Nasal Colonization with *Streptococcus pneumoniae* Includes Subpopulations of Surface and Invasive Pneumococci

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Humans acquire *Streptococcus pneumoniae* invariably by asymptomatic nasal colonization (15). Transmission to others is thought to be primarily from colonized individuals. Even so, pneumococci possess virulence factors, including capsule, PspA, PspC, and pneumolysin, that allow them to resist the opsonic effects of complement deposition and thereby to avoid phagocytosis (1, 10, 22, 26). These virulence factors enable pneumococci to cause invasive disease, which results in more human deaths in developed countries than result from any other bacterial pathogen (12). However, since the reservoir of pneumococci is maintained primarily by human nasopharyngeal colonization, virulence factors that interfere with complement and phagocytosis make little sense at the mucosal surface, where there is little complement (28) and there are few phagocytes. One possible explanation for why pneumococci contain these disease-permitting virulence factors is that long-term nasal colonization involves more than simple surface adhesion and actually requires invasion of mucosal tissue, where the pathogen has to defend itself against complement-dependent opsonization and phagocytosis.

Pneumococci have a phase shift that allows them to exist in transparent and opaque phases (36). Transparent-phase pneumococci have been shown to be released from nasal cavities of mice and infant rats by gentle washing with physiological salt solutions (36). In contrast, the majority of pneumococci recovered from the blood or lung are opaque-phase organisms (6, 19, 34). Moreover, it has been shown that nasal colonization in rodents is established more readily by transparent-phase pneumococci than by opaque phase pneumococci (36). In contrast to colonization, invasive disease in rodents has been most efficiently established by opaque-phase pneumococci (19).

Transparent pneumococci have thick cell walls but sparse capsules, which allows initial attachment (involving subcapsular molecules) to be much more efficient. Opaque pneumococci have thinner walls but denser capsules. The denser capsules are known to provide better protection of opaque pneumococci against opsonization and killing than the thin capsules of transparent-phase pneumococci provide (18, 35). The spontaneous phase shift back and forth between invasive (opaque) and noninvasive (transparent) pneumococci makes sense only if the two phases are required for different aspects of the colonization-transmission part of the pneumococcal life cycle.

For example, transparent pneumococci could be required for efficient initial attachment, but for long-term carriage it may be important for local invasion to occur by opaque-phase pneumococci. By phase shifting from the opaque phase to the transparent phase, the pneumococci residing in the local nasal tissue could continually repopulate the nasal surface, thus maintaining the reservoir of transparent pneumococci needed for the spread to other hosts. This hypothesis is consistent with the fact that expression of at least some capsule is required for nasal colonization of mice by capsular type 3 pneumococci (20). Moreover, intramuscular immunization with capsular polysaccharide-protein conjugates (which elicits good serum responses but poor mucosal antibody responses) can cause a capsular type-specific reduction in colonization in humans (13). In this study we used a mouse pneumococcal colonization model (37), histology, and the pneumococcal phase to examine...
the hypothesis that pneumococci may invade the nasal mucosa in significant numbers.

**MATERIALS AND METHODS**

**Carriage model.** In this study we used four strains of *S. pneumoniae* (Table 1) that were shown in previous studies to provide strong nasal colonization (6, 37) and that were shown in previous studies to provide strong nasal colonization (6, 37) and preliminary results (not shown) to result in carriage but no detectable bacteremia in CBA/N mice.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>Capsule type (PspA family)</th>
<th>Dose (log$_{10}$)</th>
<th>Day of assay</th>
<th>Total no. of CFU in nasal wash</th>
<th>No. of mice with Pseudomonas aeruginosa in nasal wash</th>
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<tbody>
<tr>
<td>1</td>
<td>TIGR4</td>
<td>4 (2)</td>
<td>5.78</td>
<td>6</td>
<td>4</td>
<td>4</td>
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<td>2</td>
<td>EF3030</td>
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<td>3</td>
</tr>
<tr>
<td>3</td>
<td>BG7322</td>
<td>6B (1)</td>
<td>6.33</td>
<td>7</td>
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<td>6B (1)</td>
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<td>5</td>
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<tr>
<td>5</td>
<td>LS2016</td>
<td>6B (1)</td>
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<td>6</td>
<td>5</td>
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</tr>
<tr>
<td>6</td>
<td>LS2016</td>
<td>6B (1)</td>
<td>6.66</td>
<td>6</td>
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<td>7</td>
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<td>6B (1)</td>
<td>6.80</td>
<td>5</td>
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</tr>
</tbody>
</table>

