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BACTERIAL INTERACTIONS WITH HUMAN RESPIRATORY MUCOSA *IN VITRO*

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SUMMARY

The theme of this thesis is to study the interactions of non-typable *Haemophilus influenzae* (NTHi) and *Pseudomonas aeruginosa* (PA) with intact human respiratory mucosa *in vitro*.

Recent evidence suggests that bacteria are mainly associated with respiratory mucus during exacerbation of chronic bronchitis but penetration of antibiotics into respiratory mucus is generally poor. A study was therefore performed to evaluate the effects of 0.25 and 0.5 minimal inhibitory concentrations of amoxycillin, loracarbef (a new carbacephem) and ciprofloxacin on NTHi infection of adenoid organ cultures in an agar-embedded model in which only the intact respiratory mucosa was exposed to bacteria-containing culture medium. Incubation for 24h at 37^0C in an atmosphere that contained 5% CO_2 then followed. Light and transmission electron microscopy assessment was performed to evaluate the function (ciliary beat frequency) and ultrastructure of the respiratory mucosa respectively at 0 and 24h. NTHi infection of adenoid organ cultures that did not contain antibiotics caused significant ultrastructural damage and slowing of ciliary beat compared with uninfected organ cultures. In the presence of 0.25 and 0.5 minimal inhibitory concentrations of antibiotics (amoxycillin, loracarbef and ciprofloxacin), the ultrastructural damage was reduced significantly and slowing of ciliary beat did not occur with NTHi infection. The results from this study may help explain the clinical efficacy of antibiotics in treatment of bronchial infection despite poor antibiotic penetration into respiratory secretions. By using the same organ culture model the effects of NTHi infection of intact human bronchial mucosa was also studied. NTHi infection of bronchial organ cultures was associated with ultrastructural damage compared with uninfected organ cultures after 24h incubation. This damage was similar to the pattern observed in adenoid organ cultures described earlier.

Previous organ culture methods have used bacteria-containing medium to

immerse respiratory tissue which is unphysiological. A new organ culture model of intact human respiratory mucosa with an air-mucosal interface was therefore developed using adenoid and nasal turbinate tissue. The mucosal surface was directly exposed to warm and humidified air and nutrients were supplied to the submucosal aspects of the tissue by a strip of filter paper whose ends were immersed in sterile minimal essential medium. The cut edges of the adenoid or bronchial mucosa were sealed with agar. Bacterial suspension or toxin could be applied directly onto the mucosal surface and incubation was performed at 37^0C in a humidified atmosphere that contained 5% CO_2. This organ culture model was able to maintain normal epithelial ultrastructure and function (ciliary beat frequency) for at least 24h.

By using adenoid organ cultures with an air-mucosal interface, NTHi was found to cause mild mitochondrial damage after 24h incubation *in vitro*. A new scanning electron assessment method was developed which was used to evaluate the mucosal surface morphology and bacterial adherence quantitatively. By using this method, NTHi was found to adhere to unciliated cells, mucus, cell debris and extruded cells in preference to ciliated cells. Bacterial microcolonies increased in number and size with longer incubation. NTHi infection also caused an increase in extruded cells and cell debris on the mucosal surface of adenoid organ cultures. Similar experiments using nasal turbinate tissue showed virtually no adherence of NTHi to nasal respiratory mucosa suggesting that there may be a difference in epithelial surface receptors for NTHi between adenoid and nasal turbinate mucosa.

Infection of adenoid organ cultures with an air-mucosal interface by PA caused significant ultrastructural damage (mitochondrial damage, loss of cilia, cytoplasmic blebbing and extrusion of cells from the epithelial surface) and slowing of ciliary beat when assessed by transmission electron and light microscopy respectively after 8h incubation. PA was found to cause disruption of epithelial tight junctions and adhere to basement membrane collagen. A matrix-like

material was probably produced by PA which bridged PA with respiratory mucosa and might therefore be a PA adhesin. PA formed bacterial biofilms on the surface of respiratory mucosa that might have hindered its removal by the mucociliary clearance mechanism. These findings might help explain the difficulty in eradicating PA from the lower respiratory tract of patients with cystic fibrosis and bronchiectasis.

The organ culture model with an air-mucosal interface was also used to study the effects of a bacterial toxin on intact human respiratory mucosa. An exotoxin of PA, pyocyanin was found to cause significant mucosal damage to adenoid organ cultures when assessed by transmission electron microscopy. Moreover, by using a newly developed transmission electron microscopy method to assess orientation of central microtubules of cilia and foot processes, pyocyanin was found to cause significant disorientation of the central microtubules of respiratory cilia but not the foot processes. PA pyocyanin may therefore have a role in the pathogenesis of PA infection *in vivo*.

By using these organ culture models of intact human respiratory mucosa, bacteria interactions with human respiratory mucosa can be studied using the transmission, scanning electron, and light microscopy methods described in this thesis. Potential virulent factors for NTHi and PA can be tested and the mechanisms of bacterial pathogenesis can be studied further to advance current understanding of bacterial interactions with the human respiratory tract mucosa that may lead to the development of novel therapies.

ABBREVIATIONS

AMO	amoxycillin
BHI	brain heart infusion
CBF	ciliary beat frequency
CF	cystic fibrosis
CFU	colony forming unit
CIP	ciprofloxacin
CO_2	carbon dioxide
g	gram
h	hour
HDM	Herriott's defined medium
Hib	*Haemophilus influenzae* type b
HPLC	high performance liquid chromatography
Ig	immunoglobulin
LC	loracarbef
NTHi	non-typable *Haemophilus influenzae*
PA	*Pseudomonas aeruginosa*
PBS	phosphate buffered saline
PCD	primary ciliary dyskinesia
l	litre
MEM	minimal essential medium
MIC	minimal inhibitory concentration
min	minute
ml	millilitre
n	number
SD	standard deviation
SE	standard error of the mean
SEM	scanning electron microscopy
TEM	transmission electron microscopy
μg	microgram
μl	microlitre

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INTRODUCTION

1.1 HOST DEFENCE MECHANISMS IN THE RESPIRATORY TRACT

The human respiratory tract constitutes a major interface between the environment and the internal body. During respiration, noxious agents, including respiratory pathogens, may be deposited on the mucosal surface of the airways or may even penetrate deeper into the lung parenchyma. Inhaled particles encounter a highly integrated system of defence that is designed to prevent injury, infection, and invasion of host tissue. In healthy individuals, integrated defence mechanisms that include mechanical, phagocytic and immune systems maintain the virtual sterility of the respiratory tract from trachea to alveolar spaces. However, only the mucociliary clearance mechanism, which is relevant to the studies described in this thesis, is discussed in this chapter.

1.1.1 MUCOCILIARY CLEARANCE

Mucociliary clearance is one of the most important defence mechanisms in the respiratory tract. The majority of the tracheobronchial tree is swept clean by this system which extends from the larynx proximally to the sixteenth bronchial division distally (Leeson et al., 1988). Mucociliary clearance acts as an escalator to convey a flow of mucus towards the oropharynx where it is swallowed. This mechanism operates continuously and is non-specific.

In healthy subjects, mucociliary clearance is enhanced by performing brisk exercise, and inhalation of ß-adrenergic agonists and hypertonic saline but reduced on exposure to sulphuric acid mist and cigarette smoke. The efficiency of the mucociliary clearance system depends on the integrity of ciliated epithelium, ciliary beat frequency (CBF), co-ordination of ciliary beat, and the viscoelasticity of respiratory mucus (Wanner 1977; Wilson et al., 1987). It is impaired in patients with chronic bronchitis which may be secondary to a presence of excessive secretion, an increase in mucus viscosity, a reduction in CBF, a partial loss of ciliated epithelium, or a combination of these factors (Wanner 1977). Bacterial products such as exotoxins produced

by NTHi and PA pyocyanin cause slowing of human CBF *in vitro* (Wilson et al, 1987) and guinea pig tracheal mucus transport *in vivo* (Munro et al., 1989). This slowing may reduce the efficiency of mucociliary escalator and may lead to harbouring of bacteria in the respiratory tract.

1.1.2 STRUCTURE AND FUNCTION OF HUMAN RESPIRATORY CILIATED EPITHELIUM

Each ciliated cell has approximately 200 cilia which beat at 12-17 Hz (Leeson et al., 1988). Ciliary beat commences with an active stroke towards the oropharynx, followed by an inactive recovery stroke. The ultrastructure of a cilium is conserved in all species. The shaft consists of longitudinal fibrils in a cytoplasmic matrix. There are nine pairs of outer fibrils and two inner ones that are specialised protein microtubules. Each of the peripheral nine pairs consists of one complete and one partial microtubule. Adjacent pairs are connected by nexin links, while the outer fibrils are connected to the central pair by central spokes. From each of the complete outer microtubules two dynein arms that contain ATPase extend towards the adjacent pair (Figure 1.1). Each cilium is attached to a basal body that has a triangular spur, or foot process (Sorokin 1988) (Figure 1.2). Bending of cilia is due to an active sliding movement between adjacent pairs of microtubules and is powered by an ATP-dependent process (Leeson et al., 1988). Cilia of neighbouring cells beat synchronously resulting in metachronism and the propagation of a wave over the mucosal surface (Wanner 1977). Although nerves have been observed near ciliated cells, respiratory cilia are not under direct nervous control (Sorokin 1988).

Primary ciliary dyskinesia (PCD) is a syndrome that embraces patients who have chronic rhino-sinusitis and bronchiectasis. Some of these patients also have situs inversus (Kartagener syndrome). These patients generally have no or insignificant mucociliary transport, immotile or dyskinetic airway cilia, and abnormal ciliary ultrastructure such as a lack of dynein arms, radial spoke defects,

and transposition of the double microtubules (Nielsen et al., 1983). Defects of the inner or outer dynein arms (or both) are responsible for the impaired ciliary motility in most of these patients. However some PCD patients have normal (Herzon et al., 1980) while some healthy subjects have abnormal ciliary ultrastructure (Afzelius 1981).

The central microtubules of all the cilia on a single cell usually line up along the same axis and the foot processes on the basal bodies point in the same direction. Disorientation of central microtubules has been described previously in association with other ultrastructural defects (Jonsson et al., 1982; Nielsen et al., 1983). Recently, some PCD patients who have a normal CBF and ciliary ultrastructure were found to have disorientation of the central ciliary microtubules. It has therefore been proposed that cilia can be structurally normal at electron microscopy and have nearly normal beat frequency at light microscopy, but have ineffective beating because of random direction of beating (Rutland et al., 1990). This suggests that ciliary disorientation alone may represent a variant of PCD (Rutland et al., 1990; Rutman et al., 1993). It has also been suggested that the orientation of the central microtubules alone can be used to assess ciliary orientation because the results concord with measurements of the foot process orientation (De Iongh 1989). However these hypotheses have not been validated.

The mechanisms that determine the orientation of cilia during embryonic development are poorly understood. It has been suggested that this process occurs by the 24th week of gestation in humans (Gaillard et al., 1989). Studies of the quail oviduct suggest that ciliary orientation may be related to the commencement of the ciliary beat cycle and may depend on a proper development of the apical cyto-skeleton in ciliated cells (Boisvieux-Ulrich et al., 1985). The apical cyto-skeleton, which consists of an intricate network of microtubules and microfilaments, is responsible for the anchorage of the basal body into cytoplasm. A defective development might result in abnormal

orientation of the cilia. It is also possible that abnormal ciliary orientation may occur secondary to bacterial infection or exposure to toxins.

1.2 CHRONIC BRONCHITIS AND BRONCHIECTASIS

1.2.1 DEFINITIONS AND PREVALENCE

Chronic bronchitis is defined as the production of sputum on a daily basis for at least three months a year for at least two consecutive years. A British survey of the population of age 46-60 years showed that 17% of men and 8% of women had such features (College of General Practitioners 1961). A similar prevalence was found in an American study when 22% of men and 9% of women had chronic bronchitis (Ferris et al, 1962). Bronchiectasis is present when one or more bronchi are abnormally and permanently dilated. The precise prevalence of bronchiectasis is unclear as many patients with mild bronchiectasis have a normal plain chest radiograph and are therefore undiagnosed. In the population as a whole 1.3 cases of bronchiectasis per thousand were reported in one study (Wynn-Williams 1953). Both diseases incur great morbidity and are characterised by a slowly progressive course and episodic exacerbations of symptoms including an increase in sputum purulence and quantity, worsening dyspnoea, chest pain, fever, and often deterioration of lung function parameters.

1.2.2 MICROBIOLOGY

Despite intensive studies over the last 40 years, the precise role of infection in causing exacerbation of chronic bronchitis has yet to be determined (Murphy et al., 1993). Although pulmonary function worsens acutely in chronic bronchitis during infection, the role of acute infective exacerbations in causing permanent alteration, or in accelerating the decline of pulmonary function, is uncertain. Results from longitudinal studies performed on the effects of infection on the decline of pulmonary function are conflicting and it is not entirely clear whether repeated infections cause a more rapid and permanent decline in pulmonary function (Howard 1970; Bates 1973; Fletcher et

al., 1977; Kanner et al., 1979).

Bacteria may be the primary cause of the exacerbation or may act as secondary invaders after an initial viral or mycoplasma infection. Studies using viral titres and serological assays have shown that viruses (including influenza, parainfluenza, respiratory syncytial and rhino-viruses) and *Mycoplasma pneumoniae* cause approximately one third of exacerbations (Grist 1967; Busho et al., 1978; Smith et al., 1980). In addition, isolation of *Streptococcus pneumoniae* and NTHi from the sputum and production of purulent sputum are associated phenomena in chronic bronchitis (Murphy et al., 1987). NTHi is the most commonly isolated bacteria from the sputum of patients with chronic bronchitis and bronchiectasis (Cole et al., 1986). More recently, *Moraxella catarrhalis* has emerged as a pathogen in this setting (Hager et al., 1987). One difficulty in establishing the role of bacterial infection in exacerbation is that NTHi, *S. pneumoniae* and *M. catarrhalis* are common inhabitants of the upper (Hendley et al., 1975; Murphy et al., 1987) and lower respiratory tracts of patients with chronic bronchitis between exacerbations (Murphy et al., 1993). It appears that not all exacerbations are infective in origin as pathogens were only identified in about 50% of cases (Fisher et al., 1969; Ellis et al., 1978). Exacerbations also occur during severe fog indicating that some exacerbations may be induced by exposure of airways mucosa to irritants although secondary infection may be common (Seaton et al., 1989).

In patients who have bronchiectasis, NTHi and S. pneumoniae are the most and the second most commonly isolated bacteria from sputum (Clark 1963; Hill et al., 1986). Gram-negative organisms such as PA may colonise bronchiectatic lungs. *Staphylococcus aureus* is also a frequent colonist in patients who suffer from cystic fibrosis (CF). Serum precipitins were detected with a frequency of 83% for NTHi, 35% for *S. pneumoniae*, 29% for *S. aureus* and 15% for PA (Roberts 1983) in patients who had exacerbations of bronchiectasis. The role of viral

infection in precipitating exacerbations of bronchiectasis is uncertain, although it may further impair the lung's compromised defences, thereby allowing greater bacterial proliferation (Cole 1986).

1.3 BACTERIAL INTERACTIONS WITH RESPIRATORY MUCOSA

1.3.1 BACTERIAL ADHERENCE

It is widely believed that bacterial adherence to the target mucosal surface has an important role in the pathogenesis of disease (Beachey 1981). Bacteria may achieve this process by expressing surface products (adhesins) which bind to epithelial surface structures (receptors) in a specific fashion. In the past two decades, many studies have been performed on bacterial adherence to host cells. Bacteria use a wide range of adhesion mechanisms and can produce lectin-like substances, which may take the form of pili (or fimbriae), or exopolysaccharide which often surrounds the bacterial cells (Plotkowski et al., 1993). Mammalian cells have many potential surface receptors for bacterial adhesins including cell surface glycoproteins, glycolipids and proteoglycans (Sharon et al., 1986). Bacterial adhesins may enhance contact between bacterial and epithelial cells thereby providing easier access of bacterial toxins to the mucosal surface or may themselves be toxic to the host cells (Plotkowski et al., 1993).

1.3.2 METHODS TO STUDY BACTERIAL ADHERENCE AND INTERACTIONS WITH RESPIRATORY MUCOSA

1.3.2.1 ORIGINS OF HOST TISSUE

Many studies have been performed on the interactions of bacteria with respiratory tissue. Some of these were performed on animal tissue including frog palates (Plotkowski et al., 1989), monkey nasal turbinates (Roberts et al., 1984), and tracheas obtained from chinchillas (Bakaletz et al., 1988a), hamsters (Baker et al., 1982a; Marcus et al., 1985; Grant et al., 1991), mice (Ramphal et al., 1980 & 1985a) and dogs (Hata et al., 1991). In addition to the ready

availability of tissue, the homogeneity of laboratory strains of animals allows better control of experimental conditions. Bacterial adherence to these different animal tissues differ widely. For instance, the adherence of PA to guinea pig respiratory mucosa is much greater than that to hamster and murine respiratory mucosa although these species are closely related (Marcus et al., 1989). This may be partly explained by the differences in methodologies and the strains of bacteria tested, but almost certainly also by the differences between species (Marcus et al., 1989). As NTHi is only pathogenic to humans (Murphy et al., 1993) and is generally only isolated from the respiratory tract (Turk 1984), results from *in vitro* studies on NTHi using animal tissue may not be extrapolated to humans.

To overcome these problems, many workers have used human tissue to study bacterial interactions. These studies have used buccal epithelial cells (Lampe et al., 1982), red blood corpuscles (van Alphen et al., 1986), and suspensions of nasal (Niederman et al., 1983) and tracheal cells (Franklin et al., 1987). Intact human nasal turbinate (Read et al., 1991) and adenoid tissue (Farley et al., 1986) have also been used. These studies suggest that the adherence of NTHi and PA to buccal epithelial cells and ciliated respiratory epithelium may involve different mechanisms. Apart from a recent post mortem study on airways obtained from patients with cystic fibrosis, very little is known on bacterial interactions with human bronchial tissue (Baltimore et al., 1986).

Both human (tracheobronchial mucosa or resected nasal polyps) (Plotkowski et al., 1991 & 1992; Saiman et al., 1992; Franklin et al., 1987; Niederman et al., 1983) and animal (Grant et al., 1991) respiratory epithelia in cell culture have been used to quantify bacterial adherence. Cell cultures provide a convenient source of epithelial cells for *in vitro* studies and is useful for studying the basic mechanisms of adhesin-receptor interactions, as the epithelial cell types can be selected for experiments (Wasano et al., 1988).

However there are limitations which include: an alteration in cell surface receptors during culture as suggested by lectin binding studies in hamster tracheal cell cultures (Wasano et al., 1988); alteration of the relative composition of epithelial cells after trypsinisation (Kennedy et al., 1983; Wu et al., 1985); loss of cilia from ciliated cells with prolonged culture; and absence of a mucus layer. These changes could influence the results of experiments, particularly if the epithelial monolayer is non-confluent, and results should only be extrapolated to the *in vivo* situation with caution.

Respiratory epithelium from different sites in the respiratory tract have a similar morphology but vary in the relative composition of different types of epithelial cells (Sorokin 1988). Studies of hamster bronchial and tracheal mucosae show some differences in the proportion of secretory cells, and in the secretory response to an intratracheal instillation of human neutrophil elastase (Christensen et al., 1987 & 1989). Secretory cells from different sites of animal airways also differ in their response to inhalation of sulphur dioxide (Chakrin et al., 1974); injection of salbutamol (Jones et al., 1979); and exposure to cigarette smoke (Mawdesley-Thomas et al., 1973). Some respiratory pathogens such as PA (Vishwanath et al., 1984), NTHi (Barsum et al., 1992) and *S. pneumoniae* (Feldman et al., 1992) have a high affinity for respiratory mucus, so bacterial interactions with respiratory mucosa obtained from the nose, nasopharynx and tracheobronchial tree may differ. A difference in the epithelial surface receptors may also exist in hamster tracheal and bronchial epithelial cells as they differ in lectin binding (Christensen et al., 1990). The extensive submucosal venous plexus in nasal turbinates, and lymphoid tissue in adenoid and bronchial tissue, may also affect bacterial interactions with respiratory mucosa differently (Freed et al., 1993). Functional differences also exist and there is a progressive increase in mucociliary clearance rates along the ciliated airways from terminal bronchioles to trachea both in animals (Iravani et al., 1972) and humans (Sanchis et al., 1972; Rutland et al., 1982).

Bacteria display selective tropism towards different types of epithelia. For example, the adherence of both *Neisseria meningitidis* and NTHi is greater to pharyngeal cells than buccal epithelial cells (Craven et al., 1980; Salit et al., 1981; Harada et al., 1990). This is again seen in respiratory mucosa as NTHi preferentially adhere to unciliated cells (Farley et al., 1986) while PA to ciliated cells (Plotkowski et al., 1993).

Results from these studies indicate that the target tissue i.e. intact respiratory tissue normally colonised by respiratory pathogens should be used for adherence studies. Findings from studies of bacterial interactions with respiratory mucosa that used non-bronchial tissue may not be applicable to tracheobronchial mucosa.

1.3.2.2 METHODS TO STUDY BACTERIAL INTERACTIONS WITH THE HOST (TABLE 1.2 & 1.3)

Many different methods have been developed to maintain the host epithelial and bacterial cells during their interaction. The advantages and disadvantages of each method are outlined in Tables 1.2 & 1.3. These include immersion of human or animal respiratory epithelial cells (Niederman et al., 1983; Franklin et al., 1987; Plotkowski et al., 1991; Saiman et al., 1992), red blood corpuscles and buccal epithelial cells in medium containing bacteria (Lampe et al., 1982; Niederman et al., 1983; van Alphen et al., 1986). Human and animal respiratory epithelial cells have been immersed in bacteria-containing medium (Niederman et al., 1983; Franklin et al., 1987; Plotkowski et al., 1991; Saiman et al., 1992). Some animal studies used isolated rabbit tracheal segments (embedded in nutrient agar) (Matsuyama 1974) and frog palates that were exposed to air directly (Plotkowski et al., 1989). Although these methods had established an air-mucosal interface for the respiratory mucosa, neither the respiratory tissue nor the bacteria was supplied with culture medium and only non-human tissue was used. Farley et al. described an organ

culture model using intact respiratory mucosa obtained from resected human adenoid tissue. In this study, dissected adenoid tissue was freely suspended in culture medium containing bacteria and subsequently examined by scanning electron microscopy (Farley et al., 1986). Whilst this method is simple and reproducible for studying bacterial interactions with intact human respiratory mucosa, the non-mucosal surfaces (particularly those created at dissection) were also openly exposed to bacteria.

Very recently, Read et al. described a novel organ culture model that used human nasal turbinate whose non-mucosal surfaces were sealed by agar (Read et al., 1991). This model is a significant improvement over previous methods as only the intact human respiratory mucosa is directly exposed to bacteria suspended in culture medium. Similar to most previous organ and cell culture models this method can be criticised as the respiratory tissue is immersed in cell culture medium and hence ciliary activities are rendered ineffective. Unlike the *in vivo* situation when nutrients are supplied from the subepithelial circulatory network, nutrients are supplied by the medium to the mucosal surface and diffuse in a subepithelial direction. The respiratory mucosa is continuously bathed in cell culture medium that contains high concentrations of bacteria and bacterial products that would undoubtedly favour bacteria in gaining access to the respiratory mucosa.

Although Adler et al. had described a cell culture method that involve direct exposure of animal respiratory cell culture to humidified air (Adler et al., 1986), cell culture methods present a number of problems in the study of bacterial interactions with respiratory mucosa. These include: non-confluence of the epithelial monolayer; alteration in epithelial cell compositions caused both by the preparation process and prolonged culturing (Kennedy et al., 1983; Wu et al., 1985); incoordination of ciliary beat between neighbouring cells in non-intact respiratory surface; frequent use of non-human

respiratory tissue; absence of mucus layer; alteration of epithelial cell surface receptors (Wasano et al., 1988); and loss of cilia with prolonged cell culturing (Wu et al., 1985). There is still no established organ culture method that allows direct exposure of intact human respiratory mucosa to humidified air and direct inoculation of bacteria onto mucosal surface that mimics droplet infection *in vivo*.

1.3.2.3 METHODS TO QUANTIFY BACTERIAL ADHERENCE

Studies on bacterial interactions with host cells have employed different methods to quantify bacterial adherence. These can be classified into indirect and direct methods.

Indirect methods to quantify bacterial adherence to host cells include homogenisation of host tissue after its exposure to a bacterial suspension followed by viable counting of the homogenate (Roberts et al., 1984), and radiolabelling of bacteria to quantify bacterial adherence (St Geme et al., 1990). These two methods are simple but may overestimate bacterial adherence as bacteria adhering to non-mucosal surfaces (such as the dissected edges) are also quantified. Respiratory mucosa is a heterogenous structure and different donor tissue may have different composition of mucus and epithelial cells. As bacteria display different affinities to individual mucosal components, such as mucus and cilia (Farley et al., 1986; Harada et al., 1990; Read et al., 1991; Feldman et al., 1992; Plotkowski et al., 1993), indirect methods to assess bacterial adherence are unsatisfactory in that separate assessment of bacterial adherence to individual mucosal components (which may involve different bacterial adhesins and epithelial receptors) cannot not be performed.

Direct methods have been used to assess bacterial adherence to respiratory mucosa. Light microscopy (without staining of tissue or bacteria) (Baker et al., 1990) and Gram staining (Fainstein et al., 1979) or application of florescent-antibodies (Lampe et al, 1982) have been used to study bacterial adherence to epithelial cells. Although

these techniques are simple, they are only generally applicable to epithelial cells in suspension but not intact respiratory mucosa.

Electron microscopy can provide high resolution and reproducible assessment of bacterial adherence (Read et al., 1991). Transmission electron microscopy (TEM) has been used to assess bacterial adherence when bacteria such as NTHi were only found to adhere to influenza A virus infected (Bakaletz et al., 1988b) or NTHi damaged (Read et al., 1991) respiratory epithelial cells but not to healthy cells. TEM is useful in determining bacterial adherence but may underestimate bacterial adherence as only a very small section of the respiratory mucosa is examined and the plane of TEM section may not reveal direct contact between bacteria and epithelial cells. Scanning electron microscopy (SEM) allows detailed surface assessment of respiratory mucosa and bacterial adherence but does not provide information on the ultrastructure of mucosal cells and submucosal structures (Farley et al., 1986). Bacterial adherence may also be underestimated as single bacteria may be not be readily detected and bacteria may be concealed in a lawn of cilia. Finally it is not always possible to identify a mucosal component at SEM that is obscured by a large number of adherent bacteria. Simultaneous use of TEM may overcome some of these problems. Some bacteria may be lost during processing although bacterial "foot prints" (mark left on mucosal surface after bacterial detachment) may sometimes be detectable. Although SEM may allow detailed quantitative assessment of bacterial adherence to individual mucosal components, previous SEM studies on bacterial interactions with intact respiratory mucosa were only qualitative (Farley et al., 1986; Read et al., 1991).

1.4 RESPIRATORY PATHOGENS

1.4.1 NTHi

NTHi is a part of normal human nasopharyngeal flora and is only pathogenic to humans (Murphy et al., 1987). NTHi is the most commonly isolated bacteria from the sputum of patients with chronic bronchitis

and bronchiectasis (Cole 1986). As a group NTHi plays an important role in the aetiology of otitis media, sinusitis, conjunctivitis and pneumonia (Murphy et al., 1987; Wilson et al., 1991) and is therefore responsible for a great amount of morbidity. The role of NTHi in the aetiology of exacerbation of chronic bronchitis is uncertain. NTHi tend to disappear from purulent sputum when it becomes mucoid but is also isolated during remission (Calder et al., 1968). Studies using DNA finger printing showed that exacerbation of chronic bronchitis coincided with endogenous or exogenous reinfection by NTHi and persistently infected patients harboured the same NTHi strains for long periods (Groeneveld et al., 1990a). Antibiotic treatment is not effective in eradicating NTHi from the lower respiratory tract although it often produces clinical improvement in patients with chronic bronchitis (Groeneveld et al., 1990a). An early study showed that the total serum NTHi antibody increased in patients with chronic bronchitis during an exacerbation (Gump et al., 1973). However this study was partly invalidated since only a single NTHi strain was used for testing despite the diversity of NTHi antigenicity. More recently, high titres of strain-specific serum IgG and IgA and sputum IgA were found in patients with chronic bronchitis who had NTHi in sputum (Groeneveld et al., 1990b). A role for NTHi in causing exacerbation of chronic bronchitis is also supported by a significant rise in strain-specific antibody titre that coincides with isolation of a new strain of NTHi (Groeneveld et al., 1990a).

1.4.1.1 BASIC BACTERIOLOGY

NTHi is a small coccobacillus and measures (0.3-0.5) x (0.5-1.0)μm. Colonies of non-capsulate strains are 0.5-1mm in diameter, smooth and greyish after incubation for 24h on solid media. Capsulate strains produce larger (1-3mm diameter) and slightly mucoid colonies. On transparent solid media such as Levinthal agar, capsulate strains are iridescent when viewed under obliquely transmitted light. NTHi require both X and V factors for growth and grow more readily in aerobic than in anaerobic conditions. The optimal temperature is 37^{0}C and added CO_2

does not improve growth (Slack 1990).

1.4.1.2 NTHi ADHERENCE TO RESPIRATORY MUCOSA

NTHi adhere to human buccal, pharyngeal and nasal epithelial cells more readily than encapsulated strains *in vitro* (Lampe et al., 1982; Porras et al., 1985). NTHi adhere more readily to nasal and nasopharyngeal than buccal epithelial cells, and the adherence to buccal epithelial cells obtained from children who suffer from chronic sinusitis is also greater than to epithelial cells obtained from healthy children (Harada et al., 1990). It is unclear whether this reflects an underlying intrinsic difference in cell surface receptor(s) between these children or an influence of chronic inflammation on epithelial cells rendering them more susceptible to bacterial adherence. A preferential adherence of NTHi to unciliated cells and mucus rather than to ciliated cells was reported in a qualitative study that used isolated adenoid organ cultures freely suspended in NTHi containing cell culture medium (Farley et al., 1986). It also appears that NTHi adhere preferentially to damaged epithelium. Read et al. investigated the effects of NTHi infection on human nasal turbinate in an agar-embedded organ culture model which was also used in part of this thesis (Section 3.1.2). Six strains of NTHi (clinical isolates) were used to infect human nasal turbinate organ cultures. By using TEM assessment, NTHi only adhered to damaged but not ultrastructurally normal epithelial cells (Read et al., 1991). Although Farley et al. did not assess the ultrastructure of the unciliated cells that had adherent NTHi, they had no gross abnormalities on SEM examination (Farley et al., 1986). Whilst the cell and organ culture methods, strains of NTHi, and the experimental methodologies used in these studies are different, it is possible that NTHi may adhere to respiratory mucosa derived from adenoid and nasal turbinate differently. It follows that different bacterial adhesin(s) and epithelial receptor(s) may be involved in the adherence of NTHi to adenoid and nasal turbinate.

Similar to other bacteria such as PA (Ramphal et al., 1980; Vishwanath et al., 1984), *S. pneumoniae* (Plotkowski et al., 1989) and *Vibro cholerae* (Yamamoto et al., 1988), *in vitro* studies have showed a high affinity of NTHi for respiratory mucus. By using an established method that employed microtitre plates coated with human respiratory mucin (Vishwanath et al., 1984) and viable counting to quantify bacterial adherence, a piliated strain of NTHi was found to adhere better to mucoid sputum than its non-piliated derivative (Barsum et al., 1992). In a qualitative study using intact human adenoid organ cultures suspended in bacteria-containing culture medium, NTHi adhered avidly to mucus (Farley et al., 1986). Loeb et al. made a similar observation with a strain of *H. influenzae* type b (Hib) and also found that pili conferred increased adherence for Hib (Loeb et al., 1988). Similarly, NTHi adhered to and multiplied within mucus in human nasal turbinate organ cultures (Read et al., 1991).

1.4.1.3 NTHi ADHESINS AND EPITHELIAL RECEPTORS

The molecular mechanisms that promote successful colonisation and NTHi adherence to respiratory mucosa are not fully understood. Although the role of pili in the adherence of Hib to epithelial cells is well defined, adhesin(s) and epithelial receptor(s) for NTHi have not been clearly identified.

The presence of pili has been found to correlate with the adherence of Hib to buccal epithelial cells (Pichichero 1984; LiPuma et al., 1988; van Alphen et al., 1988), human erythrocytes (Stull et al., 1984; van Alphen et al., 1988), human adenoid mucosa (Loeb et al., 1988), and monkey tracheal organ cultures *in vitro* (Smith et al., 1989). Antibodies specific for pili of a strain of Hib also inhibited the adherence of other strains of Hib to human buccal epithelial cells *in vitro* (Forney et al., 1992).

Pili may also mediate the adherence of NTHi to human respiratory mucus *in vitro* (Barsum et al., 1992). Read et al. found that piliation of

NTHi increased adherence to buccal cells but not intact respiratory mucosa in human nasal turbinate organ cultures (Read et al., 1991). Although pili appear to have adhesin properties for Hib, they do not account for NTHi adherence. The adherence of NTHi to respiratory mucosa in human adenoid (Farley et al., 1986 & 1990) and chinchilla tracheal (Bakaletz et al., 1988a) organ cultures did not correlate with the degree of piliation. Bakaletz et al. examined 60 different clinical isolates of NTHi by TEM and found that all expressed pili, although the presence of pili did not correlate with the ability to adhere to respiratory mucosal cells, nor to agglutinate human erythrocytes (Bakaletz et al., 1988a). More recently Gilsdorf et al. made similar observations. These workers found that many NTHi isolates possessed structures immunologically similar to the haemoagglutining pili of Hib; however these structures did not account for NTHi adherence to buccal epithelial cells nor erythrocyte ghosts (Gilsdorf et al., 1992). Although the prevalence of pili on clinical isolates of NTHi is almost universal, suggesting that they have an important role in the colonisation of respiratory tract (Bakaletz et al., 1988a), the role of pili in NTHi adherence to respiratory cells remains to be determined. It follows from these studies that non-pilus adhesin(s) must exist.

NTHi adhere to human conjunctival cell culture by a non-pilus dependent mechanism which depends on the presence of viable bacteria capable of *de novo* protein synthesis (St Geme et al, 1990). The cloning and sequencing of genes from a prototype NTHi strain that encode two high-molecular-weight (HMW) surface proteins (HMW-1 and HMW-2) has been reported (Barenkamp et al., 1992). As HMW-1 and HMW-2 closely resembled the *B. pertussis* haemagglutinin adhesin, they have also been postulated as NTHi adhesins. A recent study showed that the loss of HMW-1 expression by NTHi was associated with a decreased adherence to human conjunctival epithelial cells. A loss of both HMW-1 and HMW-2 was associated with a further reduction in NTHi adherence. In addition, laboratory strains of *Escherichia coli* harbouring either

the HMW-1 or HMW-2 gene clusters became adherent to epithelial cells although the wild types did not (St Gemes et al., 1993). These results show that both HMW-1 and HMW-2 surface proteins affect NTHi adherence independently and possess adhesin properties. However, their corresponding receptor(s) on the epithelial surface is still unknown, and it is also unclear whether they also mediate NTHi adherence to human respiratory mucosa.

The nature of the adhesin-receptor interaction between NTHi and epithelial cells has not been determined. Whilst the adhesin(s) for NTHi has not been clearly elucidated, even less is known on epithelial receptors for NTHi. As NTHi was found to have preferential adherence to mucus and unciliated epithelium (rather than to cilia) in adenoid organ cultures (Farley et al., 1986), epithelial receptor(s) on these mucosal components may be different (either quantitatively or qualitatively). Krivan et al. described specific binding of NTHi, PA, *S. pneumoniae* and *S. aureus* to the carbohydrate sequence GalNAcß1-4Gal sequence which is found in some glycolipids and might be a part of the receptor domain on epithelial cells (Krivan et al., 1988). However the corresponding adhesin(s) for this glycolipid sequence is still unidentified.

