

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

David E. Briles,^{1,2*} Lea Novak,³ Muneki Hotomi,¹ Frederik W. van Ginkel,^{1†} and Janice King¹

Departments of Microbiology,¹ Pediatrics,² and Pathology,³ University of Alabama at Birmingham, Birmingham, Alabama

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We demonstrated that during colonization with *Streptococcus pneumoniae* the nasal mucosal tissues of mice support two populations of pneumococci. Transparent-phase pneumococci can be readily washed from the outer surface, while a second population composed of primarily opaque-phase pneumococci is released only by homogenization of the nasal tissue. The fact that the opaque phase has previously been associated with invasion and the fact that opaque-phase pneumococci were released by homogenization of previously washed nasal tissue suggest that the opaque-phase pneumococci may have invaded the nasal tissue. Consistent with this hypothesis was our observation that there was inflammation in portions of the nasal mucosa of the colonized mice but not in the mucosa of noncolonized mice, but this observation did not prove the hypothesis. If the opaque-phase pneumococci released from the nasal tissue were from within the tissue and/or if resistance of the opaque-phase subpopulation to antibody, complement, and phagocytes is essential for long-term carriage, it seems likely that the virulence factors of *S. pneumoniae* that are necessary for killing humans exist to facilitate carriage. Although this speculation is unproven, the observation that there are separate populations of pneumococci during colonization may help guide future attempts to understand the biology of nasal colonization by this pathogen.

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 adherence 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

* Corresponding author. Mailing address: BBRB 658, 1530 3rd Avenue South, Birmingham, AL 35294-2170. Phone: (205) 934-6595. Fax: (205) 934-0605. E-mail: dbriles@uab.edu.

† Present address: Department of Pathobiology, Auburn University, Auburn, Ala.

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

Expt	Strain	Capsule type (PspA family)	Dose (log ₁₀)	Day of assay	Total no. of mice	No. of mice with ^a :				
						Carriage	CFU in olfactory bulbs	CFU in brain	CFU in lung	Bacteremia
1	TIGR4	4 (2)	5.78	6	4	4	1	0	4	0
2	EF3030	19 (1)	7.21	6	3	3	2	3	2	0
3	BG7322	6B (1)	6.33	7	3	3	2	2	ND ^b	0
4	BG7322	6B (1)	6.92	5	3	3	2	2	0	0
5	L82016	6B (1)	6.69	6	5	5	0	2	0	0
6	L82016	6B (1)	6.66	6	5	5	0	0	0	0
7	L82016	6B (1)	6.80	5	3	3	2	2	3	0

^a The lower limits of detection of pneumococci in the samples were <0.3 log₁₀ CFU for a nasal wash, <1.6 log₁₀ CFU for nasal tissue, <1.0 log₁₀ CFU for olfactory bulbs, <1.0 log₁₀ CFU for the remainder of the brain, <1.6 log₁₀ CFU for lungs, and <1.49 log₁₀ CFU for blood.

^b ND, not done.

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) in CBA/CAHN-XID (CBA/N) mice (Jackson Laboratory, Bar Harbor, ME). Mice were inoculated intranasally (i.n.) with different numbers of viable *S. pneumoniae* cells in 10 μ l of sterile Ringer's injection solution (Abbot Labs, Chicago, IL). Five to seven days later the mice were bled retroorbitally under Isoflurane (Minrad Inc., Buffalo, N.Y.) anesthesia, and the mice were then euthanized with CO₂. After the mice were euthanized, the lungs were removed. Next, the nasal cavity of each mouse was washed by flushing 100 μ l of Ringer's injection solution into the trachea and out the nostrils (33, 37).

Next, the cranium was removed to expose the brain and olfactory bulbs. The brain and its attached olfactory bulbs were slowly and carefully lifted from the skull base and removed. We removed these neuronal tissues before harvesting the nasal tissue to minimize the chance of contamination of the olfactory bulbs with pneumococci from the nasal cavity. The olfactory bulbs were separated from the remainder of the brain prior to homogenization. For these procedures all instruments were sterilized in 70% ethanol and allowed to air dry between uses to prevent contamination of subsequent samples with pneumococci from the earlier samples. Next, the nasal tissue (nasal conchae), including the olfactory epithelium, was excised as previously described (33). The brain, olfactory bulbs, nasal tissue, and lungs were then individually homogenized in defined volumes of sterile Ringer's solution as described previously (6, 33, 37).

