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Interaction of pneumococcal phase variation and middle ear pressure/gas composition: An *in vitro* model of simulated otitis media☆

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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 h 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 (~10^7 CFU/ml) of either T or O variant of *S. pneumoniae*, and then incubated for 1 h and 3 h. 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.

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 pneumoniae* (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 10^{-3}–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.

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The middle ear (ME) is a relatively non-collapsible, sterile, air-filed 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% H_2O, 8% O_2, 6% CO_2 and 80% N_2. 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 sub-ambient 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% O_2, 79% N_2). 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 Fig. 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 variant. 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

Fig. 2 shows the 3 h growth curves for the T and O variants (variant) when cultured under the three simulated ME conditions (condition) in the culture medium (medium) with or without HMEEC. Balanced block ANOVA comparing the growth rate (0–3 h) 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 variants ($t = 3.3$, $p < 0.01$).

2.3. Enhanced adherence of T variants to mucosal epithelium

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

Fig. 4 shows the results for that adherence assay at 1 and 3 h after exposure to the T and O variants under the three 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 HMEEC

Fig. 5 shows the results of HMEEC viability assay at 1 and 3 h 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 h for the different simulated ME conditions and co-cultures documented no significant effects or interactions.

2.5. Differential pH changes in culture medium at 3 h

A solution of phenol red that exhibits a gradual transition in color from yellow to red over the pH range 6.6–8.0 was used as a pH indicator (data not shown). At 3 h after exposure to the O variant, the culture media for HMEEC 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

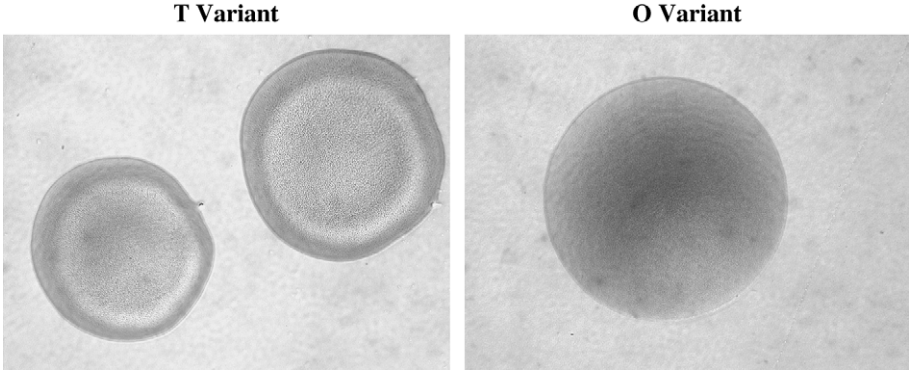

Fig. 1. Transparent (T) and opaque (O) SP colony phenotypes at 3 h after infection under the simulated ETO environment and overnight incubation on a TSA + C plate (original images were captured at 10× magnification).