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Infant rat infection modifies phenotypic properties of an invasive nontypeable *Haemophilus influenzae*

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Abstract

Enhancing the virulence trait of a specific bacterium in an animal model is often performed prior to the use of the strain for *ex vivo* human studies, such as reactivity with complement and antibody, or with phagocytic cells. For example, in *S. pneumoniae* mouse passage is used to enhance capsule production. While investigating an unusual serum-resistant unencapsulated *H. influenzae* (R2866), we found that animal passage yielded an isolate (R3392) which had decreased resistance to human serum, but increased virulence in Chang conjunctival cell monolayers, but with less invasion and transcytosis of polar H292 cells. We examined 90 colonies recovered from three infant rats for phase variants of LPS biosynthetic genes. In 88 colonies *lgtC* was OFF due to tetrameric-repeat mediated slipped-strand mispairing at the time of DNA replication, while there was no variation in *lic1A*, *lic2A*, *lic3A*, *lexA* and *oafA*. With *lgtC* OFF the LPS lacks Gal α 1-4 β Gal, an epitope mimicking the human pk^c blood group, and molecular mimicry is lost. Selection for strain susceptible to NHS in the infant rat was not antibody mediated. We conclude that the passage of pathogens virulent in humans and animals may select for phenotypes only relevant for the animal species used.

Keywords

Animal infection; *Haemophilus influenzae*; phase variation

1.0 Introduction

Animal passage is frequently used to enhance the production of bacterial virulence factors prior to the use of the organism in investigations with human cells or sera. This is particularly true with *Streptococcus pneumoniae* [1] in which mouse passage was used to enhance virulence. The implication in this approach is that virulence trait of interest, such as capsule, can phase vary, and animal passage selects for the virulence phenotype which is relevant in humans. The same approach was used for the development of a mouse model of *Campylobacter jejuni* enteritis: passage in mice enhanced the virulence of the test organism

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[2]. In contrast, in other genera, animal passage in multiple species by multiple different routes did not enhance the virulence of *Listeria monocytogenes* as measured by LD₅₀% [3]. However, increased virulence of *Salmonella typhimurium* as measured by replication in the spleen and liver was possible only with animal passage of a *dam* mutant [4]; the LD₅₀% of the *dam* mutants was two to three fold lower than wild type.

Weiser [5] first noted that opaque colonies of a type b *H. influenzae* were resistant to the bactericidal affect of low concentrations of normal adult human sera, in comparison to the transparent phenotype. Additional studies indicated that the LPS could be modified by decoration with phosphocholine (ChoP), mediated by the phase variable *lic1A* locus with the addition of ChoP making the cells more susceptible to the bactericidal affect of NHS [6]. This increased susceptibility was due to the presence of the C-reactive protein (CRP) in serum, a component of innate immunity [7]: CRP has bactericidal activity against ChoP⁺ cells. The gene responsible for generating expression of ChoP, *lic1A*, is phase variable due to the presence of tetrameric repeats (CAAT) after the start codon. These repeats permit slipped-strand mispairing at the time of replication putting the downstream orf in, or out of frame. An additional LPS biosynthetic gene, *lic2A*, which is also phase variable via the same mechanism, encodes an enzyme which adds a terminal galactose to the inner galactose on the third heptose in the LPS of most *H. influenzae*. An additional galactosyl transferase (LgtC) adds the galactose to the heptose, permitting Lic2A to generate the Gal α 1-4Gal epitope. In the panel of isolates studied by Weiser et al resistance to 10% NHS was mediated by *LgtC* when *lic2A* was OFF [6]. In the infant rat, nasopharyngeal colonization selects for ChoP⁺ cells, but not for expression of the Gal α 1-4Gal epitope. Relative resistance to 10% NHS was present when *lic1A* was off, and *lic2A* was off and *LgtC* was ON [6]. Rats do not have a homolog of CRP, while they recognize Gal α 1-4Gal as a foreign antigen. In humans Gal α 1-4Gal β 1-4Glc-ceramide is the epitope in the pc^k blood group antigen so that expression of this epitope makes the *H. influenzae* resistant to killing by NHS by molecular mimicry [8].

We had previously observed that *H. influenzae* strain R2866, an invasive nontypeable isolate isolated from the blood of a 30-month-old infant, was able to cause bacteremia in infant rats [9]. One isolate (R3392) from the blood of infant rats after infection with R2866 was also able to cause bacteremia in weanling (21 to 25 days-old) male rats, but at a reduced frequency [9]. We found that *H. influenzae* R2866 was resistant to the bactericidal activity of normal adult human blood and sera, in comparison to the unencapsulated strain Rd, but was not as resistant to the bactericidal activity of whole blood and sera as the prototypic encapsulated type b strain Eagan (E1a). When $\sim 1 \times 10^3$ CFU of strain R2866 were inoculated into three ml of defibrinated whole blood from three adult volunteers and incubated at 37°C, the organisms grew to a density of 10⁵ CFU/ml in two hours [10]. This ability to grow in blood correlated with resistance to the bactericidal affects of normal adult sera (NHS), as the serum concentration required to kill 50% of a low inoculum (2,000 CFU) was 26%. Thus the serum resistance of this strain was different from that mediated by *lic1A* and *lic2A* in other *H. influenzae*.

