Intercellular passage of epithelial cell layers: a pathogenic mechanism for Haemophilus Influenzae infections
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Citation for published version (APA):

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PARACYTOSIS OF *Haemophilus influenzae*
through Cell Layers of NCI-H292
Lung Epithelial Cells
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Received 28 March 1995/Returned for modification 11 May 1995/Accepted 25 September 1995

*Haemophilus influenzae* penetrates the respiratory epithelium during carriage and invasive disease, including respiratory tract infections. We developed an in vitro model system consisting of lung epithelial NCI-H292 cells on permeable supports to study the passage of *H. influenzae* through lung epithelial cell layers. The NCI-H292 cells formed tight layers with a Ca^2+^-dependent transepithelial resistance of around 40 Ω·cm^2. *H. influenzae* passed through the cell layers without affecting the viability of the cells and [^1^H]Hinulin penetration. The passage time was independent of the inoculum of *H. influenzae* in the apical compartment and was not influenced by the presence of capsule or fimbriae on *H. influenzae* or by the ability of the bacteria to adhere to the epithelial cells. However, highly adherent strains showed greater paracytosis. Different strains passed through the cell layer independently. The passage time was shorter for rapidly growing strains than for slowly growing strains (10 to 18 h and 30 h, respectively). Microscopic examination revealed the presence of clusters of *H. influenzae* bacteria between the epithelial cells, indicating that bacterial passage was due to paracytosis.

After the addition of chloramphenicol, no bacteria were cultured from the basolateral side, and no bacterial clusters between the epithelial cells were seen, suggesting that de novo bacterial protein synthesis was needed for the bacteria to reach the intercellular space. We conclude that *H. influenzae* passes through viable cell layers of the human lung epithelial cell line NCI-H292 by paracytosis, requiring bacterial protein synthesis.

The gram-negative bacterium *Haemophilus influenzae* is a normal part of the human upper respiratory tract and an important pathogen causing respiratory tract infections as well as systemic disease. Encapsulated strains with a serotype b polysaccharide are able to pass through the respiratory epithelium of the nose, pharynx, larynx, trachea, bronchi, and lungs; however, nontypeable *H. influenzae* strains are incapable of doing this. *H. influenzae* capsule expression is an important virulence factor that allows the capsule to shield the bacteria from phagocytosis and antibodies. *H. influenzae* is capable of adhering to mucosal epithelial cells and passing through these cell layers. *H. influenzae* can pass through viable cell layers of the respiratory tract, indicating that it is able to colonize the respiratory tract. In this study, we investigated the passage of *H. influenzae* through the respiratory epithelium using an in vitro model system consisting of lung epithelial NCI-H292 cells on permeable supports. We found that *H. influenzae* is able to pass through the cell layers without affecting the viability of the cells and [^1^H]Hinulin penetration. The passage time was independent of the inoculum of *H. influenzae* in the apical compartment and was not influenced by the presence of capsule or fimbriae on *H. influenzae* or by the ability of the bacteria to adhere to the epithelial cells. However, highly adherent strains showed greater paracytosis. Different strains passed through the cell layer independently. The passage time was shorter for rapidly growing strains than for slowly growing strains (10 to 18 h and 30 h, respectively). Microscopic examination revealed the presence of clusters of *H. influenzae* bacteria between the epithelial cells, indicating that bacterial passage was due to paracytosis.

In organ culture studies, paracytosis of *H. influenzae* was also observed (3, 17). Unlike the results of studies of infected tissue in vivo, studies with nasopharyngeal organ cultures invariably show that the respiratory tissue is functionally and structurally damaged during incubation with *H. influenzae* (3, 9, 14). To further study the interaction of *H. influenzae* with respiratory epithelium, we sought a model in which *H. influenzae* does not disrupt the epithelial cell layer integrity. In the model system described in this paper, *H. influenzae* passed through confluent layers of human lung epithelial NCI-H292 cells on permeable supports without affecting the permeability or viability of the cell layer. We show that in this model, passage of *H. influenzae* follows the paracytosis route. Adhering strains pass through the cell layers more efficiently than nonadhering strains do. In addition, we show that the time required for passage is independent of bacterial adherence properties and capsule and fimbria production but is influenced by bacterial protein synthesis.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Nontypeable *H. influenzae* d1 (6) and 148I (25) were isolated from sputum samples of patients with chronic bronchitis. A spontaneous streptomycin-resistant mutant of *H. influenzae* d1 strain d1str was isolated as a survivor of a culture of strain d1 in brain heart infusion broth (BHI; Difco) supplemented with 10 μg of NAD and hemin (Sigma) ml^-1^ and 100 μg of streptomycin ml^-1^.