1.4.1.4 OTHER MODES OF INTERACTION

NTHi produce exotoxins that interact with the respiratory mucosa. Sterile broth culture filtrates of NTHi slow CBF of human nasal epithelium *in vitro* (Wilson et al., 1985) and the onset of ciliary slowing was detected after 15min of incubation (Wilson et al., 1985). NTHi culture filtrates also caused damage to respiratory epithelium obtained from human fetus, chick embryo and several rodents (Denny 1974). The ability of NTHi to damage respiratory epithelium without initial adherence to the mucosal surface has been demonstrated using intact human respiratory mucosa (Farley et al., 1986; Read et al., 1991). Lipo-oligosaccharide from NTHi caused damage to respiratory mucosa and loss of ciliary activities in rat tracheal organ cultures

(Johnson et al., 1986). Bacterial products from *B. pertussis*, *Legionella micdadei*, PA and more recently NTHi have been reported to inhibit human neutrophil migration and chemiluminescence *in vitro* and therefore may hinder bacterial clearance *in vivo* (Groeneveld et al., 1990b; Cundell et al., 1993). Histamine is found in the sputum of patients with infective exacerbation of chronic bronchitis and NTHi is known to produce histamine *in vitro* (Devalia et al., 1989). Histamine may lead to bronchial inflammation, an increase in epithelial permeability, and damage of the tracheobronchial tree. Although IgA_1 protease is produced by NTHi, and may theoretically enhance the pathogenicity of NTHi by cleaving IgA, an *in vitro* study showed no significant difference in the adherence to respiratory mucosa or tissue destruction of IgA protease-producing and non-producing strains of NTHi (Farley et al., 1986).

1.4.2 PA

PA commonly infect the airways of CF patients and patients with other forms of bronchiectasis. Once the infection is established, it is often impossible to eradicate the bacteria even with intensive chemotherapy. Much of the morbidity and mortality of CF patients is due to chronic respiratory tract infection caused by PA (Govan et al., 1986).

1.4.2.1 BASIC BACTERIOLOGY

PA are strictly aerobic Gram negative bacilli (0.5-0.8) x (1.5-3.0)μm. Nearly all strains are motile, with a single polar flagellum, and most have long fine proteinaceous projections called pili (Liu, 1976). PA is widely distributed in nature as saprophytes or pathogens of plants, insects and animals. Instead of a hard and discrete polysaccharide capsule that coats many other gram negative bacteria, PA has an amorphous slime-like substance that is mainly composed of polysaccharide, but also contains lipid and protein (Pitt 1986).

The airways of CF patients are initially colonised by non-mucoid

strains of PA, followed by the appearance of the more typical mucoid strains (Doggett et al., 1966). The majority of isolates from the lungs of chronically infected CF patients produce a mucoid exopolysaccharide composed of an acetylated polymer of D-mannose and L-guluronic acids (Philips, 1969; Evans et al., 1973). This mucoid exopolysaccharide forms linear strands that radiate outwards from the bacterial surface and may mediate adhesion to respiratory mucus (Pier et al., 1983). It is also often referred to as alginate because of its chemical similarity to a polysaccharide found in seaweed algae. This material may increase bacterial resistance to host phagocytes (Schwarzmann et al., 1971; Costerton et al., 1979) and antibiotics (Govan et al., 1978), mediate attachment to ciliated epithelium (Baker et al., 1982b), and hinder the clearance of mucoid PA from the lungs of animals (Blackwood et al., 1981; Govan et al., 1983). Non-mucoid strains are known to change to mucoid phenotype *in vivo* and *in vitro* if the growing conditions are sub-optimal (Speert et al., 1990; May et al., 1991) and PA in the CF airways may grow under nutritional limitations *in vivo* (Anwar et al., 1984).

1.4.2.2 PA ADHERENCE TO RESPIRATORY MUCOSA

Studies on human respiratory tissue suggest that PA may have a preferential adherence to ciliated cells. PA adhere to cilia but not the body of human ciliated or unciliated epithelial cells (Franklin et al., 1987; Plotkowski et al., 1992). PA also adhere better to respiratory epithelium than other epithelial cell types. For example, adherence of PA to nasal or tracheal epithelial cells was five fold greater than that to squamous epithelial cells obtained from the same donors (Niederman et al., 1983). Mucoid strains adhere better to intact tracheal epithelium (Baker et al., 1982a) while non-mucoid strains adhere more avidly to buccal epithelial cells (Woods et al., 1980a&b).

The studies performed on adherence of PA to animal respiratory tissue show conflicting results. A non-mucoid strain of PA was found to

adhere to cilia of uninjured rat trachea but in much greater extent to injured epithelium and submucosal connective tissue (Yamaguchi et al, 1991). However, this preference of PA adherence to ciliated cells has not been confirmed in other animal studies. PA adhered to unciliated cells in cultured hamster tracheal cells (Grant et al., 1991), but mainly to mucus and damaged cells and only very occasionally to ciliated epithelium in canine tracheal organ cultures (Zoutman et al., 1991). Both mucoid and non-mucoid strains of PA adhered to ciliated and unciliated cells in canine tracheal cell culture. Although the discrepancies in these results may be partly explained by the differences in experimental methodologies and strains of bacteria used, the differences between species also appear to be an important factor. This highlights the importance of using human respiratory tissue in future studies.

Similar to NTHi, PA adhere more avidly to damaged epithelium than intact epithelial cells both in animal and human studies (Section 1.4.1.2). PA adhere to acid injured tracheal and desquamating cells of murine tracheal organ cultures infected by influenza A virus but not to intact cells (Ramphal et al., 1980 & 1985a). Non-mucoid PA adhere poorly to uninjured hamster tracheal epithelium, whereas mucoid PA adhere more avidly (Baker et al., 1982a; Marcus et al., 1985 & 1989). In the only *in vivo* study on human tracheobronchial tissue, CF lungs obtained at post mortem showed PA adherence to denuded mucosa and inflamed surface but mostly to intraluminal secretions (Baltimore et al., 1989). Disease processes such as chronic infection may alter or expose epithelial receptors rendering the damaged cells more susceptible to bacterial adherence (Doig et al., 1989).

In vitro studies have reported a high affinity of PA for respiratory mucus. These include studies that used a frog palate model (Plotkowski et al., 1989), hamster tracheal organ cultures (Ramphal et al., 1983a) and mucus-coated microtitre plates (Vishwanath et al., 1984). As described above PA also adhere avidly to bronchial secretions and

mucus in CF lungs (Baltimore et al., 1989).

1.4.2.3 PA ADHESINS AND EPITHELIAL RECEPTORS

The adhesion of PA to respiratory mucosa is complex and multiple PA adhesins and epithelial receptors appear to be involved. Several PA adhesins have been identified and these include pili (Woods et al., 1980a), exoenzyme S (Coburn et al., 1989), an exopolysaccharide produced both by mucoid and non-mucoid strains (Ramphal et al., 1985b), and other less well evaluated PA products. Less information is available on the epithelial surface receptor(s) for these adhesins but it appears that sialic acid residues are important components of epithelial cell receptors.

PA pili are highly strain specific proteinaceous appendages that have been identified as an important adhesin for buccal epithelial cells (Woods et al., 1980a), acid damaged murine tracheal mucosa (Ramphal et al., 1983a & 1984), and human tracheal epithelial cells (Doig et al., 1988). PA adherence to human buccal epithelial cells was reduced by purified and strain-specific pili but pili from a heterologous strain failed to reduce adherence in one study (Wood et al., 1980a). Similarly, PA adherence to acid-injured murine tracheal organ cultures and human buccal and tracheal cells was also reduced by both pili and strain-specific antibody against pili (Ramphal et al., 1983a & 1984; Doig et al., 1988). The C-terminal of PA pilin maintains the functionality of the binding domain (Doig et al., 1990). These results suggest an important role for pili in PA adherence to respiratory mucosa.

Exoenzyme S ribosylates several membrane-associated eukaryotic proteins and contributes to the pathogenicity of PA (Coburn et al., 1989). It has the same adherence specificity for glycosphingolipid as the whole bacteria and is found on the outer surface of the bacteria where it can function as an adhesin. Furthermore, both exoenzyme S and an antibody raised against it inhibit PA adherence to buccal

epithelial cells (Baker et al., 1991). However, it is unclear whether exoenzyme S also mediates the adherence of PA to intact respiratory mucosa.

PA adherence to respiratory mucin is also complex and may involve multiple adhesins and receptors. It has been suggested that the expression of pili and flagella are under the control of rpoN gene locus (Ishimoto et al., 1989; Totten et al., 1990). A recent study was performed to investigate the effects of inactivating the rpoN and pilin genes on PA adherence to mucin. Isogenic strains of PA with inactivated rpoN genes had significantly lower adherence to human respiratory mucin-coated microtitre plates compared with the wild type. In contrast, the adherence of a pilin structural gene mutant was similar to that of the wild type. These results indicate that there is a non-pilus mediated adherence to mucin that is also controlled by the rpoN gene (Ramphal et al., 1991a). A study on highly purified human respiratory mucus showed that PA fragments obtained by sonification adhered to mucus via a 16-kDa non-pilus protein component(s) of PA (Reddy, 1992). However, a recent study showed that the adherence of PA to highly purified mucin obtained from sputum and intestine of CF patients was non-specific and doubted the involvement of adhesin-receptor mechanism (Sajjan et al., 1992). The authors suggested that previous studies were unreliable as they used whole respiratory mucin that might have been contaminated with glycolipids derived from the surface of cells. They also suggested that the difficulty in isolating a mucin receptor may be due to the problems in isolating pure human tracheobronchial mucin.

Mucoid strains and to a lesser extent non-mucoid strains of PA produce an exopolysaccharide that has anti-phagocytic properties (Schwarzmann et al., 1971) and reduces the coating of PA by antibodies (Marrie et al., 1979). There is also evidence that this exopolysaccharide is an adhesin. Purified exopolysaccharide increased the adherence of mucoid PA to acid-injured murine trachea while an antibody against the major

epitope of the exopolysaccharide inhibited PA adherence (Ramphal et al., 1985b). Other studies showed that this exopolysaccharide bridges the organisms to cilia, and to each other in murine and hamster tracheal organ cultures (Marcus et al., 1985; Ramphal et al., 1985b) and human tracheal cells in suspension (Doig et al., 1987; Franklin et al., 1987). In primary cultures of human nasal epithelial cells this exopolysaccharide material was also found to be closely associated with bacteria (Plotkowski et al., 1991). However PA also adhered to unciliated cells in the absence of this matrix suggesting that other PA adhesins may also be present. This matrix-like material reacted with anti-mucin antibody and was therefore thought to have an epithelial origin (Plotkowski et al., 1991), although the authors did not consider that this may be the result of contamination by respiratory mucin. A non-mucoid strain of PA also produced this lace-like matrix in canine tracheal monolayer and "bridged" PA and epithelial cells (Hata et al., 1991).

Whilst bacterial adhesin(s) have received much attention, much less is known of epithelial receptors for PA. Different components and internal sequences of glycolipids have been implicated as receptor domains. Receptors for PA have been found in at least two types of glycoconjugates, namely, glycolipids and mucins. PA and other common respiratory pathogens such as NTHi and *S. pneumoniae* bind specifically to GalNAcß1-4Gal sequence found in glycolipids of human lung explants and glycosphingolipid of epithelial cell surfaces (Krivan et al., 1988). Cell surface sialic acid has been identified as a vital component of epithelial receptors for PA adhesin(s) (Ramphal et al., 1983b & 1985a; Doig et al., 1989). The adherence of PA pili to human buccal epithelial cells is inhibited by L-fucose, sialic acids and specific monoclonal antibody against pili (Doig et al., 1989). PA adherence to human buccal epithelial cells (Ramphal et al., 1983b) and acid-injured murine tracheal epithelium was inhibited by sialic acids and pre-treatment of respiratory mucosa with lipase (but not trypsin) respectively (Ramphal et al., 1985a). Ramphal et al. (1985a) therefore

concluded that a sialic acid-containing epithelial surface lipid was a PA receptor. PA bound to sialic acid-containing glycosphingolipids and lactosylceramide through independent mechanisms, and this binding correlated with the adherence to human buccal epithelial cells (Baker et al., 1990). Interesting, several other respiratory pathogens also seem to utilise sialic acid-containing glycoconjugates as receptors. *M. pneumoniae* bound to a sialic acid-containing glycophorin (Sobeslavsky et al., 1968) and influenza virus bound to a sialic acid-containing glycoprotein (Schulze 1975).

Because of the tremendous heterogeneity of the oligosaccharides found in mucins, studies of the mucin glycoproteins have not reached the oligosaccharide level (Roussel et al., 1988). It is also very difficult to purify all the hundreds of oligosaccharides found in mucins in sufficient quantity for *in vitro* studies (Ramphal et al., 1991a&b; Sajjan et al., 1992). Available data show that sialic acids and N-acetyl-glucosamine are components of mucin receptor(s) (Vishwanath et al., 1985). Although Galß1-4Glc and GalNAcß1-4Gal are not found in mucins, mucins contain many disaccharide units in the oligosaccharide backbone or periphery in either the type 1 (Galß1-3GlcNAc) or the type 2 (Galß1-4GlcNAc) forms. These chains may be substituted by sialic acids, fucose, and sulfate (Roussel et al., 1988). Monoclonal antibodies against disaccharide units partially inhibit the adherence of isogenic strains of piliated and non-piliated PA to mucin. This suggests that both type 1 (Galß1-3GlcNAc) and type 2 (Galß1-4GlcNAc) disaccharide units possess properties of epithelial receptors for PA (Ramphal et al., 1991a&b).

1.4.2.4 OTHER MODES OF INTERACTION

Pseudomonas exoproducts probably make a significant contribution to the process of airway colonisation (Saiman et al., 1992). PA produce phenazine pigments including pyocyanin and 1-hydroxyphenazine. Pyocyanin has been shown to inhibit epidermal cell growth (Cruickshank et al., 1953) and lymphocyte proliferation (Nutman et al., 1987),

exert antibiotic effects against other microorganisms (Schoental, 1941), influence the acquisition of iron by pseudomonads (Cox 1986), slow human ciliary beat and cause ciliary dyskinesia *in vitro* (Wilson et al., 1987), and slow tracheal mucus transport in guinea pigs *in vivo* (Munro et al., 1989). 1-Hydroxyphenazine slows human ciliary beat *in vitro* and inhibits mammalian cell respiration (Armstrong et al., 1971). Both pyocyanin and 1-hydroxyphenazine are found in the sputum of patients with cystic fibrosis and bronchiectasis who are colonised by PA at concentrations similar to those required to produce these *in vitro* effects (Wilson et al., 1988a). Other PA extracellular products include: proteases (elastase and alkaline proteases) and phospholipase C that disrupt respiratory epithelium *in vitro* (Amitani et al., 1991; Azghani et al., 1990); exotoxin A that inhibits protein synthesis and causes tissue necrosis (Pitt 1986); and rhamnolipid that causes ultrastructural damage and slowing of ciliary beat in human respiratory mucosa *in vitro* and tracheal mucus transport *in vivo* (Read et al., 1992). A recent study on PA adherence to epithelial cell culture derived from CF nasal polyps showed that exposure to PA exotoxin might increase epithelial receptors and modify the epithelial cell surface by exposing asialoganglioside binding sites (Saiman et al., 1992). There is, however, very little information on the effects of PA exoproducts such as pyocyanin on the ultrastructure of intact human respiratory mucosa.

1.5 ANTIBIOTIC TREATMENT OF INFECTIVE EXACERBATIONS OF CHRONIC BRONCHITIS AND BRONCHIECTASIS

1.5.1 RATIONALE AND BENEFITS OF ANTIBIOTIC THERAPY OF BACTERIAL INFECTIONS OF THE BRONCHIAL TREE

If bacterial infection plays an important role in acute exacerbations of chronic bronchitis, then a response to an appropriate antibiotic therapy would provide some evidence for its pathogenic role. Whilst there is little doubt that antibiotic treatment of acute bronchitis in persons who have no previous respiratory problems is not beneficial nor necessary (Gonzales et al., 1995), the benefits of antibiotic

treatment in acute exacerbation of chronic bronchitis is less clear. Trials of prophylactic antibiotics in these patients only showed a benefit in those who suffered from frequent exacerbations (Francis et al., 1960; Pridie et al., 1960; Johnston et al., 1961; Davis et al., 1965; Murphy et al., 1993). The studies that enroled patients with infrequent exacerbations showed no benefit from antibiotic treatment (Nicotra et al., 1982). Bacterial infection therefore only appears to play a pathogenic role in some exacerbations.

Antibiotics are used to good effect in the treatment of more severe bronchial infection such as bronchiectasis (Currie et al., 1990) and cystic fibrosis (Lebel 1991). In a study on 173 patients with exacerbations of chronic bronchitis, improvement in clinical and pulmonary function indices was found in significantly more antibiotic-treated (68%) (with amoxycillin 40%, trimethoprim/sulfamethoxazole 40%, doxycycline 20%) than placebo-treated (55%) patients (Anthonisen et al., 1987).

A striking feature of the studies on bacterial infection in patients with chronic bronchitis is that other than the introduction of the pneumococcal vaccine, very little has changed in terms of management in the past two decades. Further trials involving cultures of sputum samples and clinical observations are unlikely to yield key and new therapeutic clues (Murphy et al,. 1993). Current therapy to prevent exacerbations, prevent disease progression, and reduce the frequency of infective exacerbations is not highly effective. An improved understanding of the basic mechanisms of bacterial interactions of the human respiratory mucosa may help develop novel therapies in the future.

1.5.2 ANTIBIOTIC PENETRATION INTO BRONCHIAL SECRETIONS

The minimal inhibitory concentration (MIC) of an antibiotic for a microorganism is commonly determined as an assessment of susceptibility of a pathogen to a particular antibiotic (Hamilton-

Miller 1991). The rational goal of an effective antimicrobial drug therapy is to produce, at the site of the infection, a concentration-time profile such that free drug concentrations equal or exceed the MIC for the infecting pathogen, as determined *in vitro* (Valcke et al., 1990). The tracheobronchial mucosal surface is the site of acute bacterial infection in patients with infective exacerbation of chronic bronchitis, bronchiectasis and cystic fibrosis. In patients with bacterial pneumonia, the site of infection is in the alveolar space with its alveolar lining fluid and in the interstitium of the lung.

There are surprisingly few studies that show a positive correlation between the clinical outcome of a bacterial lower respiratory tract infection and the local concentration of an antibiotic in relation to its MIC. A more rapid clinical response to treatment with amoxycillin in patients with infective exacerbations of chronic bronchitis occurred when the sputum level of amoxycillin exceeded 0.25μg/ml (Stewart et al., 1974). A satisfactory response to treatment with either ampicillin or bicampicillin also correlated with sputum antibiotic levels (May et al., 1965; Ingold et al., 1975; Maesen et al., 1976).

There is increasing evidence that during an infective exacerbation most bacteria within the bronchial tree are associated with secretions rather than attached to the epithelium (Ramphal et al., 1984; Farley et al., 1986; Baltimore et al., 1989; Read et al., 1991; Barsum et al., 1992; Feldman et al., 1992). The penetration of an antibiotic is often expressed as the percentage ratio between the concentration in the investigated specimen and in a simultaneously obtained serum sample. Generally the penetration into sputum is poor for the penicillins and the cephalosporins, moderate for the macrolides and the aminoglycosides, and good for the quinolones (Table 1.1) (Valcke et al., 1990). As antibiotics generally penetrate poorly into respiratory secretions (Stout et al., 1987; Bergogne-Berezin, 1988), many bacteria in the respiratory tract are likely to be exposed to

subMIC of antibiotics during treatment of an infective exacerbation. However, the MIC of a presumed pathogen is only an *in vitro* finding and does not take account of *in vivo* host defence mechanisms such as mucociliary clearance and phagocytic actions. The mechanism(s) for the good clinical efficacy of antibiotics that penetrate poorly into the respiratory tract remains to be determined.

1.5.3 EFFECTS OF SUBMIC OF ANTIBIOTICS ON BACTERIAL INTERACTIONS WITH THE HOST

SubMIC antibiotics are known to influence bacterial interactions with host cells in many ways. These include bacterial synthesis of cell wall and extracellular products (Lorian et al., 1975), bacterial adherence to cells (Chopra 1986) and artificial materials (Finch et al., 1989), and changes in bacterial morphology (Lorian et al., 1975; Chopra et al., 1986). Adherence to human buccal epithelial cells, pharyngeal cells and leucocytes, and production of extracellular toxins by *E. coli* (Ofek et al., 1979; Eisenstein et al., 1982; Forestier et al., 1984), *N. meningitidis* (Kristiansen et al., 1983; Salit 1983; Stephens et al., 1984), *Streptococcus pyogenes* (Tylewska et al., 1981) and *S. aureus* (Vymola et al., 1974) are reduced in the presence of subMIC of penicillins. A reduction in the production of bacterial toxins such as ß-lactamase (Chopra et al., 1985), elastase, alkaline protease, exotoxin A, exoenzyme S and phospholipase C (Warren et al., 1985) has been observed in PA exposed to low concentrations of tetracycline, gentamicin, tobramycin (Shibl et al., 1980) and ciprofloxacin (Grimwood et al., 1989). A reduction in PA adherence to human respiratory mucin has also been reported when subMIC ceftazidime was present (Vishwanath et al., 1987). Treatment with low dose antibiotics is often effective in urinary tract infections (Rosenstock et al., 1985). Although the effects of subMIC antibiotics on a number of bacterial species including PA have been studied *in vitro*, little is known of the effects on NTHi, particularly on its interactions with human tissue.

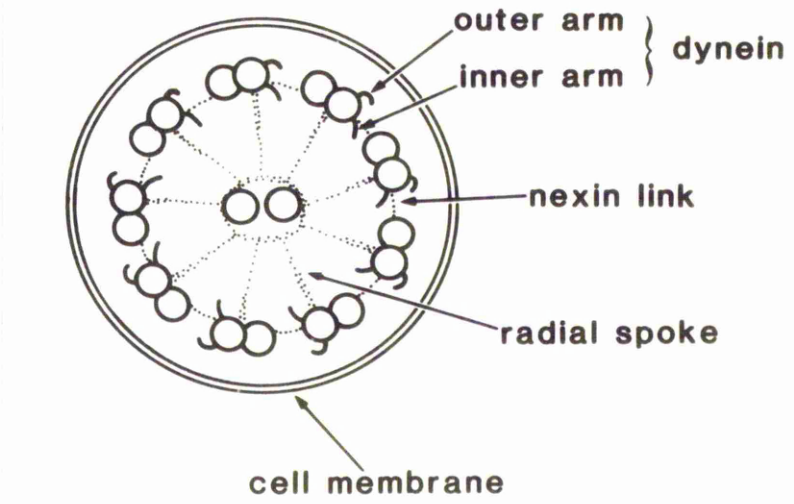


Figure 1.1 A diagram showing the ultrastructure of a cilium and its nine peripheral and two central microtubule pairs. Adjacent pairs are connected by nexin links and the outer fibrils are connected to the central pairs by central spokes.

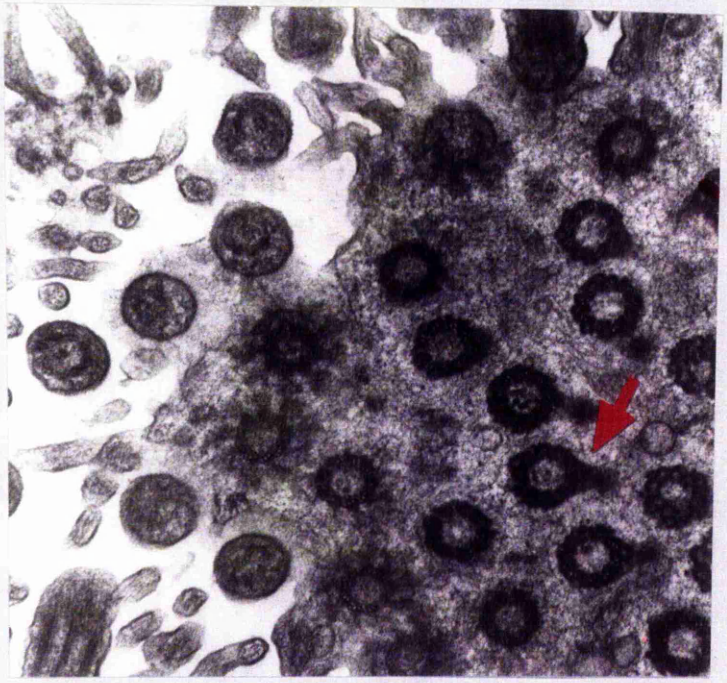


Figure 1.2 Transmission electron micrograph of a ciliated epithelial cell showing basal bodies with triangular foot processes (arrow) (15000x).

TABLE 1.1 PENETRATION OF ANTIBIOTICS INTO RESPIRATORY MUCUS EXPRESSED AS PERCENT RATIOS OF SPUTUM TO SERUM CONCENTRATIONS.

Antibiotic	sputum/serum concentration percent ratio	reference
Penicillins	2-6%	Valcke et al., 1990
Cephalosporins	15-25%	Bergogne-Berezin 1988
Aminoglycosides	20-30%	Bergogne-Berezin 1988
Tetracycline	10-30%	Campbell 1970
Macrolides	10%	Marlin et al., 1980
Quinolones	90-100%	Gartmann et al., 1972

TABLE 1.2 METHODS EMPLOYED TO STUDY BACTERIAL INTERACTIONS WITH ANIMAL TISSUE.

METHOD	ADVANTAGES	DISADVANTAGES
Intact animal respiratory tissue immersed in medium containing bacteria:		
Chinchilla trachea (Bakaletz et al., 1988) Hamster trachea (Baker et al., 1982a) Murine trachea (Ramphal et al., 1980) Dog trachea (Hata et al., 1991) Monkey nasal turbinates (Roberts et al., 1984)	1. Homogeneity of laboratory animals 2. Readily available tissue 3. No alteration of mucosal surface	1. The use of non-human tissue to study human pathogens 2. NTHi only pathogenic to man 3. Impairment of mucociliary clearance as tissue immersed 4. Tissue continuously exposed to bacteria/toxins 5. Constituents of medium may affect bacterial adherence 6. Surface mucus and components may be lost into immersing medium
Intact animal respiratory tissue exposed to air (bacteria directly inoculated onto mucosal surface):		
Frog palate exposed to air (Plotkowski et al., 1989) Rabbit trachea embedded in agar (Matsuyama 1974)	1. Air-mucosal interface achieved 2. Homogeneity of laboratory animals 3. Readily available tissue 4. No alteration of mucosal surface 5. Simple methodology	1. Non-human tissue 2. Inadequate nutrients/water supplied to tissue and bacteria

Animal respiratory epithelial cell culture immersed in medium containing bacteria:		
Hamster tracheal cells (Grant et al., 1991)	1. Convenient source of tissue 2. Greater number of cells than tracheobronchial brushing	1. Non-human source of respiratory epithelial cells 2. Alteration of cell surface receptors and loss of cilia from ciliated cells with prolonged culture 3. Problems encountered with immersion of cells as discussed above 4. Ciliary activity uncoordinated between neighbouring cells 5. Cell cultures may not be confluent 6. Absence of an intact mucus layer 7. Trypsin treatment of cells during preparation of cell cultures may alter cell surface receptors 8. Alteration of *in vivo* mucosal cellular composition and architecture
Animal respiratory cell culture with an air-mucosal interface:		
Guinea pig tracheobronchial cell culture (Alder et al., 1986)	1. air-mucosal interface established	1. Non-human tissue used 2. Problems encountered with animal cell culture as cited above

TABLE 1.3 METHODS EMPLOYED TO STUDY BACTERIAL INTERACTIONS WITH HUMAN TISSUE.

METHOD	ADVANTAGES	DISADVANTAGES
Human cells suspended in medium containing bacteria:		
Buccal epithelial cells (Lampe et al., 1982) Nasal Epithelial strips (Niederman et al., 1983) Tracheal epithelial cells (Franklin et al., 1987) Red blood corpuscles (van Alphen et al., 1988)	1. Readily available human tissue 2. Simple methodology	1. Dispersed cells with exposed non-mucosal surface 2. Original tissue architecture and functions disrupted 3. Problems encountered in immersion of tissue as cited in Table 1.2 4. Inappropriate tissue used for study of respiratory pathogens e.g. buccal epithelial cells
Human epithelial cell cultures immersed in medium containing bacteria:		
Resected nasal polyps (Plotkowski et al., 1991)	1. Readily available tissue	1. Problems encountered in immersion of tissue as cited in Table 1.2 2. Problems encountered in cell culture as cited in Table 1.2

Intact human respiratory tissue immersed in medium containing bacteria:		
Dissected adenoid tissue freely suspended in medium (Stephens et al., 1984) (Farley et al., 1986)	1. Readily available human respiratory tissue 2. Mucosal architecture preserved 3. Simple methodology	1. Dissected surface exposed directly to bacteria/toxins 2. Problems encountered in immersion of tissue as cited in Table 1.2
Intact human respiratory tissue embedded in agar and immersed in medium containing bacteria:		
Dissected nasal turbinates whose non-mucosal surfaces were embedded in agar (Read et al., 1991) (Feldman et al., 1992)	1. As cited for adenoid tissue above 2. Dissected surface sealed by agar and thus not directly exposed to bacteria/toxin(s)	1. Nasal turbinates are not usually infected by NTHi 2. Problems encountered in immersion of tissue as cited in Table 1.2

CHAPTER 2

AIMS OF THESIS

1. To study the effects of subminimal inhibitory concentrations of antibiotics (amoxycillin, loracarbef and ciprofloxacin) on NTHi infection of intact human respiratory mucosa in an agar-embedded organ culture model submerged in cell culture medium.

2. To study the effects of NTHi infection on intact human bronchial mucosa in an agar-embedded organ culture model submerged in cell culture medium.

3. To develop an organ culture model of intact human respiratory mucosa with an air-mucosal interface.

4. To study the effects of NTHi infection of intact human respiratory mucosa in the organ culture model with an air-mucosal interface and to develop a scanning electron microscopy method for the assessment of the surface morphology of the respiratory mucosa and bacterial adherence to it.

5. To study the effects of PA infection of intact human respiratory mucosa in the organ culture model with an air-mucosal interface.

6. To study the effects of a PA toxin, pyocyanin, on intact human respiratory mucosa in the organ culture model with an air-mucosal interface.

CHAPTER 3

MATERIALS

3.1 ORIGIN OF MATERIALS AND INSTRUMENTS

The media, chemicals and instruments that were used during the course of these studies were obtained from the following companies - unless otherwise indicated, chemicals were of analytical grade:

Agar Scientific, Stansted, UK
Amersham International, Amersham, UK
British Drug Houses Ltd, Dagenham, UK
Eli Lilly, Basingstoke, UK
Fisons Ltd, Loughborough, UK
Gelman Sciences, Michigan, USA
Gibco, Paisley, UK
Grant Instrument, Cambridge, UK
Hamamatsa, Tokyo, Japan
Hitachi, Ibaraki-Ken, Japan
Improvision, Coventry, UK
Kent Electronic Instruments, Kent, UK
Leec, Nottingham, UK
Leica, Milton Keynes, UK
Leitz Ltd, Luton, UK
Microtec, Oxford, UK
Oxoid, Basingstoke, UK
Perkin Elmer, Beaconsfield, UK
Polaron, Watford, UK
Sigma Ltd, Poole, UK
Sterilin, Stone, UK
Swan-Morton Ltd, Sheffield, UK
Tissue Culture Service, Buckingham, UK
Whatman Ltd, Maidstone, UK

3.2 TISSUE CULTURE MEDIUM AND BUFFER

3.2.1 MINIMAL ESSENTIAL MEDIUM (MEM)

MEM (Gibco) with Earles salts and phenol red was used for all organ culture experiments.

The composition was

INORGANIC SALTS:	**mg/l**
Calcium chloride	264
Potassium chloride	400
Magnesium sulphate	200
Sodium chloride	6300
Sodium bicarbonate	2200
Sodium hydrophosphate	158
OTHER COMPONENTS:	
D-Glucose	1000
HEPES	5960
Phenol red	10
AMINO ACIDS:	
L-Arginine HCl	126.4
L-Cystine	24.02
L-Histidine HCl.H_2O	41.92
L-Isoleucine	52.46
L-Leucine	52.46
L-Lysine.HCl	73.06
L-Methionine	14.92
L-Phenylalanine	33.02
L-Threonine	47.64
L-Tryptophan	10.2
L-Tyrosine	36.22
L-Valine	46.86
VITAMINS:	
D-Calcium pantothenate	1
Choline chloride	1
Folic acid	1
Inositol	2
Nicotinamide	1
Pyridoxal HCl	1
Riboflavin	0.1
Thiamine HCl	1

3.2.2 PHOSPHATE BUFFERED SALINE (PBS)

PBS (Oxoid) was used as the diluent for all the viable counting experiments and washing of bacteria after broth culture. It was prepared by addition of PBS tablets into distilled water (one tablet/100ml) followed by autoclaving for 15min.

3.3 BROTH CULTURE MEDIA AND BACTERIOLOGICAL AGARS

3.3.1 HERRIOTT'S DEFINED MEDIUM (HDM)

This medium was used throughout the studies described in this thesis for the cultivation of the test strain of NTHi (strain SH9). HDM contains defined quantities of the nutritional requirements of NTHi. Stock solutions of the appropriate chemicals (Sigma and British Drug Houses) were prepared and stored at 4^0C. The solution was prepared by mixing the various stock solutions (Herriott et al., 1970). The final concentrations of the components of HDM were as follows.

	mg/l
Aspartic acid	500
Monosodium glutamate	1300
Sodium chloride	5800
Potassium sulphate	1000
Magnesium chloride	200
Calcium chloride	22.7
EDTA	3.7
Ammonium chloride	220
L-Arginine	300
Glycine	30
Lysine	50
Methionine	100
Serine	100
Leucine	300
Tyrosine	200
Tween-80	20
Polyvinyl alcohol	20
Sodium lactate	800

Glycerol	3000
Uracil	100
Hypoxanthine	20
Inosine	2000
Potassium hydrophosphate	3500
Potassium dihydrophosphate	2700
Haemin	10
Histidine	10
Nicotinamide dinucleotide	2
Thiamine	2
Calcium pantothenate	2
L-Cystine	200

HDM was made in batches of 500ml and then filtered through 0.2μm Acrodisc microfilters (Gelman Sciences). Aliquots were stored at 4^{0}C.

3.3.2 BRAIN HEART INFUSION BROTH (BHI)

BHI broth was prepared by mixing 3.7g of BHI powder (Oxoid) with 100ml distilled water. Sterilisation by autoclaving (15min) was followed by storage at 4^{0}C.

3.3.3 MULLER-HINTON BROTH

Muller-Hinton broth is a commonly used medium for the determination of the MIC of most aerobic and facultatively anaerobic bacteria (National Committee for Clinical Laboratory Standards 1990a). The final concentrations of the components of Muller-Hinton broth are as follows.

Beef infusion	300mg/ml
Acid hydrolysate of casein	17.5mg/ml
Starch	1.5mg/ml

3.3.4 HAEMOPHILUS TEST MEDIUM (HTM)

HTM is the recommended growth medium for the determination of MIC of

NTHi (National Committee for Clinical Laboratory Standards 1990a). HTM compares favourably with Muller-Hinton broth and its transparent nature enables more reliable interpretation of growth end points. The final concentrations (in de-ionised water) of the components of HTM are as follows.

Beef infusion	300mg/ml
Starch	1.5mg/ml
Thymidine phosphorylase	0.2IU/ml
Magnesium chloride	0.01mg/ml
Calcium chloride	0.02mg/ml
Acid digest of casein	17.5μg/ml
Haematin	5μg/ml
Nicotinamide adenine dinucleotide	15μg/ml

3.3.5 BACTERIOLOGICAL AGARS

3.3.5.1 LEVINTHAL AGAR

Levinthal agar was used for solid medium culture of NTHi for all experiments including the viable counting.

Levinthal extract was prepared by mixing equal volume of fresh horse blood (Tissue Culture Service) with BHI broth at room temperature. The horse blood-BHI mixture was heated in a water bath maintained at 85^{0}C until the mixture just turned chocolate in colour, then centrifugated at 2000g at 4^{0}C for 30min. Supernates were obtained by decanting. Stock solution (filter-sterilised) of nicotinamide adenine dinucleotide was added to the supernates so that a final concentration of 1g/l was achieved. The supernates were transferred to 25ml universal containers for storage at -20^{0}C.

When required, 50ml of the Levinthal extract was thawed and mixed with 450ml of BHI agar. BHI agar was prepared by adding 17g BHI powder (Oxoid) and 5g of No.1 Bacteriological Agar (Oxoid) to 450ml of distilled water. This was then autoclaved for 15min and then cooled in

a water bath maintained at 56^{0}C. Mixing was followed by pouring of the mixture to form Levinthal agar plates in 8cm bacteriological petri dishes (Sterilin). The plates were stored at 4^{0}C.

3.3.5.2 NUMBER 2 AGAR

This agar was used for all PA experiments. Seventeen grams of Blood Agar Base No. 2 (Oxoid) was added to 500ml of distilled water following by autoclaving for 15min. Plates were poured after cooling of the solution in a water bath maintained at 56^{0}C. The plates were stored at 4^{0}C.