One colony isolated from the blood of an infected infant rat was retained, and designated strain R3392. In contrast to strain R2866 this strain was susceptible to the bactericidal activity of adult human sera [11]. The decreased resistance to the cidal activity of NHS was due to an apparent *in vivo* selection in infant rats for a phase variant which switched the NHS resistant phenotype to susceptible. Strain R3392 differed from R2866 in that the former lost a Gal α 1-4 β Gal epitope from an unusual LPS containing four heptose per KDO [11]. The galactosyl transferase adding the proximal galactose to Gal α 1-4 β Gal is encoded by *LgtC*. When *LgtC* is OFF the resulting structure lacks Gal α 1-4 β Gal, which is the human pc^k blood group epitope [10]. Because only one colony was retained and examined we

performed another series of experiments to determine if selection for a phase variant (*IgtC* OFF) had occurred in the infant rat. To gain insight into the selection mechanism we also assessed the cidal activity of five day-old, six week-old and adult rat sera (animals between 57 and 64 days old) against R2866 and R3392. We also sought to determine if phase variation affected the ability of virulent strain R2866, which was isolated from the blood of an immune-competent child, to adhere to and invade human epithelia cells.

2. Materials and methods

2.1 Bacteria

H. influenzae used in this study have been previously described [10]: strain R2866 is nontypeable and was isolated from the blood of a normal 30 month old who had been immunized with *H. influenzae* b conjugate vaccine. *H. influenzae* strain Rd is the unencapsulated strain sequenced by TIGR [12] and lacks recognized virulence traits; the complete genomic sequence of strains R2866 and Rd are available at NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&cmd=Retrieve&dopt=Overview&list_uids=9621). Strain E1a, is a prototypic type b which is virulent in infant rats [13]; strain R3392 is a NHS susceptible phase variant of R2866 which was isolated after infant rat infection by strain R2866 [10, 11]. Bacteria were stored at -70°C in 50% glycerol:50% BHI broth and subcultured onto chocolate agar plates and incubated at 37°C in 5% CO_2 overnight before use.

2.2 Bactericidal Assay

Blood was obtained aseptically from 7 to 10 healthy adult volunteers under protocols approved by the IRB of Seattle Biomedical Research Institute or Seattle Children's Hospital Research Institute. Blood was allowed to clot at room temperature, and human serum (NHS) was isolated aseptically, pooled, and stored at -80°C in 1 ml aliquots. Treatment of bacteria with 40% serum to select for variants with increased resistance was performed as described previously [14]. Blood was obtained by cardiac puncture from five day old rats anesthetized with Nembutal, 0.4 mg/10 gm body weight, and was processed in the same way as the human blood. Sterile, fresh frozen serum from male Sprague-Dawley rats between 57 and 64 days old was purchased from Rockland Farms, Gilbertsville, PA. Each serum sample from was assayed in duplicate and the assay repeated three times.

2.2.1 $\text{IC}_{50\%}$ assay—The microtiter plate serum bactericidal assay has been described previously [15]. Log-phase bacteria ($\sim 2,000$ CFU/ml) were incubated for 30 min at 37°C with pooled serum, serially diluted in 10 mM phosphate-buffered saline containing 4 mM KCl and then plated to determine bacterial survival. The concentration of serum that killed 50% of inoculum was calculated after fitting the data to a Boltzmann sigmoidal curve, using the program XLfit 4.1 (ID Business Solutions, Guildford, United Kingdom), and is referred to as the 50% inhibitory concentration ($\text{IC}_{50\%}$) of NHS for that strain. This program unweights the high and low values before calculating the $\text{IC}_{50\%}$. Duplicate determinations were repeated three times and the means reported.

2.2.2 Kinetic assay—The kinetic assay was modified from that previously used [14]. Briefly, bacteria were grown in sBHI in 5% CO_2 and 37°C to a density of 0.6 at $A_{600\text{nm}}$ and diluted in PBS-G to an $A_{600\text{nm}}$ of 0.2 ($\sim 2 \times 10^8$ CFU/ml); 0.2 ml of the bacterial suspension was added to 0.8 ml of 50% serum (diluted in PBS-G). Aliquots were removed immediately after mixing and after 5, 15, 30, and 45 min of incubation at 37°C in room air and the density determined. Each serum was also assayed with 5 mM EGTA to confirm complement dependence.

