THE INTERACTION OF BACTERIA

WITH THE RESPIRATORY MUCOSA

IN VITRO AND IN VIVO

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by

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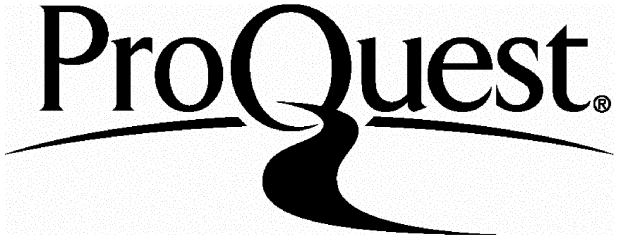

Abstract

Using a simple nasopharyngeal organ culture in which the mucosa is exposed to air, this thesis describes the interaction between two piliated and one non-piliated variants of Neisseria meningitidis and the interaction of a pneumolysin sufficient and deficient isogenic variant of Streptococcus pneumoniae with respiratory mucosa.

Piliated N.meningitidis adhered more often than the non-piliated variant to the respiratory mucosa and demonstrated tropism for non-ciliated epithelial cells and only rarely adhered to mucus. In contrast, S.pneumoniae demonstrated tropism for mucus. Infection resulted in a change in the appearance of mucus, ciliary beat slowing and epithelial damage.

To assess if other bacteria may impair mucociliary clearance by disorganising cilia the effect of pyocyanin, 1-hydroxyphenazine (1-HP) and rhamnolipid on the orientation of human ciliated cells was studied. Pyocyanin and 1-HP at pathophysiological concentrations caused ciliary slowing, dyskinesia and disorientation of the ciliary microtubular pairs. However, the orientation of basal feet did not change. Rhamnolipid at pathophysiologic concentrations caused ciliary slowing but neither dyskinesia or disorientation. Disorientation of ciliary beat as well as slowed CBF may contribute to the slowing of

mucociliary clearance in vivo.

To assess if ciliary disorientation occurs as an acquired and/or congenital abnormality, groups of patients with chronic upper respiratory tract inflammation due to infection and patients with the clinical features of primary ciliary dyskinesia but normal ciliary beat frequency and ciliary ultrastructure were studied. Ciliary disorientation was associated with slowing of nasomucociliary clearance.

The clinical features, ciliary function studies and the ciliary orientation of eleven patients with the classical features of primary ciliary dyskinesia but with normal ciliary ultrastructure were assessed. The results suggests that ciliary disorientation alone does represent a new variant of primary ciliary dyskinesia.

This thesis is dedicated to my parents, David and Brenda

"A theory has only one alternative of being right
or wrong. A model has a third possibility it
may be right but irrelevant"
(Manfred Eigen 1927).

"Faith is a fine invention for gentleman who see,
but microscopes are prudent in an emergency"
(Emily Dickenson 1830-1886).

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Abbreviations

1-HP	1-Hydroxyphenazine
α-PI	Alpha-1 protease inhibitor
CBF	Ciliary beat frequency
CFU	Colony forming units
CPS	Capsular polysaccharide
H	Hours
HZ	Hertz
H.INFLUENZAE	Haemophilus influenzae
IgA	Immunoglobulin A
IL I	Interleukin I
IL 8	Interleukin 8
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MEM	Minimal essential medium
MIN	Minutes
NMCC	Nasal mucociliary clearance
N.MENINGITIDIS	Neisseria meningitidis
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCD	Primary ciliary dyskinesia
SEM	Scanning electron microscopy
S.PNEUMONIAE	Streptococcus pneumoniae
TNF	Tumour necrosis factor
TEM	Transmission electron microscopy

1.0 General Introduction

Human evolution necessitated the development of an internal lung for gas exchange which developed from an adaptation of the primitive foregut. This adaptation has created inherent weaknesses in the defence against infection; for example the respiratory tract shares with the digestive tract the mouth and the pharynx, through which pass both food and air. Approximately 50% of normal subjects and 70% of subjects with impaired consciousness aspirate during sleep (Huxley et al 1978). Oropharyngeal secretions of the normal individual contain > 10^7 organisms per ml (Woods 1988),and aspirated bacteria are cleared much less efficiently than inhaled bacteria (Ansfield et al 1977). The large surface area (70-80 metres^2) required for gas exchange is provided by large numbers of alveoli supplied by repeated branching of the bronchial tree. Through these blind ending airways pass 10-20 x 10^3 litres of inhaled air over 24 h constantly exposing the epithelium to noxious substances and potentially infective agents from the environment (Laurenzi 1961; Lees 1959). To prevent the lungs from continually being overrun by microbes the respiratory tract possesses a complex array of defence mechanisms, the normal function of which ensures that the lung is sterile from the first bronchial division to the alveoli. Local defences include mucociliary clearance, the epithelial barrier, locally produced immunoglobulin and alveolar macrophages; systemic defences include

neutrophils, complement and immunoglobulin. Three independent processes will determine net bacterial clearance from the respiratory tract: physical transport, phagocytosis, and bacterial multiplication (Jay 1976). Respiratory tract infection may therefore result because of malfunction of the defence mechanisms, which may be inherited or acquired, or as a result of the virulence of inhaled microorganisms.

1.1 Host Defence Mechanisms of the Airways

1.1.1 The nose

The nose is the primary airway leading to the lung and inhaled air is humidified, warmed and filtered as it passes through the nose. The vestibule of the nose is lined by skin which contains sebaceous glands and coarse hairs which remove large particles. The air stream is narrow and changes direction as it passes through the folds of turbinate tissue and as a result particles are deposited onto the mucus layer lining the nasal passage. The lining mucosa is ciliated, highly vascular and rich in mucus glands and goblet cells (Proctor 1977).

1.1.2 The cough reflex

Coughing is an important protective reflex of the respiratory tract. It is probably mechanical stimulation of sensory nerves in the walls of the larynx, trachea and large intrathoracic airways by mucus or inhaled particles which triggers the cough reflex. This mechanism will also

protect the airways against foreign body inhalation. Coughing may also be induced by the production of tussive mediators released from epithelial cells as a result of an environmental change which may trigger the sensory fibres of the cough reflex (Korpas and Tomori 1979).

1.1.3 Airway epithelium

The airway epithelium forms a continuous, but heterogenous lining of the airways. The varied composition of the epithelium provides a physical and physiological barrier which performs a number of functions of host defence. A number of distinct cell types make up the pseudostratified columnar epithelium lining the conducting airways: basal, ciliated, mucous, serous, Clara, dense core granulated (DCG), special type, and brush cells (Jeffery and Corrin 1984). There is increasing evidence that the epithelium also acts as a physico-chemical barrier which is able to generate and express inflammatory eicosanoids (cell activators and chemo-attractants), pro-inflammatory cytokines, specific cell adhesion molecules and MHC class II antigens (Devalia and Davis 1993).

1.1.3.1 Tight junctions

An important part of the epithelial barrier is the intercellular tight junction which forms a seal near the apex of pulmonary epithelial cells (Schneeberger, 1984). Two functions have been attributed to the tight junction. The first, a barrier or gate function, is the result of the

tight junctions continuous belt-like structure near the apex of the epithelial cell where it forms a barrier separating the luminal from the abluminal compartments. The second function involves the maintenance of the polarised distribution of proteins and lipids in the apical and basolateral domains of the plasma membrane (Rodriguez-Goulan and Nelson, 1989). Tight junctions are highly regulated. An elevation of intracellular cyclic AMP or intracellular free calcium results in an increased transepithelial resistance which is accompanied by changes in tight junction structure (Duffey et al, 1981; Palant et al, 1983). The precise mechanism of this regulation is unclear but the cytoskeleton appears to participate in the changes. In vitro studies have shown that during acute inflammation a large number of leucocytes are able to traverse the alveolar capillary barrier along a chemotactic gradient with tight junctions being forced open by the migrating neutrophil. This causes a decrease in transepithelial and electrical resistance and an increase in transepithelial permeability (Nash et al 1987). The effect is transient and the tight junctions rapidly reseal.

1.1.3.2 Secretory cells

Epithelial cells produce a number of secretions that form a biphasic fluid composed of an aqueous sol phase containing proteins, lipids and ions, and a gel phase containing mucus. The airway secretions originate from mucous and serous cells present in both surface epithelium

and submucosal glands and Clara cells. Surface mucus secreting cells are mainly of the goblet cell form and are present throughout the bronchial airways but are uncommon in the bronchioles. Submucosal glands are numerous and are found wherever there is cartilage within the airway wall (Jeffery and Corrin 1984) and produce mixed acidic and neutral glycoprotein secretions. Clara cells are mainly found in bronchioles - these are secretory cells, but the exact nature of the secretions has not been defined.

1.1.4 Mucus

Airway mucus is a mixture of water, salts, protein and high molecular weight glycoconjugates. Mucus glycoproteins, or mucins, represent the main component of respiratory mucus. Mucin consists of a population of high molecular weight glycoproteins with different peptide cores (apomucins) to which are attached hundreds of carbohydrate side chains, each containing from 1 to 20 sugars (Lamblin et al 1991 ; Sheehan et al 1991). They are secreted by the goblet cells of the epithelium and the mucus glands of the respiratory mucosa. Mucus is formed within golgi derived vesicles of concentrated glycoprotein and is released by exocytosis as droplets 1-2 μm in diameter. It is rapidly swelled by absorption of water from serous fluid, increasing in volume by a factor of several hundred over a period of about 3 seconds to reach an equilibrium water content (Verdugo 1984).

Mucus has a number of important functions including trapping of inhaled particles, reduction of surface tension in bronchi and bronchioles, humidification of inhaled air, lubrication of mucosal surfaces and dilution of inhaled toxic substances including soluble gases such as ozone (Moorman et al 1973), ammonia (Landahl and Herrmann 1950) and sulphur dioxide (Speizer & Frank 1966). Mucus also has direct antibacterial activity, it contains alveolar macrophages, secretory IgA, transferrin and lactoferrin and lysosome.

1.1.5 Cilia

All surfaces of the upper airways are covered by ciliated epithelium except the nasal entrance, those parts of the nasopharynx and larynx covered by squamous epithelium and the olfactory area which has a specialised sensory epithelium. The tracheobronchial tree is ciliated to the level of respiratory bronchioles. Each ciliated cell has a diameter of 5 micrometres and carries some 200 cilia interspersed by microvilli (Jafek 1983; Rhodin 1966). There is a progressive decrease in the percentage of ciliated cells and the ciliary beat frequency (CBF) from the trachea to the segmental bronchi, and a positive correlation between this and the decrease in mucus velocity down through tracheobronchial tree, without this decrease in velocity the larger airways would become flooded with mucus (Morrow et al 1967; Rutland et al 1981).

1.1.5.1 Axoneme

Structurally cilia are thin longitudinal extensions from the free surface of the cell encased by the cell membrane. Each cilium is approximately 6 micrometers long reducing to 5 micrometers in the terminal bronchioles. The core of the cilia, otherwise known as the axoneme consists of nine peripheral doublets and two single central microtubules, commonly known as the nine plus two pattern (Fawcett and Porter 1954) (Figure 1). The outer doublets are composed of an "a" and "b" microtubule. The "a" microtubule has paired inner and outer dynein arms. These are high molecular weight proteins with ATPase activity and they project towards the "b" microtubule of the adjacent doublet (Gibbons 1965).

The structural proteins include nexin, the radial spoke and the central sheath. Nexin links permanently bridge the gap between the alpha and beta microtubules of adjacent peripheral doublets. Radial spoke links project inwards from "a" microtubules towards the central sheath and terminate in a bulbous enlargement known as the spoke head. The term central sheath is a misnomer because it is not a sheath but two arc-like lateral projections from each of the central microtubules forming two half circles. A line transecting the central microtubules is known as the ciliary axis. A line perpendicular to the axis of the central microtubule transects the "a" microtubule of a peripheral doublet which is conventionally labelled number

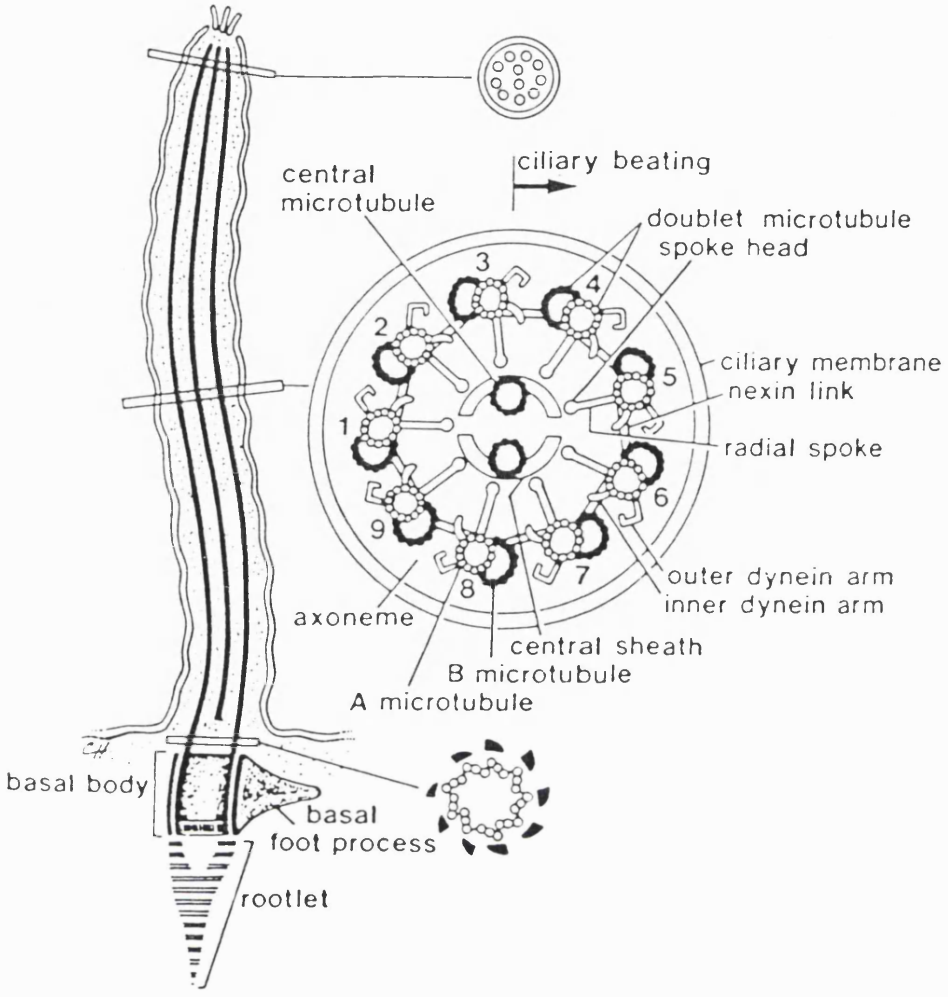


Figure 1

Schematic longitudinal and transverse sections (apex, mid portion and base) of a cilium (Edwards et al 1992)

1. Adjacent doublets are numbered 2-9 in a clockwise direction in sections viewed from the base towards the tip (Sleigh 1977).

1.1.5.2 Basal body

The intracytoplasmic extension of each cilium is a basal body. The basal body is derived from the centriole and has 9 peripheral microtubular triplets which give rise to the 9 peripheral doublets of the axoneme (Figure 1). The ciliary basal body has a basal foot and short striated rootlets and is attached to the cytoplasmic microtubules which together provide anchorage (Sleigh and Silvester 1983).

1.1.5.3 Crown

The apical structure of each cilia is composed of 3-7 bristles or claws (Floiguet and Puchelle 1986). It is thought that the claw-like process may facilitate transport of the mucus blanket.

1.1.5.4 Orientation

The basal feet tend to be at the side of the cilium towards which the effective stroke occurs (Gibbons 1961), and since all of the basal feet on a single cell are normally aligned in approximately the same direction, the effective stroke of all cilia on a cell should have a common orientation (Holley and Afzelius 1986). A line perpendicular to the ciliary axis also points in the direction of ciliary beat

(Figure 2). Therefore orientation can either be measured by studying the central microtubules or the basal feet (Rautiainen et al 1986). Quail oviduct studies suggest that orientation is determined prior to ciliogenesis (Boisvieux-Ulrich et al 1985). The process depends on the correct development of the apical cytoskeleton and is related to the commencement of the ciliary beat cycle. The apical cytoskeleton consists of a network of microtubules and microfilaments and anchors the basal body and basal foot process. Disruption of the cytoskeleton or the commencement of the ciliary beat cycle may prevent normal orientation (Boisvieux-Ulrich et al 1985; Boisvieux-Ulrich and Sandoz 1991).

1.1.5.5 Mechanism of ciliary beating

Ciliary movement is produced by sliding of adjacent peripheral microtubule doublets. This is achieved by an ATP mediated retraction of the outer dynein arms, followed by ATP hydrolysis, extension and reattachment to the adjacent microtubule (Gibbons 1965; Satir 1965). This produces sliding of adjacent peripheral doublets which is converted into a bending action by the structural proteins.

1.1.6 Mucociliary transport

Mucociliary transport is a primary defence mechanism of the respiratory tract (Sade et al 1970; Anonymous 1982) protecting the respiratory mucosa against inhaled particles by transporting them trapped in mucus towards the pharynx

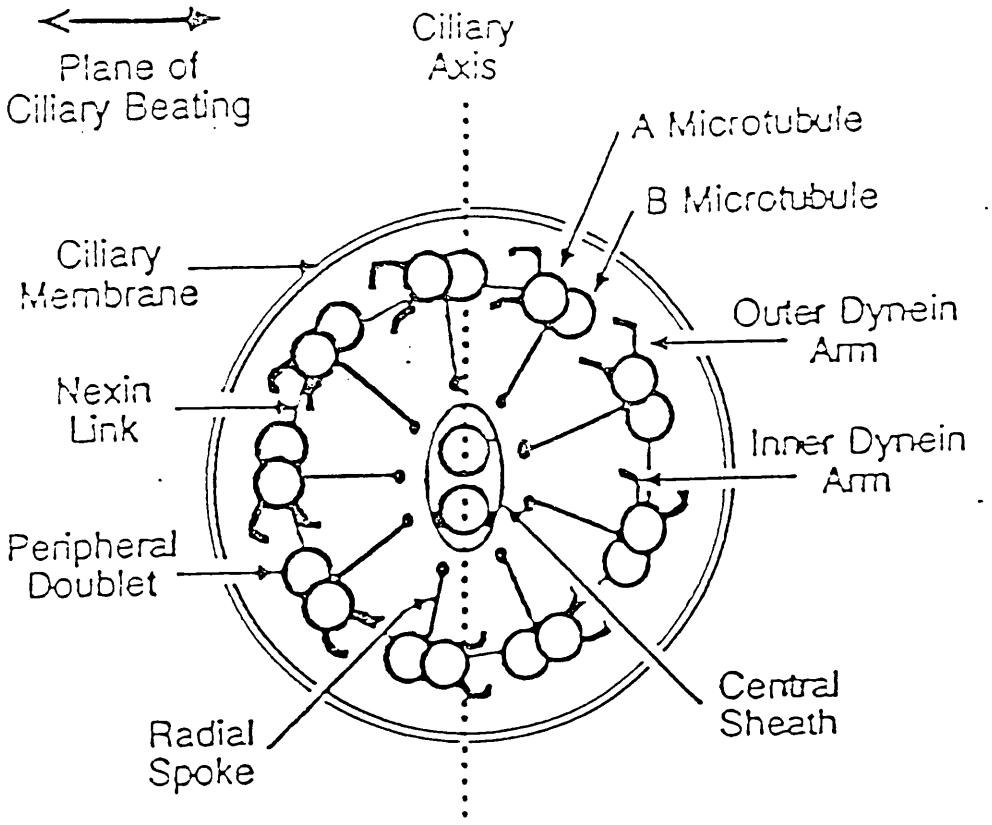


Figure 2

Schematic cross section of the mid portion of a cilium demonstrating the ciliary axis and plane of ciliary beating.

where they are swallowed or expectorated (Sleigh et al 1988) (Figure 3).

Transport depends on the characteristics and interrelations of the cilia, periciliary fluid and mucus. Cilia beat in a coordinated manner in the periciliary fluid layer beneath overlying mucus. During the arc-like effective stroke cilia contact, then propel mucus forward, they then drop below the overlying mucus in a side arm fashion for the recovery phase thus producing unidirectional mucociliary flow (Sleigh et al 1988). Coordinated patterns of ciliary activity or metachronal waves propagate across the epithelium recruiting large numbers of cilia into the process of mucus transport. Hydrated mucus droplets are exposed to the action of cilia on the epithelial surface and the droplets appear to be drawn out into short strands, which coalesce with other droplets or strands which join to form larger plaques (Iravani and van As 1972). Mucus is transported at about 5 mm per minute in the trachea, 2.5 mm per minute in the main bronchi and more slowly distally in the bronchial tree. About 10 ml of mucus per day is transported from the respiratory tract in health but this may increase to 200-300 mls in illness (Clarke 1990).

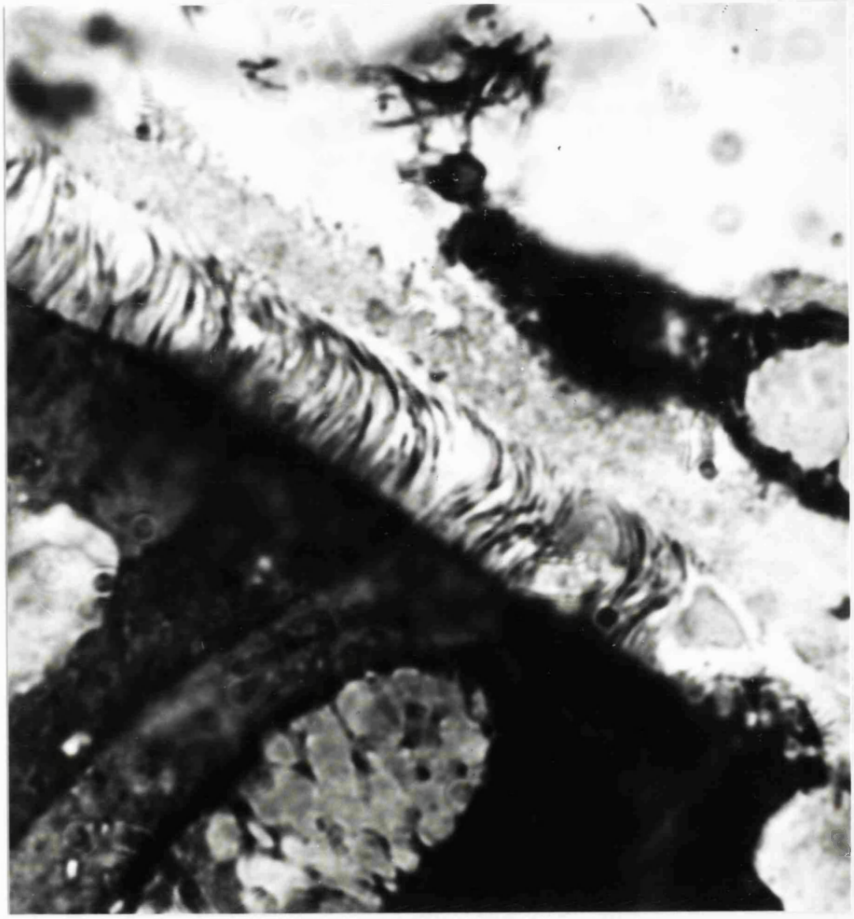


Figure 3

Light microscopy cross section of the respiratory mucosa demonstrating mucus, cilia, periciliary fluid and epithelial cells.

1.1.7 Cellular and humoral factors

When infectious agents elude the physical and mechanical defences or reach areas where these defences are not present in respiratory bronchioles and alveoli, clearance is dependent on cellular and humoral factors.

1.1.7.1 Neutrophils

Neutrophils are essential for effective host defences. The rapid localization to sites of local insult, and the ability to penetrate vessel walls and migrate into the tissues, represent key elements of neutrophil function. Neutrophils constitute approximately half the circulating white cells and are found in the bronchoalveolar lavage of normal non-smoking subjects (Reynolds 1989). The life span of the neutrophil from stem cells to its removal in tissue is approximately 12-14 days. Once neutrophils have migrated into the lung tissue they do not return into the circulation. Phagocytosis of organisms by neutrophils comprised of two steps: recognition and internalisation. Killing, neutralisation and subsequent digestion of the material generally follows (Baehner 1975). For engulfment to occur the particle must first be recognised by the neutrophils. In some cases the neutrophil may bind directly to lipopolysaccharide (LPS) on the surface of the organism. However for the most part, the particle must be opsonised by binding proteins from plasma including for example immunoglobulin and complement (Absolom 1986). Engulfment occurs through invagination of the plasma

membrane (Stossel 1988). The intracellular killing of microorganisms generally involves initiation of the respiratory burst with release of toxic oxygen metabolites, both within the phagosome and also outside of the cell (Faton and Ward 1982; Henson and Johnston 1987). Secretion from neutrophils involves a process of exocytosis, often called degranulation, which results in the release of both granules containing proteinases and other materials, including lipid mediators of inflammation and oxygen metabolites.

Neutrophil accumulation within the respiratory tract of patients with chronic bacterial colonisation has been demonstrated by indium-111 white cell isotope labelling. In severe disease, 50% of circulating white cells may cross into the respiratory tract (Currie et al 1987).

a) Neutrophil proteinases

Neutrophil proteinases are cytotoxic enzymes stored and released from neutrophils. The production of proteinases facilitates clearance of bacteria from the respiratory tract and may stimulate mucus secretion (Nadel 1991). However, inadequate inhibition of proteinase activity may lead to the generation of an excessive inflammatory response (Tetley 1993). Neutrophil elastase is a serine protease which has been shown to be present in sputum sol of patients with bronchiectasis (Stockley et al 1984) at concentrations capable of degrading a wide range of

extracellular matrix proteins. These proteins include collagen, elastin proteoglycan, fibronectin and laminin, as well as fibrinolytic and coagulation factors, complement, immunoglobulins G and M and apoprotein A. Neutrophil elastase is also capable of slowing CBF and causing epithelial disruption (Amitani et al 1991; Tetley 1993).

1.1.7.2 Alveolar macrophages

Macrophages represent a population of morphologically and functionally heterogeneous cells. Alveolar macrophages are present on the epithelial surface of the human lung and are the resident phagocyte in the alveolar space (Dubois 1986). They are derived from the monoblast series of bone marrow precursors (Hunninghake 1980). A major function of alveolar macrophages is to maintain the sterility of the lung, particularly on the epithelial surface. The alveolar macrophage may be activated by non-specific phagocytosis of microorganisms, or by recognition of specific antibodies with or without the addition of complement proteins. The recognition process is greatly enhanced by specific ligands (Sibille 1990). Following recognition, phagocytosis will occur with the formation of a phagosome. Within this structure oxidative and non-oxidative processes occur in an attempt to kill the microorganism. The primary process is an oxidative burst, which results in the production of a variety of oxygen radicals. Some of these radicals may be released outside the macrophage and therefore killing can occur both within and outside the cell (Klebanoff 1988;

Henson 1988). The macrophage may also use non-oxidative methods including proteases, lysozyme, a variety of acid hydrolases and defensins. Alveolar macrophages are also able to recruit and activate other inflammation cells. They initiate T cell processes and are able to recruit neutrophils, eosinophils and blood monocytes and can activate all classes of inflammatory cells (Sylvester 1990).

1.1.7.3 Lymphocytes

Collections of lymphocytes are found in bronchial associated lymphoid tissue (BALT), lymphoreticular aggregates and in hilar and para-tracheal lymph nodes (Moretta 1984). B lymphocytes are committed precursors of antibody secreting cells. In response to either microbes or their toxins, specific B lymphocytes recognise the antigen, proliferate and differentiate into plasma cells secreting immunoglobulin (Ig). Some undergo an Ig class switch producing antibodies of a different Ig class, and some revert to long-lived recirculating memory cells. Immunoglobulins bind to bacterial surface antigens thereby facilitating complement activation and bacterial killing through direct lysis or by opsonophagocytosis. Immunoglobulins may also bind to bacterial toxins preventing their pathological effects on target host cells (Moretta and Fauci 1984).

The two major classes of T lymphocytes, helper (TH) and

cytotoxic (CTL), are similar with respect to the determinants they recognise and their interaction with antigen presenting cells, however their effector functions are different. Broadly speaking TH cells secrete cytokines which drive differentiation of B cells into plasma cells and accelerate proliferation of T cells. CTL cells directly lyse cells and secrete cytokines resulting in eradication of pathogens. Studies have examined T-cell involvement in normal and inflamed mucosa and the ratio of TH to CTL has been found to be about 1.5:1 in normal airways and infiltration of the epithelium of both normal, and inflamed mucosa with T lymphocytes with a predominance of CD8/CD4+ cells was also found (Fournier et al 1989).

1.1.7.4 Complement

Complement consists of a sequence of proteins which are mainly synthesised in the liver and play an important role in both inflammation and immunity. The role of complement in the host defence against bacteria is two-fold, depending firstly on opsonization of bacteria for phagocytosis, by fixation of C3b and C4b, and secondly on the lysis of bacteria. Another important consequence of the fixation or activation of complement is production of mediators of acute inflammation (Brown 1991). The anaphylatoxins C3a and C5a stimulate the release of vasoactive mediators from cells bearing receptors for these two products (Hugli 1986). The C5a fragment is extremely potent at stimulating neutrophil chemotaxis, adherence, respiratory burst

generation and degranulation. Complement activity has been demonstrated in the BAL of normal rabbit bronchoalveolar fluid (Giclas 1987).

1.1.7.5 Antibacterial humoral factors in mucus

A number of antibacterial factors are present in mucus including enzymes, antimicrobial proteins, antibodies (Kaliner 1991).

a) Lysozyme

Lysosome, an enzyme which attacks the peptidoglycans in the cell walls of Gram-positive bacteria, was initially discovered in nasal secretions (Fleming 1922). Lysosome is a relatively small protein which is synthesised and released by neutrophils and serous cells of submucosal glands and represents 15-30% of the protein normally found in nasal secretions (Kaliner 1991).

b) Lactoferrin and transferrin

Lactoferrin and transferrin are antimicrobial proteins secreted by serous cells. They bind iron which is known to be important for bacterial growth and as a result may be bacteriostatic and bactericidal (Newhouse et al 1976).

c) Secretory immunoglobulins

Secretory immunoglobulins are important humoral factors in defending against bacterial invasion at the mucosal surface (Brantzaeg 1992). IgA is synthesized at the mucosal surface

and linked to a protein produced by epithelial cells to form secretory IgA which is the predominant antibody in mucus. The functions of secretory immunoglobulin are to neutralise the action of bacterial toxins and prevent bacterial adherence.

The mechanism of action of secretory IgA is thought to be by binding to bacterial surface antigens which mediate adherence thus blocking bacterial attachment. Binding to bacterial surface antigens may also facilitate complement activation and bacterial killing through direct lysis or by opsonophagocytosis (Brantzaeg 1992).

d) Protease inhibitors

A number of protease inhibitors are found in bronchial secretions which function to "balance" the effect of the proteolytic enzymes. These include α-1 protease inhibitor (α-PI), anti-leukoprotease produced by serous cells, antichymotrypsin and metalloproteases. The major inhibitor of serine proteases is α-PI which is a glycoprotein synthesised mainly in the liver and able to transverse the pulmonary endothelium and epithelium (Carrel 1986). The reactive centre of α-PI binds rapidly and irreversibly to neutrophil elastase and neutrophil elastase is inactivated at a much faster rate by α-PI than other enzymes suggesting that this is its prime function (Travis and Salvesen 1983). α-PI can be readily inactivated by oxidation and therefore the release of oxidants from neutrophils may create a zone

in which α-PI is inactive (Carrel 1986).

1.1.7.6 Cytokines

Cytokines are low molecular weight proteins which are produced by and regulate the function of many cells. They are multifunctional molecules, the biological actions of which appears to be situation specific and are chiefly involved in events in the local milieu with paracrine and autocrine functions. The effect of cytokines are mediated by binding to the high affinity receptors on the cell surface (Nicod 1993). The actions vary according to the concentration of cytokine, the state of activation and maturation of the target cell, the presence or absence of other cytokines and the composition of the surrounding matrix (Klias and Zitnak 1992). Cytokines modify many of the host responses to bacterial infection (Saukkonen et al 1990). Interleukin 1 (IL-1) and tumour necrosis factor (TNF) are early response mediators in regulating cellular functions and dictating events leading to the initiation, maintenance and repair of tissue injury. IL-1 is secreted from human monocytes in response to bacterial cell wall injury (Riesenfeld-Orn et al 1989). Although not directly chemotactic for neutrophils, (Yoshimura et al 1987) IL-1 induces interleukin 8 (IL-8) production (Matsushima et al 1989). IL-8 is a potent chemoattractant for neutrophils, has the capacity to activate neutrophils via a GTP binding protein and increases neutrophil adherence (Baggiolini et al 1989). IL-8 also activates cytokines and is a lymphocyte

chemoattractant. IL-8 is active in the presence of significant changes in PH and resists mild proteolysis suggesting that IL-8 may have prolonged in vivo biological activity in recruiting neutrophils in response to bacterial infection.

1.2 Abnormalities of the Host Defence

Abnormalities of the resident host defences whether congenital or acquired may predispose to respiratory tract infection (Table 1). Bacteria are able to take advantage of any "chink" in the intricate interrelated host defences to create an ecological niche. Some important examples of congenital and acquired defects are discussed below.

1.2.1 Cigarette smoking

Irritation by cigarette smoking may result in metaplasia of the bronchial epithelium. Irritation leads to a pronounced hypertrophy and hyperplasia of mucus glands and an increase in the number and proportion of goblet cells at the expense of ciliated cells. Goblet cells also occur in the terminal bronchioles, where they are normally absent (Niewoehner et al 1974). The excessive production of mucus combined with the loss of ciliated cells results in accumulation of mucus which may result in obstruction of bronchi. The retained secretions may be colonised by bacteria, and the lower respiratory tract, which is usually sterile, is liable to viral infection, irritation by atmospheric pollution or more extensive infection by the colonising bacteria (Kark

Table 1

Abnormalities of the physical, mechanical, cellular and humoral resident host defences which predispose to respiratory tract infection

Host defense	Abnormality
Bronchial wall and epithelium	Bronchial wall component defective Bronchial wall stenosis/compression Pulmonary sequestration Poor nutrition Smoking Inflammation Aspiration eg. gastric contents Inhalation eg. caustic gases
Mucus clearance:	Primary ciliary dyskinesia, Cystic fibrosis, Young's syndrome Viral infection Mechanical obstruction to mucociliary clearance Intrinsic: eg. Foreign body, Tumour Inspissated mucus, Stenosis Extrinsic: eg. Lymph node, Tumour
Cough:	Depressed cough reflex Endotracheal tube
Bronchoconstriction:	Asthma; Allergic bronchopulmonary aspergillosis
Immunoglobulin response:	Hypogammaglobulinaemia Panhypogammaglobulinaemia, Selective immunoglobulin deficiency Inactivation by IgA protease
Complement:	Complement deficiency (C3 and C5)
Neutrophils:	Primary or secondary neutropenia Abnormal neutrophil motility
Lymphocyte:	Primary or secondary lymphopenia
Alveolar macrophage:	Ineffective intracellular killing of intracellular bacteria

et al 1982).

1.2.2 Primary ciliary dyskinesia

Observations by Pedersen and Mygind (1976) and Afzelius (1976) led to the understanding that congenital abnormalities in the ultrastructure of the axoneme may impair mucociliary transport resulting in the clinical triad of bronchiectasis, chronic sinusitis and dextrocardia (Kartageners syndrome) (Kartagener 1933). Afzelius initially coined the term "immotile cilia syndrome" (Afzelius 1976), however subsequent reports in the medical literature have revealed a range of ultrastructural defects (Sturgess et al 1980; Sturgess et al 1979; Moreau et al 1983), a range in ciliary motility (Pedersen 1983) and that situs inversus probably occurs in approximately 50% of patients. Sleigh suggested the term "immotile cilia syndrome" should be replaced by "primary ciliary dyskinesia"(PCD) (Sleigh 1981).

Random ciliary orientation has recently been described as a possible variant of PCD (Rutland and De Iongh 1992; Rutman et al 1993). Two patients had cilia with normal ultrastructure, and normal or near normal beat frequency, but the cilia lacked efficacy because their beat direction was disorientated. The metachronal wave of ciliary beating therefore failed to propagate, and hence mucociliary transport was inefficient. It has been suggested that this may be a genetically conferred abnormality of the basal

bodies, or possibly of the anchoring mechanisms, preventing normal orientation of cilia.

The pulmonary manifestations of PCD include impairment of mucociliary clearance, airway obstruction and chronic respiratory tract infection.

1.2.3 Cystic fibrosis

Cystic fibrosis is the most common fatal inherited disease in European populations affecting about 1:2500 children. The basic defect results from a disorder of control of ion and water transport across epithelial cells. This results in increased sodium transport and chloride impermeability of the luminal surface of the airways with resultant relative deficiency of water in the airway fluids (Geddes 1990). Although no consistent abnormality of mucus viscosity or mucociliary clearance in cystic fibrosis has been demonstrated before lung damage occurs, the changes in ion transport predispose to the colonisation of the airways by bacteria.

1.2.4 Viral infection

Viral infections of the respiratory tract are extremely common. During viral respiratory tract infection, epithelial damage occurs with loss of ciliated epithelium and ciliary disorientation (Carson et al 1985). Viral infection is associated with an impairment of mucociliary clearance (Stanley et al 1985). Normal epithelial

organisation and ciliary ultrastructure return up to ten weeks after infection (Carson et al 1985). The abnormal epithelium and impaired mucociliary clearance may predispose the patient to acute bacterial infection (Harrison et al 1991) or chronic colonisation (Cole 1991).

1.2.5 Immunodeficiency disorders

Immunodeficiency disorders are a diverse group of conditions that share the common characteristic of an increased susceptibility to infection. Primary immunodeficiencies are classified based on the location of the immune defect: phagocytic, complement, B cell or T cell.

1.2.5.1 Phagocytic disorders

Phagocytic disorders may be quantitative or qualitative (Rosen et al 1984a; 1984b). Congenital granulocytopenia may be congenital or due to bone marrow dysfunction. Qualitative disorders can be extrinsic such as those that occur from deficiencies of opsonins secondary to antibody or complement abnormalities or intrinsic which result from enzyme deficiencies of the metabolic pathways necessary for killing of bacteria. Disorders may result from a defect occurring at any stage of phagocytosis by neutrophils including motility, recognition, ingestion, degranulation and intracellular killing. Pulmonary infections are common and include abscess formation, pneumonia and bronchiectasis. Bacterial infection may result from

Staphylococcus aureus, Streptococcus pneumoniae and Pseudomonas aeruginosa. Fungal infections due to Aspergillus and Candida may also occur (Rosen et al 1984a; 1984b).

1.2.5.2 Complement

Complement is necessary for opsonization (Absolom 1986), bacteria killing and neutrophil chemotaxis. Complement deficiencies predispose to recurrent pulmonary infections with S.pneumoniae, Haemophilus influenzae and susceptibility to recurrent infections with Neisseria meningitidis and Neisseria gonorrhoeae.

1.2.5.3 B cell deficiencies

The hypogammaglobulinaemic syndromes can be divided into panhypogammaglobulinaemia, selective immunoglobulin deficiency and subgroup immunoglobulin deficiency. B cell deficiencies predispose to recurrent pyogenic infections with extracellular encapsulated bacteria including S.pneumoniae and H.influenzae. The respiratory tract manifestations include otitis media, sinusitis, recurrent pneumonia and bronchiectasis (Asherton and Webster 1980).

1.2.4.4 T cell deficiencies

T cell deficiencies predispose the patient to pulmonary infection with less virulent or opportunistic organisms including fungi, mycobacteria, viruses and protozoa (Amman and Hong 1980).

1.3 The Interaction of Bacteria with the Respiratory Mucosa

From the microbe's view point, a parasitic relationship is the ideal for interaction with the respiratory tract, thereby allowing colonisation without induction of a host response (Cole and Wilson 1989). There are a variety of mechanism by which microbes can perturb the host defence mechanisms creating an environment conducive for colonisation.