3.4. INSTRUMENTS

The following instruments were also used during the course of the laboratory work described in this thesis:

Centrifuge (Coolspin, Fisons)

Critical point dryer (Polaron)

Filter manifold (Whatman)

H-7000 transmission electron microscope (Hitachi)

Humidified carbon dioxide incubator (Leec)

Image analyzer (Improvision)

Microcentrifuge (Fisons)

pH meter (Kent Electronic Instruments)

Phase contrast microscope Dialux 20 (Leitz) with photomultiplier (Hamamatsa)

S-4000 scanning electron microscope (Hitachi)

Thermostatic water bath (Grant Instrument)

UV spectrophotometer (Perkin Elmer)

Whirlmixer (MSE, Fisons)

3.5 MICROORGANISMS STUDIED

3.5.1 STRAIN SH9

Strain SH9 is a NTHi originally isolated from the sputum of a patient with an infective exacerbation of chronic bronchitis. It has previously been investigated at the Host Defence Unit (Read et al.,

1991).

3.5.2 STRAIN P455

Strain P455 is a PA originally isolated from the sputum of a patient who presented with an infective exacerbation of idiopathic bronchiectasis. It is known to produce pyocyanin and 1-hydroxyphenazine.

3.6 HUMAN ADENOID, NASAL TURBINATE AND BRONCHUS TISSUE

The human adenoid is a single pad of lymphoid tissue situated at the posterior wall of the nasopharynx. It is small at birth and begins to grow rapidly from the age of three and is maximal in size at about the age of five. Thereafter it slowly undergoes atrophy and disappears completely at puberty. Its enlargement may cause symptoms of secretory otitis media and lead to the development of acute otitis media (Snell 1981). Adenoidectomy is performed with a curette which is used to sweep down the posterior wall of the nasopharynx (Pracy et al., 1978). Adenoid tissue is covered by ciliated respiratory epithelium (Leeson et al., 1988).

The inferior turbinate is attached to the medial wall of the maxilla and is part of the lateral nasal cavity wall. It is a highly vascular structure covered with respiratory mucosa and serves to humidify and warm inhaled air. It also forms part of the baffle system of the nasal cavity, which alters the direction of airflow and encourages particle deposition onto the nasal mucosa (Snell 1981). The anterior part of the turbinate is covered by squamous epithelium but the rest is covered by ciliated respiratory epithelium (Leeson et al., 1988).

Human bronchial wall contains irregular plates of cartilage and smooth muscle layers that are arranged in a spiral fashion internal to the cartilaginous plates. The muscle coat becomes more complete distally as the cartilaginous plates become more fragmentary (Last 1981). The epithelial lining is ciliated and includes globet cells which become

less numerous peripherally. Larger bronchi also have acinar mucus-secreting glands in the submucosa. Hypertrophy of these glands is one of the features of chronic bronchitis (Leeson et al., 1988).

Human adenoid and nasal turbinate tissue was provided by Miss Valerie Lund, Consultant Surgeon to the Royal National Nose, Ear and Throat Hospital (London) who performed adenoidectomy on children who had adenoid hypertrophy and adult patients who had non-allergic nasal obstruction. Mr Peter Goldstraw, Consultant Surgeon to the Royal Brompton National Heart and Lung Hospital (London), provided bronchial tissue obtained from patients who underwent resection of bronchial carcinoma.

CHAPTER 4

METHODS

4.1 INITIAL HANDLING OF RESPIRATORY TISSUE

Adenoid tissue resected from children with adenoid hypertrophy, nasal turbinates from patients with nasal obstruction, or bronchi resected from patients undergoing thoracotomy were collected and immediately immersed in MEM which contained antibiotics (50μg/ml gentamicin, 50μg/ml streptomycin and 50IU/ml penicillin) for 4-6h to eradicate commensal organisms. The duration varied because of the time required to transport the tissue to the laboratory and the time required for dissection. The respiratory tissue was placed on the inside of an up-turned lid of a sterile 3x1cm triple vent petri dish (without MEM) to avoid excessive movement that occurred when tissue was immersed. Rapid but careful dissection was performed to avoid tissue dehydration and selected tissue pieces were immediately immersed in MEM. Smaller pieces of adenoid or bronchial tissue (approximately $4x4x4mm^3$) were obtained by dissection for the agar-embedded organ culture model. More thinly dissected adenoid or nasal turbinate pieces (approximately $3x3mm^2$ x 1-3mm in thickness) were obtained to establish the organ culture model with an air-mucosal interface. Only dissected tissue pieces with at least two completely ciliated and intact edges that had no adherent mucus were selected by light microscopy for experiments. By using these criteria, over 50% of the adenoid and 90% of the bronchial tissue that arrived at the laboratory were rejected.

Dissected pieces of adenoid, nasal turbinates, or bronchial tissue that were selected for further experiments were immersed in 30ml of MEM and incubated at 37°C for 1h to remove the antibiotics (Read et al., 1991).

4.2 PREPARATION OF THE ORGAN CULTURES

4.2.1 PREPARATION OF AN AGAR-EMBEDDED ORGAN CULTURE MODEL OF INTACT RESPIRATORY MUCOSA (READ ET AL., 1991) (FIGURE 4.1)

Following initial tissue selection (Section 4.1), approximately 0.75ml of 1% semi-molten No.1 Bacteriological Agar (Oxoid) maintained at 40°C was added to a sterile 3 x 1cm triple vent petri dish. The selected

adenoid or bronchial tissue was placed on the semi-molten agar with a plastic sterile loop (Sterilin). The mucosal surface of the tissue faced upwards so that only the ciliated but not the submucosal surface was exposed. Careful inspection of the tissue edges using a hand lens was followed by pipetting of more semi-molten agar (up to 0.1ml) directly onto the remaining exposed cut edges of the adenoid or bronchial tissue to ensure that all the cut edges were sealed by agar. Five minutes were allowed for the agar to set completely at room temperature before 3ml of MEM was added to each organ culture. The organ cultures were subsequently incubated at 37^{0}C in a humidified atmosphere that contained 5% CO_2 for 1h to allow CBF to stabilise. Assessment by light microscopy then followed (Section 4.3.1).

4.2.2 PREPARATION OF THE ORGAN CULTURE MODEL OF INTACT RESPIRATORY MUCOSA WITH AN AIR-MUCOSAL INTERFACE (FIGURE 4.2)

A 3cm petri dish (Sterilin) without its cover was placed in the centre of a 5cm petri dish (Sterilin) aseptically. Four ml of MEM without antibiotics were added to the 5cm petri dish carefully so that the inside of the 3cm petri dish remained dry. A sterile strip of filter paper measuring 5cm by 5mm (Whatman 1) was soaked in sterile MEM, then aseptically laid across the diameters of the petri dishes. The two ends of the filter paper strip were positioned by using a pair of fine forceps so that they were immersed in MEM. A piece of adenoid tissue was placed with its ciliated surface upwards onto the filter paper strip in the centre of the smaller inner petri dish. Approximately 0.25ml of 1% semi-molten agar (Oxoid) at 40^{0}C was carefully pipetted around the edges of the adenoid tissue to seal the cut edges. This produced an approximately 3mm edge of agar, and care was taken not to form a rim above the level of the tissue surface.

4.3 LIGHT MICROSCOPY ASSESSMENT OF ORGAN CULTURES

4.3.1 LIGHT MICROSCOPY ASSESSMENT OF THE AGAR-EMBEDDED ORGAN CULTURES

Organ cultures were examined with a Leitz Dialux 20 phase contrast microscope. During light microscopy assessment, the petri dish

containing the organ culture was placed on an electronically controlled warm stage (Microtec) maintained at 37°C. Ten sites, each separated from each other by at least one microscopic field (x320), were selected along one free edge of each organ culture. In order to ensure that the light microscopy assessment was done as near as possible to the same sites at 24h, a drawing of each organ culture was made. The orientation of the 10 sites to each other was aided by the natural contours of the free edge of the organ culture and markings made on the petri dish.

Each of these sites was assessed at 0h and 24h as follows:

1/. CBF measurement (Section 4.7):

CBF at 0h and 24h was assessed in two ways. Firstly the mean CBF of each of the ten sites along a free edge of the organ culture, which included scores of zero CBF at sites with absent ciliary beating where it had previously been present, was measured. Secondly the mean CBF was calculated from the CBF measured only at sites where ciliary beating was still present (excluding sites with no beating cilia).

2/. Number of sites with adherent mucus:

0 absence of adherent mucus

1 presence of adherent mucus

3/. Number of sites with disrupted epithelial integrity:

0 absence of disruption of epithelial integrity

1 presence of disruption of epithelial integrity

4/. Number of sites with ciliary activity:

At 0h all 10 sites had ciliary beating otherwise the tissue would not be used. The number of sites with ciliary beating at 24h was counted.

4.3.2 LIGHT MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE

In selected experiments, light microscopy assessment was performed using a Leitz Dialux 20 phase contrast microscope. Assessment was performed along one free edge of the adenoid tissue. In order to visualise a free edge of the organ culture without disturbing the

tissue, approximately 0.5mm of a free edge of the adenoid tissue was left overhanging the underlying strip of filter paper although the cut edges were still occluded by 1% semi-molten agar as described above. Fifty μl of sterile MEM (maintained at 37^{0}C) was then pipetted onto the organ culture which temporarily submerged the tissue to allow its visualisation with the Leitz microscope.

Five ciliated sites, separated from each other by at least one light microscopic field (320x), that were free of mucus or broken epithelial outline were selected along the overhanging free edge of the adenoid organ culture. CBF was measured at these sites at 0h and 24h as described in Section 4.7. A drawing of the epithelial contour and markings made on the outer petri dish helped identify the selected sites for repeated evaluation.

4.4 INCUBATION OF THE ORGAN CULTURES

4.4.1 INCUBATION OF THE AGAR EMBEDDED ORGAN CULTURES

After the initial light microscopy assessment (Section 4.3.1), 20μl of either a washed NTHi (strain SH9) suspension (Section 4.8) or sterile PBS was added to the MEM of the designated infected or uninfected organ cultures respectively. After 24h incubation in a humidified atmosphere that contained 5% CO_2 maintained at 37^{0}C, 50μl of the MEM from each organ culture was taken for viable counting to assess bacterial growth and purity or sterility of the respective adenoid or bronchial organ cultures (Section 4.9). Light microscopy assessment was followed by fixation and processing of tissue for TEM assessment (Section 4.5).

4.4.2 INCUBATION OF THE ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE

A pre-determined volume of a washed bacterial suspension (section 4.8) (20μl for the test strain of NTHi, SH9; 10μl for the test strain of PA, P455) or pyocyanin (10μl of 20 mg/ml) or sterile PBS alone (10 or 20μl) for control organ cultures was pipetted directly onto the centre of the designated organ culture. Pairs of organ cultures were

incubated at 37°C for up to 24h in a humidified atmosphere that contained 5% CO_2.

After incubation, each of the 4 edges of the organ culture were touched gently with a sterile plastic disposable loop and inoculated onto Levinthal or No 2 plates for NTHi and PA experiments respectively. Following inoculation, agar plates were incubated at 37°C for 24h to confirm the purity of bacterial growth or sterility in the infected and uninfected organ cultures respectively. Each of the organ cultures (with its adherent edge of agar and a small strip of filter paper) was removed from the petri dishes and processed for TEM or SEM assessment (Sections 4.5 & 4.6).

4.5 TEM ASSESSMENT OF RESPIRATORY MUCOSA

4.5.1 FIXATION AND PROCESSING OF TISSUE FOR TEM

After incubation, each of the organ cultures was carefully removed from each petri dish along with its agar edge (and a small strip of filter paper in the organ culture model with an air-mucosal interface) in both of the organ culture models. Specimens were fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.2) for 48h and post fixed in 1% osmium tetroxide (for up to 7 days), followed by standard serial dehydration in alcohols and embedding in araldite (Read et al., 1991). For TEM assessment, an ultra-thin section (70-90 nm) through the central portion of each specimen was examined. Each epithelial cell in the section was scored by Mr Andrew Rutman who is the electron microscopy technician in the Host Defence Unit, who was unaware of the experimental protocols.

4.5.2 MORPHOMETRIC MEASUREMENT OF ULTRASTRUCTURAL DAMAGE AND THE DEVELOPMENT OF A SCORING SYSTEM

Each epithelial cell seen in the randomly chosen TEM section was assessed for loss of cilia, extrusion from the epithelial surface, cytoplasmic blebbing, and mitochondrial damage (swelling and disruption of cristae).

Scoring of each cell for these parameters was performed:

1/. Loss of cilia from ciliated cell:

0 fully ciliated (Figure 4.3)

1 "mild" loss of cilia (Figure 4.4)

2 "moderate" loss of cilia (Figure 4.5)

3 "severe" loss of cilia (Figure 4.6)

2/. Extrusion of cell from the epithelial surface:

0 "no" extrusion (Figure 4.7)

1 "possible" extrusion (Figure 4.8)

2 "mild" extrusion (Figure 4.9)

3 "moderate" extrusion (Figure 4.10)

4 "complete" extrusion although contact with other epithelial cells remained (Figure 4.11)

3/. Cytoplasmic blebbing (Figure 4.12):

0 absent

1 minor

2 major

4/. Mitochondrial damage (Figure 4.13):

0 absent

1 present

As the relative significance of the TEM parameters was unclear, the total score attributed to each of these was adjusted so that each feature had a maximal adjusted score of 100. The total damage score for the tissue was calculated by summation of the 4 adjusted scores so that the maximum possible score was 400. In this way the combined damage score took account of the frequency and severity of all the TEM parameters examined. The percent of cells showing a particular parameter of damage was calculated from the sum of the cells not scoring zero for that parameter.

4.5.3 MORPHOMETRIC ASSESSMENT OF CILIARY AND BASAL BODY ORIENTATION (FIGURE 4.14)

A recently established TEM method was used to assess ciliary central

microtubule and foot process orientation (Section 1.1.2) (Rutman et al., 1993). Random sections of cilia and basal bodies were taken from each randomly chosen ciliated epithelial cell for further examination by TEM. At least 200 cilia (more than 10 cells) and 120 basal bodies (more than 15 cells) were examined in each adenoid organ culture. An image of these cilia or foot processes was taken by Mr Andrew Rutman of the Host Defence Unit, who was unaware of the experimental protocol. Processing using an image analysis system then followed (Improvision, UK).

In each cilium examined at TEM, a line was drawn electronically across through the central pairs of microtubules. The angle made by each of these lines to the vertical axis was then measured. The standard deviation (SD) of these angles for the cilia from each of the epithelial cells was calculated. A mean SD was obtained from the assessed epithelial cells in each organ culture, which represented an index of ciliary central microtubular orientation in that particular organ culture (Section 1.1.2). Similarly the angle made by a line that bisected the spur-like foot process of each basal body to the vertical axis was measured. A mean SD was calculated likewise which was taken as an index of orientation of the foot processes in a particular organ culture.

4.6 SEM ASSESSMENT

4.6.1 PROCESSING OF SEM SPECIMENS

Adenoid and nasal turbinate tissue was fixed in 2.5% glutaraldehyde in sodium cacodylate buffer at 4_0C for a minimum of 24h. Specimens were rinsed three times in sodium cacodylate buffer before post-fixed in 1% osmium tetroxide for 1h. Rinsing of specimens with distilled water was followed by dehydration through graded methanols (3x in 70%, 3x in 90%, and 3x in 100%) to 100% acetone (3x). Specimens were critically point dried in CO_2 and mounted on aluminium stubs before sputter-coated with gold. They were stored in desiccated tubes prior to SEM examination performed by myself with a Hitachi S-4000 scanning

electron microscope. The specimens were randomised and coded so that I was unaware of the treatment received by the organ cultures.

4.6.2 THE DEVELOPMENT OF A MORPHOMETRIC SEM METHOD TO ASSESS THE SURFACE MORPHOLOGY OF ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE

Adenoid or nasal turbinate tissue was placed at the centre of the SEM stage under a magnification of 50x. Two diagonal lines at right angles to each other were constructed on the video display unit. These lines intersected at the centre of the tissue and their ends were at the four corners of the tissue. The four SEM fields (25x35μm) at the centre of the adenoid or nasal turbinate tissue were examined at 2000x. Then 9 more fields of same dimensions were examined along each of the four diagonals constructed by moving away from the centre of the tissue towards each corner. Altogether 40 different fields (typically 650 epithelial cell surface area) were therefore examined.

Each SEM field was divided into 100 equal squares (0.25x0.35μm^2 i.e. similar dimension to the size of a single NTHi or PA bacterium as cited in Sections 1.4.1.1 & 1.4.2.1) by a transparent grid that rested on the video display unit of the SEM. The smallest unit for an area occupied by a mucosal component such as mucus was attributed as one square whether the square was completely occupied or not. When several mucosal components were present in the same square, an area of one square was attributed to each mucosal component (although this was an exceptional occurrence at a magnification of 2000x). By manually counting the number of squares containing mucus, extruded cells and cell debris (a granular amorphous substance that was often associated with extruded and damaged cells) (Figure 8.3), ciliated epithelium and unciliated epithelium, the percent of SEM fields containing these features was determined for each organ culture. The percent of SEM fields that contained each of the 4 mucosal components with adherent NTHi was also determined similarly (which represented the absolute adherence of NTHi to that mucosal component).

4.6.3 THE DEVELOPMENT OF A MORPHOMETRIC SEM METHOD TO ASSESS NTHi ADHERENCE TO ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE

The total area occupied by mucus that had associated NTHi on its surface, expressed as the number of unit area ($0.25 \times 0.35 \mu m^2$ for each square) i.e. similar in size to a NTHi bacterium, was determined for each organ culture to represent the bacterial density on mucus for a particular organ culture. As organ cultures differed in the quantity of surface mucus, the adherence index of NTHi for mucus (which represented the relative affinity of NTHi for mucus and allowed comparison to be made on different organ cultures) was calculated as the ratio of the percent of SEM fields that contained mucus with adherent NTHi to the percent of SEM fields that contained mucus. Likewise the adherence indices for ciliated epithelium, unciliated epithelium, cell debris and extruded cells were calculated for each organ culture.

The number and size of each bacterial microcolony was also assessed by manual counting. A bacterial microcolony was defined as a group of bacteria that were in direct contact when seen at SEM. When bacteria were seen associated with ciliated epithelium, assessment was performed separately according to whether they were adherent to the tip (upper third of a cilium), the shaft (middle third) or the base of the cilia (bottom third). The base of the cilia was usually only visible at the edge of ciliated cells.

4.6.4 PREPARATION OF PA INOCULUM FOR SEM EXAMINATION

In order to assess the production of matrix material by PA, SEM examination of PA inoculum was performed. PA inoculum was prepared as described in Section 4.8. One ml of a washed PA suspension was centrifuged at 2000g in a 1.5ml sterile Eppendorf tube. The pellet was placed on a sterile plastic loop and smeared onto a sterile rounded glass cover slip (1cm in diameter) and air-dried in a flow cabinet for 1min. The cover slip was then immersed in 2ml of 2.5% glutaraldehyde. Routine processing for SEM assessment then followed as described in

Section 4.6.1. The cover slips were mounted on aluminium stubs and examined qualitatively by using SEM.

4.7 PHOTOMETRIC MEASUREMENT OF CBF

A modified phase contrast microscope (Leitz Dialux 20) was used with a photomultiplier (Hamamatsu R928) and control panel for amplification adjustment. The power source (Leitz) generated light through a series of condensers. A diaphragm between the objective lens and the photomultiplier permitted an adjustable slit of light to enter the photomultiplier tube. The position of the slit was fixed in the middle of the field of view but it could be rotated through 360^0. When measuring the CBF of ciliated epithelium, tissue was positioned by alteration of the microscope stage so that the long axis of the light slit was parallel to the ciliary axoneme. Ciliary beat interrupted the light and the fluctuations were recorded in the photomultiplier where voltage changes were generated. The signal produced passed through a series of filters and was then frequency-to-voltage converted and passed through an analyzer which gave a cumulative mean CBF over the previous 5s (Greenstone et al., 1984). Tissue examined by light microscopy for measurement of CBF was placed on a warm stage (Microtec) maintained at 37^0C by a thermostatic device.

4.8 PREPARATION OF BACTERIAL INOCULA FOR INFECTION OF ORGAN CULTURES

The test organisms, NTHi (strain SH9) and PA (strain P455), were stored in BHI broth that contained 20% glycerol and were kept in liquid nitrogen. Bacteria were retrieved from the storage vials using standard plating techniques onto Levinthal (NTHi) or No 2 plates (PA). Following overnight incubation, a colony of the appropriate bacteria was touched with a sterile metal loop which was agitated in 5ml of HDM (NTHi) or 3.5ml of BHI (PA) in a 6ml bijou. Thorough mixing was achieved by vortexing each bijou for at least 1min. The test organisms were incubated for 4h (for strain SH9) and 6h (for strain P455) on a rolling stage (40 cycle/min) at 37^0C. After incubation, 1ml of the bacterial suspension was taken from the bijou and transferred to a

sterile 1.5ml plastic Eppendorf tube. Centrifugation for 10min at 2000g then followed in a microcentrifuge. The supernate was then pipetted off and the bacterial pellet was re-suspended in 1ml of PBS by vortexing. This washing procedure was repeated twice. The final bacterial suspension was used for inoculation of organ cultures. By using this method, the viable counts of the NTHi and PA suspensions for inoculation were generally between 10^6 and 10^7 colony forming units (cfu)/ml.

4.9 METHODS OF VIABLE COUNTING

Standard dilution techniques were used to quantify viable bacteria either in the inocula or the MEM derived from organ cultures in the agar-embedded organ culture model (Section 4.4.1).

A 50μl aliquot of bacterial suspension was added to a bijou containing 0.45ml of PBS and mixed by vortexing with a whirlmixer. This produced a 1 to 10 dilution. This process was carried out repeatedly to incur dilutions from 10^{-1} to 10^{-6} of the original suspension. When the dilution had been completed, 20μl of each were dropped onto one of 6 sectors of a marked Levinthal (for strain SH9) or No 2 plates (for strain P455) in triplicate. The plates were then incubated overnight (16-18h) at 37^{0}C. Counting of the number of colonies per sector was performed in areas with 20-60 colonies. A mean number of colonies per 20μl was then calculated. The viable count of the initial bacterial suspension was taken as the results of multiplying this mean number by 50 (dilution for 1ml) and then by the power of dilution.

4.10 DETERMINATION OF THE MIC OF AMOXYCILLIN, LORACARBEF AND CIPROFLOXACIN FOR NTHi (STRAIN SH9) (NATIONAL COMMITTEE OF CLINICAL LABORATORY STANDARDS 1990a)

The test strain of NTHi (strain SH9) was plated onto a Levinthal plate and incubated overnight. A few colonies of NTHi were touched by a sterile plastic loop (Sterilin) and then resuspended in Muller-Hinton broth. NTHi suspension of 10^8 cfu/ml was obtained by checking the

optical density with a spectrometer initially and later confirmed by viable counts. Fifty μl of the bacterial suspension in Muller-Hinton broth was added to each of the 100μl wells of a sterile microtitre plate which contained 50μl of HTM (National Committee of Clinical Laboratory Standards 1991b) with either amoxycillin (Sigma), loracarbef (Eli Lilly) or ciprofloxacin (Sigma). Eight wells in each row of the microtitre plate were used for each antibiotic tested. The concentration of an antibiotic in the HTM was half of its preceding well in the first 6 wells of the same row. The concentrations of amoxycillin, loracarbef and ciprofloxacin used were 0.64, 0.32, 0.16, 0.08, 0.04 and 0.02μg/ml; 1.24, 0.62, 0.31, 0.16, 0.08 and 0.04 μg/ml; and 0.032, 0.016, 0.008, 0.004, 0.002 and 0.001 μg/ml respectively. The last two wells of each row did not contain antibiotics and acted as positive controls. Following the addition of bacterial suspension, the microtitre plates were sealed by a sterile plastic sheet and then incubated overnight (16-18h) at 37°C. As a dilution factor of 2 was created by the addition of equal volume of antibiotic solution to the bacterial suspension, the MIC of an antibiotic for NTHi (strain SH9) was taken as half of the lowest concentration of the antibiotic that produced a clear well.

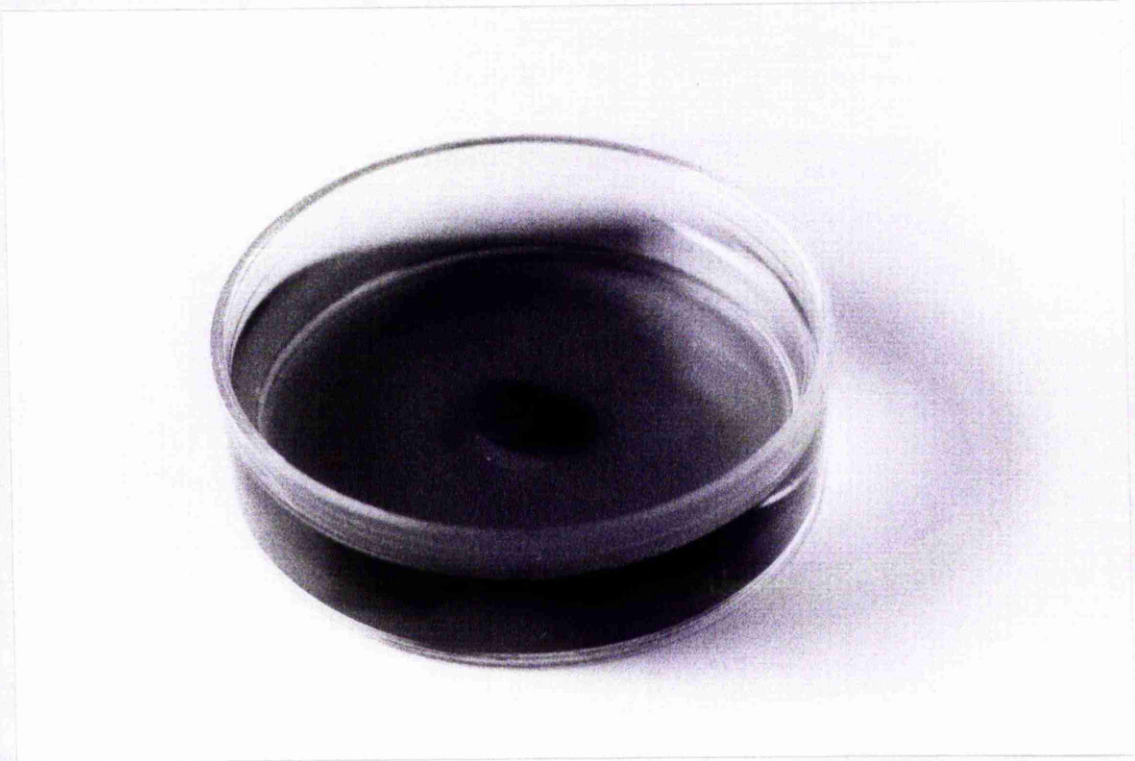


Figure 4.1 A photograph showing an agar-embedded organ culture model of intact human respiratory mucosa. Three ml of minimal essential medium was added to immerse the respiratory tissue which was embedded in 1% agar.

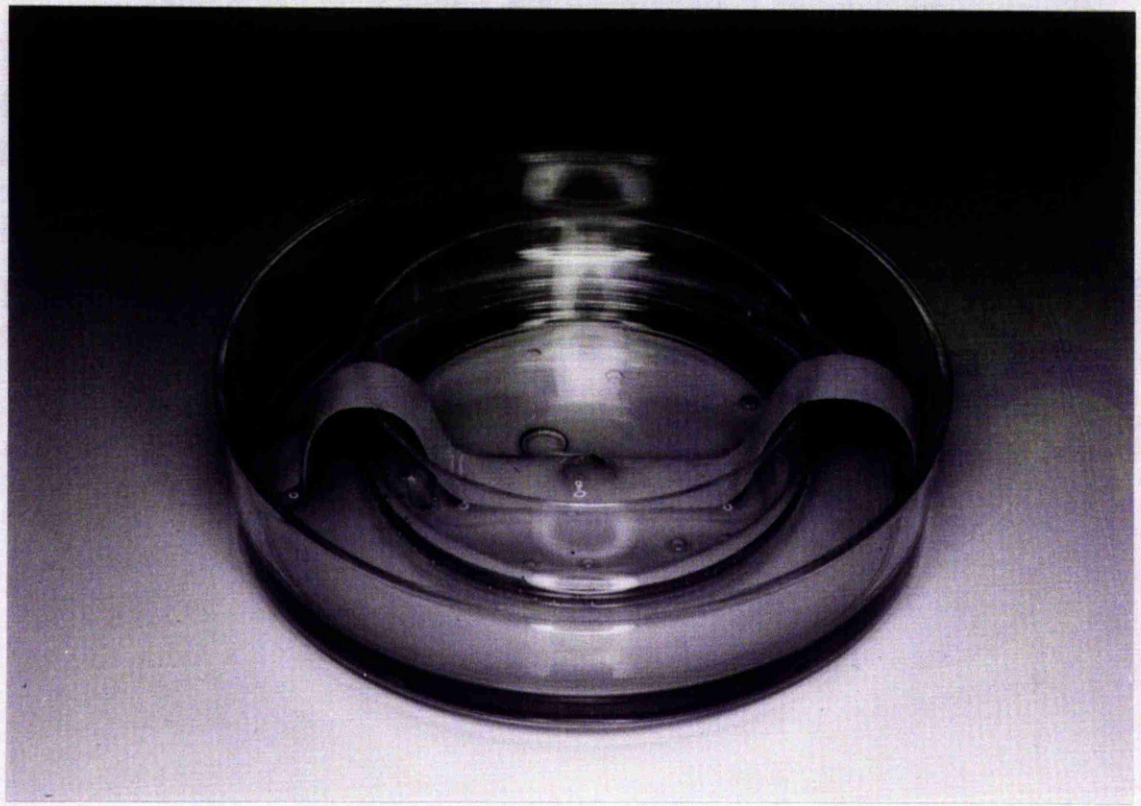


Figure 4.2 A photograph of an organ culture model with an air-mucosal interface showing the arrangement of the two petri dishes, and the adenoid tissue with its agar edge placed on a strip of filter paper whose ends were immersed in 4ml of minimal essential medium.

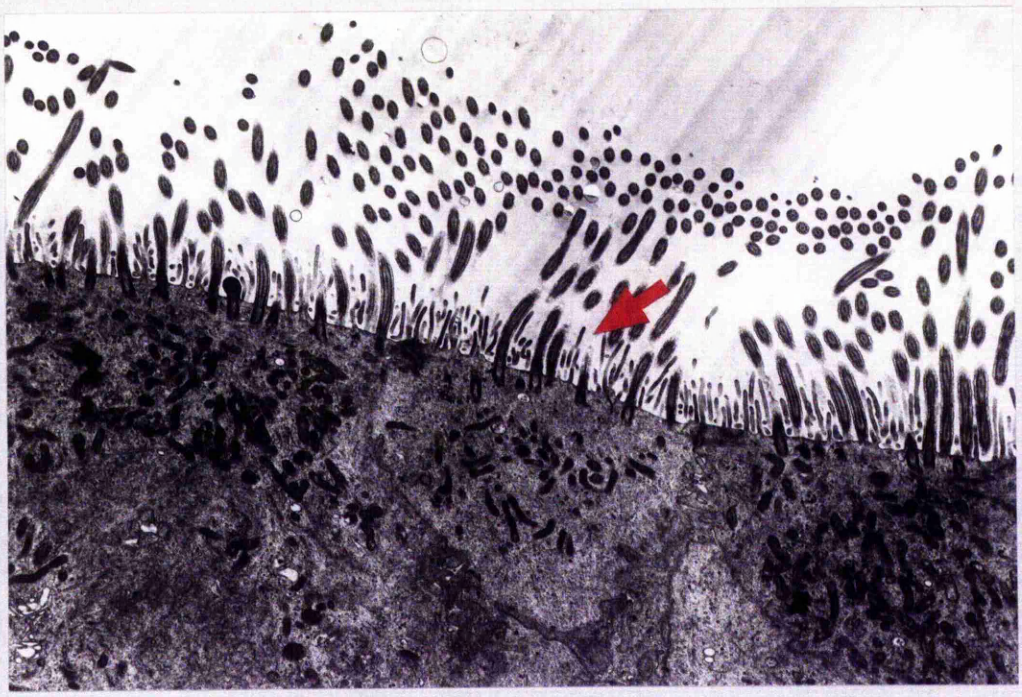


Figure 4.3 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "no" loss of cilia from an epithelial cell (arrow) (score = 0)(5250x).

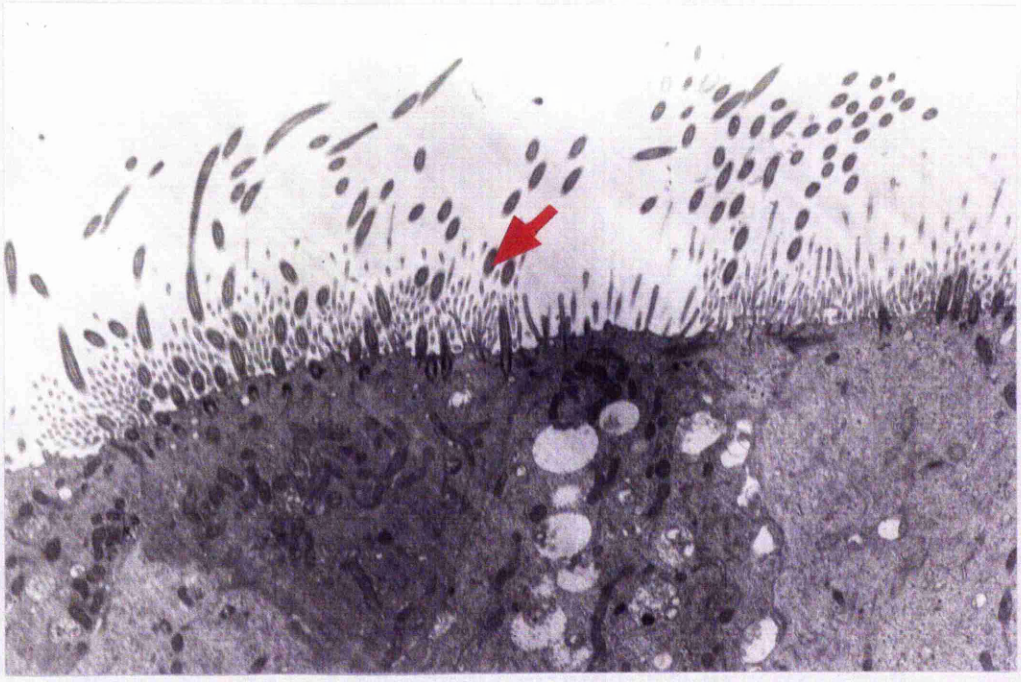


Figure 4.4 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "mild" loss of cilia from an epithelial cell (arrow) (score = 1)(5250x).

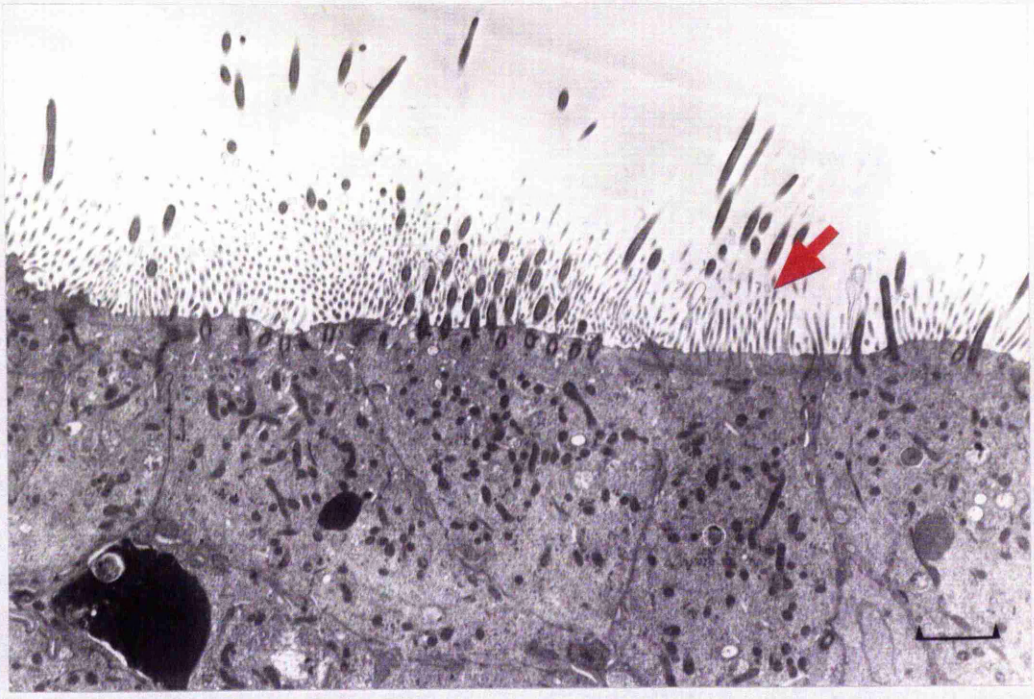


Figure 4.5 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "moderate" loss of cilia from an epithelial cell (arrow) (score = 2)(5250x).