2.3 Invasion assays

2.3.1 Submerged Monolayers—Standard assays of bacterial invasion of eukaryotic cell use a submerged monolayer. The invasion protocol described herein was adapted and modified from that previously described [16]. Colonies of *H. influenzae* were harvested from a chocolate agar plate which had been incubated overnight at 37°C in room air and resuspended in PBS-G to an OD 600nm of 0.2 ($\sim 1.0 \times 10^8$ CFU/ml). Aliquots of this suspension were used to inoculate each well of a Biocoat 12-well cell culture plate (BD catalog# 354500) coated with type I collagen with $\sim 1.5 \times 10^6$ CFU of bacteria inoculated, a MOI of $\sim 3:1$. Aliquots of the bacterial suspension were also serially diluted in PBS-G and plated on sBHI agar to enumerate the viable inoculum. The infected monolayers were incubated at 37°C in 5% CO₂ for two (HBMECs) or four hours (NHBE, A549 and Chang cells) as pilot experiments indicated that invasion had peaked at those times with those cell lines. Following the incubation, the monolayers were washed twice with 1.0 ml of Dulbecco's phosphate buffered saline (D-PBS) and then 1.5 ml of fresh medium containing 100 µg/ml gentamicin was added to the cultures to kill bacteria external to the eukaryotic cell. Gentamicin was left on the cells for 1 h at 37°C after which the gentamicin-containing media was removed and the wells were washed 3 times with 1.5 ml of Dulbecco's phosphate buffered saline (D-PBS) without magnesium or calcium. To facilitate detachment of the monolayers 1.0 ml of 1% saponin in D-PBS was added and allowed to incubate for 10 minutes at 37°C prior to scraping the cells out of the wells and vortexing vigorously to lyse the eukaryotic cells. The cell lysate was centrifuged at $500 \times g$ for 10 min and the resulting supernatant was serially diluted in PBS-G and aliquots spread on sBHI agar for quantitation of the bacteria not killed by gentamicin. Invasion was calculated as the percentage of gentamicin-resistant bacteria derived from the initial viable inoculum. Each experiment was performed in duplicate, and replicated at least three times with mean values reported.

Chang human conjunctival cells were obtained from the ATCC (CCL-20.2) and maintained in RPMI 1640 with 25 mM HEPES and 2mM L-glutamine (Invitrogen catalog #22400-105) with 50 ml heat-inactivated fetal calf serum per liter. A549 cells are a human lung epithelial carcinoma cell line and were obtained from the ATCC (CCL-185) and cultured in Ham's F12K medium (Gibco) with 2 mM L-glutamine with 100 ml heat-inactivated fetal calf serum and 1.5 g sodium bicarbonate in each liter. We also examined invasion of human brain microvascular endothelial cells (HBMECs) which were a gift to this lab from Kwang Sik Kim (Johns Hopkins University School of Medicine). These cells were maintained in HBMEC media: 760 ml RPMI 1640 with 25 mM HEPES and 2mM L-glutamine, 100 ml heat-inactivated fetal calf serum, 10 ml each of 200 mM L-glutamine, 100X MEM non-essential amino acid solution, 100X MEM vitamin solution, 100 mM MEM sodium pyruvate solution, and 100 ml heat inactivated NuSerum V (Becton Dickinson, Bedford, MA) per liter. Invasion of these cells is primarily via a pinocytotic pathway which results in transcytosis to the sub-neuronal layer [17]. These cells and the Chang and A549 were studied submerged.

Normal human bronchial tissues were obtained at autopsy as described [18] or NHBE cells were purchased from Cambrex Bio Science, Walkersville, MD. For the harvest of bronchial epithelial cells the tissue was placed in Ca-free/Mg-free Hanks' balanced salt solution containing pronase (1.5 mg/ml) and DNase (0.1 mg/ml) and incubated at 4°C for 48 hours. Fetal bovine serum was then added to a final concentration of 10% and the resulting cell suspension was washed with airway medium (equal volumes of high glucose Dulbecco's modified Eagle medium and Ham's F-12 medium). The harvested cells, or those purchased were pelleted and resuspended in airway medium at a density of 5×10^5 cells/ml; 50 µl of the cell suspension was seeded on a 25 mm diameter tissue culture insert with a 3.0 µm por

size (Biocoat, cat # 354565, Becton-Dickinson, Bedford MA) and the cells covered with BEGM media (Clonetics, San Diego, CA) and incubated submerged at 37°C in 5% CO₂.

