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Effect of lipooligosaccharide mutations of *Haemophilus influenzae* on the Middle and Inner Ears

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Summary

Objective—The purpose of this study was to determine the virulence of nontypeable *Haemophilus influenzae* 2019 (NTHi 2019) and its two lipooligosaccharide (LOS) mutant strains, B29 (gene *htrB*) and DK1 (gene *rfaD*), and compare their effect on the middle ear, round window membrane, and inner ear.

Results—Fifteen chinchillas were divided into 3 equal groups and their bullas inoculated bilaterally with 0.5 ml of 10² CFU of parent NTHi 2019, B29 or DK1 mutant strains. Two days after inoculation all animals had otitis media and inflamed middle ear mucosa. There was a trend of greater thickness and infiltration of the round window membrane in animals inoculated with the wild-type NTHi strain compared to the mutant strains and a significant increase in both inflammatory cell infiltration and bacteria presence in the scala tympani area of the inner ear. Strial edema was only observed in the wild type-inoculated group.

Conclusions—LOS mutants of NTHi appear to have a reduced ability to pass through the round window membrane resulting in less inner ear inflammation and pathological changes.

Keywords

Otitis media; chinchilla model; middle ear; round window membrane; inner ear; histopathology; nontypeable *Haemophilus influenzae*; lipooligosaccharide; mutants; B29; DK1

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a leading cause of otitis media in children [1]. Lipooligosaccharide (LOS), the major immunogen of NTHi, has a highly heterogeneous composition between strains and between bacteria within a strain [2]. LOS glycoforms, containing sialic acid and phosphorylcholine, mimic host structures to allow the organism to evade innate defenses and manipulate host cell metabolism [3,4]. NTHi was shown to adhere to and invade bronchial epithelial cells via an interaction of LOS with the PAF (platelet activating factor) receptor, and binding of LOS to the PAF receptor initiates host cell signaling

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and bacterial invasion [5]. LOS structure affects the ability of bacteria to adhere to and invade cells effectively [2]. DK1, an *rfaD* gene mutant, expresses a truncated LOS consisting of only three deoxy-D-manno-octulosonic acid residues, a single heptose and lipid A [6,⁷]. DK1, an *htrB* gene mutant, is associated with modifications of lipid A and phosphorylation of LOS [8]. The late acylation of the lipid A encoded by the *htrB* gene is important in bacterial colonization and represents a key step in LOS biosynthesis [9]. Inner ear damage, which can lead to sensorineural hearing loss, is a serious sequelae of otitis media. The round window membrane is the only soft tissue barrier between the middle ear and the inner ear and has been shown to be permeable to a variety of substances. Passage of bacterial products [10], inflammatory mediators [11], and intact bacteria [12] through the round window membrane are considered the likely cause of inner ear damage. DeMaria et al [13] showed less virulence of B29 and DK1 mutant NTHi strains in the middle ear compared to the wild-type strain. They found the incidence of labyrinthitis by 5 days postinoculation in only 5% of the B29-inoculated animals, and no labyrinthitis in those inoculated with the DK1 mutant strain.

However, detailed analysis of histopathological changes of middle and inner structures caused by the wild-type and mutant NTHi strains, including the effect of LOS mutations on bacterial invasion and recruitment of inflammatory cells in the middle and inner ears, has not been reported. The purpose of this study was to compare middle and inner ear inflammation and pathology in chinchillas following middle-ear inoculation of either the parent NTHi 2019 strain or its two LOS mutant strains, DK1 (gene *rfaD*) and B29 (gene *htrB*).

2. Materials and Methods

The parental (wild-type) NTHi 2019, an *rfaD* gene mutant (DK1) with a truncated LOS, consisting of only three deoxy-D-manno-octulosonic acid residues, a single heptose, and lipid A, and an isogenic *htrB* mutant (B29) with an altered oligosaccharide core and an altered lipid A, were used in this study [7,⁹] (all NTHi strains were provided by Dr. Michael A. Apicella, University of Iowa College of Medicine). Overnight cultures of NTHi, on chocolate agar strains, were inoculated into liquid brain-heart infusion (Difco Laboratories) supplemented with hemin/NAD (Sigma) at 37°C with 5% CO₂. We added 15 mg/ml of kanamycin to the media for the DK1 mutant that contains a kanamycin resistant cassette, and 1.5 µg/ml of chloramphenicol to the media for the B29 mutant that contains a chloramphenicol resistant gene. Bacteria at mid-log phase (O.D 600nm 0.4-0.5) were harvested by centrifugation, washed with phosphate-buffered saline and diluted to desired cell numbers as determined by colony forming units of each strain.

