Efficacy of OMP26 and Two P5-Fimbrin Derived Immunogens to Induce Clearance of Nontypeable Haemophilus Influenzae from the Rat Middle Ear and Lungs as Well as from the Chinchilla Middle Ear and Nasopharynx

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ABSTRACT

The rat middle ear and lung clearance model has been used to show that NTHi OMP26 is highly efficacious as a mucosal immunogen, inducing significantly enhanced clearance in immunized rats upon direct challenge of these two anatomic sites. Similarly, the chinchilla model of middle ear and nasopharyngeal clearance has been used to show that two P5-fimbrin adhesin-derived immunogens [LB1 and LPD-LB1(f)2,1,3] are highly efficacious as parenteral immunogens. Both induce significantly augmented clearance of NTHi upon challenge of these sites. Here, these three NTHi immunogens, in addition to six BSA or KLH conjugates of LB1(f) were assayed for relative efficacy in the reciprocal rodent model system. Thus, OMP26 was assayed in the chinchilla host using a parenteral immunization route with clearance of the middle ear and nasopharynx used as outcome measures. Further, both LB1 and LPD-LB1(f)2,1,3 were assayed in the rat host using a mucosal immunization route and clearance of NTHi from the lungs and middle ears as outcome measures. Each of the immunogens were found to induce a high titered and specific immune responses in the second host system. Moreover, each was found to be highly efficacious in the reciprocal host system, thus providing strong support for the continued development and inclusion of both OMP26 and P5-fimbrin derived peptides as candidate vaccine antigens directed at otitis media caused by NTHi.
INTRODUCTION

Developing effective immunogen(s) as well as selecting an appropriate adjuvant and dosing regimen for a vaccine to prevent otitis media is an important endeavour, particularly given the rapid increase in antibiotic resistant strains of the responsible bacteria (2, 8) and the increasing costs of treatment (9, 11, 18, 28). Several outer membrane proteins (OMPs) of nontypeable Haemophilus influenzae (NTHi), its lipoooligosaccharide (LOS), as well as synthetic peptides and recombinant proteins derived from NTHi OMPs and detoxified LOS conjugates have all been investigated to date as possible immunogens against bacterial otitis media in both rat and chinchilla models (1, 3, 5-7, 10, 17, 19, 20, 22-24, 26, 29).

The P5-fimbrin is a protein with 4 surface exposed loops, some of which appear quite heterogeneous (31), and has a role in adherence (98). Studies in the rat and chinchilla models have found this antigen is protective (4,32), but particularly when the vaccine is based on the loop 3, which can be divided into 3 groups (3). Three peptides have been designed for this region (LB11, LB12 and LB13). This antigen protects against infection via an antibody-mediated mechanism since both active immunisation and passive transfer of anti-LB1 sera significantly reduced the severity and incidence of otitis media in chinchillas (3, 22). Immunization with these peptides requires conjugation to another protein or peptide carrier to provide T-cell help and both the promiscuous T cell epitope from the measles virus protein and the H. influenzae protein, lipoprotein D, have been used in this capacity as carriers (3,4). In the chinchilla model, antibody directed against both a 40-mer synthetic chimeric peptide (LB1) and a recombinant fusion protein called LPD-LB1(f)2,1,3 provided significant protection from the development of NTHi induced otitis media (3, 22, 23). In these studies, protection was conferred in the rat and
chinchilla when either homologous or heterologous NTHi strains were used to challenge OMP26, LB1, or LPD-LB1(f)\textsubscript{2,1,3} immunized animals.

The NTHi OMP26 protein has a 26-kDa molecular mass in SDS-PAGE. The deduced sequence of this 174 aa protein (with a calculated MW of 21.73 kDa) and the pre-protein has a 23 aa N-terminal signal sequence (13). The N-terminal and C-terminal regions of the protein are hydrophobic and separated by a hydrophilic middle. This outer membrane protein is located in a highly conserved region of genetic organization and homologous sequences appear highly conserved in Gram-negative bacteria, suggesting an important function. In recent studies, we showed that OMP26 was able to significantly enhance pulmonary clearance of NTHi in a rat model in which animals were immunized via intestinal Peyer’s patches then boosted intratracheally (13, 23).

LPD is a 42-kDa membrane-associated protein (21). The N-terminal sequence contains the consensus sequence, Leu-Ala-Gly-Cys, for bacterial lipoproteins, and the protein is highly conserved across NTHi strains. The acylated form of the protein is more immunogenic than the non-acylated form (1). The efficacy of this protein in protection of a NTHi infection in animal studies has varied. In animal studies, LPD or PD was reported not to protect rats from otitis media and in the chinchilla model, LPD immunization did not significantly enhance clearance of NTHi from the nasopharynx although showed a shorter (but not significant) clearance of bacteria from the middle ear (27).

In the present study, we decided to test the efficacy of each in the reciprocal animal model systems (e.g. test LB1 and LPD-LB1(f)\textsubscript{2,1,3} in rats whereas OMP26 was to be tested in chinchillas). These two models incorporate different routes of immunization (mucosal vs. parenteral); utilize different challenge regimens; and measure different parameters of pathogenesis and protection. Thus, being able to demonstrate efficacy of these immunogens in
an additional model system would provide very strong support for their importance as candidates for a vaccine directed at otitis media caused by NTHi.
MATERIALS AND METHODS

Animals

A. Rat model  A total of 69 DA and 27 Sprague Dawley male rats, aged between 8 and 10 weeks, weighing 160-202 g and 298-352 g respectively, were used. Rats were bred and maintained under specific pathogen free conditions except for removal during immunizations and the final bacterial challenge.