1.3.1 Inhibition of mucociliary transport

Efficient mucociliary transport requires coordinated ciliary beating and also the production of the correct quantity and quality of mucus and periciliary fluid (Wanner 1977). A number of bacteria have been shown to interfere with mucociliary transport by either inhibiting ciliary beating or stimulating mucus production. P.aeruginosa produces a number of low molecular weight compounds which inhibit ciliary beating in vitro; the phenazine pigments 1-hydroxyphenazine (1-HP) and pyocyanin cause slowing and dyskinesia of the beating of human cilia (Wilson et al 1987), and a haemolysin (rhamnolipid) inhibits the beating of cilia (Read et al 1992). Pyocyanin and 1-HP have also been shown to reduce tracheal mucus velocity in the guinea pig in vivo (Munro et al 1989).

Filtered broth culture supernates of H.influenzae, (Wilson 1988) caused significant ciliary slowing of nasal

epithelium in vitro. Pneumolysin, a sulphydryl-activated haemolytic cytotoxin released by S.pneumoniae during autolysis causes ciliary slowing (Steinfort et al 1989).

Bacteria may change the quality and quantity of mucus. P.aeruginosa, H.influenzae and S.pneumoniae stimulate secretion of mucus glycoconjugates by explants of the guinea pig trachea (Adler et al 1989) and rhamnolipid increases mucus production in the cat trachea in vivo (Somerville et al 1992). Normal epithelial ion transport across cell membranes is essential for normal epithelial cell secretions, and rhamnolipid has been shown to interfere with epithelial ion transport in sheep in vitro (Graham et al 1993).

Inhibition of ciliary activity or a change in mucus production may reduce mucociliary transport.

The rheology of mucus may change during infection. Viral infection has been shown to reduce the elasticity of mucus which results in impaired transportability and bacterial infection increases mucus viscosity which reduces mucus transport.

1.3.2 Epithelial damage

Products from respiratory pathogens have been shown to interfere with the structure and integrity of the epithelium. P.aeruginosa rhamnolipid and pyocyanin cause

epithelial disruption of human ciliated epithelium in vitro (Wilson et al 1987; Read et al 1992). A peptidoglycan fragment of B.pertussis has been shown to disrupt ciliated epithelium of guinea pigs (Rosenthal et al 1987) and humans (Wilson et al 1991). Lipo-oligosaccharide from H.influenzae damages the rat tracheal mucosa (Johnson et al 1986). A cell free extract of M.pneumoniae has been demonstrated to damage the epithelium of hamster tracheal organ cultures (Chandler et al 1980). S.pneumoniae pneumolysin has been shown to be cytotoxic to the hair cells of the guinea pig cochlea, causing splaying and loss of stereocilia, dissolution of hair bundles and damaging the apical surface of hair and supporting cells (Comis et al 1993). Pneumolysin has also been shown to cause damage to alveolar epithelial cells (Rubins et al 1993) and pneumolysin installation into the rat lung produced histological changes identical to those produced by infection with a type 3 S.pneumoniae (Feldman et al 1991).

1.3.3 Bacterial adherence

During colonisation or invasion bacteria adhere to the respiratory mucosa. The ability of bacteria to adhere to mucosal surfaces is thought to be an important determinant of colonisation and the pathogenesis of most infections (Beechley 1981; Niederman 1989). Adherence offers protection from mucociliary clearance and results in the close proximity of bacteria to epithelial cells allowing toxins to reach concentrations sufficient to damage the

host cells (Middlethorpe et al 1981). Adherent bacteria have an increased ability to take up nutrients liberated by the host cells (Savage 1987) and adherence may be the first step in penetration of the mucosal surface prior to invasion.

Bacteria use a wide range of adherence mechanisms; examples include lectin-like substances which are found embedded in the outer membrane, or in the exopolysaccharide, or expressed as fimbriae (Uhlenbruck 1987). Fimbriae enhance H.influenzae adherence to buccal cells and it has been suggested that fimbriae may give H.influenzae an advantage when colonising the nasopharynx by increasing epithelial adherence (Pichichero 1984). However, it has also been demonstrated in an organ culture of human nasal turbinate tissue (Read et al 1991) that although fimbriae enhance adherence to buccal cells, they neither permit adherence to normal ciliated respiratory epithelium, nor enhance bacterial adherence to areas of epithelial damage. It is clear that some bacteria such as non-typable H.influenzae express a number of different adhesins. Streptococci pyogenes adherence to buccal cells is mediated by lipoteichoic acid (LTA) and cell wall-derived LTA binding proteins. This forms a meshwork extending away from the bacterial membrane (Ofek et al 1982). The highly reactive lipid end of the LTA molecule is orientated towards the host cell surface and interacts with fatty acid binding sites in the amino-terminal region of fibronectin molecules

that are bound to the surface of cells (Beachey et al 1988).

The interaction between bacteria and the mucosa may be complex. Bacterial adhesins may themselves act as toxins to the host cells (Hoepleman and Tuomanen 1992). Bacteria may release toxins prior to adhesion which may favour bacterial persistence by disturbing the host defence mechanisms, or toxins may unmask potential receptors for bacterial adhesins on the epithelium.

1.3.3.1 Interaction with mucus

The first interaction of bacteria with the respiratory mucosa may be with mucus. The binding of bacteria to respiratory tract mucus serves as a first line defense mechanism, as the bacteria are then conveyed out of the respiratory tract by mucociliary clearance. Histopathological studies of P.aeruginosa in lungs from patients with cystic fibrosis demonstrated that the bacteria were mainly intraluminal, associated with secretions, only occasionally forming adherent microcolonies on the epithelium in areas of epithelial damage (Baltimore et al 1989). Mucus is rich in potential carbohydrate receptors for bacteria (Lamblin and Roussel 1993). Different bacteria have been shown to demonstrate an affinity for airway mucus with preferential binding to mucus compared to normal ciliated epithelium. These include S.pneumoniae (Plotkowski et al 1989), H.influenzae (Farley

et al 1986; Read et al 1991) and P.aeruginosa (Plotkowski et al 1989).

The mechanism of P.aeruginosa interaction with mucus has been partially elucidated. P.aeruginosa has an increased affinity for the mucins from patients with cystic fibrosis compared to non-cystic fibrosis subjects (Carnoy et al 1993). Adhesion of mucoid P.aeruginosa to mucin has been reported to be mediated by the mucoid exopolysaccharide (Ramphal and Pier 1985; Marcus and Baker 1985; Hata and Fick 1991), and adhesion of non-mucoid strains is mediated by pili. However both mucoid and non-mucoid P.aeruginosa bind to type 1 (galactose B1-3 N-acetylglucosamine) and type 2 (galactose B1-4 N-acetylglucosamine) disaccharide units (Ramphal et al 1991; Vishwanath and Ramphal 1985). These workers have also postulated that sialic acid either maintains the conformation of the oligosaccharide chains in mucin or increases the affinity of adhesion of P.aeruginosa to mucin (Ramphal et al 1991). Binding can be inhibited by exposure of the mucin to the influenza virus implying that these two organisms share a common receptor (Vishwanath and Ramphal 1984; Vishwanath and Ramphal 1985). Other P.aeruginosa adhesins for mucus have been recognised (Rosenstein et al 1992; Reddy 1992), so the interaction is probably complex (Sajjan et al 1992). There are differences in the binding of P.aeruginosa to the highly glycosylated subfractions of mucins, suggesting that other receptors, such as cellular glycolipids which are shed into mucus may

be contributing to mucus adherence (Ramphal et al 1989).

Specific interactions occur between bacteria and mucins (Vishwanath and Ramphal 1984; Laux et al 1984; Levine et al 1978) which may be similar to the adhesin-receptor interactions that are responsible for bacterial adherence to epithelial cells (Beachey 1981). Adherence to mucus may also protect bacteria from phagocytosis as mucin has been shown to protect P.aeruginosa from opsonin-mediated phagocytosis (Vishwanath et al 1984).

Most organisms entering the bronchopulmonary system of the normal host will adhere to mucus and be expelled. In the host with abnormal mucociliary clearance however, mucus adherence may be of advantage to certain bacteria which are able to modify mucus production and movement.

1.3.3.2 Bacterial adhesion to normal respiratory epithelium

The net charge on the surface of bacteria and host cells is negative. This repulsive force may be overcome by the attractive forces between hydrophobic molecules present on both cell surfaces and electrostatic forces (Jones 1977). As bacteria approach the epithelial surface, specific molecular interactions may occur.

Human cells express many potential receptors for adhesion, these include saccharide residues from the cell surface,

glycoproteins, glycolipids and proteoglycans (Sharon et al 1986). However it seems that bacterial adherence to normal ciliated respiratory epithelium is rare. Mycoplasma pneumoniae and B.pertussis (Tuomanen and Hendley 1983; Wilson et al 1991; Almagor et al 1985) have been observed to adhere along ciliary membranes of functionally active ciliated cells. It has been suggested that filamentous haemagglutinin and pertussis toxin act together as adhesins by establishing a bridge between the bacteria and one or more carbohydrate-containing receptors on cilia (Tuomanen 1986).

However other airway pathogens may not bind to intact ciliated epithelium and bacterial adhesion may be dependant on prior injury to the respiratory mucosa. Studies have shown that P.aeruginosa adheres to human ciliated cells (Niederman et al 1983 ; Paranchych et al 1986; Doig et al 1987; Doig et al 1988; Johanson et al 1980; Ramphal et al 1984; Ramphal and Vishwanath 1987; Rivera and Nicotra 1982; Woods et al 1980), and H.influenzae (Bakaletz et al 1982; Lampe et al 1982; Pichichero 1984) and S.pneumoniae (Andersson et al 1981; Selinger and Reed 1979) adhere to epithelial cells. However these studies have often involved the use of dispersed cells obtained from the mouth, nose or trachea and have not examined the interaction of bacteria with intact respiratory mucosal surface. H.influenzae, S.pneumoniae and P.aeruginosa are known to bind specifically to carbohydrate sequence

GlcNAcß1-3Galß and Gal NAcß1-4Gal found in the glycolipids fucosyl-GM1-asialo-GM1 and asialo-GM2. However, these studies were performed by studying bacterial adherence to electrophoretic gels of lung extracts. Asialo-GM-1 is found in high concentration in human lung extracts (Kirvan et al 1989).

1.3.3.3 Bacterial adhesion to injured respiratory mucosa

Airway epithelial damage may be a prerequisite for the association of many bacterial pathogens including H.influenzae with respiratory epithelium (Read et al 1991). Epithelial cells from patients with recurrent respiratory infections by S.pneumoniae have been shown to be more susceptible to bacterial adhesion than cells from patients without underlying pulmonary disease suggesting that the cell content of surface receptors for S.pneumoniae adhesins may vary (Andersson et al 1981). P.aeruginosa is known to have an affinity for the mucosa of injured airways (Ramphal et al 1980). Bacterial elastase, alkaline protease and phospholipase C have been shown actively to enhance the binding of P.aeruginosa to epithelial surfaces (Saiman et al 1990; Nicas and Iglewski 1985). P.aeruginosa proteases are known to degrade fibronectin which may inhibit the blocking function of fibronectin allowing enhanced binding of the bacteria (Woods et al 1980; Woods 1987). Phospholipase C has been shown to enhance adherence of P.aeruginosa and exoenzyme S causes tissue damage allowing adherence and pulmonary invasion (Woods et al 1986; Woods

and Sokol 1985).

Adherence of P.aeruginosa to tracheal cells of patients on intensive care with tracheotomies was increased compared to controls, showed a negative correlation with nutritional status (Niederman et al 1984), and correlated with the acquisition of pneumonitis (Todd et al 1989). The binding of Pseudomonas to tracheal cells is optimal at alkaline pH (Palmer et al 1986) and related to the sputum elastase activity (Niederman et al 1986). Animal studies have also shown that illness, including uraemia and malnutrition, can lead to increase in adherence and colonisation by Gram-negative bacteria (Higuchi et al 1980).

1.3.4 Bacterial spread

Once bacteria have colonised the respiratory mucosa a balance may be established between their multiplication and elimination leading to a contrived stable colonisation. Alternatively, they may multiply and disseminate by either contiguous spreading within the respiratory tract, or by invasion through the submucosa and endothelium causing haematogenous spread. Examples of contiguous spreading within the respiratory tract are infection of the lower airways by H.influenzae in patients with exacerbations of chronic bronchitis;infection by P.aeruginosa in patients with cystic fibrosis; sinusitis, conjunctivitis and otitis media caused by S.pneumoniae and H.influenzae. Examples of invasion of the submucosa and bloodstream include

infections by N.meningitidis and S.pneumoniae which may cause bacteraemia and penetrate the blood brain barrier to cause meningitis.

1.4 Models of Bacterial Interaction with the Respiratory Tract

The interaction of bacteria with the respiratory tract can be studied in vivo or in vitro.

1.4.1 Animal models

Animal models represent an important step in the study of bacterial interaction with intact host defences. An animal model also has the advantage that it can be manipulated in ways that would not be possible in human studies. The infant rat meningitis model (Moxon et al 1974), the hamster model of M.pneumoniae infection (Collier and Clyde 1974), the mouse model of S.pneumoniae infection (Plotkowski et al 1986), the guinea pig mucociliary clearance model (Munro et al 1989) and the rat bronchiectasis model (Guerreiro 1990) are examples of in vivo models. However, respiratory tract infections with human pathogens are not easy to produce in animals, and aerosol delivery frequently produces an insufficient inoculum (Pennington, 1985). Anaesthesia, skilled intubation and surgical procedures are often required, which make the model non-physiological at the outset. Animal models are also restricted by the specificity of the host species to particular micro-organisms. Attention to detail is important when reporting

the results from any animal model. The model should mimic human disease in terms of clinical, histological and pathophysiological parameters (Pennington, 1985; Woods et al, 1989, Lapa e Silva et al, 1989), and there should be an understanding of the animal's natural defences against the pathogen (Winkelstein, 1984).

Transgenic animals may permit new infection models to be developed. These are animals in which deliberate human gene insertions and deletions create genetic lines of animals with important disabling human diseases such as cystic fibrosis (Ratcliff et al, 1993). They open up exciting new possibilities for understanding the pathogenesis of some infections eg. P.aeruginosa in cystic fibrosis, and devising new therapies for such conditions.

1.4.2 In vitro models

In vitro models may allow accurate regulation and measurement of both host and bacterial factors thought to be important in the pathogenesis of respiratory tract infections. Several in vitro model systems have been used to study bacterial properties responsible for virulence.

1.4.2.1 Suspended epithelial cells

To study the interaction of bacteria with epithelial surfaces, single or groups of epithelial cells can be obtained by brushing or scraping the mucosal surface of the respiratory tract, including the nasal cavity, the

oropharynx and the trachea. The cells obtained can be suspended in media and bacteria or bacterial products added to the suspension and the interaction assessed. Much has been learnt about the molecular interactions at the cell surface from these studies, however dispersal of cells exposes receptors on non luminal surfaces which are not normally available for bacterial interaction in vivo. Also, when studying the effect of toxins on suspended cells results must be interpreted with caution because the cell is exposed to the effect of toxin on all surfaces. The host defenses are also unphysiological, for example although cilia may be beating, the protective layer of mucus and periciliary fluid normally present will be missing.

1.4.2.2 Cell culture

Single or mixed cells can be grown in a monolayer (Wu 1986, Van Scott 1986) either from outgrowth of a tissue explant (Trump 1980; Chandler et al 1982) or by seeding from dispersed cells (Wu 1986). The monolayers may be supported on a layer of collagen (Yankaskas et al 1985), endothelial cells (Wiesel et al 1983) or fibroblasts (Lechner et al 1981). Outgrowth from an explant of airway mucosa consist of the mixed cells derived from the explant. The cell populations may be heterogenous, undifferentiated and include non epithelial cell types such as fibroblasts (Van Scott 1986). The study of bacterial interaction with explants must therefore be interpreted with caution. Cells can be obtained from airway epithelium by digestion with

protease, and monolayers of epithelial cells free of other cell types can be obtained by the use of selective media (Wu 1985). However, confluent monolayers are difficult to achieve and human cells may lose their differentiated morphology (Wu 1986). Bacteria-cell interactions have been studied with monolayers of established cell lines such as human lung fibroblasts with M.pneumoniae (Gabridge 1979). Such studies do not provide optimal models of bacterial interaction with airway mucosa in vivo because the cell lines consist of inappropriate cells with possibly inappropriate receptors. Although cell culture monolayers do not possess the complex host defences of the intact mucosa they do have the advantage of being able to study the molecular interaction between bacteria and epithelial cells in the absence confounding factors such as mucus.

1.4.2.3 Organ cultures

Organ cultures provide an intact mucosal surface with a histological cell profile similar to that found in vivo. McGee et al (1976) studied the effects of various culture media upon the performance of human fallopian tube organ cultures which remained viable for 2 weeks. However, ciliary function showed a progressive decline. M.pneumoniae has been studied in animal tracheal explant cultures (Gabridge et al 1977, 1979, Cherry et al 1973). Organ cultures prepared from human adenoids have been used to assess the interaction of H.influenzae and N.meningitidis with nasopharyngeal mucosa. The tissue was

immersed in medium supplemented with antibiotics, the organisms inoculated at known concentrations and the interaction assessed by light and electron microscopy (Stephens et al 1986; Farley et al 1986, 1990). Organ cultures constructed from foetal tracheal tissue have also been used to assess the interaction of H.influenzae (Denny 1974). Free cut surfaces may be sealed by embedding in agar (Matsuyama 1974; Gabridge et al 1979; Read et al 1991). Read (1991) quantified epithelial damage and adherence of H.influenzae in an organ culture model of nasopharyngeal tissue with cut sealed edges. Organ cultures have advantages over isolated cells or cell culture for the study of the interaction of bacteria and bacterial products with the respiratory mucosa in that they provide an intact mucosal surface with a histological cell profile similar to that found in vivo. The interaction of bacteria can therefore be studied in vitro using a system which closely pertains to the in vivo interaction. Much has been learnt about the interactions of bacteria with the respiratory tract using organ cultures including similarities and differences of adhesion to and invasion of the mucosa by different bacterial species. For example using immersed organ cultures S.pneumoniae has been shown to form a gel like layer in association with mucus (Feldman et al 1990). Non-typable H.influenzae has been shown to invade between cells through tight junctions, whilst N.meningitidis invades by parasite directed endocytosis (Stephens and Farley 1991). Although organ cultures

provide an intact mucosal surface for bacterial interactions, elements of the host defence mechanisms are absent. Although the humoral and cellular mechanism of host defence are present within the tissue the normal leucocyte response to infection and inflammation with traffic across the endothelial wall into the epithelium is absent. Immersion of the organ culture in liquid media removes the air mucosal interface and changes the dynamics of mucociliary clearance. Immersion may also alter the interaction between bacteria and bacterial products with the mucosal surface. For example, the media may support bacterial replication allowing interaction between bacteria and/or bacterial toxins and the mucosal surface. The study of the molecular mechanisms of bacterial interaction is also more difficult using organ culture than studies using isolated cells because of the numerous variables present in organ cultures.

1.5 Neisseria Meningitidis

N.meningitidis is a relatively fragile and fastidious Gram-negative bacterium that causes bacteraemia and meningitis in association with septic shock. This exclusive human pathogen, which has an affinity for certain mucous surfaces, was first identified as the causative agent of bacterial meningitis by Weichselbaum in 1887 (Weichselbaum 1887; Branham 1956). Meningococcal disease is recognised as a world-wide problem. In Third World countries more than 300,000 persons per year suffer from infectious

disease caused by N.meningitidis resulting in 35,000 deaths and occurs in epidemics across the "meningitis belt" of the Sub-Saharan Africa every seven to ten years (Greenwood 1987; Moore 1992). In addition high or increasing levels of endemic meningococcal disease have been reported from parts of the UK, Norway, Cuba and Brazil (Hart and Rogers 1993).

1.5.1 Structure

N.meningitidis is a diplococcus with a typical Gram-negative cell envelope. It has two cell membranes, one on each side of a rigid peptidoglycan layer. The outer membrane of the meningococcus continually produces blebs which are released as vesicles rich in endotoxin (Hart and Rogers 1993). Approximately 50% of the outer leaflet of the outer membrane is composed of ampiphillic lipo-oligosaccharide molecules. The hydrophobic portion of the molecule is lipid A which is the active moiety of endotoxin. The hydrophillic oligosaccharide portion is variable in structure and provides the basis for epidemiological typing (Hart and Rogers 1993).

1.5.2 Classification

Four different groups of (antigenic) surface structures can be distinguished on the meningococci: capsular polysaccharide (CPS), outer membrane proteins (OMP), LPS (located in the outer membrane) and surface appendages known as pili. An extensive phenotypic classification system has been developed on the basis of the first three

groups of surface components (Kim et al 1988; Peltola 1983; Poolman et al 1982; Verdros 1987; Zollinger and Mandrell 1977). Serogroups are based on differences in structure of the CPS, serotypes and sub-serotypes based on differences in class II/III and class I OMP, and immunotypes based on differences in the oligosaccharide structure of the meningococcal LPS. Bacteria belonging to groups A, B, C cause by far the largest number of cases (Branham 1956; Peltola 1983). Group A predominates in Africa during both epidemic and endemic periods, whereas groups B and C are the prevalent serogroups isolated during endemic periods and localised outbreaks of meningococcal disease in the Western World (Frasch 1989; Peltola 1983; Schwartz et al 1989). In England and Wales the majority of meningococcal infections are due to group B meningococci followed by group C (Jones and Kaczmarski 1991). Meningococci have also been subdivided by clonal analysis based on variation in the electrophoretic behaviour of cytoplasmic isoenzymes (Caugant et al 1986; Moore 1992). The technique is used to measure the genetic distance in meningococcal strains and demonstrate differences during epidemics which are not detected by phenotypic analysis. Large group A pandemics are generally caused by one clone.

1.5.3 Pathogenesis

The sole natural habitat and reservoir for N.meningitidis is the human upper respiratory mucosal surface, primarily the nasopharynx (Apicella 1989; Peltola 1983).

Meningococci are transmitted by large respiratory droplets or direct contact with respiratory secretions (Schwartz et al 1989). Carriage of the meningococcus in general is asymptomatic. During the non-epidemic periods 5-30% of the adult population may be colonised by the meningococcus (Peltola 1983; DeVoe 1982). Respiratory infections such as influenza may predispose individuals to systemic meningococcal disease. Meningitis patients are four times more likely to have serological evidence of a coincident infection with influenza than control patients (Young et al 1972; Harrison et al 1991).

1.5.4 Virulence Factors

1.5.4.1 IgA proteases

IgA proteases are known to be released by N.meningitidis. These enzymes selectively cleave IgA1 at the hinge region of heavy chains of the immunoglobulin molecule. The mechanism of protection has not been fully elucidated but it has been suggested that following the cleavage by IgA protease bacteria protect themselves with non-functioning antibody which would block opsonisation (Plaut 1983; Mulks et al 1980).

1.5.4.2 Pili

Pili are important adhesins of N.meningitidis and isolates from patients are almost invariably piliated. Piliation appears to be required for colonisation of host mucosal surfaces and for at least some stages of invasive disease

caused by these bacteria (Stephens et al 1985; Pinner et al 1991; Stephens et al 1984; Stephens et al 1983; Stephens and McGee 1981). Pili are filamentous protein appendages which extend a considerable distance from the bacterial surface, and are probably responsible for the initial interaction with the host epithelial cells and subsequently with endothelial cells (Virji et al 1992; Stephens et al 1983; Stephens and McGee 1981). Pili produced by pathogenic Neisseria species are composed of repeated subunits of pilin polypeptide (Meyer and van Putten 1989; Meyer 1990). Meningococci have been observed to produce either one of two types of pili class I and class II. Class I pili are similar in almost all respects to the pili produced by strains of N.gonorrhoea reacting with the monoclonal antibodies SM1 and SM2 (Virji and Heckles 1983). In contrast class II meningococci do not react with these antibodies. Class I and class II are equally adherent to human endothelial cells suggesting functional similarities (Virji et al 1991). However it has been demonstrated that class I and class II pili show differences in tropism for different epithelial cell lines (Virji et al 1992).

Expression of pili can be spontaneously turned on and off by phase variation, and a single cell can produce offspring that express structurally, antigenically and functionally distinct pilins through the process of antigenic variation. The production of the non-piliated non-attaching phase may allow the bacteria to desorb from initial sites of

infection and allow movement to a new location, for example allowing transmission of the organism from one host to another.

1.5.4.3 Blebs

Blebs are vesicle-like structures that contain lipids, LPS, OMP, and CPS which are released from the surface of N.meningitidis during growth of bacteria (Anderson and Solberg 1988; Anderson et al 1987).

1.5.4.4 Capsular polysaccharide

CPS has been shown to be antiphagocytic. In young children CPS has reduced immunogenicity. There is no memory induction and epitopes are shielded (DeVoe 1982).

1.5.4.5 Outer membrane proteins

These proteins are integral membrane proteins and act as porins transporting molecules in and out of the bacterial cell, and they also play an important role in the adhesion process. OMP demonstrate antigenic variation and phase variation (Woods and Cannon 1990). It has been demonstrated that there is a correlation between the expression of OMPs particularly Class 5c protein and adherence to and invasion of human cells by N.meningitidis (Virji et al 1992). Nasopharyngeal isolates of N.meningitidis may be relatively capsule deficient (Craven et al 1980), in addition rapidly growing bacteria or bacteria grown in iron limited conditions may be capsule deficient (Masson and Holbein

1985). If local conditions result in down regulation of capsule production, bacteria expressing Class 5c protein might colonise and invade the epithelium more efficiently.

1.5.4.6 Regulatory iron proteins

Iron is essential for the survival and growth of N.meningitidis but is not freely available in the serum of the human host because it is bound to the human iron-binding glycoprotein, transferrin. Regulatory iron proteins are OMPs which are expressed in iron-limited conditions. These proteins are able to bind to human transferrin. Although it is unclear how the process occurs, direct contact with a cell surface receptor is thought to be essential (Simonson et al 1982).

1.5.4.7 Lipopolysaccharide

The LPS of N.meningitidis are a complex group of molecules which play an important role in the pathogenesis of meningococcal disease (Verheul et al 1993). LPS is important in breaching the mucosal barrier, and also as a target molecule for sialylation by enzymes (sialyltransferases) (Mandrell et al 1991). Minor changes in primary oligosaccharide structures result in distinctly different immunological and immunochemical behaviours. Meningococcal LPS induces a cytokine cascade (Waage et al 1989; Waage et al 1989), activates complement (Brandtzaeg et al 1989), and enhances the plasminogen activated inhibitor levels (Engebretsen et al 1989). Sialylation of

the terminal lacto-N-neotetrose unit of LPS may down regulate the alternative pathway of complement, shield underlying protective epitopes,and possibly change the immunogenicity of the antigen determinants (Jennings et al 1984; Schauer 1982).

1.5.5 Interaction with respiratory mucosa

N.meningitidis is exclusively a human pathogen and the upper respiratory tract is both the principal site of colonisation and transmission (Apicella 1989; Peltola 1983). Bacterial adherence to the mucosal surface is an essential part of colonisation of the nasopharynx by N.meningitidis. Previous studies of the interaction between N.meningitidis and human ciliated nasopharyngeal epithelium have either used isolated cell systems or organ cultures immersed in media (Stevens et al 1986; Stevens and Farley 1991).

1.5.5.1 Dispersed epithelial cells

Several studies have shown that N.meningitidis pili facilitate adherence to human cells (Heckels 1986). Piliated N.meningitidis have been shown in vitro to adhere in large numbers to nasopharyngeal (McGee and Stephens 1984) and buccal (Craven and Frasch 1978; Truss et al 1983) epithelial cells, and to human umbilical vein endothelial cells (HUVEC) in culture (Virji 1992).

1.5.5.2 Ciliated epithelium

Using organ cultures immersed in media it has been

suggested that N.meningitidis penetrates the mucus barrier, attaches to non-ciliated epithelial cells which is associated with a microvillus reaction (Stephens et al 1983). N.meningitidis causes cytotoxicity with loss of ciliated cells and a reduction in ciliary activity (Stevens et al 1986; Stevens and Farley 1991). N.meningitidis then enters the cells by a process of parasite directed endocytosis (McGee et al 1983; McGee et al 1988).

1.6 Streptococcus Pneumoniae

S.pneumoniae is a human pathogen that causes life threatening, invasive diseases such as pneumonia, bacteraemia and meningitis. S.pneumoniae is the commonest cause of community acquired pneumonia in adults (Bath et al 1964; Macfarlane et al 1982; Woodhead 1990) and colonises the upper respiratory tract of up to 70% of healthy adults (Riley et al 1981). Pneumococcal carriage rates are higher in young children and in people living in crowded conditions (Riley et al 1981). S.pneumoniae is commonly isolated as a cause of otitis media, acute sinusitis and exacerbations of chronic bronchitis; and in the absence of meningococcal disease, is the commonest cause of bacterial meningitis in adults and the second most common cause in young children (Austrian 1984; Hendley et al 1975).

1.6.1 Structure and virulence factors

S.pneumoniae are Gram-positive non-motile capsulate cocci. They occur in pairs, are oval or lancelate in shape, and

are surrounded by a capsule. The surface of S.pneumoniae contains CPS and cell wall; which consists of peptidoglycan, polysaccharide and proteins.

1.6.1.1 Capsular polysaccharide

CPS is an important virulence factor of S.pneumoniae. Pneumococci capsules are complex hydrophillic polysaccharides that are expressed on the bacterial surface. These polysaccharides are antigenically distinct molecules that form the basis for classifying pneumococcus by serotypes. There are approximately 84 known serotypes and the majority of invasive infections are caused by a limited number of serotypes. CPS is known to be a virulence factor for S.pneumoniae (MacLeod and Krauss 1950). Unencapsulated pneumococci exhibit reduced virulence in humans and mice. Enzymic depolymerization of the capsule of a type 3 pneumococcus increased its LD_{50} by 10^6 (Avery and Dubos 1931), and a similar effect on virulence was achieved by transposon mutagenesis of a gene essential for capsule production (Watson and Musher 1990). Encapsulated strains cause invasive disease in humans and cause bacteraemia in mice after intraperitoneal inoculation (Watson and Musher 1990). The capsule inhibits phagocytosis in the non immune host (Wood and Smith 1949) and antibodies to capsule promote phagocytosis and confer type specific protection.

1.6.1.2 Cell wall

The peptidoglycan layer of the pneumococcal cell wall gives

the cell its shape; the peptidoglycan is covalently linked to ribitol-phosphate teichoic acid containing phosphoryl-choline (Tomasz 1981; Sorenson et al 1988). This is sometimes referred to as C-polysaccharide and is a major constituent of the cell wall (Liu and Gotschlich 1963; Gotschlich and Liu 1967). Lipid may bind to the teichoic acid forming molecules known as lipopteichoic acid (LTA). Collectively these polysaccharides are called cell wall polysaccharides to distinguish them from CPS. Both the cell wall and capsule are potent stimulators of the alternative complement pathway (Holzer et al 1984). The pneumococcal cell wall also contains protein and antigens. Pneumococcal surface protein A is expressed on the bacterial surface. Mutants lacking this antigen are less virulent in mice and antibodies to surface protein A are protective in an animal model (McDaniel et al 1984; Briles et al 1988). Installation of the pneumococcal cell wall into the sub-arachnoid space of rabbits reproduces the entire syndrome of meningitis (Wood and Smith 1949; Tuomanen et al 1985). This response is mediated by cytokines (Saukkonen et al 1990). The cell wall may interact with complement and non-complement mediated host defences to induce inflammation (Tuomanen et al 1989). Pneumococcal cell wall stimulates human macrophages to secrete early response mediators Il-1 and TNF (Riesenfeld-Orn et al 1989) and may also initiate release of Il-8 which is chemotactic for neutrophils.

1.6.1.3 Pneumolysin

Pneumolysin is a thiol-activated cytolytic toxin which is produced by S.pneumoniae (Avery and Neill 1924). It consists of a 53-kDa polypeptide chain and is produced by nearly all clinical isolates of S.pneumoniae (Paton et al 1983; Kanclerski and Mollby 1987).

The thiol activated cytolysins are believed to share a common mode of action involving two steps. The first is an interaction with cholesterol in the target cell membrane resulting in the insertion of the toxin into the lipid bilayer. The second stage involves the lateral diffusion of and oligomerization of 20-80 toxin molecules resulting in the formation of arc and ring structures visible by electron microscopy which are thought to be transmembrane pores (Bhakdi and Tranum-Jenson 1986).

Pneumolysin is a heat sensitive protein and is also susceptible to inactivation by mild oxidative conditions but has a capacity for reactivation by appropriate reducing agents (Cole 1914; Neill 1926; Neill 1927). Pneumolysin has been shown to be a potent inhibitor of the neutrophil (Ferrante et al 1984; Nandoskar et al 1986). Pneumolysin has been shown to lyse neutrophils and platelets, and at lower concentrations migration of neutrophils is inhibited and leakage of lysosome enhanced (Paton and Ferrante 1983). Pneumolysin has also been shown to activate human complement reducing the maximal opsonic activity for

S.pneumoniae (Paton and Ferrante 1983). Pretreatment of human lymphocytes with pneumolysin abrogates lymphoproliferative response to mitogens and reduces the capacity of stimulated lymphocytes to release lymphokines and all three classes of immunoglobulins(Ferrante et al 1984). These inhibitory properties of pneumolysin can be abolished by pretreatment of pneumolysin with cholesterol (Paton and Ferrante 1983; Ferrante et al 1984).

Treatment of human serum with pneumolysin results in activation of the classical complement pathway, in the absence of specific antibody, with concomitant depletion of serum opsonic activity (Paton et al 1984). Pneumolysin binds to the Fc region of IgG. Unlike the effect of pneumolysin on cells of the immune system cholesterol does not inhibit its effect on complement suggesting that activation is a feature unrelated to the toxin's cytolytic activity (Paton et al 1984).

a) Animal models

In mice immunisation with purified pneumolysin increases survival after subsequent challenge with virulent pneumococci (Paton et al 1983). A pneumolysin deficient S.pneumoniae mutant showed reduced virulence that was restored after reconstitution of the pneumolysin production (Berry et al 1992; Berry et al 1989). Pneumolysin has also been shown to cause damage to pulmonary artery endothelial cells and alveolar epithelial cells (Rubins et al 1993).

Pneumolysin installation into the rat lung produced histological changes identical to those produced by infection with a type 3 S.pneumoniae (Feldman et al 1991). This suggests that pneumolysin is produced in vivo and is important in the pathogenesis of pneumonia. Pneumolysin has also been shown to be cytotoxic to the hair cell of the guinea pig cochlea (Comis et al 1993).

b) Dispersed human epithelial cells

Pneumolysin is cytotoxic to human nasal ciliated epithelium, and has been shown to cause ciliary beat slowing, changes in epithelial cell ultrastructure and disruption of epithelial integrity (Feldman et al 1990).

c) Human ciliated epithelium in organ culture

Pneumolysin has been shown to be toxic to human respiratory epithelium in organ culture causing epithelial disruption and ciliary slowing (Feldman et al 1990).

1.6.1.4 Autolysin

Autolysin is a 36-kDa N-acetylmuramic acid, L-alanine amidase which is located in the cell envelope. The enzyme is thought to be bound to choline moieties of lipoteichoic acid which are anchored to the cell membrane (Briles and Hakenbeck 1985). In this form autolysin is inactive and the association with lipoteichoic acid may be an important means of regulating activity in vivo. When cell wall biosynthesis ceases either because of nutrient starvation

or treatment with antibiotics, this association is disrupted and the enzyme is then able to cleave the bond between the glycan chain and the peptide side chain of the choline containing cell wall, thereby bringing about cellular autolysis (Briles and Hakenbeck 1985).

Several studies have suggested that autolysin contributes (directly or indirectly) to the virulence of S.pneumoniae. Studies have shown that autolysin mediates release of highly inflammatory cell wall break down products which could contribute to pathogenesis of S.pneumoniae (Chetty and Kreger 1981; Chetty and Kreger 1980; Tuomanen et al 1985). Cell wall degradation products may not be the only substances released from pneumococci by the action of autolysin . Pneumolysin and neuraminidase are located in the cytoplasm of S.pneumoniae (Johnson 1977; Lock et al 1988). They are released when the cell undergoes autolysis, and it has been suggested that lysis of the pneumococcal cell wall by autolysin results in the release of pneumolysin and neuraminidase and therefore that autolysin contributes to the pathogenesis by releasing these potentially lethal toxins. The direct contribution of autolysin to pneumococcal virulence has been studied using the cloned gene to construct defined autolysin negative encapsulated type 2 or type 3 pneumococci by insertion-duplication mutagenesis (Berry et al 1989 and 1992). Immunisation of mice with autolysin significantly increases survival following nasal inoculation with

S.pneumoniae. Pneumococci grown in the presence of autolysin antiserum do not release significant amounts of pneumolysin (Lock et al 1988). This release is restored following exogenous addition of pneumococcal autolysin to culture medium (Berry et al 1989).

1.6.1.5 Neuraminidase

Neuraminidase is located in the cytoplasm of S.pneumoniae (Lock et al 1988). This enzyme cleaves terminal sialic acid residues from a wide variety of glycolipids, glycoproteins and oligosaccharides on cell surfaces or in body fluids (Paton et al 1993). This activity has the potential to cause great damage to the host. Neuraminidase may aid S.pneumoniae adherence to the epithelial surface by unmasking potential adhesin receptors (Howie and Brown 1985) and by decreasing the viscosity of lung mucus (Yamazaki et al 1981). All strains of S.pneumoniae have been shown to have neuraminidase activity (Kelly et al 1967). Immunisation of mice with purified neuraminidase has been shown to convey a degree of protection against intranasal challenge with virulent S.pneumoniae (Lock et al 1988).

1.6.1.6 Hyaluronidase

Hyaluronidase is an enzyme produced by the pneumococcus. Its substrate is hyaluronic acid which is found associated with connective tissue and extra cellular matrix (Humphrey 1944). Thus hyaluronidase might play a role in pneumococcal

pathogenesis by allowing microbial access to host tissue for colonization. Hyaluronidase may also act as a nutrient gaining enzyme for the pneumococcus (Ginsburg 1985).

1.6.1.7 IgA protease

S.pneumoniae like several other bacterial species that colonize mucosal surfaces produces a protease which specifically cleaves IgA1 at the hinge region of the alpha-chain releasing Fab and Fc fragments (Kilian et al 1979; Plaut 1983). The mechanism by which cleavage of IgA1 protects S.pneumoniae remains to be elucidated and to date no conclusive evidence for the involvement of any of these proteases in pathogenesis has been demonstrated. A possible mode of action for IgA proteases is that by disabling IgA bacteria are able to protect themselves with non-functioning antibody which would block opsonisation by effective antibody (Paton et al 1993). Another possible mechanism is that IgA1 protease enzyme, which is expressed on the surface of the bacterium, could act as a novel adhesin for bacteria to IgA and mucus (Plaut 1983; Moxon and Wilson 1991).

1.6.2 Interaction of Streptococcus pneumoniae with the respiratory tract

Colonisation of the human nasopharynx by S.pneumoniae is important as a source of transmission to other susceptible individuals, as a source of infection of adjacent sites, and as a site of mucosal invasion leading to systemic

disease. Colonisation of the upper respiratory tract by S.pneumoniae is common (Riley et al 1981). Nasopharyngeal colonisation can have one of two consequences : in the majority carriage results in an immune response capable of eliminating the pneumococcus; in a minority of cases the pneumococcus becomes invasive and causes disease (Gray et al 1980). Infection usually occurs within 1 month of acquiring a new pneumococcal type; prolonged carriage rarely results in invasive disease (Gray et al 1980).

1.6.2.1 Animal models

The interaction of S.pneumoniae with the respiratory mucosa of mice infected with the influenza virus demonstrated that pneumococci did not adhere to the epithelium of control animals. However pneumococci adherence was significantly increased six days after viral infection (Plotkowski et al 1986). At sites where there had been desquamation of viral infected cells pneumococci were observed adhering to the microvilli of basal cells and to exposed basement membrane (Plotkowski et al 1986).

