of 19:1; the composition of dLOS-HMP was 220 µg of dLOS and 280 µg of HMP per ml, with a molar ratio of 31:1. The conjugate vaccines were obtained and prepared as described elsewhere (26). Anti-LOS antibody titer in rabbit antiserum was 1:72,900. Sera were diluted to 50, 10, and 5 of serum IgG directed against HMP compared to the control group. 

**TABLE 1. Effect of active immunization with dLOS-TT and dLOS-HMP on bacterial recovery of homologous strain 25238 in mouse lungs**

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Anti-LOS IgG titer</th>
<th>Bacterial recovery (CFU/lung)</th>
<th>Bacterial reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLOS-TT + Ribi</td>
<td>1,223 (90–2,790)</td>
<td>(2.3 × 106) ± 0.9</td>
<td>68</td>
</tr>
<tr>
<td>dLOS-HMP + Ribi</td>
<td>1,846 (810–7,290)</td>
<td>(2.3 × 106) ± 0.8</td>
<td>68</td>
</tr>
<tr>
<td>Strain 25238</td>
<td>3,198 (810–7,290)</td>
<td>(1.3 × 106) ± 0.3</td>
<td>82</td>
</tr>
<tr>
<td>dLOS-TT + HMP + Ribi (control)</td>
<td>12 (10–30)</td>
<td>(7.1 × 105) ± 2.3</td>
<td>0</td>
</tr>
</tbody>
</table>

- Mice were challenged with 107 CFU of M. catarrhalis strain 25238 per ml in a nebulizer, and lungs were collected at 6 h postchallenge.
- Levels of serum antibody against strain 25238 LOS, expressed as reciprocal geometric mean (range) of eight mice per group, were detected by ELISA.
- Mean ± SD of eight mice.
- *P* < 0.01 compared with the control group.
- Positive controls included buffer, AP conjugate, and presera. All negative controls gave optical density readings of less than 0.1. The antibody endpoint titre was defined as the highest dilution of serum giving an A505 twofold greater than that of presera.

**Bactericidal assay.** Sera were inactivated at 56°C for 30 min and measured for bactericidal activity against M. catarrhalis strains 25238 and O35E. A complement-mediated bactericidal assay was performed as described previously (27) except that a guinea pig serum (5 µl per well; Calbiochem-Novabiochem Corp., San Diego, Calif.) was used as a source of complement and the reaction plate was incubated at 37°C for 30 min before plating onto agar plates. The bactericidal titer was determined to be the last dilution of serum causing at least 50% killing.

**Histological examination.** Two mice from each group were euthanized; their lungs were removed and fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin.

**Statistical analysis.** The vials were expressed as the mean CFU of n independent observations ± the standard deviation (SD). Geometric means of bactericidal titers and reciprocal antibody IgG titers were determined. Significance was determined by Student's *t* test.

**RESULTS**

**Effect of active immunization on bacterial clearance from mice.** An ELISA was used to determine the relative levels of M. catarrhalis strain 25238 LOS-specific and NTHi strain 12 HMP-specific IgG antibodies in the vaccinated mouse sera. The mixture of unconjugated dLOS, HMP, and TT was not immunogenic, judging by LOS antibody level after three injections (Tables 1 and 2). However, both conjugates could elicit an approximately 100-fold increase in the level of LOS antibodies compared with the mixture group. Immunization with whole cells from strains 25238 and O35E seemed to elicit a higher LOS antibody level than the conjugate groups, but no significant difference was observed (*P* > 0.05). Three injections of dLOS-HMP or HMP resulted in an 80- to 90-fold increase of serum IgG directed against HMP compared to the control animals (Table 3).

One week after the final immunization, mice from each group were challenged with M. catarrhalis homologous strain 25238, heterologous strain O35E, or NTHi strain 12. When challenged with strain 25238, the number of bacteria recovered from lungs at 6 h postchallenge was significantly (68%) re-
duced in both conjugate-immunized groups compared to the control group \((P < 0.01)\) (Table 1). The degree of clearance was similar to that observed in the group immunized with strain 25238 whole cells. As shown in Table 2, when strain O35E was used as the challenge organism, the protective effect of immunization was also significant. The number of bacteria in the lungs from both conjugate-immunized groups decreased from 35 to 41% compared to the control group at 6 h postchallenge \((P < 0.01)\). A similar protective effect was observed in the strain O35E whole-cell-immunized group. When mice were challenged with NTHi strain 12, the bacterial number recovered from the lungs of the dLOS-HMP group, but not the dLOS-TT group, was reduced significantly \((54\%)\) compared to the control group. A bactericidal test showed that all mouse sera from the preserum-treated group \((P < 0.01)\) but not for strain O35E.

