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Functions of proteins and lipids in airway secretions

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ABSTRACT: Proteins and lipids synthesized by airway secretory cells or transudated are active components in the protection of respiratory epithelium. Proteins and ions are involved in the control of mucus hydration. Secretory proteins, such as secretory immunoglobulin A (IgA), transferrin and lysozyme, participate in the airway antibacterial defence. Other biochemical components found in secretions, such as anti-inflammatory and antioxidant agents as well as antiproteases, contribute significantly to the protection of the underlying epithelium.
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Human respiratory diseases, including acute and chronic bronchitis, asthma and cystic fibrosis, are often associated with excessive airway mucus production. In the conducting airways, the submucosal glands and surface epithelial (goblet) secretory cells are the major contributors to the mucus secretion [1, 2].

Because of the cellular heterogeneity of the airway secretory apparatus, the factors that govern secretion and composition of each secretory cell type and contribute to the biochemical pattern of mucus are not completely defined. To date, mechanisms possibly responsible for the regulation in the volume and quality of the respiratory tract secretions involve the transepithelial Cl^- secretion across airway epithelium with passive diffusion of water [3, 4], secretory effects of a number of inflammatory mediators including arachidonic acid metabolites released either from airway epithelial cells [5–9] or activated inflammatory cells [10–12] and the increase in total number of mucus-producing cells [13].

The respiratory mucus is very complex and includes 95–98% of water, ions, sugars, amino acids, (1–3%) proteins, glycoproteins and lipids [14]. One of the main functions of mucus is to protect the mucosal surface from physical and chemical injuries and from inhaled microorganisms. Apart from its main participation in transport by mucociliary clearance, respiratory mucus also serves a variety of other protective purposes, including the control of airway hydration and lung tissue defence mechanisms. In this review, we will discuss recent data on potential roles of a number of ions, polypeptides, glycoproteins (other than mucins), proteases, antiproteases, plasma proteins, and lipids found in mucus and involved in: 1) the control of airway humidification and mucus hydration; and 2) the airway epithelium defence by limiting both the adhesion of bacteria and viruses to respiratory epithelial surface and protecting the airways from injury produced by proteolytic and oxidant agents.

Airway humidification and mucus hydration

The tracheobronchial epithelium is considered as an absorptive and secretory type epithelium [15]. It is now generally agreed that both trachea and bronchi are able to activate electrolyte absorption and secretion and, by osmotic coupling, water. The movement of electrolytes, more precisely the rate of transepithelial Cl^- secretion and fluid across airways, appears to play an important role in the production and quality of airway gland secretions [3, 4, 15].

Airway surface epithelial cells are polarized anatomically and functionally, joined together by tight junctions, thereby delimiting an apical membrane with cilia and microvilli and a basolateral membrane facing the interstitial space. These two membrane domains have specialized functions for the transport of ions and water. The secretion of Cl^- into the airway lumen, coupled or not with the absorption of Na^+, occurs in the apical membrane and requires energy which is supplied by the Na^+, K^+- adenosine triphosphatase (ATPase) pump localized in the basal membrane. By generating local osmotic gradients, these ion transport processes regulate the water content and, thus, the depth of the periciliary sol layer [15]. It is thought that water movement occurs in intercellular spaces. An increased rate of Na^+ absorption, coupled with fluid movement, contributes to the relative dehydration of airway secretions that characterizes the cystic fibrosis disease [16, 17].

What are the physicochemical and physiological cellular mechanisms that modulate mucus hydration?