1.6.2.2 Dispersed epithelial cells

Studies have demonstrated that the pneumococci will adhere to dispersed buccal and nasopharyngeal epithelial cells (Andersson et al 1981; Andersson et al 1983; Andersson et al 1985; Andersson et al 1988; Selinger and Reed 1989). S.pneumoniae has been shown to attach to human pharyngeal cells through the specific interaction of bacterial surface

adhesins with epithelial cell glycoconjugates containing disaccharide GlcNACß1-3Gal ß (Andersson et al 1983) or GalNACß1-Gal (Kirvan et al 1988). Work has also identified an adhesin that forms a link between components of the pneumococcal cell surface and the carbohydrate receptors on the host cell (Andersson et al 1988). Epithelial cells from patients with recurrent respiratory tract infections by S.pneumoniae have been shown to be more susceptible to bacterial adhesion than cells from patients without underlying pulmonary disease (Mbaki et al 1989).

Adhesion of S.pneumoniae to nasopharyngeal cells from children found that the bacteria adhered more frequently to desquamated cells in mucus than to normal ciliated or non-ciliated cells (Lundberg et al 1982).

1.6.2.3 Ciliated epithelium

a) Amphibian organ cultures

S.pneumoniae has been shown to rapidly adhere to mucus in an animal organ culture model of respiratory epithelium (Plotkowski et al 1989). Bacteria were never seen to adhere to ciliated cells or cilia. Even following removal of mucus by washing before application of the inoculum, the pneumococci were only seen adhering to mucus which had not been totally eliminated (Plotkowski et al 1989).

b) **Human tissue**

Very few studies have been carried out on S.pneumoniae

adhesion to human ciliated respiratory epithelium. The existing data points to a lack of adhesion to normal respiratory cells. Using a submerged organ culture model S.pneumoniae has been shown to rapidly adhere to mucus but not to ciliated cells or cilia (Feldman et al 1992). Infection demonstrated a dense collection of bacteria in a thickened gelatinous layer overlying the organ culture. The scanning electron microscopy (SEM) appearances of this layer were unusual for simple mucus and it was suggested that it was abnormal consisting of mucus with bacterial products such as capsular products. Bacteria were found in large numbers within this layer. Formation of an abnormal mucinous layer (Feldman et al 1992) and the presence of ciliary function compromised by the action of pneumolysin (Feldman et al 1990) could be a novel mechanism of bacterial colonisation.

1.7 Pseudomonas Aeruginosa

P.aeruginosa is an obligate aerobic Gram-negative bacillus with a polar flagellum and pili and is widely distributed in soil and water (Costerton 1979). In man P.aeruginosa is an opportunistic pathogen, colonising the lungs of patients with cystic fibrosis, other forms of severe bronchiectasis and severe cardiopulmonary disease. P.aeruginosa may also cause pneumonia in the immunocompromised, patients with malignancy, burns patients and patients on the intensive care unit.

1.7.1 Virulence factors

The virulence of P.aeruginosa is multi-factorial and is dependant on both host and bacterial variables. Colonization of the lungs with virtually any P.aeruginosa strain induces a strong antibody response in serum, saliva and pulmonary secretions. In patients with cystic fibrosis increased antipseudomonal antibodies titres may precede the isolation of the pathogen from the respiratory tract (Brett et al 1992). These antibodies are raised against a number of antigens including proteases and mucoid exopoly-saccharide, LPS and OMP. However once colonisation is established the bacteria is rarely eliminated, despite antibiotic treatment. The failure to eliminate P.aeruginosa despite the immune response is a result of the immunoevasive activities of P.aeruginosa (Wilson et al 1985; Buret and Cripps 1993) often combined with a pre-existing weakness in the host defense.

1.7.1.1 Adhesins

P.aeruginosa pili, mucoid exopolysaccharide, haem-agglutinins, and possibly OMP mediate adherence of P.aeruginosa to the respiratory tract (Hata and Fick 1991; Woods et al 1990). Using non-mucoid P.aeruginosa it has been demonstrated that tracheal cells bind the organism more avidly than buccal cells and that tracheal cells from tracheostomised patients bind more bacteria than cells from normal subjects (Niederman et al 1994). Other variables which influence the number of epithelial cell receptors for

P.aeruginosa include the density of cilia, structure of surface mucins and glycoproteins, and the amount of damaged epithelium. Bacterial elastase, alkaline phosphase and phospholipase C actively enhance the process of adherence, possibly by unmasking a potential receptor for P.aeruginosa (Nicas and Iglewski 1986; Saiman et al 1990).

P.aeruginosa pili are composed of pilin protein subunits which mediate adherence of non-mucoid P.aeruginosa to mucin (Ramphal 1987) and to damaged epithelial cells (Ramphal et al 1984) adherence may be blocked by pre-incubation of respiratory epithelial cells with purified pili (Woods et al 1980). In contrast the adhesion of mucoid P.aeruginosa to mucin has been reported to be mediated by the mucoid exopolysaccharide (Ramphal et al 1987).

1.7.1.2 Pigments

P.aeruginosa produces at least four distinct pigments and several precursors. The four distinct pigments are known as pyocyanin, pyoverdin, pyorubin and pyomelanin.

The distinctive blue pigment of P.aeruginosa was first noted as a blue/green discolouration of surgical dressings (Gessard 1882). Fordos first isolated a crystallised substance (Fordos 1860) and discovered the indicator and redox properties of the compound. Pyocyanin has been shown to cause slowing and dyskinesia of the beating of human cilia (Wilson et al 1987) and to inhibit epidermal cell

growth and lymphocyte proliferation (Cruikshank and Lowbury 1953; Sorenson et al 1983). Pyocyanin is also known to inactivate both the production of IL2 and the expression of its receptor on the T cell membrane (Nutman et al 1987; Muhlradt et al 1986). Pyocyanin is a redox compound (Hassett 1992) which has been previously shown to stimulate redox cycling in bacteria, liver cells and a human epithelial cell line (Armstrong et al 1971; Cohen et al 1990; Hassan and Fridovich 1980). Free radical generation has also been proposed as a mechanism of its antibacterial action (Hassat and Cohen 1989).

Pyocyanin slowly decomposes to a yellow substance 1-HP (Fordos, 1863). Synthetic 1-HP (>10μM) immediately slows and disorganises the beating of human cilia (Wilson et al 1987) and causes immediate slowing and then recovery of the tracheal transport velocity of mucus in the guinea pig (Munro et al 1989). Synthetic pyocyanin (>10μM) however causes progressive ciliary slowing of human cilia after incubation for 1 hour and slowing of tracheal transport velocity of mucus after 2 hours. 1-HP has also been shown to inhibit mammalian cell respiration (Stewart-Tull and Armstrong 1972). Pyocyanin and 1-HP are detectable in the sputum of patients with bronchiectasis at levels as high as 27.3μg/ml and 3.5μg/ml respectively (Wilson et al 1988). Therefore <u>in vivo</u> these toxins are found in concentrations above that required to cause ciliary slowing and epithelial disruption <u>in vitro</u>.

1.7.1.3 Haemolysins

Two classes of haemolytic substance are produced by P.aeruginosa. Heat stable glycolipids (rhamnolipids) and heat labile phospholipase C (lecithinase). Glycolipids are produced by clinical isolates during the stationary phase of growth. They have a detergent like structure with a polar head and a non-polar tail and their surfactant like properties may account for their known haemolytic activity. They cause ciliary stasis associated with altered ciliary membranes in rabbit tracheal epithelium (Hingley et al 1986), and interfere with epithelial ion transport in vitro in sheep in a dose dependant manner (Graham et al 1993). At pathophysiological concentrations they act as mucus secretagogues in the cat in vivo (Somerville et al 1992), slow CBF and disrupt the epithelial integrity of human epithelium in vitro (Read et al 1992).

Phospholipase C is a lecithinase which liberates phosphorylocholine from lecithin. Phosphorylocholine causes in vitro platelet aggregation (Coutinho et al 1988), the release of inflammatory mediators from human granulocytes in vitro (Bergmann et al 1989) and the accumulation of granulocytes in the peritoneal cavity of mice injected with phosphorylocholine (Meyers and Berk 1990). They have been shown to damage ciliated epithelium.

1.7.1.4 Proteases

Proteolytic enzymes from P.aeruginosa stimulate mucus

secretion from animal tissue in vitro (Adler et al 1986), in vivo in the cat (Somerville et al 1991) and in human tissue in vitro (Somerville et al 1991). This may enhance bacterial clearance since an increase in mucus load may stimulate ciliary beating. However this host defense may be ineffective if the stimulated mucus possesses abnormal rheological properties (Puchelle et al 1980) or if the increase output is sufficient to uncouple cilia from the mucus layer (Sleigh et al 1988). Other bacterial products including pyocyanin, rhamnolipid and 1-HP may join with proteases to disrupt and slow normal ciliary beating (Wilson et al 1987). Proteases of P.aeruginosa also play an important role in modulating the humoral and cellular response to infection.

1.7.2 Immunoevasion

Modulation of cellular and humoral immunity is an important feature of the immunoevasive properties of P.aeruginosa. These properties will be discussed below.

1.7.2.1 Inactivation of complement

The alginate layer of mucoid strains of P.aeruginosa may interfere with opsoninization preventing the normal antibody/complement coating (May et al 1991). P.aeruginosa has also been shown to degrade complement components at the site of colonisation with reduction of expression of the C3b receptor. This occurs as a result of cleavage by host and bacterial proteases of the C3b receptor on

bronchoalveolar polymorphonucleocytes (Berger et al 1988; Tosi et al 1988). The proteases can also inactivate the chemotactic activity of the complement component C5 (Schultz and Miller 1974). Inactivation of complement at the site of infection results in reduced phagocytosis and reduced bacterial elimination and is one method by which P.aeruginosa evades the host response.

1.7.2.2 Inactivation of cytokines

P.aeruginosa elastase and alkaline phosphatase can degrade IL2, which is known to be a primary chemotactic and growth factor for T cells and also to inactivate human interferon and human TNF alpha (Theander et al 1988; Horvat and Parmely 1988; Parmely et al 1990).

1.7.2.3 Inhibition of phagocytosis

Opsonic phagocytosis of P.aeruginosa during infection may be impaired by a number of mechanisms. Proteolysis of opsonic IgG; proteolysis of the C3b surface receptor on neutrophils; inhibition of antibody and/or complement binding to P.aeruginosa; proteolysis of fibronectin and hydrolysis of phosphatidylcholine (Buret and Cripps 1993).

Non-opsonic phagocytosis can also be impaired by P.aeruginosa. Non-mucoid strains of P.aeruginosa are phagocytosed by human neutrophils and macrophages in the absence of serum opsonins. This process is dependent on the hydrophobic interaction of phagocytic cells with pili.

Mucoid strains of P.aeruginosa are relatively resistant to non-opsonic phagocytosis and it has been suggested that the mucoid exopolysaccharide probably modulates bacterium phagocyte interactions by altering the bacterial hydrophobic characteristics (Cabral et al 1987).

1.8 Aims of Thesis

The aim of this thesis was to investigate the interaction between some bacteria and bacterial products with the respiratory mucosa in vitro and in vivo. Specifically it was intended :

1) To assess the interaction of piliated variants of N.meningitidis and pneumolysin sufficient and deficient S.pneumoniae with the respiratory mucosa using an organ culture system with an air-mucosal interface.

2) To assess the effect of P.aeruginosa toxins pyocyanin, 1-HP and rhamnolipid on the function and orientation of human nasal cilia.

3) To assess the effect of chronic inflammation due to infection on mucociliary function and ciliary orientation in vivo.

4) To assess ciliary disorientation in patients with the clinical syndrome of PCD, but with normal ciliary axoneme ultrastructure and normal or near normal CBF.

2.0 Materials And Methods

2.1 Materials

Acrodisc, Northampton, UK.

0.2 μm syringe filter discs

Agar Scientific, Stansted, Essex, UK.

Aluminum stubs 0.5 inches

Araldite

Gluteraldehyde

Paraformaldehyde

Amersham International, Buckinghamshire, UK.

Silver enhancement kit IntenSE^{tm}

Bluestar, Wakeley, UK.

Slides 76 x 26 mm

Biocell Research Laboratories, Cardiff, UK.

Protein A with a 5 nm gold particle

Bovine serum albumin (BSA)

Fish gelatin (45%)

Diagmed, Thirsk, Yorkshire, UK.

Cytology brushes with 2 mm bristles

Evans Medical Ltd, Leatherhead, Surrey, UK.

Betnesol-N

C Horwell, London, UK.

22 x 50 mm coverslips

Flow Laboratories, Irvine, Scotland.

Tissue culture Medium 199 with Earles salts and 20 mM HEPES (M199)

Gibco Life Technologies Ltd, Paisley, Scotland.

Streptomycin

Penicillin

Gentamicin

Foetal calf serum

Minimal Essential Medium (MEM) with 25 mM HEPES with Earles salts without glutamine

Image Processing and Vision Company Ltd, Coventry UK.

Improvision image analysis system

Johnson Matthey, Royston, Herts, UK.

Osmium tetroxide

Merck, Poole, UK.

Glycerol (general purpose reagents)

Acetone (general purpose reagents)

Methanol (general purpose reagents)

Propylene oxide (general purpose reagents)

Sodium cacodylate (general purpose reagents)

Sodium dodecyl sulphate (SDS)

2M Sodium hydroxide

2 ml Eppendorf safe lock tubes

Pharmax, London, UK.

Colomycin BP

Oxoid, Basingstoke, Hampshire.

Agar No 1

Blood agar No 2

Brain heart infusion agar

Brain heart infusion

RS Components, Corby, Northants, UK.

Quick set epoxy adhesive

Sigma Chemicals, Poole, Dorset, UK.

Nicotine adenosine dinucleotide (NAD)

Sterilin, Stone, UK.

Bijoux

Universal containers

Petri dishes

Whatman Scientific Ltd, Maidstone, Kent, UK.

Filter paper No3

2.2 Equipment

All glass and plastic ware was sterilised by autoclaving at

121°C for 15 min. Solutions were sterilised either by autoclaving or by filtration through a 0.2 μm filter. All water used was distilled and sterilised.

Edwards, Crawley, Sussex, UK.
Edwards auto 306 carbon sputter coater

Envair, Rossendale, Lancs, UK.
Envair class 3 convertible microbiology safety cabinet

Fisons, Loughborough, Leics, UK.
MSE Coolspin centrifuge
Polaron gold sputter coater
Polaron critical point dryer

Grant, Cambridge, UK.
Grant water bath

Gallenkamp Loughbourgh, Leicestershire, UK.
Gallenkamp CO_2 incubator

Nissei Sangyo, Wokingham, Berkshire, UK.
Hitachi 4000 S field emission scanning electron microscope
Hitachi 7000 transmission electron microscope

Leitz, Luton, UK.
Leitz Dialux 20 phase contrast microscope
Leitz microscope photometer

Reichert-Jung, Cambridge UK

Lynx EM tissue processor

Perkin-Elmer Lambda 2 UV/VIS spectrophotometer.

2.3 Commonly Used Agars

Blood agar. 40 g of blood agar (No.2) was suspended in 1 l of distilled water, this was brought to the boil to dissolve completely. The solution was sterilised by autoclaving at 121°C for 15 min. The solution was cooled to 50°C and 7% sterile horse blood was added.

Levinthal agar. Levinthal extract was prepared by preheating a water bath to 85°C. Fresh horse blood was added to sterile brain heart infusion agar in 1:2 volume:volume. The solution was placed in a water bath at 85°C until a chocolate colour began to develop. The solution was then placed in an ice bath and transferred to a centrifuge and centrifuged at 2,000g for 30 min at 4°C. The supernatant was transferred to sterile universals and filter sterilised NAD at 1 g/l added.

Levinthal agar was prepared by adding 42 g of brain heart infusion agar to 1 l of distilled water. This was brought to the boil to dissolve the solute completely. The solution was autoclaved at 121°C for 15 min. The solution was then cooled to 56°C and 100 ml of Levinthal extract added.

2.4 Bacteria

2.4.1 Neisseria meningitidis

Piliated and non-piliated variants of N.meningitidis MC58 and a polyclonal rabbit antibody raised against OMP were kindly supplied by Professor E R Moxon and Dr M Virji, Department of Paediatrics, John Radcliffe Hospital, Oxford, UK.

Two Class I piliated variants and one non-piliated variant were selected after screening colonies of N.meningitidis strain MC58 (Virji et al 1992). One of the piliated variants (PIL+A) adhered poorly to Chang and HEP-2 epithelial cells, whereas the other (PIL+B) adhered well to both cell culture lines; both piliated variants adhered well to endothelial cells in culture.

2.4.2 Streptococcus pneumoniae

The pneumolysin sufficient (PL+) and deficient (PL-) variants of a type II S.pneumoniae were kindly supplied by Dr J C Paton, (Department of Microbiology, Adelaide Children's Hospital, North Adelaide, S.A., 5006, Australia). The variants were constructed by insertion-duplication mutagenesis (Berry et al 1989).

2.5 Bacterial Manipulations

2.5.1 Neisseria meningitidis

Inoculum preparation. The three bacterial strains were coded and all the experiments were completed and analyzed

before the code was broken. Bacteria were used after a single sub-culture from a liquid nitrogen stock stored in a mixture of 80% brain heart infusion broth and 20% glycerol at -70°C. The three variants of MC58 were grown on Levinthal supplemented brain-heart infusion agar at 37°C in 5% CO_2 in air for 18 h. Twenty colonies were suspended in 1 ml of phosphate buffered saline (PBS) and the suspension centrifuged at 80 g for 3 min to remove clumped bacteria. A sample from each suspension was diluted in 1% sodium dodecyl sulphate in 0.1 M sodium hydroxide. The sample was diluted to give an optical density of 0.5 at a wavelength of 260 nm which corresponded to a viable count of approximately 5 x 10^7 cfu/ml. The three meningococcal suspensions were then diluted with PBS to the sodium dodecyl sulphate dilutions.

2.5.2 Streptococcus pneumoniae

Preparation of inoculum. A standard inoculum was prepared as follows : 10 ml of brain heart infusion was inoculated with 4-5 colonies of S.pneumoniae from an overnight culture on blood agar (No.2). This was incubated for 12 h at 37°C and then centrifuged at 2000 g for 15 min. The bacterial pellet was resuspended in 1 ml of fresh serum broth containing brain heart infusion and inactivated foetal calf serum at a ratio of 1:5. This was diluted with fresh serum broth to give an optical density of 0.7 at a wavelength of 500 nm. This was then incubated for 4 h, a viable count performed in triplicate and the bulk of the culture frozen

at -70°C. Once the viable counts were known, this was thawed and diluted in fresh serum broth to give an inoculating dose of 1 x 10^7 cfu/ml. The standard inoculum was stored in 1 ml volumes at -70°C.

2.5.3 Viable counts

The counting of viable bacteria serial dilutions of bacterial suspensions were performed in sterile PBS. 20 μl aliquots of each dilution were plated on blood agar No 2 for S.pneumoniae and Levinthal supplemented agar for N.meningitidis. Colonies were counted after overnight incubation at 37°C at the dilution that produced between 10 and 30 colonies per 20 μl aliquots. Colony forming units per ml (cfu) present in the original bacterial suspension reflect the number of viable cells present and were calculated :

$$\text{cfu/ml} = \frac{\text{number of colonies x 50}}{\text{dilution}}$$

2.6 Organ Culture With an Air Mucosal Interface

2.6.1 Tissue preparation

Human adenoids, resected at operation, were placed in MEM containing antibiotics (penicillin (50 units/ml), streptomycin (50 μg/ml) gentamicin (50 μg/ml) to eradicate commensal flora. The tissue was transported to the laboratory where it was checked by light microscopy and tissue to have a smooth surface and actively beating cilia and, if so, transferred to fresh MEM with antibiotics. The tissue was then dissected into 4 mm^2 squares. Squares with

at least one completely ciliated edge were chosen for use in the organ culture. After 4 h in MEM with antibiotics the tissue has been shown to be sterile (Read et al 1991). The tissue was then placed in antibiotic-free MEM for 1 h.

2.6.2 Organ culture preparation

To prepare the organ culture system, a 3 cm petri dish was placed inside a 5 cm petri dish and 4 ml of MEM was placed in the outer petri dish with the inner dish remaining dry. A 70 mm long and 5 mm wide strip of sterile filter paper (Whatman No 1) was immersed in MEM, and then laid across the smaller inner petri dish, with its ends immersed in the media contained in the outer petri dish. The filter paper acted as a wick to supply nutrients to the tissue. The square of adenoid tissue was placed adventitial cut surface downwards onto the centre of the filter paper. Semi-molten agar (No 1) at 40°C was pipetted around the tissue to seal all the cut edges. The organ culture was then incubated at 37°C for 1 h in a humidified atmosphere containing 5% CO_2 at 37°C (Figure 4) (Tsang et al 1994).

2.6.3 Inoculation and incubation of organ cultures

2.6.3.1 Neisseria meningitidis

For each experiment four organ cultures were prepared. The first acted as a control and was inoculated with 20 μl of PBS. The second, third and fourth organ cultures were inoculated with 20 μl of the prepared inocula of the three variants of N.meningitidis in PBS and incubated in a

humidified atmosphere of 5% CO_2 in air at 37°C for either 4, 12 or 24 h. For each time point six experiments were performed with tissue from different donors. At the end of the given time period the four edges of each culture were touched with a sterile loop and plated on levinthal supplemented brain-heart infusion agar and blood agar (No 2) to confirm a pure growth of meningococci and sterility of the control. If the cultures were contaminated or the control was not sterile the experiment was discarded.

2.6.3.2 Streptococcus pneumoniae

Prior to inoculum the 1 ml inocula was thawed and then centrifuged at 2,000g for 3 min, washed and centrifuged 3 times through 1 ml of PBS. For each experiment 3 organ cultures were prepared. The control tissue was inoculated with 20μl of PBS, the second and third organ cultures with 20μl of the prepared standard inoculum of the type II pneumolysin sufficient and deficient variants. The cultures were incubated for 4, 24 and 48 h. For each time point six experiments were performed with tissue from different donors. At the end of the given time period the four edges of each culture were touched with a sterile loop and plated onto blood agar (No 2) to confirm a pure growth of S.pneumoniae and sterility of the control. If the cultures were contaminated or the control was not sterile the experiment was discarded.

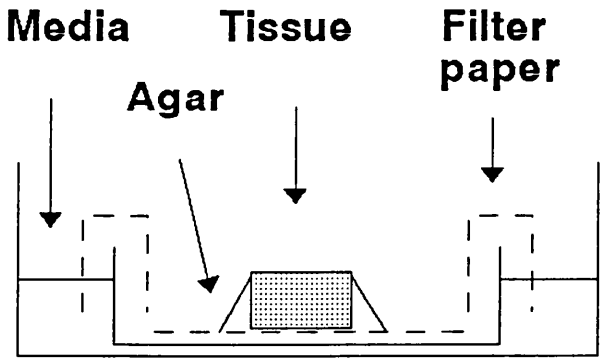


Figure 4

Schematic diagram of an organ culture with an air mucosal interface.

2.6.4 Ciliary beat frequency recording of organ cultures infected by Streptococcus pneumoniae

The CBF was recorded at the end of each incubation period. The organ culture was removed from the filter paper and placed in a 3 cm petri dish. 1 ml of MEM at 37°C was carefully pipetted along the sides of, but not covering, the organ culture. The petri dish was placed on a warm stage at 37°C. Ciliary beating could be visualized by light microscopy (x 320) along the edge of the tissue. At 24 h and 48 h ciliary beating could not be visualised in areas where mucus or cell debris had accumulated, and for this reason CBF measurements were only taken where cilia were seen beating. The CBF was measured using a photometric technique using a Leitz Dialux 20 phase contrast microscope. A Leitz microscope photometer transduced light intensity into an electrical signal. The cilia were positioned to interrupt the passage of light through a small diaphragm into the photometer. The electrical signal generated was converted into a reading of CBF (Hz) by an automated CBF processor unit (Greenstone et al 1984). The CBF was calculated as the mean of 10 separate areas of beating cilia.

2.6.5 Electron microscopy

2.6.5.1 Processing and fixation for scanning electron microscopy

Glutaraldehyde was diluted in sodium cacodylate to make a 2.5% solution, hydrochloric acid was added to adjust the pH

of the solution to 7.2, final molarity 0.5 M.

The tissue was fixed in 2.5% sodium cacodylate buffered glutaraldehyde pH 7.2 for 24 h before processing through three cacodylate buffer washes for 20 min, and one cacodylate buffer was for 60 min, using a Lynx processor. The tissue then underwent post fixation in a 1% osmium tetroxide solution in sodium cacodylate buffer for 1 h. Using the lynx processor the tissue then underwent a graded series of dehydration through 70%, 90% and 100% methanol and then 100% acetone. The tissue then underwent critical point drying in CO_2 using a Polaron critical point dryer. The tissue was mounted onto aluminium stubs using a quick set epoxy adhesive and was then given a conductive coating of gold using a Polaron gold sputter coater (Read et al 1991).

2.6.5.2 Scanning electron microscopy

Samples were coded so that the observer was blind to the experimental conditions. The samples were examined using a Hitachi 4000 S field emission scanning electron microscope. A careful morphometric assessment was made of the mucosal surface of the organ culture. The centre of the sample was positioned in the centre of the screen. A 10 x 10 cm grid of 100 squares was placed over the image at a screen magnification of x 50. The grid was used to select a representative sample of the total surface area using a standard pre-selected pattern of squares. Two diagonal

lines were followed from grid corner to grid corner, each containing 10 squares. A further 5 squares were selected from a fixed point at the centre of each of the four quadrants of the grid to make a total of 40 squares. Each of the 40 squares were then examined at a screen magnification of x 2000. The grid was again used to divide the area under examination into 100 squares. The number of these squares occupied by mucus, cell damage, ciliated and unciliated cells was counted and used to calculate the percentage surface area occupied by that mucosal feature. If more than 50% of a square was occupied by a single mucosal feature that feature was scored but if the square was equally occupied by two features each feature was scored as 0.5%. For each sample the mean percentage surface area occupied by each mucosal feature was estimated by analysis of all 40 squares. Each of which had a surface area of 800 micrometres^2. The total area of each sample examined was therefore 0.032 mm^2. The term cell damage was used to encompass extruded cells, cells with surface pitting and/or cytoplasmic surface blebbing, and cell debris. Unciliated areas were defined as areas not containing cilia, with or without microvilli and also cells with obvious loss of tight junction integrity. When bacteria were seen they were counted and their position and the presence of any interaction with respect to each mucosal feature carefully noted. For each sample the number of bacteria associated with each of the mucosal features was recorded. To estimate the density of bacteria

associated with each mucosal feature this figure was divided by the percentage of the mucosal surface occupied by that feature.

2.6.5.3 Processing and fixation for transmission electron microscopy

Tissue was fixed in 2.5% sodium cacodylate buffered glutaraldehyde at pH 7.2 for 24 h. The tissue was then fixed in a 1% osmium tetroxide solution in sodium cacodylate buffer pH 7.2 for 60 min. Using a Lynx processor the tissue was then washed 3 x 5 min in distilled water and then was exposed to a graded series of dehydration in 70% methanol for 15 min to 90% methanol for 30 min and then 100% methanol for 70 min. The tissue was then transferred to 100% propylene oxide for 60 min, then a mixture of 75% propylene oxide: 25% araldite for 30 min, then to 50% propylene oxide: 50% araldite for 30 min, and then 25% propylene oxide: 75% araldite for a further 30 min. The tissue was finally embedded in 100% araldite (Read et al 1991).

2.6.5.4 Transmission electron microscopy

Analysis was performed by Mr R Rutman. Samples were coded so that the observer was unaware of the experimental conditions. For transmission electron microscopy (TEM) assessment a single ultrathin section (70-90 nm) through the central portion of each specimen was examined. Sections typically contained 150-250 cells. Each epithelial cell

observed was scored according to the following parameters (Tsang et al 1993) :

1) Extrusion of a cell from the epithelial surface (0=no extrusion, ++=cell fully extruded but in contact with epithelium, +=intermediate).
2) Cells bearing cilia were scored for loss of cilia, as it was not possible to determine if a totally unciliated cell was originally unciliated or had lost all its cilia during the culture (0=fully ciliated, ++=sparsely ciliated, +=intermediate).
3) Numbers of unciliated cells.
4) Presence of cytoplasmic blebbing from the luminal surface of ciliated and unciliated cells (0=nil, ++=severe, +=intermediate).
5) Mitochondrial damage in ciliated and unciliated cells (present or absent).

2.7 Immunogold Labelling

Difficulties in reliably identifying bacteria, both underlying the microvilli and in association with damaged cells led to the development and use of an immunogold label using a rabbit polyclonal antibody raised against N.meningitidis OMP.

2.7.1 Solutions for immunogold labelling

Washing buffer: PBS with 0.8% bovine serum albumin and 0.1% fish gelatine.

Blocking buffer: PBS with 0.8% bovine serum albumin, 0.1%

fish gelatine and 5% fetal calf serum pH 7.4.
Primary antibody diluted using blocking buffer.
Protein A diluted using blocking buffer.

2.7.2 Immunogold experiments

Preliminary experiments were performed to determine the optimal dilutions of protein A and the specificity of the immunogold label for bacteria (method outlined in Figure 5).

Eight organ cultures were prepared. Four cultures were inoculated with PBS and four organ cultures were infected with PIL+ B bacteria (Figure 5). The cultures were incubated for 12 h which was chosen for these experiments because changes in the mucosa and bacterial adherence were already present, but mucosal damage was not severe. At the end of 12 h the tissue underwent light fixation in a solution of cacodylate buffer with 0.05% glutaraldehyde and 2% paraformaldehyde pH 7.2 for 60 min.

The tissue was washed (3 x 5 min) in washing buffer at pH 7.4 at room temperature, and was then incubated with the blocking buffer at pH 7.4 at room temperature for 60 min. The tissue was them washed (3 x 5 min) in washing buffer. Three control and 3 infected cultures were then incubated with a rabbit polyclonal antibody raised against OMP at

Figure 5

Experimental design for immunogold labelling of N.meningitidis

Organ culture

↓

Inoculum

PBS or PIL+B

↓

Incubation for 12 h

↓

Light Fixation

↓

Washing buffer

↓

Blocking buffer

↓

Primary antibody dilution

↓

Washing buffer

↓

Protein A

↓

Fixation

↓

Silver enhancement

↓

SEM +/- Backscatter

dilution of 1:50 in blocking buffer in a total volume of 200 µl for 60 min at room temperature. One control and one infected culture were incubated in 200 µl of blocking buffer only for 60 min at room temperature. After washing (3 x 5 min) in washing buffer the tissue was incubated in 200 µl of protein A solution with a 5 nm gold particle (Biocell, UK) at 1:50, 1:100, or 1:200 dilution in blocking buffer for 60 min at room temperature. The tissue was then washed (3 x 5 min) in washing buffer before fixation in sodium cacodylate buffered 2.5% glutaraldehyde pH 7.2 for 24 h.

After fixation for 24 h the tissue was washed (3 x 5 min) with high resistance water before undergoing silver enhancement using IntenSEM. 0.5 ml of enhancer solution was added to 0.5 ml of initiator solution in a 5 ml bijou bottle and 100 µl of the solution pipetted on to the surface of the tissue and then incubated at room temperature (22°) for 20 min. The tissue was immediately placed in cacodylate buffer to prevent self-nucleation.

2.7.2.1 Preparation for scanning electron microscopy

Tissue preparation for electron microscopy was performed as in section 2.6.5.1. Following critical point drying the tissue was mounted on aluminium stubs using a quick set epoxy adhesive and given a conductive coating of carbon.

2.7.2.2 Scanning electron microscopy

Only bacteria which were clearly visible were counted in

the preliminary experiment. Each square was counted with and without back scatter. The back scatter detector was adjusted to allow clear definition of particles on the surface of bacteria prior to making an assessment of background non-specific labelling.

Bacteria appeared "brighter" with the gold label despite the back scatter attachment not being connected. The blocking agents prevented non-specific labelling even at 1:50 dilutions of protein A and primary antibody. This was associated with up to 96% of bacteria taking up the label (Table 2). Protein A at a dilution of 1:50 had the highest affinity for bacteria so this dilution was chosen.

2.7.3 Preliminary experiment using the three MC58 variants

A preliminary experiment using the immunogold label and three MC58 meningococcal variants was performed to ensure that all three variants were labelled. Tissue preparation and bacterial inoculation were as described in sections 2.5.1 and 2.5.2.

At the end of 12 h the tissue underwent light fixation in a solution of cacodylate buffer with 0.05% glutaraldehyde and 2% paraformaldehyde pH 7.2 for 60 min.

2.7.3.1 Immunolabelling

Immunogold labelling was as described as in section 2.7.2 using a rabbit polyclonal antibody at a dilution of 1:50 and a solution of protein A at a 1:50 dilution in blocking buffer. 200 μl of the antibody solution was incubated with tissue for 60 min at room temperature. The tissue was washed (3 x 5 min) with 200 μl of washing buffer. The tissue was incubated with 200 μl of the protein A solution for 60 min at room temperature. Silver enhancement and preparation for electron microscopy were as described in section 2.7.2.

Table 2

The effect of varying the dilution of protein A solution on the number of clearly visible Pil B bacteria which labelled with immunogold

Primary antibody	Protein A	Clearly visible bacteria	Labelled bacteria	% labelled
1:50	1:50	120	116	96.7
1:50	1:100	115	86	74.8
1:50	1:200	112	53	47.3
0	1:50	142	0	0

2.7.3.2 Scanning electron microscopy

Using the two screens on the microscope, the tissue was examined simultaneously using the normal secondary electron mode as described above and the back scatter mode (Figure 6). In this way it was possible to both quantify and identify the location of the bacteria (Table 3). For photographic recording, the back scatter and secondary electron images were superimposed using a mixing attachment.

This experiment demonstrated that all three variants labelled with immunogold. The experiment also demonstrated that bacteria underlying microvilli and in association with debris could be located, identified and counted.

2.7.4 Immunogold label of the three MC58 meningococcal variants at 12 hours

The experiments described above confirmed that all three variants labelled with immunogold and that bacteria underlying microvilli and in association with debris could be located, identified and counted. Six experiments were then performed using the immunogold label following 12 h incubation.

Tissue preparation and inoculation were as described previously in section 2.5.1 and 2.5.2. The tissue then underwent light fixation, immunolabel and silver enhancement (section 2.7.2). Fixation, preparation for

Figure 6

Using the two screens on the microscope, the tissue was examined simultaneously using the normal secondary electron mode as described above and the back scatter mode.

Table 3

The number bacteria associated with the mucosa of one adenoid organ culture at 12 h counted without and with back scatter

	Mucus	Damaged cells	Ciliated epithelium	Unciliated epithelium	Total number
Non-piliated	4/6 (2)	8/8 (0)	6/4 (-2)	22/22 (0)	40/40 (0)
Piliated A	2/2 (0)	16/20 (4)	2/2 (0)	158/250 (92)	178/274 (96)
Piliated B	2/2 (0)	40/44 (4)	4/4 (0)	174/274 (100)	220/324 (104)

The results are shown as the number of bacteria counted in 0.032 mm^2 of mucosal surface using the normal secondary electron mode only and then with back scatter. For example for non-piliated bacteria associated with mucus 4/6 corresponds to 4 bacteria counted using the normal secondary electron mode only and 6 with the back scatter mode. The number in brackets represents the number of bacteria detected with back scatter which were not seen with the normal secondary electron mode alone.

electron microscopy and analysis by SEM was as in sections 2.7.1.3, 2.7.2.2 and 2.7.3.2 respectively.

2.8 The Effect of Pyocyanin, Rhamnolipid and 1-Hydroxyphenazine on Ciliary Orientation

2.8.1 Preparation of bacterial products

Pyocyanin, 1-HP and rhamnolipid were prepared, purified and supplied by Dr GW Taylor, Department of Clinical Pharmacology, Royal Postgraduate Medical School, Hammersmith Hospital, London.

2.8.1.1 Preparation and purification of pyocyanin

Pyocyanin was prepared by photolysis of phenazine methosulphate (Aldrich Chemical, Milwaulkee, Wis) (Knight et al 1979; (Watson et al 1986). Previous studies in our department have shown that this synthetic compound is identical to pyocyanin extracted from the sputum of patients colonised by P.aeruginosa (Wilson et al 1987 and 1988). Pyocyanin was reconstituted in Medium 199 and used at a final concentration of 25 ug/ml which has previously been shown to reduce human CBF in vitro (Wilson et al 1987).

2.8.1.2 Preparation and purification of 1-hydroxyphenazine

1-HP was prepared by photolysis of phenazine methosulphate, purity was determined by ultraviolet absorbency and thermospray mass spectometry (Watson et al 1986). 1-HP was

used at a final concentration of 20 μg/ml which has previously been shown to reduce human CBF in vitro (Wilson et al 1987).

2.8.1.3 Preparation of rhamnolipid

Rhamnolipid was prepared and purified as described by Somerville et al (1991) from P.aeruginosa strain p455. Monorhamnolipid was used for all experiments at a final concentration of 125 μg/ml, a dose which has been shown to release mucus glycoconjugates from feline trachea (Somerville et al 1991) and to slow human CBF in vitro (Read et al 1991).

2.8.2 Ciliary beat frequency assay

2.8.2.1 Method of obtaining ciliated epithelium

Ciliated epithelium was obtained from the inferior nasal turbinate of both nostrils under direct vision by a brush biopsy technique without using local anaesthetic (Rutland and Cole 1980) from healthy non-smoking volunteers. The subject blows their nose to remove excess secretions. A 2 mm diameter cytology brush was immersed in medium 199 and inserted along the side of the inferior nasal turbinate under direct vision using an auroscope. The brush is twisted twice forwards and backwards as it moved. Epithelium adherent to the brush is then dislodged into medium 199 contained in a universal tube. If either small strips of epithelium are not clearly seen to be floating in the media, or if the sample was insufficient the procedure

was repeated at the same or different site. The procedure was approved by Brompton Hospital Ethical Committee.

2.8.2.2 Preparation of epithelial suspensions

For each experiment epithelium from one volunteer was dispersed by gentle agitation in 5 mls of cell culture medium 199 with Earles salts and HEPES. The sample was divided into two equal samples in 7 ml bijou bottles. One sample acted as the control, and to the other one of the three Pseudomonas products was added to give final concentrations of pyocyanin 25 ug/l, 1-HP 20 ug/l and rhamnolipid 125 ug/l. Six experiments were performed for each agent.

2.8.2.3 The measurement of ciliary beat frequency

A sample from each epithelial suspension was removed and enclosed within a sealed microscope coverslip-slide preparation for measurement of CBF. A coverslip-slide preparation was constructed by pipetting high vacuum grease around the edge of a coverslip to create a well. 0.5 ml of epithelial suspension was then pipetted into one half of the well. A glass microscope slide was then carefully lowered into place advancing the suspension over the base of the well, removing air and sealing the preparation.

For each experiment two sealed microscope coverslip-slide preparations containing epithelium in medium 199 or medium 199 plus pyocyanin or 1-HP or rhamnolipid were prepared. The slides and bijou bottle containing the remaining

epithelial suspension were incubated at 37°C and allowed to equilibrate for 10 min. CBF was measured at 60 min intervals using a photometric technique, slides were placed on a controlled warm stage (Microtec, Oxford UK) at 37°C and viewed directly on a Leitz Dialux 20 phase contrast microscope at magnification x 320 under bright field illumination. A strip of beating cilia was chosen and the beating cilia were positioned to interrupt the passage of light through a small diaphragm into a Leitz MPV microscope photometer which converts light intensity into an electrical signal which is subsequently converted into a beat frequency in Hz (Greenstone et al 1984). For each sample CBF was calculated as the mean of readings taken from 10 separate strips of beating cilia in the control and test slide. A record of the position of the epithelial strips was taken to enable repeat measurements to be made from the same strip. Direct viewing at a magnification of x 320 allowed both assessment of epithelial integrity and assessment of ciliary dyskinesia which was defined as absence of the normal coordinated movement of the cilia. When ciliary dyskinesia or epithelial disruption was noted in 5 or more of the strips, the sample from the bijou bottle was fixed in 2.5% cacodylate buffered glutaraldehyde pH 7.2 in a round bottomed plastic tube (internal diameter 9.2 mm, length 75 mm).

