Coinfection with *Haemophilus influenzae* Promotes Pneumococcal Biofilm Formation during Experimental Otitis Media and Impedes the Progression of Pneumococcal Disease

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**Background.** Otitis media is an extremely common pediatric infection and is mostly caused by bacteria that are carried within the nasopharyngeal microbiota. It is clear that most otitis media cases involve simultaneous infection with multiple agents.

**Methods.** Chinchillas were infected with nontypeable *Haemophilus influenzae*, *Streptococcus pneumoniae*, or a combination of both organisms, and the course of disease was compared. In vitro experiments were also performed to address how coinfection impacts biofilm formation.

**Results.** The incidence of systemic disease was reduced in coinfected animals, compared with those infected with pneumococcus alone. Pneumococci were present within surface-attached biofilms in coinfected animals, and a greater proportion of translucent colony type was observed in the coinfected animals. Because this colony type has been associated with pneumococcal biofilms, the impact of coinfection on pneumococcal biofilm formation was investigated. The results clearly show enhanced biofilm formation in vitro by pneumococci in the presence of *H. influenzae*.

**Conclusions.** Based on these data, we conclude that coinfection with *H. influenzae* facilitates pneumococcal biofilm formation and persistence on the middle ear mucosal surface. This enhanced biofilm persistence correlates with delayed emergence of opaque colony variants within the bacterial population and a resulting decrease in systemic infection.

Otitis media (OM) is an extremely common pediatric infection, affecting the majority of all children [1]. Otitis media arises as a consequence of impaired drainage of the middle ear chamber, which facilitates colonization by bacterial opportunists residing in the nasopharynx, including nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* (pneumococcus) [2]. Otitis media often presents as a chronic or recurrent infection in which bacteria persist within biofilm communities [3]. Biofilms have been observed on middle ear mucosal tissues from patients undergoing tympanostomy [4], and it is now clear that biofilms promote bacterial persistence during chronic/recurrent otitis media infections [5].

Recent epidemiologic studies have shown that the majority of cases of otitis media and other opportunistic airway infections involve simultaneous infection with multiple pathogens [6]. Prior work using murine models indicates that *H. influenzae* components can prime enhanced innate responses that can help contain pneumococcal infection [7–9]. In this study, we used the chinchilla animal infection model for otitis media to test the impact of coinfection with *H. influenzae* and pneumococcus on bacterial clearance/persistence and otitis media disease. The results clearly show that co-
infection with H. influenzae promotes pneumococcal biofilm formation and persistence in localized infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. S. pneumoniae TIGR4 is a well-studied clinical isolate for which a complete genomic sequence is available [10], and we have recently demonstrated that it forms biofilms during experimental otitis media infections [11]. Pneumococci were grown on Todd-Hewitt yeast extract agar (Difco) with 4 μg/mL of gentamicin (Sigma) added, brain heart infusion agar (Difco) with 5% sheep’s blood and gentamicin, or trypticase soy agar (BD) with 315 U/mL of catalase (Worthington) added, as indicated. Nontypeable H. influenzae 86–028NP is an otitis media isolate that has been fully sequenced [12] and is known to cause otitis media featuring biofilms in the chinchilla infection model [5, 13–17]. Bacteria were grown on brain heart infusion agar supplemented with hemin (ICN Biochemicals) and nicotinamide adenine dinucleotide (Sigma) and 3 μg/mL of vancomycin (Sigma).

