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Pulmonary Clearance of Encapsulated and Unencapsulated *Haemophilus influenzae* Strains

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A mouse model system was employed to investigate the temporal pattern of pulmonary clearance of *Haemophilus influenzae* and to evaluate the effect of the type b polysaccharide capsule on this clearance pattern. The lungs of BALB/c mice were inoculated with boluses of several different *H. influenzae* strains via an endobronchial catheter. A fully encapsulated *H. influenzae* type b strain multiplied readily in the lungs for at least 6 h and then was eventually cleared from the lungs over the next 18 h. The pulmonary clearance pattern obtained with an unencapsulated variant of this *H. influenzae* type b strain was identical to that obtained with the fully encapsulated parent strain. Two nontypable *H. influenzae* strains isolated by transtracheal aspiration of patients with acute *H. influenzae* pneumonia also multiplied in the lung and resisted significant clearance for at least 6 h after inoculation. Bolus deposition of either *H. influenzae* type b or nontypable *H. influenzae* in the lungs resulted in an eventual influx of polymorphonuclear leukocytes into the alveoli. The observed delay in clearance of all these strains suggests that resident host defense mechanisms must be augmented for clearance to occur. Furthermore, these data indicate that one or more factors other than the *Haemophilus* capsule are important bacterial determinants of pulmonary clearance of *H. influenzae*.

Haemophilus influenzae is increasingly reported as a cause of pneumonia in adults in the United States (2, 12, 15, 21, 24, 26, 30, 34). Although this increase may be partly spurious and due to improved bacteriological techniques and the validation of nonbacteremic cases by transtracheal aspirates, at least one institution has reported an increased incidence of *H. influenzae* infections of the lower respiratory tract in recent years with no alteration in its diagnostic procedures (15). Most early reports of *H. influenzae* pneumonia required positive blood or pleural fluid cultures for diagnosis, and consequently, the number of reported cases was small. These early studies concluded that a preponderance of pneumonia in adults caused by *H. influenzae* was due to typable strains of this organism (24, 34), with type b strains being implicated in >80% of the reported cases.

More recent reports have stressed the importance of nontypable *H. influenzae* as a cause of serious adult infections (2, 21, 26, 29, 35). These studies have shown that greater than two-thirds of *H. influenzae* isolates from blood, cerebrospinal fluid, transtracheal aspirates, and sputum of infected adults were nontypable. These findings indicate that, despite the lack of a polysaccharide capsule, nontypable *H. influenzae* have considerable pathogenic potential. Recent genetic studies on the role of lipopolysaccharide as a virulence determinant of *H. influenzae* reinforce the fact that noncapsular antigens of *H. influenzae* may also be important in the pathogenesis of *Haemophilus* disease (38).

Previous studies have evaluated the role of the *H. influenzae* type b polysaccharide capsule in both the clearance of *H. influenzae* type b from the bloodstream and resistance of this organism to the bactericidal activity of serum complement and have firmly established that this capsule is the primary virulence factor in systemic *H. influenzae* type b disease (5, 20, 25, 31, 36). However, no studies have evaluated the effect of encapsulation on pulmonary clearance of *H. influenzae*. In the present study, we have compared the pulmonary clearance of encapsulated *H. influenzae* type b organisms with that of both an unencapsulated variant of this strain and classic nontypable *H. influenzae* strains to evaluate the effect of the capsule on this clearance process. Our results indicate that nontypable and unencapsulated *H. influenzae* strains are cleared no differently from the lung than are encapsulated *H. influenzae* strains suggesting that factors other than the capsule are important determinants of pulmonary clearance. Furthermore, our results suggest that this murine model is useful for studying the interaction of *H. influenzae* with the lower respiratory tract.

MATERIALS AND METHODS

Animals. Female BALB/c mice weighing 20 to 23 g (Cumberland Laboratories, Clinton, Tenn.) were used in all experiments.