B. Chinchilla model  A total of 44 adult (mean wt. 575 ± 78 g) chinchillas (Chinchilla laniger) with no evidence of middle ear infection by either otoscopy or tympanometry upon enrollment in the study were used. Animals were rested 10 days upon arrival and were then nominally bled by cardiac puncture for collection of pre-immune serum. Sera were stored at -70 °C until used individually to verify that no significant pre-existing immunoreactivity to any OMP of NTHi strain 86-028NP was evident in any of the 44 chinchillas.

NTHi and adenovirus isolates used in either model system

NTHi strains 86-028NP, 1128, 1729 MEE and 1715 have been described (29) and these isolates were maintained frozen in skim milk plus 20% glycerol (v/v). Strain 772 has also been described (21) and was stored frozen in Brain Heart Infusion (BHI) broth containing 25% horse blood (v/v) and 10% glycerol (v/v). Bacteria were recovered from frozen stocks, streaked onto chocolate agar (BBL, Cockeysville, MD) and incubated at 37 °C for 18 hours in a humidified atmosphere containing 5% CO₂. Adenovirus serotype 1 is a pediatric isolate and has also been described (30).
Immunogens used

A. Rats

(a) Immunization for pulmonary challenge

DA rats were immunized mucosally with either recombinant lipoprotein D (LPD) (20 μg), LPD-LB1(f)2,1,3 (10 μg) or conjugates of keyhole limpet haemocyanin (KLH) or bovine serum albumin (BSA) to a peptide representing LB1(f) group 1, group 2a or group 3 for a total of 6 immunogens as detailed in Table 1. LPD (1) and the LB1(f) peptides (3, 4, 22) have been described elsewhere. Control groups consisted of (a) untreated animals; (b) sham-treated where Incomplete Freund’s adjuvant (IFA; Sigma) emulsified in phosphate buffered saline (PBS) was used in the primary inoculation and PBS alone was used in the booster inoculation; or (c) KLH (Sigma) and BSA (Sigma) immunized administered at a dosage equivalent to the active group.

b) Immunization for middle ear challenge

Rats were immunized with either LPD (20 μg), LPD-LB1(f)2,1,3 (10 μg) or a mix comprising KLH-LB1(f)(group1), BSA-LB1(f)(group2a) and KLH-LB1(f)(group3) (Table 1). Control groups were as described above.

B. Chinchillas

As previously decided, LB1 is a 40-mer chimeric synthetic peptide that combines a B-cell epitope of P5-fimbrin [LB1(f)] with a promiscuous T-cell epitope of measles virus fusion protein. Recombinant OMP 26, called ‘rOMP26’ with the 23 amino acid leader peptide been described (13).

Active immunization

A. Rats
Immunization was essentially performed as previously described (13, 23, 25). Briefly, rats were sedated with sodium pentobarbital intraperitoneally (i.p.), the small intestine was exposed to enable inoculation of intestinal Peyer’s patches (IPP) with a 1:1 mixture of the immunogen emulsified with IFA so that each rat received the dosages described in Table 1 (total volume delivered was 50 μl). Fourteen days post-immunization, rats received a boost, delivered intratracheally (IT) to the lungs, of immunogen in 50 μl PBS at the dosage indicated in Table 1. A live NTHi challenge was delivered 21 days post-immunization either IT (pulmonary challenge) or intraauricularly (IB) (middle ear challenge). Pulmonary challenge has been described previously (21) and each rat received approximately 5 x 10⁸ CFU (viability count was confirmed by concurrent overnight culture). IB challenge was achieved by using a 26G needle to pierce the bulla which was exposed through an incision in the neck and injection of a 20 μl volume of the live NTHi at a concentration of 5x10⁸ CFU/ml. The skin wound was sutured and the animal allowed to recover. Four hours after challenge, rats were sacrificed and middle ear lavage fluid (MEL) or bronchoalveolar lavage fluid (BAL) and lung tissue were collected (where applicable).

B. Chinchillas

Four cohorts of 11 chinchillas each were established. All immunizations were delivered subcutaneously (SC) and boosting doses were received 30 days after the primary immunization. The primary dosages were as follows: 10 μg LB1 (the 40-mer chimeric peptide) in 500 μl -S5; 10 μg LB1 in 200 μl Complete Freund’s adjuvant (CFA); 10 μg rOMP26 in 500 μl S5; or 500 μl S5 only. S5 is an adjuvant formulation that includes aluminium salts, monophosphoryl lipid A
(MPL) plus QS21. The boosting dosages were identical; however, CFA was replaced by an equal volume of Incomplete Freund’s adjuvant (IFA) for delivery of LB1 in the boost.

The cohort receiving LB1 in CFA/IFA served as the positive control based on significant efficacy demonstrated previously (3, 4, 22). The adjuvant-only cohort served as a negative control. Ten days after the boost, all animals were bled by cardiac puncture for collection of immune sera. Sera were pooled by cohort and stored at −70 °C until use. At this time, chinchillas also received 6×10^6 TCID_{50} adenovirus intranasally (IN). Seven days later, chinchillas were challenged both IN and transburrally (TB) with approximately 10^8 or 2500 CFU NTHi 86-028NP, respectively. Actual challenge dosages received were confirmed by plate count. Animal procedures have been described in detail before (3,4).

