Structural Features of Tracheal Tube Biofilm Formed During Prolonged Mechanical Ventilation*

Tim J.J. Inglis, BM, DM; Lim Tit-Meng, BSc, PhD; Ng Mah-Lee, BSc (Hons), PhD; Tang Ee-Koon; and Hui Kok-Pheng, MB, BS

The dissemination of tracheal tube biofilm into the mechanically ventilated lung has been proposed as a contributory factor in the pathogenesis of ventilator-associated pneumonia. In the present study, conventional light microscopy, confocal laser scanning microscopy, and scanning electron microscopy were used to examine luminal tracheal tube biofilm in tubes from ten consecutive medical intensive care patients. Biofilms also were cultured. No tube contained a predominantly microbial aggregate. Microorganisms were either dispersed throughout the biofilm or restricted to the most superficial layer. Neutrophil polymorphonuclear cells were present in all biofilms in a pattern suggesting that a layering or stratification had taken place. The distribution of neutrophils and microorganisms was consistent with a progressive accretion of respiratory secretions, rather than formation of a predominantly microbial biofilm.

(CHEST 1995; 108:1049-52)

Key words: biofilm; confocal laser scanning microscopy; nosocomial pneumonia

Members of the group at greatest risk of nosocomial pneumonia are critically ill patients who require mechanical ventilation.1 The presence of an endotracheal tube is thought to assist the aspiration of bacteria from the oropharynx and stomach into the trachea and assist bacterial adhesion to the mucosal surface. In this way, the tube thus contributes to the eventual colonization of the ventilated lung.2-4 Bacterial colonization of the inner surface of tracheal tubes has been examined after prolonged use in the critically ill, and dislodgement of this biofilm layer during suction catheterization has been proposed as an etiological factor in nosocomial pneumonia.5 In another study, it was shown that downwind dissemination of tracheal tube biofilm could occur as a result of ventilator gas flow.6 These, and other studies, have considered the putative relationship between tracheal tube biofilm and ventilator-associated pneumonia as a purely bacteriologic phenomenon. Observations made during a recent study, however, suggested that the biofilm lining tracheal tubes from critically ill patients might form as a result of deposition of respiratory secretions during suction catheterization.7

In the current study, conventional light, scanning electron, and confocal laser scanning microscopy were combined with bacterial culture to investigate the properties of luminal biofilm from a series of tracheal tubes.

METHODS

Patients and Specimens

Tracheal tubes were collected at the time of extubation from consecutive adult medical intensive care patients. Each tube was placed in a large, prelabelled, self-seal polyethylene bag and immediately sent to the diagnostic laboratory. Samples for microscopy and culture were taken from the level of the inflatable cuff, the region of greatest biofilm accumulation.7 Clinical data were obtained after completion of laboratory investigations.

Bacterial Culture and Identification

On receipt, the outer tracheal tube surface was cleaned with cotton wool moistened in 90% ethyl alcohol (Tracheal tubes arriving in the laboratory after hours were stored at 0 to 4°C without opening the seal). A sterile 15-μL nichrome bacteriologic loop was inserted into the lower end of the tube and run around the inner circumference at the level of the lower margin of the inflatable cuff. Biofilm sampled in this way was inoculated onto fresh blood agar (5% horse red blood cells in blood agar base; Becton Dickinson, Australia) and hemolysed blood agar (Becton Dickinson, Australia), and incubated in 5% CO2 for 18 h at 37°C. Organisms isolated from the biofilm were identified according to standard laboratory procedures.8

Light Microscopy

A sterile, dry cotton swab was run around the inner circumference of the tube immediately adjacent to the culture sampling point. Biofilm was then smeared gently onto a glass microscope slide in a single smooth motion and stained using a standard Gram stain method.9 The stained specimen was preserved under a mounting agent (De Pe X, Sigma, United Kingdom) and a cover glass.

*From the Departments of Microbiology (Drs. Inglis, Mah-Lee, and Ee-Koon), Zoology (Dr. Tit-Meng), and Medicine (Dr. Kok-Pheng), National University of Singapore, Singapore.