The nontypeable *H. influenzae* strains CF2801a (32010B-10), CF300b (7920250Z-254), CF270b (33087-1), and CF753 (33087-P-2) were isolated from sputum samples of patients with cystic fibrosis. *H. influenzae* type b strain 770235b F^+^ was isolated from cerebrospinal fluid. The isolation of a heavily fimbriated variant of *H. influenzae* strain 770235b F^+^ and the capsular derivatives *H. influenzae* strain 770235b F^-^ has been described before (24, 25). *H. influenzae* reference strain 2174 (nontypeable) was...
supplied by the Quality Assurance Laboratory, Central Public Health Laboratory, London, United Kingdom, and is resistant to 10 μg chloramphenicol (CM) ml−1. All human serum samples were collected on ice at 37°C in a humidified atmosphere containing 5% CO₂. N. gonorrhoeae (ATCC 30356), isolated from a patient with a disseminated gonococcal infection (29), was cultured on GC medium (Difco) containing 10% Vitek (Oxoid) at 37°C in a humidified atmosphere containing 5% CO₂.

**Cell culture.** Epithelial cell line NCI-H292 (ATCC CRL-1848), originating from a human lung mucociliated carcinoma, was maintained in 25-cm² tissue culture flasks (Nunc) in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (Boehringer) without antibiotics. This medium is hereafter referred to as maintenance medium. Cells were passaged twice weekly with a split ratio of 1:6. Permeability studies and passage assays with the epithelial cell line were performed after culturing the cells on transparent tissue culture inserts with 1-μm pores. Originally, the tissue culture inserts contained a 0.6-mm surface area membrane; later, tissue culture inserts containing a 0.31-cm² surface area membrane were used (Costar, Cambridge, Mass.). In order to obtain confluent cell layers on the 0.31-cm² filter membranes, confluent flask cultures were tryptophanized with 0.05% trypsin plus 0.02% EDTA in Dulbecco’s phosphate-buffered saline (PBS) (137 mM NaCl, 8 mM Na₂HPO₄, 2.7 mM KCl, 0.15% Na₂HPO₄, 0.3 M KCl) diluted in 10 ml of maintenance medium and spun down at 1,000 rpm in a Heraeus minifuge RF centrifuge. The cells were resuspended in maintenance medium, and 200 μl of the cell suspension containing 3 × 10⁴ cells was added to each tissue culture insert. Tissue culture inserts were placed in 24-well plates (Falcon) containing 7 ml of maintenance medium. All culture cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The viability of the epithelial sheets was determined by trypan blue exclusion as follows: Test samples were removed from the insert (100 μl) and counted on a hemocytometer. The trypan blue dye (0.2% in Dulbecco’s PBS containing 1 μg of bovine serum albumin (BSA) ml⁻¹) was added to each well, and viable and nonviable cells were counted by light microscopy. Over 500 cells were examined for each determination.

**Electrical resistance measurement.** The transepithelial resistance (TR) of NCI-H292 fixed on the Millicell ERS Resistance System (Millipore, Bedford, Mass.) before the electrodes were sterilized in a solution of 70% (vol/vol) ethanol in H2O. After the instrument was calibrated against maintenance medium, one electrode was placed inside the tissue culture insert and the other was placed on the outside. Tissue culture inserts without cell layers served as blanks. The reading was recorded, and the TER was calculated from the equation: 

\[
\text{TER} = \frac{R_{\text{int}} - R_{\text{ext}}}{R_{\text{ext}}} \times 100\%
\]

where \(R_{\text{int}}\) is the TER of the intact TIE or epithelial layer.

**[3H]Hilum permeability measurement.** For [3H]Hilum permeability measurements of the cell layers, tissue culture inserts containing a 0.6-mm membrane were used. At time zero, 100 μl of maintenance medium containing 1.0 (0.2 to 1 mmol) of [3H]Hilum (molecular weight, 5,200; Amer sham) was added to 400 μl of maintenance medium at the apical side of the cells. The basolateral volume was 600 μl. A sample (137 μl) of the apical medium and a sample of 50 μl of the basolateral medium were taken, and in 10 ml of 100% ethanol in H₂O. After the instrument was calibrated again against PBS, the other sample was taken at the outside. Tissue culture inserts without cell layers served as blanks. The reading was recorded, and the TER was calculated from the equation: 

\[
\text{TER} = \frac{R_{\text{int}} - R_{\text{ext}}}{R_{\text{ext}}} \times 100\%
\]

where \(R_{\text{int}}\) is the TER of the intact TIE or epithelial layer.

