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Interaction of pneumococcal phase variation and middle ear pressure/gas composition: An *in vitro* model of simulated otitis media (Ms. Ref. No.: YMPAT-D-08-00046)

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Abstract

Streptococcus pneumoniae, a leading cause of otitis media (OM), undergoes spontaneous intra-strain variations in colony morphology. Transparent (T) variant is more efficient in colonizing the nasopharynx while the opaque (O) variant exhibits greater virulence during systemic infections. We hypothesized that changes in middle ear (ME) gas pressure/composition during *Eustachian* tube (ET) dysfunction and the treatment of that dysfunction, e.g., tympanostomy tube (TT) insertion, play a role in selecting the *S. pneumoniae* variant that can efficiently colonize/infect the ME mucosa. Human ME epithelial cells were preconditioned for 24 hrs under one of three conditions that simulated 1) normal ME, 2) ME with ET obstruction (ETO) and 3) ME with TT; subsequently exposed to a dose ($\sim 10^7$ CFU/ml) of either T or O variant of *S. pneumoniae*, and then incubated for 1 hr and 3 hrs. Under the simulated ETO and TT conditions, T variant exhibited a higher growth rate and greater epithelial adherence and killing than did O variants. Attachment of T variant to epithelial cells was documented by scanning electron microscopy. These results suggest that the T-variant is more highly adapted to various ME environments than the O-variants.

Keywords

Otitis media; *Streptococcus pneumoniae*; Phase variation; Simulated middle ear environment; Human middle ear epithelial cell line

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1. Introduction

With the exception of colds, otitis media (OM) is the most common disease diagnosed in infants and children, accounting for as many as 30 million office visits and annual expenditures of more than \$4 billion [1]. *Streptococcus pneumonia* (SP), a major human pathogen that is responsible for over 1 million infant deaths each year due to meningitis, pneumonia, and bacteremia, is also the leading cause of acute OM. Despite the efficacy of antimicrobial therapy for eradicating SP infection and reducing pain during acute OM, those treatments do not promote resolution of the middle ear mucosa (MEM) inflammation [2,3]. Indeed, clinical and animal studies show that killed bacteria and toxins released from both live and dead bacteria including SP can initiate and sustain MEM inflammation [4–6]. With the emergence and dissemination of antibiotic resistant SP strains, coupled to the changing patterns of SP virulence and the inadequacy of available SP vaccines, medical management has become increasingly complex and costly [7,8].

Genetic variability, adaptability, and modulated expression of virulence factors are necessary characteristics of pathogens possessing the ability to survive and prosper in different host microenvironments. SP is a genetically heterogonous species [9,10] that has the ability to quickly modify gene expression in response to changing environmental conditions. One example of the latter is the intra-strain phase variation in SP colony morphology visualized as differences in opacity [11–13]. Spontaneous, reversible phase variations among at least three discernible colony phenotypes; transparent (T), intermediate (I) and opaque (O), occur at frequencies of from 10^{-3} to 10^{-6} [13]. Most clinical SP isolates are mixed populations of T and O colony phenotypes [12], but the T variant was shown to be more efficient in colonizing the nasopharynx while the O variant exhibits greater virulence during systemic infections. Thus, variant switching may represent a viable strategy for SP adaptation to the different local environments encountered during the course of disease pathogenesis. For acute OM, these environments include the nasopharynx where colonization is first established and the MEM under normal, inflamed and treated conditions.