Manuscript received October 28, 1994; revision accepted January 27, 1995.
Table 1—Contents of Tracheal Tube Biofilm by Duration of Intubation

<table>
<thead>
<tr>
<th>Reason for ICU Admission</th>
<th>Duration of Intubation</th>
<th>Neutrophils</th>
<th>Organisms Isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary embolism</td>
<td>12 h</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Exacerbation COPD</td>
<td>1 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Status asthmaticus</td>
<td>3 d</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>4 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ascending cholangitis</td>
<td>7 d</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Viral encephalitis</td>
<td>7 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Severe salmonella colitis</td>
<td>9 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pulmonary hemorrhage</td>
<td>10 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Renal failure, sepsis</td>
<td>14 d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acute renal failure, collapse</td>
<td>17 d</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy

A 1-cm ring of tracheal tube was cut from the tube at the upper margin of the inflatable cuff with a fresh scalpel blade. The ring of tube was immersed in fresh 2% glutaraldehyde overnight. Later, it was cut longitudinally into two segments, one for electron microscopy and the other for confocal laser scanning microscopy. The former was prepared by critical point drying and gold sputter coated.1011

All the prepared specimens were viewed under the same scanning electron microscope (model 515, Philips; the Netherlands) by the same pair of operators. First, the entire specimen was scanned at low power. The edges of the biofilm and cracks or fissures on its surface were examined at progressively higher power in a systematic search for either single or clustered microorganisms. Each specimen was examined for 30 to 60 min.

Confocal Laser Scanning Microscopy

The glutaraldehyde-fixed tube segment was cut into smaller strips of about 3 mm in width with a razor blade and stained in 0.0025% acridine orange (Sigma, United Kingdom) solution for 15 min. Each strip was then mounted whole on a concave glass slide, the inner surface uppermost, protected by a plasticine-supported cover glass. To view the tube/biofilm interface, some strips were cut and mounted on one of their cut edges. Specimens were scanned under an MRC500 confocal laser scanning microscope (BioRad, United Kingdom), using the 488-nm (blue) excitation at 1 or 3% transmittance. Fluorescent images were recorded digitally using a microcomputer for analysis and later photographed with a Polaroid freeze-frame camera.

Results

Cuffed, oral endotracheal tubes from ten critically ill patients were studied (Table 1). All ten were found to have some luminal biofilm, although there was insufficient material for light microscopy in two cases. A range of microbial species was isolated from biofilms. Half of the tubes yielded more than one microbial species, and only two tubes were found to contain no viable microorganisms. No biofilm specimen in this series was found to contain Klebsiella pneumoniae, other Enterobacteriaceae, or Pseudomonas species despite the fact that these are common nosocomial pathogens in many ICUs.11 However, Candida albicans and Acinetobacter species, species commonly associated with nosocomial infections in our center, were both isolated from several tubes in use for 3 days or more.

Light Microscopy

Clumps or aggregates of bacteria were rarely seen in the material examined by light microscopy. Much more common was a random scatter of individual organisms and neutrophil polymorphonuclear cells throughout a fuchsin-stained matrix (Fig 1). Neutroph-
were Gram-stained biofilms. These preparations were identical to Gram-stained specimens of lower respiratory tract secretions.

**Scanning Electron Microscopy**

The effects of critical point drying were evident under low-power scanning electron microscope views. All biofilm specimens had an extensive network of cracks or fissures, and in most cases the biofilm was reduced to a layer of flakes. Nothing resembling a bacterial cell was observed on the luminal surface of any biofilm specimen; however, bacteria were occasionally seen at higher power in fissures. Clusters of bacterial cells were seen on only one occasion and they were partly obscured by surface charging.