**Assessment of serum titer and/or specificity by ELISA and Western blot**

**A. Rats**

ELISA assays were performed on dilutions of individual sera and BAL or pooled MEL (within group). Each were assayed against LPD and the 40-mer LB1 chimeric peptide. Polysorb microtiter wells (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μl of coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) containing either LPD (1 μg/ml) or LB1 (5 μg/ml) and the assay performed as previously described (13). HRP-conjugated immunoglobulins used were goat anti-rat IgG (1:2000) and IgA (1:1000) (Fc specific; Nordic Immunological Laboratories) which were developed with the substrate tetramethylbenzidine (Fluka, Buchs, Switzerland) in phosphate citrate buffer, pH 5, containing 0.05% (v/v) H₂O₂. The reaction was stopped with 100 μl 0.5 M H₂SO₄. Plates were read at 450 nm on a plate reader (BioRad Laboratories, Hercules, CA). Plate background was determined by rows coated with
coating buffer alone and treated the same as test wells, and between plate variation was assessed by comparison of control samples repeated on each plate. Quantitation of anti-LPD or LB1 IgG and IgA was achieved by inclusion of standards for rat IgG ranging from 31-500 ng/ml and rat IgA ranging from 7.8-125 ng/ml (Serotec, Oxford, UK). Western blots were performed as previously described (13) using pooled sera (within group).

B. Chinchillas

ELISA assays were performed using dilutions of pooled chinchilla sera from each cohort. Sera were assayed against: NTHi whole OMP preparation (from strain 86-028NP) (0.5 μg/well), rOMP26 and LB1 40 mer chimeric peptide (0.2 μg/well) in 96-well microtiter plates (Dynatech, Horsham, PA) as previously described (3, 4, 29). The titer of each serum pool was defined as the reciprocal of the dilution consistently yielding an OD_{490nm} value that showed a twofold increase over that of wells containing all components but immune serum. Western blotting was performed, also as described previously (29), using pooled immune serum diluted 1:100 as the primary antibody and HRP-Protein A (Zymed) diluted 1:200 as the secondary antibody. Color was developed with 4-chloro-1-naphthol (Sigma).

Assessment of bacterial clearance

A. Rats

Four hours after NTHi challenge, rats were killed by overdose of sodium pentobarbital (i.p.). Blood was collected by cardiac puncture for serum. The trachea was exposed and the lungs were lavaged with five 2-ml volumes of PBS and this bronchoalveolar lavage (BAL) was pooled. The lungs were removed following lavage, freed of other connected tissues and homogenized in 10 ml of PBS. BAL and lung homogenates were assessed for bacterial counts
by plating of 10-fold serial dilutions of the washings onto chocolate agar and incubated for CFU determination. At 4 hours post middle ear challenge, the rats were euthanized and the middle ear lavaged with three 50 µl volumes of PBS injected and recovered through the tympanic membrane. The pooled lavage (MEL) was assessed for bacterial counts as described. After assessment for bacterial counts, both MEL and BAL were centrifuged at 1000 rpm at 4°C for 10 minutes and the supernatants removed for storage at −20°C until required for antibody assays.

B. Chinchillas

Animals were blindly evaluated by otoscopy and tympanometry daily, or every two days, from the time of adenovirus inoculation until 35 days after NTHi challenge. Signs of tympanic membrane inflammation were rated on a 0 to 4+ scale and tympanometry was used to monitor changes in both middle ear pressure, tympanic width and tympanic membrane compliance as previously described (3, 22). Signs of respiratory tract infection, including ruffling of fur, conjunctivitis, altered character of nasal/ocular secretions, wheezing, labyrinthitis and cornering behavior were also recorded daily.

A nasopharyngeal (NP) lavage was performed on all animals on days 1, 4, 7, 10, 14, 18, 21, 28 and 35 after NTHi challenge by passive inhalation of 500 µl pyrogen-free sterile saline. Epitympanic taps were attempted on the same schedule as NP lavages when an effusion was considered to be of sufficient volume to be retrieved (any ears scored as 2.0 or greater for inflammation). NP lavage and epitympanic tap fluids were maintained on ice until serially diluted. Dilutions of NP lavage fluids were then cultured on chocolate agar plates containing bacitracin (BBL) to limit growth of other normal NP flora. Dilutions of epitympanic tap fluids were cultured on chocolate agar plates (BBL). All plates were incubated at 37 °C for 48 hours to semiquantitate CFU NTHi/ml. Animals were also tabulated as having a ‘colonized’ or ‘cleared’
status based on culture results as previously described (3, 22). Finally, blood was obtained for sera from all chinchillas via cardiac puncture, prior to sacrifice, on day 35 post-NTHi challenge.

**Statistical methods**

A. **Rat model data**

The bacterial clearance and antibody data were assessed for statistical significance by an independent *t* test on log$_{10}$ transformed data (Macintosh Systat).

