Influenza Virus Induces Bacterial and Nonbacterial Otitis Media

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Otitis media (OM) is one of the most common childhood diseases. OM can arise when a viral infection enables bacteria to disseminate from the nasopharynx to the middle ear. Here, we provide the first infant murine model for disease. Mice coinfected with Streptococcus pneumoniae and influenza virus had high bacterial load in the middle ear, middle ear inflammation, and hearing loss. In contrast, mice colonized with S. pneumoniae alone had significantly less bacteria in the ear, minimal hearing loss, and no inflammation. Of interest, infection with influenza virus alone also caused some middle ear inflammation and hearing loss. Overall, this study provides a clinically relevant and easily accessible animal model to study the pathogenesis and prevention of OM. Moreover, we provide, to our knowledge, the first evidence that influenza virus alone causes middle ear inflammation in infant mice. This inflammation may then play an important role in the development of bacterial OM.
The majority of studies investigating pneumococcal-influenza OM have been performed in chinchillas [11, 16, 17] and, to a lesser extent, in ferrets [13]. Chinchillas are a useful animal model to study OM because of their enlarged cephalid bulla [18]. This facilitates transbulla inoculation and reduces difficulties associated with obtaining samples of the middle ear fluid [18]. However, the availability and costs associated with the use of such large animals can be prohibitive and knockout strains of these animals are not available, making it difficult to elucidate the specific host factors important in OM pathogenesis [18]. Moreover, the majority of experimental reagents have been developed for use in the mouse.

Studies from our group [10] and those of others [12] show that intranasal infection of mice with IAV facilitates pneumococcal OM. However, this supposition is based solely on the presence of a luminescent signal in the ears of mice infected with IAV and a bioluminescent strain of S. pneumoniae, and neither study comprehensively characterized the murine model. The thorough development of this model would represent a relatively inexpensive and easily accessible means for understanding in vivo viral-bacterial interactions.

The aim of this study was therefore to examine the effect of IAV infection on S. pneumoniae in infant mice. Infant mice were selected to reflect the underdeveloped immune system of children, who have the greatest burden of OM [2]. Similar to human infants, murine infants show an inability to respond to T-independent antigens (such as the pneumococcal polysaccharide) and display a variety of different defects in components of the innate immune response [19–23]. Such features may leave children vulnerable to OM by, for example, preventing the development of antibodies necessary for the opsonophagocytosis of the bacteria or reducing the functionality of cell types (such as neutrophils) that may be necessary for the clearance of infection. Thus, using infant mice as a clinically relevant model, we show that coinfection with S. pneumoniae and IAV results in a significant increase in middle ear bacterial titers, middle ear inflammation, and temporary hearing loss. We also provide evidence that intranasal infection of infant mice with IAV alone can result in inflammation in the middle ear cavity. This may suggest that virus-induced damage in the middle ear cavity plays a previously unappreciated role in the pathogenesis of pneumococcal OM.

METHODS

Viral and Bacterial Strains

The bioluminescent S. pneumoniae strain EF3030lus (type 19F) [10] or the S. pneumoniae strain ML-2302 (type 23F; kindly donated by Prof. Susan Hollingshead, University of Alabama at Birmingham) were used in all experiments. For infection experiments, pneumococci were cultured and stored as described elsewhere [10]. Influenza virus strain A/Udom/307/72 (H3N2) was used to model infection with IAV. Virus stocks were prepared in embryonated eggs, and titers of infectious virus were determined using 3 independent plaque assays on Madin-Darby Canine Kidney cells [10].

