

**SERIES: 'INTERACTION OF BACTERIA AND AIRWAY EPITHELIAL CELLS'**  
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## The biology of bacterial colonization and invasion of the respiratory mucosa

R. Wilson, R.B. Dowling, A.D. Jackson

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**ABSTRACT:** Despite being regularly exposed to particulate matter during breathing, which contains bacteria from the commensal flora in the nasopharynx and from the environment, the healthy lung is kept sterile by efficient defence mechanisms. Bacterial infections of the respiratory mucosa represent a dynamic interaction, to which both host and bacterial factors contribute.

The abnormal host defences associated with chronic respiratory infections (e.g. cystic fibrosis and other forms of bronchiectasis) serve to emphasize their permissive role. The bacteria that cause bronchial infections possess a wide array of potential virulence factors that contribute to their pathogenicity. Many of these factors influence the mucociliary system, an important first-line defence mechanism. The multiplication, spread and persistence of bacteria within the bronchial lumen, and consequent damage to the epithelium, stimulates a chronic inflammatory response, which also impairs mucociliary clearance and damages lung tissue.

A greater understanding of host-bacterial interactions during mucosal infections should in the future lead to the development of new therapies and treatment strategies.

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Host Defence Unit, Imperial College of Science, Technology and Medicine, National Heart and Lung Institute, London, UK.

Correspondence: R. Wilson  
Host Defence Unit  
Imperial College of Science, Technology and Medicine  
National Heart & Lung Institute  
Emmanuel Kaye Building  
Manresa Road  
London SW3 6LR  
UK

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The lung is regularly exposed to particulate matter during breathing, which contains bacteria from the commensal flora in the nasopharynx and from the environment. However, in health, the lung is kept sterile by efficient defence mechanisms. There are at least four possible outcomes for bacteria inhaled into the bronchial tree: 1) immediate clearance by first-line defence mechanisms, such as mucociliary clearance [1]; 2) asymptomatic carriage, which occurs for example in some chronic bronchitis patients between exacerbations [2, 3]; 3) infection which remains localized on the mucosa and spreads contiguously through the airways inciting an inflammatory response; and 4) invasion of the mucosa or parenchyma. In health, bacteria are confined to the upper respiratory tract. Their presence elsewhere, for example the middle ear, the lower respiratory tract or the bloodstream, reflects a failure of host defence mechanisms that could be ascribed either to the virulence of the bacterium and its ability to overcome the host's defences or to a deficiency of one or more of these defences. The relative contribution of host and microbial determinants need to be considered, since mutuality is of the essence in understanding bacterial pathogenesis.

The bacteria that cause bronchial infections are less virulent, in the usually accepted sense of the word, than those causing invasive diseases, such as pneumonia, that can occur in previously healthy people. For example: nontypable unencapsulated *Haemophilus influenzae* forms part of the commensal flora in the nasopharynx and also commonly causes lower respiratory tract infections in

chronic bronchitis; *Pseudomonas aeruginosa* is exclusively an opportunistic pathogen that causes bronchial infections in cystic fibrosis and bronchiectasis. The abnormal state of the host defences associated with these chronic respiratory conditions serves to emphasize their permissive role in the pathogenesis of bronchial infections [4]. The pathogenic mechanisms of bacteria that colonize the respiratory mucosa need to be considered in the context of how they facilitate persistence in the bronchial tree.

An abnormality in the host defences may be hereditary, such as in cystic fibrosis or primary ciliary dyskinesia, or acquired, for example, after a viral infection or with chronic cigarette smoking. The synergistic role of viruses in predisposition to bacterial infection of the airways has been investigated, and may be due to a number of possible mechanisms: loss of ciliated epithelial cells; slowing of the ciliary beat; increase in mucus production; alteration in mucus rheology and ion transport; and change in epithelial cell receptors for bacterial adherence [5, 6].

It has been shown in an animal model that the population of *H. influenzae* within the upper respiratory tract is often the progeny of the successful survival and replication of a single, or a very small number of organisms. This was shown by experiments in which rats were challenged intranasally with a mixture of isogenic variants differing in their antibiotic resistance phenotype. Providing that the inoculum was close to the dose required to colonize 50% of the population, more than half of the rats

had nasopharyngeal cultures containing a pure growth of one or the other, but not both, mutants [7]. This suggests that the host nasopharyngeal environment is able to suppress the survival and growth of all but a few bacteria. This may relate to, or depend on, the availability of host cell attachment sites on mucus or epithelial cells or the supply of nutrients. A challenging concept raised as a result of this experiment is that genetic and/or phenotypic heterogeneity within the bacterial population imparts a distinct survival advantage to only a few bacteria.