B. **Chinchilla model data**

A log-rank test was used to compare cohorts for relative time to bacterial clearance of the NP, as determined by culture negative status, and illustrated with Kaplan-Meier survival analysis curves. Cox proportional hazards regression analyses were additionally performed to further elaborate the differences between the cohorts. An alpha level of 0.05 was accepted as significant and was adjusted for multiple comparisons using the method of Bonferroni.
RESULTS

Characterization of antisera

A. Rat model

Antisera generated against LB1(f) peptide conjugates or LPD were quantitated by ELISA by measurement of the reciprocal dilution that gave an optical density within the range of the rat immunoglobulin standards assayed on the same plate. LPD-LB1(f)_{2,1,3} was also assessed but was not different to results generated by LB1(f) immunogens or LPD alone. Levels of IgG specific to the 40-mer LB1 in the sera were very low for all animals in the groups immunized with either the BSA or KLH conjugates. Significant titres ($P<0.05$) were detected following either immunization with the LPD-LB1(f)_{2,1,3} or combination of KLH-LB1, BSA-LB1_{2a} and KLH-LB1_{3} (Figure 1). The only detectable LB1-specific antibody in the lavage were in two rats in the LPD-LB1(f)_{2,1,3} group for IgG and no rats had detectable levels of IgA (data not shown). LPD and LPD-LB1(f)_{2,1,3} immunized groups had high levels of LPD-specific antibody in both sera and lavage (Table 2). There were low levels of anti-LPD IgG in both the sera and the lavage of some non-immune rats, however, there was a $>1,000$ fold increase in the levels measured in the immunized groups. There was no significant difference in the levels of IgG or IgA to LPD following immunization with either LPD alone or the LPD-LB1(f)_{2,1,3} group construct.

B. Chinchilla model

Antisera generated against LB1 or rOMP26 were characterized by ELISA and Western blotting for titer and specificity. Reciprocal serum antibody titers were determined in triplicate for each serum pool (Table 3) and showed that a high-titered and specific immune response had been elicited against each immunogen preparation. Animals receiving adjuvant only did not
demonstrate a reciprocal immune titer greater than $1 \times 10^3$ against either a whole OMP preparation, LB1 or to rOMP26. As confirmation, by Western blotting (Figure 2), no reactivity was shown by the anti-SBAS5 serum pool (panel A) against either LB1 or rOMP26. Chinchillas immunized with this strong adjuvant do recognize several OMPs within the NTHi whole OMP preparation (lane 3) as we have previously described for other strong adjuvants (22). However, anti-LB1/CFA (panel B) and anti-LB1/SBAS5 (panel C) serum pools both recognized LB1 (lane 1); as well as the fully and partially denatured species of P5-fimbria in whole OMP preparation from the NTHi challenge strain 86-028NP (lane 3) as reported previously (3). Anti-rOMP26/SBAS5 (panel D) recognized rOMP26 (lane 2) and a band of approximately 28 kDa (lane 3) in the 86-028NP OMP preparation which is presumed to be native OMP26. Anti-rOMP26/SBAS5 did not recognize LB1 in lane 1 of panel D as expected.

**Clearance of the rat lung**

Bacterial clearance was measured in the rats at 4 hours post challenge as has been described previously for this infection model (13, 14). There were differences in the ability of the different LB1(f) conjugates to enhance clearance of their homologous NTHi strains (note: immunization with either BSA or KLH alone does not enhance clearance of NTHi; data not shown). LB1$_1$ was most protective when conjugated to KLH, whereas LB1$_{2a}$ only significantly cleared the NTHi when conjugated to BSA (Table 4). LB1$_3$ was the least protective of the peptides and only significantly enhanced clearance of the bacteria from the lung tissue when conjugated to KLM. The degree of clearance from the lung tissue was comparable to that observed for the other two protective LB1(f) conjugates. KLH-LB1$_1$ and BSA-LB1$_{2a}$ enhanced clearance more effectively from the bronchoalveolar area than from the lung tissue, as demonstrated by the percent clearance in the BAL. There was an 84.5% and a 91.5% reduction in the bacterial load in the BAL for the KLH-LB1$_1$ and BSA-LB1$_{2a}$ immunized groups,
respectively, compared with 74.9% and 72.4% enhanced clearance in the lung tissue during the first 4 hours of infection. The three conjugates of LB1(f), KLH-LB11, BSA-LB12, and KLH-LB13, were combined for immunization and the rats challenged with the unrelated strain, NTHi 772. This combination significantly enhanced clearance from both the BAL and lung tissue ($P=0.018$ and $P=0.001$, respectively) and represented reductions of 85.9% and 87.1%, respectively, in bacterial load over 4 hours compared to that of the non-immune group.

Immunization with LPD or LPD-LB1(f)$_{2,1,3}$ resulted in significant clearance ($P<0.001$) in both the BAL and lung tissue (Table 4). The bacterial recovery corresponds to an enhanced reduction in bacterial load of 96.7% for LPD and 97.6% for LPD-LB1(f)$_{2,1,3}$ in the BAL and 93.1% and 94% in the lung tissue, respectively, compared to the non-immune rats during the first 4 hours of infection. This level of clearance was greater than that observed for LB1(f) peptides in this model.