The nasal wash and homogenized nasal epithelium were serially diluted, and 50- μ l aliquots were spread on blood agar plates (containing 4.9% blood agar base no. 2 [Becton Dickinson, Sparks, MD] and 3% defibrinated sheep blood [Colorado Serum Co., Denver, CO]) supplemented with 4 μ g/ml of gentamicin (Roche, Indianapolis, IN) and on blood agar plates supplemented with 4 μ g/ml gentamicin and 5 μ g/ml optochin (ethylhydrocupreine hydrochloride; Sigma, St. Louis, MO) (5). Gentamicin prevented growth of most contaminants in the nasal wash (8). The *S. pneumoniae* strains used did not grow on the plates with optochin and gentamicin. Using these two types of agar plates, we determined that the organisms observed on the gentamicin plates were pneumococci. Although small numbers of bacterial contaminants were occasionally seen in nasal washes, they were never observed in homogenized nasal tissue, nasal bulbs, brains, lungs, or blood.

Although generally there were few contaminants in the nasal wash, there were a few mice for which pneumococci accounted for less than 90% of the bacteria recovered. Data for these mice were omitted from the analysis. In some shipments of CBA/N mice from Jackson Laboratory most mice had nasal contaminants, and mice from these shipments were not used for these studies.

Transparent- and opaque-phase pneumococci. To identify transparent- and opaque-phase colonies, samples of pneumococci were plated on tryptic soy agar (TSA) made with 1.5% tryptic soy broth (Becton Dickinson, Sparks, MD), 1.5% agar (Becton Dickinson), and 315 U of catalase (Worthington, Lakewood, N.J.) per ml (34). TSA contained no added antibiotics to avoid reducing the colony size and interfering with phase determination. Colonies growing on the TSA plates were scored as transparent or opaque. Counts were obtained in a blinded manner. The tissues were obtained, coded, and homogenized by one individual.

The colonies were always counted and classified for opacity by another individual before the code was broken.

Histology. Hematoxylin and eosin staining was carried out on slides of excised nasal tissue (nasal conchae) from four CBA/N mice infected intranasally 6 days previously with strain 10⁷ 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 eosin, mounted, and examined microscopically.

In a separate study nasal tissues were obtained from mice that were infected with 5 \times 10⁵ 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 Biotechnology 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 \times 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 L82016, 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).

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

TABLE 2. CFU in nasal washes, nasal tissues, brains, and lungs of mice inoculated i.n.

Expt	Inoculum		No. of mice	Log ₁₀ CFU in ^a :				
	Strain	Dose (log ₁₀)		Nasal wash	Nasal tissue	Olfactory bulb ^b	Brain ^b	Lung ^b
1	TIGR4	5.78	4	3.55 (0.35)	2.63 (0.06)	1.0 (0.00) ^c	<1.0	2.66 (0.39)
2	EF3030	7.21	3	3.98 (0.07)	6.63 (0.16)	5.50 (1.60)	2.68 (0.47)	2.1 (0.1)
3	BG7322	6.33	3	3.17 (0.16)	3.93 (0.08)	2.37 (0.47)	1.34 (0.04)	ND
4	BG7322	6.92	3	4.03 (1.48)	5.78 (1.88)	2.53 (0.07)	1.63 (0.048)	<1.6 ^c
5	L82016	6.69	5	2.28 (0.23)	2.27 (0.32)	<1.0	1.5 (0.50)	<1.6
6	L82016	6.66	5	2.78 (0.30)	3.81 (0.31)	<1.0	<1.0	<1.6
7	L82016	6.80	3	4.067 (0.35)	4.953 (0.10)	0.55 (0.25)	0.30 (0.00)	2.67 (0.07)

^a Mean (standard error). The values indicate the total numbers of CFU in the tissues.

^b The values for the average numbers of CFU in the olfactory bulb, the remainder of brain, and the lung are averages for positive mice only. ND, not done.