Bacterial strains and culture media. The strains employed in this study were: *H. influenzae* type b strain DL26; DL26B, an unencapsulated variant of strain DL26 that produces very little detectable capsular polysaccharide; TN100, a nontypable *H. influenzae* strain; and TN104, another nontypable *H. influenzae* strain. Strains DL26 and DL26B were described previously (11). Strains TN100 and TN104 were isolated from transtracheal aspirates obtained from patients with acute *H. influenzae* pneumonia (2). All bacterial strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal base (BHIs) (1) as a source of hemin and nicotinamide adenine dinucleotide. Before each experiment, a flask containing 50 ml of BHIs was inoculated with a 5-ml sample of a mid-logarithmic-phase culture and incubated at 37°C until the bacteria were again in the mid-logarithmic phase of growth. The *H. influenzae* cells were then harvested by centrifugation at 7,000 × *g* for 10 min at 4°C, and the resultant cell pellet was resuspended in a volume of sterile, cold phosphate-buffered saline (PBS) (pH 7.2) to yield the

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desired concentration of organisms. The bacterial suspension was kept on ice throughout the experiment. Each final bacterial suspension was quantitated by serial 10-fold dilution followed by plating on chocolate agar plates.

Method of bacterial inoculation. The method used to deposit bacteria into the lung was previously described (22). Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (82 mg/kg of body weight). After tracheal exposure, mice were intubated trans-orally with a blunt 20-gauge needle which was guided along the trachea to a point just within the intrathoracic cavity. A PE-10 polyethylene catheter (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, N.J.) containing 5 μl of bacterial suspension was passed through the needle into the lung where the bacteria were deposited with 0.1 ml of air. This technique reproducibly delivered the inoculum to a localized peripheral segment of lung. The inocula were delivered to the lower third of the left lung in 85% of animals, with the remainder being delivered to the right inferior lobe. All animals recovered from anesthesia within 1 h.

Clearance. In each experiment, 29 BALB/c mice were inoculated with bacteria and divided into four groups. Immediately after each bolus challenge, five mice (0-h mice) were killed to determine the initial deposition, and groups of eight mice were subsequently killed at 4, 6, and 24 h after inoculation by cross-clamping of the neck to prevent aspiration of pharyngeal contents. The lungs were removed aseptically, added to 4 ml of sterile PBS, and homogenized in a tissue homogenizer (VirTis 45; The VirTis Co., Gardiner, N.Y.). After further grinding in a Broeck tissue grinder (Corning Glass Works, Corning, N.Y.), a portion of the homogenate was serially diluted in BHIs broth, plated on chocolate agar, and incubated at 37°C in a candle extinction jar. Colony counts were determined with a dark-field Quebec colony counter at 24 h. Cultures of each lung homogenate were expressed as CFU per lung. To compare test groups, a percentage was obtained by dividing the number of CFU from each mouse at each time point by the mean CFU in the lungs of all 0-h mice for that experiment and then multiplying by 100. The resultant number represented the percentage of viable bacteria that remained in the lung at each time.

Bronchoalveolar lavage and phagocytic cell response. The phagocytic cell response in the lung was determined by bronchoalveolar lavage after inoculation of 2.0×10^5 CFU of *H. influenzae* type b strain DL26 and 3.1×10^5 CFU of nontypable *H. influenzae* TN100. PBS-challenged mice were employed as controls. Five mice were killed immediately after inoculation, and the lungs were cultured quantitatively to determine the bacterial deposition. Bronchopulmonary lavage was performed on separate groups of eight mice at 0, 4, 6, and 24 h after a bacterial challenge. Mice in each group were killed by intraperitoneal injection of pentobarbitol (160 mg/kg of body weight). The trachea was exposed and cannulated with PE-50 polyethylene tubing (Clay Adams) attached to a 20-gauge needle. The lungs were lavaged with 0.6-ml portions of heparinized saline until a final volume of 5 ml was obtained. Lavage fluid was collected on ice and subjected to centrifugation at 4°C for 10 min at $150 \times g$. The supernatant fluid was removed, and the cell pellet was resuspended in 1 ml of Hanks balanced salt solution without calcium or magnesium. Cells were counted in a Coulter Counter model ZBI (Coulter Electronics, Inc., Hialeah, Fla.). Differential counts of 200 cells each were performed on duplicate Wright-stained cytocentrifuge preparations.