The middle ear (ME) is a relatively non-collapsible, sterile, air-filled cavity that communicates with the nasopharynx during the periodic and transient openings of the ET. The healthy ME is maintained at near ambient pressure with a represented gas composition of approximately 6% H₂O, 8% O₂, 6% CO₂ and 80% N₂. The initial stage in the pathogenesis of pneumococcal acute OM is the transfer of SP to the ME via the ET with subsequent infection of the MEM. As the acute OM progresses, ME pressure becomes subambient as the ET becomes obstructed (ETO) but the gas composition remains unchanged. A common treatment for recurrent acute OM is to bypass the ETO by inserting a tympanostomy tube (TT) through the eardrum which exposes the ME to the ambient environment, re-establishes ambient ME pressure but creating an abnormal gas composition (21% O₂, 79% N₂). Thus, in the pathogenesis of acute OM, SP must acclimate to the pressure/gas environments of the nasopharynx, the normal ME, the infected ME and the ME with functioning TT. We hypothesize that pneumococcal phase variations that adapt to these environments may underlie SP infection/colonization. This study was designed to test that hypothesis using *in vitro* models that simulate the normal, abnormal and pathological ME pressure/gas conditions.

2. Results

2.1 Pneumococcal phase variants

The *S. pneumoniae* 6A T and O colony morphologies under light microscopy after overnight incubation on a TSA+C plate are shown in Figure 1. T colonies appear smaller in size, concave (umbilicated) and transparent in the center. The opaque colony is larger, more uniformly whitish throughout and with a domed center as previously described [14]. The edge of the

colony is slightly irregular in the T variant, but smoother in the opaque variant. The transparent center of the T-phenotypic colony is due to autolysis during the early stage of bacterial growth [13]. This is a unique characteristic of T variants. In the experiments described below, the SP variants (T or O phenotype) used for challenge did not exhibit significant phase switching over time, but intermediate phenotypic variants were observed (data not shown).

2.2. Effect of culture medium and simulated ME conditions on SP variant growth

Figure 2 shows the 3 hrs growth curves for the T and O variants (variant) when cultured under the three simulated ME conditions (condition) in the HMEEC culture medium and in that medium with HMEECs (medium). Balanced Block ANOVA comparing the growth rate (0 to 3 hrs) divided by the initial cell count among the three factors (variant, condition, medium) showed that the T variant was favored over the O variant under all conditions and media ($F = 10.5$, $p < 0.01$). The ME condition and the growth medium did not exhibit a significant effect on SP growth and there were no significant interactions among the variables. *Post hoc* testing showed that the T variants exhibited a significantly faster growth than the O variant ($t = 3.3$, $p < 0.01$).

2.3. Enhanced adherence of T variants to mucosal epithelium

SEM documented few bacteria (T-variant) attached to the mucosal surface at 1 hr after exposure but a higher attachment rate for that variant at 3 hrs after exposure (data not shown). As shown in Figure 3, an intimate contact between the T-variant and HMEECs was often observed at 3 hrs. In the limited number of SEM preparations studied, no instance of O-variant attachment to HMEECs was observed.

Figure 4 shows the results for that adherence assay at 1 and 3 hrs after exposure to the T and O variants under the 3 simulated ME conditions. Balanced block ANOVA operating on the number of adhering cells divided by the challenge number (fractional adherence) documented a significant effect of variant ($F = 23.5$, $p < 0.01$), simulated ME condition ($F = 4.2$, $p = 0.04$) and the variant-condition interaction ($t = 4.4$, $p = 0.04$). *Post hoc* analysis showed that adherence was greater for the T variant ($t = 4.9$, $p < 0.01$). Compared to the simulated normal ME condition, the simulated ETO condition ($t = 2.7$, $p = 0.02$), but not the simulated TT condition ($t = 0.51$) had higher adhesion rates.

2.4. Cell viability in T and O-variant infected HMEECs

Figure 5 shows the results of HMEEC viability assay at 1 and 3 hrs after co-incubation with T and O variants or PBS under the three simulated ME conditions. Although it appears that exposure to T variants potentiates HMEEC killing under both ETO and TT conditions, balanced block ANOVA operating on the fractional HMEEC survival at 3 hrs for the different simulated ME conditions and co-cultures documented no significant effects or interactions.