The lower limits of detection of pneumococci in the samples were <0.3 log$_{10}$ CFU for a nasal wash, <1.6 log$_{10}$ CFU for nasal tissue, <1.0 log$_{10}$ CFU for olfactory bulbs, <1.0 log$_{10}$ CFU for the remainder of the brain, <1.6 log$_{10}$ CFU for lungs, and <1.49 log$_{10}$ CFU for blood.

**RESULTS**

From mice nasally colonized with *S. pneumoniae*, it is possible to recover similar (within 1 order of magnitude) numbers of pneumococcal CFU by washing the nasal passages of a mouse and by homogenizing the nasal tissue excised after a nasal wash (37). In the present studies we determined whether the pneumococci obtained from homogenization of the washed nasal tissue were transparent-phase organisms, as is the case for pneumococci washed from the nose (36), or opaque-phase organisms, as has been reported for pneumococci from invasive infections (6, 34).

**Colonization and invasion of pneumococci following nasal inoculation.** Nasal colonization studies were conducted with four strains of *S. pneumoniae*. Table 1 shows the numbers of CFU of pneumococci recovered in the nasal washes, excised nasal tissues, blood, lungs, and olfactory bulbs of mice colonized with these strains. All 26 mice challenged at the inoculation doses used showed nasal carriage, but none of these mice were bacteremic (Table 1). This result was anticipated from previous studies (6, 37) and preliminary results (not shown) that revealed the optimal range of CFU given i.n. that resulted in carriage but no detectable bacteremia in CBA/N mice. The colonies were always counted and classified for opacity by another individual before the code was broken.

**Histology.** Hematoxylin and cosin staining was carried out on slides of excised nasal tissue (nasal conchae) from four CBA/N mice infected intranasally 6 days previously with strain 10$^7$ CFU of EF3030. Nasal tissue from two uninfected CBA/N mice from the same shipment was similarly examined. Tissues were fixed in 10% formalin, embedded in paraffin, stained with hematoxylin and cosin, mounted, and examined microscopically.

In a separate study nasal tissues were obtained from mice that were infected with 5 x 10$^7$ CFU of TIGR4 and euthanized 10 days later. Four-millimeter frozen sections were stained with rabbit anti-PspA family 2 at a 1:200 dilution (9), followed by anti-immunoglobulin G-biotin at a dilution of 1:200 (Southern Technology Associates, Birmingham, AL) and finally anti-streptavidin-fluorescein isothiocyanate (BD Pharmingen, San Diego, CA) at a dilution of 1:100. Between the incubation steps tissues were washed with four changes of cold phosphate-buffered saline over a 20-min period. The pneumococci were visualized by fluorescence microscopy at a magnification of ×400.

**Statistics.** Statistical comparisons of the percentages of the transparent- and opaque-phase organisms for the bacteria recovered from the nasal washes and nasal tissues were performed by a paired two-tailed Student's t test. In the case of strain LS2016, for which the samples were scored as either >90% transparent or <10% transparent, P values were calculated using the Fisher exact test. Statistical values were calculated with InStat (GraphPad Software Inc., San Diego, CA).

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**TABLE 1. Presence of pneumococci in the noses, brains, and lungs of mice inoculated i.n.**

<table>
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<td>5</td>
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<td>5</td>
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<tr>
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<td>LS2016</td>
<td>6B (1)</td>
<td>6.80</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
mice. For all mice we observed pneumococci in both the nasal wash and the homogenized nasal tissue. For most mice there was between $10^9$ and $10^{10}$ CFU in the nasal wash (Table 2). For strain TIGR4 we generally observed more CFU in the nasal wash than in the nasal tissue, and for BG7322, EF3030, and L82016 the opposite was true.