Preparation and gel electrophoretic analysis of outer membrane vesicles. Outer membrane vesicles were prepared from the nontypable *H. influenzae* strains as described previously (9). Protein was determined by the method of Markwell et al. (17). A portion of outer membrane vesicles (40 to 50 μg of protein) from each strain was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein bands in the gel were visualized by staining with Coomassie brilliant blue exactly as described by Gulig et al. (9).

Quantitative radioimmunoassay of cell-associated polysaccharide capsule. *H. influenzae* cells were collected from BHIs broth cultures by centrifugation at $7,000 \times g$ for 10 min at 4°C and were resuspended in PBS containing 10% (vol/vol) fetal calf serum to a final concentration of 10^8 CFU/ml. All subsequent operations were performed at 4°C. A 0.2-ml portion of these cells was mixed with 10^6 cpm of radioiodinated mouse immunoglobulin M monoclonal antibody (specific activity = 10^6 cpm/μg of protein) directed against the type b capsule (8). This amount of antibody was shown by quantitative titration experiments to represent antibody excess relative to the type b polysaccharide associated with the *H. influenzae* cells. This suspension was agitated gently for 2 h, and then the cells were collected by centrifugation at $12,000 \times g$ for 2 min. The supernatant fluid containing unattached monoclonal antibody was removed by aspiration, and the cell pellet was resuspended in 1 ml of PBS-fetal calf serum. At this time, 10^9 CFU of *Escherichia coli* HB101 was added to the tube as carrier for the relatively small number of *H. influenzae* cells. The cells were then washed 3 times with 1 ml of PBS-fetal calf serum by repeated centrifugation and resuspension. The final cell pellet was resuspended in solubilization buffer (9), and the counts per minute of radioiodinated antibody attached to capsular polysaccharide on the cells were measured in a gamma counter (Searle Corp., Chicago, Ill.). The data presented in Table 2 represent the mean duplicate tubes from three separate experiments.

Statistical analysis. The clearance data were analyzed with the Mann-Whitney U test for nonparametric analysis. A probability value of <0.05 was considered significant (37).

RESULTS

Clearance of *H. influenzae* type b from the lungs. The clearance of the various *H. influenzae* strains from the lungs of normal BALB/c mice was quantitated at 4, 6, and 24 h after bolus deposition of organisms in the lungs via an endotracheal catheter. After a bolus deposition of 10^5 CFU of the fully encapsulated *H. influenzae* type b strain DL26, multiplication of these organisms occurred in the lung to such an extent that the number of organisms present at 6 h post-inoculation was fivefold greater than the number of organisms present at 0 h (Table 1). These organisms were then progressively cleared from the lung over the next 18 h. When the inoculum of strain DL26 was lowered to 10^4 CFU, the resultant multiplication and subsequent clearance pattern were essentially identical to those seen with the higher inoculum (Table 1).

To evaluate the effect of the presence of the type b polysaccharide capsule on clearance of *H. influenzae* from the lung, a spontaneously occurring unencapsulated variant of *H. influenzae* type b strain DL26 was employed in this mouse model system (11). This variant strain (DL26B) possesses the same outer membrane protein profile as its fully encapsulated parent strain, but this unencapsulated variant does not agglutinate with type b-specific antiserum and also lacks the classic colony iridescence associated with

TABLE 1. Clearance of *H. influenzae* from the lower respiratory tract[a]

H. influenzae strain	Deposition (CFU) (mean ± SEM)	% Bacteria remaining (mean ± SEM) at:		
		4 h[b]	6 h	24 h[b]
DL26	1.5 (±0.2) × 10^5	153 ± 12[b]	505 ± 37[b]	0[b]
DL26	1.4 (±0.2) × 10^4	156 ± 13[b]	572 ± 41[b]	0[b]
DL26B	2.1 (±0.2) × 10^5	165 ± 11[b]	455 ± 27[b]	0[b]
DL26B	4.3 (±0.3) × 10^4	136 ± 12[b]	556 ± 51[b]	0[b]
Nontypable TN104	6.8 (±0.5) × 10^5	144 ± 11[b]	89 ± 9[c]	0[b]
Nontypable TN100	1.4 (±0.2) × 10^5	230 ± 17[b]	395 ± 32[b]	2[b]

[a] Each value represents a mean of six to eight animals at each time.
[b] $P < 0.05$ compared with 0 h.
[c] Not significantly different.

encapsulated *H. influenzae* type b strains when viewed by obliquely transmitted light (3, 11). Quantitative radioimmunoassay of the residual cell-associated capsular polysaccharide on strain DL26B established that this variant possesses barely detectable amounts of capsular polysaccharide on its cell surface (Table 2). These quantitative and qualitative data concerning the lack of cell-associated capsular material on strain DL26B correlate with the previously reported ability of this strain to be radioiodinated to a much higher specific activity than that obtained with strain DL26 (11).