2.6. Differential pH changes in culture medium at 3 hrs

A solution of phenol red that exhibits a gradual transition in color from yellow to red over the pH range 6.6 to 8.0 was used as a pH indicator (Data not shown). At 3 hrs after exposure to the O-variant, the culture media for HMEECs was not changed from physiological pH. However, for T-variant treated cell cultures, the color of the media changed to an orange yellow, indicating acidification. The acidification of the media in T-variant infected cultures may be caused by accumulation of waste products produced by an overgrowth of T-variants and/or increased cell death. This unique phenomenon was observed in T-variant/HMEEC co-cultures under all three simulated ME conditions at 3 hrs after bacteria challenge. Interestingly, bacteria-alone culture of T variants was less acidic than T-variant/HMEEC co-cultures, indicating a possible role of the pathogen-host interaction in the acidification of the culture medium.

3. Discussion

The *in vivo* host ME responses to SP opacity variants were first reported by Dr. DeMaria's group [15–17]. In those studies, O variants were more efficient at survival and multiplication in the ME, caused the accumulation of more inflammatory cells and provoked higher concentrations of inflammatory mediators. However, T variants were more potent in inducing inflammation when the data were normalized to account for differences in ME bacterial titers [15]. Our results demonstrate differential adaptability and virulence between the T- and O-variants of SP 6A in the context of the host under simulated ME microenvironments. Specifically, T-variants clearly showed favorable growth in the culture medium, with and without HMEECs, under all three simulated ME conditions. T-variants also showed greater HMEEC adhesion that was differentially moderated by the ME microenvironment. These results suggest that the T variant is better adapted to survival and reproduction in the normal and pathological microenvironments of the ME, and consequently, that the T-variant is a key player in the pathogenesis of pneumococcal OM.

A previous study reported that the *in vitro* growth of SP phenotypic variants was not differentially affected by differences in pH, temperature, and osmolarity [12]. In our study, growth curves were similar in both variants under conventional culture conditions (data not shown) but, T-variants grew better and enhanced virulence (adhesion) in the simulated ME environments. These results suggest that the factors of the ME microenvironment to which pathogens respond are much more complex than can be represented under simple culture conditions.

A unique phenomenon observed only in T-variant infected HMEEC cultures was the progressive acidification of the culture media during 3 hours of growth. Because acidification was much greater for HMEECs in medium when compared to medium alone, the effect appears to be driven by the host-pathogen interaction. If this finding proves to have a role in the pathogenesis of pneumococcal OM, it may have potential clinical applications. For example, modulation of ME pH early in an OM episode could prove to be an effective approach for disease management, limiting bacteria growth and improving survival of HMEECs. Thus, drugs such as proton-pump inhibitors, with such an effect, are currently marketed for treating laryngopharyngeal reflux [18,19].

The model system used in this study is an oversimplified representation of the complex microenvironments potentially presented by the ME as it transitions from a normal to pathological state. However, as configured, the model allowed for control over two factors (total pressure and gas composition) that are known to be changed during the pathogenesis of pneumococcal OM. As such, the model provides a unique tool for dissecting the role of specific factors on the growth and virulence of potential ME pathogens under controlled conditions. The system allows for the assay of multiple outcomes in addition to those presented here and is adaptable to modifications that simulate other aspects of the ME microenvironment that affect pathogen survival and reproduction.

In conclusion, the ME mucosal response to pneumococcal infection is a complex process involving host-pathogen interactions under the extant local pressure/gas environment. A phase variant, T-phenotype, that dominates nasopharyngeal SP colonization has a survival advantage in the human ME under normal and pathological conditions. This is expressed as significantly more rapid growth and adherence to HMEECs and an apparently greater ability to kill HMEECs. Our study suggests that the T-variant is a key player in the pathogenesis of pneumococcal OM. Identification of pneumococcal variants that are preferentially expressed during the interaction with host epithelial cells under altered pathologic ME environments is central to our understanding of how pneumococcus adapts to and infects the ME.