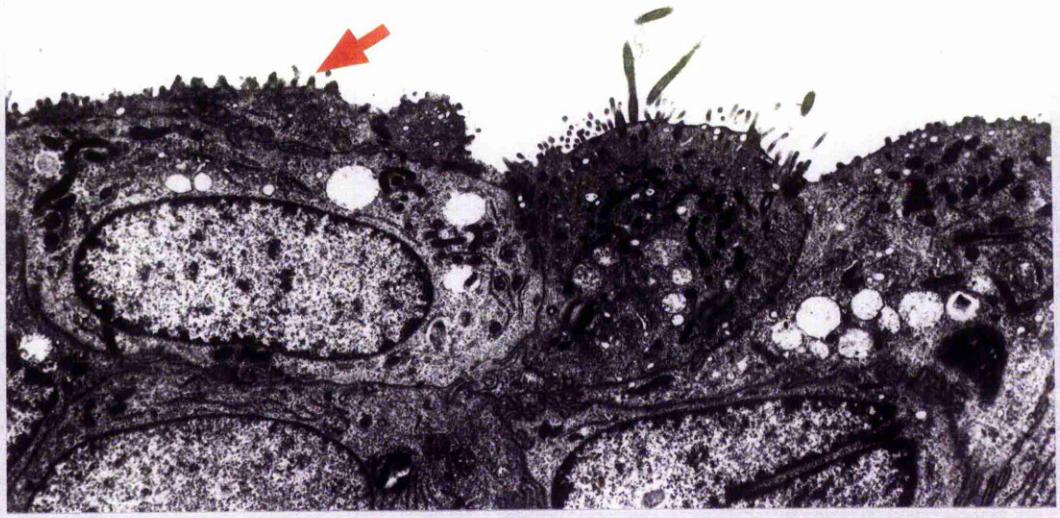


Figure 4.6 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "severe" loss of cilia from an epithelial cell (arrow) (score = 3)(5250x).

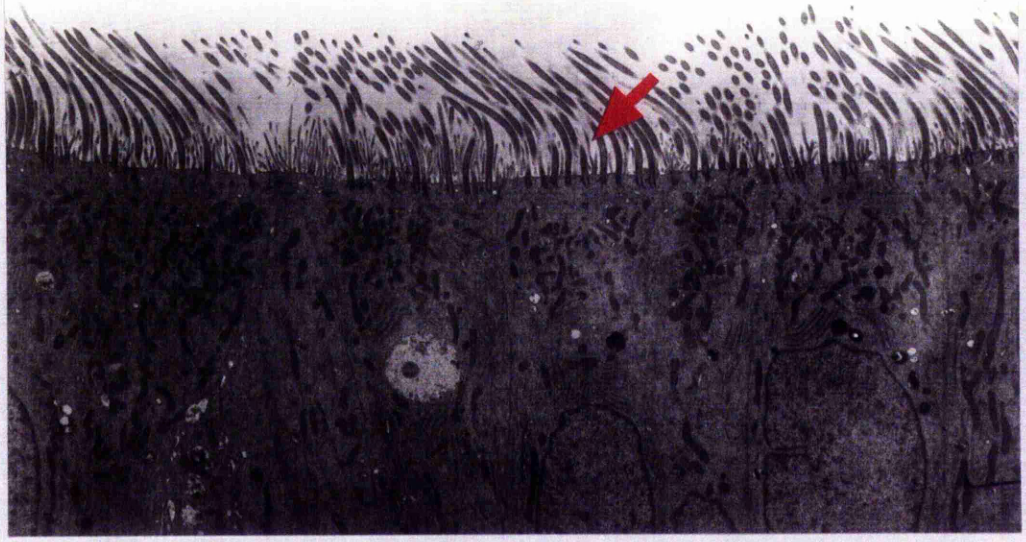


Figure 4.7 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "no" extrusion of a cell (arrow) from the epithelial surface (score = 0) (3500x).

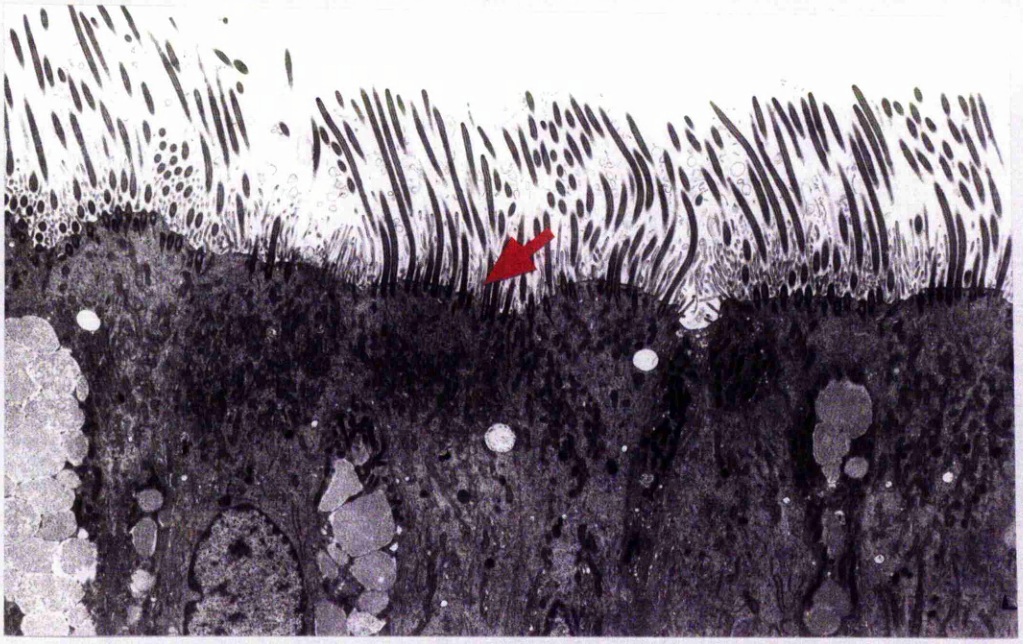


Figure 4.8 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "possible" extrusion of a cell (arrow) from the epithelial surface (score = 1) (3500x).

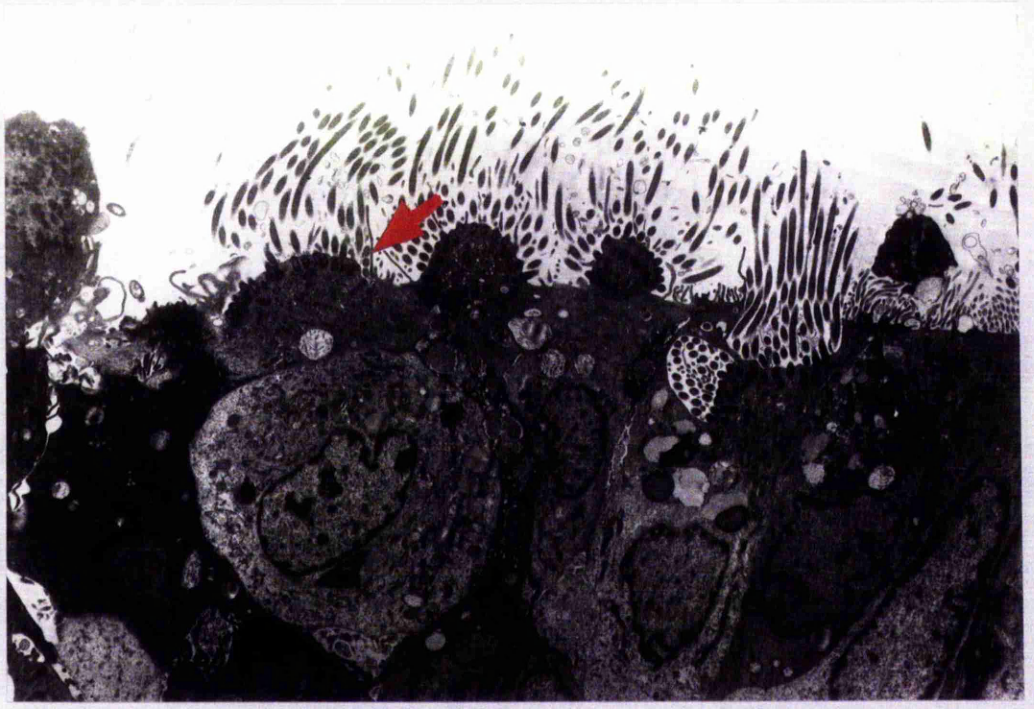


Figure 4.9 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "mild" extrusion of a cell (arrow) from the epithelial surface (score = 2)(3500x).

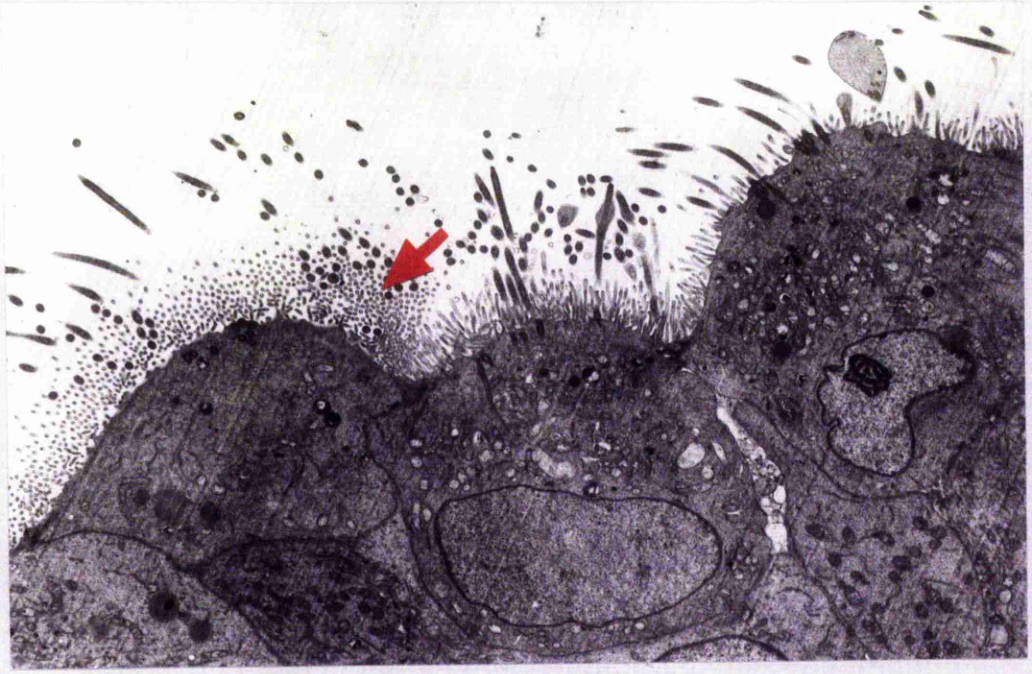


Figure 4.10 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "moderate" extrusion of a cell (arrow) from the epithelial surface (score = 3)(3500x).

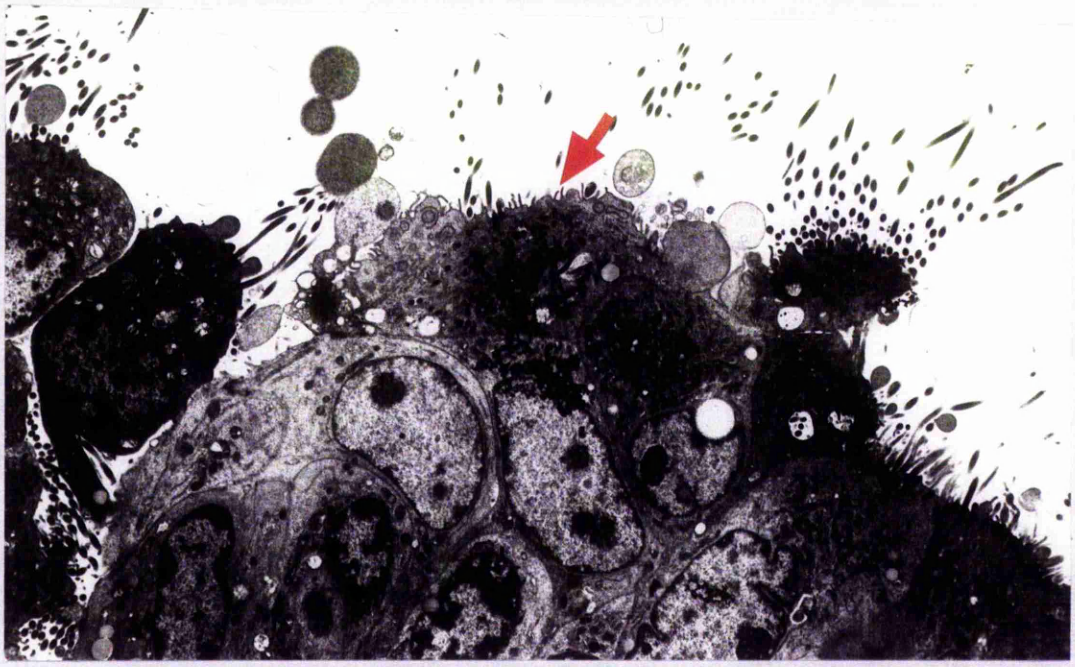


Figure 4.11 Transmission electron micrograph of human adenoid mucosa demonstrating the scoring system for "complete" extrusion of a cell (arrow) from the epithelial surface (score = 4)(3500x).

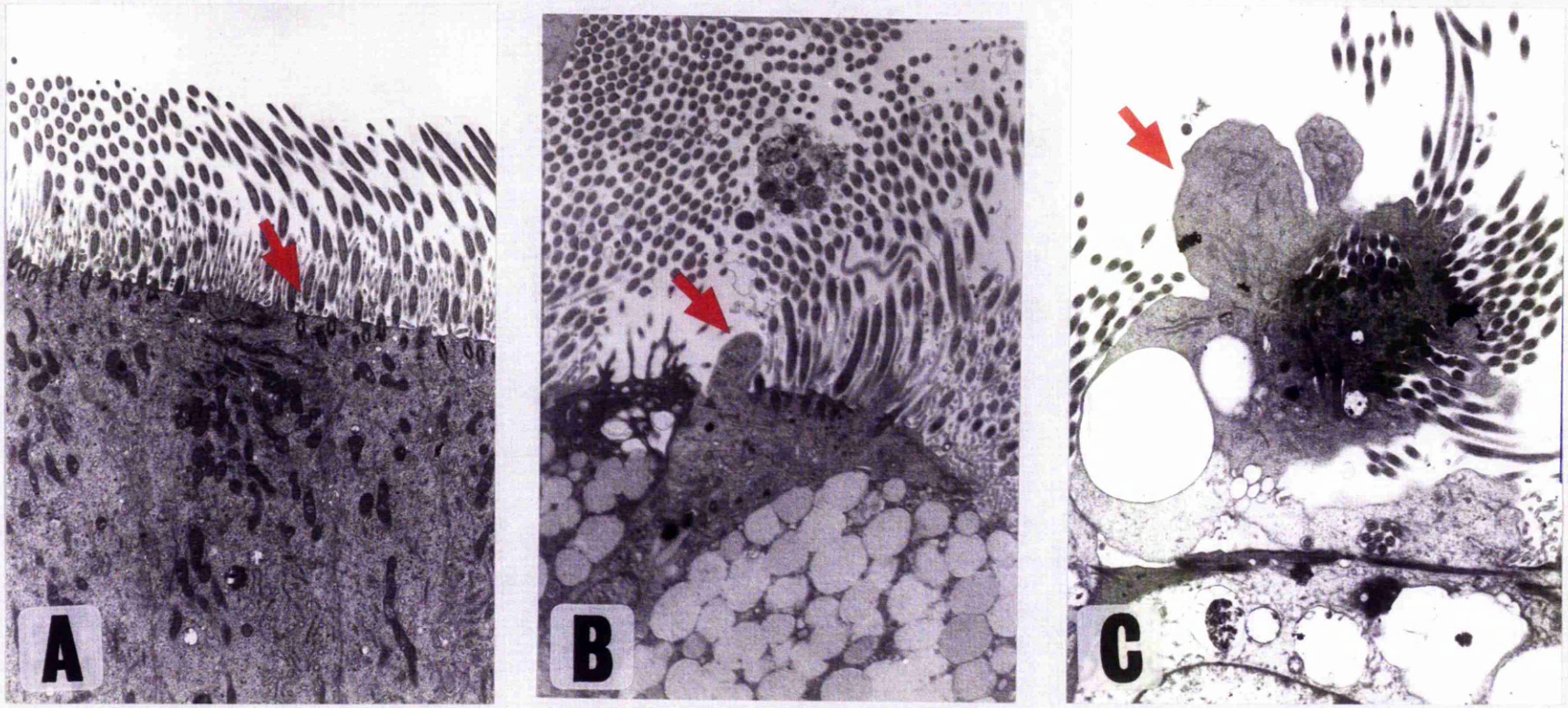


Figure 4.12 Transmission electron micrographs of human adenoid mucosa demonstrating the scoring system for: (A) "no" cytoplasmic blebbing (arrow) (score = 0); (B) "minor" cytoplasmic blebbing (arrow) (score = 1); (C) "major" (arrow) cytoplasmic blebbing (score = 2)(5250x).

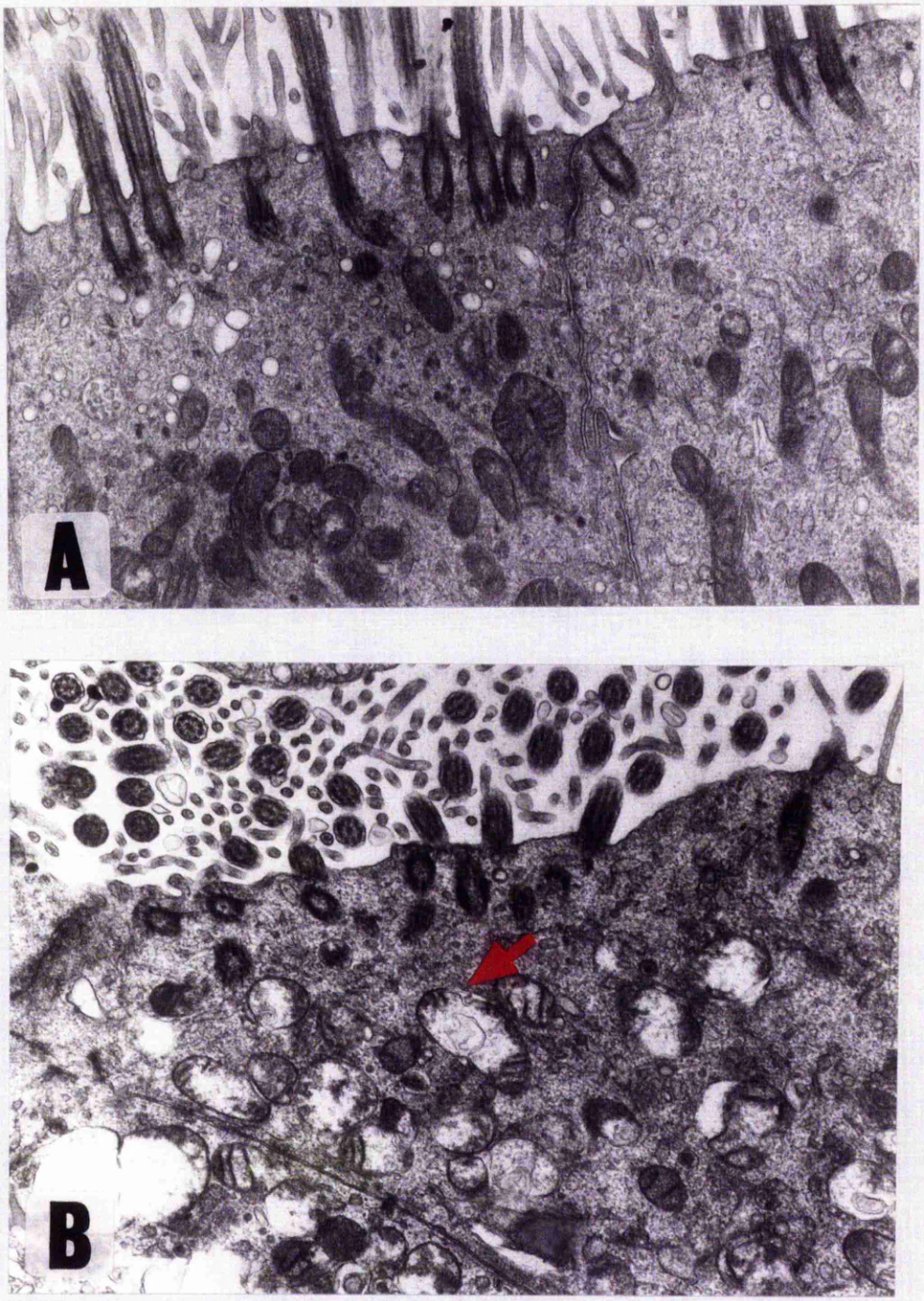
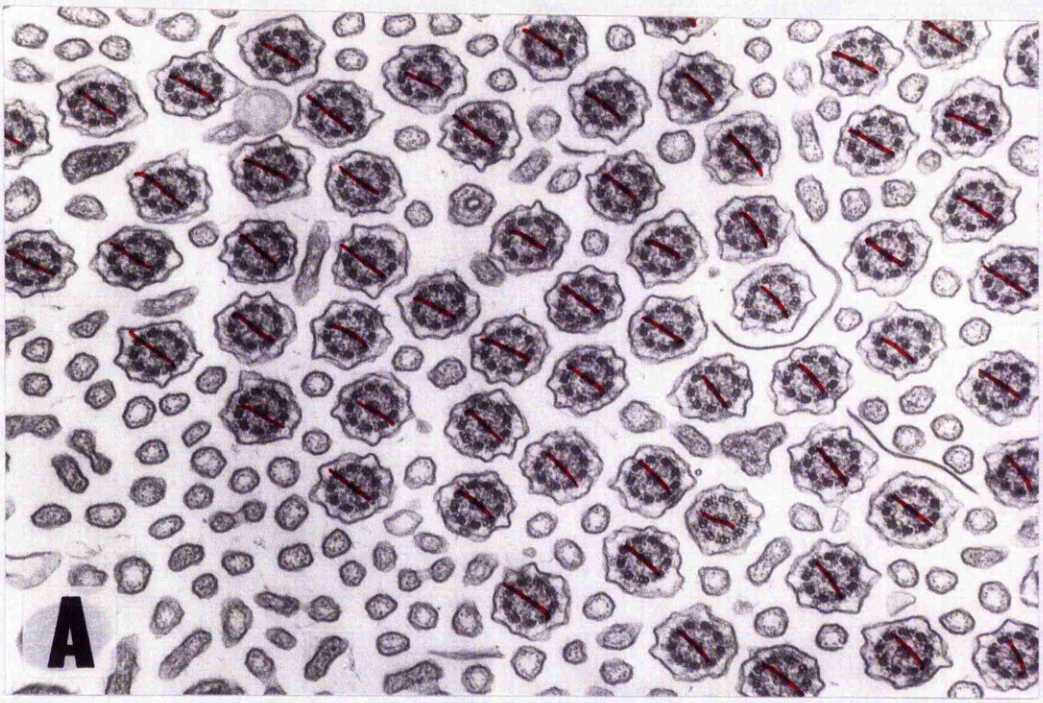


Figure 4.13 Transmission electron micrographs of human adenoid mucosa demonstrating the scoring system for (A) "absence of" mitochondrial damage (arrow) (score = 0); and (B) "presence of" mitochondrial damage (arrow) (score = 1)(17500x).



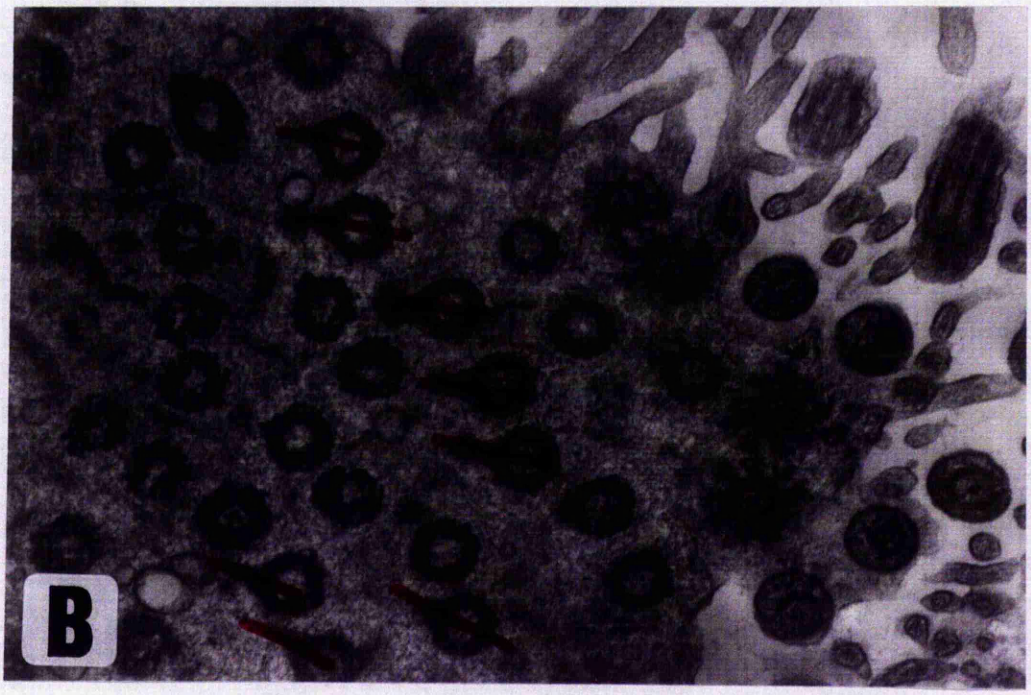


Figure 4.14 Transmission electron micrographs of normal adenoid mucosa showing: (A) cilia (with a line drawn through the central pairs of microtubules) with normal orientation of central pairs of microtubules; (B) basal bodies (with a line drawn bisetting the spur-like foot processes) with normal orientation (35000x).

CHAPTER 5

EFFECTS OF SUBMINIMAL INHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON NON-TYPABLE *H. INFLUENZAE* INFECTION OF HUMAN RESPIRATORY MUCOSA *IN VITRO*

5.1 AIM OF STUDY

This study was performed to evaluate the effects of subMIC antibiotics on the interactions of NTHi (strain SH9) with intact human respiratory mucosa in adenoid organ cultures. The antibiotics tested were amoxycillin, loracarbef and ciprofloxacin that are commonly prescribed for patients who have infective exacerbations of chronic bronchitis. Amoxycillin and ampicillin are the most commonly prescribed antibiotics in the UK. Loracarbef is a carbacephem, a new class of synthetic beta-lactam antibiotics, that is effective in the treatment of lower respiratory infections (Muller et al., 1992). Ciprofloxacin, a quinolone, is commonly prescribed for lower respiratory infections and has good penetration into respiratory mucosa and secretions (Table 1.1).

Functional (measured as CBF) and ultrastructural assessment of the respiratory epithelium were performed using light and transmission electron microscopy respectively.

5.2 STUDY DESIGN

The MIC values of NTHi (strain SH9) for each of the antibiotics were determined by microdilution method (Section 4.10). Eight agar-embedded adenoid organ cultures were established for each experiment (n=6 and obtained from 6 donors) (Section 4.2.1) containing: 3ml of MEM with no antibiotics (two organ cultures), 0.25 MIC of amoxycillin, 0.5 MIC of amoxycillin, 0.25 MIC of loracarbef, 0.5 MIC of loracarbef, 0.25 MIC of ciprofloxacin, and 0.5 MIC of ciprofloxacin. Light microscopy assessment was performed on each organ culture after 1h incubation for CBF to stabilise (Section 4.1.2). Washed NTHi (Section 4.8) was added to the MEM of each of the organ cultures except one of the two without antibiotic that acted as the uninfected control. The organ cultures (n=6) were then incubated in a humidified atmosphere that contained 5% CO_2 for 24h (Section 4.4.1). Fifty μl of the MEM was removed at 24h for a viable count of bacteria from each organ culture. Immediately after the 24h light microscopy assessment, the organ cultures were fixed for

TEM assessment (Section 4.5.1).

In a separate experiment in which 8 sets of adenoid organ cultures (obtained from the same donor) were established as above, a viable count of bacteria from the MEM was performed on each organ culture at 0, 1, 2, 4, 8, 12, 16, and 24h to assess bacterial growth rate (Section 4.4.1).

5.3 PARAMETERS MEASURED

By using the methods described in Sections 4.3.1 and 4.5.2, the following parameters were measured by light microscopy and TEM for each organ culture:

5.3.1 LIGHT MICROSCOPY ASSESSMENT

1. CBF
2. Number of sites with adherent mucus
3. Number of sites with ciliary activity
4. Number of sites with epithelial disruption

5.3.2 TEM ASSESSMENT

1. Percent of cells displaying
   1/. Loss of cilia
   2/. Extrusion from the epithelial surface
   3/. Cytoplasmic blebbing
   4/. Mitochondrial damage
2. Combined mucosal damage score

5.4 STATISTICAL METHOD

Due to the small sample size and variance heterogeneity of the groups, a non-parametric multiple comparisons method based on Friedman's test was used (Hollander et al., 1974). A p value of less than 0.05 was taken as significant.

5.5 RESULTS

5.5.1 BACTERIOLOGY (TABLE 5.1)

The MIC values of strain SH9 for amoxycillin, loracarbef and ciprofloxacin were 0.16 μg/ml, 0.31 μg/ml and 0.008 μg/ml respectively. These were consistent at the beginning and the end of each experiment.

The mean viable count (± standard error of mean) at the beginning of the experiments was $5.1\pm1.1 \times 10^5$ cfu/ml. After 24h incubation mean viable counts were $1.4\pm0.9 \times 10^8$ cfu/ml in organ cultures without antibiotics and $1.1\pm0.3 \times 10^8$ cfu/ml in organ cultures with antibiotics. There was no significant difference between the final viable counts in infected organ culture with and without antibiotics.

5.5.2 ASSESSMENT OF NTHi GROWTH RATE (TABLE 5.2)

The initial concentration of the 8 adenoid organ cultures was 4.8×10^5 cfu/ml. Viable counts performed from 1 to 24h did not show a significant difference in NTHi growth rate between different organ cultures.

5.5.3 LIGHT MICROSCOPY ASSESSMENT (TABLES 5.3. & 5.4)

In the uninfected organ cultures, CBF and the number of sites with beating cilia and epithelial disruption were not significantly different at 0h and 24h. However, there were significantly more sites with adherent mucus at 24h than 0h ($p<0.05$).

Infected organ cultures without antibiotics had a significant reduction in the number of sites with ciliary beating. Two measures of CBF were made (Section 4.3.1). Firstly, the mean CBF of all ten selected sites was measured which included sites at which ciliary beating could no longer be identified. Secondly, the mean CBF was calculated from only those sites at which ciliary beating could be seen. The mean CBF at all ten sites and mean CBF at sites with remaining ciliary beating at 24h were significantly reduced compared with 0h ($p<0.05$). Also the number of sites with disruption of

epithelial integrity increased significantly ($p<0.05$), but there was no significant change in the number of sites with adherent mucus after 24h incubation compared to control organ cultures at 24h.

When compared with the uninfected organ cultures, NTHi infected organ cultures that contained both 0.25 and 0.5 MIC antibiotics showed significant deterioration of all the light microscopy parameters ($p<0.05$) apart from the mean CBF at sites with remaining ciliary beating which did not change significantly after 24h incubation. However, the slowing of mean CBF (mean of ten sites), reduction in the number of sites with ciliary beating, and disruption of epithelium were significantly less in organ cultures that contained antibiotics than those without antibiotics at 24h ($p<0.05$). There was no significant difference in the number of sites with adherent mucus between the infected and uninfected organ cultures at 24h whether or not antibiotics were present (Tables 5.3 & 5.4).

The light microscopy parameters were not significantly different between 0.25 and 0.5 MIC of either amoxycillin, loracarbef or ciprofloxacin indicating a lack of dose dependent response (Table 5.4).

5.5.4 TEM ASSESSMENT (TABLES 5.5 & 5.6; FIGURE 5.1)

At 24h the infected organ cultures that did not contain antibiotics displayed a significant increase in combined mucosal damage score and percent of cells displaying loss of cilia, extrusion from the epithelial surface, cytoplasmic blebbing and mitochondrial damage when compared with uninfected organ cultures ($p<0.05$).

Infected organ cultures that contained 0.25 and 0.5 MIC amoxycillin and ciprofloxacin had a significantly higher combined mucosal damage score but the other TEM parameters were not significantly different from the uninfected organ cultures at 24h. In the presence of 0.25 and 0.5 MIC loracarbef, infected organ cultures showed a significant

increase in mucosal damage score and percent of cells displaying loss of cilia, extrusion from epithelial surface and mitochondrial damage when compared with uninfected controls at 24h ($p<0.05$).

The amoxycillin and ciprofloxacin-containing infected organ cultures had a significantly lower combined mucosal damage score and percent of cells displaying all the TEM parameters than the infected organ cultures without antibiotics at 24h ($p<0.05$). In the presence of 0.25 and 0.5 MIC loracarbef, infected organ cultures had a significant lower mucosal damage score and percent of cells displaying loss of cilia, cytoplasmic blebbing and mitochondrial damage when compared with infected organ cultures without antibiotic at 24h ($p<0.05$).

Similar to the light microscopy findings, no dose-dependent response was demonstrated by the TEM parameters between 0.25 and 0.5 MIC of each antibiotic-containing organ cultures at 24h.

5.5.5 BACTERIA ASSOCIATED WITH THE RESPIRATORY MUCOSA

Bacteria were found to be closely associated with the mucosal surface in some TEM sections obtained from the infected organ cultures. For each series of 6 TEM sections, bacteria were seen associated with the mucosal surface in: all 6 of the infected organ cultures without antibiotics; 3 of the infected organ cultures with 0.25 MIC loracarbef; 2 of the infected organ cultures with 0.25 MIC amoxycillin, 0.5 MIC loracarbef, and 0.5 MIC ciprofloxacin; 1 of the infected organ cultures with 0.25 MIC ciprofloxacin and 0.5 MIC amoxycillin; and in none of the uninfected organ cultures.

5.6 DISCUSSION

The organ culture model used in this study has previously been described by Read et al. although adenoid tissue was used in this thesis instead of nasal turbinates because it was more readily available. Embedding of tissue in agar occluded the cut surgical surfaces leaving only the mucosal surface exposed to infection (Read

et al., 1991). As previously reported in nasal turbinate tissue, infection by NTHi for 24h caused patchy damage to the epithelium and a loss of ciliary epithelium. NTHi infection also caused slowing of CBF in those areas in which ciliary beating remained. Bacterial infection of the lower respiratory tract causes increased mucus production both in patients with chronic bronchitis. In the study described in this chapter light microscopy assessment did not show an increase in mucus production in the infected compared with the uninfected organ cultures. This may either be a genuine finding or possibly due to the fact that light microscopy assessment performed along one free edge of the organ cultures is not an accurate assessment of the mucus content of an organ culture.

The extent of disruption of epithelial integrity, ultrastructural damage to the epithelial cells, slowing of CBF, and bacterial association to mucosal surface were all significantly reduced in the presence of subMIC antibiotics. No significant dose dependent effect of the three antibiotics was observed when the data obtained from 0.25 MIC was compared to that obtained from 0.5 MIC. This may be explained by a small difference between 0.25 and 0.5 MIC, a genuine lack of dose dependent response, or a lack of accuracy of the assessment methods.

The MIC is widely used to judge the effectiveness of an antibiotic, and treatment should aim to achieve a high concentration of antibiotic at the site of infection compared with the MIC of the presumed pathogen (Eller et al., 1981). However, the concentration of antibiotics in respiratory mucus is frequently much lower than that in serum (Stout et al., 1987; Bergogne-Berezin, 1988; Valcke et al., 1990), and the concentration may fall in the intervals between administration (Beet et al., 1990).

Recent studies (Farley et al., 1986; Baltimore et al., 1989; Read et al., 1991; Feldman et al., 1992) have suggested that during bronchial infections the majority of bacteria such as NTHi, PA and *S. pneumoniae*

are in the lumen of the airway associated with secretions. By using the same agar-embedded organ culture model described in this thesis, Read et al. (1991) found that NTHi adhered virtually exclusively to damaged nasal turbinate mucosa and mucus. Feldman et al. (1992) made similar findings on *S. pneumoniae* in the same nasal turbinate organ cultures. By using SEM to assess adenoid organ cultures freely suspended in MEM that contained NTHi, Farley et al. (1986) observed that NTHi predominantly adhered to mucus on the surface of the organ cultures. In a post mortem study performed on CF lungs, Baltimore et al. (1989) discovered that PA was frequently associated with intraluminal secretions and areas of damaged bronchial mucosa. Antibiotics generally penetrate poorly into secretions and it is very likely that bacteria in the bronchial tree would, at least for part of the time during treatment, only be exposed to subMIC of antibiotics (Stout et al., 1987; Bergogne-Berezin, 1988; Valcke et al., 1990). Secretions may also contain factors such as beta-lactamase which inhibit the activity of beta-lactam antibiotics and further reduce their effective concentrations.