Infection of Mice

C57BL/6 mice were bred and housed under specific pathogen-free (SPF) conditions at the animal facility of the Department of Microbiology and Immunology at The University of Melbourne, Australia. Germ-free mice were bred and housed in germ-free isolator units at the Bioservices Department of The Walter and Eliza Hall Institute for Medical Research (Kew, Victoria, Australia). Five-day-old C57BL/6 mice were colonized intranasally with $2 \times 10^7$ colony-forming units of S. pneumoniae in 3 µL. Alternatively, mice were mock infected with an equivalent volume of phosphate-buffered saline (PBS). At 14 days of age, infant mice were infected intranasally with $10^{2–5}$ plaque-forming units of egg-grown IAV in 3 µLs or were mock treated with a similar volume and dilution of noninfected allantoic fluid. Six days after IAV or mock infection, mice were killed and the entire bulla from each ear was dissected. All animal experiments were approved by the Animal Ethics Committee of the University of Melbourne and were conducted in accordance with the Prevention of Cruelty to Animals Act (1986) and the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

Enumeration of Bacterial Load

Tissues used to quantify bacterial load were homogenized (Polytron PT2100; Kinematica), and serial dilutions of tissue homogenates were prepared in PBS and cultured on horse blood agar containing 5 µg/mL of gentamicin (Pfizer).

In Vivo Imaging

In vivo imaging was performed using an IVIS-Spectrum camera (Caliper Life Sciences) as described elsewhere [10]. Imaging was performed for 7 minutes for each ear, and images were edited using the Living Image software package, version 3.0 (Caliper Life Sciences).

Auditory Brainstem Response

The hearing status of each mouse was evaluated using a click-evoked auditory brainstem response (ABR) [24]. In brief, each mouse was anesthetized [10] and placed in a sound-attenuated room. A loud speaker was placed 10 cm from the pinna of the test ear, and computer-generated clicks over a range of 0–100 dB peak equivalent sound pressure level were presented randomly. The contralateral ear was plugged with an ear mould compound (Ottoform), and percutaneous recording electrodes were positioned at the vertex of the skull (positive) and on the nape of the neck (negative), with a ground on the snout. ABRs were differentially recorded, and the signals were amplified by 10^5 and...
bandpass filtered (150 Hz–3k Hz). Stimulus intensity was adjusted in 5-dB steps around threshold levels. The signals were digitized, and the mean value from 2 sets of 100 trials were determined. The 2 mean ABRs were subsequently analyzed to determine threshold with use of Igor Prov6.04 software (WaveMetrics). Threshold was defined as the minimum stimulus intensity that produced a response amplitude of at least 0.2 mV in the 2–3-ms period after the click stimulus [25]. The threshold increase observed as a result of infection was calculated by subtracting the mean threshold of mock-infected, age matched mice (n = 20) from each data point. When this resulted in a negative value, the threshold increase was assigned a value of 0.

Middle Ear Inflammation
To collect the middle ears for a histological examination of inflammation, mice were killed by carbon dioxide asphyxiation and intracardiacally perfused with 3 mL of 4% paraformaldehyde (PFA). Middle ear tissues were subsequently dissected and fixed for a minimum of 4 hours in 4% PFA. Middle ears were decalcified in Formalin-EDTA [26], embedded in paraffin, sectioned at 6–8 μm, and stained with hematoxylin and eosin. The percentage of the middle ear cavity containing an inflammatory cell infiltrate was scored by a pathologist (JP) who was blind to the study design.

Immunofluorescence
Heat treatment (10 mmol/L citrate buffer; pH, 6.0) was used for antigen retrieval, and sections were blocked for 10 minutes (Protein block; Dako). Sections were labeled for 1 hour with either S. pneumoniae type 19F antiserum samples (Statens Serum Institut) diluted 1:100 in TrueVision reagent (Sapphire Bioscience) or the immunoglobulin fraction from nonimmunized rabbit serum (Rabbit universal negative control; Dako). Sections were subsequently washed with PBS and incubated with sheep anti-rabbit IgG FITC (Silenus Laboratory) diluted 1:300 in PBS and 0.05% bovine serum albumin. Sections were then washed with PBS and incubated in 0.01% Evans blue (Sigma-Aldrich) for 1 minute at room temperature. A final PBS wash was performed, and sections were mounted with 10% (w/v) Mowiol 4–88 and visualized.