4. Materials and Methods

4.1. Study design

Human middle ear epithelial cells (HMEECs) were preconditioned for 24 hrs to simulated ME environments of: A) normal middle ear (NME): approximately physiological ME gas composition (8% O₂, 6% CO₂, 86% N₂) and ambient pressure; B) ET obstruction (ETO): physiological ME gas composition and subambient pressure (ambient-250 mm H₂O); and C) tympanostomy tube (TT): approximately ambient gas composition (21% O₂, 5% CO₂, and 74% N₂) and pressure. Cells were exposed to equal aliquots of relatively uniform populations of T or O variants of the same strain (*S. pneumoniae* 6A, ~10⁷ CFU/ml) and incubated for 1 and 3 hrs under the three simulated ME environments as described above. Cell viability, bacterial growth, phenotypic variation, and adhesion were evaluated at 1 hr and 3 hrs, respectively. Bacterial morphology was assessed by light microscopy and SEM. Three independent experiments for each simulated ME condition at each time were performed. For each assay, this resulted in 3 replicates for each condition and time point.

4.2. Bacterial culture and growth curve

Two phenotypic variants, opaque (O) and transparent (T) of *S. pneumoniae* 6A, that were passage one of the Weiser strains originally identified by Dr. Jeffrey N. Weiser (University of Pennsylvania) [13] and kindly provided by Dr. Thomas F. DeMaria (Ohio State University) were used. Bacteria from frozen stocks were streaked on tryptic soy agar plates (Becton Dickinson, Cockeysville, MD) pretreated by spread of 100 µl of catalase (5000 U) (Worthington Biochemical Co., Freehold, NJ) (TSA+C plate) and incubated overnight at 37°C and 7% CO₂. A single typical colony of each variant was selected and restreaked on a chocolate agar plate and incubated for 16 hrs at 37°C and 7% CO₂. A broth culture containing colonies from the whole plate was then grown in Todd-Hewitt broth (Becton Dickinson) supplemented with 0.5% yeast extract (Difco laboratory, Detroit, MI) (TH+Y broth) at 37°C and 7% CO₂ without shaking. The number of viable bacteria was determined by serial dilutions and plate counts. The log phase of exponential growth of both variants of the *S. pneumoniae* 6A strain reached an equal level at 3 hrs when the bacteria were collected for use in the experiments.

4.3. Middle ear epithelial cell culture

The HMEEC line was previously established using a retrovirus containing the E6/E7 genes of human papilloma virus type 16 [20] and generously provided by Dr. David Lim (House Ear Institute, Los Angeles, CA). HMEEC cells were seeded into 12-well plates at a density of 3.5×10⁵ cells/well in a humidified 5% CO₂ incubator at 37°C, in a medium composed of Dulbecco's Modified Eagle Media (Invitrogen Corporation, Carlsbad, CA) and Bronchial Epithelial Basal Medium (Clonetics, Walkersville, MD), supplemented with bovine pituitary extract (52 µg/ml), hydrocortisone (0.5 µg/ml), hEGF (0.5 ng/ml), epinephrine (0.5 µg/ml), transferrin (10 µg/ml), insulin (5 µg/ml), triiodothyronine (6.5 ng/ml), retinoic acid (0.1 ng/ml), gentamicin (50 µg/ml), and amphotericin-B (50 ng/ml) following a previously published protocol [20].

4.4 Exposure to simulated ME conditions

HMEEC cultures were placed in a humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) that was then placed in an incubator with 7% CO₂ at 37°C. The chamber gas composition, either ambient (21% O₂, 5% CO₂, and 74% N₂) or ME (8% O₂, 6% CO₂, and 86% N₂) came from Certified Standard Spec cylinders (Airgas Great Lakes Inc., Royal Oak, MI). The chamber gas composition was monitored by an O₂ analyzer (Analytical Industries, Inc., Pomona, CA). The pressure of the incubator chamber was monitored and

controlled by a custom-written software program (GasContr, VEE Pro 7.0 from Agilent Technologies, Santa Clara, CA). Twelve-well plates with monolayers of 80–90% confluence were preconditioned to the three simulated ME conditions, as described above, for 24 hrs.