Chinchilla infections. Healthy adult chinchillas (Chinchilla lanigera) were purchased from Rauscher’s chinchilla ranch and were allowed to acclimate to the vivarium for 1 week prior to infection. All animals were examined by otoscopy prior to infection, and none had any clinical signs of middle ear infection or other overt disease. The chinchilla infection protocols were performed essentially as described elsewhere [5, 11, 13, 14]. S. pneumoniae TIGR4 and/or nontypeable H. influenzae 86–028NP were diluted using sterile phosphate-buffered saline (PBS), and the bacterial density was confirmed by plate count. Chinchillas (3–5 animals/group/time point) were anesthetized with isofluorane and were inoculated via transbullar injection with 0.1 mL of bacterial suspension containing either S. pneumoniae, H. influenzae, or both bacterial species, as indicated. Infectious doses ranged from 1 × 10^2 to 1 × 10^4 colony-forming units (CFU), as indicated in the individual experiments. Groups of animals were euthanized at 3, 7, 14, or 21 days postinfection. Animals exhibiting overt symptoms of systemic disease were euthanized. Blood was collected at euthanasia and was plated to determine the presence of bacteremia. After euthanasia, the superior bullae were opened to expose the middle ear cavity as described elsewhere [14], and the presence of visible biofilm was assessed. If present, middle ear effusion fluids were collected. The middle ear cavity was then lavaged with 1 mL of sterile PBS. Effusion and lavage fluids were serially diluted and assessed by plate count. For animals that received both bacteria, fluid was plated on 2 separate plates, 1 selective for H. influenzae (supplemented brain heart infusion plus vancomycin) and 1 selective for S. pneumoniae (Todd-Hewitt yeast extract plus gentamicin or TSB plus catalase). Middle ear bullae were aseptically removed and homogenized using a Power Gen 1000 homogenizer (Fisher Scientific); the bullar homogenates were plated to assess tissue-associated bacterial load. Representative bullae were fixed in 4% paraformaldehyde for microscopy studies. All of the chinchilla infection protocols were approved by the Wake Forest University Health Sciences Institutional Animal Care and Use Committee.

Microscopy. Biofilms were excised from the middle ear chamber, were rinsed with PBS, were then placed in Cryomolds (Sakura Finetek) with OCT compound (Sakura Finetek), and were frozen at −70°C. Serial 5-μm sections were cut with Accu-Edge Low Profile Blade (Feather Safety Razor) at −20°C, were placed on adhesive slides, and were stored at −70°C. For immunofluorescent staining, slides were brought to room tem-

Table 1. Quantification of Systemic Disease

This table is available in its entirety in the online version of the Journal of Infectious Diseases

Figure 1. Bacterial competition during experimental otitis media coinfections. Symbols represent the total recovered colony-forming units (CFU) from the middle ears of infected animals on days 3 and 7 postinfection. All animals received inocula of ∼10^6 CFU of Haemophilus influenzae (Hi) and/or ∼150 CFU of Streptococcus pneumoniae (Sp) as indicated. Each data point represents 1 ear. The dashed line indicates the limit of detection; the short solid and dashed lines represent the mean CFU for H. influenzae and S. pneumoniae for each group, respectively.
perature, were washed with PBS, were blocked with 1% bovine serum albumin (Sigma), and were then stained with monoclonal antibody specific for pneumococcal type 4 capsule (kindly provided by Dr Moon Nahm, UAB Bacterial Pathogen Reference Laboratory) and rabbit anti–*H. influenzae* antiserum [13] as primary antibodies. Donkey anti–rabbit immunoglobulin G AlexaFluor 488 (Invitrogen) or goat anti–rabbit immunoglobulin G Texas Red (Invitrogen) were used as secondary antibodies. Stained slides were mounted with Prolong Gold antifade reagent (Invitrogen). Samples were visualized using a Zeiss LSM510 confocal laser-scanning microscope.

**Static biofilms.** Assessment of biofilm formation in static cultures was performed as described elsewhere [11, 18]. *H. influenzae* 86–028NP (~1 × 10^7 CFU) was plated in each well of a 24-well plate in a total volume of 1.5 mL of supplemented brain heart infusion or Morse’s minimal media [19]. After 24 h, supernatants were removed and replaced with 1.5 mL of supplemented Morse’s minimal media plus 10% horse serum. *S. pneumoniae* TIGR4 (~1 × 10^7 CFU) was added to the coinfection and *S. pneumoniae* wells. After 48 h, the supernatant was removed and the surface-attached bacteria (biofilm) were collected by scraping and were resuspended in 0.2 mL of sterile PBS; this suspension was then diluted and plated for bacterial counts.

**RESULTS**

**Competitive infections.** The impact of *H. influenzae*-pneumococcal coinfection on bacterial persistence and otitis media disease was assessed using infectious doses previously established for monospecies infections with each organism [11, 13]. Groups of chinchillas were inoculated with either ~1 × 10^3 CFU of *H. influenzae*, ~150 CFU of *S. pneumoniae*, or the same infectious doses of both species. On day 3, equivalent bacterial counts for both bacterial species were obtained from both middle ear fluids (Figure 1A) and bullar homogenates (Figure 1B).