**Passage assay.** Bacteria of a culture grown overnight on chocolate agar plates were suspended in RPMI 1640 medium with 25 mM HEPES (N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid) buffer (Gibco) supplemented with 10% fetal calf serum (assay medium) to the appropriate concentration (10⁴ CFU ml⁻¹, unless stated otherwise). At time zero, the apical medium of the tissue culture inserts was replaced by 200 μl of bacterial suspension in assay medium. The cell culture inserts were transferred to a 24-well plate containing 200 μl of assay medium per well and incubated at 37°C in a CO₂ incubator unless stated otherwise. The apical medium was replaced with fresh 37°C medium after 6 h and then every subsequent 48 h to avoid acidification. After 8 h of incubation, the basolateral medium was replaced with fresh 37°C medium every 48 h and cultured on chocolate agar plates to determine the numbers of bacteria in each sample by colony counting. The passage time was determined as the time point that a positive sample was taken for the first time. Alternatively, the tissue culture inserts were placed in 700 μl of assay medium, and the bacterial number was determined from aliquots of 50 to 100 μl that were taken at regular time intervals. Since the bacteria that passed through the cell layer started to grow in the basolateral compartment with the rate of the exponential phase of the growth curve (data not shown), the passage time of the bacteria was determined by extrapolation of the curve from the number of bacteria in the basolateral compartment to the time point that two bacteria were in the basolateral compartment. The passage times calculated in this manner correlated with the passage times of the experiments, in which the basolateral medium was changed every 30 or 60 min. Inhibition of bacterial protein synthesis in the passage assay was obtained by the addition of the same concentration of CAM to the apical and basolateral medium to a final concentration as indicated. The batch number of the apical medium was determined by colony counting at various time points to assure cholera toxin activity.

**Adherence of H. influenzae to NCI-H292 cells.** Cells were grown to confluence on round glass coverslips (Menzel-Gläser, Germany) with a diam of 12 mm in 24-well plates with 1 ml of maintenance medium. The medium was replaced by 1 ml of maintenance medium containing bacteria in a concentration of 10⁴ CFU ml⁻¹. After 5 h of incubation at 37°C in a humidified atmosphere containing 5% CO₂, the cells were washed three times with PBS (10 mM sodium phosphate, 140 mM NaCl, 7.4). The cells were fixed with 0.5 ml of 4% paraformaldehyde (PFA; Merck) 1% glutaraldehyde (GA; Merck) in Dulbecco’s PBS for 30 min at room temperature and stained overnight with 0.007% crystal violet solution. To detect adherence, the number of adherent bacteria per cell was counted on an apparatus 50 cells by light microscopy. Alternatively, cells were treated with 1% saponin in Dulbecco’s PBS for 15 min to release them from the f coverslips and dilutions were plated on chocolate agar plates to yield the number of adherent bacteria per microorganism. The number of adherent bacteria per cell was calculated by dividing this number of CFU per cell by the number of epithelial cells, as determined by counting the number of cells of a trypan blue-positive cell layer.

**Light microscopy and TEM.** Cell layers on tissue culture inserts were fixed in PBS containing 4% PFA and 1% GA, postfixed with 1% mercuric sodium cacodylate buffer (pH 7.4) (7), dehydrated via graded alcohol treated with propylene oxide, and embedded in Epon 1111 (Ladd). For 1-micron sections, sections, 1 and 2 μm were thick for 1 min with each blue; 0.5% methylene-blue, 0.5% Araldite II, 0.5% borax in distilled water; transmission electron microscopy (TEM), ultrathin sections approximately 70-80 nm were mounted on grids, stained with aqueous uranyl acetate and lead citrate, and analyzed with a Zeiss transmission electron microscope. In some cases, gold probes were added to the cell layers during fixation in the PFA solution. Gold particles with a diameter of 2 nm were prepared as described Steer and Giese (16). Because of the thick staining of the lamina propria of the tissue layer, this technique made it possible to distinguish between bacterial presence and bacterial presence of the lamina propria and those located in compartments between cells.

**RESULTS**

Characteristics of NCI-H292 lung epithelial cell layers tissue culture inserts. One day after seeding, the NCI-HII lung epithelial cells formed confluent cell layers on permeable supports as observed by inverse light microscopy. Examination across sections of the cell layers on permeable supports by light microscopy (Fig. 1a) and TEM showed that the cell lay consisted of one to three layers of cells. Large intercellular spaces were observed between the lower layers of the cells. Desmosomes were abundantly present. The cells of the upper layer were tightly connected by desmosomes and tight junctions.

