Neutrophil Response to Nontypable Haemophilus influenzae in Respiratory Infections

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Abstract: Sputa from patients with respiratory infections by nontypable Haemophilus influenzae (H. influenzae) were investigated by electron microscopy. The cell wall of H. influenzae appeared wavy and nonwavy. In the cell wall the peptidoglycan layer was ill-defined. These patients had adequate IgG response in the serum against H. influenzae. However neither capsule nor fimbriae were found. Different stages of phagocytosis and destruction of the bacteria by polymorphonuclear neutrophils (PMN) were observed. PMNs were also found to phagocytose the debris. Evidences were found that the debris is formed mainly by the destruction of polymorphonuclear neutrophil. Extracellular lysosomes were also observed, which may have a role in destruction of both bacteria and host tissue. It was concluded that nontypable H. influenzae are nonfimbriated and noncapsulated during infection. Debris are the end product of PMN destruction, and phagocytosis of debris by PMNs has a role in the pathogenesis of chronic respiratory diseases.

Key words: Sputum, Electron microscopy, Neutrophil, Haemophilus influenzae

At present in Japan, the United States and Europe, the principal pathogen in respiratory infection is Haemophilus influenzae (16). Our experience shows that in adults almost all respiratory infections by H. influenzae are caused by nontypable strains. There are many effective antibiotics for the treatment of H. influenzae respiratory infections. However, the most difficult aspect is the recurrence of infections by H. influenzae. Heterogeneity in outer membrane protein (OMP) components is proposed to be very important for the escape of H. influenzae from antibody-dependent host defense (24).

Neutrophil and debris in sputum are excellent indicators of the status of bacterial infection of respiratory tract. Additionally, the presence of large amounts of cell debris suggests an accumulation of inflammatory cells in the respiratory tract for long time (16). Routinely we follow the disease course of chronic respiratory infections by inflammatory cells in sputum. Most of the studies concerning the pathogenesis of H. influenzae were done in vitro. Therefore the in vivo studies are important to obtain insight into the interplay between H. influenzae and the ineffective opsonophagocytic defense mechanisms of the host, despite the influx in the infection area of large number of granulocytes, especially during chronic and recurrent infections (24).

Bacterial adherence on the surface of oral mucosa has been regarded as an important factor for pathogenic bacteria to invade lower respiratory tract (14). Interestingly we found that 37-40% of children aged 4-6 years old are carriers of H. influenzae, and among these out of 87 strains of H. influenzae, 86 were nontypable and one strain was type b (23). In many species of bacteria, fimbriae are the mediator of adherence, while in others the capsule mediates the adherence (1). It is reported that all uncapsulated H. influenzae isolated from the upper respiratory tract are fimbriated, although the degree of fimbriation does not correlate with the ability to adhere to respiratory epithelial cells (5). While another report states that when fresh clinical

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; HTLV-1, human T-cell leukemia virus type 1; IgG, immunoglobulin G; OMP, outer membrane protein; PMN, polymorphonuclear neutrophil; RR, ruthenium red.
isolates of *H. influenzae* were observed under the electron microscope (EM), only three of the 15 strains of nontypable *H. influenzae* were fimbriated and the fimbriae were mainly in polar distribution (4). The culture condition has a significant effect on the expression of the surface structure of bacteria. As sputum originate from the site of infection, the observation of sputum under the EM may solve some controversies regarding fimbriation.

The expression of surface structures of bacteria during infection is very important to understand the process of attachment of the bacteria to epithelial cells and the initiation of infection. The interaction of PMNs with *H. influenzae* is important to know the host cell response during infection. This knowledge can be readily applied to the understanding of pathogenesis. This will further direct the subsequent means of experiments to design new preventive measures, treatment, and management of infections by *H. influenzae*. Therefore, in this study we observed the sputum of patients infected with *H. influenzae* under the EM, to know the expression of the surface structures of *H. influenzae* and the interaction of neutrophil with *H. influenzae* and the composition of sputum.

**Materials and Methods**

*Selection of sputum.* Expectorated sputa were collected from patients during the acute exacerbation of respiratory infections by *H. influenzae*. Only purulent sputum were selected for Gram-staining and quantitative culture to determine the acute exacerbation of infection by *H. influenzae*. From culture of sputum, *H. influenzae* were identified by standard methods (8). The serotyping was done by slide-agglutination test using Bacto-Haemophilus Influenzae Antiserum Poly (Difco Laboratories, Detroit, Mich., U.S.A.), according to the instructions of the manufacturer.