^c <1.0 or <1.6 log₁₀ CFU are the limits of detection. These values are given when there were no colonies in the tissue of any of the inoculated mice.

mice. For all mice we observed pneumococci in both the nasal wash and the homogenized washed nasal tissue. For most mice there was between 10³ and 10⁴ CFU in the nasal wash (Table 2). For strain TIGR4 we generally observed more CFU in the 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.

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 × 10⁻⁸. For the mice colonized with each of the four strains 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

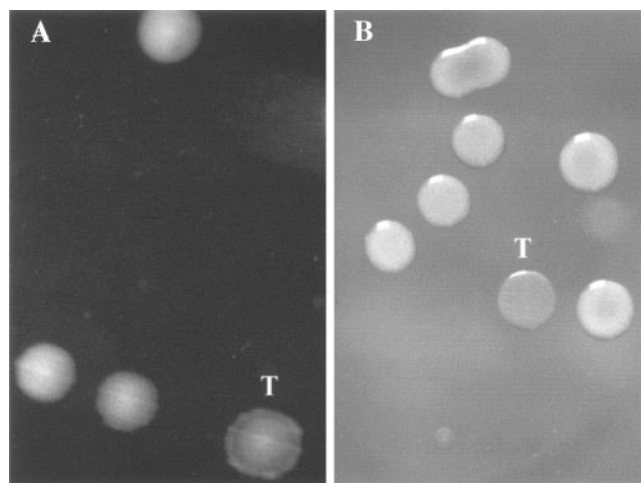


FIG. 1. Transparent and opaque colonies of strains TIGR4 (A) and L82016 (B) growing on TSA supplemented with cysteine. A “T” indicates transparent colonies. All other colonies were classified as opaque.

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

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

^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.

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 magnifica-

tion 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 10⁴ 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 × 10⁵ 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

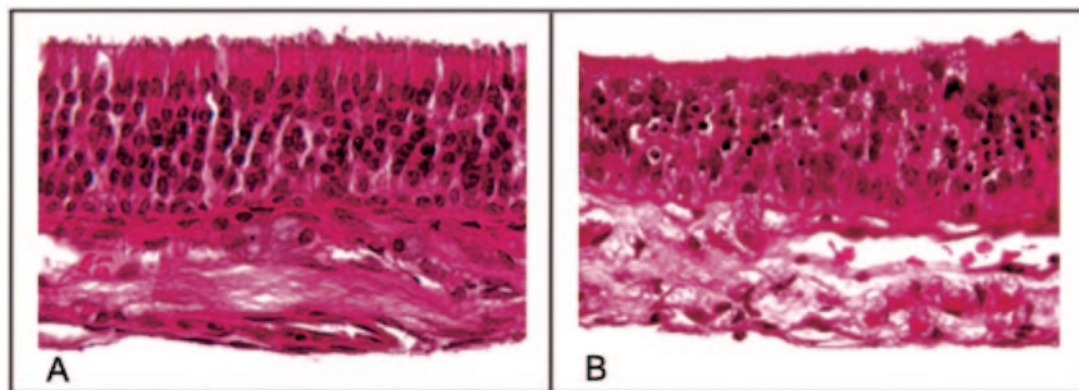


FIG. 2. Nasal tissue from a noninoculated CBA/N mouse (A) and a mouse inoculated i.n. 6 days previously with 2.4×10^6 CFU of EF3030 (B). Tissues were stained with hematoxylin and eosin and examined microscopically at a magnification of ×200. The tissue from the normal mouse is representative of all fields 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.