**TER:** A property of tight epithelia, reached its maximum 38.6 ± 4.0 Ω·cm⁻² within 4 days of seeding and remained stable until at least 7 days after seeding (Fig. 2). When EDTA was used to chelate the free calcium, TER decreased to 17.9 ± 1 Ω·cm⁻². Tightness of the cell layer was further demonstrated by [3H]Hilum permeation assay. Four days after seeding, permeation rate was 1 to 3% h⁻¹ (Fig. 3). After the addition EDTA, the permeation rate increased to 4 to 9% h⁻¹. The fore, the NCI-H292 lung epithelial cells formed sealed, tically tight layers on permeable supports.

**Passage of H. influenzae through cell layers.** The NCI-HII cells were grown for 4 days on filters with 1-μm pores. *influenzae* d1, which caused a persistent infection in a patient with chronic obstructive pulmonary disease, was used to determine bacterial passage through this barrier. Figure 4 shows numbers of bacteria per hour in the basolateral compartment after passage through the NCI-H292 cell layer. Occasionally, few bacteria passed through the cell layers during the first postinfection, as in one of the experiments with *H. influenzae* d1 shown in Fig. 4. In all experiments, the number of bacteria cultured from the basolateral compartment was very small until 16 to 18 h postinfection. Then, the number of bacteria cultured from the basolateral medium started to increase.
proximately 1,000 bacteria had passed through the cell layers in 1 hour at 24 h.

To examine the effect of *H. influenzae* infection on the integrity of the cell layers, trypan blue exclusion and \[^{3}H\]inulin permeability assays were performed. Trypan blue exclusion showed that 85 to 95% of the epithelial cells remained viable 6 h postinfection with *H. influenzae* d1 compared with 92% ble cells in an untreated cell layer. The \[^{3}H\]inulin permeation rate from 14 to 20 h postinoculation of the cells with *H. influenzae* d1, resulting in passage, was essentially the same as the permeation rate of untreated cell layers, namely, 1 to 3% (Fig. 3). These results indicated that *H. influenzae* passed through cell layers of NCI-H292 cells without loss of cell layer integrity.

**Effect of the bacterial inoculum on the passage time of *H. influenzae*.** The passage times of *H. influenzae* d1 after inoculation with various numbers of bacteria at the apical side of the 1 layers \((10^{7} \text{ to } 10^{11} \text{ CFU ml}^{-1})\) are shown in Fig. 5. The passage time varied in individual experiments. For *H. influenzae* d1, the shortest observed passage time was 4.5 h and the longest was 19 h, but this variation in passage time was independent of the inoculum. Since the growth curve of *H. influenzae* d1 in the apical compartment showed that 9 h was required before an inoculum of \(10^{7} \text{ CFU of bacteria ml}^{-1}\) increased to \(10^{8} \text{ CFU ml}^{-1}\), it is not likely that a high concentration of bacteria is required at the first onset of passage. This indicates that only a small number of bacteria was required to start passage of the cell layer and that passage started shortly after inoculation of the bacteria.

**Fig. 2.** TER across uninfected NCI-H292 cell layers in the first 7 days after seeding the cells on cell culture inserts. Symbols: ● untreated cell layer; ○ same cell layer after 30 min of incubation in 10 mM EDTA in maintenance medium. Measurements are mean values of 10 tissue culture inserts ± standard deviations.

**Fig. 3.** \[^{3}H\]inulin permeability of NCI-H292 cell layers. The ratio of counts at the basolateral side to the counts at the apical side at time zero was calculated. Mean values ± standard deviations of five tissue culture inserts are shown. Symbols: ● untreated cell layer; ○ cell layer 14 h postinoculation with *H. influenzae* d1 at the apical side; ◆ cell layer after the addition of maintenance medium with 30 mM EDTA. dpm, disintegrations per minute.
Effect of adherence of other strains. Dual infections with *H. influenzae* d1 and a streptomycin-resistant mutant of this strain (d1str) were performed to study whether individual bacteria invaded independently of each other. The passage times of the two variants were compared after inoculation of the two variants in one tissue culture insert either at the same time (controls; n = 2) or 4 h after each other (n = 2). In the controls, the mean passage time of strain d1 was 9 h and the mean passage time of strain d1str was 10 h. When the two variants were inoculated 4 h after each other, the mean passage time of strain d1 was 12 h and the mean passage time of strain d1str was 10.5 h, which was 1 and 4 h after passage of strain d1. These results showed that preincubation with strain d1 for 4 h did not alter the passage time of strain d1str. Because the passage time of one strain may vary up to 4 h in one experiment, other experiments were done in which d1str was inoculated 6 h after strain d1. At time zero, 10^5 CFU of *H. influenzae* d1 ml⁻¹ was inoculated at the apical side of NCI-H292 cell layers, and after 6 h of incubation at 37°C, 10^5 d1str was added so that both bacterial variants were present in the same numbers. The passage time of *H. influenzae* d1 was 6.5 to 7 h compared with 7 h when no d1str was added. The passage time of the streptomycin-resistant bacteria was 9.5 to 13 h after inoculation of d1 compared with 5 h without preincubation with strain d1. These results showed that incubation of the cell layer with *H. influenzae* d1 for 6 h did not induce accelerated passage of strain d1str. Moreover, a long preincubation with strain d1 may even prolong the passage time of strain d1str, indicating that the different bacteria passed through the cell layers independently.