The criteria for respiratory infection by pathogenic bacteria are: (i) Gram-staining reveals many bacteria inside and outside the polymorphonuclear neutrophil (PMN); (ii) quantitative sputum culture reveals bacterial count equal to or more than $10^7$ cfu/ml; (iii) deterioration of patient's clinical condition (16). Four sputum specimens from four patients with acute exacerbation of chronic respiratory diseases by *H. influenzae* were used in this study. Three patients were male and one patient was female; ages ranged from 37 years to 82 years; their mean age was 59.3 years old. Two patients were suffering from bronchiectasis and chronic bronchitis, one from Kartagener's syndrome and the female patient was suffering from bronchiectasis, old pulmonary tuberculosis, chronic renal failure and she was human T-cell leukemia virus type 1 (HTLV-1) antibody positive. In this study all patients had adequate amount of serum immunoglobulin and cells. No patient received any known immunosuppressive drugs or radiation therapy.

**Determination of antibodies against H. influenzae in patients' serum.** Antibodies in patients' serum against *H. influenzae* were determined. The serum from patients was taken during the infection or at the convalescent stage. OMP of *H. influenzae*, isolated from each patient, was separated by the method of Barenkamp et al (6). Using these OMPs at a concentration of 20 µg/ml, ELISA was done by the method described by Karasic et al (15). Readings were monitored at a wavelength of 405 nm (Microelisa reader; Dynatech).

**Electron microscopy.** Only a portion of high quality purulent sputum was fixed in a solution of 2% glutaraldehyde and 0.01% calcium chloride in cacodylate buffer (0.1 M sodium cacodylate, pH 7.2). Then three times washing was done with cacodylate buffer by centrifugation at 450 × g for 15 min each time. Postfixation was done in 1% osmium tetroxide in cacodylate buffer for 1 hr at room temperature. The fixed specimens were dehydrated through graded ethanol solutions and finally in absolute acetone, then embedded in epoxy resin. Sections were cut with a Reichert Ultra Cut E and stained with uranyl acetate and lead citrate. All specimens were examined with a JEM 100CX electron microscope (JEOL Ltd., Tokyo) operated at 80 kV.

**Results**

**Results of Gram-Staining and Quantitative Culture of Sputum**

Gram-stained sputum smear showed plenty of gram-negative bacilli, characteristics suggestive of *H. influenzae*, inside and outside the PMN. *H. influenzae* are relatively smaller and slenderer than other gram-negative bacilli found in the sputum. Culture revealed $10^7$ cfu/ml or more *H. influenzae* in all sputum. Tested strains were nontypable capsular serotype of *H. influenzae* in slide-agglutination test. Table 1 shows patients' profiles and the result of sputum culture in detail.
Level of Antibodies against *H. influenzae* in Patients' Serum

All patients had high titer (1 : > 1,024) of serum IgG against *H. influenzae* isolated from the same patient or from other patient selected for this study.

Features of *H. influenzae* in Sputum

Many bacillus-shaped bacterial cells were observed in the thin-sectioned profiles of the sputum. Culture of sputum revealed 10^7 cfu/ml or more *H. influenzae*. This is one of the findings which proves the bacteria observed under the EM were *H. influenzae*. Both wavy (Fig. 1, patient # 2) and nonwavy (Fig. 2, patient # 3) pattern of cell wall were observed. A thin inner membrane and an outer membrane were observed. The peptidoglycan layer was ill-defined. Outside the cell wall, no thin granular electron-dense layer, suggestive of the capsule, was observed in the *H. influenzae*. Fimbriae were also not observed. Blebs were observed in some of the bacteria (Fig. 1).

Features of Polymorphonuclear Neutrophils (PMN) in Sputum

According to the level of section, different pattern of nucleus was observed. Inside the PMN, phagosome and lysosome were abundant. The nucleus, phagosome and lysosome were bound by membrane.

Different stages of phagocytosis were observed. In some phagosomes, the membrane was separated from the phagocytosed bacteria (Fig. 3, patient # 1) while in others the bacteria were in close contact.