2.8.3 Orientation and ultrastructure assessment

2.8.3.1 Fixation and processing

The epithelium was fixed in cacodylate buffered 2.5%

glutaraldehyde pH 7.2 for 24 h during which time the strips settled to the bottom of the tube. Supernatant gluteraldehyde was removed and the strips suspended in cacodylate buffer pH 7.2 (3 x 5 min). The strips were then post fixed in 1% osmium tetroxide for 1 h. The tubes were then wedged into a rack to prevent floating and placed in a water bath at 42°C. Using a pasteur pipette one drop of 2% agar at 42°C was mixed with the specimen and left to settle for 3 min before being solidified at 4°C(Rutland et al 1982). The agar embedded tissue underwent processing for electron microscopy. Using a lynx processor the tissue then underwent a graded series of dehydration in 70% methanol for 15 min to 90% methanol for 30 min and then 100% methanol for 70 min. The tissue was then transferred to 100% propylene oxide for 60 min, then a mixture of 75% propylene oxide : 25% araldite for 30 min, then to 50% propylene oxide : 50% araldite for 30 min and then 25% propylene oxide : 75% araldite for a further 30 min. The tissue was finally infiltrated with 100% araldite. Prior to araldite embedding any excess agar was trimmed.

2.8.3.2 Transmission electron microscopy

Using TEM at a magnification of x 3,000, continuous strips of epithelium were examined and groups of cilia originating from a single cell were chosen for further evaluation. At a magnification of x 36,000, fields with a minimum of 10 cross sections of cilia in which central pairs could be clearly seen were captured on to disc. Continuous strips of

epithelium were examined and cells in which basal foot processes were clearly visible were chosen for further examination. At a magnification of x 24,000, cells with at least 10 basal foot processes visible were captured onto disc.

2.8.3.3 Measurement of ciliary orientation

Ciliary orientation was investigated using an Improvision image analysis system on an Apple Macintosh II-fx computer. The image captured onto disc was displayed onto the computer screen. On each image a line was electronically drawn through the central pair of each cross section and the angle of each line was measured by the computer. Vertical up= 0°, horizontal right=90,° vertical down=180° (Figure 7). Thus within each image the angle of orientation of each cilium was obtained and the standard deviation of the angles per cell calculated. The mean standard deviation of the cells represented the overall measurement of ciliary orientation for that experiment.

For each experiment basal foot orientation per cell was also measured. Sections of cells with basal foot processes visible were examined. A line was electronically drawn which transected the mid-point of the base and the apex of the basal foot (Figure 8). The angle of each line was measured by the computer. Vertical up= 0°, horizontal right=90°, and vertical down=180°. Thus within each image the angle of orientation of each basal foot was obtained

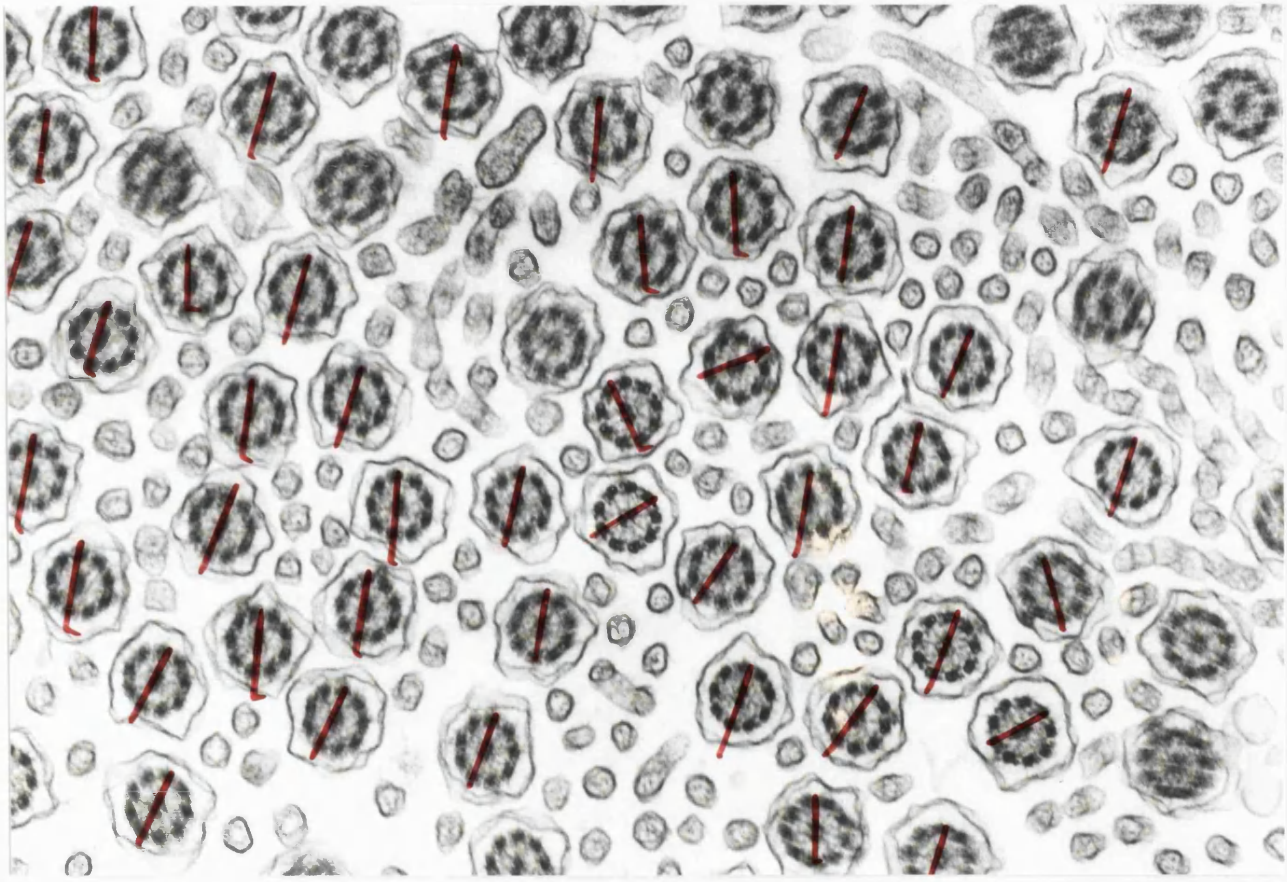


Figure 7

On each image a line was electronically drawn through the central pair of each cross section. Magnification x 50,000.

and the standard deviation of the angles per cell calculated.

2.9 Clinical Study Of Ciliary Orientation In Patients With Chronic Inflammation Due To Infection And Patients With The Clinical Features Of Primary Ciliary Dyskinesia But Normal Ciliary Beat Frequency And Ultrastructure

2.9.1 Orientation in chronic mucopurulent sinusitis

2.9.1.1 Study population

The study population consisted of 10 healthy non-atopic, non-smoking volunteers, 15 patients with idiopathic bronchiectasis and chronic mucopurulent sinusitis, 12 patients with cystic fibrosis and 2 patients with the clinical features of PCD. These features included bronchiectasis, chronic mucopurulent sinusitis, situs inversus and absent nasal mucociliary clearance (NMCC). However, they had a normal CBF and ciliary ultrastructure. The 15 patients with bronchiectasis, 5 men, age range 17-56, under the care of Professor Cole who regularly attended a clinic for patients with bronchiectasis at The Royal Brompton Hospital, London. All had undergone computerised tomography to confirm the diagnosis of bronchiectasis and all had undergone the investigations in Table 4 which had failed to reveal an aetiology for the bronchiectasis. Chronic mucopurulent sinusitis was diagnosed on clinical and radiological grounds. This included a six month history of post nasal drip with purulent nasal secretions and

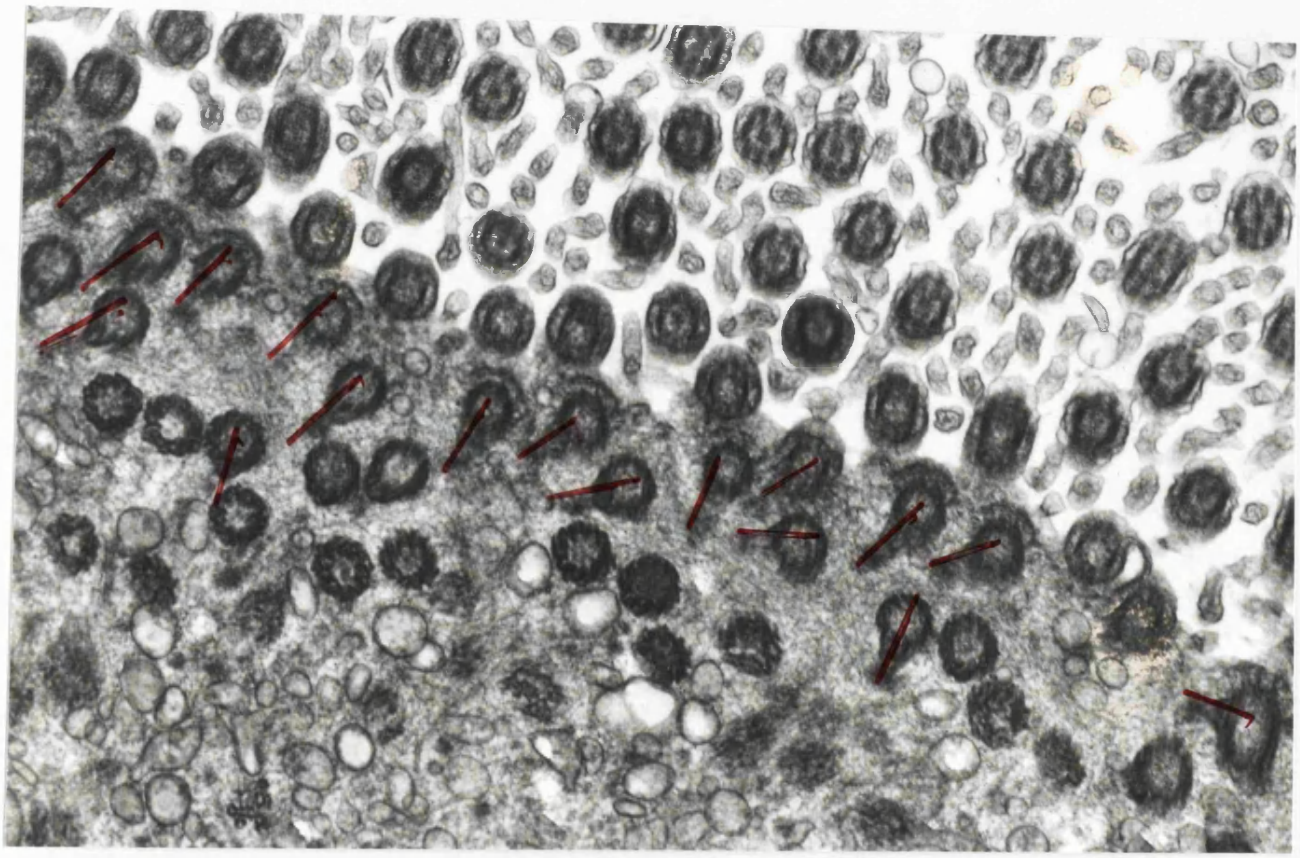


Figure 8

Basal foot orientation per cell was measured by drawing a line transecting the mid-point of the base and the apex of the basal foot. Magnification x 35,000.

Table 4

Investigations performed in patients with chronic bronchial sepsis

Blood

- Differential white cell count
- IgG, IgG subclasses, IgA, IgM and IgE
- Aspergillus precipitins
- Rheumatoid factor and antinuclear antibodies
- Alpha-1 antitrypsin

Sputum

- Differential white cell count
- Culture and sensitivities
- AFB smear and culture

Chest radiographs (PA and lateral)

Sinus radiographs

Immediate hypersensitivity skin tests

High resolution thin-section CT scan of thorax

Sweat electrolyte excretion

NMCC by saccharin test

CBF (if NMCC >30 min) ± electron microscopy of cilia ultrastructure

radiological evidence of sinus mucosal thickening.

The 12 patients with cystic fibrosis, 6 men, age range 14-26, were under the care of Dr M Hodson and regularly attended a cystic fibrosis clinic at The Royal Brompton Hospital, Sydney Street, London, SW3. All 12 were stable at the time of assessment and free from an infective exacerbation.

2.9.1.2 Measurement of nasal mucociliary clearance

NMCC was measured with the saccharin method (Rutland and Cole 1981). Before starting the procedure subjects spend at least 1 h in a stable environment (temperature 21-24°C). The subject blows the nose gently to remove any excess secretions and a saccharine particle 0.5 mm diameter was gently placed on the medial aspect of the inferior turbinate 5 mm behind the turbinate's anterior end to avoid the area of mucosa where cilia beat in an anterior direction. The patient's head is bent forward and the patient was requested not to sniff or sneeze and to sit still. If the patient sneezed or sniffed then the procedure must be repeated on another day. The time from particle placement until the first sensation of a sweet taste was measured using a stop-watch. Results are expressed as a clearance time. If the patient fails to taste saccharin before 60 min then they were checked to ensure that they could taste saccharine by placing it on the tongue.

2.9.1.3 Method of obtaining human nasal ciliated epithelium

Ciliated epithelium was obtained from both nostrils by a brush biopsy technique as described in section 2.8.2.1 approved by the Royal Brompton Hospital Ethical Committee.

2.9.1.4 Ciliary beat frequency assay

Using agitation of the cytology brush, epithelium from the first nostril was gently dispersed in Medium 199. Each suspension was transferred to a sealed microscope coverslip-slide preparation for measurement of CBF at 37°C as described in section 2.8.2.3.

2.9.1.5 Ultrastructure assessment

The epithelium from the second nostril was dislodged by gentle agitation directly into cacodylate buffered 2.5% glutaraldehyde and processed for TEM as in section 2.8.3.1. Transversely sectioned cilia were assessed in detail at magnification of x 30,000 as previously described by Greenstone et al (1988). Sections for assessment were selected at random from the epithelial strip. Microtubule abnormalities and compound cilia and dynein arm numbers were quantified. The presence of microtubule and compound defects were assessed from sections not cut in perfect cross-section, however, cilia cut in near perfect cross-sections were only used to detect the presence of dynein arms. Cross-sections were recorded as showing both dynein arms, inner dynein arms only, outer dynein arms only or no

arms.

2.9.1.6 Measurement of orientation

Using TEM and an improvision image analysis system ciliary orientation of the central pairs was measured as describe in section 2.8.3.3. In three subjects basal foot orientation per cell was also measured by drawing a line transecting the mid-point of the base and the apex of the basal foot.

2.9.1.7 Microbiology

At the time of the nasal brush biopsy nasal swabs were taken from each patient and the cultures identified after overnight incubation at 37°C. One patient whose original nasal swabs grew P.aeruginosa underwent repeat studies following a three month course of nebulised colomycin and topical Betnesol-N during which time nasal cultures became negative.

2.9.2 Ciliary orientation in patients with the clinical features of primary ciliary dyskinesia but normal ciliary beat frequency and ultrastructure

2.9.2.1 Study population

The eleven patients were under the care of Professor P Cole and Dr R Wilson and attended out patient clinics at the Royal Brompton Hospital. Each patient had previously or was currently being investigated for chronic bronchial sepsis. In each patient the clinical and radiological features in

association with absent NMCC suggested a diagnosis of PCD. However in some the CBF was normal and ciliary axonemal ultrastructure was normal in all. Dr M Greenstone had previously described 30 patients with the clinical features of PCD, 5 of these had normal ultrastructure (Greenstone et al 1988). One of the five was reassessed in this study.

Eleven patients with the clinical features of PCD but normal axonemal ultrastructure were assessed. The clinical features of PCD included bronchiectasis, chronic mucopurulent sinusitis, absent NMCC with or without situs inversus. Within the above group was a brother and sister, so the parents and a non-affected female sibling were also assessed.

2.9.2.2 Clinical assessment

All patients were assessed clinically. The following features were recorded: age; sex; history of rhinitis and sinusitis; history of otitis; history of chronic sputum production; fertility; the situs of the heart and abdominal organs; CXR; pulmonary function tests; presence and distribution of bronchiectasis.

2.9.2.3 Measurement of nasal mucociliary clearance and ciliary beat frequency

NMCC was measured as described in section 2.9.2.2. Ciliated epithelium was obtained by a brush biopsy technique as described in section 2.8.2.1. Two affected siblings also

had assessment of orientation from a nasal brush biopsy taken by an identical method for assessment of ultrastructure in 1983. Right and left bronchial brushings were available from one patient who underwent bronchoscopy during investigation of bronchiectasis and possible PCD in 1988. CBF in each subject was assessed as described in section 2.8.2.3.

2.9.2.4 Ultrastructure and orientation assessment

Ultrastructure was assessed as described in section 2.9.5. Using TEM and an improvision image analysis system, ciliary orientation of the central pairs and basal feet was measured as described in section 2.8.3.3.

2.9.2.5 Assessment of fertility

One male patient underwent analysis of fertility. These studies were performed by Dr K Linsey at Queen Charlottes Hospital, London. Seminal fluid was assessed for volume, viscosity, pH, and sperm viability, number and motility. TEM was used to assess the ultrastructure of the sperm tails.

2.9.2.6 Repeat studies

Studies were repeated in 2 patients following three months of antibiotics and topical nasal corticosteroid treatment.

2.10 Statistical Analysis

A p value <0.05 was taken as significant. All values are

quoted as mean ± the standard deviation.

2.10.1 Organ cultures

Viable counts of bacteria prepared for inoculation on to the organ cultures and bacterial adherence to the organ cultures were compared using the Mann-Whitney U-test (Altman 1991). The Kruskal-Wallis analysis of variance was used to determine if there was any difference in the area occupied by each of the mucosal features between the control and infected organ cultures at each time point (Altman 1991). To investigate the nature of any differences found, the percentage surface area of a given mucosal feature of infected tissue was compared to control using the Mann-Whitney U-test.

2.10.2 The effect of pyocyanin, rhamnolipid and 1-hydroxyphenazine on ciliary orientation

Statistical analysis of CBF was investigated by comparing the mean CBF of toxin treated epithelium with control using the Mann-Whitney U-test. Ciliary orientation data was investigated using a specific comparison of the mean cell count, mean field count and mean ciliary orientation of toxin treated epithelium with control.

2.10.3 Ciliary orientation <u>in vivo</u>

Statistical analysis of CBF was investigated by comparing mean CBF of patient group with control using the Mann-Whitney U-test. Ciliary orientation data was investigated

using a specific comparison of the mean cell count, mean field count and mean ciliary orientation of patient group with control group using the Mann-Whitney U-test.

3.0 The Interaction Of Neisseria Meningitidis And Streptococcus Pneumoniae With Human Nasopharyngeal Epithelium

N.meningitidis and S.pneumoniae are human pathogens which are important causes of mortality and morbidity and both may colonise and invade the upper respiratory tract. Previous studies investigating the interaction between N.meningitidis and human ciliated nasopharyngeal epithelium have either used isolated cell systems or organ cultures immersed in media (Stevens et al 1986; Stevens and Farley 1991). Few studies have studied the interaction of S.pneumoniae with human ciliated epithelium but again these studies have used isolated ciliated cells or organ cultures immersed in media (Feldman et al 1992; Steinfort et al 1989). Although much has been learnt from these studies, they may not represent physiological conditions. Immersion of tissue in media removes the air-mucosal interface and thus changes the dynamics of mucociliary clearance. The media may either support replication of bacteria allowing continuous interaction between high numbers of bacteria growing in the medium and the epithelium; or the media may dilute the concentration of bacterial products in the microenvironment on the mucosal surface decreasing their effect on the epithelium.

This study, for the first time, assessed the interaction of

these two human pathogens using an organ culture with an air interface.

Each of these two pathogens have a number of virulence factors which are important for colonisation and invasion. Clinical isolates of N.meningitidis are almost invariably piliated which mediate adherence to epithelial and endothelial cells in culture (Virji et al 1991 and 1992). Pili also appear to be required for colonisation of host mucosal surfaces and for at least some stages of invasive disease caused by these bacteria (Stephens et al 1985; Pinner et al 1991; Stephens et al 1984; Stephens et al 1983; Stephens et al 1983; Stephens and McGee 1981).

The cytolytic toxin pneumolysin is an important virulence factor for S.pneumoniae (Paton et al 1993). Pneumolysin causes epithelial (Feldman et al 1991; Rubins et al 1993) and endothelial cell damage (Rubins et al 1992), slows CBF (Feldman et al 1991), and adversely effects the function of cellular and humoral components of the host defences (Paton and Ferrante 1983; Ferrante et al 1984).

Pili and pneumolysin represent key virulence factors which have markedly different functions for N.meningitidis and S.pneumoniae respectively. This study will examine the effect of variation of each of these key virulence factors on the interaction of each pathogen with the organ culture.

The aims of this chapter were to :

1) Assess the interaction of meningococci with nasopharyngeal mucosa in an organ culture system with an air-mucosal interface using SEM.

2) Assess the interaction of pili variants of meningococcus MC58 (two class I piliated variants and one non-piliated variant with known functional variations in adherence to epithelial cells) with the same model.

3) To investigate the interaction between S.pneumoniae and human respiratory mucosa in an organ culture with an air interface.

4) To determine using the same model whether pneumolysin is an important virulence factor affecting colonisation and invasion of the mucosa, using isogenic variants of a type 2 pneumococcus which are sufficient or deficient in the production of pneumolysin.

3.1 Neisseria Meningitidis Results

3.1.1 Bacterial viable counts

For comparable studies on the interaction of the three meningococcal variants with the respiratory mucosa, it was necessary to ensure that the viable counts of the inocula

were equivalent. The mean viable counts of the inocula are given in Table 5. Kruskal-Wallis analysis of variance showed no difference in the viable counts of each of the three variants in each series of experiments, nor for a given variant in experiments conducted for different lengths of time. Equivalent inocula allowed comparison of the interaction of the three variants with the organ culture.

Table 5

The viable count of the inocula for the three strains for each series of experiments.

Strain	4 h	12 h	12 h (gold label)	24 h
MC58 PIL-	4.8 ± 1.2	4.8± 0.7	5.0 ± 0.1	5.3 ± 1.5
MC58 PIL+A	5.8 ± 1.4	4.7±0.4	4.9 ± 0.5	5.6 ± 1.0
MC58 PIL+B	4.7 ± 0.7	5.1±0.7	5.1 ± 0.9	5.4 ± 1.3

The results are the mean viable count ± standard deviation ($x10^7$ per ml) for six experiments

3.1.2 Scanning electron microscopy results

To assess the interaction of the three variants with respiratory mucosa, experiments were performed at 4 h, 12 h and 24 h. High resolution SEM was used for quantitative and qualitative analysis. The percentage surface area occupied by mucus, cell damage, unciliated and ciliated cells are shown in Table 6 for the three series of experiments (4, 12 and 24 h).

3.1.2.1 Scanning electron microscopy results at 4 hours

At 4 h there was no difference in the measured parameters between control and those infected with the three strains. Occasionally isolated diplococci of both MC58 piliated variants were seen in association with unciliated areas but MC58 PIL- bacteria were not seen.

3.1.2.2 Scanning electron microscopy results at 12 hours and 24 hours

a) Mucus

There was no difference in mucus coverage of organ cultures for all three variants. Bacteria were very rarely associated with mucus at any time point, but this occurred more commonly with the non-piliated variant than the two piliated variants. When bacteria were observed they tended to be at the edge of a mucus sheet.

b) Ciliated cells

At 12 h there was a suggestion of a fall in ciliated cells in MC58 PIL+B compared to control, but this did not reach significance ($p<0.06$). At 24 h there was a significant fall in the area covered by ciliated cells for both piliated variants ($p<0.02$) compared to control, but not compared to MC58 PIL-. At 12 h with the two piliated variants, and at 24 h with MC58 PIL-, cilia appeared to be disorganised and were bent in different directions (Figure 9), this compares to control cultures in which cilia appear straight and

point in the same direction.

c) Unciliated cells

There was no difference in the percentage surface area occupied by unciliated cells between the three strains and control at 12 h, by 24 h there was a suggestion of a fall in unciliated areas in MC58 PIL+B, but this did not reach significance ($p<0.08$).

By 12 h piliated bacteria could be seen adhering to unciliated areas, and there were depressions in the mucosal surface which gave a "foot print" appearance that might have been created by adherent bacteria that had been dislodged during processing (Figure 10). Blebs were seen on the surface of adherent bacteria (Figure 11a). Bacterial adherence was associated with a change in the appearance of the microvilli, which became longer and developed branches (Figures 10, 11a, 11b). These changes occurred in areas closely associated with bacteria, but also in areas quite distant from any adherent bacteria. The changes in the microvilli varied considerably in intensity, but could occur to such an extent that bacteria appeared to be covered by the cross-linkages (Figure 11c). Identification of bacteria underlying the cross linkages was difficult, and led to the use of immunogold labelling to confirm their presence.

Table 6

Scanning electron microscopy of the interaction between *Neisseria meningitidis* and adenoid organ culture

Strain	Mucus	Damaged cells	Ciliated epithelium	Unciliated epithelium
4 h				
Control	55.6 ± 25.8	4.7 ± 1.7	18.4 ± 11.1	20.5 ± 13.1
MC58 PIL-	51.9 ± 29.1	4.2 ± 1.8	17.3 ± 15.3	25.5 ± 13.4
MC58 PIL+A	56.6 ± 17.4	5.2 ± 2.1	12.7 ± 6.7	25.6 ± 14.6
MC58 PIL+B	47.5 ± 20.3	7.1 ± 1.4	28.3 ± 11.9	17.8 ± 10.2
12 h				
Control	43.2 ± 9.7	5.3 ± 1.2	24.3 ± 3.1	31.2 ± 9.9
MC58 PIL-	55.5 ± 6.5	4.9 ± 1.9	23.1 ± 4.4	16.4 ± 7.3
MC58 PIL+A	46.0 ± 12.1	10.3 ± 3.9*	19.5 ± 4.5	24.2 ± 4.7
MC58 PIL+B	36.8 ± 11.6	15.6 ± 4.6**	14.1 ± 8.8	33.7 ± 10.9
24 h				
Control	54.3 ± 14.8	4.9 ± 1.7	14.8 ± 7.9	24.8 ± 12.9
MC58 PIL-	56.6 ± 23	10.9 ± 6.6▲	13.1 ± 12.8	19.8 ± 14.2
MC58 PIL+A	52.4 ± 17.8	15.9 ± 6.5▲▲	6.3 ± 2.7#	25.4 ± 14.1
MC58 PIL+B	53.9 ± 14.3	24.2 ± 9.4+	5.4 ± 4.0#	15.7 ± 9.5

* = MC58 PIL+A versus control and versus MC58 PIL- $p<0.05$
** = MC58 PIL+B versus control and versus MC58 PIL- $p<0.01$
▲ = MC58 PIL- versus control $p<0.02$
▲▲ = MC58 PIL+A versus control $p<0.002$
\+ = MC58 PIL+B versus control $p<0.001$;MC58 PIL+B versus MC58 PIL- $p<0.02$
\# = MC58 PIL+A and MC58 PIL+B versus control $p<0.02$

The results are the mean percentage surface area of an organ culture occupied by each mucosal feature ± standard deviation. Each experiment (n=6) consisted of four organ cultures constructed from the same adenoid tissue.

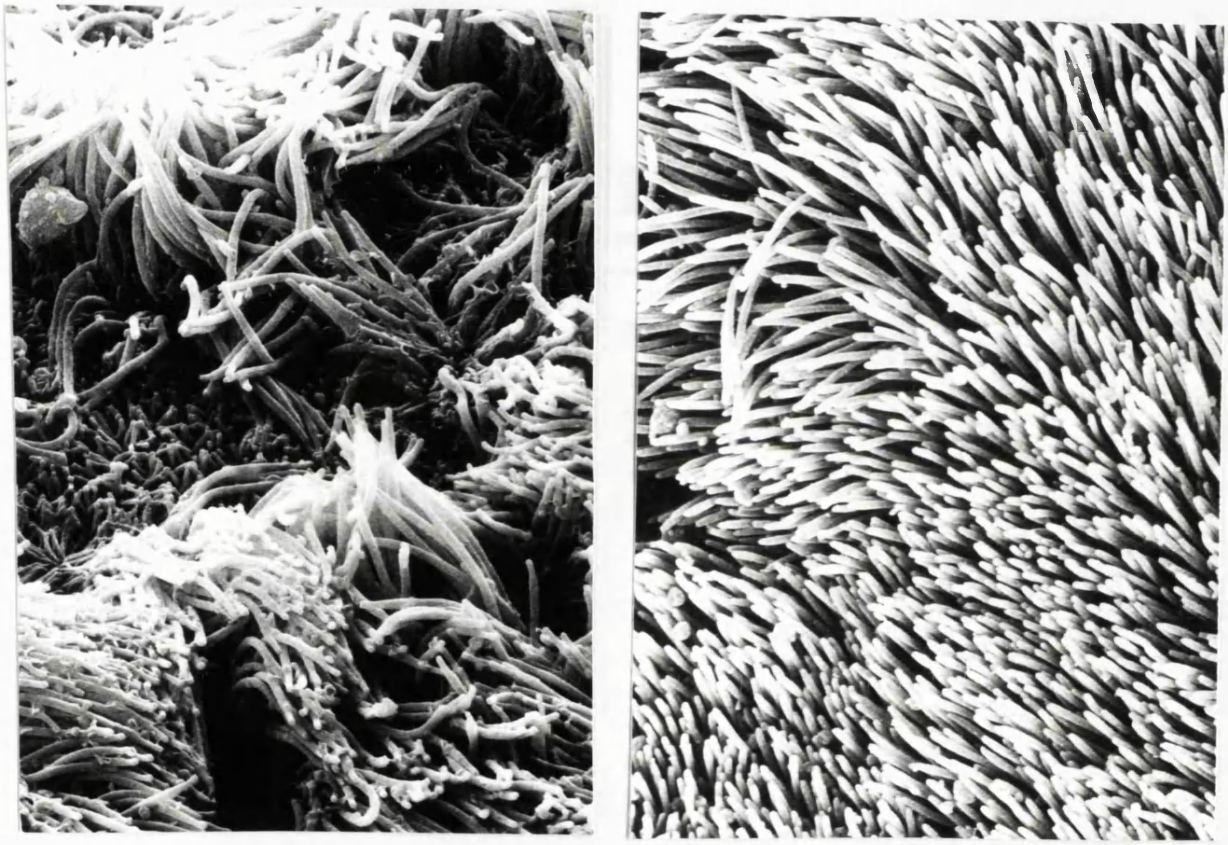


Figure 9

Organ culture infected with Neisseria meningitidis PIL+A for 12 h (9a). The cilia of infected organ cultures appear disorganised and bent in different directions compared to those in control organ cultures (9b).

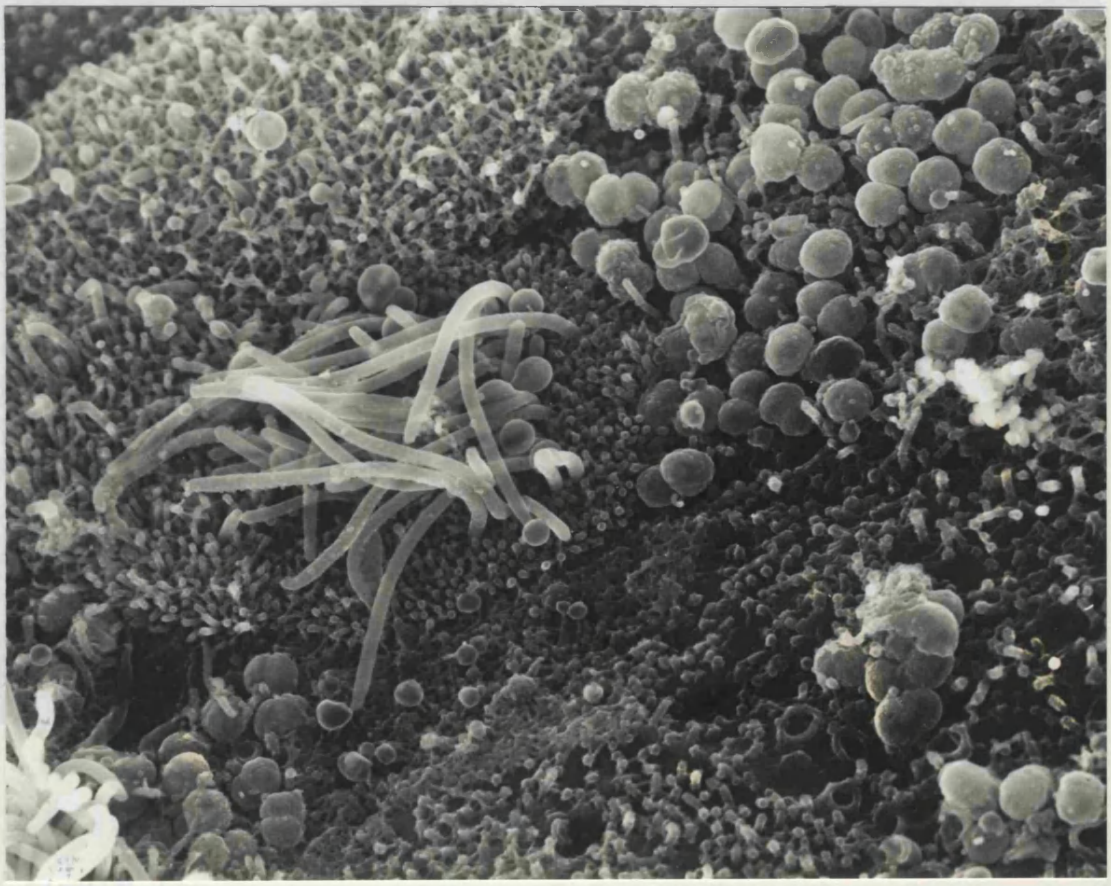
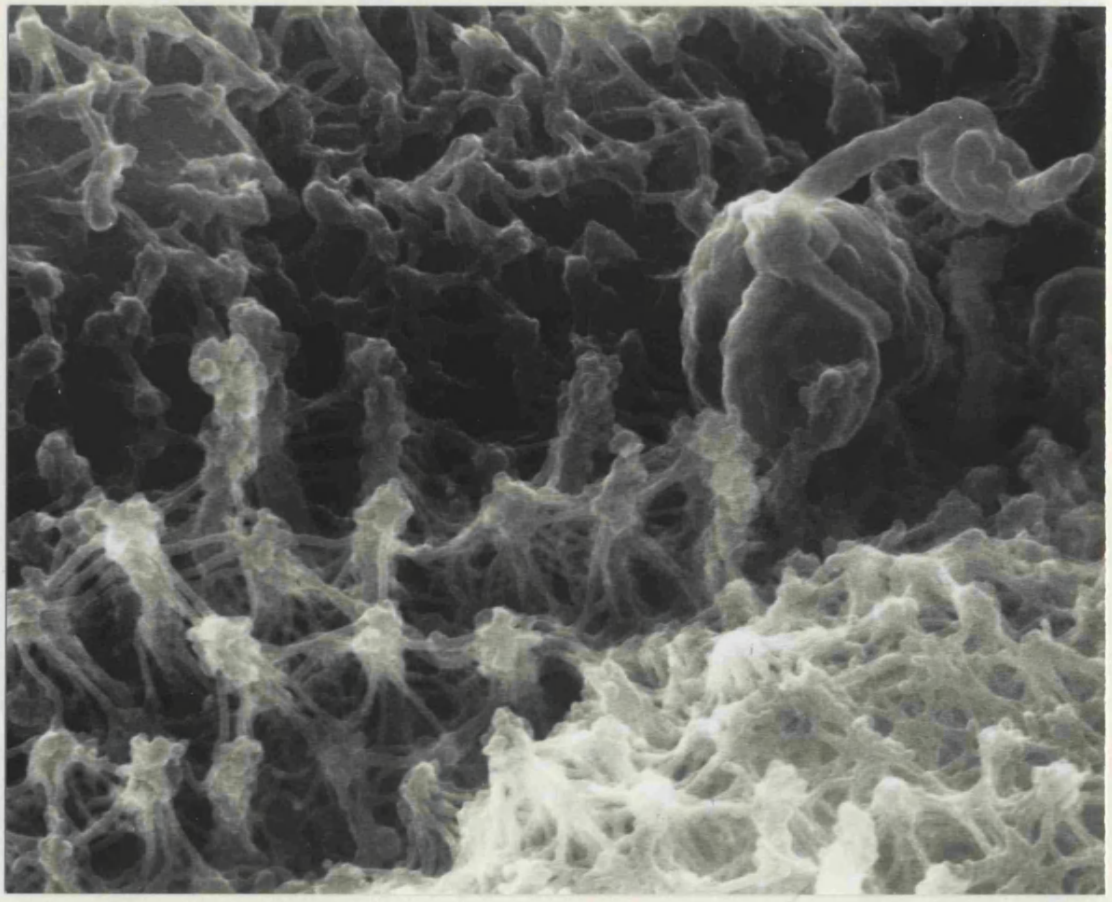


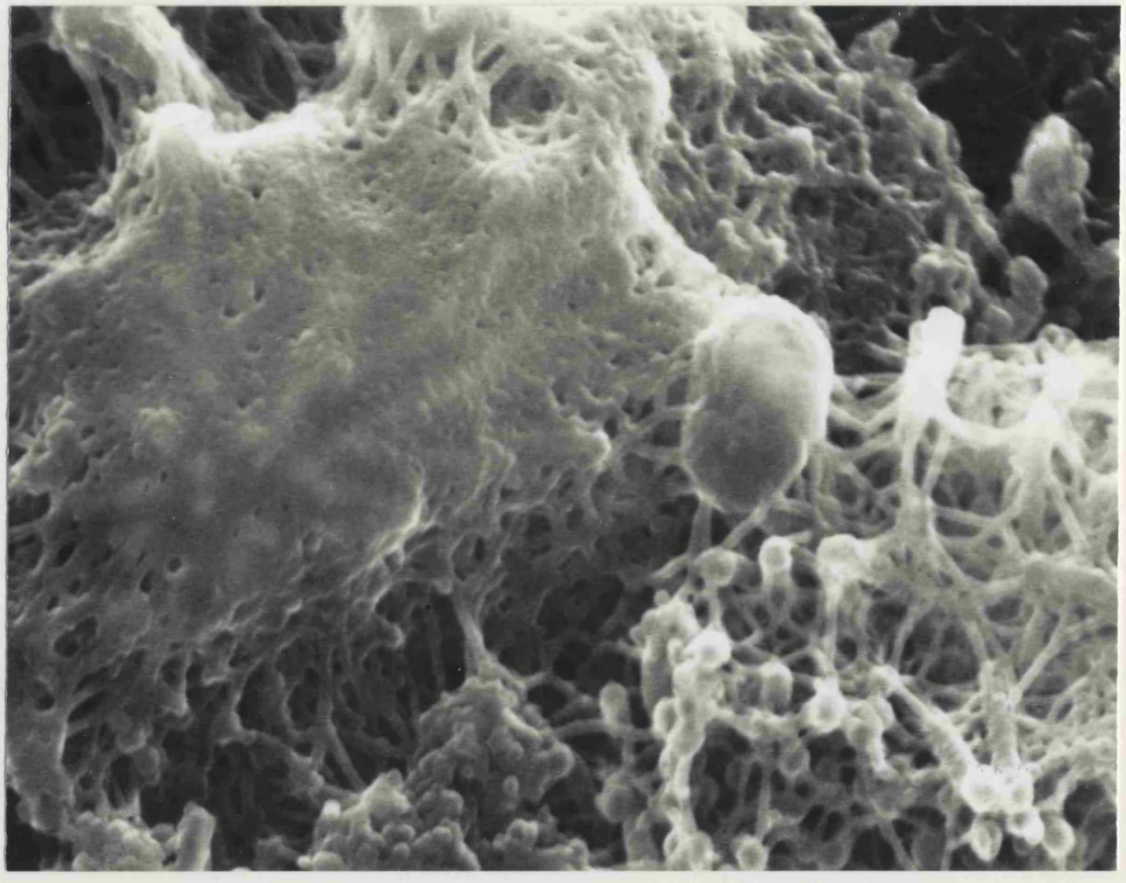
Figure 10

Neisseria meningitidis PIL+A adhering to unciliated epithelium in an organ culture infected for 12 h. Early changes in microvilli are seen, and folds of mucosa ("foot prints") are visible adjacent to adherent bacteria suggesting that bacteria may have been dislodged during processing.