**Effect of adherence on passage.** The passage times of *influenzae* strains differing in adherence characteristics were determined. *H. influenzae* d1 did not adhere to the NCI-H cells after 6 h in contrast to *H. influenzae* 1481, which adhered strongly (>100 bacteria per cell), as determined by light microscopy. The number of adherent bacteria per cell as detected by colony counting (31 ± 15 bacteria per cell for str 1481) was lower than determined by light microscopy (Ta 1). This difference in counting methods was a general phenomenon (Table 1) which may be caused by incomplete solubilization of the adherent strain 1481, which were not cultured from the basolateral compartment in a shorter time period than bacteria from the nonadherent strain d1 but passed through the cell layers in larger numbers. Strain CF3001 (O, •) which grew slower than strains d1 and 1481, passed through the cell layers more than 15 h later than these strains.

**TABLE 1.** Effects of adherence of different *H. influenzae* strains passage through NCI-H292 cell layers on filter supports

<table>
<thead>
<tr>
<th><em>H. influenzae</em> strain</th>
<th>Adherence</th>
<th>Mean generation time* (min)</th>
<th>Passage time (h) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d1</td>
<td>++</td>
<td>0.4 ± 0.4 (4)</td>
<td>10.4 ± 2.7 (</td>
</tr>
<tr>
<td>1481</td>
<td>++</td>
<td>0.4 ± 0.9 (4)</td>
<td>12.3 ± 1.2 (</td>
</tr>
<tr>
<td>770235h&quot;p&quot;</td>
<td>++</td>
<td>0.6 ± 0.6 (4)</td>
<td>8.1 ± 2.7 (</td>
</tr>
<tr>
<td>770235h&quot;p&quot;</td>
<td>-</td>
<td>0.002 (2)</td>
<td>10.1 ± 3.4 (</td>
</tr>
<tr>
<td>770235h&quot;t&quot;</td>
<td>+</td>
<td>ND*</td>
<td>9.6 ± 3.3 (</td>
</tr>
<tr>
<td>770235h&quot;t&quot;</td>
<td>-</td>
<td>ND*</td>
<td>11.4 ± 3.4 (</td>
</tr>
</tbody>
</table>

*Mean generation time in the exponential growth phase in the apical compartment of the cell culture.
* Passage time was determined after inoculation of 10^6 CFU/ml in the apical compartment at time zero.
* Adherence as determined by light microscopy, as described previously (Symbols: ++, 50 to 200 bacteria bound per epithelial cell; +, 5 to 50 bacteria bound per epithelial cell; -, less than 5 bacteria bound per epithelial cell.
* ND, not determined.
TABLE 2. Effects of the mean generation times of different H. influenzae strains on passage through NCI-H292 cell layers on filter supports

<table>
<thead>
<tr>
<th>strain</th>
<th>Inoculum</th>
<th>Mean generation time (min)</th>
<th>Passage time (h) (mean ± SD [n])</th>
</tr>
</thead>
<tbody>
<tr>
<td>2301a</td>
<td>10^5</td>
<td>30</td>
<td>15.6 ± 2.7 (3)</td>
</tr>
<tr>
<td>3001f</td>
<td>10^4</td>
<td>114</td>
<td>22 (1), &gt;22 (3)</td>
</tr>
<tr>
<td>2701h</td>
<td>10^6</td>
<td>105</td>
<td>&gt;30 (3)</td>
</tr>
<tr>
<td>47A</td>
<td>10^6</td>
<td>130</td>
<td>&gt;30 (3)</td>
</tr>
</tbody>
</table>

Number of CFU inoculated per milliliter at time zero.