SubMIC antibiotics are known to influence bacterial interactions with host cells in many ways. These include bacterial synthesis of cell wall and extracellular products (Lorian et al., 1975), bacterial adherence to cells and artificial materials (Finch et al., 1989), and changes in bacterial morphology such as elongation of *E. coli* and the development of spherical shape of staphylococci (Lorian et al., 1975; Chopra et al., 1986). Adherence to human buccal epithelial cells, pharyngeal cells and leucocytes, and production of extracellular toxins are reduced when *E. coli* (Ofek et al., 1979; Eisenstein et al., 1982; Forestier et al., 1984), *N. meningitidis* (Kristiansen et al., 1983; Salit, 1983; Stephens et al., 1984), *S. pyogenes* (Tylewska et al., 1981) and *S. aureus* (Vymola et al., 1974) are exposed to subMIC of penicillins. A reduction in the production of bacterial toxins such as elastase, alkaline protease, exotoxin A, exoenzyme S and phospholipase C (Warren et al., 1985) has been observed in PA exposed

to low concentrations of tetracycline, gentamicin, tobramycin (Shibl et al., 1980) and ciprofloxacin (Grimwood et al., 1989). SubMIC ceftazidime has also been shown to reduce the adherence of PA to human respiratory mucin *in vitro* (Vishwanath et al., 1987). Whilst the effects of subMIC antibiotics on many bacteria have been investigated, little is known of the effects on NTHi, particularly on its interaction with human respiratory tissue.

The final viable counts and NTHi growth rates were not significantly different whether or not subMIC antibiotics were present in the organ cultures. This indicates that a reduction in NTHi multiplication in the organ cultures was unlikely to be a mechanism for the observed subMIC protective effects described in this thesis. However, association of bacteria with the mucosal surface was seen less commonly in the infected organ cultures with antibiotics. It is possible that the lower incidence of bacteria seen in section when subMIC antibiotics were present was the result of preserved ciliary activity which protected the respiratory surface. Alternatively, NTHi might be less adherent in the presence of subMIC antibiotics. This might relate to changes in bacterial morphology (Lorian et al., 1975), expression of bacterial adhesins (Eisenstein et al., 1982; Chopra, 1986), or to changes in the epithelium itself. Damage of the respiratory epithelium was less when the infected organ cultures contained subMIC antibiotics. NTHi release factors (Section 1.4.1.4) which cause slowing of ciliary beating (Wilson et al., 1988b) and damage to epithelial cells (Denny, 1974; Farley et al., 1986; Johnson et al., 1986; Read et al., 1991; Wilson et al., 1991). Culture filtrates obtained from NTHi (strain SH9) in the presence of 0.25 MIC of amoxycillin, loracarbef and ciprofloxacin cause significantly less slowing of CBF in human nasal epithelium than culture filtrate obtained without antibiotics (Tsang et al., 1995). This suggests that a reduced production of NTHi toxin(s) might have played an important role in providing the subMIC protective effects reported in this thesis.

When bacteria were seen in association with the respiratory mucosa they were mainly seen to be associated with areas of epithelial damage. These findings agree with those of Read et al. in that NTHi adhered more avidly to damaged than healthy epithelial cells (Read et al., 1991). However, further studies (described in Chapter 8) on NTHi adherence using a more physiological organ culture model with an air-mucosal interface and SEM suggest that NTHi also adhere to apparently healthy unciliated cells. Epithelial damage may expose receptors for bacterial adhesins that are otherwise not exposed. Thus, reduced damage could be a further explanation for less bacterial association with the mucosal surface when subMIC antibiotics were present.

SubMIC of two beta-lactam antibiotics and a quinolone antibiotic protected epithelium against damage caused by NTHi infection. The similarity in the protective effects of amoxycillin, loracarbef and ciprofloxacin is intriguing in view of the difference in mechanism of action of these families of antibiotics. This similarity in the effects of sub-MIC of beta-lactams and quinolones, and also aminoglycosides, has also been observed for killing of aerobic Gram negative bacilli by human polymorphonuclear leucocytes (Mandell et al., 1989 & 1991). The effect may involve a general action of subMIC antibiotics rather than relate to the mechanism of bacterial killing by the antibiotic. For example, subMIC antibiotics of a number of different classes alter bacterial morphology (Lorian et al., 1975) and toxin production (Chopra et al., 1985 & 1986; Grimwood et al., 1989; Honma et al., 1991). It is also possible that antibiotics could directly affect adenoid tissue and protect it in some way against the effects of infection. For example, erythromycin has been shown to reduce mucus production by respiratory mucosa (Marom et al., 1991), although this effect has been shown to be dose dependent and occur at higher concentrations of antibiotic.

Antibiotics are used to good effect in the treatment of infective exacerbations of chronic bronchitis in which patients have increased

dyspnoea and an increased volume of purulent sputum (Anthonisen et al., 1987), and in more severe bronchial infections such as bronchiectasis (Currie et al., 1990) and cystic fibrosis (Lebel, 1991). Recovery from a bronchial infection involves the local and systemic host defences, as well as the action of the antibiotic. In diseases where the host defences are severely impaired such as cystic fibrosis, it is often impossible to eradicate bacterial infection of the airways despite prolonged high dosage antibiotic therapy. As discussed above, it is likely that some bacteria in the bronchial tree are not exposed to antibiotics at concentrations above the MIC during treatment. This will be particularly true for beta-lactam antibiotics that do not penetrate into secretions as well as quinolone antibiotics. This study suggests that subMIC of antibiotics protect the respiratory mucosa from damage during infection. In certain circumstances, preservation of local host defences, and effects of subMIC on phagocytosis and other aspects of the host interaction with bacteria, may lead to clearance of the infection without the need to achieve bactericidal or even bacteriostatic levels of antibiotic.

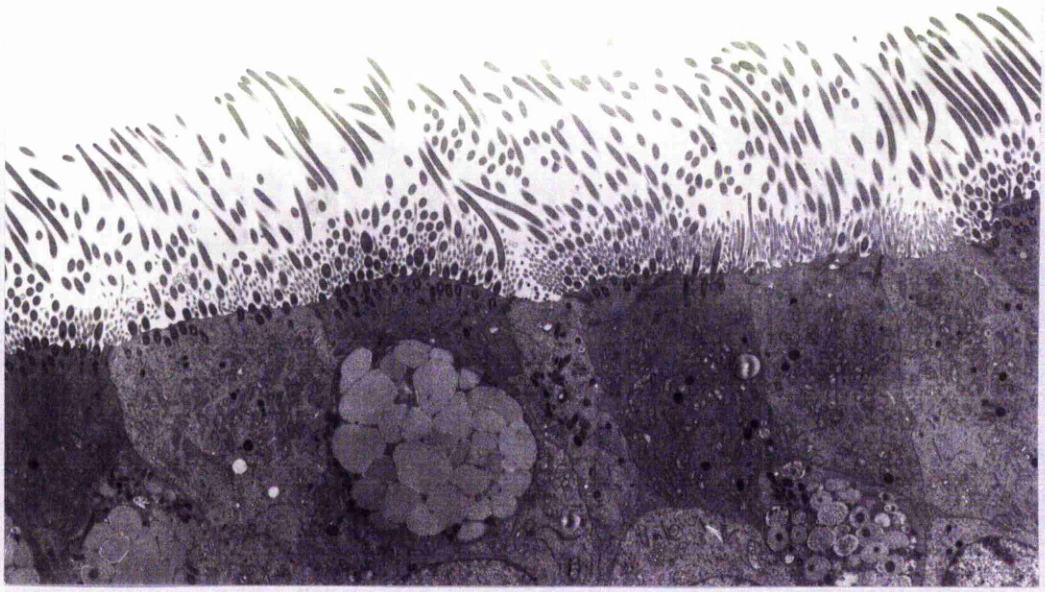


Figure 5.1 Transmission electron micrograph showing normal ciliated epithelium in an uninfected adenoid organ culture after 24h incubation (3500x).

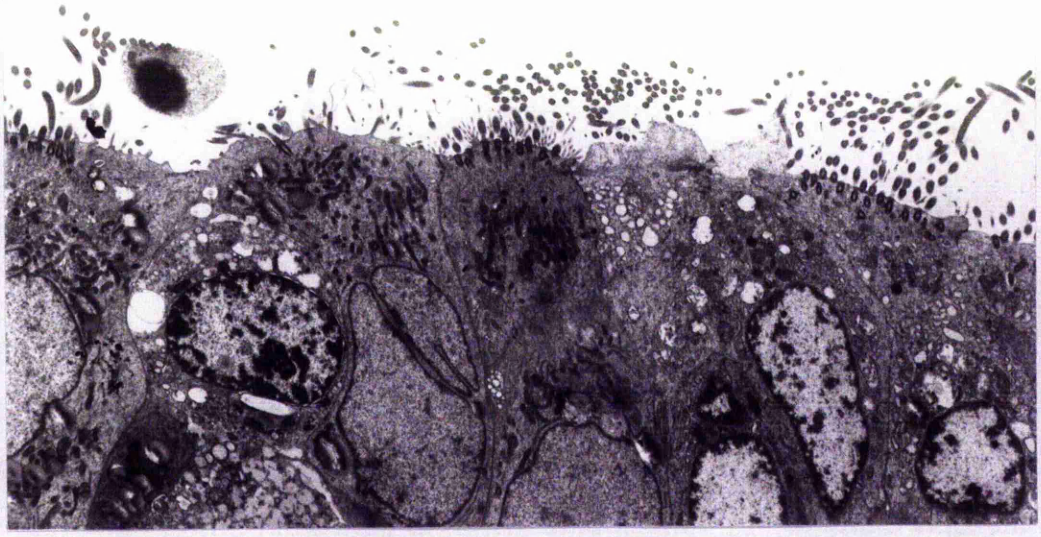


Figure 5.2 Transmission electron micrograph showing loss of cilia, extrusion of a dead cell, cytoplasmic blebbing and mitochondrial damage in a NTHi (strain SH9) infected adenoid organ culture that did not contain antibiotics after 24h incubation (3500x).

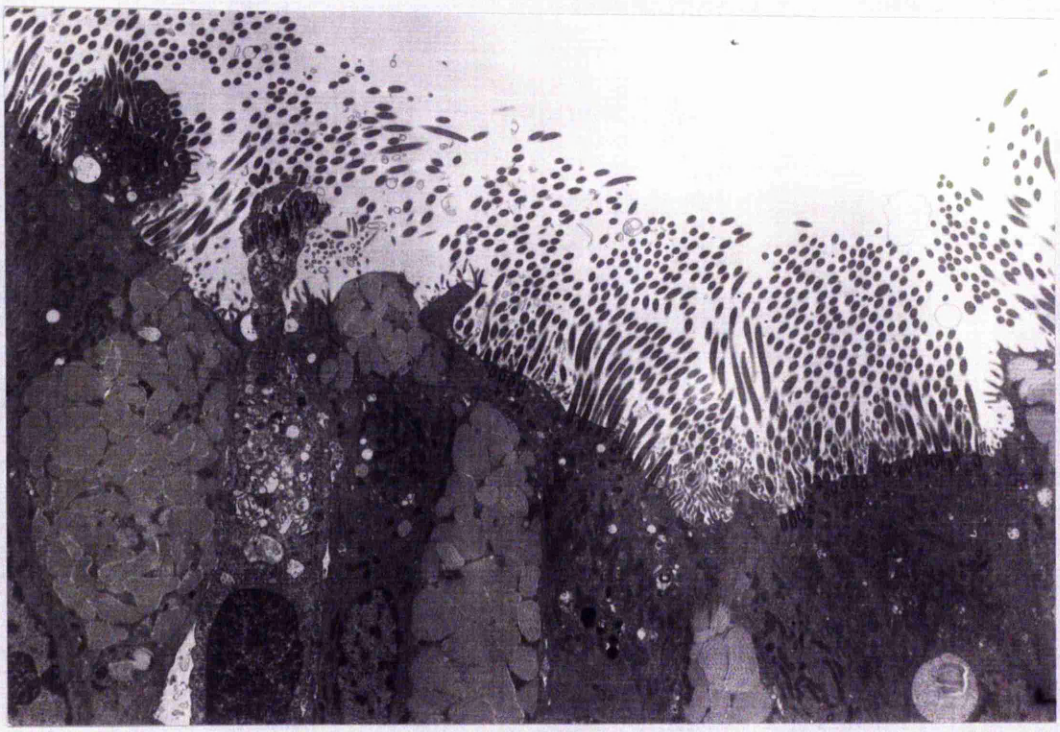


Figure 5.3 Transmission electron micrograph showing less severe loss of cilia, extrusion of epithelial cells, cytoplasmic blebbing and mitochondrial damage in a NTHi (strain SH9) infected adenoid organ culture that contained 0.25 MIC amoxycillin after 24h incubation (3500x).

TABLE 5.1 VIABLE COUNTS OF NTHi (STRAIN SH9) IN INFECTED ADENOID ORGAN CULTURES AT 24H.

Adenoid Organ culture	NTHi viable count (cfu/ml)
Infected + no antibiotics	$1.4 \pm 0.89 \times 10^8$
Infected + 0.25 MIC amoxycillin	$8.1 \pm 0.42 \times 10^7$
Infected + 0.50 MIC amoxycillin	$8.4 \pm 0.48 \times 10^7$
Infected + 0.25 MIC loracarbef	$8.1 \pm 0.49 \times 10^7$
Infected + 0.50 MIC loracarbef	$2.1 \pm 1.3 \times 10^8$
Infected + 0.25 MIC ciprofloxacin	$1.3 \pm 0.72 \times 10^8$
Infected + 0.50 MIC ciprofloxacin	$6.6 \pm 0.37 \times 10^7$

All data is mean of 6 separate experiments ± standard error of mean. No statistical difference was found between the viable counts. MIC = minimal inhibitory concentration. cfu= colony forming unit. NTHi = non-typable *Haemophilus influenzae*.

TABLE 5.2 BACTERIAL VIABLE COUNTS IN ADENOID ORGAN CULTURES (COLONY FORMING UNITS/ML).

Incubation time (h)	Viable count in NTHi infected organ culture						
	No antibiotic	0.25 MIC AMO	0.5 MIC AMO	0.25 MIC LC	0.5 MIC LC	0.25 MIC CIP	0.5 MIC CIP
1h	$3.0x10^5$	$3.7x10^5$	$2.7x10^5$	$2.2x10^5$	$1.8x10^5$	$2.5x10^5$	$2.8x10^5$
2h	$1.3x10^6$	$7.3x10^6$	$5.3x10^6$	$7.0x10^6$	$1.0x10^6$	$8.2x10^6$	$1.0x10^6$
4h	$1.2x10^7$	$3.2x10^7$	$2.7x10^7$	$5.7x10^7$	$4.2x10^7$	$5.5x10^7$	$5.3x10^7$
8h	$4.8x10^7$	$5.3x10^7$	$10.2x10^7$	$6.0x10^7$	$7.2x10^7$	$10.7x10^7$	$9.5x10^7$
12h	$1.2x10^8$	$1.0x10^8$	$1.1x10^8$	$9.5x10^8$	$1.2x10^8$	$1.2x10^8$	$1.4x10^8$
16h	$1.1x10^8$	$1.4x10^8$	$1.5x10^8$	$1.2x10^8$	$1.0x10^8$	$1.8x10^8$	$1.4x10^8$
24h	$1.2x10^8$	$1.5x10^8$	$1.3x10^8$	$1.2x10^8$	$1.5x10^8$	$1.3x10^8$	$1.2x10^8$

Data shown are viable counts (colony forming units/ml) obtained from one single experiment. MIC = minimal inhibitory concentration. NTHi = Non-typable *Haemophilus influenzae* (strain SH9). AMO = amoxycillin. LC = loracarbef. CIP = ciprofloxacin.

TABLE 5.3 LIGHT MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES.

Organ Culture	Mean CBF (Hz) of organ cultures 0h	24h	Mean CBF (Hz) of sites with ciliary beating 0 h	24h	Mean number of sites with beating cilia at 24 h (=10 at 0h)	Mean number of sites with disruption of epithelium at 24h (=0 at 0h)	Mean number of sites with adherent mucus at 24h (=0 at 0h)
Uninfected	10.7±0.38	10.8±0.25	10.7±0.38	10.8±0.25	10 ± 0	0 ± 0	3.7 ± 0.3
Infected	10.8±0.27	0.5±0.27*†	10.8±0.27	3.9±1.77*	0.7 ± 0†	9.5 ± 0†	3.8 ± 0.8
Infected plus 0.25 MIC amoxycillin	11.1±0.28	4.5±0.75*†‡	11.1±0.28	10.0±0.78‡	5.3 ± 0.7†‡	5.0 ± 0.7†‡	3.5 ± 1.1
Infected plus 0.25 MIC loracarbef	11.1±0.26	3.3±0.35*†‡	11.1±0.26	10.2±0.16‡	3.5 ± 0.4†‡	5.7 ± 0.5†‡	4.8 ± 0.9
Infected plus 0.25 MIC ciprofloxacin	10.5±0.38	3.6±0.50*†‡	10.5±0.38	9.9±0.29‡	4.0 ± 0.4†‡	5.7 ± 0.9†‡	5.0 ± 0.6

All data is mean of six separate experiments ± standard error of mean. Observations were made at ten sites along one edge of the organ culture at 0h and 24h. * $p<0.05$ at 24h compared to 0h. † $p<0.05$ versus uninfected control. ‡ $p<0.05$ versus non-typable *Haemophilus influenzae* (strain SH9) infected organ culture without antibiotics. CBF = ciliary beat frequency. MIC = minimal inhibitory concentration.

TABLE 5.4 LIGHT MICROSCOPY ASSESSMENT OF NTHi INFECTED (STRAIN SH9) ADENOID ORGAN CULTURES CONTAINING 0.5 MIC OF ANTIBIOTICS AFTER 24 HOURS INCUBATION.

Organ Culture	Mean CBF (Hz) of organ cultures 0h	24h	Mean CBF (Hz) of sites with ciliary beating 0h	24h	Mean no. of sites with beating cilia at 24h	Mean no. of sites with disruption of epithelium at 24h	Mean no. of sites with adherent mucus at 24h
Infected plus 0.5 MIC amoxycillin	11.1±0.39	3.6±0.78*	11.1±0.39	9.1±0.56‡	3.8 ± 0.8†‡	4.5 ± 1.0†‡	4.2 ± 1.2
Infected plus 0.5 MIC loracarbef	10.3±0.29	3.3±0.63*	10.3±0.29	10.1±0.90‡	4.2 ± 0.8†‡	6.3 ± 0.7†‡	4.2 ± 0.7
Infected plus 0.5 MIC ciprofloxacin	10.9±0.29	4.2±0.91*	10.9±0.29	9.8 ± 0.65‡	4.7 ± 0.7†‡	5.3 ± 0.8†‡	5.0 ± 1.1

All data is mean of six separate experiments ± standard error of mean. * $p<0.05$ at 24h compared to 0h. † $P<0.05$ versus uninfected control. ‡ $p<0.05$ versus NTHi infected organ cultures without antibiotics. There was no statistical difference between results of 0.5 MIC and 0.25 MIC. CBF = ciliary beat frequency. MIC = minimal inhibitory concentration.

TABLE 5.5 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES AFTER 24H INCUBATION.

Organ culture	Percent of cells with loss of cilia	Percent of cells extruding from the epithelial surface	Percent of cells with cytoplasmic blebbing	Percent of cells with mito-chondrial damage	Mucosal damage score
Uninfected	13.4 ± 1.2	19.6 ± 2.1	3.5 ± 0.6	4.6 ± 1.1	17.6 ± 2
Infected	42.0 ± 12.3*	57.5 ± 10.4*	36.8 ± 13.4*	38.3 ± 13.1*	132.4 ± 55*
Infected plus 0.25 MIC amoxycillin	15.2 ± 1.9†	28.1 ± 4.3†	3.6 ± 0.9†	6.4 ± 1.7†	25.6 ± 5.1*†
Infected plus 0.25 MIC loracarbef	16.1 ± 1.8*†	29.1 ± 2.8*	6.1 ± 1.4†	10.0 ± 1.6*†	31.6 ± 3.7*†
Infected plus 0.25 MIC ciprofloxacin	15.3 ± 1.3†	23.4 ± 2.5†	2.0 ± 0.8†	9.8 ± 4.3†	22.2 ± 1.9*†

All data is mean of six separate experiments ± standard error of mean. * $p<0.05$ versus uninfected organ culture. † $p<0.05$ versus NTHi (strain SH9) infected organ culture. CBF = ciliary beat frequency. MIC = minimal inhibitory concentration.

TABLE 5.6 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF NTHi (STRAIN SH9) INFECTED ORGAN CULTURES CONTAINING 0.50 MIC ANTIBIOTICS AFTER 24H INCUBATION.

Organ culture	Percent of cells with loss of cilia	Percent of cells extruding from the epithelial surface	Percent of cells with cytoplasmic blebbing	Percent of cells with mito-chondrial damage	Mucosal damage score
Infected plus 0.50 MIC amoxycillin	12.8 ± 1.0†	23.8 ± 1.5†	3.8 ± 1.3†	10.5 ± 3.1†	23.2 ± 2.2*†
Infected plus 0.50 MIC loracarbef	17.8 ± 1.9*†	28.7 ± 4.1*	7.0 ± 1.6†	9.5 ± 1.4*†	32.4 ± 4.2*†
Infected plus 0.50 MIC ciprofloxacin	18.4 ± 2.0†	35.5 ± 6.8†	8.9 ± 3.3†	15.5 ± 4.7†	39.7 ± 7.7*†

All data is mean of six separate experiments ± standard error of mean. * $p<0.05$ versus uninfected organ culture. † $p<0.05$ versus NTHi (strain SH9) infected organ culture. CBF = ciliary beat frequency. MIC = minimal inhibitory concentration.

CHAPTER 6

INTERACTIONS OF NON-TYPABLE *H. INFLUENZAE* WITH HUMAN BRONCHIAL MUCOSA *IN VITRO*

6.1 AIM OF STUDY

This study was performed to evaluate the interactions of NTHi (strain SH9) with intact human bronchial mucosa in an agar-embedded organ culture model (Section 4.2.1).

6.2 STUDY DESIGN

A pair of identical organ cultures was established for each experiment (n=4) as described in Section 4.2.1. Twenty μl of a washed NTHi suspension (strain SH9) suspension or PBS was added to the MEM of the designated infected and uninfected organ cultures respectively (Section 4.4.1). Incubation then followed at 37^{0}C in a humidified atmosphere that contained 5% CO_2. After 24h incubation, viable count of the MEM was obtained by standard dilution techniques (Section 4.9). Bronchial tissue was processed for TEM examination (Sections 4.5.1 and 4.5.2). As healthy bronchial tissue was not readily available only 4 experiments were performed.

6.3 PARAMETERS MEASURED

6.3.1 ULTRASTRUCTURAL ASSESSMENT

By using TEM morphometric assessment (Section 4.5.2), the mean combined mucosal damage score and the percent of cells displaying each of the following parameters were assessed for each organ culture:

1. extrusion from the epithelial surface
2. loss of cilia
3. cytoplasmic blebbing
4. mitochondrial damage

6.3.2 BACTERIAL GROWTH

Viable counts of the MEM that immersed the bronchial tissue were performed at 0 and 24h (Section 4.9).

6.4 STATISTICAL ANALYSIS

Statistical analysis was not performed as only 4 experiments were conducted.

6.5 RESULTS

6.5.1 BACTERIOLOGY

The uninfected bronchial organ cultures remained sterile after 24h. In the infected organ cultures, the mean (± standard error of mean) viable counts of NTHi in MEM were $4.1\pm0.4 \times 10^5$ and $4\pm0.9 \times 10^7$ cfu/ml at 0 and 24h respectively.

6.5.2 ULTRASTRUCTURAL ASSESSMENT (FIGURES 6.1 & 6.2)

No bacteria were seen in the TEM sections obtained from uninfected organ cultures although they were seen closely associated (i.e. within the diameter of one epithelial cell) with the mucosal surface in all the infected organ cultures. Direct contact between bacteria and epithelial cells was not detected at TEM.

After 24h incubation, NTHi infected organ cultures had a higher percent of cells displaying the four parameters of ultrastructural damage and mean combined mucosal damage score (± standard error of mean) (50.9±6.0) when compared with uninfected organ cultures (mean combined mucosal damage score 18.7±3.5) (Table 6.1). Separate TEM assessment was performed on epithelial cells according to whether they were within one epithelial cell diameter from the mucosally-associated bacteria (Table 6.2). Ciliated cells within one epithelial cell distance from the bacteria displayed more severe ultrastructural damage (mean combined mucosal damage score 65.7±7.4) than those farther away (mean combined mucosal damage score 40.0±8.2).

6.6 DISCUSSION

In this thesis, intact human bronchial mucosa was used to study the effects of NTHi infection *in vitro*. This method has also been used to study the interactions between NTHi, Hib (Read et al., 1991 & 1992), and *S. pneumoniae* (Feldman et al., 1992) with human nasal turbinate and NTHi with adenoid tissue (Chapter 5). NTHi is found in the bronchi of patients with chronic bronchitis and the nasopharynx of 75% of healthy population but only in 0-6% of the nasal cavity of healthy

objects (Jousimies-Somer et al., 1989). The regional differences in airways mucosal cells have already been discussed in Section 1.3.2.1. Briefly, mucosa from different sites in the lower respiratory tract differ in: their response to intratracheal instillation of neutrophil elastase (Christensen et al., 1989); exposure to cigarette smoke (Mawdesley-Thomas et al., 1973) and sulphur dioxide (Lamb et al., 1968); the proportion of secretory cells (Christensen et al., 1987); binding to lectins (Christensen et al., 1990); and in ciliary beat frequency (Rutland et al., 1982). By using intact human bronchial mucosa, the effects of alteration in the proportion of epithelial cell types and cell surface receptors which may occur in sub-cultures of epithelial cells could also be avoided (Wasano et al., 1988). As NTHi is only pathogenic to human beings and usually infect the respiratory tract (Section 1.3.2.1), studies of the interactions between bacterial and host cells using buccal epithelial cells (Woods et al., 1980a; Franklin et al., 1987) and animal respiratory tissue (Baker et al., 1982a; Ramphal et al, 1983a; Plotkowski et al., 1986 & 1989; Zoutman et al., 1991) may be inappropriate.

Whilst damaged human bronchial tissue may be available from resected and diseased lungs, well preserved and normal bronchial tissue suitable for *in vitro* studies was not readily available. Although only 4 experiments were performed, a pattern of ultrastructural damage incurred by NTHi infection of bronchial mucosa was consistently found. In this study, NTHi infection was found to cause substantial but patchy damage to epithelial cells with loss of cilia, extrusion of cells from the epithelial surface, cytoplasmic blebbing and mitochondrial damage. Damage of ciliated columnar cells had also been observed in sputum of patients with infective exacerbation of chronic bronchitis (Chodosh et al., 1971). By using the same TEM assessment and organ culture methods, this ultrastructural damage pattern is similar to that found in NTHi infection of human nasal turbinate (Read et al., 1991) and adenoid tissue (Chapter 5). Results of studies performed on respiratory mucosa such as the protective effects of

subMIC antibiotics on NTHi infection of human adenoid mucosa may therefore be applicable to bronchial mucosa (Chapter 5). However the strength of this extrapolation is limited by the small number of experiments performed.

TEM employed in the study described in this thesis only showed close association of NTHi with the mucosal surface (i.e. within one epithelial cell diameter) but not direct contact with the mucosal cells. It was likely that NTHi did adhere to the mucosal cells and their direct contact was not detected by TEM due to the three dimensional nature of the interaction (Section 1.3.2.3). Ciliated cells with closely associated NTHi appeared to have a higher combined mucosal damage score than cells further away from NTHi (Section 6.5.2 and Table 6.2). Interestingly, Read et al. (1991) reported that NTHi only adhered to damaged epithelial cells but not healthy ones in a study that used the same organ culture model and nasal turbinate tissue. From the study described in this thesis and the findings of Read et al. (1991), it would appear that NTHi may adhere more readily to damaged respiratory epithelial cells than healthy cells. SEM study of NTHi infection of bronchial tissue would be more helpful in this situation and would be important for comparative studies of NTHi adherence to bronchial, adenoid and nasal turbinate tissue.

In summary the effects of NTHi infection of intact human bronchial mucosa was studied and a substantial ultrastructural damage was found after 24h incubation. By using this TEM assessment protocol, the damage pattern is similar to that observed in adenoid and nasal turbinate tissue.

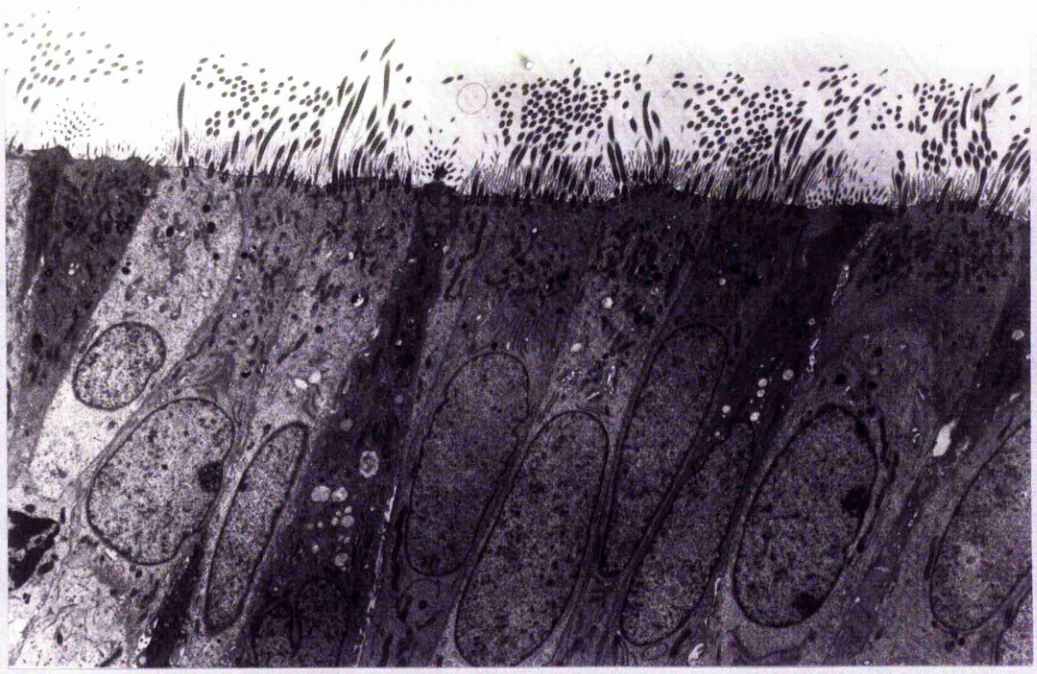


Figure 6.1 Transmission electron micrograph of an uninfected bronchial organ culture at 24h showing well ciliated normal epithelium (2625x).

Figure 6.2 Transmission electron micrograph of a bronchial organ culture infected by NTHi (strain SH9) at 24h showing loss of cilia, extrusion of cells from the epithelial surface, cytoplasmic blebbing and mitochondrial damage (2625x).

TABLE 6.1 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF BRONCHIAL ORGAN CULTURES AT 24H.

	Organ culture	
Percent of cells displaying	uninfected	NTHi infected
Loss of cilia	8.3±0.7	10.9±1.3
Extrusion from epithelial surface	16.6±3.7	36.3±3.6
Cytoplasmic blebbing	2.2±0.8	7.4±2.0
Mitochondrial damage	7.5±2.1	25.2±3.4

Data shown is mean ± standard error of mean of four separate experiments. NTHi = non-typable *H. influenzae* (strain SH9).

TABLE 6.2 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF BRONCHIAL EPITHELIUM NEAR (WITHIN ONE EPITHELIAL CELL DIAMETER) AND AWAY FROM BACTERIA IN ORGAN CULTURES AT 24h.

	Respiratory epithelial cell	
Percent of cells displaying	Near bacteria	Away from bacteria
Loss of cilia	12.1±0.6	9.4±2.5
Extrusion from epithelial surface	44.9±3.4	30.7±5.7
Cytoplasmic blebbing	12.2±5.1	3.9±1.2
Mitochondrial damage	31.6±2.9	20.8±4.7

Data shown is mean ± standard error of mean of four separate experiments.

CHAPTER 7

AN ORGAN CULTURE MODEL OF INTACT HUMAN RESPIRATORY MUCOSA WITH AN AIR-MUCOSAL INTERFACE

7.1 AIM OF STUDY

The aim of this study was to develop an organ culture model of intact human respiratory mucosa with an air-mucosal interface i.e. intact human respiratory mucosa exposed to humidified warm air.

7.2 STUDY DESIGN

Three experiments were performed using well ciliated human adenoid (n=2; 2 different donors) and nasal turbinate (n=1; one single donor) tissue (Section 4.1). Each piece of tissue (measuring 3x3mm^2 and 1-3mm in thickness) was dissected carefully and placed on a strip of filter paper that was laid across two petri dishes as described in Section 4.2.2. Organ cultures were incubated at 37^{0}C in a humidified atmosphere that contained 5% CO_2 for 24h. Sterility was checked by gently touching the 4 edges of the tissue with a sterile loop (Section 4.4). Functional assessment was made by measuring CBF as outlined in Section 4.7. Specimens were then processed (Section 4.5.1) and ultrastructural assessment was made using TEM protocol as described in Section 4.5.2.

7.3 PARAMETERS MEASURED

7.3.1 TEM ASSESSMENT

The percent of cells displaying each of the following features was measured (Section 4.5.2):

1. Extrusion from the epithelial surface
2. Loss of cilia
3. Mitochondrial damage
4. Cytoplasmic blebbing

7.3.2 CBF MEASUREMENT

CBF of an adenoid organ culture (n=1) was measured along 5 selected sites on the free edge of the tissue that overhung the filter paper (Sections 4.3.2 & 4.7) at 0 and 24h. These selected sites were identified by a sketch of the natural contour of the free edge of the adenoid tissue and markings made on the outer petri dish (Section 4.3.2).

7.4 RESULTS

The four edges of one of the adenoid and the nasal turbinate organ cultures were sterile after 24h indicating no bacterial contamination (Section 4.4.2). One of the adenoid organ cultures yielded gram negative bacilli from its four edges and was therefore excluded from further TEM and CBF analysis.

7.4.1 TEM ASSESSMENT (FIGURE 7.1)

TEM assessment of the uncontaminated adenoid organ culture (n=1) showed that 14.3%, 11.3%, 1.5% and 2.7% of epithelial cells displayed extrusion from the epithelial surface, loss of cilia, cytoplasmic blebbing and mitochondrial damage respectively.

7.4.2 CBF MEASUREMENT

CBF of the uncontaminated adenoid organ culture remained unchanged and the mean CBF were 11.7 and 11.9Hz at 0 and 24h respectively.

7.5 DISCUSSION

Over the last few decades, a lot of work has been performed on bacterial adherence to host cells (Section 1.3). The advantages and disadvantages of some of the study methodologies have been cited in Tables 1.2 and 1.3. Many previous studies, particularly those performed on respiratory pathogens such as NTHi, PA, and *S. pneumoniae*, used suspension of buccal epithelial cells (St Geme et al., 1993), red blood corpuscles (van Alphen et al., 1986) or respiratory epithelial cells obtained from brushing of the nasal turbinate (Niederman et al., 1983) or trachea (Franklin et al., 1987) whose "non-luminal" surfaces were also exposed. These "non-luminal" cell surfaces might bind bacteria independently. Bacterial adherence to more intact animal tissue such as chinchilla and murine tracheal rings have also been studied (Ramphal et al., 1983; Marcus et al., 1985; Bakaletz et al., 1988a). However, these studies generally also involved immersion of respiratory tissue in culture media containing bacteria. Conflicting results have been obtained from different animal

studies which may be explained by tissue tropism, species differences or the use of different experimental protocols (Section 1.3.2.1). The use of tissue other than human respiratory mucosa may not provide relevant information for the interactions of bacteria with human respiratory tract.