Despite the fact that strain DL26B possesses only 1 to 2% the amount of cell-associated capsular polysaccharide found on the parent strain DL26, this unencapsulated strain was not cleared from the lungs of mice any more readily than its fully encapsulated parent strain. After a bolus deposition of 10^5 CFU, strain DL26B grew in the lungs during the first 6 h post-inoculation, and similar to the situation with strain DL26, this organism was then gradually cleared from the lung over the ensuing 18-h period. When the inoculum of strain DL26B was lowered to 10^4 CFU, the clearance pattern once again was similar to that seen with the higher inoculum. Similarly, when the inocula of DL26 and DL26B were increased to 10^7 CFU, both strains were cleared from the lower respiratory tract at the same rate (data not shown). No statistically significant differences in clearance were noted at any period when the encapsulated strain DL26 was compared with the unencapsulated strain DL26B.

Clearance of nontypable *H. influenzae*. The apparent lack of effect of the type b capsular polysaccharide on pulmonary clearance of *H. influenzae* strains prompted us to evaluate clinically relevant unencapsulated *H. influenzae* strains in this mouse model system. Two different nontypable *H. influenzae* strains isolated from patients with *H. influenzae* pneumonia were chosen for further study. That these two naturally occurring unencapsulated *H. influenzae* strains are truly different from each other was established by examination of the outer membrane protein profile of each strain (Fig. 1). These two different nontypable *H. influenzae* strains are not agglutinated by either polyvalent (serotypes a through f) or type b-specific antisera, and neither strain reacted with the radioiodinated type b capsule-specific monoclonal antibody (Table 2).

After bolus deposition of 10^5 CFU of nontypable strain TN104, there was significant bacterial growth for 4 h and only by 6 h did minimal clearance of this organism become

TABLE 2. Quantitation of cell-associated type b polysaccharide capsule on *H. influenzae* strains DL26, DL26B, TN100, and TN104[a]

Bacterial strain	cpm of probe bound[b]
DL26	3,726
DL26B	68
TN100	0
TN104	0

[a] Cell-associated capsular polysaccharide was quantitated as described in the text.
[b] Counts per minute of radioiodinated capsule-specific monoclonal antibody bound to the bacterial cells.

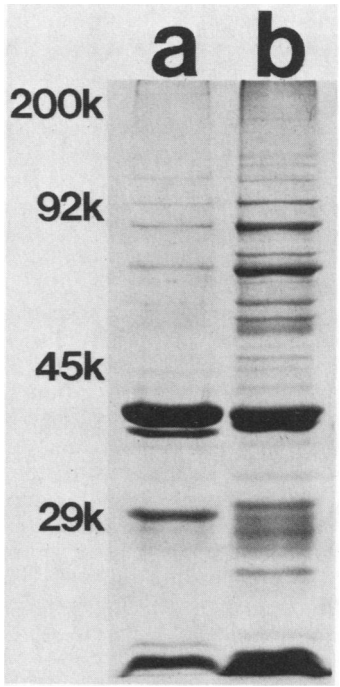

FIG. 1. Outer membrane protein profiles of the nontypable *H. influenzae* strains employed in this study. Outer membrane vesicles were extracted from intact cells of each strain by the lithium chloride-based extraction method and were collected by differential centrifugation as described previously (9). Proteins present in these outer membrane vesicles were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lanes a and b contain the proteins present in the outer membrane vesicles prepared from *H. influenzae* strains TN100 and TN104, respectively. Coelectrophoresis of purified myosin, phosphorylase *b*, ovalbumin, and carbonic anhydrase (with molecular weights of 200,000, 92,000, 45,000, and 29,000, respectively) was used to determine the positions of the molecular-weight reference markers shown on the left side of the figure.