As in Table 1, footnote a.

The number of bacteria of strain 1481 that passed through cell layers per hour was similar to the number of bacteria strain d1 passing through the cell layers per hour until 15 to 18 h after inoculation (Fig. 4). Then, the number of bacteria of strain 2301b passing through the cell layers per hour started to increase much faster than the number of bacteria of strain d1 passing through per hour. The passage times of these strains do not differ significantly though, as shown in Table 1 and Fig. 4, indicating that the ability to pass was independent of adherence to the epithelial cells. Next, four different variants of strain 2301b were compared. The encapsulated H. influenzae 3235b(fimbriated) and 770235b(nocombriated) did not adhere after 6 h. The nonencapsulated variants H. influenzae 3235b' and 770235b' adhered in an overall pattern of more than 50 bacteria per cell and 5 to 10 bacteria per 1, respectively, as determined by light microscopy after 6 h of culture on the cells (Table 1). The passage times of the variants of strain 770235 were similar for each variant and were comparable to the passage times of the nonencapsulated H. influenzae d1 and 1481, as summarized in Table 1. These results indicate that the passage time of H. influenzae is unrelated to the presence of capsular, fimbrial, or other adhesins promoting adherence of H. influenzae to NCI-H292 cells, although the number of bacteria passing through the cell layer is higher for adherent strains.

The influence of bacterial metabolism on the passage time H. influenzae. As shown in Fig. 4 and Table 2, the mean generation time of strain CF3001 after inoculation of 10^3 CFU was 29.5 h. To determine if this long passage time was characteristic for cystic fibrosis isolates, the passage times of several H. influenzae strains isolated from sputa of cystic fibrosis patients were determined. Table 2 shows the growth rates of the passage times of these strains after inoculation of 10^5 CFU ml^(-1) in the apical compartment at time zero. The slowly growing strains (generation times of 100 min or more) needed 22 h to pass through the cell layers, while strain CF2301a h a generation time of 30 min passed through the cell layers in 12 to 18 h (Table 2). To analyze whether a long passage time of slowly growing H. influenzae strains was characteristic for slowly growing strains or due to slow growth rates, the strain rate of H. influenzae d1 was influenced by lowering the incubation temperature or by adding CAM to the bacteria, passage assays at 37°C. The growth curves of H. influenzae d1 in the apical compartment showed that the mean generation time increased from 28 to 67 min by incubation at 37°C. Concomitantly, the passage time increased from 10.4 ± h to 26.3 ± 4.0 h (n = 4). Because incubation at 28°C affects the metabolism and the motility of both the bacteria and epithelial cells, passage experiments at 37°C were performed in which either the cell metabolism or bacterial growth was delayed at 37°C to study the role of protein synthesis during passage. Addition of 5.6 μg of cycloheximide ml^(-1) during the passage assay, a concentration sufficient to block the protein synthesis of mouse fibroblasts, delayed cells by at least 97% (8). This did not significantly affect the passage time of H. influenzae. The same results were obtained by using anisomycin as inhibitor of the protein synthesis of the epithelial cells at the concentrations of 0.5 and 10 μM. When 1.5 μg of CAM was added to the medium during the passage experiment with H. influenzae d1, it was bacteriostatic for the first 6 h. The bacterial number in the apical compartment decreased approximately 50% after 15 h. No bacteria were cultured from the basolateral compartment after 24 h (n = 2) or 27 h (n = 4). Addition of CAM to the medium did not affect the passage time of H. influenzae 2174, a CAM-resistant strain, thereby excluding an effect of CAM on the epithelial cells. These results showed that bacterial protein synthesis, and not epithelial cell protein synthesis, was important during the passage of H. influenzae.

Features of bacterium-cell interactions. Sections of cell layers incubated with bacteria were screened by light microscopy for the presence of bacteria. H. influenzae d1 was not found adherent to the surface of NCI-H292 cells. The absence of adherent bacteria to the epithelial cells in sections of cells grown on filters is in agreement with the data obtained from incubations of cells on glass slides. After 17 or 26 h of incubation but not after 6 h, clusters of bacteria were occasionally seen between the cells of the cell layer (Fig. 1b). These clusters had the appearance of microcolonies. After incubation of the cell layers with the adherent H. influenzae 1481 or 770235b' at 37°C for 24 to 26 h, a much higher number of bacterial clusters at these conditions than that after incubation with the nonadherent H. influenzae d1 was seen as shown in Fig. 1c and Table 3.

