A Novel Approach to Bacterial Vaccines: *Haemophilus influenzae* as a Paradigm

Paul W. Whitby; Daniel J. Morton; Huda J. Mussa; Lucia Mirea and Terrence L. Stull.

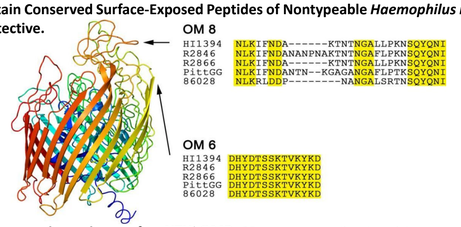
Phoenix Children's Hospital, Phoenix, AZ, United States And University of Arizona COM-P, Phoenix, AZ, United States.

Background: The *H. influenzae* type b vaccines target the type b capsule and therefore have no impact on the nontypable (unencapsulated) *H. influenzae* (NTHi). NTHi has become the most common cause of otitis media and is the most common isolate from patients with exacerbations of Chronic Obstructive Pulmonary Disease (COPD). Therefore, NTHi is an appropriate target for vaccine development.

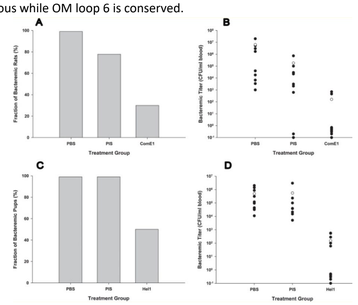
Objective: We used the methods of genomics, structural biology, and immune protection models, to identify specific peptide targets in the NTHi surface proteins, to develop a method for delivery of the targets, and to test the effectiveness in a relevant preclinical model.

Design/Methods: To characterize potential vaccine targets, the core outer proteins of NTHi present in the available sequenced genomes were identified through genomic bioinformatics. The structures of the outer proteins were analyzed through comparison with the available structures of homologues characterized by X-ray crystallography. Sequenced conserved outer regions of these proteins were selected by analyzing for their protective capacity in the infant rat model of *H. influenzae* infection. A novel Bacterial Vaccine Polypeptide (BVP) was used to deliver the targets and test for protection.

Certain Conserved Surface-Exposed Peptides of Nontypeable *Haemophilus influenzae* are protective.



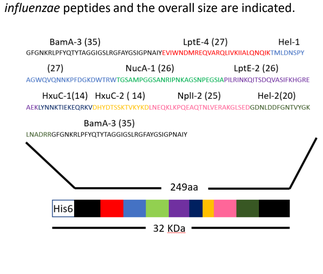
Outer membrane loops of an NTHi OMP. 3D computer-predicted spatial topography of the gated TonB-dependent porin HxuC. The ribbon area corresponds to the external membrane embedded barrel. Also shown are surface exposed external loops. Arrows point to two specific loops designated OM loops 6 and 8. Alignments of both loop regions from five NTHi strains are shown. OM loop 8 is highly heterologous while OM loop 6 is conserved.



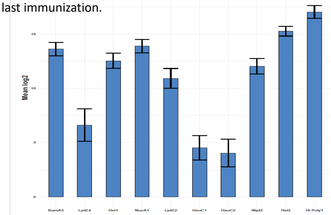
Protection afforded by antisera raised against ComE and Hel derived peptides in the infant rat model of NTHi bacteremia. Twenty-four hours prior to infection cohorts of infant rats were pretreated with PBS, pre-immune serum (PIS) or anti-peptide antiserum. Panel A) Percent infected infant rats pretreated with anti-ComE1 antiserum with detectable bacteremia 24 hours after infection. Using Fisher's exact test, P = 0.0031 for PBS vs ComE1 and P = 0.0698 for PIS vs ComE1. Panel B) Bacteremic titers. Using the Kruskal-Wallis test P = 0.07 for PBS vs PIS, P = 0.0002 for PBS vs ComE1 and P = 0.01 for PIS vs ComE1. Panel C) Percent infected rats pre-treated with anti-Hel1 antisera with detectable bacteremia 24 hours after infection. Using Fisher's exact test, P = 0.0325 for both PBS vs Hel1 and PIS vs Hel1. Panel D) Bacteremic titers Using the Kruskal-Wallis test to compare bacteremic titers (mean ±SD) P = 0.15 for PBS vs PIS, P = 0.0003 for PBS vs Hel1 and P = 0.0005 for PIS vs Hel1.