The rate determining step in secretion, as for absorption, lies at the apical membrane where the activity of Cl^- channels controls, in part, the transepithelial Cl^- secretion [18]. Several intracellular second messenger pathways that activate apical membrane Cl^- channels have recently been described in normal airway cells: 1) intracellular complementary adenosine monophosphate (cAMP) stimulates Cl^- secretion and, therefore, the Cl^- channel can be opened through phosphorylation brought about by protein kinase A (PKA), or at low intracellular Ca^{++} concentration, by protein kinase C (PKC). This activation (phosphorylation) by both kinases is defective in cystic fibrosis airway cells [19, 20]; 2) several secretory hormones, such as bradykinin, stimulate Cl^- secretion in canine airway cells by activating phospholipase A2 and phospholipase C [21]; and 3) extracellular adenosine triphosphate (ATP) stimulates Cl^- channels in human airway epithelial cells [22]. ANDERSON and WELSH [23] have recently shown that addition of arachidonic acid in cultured airway epithelial cells from normal and cystic fibrosis inhibits apical membrane Cl^- channels. Fatty acids (arachidonic acid) would, therefore, directly interact with the Cl^- channels or an associated protein to alter the Cl^- channels.

Roles of proteins in mucus hydration

Several proteins in respiratory mucus are considered to be active components in mucus hydration. According to TAM and VERDUGO [24], mucus hydration and swelling are governed by a Donnan equilibrium process and not by simple osmosis. Therefore, the concentration of free ions, pH, ionic strength and small polyionic proteins, such as proline-rich proteins, lysozyme and albumin, in the fluid on airway surfaces can modulate mucus hydration and its swelling rate [25, 26]. WIDDICOMBE [27] reported that airway surface fluid is hyperosmolar, and possesses an electrolyte composition different from that of interstitial fluid. He suggested that a homeostatic mechanism in mucosa may regulate the pH of the periciliary fluid layer. Recently, DEFFEBACK *et al.* [28] showed that different prostaglandins can selectively modify *in vitro* the volume and quality of secretions in the ferret trachea. They demonstrated that prostaglandin E_1 (PGE_1) inhibited lysozyme release, a protein marker for serous cell secretion [29], whereas PGD_2 reduced secretion volume and $PGF_{2\alpha}$ inhibited albumin transport. Conversely, PGE_1 was a potent, and PGD_2 a weaker, stimulant of albumin transport, whereas $PGF_{2\alpha}$ increased the secretion volume, and was a potent stimulant of lysozyme release. The presence of albumin in airway and alveolar fluid has been attributed to either passive serum-transudation from local vessels [30] or active transport across tracheal epithelial cells to the airway lumen [28]. JOHNSON *et al.* [31] have also shown that albumin may be actively absorbed from the lumen to interstitium by canine bronchial epithelium, probably involving specialized vesicular transport mechanisms. Using *in vitro* culture techniques, we have also demonstrated that an albumin-like protein can be synthesized *de novo* by bovine tracheal gland serous cells [32], and that the amounts of albumin-like protein released by these cells were dependent on the composition of culture medium [33]. Thus, the discovery that albumin can be actively transported bidirectionally through airways, and also synthesized by airway gland cells, suggests that the albumin protein may contribute to the regulation of lung fluid balance and, consequently, may be an important component in the control of mucus hydration. Other properties for albumin in the airways have been reported, including the increase of mucus viscosity [34], the binding of drugs, ions and a potential scavenger for free radicals [35].

Mucus components involved in the airway epithelium defence systems

Apart from bronchial mucins, which by their heterogeneity of carbohydrate structures [36] are able to entrap, and thus eliminate, various bacteria and viruses by means of the mucociliary escalator, other components found in secretions (glycoproteins, peptides, lipids and protease inhibitors) also play important roles in the protection of the underlying epithelium.

Protective functions of lipids

It has long been recognized that airway secretions contain noticeable amounts of lipids [37–42]. As reviewed extensively by WIDDICOMBE [43], different studies showed not only the variations in the overall amount of lipids, but also the variabilities in content of the different components, which include phospholipids, glycerides, cholesterol and its esters, glycosphingolipids, glyceroglycolipids [41], and free fatty acids. The major phospholipid component is phosphatidylcholine (PC), which generally contains less saturated fatty acids (palmitic acid) [39–42] than PC coming from the alveolar surfactant. Lipids are closely associated with the mucus glycoproteins [44–48] mostly *via* non-covalent binding [48, 49]. The amount of associated lipids may represent up to 49% of the weight of the solids in mucin preparations obtained from pathological secretions [47, 48]. In infected purulent secretions, such as in cystic fibrosis and bronchiectasis, the amount of lipids is largely increased [48].