Fluorescent In Situ Hybridization (FISH)
FISH was conducted essentially as described elsewhere [27], with use of 16S rRNA probes labeled with AlexaFluor 546 (Invitrogen). In brief, sections were incubated with 10 mg/mL lysozyme (Sigma-Aldrich) in 0.1M Tris (hydroxymethyl) significant difference (P < .01) between treatment groups using a 2-tailed Mann–Whitney U test. The geometric mean is represented by a horizontal line, and dashed lines indicate the detection limit of the assay.
aminomethane hydrochloride and 0.05 M Na₂ EDTA at 37°C for 2 hours and washed with PBS. FISH was performed on sections in 20% formamide (Amresco) with use of 50 ng/μl Lo f 16S ribosomal probe sequence EUB338 (for the domain bacteria): 5′-GCTGCCTCCCGTAGGAGT-3′ [28]. Autofluorescence was quenched using 2.5 mM CuSO₄ in 50 mmol/L CH₃COONH₄ (Sigma-Aldrich). Nuclei were stained using 1.2 ug/mL Hoechst 33342 (Invitrogen). Sections were mounted using low-fade mounting media and were imaged using a Nikon A1Si confocal laser scanning microscope and high-resolution Plan Apo objectives.

Statistical Analysis
Statistical analyses were performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software).

RESULTS

IAV Facilitates Acute Pneumococcal OM
To investigate the effect of IAV on the development of pneumococcal OM, infant mice were colonized with a bioluminescent 19F strain of S. pneumoniae [10]. At 14 days of age, mice were infected via the intranasal route with IAV or mock. The development of pneumococcal OM was assessed 6 days later by in vivo imaging and enumeration of bacterial load in the middle ear. A strong luminescent signal was observed from the ears of mice coinfected with S. pneumoniae and IAV (Figure 1A). This was not seen in mice infected with S. pneumoniae alone (Figure 1A). Enumeration of bacterial titers in the middle ear showed that ~90-fold more bacteria were recovered from the middle ear of coinfected mice than from mice infected with S. pneumoniae alone (P < .001, by Mann–Whitney U test) (Figure 1B). These findings were not strain dependent, because similar differences in bacterial load were obtained using a nonbioluminescent serotype 23F S. pneumoniae strain (P = .003, by Mann–Whitney U test) (Figure 1C). Similarly, these data were not attributable to a systemic infection in coinfected mice, because no bacteria could be detected in blood samples from coinfected mice 6 days after IAV infection (n = 16; data not shown).

In vivo imaging was subsequently used to track the infection over time in each individual mouse. The luminescent signal from the middle ears of coinfected mice resolved 11–16 days after IAV infection (Figure 2). Consistent with these data, 16 days after infection with IAV, bacterial titers in the middle ears of coinfected mice had reduced to ~10³ colony-forming units (data not shown). No mice became moribund during the infection period, indicating the localized nature of the infection.

Mice Infected With IAV Show Middle Ear Inflammation
To determine whether the increased bacterial load in the middle ears of coinfected mice had pathological consequences, inflammation in the middle ear was assessed. No obvious inflammation was detected in the middle ear cavity of mock-colonized mice (Figure 3A and 3B) or mice colonized with S. pneumoniae alone (Figure 3A and 3B). In contrast, middle ear sections from mice coinfected with S. pneumoniae and IAV showed submucosal edema and a large influx of inflammatory cells (predominately neutrophils) into the lumen of the middle ear cavity (Figure 3A). The inflammation observed in these mice was significantly greater than that seen in mock-infected mice or...
Figure 3. Middle ear inflammation in infected mice. Middle ear inflammation in mock- or *Streptococcus pneumoniae* (SP)—colonized mice 6 days after infection with influenza A virus (IAV) or mock. A, Representative images of middle ear paraffin sections stained with hematoxylin and eosin. Images were taken at 40× magnification. The Eustachian tube (ET), tympanic membrane (TM), middle ear cavity (MEC), and cochlear (Co) are labeled. Insert panels show the inflammatory cell infiltrate of “SP + IAV” and “Mock + IAV” mice at increased magnification (100×). B, Scoring of middle ear inflammation observed by histology 6 days after infection with IAV or mock. Data are pooled from at least 2 independent experiments, and each data point represents
mice colonized with S. pneumoniae alone (P < .001, by Kruskall–Wallis test) (Figure 3B). Unexpectedly, mice infected with IAV alone also showed submucosal edema and an influx of predominately neutrophils into the middle ear cavity (Figure 3A). The mean inflammatory score for these mice was lower than that observed in coinfected mice, although there was not a statistically significant difference (Figure 3B).