With strains BG7322, EF3030, and L82016 we observed either no bacteria or only a few hundred bacteria in the lungs (Table 2). This was reminiscent of previous findings for EF3030 and L82016 (6, 33, 37), which showed that the numbers of bacteria in the lungs were low unless the mice were anesthetized during i.n. inoculation. In the case of TIGR4, however, each of the four mice inoculated i.n. had from 100 to 2,000 CFU of pneumococci in its lungs (Tables 1 and 2).

We observed bacteria in the olfactory bulbs and/or brain in over 40% of the 26 mice inoculated with one of the four strains of pneumococci (Table 1). Each of the four strains reached the olfactory bulbs or brain of at least one mouse. The presence of bacteria in the olfactory bulbs and brains of nonbacteremic mice inoculated i.n. with pneumococci was anticipated from previous studies in which mice were inoculated i.n. with TIGR4 or EF3030 (33). In these studies it was argued that the bacteria entered the brain directly via the olfactory nerves (33), as has previously been shown to occur when cholera toxin (32) or vesicular stomatitis virus (16, 25) is given i.n. or vesicular stomatitis virus (16, 25) is given i.n.

**Phase of pneumococci in the nasal wash and nasal tissue.** It has been demonstrated that opaque-phase pneumococci exhibit a range of opacities (34). In the present study only the most transparent colonies were scored as transparent, and all others were scored as opaque. Figure 1 shows transparent and opaque colonies from stock cultures of TIGR4 (Fig. 1A) and L82016 (Fig. 1B). The percentages of transparent and opaque colonies were determined for each inoculum, nasal wash, or tissue homogenate by examining at least 100 colonies grown on TSA. Each colony was visually assigned to the “transparent” or “opaque” phenotype. The results were expressed as percentages of transparent colonies (Table 3). In the case of L82016, exact counts of colonies of pneumococci from the nasal washes and homogenates of the nasal tissue were not obtained. Each sample of L82016 from each mouse tissue yielded either virtually all transparent colonies or virtually all opaque colonies. No washes or tissue homogenates of L82016-infected mice had intermediate values for the percentage of transparent-phase colonies. As a result, Table 3 conservatively lists the data for L82016 bacteria recovered from nasal washes or nasal tissues as either “>90% transparent” or “<10% transparent.”

The relative percentages of transparent colonies in the nasal washes and the nasal tissues differed somewhat for the different challenge strains (Table 3) and from mouse to mouse for inoculations with some strains (data not shown). However, for each of the 26 mice infected with pneumococci, we observed a higher percentage of transparent-phase CFU for the nasal wash than for the homogenized nasal tissue. The fact that we observed a higher percentage of transparent colonies for the nasal wash than for the washed nasal tissue of each of the 26 mice was significantly different from random at a $P$ value of $<1.5 \times 10^{-8}$. For the mice colonized with each of the four stains of pneumococci, the greater frequency of transparent colonies in the nasal wash than in the tissue homogenate was also statistically significant (Table 3).

These results indicated that the bacteria recovered in the tissue homogenate were not just leftover surface bacteria that had not been washed off but were a separate population of bacteria that were more intimately associated with the nasal mucosa. Since the majority of the bacteria retrieved from the tissue homogenates were opaque, as are pneumococci from...
blood or lungs (6, 19, 34), it seemed likely that the bulk of the bacteria released by homogenization may have been living within the nasal tissue.

Opacity of pneumococci in the lungs, olfactory bulbs, and brain. As expected (6), whenever bacteria were observed in the lung, they produced predominantly opaque colonies. The small numbers of pneumococci from the brain and olfactory bulbs all produced opaque colonies (data not shown). These observations are consistent with the original reports that the opaque phenotype is associated with invasion (34–36).

