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Comparison of *Haemophilus influenzae* Type b Interaction with Respiratory Mucosa Organ Cultures Maintained with an Air Interface or Immersed in Medium

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Haemophilus influenzae **type b infection of immersed and air interface organ cultures for 24 h caused significant epithelial damage. Bacterial association with mucus, damaged epithelium, and unciliated cells was significantly higher in air interface than immersed organ cultures, and total bacterial association was 55.8 times greater. Bacteria exhibited tropism for mucus only on explants maintained with an air interface. We conclude that immersion of nasopharyngeal tissue in medium may influence bacterial interaction with the mucosal surface.**

Bacterial association with the respiratory mucosa is considered to be an important event in the development of invasive and noninvasive disease states (2, 11), and organ cultures utilizing animal or human tissue provide an important tool with which to study it. However, respiratory tissue organ cultures immersed in medium are unphysiological because they lack the air interface present in vivo and the tissue is continuously exposed to an autonomous replicating bacterial population. Furthermore, bacteria (3, 20) and respiratory cells (9, 19) may alter their surface composition in response to environmental factors, and the pH (12) and ionic composition (8) of the microenvironment can influence the interaction of bacteria with the mucosal surface. We have examined the interaction between *Haemophilus influenzae* type b (Hib) strain Eagan (RM153) and human nasal turbinate tissue organ cultures to determine whether it is influenced by immersion of tissue in culture medium.

Immersed (15) and air interface (18) organ cultures were prepared according to previously published methods, except that 4 ml of medium was used in both models. Control and experimental immersed and air interface organ cultures in each experiment were prepared from tissue resected from a single patient, and each experiment utilized tissue from a different patient. RM153 was cultured overnight on Levinthal agar, inoculated into 5 ml of brain heart infusion broth supplemented with NAD (10 μg ml^{-1}; Sigma, Poole, Dorset, United Kingdom) and hemin (5 μg ml^{-1}; Sigma), and then incubated for 8 h at 37°C in 5% CO_2. Bacteria were centrifuged at $2,000 \times g$, washed twice in 10 ml of phosphate-buffered saline (PBS) (Oxoid, Basingstoke, United Kingdom), and resuspended in 150 μl of PBS. Viable counts were estimated in triplicate. Two microliters of this stock containing 1.0×10^7 to 2.6×10^7 CFU was inoculated gently onto the tissue surface of air interface organ cultures and into the medium directly overlying the tissue in immersed organ cultures. After infection for 24 h at 37°C in a humidified atmosphere containing 5% CO_2, counts of viable bacteria were conducted on immersed organ cultures, and all organ cultures were examined for microbial contamination (15, 18). When contamination occurred, the entire experiment was discarded. Tissue was removed from the organ cultures along with the surrounding agar or the filter paper wick, washed gently in PBS, and prepared for scanning electron microscopy (SEM) by standard techniques (15, 18).

The percentages of the tissue surface occupied by mucus, damaged epithelium, ciliated cells, and unciliated cells were estimated by SEM with a counting system described previously (13). The category of damaged epithelium included extruding cells, cell debris, ruptured cells, basal cells, and basement membrane. The number of bacteria associated with each mucosal feature was assessed at a magnification of $\times$13,750. Bacterial densities were calculated by dividing the total number of bacteria on each feature by the percentage of the mucosal surface occupied by that feature. Statistical comparisons were made by using the Wilcoxon signed ranks paired test. Differences were considered significant when P was < 0.05. All specimens were coded and randomized prior to analysis.

The percentage of the organ culture surface occupied by each mucosal feature is shown in Table 1. There were lower levels of mucus on immersed organ cultures than on air interface organ cultures, possibly due to loss into the bathing medium (1) and higher levels of unciliated cells. Infection with Hib for 24 h was associated with a decrease in the percentage of the tissue surface exhibiting mucus, whereas other reports have suggested that infection would increase mucus production (1). It is possible that Hib may have released factors which modified the mucus making it less stable during processing (17).

Infection caused epithelial damage in both organ culture models, and these results are consistent with previous studies (5, 6, 14). The number of ciliated cells on the tissue surface decreased significantly in infected immersed but not air interface organ cultures. *H. influenzae* releases cytotoxic products as it grows (4), which might be responsible for the observed epithelial damage. We have previously found that without replenishment of nutrients, the ciliated cells of an air interface organ culture maintained for 10 days were most susceptible to ultrastructural disruption (8a). In the present study the large population of Hib replicating in the culture fluid of the immersed organ cultures may have placed the tissue under conditions of nutritional stress, which may have increased the susceptibility of the more metabolically active ciliated cells to bacterial products.