**IAV Causes Middle Ear Inflammation in the Absence of Normal Flora**

The development of middle ear inflammation in mice infected with IAV alone may indicate that the viral infection induces inflammation in the middle ear. Alternatively, these data may reflect the ability of normal nasopharyngeal flora, in the presence of an IAV infection, to ascend the Eustachian tube and induce middle ear inflammation. To differentiate between these hypotheses, 14-day-old C57BL/6 germ-free mice (which lack commensal flora) were mock or IAV infected. Low-level inflammation was observed in the middle ear cavity of mock-infected, germ-free mice 6 days after mock infection (Figure 4A). Nevertheless, IAV infection of germ-free mice induced a significant increase in middle ear inflammation (P = .04, by Mann–Whitney U test) (Figure 4A). These data suggest that IAV-induced middle ear inflammation is not dependent on the presence of normal nasopharyngeal flora and may be caused by the virus. To verify these findings, FISH for 16S ribosomal RNA (rRNA) was performed on middle ear sections taken from IAV-infected SPF-housed C57BL/6 mice. No bacterial rRNA was detected in these sections (Figure 4B). In contrast, 16S rRNA was detected in the middle ear cavity of mice coinfected with S. pneumoniae and IAV (Figure 4B). Bacteria in these sections were restricted to the inflammatory cell infiltrate. To confirm the localization of S. pneumoniae in the ears of coinfected mice, immunofluorescence for pneumococcal antigens was performed. As seen with FISH, bacteria localized to the inflammatory cell infiltrate (Figure 4C). In summary, these data demonstrate that the middle ear inflammation observed in IAV-infected mice is not the result of bacterial antigens in the middle ear. Furthermore, in coinfected mice, pneumococcal antigens are found in the lumen of the middle ear cavity.

**Hearing Loss in Infected Mice**

In humans, the middle ear inflammation associated with OM can lead to hearing loss [2, 29]. Thus, the hearing status of infected mice was measured using a click-evoked ABR [24]. Hearing thresholds of infected mice were compared with thresholds of mock-infected mice, in which higher hearing threshold differences were indicative of greater hearing loss. Six days after IAV infection, mice infected with S. pneumoniae alone showed a mild increase in hearing thresholds, compared with mock-infected mice (Figure 5). Conversely, hearing thresholds for mice coinfected with S. pneumoniae and IAV were ~20 dB higher than those in mock-infected mice. This was significantly higher than any hearing loss observed in mice infected with S. pneumoniae alone (P < .001, by Kruskall Wallis test). Mice infected with IAV alone also displayed hearing loss, consistent with our previous observations that IAV induces middle ear inflammation. However, the threshold increase detected in these mice was significantly lower than that observed in coinfected mice (P < .05, by Kruskall Wallis test). The hearing loss observed in coinfected and IAV-infected mice returned to similar levels to those in mock-infected mice within 3 weeks (data not shown).

**DISCUSSION**

OM is the most frequently diagnosed illness in children [2]. OM can arise when a viral infection enables bacteria from the nasopharynx to replicate in the middle ear. Here we have presented the first comprehensive characterization of an infant murine model for influenza-pneumococcal OM. Similar to children, infant mice have an incompletely developed immune system [19, 20]. Thus, infant mice were used to model the underdeveloped immune system of children, who have the greatest burden of OM [2].