### Table 2. Effect of active immunization with dLOS-TT and dLOS-HMP on bacterial recovery of heterologous strain O35E in mouse lungs

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Anti-LOS IgG titer</th>
<th>Bacterial recovery</th>
<th>Bacterial reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLOS-TT + Ribi</td>
<td>1,223 (90–7,290)</td>
<td>((4.0 \times 10^4) ± 1.6)</td>
<td>44</td>
</tr>
<tr>
<td>dLOS-HMP + Ribi</td>
<td>2,380 (90–7,290)</td>
<td>((4.0 \times 10^4) ± 1.3)</td>
<td>35</td>
</tr>
<tr>
<td>Strain O35E</td>
<td>2,788 (810–7,290)</td>
<td>((4.4 \times 10^4) ± 1.4)</td>
<td>35</td>
</tr>
<tr>
<td>dLOS-TT + HMP + Ribi (control)</td>
<td>15 (10–30)</td>
<td>((6.8 \times 10^3) ± 1.5)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\) Mice were challenged with \(4 \times 10^9\) CFU of \(M. catarrhalis\) strain O35E per ml in a nebulizer, and lungs were collected at 6 h postchallenge.

\(b\) Levels of serum antibody against strain 25238 LOS, expressed as reciprocal geometric mean (range) of eight mice per group, were detected by ELISA.

\(c\) Mean ± SD of eight mice.

\(d\) Compared with the control group.

\(e\) \(P < 0.01\) compared with the control group.

### Table 3. Effect of active immunization with dLOS-TT and dLOS-HMP on bacterial recovery of NTHi strain 12 in mouse lungs

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Anti-HMP IgG titer</th>
<th>Bacterial recovery</th>
<th>Bacterial reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dLOS-TT + Ribi</td>
<td>10 (10–30)</td>
<td>((1.1 \times 10^5) ± 3.1)</td>
<td>8</td>
</tr>
<tr>
<td>dLOS-HMP + Ribi</td>
<td>1,066 (90–2,430)</td>
<td>((5.5 \times 10^4) ± 1.4)</td>
<td>54</td>
</tr>
<tr>
<td>Strain 12</td>
<td>1,403 (270–7,290)</td>
<td>((4.7 \times 10^4) ± 2.2)</td>
<td>61</td>
</tr>
<tr>
<td>HMP + Ribi</td>
<td>929 (90–2,430)</td>
<td>((6.2 \times 10^4) ± 2.2)</td>
<td>48</td>
</tr>
<tr>
<td>dLOS-TT + HMP + Ribi (control)</td>
<td>12 (10–30)</td>
<td>((1.2 \times 10^4) ± 3.5)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\) Mice were challenged with \(6 \times 10^8\) CFU of NTHi strain 12 per ml in a nebulizer, and their lungs were collected at 6 h postchallenge.

\(b\) Levels of serum antibody against strain NTHi strain 12 HMP, expressed as reciprocal geometric mean (range) of eight mice per group, were detected by ELISA.

\(c\) Mean ± SD of eight mice.

\(d\) Compared with the control group.

\(e\) \(P < 0.01\) compared with the control group.

### Table 4. Effect of passive immunization with rabbit antisera elicted by dLOS-TT on bacterial recovery of homologous strain 25238 in mouse lungs

<table>
<thead>
<tr>
<th>Time (h) postchallenge</th>
<th>Bacterial recovery</th>
<th>Bacterial reduction (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(CFU/lung)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preserum</td>
<td>Antiserum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>( (5.4 \times 10^4) ± 1.2 )</td>
<td>( (3.6 \times 10^4) ± 1.1 )</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>( (1.7 \times 10^4) ± 0.5 )</td>
<td>( (8.7 \times 10^3) ± 3.6 )</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>( (3.8 \times 10^4) ± 1.1 )</td>
<td>( (1.3 \times 10^4) ± 0.2 )</td>
<td>66</td>
</tr>
</tbody>
</table>

\(a\) Mice were injected intraperitoneally with 1 ml of 50% antisera or preserum 17 h prior to a bacterial aerosol challenge with \(4 \times 10^6\) CFU of \(M. catarrhalis\) strain 25238 per ml in a nebulizer.

\(b\) Mean ± SD of eight mice.

\(c\) Compared with the preserum-treated group.

### Table 5. Effect of passive immunization with different doses of antisera elicted by dLOS-TT on bacterial recovery of the homologous strain 25238 in mouse lungs

<table>
<thead>
<tr>
<th>Dose (1 ml/mouse)</th>
<th>Anti-LOS IgG titer</th>
<th>Bacterial recovery</th>
<th>Bacterial reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% antisera</td>
<td>14,485 (65,610–7,290)</td>
<td>((2.1 \times 10^4) ± 0.9)</td>
<td>67</td>
</tr>
<tr>
<td>10% antisera</td>
<td>4,828 (7,290–2,430)</td>
<td>((4.6 \times 10^3) ± 1.5)</td>
<td>39</td>
</tr>
<tr>
<td>5% antisera</td>
<td>205 (270–90)</td>
<td>((6.7 \times 10^2) ± 2.1)</td>
<td>5</td>
</tr>
<tr>
<td>50% preserum</td>
<td>&lt;30</td>
<td>((6.4 \times 10^3) ± 3.1)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(a\) Mice were challenged with \(5 \times 10^9\) CFU of \(M. catarrhalis\) strain 25238 per ml in a nebulizer, and lungs were collected at 6 h postchallenge.