TABLE 3. Recruitment of PMN to the alveoli after challenge with *H. influenzae* type b and nontypable *H. influenzae*

Inoculum	Time (h)	Total cells × 10^6 ± SD[a]	PMN × 10^6 ± SD[a]	% PMN
H. influenzae type b strain DL26	0	1.03 ± 0.06	0.003 ± 0.0001	0.3
	4	1.68 ± 0.07	0.43 ± 0.02	26
	6	1.86 ± 0.07	0.92 ± 0.03	49
	24	2.18 ± 0.10	0.78 ± 0.03	35
Nontypable *H. influenzae* strain TN100	0	1.00 ± 0.02	0	0
	4	1.61 ± 0.06	0.38 ± 0.01	24
	6	1.92 ± 0.04	1.10 ± 0.06	55
	24	2.54 ± 0.09	1.70 ± 0.20	63
PBS (control)	0	1.04 ± 0.07	0.004 ± 0.001	0.4
	4	1.03 ± 0.08	0.03 ± 0.01	3
	6	1.10 ± 0.08	0.05 ± 0.01	5
	24	1.01 ± 0.06	0.03 ± 0.01	3

[a] Each value represents the mean of six to eight animals at each time.

evident. A similar inoculum of nontypable strain TN100 multiplied at both 4 and 6 h post-inoculation. Clearance of TN100 was significantly different ($P < 0.05$) from that of TN104 at 6 h, but it is important to note that no differences in clearance exist at 4 and 6 h when strain TN100 is compared with either the encapsulated *H. influenzae* type b strain DL26 or its unencapsulated variant strain DL26B.

Changes in alveolar cell populations after challenge with *H. influenzae*. The delay in clearance of these *H. influenzae* strains from the lower respiratory tract suggested that resident host defense mechanisms had to be augmented for clearance to occur. To determine whether the populations of phagocytic cells in the lung are altered in response to challenge with *H. influenzae*, 10^5 CFU of *H. influenzae* type b strain DL26 and 10^5 CFU of nontypable *H. influenzae* TN100 were independently deposited in the lungs of mice, and bronchoalveolar lavage of the infected animals was performed at several times after inoculation. Control animals were inoculated with an equivalent volume of sterile PBS.

The vast majority of the cells obtained in the bronchoalveolar lavage fluid at the time of inoculation were mononuclear cells which were mostly alveolar macrophages (Table 3). However, by 4 h post-inoculation, nearly a 100-fold increase occurred in the number of polymorphonuclear leukocytes (PMN) present in bronchoalveolar lavage fluid recovered from *H. influenzae* type b-inoculated animals. Fully one-half of the cells recovered in the bronchoalveolar lavage fluid at 6 h post-infection were PMN. Even 24 h after inoculation with *H. influenzae* DL26, one-third of the cells recovered from the alveoli were PMN. Essentially identical results were obtained when the challenge organism was the nontypable *H. influenzae* TN100 (Table 3). In contrast, the bronchoalveolar lavage fluids recovered from the control animals contained only slightly increased numbers of PMN over a 24-h period post-inoculation.

Incidence of bacteremia. Blood cultures were negative in all animals at each time interval (0 to 24 h) after inoculation with strain DL26, strain DL26B, nontypable strain TN104, and nontypable strain TN100. Long-term experiments in which animals were examined for the presence of bacteremia at 48, 96, and 144 h post-infection showed that bacteria were not detectable in the blood at any of these times.

DISCUSSION

H. influenzae type b is the leading cause of endemic bacterial meningitis in infants and young children (4). Accordingly, most experimental studies of *H. influenzae* disease have used a model of meningitis or bacteremia in which infant rats are inoculated intraperitoneally (27) or by the intranasal route (19). Recently, however, serious adult respiratory tract infections due to *H. influenzae* have received increased attention (2, 12, 15, 21, 24, 26, 30, 34). Whereas considerable data exist regarding the clearance of *H. influenzae* from the bloodstream (20, 25, 36), there have been no published reports concerning the clearance of this organism from another important site of infection, the lower respiratory tract.