However, by day 7 postinfection, no pneumococci were recovered from the coinfect ed animals (Figure 1). Notably, although a significant percentage of animals infected with pneumococci developed systemic disease, no systemic disease was apparent in the coinfected animals (Table 1). To determine whether polymicrobial biofilms were formed and whether there was a difference in biofilm formation in animals infected with *H. influenzae*, pneumococcus, or both, the numbers of ears containing biofilm were enumerated, and biofilms were removed and analyzed by confocal laser scanning microscopy. There was no difference in the number of ears with biofilms in coinfection versus single infection animals, although animals infected with *S. pneumoniae* alone tended to form small biofilms, whereas coinfected animals formed large biofilms that filled the majority of the middle ear space (data not shown). Based on these data, we conclude that in the conditions of this experiment, *H. influenzae* and pneumococcus can establish a competitive relationship during otitis media infection. Moreover, coinfection appeared to moderate the progression of pneumococcal disease to fatal systemic infection (Table 1).

**Stable coinfections.** Although the above results clearly showed that *H. influenzae* could outcompete pneumococci in some infection conditions, it should be noted that the infec-
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Figure 4. Imaging of polymicrobial biofilms. A–C, Gross images of sectioned bullae removed from mock (A) and coinfected animals at 14 (B) and 21 (C) days postinfection. D–F, Confocal laser scanning microscopy images of biofilms removed from mixed infection animals at 7, 14, and 21 days postinfection. Green is staining for Haemophilus influenzae, and red is staining of the capsule of Streptococcus pneumoniae. Yellow indicates colocalization of the 2 bacterial species.

tious doses used may have afforded a competitive advantage to H. influenzae simply because of greater numbers of these bacteria. Therefore, groups of animals were infected with either 1:1 or 10:1 ratios of H. influenzae to pneumococcus. No animals were given S. pneumoniae alone, because at these doses, the majority of animals would be expected to rapidly develop systemic disease (data not shown). Notably, at 3 and 7 days postinfection, H. influenzae and S. pneumoniae were present in equivalent numbers in the middle ears of mixed infection animals from both the 1:1 ratio and 10:1 ratio groups (Figure 2). At these inoculating doses, there was a marked difference in the prevalence of visible biofilm in the infection groups; 89% of the ears of mixed infection animals (16 of 18) contained biofilm, whereas biofilms were observed in 6 (50%) of 12 ears from animals infected with either species alone. In addition, biofilms from coinfected animals were much larger and generally filled the entire middle ear chamber (data not shown). Confocal images of biofilms from mixed infection animals were very similar to those seen in the previous experiment, where both bacterial species are present in close proximity to one another.

With the results of these infection studies in hand, we asked how long a stable coinfection was maintained. Groups of chinchillas were infected with H. influenzae alone or with a 10:1 ratio of H. influenzae to S. pneumoniae and were euthanized at 7, 14, and 21 days postinfection. As in the preceding experiment, equivalent numbers of H. influenzae and S. pneumoniae were recovered from the middle ears of single- and mixed-infection animals at 7 days postinfection (Figure 3). On day 14, S. pneumoniae was not recovered from the middle ears of every animal except one, where it was found in both ears. Interestingly, the only animal with S. pneumoniae recovered from its ears had the lowest levels of H. influenzae (light and dark blue circles and squares, respectively; Figure 3). In contrast to day 14, on day 21, the majority of coinfected animals had higher numbers of S. pneumoniae than H. influenzae. The most likely explanation for this is that although S. pneumoniae was undetectable in the effusion, it was able to persist within biofilm communities or at a second site within the upper airway at day 14, and it reestablished an infection when the conditions were more favorable.

There were equivalent numbers of ears with biofilm recovered from single infection and coinfected animals on days 7 and 14; however, on day 21, 4 of 6 ears from animals with
mixed infection contained biofilm, whereas no biofilms were observed in animals infected with a single species (data not shown). Figure 4 shows the amount of biofilm recovered within the middle ear chamber of animals at 14 and 21 days postinfection (Figure 4A–4C). Cryosections of biofilms excised from the middle ear chamber were stained with mouse monoclonal anti-serotype 4 capsular antibody to detect *S. pneumoniae* or a polyclonal rabbit anti-*Haemophilus* antibody and were analyzed by confocal laser scanning microscopy. Confocal laser scanning microscopy images from coinfected animals showed the presence of both bacterial species at 7, 14, and 21 days postinfection (Figures 4D–4F). Three-dimensional images of polymicrobial biofilms recovered from infected chinchillas at 7, 14, and 21 days postinfection were constructed from stacked Z-series images collected by confocal laser scanning microscopy (Figure 5; see also Videos 1 [day 7], 2 [day 14], and 3 [day 21], which are available in the online version of the *Journal*).