In the new organ culture model described in this chapter, the respiratory mucosa was directly exposed to humidified air. The tissue derived its nutrients from its submucosal aspects via the filter paper whose ends were immersed in fresh culture medium. As the culture medium would not be directly contaminated by the bacterial or chemical inoculum, relatively fresh cell culture medium would be available to the respiratory mucosa. Immersion of the respiratory mucosa with culture medium that contained high concentrations of bacteria and their toxic products could therefore be avoided which might severely impair ciliary functioning and favour bacteria in the infective process. In this model, bacterial suspension or chemicals can be directly applied onto the mucosal surface. The cut edges of the respiratory mucosa were sealed with agar and only the ciliated epithelial surface was exposed to bacteria. Detailed studies on the surface morphology of the respiratory mucosa would be interesting as surface mucus and cell debris were not directly lost to the immersing culture medium and the mucosal surface was undisturbed. The advantages and disadvantages of this new organ culture model are also outlined in Table 7.1.

Bacterial adherence to host cells is dependent on the interactions between bacterial adhesins and epithelial receptors and the microenvironment in which the interactions take place. Thus local pH, concentrations of divalent cations, and the presence of host airway secretions and bacterial exoproducts may affect bacterial adherence (Beachey 1981). For example, the adherence of PA to hamster tracheal cell culture is both pH sensitive and Ca^{2+} dependent (Marcus et al., 1989; Grant et al., 1991). Different culture media used to immerse

respiratory mucosa may therefore affect bacterial adherence *in vitro*. In this organ culture model, the problem of choosing the immersing cell culture medium has been eliminated as bacteria or bacterial exoproducts can be applied directly onto the mucosal surface. This direct inoculation mimics *in vivo* droplet infection in the respiratory tract *in vitro*.

In order to obtain healthy and well ciliated tissue for experiments very strict tissue selection criteria were followed. Firstly, tissue was dissected immediately on arrival at the laboratory. Secondly, only well ciliated tissue was selected i.e. dissected pieces that had 2 completely ciliated edges. Thirdly, tissue was only chosen if there was no adherent mucus at the free edges so that the epithelial outline could be seen to be intact. By using these criteria, at least half of the adenoid tissue obtained from adenoidectomies was rejected after initial light microscopy assessment.

This study shows that the organ culture model with an air-mucosal interface described in this thesis can maintain normal ultrastructure and function (CBF) of intact human respiratory mucosa derived from adenoid tissue for at least 24h. By using this model, further studies of the effects of bacterial infection or bacterial toxins on respiratory mucosa by TEM and SEM were performed. The following chapters describe studies that employed this organ culture model on the interactions between intact human respiratory mucosa and NTHi (strain SH9), PA (strain P455) and the PA exotoxin pyocyanin.

Figure 7.1 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface showing normal and densely ciliated epithelial cells after 24h incubation (2000x).

TABLE 7.1 THE ADVANTAGES AND DISADVANTAGES OF THE ORGAN CULTURE MODEL WITH AN AIR-MUCOSAL INTERFACE.

ADVANTAGES	DISADVANTAGES
1. An air-mucosal interface is established	1. Pooling of bacteria and mucus may occur at the edges of the organ cultures without any removal mechanism(s) for them
2. Medium is supplied to the submucosal aspects of the organ cultures	2. Only human adenoid and nasal turbinate tissue (but not bronchial) studied
3. Simple methodology and equipment needed to establish organ cultures	3. Availability of adenoid and nasal turbinate tissue could be inconsistent making planning of experiments rather difficult
4. Continued exposure to bacteria/toxin(s) can be avoided (after initial inoculation)	
5. CBF of organ cultures can be assessed	

6. Mucus and surface debris are not directly lost into the immersing medium

7. Abolition of the effects of the constituents of culture medium on bacterial adherence to mucosa

8. Bacterial inoculation onto mucosal surface mimics *in vivo* droplet infection

9. Pharmacological agents/toxins can be applied directly onto organ culture surface for evaluation of CBF and ultrastructure

10. Dissected edges of organ cultures partially sealed by agar

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CHAPTER 8

INTERACTION OF NON-TYPABLE *HAEMOPHILUS INFLUENZAE* WITH HUMAN RESPIRATORY MUCOSA IN AN ORGAN CULTURE MODEL WITH AN AIR-MUCOSAL INTERFACE

8.1 AIM OF STUDY

The aim of this chapter was to evaluate the effects of infection by a clinical isolate of NTHi (strain SH9) on intact human respiratory mucosa (adenoid and nasal turbinate) in an organ culture model with an air-mucosal interface. TEM was used to assess ultrastructural changes. A new SEM quantitative method was also developed to assess the surface morphology of the respiratory mucosa and bacterial adherence to the mucosal surface.

8.2 STUDY DESIGN

Human adenoid and nasal turbinate organ cultures with an air-mucosal interface and washed SH9 suspensions were prepared as described in Sections 4.2.2 and 4.8 respectively. Pairs of identical organ cultures were established from the same donor tissue and were incubated and processed simultaneously (Sections 4.4.2). Twenty μl of a washed NTHi (strain SH9) suspension and sterile PBS were inoculated onto the surface of designated infected and uninfected organ cultures respectively. The size of the NTHi inocula was assessed by a viable count (Section 4.9). Incubation at 37^{0}C in a humidified atmosphere that contained 5% CO_2 then followed.

Four series of experiments were performed:

1/. TEM and SEM assessment was performed on 12 pairs of adenoid organ cultures (obtained from 12 donors) that were incubated for 24h (SEM n=6; TEM n=6).

2/. TEM assessment was performed on 3 pairs of adenoid organ cultures (obtained from 1 donor) that were incubated for 4, 14 and 24h.

3/. A single experiment was performed using an exceptionally well ciliated and large piece of adenoid tissue obtained from one donor. Six pairs of adenoid organ cultures were established and were incubated for 0, 1, 2, 4, 8 and 14h for SEM examination.

4/. SEM assessment was performed on 2 pairs of nasal turbinate organ cultures (obtained from 2 donors) that were incubated for 24h.

After incubation, the sterility of the uninfected organ cultures and the purity of bacterial growth in the infected organ cultures were assessed (Section 4.4.2). Specimens were processed and examined using TEM (Sections 4.5.1 and 4.5.2) and SEM (Sections 4.6.1, 4.6.2, and 4.6.3).

8.3 PARAMETERS MEASURED

TEM and SEM assessments were performed as follows.

8.3.1 TEM ASSESSMENT (SECTION 4.5.2)

The combined mucosal damage score and the percent of cells displaying each of the following were measured:

1/. Extrusion of cells from the epithelial surface

2/. Loss of cilia

3/. Mitochondrial damage

4/. Cytoplasmic blebbing

8.3.2 SEM ASSESSMENT (SECTIONS 4.6.2 AND 4.6.3)

1. The percent of SEM fields displaying each of the following was measured:

1/. Mucus

2/. Ciliated epithelium

3/. Unciliated epithelium

4/. Extruded cells and cell debris

2. The percent of SEM fields displaying each of the following that had associated bacteria was measured:

1/. Mucus

2/. Ciliated epithelium

3/. Unciliated epithelium

4/. Extruded cells and cell debris

3. The adherence index (defined as the ratio of the percent of SEM fields displaying a mucosal component that had associated bacteria to

the total percent of SEM fields that displayed that mucosal component) was determined for each of the following:

1/. Mucus

2/. Extruded cells and cell debris

3/. Ciliated epithelium

4/. Unciliated epithelium

The adherence indices represented the relative affinity of NTHi for each of the mucosal components and therefore allowed comparisons to be made between different organ cultures that had different surface compositions.

8.4 STATISTICAL METHOD

Wilcoxon signed ranked test was employed to analyze the data (Hollander et al., 1974). A p value less than 0.05 was taken as a statistical significant difference between two groups of data.

8.5 RESULTS

8.5.1 MACROSCOPIC APPEARANCE OF ORGAN CULTURES

There was no difference between the macroscopic appearance of the uninfected and NTHi infected organ cultures after incubation for 24h.

8.5.2 MICROBIOLOGY

The mean (± standard error of mean) NTHi inocula for the adenoid (n=14) and nasal turbinate (n=2) organ cultures were 4.3±2.1 and 5.7 (standard error of mean not calculated) x 10^6 cfu respectively. The infected organ cultures had a pure growth of NTHi from their four edges and the uninfected organ cultures remained sterile after incubation.

8.5.3 ULTRASTRUCTURE

8.5.3.1 TEM ASSESSMENT OF ADENOID ORGAN CULTURES (TABLES 8.1 & 8.2; FIGURE 8.1)

TEM assessment was only performed on adenoid organ cultures. There was

no difference in the percent of cells displaying the TEM parameters between the infected and the uninfected adenoid organ cultures after 4 and 14h incubation (Table 8.1).

However, a significantly higher combined mucosal damage score and a higher percent of cells displaying mitochondrial damage were found in the infected than the uninfected adenoid organ cultures after 24h incubation ($p<0.05$) (Table 8.2). There was no significant difference in the percent of cells displaying loss of cilia, extrusion from the epithelial surface and cytoplasmic blebbing after 24h. Bacteria were found to be within one epithelial cell diameter from the mucosal surface in four of the six infected adenoid organ cultures but none of the uninfected organ cultures. The sum of the adjusted damage scores ($n=6$) on mitochondrial damage and cytoplasmic blebbing (Section 4.5.2) for unciliated cells in the infected organ cultures (16.1 ± 3.3) was higher but not significantly different from that of the uninfected adenoid organ cultures (8.2 ± 2.9) at 24h ($p=0.142$).

8.5.3.2 SEM ASSESSMENT OF THE SURFACE MORPHOLOGY OF ADENOID AND NASAL TURBINATE ORGAN CULTURES (FIGURE 8.2; TABLES 8.3 & 8.4)

Twenty four h after incubation, the NTHi infected organ cultures showed a significantly higher percent of SEM fields displaying extruded cells and cell debris than the uninfected adenoid organ cultures ($p<0.05$). However, no significant difference was found in the percent of SEM fields displaying mucus, ciliated epithelium and unciliated epithelium. A similar pattern was observed in the nasal turbinate organ cultures after 24h incubation although there was less mucus.

8.5.3.3 SEM ASSESSMENT OF BACTERIAL ADHERENCE TO ADENOID AND NASAL TURBINATE ORGAN CULTURES (FIGURES 8.3 TO 8.6; TABLE 8.5)

No bacteria were seen in the uninfected adenoid and nasal turbinate organ cultures. In the adenoid organ cultures, bacteria were first detected on the mucosal surface 1h after inoculation and were adherent

to mucus, ciliated cells, unciliated cells, and extruded cells and cell debris. The adherence indices for mucus showed a decreasing trend from 4 to 14h while that for cell debris and extruded cells and unciliated cells increased with longer incubation period. The adherence indices for ciliated cells remained relatively low and did not change remarkably between 0 and 14h of incubation (0-5.1%) (Table 8.5).

After 24h incubation, the mean (± standard error of mean) adherence indices (n=6) of NTHi for mucus, extruded cells and cell debris, and unciliated cells were 50.4±4.5, 44.8±11.0, 51.0±11.9% respectively which were significantly higher than that for ciliated cells (13.3±2.7%) ($p<0.05$). In the infected nasal turbinate organ cultures (n=2) no bacteria were found in the 40 designated SEM fields although some were found at the periphery of the organ cultures. After 24h incubation (n=6), 85.4% of NTHi colonies associated with ciliated epithelium were associated with the ciliary tips, 0% with the ciliary shaft and 14.6% with the base of cilia in the infected adenoid organ cultures.

8.5.3.4 SEM ASSESSMENT OF BACTERIA IN ADENOID ORGAN CULTURES (TABLES 8.6 & 8.7)

Zero to 4h after inoculation with NTHi (n=1), the number of adherent bacterial microcolonies increased for unciliated cells, remained relatively unchanged for ciliated cells and showed no obvious trend for mucus, and cell debris and extruded cells. From 0 to 14h after incubation, the mean number of bacteria in each microcolony increased with time for mucus, unciliated cells, and extruded cells and cell debris but not for ciliated cells.

8.6 DISCUSSION

Previous studies on bacterial adherence have used many different methods to signal adherence of bacteria to respiratory tissue or human buccal epithelial cells (Section 1.3.2.3). These include the use of

radiolabelling of bacteria (St Geme et al., 1990), bacterial viable counting of homogenised tissue (Roberts et al., 1984), light microscopy (Fainstein et al., 1979), and immunolabelling techniques (Lampe et al., 1982). SEM has been used to study the adherence of PA to murine and canine tracheas (Ramphal et al., 1980; Zoutman et al., 1991), and that of *N. meningitidis* and Hib to human adenoid tissue (Stephens et al., 1984; Farley et al., 1986). However previous studies were essentially descriptive and did not include morphometric assessment of the respiratory mucosa. TEM assessment of bacterial adherence (Read et al., 1991; Feldman et al., 1992) may not accurately assess adherence due to the small area examined and the three dimensional nature of the interaction. Tissue processing for SEM involves gradual dehydration in alcohols leading to shrinkage of the mucus layer which only covered about 30% of the tissue surface of the uninfected organ cultures. This permitted detailed assessment of bacterial interactions with cilia and the cell surface. Adherence of bacteria such as PA and NTHi to respiratory mucosa probably involves the use of different adhesins and receptors for mucus and different cell types (Plotkowski et al., 1993). Studies that use a single individual mucosal component such as mucus, or ciliated epithelium in cell culture, therefore could not provide a "true" mucosal surface, as the presence of different mucosal components may affect bacterial adherence.

The SEM method described in this thesis allows simultaneous assessment of bacterial adherence to different mucosal components of an intact respiratory mucosa. However, SEM could not assess bacterial interactions with structures underneath the mucus layer, although TEM could achieve this. Bacterial interactions with ciliated cells were also difficult to assess. Bacterial attachment to the ciliary shaft and ciliary base could only be assessed at the edge of ciliated cells, or when some loss of cilia had occurred so that these sites could be seen. Furthermore, the adherence indices determined by using the SEM protocol described in this thesis only represented the "area" occupied

by mucosal components that had associated NTHi (Section 4.6.3). As the size of each unit area was similar to that of a NTHi bacterium, the area occupied by NTHi associated mucosal components would therefore represent the bacterial density for that mucosal component. Other limitations would include the inability to count the bacterial cells that were hidden underneath other bacteria or mucosal structures, poor visualisation of the point of contact between NTHi and the mucosal surface, and the labour intensive nature of the manual counting. Despite these limitations, calculation of the adherence indices shows a preferential adherence of NTHi to mucus, unciliated cells and extruded cells and cell debris but not to ciliated cells.

NTHi also attached to mucus and unciliated cells but not to ciliated cells of adenoid tissue freely suspended in bacteria-containing cell culture medium after 12h incubation (Farley et al., 1986). Similarly Hib were found to adhere preferentially to unciliated cells (Loeb et al., 1988; Farley et al., 1986 & 1992). Other bacterial species also show preferential affinities to components of the mucosal surface. *N. meningitidis* (Stephens et al., 1984) adhere preferentially to unciliated cells and mucus but not to ciliated cells. Many respiratory pathogens including NTHi, Hib, *B. pertussis* and *M. pneumoniae* adhere to cilia of respiratory mucosa *in vitro* (Fernald et al., 1976; Tuomanen et al., 1983; Roberts et al., 1984; Franklin et al., 1987).

In this thesis, adherence of NTHi to the mucosal surface was infrequently found until 4h after incubation. An increase in NTHi adherence to human conjunctival cell culture (St Geme et al., 1990), and human nasal turbinate (Read et al., 1991) and adenoid organ cultures (Farley et al., 1986) with longer incubation period has also been reported. Detection of PA on mucosal surface also showed a latency in the same organ culture model (Chapter 9). This may reflect a genuine need for bacteria to adapt to a new environment before adherence is secured or a failure to detect small NTHi microcolonies on specimens which had a shorter incubation period. The latter is

unlikely to be important as many single bacteria were detected frequently. The number and size of bacterial microcolonies also increased with longer incubation period. These observations suggest that most of the NTHi inoculated onto the mucosal surface were removed by the intact mucociliary clearance mechanism and only a small number of bacteria were able to secure themselves on the mucosal surface. These bacteria may multiply leading to the formation of larger and more numerous microcolonies that spread contiguously on the mucosal surface.

Preliminary data from the single experiment suggests that the adherence indices for unciliated cells, and extruded cells and cell debris might increase with longer incubation. This suggests that NTHi might have an increased affinity to these mucosal components with longer incubation. However these may also be explained solely by an increase in the size and number of NTHi microcolonies, and that the indices were indirect measurements of bacterial adherence (Section 4.6.3). The possible decrease in adherence index for mucus with incubation might be explained by a gradual reduction in NTHi affinity for mucus, a transfer of mucus-bound bacteria to other mucosal components, or the clearing of "older" mucus by the intact mucosal surface that only left more freshly formed mucus which had fewer adherent bacteria. However, interpretation of the data, which was derived from only one single experiment, should only be made with caution.

Read et al. described a preferential adherence of NTHi to mucus and damaged epithelial cells (ciliated and unciliated), but adherence to normal epithelial cells was not detected in nasal turbinate organ cultures in a agar-embedded model employed in some of the studies described in this thesis (Section 4.2.1) (Read et al., 1991). Although adherence to mucus and damaged cells was also detected in this study, NTHi also frequently adhered to apparently healthy unciliated cells in an adenoid organ culture with an air-mucosal interface. Probably due

to the small area sampled, TEM did not confirm this, in that bacteria were not seen directly adherent to the cell surface. There are a number of possible explanations for this difference between the studies of Read et al. and those reported in this thesis. The unciliated cells with adherent bacteria observed by SEM may have been ultrastructurally abnormal if they had been examined by TEM. However this seems unlikely as the overall damage to the tissue caused by NTHi infection was small. When unciliated cells of uninfected and NTHi infected organ cultures were compared, no significant difference was found in the adjusted damage score computed from cytoplasmic blebbing and mitochondrial damage (Section 8.5.3.1). Another explanation would be that there is a difference between the adherence of NTHi to epithelial cells depending on whether they are immersed in culture medium or not i.e. cell culture medium might interfere with bacterial adherence to host cells. Although the amount of damage to tissue was probably greater in the experiments with adenoid organ cultures reported by Farley et al., their results also suggest adherence of NTHi to normal unciliated cells (Farley et al., 1986). The most likely explanation of these results is that adenoid and nasal turbinate tissue express different surface receptors for NTHi adhesins. Studies of hamster trachea and bronchus with a panel of lectins have also shown differences in the surface exposed glycoconjugates (Christensen et al., 1990). This study suggests that unciliated cells of adenoid tissue may express receptors for NTHi, but turbinate cells do not unless they are damaged (Read et al., 1991). This could also explain why NTHi is a common nasopharyngeal commensal, but is rarely isolated from the nose (Jousimies-Somer et al., 1989).

It is generally assumed that the first interaction of inhaled bacteria with the respiratory mucosa is with mucus. In the presence of normal mucociliary clearance, bacteria are transported with secretions to the nasopharynx where they are expectorated or swallowed. Consistent with previous studies, a high affinity of NTHi for mucus was also found in this study. NTHi (Farley et al., 1986; Read et al., 1991; Barsum et

al., 1992), *S. pneumoniae* (Plotkowski et al., 1986; Feldman et al., 1992), and PA (Vishwanath et al., 1984) all adhere to respiratory mucus *in vitro*. Association of PA with lower respiratory mucus has also been reported in a post mortem study of lungs obtained from patients with cystic fibrosis (Baltimore et al., 1989).

Many previous studies on bacterial adherence to respiratory mucosa practised "washing" of host tissue to remove "passively adherent" bacteria prior to quantification of bacterial adherence. This was not practised in the studies described in this thesis (please also refer to Chapter 9) as the rationale for the establishment of an air-mucosal interface was to have an undisturbed mucosal surface. As immersion of respiratory mucosa with bacteria-containing culture medium did not occur, bacteria would not be left on the mucosal surface simply because they were passively suspended in the medium above the mucosa. Instead bacteria "fixed" by glutaraldehyde on mucosal surface should have been in direct contact with the mucosa. It therefore appears inappropriate to "wash" the respiratory mucosa prior to SEM processing for the organ culture model with an air-mucosal interface.

NTHi colonise the human respiratory tract and cause disease by contiguous spread or invasion (Wilson et al., 1992). NTHi is a common commensal of the normal human upper respiratory tract, being present in up to three quarters of healthy adults (Murphy et al., 1987). Recently, it has been shown that NTHi persist in the lower respiratory tract of patients with chronic bronchitis despite clinical improvement and treatment with appropriate antibiotics (Groeneveld et al., 1990a&b). The exact location of bacteria in the upper and lower respiratory tract during health or disease is unknown. Common human respiratory pathogens including NTHi, *S. pneumoniae*, *Staphylococcus aureus* and PA bind specifically to the GalNAcß1-4Gal sequence found in various glycosphingolipids that are located in human lung tissue (Krivan et al., 1988), but whether these receptors are expressed on the epithelial surface is unknown. The results in this thesis suggest

that in health NTHi associate with mucus and can adhere to normal unciliated epithelial cells in some sites (adenoid) but not others (turbinate). NTHi adherence to cilia or normal ciliated cells is less usual. The association of NTHi with mucus in disease has important implications for antibiotic therapy because antibiotics in general penetrate poorly into mucus. The results of this study do not agree with the observation made by Read et al. in the agar embedded nasal turbinate model when NTHi infection increased mucus production by the organ cultures (Read et al., 1991). NTHi produce factors which stimulate mucus production by guinea pig tracheal rings *in vitro* (Adler et al., 1986), and the failure to show an increase may relate to the fixation for SEM which leads to contraction of the mucus layer, or to clearing of mucus by an intact mucociliary system. The difference may also relate to the lack of culture medium containing bacterial products and the less severe damage caused by infection of the air-mucosal interface model. NTHi adhere well to damaged cells, which may be caused by the NTHi infection itself or could be caused by prior damage from viral infection or cigarette smoking (Fainstain et al., 1980; Ramphal et al., 1980; Michaels et al., 1977 & 1983).

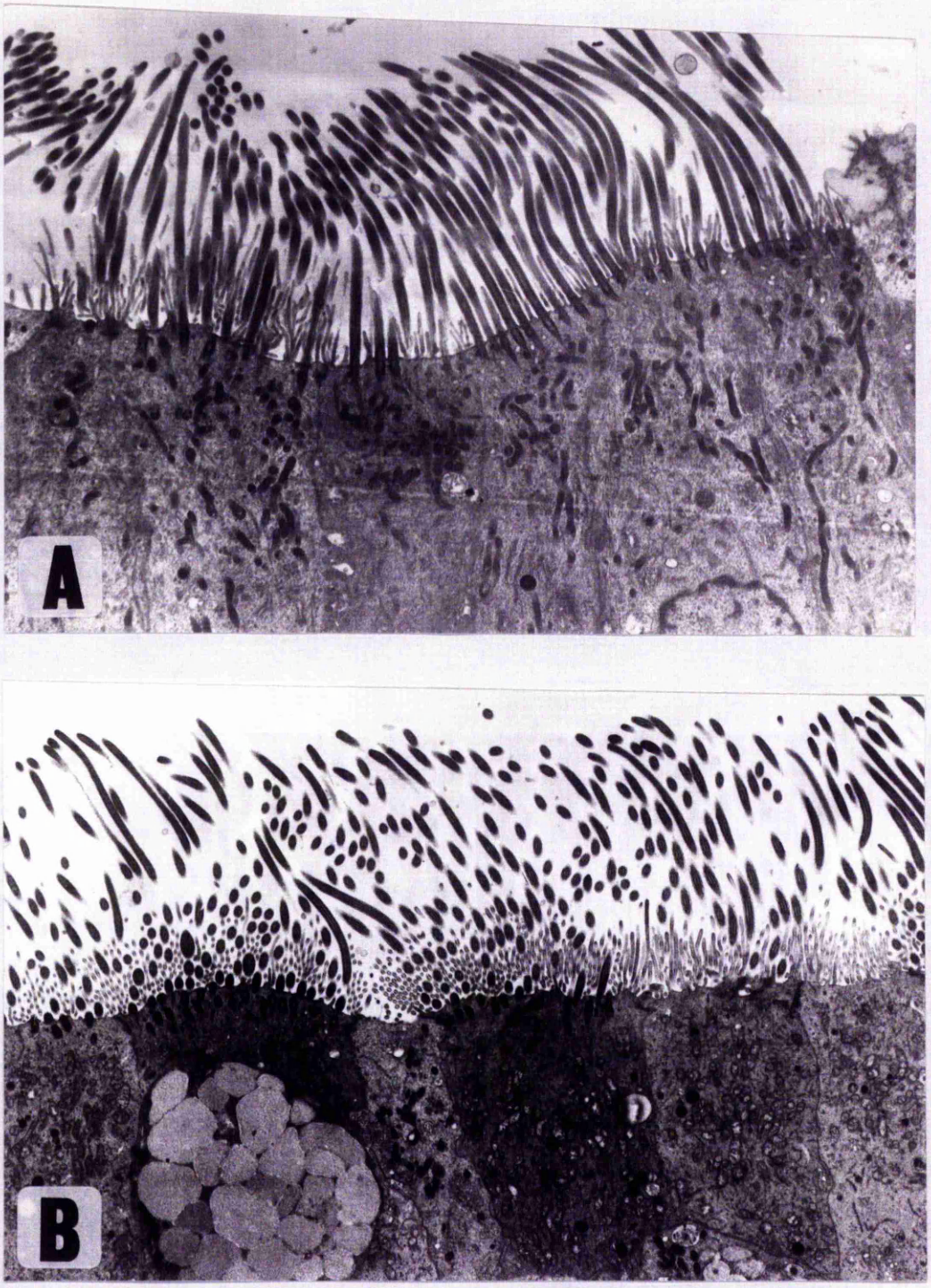

Figure 8.1 Transmission electron micrographs of adenoid organ cultures with an air-mucosal interface showing (A) normal and well ciliated cells in an uninfected organ culture; and (B) mitochondrial damage in a NTHi infected organ culture after 24h incubation (5250x).

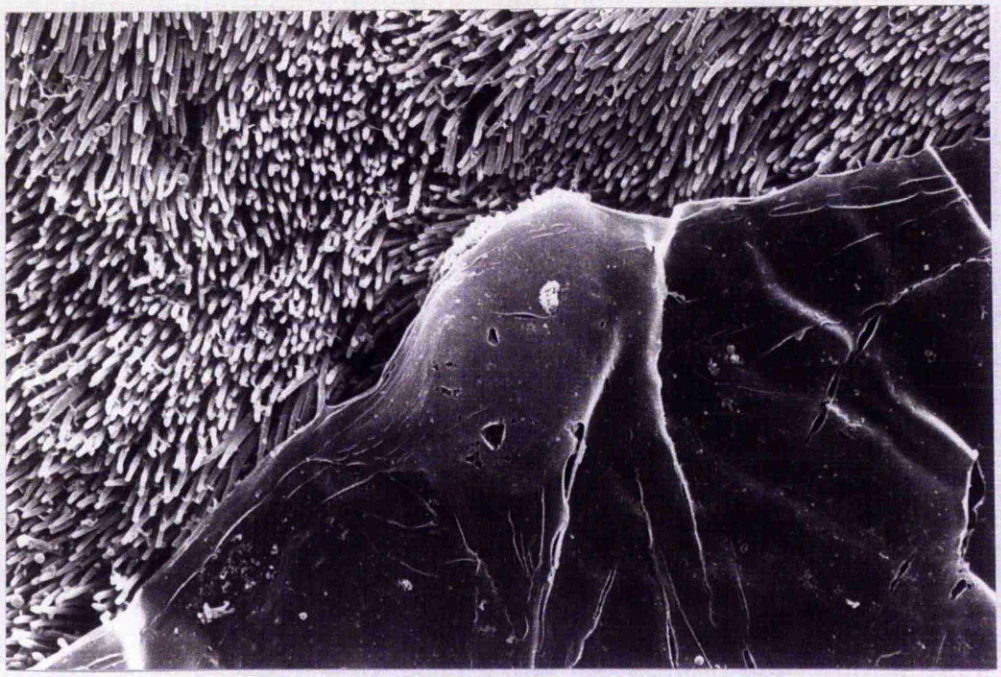

Figure 8.2 Scanning electron micrograph showing well ciliated epithelial surface with associated mucus in an uninfected adenoid organ culture with an air-mucosal interface after 24h incubation (5000x).

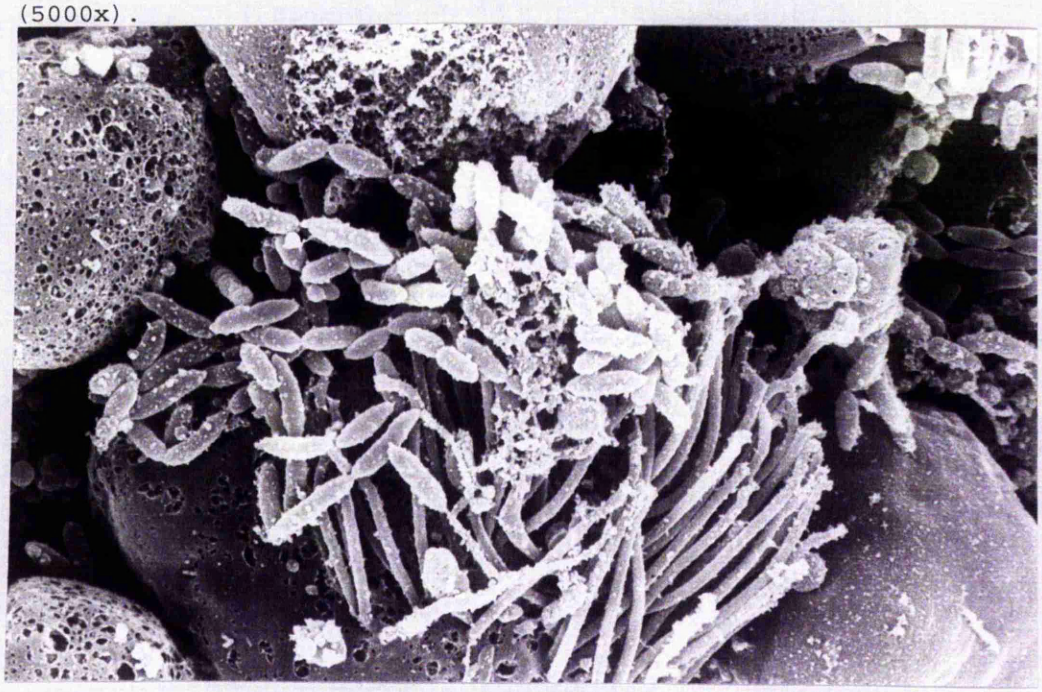

Figure 8.3 Scanning electron micrograph showing adherence of bacteria to extruded cells and cell debris in an adenoid organ culture with an air-mucosal interface infected with NTHi (strain SH9) after 24h incubation (7500x).

Figure 8.4 Scanning electron micrograph showing adherence of bacteria to mucus in an adenoid organ culture with an air-mucosal interface infected with NTHi (strain SH9) after 24h incubation (4500x).

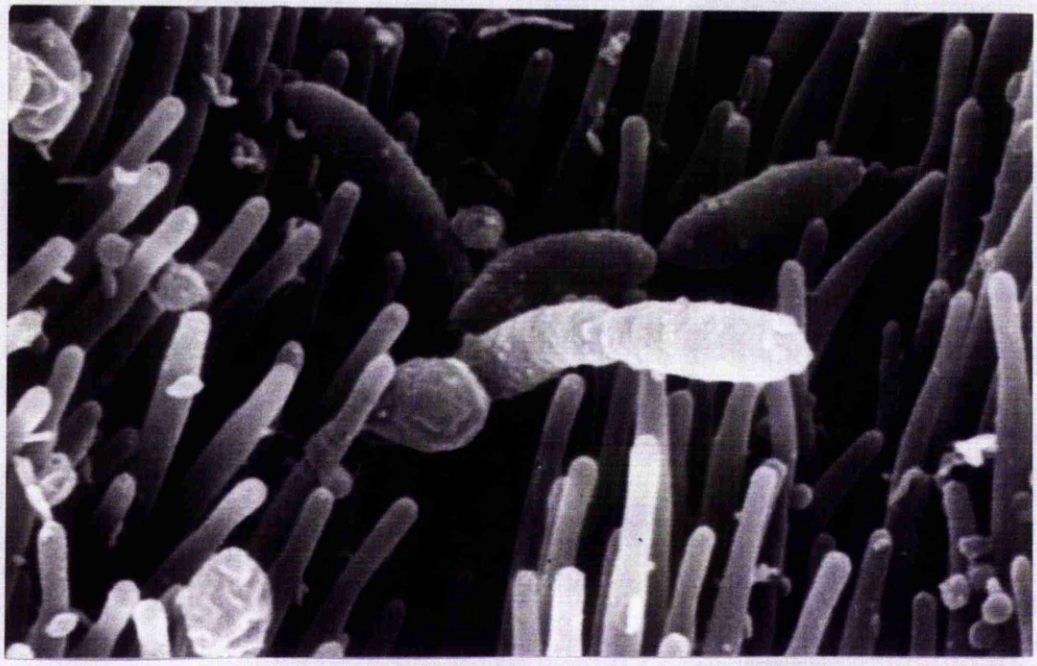

Figure 8.5 Scanning electron micrograph showing adherence of bacteria to cilia in an adenoid organ culture with an air-mucosal interface infected with NTHi (strain SH9) after 24h incubation (25000x).

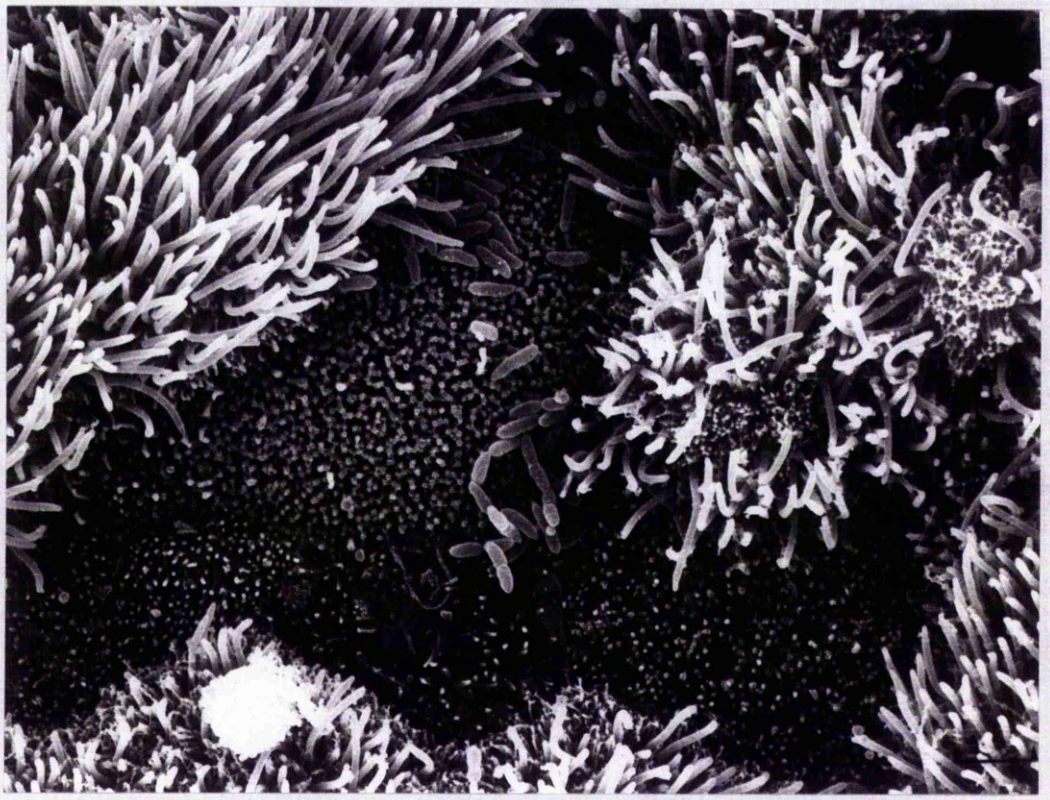

Figure 8.6 Scanning electron micrograph showing adherence of bacteria to unciliated cells in an adenoid organ culture with an air-mucosal interface infected with NTHi (strain SH9) after 24h incubation (5000x).

TABLE 8.1 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE AFTER INCUBATION.

Time	organ culture	% of cells extruding from the epithelial surface	% of cells with loss of cilia	% of cells with mito-chondrial damage	% of cells with cytoplasmic blebbing	Combined mucosal damage score
4h	uninfected	18.5	9.9	2	1	12.6
4h	NTHi infected	15.3	7.8	1.7	1.7	10.7
14h	uninfected	13	13	2.9	2.2	12.9
14h	NTHi infected	11.2	6.8	2.8	2.1	10.2
24h	uninfected	16.7	14.2	1.5	2.5	12.9
24h	NTHi infected	15.8	11.8	2.6	1.5	12.4

All data is result of one single experiment. NTHi = non-typable *Haemophilus influenzae* (strain SH9).

