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Evaluation of the Virulence of Nontypeable *Haemophilus influenzae* Lipooligosaccharide *htrB* and *rfaD* Mutants in the Chinchilla Model of Otitis Media

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Considerable evidence has implicated nontypeable *Haemophilus influenzae* (NTHi) lipooligosaccharide (LOS) in the pathogenesis of otitis media (OM); however, its exact role has not been conclusively established. Recently, two NTHi LOS-deficient mutants have been created and described. Strain 2019-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. Strain 2019-B29, an isogenic *htrB* mutant, possesses an altered oligosaccharide core and an altered lipid A. Each strain's ability to colonize the nasopharynx and to induce OM subsequent to transbullar inoculation was evaluated in the chinchilla model. Nasopharyngeal colonization data indicate that the parent strain and both mutants are able to colonize the nasopharynx and exhibit comparable clearance kinetics. Compared with the parent and each other, however, the mutants demonstrated marked differences in virulence regarding their relative abilities to induce OM and persist in the middle ear post-transbullar inoculation. Strain B29 required a 3-log-greater dose to induce OM than the parent strain and did not exhibit evidence of sustained multiplication but persisted for the same duration as the parent. Conversely, strain-DK1, even when inoculated at a dose 4 logs greater than the parent dose, was eliminated from the middle ear 72 h after challenge. A comparison of the relative pathogenicities of these isolates provides the opportunity to address fundamental questions regarding the contribution of LOS to pathogenesis issues at the molecular level. Specifically, the impact of these LOS gene disruptions on OM pathogenesis can be defined and may thus provide potential new targets for future protection and intervention strategies.

Nontypeable *Haemophilus influenzae* (NTHi) strains have emerged in recent years as significant pathogens, particularly with regard to respiratory tract disease, and may cause more types of invasive disease than previously recognized. One of the most extensively characterized NTHi-induced diseases is otitis media (OM). NTHi strains account for 25 to 30% of all cases of OM and 53% of recurrent OM (29) and are the primary pathogens isolated from 62% of cases of chronic OM with effusion (9).

The pathogenesis of NTHi-induced disease is not well defined; however, endotoxin or its subcomponent lipooligosaccharide (LOS) has been suggested as an important virulence factor for these bacteria (17). *H. influenzae* LOS is analogous to the lipopolysaccharide (LPS) of enteric gram-negative bacteria in that it contains lipid A linked by 3-deoxy-D-manno-octulosonic acid (KDO) to a heterogeneous sugar polymer (10). *Haemophilus* LOS, however, differs from classic enterobacterial LPS in that it does not contain repeating O-antigen units and is thereby more similar to that derived from *Neisseria* and *Bordetella* species (18). Endotoxin is present in the bacterial outer membrane but is also released in a biologically active form during growth or as a result of antibiotic- or immune response-mediated death of the organism (11). Endotoxin is a potent, biologically active mediator known to effect the release of various vasoactive amines and other mediators of inflammation by direct interaction with macrophages, polymorphonuclear neutrophils, and other cell types (19).

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Endotoxin is not readily eradicated by local host defense mechanisms and has been shown to persist in the middle ear for 3 months, even after effective antibiotic treatment (3, 7). Data from our laboratory have shown that endotoxin is present in 80% of all middle ear effusions (MEEs) and in 67% of culture-negative MEEs from patients with chronic OM with effusion. Others have confirmed these findings, reporting a >65% incidence of endotoxin in sterile MEEs (12, 22).

Many of the histopathological changes observed during the course of experimental OM in chinchillas can be elicited solely by isolated NTHi LOS (6). In addition to classic inflammatory responses, extensive proliferation of the chinchilla middle ear epithelium and fibrosis has also been observed (23). Previous reports from this laboratory have demonstrated that the transbullar (TB) injection of as little as 1 ng of NTHi LOS induces middle ear inflammation and histopathological changes (6).

Recently, two NTHi LOS-deficient mutants have been created and described. Strain 2019-DK1 is an *rfaD* gene mutant which possesses a truncated LOS consisting of only lipid A and KDO with a single heptose moiety (15, 20). The *rfaD* gene was originally described for *Escherichia coli* (24), was subsequently identified in *Salmonella typhimurium* (28), and has been recently described in detail for NTHi by Nichols et al. (20). The *rfaD* gene encodes the enzyme ADP-L-*glycero*-D-*manno*-heptose-6-epimerase, which is responsible for the epimerization of ADP-D-glycero-D-*manno*-heptose to ADP-L-*glycero*-D-mannose. Without the activity of this enzyme, the LOS structure truncates at the first heptose and has hydrophobic characteristics. The LOS derived from NTHi *rfaD* mutants is severely truncated, as demonstrated by gel chromatography and mass spectroscopy. The organism appears to be otherwise unaffected by this mutation; it grows as well on solid and liquid media as the wild-type strain (20).