Net pulmonary bacterial clearance is determined by the interaction between in vivo bacterial multiplication and bacterial killing (13). After inoculation of ca. 10^5 CFU of *Staphylococcus aureus* or *Streptococcus pneumoniae*, pulmonary defenses are rapidly mobilized, and pulmonary bacterial counts diminish within the first 4 h after inoculation (22, 32). Clearance of *H. influenzae*, however, differs not only quantitatively but also qualitatively from the clearance patterns obtained with these other bacteria. Clearance of both encapsulated and unencapsulated *H. influenzae* strains occurred in a biphasic pattern. During an initial phase of 4 to 6 h, bacterial multiplication exceeded killing, and the number of bacteria in the lung increased ca. fivefold. During the second phase, clearance occurred and the bacteria were eradicated over an 18-h period. This pulmonary clearance pattern resembles the pattern found after aerosol inoculation of certain virulent *Pseudomonas aeruginosa* strains into the lung (28) but differs from that seen after inoculation with *Staphylococcus aureus* and *Streptococcus pneumoniae* strains. It should be noted that this latter organism, like *H. influenzae* type b, possesses an antiphagocytic polysaccharide capsule.

The reason(s) for the biphasic clearance pattern observed with these *H. influenzae* strains is unknown. These results indicate, however, that resident pulmonary defenses in mice are unable to effectively eradicate *H. influenzae* and must be augmented for clearance to occur. Alveolar macrophages are thought to be involved in the early clearance of most bacteria, but in vitro studies have shown that murine alveolar macrophages possess significantly less bactericidal activity against *H. influenzae* than that expressed by peritoneal macrophages (6). Clearance of most encapsulated organisms, like the pneumococcus, involves recruitment of PMN to the lung (33). It is likely that a portion of the augmentation in clearance of *H. influenzae* which occurs between 6 and 24 h post-inoculation is related to the recruitment of PMN to the alveoli (Table 3). Since PMN are more efficient at phagocytosis than macrophages in vitro (14), the arrival of the former cells in the lung could account for the observed increase in bacterial clearance between 6 and 24 h post-inoculation.

It is of particular interest to note that the presence of a capsule had no apparent effect on the pattern or rate of pulmonary clearance of *H. influenzae*. An unencapsulated variant of *H. influenzae* type b and one of the nontypable strains were cleared at the same rate at each time interval as the fully encapsulated *H. influenzae* type b strain (Table 1). Previous studies evaluating the role of the type b capsule in systemic *Haemophilus* disease have used a model of bloodstream clearance and have firmly established the capsule as an important virulence factor in this system (20, 25, 36). Even partial removal of the capsule by mechanical means

has been shown to result in enhanced clearance of *H. influenzae* type b from the blood (36). Furthermore, other studies have shown a role for the type b capsule in promoting bacterial entry or survival (or both) in both blood and the central nervous system (5, 20, 25, 31). Interestingly, bacteremia was not noted at any time with any of the organisms employed in our study, despite bacterial multiplication in the lung. Thus, our results suggest that virulence factors may differ for pulmonary and bloodstream clearance of *H. influenzae* and that bacterial factors other than the capsule are important determinants of pulmonary clearance.

It must also be emphasized that encapsulation may not be the sole determinant of virulence for *H. influenzae* type b. Recent genetic studies of *H. influenzae* type b have shown the importance of other cell surface components, such as lipopolysaccharide, in the pathogenesis of *H. influenzae* disease (38). Lipopolysaccharide is clearly a major determinant of virulence in other gram-negative bacteria (16, 18), and the endotoxic activities of *H. influenzae* lipopolysaccharide are similar to those of lipopolysaccharide from other gram-negative bacteria (7). *H. influenzae* type b also produces extracellular proteins which could be involved in virulence (10, 23). Although the nature of the virulence factors involved in the pathogenesis of nontypable *H. influenzae* disease is not addressed by the present study, the data presented in this report clearly indicate that nontypable *H. influenzae* strains resist ready clearance from the lower respiratory tract.

The present study is the first to quantitatively define the pulmonary clearance of various *H. influenzae* strains. The demonstrated ability of unencapsulated and nontypable *H. influenzae* to initially evade pulmonary clearance indicates that factors other than the capsule are of primary importance as determinants of clearance of *H. influenzae* from the lung. These findings reinforce the importance of studying certain aspects of host defense, not only in intact animals but in each relevant organ system.

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