Results: Nine peptides that were protective in the infant rat model were used in a novel vaccine to immunize chinchillas, the most established animal model of otitis media. Chinchillas (40 vaccinated and 41 controls) were infected with NTHi 86-028NP. The vaccinated group cleared infection more quickly than the control group as indicated by significantly decreased positive findings on video-otoscopy (p<0.0001) and tympanometry (p=0.0002) on day 7, and presence of middle ear fluid obtained by aspiration (p=0.0001) on day 10 post infection. Similarly, in the mouse model of NTHi pulmonary clearance, the vaccinated group (n=5) reduced infection more rapidly than the control group (n= 5), p=0.008.

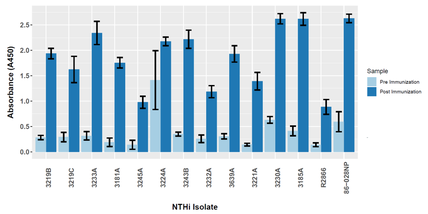
Design and purification of the NTHi BVP. Hi Poly 1 was designed as a linear sequence of *H. influenzae* peptides with BamA-3 at each terminus with a His-Tag at the N-terminus as shown. Individual peptides are delineated by alternating bold type face. The length in amino acids of the combined *H. influenzae* peptides and the overall size are indicated.



Purification of Hi Poly 1. Hi Poly1 was eluted from a nickel affinity column and a fraction of the eluate examined by denaturing SDS-PAGE. Molecular weight markers (lane A) were used to estimate the size of the polypeptide (Lane B). Cohorts of chinchillas were immunized three times at two-week intervals with either Hi Poly 1 in alum or PBS-alum as a control. Antisera were collected 2-weeks post last immunization.

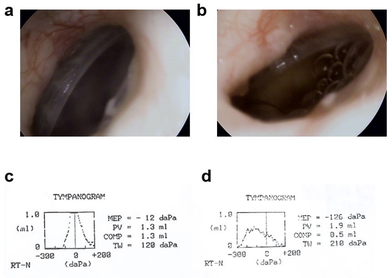


ELISA of antisera from chinchillas immunized with Hi Poly 1. Antisera were tested against the whole Hi Poly 1 polypeptide and the individual component peptides (data are shown in the same order as the order of the peptides in Hi Poly 1). The numbers indicate the mean $\log_2$ transformed titers of the 40 chinchilla antisera samples collected 14 days after the final immunization with Hi Poly 1.

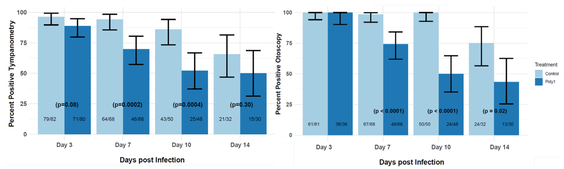


Cross reactivity of Anti Hi Poly 1 antisera across a genetically diverse panel of NTHi. A genetically diverse panel of NTHi isolates were examined in a Live Cell Elisa with pre and post-immune chinchilla anti Hi Poly 1 antisera. ELISA absorbance was significantly greater from post- compared to pre-immunization sera (Wilcoxon-Mann-Whitney p = 0.02).

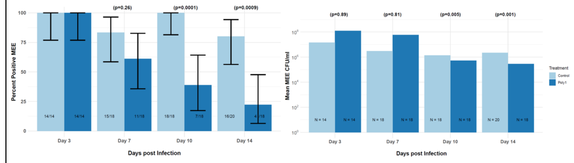
Representative normal and abnormal otoscopy and tympanometry of the chinchilla ear. Panel a) normal chinchilla tympanic membrane at day 0 of the experiment and panel b) on day 3, showing fluid behind the membrane. Panel c) tympanometry of the same ear displayed in panel a. Panel d) tympanometry of the ear from panel b showing abnormal compliance (COMP) and TW.



Immunization of Chinchillas with the NTHi BVP protects against Otitis Media following challenge with NTHi isolate 86-028NP. Cohorts of chinchillas were immunized three times at two-week intervals with either Hi Poly 1 in alum or PBS-alum as a control. Two weeks post immunization all animals were challenged by injection of 1500 cfu of NTHi strain 86028NP.