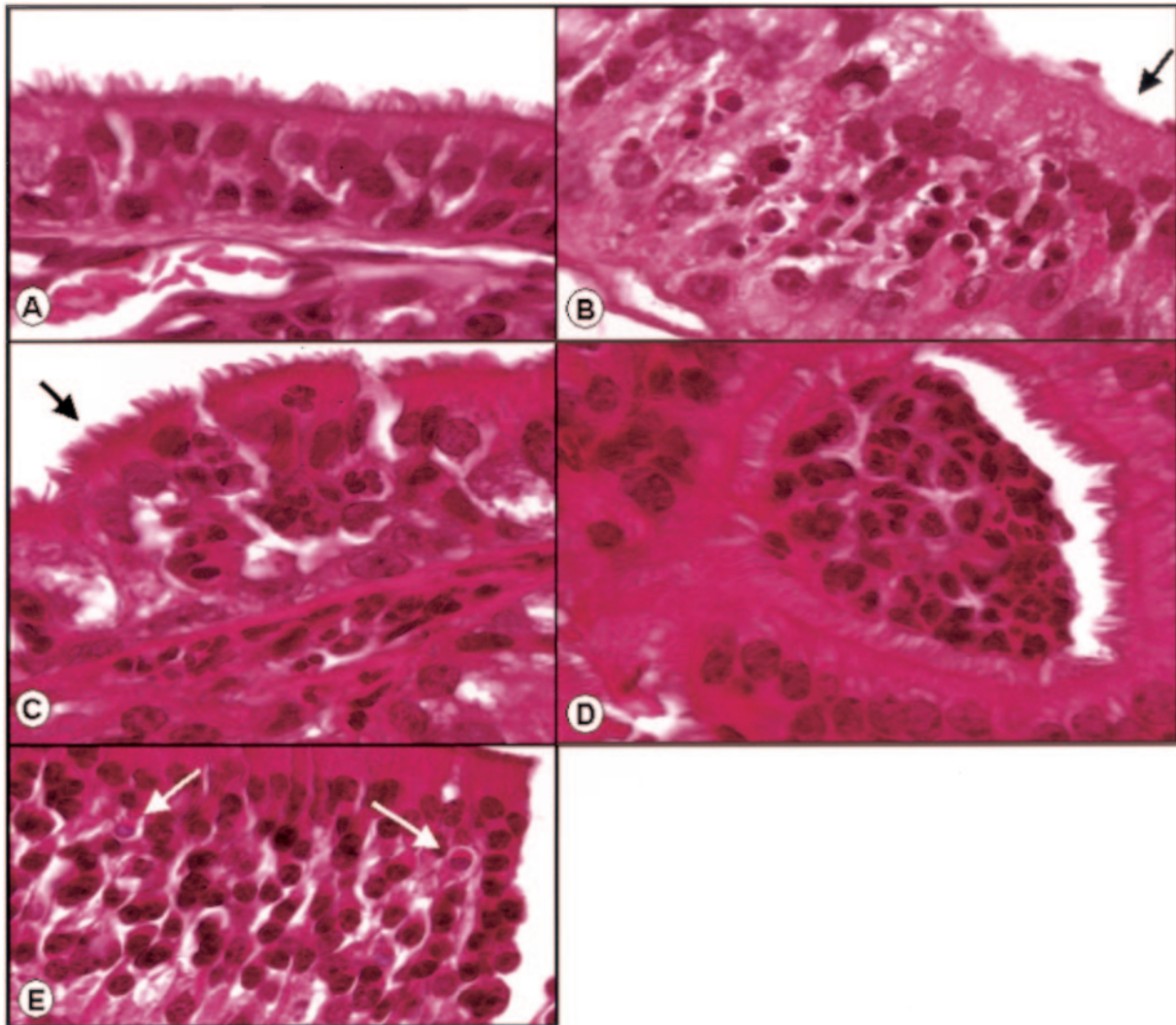


FIG. 3. Nasal tissue from a noninoculated CBA/N mouse (A) and from mice inoculated i.n. 6 days previously with 2.4×10^6 CFU of EF3030 (B to E). All fields were examined microscopically at a magnification of $\times 400$ after staining with hematoxylin and eosin. (B) Acute inflammation of the mucosa with loss of cilia (arrow); (C) acute inflammation of mucosa and submucosa with the cilia preserved (arrow); (D) neutrophils filling a crypt in the mucosal surface; (E) scattered inflammatory cells (arrows) in the inflamed mucosa of a colonized mouse.

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 defenses. However, to invade local tissue, they needed to be able to breach epithelial barriers and to deal 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 transparent phase. Support for this hypothesis comes from observations that capsule (20), PspC (2), and PspA (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, 27). 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.

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