Animals were housed and fed under standard conditions at our institutional animal care facility. Experiments were performed on young (~ 1-year-old) chinchillas weighing, 250-350 g that had normal external auditory canals and tympanic membranes. The care and use of animals was approved by the Institutional Animal Care and Use Committee of the University of Minnesota. All animals were anesthetized prior to intrabullar inoculations with a combination of ketamine (100 mg/kg) and acepromazine (10 mg/kg). Animals were sacrificed by overdose of sodium pentobarbital.

A total of 15 chinchillas were given bilateral intrabullar inoculations of 0.5 ml of 10² CFU of NTHi 2019, B29, or DK1 strains (5 animals for each strain). Two days after inoculation, animals were euthanized, bullas removed, and the cochlea perfused via the apex and oval window with 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). Fixation was continued by emersion for 2 hours. Samples were decalcified in 10% EDTA on a rotator in a cold room for 3 days. EDTA was changed daily. Samples were washed in phosphate buffer and post-fixed in 1% OsO₄ in phosphate buffer (pH 7.4) for 1 hour. They were washed again in buffer, dehydrated in a graded series of ethanol, followed by propylene oxide, and embedded in epoxy

resin. Samples were cut at a thickness of 1 μm and stained with Toluidine blue for light microscopic assessment.

Statistical analysis

Thickness of the middle ear mucosa was measured at the promontory. Measurements of the thickness of the middle ear mucosa and round window membrane were made at the midpoint of the sample and midway between the midpoint and the edge of the sample on each slide, using a 10×10 unit eye piece grid calibrated in units of 0.16 μm . Measurements were averaged. The areas of greatest inflammatory cell infiltration in the middle ear mucosa, round window membrane, and scala tympani were counted within the 10×10 unit grid of for each sample. Thickness and inflammatory cell infiltration were assessed at a magnification of 600 $\times$. Multiple slides from each ear were averaged and the data presented per ear. Bacterial infiltration of the scala tympani was determined at a magnification of 1000 $\times$. All results were expressed as mean $\pm$ SE. Standard software SPSS 11 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) with LSD post-hoc test was applied for pairwise multiple comparisons between the treatment groups. Differences were considered to be significant if $P \leq 0.05$.

3. Results

Forty-eight hours after inoculation, all of the animals inoculated with wild-type and two mutant NTHi strains, B29 and DK1, had middle ear effusions. The middle ear mucosa of animals inoculated with all three NTHi strains showed infiltration of inflammatory cells (predominantly polymorphonuclear leukocytes), mucosal thickening due to inflammatory cell infiltration in the subepithelial space, vascular dilation, and hemorrhage. Fig. 1,a-c shows examples of the most severe inflammation of the middle ear mucosa observed in the B29, DK1, and wild-type NTHi-inoculated groups. Inflammatory cell infiltration of the round window membranes (Fig. 2,a-c) was observed in all three groups. Fig. 3,a-c represents examples of the most severe inflammatory and pathological changes of the inner ear observed in each group. In some animals, inflammatory cell infiltration and bacterial invasion in the inner ear was accompanied by changes of cochlear structures. There was hair cell loss or damage, and severe strial edema in two ears of the wild-type NTHi-inoculated animals. One ear in the DK1-inoculated group had distortion of both inner and outer hair cells; however, there was no loss of hair cells and no strial edema. No damage to the inner ear was observed in the B29-inoculated group. Bacterial cells of the wild-type and both mutant strains, B29 and DK1, could be seen to pass into the scala tympani area of the inner ear. Figure 4 shows an example of bacterial passage through all three layers of the round window membrane in a DK1-inoculated animal. Bacteria were seen inside cells and outside of cells in wild-type and both mutant groups. We also observed that in some cases, polymorphonuclear leukocytes replaced the epithelial layer of the round window membrane. In some animals inoculated with the wild-type NTHi strain, bacteria and inflammatory cells were seen in the organ of Corti. The number of ears with bacteria in the scala tympani was greater in the wild-type NTHi-inoculated group compared to the mutant-inoculated groups (Table 1).