2.3.2 Cells at a air-liquid interface—To determine if the ability to adhere and invade human derived cells was affected by growing the cells under conditions which permit the formation of tight junctions, we used NCI-H292 human mucoepidermoid pulmonary carcinoma cells grown at air-liquid interface [19, 20]. Under this condition NCI-H292 cells form tight junctions, with transepithelial resistance (TER) 500 Ω·cm². NCI-H292 cells were obtained from the ATCC (CRL-1848) and maintained in RPMI 1640 medium (Invitrogen, catalog #22400-105) with 100 ml heat-inactivated fetal bovine serum, 0.075% sodium bicarbonate (Gibco), and 1mM sodium pyruvate (Cellgro, Mediatech, Manassas, VA) per liter. The cells were first grown submerged at 37°C in 5%CO₂ on 25 mm diameter, 3 μm pore size in 12-well Biocoat tissue culture inserts (cat # 354565, Becton-Dickinson). Upon reaching confluence the NCI-H292 cells were placed at ALI until the TER had stabilized, between seven to 10 days and challenged with *H. influenzae*. The bacteria are suspended in ice cold PBS-G from a fresh overnight growth on a chocolate agar plate, and diluted to an A600nm of 0.2 to 0.5 (0.2 assumes ~10⁸ CFU/ml). Aliquots of this suspension were used to inoculate the apical side of each tissue culture insert with ~1.0 to 5×10⁵ CFU of bacteria, a MOI of ~3:1. The remaining bacterial suspension was diluted and plated on chocolate agar to determine actual bacterial concentration in the inoculum. The infected respiratory epithelial cells were incubated at 37°C in 5% CO₂ for one, three, or 24 hours. Following incubation, the apical fluid, and the basal fluid was sampled to determine bacteria density. The apical surface was then washed twice with 1 ml D-PBS; and the basal compartment washed twice with 2 ml. These volumes were replaced with an equivalent volume of D-PBS containing 100 μg/ml gentamicin. The gentamicin treated cells were incubated at 37°C for an additional hour prior to being washed three times with D-PBS without magnesium or calcium, using 1 ml for the apical surface and 2 ml for the basal compartment. After washing 1 ml of a 1% saponin solution in PBS is added to the apical side and the cells were scraped from inserts, and the collected suspension was vortexed for 1 minute. The resulting lysed cell supernatant was serially diluted and plated on sBHI agar and incubated at 37°C in 5%CO₂ to determine the number of bacteria not killed by the gentamicin, and the number associated with the cells (not gentamicin treated). The number of adherent bacteria was calculated as the percentage of bacteria derived from the initial viable inoculum which adhered to cells, while invaded bacterial data was the percentage of bacteria in the inoculum which survived gentamicin treatment. The percentage of the inoculum recovered from the basal compartment were those transcytosed. At each time point three wells were processed with the bacterial challenge performed in duplicate. The geometric mean bacterial density was calculated for each strain at each time point.

2.4 Animal Infection

The infant rat model is essentially that previously described [13] and its use was approved by the Institutional Animal Care and Use committees of the University of Missouri, the Seattle Biomedical Research Institute and the Seattle Children's Research Institute. Outbred, pathogen free, Sprague-Dawley COBS/CD rats were obtained from Charles River Laboratories. Timed pregnant females were housed individually at 21 ± 2°C in 50% relative humidity on a 7:00 am to 7:00 pm lighting schedule. Twenty-four hours after delivery the pups were randomly assigned to each dam limiting the number of pups in each litter to 10. When the pups were five days old, the bacteria were harvested from chocolate agar plates, inoculated into BHI broth supplemented with hemin (10 μg/ml) and β-NAD⁺ (10 μg/ml) and incubated with shaking (200 rpm) at 37°C in room air until the A600nm was 0.20–0.25 (~ 10⁸ CFU/ml). The bacteria were pelleted by centrifugation at 10,000 × g for 5 min and diluted in phosphate buffered saline containing 0.1% gelatin (PBS-G) to obtain the

desired inoculum which was confirmed by plating serial dilutions on chocolate agar: bacteria were inoculated intraperitoneally in a volume of 0.1 ml. Forty-eight hours after intraperitoneal inoculation the pups were anesthetized with Nembutal (0.4mg/10 gm body weight) and two 0.1ml aliquots of blood were collected from the external jugular vein; one aliquot was diluted in PBS-G for subsequent dilution and quantitative plating to determine the density of bacteremia, while the other was directly plated on Chocolate agar containing 5 units bacitracin/ml. All plates were incubated at 37°C in 5% CO₂ for 24 hours before the colonies were counted.