**Confocal Laser Scanning Microscopy**

By using a confocal laser scanning microscope, it was possible to examine biofilms without significant prior dehydration, even when they were only a few cells thick. At lower powers (eg, ×100), a layering or stratification was seen (Fig 2), which at higher power (×1,000) was seen to be caused by variations in the density of nucleated cells. Eukaryotic cells, some clearly recognizable as polymorphonuclear cells (Fig 3), were present throughout all ten biofilm specimens. Smaller lucent bodies of around 1-5 μm, consistent with bacteria, were also scattered throughout most of the specimens. Bacteria were restricted to the uppermost biofilm layer in one specimen. There was evidence of microbial aggregation in only one sample. The densely packed microbial consortia expected from the results of previous studies were not observed. However, one specimen taken from the longest used tracheal tube comprised a densely packed layer of effete nucleated cells (Fig 4). *Flavobacterium meningosepticum*, coagulase-negative staphylococci, *Mora-

**xella catarrhalis*, and *C albicans* all were isolated in the culture from the same tracheal tube.

**Discussion**

In this study, the structural features of tracheal tube biofilm were examined using a series of microscopic techniques and culture. It was suggested previously that tracheal tube biofilm might be formed as a result of an accumulation of respiratory secretions deposited on the inside of the tracheal tube during the withdrawal of successive tracheal suction catheters. The similarities between Gram-stained tracheal tube biofilm and respiratory tract secretions on light microscopy support this view. However, conventional light microscopy does not allow *in situ* examination of either the surface or deeper layers of tracheal tube biofilm.

In earlier studies, scanning electron microscopy has been used to obtain views of tracheal tube biofilm. If, as just suggested, the biofilm is composed of predominantly respiratory secretions, a water content of approximately 90 to 95% would be expected. During critical point drying, a substantial proportion of the volume of tracheal tube biofilm would have been removed in preparation for scanning electron microscopy. Dehydration would have collapsed the luminal surface of the biofilm onto underlying solids and destroyed the structural architecture of the biofilm layer and any nucleated cells it might have contained. It is not surprising, then, that scanning electron microscopy has tended to emphasize the bacterial component of the biofilm. In at least one of the previous studies, the reported location of the bacteria was in cracks or fissures in the biofilm. In the present study, very few aggregates of bacteria were observed despite a more systematic search of each specimen.

Confocal laser scanning microscopy is a novel form of image analysis-augmented microscopy that enables the study of intact biofilms. By cross-sectional views
and optical slicing, it was possible to examine the deeper layers of biofilm, including the biofilm-tracheal tube interface, in their original state. As in the Gram-stained material, nucleated cells were found in all specimens. Where identifiable, these cells were polymorphonuclear. Only rarely were epithelial cells found. The layering or stratification of the biofilm is yet another observation that supports the formation of this material by progressive accretion of respiratory secretions during suction catheterization. The random scattering of single microorganisms throughout most biofilms is not consistent with a predominantly microbial origin for tracheal tube biofilm.

The distribution of bacteria throughout the biofilm layer, rather than in dense microbial aggregates, means that the material scattered into the lungs during mechanical ventilation is unlikely to contain an aerosol of bacterial microaggregates. However, material disseminated from the lower end of the tracheal tube as a result of ventilator gas flow over the luminal biofilm surface cannot be an aerosol of monodispersed bacteria, since neutrophils, their contents, and respiratory mucus also may be present. It remains to be shown whether aerosolization of tracheal tube biofilm poses an increased risk of pulmonary damage.

The present investigation of the structure and cellular content of tracheal tube biofilm has resulted in several unexpected findings, most notably the occurrence of human inflammatory cells in the biofilm and the absence of extensive microbial aggregates. Recognition of the prominence of host-derived components of tracheal tube biofilm raises questions regarding their role in the pathogenesis of ventilator-associated pneumonia. An investigation of the relationship between the composition of tracheal tube biofilm and the clinical consequences of biofilm dissemination into the ventilated lung is now required.

ACKNOWLEDGMENTS: We gratefully acknowledge the assistance of the staff of the Medical Intensive Care Unit, National University Hospital, Singapore.

REFERENCES