We performed one-way ANOVA statistical analysis to compare the number of inflammatory cells, predominantly polymorphonuclear leukocytes (PMNs), in the middle ear mucosa, round window membrane, and scala tympani, and the thickness of the middle ear mucosa and round window membrane, as well as bacterial presence in the scala tympani area of the inner ear, adjacent to the round window membrane, for animals infected with all three NTHi strains. These data are summarized in the Table 1. Although the mean numbers (Table 1) and ANOVA showed a trend of a greater number of inflammatory cells and an increased thickness of the round window membrane in the wild-type NTHi-inoculated animals compared to the mutant-

inoculated animal groups, P-values were higher than 0.05. There was no significant difference between the number of inflammatory cells, in the scala tympani, in animals inoculated with B29 and DK1 strains ($P = 0.873$); however, significant differences were found between animal groups inoculated with the parent NTHi strain and each of its isogenic mutants ($P = 0.032$ for B29 mutant and $P = 0.044$ for DK1 mutant).

4. Discussion

Streptococcus pneumoniae and nontypeable *Haemophilus influenzae* (NTHi) are two of the major pathogens of acute otitis media. Current polysaccharide-based vaccines against *Streptococcus pneumoniae* contain the most common serotypes causing pneumococcal infection. While the incidence of pneumococcal otitis media decreased since the introduction of pneumococcal conjugate vaccines, the incidence of otitis media attributed to NTHi has been on the rise [14,15]. Progress in developing a vaccine for NTHi has been hampered by the antigenetic heterogeneity of surface molecules among strains. The pathology from NTHi infection is the result of host response to bacterial factors, including lipooligosaccharide (LOS), a major antigenic bacterial component. LOS of NTHi has been shown to elicit many of the histopathological changes observed during experimental NTHi-induced otitis media [13].

We studied two LOS mutants of NTHi, DK1 and B29, and compared them to the wild-type strain in an effort to better understand the role of LOS components in the pathology of otitis media and inner ear complications secondary to otitis media. DK1, an *rfaD* gene mutant, expresses a truncated LOS consisting of only three deoxy-D-manno-octulosonic acid residues, a single heptose and lipid A [6,7]. B29, an isogenic *htrB* mutant, possesses an altered oligosaccharide core and an altered lipid A [8]. Our findings demonstrate, that both B29 and DK1, like the wild-type strain bacteria, were able to cause otitis media and inflammation of the middle ear mucosa; however, they were significantly less likely to pass through the round window membrane into the inner ear or cause inflammatory cell infiltration in the inner ear.

We cannot comment on the persistence of the mutants to colonize the middle and inner ear, because the survival time of our animals was of short duration. Although, approximately, the same number of colony forming units (CFUs) of each strain were inoculated, the relative fitness of these strains and their ability to multiply within the middle ear space, which could be bacterial intrinsic and/or also a consequence of host immune response, were not known.

Our study showed that NTHi LOS mutants caused less severe inflammatory and pathological changes to the inner ear than the wild-type strain, providing histological evidence that LOS plays a role in inner ear inflammation and pathology. The presence of bacterial and inflammatory cells in the scala tympani was significantly greater and resulted in more pathological damage of the cochlea in animals inoculated with the wild-type strain than in the B29 or DK1 mutant-inoculated animals. Most of the endotoxic activity of LOS is attributed to lipid A, and mutation of the *htrB* gene in NTHi B29 is associated with modifications of lipid A and phosphorylation of LOS [8]. Interestingly, we did not find any significant differences between the DK1-inoculated and the B29-inoculated animal groups.

Studies of other mutations involved in LOS biosynthesis or their combination may be necessary to determine those bacterial components that can be used for therapeutic targeting of NTHi infection.