TABLE 8.2 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE AFTER 24H INCUBATION.

Organ culture	% of cells extruding from the epithelial surface	% of cells with loss of cilia	% of cells with mito-chondrial damage	% of cells with cytoplasmic blebbing	Combined mucosal damage score
Uninfected	13.4 ± 2.2	12.1 ± 2.6	2.3 ± 0.4	2.0 ± 0.6	11.8 ± 2.0
Infected with NTHi	19.3 ± 3.0	15.2 ± 2.3	5.0 ± 1.2*	3.3 ± 0.9	18.9 ± 2.9*

All data is mean of six separate experiments ± standard error of mean. * = $p<0.05$ versus uninfected organ culture. NTHi = non-typable *Haemophilus influenzae* (strain SH9).

TABLE 8.3 SCANNING ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE AFTER 24H INCUBATION.

Percent of SEM fields displaying	Organ culture: uninfected	Organ culture: NTHi infected
Mucus	31.6±6.7	29.1±7.4
Ciliated epithelium	56.3±6.6	40.7±11.3
Unciliated epithelium	10.3±2.4	17.5±4.7
Extruded cells and cell debris	3.6±1.7	12.3±3.7*

All data is mean ± standard error of mean of six separate experiments.

* = $p<0.05$ when compared with uninfected organ cultures. NTHi = non-typable *Haemophilus influenzae* (strain SH9).

TABLE 8.4 SCANNING ELECTRON MICROSCOPY ASSESSMENT OF NASAL TURBINATE ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE AFTER 24H INCUBATION.

	Organ culture	
Percent of SEM fields displaying	uninfected	NTHi infected
Mucus	4.7±0.8	2.5±0.5
Ciliated epithelium	37.0±14.5	54.5±20.1
Unciliated epithelium	55.8±11.70	40.8±20.0
Extruded cells and cell debris	2.8±1.9	2.3±0.4

All data is mean ± standard error of mean of two separate experiments. NTHi = non-typable *Haemophilus influenzae* (strain SH9).

TABLE 8.5 SCANNING ELECTRON MICROSCOPY ASSESSMENT OF ADHERENCE INDICES IN NTHi (STRAIN SH9) INFECTED ADENOID ORGAN CULTURES.

	Incubation time					
Adherence Index for	0h	1h	2h	4h	8h	14h
Mucus	0	14	0	45.5	32.3	25
Ciliated cells	0	2.8	2.6	3.4	0	5.1
Unciliated cells	0	26.7	16.7	41.9	25	58.6
Extruded cells and cell debris	0	26.3	8.3	15.4	18.8	25

Data shown are the results of one experiment performed on adenoid tissue obtained from one donor.

TABLE 8.6 SCANNING ELECTRON MICROSCOPY ASSESSMENT OF THE NUMBER OF NTHi (STRAIN SH9) MICROCOLONIES IN ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE.

	Incubation time					
Total no of NTHi microcolonies adherent to	0h	1h	2h	4h	8h	14h
Mucus	0	5	0	49	40	15
Ciliated cells	0	3	1	1	0	2
Unciliated cells	0	22	7	45	22	54
Extruded cells and cell debris	0	15	2	7	8	7

Data shown are the results of one experiment performed on adenoid tissue obtained from one donor.

TABLE 8.7 SCANNING ELECTRON MICROSCOPY ASSESSMENT OF THE SIZE OF NTHi (STRAIN SH9) MICROCOLONIES ON THE SURFACE OF ADENOID ORGAN CULTURES.

Mean (median) no of NTHi in each microcolony adherent to	Incubation time 0h	1h	2h	4h	8h	14h
Mucus	0 (0)	3.0±0.84 (3)	0±0 (0)	6.5±1.14 (3)	9.0±1.83 (4)	10.9±3.15 (7)
Ciliated cells	0 (0)	6.7±4.70 (2)	9±0 (9)	16.0±0.00 (16)	0±0 (0)	4.0±1.00 (4)
Unciliated cells	0 (0)	2.9±0.54 (2)	3.9±0.83 (3)	6.6±0.97 (4)	6.6±1.82 (3)	17.3±4.74 (6)
Extruded cells and cell debris	0 (0)	5.7±1.50 (3)	6.5±2.50 (6.5)	8.4±4.70 (5)	5.5±2.41 (3)	14.9±6.65 (6)

Data shown are the mean (median) ± standard error of mean from one experiment performed on adenoid tissue obtained from one donor. NTHi = Non-typable *Haemophilus influenzae* (strain SH9).

CHAPTER 9

INTERACTION OF *P. AERUGINOSA* WITH HUMAN RESPIRATORY MUCOSA *IN VITRO*

9.1 AIM OF STUDY

The aim of this chapter was to study the interactions of a clinical isolate of PA (strain P455) with intact human respiratory mucosa in an organ culture model with an air-mucosal interface. This involved the use of light microscopy to assess CBF, TEM to study the epithelial ultrastructure, and SEM to make qualitative assessment of PA adherence to the respiratory mucosa. Qualitative rather than quantitative SEM assessment was performed as most of the mucosal surface was obscured by bacterial biofilm rendering quantification of surface morphology and PA adherence impracticable.

9.2 STUDY DESIGN

Organ cultures and washed suspensions of PA were prepared as described in Sections 4.2.2 and 4.8 respectively. For each experiment, pairs of organ cultures were established from the same piece of adenoid tissue. Each pair of adenoid organ cultures was incubated and processed simultaneously. From each pair of organ cultures, one organ culture was inoculated with PA suspension as described in Section 4.4.2 and the other with sterile PBS which acted as the uninfected control. The size of PA inoculum was quantified by performing a viable count (Section 4.9). Organ cultures were then incubated at 37^0C in a humidified atmosphere that contained 5% CO_2.

Four series of experiments were performed:

1. TEM and SEM assessment on 8 pairs of organ cultures (obtained from 8 donors) that were incubated for 8h (TEM, n=6; SEM, n=2). Light microscopy assessment was also performed on 2 pairs of these organ cultures.

2. SEM examination (n=2) was performed on pairs of organ cultures that were obtained from 2 different donors after incubation for 15min, 1h, 2h, 4h and 8h.

3. TEM assessment was performed on organ cultures after incubation for 12 (n=3, 3 donors), 16 (n=3, 3 donors), and 24h (n=4, 4 donors).

4. Qualitative SEM examination (n=1) was performed on a PA inoculum

prepared and processed as described in Sections 4.6.1 and 4.6.4.

After incubation, the sterility of the uninfected control and the purity of growth of PA in the infected organ cultures were checked (Section 4.4.2). Specimens were then processed for TEM and SEM assessment as described in Sections 4.5.1 and 4.6.1 respectively. In addition, light microscopy assessment was performed on control and PA infected organ cultures after 8h incubation in 2 separate experiments as described in Sections 4.3.2 and 4.7.

9.3 PARAMETERS MEASURED

The following assessment was performed on adenoid organ cultures by TEM, SEM and light microscopy.

9.3.1 TEM ASSESSMENT (SECTION 4.5.2)

The combined mucosal damage score and the percent of cells displaying each of the following were determined:

1. Extrusion from the epithelial surface
2. Loss of cilia
3. Mitochondrial damage
4. Cytoplasmic blebbing

9.3.2 SEM ASSESSMENT (SECTION 4.6.2)

Qualitative assessment on each of the following was made:

1. Mucosal surface
2. Bacterial morphology
3. Bacterial adherence to mucosal surface

9.3.3 LIGHT MICROSCOPY ASSESSMENT (SECTIONS 4.3.2 & 4.7)

1. CBF
2. Number of sites with ciliary activity

9.3.4 SEM EXAMINATION OF PA INOCULUM (SECTION 4.6.4)

Qualitative assessment was made on PA (strain P455) cultured in BHI

broth to assess whether there was any extracellular matrix.

9.4 STATISTICAL METHOD

Wilcoxon signed ranked test was employed to analyze the data (Hollander et al., 1974). A p value less than 0.05 was taken as statistical significant difference between two groups of data.

9.5 RESULTS

9.5.1 MACROSCOPIC APPEARANCE OF ORGAN CULTURE

After incubation, naked eye appearances of the uninfected organ cultures remained unchanged. PA infected organ cultures developed a greenish blue colour that was detectable from 12h.

9.5.2 BACTERIOLOGY

The mean (± standard error of mean) inoculum of PA for the infected organ cultures was $5.9\pm0.9 \times 10^6$ cfu. PA infected organ cultures produced pure growth of PA from all four edges after incubation and the uninfected organ cultures were sterile.

9.5.3 ULTRASTRUCTURE

9.5.3.1 TEM ASSESSMENT (TABLE 9.1)

The uninfected organ cultures had normal ultrastructure in all the experiments. The infected organ cultures showed a significantly ($p<0.05$) higher percent of cells displaying extrusion from the epithelial surface, loss of cilia, mitochondrial damage and cytoplasmic blebbing, and combined mucosal damage scores compared with the uninfected organ cultures after 8h incubation (Figure 9.1). However, damage to the epithelium was patchy, and some areas examined had relatively normal ultrastructure. This is reflected in the combined mucosal damage score which has a possible maximum value of 400.

Bacteria were seen closely associated with the mucosal surface in all the infected organ cultures after 8h incubation. PA was found adherent

predominantly to damaged epithelial cells and mucus. In the infected organ cultures, large colonies of bacteria were found infiltrating the epithelium and adhering to the basement membrane collagen layer (Figure 9.2).

In the organ cultures infected by PA for 12, 16 and 24h, complete disintegration of the epithelial structure and subepithelial collagen layer was seen. Bacteria were seen infiltrating the disintegrated mucosa. No intracellular bacteria were detected.

9.5.3.2 SEM ASSESSMENT

From 15min to 2h after inoculation, small microcolonies of PA were only found infrequently. PA was associated with mucus, and extruded cells and cell debris although the latter were only found occasionally. The number of bacteria adherent to the mucosal surface had increased substantially and mucosal damage was more apparent after 4h incubation when most of the surface mucus, cell debris and extruded cells had associated PA (Figure 9.3). PA microcolonies associated with mucus usually contained many bacteria. PA were found occasionally associated with cilia (Figure 9.3) and only rarely with unciliated cells. When PA were seen associated with ciliated epithelial cells, they were usually associated with the tips of cilia. Separation of epithelial cell junctions was evident on some parts of the mucosal surface. Frequently, but not always, microcolonies of PA were seen in the gaps formed between epithelial cells and appeared to be migrating in a subepithelial direction (Figure 9.4). After 8h, most of the surface of the organ cultures was covered with bacteria that formed continuous sheets covering the surface of a large proportion of the organ culture (Figure 9.5). This concealed the structures that the bacilli were associated with and made quantitative SEM assessment difficult. Bacteria in these large colonies were often seen dividing.

In bacterial microcolonies, particularly those with large numbers of bacteria, an extracellular matrix material was seen closely associated

with bacteria. This material was not seen in uninfected organ cultures, but was seen associated with PA grown in BHI broth after 6h suggesting that it had a bacterial origin (Section 9.5.5). When PA was seen associated with the mucosal surface components (including mucus, cilia, unciliated cells and extruded cells), this matrix material was often seen "bridging" the gap between bacteria and the mucosa, and between the bacteria themselves (Figures 9.4).

9.5.4 LIGHT MICROSCOPY ASSESSMENT (TABLE 9.2)

CBF and number of sites with ciliary activity remained stable in uninfected organ cultures after 8h incubation (n=2). CBF and the number of sites with ciliary activity were reduced in PA infected compared with uninfected organ cultures.

9.5.5 SEM EXAMINATION OF PA INOCULUM

Bacteria appeared to be uniform in morphology (bacilliform) and clustered together. Some bacteria were seen dividing and linear strands of matrix material was found bridging some of them together. This matrix material that surrounded PA was less abundant in PA obtained from broth than PA found on the surface of infected organ cultures.

9.6 DISCUSSION

In this study, an organ culture with an air-mucosal interface was used to study the interaction of a non-mucoid strain of PA with human respiratory mucosa (Chapter 7). A non-mucoid PA (strain P455) was chosen partly because this strain has previously been investigated at the Host Defence Unit (Wilson et al., 1987; Lapa de Silva et al., 1989), and partly because the initial colonisation of the respiratory mucosa is usually by non-mucoid strains which become mucoid with chronic infection (Doggett et al., 1966).

Previous studies have mainly concentrated on the adherence of PA to epithelial cells in the absence of an intact mucociliary system, and

little is known of the effects of PA infection on the ultrastructure of the respiratory mucosa. In this study, PA infection caused slowing of CBF and reduction in number of sites with ciliary activity after 8h. By using TEM examination, loss of cilia, extrusion of cells from the epithelial surface, cytoplasmic blebbing and mitochondrial damage were evident in the respiratory mucosa after 8h incubation with PA. SEM only detected PA infrequently on the mucosal surface in the first 2h after inoculation despite a relatively large bacterial inoculum. This suggests that although PA shows a high affinity for mucus, washed bacteria from broth culture do not immediately adhere to mucus avidly. However a substantial increase in bacterial density occurred by 4h which was associated with mucosal damage. This latency for the detection of PA on the mucosal surface has also been reported previously in hamster and mouse tracheal organ cultures (Baker et al., 1982a&b; Ramphal et al., 1983A; Marcus et al., 1985) and is similar to that found in NTHi infection of the same organ culture model (Chapter 8).

In this study, PA has been shown to adhere preferentially to mucus and extruded and damaged epithelial cells, in preference to cilia and unciliated cells (Section 1.4.2.2). Adherence to damaged epithelial cells has previously been shown in hamster trachea organ culture in which adherence only occurred after influenza A virus infection or treatment with acid that injured the epithelium (Ramphal et al., 1980 & 1983a&b). These studies have suggested that both mucoid and non-mucoid strains of PA only adhered to damaged but not normal cells (Ramphal et al., 1985a&b). Recently Baltimore et al. reported adherence of PA to intraluminal secretions, damaged epithelium and exposed connective tissue but not to normal epithelium in a post mortem study of CF airways (Baltimore et al., 1989). The results from this study agree with these observations. PA also less frequently adhered to ultrastructurally normal cilia which has been observed previously with human respiratory epithelial cells (Niederman et al., 1983; Franklin et al., 1987; Plotkowski et al., 1991). Although the

cilia were ultrastructurally normal, their functioning was unclear at the time of bacterial adherence. PA produces a number of factors which slow or stop ciliary beating (Wilson et al., 1987; Read et al., 1991), which may precede bacterial adherence to cilia.

In studies using hamster tracheal organ cultures, mucoid strains of PA were found to adhere as aggregates, primarily to ciliated cells, with a bacterial extracellular matrix binding directly to the cilia (Baker et al., 1982a&b; Marcus et al., 1985). Non-mucoid PA adhered primarily to unciliated cells in a study using hamster tracheal cell cultures (Grant et al., 1991); to mucus and damaged cells, and very uncommonly to ciliated epithelium in sulphur dioxide injured canine tracheal organ cultures (Zoutman et al., 1991); and to both ciliated and unciliated cells in canine tracheal cell cultures (Hata et al., 1991).

PA has a high affinity for human tracheobronchial mucin (Vishwanath et al., 1984 & 1985; Ramphal et al., 1991a; Reddy 1992; Sajjan et al., 1992), and this study shows that PA also has a high affinity for mucus in adenoid organ cultures. PA also produce a number of toxins which stimulate mucus production (Klinger et al., 1984; Somerville et al., 1991 & 1992).

In the organ cultures incubated for 8h, PA formed continuous sheets over the mucosal surface. It has been suggested that the persistence of PA in the lower respiratory tract may be helped by the formation of such biofilms. Biofilms may protect the bacteria against host defences (Costerton 1984) such as opsonophagocytic killing by neutrophils (Jensen et al., 1990).

In this thesis PA was found to adhere to the basement membrane collagen. Adherence of a non-mucoid strain of PA to the tracheal collagen layer was also found in rat trachea injured by brushing (Yamaguchi et al., 1991) and in ferrets (Ramphal et al., 1980). Plotkowski et al. have also reported adherence of non-mucoid PA to

type I collagen matrix (Plotkowski et al., 1991), and to submucosal connective tissue obtained from frog palates (Plotkowski et al., 1989). This adherence to subepithelial collagen may help explain the persistence of PA in the lower respiratory tract of patients who have cystic fibrosis or bronchiectasis whose bronchial tree is damaged by the disease process.

Pili have been identified as an important adhesin for PA to buccal cells (Woods et al., 1980b), damaged tracheal epithelial cells (Ramphal et al., 1984), and mucin (Ramphal et al., 1987), but do not account for all the adhesive properties of PA (Ramphal et al., 1991b), and other adhesins such as exoenzyme S have been identified (Baker et al., 1991).

The exopolysaccharide of PA may be another adhesin and is produced by both mucoid strains and non-mucoid strains (Anastassiou et al., 1987). PA produce an exopolysaccharide that forms a loose capsule of organised linear strands of exopolysaccharide radiating outwards from the cell surface seen at SEM although this may be an artefact and appears as a result of dehydration of the exopolysaccharide. This exopolysaccharide appears to mediate adherence to human respiratory epithelium (Ramphal et al., 1985b; Doig et al., 1987), and an antibody raised against it reduces PA adherence to acid-injured murine trachea (Ramphal et al., 1985b). In the SEM study described in this thesis, a matrix-like material that formed organised linear strands was also found to be closely associated with PA biofilms, and present at the point of contact between bacteria and mucosal surface. A similar matrix-like material was also seen closely associated with colonies of aggregated bacteria in a recent study performed with primary cultures of human respiratory epithelium obtained from nasal polyps (Plotkowski et al., 1991). Plotkowski et al. suggested that because the matrix-like material reacted with antimucin antibody it might have an epithelial origin (Plotkowski et al., 1991). However, in the study described in this thesis, a similar material was also found to be

closely associated with PA cultured in broth, and this observation suggests that this matrix could have a bacterial origin. It is therefore possible that this material may act as a PA adhesin for human respiratory mucosa. As this matrix-like material appears to mediate PA adherence to several different mucosal components, it may actually mediate bacterial adherence non-specifically by a "glue-like" action rather than a specific adhesin-receptor fashion. The florid production of this matrix by the test strain of non-mucoid PA (P455) suggests that it might have converted to a mucoid phenotype during organ culture which has also been reported to occur *in vivo* and *in vitro* (Speert et al., 1990; Dunne et al., 1985) although non-mucoid strains also produce exopolysaccharide (Pitt 1986; Anastassiou et al., 1987).

Tight junctions between cells are important in maintaining the integrity of epithelial surface. They form a barrier to the diffusion of molecules and ions across the epithelial cell layer and their loss may lead to changes in transepithelial electrical resistance, exposure of subepithelial structures to bacteria and their toxins, and leakage of tissue fluid which contributes to the increased secretions during infection (Gumbiner 1987). In this study, both TEM and SEM examination of tissue infected by PA showed separation of epithelial cells from their neighbours, and bacteria were sometimes seen invading the epithelium by this route. The separation of epithelial cells is consistent with a disruption of epithelial cell tight junctions and may precede the extrusion of cells from the epithelial surface. Current knowledge on the effects of bacterial infection on human epithelial tight junctions is lacking, although animal studies show that exposure to cigarette smoke (Simani et al., 1974; Boucher et al., 1980), nitrous oxide (Ranga et al., 1980; Gordon et al., 1983), PA elastase (Azghani et al., 1990), human neutrophils (Nash et al., 1987; Parkos et al., 1992), and reduced Ca^{2+} concentration in culture medium (Cereijido et al., 1978) disrupts epithelial tight junctions.

Chronic infection of the respiratory tract by PA is difficult to eradicate even with prolonged use of potent antibiotics. DNA fingerprinting techniques suggest that most CF patients harbour genetically related PA strains in their respiratory tract over long periods of time (Kubesch et al., 1988). This thesis has shown that during infection PA damages the respiratory mucosa and adheres to secretions, damaged epithelial cells and basement membrane collagen, and slows ciliary beat in vitro. PA adherence to mucus, and its lack of adherence to normal epithelium, may explain why it does not infect the normal airway which has efficient mucociliary defences. However in bronchiectasis and cystic fibrosis, in which mucus is poorly cleared, PA colonises static secretions and produces toxins which further disable remaining host defences. PA infection stimulates a florid chronic inflammatory response which is ineffective in clearing PA and damages the lung, encouraging persistence and spread of the infection in the airways (Piers 1985). In Chapter 10, the effects of pyocyanin, one of the PA exotoxins, on the ultrastructure and ciliary orientation of intact human respiratory mucosa is described.

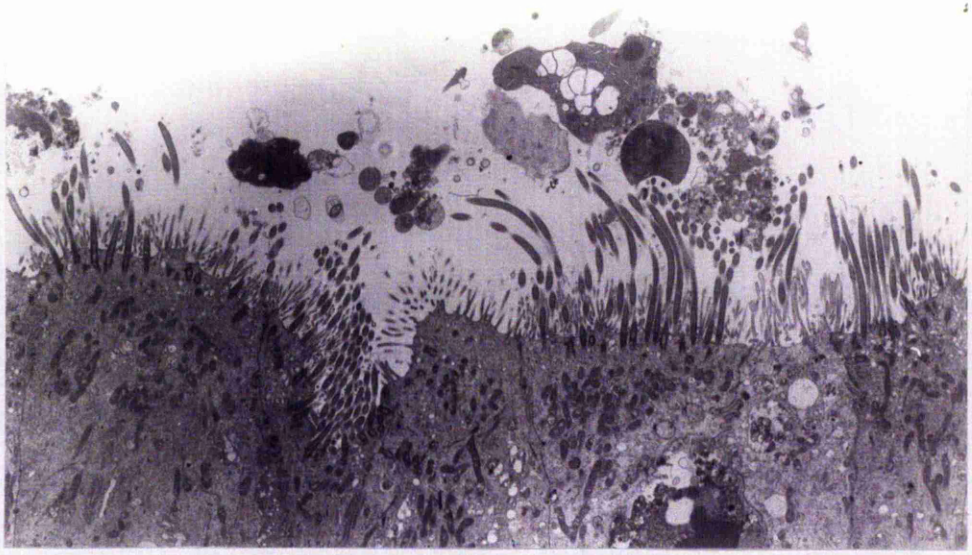

Figure 9.1 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface infected with PA (strain P455) for 8h showing loss of cilia, extrusion of cells from the epithelial surface, mitochondrial damage and cytoplasmic blebbing (3500x).

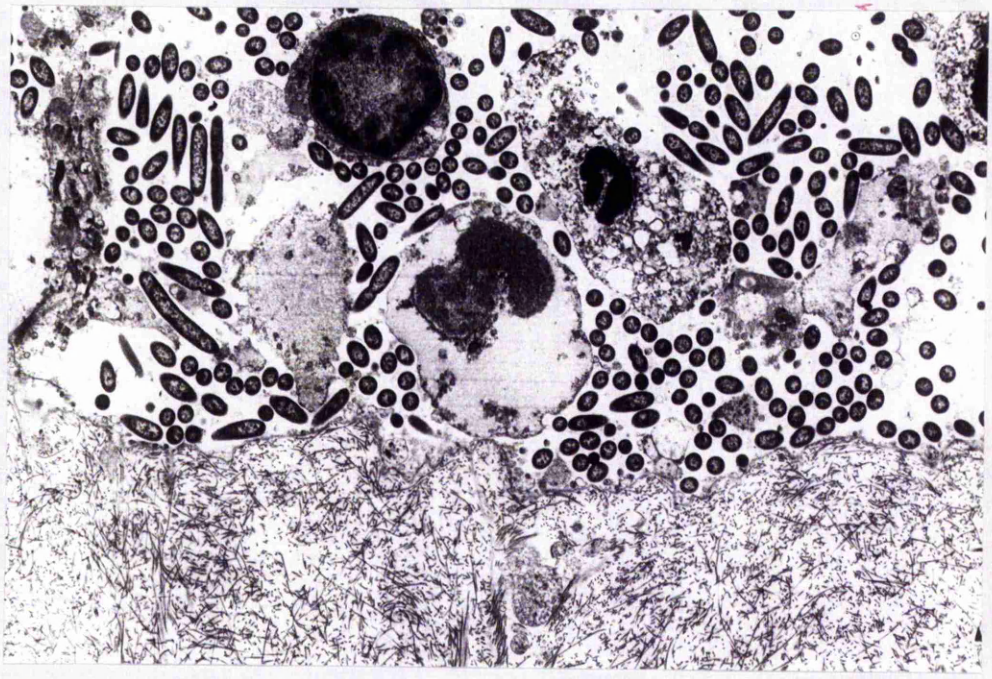

Figure 9.2 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface infected with PA (strain P455) for 8h showing infiltration of subepithelial structures and adherence of bacteria to basement membrane collagen (3500x).

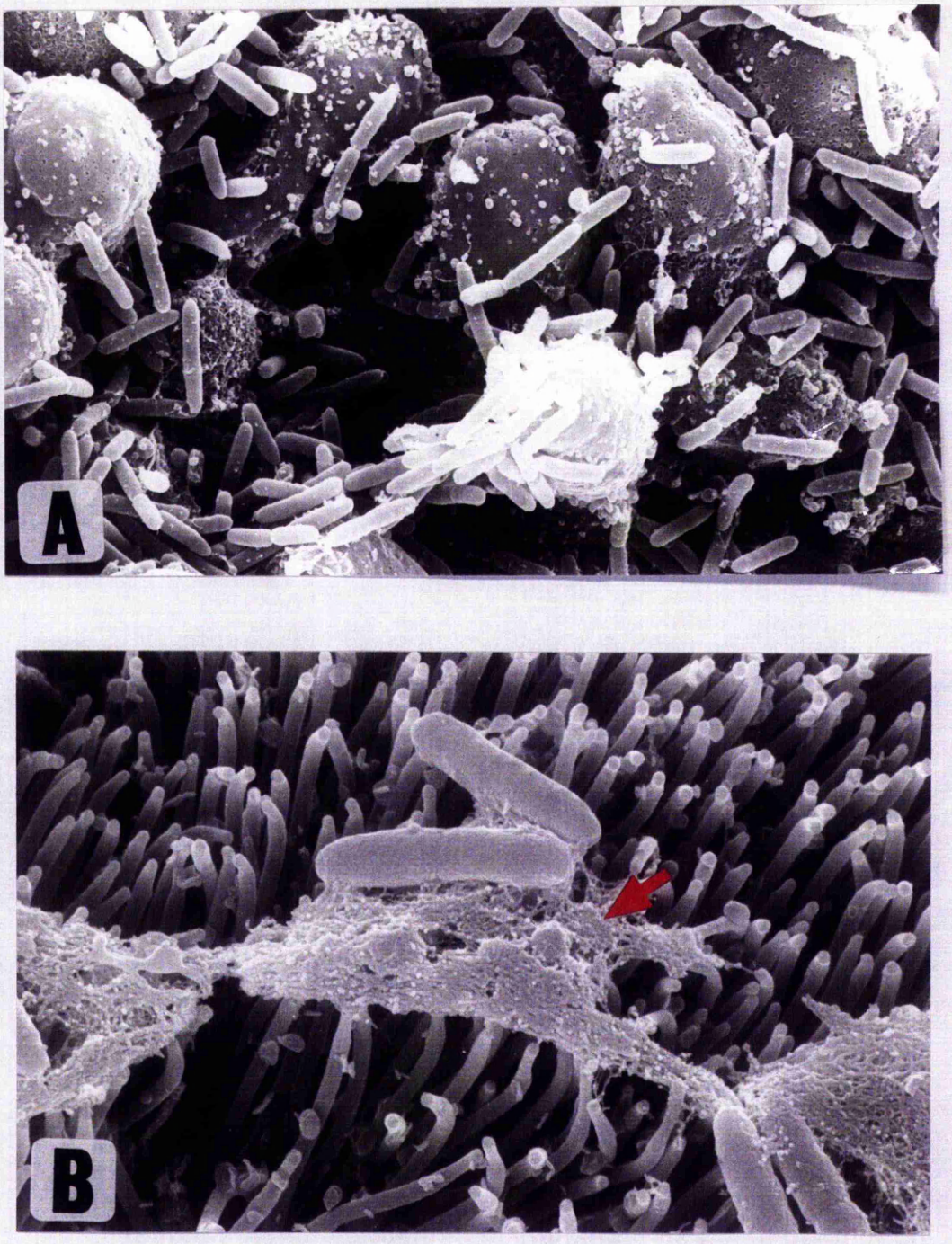

Figure 9.3 Scanning electron micrographs of an adenoid organ culture with an air-mucosal interface infected with PA (strain P455) for 4h showing bacteria adhering to (A) extruded and damaged epithelial cells and (5000x) (B) cilia with an amorphous matrix-like material (15000x).

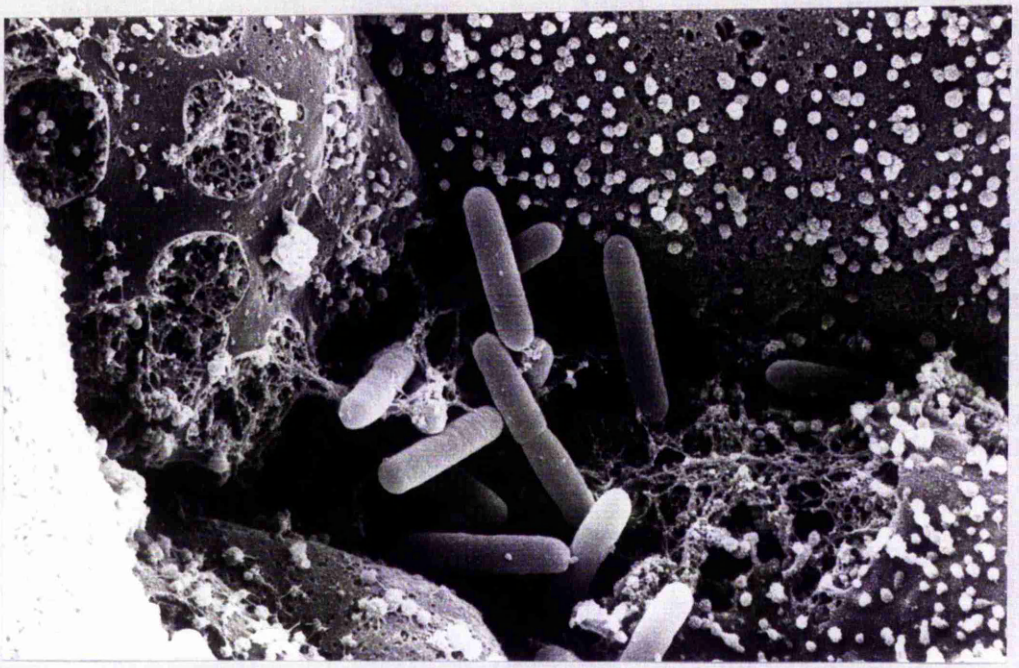

Figure 9.4 Scanning electron micrograph of an adenoid organ culture with an air-mucosal interface infected with PA (strain P455) for 4h showing bacteria at a gap formed between adjacent epithelial cells. There was an extracellular matrix-like material that bridged the space between bacteria and the mucosal surfaces (11250x).

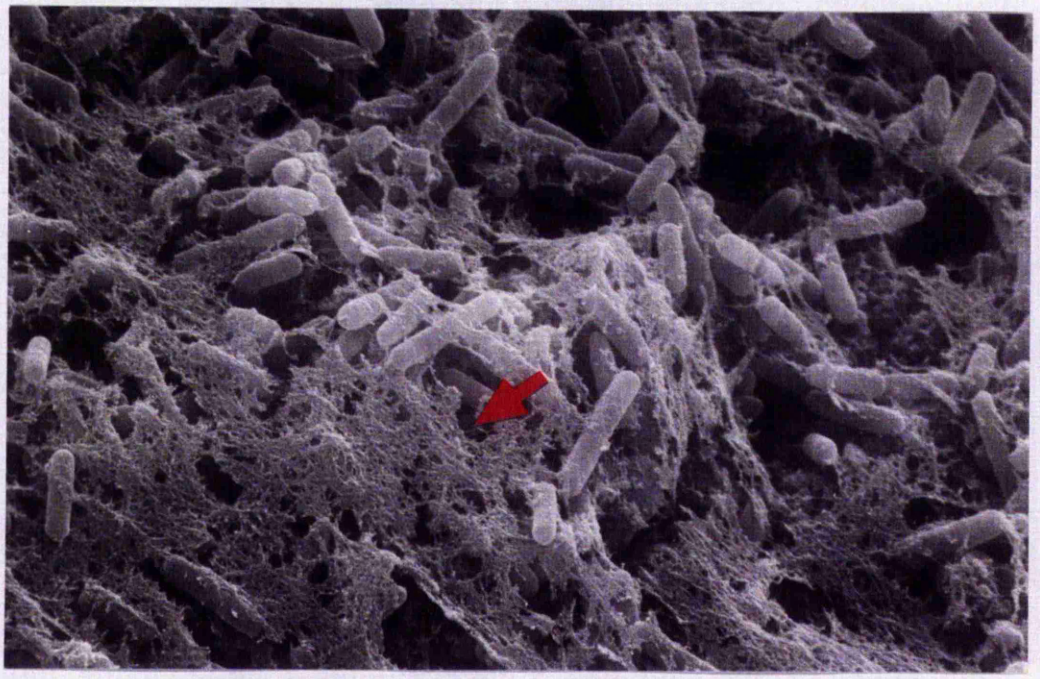

Figure 9.5 Scanning electron micrograph of an adenoid organ culture with an air-mucosal interface infected with PA (strain P455) for 8h showing a biofilm of bacteria with a lace-like extracellular matrix material that obscured most of the organ culture surface (7500x).

TABLE 9.1 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES AFTER 8H INCUBATION.

Organ culture	% of cells extruding from the epithelial surface	% of cells with loss of cilia	% of cells with mito-chondrial damage	% of cells with cytoplasmic blebbing	Combined mucosal damage score
Uninfected	13.1 ± 1.2	7.8 ± 1.1	1.8 ± 0.5	1.7 ± 0.4	9.5 ± 1.0
Infected with PA	43.9 ± 10*	17.7 ± 3.0*	32.9 ± 10.2*	11.6 ± 3.0*	32.9 ± 18*

All data is mean of six separate experiments ± standard error of mean. * $p<0.05$ versus uninfected organ culture. PA = *Pseudomonas aeruginosa* (strain P455).

TABLE 9.2 LIGHT MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES WITH AN AIR-MUCOSAL INTERFACE AFTER 8H INCUBATION.

Organ culture	Mean CBF		CBF at sites with ciliary activity		No. of sites with ciliary activity	
	0h	8h	0h	8h	0h	8h
Uninfected	11.3±0.2	11.1±0.5	11.3±0.2	11.1±0.5	10±0	10±0
Infected with PA	10.8±0.1	1.9±0.5	10.8±0.1	6.2±0.8	10±0	3±1

All data is mean of two separate experiments ± standard error of mean. PA = *Pseudomonas aeruginosa* (P455), CBF = ciliary beat frequency.

CHAPTER 10

THE EFFECTS OF *PSEUDOMONAS AERUGINOSA* PYOCYANIN ON HUMAN RESPIRATORY MUCOSA *IN VITRO*

10.1 AIM OF STUDY

The aim of this chapter was to investigate the effects of PA pyocyanin on intact human respiratory mucosa in adenoid organ cultures with an air-mucosal interface. In the study described in Chapter 9, PA infected adenoid organ cultures became blue after incubation indicating the production of pyocyanin *in vitro* which might have contributed to the pathogenicity of PA. TEM was therefore used to study the effects of a single application of pyocyanin on the ultrastructure and ciliary orientation of adenoid organ cultures.

10.2 STUDY DESIGN

Adenoid organ cultures with an air-mucosal interface were prepared as described in Section 4.2.2. Six pairs of organ cultures (each prepared from a different donor) were established and incubated at 37^0C for 24h in a humidified atmosphere that contained 5% CO_2. Ten μl of PBS or pyocyanin solution (20mg/l in PBS) i.e. 200ng were gently pipetted onto the surface of the organ cultures. After 24h incubation, each of the 4 edges of the organ cultures was touched with a plastic loop to check for sterility as described in Section 4.4.2. Processing of the organ cultures for TEM assessment then followed (Section 4.5.1). TEM assessment of the ultrastructure and orientation of ciliary microtubules and foot processes was then performed (Sections 4.5.2 and 4.5.3).