The bacteria that cause bronchial infections possess a wide array of potential virulence factors (table 1) that contribute to their pathogenicity. However, their failure, in general, to infect the healthy bronchial tree means that no single virulence factor is by itself omnipotent, but that the different virulence factors should be viewed together as contributing to the "pathogenic personality" which enables the bacterium to exploit deficiencies in the host defences.

The mucociliary system is the most important first-line defence mechanism of the bronchial tree against bacterial infection. In this review, we will discuss bacterial interactions with the different parts of this system: mucus, cilia, periciliary fluid and epithelial cells. We will mainly use *H. influenzae*, *P. aeruginosa* and *Streptococcus pneumoniae*, to illustrate the different pathogenic mechanisms.

Table 1. – Bacterial strategies to evade clearance from the airways

<b>Exoproducts that impair mucociliary clearance</b>
Stimulate mucus production [8–10]
Slow and disorganize ciliary beat [1, 11–13]
Affect ion transport [14–17]
Damage epithelium [1, 11, 13, 17–19, 23]
<b>Enzymes that break down local immunoglobulin</b> [24, 25]
Proteases cleave antibodies to create nonfunctional "blocking antibodies"
IgA <sub>1</sub> proteases
<b>Exoproducts that alter immune effector cell function</b> [24]
Neutrophils: inhibit chemotaxis and phagocytosis, enhance oxidative metabolism
Lymphocytes: impair cytokine production, activate suppressor T-cells
Macrophages: reduce viability
<b>Bacterial adherence to epithelium</b> [20–22]
May be increased by environmental factors and in certain disease states
Increased by epithelial damage
Avoids clearance in secretions
Enhances the effect of toxins released in the microenvironment of the epithelium
Increases the availability of nutritional factors for bacterial growth
<b>Avoid immune surveillance</b> [24, 25, 37, 38]
Antigenic heterogeneity of the bacterial surface
Growth in biofilms
Microcolonies of bacteria surround themselves with a polysaccharide gel
Endocytosis, bacteria "hide" within epithelial cells

IgA<sub>1</sub>: immunoglobulin A<sub>1</sub>. References are given in square brackets.

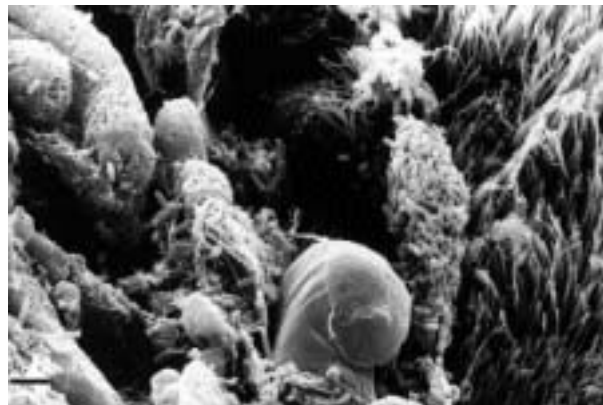


Fig. 1. – *Pseudomonas aeruginosa* infection of human respiratory mucosa in an organ culture caused patchy epithelial damage after 8 h. *P. aeruginosa* adhered to mucus and damaged epithelium, but not to normal epithelium. (scale bar = 2.67 µm).

### Bacterial interactions with mucus

The first interaction of inhaled bacteria with the airway mucosa is with mucus. *H. influenzae*, *P. aeruginosa* and *S. pneumoniae* have a high affinity for mucus *in vitro* [26–30], although this is not true for all bacteria that have been investigated [31]. In a histological study of the lungs of patients with cystic fibrosis, it was found that *P. aeruginosa* predominantly associated with secretions, and only adhered to the epithelial surface when there was erosion of, or damage to, the epithelium [32]. Similar observations (fig. 1) have been made with *P. aeruginosa* infection of organ cultures [27]. Bacterial adherence to mucus probably involves both specific (adhesin-receptor) and nonspecific interactions [28, 33–36]. In organ cultures, *P. aeruginosa* are seen to grow as continuous sheets over the mucus surface [27], and it has been shown that growth in such biofilms is resistant to opsonophagocytic killing by neutrophils [37, 38].