Figure 11

Neisseria meningitidis PIL+B adhering to microvilli in an organ culture infected for 12 h. An intense microvillus reaction develops. This begins as an elongation and branching of the microvilli (Figure 11a and 11b) which are seen to envelop bacteria (Figure 11c). Bacteria are seen to have surface blebs (Figure 11a). (Figure 11b and 11c overleaf).





d) Cell damage

By 12 h there had been a significant increase in cell damage (Figure 12) associated with MC58 PIL+A compared to control ($p<0.05$) and compared to MC58 PIL- ($p<0.05$), and for MC58 PIL+B compared to control ($p<0.01$) and compared to MC58 PIL- ($p<0.01$), but there was no significant difference between the two piliated strains. The results from the immunogold label experiments (Table 7) confirmed a significant increase in the amount of cell damage for MC58 PIL+A compared to both control and MC58 PIL- ($p<0.01$); and for MC58 PIL+B compared to control and MC58 PIL- ($p<0.005$). At 12 h both piliated variants were associated with areas of cell damage, but it was often difficult to reliably distinguish the meningococci from components of cell damage (Figures 12 and 13). Breaks in the epithelial integrity, caused by cell separation due to loss of tight junctions were seen, and piliated bacteria adhered to these damaged areas (Figure 14). By 24 h there was a significant increase in the percentage surface area covered by cell damage for MC58 PIL- ($p<0.02$), as well as for MC58 PIL+A ($p<0.002$) and for MC58 PIL+B ($p<0.001$) compared to control. The cell damage cause by MC58 PIL+B remained significantly greater than MC58 PIL- ($p<0.02$). The increase in cell damage at 24h in both MC58 PIL+A and MC58 Pil+ B infected cultures was associated with a significant fall in ciliated ($p<0.02$) epithelium but not unciliated epithelium.

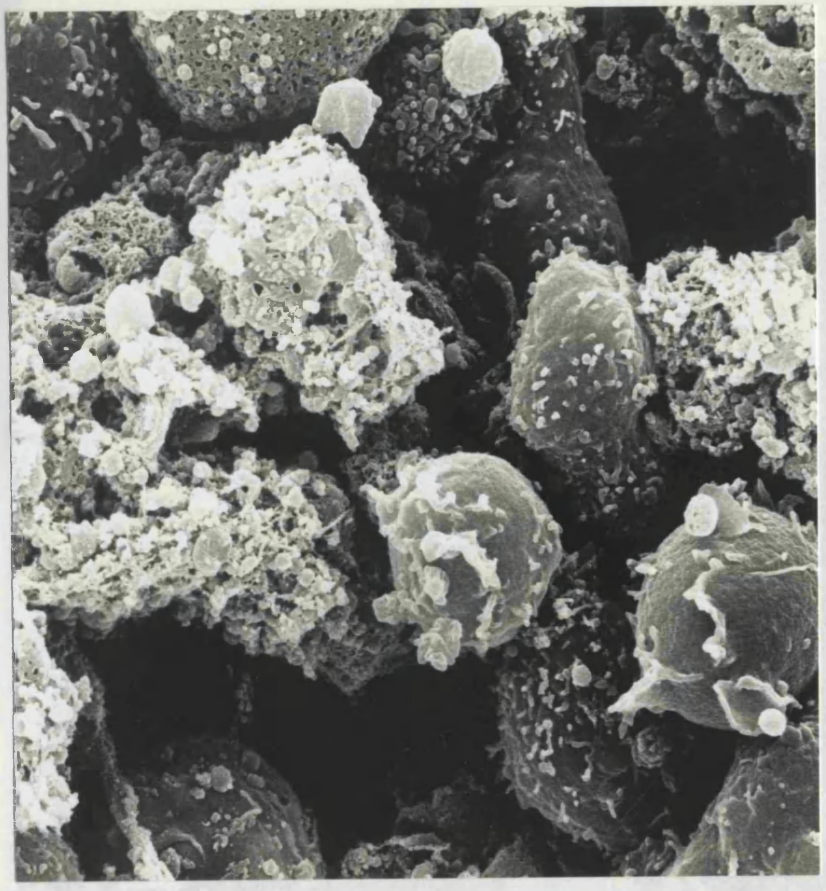


Figure 12

Cell damage and cell debris in an organ culture infected by Neisseria meningitidis PIL+B for 12 h. The appearance of the cellular damage and debris made reliable identification and counting of meningococci difficult.

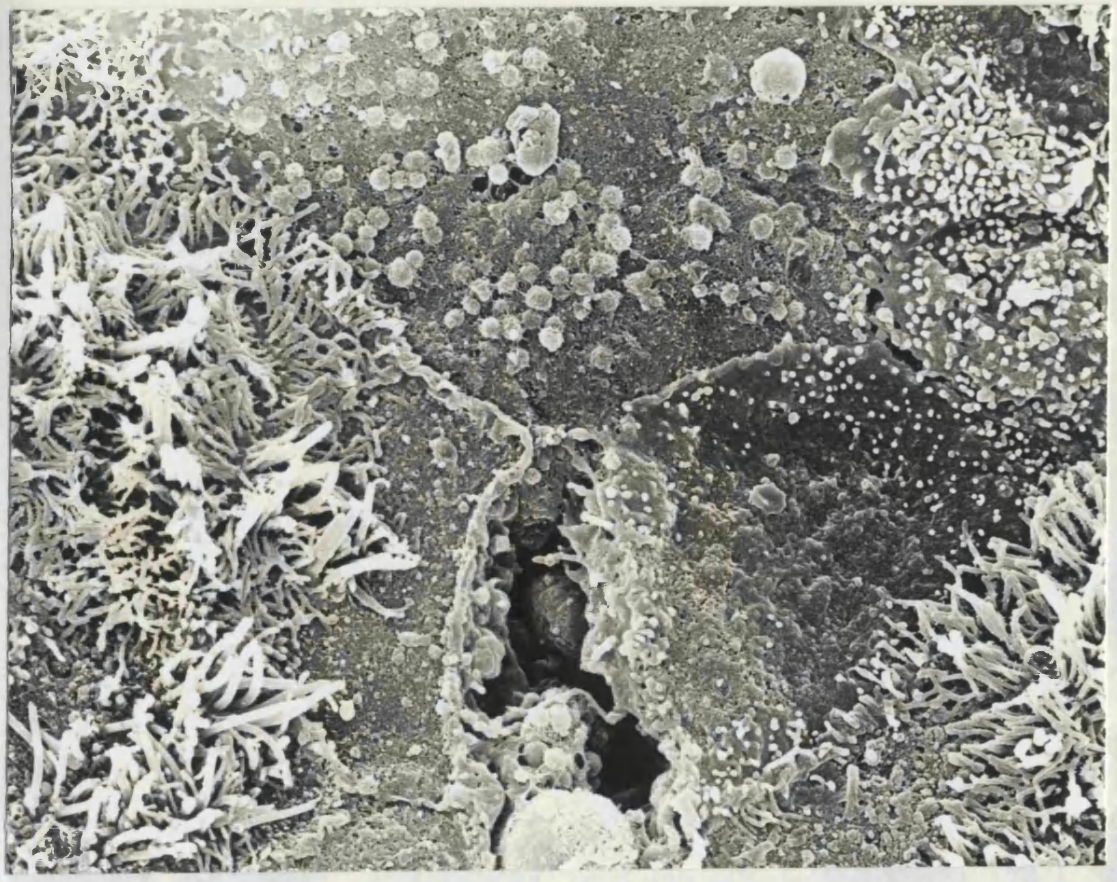


Figure 13

Organ culture infected with Neisseria meningitidis PIL+B for 12 h. Bacteria are seen adhering to unciliated cells and there is a microvillus reaction.

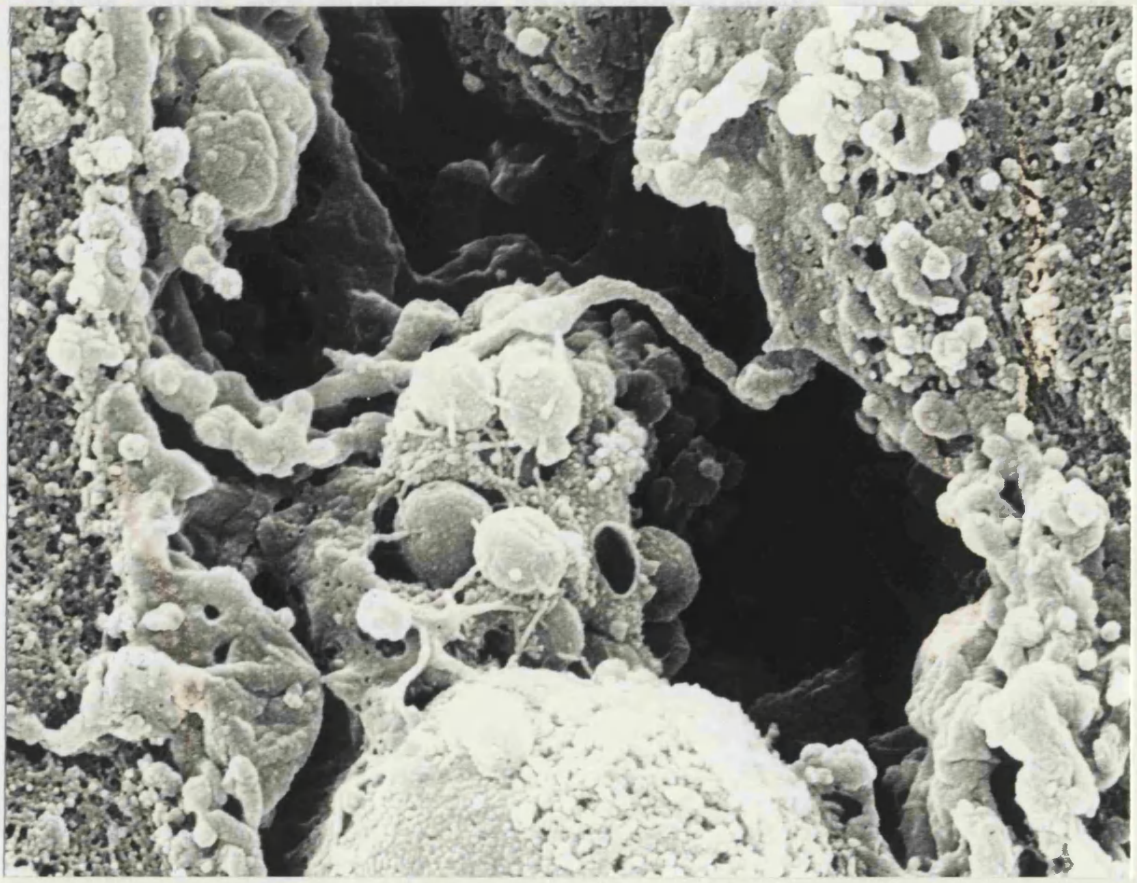


Figure 14

Bacteria are seen adhering to a break in the integrity of the epithelial surface where a tight junction has separated.

3.1.3 Immunogold labelling

In view of the difficulties in reliably identifying bacteria, both underlying the microvillus reaction and in association with damaged cells and cell debris, immunogold labelling was used with a rabbit polyclonal antibody raised against N.meningitidis OMP. The aim was to confirm the location and quantify the numbers of bacteria; the 12 h time-point was chosen for these experiments because changes in the mucosa and bacterial adherence had already been observed to be present but damage was not severe.

3.1.3.1 Immunogold label results at 12 hours

The results from the six experiments can be seen in Table 7. These show a significant increase in the amount of cell damage for MC58 PIL+A compared to both control and MC58 PIL- ($p<0.01$); and for MC58 PIL+B compared to control and MC58 PIL- ($p<0.005$). These results are similar to the previous 12 h results described above.

Table 7

Scanning electron microscopy of the interaction between Neisseria meningitidis and adenoid organ culture at 12 h used in gold labelling experiments

Strain	Mucus	Damaged cells	Ciliated epithelium	Unciliated epithelium
Control	34.2 ± 9.6	3.4 ± 0.76	28.6 ± 11.7	35.0 ± 9.9
MC58 PIL-	28.3 ± 9.8	3.9 ± 0.7	37.2 ± 14.8	30.7 ± 8.9
MC58 PIL+A	30.9 ± 19.2	6.7 ± 1.4*	29.3 ± 14.8	31.6 ± 10.7
MC58 PIL+B	32.5 ± 10.3	9.7 ± 2.8**	22.5 ± 6.1	35.5 ± 12.2

* MC58 PIL+A versus Control $p<0.01$ and versus MC58 PIL- $p<0.01$

** MC58 PIl+B versus Control $p<0.005$ and versus MC58 PIL- $p<0.005$

The results are the mean percentage surface area of an organ culture occupied by each mucosal feature ± standard deviation. Each experiment (n=6) consisted of four organ cultures constructed from the same adenoid tissue.

3.1.3.2 Bacterial adherence

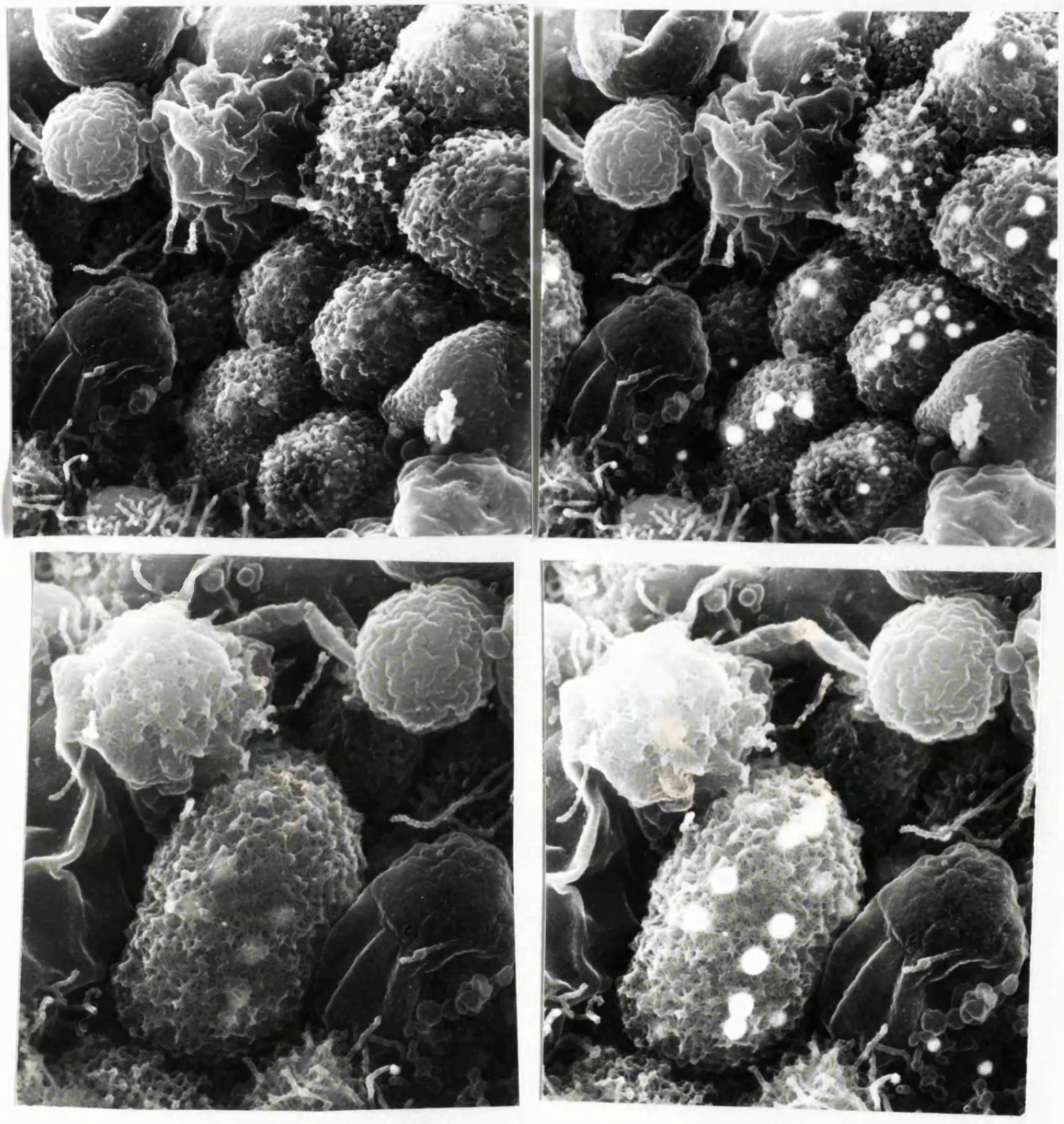
Using the gold label, bacteria were confirmed as adhering to areas of cell damage. The presence of bacteria underlying the microvillus reaction was confirmed (Figure 15a, 15b, 16a, 16b). It was possible to quantify bacterial adherence (Table 8). The density of bacteria adhering to each mucosal feature has been calculated by dividing the total number of bacteria by the percent of the mucosal surface occupied by each feature and these results are shown in Table 9.

a) Mucus and cilia

The association of bacteria with mucus and cilia was extremely rare but was more common for the non-piliated variant compared to the piliated variants ($p<0.05$) (Tables 8 and 9).

b) Unciliated and damaged cells

There was tropism of both piliated strains for unciliated cells with microvilli and damaged cells which was significantly greater than the non-piliated variant ($p<0.01$). MC58 PIL+B showed an increase in the numbers of bacteria associated with damaged cells compared to MC58 PIL+A (Tables 8 and 9).



Figures 15 and 16

Organ culture infected with PIL+B for 12 h. The presence of bacteria underlying the intense microvillus reaction (15a, 16a) is confirmed by immunogold labelling (15b, 16b).

Table 8

The number of gold labelled bacteria associated with the mucosa of adenoid organ cultures at 12 h

	Mucus	Damaged cells	Ciliated epithelium	Unciliated epithelium	Total no.
MC58 PIL-	7.6± 2.6§	6.3± 3.7	32 ± 24†	35 ± 17	81
MC58 PIL+A	3.2± 1.4	31.7± 9.5*	11.7± 6.6	169.7± 69+	216
MC58 PIL+B	2.1± 1.2	37.4±12.0*	3.5± 1.4	361.6± 204+	403

§ MC58 PIL- versus MC58 PIL+A and MC58 PIL+B $p<0.05$

† MC58 PIL- versus MC58 PIL+A and MC58 PIL+B $p<0.05$

* MC58 PIL+A and MC58 PIL+B versus MC58 PIL- $p<0.02$

+ MC58 PIL+A and MC58 PIL+B versus MC58 PIL- $p<0.01$

The results are the mean number of bacteria counted in 0.032 mm^2 of mucosal surface ± standard deviation. Each experiment (n=6) consisted of four organ cultures constructed from the same adenoid tissue.

Table 9

Bacterial density on each mucosal feature of adenoid organ culture

	Mucus	Damaged mucosa	Ciliated epithelium	Unciliated epithelium
PIL-	0.7±0.3§	4.8±3.1	1.7±0.5§	2.1±0.6
PIL+A	0.3±0.2	12.5±4.6♦	1.0±0.3	13.0±4.5 †
PIL+B	0.1±0.1	18.4±8.2♦	0.4±0.2	26.6±12.4*

§ MC58 PIL- versus MC58 PIL+A and MC58 PIL+B $p<0.05$

♦ MC58 PIL+A and MC58 PIL+B versus MC58 PIL- $p<0.01$

† MC58 PIL+A versus MC58 PIL- $p<0.005$

* MC58 PIL+B versus MC58 PIL+A $p<0.05$ and versus MC58 PIL- $p<0.005$

The results are the mean (n=6) total number of bacteria adhering to each mucosal feature divided by the percent of the mucosal surface occupied by that feature ± standard deviation.

3.2 *Streptococcus Pneumoniae*

3.2.1 Viable counts

The mean viable counts of the inoculum for PL+ and PL- are shown in Table 10. Kruskal-Wallis analysis of variance showed no difference in the inocula of each strain in experiments conducted for different lengths of time. There was also no statistical difference between the inocula of the two variants at the start of experiments conducted for 4 h ($p<0.4$), 24 h ($p<0.8$) and 48 h ($p<0.9$).
Equivalent inocula allowed comparison of the interaction of the two variants with the organ culture.

Table 10
The mean viable count ± sd of the inocula for each series of experiments conducted for different lengths of time x 10^8 per ml (n=6)

Time	4	24	(24 TEM)	48
PL+	1.0±0.4	1.1±0.3	1.1±0.5	1.1±0.3
PL-	1.0±0.3	1.1±0.3	1.2±0.4	1.0±0.5

3.2.2 Ciliary beat frequency

CBF of adenoids are shown in Table 11. At 4 h (n=6) there was no difference in the CBF of the control and the two pneumolysin variants. By 24 h CBF of PL+ had fallen to 8.8 ± 0.9 Hz which was significantly slower than that of the

control, 10.7 ± 0.7 Hz ($p<0.01$). PL- at 24 h was not significantly different from the control ($p<0.06$).

The CBF of PL+ infected tissue prepared for TEM analysis showed a similar fall to 9.1 Hz which was significantly different from control ($p<0.01$). By 48 h there was a further fall in the CBF of PL+ to 5.6 ± 1.9 Hz and of PL- to 7.4 ± 1.3 Hz and both were significantly different compared to control 10.5 ± 0.54 Hz.

Table 11

Ciliary beat frequency ± standard deviation of adenoid organ cultures incubated alone or infected with pneumolysin sufficient or deficient Streptococcus pneumoniae

	CBF (Hz) mean ± sd			
Time h	4	24	24 TEM	48
Control	12.1±0.8	10.7±0.7	11.1±1.3	10.5±0.5
P+	10.8±1.1	8.8±0.9*	9.1±1.0*	5.6±1.9§
P-	11.9±0.8	9.7±0.8	10.4±0.5	7.4±1.3†

* = P+ at 24 h compared to control $p<0.01$

§ = P+ at 4 h compared to control $p<0.005$

† = P- at 48 h compared to control.

3.2.3 Scanning electron microscopy

3.2.3.1 Scanning electron microscopy at 4 hours

At 4 h there was no difference in the measured parameters between control and PL+ and PL- and no difference between PL+ and PL-. Bacteria were not seen in association with the mucosa at this time point despite the inoculation of large numbers of bacteria at the start of the experiment (Table 12).

3.2.3.2 Scanning electron microscopy at 24 and 48 hours

a) Mucus

There was no difference in the surface area covered by mucus for either PL+ or PL- infected cultures compared to control. However the appearance of mucus in the infected organ cultures changed becoming fibrogranular.

b) Damaged cells

At 24 h the surface area covered by damaged cells had increased to 21.7 $\pm$ 6% for PL+ compared to 4.3 $\pm$ 1.4% for control tissue ($p<0.01$) (Figure 17). PL+ also demonstrated a significant increase in damaged cells compared to PL- 8.8 $\pm$ 2.8% ($p<0.02$). By 48 h there had been a significant increase in the surface area covered by cell damage for both PL+ 30.0 $\pm$ 4.7% ($p<0.005$) and PL- 23.4 $\pm$ 10.5% ($p<0.05$) compared to control 5.0 $\pm$ 2.6%. There was no significant difference at 48 h between PL+ and PL-.

c) Cilia

At 24 h there was a decrease in the surface area covered by ciliated epithelium to 8.8 ± 6.5% for PL+ compared to control 25.1 ± 9.1% ($p<0.05$). However no difference in the mucosal surface covered by cilia was observed for PL- 16.2 ± 13.2% compared to control ($p<0.22$). By 48 h a significant decrease in ciliated surface area for both PL+ and PL- at 4.9 ± 2.7% and 7.5 ± 5.7% respectively compared to control 19.5 ± 6.2% ($p<0.01$) was found.

At 24 h and 48 h in the PL+ infected organ cultures cilia appeared to be disorganised (Figure 18) with an increased granularity on the surface of the cilia shafts and extruded ciliated cells were seen (Figure 19); in the PL- infected cultures cilia were not disorganised.

3.2.4 Bacterial adherence

3.2.4.1 Bacterial adherence at 4 hours

At 4 h bacteria were not seen in association with the mucosal surface.

3.2.4.2 Bacterial adherence at 24 hours and 48 hours

The total number of bacteria adhering to the mucosal surface at 24 h and 48 h are shown in Table 13. The density of bacteria adhering to each mucosal feature has been calculated by dividing the total number of bacteria by the percent of the mucosal surface occupied by each feature and these results are shown in Table 14.

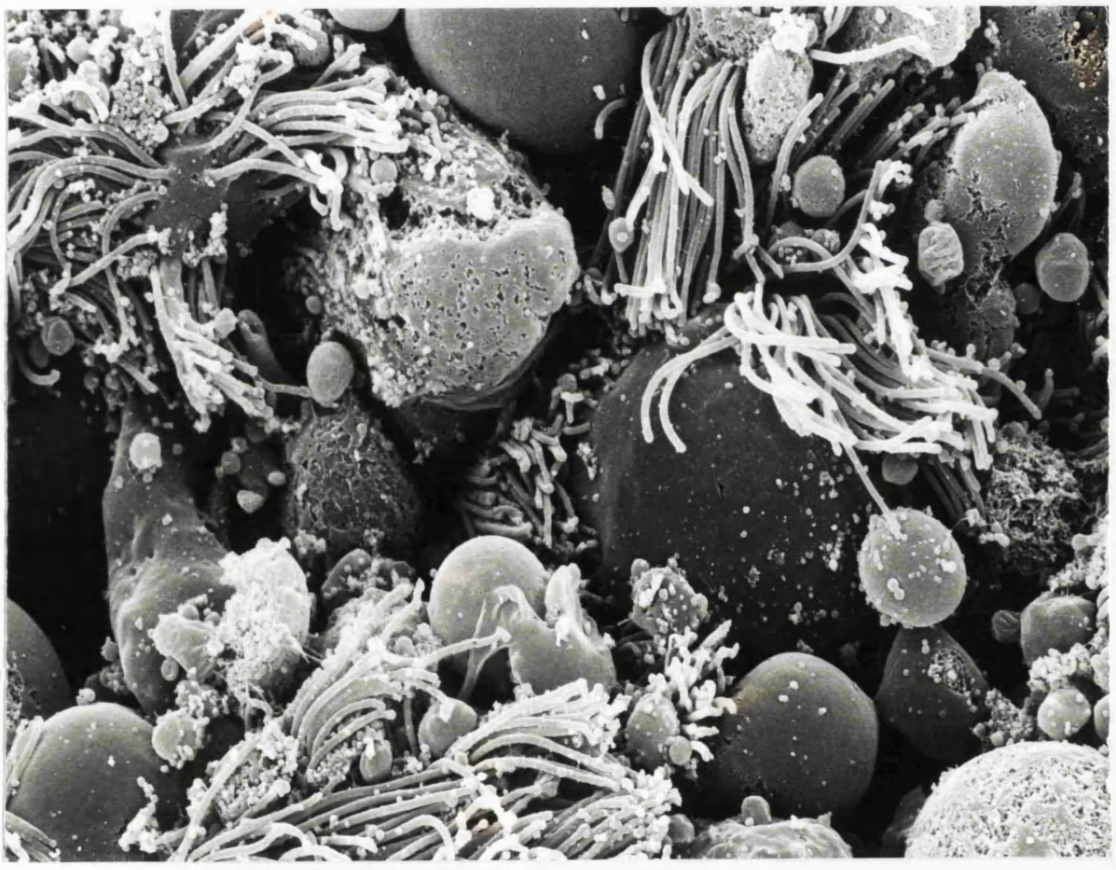


Figure 17

Cell damage with cell debris in an organ culture infected by PL+ at 24 h.

Figure 18

Organ culture infected with Streptococcus pneumoniae PL+ for 24 h. The cilia of PL+ infected organ cultures appear disorganised and bent in different directions compared to those in control organ cultures.

Figure 19

Organ culture infected with Streptococcus pneumoniae PL+ for 24 h showing extrusion of ciliated cells.

Table 12

Scanning electron microscopy of the interaction between Streptococcus pneumoniae and adenoid organ culture

Strain	Mucus	Damaged mucosa	Ciliated epithelium	Unciliated epithelium
4 h				
CONTROL	48.6±7.6	4.3±0.9	22.2±16.0	24.7±11.7
PL+	32.3±16.7	8.4±2.9	21.2±10.5	38.1±16.9
PL-	33.0±17.6	6.1±2.8	25.4±18.8	35.5±7.4
24 h				
CONTROL	48.4±20.6	4.3±1.4	25.1±9.1	22.2±12.5
PL+	31.4±15.5	21.7±6.8*+	8.0±6.5*	38.9±11.8
PL-	41.8±21.2	8.8±2.8	16.2±13.2	33.2±19.6
48 h				
CONTROL	39.5±22.3	5.0±2.6	19.5±6.2	36.0±12.3
PL+	45.4± 7.8	30.0±4.7**	4.9±2.7*	19.7±2.1♦
PL-	36.0±19.2	23.4±10.5♦	7.5±5.7♦	33.1±17.0

*p<0.01, PL+ cf control

+ p<0.02, PL+ cf PL-

**p<0.005, PL+ cf control

♦p<0.05, PL- cf control; PL+ cf control

The results are the mean percentage surface area of an organ culture occupied by each mucosal feature ± standard deviation. At each time point an experiment (n=6) consisted of three organ cultures constructed from the same adenoid tissue. PL+ and PL- are pneumolysin sufficient and deficient S.pneumoniae respectively.

a) Mucus

At 24 h PL+ and PL- were most commonly found in association with mucus, and there was no significant difference in adherence to mucus for the two variants ($p<0.7$). PL+ adherence to mucus was significantly increased compared to ciliated and unciliated tissue ($p<0.05$). In infected cultures the mucus became fibrogranular and appeared to contain both cellular material and bacteria, which was not seen in control cultures (Figure 20). By 48 h the two variants again demonstrated tropism for mucus compared to ciliated and unciliated tissue (Tables 13 and 14). Projections from bacteria were seen (Figure 21).

b) Damaged cells

At 24 h PL+ and PL- were seen to adhere to damaged cells. Although the total number of PL+ adhering to areas of damage was greater than PL-. When the bacterial density was measured there was no difference suggesting that there was no difference in adherence of PL+ and PL- to damaged cells (Figure 22). Bacteria also adhered to damaged ciliated cells which appeared to be undergoing extrusion (Figure 23).

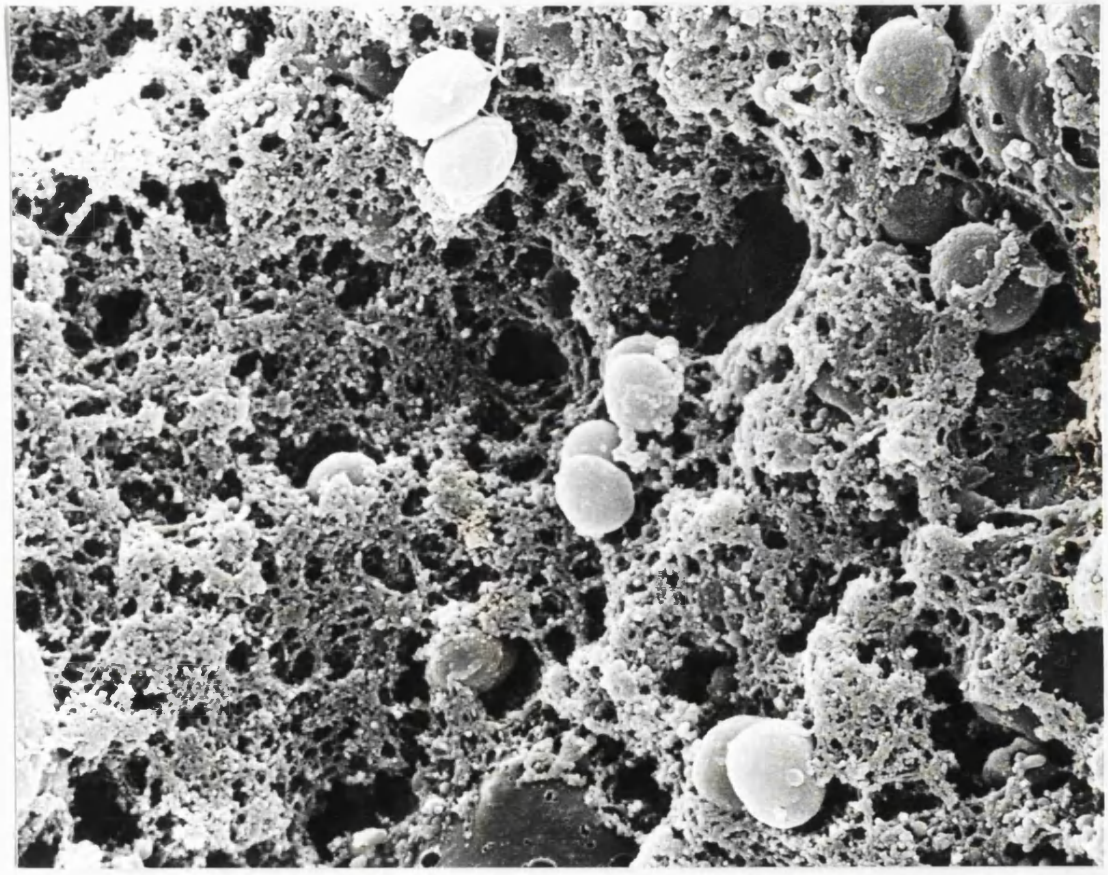


Figure 20

Organ culture infected by Streptococcus pneumoniae PL+ for 24 h. The mucus appears fibrogranular and appears to contain both cellular material and bacteria.

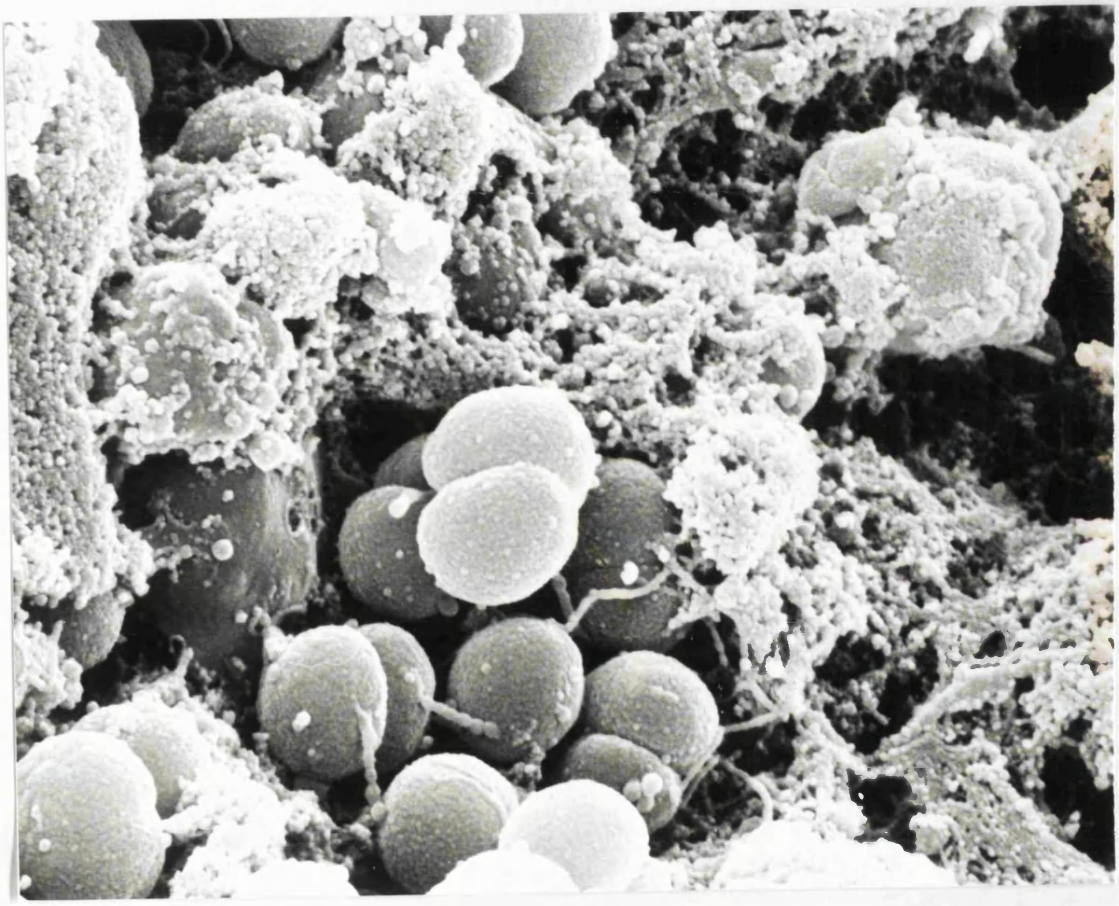


Figure 21

Organ culture infected by Steptococcus pneumoniae PL+ for 48 h. The mucus appears fibrogranular and projections from bacteria are seen.

c) Cilia

PL+ were only rarely seen in association with cilia at 24 h and 48 h. PL- bacteria were not seen in association with cilia at 24 h but were occasionally seen at 48 h.

d) Unciliated epithelium

Adherence to unciliated epithelium was uncommon for both variants, although the total number of PL+ adhering to unciliated epithelium was greater than PL- ($p<0.05$). The reason for this was PL+ adherence to normal unciliated cells at sites where separation of tight junctions had occurred. This separation of cells, that otherwise appeared normal, was seen exclusively in PL+ infected cultures.

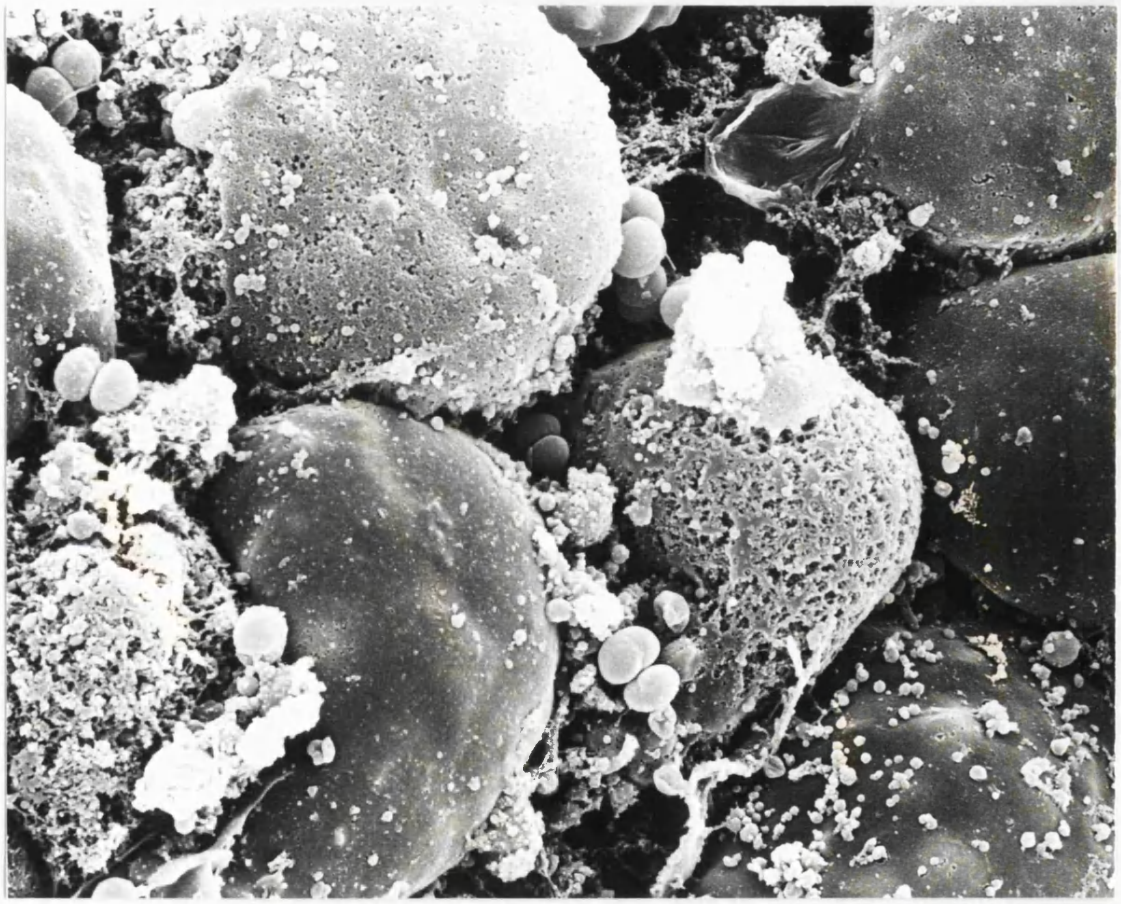


Figure 22

Organ culture infected by Streptococcus pneumoniae PL+ for 48 h. PL+ bacteria were seen adhering to both areas of cell damage and to areas of unciliated epithelium where a separation of tight junctions integrity could be seen.