2.5 Determination of ON/OFF state of LPS biosynthetic genes

Genomic DNA for sequencing the 5' end of phase variable genes was prepared from individual colonies of strains R2866 and R3392 after overnight growth on chocolate agar at 37°C in 5% CO₂ using the DNeasy kit (Qiagen, Valencia, CA). DNA from individual colonies was obtained by using a toothpick to sample a colony and suspend bacteria in 100 µl of a 10% suspension (wt/vol) of Chelex resin (Bio-Rad, Hercules, CA). After vortexing the suspension for 15 s, the samples were heated to 100°C for 10 min in a thermo cycler, placed on ice for 2 min, and centrifuged for 2 min at 8,000 × g. Fifty microliters of supernatant was saved and used as a PCR template. Primers for the phase variable genes were designed using the R2866 genome sequence, available through the microbial genome database of NCBI and described in [10, 11]. In order to evaluate the heterogeneity of the phase variable genes all amplicons were analyzed with GeneScan, sequencing with forward primer labeled with 6-carboxyfluorescein (6-FAM) to allow detection of products within gels by fluorescence. The number of tetrameric repeats after the start codon could easily be distinguished and the expression of the corresponding gene was predicted by determination of the number of tetrameric repeats after the start codon using GeneScan or GeneMapper systems. We PCR amplified the 5' region of *IgtC*, *lic1A*, *lic2A*, *lic3A*, *lex2A* and *oafA* and determined the OFF/ON status. All primers were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR conditions were 95°C for 12 min; 10 cycles of 94°C for 15 s, 50°C for 15 s, and 72°C for 15 s; and 20 cycles of 89°C for 15 s, 50°C for 15 s, 72°C for 15 s, and 72°C for 10 min. Analysis of these PCR products using the GeneScan or GeneMapper systems (Applied Biosystems, Inc., Foster City, CA) resulted in accurate size determination of the repeat region with a precision of 1 bp.

2.6 Trans-complementation of R3735

A PCR fragment containing *IgtC* was amplified from R2866 genomic DNA using primers *IgtC* CompF *NheI* 5'-CGGCTAGCACAGACAGACAGACGGACT-3' *IgtC* CompR *NheI* 5'-CGGCTAGCAATAGGCAATAAAGCTATTGATCTAATCCAG-3' with the PCR conditions as described above. The *NheI* sites are underlined with forward primer amplifying three tetrameric repeats and was cloned into the *NheI* site of pDD514 which was electroporated into *E.coli* DD12 and plated on LB agar containing gentamicin at 10 µg/l [21]. Wild type *IgtC* in strain R2866 has 27 GACA repeats after the start codon, with three repeats not permitting slipped strand mispairing [22]. Two × 10⁸ CFU of *E.coli* DD12/pDD514::*IgtC* were mixed with ~ 2 × 10⁸ CFU of *H. influenzae* R3735 (R2866::*IgtC*::*cat*) [10], plated on chocolate agar and incubated at 37°C in 5% CO₂ for four hours. The entire plate was then harvested and spread on chocolate agar containing gentamicin at 5 µg/ml and 10¹⁰ PFU of bacteriophage T4 and incubated overnight at 37°C in 5% CO₂. Several colonies which have *H. influenzae* morphology were picked and plated on chocolate agar containing 4 µg/ml chloramphenicol and 5 µg/ml gentamicin. Plasmid Mini Preps were prepared and analyzed by PCR for the presence of the gentamicin resistance cassette (an *aacC1*) and the 5' end of *IgtC* using primer *aacC1* For 5'-AGTTGGGCATACGGGAAGAAGTGA-3' and *IgtC* Rev 5'-TTCCGCTAAGTGGTTGGAGCCATA-3'. These primers yield a 360 bp

amplicon which covers the 3' end of *aacC1* and 280 bp of the 5' end of *lgtC*. One colony containing the wild-type *lgtC* expressed from pDD514 was retained and designated R4001.

2.7 Statistical analysis

Significant differences among the data in the different assays were determined with the Mann-Whitney rank sum test as the data were not normally distributed.

3. Results

3.1 Serum susceptibility

To gain insight into the mechanism of increased virulence of the animal-passaged strain R3392 we determined the $IC_{50\%}$ for NHS, adult rat serum and five-day-old rat sera (Table 1). Strain R2866 was relatively resistant to all sera, while strain Rd was susceptible to all sera tested: strain R3392 was susceptible to NHS as we had found previously [11], and it was resistant to the cidal action of infant rat serum, but susceptible to NHS and adult rat sera. The strain trans-complemented with *lgtC* (R4001) was as resistant to NHS as the wild-type, strain R2866. This data indicates that in infant rats immune mechanisms other than antibody directed against strain R2866 selects for the phase variant, R3392 with *lgtC* OFF. In weanling rats fixed macrophages contribute to the clearance of *H. influenzae* type b bacteremia [23]. The increase in cidal activity against R3392 in 42 day-old rats but preservation of serum resistance against R2866 suggests that there is an age-dependent acquisition of antibodies reacting with other bacterial epitopes. Strain E1a is an encapsulated type b, is virulent in infant and adult rats, and is resistant to the cidal activity of all sera tested.