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**Table 1. Patients' profiles with bacteria isolated from sputum**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Underlying diseases</th>
<th>Bacteria isolated from sputum (cfu/ml)</th>
</tr>
</thead>
</table>
| 1       | 82  | M   | Bronchiectasis, Chronic bronchitis | *H. influenzae* 1×10^9  
*α*-Streptococcus 3×10^7  
*Neisseria* spp. 1×10^7  
Gram (+) bacillus 3×10^6 |
| 2       | 66  | M   | Bronchiectasis, Chronic bronchitis | *H. influenzae* 4×10^9  
*α*-Streptococcus 2×10^4  
Gram (+) bacillus 3×10^3  
*Neisseria* spp. 4×10^4  
Micrococcus 2×10^4 |
| 3       | 37  | M   | Kartagener's syndrome | *H. influenzae* 8×10^8  
*α*-Streptococcus 2×10^6  
*B. catarrhalis* 1×10^6 |
| 4       | 52  | F   | Bronchiectasis, Old pulmonary tuberculosis, Chronic renal failure, HTLV-I antibody positive | *H. influenzae* 1×10^7  
*α*-Streptococcus 1×10^7  
*Neisseria* spp. 1×10^8  
Gram (+) bacillus 1×10^5 |

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**Fig. 1.** A thin section of *Haemophilus influenzae* in the sputum. The cell wall is wavy. The peptidoglycan layer is ill-defined. Structure suggestive of capsule is not seen. Arrowheads indicate cell wall associated and free blebs. Bar, 400 nm.

**Fig. 2.** A thin section of *Haemophilus influenzae* observed in the sputum. The cell wall is nonwavy. The peptidoglycan layer is ill-defined. Structure suggestive of capsule is not seen. Bar, 400 nm.
with the membrane (Figs. 4 and 5, patient #1). Different stages of destruction of bacteria were observed inside the phagosome. Lysosomes were found in contact with the cell wall of the bacteria. Inside the phagosome, bacteria with destroyed cell wall were observed (Fig. 6, patient #3). Therefore, it is assumed that the destruction of the bacterial cell by lysosomal enzymes started on the cell wall of *H. influenzae*. *H. influenzae* was found inside the nucleus of PMN (Fig. 7, patient #4). Round, membrane bound structures similar to lysosomes were found extracellularly (Fig. 8, patient #2).

The PMN were found also to phagocytose debris (Fig. 4). But the rate of phagocytosis of debris varied greatly among different sputum specimens. In some specimens, the phagosomes containing debris were fewer in number and smaller in size,

![Fig. 3. Polymorphonuclear neutrophil in the sputum. Arrowheads indicate phagocytosed *Haemophilus influenzae* inside the phagosome. The phagosomal membrane is separated from the bacteria. Bar, 2.5 μm.](image)

![Fig. 4. A section of polymorphonuclear neutrophil in the sputum. In the center there is a large phagosome containing phagocytosed debris. Phagocytosed *Haemophilus influenzae* are shown by arrowheads. The membrane is associated with the bacteria. Bar, 3 μm.](image)

![Fig. 5. Higher magnification of Fig. 4. A phagocytosed *Haemophilus influenzae* is seen, near the nucleus of the polymorphonuclear neutrophil. The bacteria is not in the vacuole of the phagosome. The phagosomal membrane is associated with the bacteria. Bar, 400 nm.](image)

![Fig. 6. A *Haemophilus influenzae* inside the phagosome. The activity of the lysosome started on the cell wall; as a result the cell wall is destroyed (indicated by arrowheads). Bar, 400 nm.](image)

![Fig. 7. Arrowhead indicates invasion of *Haemophilus influenzae* inside the nucleus of polymorphonuclear neutrophil. Bar, 2 μm.](image)
while in others, the phagosomes containing debris were greater in number and larger in size.

**Debris**

Debris was found in many different forms (Fig. 9, patient # 4). The structure of debris corresponded with the subcellular structures of PMN. By the destruction of PMN, debris was formed (Fig. 10, patient # 3). The amount of debris varied in different sputum specimens.

**Discussion**

In this study, we have examined the expression of surface structures of *H. influenzae* in sputum, the composition of sputum during acute exacerbation, and the interaction between the host and nontypable *H. influenzae*. Our experience showed that 98% of *H. influenzae* isolated from sputum of patients with respiratory infections are nontypable, suggesting that they do not express capsule. The strains of *H. influenzae* isolated in this study were also nontypable serotype by slide-agglutination method. There is much controversy about the capsule of *H. influenzae*. It was found that capsulated strains of *H. influenzae* type b spontaneously mutate in vitro to nontypable form (18). On the other hand, it is interesting that DNA from a capsulated strain could transform a noncapsulated strain into the serotype of the donor (3). In the case of EM observation, capsular material must be fixed either with ruthenium red (RR) or anticapsular antibody (7). The patients in this study had no deficiency of immunoglobulin in the serum. Moreover, the serum of affected patients had an adequate immunoglobulin response against *H. influenzae*. However, in this study, structure suggestive of capsule of *H. influenzae* was not found in the sputum. Gyorkey et al also reported that *H. influenzae* are unencapsulated in sputum by using RR (12). This is consistent with our result. By the same method used in the present study, structures suggestive of capsule were observed in *Pseudomonas aeruginosa* by the EM observation of sputum (2).