**Clearance of the rat middle ear**

Unlike in the pulmonary model, there was no significant clearance of a middle ear infection in LPD or LPD-LB1(f)$_{2,1,3}$ immunized rat (Table 5), but there was for the LB1-combination immunized group ($P<0.05$) with an 82% reduction in bacterial load. LPD immunization enhanced clearance of the infection in 3 of the 6 rats, indicating a tendency towards a protective response. However, immunization with LPD-LB1(f)$_{2,1,3}$ did not result in clearance in any of the rats in the group. LPD-LB1(f)$_{2,1,3}$ rats were immunized with two 10 μg doses, whereas LPD was administered in two 20 μg doses. The results would suggest that perhaps higher immunization doses or additional immunization boosts may result in a protective immune response in the middle ear in the rat model.
Clearance of the chinchilla nasopharynx

Intranasal challenge of chinchillas with NTHi strain 86-028NP (actual dose: 9.4 x 10⁷ CFU/animal) resulted in initial colonization of the nasopharynx within all cohorts, as verified by positive plate counts obtained from 95% of the nasal lavage fluids collected one day after challenge. Thereafter, the effect of immunization became evident as enhanced clearance in selected cohorts. The LB1/CFA cohort cleared NTHi from the nasopharynx at least 17 days before the control cohort that received only SBAS5 (Figure 3A). The rOMP26/SBAS5 cohort also rapidly cleared NTHi from the nasopharynx. NTHi were not detected in NP lavage fluids of any animal in the rOMP26/SBAS5 cohort by 21 days after challenge, two weeks earlier than the adjuvant-only cohort. The LB1/SBAS5 cohort completely cleared NTHi 28 days after challenge. The SBAS5 cohort remained colonized 35 days after challenge, with 20% (2/10) of these animals remaining culture positive at this time point.

Cox regression analysis was used to compare the time to clearance of NTHi from the nasopharynx among the cohorts. When each cohort was compared to the sham (SBAS5) cohort, the LB1/CFA and rOMP26/SBAS5 cohorts were significantly more likely to clear NTHi from this site (P ≤ 0.01). There was no statistically significant difference noted for the LB1/SBAS5 cohort (P = 0.07) however this cohort yielded clearance kinetics that were likely biologically relevant as NTHi was eradicated from the NP at least 7 days earlier than the SBAS5 cohort. Moreover, by day 18 after challenge, only 22% of animals were culture positive in the LB1/SBAS5 cohort versus 44% of the controls.
Clearance of the chinchilla middle ear

Chinchillas were also challenged transbullarly with an actual dose of $1.8 \times 10^5$ CFU/ear. Figure 3B illustrates the percentage of culture positive middle ear fluids retrieved by epitympanic taps. The LB1/SBAS5 cohort completely cleared NTHi from the middle ears on day 28, however by day 10 after challenge, 78% of all ears in this cohort (14/18) were already culture negative for NTHi. The middle ear fluids in the LB1/CFA cohort were culture negative for NTHi for all animals in the cohort only upon the final day of the study, however on day 28 this cohort had only 8% of ears still culture positive for NTHi versus 50% of the controls. The rOMP26/SBAS5 and SBAS5 cohorts remained culture positive on day 35, however only 5% of ears in the rOMP26/SBAS5 cohort remained culture positive compared to 22% in the SBAS5 cohort at this time point. By day 18 after challenge, 60% of the rOMP26/SBAS5 ears were already culture negative for NTHi versus 28% of the SBAS5 cohort animal’s ears.

Cox regression analysis was used to compare the time to clearance of NTHi from middle ear fluids among the cohorts. When each cohort was compared to the sham (SBAS5) cohort, the LB1/SBAS5 and LB1/CFA cohorts were found to be significantly more likely to clear NTHi from this site ($P \leq 0.001$ and 0.005 respectively). The rOMP26/SBAS5 cohort data yielded a $P$-value of 0.02 that was determined to be not statistically significant due to the adjustment for multiple comparisons, however the clearance kinetics generated in this cohort would suggest biological relevance.
DISCUSSION

Many factors contribute to both the pathogenesis of bacterial otitis media as well as to the host’s ability to resolve these infections and return the tympanum to homeostatic conditions. It has become apparent, through the efforts of many laboratories, that the greatest likelihood for the successful development of an NTHi-directed vaccine relies upon the careful selection of conserved and protective immunogens (16, 27). Ideally, the vaccine must induce a balanced and broadly protective immune response in very young children.

Toward this goal, our laboratories have focused recently on two NTHi OMPs that have the shared qualities of at least partial surface exposure, relative conservancy, and the ability to induce an immune response that results in significantly augmented clearance of bacteria upon challenge. OMP26 is a well-conserved surface protein of approximately 26 kDa with homology to other SKP transporter proteins and to OmpH (13, 23). Rats immunized with OMP26 via a mucosal route respond in a dose-dependent fashion (21). Following homologous or heterologous challenge, OMP26 immunized animals demonstrate significantly enhanced pulmonary clearance 4 hours after challenge. Recombinant forms of OMP26 (one possessing and the other lacking the 23 aa leader peptide) induce significant IgG and IgA titers in BAL and serum, however the larger pre-protein is more efficacious at enhancing pulmonary clearance and inducing a greater cell-mediated immune response (13). Moreover the larger 28 kDa rOMP26 induces higher overall levels of systemic and mucosal antibodies equivalent to those induced by the isolated native protein.