To confirm the serum susceptibility as assessed by $IC_{50\%}$, we measured the survival of each of the test strains in 40% NHS (Fig 1A) and infant rat serum (Fig 1B). Strain E1a had the greatest survival in all sera tested, while R2866 was more slowly killed in NHS in comparison to R3392. Infant rat sera had minimal bactericidal activity against all of the test strains, except strain Rd whose density decreased 100-fold during the 60 min incubation. Strain Rd is susceptible to serum-mediated killing independent of the assay used. In infant rat sera strain R3392 density increased by 1 log during the 60 min incubation period. This increase is significantly different than R2866 which decreased by 1 log during the 60 min incubation. The growth of R3392 in comparison to the loss of viability of strain R2866 was significantly different, $p < 0.03$. The growth of R3392 was not significantly different, $p > 0.05$ in comparison to strain E1a. This Data indicates that the kinetic assay which uses a large inoculum ($\sim 5 \times 10^8$ CFU) measures a phenotypic property which is not present in the $IC_{50\%}$ assay, which uses a low inoculum ($\sim 2,000$ CFU).

3.2 *lgtC* expression in vivo

In prior experiments in which only one colony recovered from the blood of infants rats was retained (R3392), after the intraperitoneal inoculation of strain R2866. We repeated the experiment and examined 30 colonies from each of the three rats which were bacteremic after intraperitoneal inoculation of strain R2866. DNA was prepared from the 90 colonies and was analyzed with GeneScan for the expression status of *lgtC*, *lic1A*, *lic2A*, *lic3A*, *lex2A* and *oafA* [11]. Fragment size of the *lgtC* amplicon could not be determined with one colony, while in 88 colonies *lgtC* was OFF; *lgtC* was ON in one colony due to the deletion of one tetrameric repeat. In parallel we processed 30 individual colonies of strain R2866 grown on chocolate agar over night at 37°C in 5% CO₂. One colony yielded two amplicons, one compatible with *lgtC* ON, the other *lgtC* OFF while another colony did not produce an interpretable amplicon. In the remaining 28 colonies the amplicon size indicated that

indicated that *lgtC* was ON. Thus the strain expressed genes which permitted it to reside and multiply in human blood.

3.2 Adherence and Invasion of Human cells

3.2.1 Submerged Monolayers—To determine if the difference in susceptibility to NHS in the IC_{50%} assay was correlated with an accepted virulence property, invasion of human cells, we studied invasion of A549, Chang and human bronchial epithelial cells grown as a monolayer. Differences might be expected as the phase variant of *H. influenzae* strain R2866, strain R3392 differed in lipopolysaccharide surface structure which is manifested in a decrease in the resistance to NHS. One mechanism of Invasion of eukaryotic cells is via the PAF receptor and LPS [24], but the specific epitope is unknown. With cells grown as monolayers strain R3392 was more invasive than R2866 with A549 cells, but the difference was not statistically significant (Table 2). There was increased invasion of Chang cells by the phase variant, R3392, and the difference was statistically significant, $p = 0.027$ (Table 2). There was no difference in adherence and invasion of primary human bronchial epithelial cells in this assay. We also examined immortalized normal human brain microvascular endothelial cells (HBMECs) and did not find a difference in adherence and invasion between the strains tested (data not shown).

3.2.2 Cells at air-liquid interface—Because *H. influenzae* colonizing the airway are on the surface of polar cells, we studied the adherence, invasion and transcytosis with polar HC292 cells grown at liquid-liquid interface over a 24 hour period. Invasion by R3392 was greater than that with strain Rd, but was not statistically different because of the wide variance found in replicate experiments. The number of bacteria which migrated across the epithelium to the basal compartment by R2866 was significantly greater than that occurring with strain Rd KW20 ($p < 0.01$) and greater than its phase variant R3392 ($p < 0.05$). This observation is not unexpected as others have observed the paracytosis of a panel of *H. influenzae* cells with NCI-H292 cells [25].

3.3 Animal infection

Intraperitoneal inoculation of infant rats with *H. influenzae* strains R2866 and the animal-passed derivative (strain R3392) indicated that at equivalent inoculae, 10^6 CFU there were more deaths with the animal passed strain, 15/20, compared to none of 30 receiving strain R2866. At a higher inoculum, 10^7 CFU the prevalence of death was the same, 50%, but those animals surviving infection with R3392 had very low levels of bacteremia (Table 4). It appears that in the infant rat R3392 either causes a fulminate infection or is rapidly cleared from the blood stream.

4.0 Discussion

Haemophilus influenzae and *Neisseria meningitidis* are restricted to the upper airway of humans so that a natural animal paralog does not exist. Of the known virulence factors of *H. influenzae* and *N. meningitidis* the presence of a capsule will permit ready airway colonization and produce a bacteremic infection in laboratory animals after intra-nasal instillation or intraperitoneal inoculation.[26, 27]. The most prevalent *H. influenzae* in the nasopharynx are unencapsulated and therefore nontypeable (NTHi) and can cause infections of the mucosa of the respiratory tract. NTHi do not usually produce a systemic infection in animals after inoculation by any route. The prevention of *H. influenzae* type b sepsis in infant rats by anti-type b capsular immunoglobulin provided a basis for the development of protein-carbohydrate conjugate vaccines which elicited protective anti-capsular immunity. Thus the presence of capsule appears to be a determinant which facilitates animal infection.