Inflammation of mucosa and submucosa during colonization. The conclusion that pneumococci did more than cause “asymptomatic colonization” was apparent from our observation that there was evidence of inflammation in the nasal tissue of mice colonized for 6 days with EF3030 pneumococci. Inflammation was not seen in any of the tissues of mice lacking pneumococcal colonization (Fig. 2 and 3). The majority of the nasal epithelium of the infected mice appeared to be normal, but three of four mice colonized with EF3030 had localized regions of inflammation characterized by scattered lymphocytes within the mucosa, a lack of normal tissue architecture, and/or the loss of cilia. Figure 2 shows the loss of cilia and modified tissue architecture in tissue from an infected mouse (Fig. 2B) compared with tissue from a normal mouse (Fig. 2A) (both at a magnification of ×200). This low-magnification image emphasizes the size of the altered areas in the colonized animals. The micrographs in Fig. 3 were taken at a magnification of ×400 to visualize inflammatory cells. Figure 3A shows a preparation from a noncolonized mouse, and Fig. 3B to D show preparations from a colonized mouse. Figure 3B and C show leukocytes that have entered the mucosa and submucosa. Figure 3B shows that there was a loss of cilia. Figure 3C shows inflammation with retention of cilia. In the colonized mice nasal crypts were often observed to be filled with neutrophils (Fig. 3D).

Visualization of pneumococci within the nasal mucosa. Because the numbers of pneumococci in the nasal tissue were seldom greater 10^4 CFU, the chances of visualizing the bacteria microscopically in convincing numbers were small. However, when we used immunofluorescence to examine nasal tissue of mice inoculated i.n. with 5 × 10^5 CFU of TIGR4, we detected individual diplococci in some fields of some tissue sections. The bacteria were visualized with polyclonal antibody to PspA, a surface molecule known to be expressed by pneumococci in vivo (21, 29).

**DISCUSSION**

In contrast to the transparent-phase pneumococci that can be washed from the nasal surface, we observed that the pneumococci released from the washed nasal tissues by homogenization are largely opaque-phase (invasive) organisms. This finding suggests that these opaque-phase pneumococci invaded the tissues. Although occasional diplococci were observed in

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**TABLE 3. Levels of transparent-phase organisms in nasal washes and nasal tissues**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Strain</th>
<th>% of transparent phase in inoculum</th>
<th>No. of mice</th>
<th>% of transparent phase in nasal wash</th>
<th>% of transparent phase in nasal tissue</th>
<th>P for nasal wash versus tissue^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TIGR4</td>
<td>87</td>
<td>4</td>
<td>76.8 ± 4.9</td>
<td>39.7 ± 7.0</td>
<td>0.0048</td>
</tr>
<tr>
<td>2</td>
<td>EF3030</td>
<td>83</td>
<td>3</td>
<td>96.7 ± 0.9</td>
<td>6.3 ± 1.9</td>
<td>0.0009</td>
</tr>
<tr>
<td>3</td>
<td>BG7322</td>
<td>3</td>
<td>3</td>
<td>96.0 ± 0.6</td>
<td>2.0 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>4</td>
<td>BG7322</td>
<td>5</td>
<td>3</td>
<td>61.7 ± 3.3</td>
<td>26.3 ± 5.2</td>
<td>0.046</td>
</tr>
<tr>
<td>5–7</td>
<td>L82016</td>
<td>50</td>
<td>13</td>
<td>&gt;90^c</td>
<td>&lt;10^c</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

^a In each case the percentage of the opaque phase is 100% minus the percentage of the transparent phase.

^b The Student’s `t` test was used for TIGR4, EF3030, and BG7322, the Fisher exact test was used for L82016.

^c For 13 mice the nasal wash bacteria were >90% transparent, and for 13 mice the nasal tissue bacteria were <90% transparent. The L82016 data were calculated from a pool of the data from experiments 5, 6, and 7, as shown in Tables 1 and 2.

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**FIG. 2.** Nasal tissue from a noninoculated CBA/N mouse (A) and a mouse inoculated i.n. 6 days previously with 2.4 × 10^6 CFU of EF3030 (B). Tissues were stained with hematoxylin and eosin and examined microscopically at a magnification of ×200. The tissue from the normal mouse is representative of all fields examined for the noninfected mouse. Most fields of infected tissue were similar to the field of tissue from the noninfected mouse. Panel B shows an inflamed area of the mucosa of a colonized mouse. Note the lack of cilia and the disorganization of the submucosal cells.
tissue by fluorescence microscopy, the numbers of CFU in the tissues were too small for convincing numbers of CFU to be visualized in a single field. As a result, our results did not rule out the possibility that extracellular products of surface pneumococci caused the inflammation.