The depth of the mucous layer may influence its transport by cilia. If the mucous layer is too thick, uncoupling may occur within it, so that the innermost part is moved forward by the beating cilia but the outer part, on which particles are trapped, remains stationary [39]. Thus, it may be significant that a number of bacterial species causing bronchial infections elaborate extracellular substances which stimulate mucus secretion *in vitro* [8]. *P. aeruginosa* proteases and rhamnolipid have also been shown to stimulate mucous production *in vivo* in an animal model [9, 10].

The affinity of bacteria for mucus, and their relative lack of adherence to healthy epithelium [27, 29, 30, 32] may explain why they do not infect normal airways, which have efficient mucociliary clearance. Whereas in chronic bronchitis [39], bronchiectasis [40] and cystic fibrosis [41], mucociliary clearance is delayed, giving bacteria that have adhered to mucus time to produce virulence factors (table 1) in sufficient quantities to establish the infection. Bacterial infection attracts leucocytes into the airways, many of which eventually degenerate releasing deoxyribonucleic acid (DNA) into the secretions making them more viscous and difficult to clear [42, 43]. Recent studies have shown that the sputum of patients is poorly transported by cilia compared to healthy mucus, and the cause of this needs further investigation [44].

### Bacterial interactions with cilia

Some bacteria produce factors which disturb the mucociliary system by slowing and disorganizing the beating of cilia [1]. This has the effect of delaying mucus clearance, and also removes a physical barrier that prevents bacteria binding with receptors on the epithelial surface. Some of these cilioinhibitory factors have been characterized: *P. aeruginosa* produces pyocyanin, 1-hydroxyphenazine [11] and rhamnolipid [12]; *H. influenzae* produces low molecular weight glycopeptides [4]; and *S. pneumoniae* produces pneumolysin [13].

We have investigated the phenazine pigments of *P. aeruginosa*, pyocyanin and 1-hydroxyphenazine [11]. We first noted that 18 h culture filtrates of *P. aeruginosa* slowed and disorganised human ciliary beating *in vitro*. Prolonged incubation caused ciliary stasis and disruption of epithelial integrity. Assays were developed to measure the amount of known virulence factors in the filtrates, and these levels were then correlated with ciliary slowing activity. Only the phenazine pigment content of the filtrates correlated. Gel filtration was then performed on culture filtrates and yielded only one peak of ciliary slowing activity, which co-eluted with the pigments. Finally, the accumulation of pigment during bacterial culture correlated with an increase in ciliary slowing activity.

Pyocyanin and 1-hydroxyphenazine were extracted from cultures and purified by high performance liquid chromatography. They were then characterized by mass spectrometry and, subsequently, synthesized [11]. 1-hydroxyphenazine caused immediate onset of ciliary slowing and dyskinesia, which was not associated with epithelial disruption. Pyocyanin caused gradual slowing of ciliary beating which was associated with epithelial disruption later in the experiment. Both of these compounds have been extracted from the sputum of patients infected by *P. aeruginosa* at concentrations similar to those required to slow ciliary beat *in vitro* [45], and both slowed mucociliary transport in the guinea-pig *in vivo* [46]. A bolus dose of 1-hydroxyphenazine slowed mucociliary transport immediately, although it subsequently recovered, whilst a bolus dose of pyocyanin had no immediate effect, but later transport rate fell without any recovery. When both compounds were introduced simultaneously, there was an additive effect.

We have recently shown that the mechanism of action of pyocyanin on ciliary beat is cyclic adenosine monophosphate (cAMP) dependent [47]. The long-acting  $\beta_2$ -agonist salmeterol raises the level of cAMP in epithelial cells [48], and partially inhibits the action of pyocyanin *in vitro* [49]. Therefore, salmeterol may benefit patients colonized by *P. aeruginosa* not only by its bronchodilating action but also by protecting cilia from the cAMP-dependent effects of pyocyanin.

For efficient mucociliary transport to occur, cilia must beat in the same direction in a co-ordinated fashion with their neighbours [1]. We have recently shown that the beat direction of cilia on biopsies taken from sites of infection is disorientated [50]. This was particularly the case in patients infected with *P. aeruginosa*, and the degree of ciliary disorientation correlated closely with the delay in mucociliary clearance. Furthermore, treatment with antibiotics and topical corticosteroids improved mucociliary clearance and decreased ciliary disorientation. This study

Table 2. – Bacteria that cause separation of tight junctions of epithelium and endothelium