It is reported that nontypable *H. influenzae* has undulant cell wall (12). However, we could find both wavy and nonwavy cell wall; this difference among bacteria might be due to the different phases of development of *H. influenzae* in the sputum. In the cell wall of *H. influenzae*, the peptidoglycan layer was not observed under the magnification used in this study. Reyn and Birch-Andersen were
unable to visualize a typical peptidoglycan layer in *H. influenzae* (20). However, another report stated that a peptidoglycan layer was rarely seen using different preparative procedures (10). The visualization of the peptidoglycan layer in *H. influenzae* must have some relation with the method used.

In this study, fimbriae were not observed in any strain of nontypable *H. influenzae*. It is necessary to mention here that in this study only a thin section was viewed. If fimbriae are few in number, they may not be apparent by this method. Fimbriae may be observed more clearly by negative stain. It is reported that fimbriated nontypable *H. influenzae* did not facilitate attachment to respiratory epithelium (19). On the other hand, nonfimbriated *H. influenzae* could adhere and invade into cultured epithelial cells (22). When fresh clinical isolates of *H. influenzae* were observed by EM, only three of the 15 strains of nontypable *H. influenzae* were found to be fimbriated and the fimbriae were primarily polar distribution (4). The importance of fimbriae during infection by nontypable *H. influenzae* needs further investigation.

The main task for the phagocytic cells is to eradicate the invading microbes, either by extracellular or intracellular killing (17). If the bacteria interacting with the phagocytes resist ingestion and killing, a sustained activation with release and leakage of toxic oxygen metabolites, lysosomal enzymes and inflammatory mediators will occur (17). We could provide here the evidence that structures suggestive of lysosome were found extracellularly with intact membrane. No evidence was found whether these lysosomes were released from the PMN to kill *H. influenzae* extracellularly or were released after the disintegration of PMN. As the lysosomes were within the vesicle, they can release enzymes after contact and can kill bacteria, as well as cause host cell damage. In the respiratory tract, host tissues are relatively more abundant than bacteria. Therefore, the probability to cause host tissue destruction is more than that of killing bacteria by these extracellular lysosomes. It is a very important evidence that the tissue damage occurs in patients with chronic respiratory diseases, often with repeated infections.

In 1953, Hers and Mulder demonstrated by light microscopy the presence of nontypable *H. influenzae* in epithelium and submucosa of bronchi and intralobular bronchioles of patients with chronic and acute bronchitis (13). Moreover, the invasion by nontypable *H. influenzae* to cultured epithelial cells has been demonstrated by EM (22). Another study showed both typable and nontypable *H. influenzae* could enter the epithelial layer of monkey respiratory tissue in organ culture (21). All these papers described the ability of *H. influenzae* to invade the epithelium and submucosa. However, we could observe *H. influenzae* inside the nucleus of the PMN. The presence of *H. influenzae* inside the nucleus may have some relation to its ability to escape killing by the PMN and persists during infection.

The structure of debris is similar to the subcellular structures of PMN. We showed here the evidence that debris is mainly formed by the destruction of PMN. In the sputum of *H. influenzae*, debris formation and phagocytosis of debris varied among sputum samples. It was found that when the debris increases in the sputum, PMN with phagocytosed debris also increase. We can speculate that if debris formation is increased, phagocytosis of debris by PMN is also increased. In other words, the number of PMN destruction varies during infection by *H. influenzae*.

The observation of blebs indicates that the bacteria are in an active physiologic state. In many bacteria, endotoxin is released in the form of blebs (9). Moreover, the DNAs of *Neisseria gonorrhoeae* were associated with blebs (11). Interestingly, export and intercellular transfer of DNA also occurred via the blebs (11). Further investigations are necessary to elucidate the role of blebs in the pathogenesis of infections by *H. influenzae*.

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References