Our observations do indicate, however, that even in mice lacking bacteremia or significant lung infections, “asymptomatic” nasal colonization involves two populations of pneumococci, a transparent-phase population loosely associated with the nasal surface and an opaque population more intimately associated with or within the nasal mucosa and submucosa. It is possible that some of the opaque pneumococci were within the granulocyte-filled nasal crypts. If they were in the nasal tissue or in inflamed nasal crypts with granulocytes, they were in locations where they would need to protect themselves from phagocytosis and killing.

*S. pneumoniae* is unusual compared to its relatives the oral streptococci, in that it is able to cause serious invasive disease and even sepsis in a significant number of patients. It is well known that pneumococci have virulence factors, such as capsule, PspA, PspC, and pneumolysin, that act in part by minimizing complement-dependent opsonophagocytosis and killing (7, 11, 17, 20, 22, 26, 38). These virulence factors are important for the survival of pneumococci in the blood, lungs, and tissues of the host. It is also known that pneumococci have virulence factors that allow them to cross cellular barriers and move from the mucosa to the blood and tissue (31).

*S. pneumoniae* is able to establish asymptomatic carriage that can last for months (4, 14). It is generally believed that most transmission of pneumococci is from carriers with nasal colonization and that invasive disease is not necessary for this organism to be maintained as a parasite in humans. In fact, it seems likely that instances of fatal pneumococcal disease are probably counterproductive from the point of view of the pneumococci, as the death of a carrier would shorten the period during which colonizing pneumococci could spread to...
other humans. Moreover, the opaque phase of pneumococci coughed from the lungs would not be optimal for establishing carriage in another individual.

The data presented here help provide a rationale to explain why an organism that wants to exist as an asymptomatic colonizer has evolved virulence factors that enable it to occasionally invade and kill its hosts. Pneumococci may have chosen to avoid competing with noninvasive nasal flora by moving a short distance into sterile tissue, where they compete instead with the host’s phagocytes, C-reactive protein, complement, and antigen-specific immune mechanisms (7, 11, 17, 26, 30). Thus, although pneumococci generally manage not to successfully invade the blood of their human (or mouse) hosts in large numbers, they need the tools for invasion and survival within vascularized tissues in order to be able to penetrate and survive within the nasal submucosa. The same virulence factors would permit them to invade and occasionally kill their hosts if there is a temporary or permanent deficit in immunity. In fact, it seems likely that pneumococci may depend on normal host immunity to help them restrict their invasion to the local nasal tissue.

Based on the findings that we present here, we hypothesize that invasion of the local nasal mucosa may be required for maintenance of long-term carriage in most individuals. It is possible that by being able to invade the mucosa, the pathogen can develop a reservoir of pneumococci that is not affected by mucus flow, secreted lactoferrin, and competition from normal nasal flora or other pathogens. Pneumococci from this reservoir should be able to continually repopulate the nasal surface over time because of their ability to switch back to the translucent phase. Support for this hypothesis comes from observations that capsule (20), PspC (Hotomi and Briles, unpublished), which are important for nasal colonization of mice by at least some pneumococcal strains, are also virulence factors for invasive disease (2, 3, 20). Additional studies are required to completely test this hypothesis.

If true, this hypothesis might also explain why other bacteria that frequently colonize humans, such as Haemophilus influenzae and Neisseria meningitidis, have virulence factors that allow them to invade and occasionally kill their hosts. Finally, if this hypothesis is true, it is possible that some of the competition between pneumococci and other pathogenic bacteria (23, 24) may take place within the mucosa and submucosa and not just at the mucosal surface.

ACKNOWLEDGMENTS

We acknowledge the assistance and interest of Lindsay Parish, Michelle Darrieux Sampaio, and William Benjamin in these studies. Yvette Hale and Flor Gathof are acknowledged for technical and administrative assistance, respectively. Kevin M. Drace is gratefully acknowledged for assistance with digital photographs of transparent and opaque colonies.

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