Strain 2019-B29 is an isogenic *htrB* mutant which possesses an altered LOS core as well as an altered lipid A (16). The *htrB* gene was originally described in *E. coli* and encodes a protein essential for cell viability at temperatures above 33°C (13). The *htrB* protein acts as a specific acyltransferase in the late stage of lipid A biosynthesis. Recent data demonstrate that the *H. influenzae htrB* mutant strain expresses an altered lipid A that lacks the secondary myristoyl groups that are usually esterified to the 3-hydroxyl acyl moieties of the distal glucosamine (21). Mass spectrometric analysis indicates that 90% of the lipid A expressed by the *H. influenzae htrB* mutant has no myristoyl groups while the remaining 10% bears a single myristoyl moiety (16).

The purpose of this study was to compare the virulence of the parent strain with these two mutants in the chinchilla model of nasopharyngeal colonization and OM in order to begin to evaluate and dissect the individual roles of LOS and its lipid A and oligosaccharide (OS) moieties in the entire spectrum of OM pathogenesis.

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MATERIALS AND METHODS

Bacterial strains. The *H. influenzae* strains used in this study were NTHi 2019, 2019-B29, and 2019-DK1. NTHi 2019 is the parental isolate (wild type) and has been previously described (4, 10, 15, 16, 25).

Strain 2019-B29 is an isogenic *htrB* mutant generated by shuttle mutagenesis using a mini-Tn*3* (16). The biological consequences of mutation in the *htrB* locus of strain 2019-B29 include (i) modification of the OS core, reflected, in part, by a net loss in phosphoethanolamine, (ii) loss of myristic acid substitutions of the lipid A, and (iii) lost reactivity to monoclonal antibody 6E4, which binds to wild-type NTHi 2019 LOS (16). Strain 2019-B29 has a chloramphenicol resistance gene in the *htrB* gene and can be differentiated from the parent and 2019-DK1 by its ability to grow on brain heart infusion agar supplemented with 2% Fildes reagent (Difco Laboratories) containing 1.5 μg of chloramphenicol/ml.

Strain 2019-DK1 is an *rfaD* gene mutant. This gene encodes the enzyme ADP-glycero-D-*manno*-heptose-6-epimerase, and the DK1 mutant was produced by means of complementation studies with *S. typhimurium* LPS mutants with defined enzymatic defects (15, 20). The 2019-DK1 strain's LOS is truncated and consists only of lipid A and KDO with a single heptose moiety (20). It is devoid of other core OSs. Strain 2019-DK1 contains a kanamycin resistance cassette and can thus be differentiated from wild-type strain 2019 or 2019-B29 by its ability to grow on brain heart infusion containing 15 μg of kanamycin/ml.

Animals. A total of 84 chinchillas (*Chinchilla lanigera*) (350 to 650 g) free of middle ear disease, as determined by otoscopy and tympanometry, were used in these studies.

i.n. challenge and assessment of nasopharyngeal colonization. The biological consequences of NTHi *htrB* and *rfaD* gene disruption on the induction and persistence of nasopharyngeal colonization in the chinchilla model were assessed subsequent to intranasal (i.n.) challenge. While colonization by NTHi is an important step in OM pathogenesis, it requires analysis separate from OM. Three cohorts of seven chinchillas each were challenged i.n. with 300 μl of a suspension of parent strain 2019 or either LOS-deficient mutant containing approximately 5×10^8 CFU of NTHi per ml of sterile pyrogen-free saline. The inoculum was divided equally between the nares. Nasopharyngeal lavage was performed on each chinchilla every 4 to 7 days for semiquantitative assessment of numbers of CFU of NTHi per ml of lavage fluid as previously described (27, 30). All inoculations, nasopharyngeal lavages, and other assessments were made blindly by the same person throughout this study.

TB middle ear challenge and induction of OM. TB injection of an inoculum directly into the middle ear of the chinchilla is the method most widely used to induce NTHi OM (5). i.n. inoculation of the chinchilla with NTHi strains alone typically induces only inconsistent tympanic membrane inflammation and does not result in culture-positive OM with middle ear fluid (MEF) or sustained presence of the organism (2).