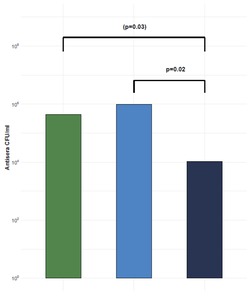
Tympanometric and Otoscopic measurements of protection afforded by Hi Poly 1 in the chinchilla model of OM. The presence of OM was monitored by Tympanometry (panel a) and blinded video otoscopy (panel b). Values inside each bar indicate the number of ears determined to be positive for OM and the cohort size. P-values were obtained from Fisher's exact test comparing the counts of positive ears of the total as indicated for each group.



Clearance and protection against NTHi 86-028NP afforded by Hi Poly 1 in the chinchilla model of OM. Middle ear effusions (MEE) were collected from random chinchillas in each cohort at each time point. Ears were determined as dry if no fluid was observed following three separate taps. The values inside each bar indicate the number of ears with detectable MEE and the sampled cohort size. Percent (±95% CI) of middle ears with detectable MEE in Hi Poly 1 vaccinated and control chinchillas are shown in panel a. P-values were obtained using the Fisher's exact test, comparing counts of positive MEE ears of the total sample, as indicated for each group. Panel b) mean bacterial titer of MEE in Hi Poly 1 vaccinated and control chinchillas. Data with no detectable middle ear fluid were imputed as 0 cfu/ml. P-values were computed using the Wilcoxon-Mann-Whitney test based on total samples of ears, as indicated for each group.

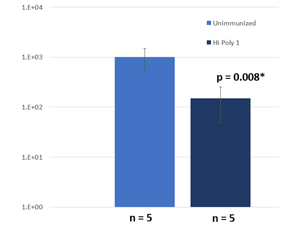
Conclusion(s): These data demonstrate the effectiveness of the Bacterial Vaccine Polypeptide methodology in development of a vaccine against NTHi with protection in relevant preclinical models of both otitis media and pulmonary clearance. The methods are applicable to other bacteria, and this approach to a Bacterial Vaccine Polypeptide against NTHi serves as a paradigm for development of similar vaccines to protect against other bacterial infections.

Passive immunization of infant rats with the chinchilla anti-NTHi BVP sera protects against bacteremia following challenge with NTHi isolate R2866. To investigate the protective capacity of Hi Poly 1 in bacteremia, pooled chinchilla post-immunization antisera were compared to PBS and the chinchilla pre-immunization sera for passive protection of infant rats against strain NTHi R2866. Twenty-four hours prior to infection by NTHI R2866, individual cohorts of infant rats were mixed and reassigned to individual dams and pretreated with 100 µl phosphate-buffered saline (PBS), pre-immune serum (PIS) or chinchilla antiserum.



Protection afforded by antisera raised against Hi Poly 1 in the infant rat model of bacteremia. Infant rats (n = 10 per cohort) were passively immunized i.p. with chinchilla anti-Hi Poly 1 BVP antisera (BVP), matched pre-immune sera (PIS), or PBS alone one day prior to challenge with $1.5x10^5$ cfu NTHi R2866 . Blood was collected 24hrs post challenge for quantitative plating. The Wilcoxon-Mann-Whitney test compared bacteremic titers between PBS vs BVP (p = 0.03) and PIS vs BVP (p = 0.02).

Immunization of mice with the NTHi BVP leads to increased clearance of bacteria from the lungs following intranasal challenge with NTHi isolate R2866. Cohorts of mice (n=5) were immunized three times at two weeks intervals with 10 µg of the BVP Hi Poly 1 adsorbed to alum and 2 µg of MPLA in 50 uL of sterile saline. At 5 months after the last immunization, a group of 5 mice immunized with Hi Poly 1 and a group of 5 unimmunized mice were anesthetized with isoflurane and then intranasally infected



with isoflurane and then intranasally infected with 3.4×10^6 CFU of *H. influenzae* strain 86-028 NP in 20 µL of PBS. At 24-hours after infection, mice were sacrificed and lungs were removed. Lungs were homogenized in sterile PBS, serially diluted, and quantitatively plated on BHI agar supplemented with 10µg/mL heme, 10µg/mL NAD and 20U/mL bacitracin.

In Summary: Using NTHi as a test, the Bacterial Vaccine Polypeptide methodology led to the development of a single, easily purified, recombinant antigen. This BVP proved to be highly immunogenic resulting in antisera with cross reactivity against a broad panel representative of the species and provided significant protection in preclinical models of otitis media, bacteremia and pulmonary clearance using two different strains of NTHi.