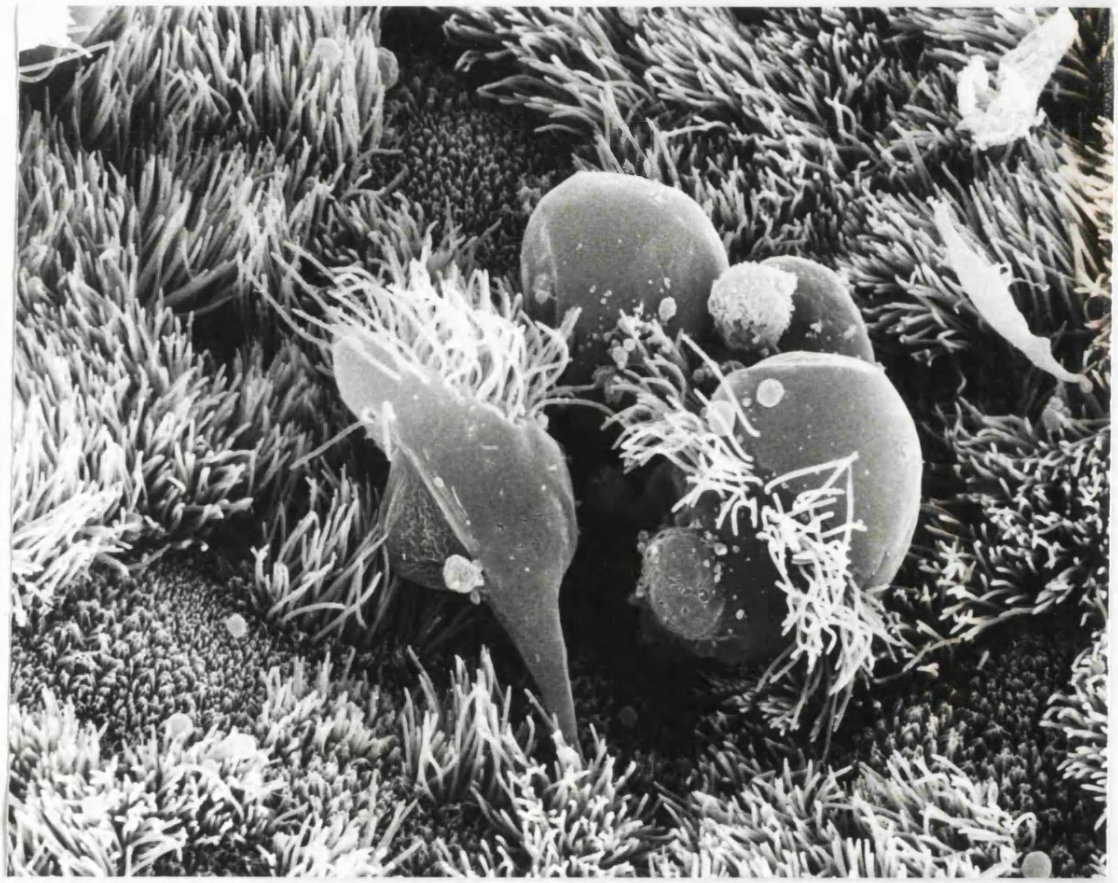


Figure 23

Organ culture infected by Streptococcus pneumoniae PL+ for 48 h. PL+ adherence to damaged ciliated cells which appear to be undergoing extrusion.

Table 13

Bacterial adherence to the mucosal surface of adenoid organ culture assessed by scanning electron microscopy

	Mucus	Damaged mucosa	Ciliated epithelium	Unciliated epithelium
24 h				
PL+	19.2±8.9	9.8*± 4.5	1.1±1.6	6.2*±2.7
PL-	16.7±9.9	4.8 ± 3.0	0	2.3 ±2.3
48 h				
PL+	142.0±14.7	121.7*± 8.4	2.7±2.4	23.8*±8.7
PL-	128.7±23.6	100.1 ±26.6	1.0±2.7	14.2 ±8.5

* = $p<0.05$, PL+ cf PL-

The results are the mean number of bacteria adhering to each mucosal feature ± standard deviation. At each time point an experiment (n=6) consisted of three organ cultures (including control) constructed from the same adenoid tissue. PL+ and PL- are pneumolysin sufficient and deficient variants respectively.

Table 14

Bacterial density on each mucosal feature of adenoid organ culture

	Mucus	Damaged mucosa	Ciliated epithelium	Unciliated epithelium
24 h				
PL+	3.7±1.0*	2.0±1.8*	0.8±1.7	0.43±0.27
PL-	2.3±2.2	1.5±1.4	0	0.16±0.19
48 h				
PL+	7.7±2.0†	10.3± 1.5†	1.3±1.0	3.0 ± 1.2
PL-	8.6±6.0♦	8.9 ±0.7	0.4±0.8	1.7 ± 1.4

* PL+ adherence to mucus and damaged cells compared to ciliated and unciliated tissue $p<0.05$

† PL+ adherence to mucus and damaged cells compared to ciliated and unciliated tissue $p<0.02$

♦ Pl- adherence to mucus and damaged cells compared to ciliated and unciliated tissue $p<0.05$

The results are the mean (n=6) total number of bacteria adhering to each mucosal feature divided by the percent of the mucosal surface occupied by that feature ± standard deviation. At each time point an experiment consisted of three organ cultures (including control) constructed from the same adenoid tissue. PL+ and PL- are pneumolysin sufficient and deficient variants

3.2.5 Transmission electron microscopy results

The effects of S.pneumoniae infection on epithelial ultrastructure are shown in Table 15. Both PL+ and PL- caused an increase in the number of cells extruding from the epithelial surface compared to control (PL+ $p<0.01$, PL- $p<0.02$) and an increase in the number of poorly ciliated cells ($p<0.01$). Infected organ cultures also had an increase in the number of unciliated cells with mitochondrial damage compared to control (PL+ $p<0.01$, PL- $p<0.05$). Cell extrusion and toxic changes in cells were greater for PL+ than PL- infected tissue, but this was not significant except for a significant increase in cytoplasmic blebbing on unciliated cells ($p<0.05$).

Table 15

Transmission electron microscopy of adenoid organ culture infected by Streptococcus pneumoniae for 24 h

	Percentage of cells showing indicated degree of given histological feature															
	Cells extruding from cell surface			Loss of cilia				Blebbing on :						Mitochondrial damage in:			
								Ciliated cells			Unciliated cells			Ciliated cells		Unciliated cells	
	0	+	++	0	+	++	Unciliated	0	+	++	0	+	++	Absent	Present	Absent	Present
C *n=297.2*	89.2	9.3	1.5	69.4	6.4	0.6	23.6	98.7	1.3	0	97.2	2.8	0	97.5	2.5	94.3	5.7
PL+ *n=254.2*	80.0	12.2	7.8♦	66.7	11.1♦	1.8♦	20.4	96.3	2.2	1.5	92.5	6.8♦♦	0.7	93.2	6.8	80.7	19.3♦
PL- *n=298.7*	84.5	11.0	4.5*	74.2	10.5♦	0.8	14.5	97.5	2.1	0.4	98.2	1.8	0	94.6	5.4	86.8	13.2*

* = $p<0.05$, cf control ♦ = $p<0.01$, cf control ♦♦ = $p<0.05$, cf control and PL-
n = mean number of cells examined per experiment

Each cell in a tissue section was examined for extrusion from the epithelial surface (a score of 0 indicates normal positioning in the epithelium), the presence of cilia (0 indicates a full compliment of cilia on the cell surface), cell blebbing (cytoplasmic projections from the luminal cell surface) and mitochondrial damage. PL+ and PL- are pneumolysin sufficient and pneumolysin deficient variants respectively. The cells were from six separate experiments, each consisting of three organ cultures constructed from the same adenoid tissue.

3.3 Summary

1) Pili increased adherence of meningococci to the mucosa of adenoid tissue.

2) There was marked tissue tropism of piliated strains for non-ciliated cells containing microvilli, and to a lesser extent for cell debris and damaged cells.

3) The number of meningococci adhering to cilia and mucus was very low.

4) Meningococcal infection of organ cultures caused epithelial damage, loss of ciliated epithelium and ciliary disorganisation which was greater with piliated strains.

5) Meningococcal interaction with the mucosa stimulated a florid reaction in the microvilli of unciliated cells which enveloped bacteria.

6) Meningococci adhered to areas where the integrity of the epithelium was interrupted.

S.pneumoniae

1) S.pneumoniae infection of organ cultures caused ciliary beat slowing, disorganisation of cilia, epithelial damage with separation of epithelial cells.

2) During infection of organ cultures by S.pneumoniae the appearance of mucus changed becoming fibrogranular.

3) There was marked tissue tropism of S.pneumoniae for mucus and damaged cells.

4) PL+ caused significant ciliary slowing and epithelial damage compared to control at 24 h and 48 h. PL- had a delayed onset and reduced severity of slowing compared to PL+. PL- caused significant ciliary slowing compared to control at 48 h.

5) Separation of epithelial cell tight junctions may provide a route of invasion by S.pneumoniae. The separation of tight junctions occurred more frequently in PL+ infected culture than PL-.

6) PL+ and PL- adhered to mucus and damaged epithelial cells with equal density. PL+ also adhered to the edge of unciliated cells and in the region of tight junction separation.

4.0 The Effect Of Pyocyanin, Rhamnolipid And 1-Hydroxyphenazine On Ciliary Orientation

Early in an infection it would be an advantage for bacteria to produce virulence factors which compromise ciliary function allowing the organism to establish itself with in the respiratory tract. There have been a number of observations showing that transitory ultrastructural abnormalities of cilia including disorientation of cilia occur in the presence of inflammation in vivo (Rutland 1982; Carson et al 1987; Lee 1984). We have shown that ciliary slowing and disorganisation occurs in organ cultures infected by S.pneumoniae, and that ciliary disorganisation and ciliated cell sloughing occurs in organ cultures infected by N.meningitidis. The mechanism of ciliary disorganisation and disorientation in vitro and in vivo has not been elucidated and may be caused by either bacterial factors or host derived inflammatory mediators. Pyocyanin, 1-HP and rhamnolipid are well characterised in vitro and in vivo ciliary toxins which exert their effect at pathophysiological concentrations (Wilson et al 1987; Munro et al 1989; Read et al 1992). Pyocyanin and 1-HP cause dyskinesia in association with ciliary slowing (Wilson et al 1987); and Rhamnolipid and Pyocyanin cause ciliary slowing and epithelial disruption (Wilson et al 1989; Read et al 1992). If these ciliary toxins cause ciliary disorientation at the time point that dyskinesia or epithelial disruption occurs in vitro this would provide

information on the mechanism of ciliary disorientation in vivo.

The aims of the following section was to investigate the effect of pyocyanin, 1-HP and rhamnolipid at concentrations similar to those found in patients sputa colonised with P.aeruginosa on the orientation of cilia in vitro.

4.1 Ciliary Beat Frequency

The effect of pyocyanin, 1-HP and rhamnolipid on CBF are shown in Table 16.

4.1.1 1-Hydroxyphenazine

Following 15 minutes equilibration after coverslip-slide preparation 1-HP had already caused a rapid onset of ciliary slowing with the CBF falling to 8.4 ± 1.3 Hz. This was significantly different from the control value of 13.2 ± 1.9 Hz ($p<0.05$). The ciliary slowing was associated with an absence of the usual coordinated movement, with cilia on the same strip beating in opposite directions. This was termed ciliary dyskinesia.

4.1.2 Pyocyanin

Pyocyanin showed no difference from control CBF at time 0: 13.4 ± 1.4 Hz compared to 13.7 ± 1.5 Hz for control. By 1 h CBF had slowed to 11.3 ± 1.6 Hz compared to the control value of 13.8 ± 1.4 Hz ($p<0.05$). At 2 h there had been further slowing to 8.0 ± 1.7 Hz compared to a control value

of 13.1 ± 0.9 Hz ($p<0.02$). At 2 h in the pyocyanin treated experiments ciliary dyskinesia was seen in at least 50% of the epithelial strips, epithelial disruption was also occasionally seen.

4.1.3 Rhamnolipid

Rhamnolipid showed no difference from control CBF at time 0: 13.3 ± 1.1 Hz compared to 13.0 ± 1.7 Hz. By 1 h CBF had slowed to 10.0 ± 1.1 compared to control 12.3 ± 1.2 Hz ($p<0.05$). At 2 h CBF had fallen to 8.9 ± 1.2 Hz compared to control 12.2 ± 1.0 Hz ($p<0.05$). At 3 h CBF had fallen to 8.6 ± 1.1 Hz compared to control 12.6 ± 1.4 Hz. By 3 h in the rhamnolipid treated epithelium there was also evidence of epithelial disruption of at least 50% of the epithelial strips examined in each experiment, but there was no evidence of ciliary dyskinesia.

4.2 Ciliary Orientation

4.2.1 Fields and cilia assessed for orientation

There was no difference in the number of fields or total number of cilia assessed for measurement of the orientation of central pairs or basal feet for controls compared to pyocyanin, 1-HP or rhamnolipid (Table 17).

4.2.2 The effect of pyocyanin, 1-hydroxyphenazine and rhamnolipid on ciliary orientation

The effect of pyocyanin, 1-HP and rhamnolipid on ciliary orientation are shown in Table 18 and Figure 24.

Table 16

Mean ± standard deviation of ciliary beat frequency of epithelium treated with pyocyanin, 1-hydroxyphenazine and rhamnolipid (n=6)

Time (h)	CBF Mean ± sd (Hz) 0	1	2	3
Control	13.7±1.5	13.8±1.4	13.1±0.9	
Pyocyanin	13.4±1.4	11.3±1.6*	8.0±1.7**♦	
Control	13.2±1.9			
1-HP	8.4±1.3*♦			
Control	13.0±1.7	12.3±1.2	12.2±1.0	12.6±1.4
Rhamnolipid	13.3±1.1	10.0±1.1*	8.9±1.2*	8.6±1.1**#

* p<0.05 , ** p<0.02
♦ Dyskinesia
Epithelial disruption

Table 17

Number of fields and cilia assessed for measurement of orientation via central pair and basal feet for pyocyanin, 1-hydroxyphenazine and rhamnolipid (n=6)

	Central pair		Basal feet	
	fields mean sd	cilia mean sd	fields mean sd	cilia mean sd
Control	13.3±3.6	189.7±81.5	12.3±3.4	128.5±50.3
Pyocyanin	12.2±6.4	184.7±98.4	12.3±3.4	110.6±44.9
Control	19.0±6.9	277.8±143.2	12.4±4.9	185.2±94.1
1-HP	15.0±4.8	225.5±70.4	14.5±5.5	161.3±49.5
Control	15.8±4.4	245.0±94.0	16.2±3.5	144.8±48.4
Rhamnolipid	12.7±3.4	172.0±50.7	13.0±3.7	117.0±26.1

4.2.2.1 1-hydroxyphenazine

1-HP caused a significant increase in mean ciliary central pair disorientation to 17.9 ± 3.7° ($p<0.05$) compared to 13.4 ± 1.8° for control. There was no significant difference for basal feet orientation for 1-HP 14.0 ± 1.5° compared to the control 14.9 ± 2.1°.

4.2.2.2 Pyocyanin

Pyocyanin caused a significant increase in mean ciliary central pair disorientation at 20.1 ± 4.9° ($p<0.005$) compared to control 13.5 ± 1.5. There was no significant change in the basal feet orientation at 14.7 ± 2.5° compared to 15.2 ± 1.5° for the control.

4.2.2.3 Rhamnolipid

Although rhamnolipid caused an increase in the mean ciliary central pair disorientation to 17.4 ± 3.5°, this was not significantly different from the control at 14.4 ± 2.6°. There was no difference in basal feet orientation for rhamnolipid at 13.8 ± 0.9° compared to control 13.9 ± 1.99° (Figure 24).

Table 18

Mean ciliary orientation ± standard deviation measured via the central pair and basal feet for pyocyanin, 1-hydroxyphenazine and rhamnolipid (n=6)

	n	Central pair mean±sd	Basal feet mean±sd
Control	6	13.5±1.5	15.2±1.5
Pyocyanin (25μg/ml)	6	20.1±4.9*	14.7±2.5
Control	6	13.4±1.8	14.9±2.2
1-HP (20μg/ml)	6	17.9±3.7♦	14.0±1.5
Control	6	14.4±2.6	13.8±0.9
Rhamnolipid (100μg/ml)	6	17.4±3.5	13.9±2.0

* $p<0.005$ ♦$p<0.02$

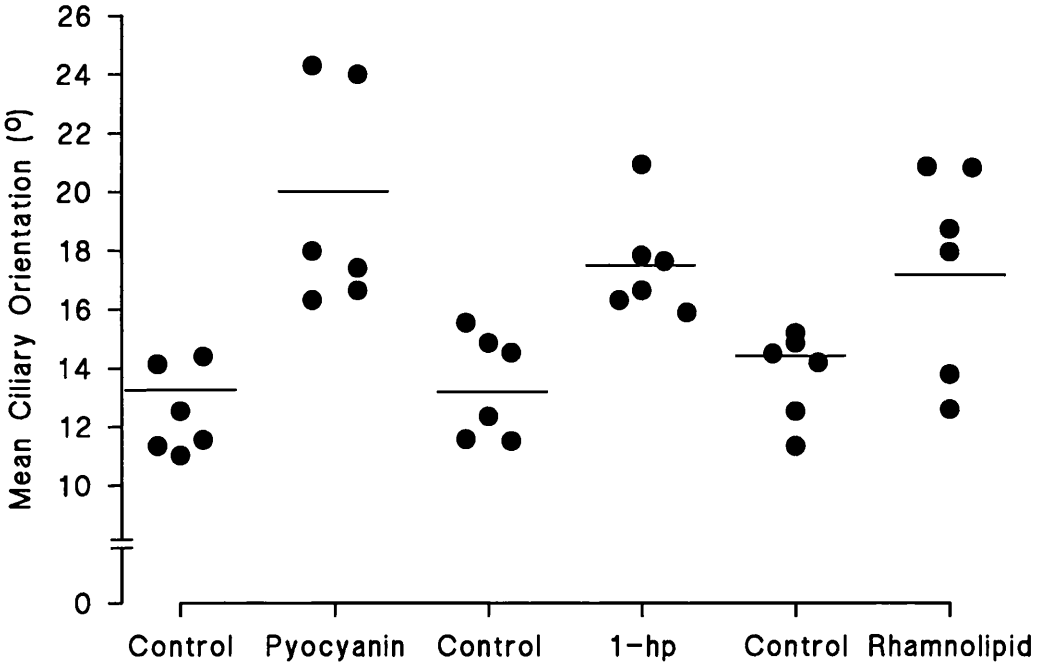


Figure 24

The effect of pyocyanin, 1-hydroxyphenazine and rhamnolipid on ciliary orientation measured through the central pairs.

4.3 Summary

1) Pyocyanin and 1-HP at pathophysiological concentrations caused ciliary slowing and dyskinesia. The onset of slowing and dyskinesia was immediate with 1-HP and delayed with pyocyanin.

2) Rhamnolipid at pathophysiological concentrations caused ciliary slowing and epithelial disruption but not dyskinesia. The onset of ciliary slowing was delayed.

3) Pyocyanin and 1-HP at pathophysiological concentrations caused disorientation measured through the ciliary central pairs, and this occurred at a time point when ciliary dyskinesia was seen via the microscope.

4) Pyocyanin and 1-HP at pathophysiological concentrations did not cause disorientation measured through the basal feet.

5) Disorientation of the central pair but not basal feet suggested that pyocyanin and 1-HP caused twisting of the ciliary shaft. The dyskinesia associated with ciliary slowing may result in ciliary twisting.

6) Rhamnolipid did not cause disorientation of the central pairs or basal feet.

5.0 Clinical Study Of Ciliary Orientation In Patients With Chronic Inflammation Due To Infection And Patients With The Clinical Features Of Primary Ciliary Dyskinesia But Normal Ciliary Beat Frequency And Ultrastructure

There have been a number of observations showing that disorientation of cilia occurs secondary to respiratory tract viruses (Carson et al 1985), in patients with asthma (Laitinen et al 1985), and in rats with experimental bronchitis (Iravani and van As 1972). This work has shown that pyocyanin and 1-HP cause disorientation of the ciliary shaft and that organ cultures infected with S.pneumoniae and N.meningitidis demonstrate damage of ciliated epithelium.

The aim of the following study was to investigate a group of patients with chronic upper respiratory tract inflammation secondary to infection to determine if ciliary disorientation was present.

5.1 Ciliary Orientation in Patients with Chronic Inflammation Due to Infection

Nasal brushings adequate for functional and electron microscopy evaluation were obtained from 10 healthy volunteers with no recent history of upper respiratory

tract infection, 9 patients with cystic fibrosis, 12 patients with idiopathic bronchiectasis and chronic sinusitis, and 2 patients with clinical features of PCD. Three patients with bronchiectasis and 3 patients with cystic fibrosis were excluded because nasal brushings provided inadequate samples for evaluation.

5.1.1 Microbiology

The 12 patients with idiopathic bronchiectasis all had positive cultures from nasal swabs: H.influenzae 4, S.pneumoniae 3, P.aeruginosa 5. None of these patients were taking antibiotic therapy at the time of the study. The 9 patients with cystic fibrosis had 2 positive cultures for P.aeruginosa, all the other 7 were sterile. All 9 cystic fibrosis patients were either taking a nebulised aminoglycoside antibiotic with oral flucloxacillin, or parenteral antipseudomonal antibiotics and oral flucloxacillin. The two patients with clinical PCD had negative nasal swabs and both were taking oral amoxycillin.

5.1.2 Mucociliary function

5.1.2.1 Nasal mucociliary clearance

NMCC was absent (>60 min) in both cases with clinical PCD and was prolonged in the patients with idiopathic bronchiectasis (Table 19). When this latter group was divided into those with P.aeruginosa compared to H.influenzae and S.pneumoniae, the NMCC was greatest in those with P.aeruginosa infection at 50 min $\pm$ 12.25

compared to 30.23 ± 1.52 min in patients with S.pneumoniae and 25 ± 7.23 in patients with H.influenzae. The cystic fibrosis group had a mean NMCC within the normal range (Table 19).

5.1.2.2 Ciliary beat frequency

CBF was within the normal range (11-16 Hz) (Rutland et al 1982) in each group (Table 20). Analysis of variance showed no difference in CBF between groups. The ciliary beat pattern appeared normal in all patients.

5.1.3 Ultrastructure

The total number of cilia assessed and percentage of ultrastructural abnormalities are shown in Table 21. There was no difference in the mean number of cilia assessed in each group for either microtubular and compound ultrastructural abnormalities ($p< 0.22$) or for dynein arm abnormalities ($p<0.85$). There was no difference between groups in the percentage of compound cilia ($p<0.06$), the number of outer microtubular defects ($p<0.4$), or for the number of dynein arm defects ($p<0.37$).

Table 19

The nasal mucociliary clearance for normal volunteers, clinical primary ciliary dyskinesia and patients with bronchiectasis and cystic fibrosis

	Nasal Mucociliary Clearance (min)		
	n	mean ± sd	(95% CI)
Controls	10	12.6 ± 4.6	(9.0-16.2)
Clinical PCD	2	> 60	
Bronchiectasis	12	38.8 ± 16.4	(28-49)
Cystic Fibrosis	9	18.1 ± 7.0	(12.7-23.5)

Table 20

The ciliary beat frequency for normal volunteers, clinical primary ciliary dyskinesia and patients with bronchiectasis and cystic fibrosis

	Ciliary Beat Frequency (Hz)		
	n	mean ± sd	(95% CI)
Controls	10	12.2 ± 1.3	(11.3-13.1)
Clinical PCD	2	13.8 ± 1.0	(11.3-16.3)
Bronchiectasis	12	12.4 ± 0.9	(11.8-13.0)
Cystic Fibrosis	9	11.4 ± 1.3	(10.4-12.4)

Table 21

Ultrastructure of cilia for normal volunteers, clinical primary ciliary dyskinesia and patients with bronchiectasis and cystic fibrosis

	N	Number of cilia	Compound cilia %	Microtubular defects %		Number of cilia	Absent dynein arms %		
				outer	central		inner	outer	inner & outer
Control	10	2722	1.2	0.6	0	911	0.4	0	0.7
Clinical PCD	2	728	0.27	1.8	0	301	1	0	1.6
Bronchiectasis	12	3714	0.2	1.4	0	1200	1.2	0	0.83
Cystic Fibrosis	9	2783	0.6	0.91	0	1175	0.3	0	1.4

5.1.4 Orientation

The mean number of fields, cilia per field and total cilia per case assessed for orientation is shown in Table 22. The Kruskal-Wallis analysis of variance showed no difference in the number of fields ($p<0.1$), cilia per field ($p<0.3$) or cilia per case ($p<0.2$).

5.1.4.1 Orientation in controls and the three patient groups

In all three patient groups the mean ciliary orientation was significantly increased as compared to the control mean of $10.47\pm 0.53°$: $22.1 \pm 0.26°$ ($p<0.05$) in the two patients with clinical PCD, $17.72 \pm 4.15°$ ($p<0.001$) in the bronchiectatic group and $12.3\pm 2.17°$ ($p<0.05$) in the cystic fibrosis group (Figure 25).

5.1.4.2 The effect of bacteriology on mean ciliary orientation

Compared to controls the five patients with bronchiectasis and P.aeruginosa had the greatest sub group mean ciliary orientation at $21.9°\pm 1.7$ ($p<0.002$), the 4 subjects with H.influenzae $14.0° \pm 1$ ($p<0.005$) and the 3 subjects with S.pneumoniae $15.5° \pm 1.9$ ($p<0.02$) (Figure 26). However the P.aeruginosa result was not statistically different from the seven other patients in this group ($p<0.1$). The mean ciliary orientation for the 6 cystic fibrosis patients with sterile nasal culture was $11.3° \pm 1$ which was not significantly different from controls whereas the 2 with

Table 22

Number of fields, cilia per field and cilia per case measured for orientation

	n	Number of fields		Number of cilia per field		Number of cilia per case	
		mean	range	mean	range	mean	range
Control	10	8.5	7-10	17.4	10-34	141	113-171
Clinical PCD	2	15.5	12-19	16.7	10-38	262.5	204-321
Bronchiectasis	12	12.2	5-21	17.0	10-45	213	78-400
Cystic Fibrosis	9	13.8	4-20	17.8	10-38	237.1	53-464

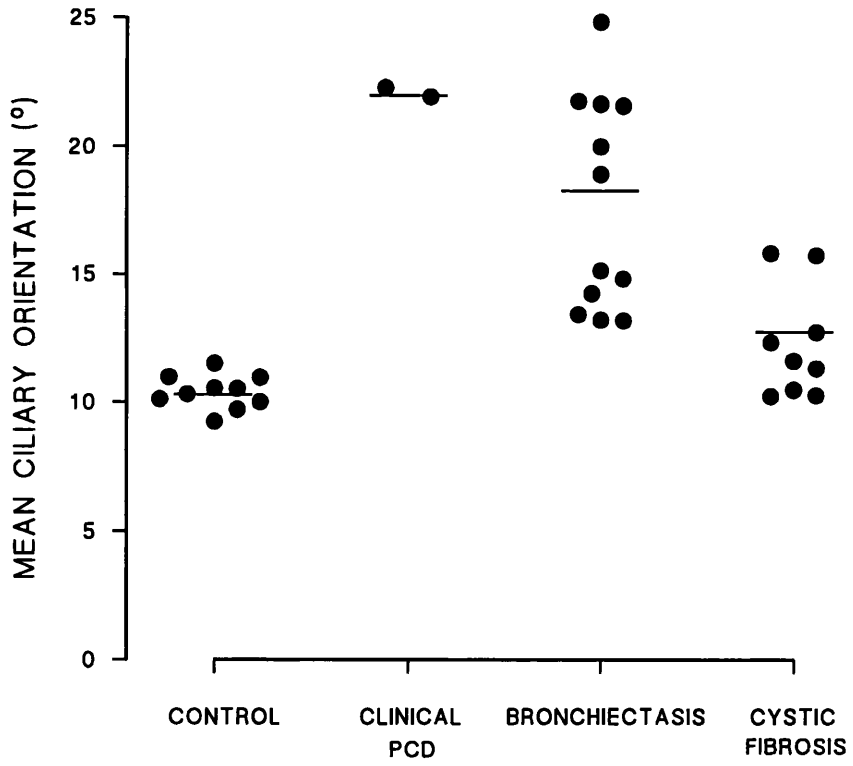


Figure 25

The mean ciliary orientation in three patient groups with chronic mucopurulent sinusitis and a normal control group. The mean ciliary orientation was significantly increased as compared to controls in all three patient groups.

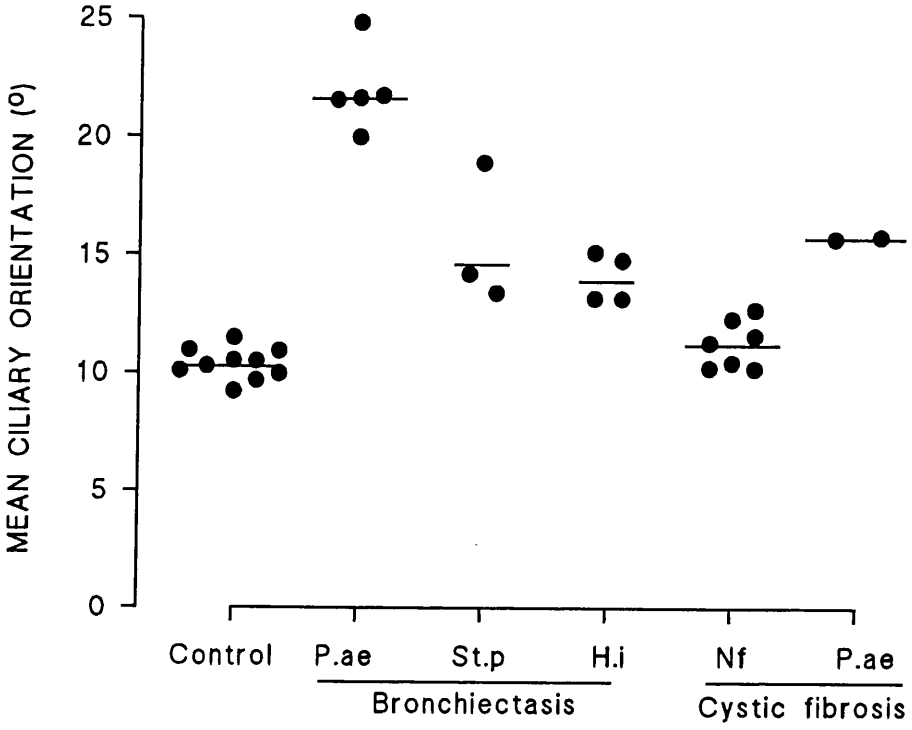


Figure 26

The mean ciliary orientation in bronchiectasis and cystic fibrosis patients showing bacteriology of nasal swab cultures. All infected patients had a mean ciliary orientation which was significantly increased as compared to controls. P.ae = Pseudomonas aeruginosa, St.p = Streptococcus pneumoniae, H.i = non-typable Haemophilus influenzae, N.F = normal flora.

P.aeruginosa were significantly different 15.8° ± 0.6 ($p<0.02$) (Figure 25).

5.1.4.3 Orientation of basal feet compared to central pairs

In three subjects the ciliary orientation per cell was obtained for both the central pair and the lateral basal foot process. The three patients were: one control, one patient with clinical PCD, and one bronchiectatic patient with a positive nasal culture for P.aeruginosa (Figure 27). There was no significant difference between the assessment of ciliary orientation from the central microtubules and the basal feet ($p<0.35$).

5.1.4.4 Correlation between ciliary beat frequency and mean ciliary orientation

There was no correlation between CBF and mean ciliary orientation for the patients with bronchiectasis and cystic fibrosis $r = 0.3$ ($n = 21$) (Figure 28).

5.1.4.5 Correlation between nasal mucociliary clearance and mean ciliary orientation

Comparison of NMCC and mean ciliary orientation for the same subjects demonstrated a positive correlation $r = 0.9$ ($n = 21$) (Figure 29).

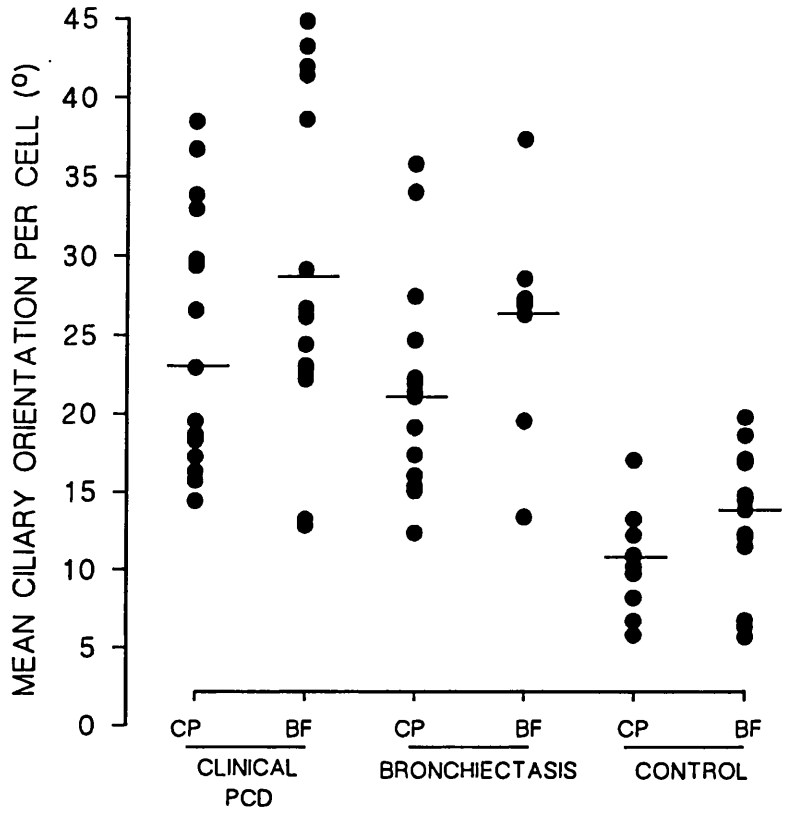


Figure 27

The mean ciliary orientation per cell measured via the central microtubules and basal feet in samples from 3 patients. One patient had clinical primary ciliary dyskinesia, one had bronchiectasis and was colonised by Pseudomonas aeruginosa, and one was a normal volunteer. There was no significant difference between assessment of ciliary orientation from the central microtubules and the basal feet.

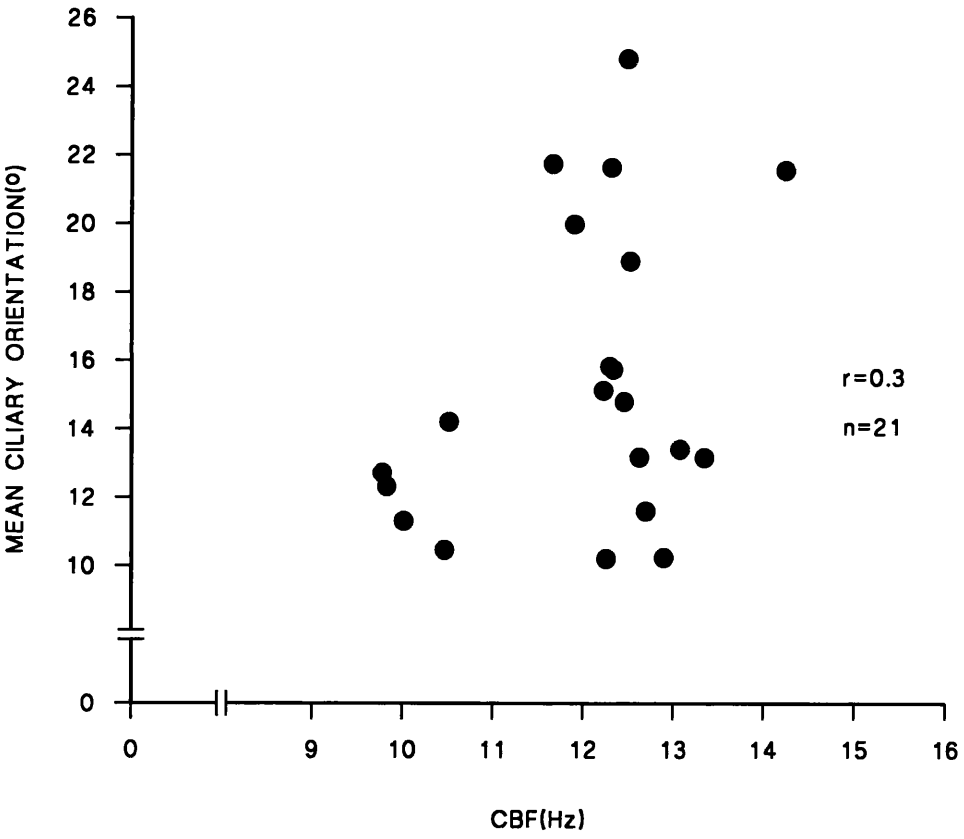


Figure 28

Correlation between ciliary beat frequency and mean ciliary orientation in patients with bronchiectasis and cystic fibrosis.

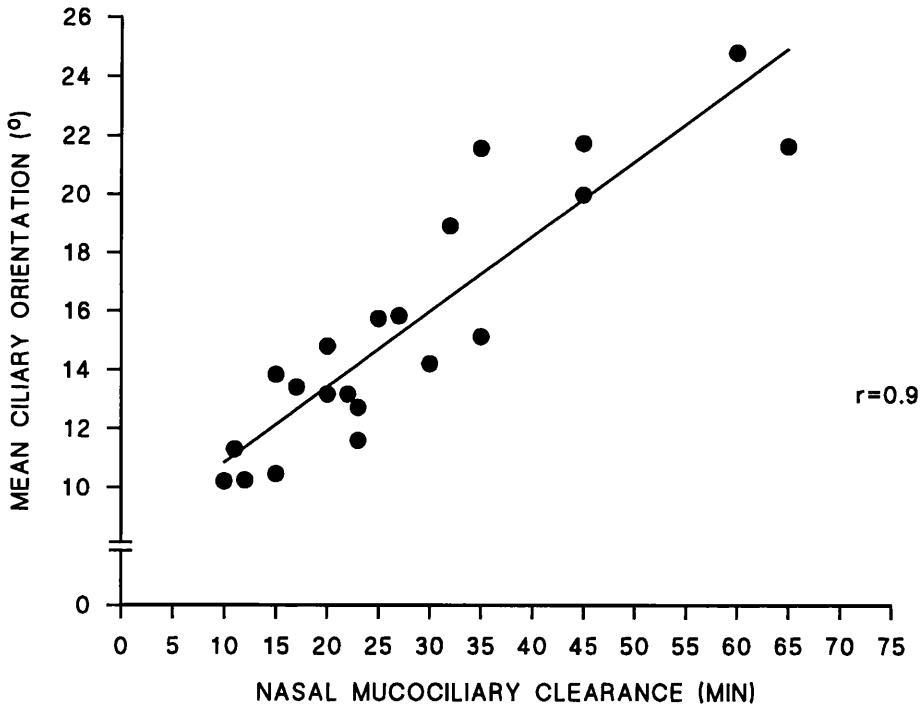


Figure 29

Correlation between the mean ciliary orientation and nasal mucociliary clearance in bronchiectasis and cystic fibrosis.