Using this model, we have shown that infection with IAV significantly increased pneumococcal bacterial counts in the middle ear. These data closely mimic the association seen in children between the presence of an IAV infection and the detection of S. pneumoniae in the middle ear [8]. The acute and self-limiting nature of this infection also accurately reflects the clinical course of acute OM, which typically resolves in the absence of treatment [30].

In children, the presence of bacteria in the middle ear can lead to inflammation [31]. We reasoned that the increased bacterial load seen in coinfected mice would similarly lead to increased middle ear inflammation. Indeed, co-infected mice had significantly greater middle ear inflammation, compared with mock-infected mice or mice infected with S. pneumoniae alone. There was also a trend toward increased inflammation in co-infected mice, compared with mice infected with IAV alone. Although the

Figure 3 continued. a single ear from an infected mouse (12 mice treated with S. pneumoniae alone, 11 mock-treated mice, 17 mice treated with IAV alone, and 16 mice treated with S. pneumoniae and IAV). Triple asterisks indicate a highly statistically significant difference (P < .001) between treatment groups using a Kruskall Wallis test accompanied by Dunn’s posttest. The arithmetic mean is represented by a horizontal line. “Mock + Mock” and “SP + Mock” images shown in A were given a score of 0. The “SP + IAV” image in A was given a score of 75, and the “Mock + IAV” image was given a score of 20.
difference between these 2 treatment groups was not statistically significant, this may simply reflect the strain of *S. pneumoniae* that was used for these studies. Different *S. pneumoniae* strains show a differential ability to induce middle ear inflammation [32]. Thus, it is possible that, if another *S. pneumoniae* strain were used, the difference observed in middle ear inflammation between treatment groups would be more striking. Of importance, consistent with the observed middle ear inflammation, coinfected mice also showed significantly higher hearing loss, compared with mice infected with *S. pneumoniae* alone or IAV alone.

Results from animal models must be interpreted with caution, because an animal model of disease can never fully represent the complexity of disease that occurs in humans. However, our novel infant mouse model mimics many aspects of human OM and offers a clinically relevant animal model to understand pneumococcal-influenza interactions. Unlike previous studies, the model presented here provides a unique opportunity to evaluate novel preventative and therapeutic measures against OM in an easily accessible animal model using functionally relevant readouts (ie, hearing loss). This is particularly pertinent, because at present, the ability of vaccination (either against *S. pneumoniae* or IAV) to protect against OM remains controversial [33].

In this study, we have also provided the first evidence to our knowledge that intranasal infection of infant mice with IAV alone results in middle ear inflammation and temporary hearing loss. IAV is able to infect human middle ear epithelial cells in vitro [34, 35], and a tympanic infection with IAV in chinchillas or guinea pigs can result in tissue edema and an inflammatory cell infiltrate [36, 37]. However, the ability of influenza virus to induce inflammation in the middle ear cavity after intranasal infection has not been well documented. Previous studies in animal models have either not investigated middle ear inflammation in animals infected with IAV alone or has shown, at most, only a low incidence of inflammation [11, 38]. In contrast, Rarey et al [39] showed that ferrets infected with IAV developed OM and hearing loss. This later finding reflects studies in human adults, whereby experimental intranasal infection with IAV results in the development of OM and the presence of viral RNA in the middle ear fluid [15]. This would suggest that the inflammation seen in IAV-infected mice here might also reflect the clinical situation in human adults. In children with OM, viral antigens are most frequently isolated from the middle ear in conjunction with bacterial antigens [8, 40]. However, children have a high rate of nasopharyngeal carriage of the 3 leading causes of bacterial OM; *S. pneumoniae*, and IAV. Sections were stained with universal rabbit negative control (Control-Ab) or with *S. pneumoniae* serotype 19f rabbit antisera (aSP-Ab), followed by a FITC-conjugated secondary anti-rabbit antibody. The MEC is shown. Pneumococci in the MEC are predominantly localized around the inflammatory cell infiltrate. Images were taken at 60× magnification.