**Temporal variation of inoculation has no effect on bacterial persistence.** We next wanted to determine whether a preexisting infection with one of the bacterial species would prevent colonization with the other bacterial species or alter the type of coinfection. To address this question, 2 sets of experiments were performed. In the first experiment, 3 groups of chinchillas were infected. The first received *H. influenzae* on day 0 and PBS on day 7, the second received *H. influenzae* on day 0 and *S. pneumoniae* on day 7, and the last received PBS on day 0 and *S. pneumoniae* on day 7. All animals were euthanized 14 days after the initial infection. One animal (dark and light green symbols; Figure 6A) had no recovery of *S. pneumoniae* and had high levels of *H. influenzae*, similar to results seen in Figure 1. At the other end of the spectrum, 1 animal (light and dark orange symbols; Figure 6A) had no *H. influenzae* in either ear but had high numbers of *S. pneumoniae*. The other 3 coinfected animals had equivalent numbers of both bacterial species in each ear. Although the outcome varied with each animal, the experiment clearly shows that a preexisting *H. influenzae* infection does not prevent *S. pneumoniae* from establishing an infection. However, no systemic disease was observed in animals with preexisting *H. influenzae* infection (Table 1).

All ears from coinfected animals contained biofilm (10 of 10), compared with 4 of 8 ears from *S. pneumoniae*-infected animals and 6 of 8 ears from *H. influenzae*-infected animals. As observed in other experiments, biofilms from coinfected animals consistently appeared larger than those from animals infected with either species alone. In the second set of experiments, we wanted to determine whether a preexisting *S. pneumoniae* infection could prevent *H. influenzae* from establishing an infection. The same experimental groups were used, except the timing of inoculation of *H. influenzae* and *S. pneumoniae* was reversed. Groups of animals were given either *S. pneumoniae* or PBS on day 0. The majority of animals infected with *S. pneumoniae* developed systemic disease and were, thus, euthanized before day 7 (Table 1). The 2 surviving animals that received *S. pneumoniae* and the 4 animals given PBS on day 0 were inoculated with *H. influenzae* on day 7. All animals were euthanized on day 14. The 2 coinfected animals established stable coinfections containing equivalent numbers of *H. influenzae* and *S. pneumoniae* in their middle ears (Figure 6B), showing that a preexisting *S. pneumoniae* infection does not prevent *H. influenzae* colonization. All 4 ears from the coinfected animals contained visible biofilms, and immunofluorescence showed the presence of both bacterial species (data not shown).

**The presence of *H. influenzae* increases pneumococcal biofilm formation in vitro.** One observation made during the in vivo studies was that *S. pneumoniae* formed biofilms with a greater frequency and of a larger size in the presence of *H. influenzae* than when alone. Thus, the impact of *H. influenzae* on pneumococcal biofilm formation was assessed using an in vitro static biofilm assay. The results show significantly greater numbers of *S. pneumoniae* in surface-attached communities containing *H. influenzae* than in those containing pneumococcus alone (Figure 7A). Of note, these results were more dramatic when performed in chemically defined minimal media (Figure 7B).

**Coinfection increases the translucent colony type within pneumococcal populations in vivo.** Pneumococci undergo phase-variation between 2 distinct colony phenotypes; opaque colonies produce greater amounts of capsular polysaccharide and are associated with systemic infections, whereas translucent colonies have less capsular polysaccharide and more choline-containing cell wall teichoic acid on their surfaces. Notably, the translucent populations predominate during the early stages of
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Figure 6. Temporal variation of coinfections. Counts were obtained from middle ear fluids or bullar homogenates 14 days after initial infection. A, Animals received either Haemophilus influenzae (Hi) or phosphate-buffered saline (PBS) on day 0 and Streptococcus pneumoniae (Sp) or PBS on day 7. B, Animals received either S. pneumoniae or PBS on day 0 and H. influenzae or PBS on day 7. Each point represents 1 ear. Dark and light colored symbols represent the right and left ears of 1 animal with respect to both bacterial species. The dashed line indicates the limit of detection; the short solid and dashed lines represent the mean colony-forming units (CFU) for H. influenzae and S. pneumoniae for each group, respectively.