5.1.5 Repeat studies following antibiotic treatment

Studies were repeated in one patient with bronchiectasis and P.aeruginosa following three months of antibiotic and topical nasal corticosteroid treatment. During that time nasal culture became sterile, the NMCC fell from 37 to 20 min and the mean ciliary orientation fell from 21.57° to 10.25° ($p<0.05$).

5.1.6 Summary

1) Two patients with the clinical syndrome of PCD had absent NMCC. They had normal CBF and ciliary beat pattern, normal ciliary ultrastructure, but did have ciliary disorientation.

2) In 21 patients with chronic mucopurulent sinusitis and idiopathic bronchiectasis or cystic fibrosis, ciliary disorientation correlated with nasal mucociliary clearance.

3) Ciliary disorientation without ultrastructural defects of cilia and with normal CBF occurred in patients with chronic inflammation caused by infection. In one patient the delayed NMCC and slow CBF were reversed with appropriate and prolonged antibiotic therapy and anti inflammatory medication, which suggests that the changes are secondary to chronic inflammation due to infection.

4) Ciliary disorientation was greatest in patients infected with P.aeruginosa.

5) Measurement of orientation must be made through the basal feet and central pairs because these results may be discordant due to twisting of the ciliary shaft.

5.2 Ciliary Disorientation As A New Variant Of Primary Ciliary Dyskinesia

It has been demonstrated that ciliary disorientation occurs in association with chronic inflammation and that this correlated with delayed mucociliary transport. The two index patients described in the previous chapter had the classical features of PCD including dextrocardia and absent NMCC, however they had normal ciliary ultrastructure and normal CBF. Disorientation of their cilia was the only abnormality demonstrated. PCD encompasses a range of congenital abnormalities in the ultrastructure of the axoneme which may impair CBF or ciliary beat pattern and thus mucociliary transport. Random ciliary orientation has been described as a possible variant of PCD (Rutland and De Iongh 1992; Rutman et al 1993).

The aim of this study was to :

a) Assess the clinical findings, nasomucociliary function and ciliary orientation in a group of patients with the classical clinical features of PCD but normal ciliary ultrastructure.

b) To Study a family in which two siblings have the clinical features of PCD without axonemal ultrastructural abnormalities.

5.2.1 Study population

The Host Defence Unit has an interest in PCD and over several years, 11 patients with the clinical features of PCD but normal axonemal ultrastructure were identified. The mean age of the 11 patients was 23.6 ± 15.1 (range 12-38 years), 6 patients were women. All patients had a documented history of recurrent otitis media and 8 glue ear in childhood, all had a history of a persistent runny nose, chronic sinusitis and a constant productive cough since childhood (Table 23).

5.2.2 Radiology

Five of the eleven had situs inversus. Bronchiectasis was diagnosed in all 11 patients, 8 following high-resolution computerised tomography of the chest and in three by clinical signs and chest radiographs alone. In 4 patients, including the three in whom a diagnosis was made by X-ray and clinical findings alone, bronchiectasis was thought to be confined to one lobe only (three in the right middle lobe and one in the lingula). In 4 bronchiectasis was confined to three lobes and in 3 all lobes.

Table 23 The clinical findings, nasal mucociliary clearance, ciliary beat frequency and ciliary orientation of the eleven cases with clinical primary ciliary dyskinesia and normal ciliary ultrastructure

Subject Number	Sex	Age	Sinusitis	Otitis	Situs inversus	Bx	NMCC (mins)	CBF (Hz)	Orientation CP	Orientation BF
1	F	44	+	+	+	+	>60	14.2	22.2	20.8
2	F	38	+	+	-	+	>60	9.7†	21.8	22.7
3	M	29	+	+	-	+	>60	11.6	24.2	23.4
4*	M	16	+	+	-	+(X)	>60	12.6†	22.6	22.9
5*	F	14	+	+	-	+(X)	>60	8.9†	26.4	25.2
6	M	15	+	+	+	+	>60	14.9	23.4	28.9
7	F	10	+	+	+	+	>60	13.5	21.9	20.6
8	F	53	+	+	-	+	>60	8.6†	21.9	24.9
9	F	14	+	+	-	+	>60	8.6†	24.3	22.9
10	M	10	+	+	+	+(X)	>60	8.8†	23.4	21.1
11	M	18	+	+	+	+	>60	8.4†	25.0	25.2

BX = bronchiectasis. † = "stiff" beat pattern. * = sibling. X = x-ray diagnosis alone.

5.2.3 Pulmonary function

The percent predicted values of pulmonary function for the 11 patients are shown in Figure 31. The mean ± sd predicted values were as follows: FEV1 81.6 ± 16.1%, FVC 93.9 ± 16.7%, PEFR 100.2 ± 12.5%, TLC 100.2 ± 17.2%, RV 129.9 ± 33.0 and TLCO 89.6 ± 13.9%. The 11 patients therefore had mild impairment of lung function.

5.2.4 Mucociliary clearance

The results of NMCC and CBF for all 11 patients are shown in Table 23. NMCC was greater than 60 min in all 11 patients. The mean CBF was 11.2 ± 2.8 Hz, range 8.4 - 15.6 Hz. Six patients had a CBF which would considered below the normal range (11-16 Hz) (Rutland et al 1982). The ciliary beat pattern appeared synchronous in all eleven patients. However the beat pattern in 7 patients appeared "stiff", and the other 4 completely normal. The "stiff" beat pattern was present in all 6 patients with a CBF below the normal range, and in 1 patient with a normal CBF.

5.2.5 Ultrastructure

Nasal brushings adequate for functional and electron microscopy evaluation were obtained from all subjects. The mean number of cilia assessed for dynein arm defects was 151.1 ± 60 range 76-234; 97.8 ± 1.4 % were normal, 0.5 ± 0.6% had no inner arms and 1.7 ± 1.3% had absence of inner and outer dynein arms. The mean number of cilia assessed for microtubular defects and compound cilia was 411.2 ± 170

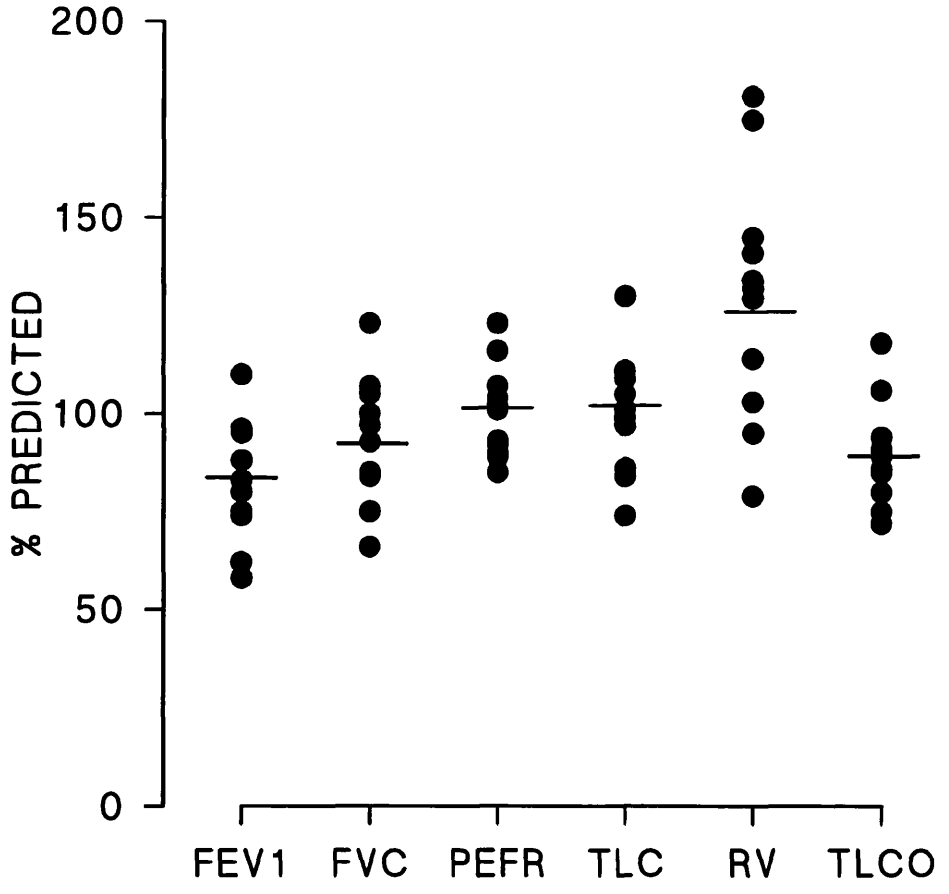


Figure 30

The percent predicted values of pulmonary function for the 11 patients.

range 198-600; 97.8% ± 0.9% were normal, 1.5% ± 0.9 had microtubular abnormalities and 0.7 ± 1.0 were compound in appearance. This frequency of abnormal ciliary ultrastructure has been described in healthy normals (Fox et al 1983; Rutland et al 1993).

5.2.6 Orientation

5.2.6.1 The number of fields and cilia assessed for orientation

The mean number of fields, cilia per field and cilia per case that were assessed for orientation of the central pair were 22.3 ± 6.8, 17.22 ± 5.5 and 435.1 ± 227.9 respectively, and for basal feet 18.8 ± 5.24, 12.25 ± 5.3 and 247.3 ± 109.4 respectively.

5.2.6.2 The mean ciliary orientation

The mean ciliary orientation as measured via central pairs and basal feet was 23.3 ± 1.5° and 23.5 ± 2.5° respectively (Figure 31). The results were compared to the mean ciliary orientation in 16 non-smoking non-atopic volunteers with normal axonemal ultrastructure. The mean ciliary orientation for the 16 subjects was 12.8 ± 1.5 for central pairs and 14.8 ± 1.6° for basal feet. Comparison of the mean ciliary orientation of basal feet and central pairs for the PCD group to the normal subjects show a significant difference ($p<0.001$) (Figure 31).

5.2.7 Orientation of bronchial brushings

Studies of ciliary orientation of right and left bronchial brushings on patient no 1 demonstrated a ciliary orientation as measured via central pairs and basal feet on the right of 23.5° and 20.7° respectively and on the left 23.7° and 21.8° which compared to nasal ciliary orientation of 22.2° and 20.7.°

5.2.8 Correlation between ciliary beat frequency and ciliary orientation

There was no correlation between CBF and mean ciliary orientation of cental pair (r=-0.28) or basal feet (r=-0.2) for the patients with clinical PCD (n=11).

5.2.9 Repeat studies following treatment

Two patients with positive sputum and nasal cultures for H.influenzae underwent repeat studies following three months of oral amoxycillin and topical nasal corticosteroid treatment. Treatment resulted in a symptomatic improvement of cough and a decrease in the volume of sputum produced. Sputum and nasal cultures became sterile. There was no change in NMCC (>60 min) and no change in the mean ciliary orientation for central pairs from 25.2° to 24.5° and basal feet from 23.8° to 23.05°.

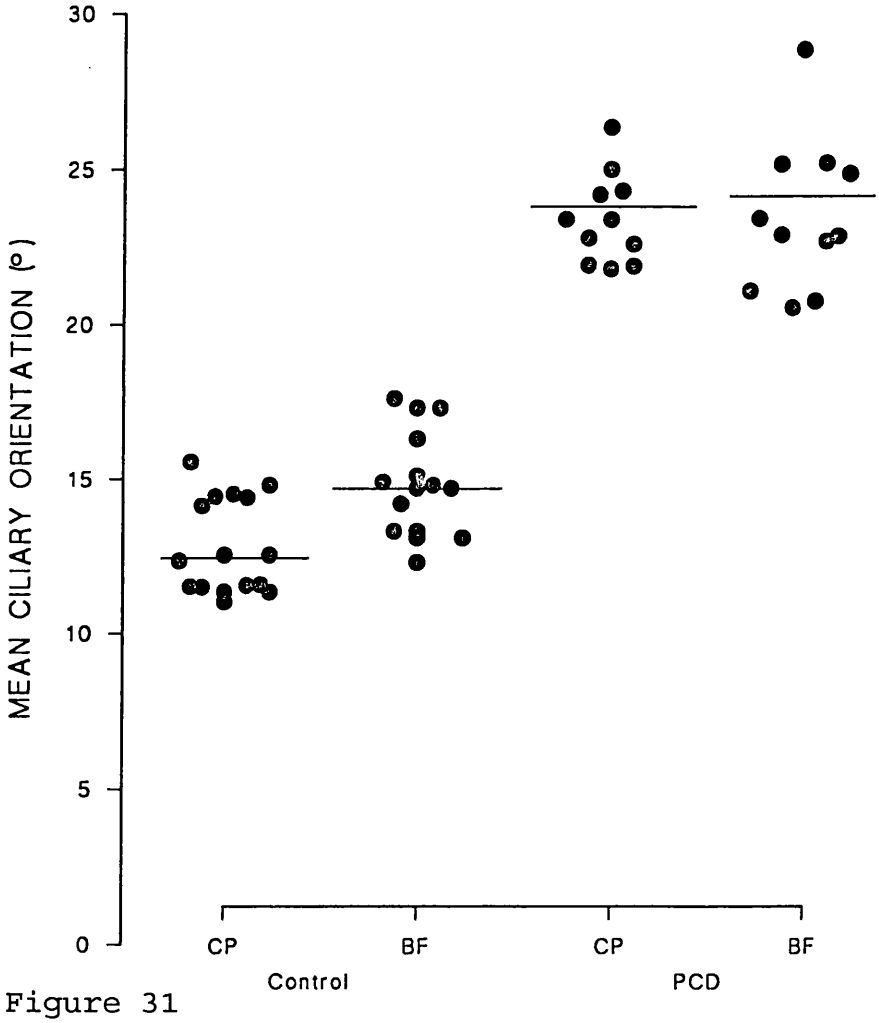


Figure 31

The mean ciliary orientation as measured via central pairs and basal feet for the 11 patients with clinical primary ciliary dyskinesia and 16 controls.

5.2.10 Family studies

The results from the family with two affected siblings are shown in Table 24. Brushings from the affected siblings in 1983 and 1994 were available for assessment. In the parents and the non-affected sibling NMCC was normal, the CBF and ciliary beat pattern were normal, and ciliary orientation of the central pair and basal feet was comparable to the 16 control subjects. The ciliary orientation of the central pairs and the basal feet of the two affected siblings was similarly abnormal when assessed at two time points 11 years apart.

Table 24

The ciliary beat frequency, nasal mucociliary clearance and ciliary orientation of the family with two affected siblings

Family member	Sex	Age	NMCC (min)	CBF (Hz)	Orientation CP	Orientation BF
Father	M	46	8	13.2	13.1	12.9
Mother	F	37	7	14.2	15.6	16.0
Normal sibling	F	17	6	13.6	13.4	18.2
Affected Sibling (1983)	M	16	>60	12.6	22.6	22.9
		5	-	10.0	26.7	25.8
Affected Sibling (1983)	F	14	>60	8.6	26.4	25.2
		3	-	9.3	25.0	24.4

5.2.11 Fertility assessment

Of the 11 patients 7 were either sexually inactive or using contraception. Patients 1, 2 and 8 had failed to conceive but had not undergone investigation. Fertility was investigated in patient 3 (Table 23). Seminal analysis demonstrated oligospermia and was as follows : Volume 1.5 ml; count 2 x 10^6; pH 7.3; viability 93%; total motility <1%; degenerate sperm, only a few sperm were suitable for tail ultrastructure assessment all of which demonstrated 9 plus 2 microtubule pattern with dynein arms.

5.2.12 Summary

1) The clinical findings in this group of 11 patients were: otitis media, chronic sinusitis, bronchiectasis with chronic productive cough, with situs inversus occurring in 5. Symptoms had been present since childhood.

2) Mucociliary clearance was absent.

3) Five of the 11 patients had a normal CBF (11-16 Hz) and in 6 the CBF was slow. In 4 patients (all normal CBF) the ciliary beat pattern was completely normal, but in 7 patients (6 with slow CBF) the beat pattern appeared "stiff" but ciliary dyskinesia (loss of synchronous beat pattern) did not occur.

4) Ciliary ultrastructure was normal in all 11 patients.

5) Ciliary orientation of both the central pair and basal feet was abnormal in all 11 patients compared to 16 normal volunteers.

6) In 2 patients the abnormalities were not reversed with appropriate and prolonged antibiotic and anti-inflammatory therapy.

7) In 1 patient the orientation of cilia obtained from three sites in the respiratory tract were similar.

8) Cilia from the affected siblings were similarly abnormal 11 years apart. The parents and the non-affected sibling had normal NMCC, CBF, ciliary beat pattern and ciliary orientation.

6.0 Discussion

The human respiratory tract is continually exposed to a variety of inhaled particles including bacteria, and yet infection rarely occurs because an elaborate array of host defense mechanisms exist which coordinate to remove potentially infectious agents (Newhouse et al 1976; Green et al 1977). Bacteria utilize a number of mechanisms to overcome host defences and to facilitate their own persistence (Cole and Wilson 1989). Health depends on the balance between bacterial factors and the ability of the host defences to clear bacteria from the respiratory tract favouring the host so preventing bacterial colonisation, proliferation and infection. Respiratory tract infections may therefore occur in the context of weakened host defences (which may be inherited or acquired), or conversely bacteria may cause infection in the presence of intact host defences (Reynolds 1988).

To study the interaction of bacteria with mucosal surfaces, either animal models, cell cultures, suspended epithelial cells or organ cultures can be used. Animal models represent an important step in the study of bacterial interaction with intact host defences and have the advantage that they can be manipulated in ways that would not be possible in human studies. The models may however differ substantially from conditions which pertain in humans (Pennington 1985; Wilson and Rayner 1994). In vitro

models may allow accurate regulation and measurement of both host and bacterial factors thought to be important in the pathogenesis of respiratory tract infections. For example cell culture monolayers allow the study of molecular interactions between bacteria or bacterial products and a specific cell type in the absence confounding factors such as mucus. Proposed mechanisms of interaction between bacteria or bacterial products and the mucosa that have arisen from experiments using more complex systems such as organ cultures can be confirmed or refuted using monolayers. For example, pyocyanin induced ciliary slowing of ciliated cells in organ culture was proposed to be caused by stimulation of neutrophils and generation of toxic oxygen radicals (Jackowski et al 1991). Using epithelial cell culture monolayers the ciliary slowing was shown still to occur in the absence of neutrophils suggesting that they were not involved (Kanthakumar et al 1993). So although monolayers provide a perpetual supply of cells for experiments respiratory cells may not maintain all the characteristics of their in vivo counterparts, for example there is often irreversible loss of cilia (Jorrisen and Van den Berghe 1991).

Although all the normal host defence mechanisms are not intact, and dispersal of cells may expose receptors which are not normally available in vitro, suspended cell culture models provide a powerful tool with which to initiate both studies and further investigate the interaction of bacteria

with the respiratory mucosa. For example, S.pneumoniae has been shown to attach to human pharyngeal cells through the specific interaction of bacterial surface adhesins with epithelial cell glycoconjugates containing disaccharide GlcNACß1-3Gal ß (Andersson et al 1983) or GalNACß1-Gal (Kirvan et al 1988). Epithelial cells obtained by scraping or brushing the respiratory mucosa are also relatively easy to obtain.

Organ cultures offer an insight into the interaction between bacteria and the intact respiratory mucosa. Normal epithelial function depends upon the retention of cell junctions, the polarity of the epithelium, its orientation on the connective tissue substratum (Chambard et al 1984) and the maintenance of the different cell types. In these respects, the intact mucosal surface of organ cultures is more physiological than isolated cell systems and cell culture monolayers. Organ cultures may utilise animal or human respiratory tissue, and provide one way of studying the effects of airborne agents on the structure and function of the respiratory epithelium. For example, they have been used to study the effects of oxygen metabolites (Adler et al 1990), cigarette smoke (Keeling et al 1993), bacteria (Read et al 1991, Stephens et al 1986), and their toxins (Johnson et al 1986), and the interactions between viruses and bacteria (Bakaletz et al 1988). The production of mucus (Adler et al 1990) and mediators (Kelsen et al 1993) can be measured, and inflammatory processes studied

or manipulated (Wills-Karp et al 1993).

Organ cultures are usually immersed in medium and therefore the models may differ substantially from conditions that pertain to natural infection. Unsealed cut edges present receptors not normally available for bacterial interaction and provide alternative routes for invasion of the epithelium. Immersion of tissue in media removes the air-mucosal interface and thus changes the dynamics of mucociliary clearance and the state of mucus hydration; also the media may support replication of bacteria allowing continuous interaction between high numbers of bacteria growing in the medium and their products with the epithelium. Conversely the media may dilute toxins and prevent bacteria coming into close proximity with the epithelium. The development of an organ culture with an air mucosal interface, which has been shown to have a viability of 5 days (Jackson et al 1994), may allow study of the interaction of bacteria with the respiratory mucosa under physiological conditions.

This thesis describes a simple nasopharyngeal organ culture in which the mucosa is exposed to air. This is achieved by maintaining the organ culture in a humidified atmosphere, sealing the surgically cut surfaces, and feeding the tissue from below via a filter paper wick whose ends are in medium.

6.1 The Pathogenesis Of Colonisation And Invasion Of The Respiratory Mucosa By Neisseria Meningitidis And Streptococcus Pneumoniae

The ability of different bacteria to adhere to mucosal surfaces is essential for host colonisation and often correlates closely with disease pathogenesis (Beachey 1981; Niederman 1989).

S.pneumoniae and N.meningitidis are exclusively human pathogens and may colonise the respiratory tract or cause life threatening invasive diseases.

The upper respiratory tract is the principal site of asymptomatic carriage and transmission to other individuals for N.meningitidis (Apicella 1989; Peltola 1983). The main mechanisms of transmission are by direct contact with respiratory secretions and by respiratory droplets (Shwatz et al 1989). Transmission of meningococci is facilitated by close contact, crowding and poverty and may be influenced by coexisting viral infection (Wall 1988). N.meningitidis is carried by 2-10% of the adult population during non-endemic periods (Greenfield et al 1971) although higher rates of carriage have been documented in household contacts of cases (17-50%), cohorts of military recruits (40-80%) and hospital personnel (Broome 1986).

S.pneumoniae colonises the upper respiratory tract of up to 70% of healthy adults, although carriage rates are higher

in young children and in people living in crowded conditions (Riley et al 1981). Colonisation of the nasopharynx may be asymptomatic, however S.pneumoniae may also spread contiguously to cause upper and lower respiratory disease including sinusitis, otitis media and pneumonia. S.pneumoniae is the commonest cause of community acquired pneumonia (Bath et al 1964; Macfarlane et al 1982; Woodhead 1990). S.pneumoniae also colonises the lower respiratory tract of patients with impaired host defences such as chronic obstructive airways disease (Laurenzi et al 1961) and this may result in repeated infective exacerbations.

N.meningitidis and S.pneumoniae may invade the upper respiratory tract resulting in bacteraemia and meningitis, and S.pneumoniae may also invade through the lower respiratory tract. The mechanisms that regulate S.pneumoniae and N.meningitidis on the nasopharyngeal surface and determine whether acquisition results in carriage, local disease or invasion are dependant on the balance between bacterial virulence and the host defense mechanisms. Group B stains of N.meningitidis are responsible for the majority of endemic disease. Certain group B strains that are genetically closely related appear to possess greater pathogenic potential than others, often causing prolonged outbreaks of meningitis (Poolman et al 1986). Studies have also shown that a minority of the 84 serotypes of S.pneumoniae cause invasive disease (Mufson

1990). For example, 30% of pneumococcal meningitis is caused by types 3, 4 and 5 (Wasilauskas and Hampton 1982).

The initial barriers to bacterial colonisation of the respiratory mucosa include mucociliary clearance, alveolar macrophages and a local immune system including secretory IgA (Reynolds 1988). Efficient mucociliary transport depends on the normal function and interrelations of cilia, mucus and periciliary fluid (Sleigh et al 1988). N.meningitidis and S.pneumoniae may delay mucociliary transport. S.pneumoniae has been shown to stimulate the secretion of mucus glycoconjugates (Adler et al 1986), and to release pneumolysin which slows CBF and causes damage to ciliated epithelia (Feldman et al 1990); N.meningitidis has not been shown to change mucus secretion but has been shown to have cytotoxic effects with loss of ciliated cells and a reduction in ciliary activity (Stephens et al 1986; Stephens and Farley 1991).

Bacteria may demonstrate tropism for both a particular area of the respiratory tract and for a particular mucosal feature. S.pneumoniae has been shown to demonstrate tropism for mucus (Plotkowski et al 1989, Feldman et al 1992). In contrast N.meningitidis has been shown to demonstrate tropism for non-ciliated cells interacting with microvilli (Stephens et al 1986), and may then enter the cell by a process of parasite directed endocytosis (McGee et al 1983; McGee et al 1988).

Pili are important adhesins of N.meningitidis and organisms isolated from patients are often piliated. Piliation appears to be required for colonisation of host mucosal surfaces and for at least some stages of invasive disease (Stephens et al 1985; Pinner et al 1991; Stephens et al 1984; Stephens et al 1983; Stephens and McGee 1981). N.meningitidis can also exploit their ability to produce a non-piliated, non-attaching phase to desorb from initial sites of infection and possibly allow movement to other locations. Expression of outer membrane protein may also mediate adherence and invasion of human cells by N.meningitidis. Strains of N.meningitidis which are capsule deficient and lack assembled pili have been shown to adhere to epithelial and endothelial cells if they express the Opc protein (Virji et al 1992). In contrast S.pneumoniae has been shown to attach to human pharyngeal cells through the specific interaction of bacterial surface adhesins with epithelial cell glycoconjugates (Andersson et al 1983; Kirvan et al 1988). Work has also identified an adhesin that forms a link between components of the pneumococcal cell surface and the carbohydrate receptors on the host cell (Andersson et al 1988).

Adherence to the mucosa places the bacteria in an ideal situation not only for invasion but also for the release of toxins which cause epithelial damage and impair host defences (Beachey 1981) S.pneumoniae releases a number of toxins which may cause epithelial damage including

pneumolysin, autolysin, and neuraminidase (Paton et al 1993). Nearly all clinical isolates of S.pneumoniae release the cytolytic toxin pneumolysin (Paton et al 1983; Kanclerski and Mollby 1987) which has been shown to cause ciliary slowing and epithelial damage (Feldman et al 1990). During growth of N.meningitidis vesicle-like structures are released from the surface which contain lipids, LPS, OMP, and capsular polysaccharide (CPS) (Anderson and Solberg 1988; Anderson et al 1987). CPS has been shown to be antiphagocytic (DeVoe 1982). The LPS of N.meningitidis may be important in breaching the mucosal barrier by inducing the host inflammatory response and following sialylation of LPS the immunogenicity of the antigenic determinants may be changed (Jennings et al 1984; Schauer 1982). Epithelial injury may also unmask potential receptors for bacterial adherence. S.pneumoniae demonstrates tropism for damaged cells compared to normal ciliated epithelium (Plotkowski et al 1989).

S.pneumoniae and N.meningitidis may also take advantage of impaired host defences. The antiphagocytic properties of the pneumococcal capsule are important virulence factors which can be overcome by type specific antibodies that activate the complement system resulting in opsonisation and removal of the pneumococcus by the liver and spleen (Landesman et al 1982). Splenectomy and complement deficiency are therefore important predisposing factors to pneumococcal disease. Splenectomy (Francke and Neu 1981)

and complement deficiency (Ross and Densen 1984) are also well known predisposing factors to meningococcal disease however the ability to mount a relevant functional antibody response is the major barrier to disease (Goldschneider et al 1969). Specific antibody to the CPS is an important defence against pneumococcal disease, and IgG2 subclass deficiency will predispose to recurrent sino-bronchial infection (Umetsu et al 1985). Acquired defects may also predispose to infection with N.meningitidis and S.pneumoniae. Concurrent viral respiratory infections may predispose individuals to systemic meningococcal disease (Young et al 1972; Harrison et al 1991) and the adherence of S.pneumoniae to animal tracheae significantly increases after viral infection (Plotkowski et al 1986).

If the initial epithelial barrier to infection is breached a cascade of events is set in motion resulting in the recruitment of circulating inflammatory cells and proteins including cytokines, complement and immunoglobulins (Toews 1988; Newhouse et al 1976; Green et al 1977; Currie et al 1987). S.pneumoniae and N.meningitidis use both different and similar methods to evade these cellular and humoral host defence mechanisms. The cell wall of S.pneumoniae may interact with complement and non-complement mediated host defences to induce pulmonary inflammation (Tuomanen et al 1989). IL1 and TNF are central mediators of inflammation for Gram-negative bacteria and endotoxin. In contrast to Gram-negative bacteria the pneumococcal cell wall does not

induce the production of TNF (Riesenfeld-Orn et al 1987). This suggests that the mechanism by which Gram-positive and Gram-negative bacteria induce an inflammatory response are fundamentally different. Both N.meningitidis and S.pneumoniae produce IgA protease which may protect the bacteria from opsonisation (Plaut 1983; Mulks et al 1980). Pneumolysin activates human complement reducing the maximal opsonic activity for S.pneumoniae (Paton and Ferrante 1983) and pretreatment of human lymphocytes with pneumolysin reduces the capacity of stimulated lymphocytes to release lymphokines and all three classes of immunoglobulins (Ferrante et al 1984).

6.1.1 The interaction of Neisseria meningitidis with nasopharyngeal tissue

Inocula of 10^6 of N.meningitidis reproducibly caused infection of the organ culture in this thesis. Previous studies using adenoid tissue suspended in medium reported that inocula of less than or equal to 10^3 bacteria did not reliably produce infection, but that this was achieved by inocula of 10^6 bacteria or more (Stephens et al 1991).

Adherence to mucus may be the first step in colonisation for a number of bacteria (Lamblin and Roussel 1983). It has been suggested that N.meningitidis penetrates the mucus barrier prior to epithelial attachment (Stephens et al 1991), but no quantitative assessment of N.meningitidis interactions with mucus has previously been reported. In

this thesis bacteria were rarely seen in association with mucus at any of the time-points studied, although this was more common for the non-piliated variant than the two piliated variants. When bacteria were seen they were usually at the edge of the mucus sheet, possibly due to build up of bacteria cleared from the organ culture surface by ciliary beating. The finding of low adherence to mucus may partly explain the observation that meningococcal epidemics occur in the dry season in sub-Sahara Africa (Moore 1992; Greenwood et al 1985). The upper respiratory tract mucus membranes are dried by low humidity, and this may expose larger areas of the epithelium to interaction with inhaled meningococci, or alter the rheology or adherence properties of mucus, because in this thesis the cultures were maintained in a humidified atmosphere. In contrast to N.meningitidis, lower respiratory tract pathogens such as P.aeruginosa and non-typable H.influenzae have been shown to avidly adhere to mucus (Vishwanath and Ramphal 1984; Read et al 1991; Farley et al 1986) and specific adhesin receptor interactions have been characterised (Ramphal et al 1989). The poor adherence of N.meningitidis to mucus could contribute to its failure to colonise the lower respiratory tract.

N.meningitidis were only very occasionally seen associated with cilia, although this was more common for the non-piliated variant compared to the piliated strains. There was a decrease in the surface area covered by cilia at 24

h in organ cultures infected by both piliated strains. Extruded ciliated cells were frequently seen and this may account for loss of ciliated epithelium, but it is also possible that the infection caused loss of cilia from cells. This has been observed with B.pertussis (Wilson et al 1991) and with viral infections (Carson et al 1985). Cilia appeared collapsed and bent in different directions in infected organ cultures compared to controls (Figure 9). This occurred at 12 h for both piliated variants and at 24 h for the non-piliated variant. The disorganisation of cilia and loss of ciliated cells would impair mucociliary clearance and facilitate bacterial colonisation. Previous studies have reported a significant fall in the CBF of human nasopharyngeal mucosa infected with N.meningitidis (Stephens et al 1986). The mechanism of slowed ciliary beat and damage to ciliated cells may be due to soluble bacterial toxins such as those described for other bacterial species (Wilson et al 1987; Read et al 1992; Steinfort et al 1989). Previous studies have reported loss of ciliated cells after meningococcal infection for 30 h (Stephens et al 1986), but this study used a model with tissue submerged in media. Experiments described in this thesis have shown that cytotoxicity occurs at an earlier time-point under more physiological conditions. This suggests that toxins are released in the micro-environment of the mucosa, and that they are more damaging in the air-mucosal interface model because they are not diluted by surrounding media. Ciliated cells seemed more vulnerable

to the toxic effects of meningococcal infection than non-ciliated cells.

Adhering bacteria were seen to produce blebs (Figure 11a) which are known to contain LPS, OMP and CPS (Anderson et al 1988; Anderson and Solberg 1988). Purified meningococcal LPS has been shown to slow CBF of fallopian tube cilia but not of nasopharyngeal cilia (Stephens 1986). During systemic infection meningococcal LPS induces a cytokine cascade and activates complement (Waage et al 1989a; Waage et al 1989b). Although LPS may have a direct cytotoxic effect, cytokines and complement may also be involved in the mucosal changes seen during meningococcal infection.

Previous studies using tissue immersed in medium have shown that meningococci appear to attach in large numbers to the microvilli of non-ciliated mucosal cells within 4 h of infection (Stephens et al 1991). Using this organ culture system with an air-mucosal interface, Piliated bacterial adherence was only rarely seen at 4 h, and at this time the mucosa was not significantly different from controls. This lack of adherence, despite a large inoculum of bacteria, might be explained by the protective effect of mucus to which the bacteria do not adhere. In the air mucosal interface model piliated bacteria were frequently seen adhering to unciliated cells at 12 h. Bacterial adherence to unciliated cells was associated with changes in the appearance of microvilli, both in direct opposition to the

bacteria and also on adjacent cells (Figures 10 and 11a, 11b & 11c). The changes in the microvilli progressed from elongation and single cross links between microvilli, to almost complete coverage of bacteria by a complicated mesh-work appearance (Figure 11c). The use of a gold label confirmed that bacteria were underlying this extensive cross-linking (Figures 15a & 15b and 16a & 16b) The reaction of the microvilli to meningococcal infection followed bacterial adherence and may precede invasion of the epithelium by endocytosis. However, changes in the microvilli occurred in adjacent cells lacking adherent bacteria which suggests that either soluble mediators stimulate the response on the mucosal surface, or that intercellular communication occurs resulting in a change in the cell cytoskeleton. Invasion of the epithelial cells in culture by N.meningitidis has been shown to be inhibited by cytochalasin D which suggests that invasion is dependant on changes in the cytoskeleton of the cell (Virji et al 1992).

Bacteria were also found to associate with dead cells and damaged cells overlying the epithelial surface, and to cells in areas where a break in the integrity of the epithelium had occurred due to disruption of the tight junctions between cells (Figure 14). This suggests that there may be an alternative method of epithelial invasion by an intercellular route in contrast to the parasite directed endocytosis which has previously been suggested as

the sole mechanism of mucosal invasion by N.meningitidis (McGee et al 1988). Respiratory infections such as influenza may predispose individuals to systemic meningococcal disease. Meningitis patients are four times more likely to have serological evidence of a coincident infection with influenza than control patients (Young et al 1972; Harrison et al 1991). Upper respiratory tract viral infections are known to damage ciliated epithelium (Carson et al 1985), and therfore adherence of N.meningitidis to dead and damaged cells may predispose to colonisation and invasion.

Piliated meningococci have been shown to adhere in large numbers to nasopharyngeal and to buccal epithelial cells in vitro compared with non-piliated bacteria (Stephens et al 1981; Craven and Frasch 1978). The non-piliated variant and piliated variants of strain MC58 that were used in this work have previously been shown to demonstrate variable adherence to Chang and HEP-2 epithelial cells in culture (Virji et al 1992). PIL+B adhered to epithelial cells more commonly than PIL+A, however there was no difference in the adherence of the two piliated strains to Huvec endothelial cells (Virji et al 1991). This suggests that there are different receptors for bacterial adhesins on epithelial and endothelial cells. The study in this thesis demonstrated greater adherence of both piliated variants to unciliated and damaged cells compared to the non-piliated variant and an increased density of PIL+B compared to PIL+A

on unciliated cells. While PIL+B did adhere in greater numbers to the other mucosal features, these differences did not reach statistical significance. The damage caused by meningococcal infection to the mucosa was greater for both piliated variants. This may be explained by their increased adherence to unciliated cells, which would increase the concentration of bacterial toxins in the micro-environment on the epithelial surface. Cell damage and separation of tight junctions may expose receptors for bacterial adherence which were not previously accessible. Previous studies have shown N.meningitidis pili facilitate adherence to human endothelial and epithelial cells (Heckels 1986), and piliated N.meningitidis have been shown in vitro to adhere in large numbers to unciliated nasopharyngeal cells (McGee and Stephens 1984). Damage to ciliated cells have been shown to occur more rapidly for piliated compared to non-piliated bacteria (Stephens et al 1986). These observations were similar to those noted in this thesis.

In summary the work in this thesis showed that pili increase adherence of meningococci to the mucosa of adenoid tissue. There is marked tissue tropism of piliated strains for non-ciliated cells containing microvilli, and to a lesser extent for damaged cells. The number of bacteria adhering to cilia and mucus was very low, but was significantly greater with the non-piliated variant. Meningococcal infection caused epithelial damage, loss of

ciliated epithelium and ciliary disorganisation which was greater with piliated strains. Bacterial adherence stimulated a florid reaction in the microvilli which enveloped bacteria. Bacteria also adhered to areas where the integrity of the epithelium was interrupted by separation of tight junctions which suggests that bacteria might also invade the epithelium by an intercellular route.

6.1.2 The interaction of Streptococcus pneumoniae with nasopharyngeal tissue

Adherence to mucosal surfaces is thought to be an important determinant of colonisation and the pathogenesis of most bacterial infections (Niederman 1989; Beachey 1981). Previous studies have shown that S.pneumoniae adhere to suspended buccal and nasopharyngeal cells (Andersson et al 1983; Andersson et al 1981; Andersson et al 1985; Selinger and Reed 1979) S.pneumoniae has been shown to attach to human pharyngeal cells through the specific interaction of bacterial surface adhesins with epithelial cell glycoconjugates and an adhesin that forms a link between Pneumococci and the carbohydrate receptors on the host cell has been identified (Andersson et al 1988). However little is known about the interaction of S.pneumoniae with intact respiratory mucosa.

Respiratory mucus contains a heterogeneous mixture of mucus glycoprotein molecules which may differ in the amount and extent of sialylation and sulphation of the constituent

oligosaccharides (Lamblin et al 1991; Sheehan et al 1991). The multiple carbohydrate chains of the mucin molecule may represent sites for adhesion of microorganisms (Lamblin et al 1991). Using the frog palate it has been demonstrated that S.pneumoniae adheres rapidly to mucus but never to ciliated cells or cilia, even after attempts to remove the mucus by washing prior to bacterial application (Plotkowski et al 1989). This suggests that Pneumococci cannot adhere to normal ciliated mucosa. Bacterial infection of guinea pig organ cultures has been shown to increase mucus production (Adler et al 1986). In the study in this thesis S.pneumoniae demonstrated tropism for mucus, however there was no change in the surface area covered by mucus. The absence of an increase in mucus production may be explained by either the effect of mucociliary clearance removing mucus from the organ culture or a difference in response to pneumococci infection of animal tissue compared to human tissue. The appearance of the mucus changed in the infected organ cultures. The mucus appeared granular and bacteria were seen in association with the mucus. These appearances were similar to those seen previously in a submerged model of nasal turbinates in organ culture in which S.pneumoniae was shown to occupy a gelatinous layer formed above the epithelial surface (Feldman et al 1992). The nature of the gelatinous layer was uncertain, although it was postulated that it might contain both mucus and bacterial products. This may be mediated by bacterial products (Somerville et al 1991) or host inflammatory

mediators such as proteases (Nadel 1991). The character of the mucus may also be changed by the infection (Griod et al 1992). The pneumococcal toxin, neuraminidase, might be expected to decrease the viscosity of mucus through cleavage of sialic acid residues (Paton et al 1993).