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**Figure 4.** Infection with influenza virus alone causes middle ear inflammation. 

**A.** Scoring of middle ear inflammation in germ-free mice 6 days after infection with influenza A virus (IAV) or mock. Each data point represents either the left or right ear of an infected mouse (6 mock-treated mice and 8 mice treated with IAV alone). The asterisk indicates a statistically significant difference (*P* < .05) between treatment groups using a 2-tailed Mann–Whitney *U* test. The arithmetic mean is represented by a horizontal line. 

**B.** Fluorescent in situ hybridization (FISH) analysis of 16S ribosomal RNA (red) in the middle ear. Sections were obtained from SPF-housed C57BL/6 mice infected with IAV or mock. Images were taken at 40× magnification, and cell nuclei are shown in blue. Arrows indicate the presence of 16S ribosomal RNA. In each image, the middle ear cavity (MEC) is labeled. 

**C.** Representative immunofluorescent images (n = 5) of the middle ear of a mouse infected with *S. pneumoniae* (SP) EF3030lux and IAV. Sections were stained with universal rabbit negative control (Control-Ab) or with *S. pneumoniae* serotype 19f rabbit antisera (aSP-Ab), followed by a FITC-conjugated secondary anti-rabbit antibody. The MEC is shown. Pneumococci in the MEC are predominantly localized around the inflammatory cell infiltrate. Images were taken at 60× magnification.
**Figure 5.** Coinfected and IAV-infected mice display hearing loss. Hearing loss in mock- or Streptococcus pneumoniae (SP)—colonised mice 6 days after infection with influenza A virus (IAV) or mock. Data are pooled from at least 2 independent experiments, and each data point represents the hearing threshold increase of either the left or right ear of an infected mouse (10 mice infected with S. pneumoniae alone, 12 mice infected with S. pneumoniae and IAV, and 17 mice infected with IAV alone). The threshold increase was calculated by subtracting the mean hearing loss of mock-infected, age-matched mice from each data point. Triple asterisks indicate a highly statistically significant difference ($P < .001$) between treatment groups using a Kruskall-Wallis test accompanied by Dunn’s posttest. Single asterisks indicate a statistically significant difference ($P < .05$) between treatment groups. The arithmetic mean is represented by a horizontal line.

**Moraxella catarrhalis** and **Haemophilus influenzae** [2]. Thus, if these individuals contract IAV, they are likely to develop viral-bacterial OM. This may account for the lower rate of virus-alone middle ear inflammation observed in the pediatric population.

At present, the mechanisms by which IAV facilitates middle ear replication of *S. pneumoniae* remain unclear. Previous studies have suggested that the ability of IAV to perturb Eustachian tube function may facilitate bacterial replication in the middle ear [41]. In the present study, minimal Eustachian tube damage was observed (data not shown). However, the ability of IAV to induce middle ear inflammation was striking. We therefore postulate that IAV-induced inflammation in the middle ear facilitates bacterial replication. The role of IAV-induced inflammation in pneumococcal pneumonia has been well documented [42, 43]. It has been suggested that the pro-inflammatory response to IAV increases the expression of pneumococcal receptors on epithelial cells and, thus, facilitates bacterial outgrowth [44]. However, in our study, pneumococci were localized to the inflammatory cell infiltrate, rather than the middle ear epithelium. Instead, a possible scenario is that the neutrophil influx into the middle ear cavity after IAV infection results in damage to the cells of the middle ear. This would increase the amount of free nutrients available and help support increased bacterial growth. Neutrophils may not be able to control this bacterial outgrowth, because IAV is known to suppress neutrophil function [45, 46]. Determining the precise mechanisms underlying disease development in our model remains a key area for further study.

**Notes**

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