Bacteria	Tissue
<i>Pseudomonas aeruginosa</i>	Sheep tracheal epithelium [14]
<i>Pseudomonas aeruginosa</i>	Canine renal epithelial cell monolayer [17]
<i>Pseudomonas aeruginosa</i>	Human nasopharyngeal epithelium [27]
<i>Streptococcus pneumoniae</i>	Human nasopharyngeal epithelium [18]
<i>Streptococcus pneumoniae</i>	Rat blood/brain barrier [54]
<i>Haemophilus influenzae</i>	Human nasopharyngeal epithelium [29, 53, 55]
<i>Haemophilus influenzae</i>	Rat and human blood/brain barrier [54, 56]

References are given in square brackets.

suggests that the growth of cilia may be affected by bacterial infection, and this could be due to either bacterial products or to the inflammation that they induce.

### The effect of bacteria on ion transport and epithelial cell tight junctions

The depth and constitution of the periciliary fluid, and the ionic content of secretions, may both affect mucociliary transport [1, 39, 44]. Regulation of these features relies on active ion transport across a continuous epithelial layer with intact tight junctions. Considerable attention has been focused upon the role of epithelial integrity in bacterial diseases of the gastrointestinal tract, and specific bacterial toxins have been identified which interfere with ion transport, *e.g.* *Vibrio cholerae* cholera toxin which alters ion transport across the microvillar membrane [51] and *V. cholerae* zonula occludens toxin, which disrupts epithelial tight junctions [52]. These toxins, along with those of other gastrointestinal tract pathogens, have been linked to pathological features *in vivo* and their mechanisms of action are beginning to be understood. However, although a number of respiratory pathogens have been associated with the disruption of tight junctions in the epithelium and the endothelium (table 2), very little information is available on the specific bacterial factors involved or the mechanisms by which they cause changes in the epithelium.

*P. aeruginosa* rhamnolipid has been shown to inhibit transcellular ion transport in sheep tracheal epithelium at low concentrations and to increase paracellular permeability at higher concentrations by disrupting the epithelial tight junctions [14–16]. *Pseudomonas elastase* has also been shown to disrupt epithelial tight junctions, a lesion that in this study was not reproduced by human leucocyte elastase [17]. We have also observed the separation of epithelial tight junctions in human nasopharyngeal organ cultures infected with *H. influenzae* [29] and pneumolysin positive, but not pneumolysin negative, isogenic strains of *S. pneumoniae* [18].

### Bacterial adherence and cell damage

The attachment of bacteria to mucosal surfaces is considered to be an important event in the pathogenesis of

most infectious diseases, and has been shown to be essential to the production of epithelial damage in some cases [57]. In a number of studies, bacteria have not adhered well to normal epithelium *in vitro*, whilst epithelial damage has been noted to increase bacterial adherence [27, 29, 30, 58, 59]. However, the source of the tissue used in these experiments might be important, because nontypable *H. influenzae* do not adhere to normal nasal turbinate epithelium [29], but do adhere to normal epithelium of adenoid tissue [53]. Cell damage might remove defence mechanisms, such as ciliary beating, which would otherwise prevent bacteria approaching the epithelial surface, and might also expose new receptors to which bacteria can adhere on damaged cells, on newly exposed nonluminal cell surfaces, and on cells that migrate and differentiate to repair the damage [27, 58, 59].

A number of bacterial products have been shown to damage epithelial cells, such as the protease enzymes of *P. aeruginosa* [19]. However, *in vivo* and *in vitro* the distribution of epithelial damage is patchy [18, 29, 32], and much of the epithelium must be protected from the effect of bacterial toxins. This is achieved *in vivo* partly by antibodies that develop against bacterial toxins and neutralize their effects. The influence of mucus on the effect of bacterial toxins has not been studied, but this may be another way in which the potency of bacterial toxins is reduced.

Although, compared to gastrointestinal tract pathogens, little research has been undertaken into the mechanisms of action of the toxins produced by respiratory pathogens, the mediation of cell damage by some respiratory bacterial toxins has been elucidated. *Bordetella pertussis* tracheal cytotoxin (TCT), a muramyl peptide fragment secreted during bacterial growth, is responsible for the respiratory epithelial pathology of pertussis. TCT has been shown to induce interleukin-1 (IL-1) production by the hamster tracheal epithelium and exogenous IL-1 reproduced the cytopathology caused by TCT [60]. Both TCT and IL-1 induced high levels of nitric oxide production by epithelial cells, and inhibition of nitric oxide synthase prevented the destruction of ciliated cells in hamster tracheal organ cultures [61]. These observations suggest that TCT triggers the production of IL-1, which in turn stimulates nitric oxide production leading to epithelial cell damage.