4.5 S. Pneumoniae challenge

After 24 hrs exposure to the selected gas/pressure, monolayers were washed 3 times with Dulbecco's Phosphate-Buffered Saline (1X) (Invitrogen) and replaced with culture medium without antibiotics. Four/12 wells from each plate were treated with either 25 μ l PBS or 25 μ l PBS containing 10^7 CFU/ml of T or O variants. The plates were returned to the incubator chambers at the selected gas/pressures, along with TSA+C plates containing either T or O variant alone (for evaluation of phenotypic changes under the simulated ME environments without the interaction with HMEECs). All plates were placed in the incubator with 7% CO₂ at 37°C for 1 hr and 3 hrs. At the end of each time point, monolayers of one set 12-well plate were washed 3 times and detached from the surface by adding 100 μ l of 0.25% trypsin/0.02% EDTA. Cells were collected by centrifugation and stored at –80°C for future assays. Supernatant was collected and the number of viable bacteria in the supernatant was determined by serial dilutions and plate counts. Bacteria/cell debris from the supernatant was collected and saved at –80°C.

4.6 Adhesion assays

Assays followed published protocols [21] with modifications. The monolayers of one set 12-well plate with the culture media without antibiotics were washed and detached as described above. Cells were lysed by 1 ml of 0.025% ice-cold Triton X-100 and serial dilutions were placed on TSA+C plates to determine the total number and variants of both adherent and intracellular bacteria.

4.7. Epithelial cell viability

The monolayers were washed 3 times with PBS to remove unbound bacteria and detached from the surface by adding 100 μ l of 0.25% trypsin/0.02% EDTA. The cell viability was determined by trypan blue dye exclusion and a hemacytometer.

4.8. Identification of phase variations

Identification of phase variations was performed as described previously [22,13]. Briefly, colony morphology on the TSA+C plates was evaluated under a stereo-dissecting microscope equipped with double oblique, transmitted and adjustable-angle lights.

4.9. Scanning electron microscopy (SEM)

At the end of the experiment, cells were rinsed with cacodylate buffer (0.1 M) and fixed overnight with 2.5% glutaraldehyde. SEM was performed on 2.5% glutaraldehyde/PBS-fixed cells to evaluate bacteria-epithelial cell interaction using a JEOL Field Emission gun 6330F SEM (JEOL, Peabody, MA) as described previously [23].

4.10. Statistical analysis

Balanced Block ANOVA was used to compare the results for summary variables among the different ME conditions, bacterial variants and growth media. If significance effects were identified, *post hoc* testing was done with appropriate corrections for multiple comparisons.

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Figure 1. Transparent (T) and opaque (O) SP colony phenotypes at 3 hrs after infection under the simulated ETO environment and overnight incubation on a TSA+C plate (original images were captured at 10X magnification).

Figure 2.

Three hour growth curves for T and O variants cultured in HMEEC medium with and without HMEECs for the three simulated ME conditions. Each point is the average value for 3 experiments.

Figure 3.
SEM of HMEEC cells 3 hrs after exposure to T-variants under the simulated ETO condition (8% O₂, 6% CO₂, 86% N₂, -250 mm H₂O).

Figure 4. Results for adherence assay of T and O variants to HMEECs (Log CFU/well) at 0 (no adherence), 1 and 3 hrs after bacterial exposure under the three simulated ME conditions (mean $\pm$ SD).

Figure 5. HMEEC cell viability (Log Cell/well) at 1 hr and 3 hrs after exposure to PBS, T or O variants under the three simulated ME conditions (mean $\pm$ SD). Cell counts at 0 hr were depicted as references.