The surface of unencapsulated *H. influenzae* is composed of proteins and a lipopolysaccharide, with the latter composed of numerous short glycoforms, and called LPS [28]. Six to eight phase variable genes, depending on the strain, encode enzymes which modify the LPS creating a polymorphic surface side chain. The ON and OFF switching of these genes is stochastic and permits the bacterium to express progeny with a surface structure advantageous for its current environment. Most adult humans have bactericidal antibodies directed against *H. influenzae* LPS which are postulated to arise by repeated subclinical respiratory tract infection [29].

The isolation of strain R2866 from the blood of an anatomically and immunologically 30 month-old normal child who had been immunized with the *H. influenzae* type b conjugate vaccine suggested that this strain had novel properties. Early studies indicated that strain R2866 was resistant to the bactericidal activity of NHS [11], and at a level displayed by encapsulated type b isolates, but the conserved capsule locus could not be detected. In an effort to increase expression of the serum-resistance trait, five day old rats were inoculated with strain R2866 and the majority of the pups were bacteremic 48 hours later. One of those isolates was retained and identified as strain R3392, which had decreased resistance to NHS [10], rather than having increased serum resistance which we were seeking. Since bactericidal antibodies react with the surface of the bacterium we examined the LPS with monoclonal antibodies and mass spectroscopy. We determined that the target of the cidal antibodies was LPS and outer membrane proteins were not involved. Examination of the LPS biosynthetic genes in strains R2866 and R3392 indicated that the phase variable LPS biosynthetic gene *lgtC* was predicted to be OFF in R3392, while in the parent R2866 the same gene was predominantly ON. Monoclonal antibody analysis indicated that R2866 possessed Gal α 1-4 β Gal while R3392 lacked that epitope. This digalactoside is synthesized by Lic2A, adding the proximal galactose to the fourth heptose, while LgtC adds the terminal galactose forming Gal α 1-4 β Gal. Because we only examined one blood isolate after infection with strain R2866 it was not clear that the OFF state of *lgtC* was selected in the infant rat. In this work we showed that selection of *lgtC* OFF occurred in the majority of the pups inoculated with strain R2866. Because the modification involved surface LPS, we examined the adherence and invasive capacity of this panel with human cells as *H. influenzae* invade human epithelial cells through interaction of LPS with the PAF receptor [24]. We examined the adherence and invasion of a variety of cells and cell lines which we and others had used previously. We found that R3392 had increased invasion of Chang conjunctival cells in comparison to strain R2866 (table 2). To determine if this was a property of the bacterium or the cell line we repeated invasion of NCI-H292 cells grown under air-liquid interface conditions in which the cells are polar and form tight junctions. In this system strain R2866 was more adherent, invasive and able to penetrate the cell layer into the basal compartment (Table 3).

Intraperitoneal inoculation of the prototypic type b strain, E1a produced bacteremia or death at a low inoculum, Strain R2866 produced bacteremia in the majority of the animals with few deaths while strain R3392 caused more deaths than bacteremia (table 4). To gain insight in to the mechanism of virulence of R3392 we compared the bactericidal activity of infant rat sera in the semi-quantitative IC₅₀% assay. This assay calculates the concentration of sera which kills one-half of a low (~2,000CFU) inoculum. Strain Rd is very susceptible to all sera tested, while, not unexpectedly, the encapsulated strain E1a was resistant to the cidal action of all sera (table 1). As reported previously, the animal passed strain R3392 had decreased resistance to both human and adult rat sera (table 1). An alternative way of examining the susceptibility of bacteria to the cidal activity of sera is incubation in 40% sera over time using a high inoculum (~ 10⁸ CFU). In NHS strain Rd is rapidly killed, while E1a is slowly killed and R2866 persists at high density throughout the incubation period (Fig 1A) Strain R3392 is also killed by NHS in this assay correlating with the IC₅₀% data. All

clinical strains were minimally affected by infant rat sera, with strain R3392 replicating during the assay (Fig 1B). Although this growth was significantly different than the loss of viability of strain Rd, the physiological relevance is not clear. Strain R3392 is a clinical strain passed once in the infant rat, while strain Rd was passed in laboratories for approximately 30 years. Accepted virulence factors, such as the capsule gene cluster was lost in *H. influenzae* strain Rd. The observed differences may be due to different nutritional requires rather than being based on immunological differences.

Future experiments which seek to indentify novel virulence determinants of *H. influenzae* should use human cells or tissues to obtain results relevant to the clinical situation.

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Figure 1A

Figure 1B

Figure 1.