To assess the role of LOS in the multiplication, persistence, induction of disease, and clearance of NTHi from the middle ear cleft, a comparison of the survival rates of the strains was performed. Three cohorts of seven chinchillas each were inoculated TB with 50, 500, or 5,000 CFU of the parent strain 2019. The bullae were vented with a sterile 26-gauge needle placed near the inoculation site. The chinchillas were evaluated daily for tympanic membrane inflammation and middle ear pressure changes. Tympanic membrane inflammation was rated on a scale of 0 to 4+ as previously described (27). Normal chinchilla middle ear pressure was considered to be between −60 daPa and +40 daPa (26). Middle ear fluid was aspirated by means of epitympanic taps of the inferior bulla from each inoculated ear, which were performed serially every 4 to 7 days postchallenge for the semiquantitative determination of the number of viable CFU of NTHi per milliliter of MEF. The uninoculated contralateral ears were inspected, and MEF was retrieved when present.

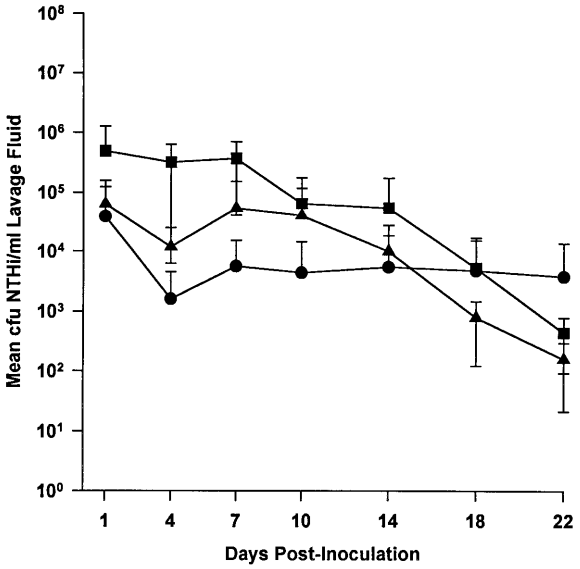

FIG. 1. Nasopharyngeal colonization dynamics in chinchillas challenged intranasally with parent strain NTHi 2019 (▲), the B29 *htrB* mutant (■), or the DK1 *rfaD* mutant (●). Each datum point represents the mean number of CFU of NTHi ± standard deviation (error bar)/milliliter of nasal lavage fluid from seven chinchillas.

The same experiment was repeated for the *htrB* mutant strain 2019-B29. Three cohorts of seven chinchillas each were inoculated TB with approximately 10^4, 10^5, and 10^7 CFU, respectively, of strain B29 and monitored as described above.

Finally, in a third experiment, the survival of strain DK1 was assessed after the TB inoculation of seven chinchillas with 10^8 CFU/ear. All inoculations and assessments were made blindly and by the same observer throughout the TB challenge study.

MEF is not typically present in a sufficient quantity to be aspirated prior to 4 days postinoculation. In those cases, or when no aspiratable MEF was present, the bullae were lavaged with 0.5 ml of sterile pyrogen-free saline. The MEF or lavage fluids were cultured on chocolate agar (Choc II; BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C in a humidified atmosphere of 5% CO_2 for up to 72 h to detect and semiquantitate the number of CFU of NTHi per milliliter.

Animals were additionally evaluated to determine the incidence of labyrinthine involvement to assess the effect of the induced OM on the inner ear and related balance function.

Light microscopic evaluation of histopathology. Two cohorts of three chinchillas each were used for an evaluation of histopathological changes induced by each strain on days 7 and 14 post-TB inoculation. Evaluation of the middle ear mucosae was performed as previously reported (1).

Statistical analyses. Mean numbers of CFU of NTHi/milliliter were compared among cohorts at each sample by means of a one-way analysis of variance. The Tukey test was utilized for a pairwise multiple comparison. A P value of <0.05 was accepted as the minimal level of significance.

RESULTS

Effect of LOS gene disruption on nasopharyngeal colonization. The relative abilities of the parent and both LOS-deficient mutants to colonize the nasopharynx for up to 22 days post-i.n. challenge is shown in Fig. 1. There was no significant difference, at any time point, between the ability of the parent strain and the two LOS-deficient mutants to colonize or persist in the nasopharynx.