Likewise, immunogens derived from an area of limited diversity (4, 12, 31) within the OMP P5-homologous adhesin, P5-fimbrin, induce the production of antibodies, upon parenteral
immunization, that significantly augment clearance of a homologous challenge isolate from the middle ears and nasopharynges of chinchillas (3, 4). Moreover, passive transfer of P5-fimbria directed antibodies significantly prevents the development of otitis media in animals receiving either anti-LB1 or anti-LPD-LB1(f)2,1,3. This protection is conferred against both homologous as well as heterologous challenge (3, 4, 22, 27).

While both the rat and the chinchilla model systems measure reduction in bacterial counts from recovered lavage fluids as one outcome for judging efficacy, each model is also unique in multiple ways. Advantages of the chinchilla model of otitis media include the sustainability of NTHi in the nasopharynx and middle ear over several weeks. In contrast, the rat model investigates both lung and middle ear infections, focusing on the capacity of the immune system to invoke an early response to the presence of NTHi. The rat model is one of mucosal immunization with the primary dose delivered directly to the Peyer’s patches followed by a boost via the trachea 14 days later (23, 25). Direct challenge of the lungs or middle ears occurs 7 days after the boost. Bacteria recovered in BAL, MEL and lung homogenates can be measured 4 or 6 hours after challenge of the lung or middle ear respectively. Also, absolute and differential counts of white blood cells recruited to these sites can be made, the induced immunoglobulins can be isotyped and specific cytokines produced during infection can be measured (14, 15).

Conversely, the chinchilla model is one of either active parenteral immunization as described here or can be one of passive transfer. Animals are then either directly challenged in the middle ears and NP or, after passive transfer, juvenile animals are challenged exclusively IN, allowing the bacteria to ascend the virus-compromised ET tube to induce otitis media (3, 4, 22). Bacterial counts from middle ear and NP lavage fluids are determined over a 5 week period of observation and total immunoglobulin titer (predominantly IgG) is assayed. Tympanic membrane inflammation and changes in middle ear pressure are recorded for the entire disease course.
Histopathological assessment of the tympanic membrane and inferior bulla mucosa are also typically evaluated (29). Thereby, to further strengthen the argument for development of each of these immunogens, we wanted to evaluate each in the reciprocal host system. Our hypothesis was that demonstrated efficacy in two rodent models, each using a different immunization regimen; adjuvant and schedule of dosing, boosting, challenging and assessing would be extremely supportive of data gathered previously in a single host model.

In the study of OMP26 in the chinchilla model, we have shown that rOMP26 induced a high titered and specific immune response that was highly efficacious in the chinchilla model. Delivery with the adjuvant SBAS5 resulted in significantly earlier clearance of NTHi from the NP of all animals in the cohort. While clearance of NTHi from the directly challenged middle ear of rOMP26 immunized chinchillas was determined to be not significantly enhanced (a $P$-value of 0.0167 was required when the adjustment was made for multiple comparisons), a $P$-value of 0.02 was obtained and is highly likely to be biologically significant nonetheless. Due to the observed significantly enhanced clearance of NTHi from the NP following immunization with rOMP26, future testing of this immunogen for efficacy would be more relevant if conducted in a model wherein bacteria are first allowed to colonize the NP prior to ascending the Eustachian tube. The ability of this immunogen to rapidly clear NTHi from the NP colonization site would likely greatly diminish the incidence of OM that developed in the latter model.

Likewise P5-fimbrin derived immunogens proved efficacious in the rat lung and middle ear clearance studies following a mucosal immunization regimen. Demonstrated ability to enhance pulmonary clearance is a pre-requisite in our laboratory for further assessment of any immunogen in the more rigorous and tedious rat middle ear challenge model. Here both LB1(f) conjugates and LPD-LB1(f)$_{2,1,3}$ were shown to induce significantly augmented clearance of NTHi from both BAL fluids as well as lung homogenates following mucosal immunization.
There was no significant difference noted between the two immunogens and clearance compared with that observed following LPD immunization. The 40-mer chimeric peptide immunogen LB1 has been previously characterized (3,4,20) and shown to clear NTHi from the middle ear and nasopharynx of chinchillas. In LB1, a 19-mer putative B-cell epitope from the NTHi isolate 1128 (called LB1(f) and incorporated into the group 1 peptide conjugates used here to immunize rats) has been fused to a "promiscuous" T-cell epitope from the measles virus protein to create a 40-mer peptide. This construct was efficacious in clearing the chinchilla NTHi infection.

However, this group 1 peptide-containing immunogen potentially, if no cross protection access, only protects against approximately 76% of strains according to the distribution in 99 clinical isolates that were sequenced (3). In that study the LPD-LB1(f)\textsubscript{2,1,3} protein was constructed to confer broader coverage by the anti-LB1(f) response. In the current study, we have constructed BSA and KLH conjugates of each LB1(f) peptide from the three groupings and investigated their efficacy against homologous NTHi pulmonary challenge following mucosal immunization in the rat model. The most protective conjugates from each group were then combined as immunogens for heterologous NTHi pulmonary and middle ear challenges. These results were compared with that achieved by the immunogens LPD-LB1(f)\textsubscript{2,1,3} and LPD.