Bacterial numbers in the medium of the immersed organ

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TABLE 1. SEM of human nasal turbinate tissue in immersed and air interface organ cultures following infection with Hib[a]

Type of organ culture	% Organ culture occupied by:			
	Mucus	Damaged epithelium	Ciliated cells	Unciliated cells
Immersed				
Control	28.1 ± 9.9	3.9 ± 1.2	15.9 ± 5.1	52.1 ± 4.9*
Infected	18.3 ± 5.8	18.5 ± 4.6‡	7.9 ± 3.3§	55.3 ± 7.6*
Air interface				
Control	65.9 ± 7.1‖	4.3 ± 1.1	11.1 ± 3.5	18.7 ± 6.5
Infected	40.8 ± 9.1§	14.5 ± 3.8§	12.3 ± 3.6	32.3 ± 6.9

[a] Data are means ± standard errors of the means of 14 experiments. Each experiment utilized tissue from a different patient. Immersed and air interface organ cultures were constructed from tissue from the same patient. Tissue was infected with Hib for 24 h. Significant differences between results were as follows. *, air interface versus immersed organ culture ($P < 0.05$). ‡, infected versus control organ cultures ($P < 0.005$). §, infected versus control organ cultures ($P < 0.05$). ‖, air interface versus immersed organ culture ($P < 0.005$).

cultures increased from $(4.5 \pm 1.9) \times 10^6$ CFU ml^{-1} at time zero to $(4.7 \pm 2.6) \times 10^8$ CFU ml^{-1} at 24 h. In the two experiments examined, bacterial numbers reached 1×10^8 and 2.3×10^8 CFU ml^{-1}, respectively, after 6 h. No correlation was observed between total numbers of bacteria associated with the mucosal surface and bacterial numbers in the inoculating dose or in the culture fluid of immersed organ cultures after 24 h of infection. Bacterial association with the mucosal surface of immersed explants was rare, in agreement with previous studies (5, 10, 14), and there was no significant difference between bacterial densities on mucus, damaged epithelium, ciliated cells, and unciliated cells (Table 2). We have found that an inoculating dose containing 10^6 CFU of Hib was the minimum required for consistent infection of the air interface organ culture model. For bacterial numbers above this critical inoculating dose, no correlation was observed between overall bacterial density on the mucosal surface of air interface organ cultures and the number of bacteria in the inoculating dose. Significantly greater Hib densities were associated with mucus, damaged epithelium, and unciliated epithelial cells on explants maintained with an air interface than with these respective mucosal features on tissue immersed in culture medium (Table 2). Bacterial association with ciliated cells was rare in both models. The large numbers of Hib organisms observed on the air interface organ cultures were predominantly associated with mucus and less frequently associated with damaged epithelium and unciliated cells. The total bacterial density on the mucosal surface of the air interface organ cultures was 55.8 times greater than on immersed organ cultures. Where bacteria were associated with apparently healthy unciliated cells, they were present predominantly at or near cell junctions. Bacteria appeared to penetrate deep between separated epithelial cells and in cases of severe separation were observed on the underlying basal cells and collagen matrix. These features were substantially more prominent on tissue maintained with an air interface than on immersed tissue.

TABLE 2. SEM of the density of Hib on nasal turbinate tissue in immersed and air interface organ cultures[a]

Type of organ culture	Bacterial density			
	Mucus (n = 14)	Damaged epithelium (n = 14)	Ciliated cells (n = 11)	Unciliated cells
Immersed	0.3 ± 0.2	1.6 ± 0.8	0.6 ± 0.6	0.7 ± 0.5
Air interface	88.1 ± 32.7*	29.3 ± 20.2‡§	0.25 ± 0.15§‖¶	5.2 ± 1.6‡§

[a] Data are means ± standard errors of the means of the densities of bacteria associated with each mucosal feature. Data were obtained in 14 experiments; data for ciliated cells were recorded in 11, and data for unciliated cells for the air interface were recorded in 13. Bacterial densities were calculated as described in the text. Each experiment utilized tissue from a different patient. Immersed and air interface organ cultures were constructed from tissue from the same patient. Tissue was infected with Hib for 24 h. Significant differences between results were as follows. *, air interface versus immersed organ culture ($P < 0.005$). ‡, air interface versus immersed organ culture ($P < 0.05$). §, mucosal feature versus mucus ($P < 0.05$). ‖, ciliated cells versus damaged epithelium ($P < 0.05$). ¶, ciliated cells versus unciliated cells ($P < 0.05$).

The present study, together with previous work, suggests that mucus is important in the interaction of Hib and other respiratory pathogens with the respiratory mucosal surface (7, 16, 18). Our results show that immersion of respiratory tissue during experimental infection can substantially influence the results obtained.

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