Effect of LOS gene disruption on induction of OM subsequent to TB inoculation. Dose-response data derived from the TB challenge studies indicated marked differences between the parent and each mutant relative to the dose of each strain

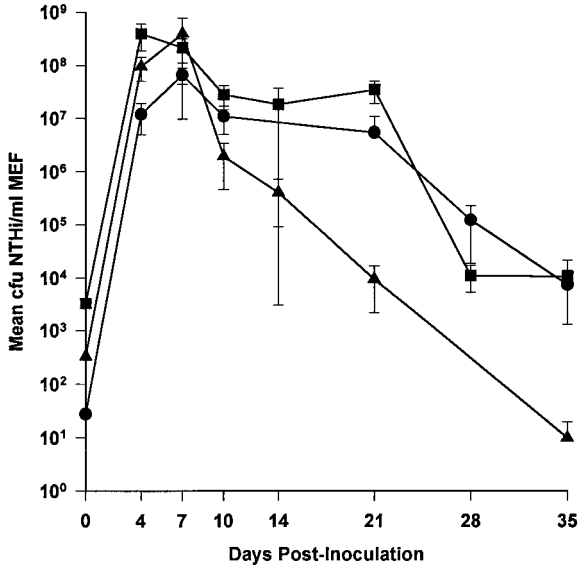

FIG. 2. Survival of parent strain 2019 in the middle ear among cohorts inoculated TB with 50 (●), 500 (▲), or 5,000 (■) CFU of NTHi. Each datum point represents the mean number of CFU of NTHi ± standard deviation (error bar)/milliliter of MEF from seven chinchillas.

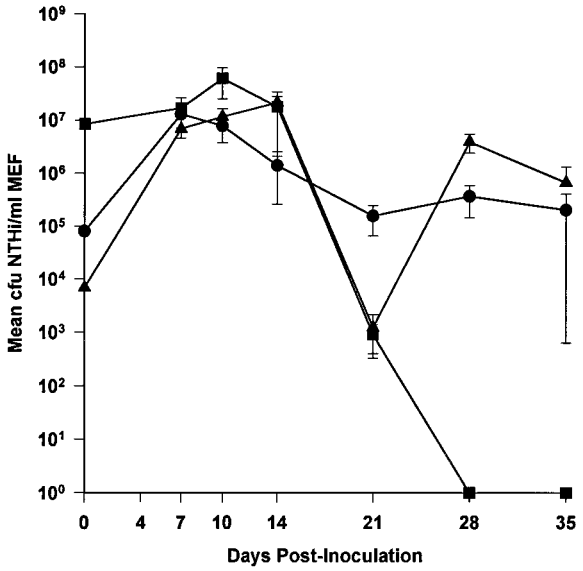

FIG. 3. Survival of the B29 *htrB* mutant in the middle ear among cohorts inoculated TB with 7×10^3 (▲), 8×10^4 (●), or 8×10^6 (■) CFU of NTHi. Each datum point represents the mean number of CFU of NTHi ± standard deviation (error bar)/milliliter of MEF from seven chinchillas.

required to infect the middle ear, multiply, induce OM, and persist in the middle ear cleft.

Inoculation of 50, 500, or 5,000 CFU of the parent strain, 2019, resulted in culture-positive OM in all animals in each cohort. A 6-log increase in the concentration of CFU of NTHi/milliliter of MEF and persistence of the bacteria were demonstrable with each dose between 4 and 7 days post-TB inoculation, after which the number of CFU of NTHi/milliliter steadily declined over the remaining 28 to 31 days of the experiment (Fig. 2). However, at the termination of the experiment, the level of strain 2019/milliliter of MEF in each cohort remained greater than or equal to the concentration of the original inoculum.

NTHi B29, the *htrB* mutant, was less virulent than the parent, and a dose of $>10^4$ CFU of NTHi B29 per ear was required to induce OM with culture-positive MEFs (Fig. 3). Between 7 and 10 days post-TB challenge, only a 2- to 3-$\log_{10}$ increase in the number of CFU/milliliter of MEF was demonstrable at the two lower inoculum doses. There was no similar 2- to 3-log increase noted at the highest dose (10^7 CFU of NTHi/ear). Similar to the parent strain, the B29 mutants persisted for the duration of the experiment in the MEFs of two of the three cohorts inoculated with this strain.