In a submerged model of S.pneumoniae infection bacteria within the gelatinous layer were found in large numbers and appeared in long chains (Feldman et al 1992), whereas in the model described in this thesis bacteria were in smaller numbers and appeared as diplococci. In vivo pneumococci are normally found as diplococci or occasionally as short chains whereas when replicating in broth culture or after prolonged incubation on agar they are often found in chains (White 1979). This suggests the air mucosal model provides conditions for bacterial growth which resemble the in vivo situation more closely than submerged models.

The isogenic variants of S.pneumoniae both failed to adhere to intact epithelium. This failure of S.pneumoniae to adhere to intact epithelium has been noted previously (Feldman et al 1992, Plotkowski et al 1989). Pneumococci have been shown to bind to pharyngeal epithelial cells (Andersson et al 1983; Andersson et al 1981; Andersson et al 1985; Selinger and Reed 1979). The adherence of pneumococci to pharyngeal cells is lower than that seen for Streptococcus pyogenes (Selinger and Reed 1979).

In the studies reported in this thesis, the pneumolysin sufficient S.pneumoniae caused a progressive slowing of CBF over 48 h and there was loss of ciliated epithelium. The cilia appeared disorganised (Figure 18) with an increased granularity on the surface of the cilia shafts and microvilli, similar to that produced by pneumolysin in the guinea pig cochlea (Commis et al 1993). Pneumolysin causes ciliary beat slowing and ciliostasis, (Feldman et al 1990) and has been shown to be toxic for the guinea pig cochlea causing disorganisation and loss of cilia, damage to the hair bundles and change in the surface of the cilia (Commis et al 1993). By 48 h PL-infected organ cultures also showed slowing of CBF and an increase in the surface area associated with cell damage, suggesting that other factors besides pneumolysin cause ciliary slowing. Respiratory pathogens are also known to release a number of products which interfere with mucosal defences by slowing ciliary beat, causing ciliary dyskinesia (Johnson and Inzana 1986; Read et al 1992; Steinfort et al 1989; Wilson et al 1988) and damaging epithelial cells (Wilson et al 1988; Feldman et al 1990, Feldman et al 1991. Rubbins et al 1993). PL+ infected organ cultures showed a progressive increase in the surface area covered by damaged cells, cell surface damage included pitting and craters (Figure 17) and extruded ciliated and unciliated cells were seen. S.pneumoniae adherence to the mucosa may change if the epithelium is damaged, this may be due to a change in the receptors that were previously unavailable. Both variants

of S.pneumoniae were seen to adhere to damaged cells and extruded cells (Figure 22). Influenza A infection of murine respiratory epithelium caused damage which led to a significant increase in S.pneumoniae adherence compared to non-viral infected tissue (Plotkowski et al 1986). Andersson reported the binding of pneumococci to the collagen-binding region of fibronectin present on the surface of the human pharyngeal cells (Andersson et al 1983). Fibronectin is a glycoprotein present in interstitial connective tissue and which may be found in an insoluble form on epithelial cells (Ruoslahtie et al 1983). Damage to epithelial cells may enable S.pneumoniae to bind to fibronectin. Terminal sialic acid residues in oligosaccharide chains of membrane glycoproteins may mask cellular antigens. Such antigens may be revealed by treating cells with neuraminidase (Howie and Brown 1985). Separation of tight junctions between apparently normal unciliated cells, and bacterial adherence at this site, was seen exclusively in PL+ infected organ cultures. This might represent an important mechanism of invasion which appears to be dependent on pneumolysin. Pneumolysin is easily oxidised (Avery and Neill 1924) and this inactivates the toxin, but despite the air interface the damage caused by the PL+ variant was greater than PL-, suggesting that pneumolysin is active in the microenvironment on the mucosal surface. TEM also showed toxic changes to cell ultrastructure including mitochondrial damage. Mucosal damage occurred in the absence of bacterial adherence which

suggests that a diffusible bacterial factor released on the mucosal surface may mediate these changes. Although the damage to the mucosa at 24 h assessed by TEM was significantly greater for the PL+ variant, TEM of organ cultures infected by PL- showed that other factors beside pneumolysin cause cell damage. Studies have shown that autolysin mediates release of highly inflammatory cell wall break down products which could contribute to epithelial cell damage of S.pneumoniae (Chetty and Kreger 1981; Chetty and Kreger 1980; Tuomanen et al 1989). Hyaluronidase and neuraminidase may also cause epithelial cell damage (Paton et al 1993). A pneumolysin sufficient type III S.pneumoniae infection of an immersed organ culture caused slowing of CBF, but only minor damage to the epithelial surface at 24 h (Feldman et al 1992). This was despite large numbers of bacteria in the mucus layer overlying the epithelium. The difference between their study and that reported in this thesis may be due to immersion of tissue in culture medium which may dilute the concentration of bacterial toxins produced on the mucosal surface. An alternative explanation might be that pneumococci express factors which damage the epithelium differently depending on whether they are surrounded by liquid or at an air liquid interface. Bacteria may express highly variable virulence factors. For example N.meningitidis may switch reversibly between a piliated and non-piliated state which allows the pathogen to absorb and desorb from epithelial and endothelial cells (Saunders et al 1993) and

H.influenzae may switch from a fimbriated to a non-fimbriate strain during invasion of the infant rat nasopharynx (Kaplan et al 1983). Pneumolysin has previously been shown by electron microscopy to exert a toxic effect on epithelial cells (Commis et al 1993; Steinfort et al 1989) and has also been shown to cause endothelial and alveolar epithelial cell injury (Rubbins et al 1992; Rubbins et al 1993).

Pneumolysin is thought to be directly involved in the pathogenesis of pneumococcal disease (Bhakdi et al 1988) and instillation of pneumolysin into the rat lung induces the salient histological features of a pneumococcal pneumonia (Feldman et al 1991). However after 48 h PL-infected organ cultures showed an increase in the surface area associated with cell damage, suggesting that other bacterial factors besides pneumolysin damage the respiratory mucosa. However, mucosal damage was less severe in PL- infected organ cultures, and it is possible that host inflammatory mediators released by the organ culture in response to the infection, such as cytokines, nitrogen oxides or prostaglandins, could be involved in causing epithelial cell damage (Gaston et al 1994; Nicod 1993; Wanner et al 1983) or other pneumococcal products in addition to pneumolysin are cytotoxic (Paton et al 1993).

Using an organ culture with an air interface the study has shown that ciliary beat slowing, epithelial damage with

separation of epithelial cells, and abnormal mucus occur during infection of the respiratory mucosa by S.pneumoniae in vitro. A isogenic variant deficient in pneumolysin had delayed onset and reduced severity of ciliary slowing and epithelial damage compared to a pneumolysin sufficient strain. Bacterial adherence occurred to mucus, damaged epithelial cells and the edge of unciliated cells when separation of tight junctions had occurred. Separation of epithelial cell tight junctions between apparently normal unciliated cells occurred exclusively with the pneumolysin sufficient strain and may provide a route of invasion by S.pneumoniae.

6.1.3 Comparison of the interaction of Streptococcus pneumoniae and Neisseria meningitidis with nasopharyngeal tissue

The viable count of the inoculum for N.meningitidis and S.pneumoniae were similar at 5 x 10^7 and 1 x 10^8 respectively. At 4 h there was no significant change in the measured mucosal parameters and despite the high inoculum of both pathogens bacterial adherence was not seen. Adherence to mucus may be the fist step in colonisation of the respiratory tract and S.pneumoniae demonstrated tropism for mucus which was associated with a change in the appearance of the mucus. S.pneumoniae is both a lower and upper respiratory tract pathogen and the ability to adhere to mucus may aid colonisation. However in contrast N.meningitidis was only rarely seen in association with

mucus. N.meningitidis demonstrated tropism for non-ciliated epithelium and the interaction resulted in a change in the appearance of microvilli. The adherence of N.meningitidis to unciliated cells appeared to be mediated by pili. In contrast S.pneumoniae was only rarely found in association with unciliated epithelium unless there was evidence of loss of tight junction integrity. Both pathogens caused disorganisation of cilia, and damaged ciliated epithelium but did not adhere to cilia suggesting that both release diffusible factors which are toxic to cilia. The isogenic variant of S.pneumoniae sufficient in pneumolysin caused an earlier and greater reduction in CBF and epithelial damage compared to the pneumolysin deficient variant. This suggests that pneumolysin demonstrates toxicity on release and confirms previous reports that it causes epithelial damage and slows CBF (Feldman et al 1990; Rubbins et al 1993). Ciliated cells appeared more vulnerable to the toxic effects of N.meningitidis and S.pneumoniae than non-ciliated cells. Ciliary slowing and damage to ciliated epithelium has been previously described for both pathogens in submerged organ cultures (Feldman et al 1992; Stephens et al 1986). Damage to ciliated epithelium and slowing of CBF may result in slowing of mucociliary clearance and hence decrease the rate of bacterial clearance. In the respiratory tract initial adherence of these two pathogens to epithelial cells may be less important than slowing of mucociliary clearance. Both S.pneumoniae and N.meningitidis caused a progressive

increase in epithelial damage and both pathogens adhered to areas of damaged epithelium. The breeches in epithelial integrity may represent a route for S.pneumoniae invasion and an alternative route for N.meningitidis other than parasite directed endocytosis has been proposed as the sole mechanism for invasion (McGee et al 1983; Stephens and Farley 1991).

In summary S.pneumoniae demonstrates tropism for mucus whereas piliated N.meningitidis demonstrates tropism for intact non-ciliated epithelial cells and only rarely adhere to mucus.
S.pneumoniae only rarely adheres to intact epithelium.
N.meningitidis and S.pneumoniae do not bind to ciliated epithelium but do cause damage to ciliated epithelium.

Epithelial damage may provide a route for invasion for both N.meningitidis and S.pneumoniae.

6.2 The Effect Of Pyocyanin, 1-Hydroxyphenazine And Rhamnolipid On Ciliary Orientation In Vitro

Disorganisation of cilia slowing of CBF and damage to ciliated epithelium may result in slowing of mucociliary clearance and hence decrease the rate of bacterial clearance. In the respiratory tract slowing of mucociliary clearance may be an important step in the initial colonisation by pathogens. Both N.meningitidis and S.pneumoniae caused disorganisation of cilia, and damaged

ciliated epithelium but did not adhere to cilia suggesting that both release diffusible factors which are toxic to cilia. Bacterial toxins may aid organisms to persist in colonised airways by slowing ciliary beating and also by delaying mucociliary transport (Wilson et al 1987; Wilson et al 1988; Munro et al 1989; Read et al 1992; Pier 1985; Pitt 1986). Ciliary dyskinesia has been reported as to occur in association with ciliary slowing following the application of pyocyanin and 1-HP toxins to ciliated epithelium (Wilson et al 1988). However, the effect of bacteria or bacterial products on the organisation of cilia has not previously been reported. Although ciliary dyskinesia has been described in patients with ultrastructural abnormalities in PCD (Pedersen et al 1982; Greenstone et al 1988) the association between dyskinesia and ciliary organisation is unknown.

P.aeruginosa produces the phenazine pigments pyocyanin and 1-HP and also a haemolytic glycolipid, rhamnolipid. These three products of P.aeruginosa have been shown to be ciliotoxic in vitro (Wilson et al 1987) and in vivo and to slow mucociliary clearance in vivo (Munro et al 1989; Read et al 1992) at concentrations found human sputum (Wilson 1988). These three products were therefore chosen to investigate further if a change in ciliary organisation occurred in association with ciliary slowing or dyskinesia. All cilia on a single cell should have a common orientation which can be measured by drawing a line transecting the

central pair or basal foot (De Iongh and Rutland 1989). The orientation of cilia was therefore used as a measure of ciliary organisation.

The work in this thesis has shown that 1-HP causes an immediate reduction in CBF associated with ciliary dyskinesia, and that pyocyanin causes a progressive slowing of CBF which at 2 h was associated with ciliary dyskinesia. Rhamnolipid caused slowing of CBF at 60 min ($p<0.05$) and the slowing remained significantly different from control but dyskinesia was not seen. By 3 h there was also disruption of the epithelial strips. These findings concur with previous studies which demonstrated a reduction in CBF, epithelial disruption but not dyskinesia following addition of rhamnolipid to nasal epithelium in vitro (Read et al 1992).

Analysis of orientation showed a significant increase in disorientation of the central pairs of 1-HP treated cilia compared to controls ($p<0.02$), and a greater increase in disorientation for pyocyanin treated cilia ($p<0.005$) compared to controls. There was no change in basal feet orientation for 1-HP or pyocyanin treated cilia. This suggests that the shafts of the cilium may be twisting with respect to the anchored basal feet (Sleigh and Silvester 1983). Pyocyanin enhances the oxidative metabolism of neutrophils (Ras et al 1990), and it has been suggested that the action of pyocyanin on ciliary beating may be by

the activation of mucosal neutrophils and generation of toxic oxygen radicals (Jackowski et al 1991). However recently it has been shown that the pyocyanin slowing of CBF is accompanied by a fall in both intracellular cyclic AMP and ATP and is prevented by the cyclic AMP analogue dibutyryl cyclic AMP, the phosphodiesterase inhibitor isobutyl methylxanthine, and the adenylate cyclase stimulant forskolin (Kanthakumar et al 1993). In the same study it was shown that slowing of CBF by pyocyanin occurred in cell culture monolayers in the absence of neutrophils. $ß_2$ adrenoreceptor agonists have been shown to increase cyclic AMP levels in both animal and human respiratory epithelial cells (Lansley et al 1992; Devalia et al 1992).The $ß_2$ adrenoreceptor agonist salmeterol has been shown to protect against the pyocyanin-induced fall in intracellular adenosine nucleotides and this was associated with a reduction in pyocyanin-induced ciliary slowing (Kanthakumar et al 1994), which suggests that these events could be mediated via a common mechanism. As ATP is an essential energy source for beating cilia (Satir 1989; Satir and Sleigh 1990), it is possible that the effects of pyocyanin on CBF and coordination of ciliary beat are directly mediated through the fall in intracellular ATP levels. Cyclic AMP may affect the availability and usage of ATP by the ciliary axoneme (Lansley et al 1992). Ciliary movement is achieved by an ATP mediated retraction of the outer dynein arms, followed by ATP hydrolysis, extension and reattachment to the adjacent microtubule (Gibbons 1965;

Satir 1965). Reduction in intracellular cyclic AMP and ATP may result in disruption of normal coordinated ciliary movement. Cilia may therefore be at different points in their cycle of ciliary movement and may therefore appear disoriented with respect to each other as measured by the central pair, but not as measured by basal feet. Ciliary disorganisation assessed by electron microscopy of tissue infected by N.meningitidis and S.pneumoniae in this work, may represent ciliary dyskinesia seen by light microscopy of living cells. The measurements made in this work allow an assessment of the severity of disorganisation.

1-HP is a base hydrolysis product of pyocyanin (Watson et al 1986), and its mechanism of action has been suggested to involve blocking of the electron transport chain in mitochondria. It has been proposed that 1-HP accepts electrons from but fails to donate electrons back to the chain. Electron transport is impaired which may in turn impair ATP generation (Armstrong and Stewart-Tull 1971). It is possible that reduction of intracellular ATP may cause the effects of 1-HP on cilia and disorientation of cilia as measured by the central microtubules.

Rhamnolipid is a number of glycolipids with a detergent-like structure with a polar head and a non-polar tail, and their surfactant-like properties may account for their known haemolytic activity. They have been shown to interfere with epithelial ion transport in sheep

respiratory epithelium in a concentration-dependant manner (Graham et al 1993), to slow CBF and disrupt the integrity of human epithelium (Read et al 1993). Although rhamnolipid is known to cause ciliary slowing it has not been observed to cause ciliary dyskinesia (Read et al 1992). Measurement of orientation showed that although this was 17.4 $\pm$ 3.5° compared to control 14.4 $\pm$ 2.6°, this was not significantly different. The disorientation of ciliary central microtubes in rhamnolipid treated epithelium may be due to epithelial disruption caused by the toxin. The orientation of control cilia measured by the standard deviation of central pairs at three hours was increased compared to the control cilia fixed immediately in the 1-HP experiments, which probably accounted for the lack of significance. Ciliated epithelial strips become curved in appearance if left in suspension and it is possible that this change in appearance is associated with an increase in the standard deviation of orientation seen in the control cilia. However, because orientation is measured from cilia on individual cells this not likely to be the case. Control epithelial strips do demonstrate some disruption after three hours and this may be contributing to the increase in ciliary disorientation.

These experiments show that bacterial products cause ciliary slowing and dyskinesia which is associated with disorientation of their cilia as measured by the central pairs but not their basal feet. This suggests that there is twisting of the axoneme. The mechanism of the

disorientation has not been elucidated but may involve a reduction in intracellular ATP.

In summary :

1) Pyocyanin and 1-HP at pathophysiological concentrations cause ciliary slowing and dyskinesia.

2) Pyocyanin and 1-HP at pathophysiological concentrations causes disorientation of the ciliary microtubular pairs and this occurs at time points when ciliary dyskinesia is visible by light microscopy.

3) Although pyocyanin and 1-HP at pathophysiological concentrations cause disorientation of the ciliary microtubular pairs, the orientation of basal feet did not change this suggests that the shafts of the cilium may be twisting with respect to the anchored basal feet.

4) These products are known to cause slowing of mucociliary clearance <u>in vivo</u>. Disorientation of ciliary beat as well as slowed CBF may contribute to the slowing of mucociliary clearance <u>in vivo</u>.

5) Rhamnolipid at pathophysiologic concentrations caused ciliary slowing but not dyskinesia. Disorientation of ciliary central microtubules was increased possibly following disruption of epithelial integrity but this

was not significantly different from controls.

6.3 Ciliary Orientation In Patients With Chronic Inflammation Due To Infection And Patients With The Clinical Features Of Primary Ciliary Dyskinesia But Normal Ciliary Beat Frequency And Ultrastructure

6.3.1 The effect of chronic inflammation on ciliary orientation <u>in vivo</u>

Patients with chronic infection have delayed mucociliary transport for which there are likely to be several causes, including increased mucus production, abnormal mucus rheology, cilia slowing and loss of ciliated cells (Griod et al 1992; Wilson 1988).

This work confirms previous reports (Rashad et al 1983) that patients with chronic mucopurulent sinusitis have slow mucociliary clearance. The results also suggest that ciliary disorientation without other ciliary ultrastructural defects may occur secondary to chronic inflammation. In the 12 patients with chronic mucopurulent sinusitis and bronchiectasis, and in the cystic fibrosis group with positive nasal cultures, the ciliary disorientation was significantly greater than controls. There was a strong correlation between NMCC and the mean ciliary orientation, which suggests that ciliary disorientation contributes to slowed NMCC. However ciliary disorientation did not affect the appearance or rate of

cilia beating by light microscopy.

Rautiainen (1988), and De Iongh and Rutland (1989) demonstrated that the orientation of cilia may vary in normal subjects. Rautiainen (1988) found a ciliary orientation of 27.3 ± 7.4° and De Iongh and Rutland 14.4 ± 4.0° (1989) which are both greater than 10.47 ± 0.53° in the normal subjects in this work. This may be explained by a difference in the type of field used to measure orientation. In this work fields were chosen from a continuous strip of cells originating from single cells and represents an assessment of intracellular orientation. Rautiainen (1988) and De Iongh and Rutland (1989) used fields containing larger numbers of cilia which may have originated from more than one cell. It seems likely that although in vivo cilia on adjacent cells should have a common orientation, intercellular variation may be greater than intracellular variation. In addition tissue biopsy and preparation may increase intercellular variation resulting in a greater standard deviation. Rautiainen (1988) also included biopsies from six patients undergoing transphenoidal hypophysectomy for hypophyseal tumours which may not represent healthy controls.

Previous studies have suggested that in some patients with respiratory tract disease, ciliary orientation may be abnormal. Ciliary disorientation has been described in patients with asthma (Laitinen et al 1985), and in rats

with experimental bronchitis (Iravani and van As 1972). Viral infection in children causes a range of abnormalities of the respiratory epithelium, from being relatively normal to one of marked cytopathic effect. Dysmorphic cilia are found, with the most common abnormality being that of altered configuration of microtubules. However, within focal areas, cilia with normal axoneme ultrastructure were observed which often appeared to show disorientation when compared to their neighbouring cilia (Carson et al 1985). However Rautiainen et al (1992) found the most common ultrastructural change in adult nasal cilia caused by the common cold was loss of cilia and ciliated cells without an increase in microtubular abnormalities, and ciliary orientation was normal. The difference in results from these two studies may be explained by the fact that Carson et al (1988) confirmed the diagnosis of viral infection by culture and Rautiainen (1992) made the diagnosis by symptomatology alone. Abnormalities in the ultrastructure of bronchial cilia have been reported in patients with chronic bronchitis, and disorientation was reported as occurring frequently in these patients but the degree was not quantified (Lunggarella et al 1983).

De Iongh and Rutland (1989) examined the orientation of nasal cilia in seven patients with bronchiectasis and reported orientation as being normal. However they did not report whether the patient had evidence of upper airway inflammation or infection. This may explain why their

results differed from those reported in this work. It has also been suggested that measurements of ciliary orientation made from the central microtubules might be open to error because the central microtubules may rotate within the axoneme during bending, or that the cilia may twist during fixation and proeessing for electron microscopy. If this were the case, measurement of ciliary orientation would only be valid if it were made at the level of the basal foot (Holley and Afzelius 1983; Sleigh 1983). In the previous section of this thesis it was shown that bacterial toxins can cause twisting of the axoneme *in vitro*. De Iongh and Rutland (1988) measured ciliary orientation at three levels along the length of the cilium and showed that there was little variation in the mean orientation at these three levels. They suggested that representative measurements may be made at almost any point along the cilium. In this work the orientation of the basal feet and central microtubules of two patients with disorientation and one control subject was measured. In each case the orientation was similar, suggesting that disorientation in these cases measured from the central microtubules had not resulted from the twisting of the axoneme.

Quail oviduct studies suggest that although orientation is determined prior to ciliogenesis, the process depends on the correct development of the apical cytoskeleton and is related to the commencement of the ciliary beat cycle

(Boisvieux-Ulrich et al 1985). The apical cytoskeleton consists of a network of microtubules and microfilaments which anchor the basal body and basal foot process. Disruption of the cytoskeleton or the commencement of the ciliary beat cycle may prevent normal orientation (Boisvieux-Ulrich et al 1985; Boisvieux-Ulrich and Sandoz 1991). The results from this work suggest that chronic inflammation secondary to infection is interfering with the normal mechanism of cilia alignment. Inflammation during infection may be mediated by either bacterial or host derived factors. The cytokines IL1 and TNF are released in response to bacterial infection (Riesenfeld-Orn et al 1989) and mediate the host response, including the release of IL8 with resultant recruitment of inflammatory cells including neutrophils. (Matsushima et al 1989). Neutrophil proteinases are cytotoxic and the production of proteinases normally facilitates clearance of bacteria from the respiratory tract. However, inadequate inhibition of proteinase activity spilled by neutrophils during phagocytosis may lead to the generation of an excessive inflammatory response (Tetley 1993). Neutrophil elastase has been shown to be present in sputum sol of patients with bronchiectasis (Stockley et al 1984) at concentrations capable of degrading a wide range of extracellular matrix proteins. Neutrophil elastases may therefore possibly disrupt the normal development of the cytoskeleton. Chronic inflammation may also result in an increased rate of cell turn over, and the immature cells undergoing ciliogenesis

could possibly possess cilia which are not yet correctly aligned.

Respiratory pathogens are known to release products which are thought to interfere with mucociliary transport by slowing ciliary beat, causing ciliary dyskinesia or by causing epithelial disruption or cell death (Feldman et al 1991; Johnson and Inzana 1986; Munro et al 1989; Read et al 1992; Wilson et al 1987; Wilson 1988). The studies in this thesis using an organ culture have shown that N.meningitidis causes ciliary disorganisation, loss of ciliated cells, epithelial damage; and S.pneumoniae causes ciliary slowing, disorganisation of cilia and epithelial damage. Pneumolysin from S.pneumoniae has been shown to cause ciliary slowing and epithelial cell damage in vitro (Steinfort et al 1989) and in vivo (Feldman et al 1991). The phenazine pigments pyocyanin and 1-HP have been shown to slow and disorganise human ciliary beating in vitro (Wilson et al 1987) and slow guinea pig tracheal mucus velocity in vivo (Munro et al 1989), and in this work have been shown to cause ciliary shaft twisting. Pyocyanin also causes epithelial disruption (Wilson et al 1987). The effects of pyocyanin and 1-HP occur at concentrations similar to those found in sputum in vivo (Wilson et al 1988). H.influenzae lipo-oligosaccharide has also been shown to cause loss of ciliary activity (Johnson and Inzana 1986) and a supernatant fluid from H.influenzae has been shown to cause ciliostasis, loss of cilia and cell

sloughing (Denny 1974). Disorientation in chronic inflammation may therefore be caused by bacterial factors or host mediators of inflammation.

This work has shown that the bacterial products pyocyanin and 1-HP cause twisting of the ciliary shaft as assessed by electron microscopy, in association with slowing of CBF and ciliary dyskinesia assessed by light microscopy. The ex-vivo study has shown that chronic inflammation results in disorientation of cilia which may be associated with impaired mucociliary clearance. Disorientation of cilia or twisting of the ciliary shafts in vivo may impair mucociliary clearance. Impaired mucociliary clearance may promote chronic bacteria colonisation resulting in a continued inflammatory response and lung injury.

There is no published data that documents the time course for reversal of ciliary abnormalities due to chronic inflammation of the airways. Following acute viral infection normal epithelium and ciliary ultrastructure returned between two and ten weeks after infection (Carson et al 1985). To assess if disorientation was reversible following eradication of infection and hence reduction in inflammation, one patient with P.aeruginosa infection was treated with antibiotics and topical corticosteroids for three months. The mean standard deviation of orientation returned to the normal range suggesting that disorientation due to inflammation is reversible. Also the cystic

fibrosis patients receiving antibiotic therapy, who had sterile nasal cultures, had ciliary orientation similar to the normal subjects.

In summary :

1) Patients with chronic mucopurulent sinusitis have slow mucociliary clearance.

2) Ciliary disorientation without other ciliary ultrastructural defects may occur secondary to chronic inflammation.

3) Slowing of NMCC and ciliary disorientation due to inflammation can be reversible.

6.3.2 Ciliary orientation as a new variant of primary ciliary dyskinesia

PCD encompasses a range of congenital abnormalities in the ultrastructure of the axoneme which may impair mucociliary transport resulting in bronchiectasis and chronic sinusitis with situs inversus occurring in approximately 50% of patients (Sleigh 1981). Herzon (1980) reported that some individuals with the classical Kartagener's triad had normal ciliary ultrastructure, implying that either the association of the clinical syndrome with the abnormal cilia does not always hold, or that functional abnormalities of cilia may occur in the absence of ultrastructural defects. Greenstone et al (1988) reported

two cases in which cilia dyskinesia was seen with normal ultrastructure. Both had sinusitis and bronchiectasis, one had normal situs and the other situs inversus. Recently random ciliary orientation has been suggested as a new variant of PCD (Rutland 1992; Rutman et al 1993). These patients had cilia with normal ultrastructure, and normal or near normal CBF, but the cilia lacked efficacy because their beat direction was disorientated. When cilia are not orientated with respect to each other there can be no coordination of ciliary beating, and individual cilia may impede the beating of neighbouring cilia. The metachronal wave of ciliary beating therefore fails to propagate, and hence mucociliary transport is inefficient.

Quantification of the degree of ciliary disorientation allows reliable detection of this defect. It has been suggested that random ciliary orientation may be a genetically conferred abnormality of the basal bodies, or possibly of the anchoring mechanisms, preventing normal orientation of cilia. The case for a primary defect would be strengthened if inheritance of disorientated cilia could be shown, if the abnormality was demonstrated in a large number of cells from more than one site in the respiratory tract, and if the abnormality was shown to be irreversible despite effective antiinflammatory and antibiotic therapy.

In an attempt to address whether random orientation is a new variant of PCD the clinical findings, nasomucociliary

clearance, ciliary orientation, fertility and the effect of anti-inflammatory and antibiotic therapy, was assessed in a group of patients (including two siblings) with the clinical feature of PCD without axonemal ultrastructural abnormalities.

In the study described in this work the clinical symptoms and findings of 11 patients with ciliary disorientation and normal ciliary ultrastructure were similar to those reported in a previous series of patients with PCD resulting from axoneme ultrastructural abnormalities (Greenstone et al 1988; Pedersen 1983; Levison et al 1983; Mygind et al 1983). The common clinical symptoms and findings in a total of 90 patients with PCD described in the above studies included a nasal discharge from birth, often with neonatal chest infections requiring prolonged admission to hospital, followed by chronic rhinosinusitis with chronic secretory otitis media manifested by a persistently runny nose and hearing loss. Bronchitis was an early and common feature. These symptoms are similar to those described in the 11 patients with disorientation. Bronchiectasis was diagnosed in 51 of the 90, 29 were diagnosed on CXR alone, 4 from lobectomy specimens and 18 from 26 patients who underwent bronchography. In the 11 patients in this work thin-section high resolution CT was used in 8 of the 11 to confirm the diagnosis of bronchiectasis. This type of cut CT has a high sensitivity and specificity for the diagnosis of bronchiectasis and is

far better tolerated than bronchography (Cole et al 1993; Joharjy et al 1987; Munro et al 1990; Gremier et al 1986). The mean age of the 11 patients was higher than the 90 previously reported and this may account for increased proportion of patients with bronchiectasis.

In patients with PCD pulmonary function tests demonstrate comparatively well preserved lung function. Patients may demonstrate small airways disease associated with increased gas trapping (Evander et al 1983) and also bronchial hyperreactivity with a decrease in FEV1 (Mossberg et al 1983). The results in the 11 patients with ciliary disorientation were similar. An increase in RV and decrease in FEV1, FVC, and VC has previously been described in patients with bronchiectasis, and these may improve following antibiotic therapy (Rayner et al 1994; Sheldon et al 1993; Hill and Stockley 1986).

Assessment of fertility in both groups was difficult because a high proportion of the subjects were pre-pubescent or sexually inactive. Two recent studies, one of patients presenting with infertility (Escudier et al 1993) and one with rhinosinusitis and chronic bronchial sepsis (Munro et al 1994), have shown that there is dissimilar expression of axoneme abnormalities in respiratory cilia and spermatozoa, suggesting axonemes are controlled both by common and by different groups of genes. One patient of the 11 with disorientation underwent fertility studies, and was

found to have oligospermia and immotile sperm with normal tail ultrastructure.

NMCC was absent in all 11 patients with disorientation and CBF varied from 8.4-15.6 Hz. Absent NMCC is usual in patients with PCD. The absence of NMCC, as opposed to a prolonged NMCC as seen in the patients with mucopurulent sinusitis, represents an important diagnostic difference in these patients. An absent NMCC indicates the need to perform an epithelial biopsy to study ciliary function (Cole and Wilson 1994).

The CBF and beat pattern in patients with PCD may vary according to the type of axoneme abnormality present (Pedersen and Stafanger 1983; Rossman 1983; Greenstone et al 1988). For example, absence of both dynein arms is associated with immotile cilia, and radial spoke defects with asynchronous motility. Pedersen and Stafanger (1983) also described asynchronous beating in 2 patients with random orientation of axis. Rutland and De Iongh (1992) reported a reduction of CBF in one patient with ciliary disorientation, whereas Rutman et al (1993) reported a normal CBF in two patients with ciliary disorientation. In this work ciliary disorientation did affect the appearance and rate of cilia beating in six patients. However immotile cilia were never seen, and the beat pattern was not thought to be dyskinetic. In these six patients the beat pattern appeared "stiff" with the CBF ranging between

8.4-9.7 Hz. This suggests that disorientation alone may alter the function of cilia as assessed by light microscopy. This "stiff" appearance of ciliary beat also contrasts with the appearance of ciliary dyskinesia, and the results presented here suggest that disorientation of the central microtubules can be due either to twisting of the axoneme - ciliary dyskinesia by light microscopy and normal orientation of basal feet; or due to true disorientation of the cilia - ciliary beat may sometimes appear stiff and the basal feet are disorientated. In the remaining five patients the CBF and ciliary beat pattern were indistinguishable from controls. There was no correlation between disorientation and CBF. The measurement of orientation of the basal feet and central microtubules in all the 11 patients showed in each case the orientation was similar, even in the patients with an abnormal beat frequency and beat pattern. This suggests that disorientation in these patients did not result from the twisting of the axoneme, but was due to true ciliary disorientation. The results of this work suggest that orientation should be assessed from both the central microtubules and basal feet.

Disorientation may result in failure of propagation of the metachronal wave and hence failure of mucociliary transport resulting in absent NMCC. Some of the patients with ciliary disorientation secondary to chronic inflammation had prolonged but not absent NMCC. The orientation values in

patients colonised by P.aeruginosa were as high as the 11 PCD patients and yet NMCC was not absent. The explanation for this is not apparent from the studies made in this work. Orientation of cilia depends on the correct development of the apical cytoskeleton which consists of a network of microtubules and microfilaments and anchors the basal body and basal foot process. Failure of correct development of the components of the cytoskeleton may prevent normal orientation. It is possible that the disorientation in the patients with the clinical features of PCD but normal ciliary ultrastructure is associated with other functional, chemical or structural abnormalities of the cilia or cell which have not yet been identified. Abnormalities in cytoplasmic dynein rather than ciliary dynein has been proposed as the cause of situs inversus in patients with Kartagener's syndrome (Brown et al 1991). Studies looking at fertility in patients with ciliary ultrastructural defects (Munro et al 1994) and the ciliary structure in patients with infertility (Escudier et al 1990) suggest that ciliary and flagellar axonemes may be controlled by different and similar groups of genes. Previous studies have shown that abnormal orientation may occur in association with abnormalities of axoneme ultrastructure (Holley and Afzelius 1986; Pedersen 1983). These findings support the hypothesis that other abnormalities affecting ciliary function and hence mucus transport may be present in addition to ciliary disorientation in the patients with the clinical features

of PCD.

The prevalence of PCD has been estimated at 1:16,000 (Rott 1993) Segregation analysis of proband sibships is consistent with autosomal recessive inheritance (Sturgess et al 1986), although a recent case report supports the hypothesis that PCD does not follow a simple genetic pattern (Santosh et al 1994). Indeed a feature of PCD is phenotypic heterogenicity and there are likely to be several genes controlling the different axoneme and basal feet structures. The presence of disorientation in the two siblings and the finding of disorientation at more than one site in the respiratory tract described in this thesis supports the hypothesis that disorientation is a genetically inherited variant of PCD.

Respiratory tract inflammation was shown to cause ciliary disorientation in patients with chronic mucopurulent sinusitis (section 7.3). Disorientation has also been described in patients with asthma (Laitinen 1985), following viral infection (Carson et al 1985), with bronchitis (Lunggarella et al 1983) and in rats with experimental bronchitis (Iravani and van As 1972). Disorientation was shown to be reversible following treatment of chronic inflammation and following recovery after acute viral infection (Carson et al 1985). However in 2 of the 11 patients disorientation was not reversible despite three months treatment with antibiotics and topical

nasal corticosteroids. Also the orientation of the two siblings was similar after an interval of 10 years. This again suggests that ciliary disorientation alone may be partly or wholly responsible for patients having the clinical features of PCD.

Investigation of patients with possible PCD should therefore include :

1 NMCC by saccharin test.

2 Result >60 minutes perform nasal brush biopsy of inferior turbinate.

3 Light microscopy measurement of CBF and assessment of beat pattern.

4 If CBF <11 Hz and/or ciliary dyskinesia send specimen for electron microscopy assessment of cilia ultrastructure.

5 Ciliary orientation studies may need to be repeated after an interval of treatment of chronic inflammation, and the evidence of primary rather than secondary disorientation will be strengthened if it is present in biopsies taken from two sites in the respiratory tract.

6 If CBF normal but strong clinical suspicion of PCD and NMCC >60 minutes send for ciliary ultrastructure and orientation studies.

In summary :

1) The study presented in this thesis suggests that

ciliary disorientation alone does represent a new variant of PCD. The features which support this hypothesis are : the clinical symptomatology and clinical findings including the presence of situs inversus; the NMCC is invariably prolonged; the findings of disorientation in two siblings; disorientation cannot be reversed by appropriate and prolonged antibiotic and anti-inflammatory treatment.

2) A normal CBF and ciliary beat pattern by light microscopy does not exclude random ciliary orientation.

3) A diagnosis of ciliary disorientation should be considered in patients with the clinical symptoms of PCD who have absent NMCC.

7.0 Suggestions For Further Work Arising Out Of This Thesis

7.1 *Neisseria Meningitidis Pathogenesis*

a) Characterisation of the factor causing changes in microvilli structure

b) Characterisation of factor causing ciliary damage

c) Investigate the adherence of N.meningitidis to mucus

d) Study the invasion of N.meningitidis into respiratory epithelium using the organ culture with gold labelling and transmission electron microscopy

e) Use the organ culture with SEM and TEM to investigate the effect of intercurrent viral infection on adherence and invasion of N.meningitidis

7.2 *Streptococcus Pneumoniae Pathogenesis*

a) Study the interaction of purified pneumolysin and respiratory epithelium to identify possible binding sites and the mechanisms of action of pneumolysin on tight junctions

b) Study the interaction autolysin isogenic variants of

S.pneumoniae with human respiratory mucosa in an organ culture with an air mucosal interface to investigate the effect of autolysin on adherence and invasion of S.pneumoniae

c) Study the interaction of hyaluronidase isogenic variants of S.pneumoniae with human respiratory mucosa in an organ culture with an air mucosal interface to investigate the effect of hyaluronidase on adherence and invasion of S.pneumoniae

7.3 Ciliary Disorientation

a) Investigate if pyocyanin, 1-HP and rhamnolipid cause ciliary disorientation in vivo

b) Assess if ciliary disorientation occurs in the lower respiratory tract during respiratory infection

c) Identify if host factors contribute to ciliary disorientation in respiratory tract infection

8.0 References

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9.0 Publications Arising Out of Thesis

1 Wilson R, Rayner C. Animal models of respiratory infection. J Antimicrob Chemother 1994;33:381-386.

2 Rayner CJF, Tillotson G, Cole PJ, Wilson R. Efficacy and safety of long term ciprofloxacin in the management of severe bronchiectasis. J Antimicrob Chemother 1994;34:149-156.

3 Rayner CFJ, Rutman A, Dewar A, Cole PJ, Wilson R. Ciliary disorientation in patients with chronic upper respiratory tract infection. Am J Respir Crit Care Med In Press

4 Jackson AD, Rayner CFJ, Dewar A, Cole PJ, Wilson R. A human respiratory tissue organ culture incorporating an air interface. Submitted Am J Respir Crit Care Med

5 Rayner CFJ, Jackson AD, Rutman A, Dewar A, Mitchell TJ, Andrew PW, Cole PJ, Wilson R. The interaction of pneumolysin sufficient and deficient isogenic variants of Streptococcus pneumoniae with human respiratory mucosa. Infect Immun 1995;63:442-447.

6 Rayner CJF, Dewar A, Moxon ER, Virji M, Wilson R. The effect of variations in the expression of pili on the interaction of Neisseria meningitidis with human nasopharyngeal epithelium. J Infect Dis 1995;171:113-121.

7 Rayner CFJ, Rutman A, Dewar A, Greenstone MA, Cole PJ, Wilson R. Ciliary disorientation alone as a cause of primary ciliary dyskinesia syndrome. Submitted Am J Respir Crit Care Med

8 Rayner CFJ, Cole PJ, Wilson R. The management of chronic bronchial sepsis due to bronchiectasis. Clin Pulm Med 1994;1(6):348-355.

9 Wilson R, Rayner CFJ. Bronchitis. Current Science In Press

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Abstracts

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