10.3 PARAMETERS MEASURED

10.3.1 ULTRASTRUCTURE

By using TEM, the mean combined mucosal damage score and the percent of cells displaying each of the following parameters were assessed:

1. Extrusion from the epithelial surface
2. Loss of cilia
3. Mitochondrial damage
4. Cytoplasmic blebbing

10.3.2 CILIARY ORIENTATION

TEM images of cilia and basal bodies were taken and analyzed as described in Section 4.5.3 and the following indices were calculated for each organ culture:

1. Index of ciliary orientation : mean SD of the angles formed by a line drawn through the central microtubules of each cilium with the vertical axis

2. Index of foot process orientation : mean SD of the angles formed by a line bisecting the foot processes of each cilium with the vertical axis

10.4 STATISTICAL METHOD

Wilcoxon signed ranked test was employed to analyze the data (Hollander et al., 1974). $P<0.05$ was taken as a statistical significant difference between any two groups of data.

10.5 RESULTS

All the organ cultures remained sterile after 24h incubation.

10.5.1 TEM ASSESSMENT OF ULTRASTRUCTURE (TABLE 10.1; FIGURE 10.1)

After 24h incubation, the organ cultures treated with PBS (n=6) had normal ultrastructure. Pyocyanin treated organ cultures (n=6) had a significant increase in the percent of cells displaying extrusion from the epithelial surface, mitochondrial damage, cytoplasmic blebbing and combined mucosal damage scores ($p<0.05$), and an insignificant increase in the percent of cells displaying loss of cilia ($p>0.05$) when compared with PBS treated organ cultures ($p<0.05$). The damage detected by TEM was patchy and some cells had relatively normal ultrastructure, which is reflected in the combined mucosal damage score of 74.7 compared to a maximum possible score of 400. Although treatment with pyocyanin caused blebbing of the cytoplasmic membrane, this disruption was not evident in the ciliary membrane.

10.5.2 TEM ASSESSMENT OF CILIARY ULTRASTRUCTURE AND ORIENTATION (TABLE 10.2; FIGURES 10.2 TO 10.4)

All the cilia examined had normal ultrastructure irrespective of treatment. A mean of (± standard error of mean) 261.9±13.9 cilia (15±0.44 cells) and 165.7±12.1 foot processes (19.3±1.5 cells) were examined for each organ culture. Pyocyanin treated organ cultures (n=6) had significantly higher indices of ciliary orientation than PBS treated organ cultures ($p<0.05$). However, there was no significant difference in the indices of foot process orientation between the pyocyanin and PBS treated organ cultures.

10.6 DISCUSSION

PA commonly infects the airways of patients with cystic fibrosis and bronchiectasis, and its exoproducts probably make a significant contribution to the process of airway colonisation (Pier 1985). Pyocyanin is a PA exotoxin which may have an important role in the pathogenesis of airway colonisation. Pyocyanin can exist in either oxidised or reduced form, the latter being an unstable free radical which reacts rapidly with molecular oxygen. This auto-oxidation leads to the formation of superoxide, hydrogen peroxide, or in the presence of metal catalysts such as iron, hydroxyl radical. A diversion of electron flow from biological pathways to pyocyanin may increase the production of intracellular reduction products and ultimately may lead to cell death (Hassan et al., 1980). It is of particular interest that PA, a strict aerobe, is itself insensitive to pyocyanin and seemingly escapes free-radical injury during production of or exposure to pyocyanin (Baron et al., 1981).

Pyocyanin has been shown to inhibit epidermal cell growth (Cruickshank et al., 1953) and lymphocyte proliferation (Nutman et al., 1987), have antibiotic effects against other microorganisms (Schoental 1941), influence the acquisition of iron by PA (Cox 1986), slow human ciliary beat and cause ciliary dyskinesia *in vitro* (Wilson et al., 1987), slow tracheal mucus transport in guinea pigs *in vivo* (Munro et al., 1989)

and enhance the oxidative metabolism of neutrophils (Ras et al., 1990). However, the effects of pyocyanin on the ultrastructure of intact human respiratory mucosa have not been investigated.

In this study, an organ culture model with an air-mucosal interface was used to investigate the effects of a single application of pyocyanin onto intact human respiratory mucosa. Because mucus is cleared from the surface of the organ culture by ciliary beating, this organ culture is more physiological than those in which tissue is bathed in medium containing pyocyanin, when the tissue is continually exposed to the toxin. Previous studies have shown that pyocyanin slows ciliary beat within 15min (Wilson et al., 1987; Kanthakumar et al., 1993). This thesis shows that one single application of pyocyanin to the mucosal surface causes tissue damage. While excessive pyocyanin solution would be removed by mucociliary action, some may remain in the epithelial lining fluid or within some epithelial cells to cause continued damage. As pyocyanin is a redox compound the availability of air (oxygen) to the site of contact between the target tissue (respiratory mucosa) and itself would be important.

In this study, light microscopy assessment was not performed on tissue as the effects of pyocyanin on causing ciliary beat slowing, ciliary dyskinesia and disruption of human nasal mucosa have been thoroughly studied (Wilson et al., 1987; Kanthakumar et al., 1993). SEM assessment was not indicated although it would be valuable in further studies of the effects of pyocyanin on bacterial adherence. Interestingly, exotoxins of PA have been reported to increase the adherence of *Pseudomonas cepacia* to bovine tracheal cells (Saiman et al., 1990). It is possible that *P. cepacia* also adhere more readily to damaged epithelium, and the enhanced adherence of *P. cepacia* to respiratory mucosa found by Saiman et al. might be explained by the tissue damaging effects of PA exotoxins such as pyocyanin.

Pyocyanin is found in the sputum of patients with cystic fibrosis and

bronchiectasis who are infected by PA at concentrations between 0.2 - 27.3 mg/l (Wilson et al., 1988a). These concentrations are sufficient to slow the ciliary beat of human nasal epithelial cells *in vitro* (Wilson et al., 1987). The concentration of pyocyanin (20mg/l) used in this study is therefore similar to that found in the sputum of patients. Although only a single application of 10μl of this solution was gently pipetted onto the mucosal surface, the exact concentrations of pyocyanin in the mucus, periciliary fluid and within the cytoplasm of the respiratory mucosa could not be determined, nor could the time of exposure of the tissue to the toxin. A single application of 300ng of pyocyanin, similar to the 200ng used in the current study, onto the tracheal mucosa of anaesthetized guinea pig slowed mucociliary transport by 38% after 120min, although subsequent TEM examination did not show any ultrastructural abnormality of cells at that time (Munro et al., 1989). In Chapter 9, PA infection of the same organ culture model produced a blue discolouration after 12 hours although a single application of pyocyanin did not change the macroscopic appearance of the organ cultures. Pyocyanin-induced slowing of ciliary beat has recently been shown to be reversible by washing the cells 2h after exposure to the toxin (Kanthakumar et al., 1993). Ciliary beat slowing was associated with a fall in intracellular ATP and cAMP, and agents which increase intracellular cAMP inhibit the effects of pyocyanin on ciliary beat (Kanthakumar et al., 1993). The ultrastructural changes present in some pyocyanin-treated cells in the present study suggest more permanent damage after 24h which may be mediated by substrate cycling and generation of hydroxyl radicals.

Whilst light microscopy can accurately measure CBF by various techniques, the speed of beating coupled with the lack of resolution of light microscopy make assessment of the direction of beating difficult (Rutland et al., 1990; Rutman et al., 1993). Previously, measurement of the orientation of the central microtubules, and the axis of the ciliary foot processes have been thought of as identical measurements of the orientation of ciliary beat (Rutland et al., 1990;

Rutman et al., 1993). It has also been suggested that disorientation of ciliary beat may be a primary abnormality, presumably under genetic control, and that these changes occur during ciliogenesis. However, this thesis shows that disorientation of the central microtubules can be caused by a bacterial toxin. When pyocyanin slows CBF *in vitro* it also causes ciliary dyskinesia, so that the normal coordinated beating of the cilia becomes disorganised and the cilia beat in different directions and in different planes (Wilson et al., 1987). The results in this thesis suggest that pyocyanin causes twisting of the ciliary axoneme, which is a more likely explanation of the disorientation of the central microtubules than an effect of pyocyanin on ciliogenesis over a 24h period. This is confirmed by the lack of disorientation of the basal feet in pyocyanin-treated organ cultures. The twisting of the axoneme might result from the pyocyanin-induced fall in intracellular ATP affecting the sliding of microtubules in the ciliary axoneme (Kanthakumar et al., 1993). Therefore it is essential to examine both the central microtubules and the basal feet when assessing ciliary disorientation.

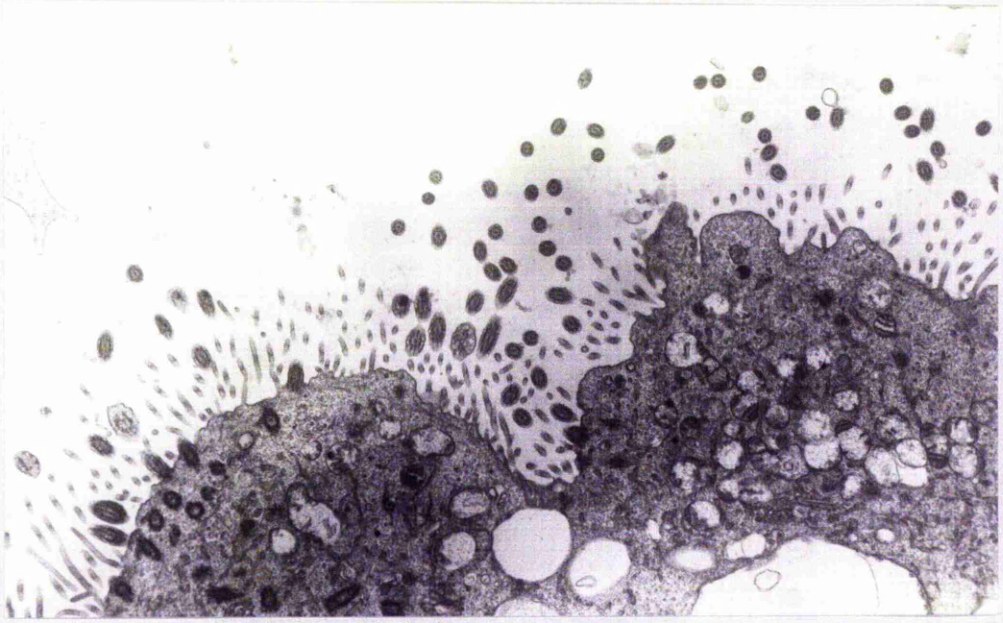

Figure 10.1 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface treated with pyocyanin (200ng) at 24h showing mitochondrial damage, cytoplasmic blebbing and extrusion of cells from the epithelial surface (8750x).

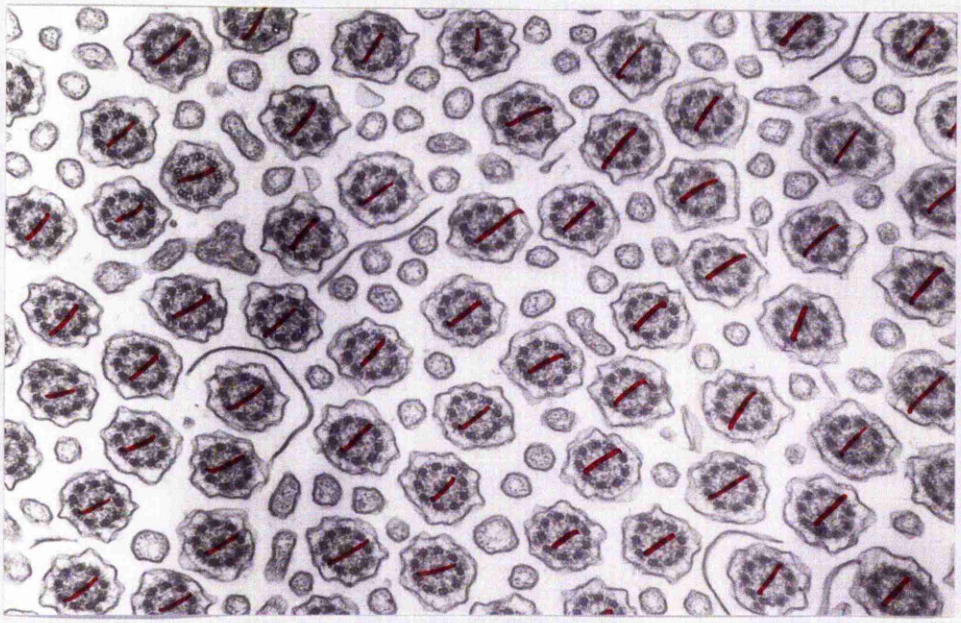

Figure 10.2 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface treated with phosphate buffered saline showing normal orientation of the ciliary central microtubules (with a line drawn through the central pairs of microtubules) after 24h incubation (35000x).

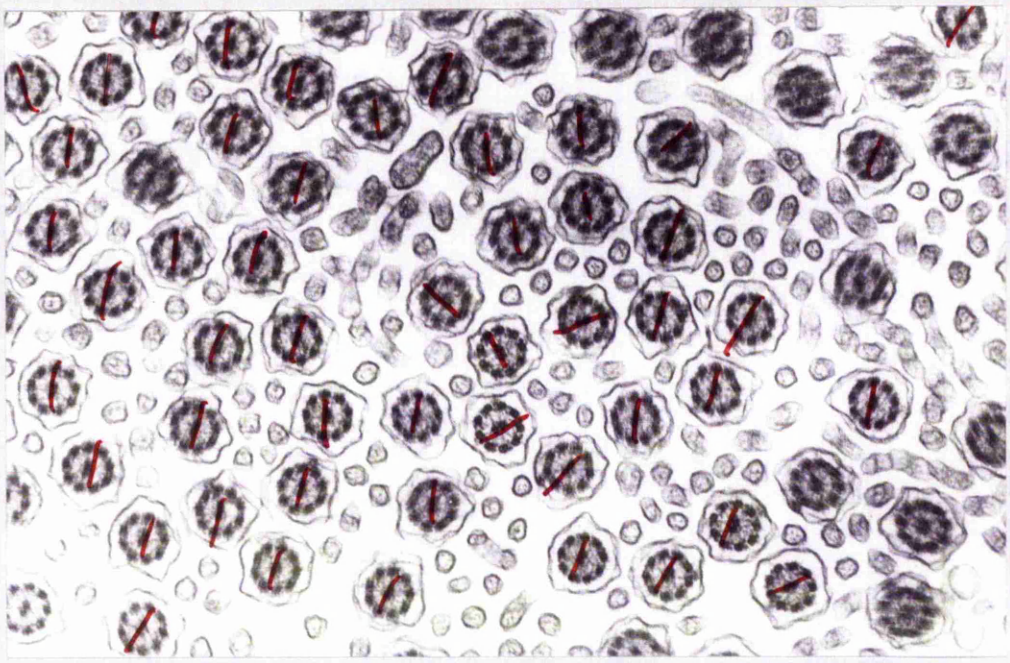

Figure 10.3 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface treated with pyocyanin (200ng) showing disorientation of the ciliary central microtubules (with a line drawn through the central pairs of microtubules) after 24h incubation (35000x).

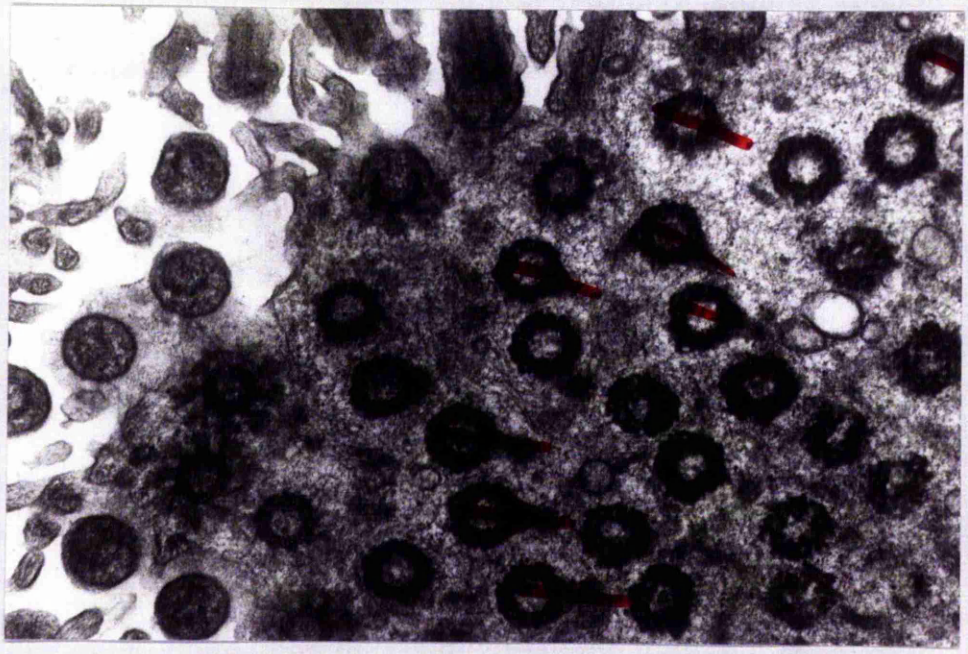

Figure 10.4 Transmission electron micrograph of an adenoid organ culture with an air-mucosal interface treated with pyocyanin (200ng) showing normal orientation of the basal bodies (with a line drawn through the spur-like foot processes) after 24h incubation (35000x).

TABLE 10.1 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF ADENOID ORGAN CULTURES AFTER 24H INCUBATION.

Organ culture	% of cells extruding from the epithelial surface	% of cells with loss of cilia	% of cells with mito-chondrial damage	% of cells with cytoplasmic blebbing	Combined mucosal damage score
Control	12.9 ± 3.1	7.2 ± 1.8	3.4 ± 0.4	1.3 ± 0.7	12.3 ± 1.7
Pyocyanin treated	40.1 ± 6.6*	19.1 ± 5.5	35.6 ± 9.9*	13.1 ± 2.8*	74.7 ± 18.3*

Data shown is mean ± standard error of mean of six separate experiments. * = $p<0.05$ when compared with control.

TABLE 10.2 TRANSMISSION ELECTRON MICROSCOPY ASSESSMENT OF CILIARY CENTRAL MICROTUBULES AND FOOT PROCESSES IN ADENOID ORGAN CULTURES AFTER 24H INCUBATION.

	Organ culture	
	PBS treated	pyocyanin treated
No of central pair of microtubules counted	256.7±18	267.2±22.7
Angle of orientation	5.3-17.1^0	6.9-59.5^0
Mean SD of angles	11.3^0	22.6^{0*}
No of foot processes counted	167.5±18.3	164.0±17.4
Angle of orientation	3.9-34.9^0	2.6-40.7^0
Mean SD of angles	13.5^0	14.2^0

Data are mean ± standard error of mean of six separate experiments. * = $p<0.05$ when compared with control.

CHAPTER 11

GENERAL DISCUSSION

11.1 CONCLUSIONS

1. NTHi infection of adenoid and bronchial organ cultures in an agar-embedded model produces a similar pattern of ultrastructural damage including loss of cilia, extrusion of cells from the epithelial surface, mitochondrial damage, and cytoplasmic blebbing.

2. In the presence of subMIC antibiotics (amoxycillin, loracarbef and ciprofloxacin), NTHi infection of adenoid organ cultures in an agar-embedded model is associated with less ultrastructural damage, ciliary beat slowing, and bacterial association with the mucosal surface when compared with organ cultures that do not contain antibiotics.

3. An organ culture model of intact respiratory mucosa with an air-mucosal interface, described in this thesis, can maintain normal ultrastructure and function of adenoid tissue for at least 24h.

4. Morphometric SEM and TEM methods described in this thesis show that NTHi infection of adenoid organ cultures with an air-mucosal interface only causes mitochondrial damage, and a small but significant increase in combined mucosal damage score. NTHi has a preferential adherence to mucus and damaged cells, and also frequently adhere to apparently healthy unciliated cells.

5. PA infection of adenoid organ cultures with an air-mucosal interface causes severe ultrastructural damage, bacterial infiltration of mucosa, and separation of epithelial tight junctions. PA also has a preferential adherence to mucus, damaged epithelial cells, and cilia but not to unciliated cells. PA also adhere to subepithelial collagen layer. An extracellular matrix-like material, probably produced by PA, may act as a bacterial adhesin.

6. A single application of PA pyocyanin to adenoid organ cultures with an air-mucosal interface causes ultrastructural and particularly mitochondrial damage, and disorientation of the central ciliary

microtubules but not the basal feet.

7. TEM studies of intact respiratory mucosa described in this thesis show that application of NTHi, PA and pyocyanin to adenoid and bronchial organ cultures causes patchy ultrastructural damage. SEM studies described in this thesis show that both NTHi and PA adhere to some apparently healthy epithelial cells but not to others. It is unclear from these studies whether the ultrastructurally damaged cells are also those with adherent bacteria but preliminary analysis does not suggest this is the case (Section 8.5.3.1). The underlying mechanism(s) for the patchy distribution of ultrastructural damage is unclear.

11.2 CRITICISMS

Before attempting to correlate the *in vitro* findings described in this thesis with human disease, it is important to note the potential shortcomings of the models and methods used.

1. In order to eradicate commensal organisms, the respiratory tissue (adenoid, nasal turbinate and bronchus) was immersed in minimal essential medium that contained gentamicin, benzylpenicillin and streptomycin. Although this is a widely accepted practice for the eradication of commensal organisms in adenoid and nasal turbinate tissue for *in vitro* studies (Stephens et al., 1984; Farley et al., 1986; Loeb et al., 1988; Read et al., 1991 & 1992; Feldman et al., 1992), and rinsing of tissue took place after 4-6h, it is possible that some antibiotics remained on the surface of or within the epithelial cells. Although the precise concentrations of these remaining antibiotics are unknown, the studies described in this thesis showed that bacteria multiplied well in the organ cultures. It would therefore appear that the MIC of NTHi and PA had not been exceeded by the residual antibiotics. As described in Chapter 5, low concentrations of these antibiotics might have affected bacterial interactions with respiratory mucosa in the studies described. It

would be interesting to determine the concentrations of these antibiotics in the "washed tissue". This may be achieved by homogenisation of tissue followed by assaying of the homogenate for these antibiotics using conventional analytical methods such as high performance liquid chromatography (HPLC).

2. In both of the organ culture models described in this thesis, it was assumed that the agar edge completely sealed the non-mucosal surfaces of the respiratory tissue. However agar is permeable and might have been penetrated by the bacteria and pyocyanin.

3. Only well ciliated tissue with two ciliated edges that had no adherent mucus was selected for experiments. This might have led to a selection of tissue with less than average amount of surface mucus. As the respiratory mucosa is normally covered by mucus *in vivo*, it might have been more appropriate to pre-incubate the established organ cultures for longer than one hour to allow surface mucus to re-accumulate prior to inoculation with bacteria or toxins.

4. In the studies described in this thesis, comparisons were made between uninfected and infected organ cultures that were assumed to be identical at the beginning of experiments. This might not be true, although every effort was made to ensure that this was the case. Each pair of organ cultures (i.e test and control) was obtained from immediately adjacent areas on the same respiratory tissue, and incubation, processing and assessment of specimens were performed simultaneously. The thickness of the remaining submucosal layer in the organ cultures with an air-mucosal interface might not be identical but this was unlikely to be greatly different or have any significance. In addition, six different donors were used for most of the series of experiments to ensure that the observations made in this thesis were consistent.

5. Although the studies described in this thesis on NTHi and PA

interactions with respiratory mucosa employed both TEM and SEM assessment, mucus and serous producing cells and other epithelial cell types were not studied. Bronchial and adenoid mucosa appear to have similar TEM patterns of damage when infected with NTHi. A similar pattern was also found with PA infection although it was more severe. This suggests that the TEM protocol described in this thesis is reliable and reproducible but it does not differentiate the damage patterns caused by different bacterial infections. On the other hand, the SEM assessment method for mucosal surface morphology and bacterial adherence described in this thesis clearly differentiates the adherence patterns of NTHi and PA.

6. NTHi is a normal upper respiratory tract commensal and PA do not infect the airways of normal individuals, it is unclear why these organisms cause ultrastructural damage to respiratory mucosa *in vitro*. Possible mechanisms would include: a lack of circulating neutrophils and antibodies; large bacterial inoculum; and ineffective mucociliary clearance that does not remove all the bacteria and mucus particularly from the edges of the organ cultures.

7. As NTHi are usually only isolated from the airways of patients who have bronchitis, and PA from that of patients with bronchiectasis or cystic fibrosis, the use of respiratory tissue from healthy donors may be inappropriate. The disease process may cause qualitative or quantitative changes in epithelial surface receptors which would affect bacterial adherence. A significant proportion of epithelial cells in patients with bronchial disease would be damaged prior to infection. As NTHi and PA adhere avidly to damaged respiratory epithelial cells it can be speculated that NTHi and PA adherence to diseased respiratory tissue may be different from that to healthy tissue. The observation that PA and NTHi were only infrequently detected on mucosal surface of adenoid organ cultures at the beginning of the experiments described in Chapters 8 and 9 may be partly explained by the low incidence of damaged epithelial cells at the time

of inoculation.

8. Only one strain of NTHi (strain SH9) and one of PA (strain P455) were studied in the work described in this thesis. This may limit the extrapolation of the conclusions to other strains of NTHi and PA. However, both of the test strains were originally clinical isolates (Section 3.5) and the labour intensive nature of the work made it only feasible to test one strain of each bacteria within the study period. Further work should be performed to evaluate whether or not other clinical isolates of NTHi and PA have the same patterns of interactions with human respiratory mucosa.

11.3 APPLICATION OF IN VITRO RESULTS TO HUMAN DISEASE

Not withstanding the difficulties of deriving *in vivo* relevance from *in vitro* results, it is a useful exercise to speculate how the conclusions of Section 11.1 may be applied to human disease.

1. As cited in Section 1.4.1, the role of NTHi in infective exacerbations of chronic bronchitis is still uncertain. However the studies described in this thesis have identified some mechanisms that would support a pathogenic role of NTHi in causing exacerbations of chronic bronchitis. These include loss of cilia, development of mitochondrial damage and cytoplasmic blebbing, extrusion of cells from the epithelial surface, and slowing of ciliary beat in NTHi infection of respiratory mucosa *in vitro*. These changes would certainly disrupt the mucociliary system *in vivo* and the high affinity of NTHi for respiratory mucus demonstrated in this thesis would lead to stagnation of respiratory mucus that contained numerous NTHi. The accumulation of NTHi and its exotoxins in the lower respiratory tract (Section 1.4.1.4) would lead to a vicious circle of events (Cole 1986) that may further damage the respiratory mucosa *in vivo*.

2. The mechanism(s) for the persistence of NTHi in the lower respiratory tract of patients with chronic bronchitis is poorly

understood. However the studies described in this thesis show that NTHi adhere well to apparently healthy unciliated respiratory epithelial cells and respiratory mucus in an organ culture model with an air-mucosal interface. NTHi is generally not isolated from the nose (Jousimies-Somer et al., 1989) and the preliminary experiments described in this thesis show that NTHi do not readily adhere to nasal turbinate tissue. NTHi is often isolated from the nasopharynx and the tracheobronchial tree of patients with chronic bronchitis and bronchiectasis (Section 1.4.1). Results described in this thesis suggest that NTHi may persist in the nasopharynx by adhering to unciliated cells, damaged cells and mucus. Studies described in this thesis have also identified a similar TEM damage pattern for adenoid and bronchial mucosa with NTHi infection. These observations suggest that NTHi may also be adherent to unciliated cells in the tracheobronchial tree of patients with chronic bronchitis *in vivo* which may be a mechanism for its persistence and pathogenesis in the lower respiratory tract.

3. In patients with cystic fibrosis and bronchiectasis, PA infection of the respiratory tract is often impossible to eradicate despite intensive and prolonged therapy with potent antibiotics. By using an organ culture model with an air-mucosal interface, PA infection has been shown to cause severe ultrastructural damage to respiratory mucosa including loss of cilia, extrusion of cells from the epithelial surface, mitochondrial damage, and cytoplasmic blebbing. In addition, PA infection slows ciliary beat and PA adhere avidly to damaged cells and mucus. This would lead to stagnation of mucus that contains numerous PA and its exotoxins in the lower respiratory tract. Other possible mechanisms of PA avoiding host defences described in this thesis include: the formation of bacterial biofilm; production of extracellular matrix that may act as a bacterial adhesin; production of pyocyanin that causes slowing of ciliary beat and microtubular disorientation; separation of epithelial tight junctions; and adherence to subepithelial basement membrane collagen layer.

4. The studies on subMIC antibiotics have highlighted the importance of host defence mechanisms in the respiratory tract and help explain the clinical efficacy of commonly prescribed antibiotics that penetrate into respiratory mucus poorly. A recent Japanese study has also shown that low dose erythromycin is effective in treatment of patients with chronic bronchial sepsis who are colonised by PA (Ichikawa et al., 1992). Tanaka et al. also demonstrated that erythromycin at concentrations well below the MIC of PA also reduced PA exotoxin production (Tanaka et al., 1994). Although low dose long term antibiotic therapy in these patients may be an useful therapeutic option, the studies described in this thesis show that NTHi infection of respiratory mucosa in the presence of sub-MIC antibiotics still cause significant tissue damage.

5. Ciliary disorientation, as measured by the central microtubules, has been shown in the study described in this thesis to be induced by treatment of respiratory mucosa with pyocyanin, a bacterial toxin. This would suggest that twisting of central microtubules can occur as a secondary phenomenon, although it has been suggested that ciliary disorientation may also occur as a primary phenomenon. The results presented in this thesis show that the diagnosis of primary ciliary disorientation may be very complicated, and must include assessment of both the central microtubules and basal feet.

11.4 FUTURE PROJECTS

From the results of the studies described in this thesis, the following future projects appear important for further evaluation of the interactions of NTHi and PA with human respiratory mucosa.

1. To evaluate how subMIC antibiotics protect the respiratory mucosa during NTHi infection in an agar-embedded organ culture model, the culture medium can be obtained after 24h incubation and filter-sterilised. Analytical tests such as HPLC can be performed to compare the biochemical contents of uninfected and infected organ cultures

(with and without antibiotics). The difference(s) in the biochemical contents (e.g. lipopolysaccharide), detected both quantitatively and qualitatively, may give valuable clues to the protective effects of subMIC antibiotics on the respiratory mucosa in NTHi infection. Moreover, NTHi can be collected from the infected organ cultures by centrifugation and examined by SEM. Measurements of bacterial dimensions can be made to determine the effects of subMIC antibiotics on NTHi morphology. *In vitro* studies on the effects of subMIC antibiotics on exotoxin(s) production can be performed by inoculating NTHi in a simple broths that contain subMIC antibiotics. Then sterile culture filtrates can be obtained and the effects of these on the CBF of respiratory mucosa can be evaluated. HPLC can also be used to assess the effects of subMIC antibiotics on NTHi production of exotoxin(s).

2. More experiments should be performed to evaluate NTHi infection of bronchial organ cultures in an agar-embedded model so that statistical analysis can be performed to compare the ultrastructural measurements. Although it may be difficult to dissect bronchial mucosa from its underlying cartilaginous layer using the current methodology, careful dissection using extra fine instruments and a dissecting microscope may allow separation of bronchial mucosa from its cartilaginous layer without causing damage. Dissected bronchial mucosa can then be used to establish organ cultures with an air-mucosal interface using current methodology. Further experiments as outlined in this thesis may be performed.

3. By using the organ culture model with an air-mucosal interface and the SEM morphometric method described in this thesis, potential NTHi adhesin(s) identified for human buccal and conjunctival epithelial cells and animal respiratory tissue can be evaluated using intact human respiratory mucosa. Isogenic strains of NTHi with or without the potential adhesins for NTHi can then be tested on this model. These include piliated and non-piliated NTHi, capsulated and non-capsulated

NTHi, and HMW-1 producing or HMW-2 producing or non-producing NTHi isogenic strains (St Gemes et al., 1993) (Section 1.4.1.3). By performing these projects, the role of these potential adhesins on intact human respiratory mucosa can be studied in a physiological organ culture model with an air-mucosal interface and bacterial adherence can be assessed by a direct SEM morphometric method as described in this thesis. Purified NTHi pili and monoclonal antibody raised against strain-specific pili can be used to determine whether they also inhibit NTHi adherence to respiratory mucosa which would confirm the role of pili as an adhesin. Confirmation of the role of these adhesin(s) could be followed by animal studies to evaluate the effects of either adhesin analogues or monoclonal antibodies raised against these adhesins on bacterial adherence *in vivo*. These projects may lead to improved understanding of bacterial interactions with respiratory mucosa and possibly development of novel therapies in the future. Similar future projects can be performed with PA using the known adhesin(s) identified by previous studies such as pili, exoenzyme S, and mucopolysaccharide (Section 1.4.2.3).

4. The effects of PA infection on disruption of epithelial tight junctions should also be studied further by using freeze-fracture TEM methods (Gumbiner 1987). PA adherence to purified human basement membrane collagen should also be studied *in vitro* as this may also help explain the persistence of PA in the lower respiratory tract.

5. Patchy ultrastructural damage was found at TEM assessment of NTHi, PA and pyocyanin treatment of adenoid organ cultures. NTHi and PA also show preferential adherence to different epithelial cell types. The underlying mechanism(s) for some cells being more susceptible to damage and bacterial adherence than others is unclear. This may not be a random phenomenon but a specific process against some epithelial cells that differ from others in surface receptors, age, distribution or metabolic activity. Further projects to study the biology and to classify respiratory epithelial cell types may help explain this

phenomenon and further the understanding of pathogenesis of respiratory infections that may lead to the development of novel therapies.

CHAPTER 12

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CHAPTER 13

PUBLICATIONS ARISEN FROM THIS THESIS

13.1 ABSTRACTS

1. **Tsang K**, Rutman A, Lund V, Roberts D, Cole P, Wilson R. Interaction of non-typable *Haemophilus influenzae* with human respiratory epithelium in the presence of subminimal inhibitory concentrations of amoxycillin, loracarbef and ciprofloxacin. (**Am Rev Respir Dis** 1992;145(4):A804)

2. **Tsang K**, Rutman A, Belcher J, Roberts D, Cole PJ, Wilson R. The protective effects of subminimal inhibitory concentrations of antibiotics on non-typable *Haemophilus influenzae* infection of human respiratory epithelium *in vitro*. (**Thorax** 1992;48:859)

3. **Tsang KWT**, Rutman A, Kanthakumar K, Cole PJ, Wilson R. The interaction of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*. (**Thorax** 1993;48:426)

4. **Tsang KWT**, Rutman A, Kanthakumar K, Cole PJ, Wilson R. Interaction of *Haemophilus influenzae* with human respiratory mucosa in an organ culture model with an air-mucosal interface. (**Thorax** 1993;48:443)

5. **Tsang KWT**, Rutman A, Kanthakumar K, Taylor G, Cole PJ, Wilson R. The effects of pyocyanin administered onto the surface of intact respiratory mucosa in an organ culture with an air-mucosal interface. (**Thorax** 1993;48:426)

6. **Tsang KWT**, Rutman A, Dewar A, Cole PJ, Wilson R. *Pseudomonas aeruginosa* infection of human respiratory mucosa. (**Am Rev Respir Dis** 1994;149:A121)

7. **Tsang KWT**, Rutman A, Dewar A, Taylor G, Cole PJ, Wilson R. The effects of pyocyanin on the ultrastructure and ciliary orientation of human respiratory mucosa in organ culture. (**Am Rev Respir Dis** 1994;149:A122)

8. **Tsang KWT**, Rutman A, Kanthakumar K, Belcher J, Roberts D, Lund V, Cole PJ, Wilson R.
Haemophilus influenzae infection of human respiratory mucosa in low concentrations of antibiotics. (**Clinical Digest** 1995 -- in press)

9. **Tsang KWT**, Rutman A, Cole PJ, Wilson R.
Haemophilus influenzae infection of human adenoid and nasal turbinate mucosa. (**Eur Respir J** 1995 -- in press)

13.2 PAPERS PUBLISHED

1. **Tsang KWT**, Rutman A, Kanthakumar K, Belcher J, Roberts D, Lund V, Cole PJ, Wilson R.
Haemophilus influenzae infection of human respiratory mucosa in low concentrations of antibiotics. (**Am Rev Respir Dis** 1993;148:201-297)

2. **Tsang KWT**, Rutman A, Tanaka E, Lund V, Cole PJ, Wilson R.
Interactions of *Pseudomonas aeruginosa* with human respiratory mucosa *in vitro*. (**Eur Respir J** 1994;7:1746-53).

13.3 PAPERS IN PREPARATION FOR SUBMISSION

1. **Tsang KWT**, Rutman A, Kanthakumar K, Taylor G, Cole PJ, Wilson R.
The effects of pyocyanin on intact human respiratory mucosa.

2. **Tsang KWT**, Rutman A, Lund V, Cole PJ, Wilson R.
Interaction of *Haemophilus influenzae* with human respiratory mucosa in an organ culture model with an air-mucosal interface.

3. **Tsang KWT**, Rutman A, Cole PJ, Wilson R.
Interaction of *Haemophilus influenzae* with intact human bronchial mucosa *in vitro*.