Figure 7. Haemophilus influenzae (Hi) promotes pneumococcal biofilm formation in vitro. Total recovered Streptococcus pneumoniae colony-forming units (CFU) from 48 h static biofilms in supplemented brain heart infusion (A) or minimal media (B). The black bars represent the S. pneumoniae recovered from coinfection with H. influenzae and the white bars represent the S. pneumoniae recovered from S. pneumoniae alone static biofilms. Bars represent the mean ± the standard error of the mean from 4 independent wells from 1 representative experiment. Statistical significance was assessed using a Mann-Whitney nonparametric t test. **P < .005.

infection and within the tissue-associated populations that presumably include biofilms. Thus, we hypothesized that H. influenzae may influence pneumococcal colony phenotype. If this were the case, we would expect to see a higher percentage of translucent colonies in S. pneumoniae removed from mixed infection animals than in animals that received S. pneumoniae alone. To address this hypothesis, animals were infected with H. influenzae alone, S. pneumoniae alone, or both organisms. The colony type within the pneumococcal inocula was determined to be ~40% translucent. Animals were euthanized 7 days postinfection, and colony counts and opacity from both the effusion (planktonic bacteria) and homogenized bullae (surface-associated bacteria) were assessed. All infection groups established stable middle ear infections with equivalent numbers of both bacterial species in both the effusion and homogenized bullae (Figures 8A and 8B). In the effusion, the percentage of translucent colonies decreases to ~20% for both S. pneumoniae alone and S. pneumoniae plus H. influenzae animals (Figure 8C). However, in the bullae, the percentage of translucent colonies increased significantly to ~50% in mixed infection animals and decreased to ~20% in animals infected with S. pneumoniae alone (Figure 8C; P = .015).

DISCUSSION

Although it is clear that the majority of otitis media infections involve multiple species, most of our current knowledge regarding the bacterial pathogenesis of otitis media has been derived from infections using pure cultures of single organisms. Because H. influenzae and pneumococcus collectively account for the majority of otitis media infections [2], it is of particular importance to understand how these species influence one another. Other work has provided clear evidence for modulation of pneumococcal disease by H. influenzae, most notably by
priming enhanced host innate responses to clear the pneumococcal infection [7–9]. The results of our work clearly establish that preceding or concurrent infection with \textit{H. influenzae} moderates the progression and severity of pneumococcal disease in chinchilla. Additional studies with phagocytes in vitro showed no difference in pneumococcal killing with coinfection (data not shown).

Our data also clearly show that the presence of \textit{H. influenzae} significantly increases the percentage of translucent pneumococcal colonies, compared with the populations recovered from animals that received pneumococcus alone. Translucent pneumococcal variants have a decreased propensity toward systemic infections [20] and instead are more adherent to abiotic and host cell surfaces [21]. Work from a number of groups has established that biofilm communities contain predominantly translucent colony variants [22–26]. Notably, the \textit{H. influenzae} biofilm matrix consists of sialylated lipoooligosaccharides [15, 18, 27]. Recent work has clearly established that pneumococcal neuraminidases promote biofilm formation [28], a result that is recapitulated in the presence of free sialic acid [29]. The observed enhancement of pneumococcal biofilm formation by \textit{H. influenzae} was unaffected in neuraminidase mutant strains or \textit{H. influenzae siaB} mutants lacking sialylated matrix [18], which would seem inconsistent with a specific role for sialic acid in this phenotype. It may be that \textit{H. influenzae} plays a more generic role, such as enhanced retention of pneumococci on a surface with an established biofilm matrix scaffold. Based on our results, we conclude that enhancement of pneumococcal persistence within the biofilm phase on mucosal surfaces can delay or even ablate the progression to systemic disease. It is important to note that in contrast to the systemic infections observed in most animal model systems, the majority of pneumococcal infections in patients are localized mucosal infections. It is clear from many studies that particular host immunodeficiencies (most notably, complement deficiencies) can predispose to systemic infection with pneumococci and other bacteria.
[30, 31]. In light of the results of this study, it may be important to consider the possibility that systemic pneumococcal infection results not only from host susceptibility but also from infection and colonization by a bacterial population in which pneumococci predominate. It is equally important to consider the possibility that elimination of one or more components of the nasopharyngeal microbiota by vaccination or therapy may have unforeseen sequelae, not only in terms of opening host niches for colonization but also, potentially, by changing the course of infection by opportunists within this population.

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