The results showed that the KLH conjugates were better for the first and third peptide groups, whereas BSA was the preferred conjugate for the second group peptide. While each of these peptides enhanced clearance of the homologous NTHi strain, there were no significant titres of IgG or IgA detected to either the 40-mer peptide LB1 (Figure 1) or LPD-LB1(f)\textsubscript{2,1,3} (data not shown). When the KLH-LB1\textsubscript{1}, BSA-LB1\textsubscript{2a} and KLH-LB1\textsubscript{3} were combined, there was significant clearance of NTHi following both pulmonary and middle ear challenge with a heterologous strain and measurable titres of IgG to LB1 in the serum. The titres of anti-LB1 induced by the combination immunizations were not different from the anti-LB1 induced by the
LPD-LB1(f)_{2,1,3} protein. These results support the concept of inclusion of peptides from the three groups in a vaccine. Mucosal immunization with either LPD or LPD-LB1(f)_{2,1,3} enhanced clearance of a pulmonary NTHi challenge, however, neither achieved significant clearance of the middle ear challenge, although LPD did show a trend towards clearance. This may be a dosage effect for efficacy in the middle ear since LPD was administered in 20 \( \mu \)g doses and LPD-LB1(f)_{2,1,3} in 10 \( \mu \)g doses, being the doses that enhanced the pulmonary NTHi challenge. Increasing the doses of these immunogens may be required to enhance clearance in the middle ear of rats.

In summary, NTHi OMP26 has previously been shown to be a highly efficacious mucosal immunogen, inducing significantly augmented clearance of bacteria from the rat lung. Here we showed that when used as a parenteral immunogen, rOMP26 is also highly effective at inducing an immune response that leads to significantly enhanced clearance of the chinchilla nasopharynx. Similarly, LPD and the P5-fimbrin based immunogens LPD-LB1(f)_{2,1,3} and several BSA- or KLH-conjugates of LB1(f) delivered singly, as well as in combination, proved to be protective in a rat model of pulmonary and/or middle ear clearance following mucosal immunization. This study has thereby demonstrated that: (1) both protein and peptide constructs can confer protection against NTHi infection; (2) these immunogens were highly efficacious in reciprocal animal systems; and (3) the immunogens can confer protection when delivered both mucosally or parenterally.
ACKNOWLEDGEMENTS

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interstrain variability in the P5 outer-membrane protein of non-typeable *Haemophilus

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influenzae* from acutely infected rat lung in the absence of detectable peptide-specific
FIGURE LEGENDS

Figure 1: LB1-specific levels of IgG in the serum of rats immunized with KLH- or BSA-conjugates of the different LB1(f) peptides or LPD-LB1(f)2,1,3.

Figure 2: Western composite showing reactivity of chinchilla: (A) anti-SBAS5; (B) anti-LB1/CFA; (C) anti-LB1/SBAS5 or (D) anti-OMP26 against LB1 in lane 1, rOMP26 in lane 2 or a whole OMP preparation from NTHi strain #86-028NP in lane 3.

Figure 3: A - three-dimensional bar graph showing percentage of animals per cohort that were culture-positive for NTHi in nasopharyngeal lavage fluids over time. B - percentage of ears per cohort that were culture-positive for NTHi in middle ear fluids over time.
Table 1. Immunogens and dosages used in the rat studies.

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>NTHi strain from which Immunogen was derived</th>
<th>Immunization Dosage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Primary (µg)</td>
</tr>
<tr>
<td>BSA-LB1₁</td>
<td>NTHi 1128</td>
<td>10</td>
</tr>
<tr>
<td>KLH-LB1₁</td>
<td>NTHi 1128</td>
<td>10</td>
</tr>
<tr>
<td>BSA-LB1₂a</td>
<td>NTHi 1729</td>
<td>10</td>
</tr>
<tr>
<td>KLH-LB1₂a</td>
<td>NTHi 1729</td>
<td>10</td>
</tr>
<tr>
<td>BSA-LB1₃</td>
<td>NTHi 1715</td>
<td>10</td>
</tr>
<tr>
<td>KLH-LB1₃</td>
<td>NTHi 1715</td>
<td>10</td>
</tr>
<tr>
<td>LB1(f) combin.:</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>KLH-LB1₁</td>
<td></td>
<td>(8.3)</td>
</tr>
<tr>
<td>BSA-LB1₂a</td>
<td></td>
<td>(8.3)</td>
</tr>
<tr>
<td>KLH-LB1₃</td>
<td></td>
<td>(8.3)</td>
</tr>
<tr>
<td>LPD-LB1(f)₂,₃</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>LPD</td>
<td>NTHi 772</td>
<td>20</td>
</tr>
</tbody>
</table>

a The LB1(f) combination was a mix of three of the protein-LB1 conjugates as indicated and belonging to the different strains as listed in the table.
Table 2. Comparison of LPD-specific antibody in serum and BAL.

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Anti-LPD as determined by ELISA(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum ((\mu)g/ml)(^c)</td>
</tr>
<tr>
<td>Group</td>
<td>IgG</td>
</tr>
<tr>
<td>Non-immune</td>
<td>5.4±3.4</td>
</tr>
<tr>
<td>LPD</td>
<td>14,200±1,142(^b)</td>
</tr>
<tr>
<td>LPD-LB1(f)(_2,1,3)</td>
<td>11,472±907(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Antibody in serum or BAL of rats post mucosal immunization with either LPD or LPD-LB1(f)\(_2,1,3\) and at time of bacterial challenge.

\(^b\) Titer significantly different to the non-immune group (\(P<0.005\)). There was no significant difference between the LPD and LPD-LB1(f)\(_2,1,3\) groups.