Acknowledgments

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References

1. Bluestone CD. Clinical course, complications and sequelae of acute otitis media. *Pediatr Infect Dis J* 2000;19(5 Suppl):S37–S46. [PubMed: 10821471]
2. Erwin AL, Smith AL. Nontypeable *Haemophilus influenzae*: understanding virulence and commensal behavior. *Trends Microbiol* 2007;15:355–362. [PubMed: 17600718]
3. Mandrell RE, Apicella MA. Lipo-oligosaccharides (LOS) of mucosal pathogens: molecular mimicry and host-modification of LOS. *Immunobiology* 1993;187:382–402. [PubMed: 8330904]
4. Swords WE, Jones PA, Apicella MA. The lipo-oligosaccharides of *Haemophilus influenzae*: an interesting array of characters. *J Endotoxin Res* 2003;9:131–144. [PubMed: 12831454]
5. Swords WE, Ketterer MR, Shao J, Campbell CA, Weiser JN, Apicella MA. Binding of the non-typeable *Haemophilus influenzae* lipooligosaccharide to the PAF receptor initiates host cell signaling. *Cell Microbiol* 2001;3:525–536. [PubMed: 11488814]
6. Lee NG, Sunshine MG, Apicella MA. Molecular cloning and characterization of the nontypeable *Haemophilus influenzae* 2019 rfaE gene required for liposaccharide biosynthesis. *Infect Immun* 1995;63:818–824. [PubMed: 7868252]
7. Nicols WA, Gibson BW, Melaugh W, Lee NG, Sunshine M, Apicella M. Identification of the ADP-L-glycero-D-manno-heptone-6-epimerase (rfaD) and heptosyltransferase II biosynthesis genes from nontypeable *Haemophilus influenzae* 2019. *Infect Immun* 1997;65:1377–1386. [PubMed: 9119477]
8. Lee NG, Sunshine MG, Engstrom JJ, Gibson BW, Apicella MA. Mutation of the htrB locus of *Haemophilus influenzae* nontypeable strain 2019 is associated with modifications of lipid A and phosphorylation of the lipo-oligosaccharide. *J Biol Chem* 1995;270:27151–27159. [PubMed: 7592970]
9. Swords WE, Chance DL, Cohn LA, Shao J, Apicella MA, Smith AL. Acylation of the lipooligosaccharide of *Haemophilus influenzae* and colonization: an htrB mutation diminishes the colonization of human airway epithelial cells. *Infect Immun* 2002;70:4661–4668. [PubMed: 12117980]
10. Lundman L, Juhn SK, Bagger-Sjöbäck D, Svanborg C. Permeability of the normal round window membrane to *Haemophilus influenzae* type b endotoxin. *Acta Otolaryngol* 1992;112:524–529. [PubMed: 1441995]
11. Juhn SK, Jung TTK, Lin J, Rhee CK. Effect of inflammatory mediators on middle ear pathology and inner ear function. *Ann NY Acad Sci* 1997;830:130–142. [PubMed: 9616673]
12. Schachern PA, Paparella MM, Hybertson R, Sano S, Duvall AJ 3rd. Bacterial tympanogenic labyrinthitis, meningitis, and sensorineural hearing loss. *Arch Otolaryngol Head Neck Surg* 1992;118:53–57. [PubMed: 1728278]
13. DeMaria TF, Apicella MA, Nichols WA, Leake ER. Evaluation of the virulence of nontypeable *Haemophilus influenzae* lipooligosaccharide htrB and rfaD mutants in the chinchilla model of otitis media. *Infect Immun* 1997;65:4431–4435. [PubMed: 9353016]
14. Casey JR, Pichichero ME. Changes in frequency and pathogens causing acute otitis media in 1995–2003. *Pediatr Infect Dis J* 2004;23:824–828. [PubMed: 15361720]
15. Block SL, Hedrick J, Harrison CJ, et al. Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. *Pediatr Infect Dis J* 2004;23:829–833. [PubMed: 15361721]

Fig 1.

These figures represent examples of the most severe changes of the middle ear mucosa in animals inoculated with the B29 mutant (a); DK1 mutant (b); and wild-type (c) NTHi.

Fig 2.

Histopathological changes of the round window membranes were variable both in and between groups. These images represent examples of the most severe samples in the B29 (a), DK1 (b) and wild-type (c) NTHi-inoculated groups.

Fig. 3.

These images represent examples of the most severe animals with inflammatory cell infiltration in the B29 (a), DK1 (b) and wild-type (c) NTHi-inoculated groups. Although, inflammatory infiltration of the scala tympani occurred in the B29 (a) and DK1 (b) mutant NTHi-inoculated groups, it was more frequent and severe in the wild-type NTHi-inoculated animals (c). Note severe edema of the stria vascularis (arrow) in the wild-type NTHi-inoculated group. ME = middle ear, ST = scala tympani.

Fig. 4.
High-power images showing bacteria (arrows) of an animal inoculated with the mutant DK1 strain in the middle ear (ME), all three layers of the round window membrane, and scala tympani (ST).

Table 1
Comparison of ears inoculated with wild-type, B29, and DK1 NTHi strains

Type of inoculated bacteria	MEM inflammatory cells/area mean	MEM Thickness (µm) mean	RWM inflammatory cells/area mean	RWM thickness (µm) mean	Inflammatory cells/area in the ST mean ± SE	Bacterial infiltration in the ST (ears)
B29	28	40	26	32	5 ± 4	1 of 10
DK1	45	43	19	30	9 ± 8	1 of 10
Wild-type	43	42	58	42	56 ± 25	5 of 10
control				14		

RWM = round window membrane; MEM = middle ear mucosa; ST = scala tympani