\(b\) Levels of serum antibody against \(M. catarrhalis\) strain 25238 LOS, expressed as reciprocal geometric mean (range) of eight mice per group, were detected by ELISA.

\(c\) Mean ± SD of eight mice.

\(d\) Compared with 50% preserum-treated group.

\(e\) \(P < 0.01\) compared with any other group.

\(f\) \(P < 0.05\) compared with 50% preserum-treated group.
Histopathologic lesions of lungs. The lungs demonstrated capillary congestion with widened alveolar septa. In the alveolar space there was substantial exudate with focal hemorrhage. The exudate was comprised of macrophages, polymorphonuclear neutrophils, and proteinaceous fluid. These pathological changes existed throughout the course of the 6-h postchallenge in both antiserum-treated and preserum-treated groups. However, the pathological changes in the antiserum-treated group appeared earlier. The peak of the pathological changes was 3 h postchallenge for the antiserum-treated group and 6 h postchallenge for the preserum-treated group (Fig. 1).

In the active immunization protocol, the inflammatory changes of lungs in each conjugate-immunized group were decreased at 6 h postchallenge compared with the control group except for the LOS-TT-treated group challenged with NTHi strain 12, in which there were no obvious differences from the control group (data not shown).

DISCUSSION

The active immunization of mice with either of two dLOS-protein conjugates elicited a significant rise of anti-LOS IgG in the sera and resulted in an enhanced clearance of bacteria from the lungs following an aerosol challenge with the homologous strain 25238 or the heterologous strain O35E. Since there was a significant correlation between serum anti-LOS antibody levels and bacterial CFU recovered from the lungs, it is postulated that the accelerated removal of *M. catarrhalis* from the lungs after active immunization with the conjugates was specifically due to the anti-LOS antibody. To address this question directly, mice were passively immunized with a rabbit antiserum to dLOS-TT, and a remarkable enhancement of strain 25238 clearance was observed in the lungs in a dose- and time-dependent manner. These results suggest that the conjugate-induced serum antibodies played a pivotal role in the observed immunoprotection. In mammals, IgG predominates in the alveolar lining liquid of the normal lungs and increases during acute inflammation (14). It is possible that in the present study the specific IgG antibody contributed to the bacterial clearance by passing from the blood into the lungs before or soon after an aerosol challenge. Increased pulmonary levels of antibody would be expected with the rapid inflammatory response following an aerosol challenge seen in antiserum-treated mice.

Antibody in the serum kills these bacteria, or at least inhibits the amount of bacterial growth, primarily by activation of the complement system and promotion of the opsonophagocytosis of the bacteria (18, 23). In our study, we found that most of the immune sera showed complement-mediated bactericidal activity against *M. catarrhalis* and the correlation between serum antibody levels and its bactericidal titers was significant, indicating that bactericidal activity of serum anti-LOS antibody takes part in enhancement of bacterial clearance from the lungs.

Histopathological examination of both antiserum-treated and preserum-treated mice showed an acute inflammatory response within the lungs. Both macrophages and neutrophils were found as major components of the exudate within the inflammatory lungs. These two kinds of phagocytic cells are important in the pulmonary clearance of aerosolized bacteria in mice (42). Phagocyte recruitment into the lungs is considered to be the result of chemotaxis due to the challenge bacteria. The chemotaxis may have been enhanced by the anti-LOS IgG-bacterium complex, because this complex may trigger the complement cascade and activate macrophages, which bear receptors for IgG, via the Fc fragment of the IgG (7). Some chemotaxins could result from the activation of complement, such as C5a and C3a (31, 45). Meanwhile, the activated macrophages may secrete many cytokines, including macrophage inflammatory peptides 1 and 2, which are chemotaxins (15). The bacterium-specific antibody in the lungs could increase opsonization and phagocytosis by macrophages and neutrophils to destroy the bacteria (37, 38). This is partly because these phagocytes are able to adhere to the antibody or complement component-coated bacteria by virtue of their IgG Fc or complement receptors (1, 10, 20). In this study, the inflammatory response was found to be regulated upward and then downward in antiserum-treated mice earlier than in preserum-treated mice. It is speculated that antiserum treatment...
primates clearance and also sets the scene for a faster decline of the inflammatory response than in the preserum-treated mice.

TT is a common and useful protein carrier for conjugate vaccines due to its safety, stability, and immunogenicity. However, suppression of the immune response could also occur when many conjugate vaccines containing the same protein component, TT, are administered simultaneously (19, 39). In a covalent reaction between dLOS and HMP, there is a risk of destroying or/and modifying essential epitopes of (22). In a covalent reaction between dLOS and HMP, there is a risk of destroying or/and modifying essential epitopes of (22).

We thank E. J. Hansen for providing M. catarrhalis strain O35E and S. J. Barenkamp for providing NTHi strain 12 and HMP.

REFERENCES