NTHi strain DK1, the *rfaD* mutant, was the least virulent and required a dose of approximately 10^8 CFU of NTHi/ear to establish OM with culture-positive middle ears. No retrievable MEFs were present, and all counts were derived from bulla lavages. There was no evidence of multiplication or persistence of the inoculum (Fig. 4), which decreased by 2 logs within 24 h and was completely eliminated from the middle ear cleft by 7 days post-TB inoculation.

Clinical signs of OM. The mean tympanic membrane inflammation scores did not reflect the survival of each strain in the middle ear and were comparable during the OMs induced by the parent and both LOS-deficient mutants (not shown). In each cohort, these scores rose to maximum levels (+3) by day 4 and plateaued for the duration of the experiment, despite declining concentrations of bacteria in the middle ear cleft.

Similarly, although a quantitative morphometric analysis was not performed, there were no qualitative differences in the abilities of the parent and both LOS-deficient mutants to induce histopathological changes in the middle ear epithelium. Specimens from all three cohorts demonstrated moderate thickening of the mucosa, evidence of osteoneogenesis, and the presence of both erythrocytes and polymorphonuclear inflammatory cells in the subepithelial space.

Labyrinthitis was more prevalent and severe in the cohort inoculated with the parent strain, indicating the diminished

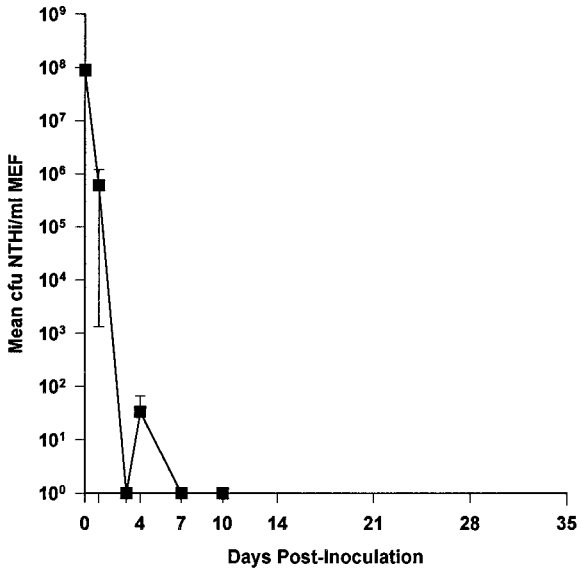

FIG. 4. Survival of the DK1 *rfaD* mutant in the middle ears of chinchillas inoculated TB with 10^8 CFU of NTHi. Each datum point represents the mean number of CFU of NTHi ± standard deviation (error bar)/milliliter of bulla lavage fluid from seven chinchillas.

virulence of each mutant to induce an effect on the inner ear and related balance function subsequent to OM. By 5 days postinoculation, the incidence of labyrinthitis was 18% in the parent cohort, peaking at 81% by 17 days postinoculation. Labyrinthitis was initially evident in only 5% of the chinchillas in the B29 cohort, and the incidence of labyrinthitis did not exceed 14% for the duration of the study. No labyrinthitis was observed in the DK1 cohort.

Invasion of the contralateral ear. Invasion of the contralateral ear by strain 2019, as determined by the presence of a retrievable NTHi-positive MEF, was comparable to that by the B29 mutant. By day 7 postinoculation, 25 and 30%, respectively, of the culture-positive MEFs in each cohort were obtained from the uninoculated right ears. By 30 to 35 days, when the experiment was terminated, though the incidence of culture-positive MEFs had declined overall, three of the five MEFs from the cohort inoculated with strain 2019 and three of the six MEFs obtained from the B29-injected animals were aspirated from the right ears. Culture-positive MEFs were not present, however, in any of the contralateral ears of the cohort receiving the DK1 mutant.

DISCUSSION

Endotoxin has been suggested as an important virulence factor for NTHi-induced disease (17). Considerable evidence has implicated NTHi endotoxin or LOS in the pathogenesis of OM. Experimental studies to date, however, have all utilized the injection of purified LOS or LPS to induce an effect and have primarily served as models for only that narrow clinical window of OM when the acute inflammatory phase is resolving. By this point in the clinical course of OM, viable bacteria have been eliminated by either antibiotic treatment or host immune defenses, and only free and/or shed endotoxin is present in the MEEs. Thus, obtaining definitive evidence that endotoxin or LOS is involved in the complete spectrum of OM pathogenesis, beginning with bacterial colonization of the nasopharynx and followed by retrograde ascension to the Eustachian tube, multiplication of the organisms in the middle ear cleft, induction of disease, and eventual elimination of viable NTHi from the middle ear, has not been possible. The results of the present study indicate that alteration of the LOS phenotype does have an impact on the virulence of NTHi during experimental OM in the chinchilla and modifies some, but not all, of the progressive stages of the disease course and its inner ear sequelae.