Intriguingly, this is not the only case in which host factors have been implicated in the mediation of respiratory disease pathology. Tumour necrosis factor (TNF) and IL-1 are released in the respiratory tract in response to a number of bacterial pathogens and/or their products. These cytokines have been shown to induce the breakdown of tight junctions in the blood/brain barrier *in vivo* [62], and a combination of anti-TNF and anti-IL-1 antibodies completely neutralized cell separation in the vascular endothelium that was induced by *S. pneumoniae* [63].

Bacterial adherence to mucosal features (*e.g.* mucus, cilia or epithelial cells) occurs *via* specific interactions between adhesin structures on the bacterial surface and receptors on the mucosal surface. Pili have been identified as an important adhesin of *P. aeruginosa* [20, 64], but do not account for all of the adhesive properties of this bacterium, and other adhesins such as exoenzyme S [21] and alginate [22] have been identified. Multiple

adhesins on the bacterial surface and multiple mucosal receptors have been found for most pathogens that have been studied, and the number of adherence interactions that can occur makes this an unlikely target for therapeutic intervention [65].

A number of oligosaccharides have been identified which bind various bacteria, *e.g.* GalNAc $\beta$ 1-4Gal sequences found in glycosphingolipids extracted from lung tissue [66]. However, both the location of these receptors *in vivo*, and the factors which might influence their accessibility need further investigation. It has been suggested that the number of potential binding sites for bacterial pathogens can be influenced by environmental factors or disease states. There is a special association between cystic fibrosis and *P. aeruginosa*, and infection can occur before there is significant lung damage [67]. Cystic fibrosis epithelial cells in primary culture bind approximately twice the number of *P. aeruginosa* compared to normal cells [68], and subsequent work has suggested that this is due to alteration in the number of receptors for *P. aeruginosa* adhesins on the cell surface [69]. Although such an increase in *P. aeruginosa* binding seems unlikely to be the sole explanation of the susceptibility of cystic fibrosis patients to this infection, it may be important when it occurs with slow mucociliary clearance [41]. Recently, the cystic fibrosis transmembrane regulator (CFTR) has been implicated in the susceptibility of cystic fibrosis patients to *P. aeruginosa* infection. The type of defect in CFTR correlates both with the age of the patient when *P. aeruginosa* colonization occurs [70], and the number of *P. aeruginosa* binding to epithelial cells of cystic fibrosis patients [71]. *P. aeruginosa* bind to the glycolipids asialoganglioside 1 (aG<sub>M1</sub>) and aG<sub>M2</sub> but not to their sialylated homologues [66]. This research has led to the hypothesis that glycosylation and sulphation of superficial glycoconjugates may be altered in cystic fibrosis, and that this could be due in some way to abnormal intracellular chloride transport as a consequence of abnormal CFTR function [72–74].

#### The effect of chronic inflammation on bacterial interactions with the mucosa

The multiplication and spread of bacteria within the bronchial lumen, and consequent damage to the epithelium, stimulates the host to mount an inflammatory response. If this fails to clear the bacteria and bacterial infection continues, the inflammatory response becomes chronic. Large numbers of activated neutrophils are attracted into the airway [75] by host (*e.g.* complement factor 5a (C5a), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), interleukin-8 (IL-8)) and bacterial chemotactic factors [76]. Activated neutrophils do not differentiate between bacteria and bystander lung tissue. They spill proteinase enzymes and reactive oxygen species and, because of the large number of neutrophils present, lung defences, such as antiproteinases, are overwhelmed. High levels of biologically active neutrophil elastase have been measured in the sputum of chronically infected patients [77, 78]. Proteinase enzymes [23] and reactive oxygen species [79] both cause epithelial damage, and stimulate mucus production [80, 81]. These changes promote continued bacterial infection by