Figure 1A. Survival of *H. influenzae* strains in 40% normal human serum after inoculation of 9×10^6 to 7×10^7 CFU in 1.0 ml. Aliquots were removed at the times indicated and plated on chocolate agar to determine the number of surviving bacteria. Strains E1a, ◆; R2866, ■; R3392, ▲; and Rd Kw20 ●

Figure 1B. Survival of *H. influenzae* strains in 40% infant rat serum after inoculation of 9×10^6 to 7×10^7 CFU in 1.0 ml. Aliquots were removed at the times indicated and plated on chocolate agar to determine the number of surviving bacteria. Strains E1a, ◆; R2866, ■; R3392, ▲; and Rd Kw20 ●

Table 1Bactericidal activity of sera against *H. influenzae* expressed as the IC₅₀%

Strain	NHS ^a	IC ₅₀ % mean (range)	
		Aa adult rat sera ^b	Infant rat sera ^c
Rd	0.71 (0.37 – 3.01)	0.92 (1.4 – 0.66)	1.6 (0.88 – 3.0)
Ela	22.4 (18 – 33)	20.6 (17.1 – 24)	18.0 (22 – 16.4)
R2866	19.6 (10.4 – 25.0) *	11.5 (9.4 – 13.0) †	10.4 (8.6 – 12.4)
R3392	4.76 (2.14 – 9.38)	1.59 (1.3 – 1.91)	12.6 (10.2 – 16.4)
R4001	15.5 (11.2 – 18.3) *	12.4 (9.8 – 14.2) †	Not performed

^a = normal adult human sera^b = sera from 42-day-old male rats^c = sera from 5-day-old rats

* Strains R2866 and R4001 are significantly greater than R3392 at p <0.018 with NHS.

† The IC₅₀% with adult rat sera for strains R2866 and R4001 is significantly greater than R3392 at p <0.05

Table 2
Adherence and invasion of human cells grown as a monolayer by *H. influenzae* strains^a

Strain	A549 cell-associated (CFU)	A549 percent invaded	Chang cell-associated (CFU)	Chang percent invaded	HBE cell-associated	HBE percent invaded
R2866	3.95 × 10 ⁶	0.042	5 × 10 ⁷	0.05	4.20 × 10 ⁶	5.34
R3392	9.25 × 10 ⁵	0.148 ^b	3.08 × 10 ⁷	0.37 ^c	8.40 × 10 ⁵	6.32

^a = Cell-associated CFU were calculated as the geometric mean of three replicates. Invaded CFU were those present after gentamicin treatment and lysis of the monolayer.

^b = The increased invasion by R3392 is not significantly different than strain R2866; p = 0.183.

^c = Increased invasion by R3392 is significantly different from strain R2866 at p = 0.027.

Table 3*H. influenzae* interaction with H292 cells at air-liquid interface

Strains	Percent of inoculum		
	Rd	R2866	R3392
Adherent ^a			
1 hr	0	0.06	0.06
3 hr	0.13	0.09	0.06
24 hr	0.74	7.24	3.33
Invaded ^b			
1 hr	0	2.21	0.48
3 hr	0.62	11.55	7.97
24 hr	0.16	8.54	2.67
Transcytosis			
1 hr	0.001	0.002	0.001
3 hr	0.0036	0.253	0.0189
24 hr	2.74	62.264 [*]	47.428 [†]

^alimit of detection was 25 CFU, 0.005 – 0.025% of the inoculum^blimit of detection was 25 CFU, 0.005 – 0.025% of the inoculum.^{*}transcytosis is significantly greater than that observed with strain Rd (P<0.001)[†]Transcytosis is significantly greater than that observed with strain Rd (P<0.05)

Table 4Virulence of *H. influenzae* in infant rats after intraperitoneal inoculation

<u>Strain</u>	<u>Inoculum (cfu)</u>	<u>Deaths</u>	<u>Bacteremia incidence (48 hrs)</u>	<u>Bacteremia (cfu/ml blood)</u>
Ela	4×10^3	4/10	6/6	9.40×10^4
				1.02×10^5
				1.56×10^5
	2×10^3	8/10	2/10	$> 10^7$
				2.8×10^5
				6.00×10^5
Rd	6×10^6	0/10	0/10	
	1.7×10^7	1/10	0/9	Not performed
R2866	5×10^6	0/10	5/10	3.5×10^5
				6.0×10^2
				2.4×10^4
	1.1×10^7	1/10	2/9	1.2×10^2
				2.5×10^5
				1.26×10^5
R3392	6.5×10^6	8/10	2/2	2.10×10^4
				1.93×10^3
	2.3×10^7	5/10	0/5	70, 20
				Not performed
				Not performed
	2.3×10^6	7/10	0/3	Not performed
	2.3×10^5	3/10	2/7	2.4×10^3
				$> 1 \times 10^7$