TEM analysis of ultrathin sections of filters incubated with H. influenzae strains (Fig. 6) showed that adherent bacteria appeared to be attached to microvilli on the surface of the epithelial cells (Fig. 6a). Clusters of bacteria were localized in the large intercellular spaces of the cell layer (Fig. 6). These intercellular spaces were separated from the external surface, since the dense staining on the surface of the cell layer of gold particles added to the apical medium of the cell layers before staining the filters, was not seen in the intercellular spaces (Fig. 6b and c). Tight intercellular junctions were apparent above the bacterial clusters (Fig. 6b), indicating that the cells were not damaged by the bacterial passage. Sometimes intracellular bacteria were seen, but these seemed to be partially degraded (Fig. 6c).

To analyze whether intercellular localization was specific for H. influenzae or a common phenomenon for bacteria passing through NCI-H292 cell layers, passage of N. gonorrhoeae 830563 was studied. This strain was shown to invade Chang epithelial cells (28, 29). The passage time of N. gonorrhoeae 830563 at 37°C was more than 22 h (n = 4). After incubation of the cell layer at 37°C for 6 or 26 h with N. gonorrhoeae 830563, diploccoci were found adherent on the surface of the cells (Fig. 7a) or intracellularly in the upper cells of the cell layer (Fig. 7b). Intracellular diploccoci were not found. These results indicate that passage of N. gonorrhoeae occurred...
ough invasion into the epithelial cells, whereas paracytosis perked to be the major route employed by *H. influenzae*. With light microscopy and TEM analysis of slices of the cells after incubation with *H. influenzae* d1 at 28°C, clusters in intercellular spaces were still found. In contrast, no clusters were found after incubation of the cell layers with either *H. influenzae* d1 or 770235b"T" at 37°C in the presence of CAM (able 3), which did not inhibit cluster formation by the CAM-distant strain 2174.

**DISCUSSION**

We have developed an in vitro model to study the passage of *influenzae* through lung epithelial cell layers. In this model, the human epidermal lung epithelial cells formed layers with a thickness of one to three cells on filter supports. Although large intercellular spaces gave the cell layer an appearance of loosely connected cells, the cells were joined by many cell-to-cell contacts, such as tight junctions and desmosomes. This morphology is similar to that described previously for bronchial epithelial cells in primary culture (20). The [3H]ulin permeability and TER measurements showed that the cell layers were tight barriers, with a low TER compared with that of polarized monolayers of MDCK or Caco-2 epithelial cells that have been used in transcytosis studies of *Salmonella choleraeus* and *Escherichia coli* (2, 4). The decreased TER and increased paracellular flux of [3H]ulin after the addition of EDTA is indicative of the function of tight junctions in intercellular contacts.

TEM examination of thin sections of infected-cell layers revealed that *H. influenzae* bacteria passed predominantly between the lung epithelial cells. Occasionally, intracellular bacteria were seen, but these seemed to disintegrate. Since *N. gonorrhoeae* entered the epithelial cells and was not found between the epithelial cells, these data indicate that the passage route of *H. influenzae* through the cell layers of respiratory epithelial cells was specific for this bacterium. Our data are in line with those described previously in organ model studies of *H. influenzae* infection (3, 17). The passage route of *H. influenzae* seems to depend on the cell type used, since in human umbilical cord endothelial cells, *H. influenzae* was mainly taken up by the cells and translocated within vacuoles in the cells (27). Taken together, these results indicate that the route followed by the bacteria is influenced by the bacterial species and cell type.

Passage of *H. influenzae* from the apical to the basolateral side did not result in cell death, as determined by the trypan blue exclusion test, and also did not lead to increased [3H]ulin permeability. The permeability of the cell layer for [3H]ulin (an inert compound with a molecular weight of 5,200) generally provides a definition of the magnitude of the paracellular permeability. Since it has been shown that energy depletion of MDCK cells abolishes the gate function of their tight

<table>
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<th>TABLE 3. Effects of temperature and inhibition of bacterial protein synthesis on bacterial clustering in NCI-H292 cell layers</th>
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<tr>
<td><strong>H. influenzae</strong> strain</td>
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CAM was bacteriostatic at the concentrations used, except for *H. influenzae* 2174, which is resistant to concentrations up to 8 µg/ml.