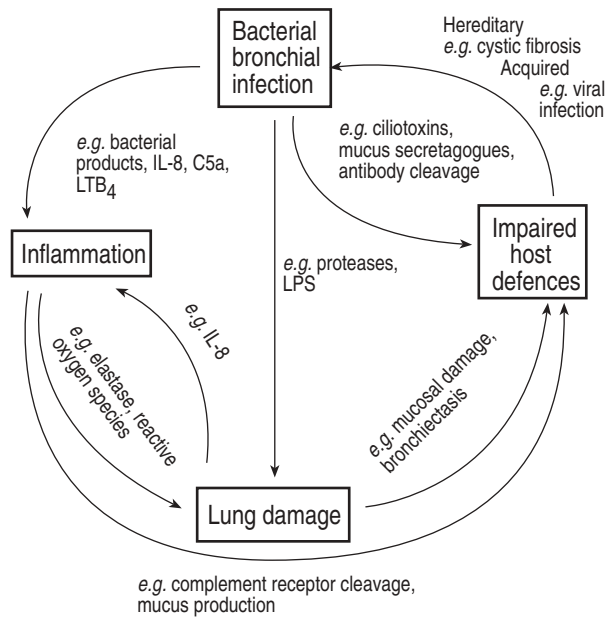


Fig. 2. — Schematic representation of a self-perpetuating "vicious circle" of events which occur during chronic bronchial infection. LPS: lipopolysaccharide; IL-8: interleukin-8; C5a: complement factor 5a; LTB<sub>4</sub>: leukotriene B<sub>4</sub>.

impairing mucociliary clearance. Neutrophil elastase present in secretions attracts additional neutrophils into the airway by inducing production of the powerful chemoattractant IL-8 by epithelial cells [82], and may impair phagocytosis by cleaving complement receptors from neutrophils [83] and complement components from bacteria [84]. Thus, a self-perpetuating cycle of events (fig. 2) may develop.

Another marker of chronic inflammation is the strong antibody response to bacterial antigens, which can be detected in serum, saliva and pulmonary secretions [24, 25]. Immune complexes are found in the bloodstream and in sputum [85, 86], and probably have a role in causing lung damage as suggested by the strong correlation between severity of lung disease and the titre of anti-*Pseudomonas* antibodies in cystic fibrosis [87].

Whilst the major inflammatory cell in the airway lumen of patients with chronic infection is the neutrophil, there is also an expansion of the lymphoid cell population in the bronchial wall. Many of these are T-cells with a suppressor phenotype [88]. Although they could simply represent a secondary response to chronic antigenic stimulation, mononuclear cells probably play an important role in orchestrating the inflammatory response. There are high levels of a number of cytokines in the sputum of chronically infected patients, and the levels are higher than those found in serum, which suggests local production. Whether host cytotoxic T-cell-mediated lung damage also occurs requires investigation [88].

Some bacterial toxins disable the inflammatory response, for example by inhibiting phagocyte function or cleaving antibodies, whilst others enhance inflammation, for example by inactivating  $\alpha_1$ -antiproteinase or enhancing neutrophil oxidative metabolism [24, 25, 89]. Similarly infection may result in the release of a mixture of pro- and anti-inflammatory cytokines [90]. The relative importance of these different bacterial and host factors

may change depending on the stage of the infectious process. Bacterial factors which disable host defences may compete with proinflammatory host factors early in the infectious process, whereas later, when airway damage has occurred and chronic bacterial infection is established, proinflammatory bacterial factors may subvert the host defences to promote continued bacterial infection by increasing inflammation, which causes lung damage. In these circumstances, the anti-inflammatory cytokines will curtail the exuberant chronic inflammatory response and in this way protect lung tissue.

In conclusion, bacterial pathogenicity in chronic infections of the respiratory tract must be examined in a wider context, to include investigation of the conditions which are permissive for their perpetuation in the respiratory tract. Bacterial virulence factors and their interaction with the host defences reflect a co-evolution of microbe and host, particularly for pathogens, such as *H. influenzae* in which man is the sole host. For bacteria such as *P. aeruginosa*, that exist naturally in the environment, mechanisms that allow the organism to survive in nature may fortuitously give it benefit during infection of man. For example, alginate is produced in nature to help *P. aeruginosa* to attach to solid objects in watery environments while in man it acts as an adhesin to epithelium, and surrounds microcolonies of bacteria acting as a barrier to phagocytes and antibiotics [24].

The biology of bacterial colonization and invasion of the respiratory mucosa is complex, and the dynamic nature of the host-bacterial relationship is evidenced by the large number of bacterial products and the wide spectrum of their actions, and by the polymorphic nature of the immune responses to bacterial infection. The encounters between bacteria and man are resolved in outcomes which range from the establishment of a commensal relationship (carrier state) to potentially lethal disease, circumstances under which the role of specific microbial determinants may differ and phenotypic expression may vary. The effect of host cells and/or the local environment in the airway on the expression of different virulence factors needs investigation. A greater understanding of host-bacterial interactions should, in the future, lead to the development of new therapies and treatment strategies.

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