The LOS-deficient mutants were as adept as the parent at colonizing the nasopharynx. Thus, the variable virulence observed in the middle ear is the direct result of the specific LOS gene disruptions rather than a global impairment of the mutants' ability to adhere, colonize, or multiply in the chinchilla.

It is noteworthy that, while all three strains were equally adept at colonizing and persisting in the nasopharynx, each exhibited a substantially different virulence when used to induce OM after TB inoculation. This was most clearly evidenced by the higher doses required to induce OM and the lack of sustained multiplication by both the B29 and DK1 LOS-deficient mutants. We have been able to demonstrate that all three strains are equally susceptible to both human and chinchilla serum bactericidal activities in vitro (unpublished observations), which suggests that alteration of the LOS phenotype does not result in an increased sensitivity to serum bactericidal activity and a subsequent decrease in the virulence of the mutants.

It is of interest that each mutation also resulted in a unique change in pathogenesis as defined by the parameters assessed in this study. Compared to the 2019 parent, NTHi DK1, the *rfaD* mutant, colonized the nasopharynx but did not induce MEF formation, multiply, or persist in the middle ear. Also, it neither invaded the contralateral ear nor induced labyrinthitis.

Compared to the 2019 parent, NTHi B29, the *htrB* mutant, also colonized the nasopharynx, persisted in the middle ear, and invaded the contralateral ear, but a significantly larger inoculum was required to induce culture-positive MEF, and it exhibited only limited multiplication and a reduced incidence of labyrinthitis.

The ability of NTHi to induce OM in the contralateral ear was first described by Doyle et al. (8), although the exact mechanism whereby this phenomenon takes place has not been defined. The fact that the B29 mutant was as competent as the parent at invading the contralateral ear indicates that disruption of the *htrB* gene does not inhibit the ability of NTHi to invade and colonize other anatomic niches, whereas the disruption of the *rfaD* gene clearly inhibits the ability of NTHi to do so.

The tympanic membrane inflammation scores and histopathological changes for all three strains examined were comparable and persisted even as the concentration of viable bacteria in the middle ear cleft declined. This is not totally unexpected. Previously, our laboratory demonstrated that killed NTHi cells or LOS (6) and isolated NTHi peptidoglycan, free of LOS contamination (14), all induce severe inflammatory changes, MEF, and histopathology in the chinchilla OM model. The fact that the tympanic membrane inflammation is the same after challenge with each of the three strains suggests that the disruption of the NTHi *htrB* and *rfaD* genes does not impact tympanic membrane inflammation or histopathology in the middle ear. Alternatively, any differences between the wild type and mutants may be potentially masked by activation of inflammatory pathways by other NTHi cellular components, such as peptidoglycan. At this point, it is not entirely clear why there is no demonstrable difference between the wild type and both mutants with regard to these parameters.

The data generated with B29, the *htrB* mutant, suggest that the products of its mutant gene impact the virulence of this strain in a variable fashion, depending on the anatomical niche being colonized or infected. This is in contrast to strain DK1, which contains a highly truncated LOS and, in spite of being able to colonize and persist in the nasopharynx, is unable to persist in the middle ear, invade the contralateral ear, or induce labyrinthitis. A recent report by Nichols et al. indicates that wild-type NTHi LOS has been shown by *Limulus* assay to have eightfold more endotoxin activity than the LOS derived from the isogenic *htrB* mutant and that the *htrB* mutants demonstrate a reduced ability to induce tumor necrosis factor production in vitro (21). Moreover, in vivo assays using i.n. or intraperitoneal inoculation of suckling rats with an NTHi *htrB* mutant result in a 40% reduction in the incidence of bacteremia (21). These data suggest that the *htrB* gene of *H. influenzae* is important for virulence and that the host's tumor necrosis factor alpha expression is attenuated in response to NTHi *htrB* mutant strains.

The results from the present study indicate that the disruption of genes which alter the phenotype of NTHi LOS also impacts various aspects of this organism's virulence in the chinchilla model of nasopharyngeal colonization and OM. These and other LOS-deficient mutants provide, for the first time, an opportunity to evaluate the role of LOS pathogenesis of NTHi-induced disease.

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