Mean number of bacterial clusters per slice, as observed by light microscopic analysis of four slices. The absence of clusters after incubation with CAM was confirmed by TEM analysis.
junctions, thereby leading to an insulin flux (10), the lack of an insulin flux would also suggest that the cells are alive and energetically active (2, 10). Therefore, it was considered a sensitive assay for the integrity of the cell layer. The maintenance of the integrity of the epithelial cell layer during passage of *H. influenzae* is in contrast with the extensive damage observed in organ culture models after infection (14, 30). Such a damage, however, does not seem to reflect the in vivo situation, since Hors and Mulder (7) described subepithelial localization of *H. influenzae* while the epithelium was uninjured. In addition, the epithelial cell layer is not damaged during carriage with *H. influenzae*, although bacteria were observed in the subepithelial layers (5). Therefore, we are of the opinion that the para-cytosis model described in this paper is more representative for in vivo penetration of *H. influenzae* than the organ culture models.

The results of the permeability measurements and TLM analysis suggest that the bacteria penetrated the cell layers by a selective disclosure of the intercellular junctions. This concept is supported by the results of mixed-infection experiments. Inoculation of *H. influenzae* d1 4 to 6 h prior to inoculation of a streptomycin-resistant mutant did not result in accelerated passage of the streptomycin-resistant bacteria, indicating that during the first 6 h of incubation with *H. influenzae*, the epithelium was not activated such that the permeability for the *H. influenzae* strain added second increased. In addition, both cycloheximide and ansomycin had no effect on the passage when added in different concentrations. Sometimes, higher concentrations led to accelerated passage of *H. influenzae* through the cell layers, probably because of loss of cell layer integrity (data not shown). Since active translocation of *H. influenzae* bacteria through the cells would require cellular protein synthesis, it is unlikely that transcytosis was the main route for passage.

The time *H. influenzae* required to pass the epithelial cell layers appeared to be independent of the number of bacteria in the starting inoculum. Since it took 9 h before an inoculum of 10⁵ CFU of *H. influenzae* bacteria ml⁻¹ increased to 10⁹ CFU ml⁻¹, the absence of an inoculum effect indicates that passage required only a relatively small number of bacteria and started shortly after inoculation of the bacteria on the cell layers. Indeed, a very small number of bacteria was found to pass the epithelial cell layer during the first 16 to 18 h of incubation. By comparing the passage times of the nontypeable *H. influenzae* d1 and 1481 that differed in adherence characteristics, we showed that the passage time was independent of adherence to the cell layer. However, a comparison of the number of bacteria that passed through the cell layers per hour showed that more bacteria per hour passed the cell layers after inoculation with the adherent strain 1481 than after inoculation with the nonadherent strain d1. Since microscopic analysis of the cell layers after incubation also showed that many more bacteria were located in the paracellular spaces of the cell layer after inoculation with strain 1481 than after inoculation with strain d1, we suggest that adherence contributes to the degree of passage. This is probably important when the number of bacteria infecting the host is small. Furthermore, these data indicate that passage of *H. influenzae* bacteria is a result of paracytosis.

The passage time appeared to be independent of the presence of a capsule and/or fimbriae on the surface of the bacteria as obtained after comparison of the passage times of four variants of one strain that differed in expression of capsule and/or fimbriae. As described by St. Gemes III and Falkow (19), who observed that the expression of the capsule was switched off during *H. influenzae* adherence and invas of Chang epithelial cells, van Ham et al. (26) showed that synthesis of *H. influenzae* fimbriae is subject to phase variation at the transcriptional level. It is unlikely though that the pressure of the capsule and/or fimbriae was switched off during the passage experiments, since the bacteria recovered from basolateral compartment had the same phenotype as the bacteria inoculated on the apical side of the filter.

The passage time of *H. influenzae* appeared to be related to the growth rate of the bacteria, since slowly growing strains needed more time to pass through the cell layers than normally growing bacillar strains. The slower passage is likely due to lower protein synthesis rate of these bacteria, since both incubation temperature of 28°C and inhibition of bacterial protein synthesis at 37°C by the addition of CAM resulted in an increase in the passage time. We suggest that de novo bacterial protein synthesis is needed for passage through cell layers. This de novo synthesis is probably required penetration of *H. influenzae* between the cells and subsequently multiplication and passage, since CAM inhibited these processes. Earlier studies have shown that the expression of *H. influenzae* bacteria varied when the bacteria were grown under different conditions and that the expression of several proteins may be induced (18, 23). Identification the proteins that are involved in the interaction between *influenzae* bacteria and NCI-H1292 cells will be the focus of further investigations.

ACKNOWLEDGMENTS

We thank René Lutter for sharing the NCI-H1292 cell line, help with cell culture, and stimulating discussions; Anneke Nichol for help with preparation of the slides for light microscopy and TEM analyses; Wim van Est and Carls Graassmeyer for photographic assistance; Arije van den End for critically reading the manuscript.

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