\(^c\) Data expressed as the mean ± standard error of the mean for \(n = 5\) animals per group.
Table 3. Reciprocal titers in chinchilla serum.

<table>
<thead>
<tr>
<th>Cohort</th>
<th>LB1</th>
<th>rOMP26</th>
<th>86-028NP OMP&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBAS5</td>
<td>$1 \times 10^3$</td>
<td>100</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>rOMP26/ SBAS5</td>
<td>550</td>
<td>$5 \times 10^4$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>LB1/ CFA</td>
<td>$5 \times 10^4$</td>
<td>$1 \times 10^3$</td>
<td>$1 \times 10^3$</td>
</tr>
<tr>
<td>LB1/ SBAS5</td>
<td>$5 \times 10^4$</td>
<td>100</td>
<td>$1 \times 10^3$</td>
</tr>
</tbody>
</table>

<sup>a</sup> Titers of $\geq 10^4$ are shown in bold type.

<sup>b</sup> OMP, whole outer membrane protein preparation
Table 4. Bacterial clearance in rats following immunization with LPD or LB1(f) peptides conjugated to either BSA, KLH or LPD and pulmonary challenge with NTHiNTHi.

<table>
<thead>
<tr>
<th>Immunization Group</th>
<th>Challenge Strain</th>
<th>Bacterial Clearance (%)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>BAL&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Lung homogenate&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td>NTHi 1128</td>
<td>0±17.8</td>
<td></td>
<td>0±13</td>
</tr>
<tr>
<td>BSA-LB1&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>45±18</td>
<td>22.4±14</td>
<td></td>
</tr>
<tr>
<td>KLH-LB1&lt;sub&gt;1&lt;/sub&gt;</td>
<td></td>
<td>84.5±13.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td>74.9±17.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Non-immune</td>
<td>NTHi 1729</td>
<td>0±14</td>
<td></td>
<td>0±12.9</td>
</tr>
<tr>
<td>BSA-LB1&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>4</td>
<td>91.5±20.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>72.4±14.8&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>KLH-LB1&lt;sub&gt;2a&lt;/sub&gt;</td>
<td>5</td>
<td>48.7±17.3</td>
<td>25.8±13.8</td>
<td></td>
</tr>
<tr>
<td>Non-immune</td>
<td>NTHi 1715</td>
<td>0±14.8</td>
<td></td>
<td>0±13.1</td>
</tr>
<tr>
<td>BSA-LB1&lt;sub&gt;3&lt;/sub&gt;</td>
<td>5</td>
<td>36.9±15.4</td>
<td>-2.3±13.5</td>
<td></td>
</tr>
<tr>
<td>KLH-LB1&lt;sub&gt;3&lt;/sub&gt;</td>
<td>5</td>
<td>33.9±17</td>
<td>71.2±15.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Non-immune</td>
<td>NTHi 772</td>
<td>0±13.8</td>
<td></td>
<td>0±10.9</td>
</tr>
<tr>
<td>LB1 combination&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5</td>
<td>85.9±17.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>87.1±13.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Non-immune</td>
<td>NTHi 772</td>
<td>0±12.5</td>
<td></td>
<td>0±12</td>
</tr>
<tr>
<td>LPD</td>
<td></td>
<td>96.7±19&lt;sup&gt;g&lt;/sup&gt;</td>
<td>93.1±12.3&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>LB1-LPD</td>
<td></td>
<td>97.6±13.5&lt;sup&gt;g&lt;/sup&gt;</td>
<td>94±0.11&lt;sup&gt;g&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of rats per group.
\[ b\] BAL: Bronchoalveolar lavage of lungs.

c Lung were collected after lavage, homogenised in PBS and aliquoted for presence of bacteria.

d Percentage clearance in the immune groups was calculated as 100 minus of the mean CFU recovered from the immunized group divided by the mean of the non-immune group. The mean for the non-immune group was assigned as 0%. The error represents the standard error of the mean expressed as a percentage.

e LB1 combination comprised immunization with KLH-LB1, BSA-LB1, and KLH-LB1, and as described in Table 1.

f Significance of \( P<0.05 \) when compared to the non-immune group on log_{10} CFU transformed data

g Significance of \( P<0.001 \) when compared to the non-immune group on log_{10} CFU transformed data
Table 5. Bacterial clearance following middle ear challenge of rats with NTHi.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Immunization</th>
<th>Challenge</th>
<th>Bacterial Clearance in MEL&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group</td>
<td>N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Strain</td>
</tr>
<tr>
<td>1</td>
<td>Non-immune</td>
<td>6</td>
<td>NTHi 772</td>
</tr>
<tr>
<td></td>
<td>LPD</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LPD-LB1(f)&lt;sub&gt;2,1,3&lt;/sub&gt;</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Non-immune</td>
<td>5</td>
<td>NTHi 772</td>
</tr>
<tr>
<td></td>
<td>LB1 combination&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of rats per group.

<sup>b</sup> MEL: Middle ear lavage.

<sup>c</sup> Percentage clearance and data expressed as described for Table 4.

<sup>d</sup> LB1 combination comprised immunization with KLH-LB1<sub>1</sub>, BSA-LB1<sub>2a</sub> and KLH-LB1<sub>3</sub> and as described in Table 1.

<sup>e</sup> Significance of P<0.05 when compared to the non-immune group on log<sub>10</sub> CFU transformed data.