It has generally been assumed that the lipids found in airway secretions originate from alveolar surfactant, from serum transudate, or from degradation products of shed epithelial cells, macrophages, inflammatory cells and bacteria [49]. In fact, the alveolar surfactant is unlikely to be the main source of lipids in the upper airways, since it was reported that only a very small

amount could reach the trachea [50, 51]. On the other hand, serum transudation and breakdown of membrane debris from various cells are certainly a major source of lipids in inflammatory and infected secretions. To date, it has become evident that lipids normally found in airway secretions are locally produced and do not simply represent the transport of alveolar surfactant from the periphery. Several reports [52–57] have demonstrated that phospholipids, glycosphingolipids, glycerides and sterols were associated to mucins isolated from supernatants of animal and human tracheal explants. According to these studies [46, 53], the amount of lipids ranges from 5–15% of the dry material. Recently, KIM and SINGH [58, 59] demonstrated that the lipids were synthesized and secreted in association with mucus glycoproteins in hamster cultured tracheal epithelial cells, which could explain the extreme hydrophobicity of the secreted mucins. Recently, GIROD *et al.* [60], using a cytochemical enzyme-gold technique, localized intragranular phospholipids in serous and mucous cells of human tracheobronchial gland. BARROW [61] compared the phospholipid secretion at the bronchoalveolar, bronchial and tracheal levels in sheep and suggested a local synthesis and release of phospholipids by tracheal epithelial cells. These convergent results suggest the production and secretion of surfactant material at the bronchiolar and tracheobronchial levels and support the hypothesis that mucosal airway secretory cells produce phospholipids which may be secreted in the airway lumen.

The first functional role of lipids in tracheobronchial secretions may be to participate in mucosal protection. It is well established that the protective function of the mucus lining the airway luminal surface is due mainly to the physicochemical properties of mucus glycoproteins (mucins). The fact that mucins are associated with a significant amount of lipids may increase their hydrophobicity and, consequently, modulate their physicochemical characteristics. Although experimental data are lacking for bronchial secretions in this field, phospholipids have been shown to influence ion diffusion in other mucous secretions: in gastric secretion, an adsorbed layer of surface active phospholipids may be an essential component to maintain the hydrophobicity of the mucosa [62, 63]. SAROSIEK *et al.* [64] and SLOMIANY *et al.* [65] have shown that lipids bound to gastric mucus contribute significantly to the retardation of hydrogen ion diffusion *in vitro*.

Antibacterial components

Glycoproteins other than mucins. The airways are protected by immunity mechanisms, including a combination of local mucosal immunity especially characteristic of the upper respiratory tract, and peripheral type immune reactions which characterize the alveolar level [66]. In humans, IgA represents about 10% of the total protein recoverable in bronchoalveolar lavage fluids [67]. The secretory IgA (sIgA), the major immunoglobulin in bronchial secretions and other external body fluids [68–71] forms, along with the polymeric immunoglobins (IgG, IgM), a very early specific immunological defence against infection. Their synthesis and transport through the bronchial epithelium are mediated by specific transcytotic events [69, 72].

The IgA molecule is produced by submucosal plasma cells that are often localized in bronchus-associated lymphoid tissue (BALT). After secretion, two IgA molecules are covalently linked by a J-chain glycoprotein (15 kDa), also synthesized within the plasma cells. Then, a 70 kDa glycoprotein, called the secretory component, synthesized separately in gland epithelial cells and localized in their basolateral surfaces, acts as a membrane receptor for dimeric IgA (or polymeric IgM), now known as the polymeric immunoglobulin receptor (pIg-R). According to BRIETFELD *et al.* [72], the pIg-R and ligand complex is then endocytosed in coated vesicles and transported by a variety of vesicles and tubules to the apical membrane surface, and extruded by exocytosis into the bronchial secretions. The extracellular portion of the pIg-R is proteolytically cleaved and remains associated with the IgA. By using electron microscopy, GOODMAN *et al* [73] have shown that in human bronchial submucosal glands, more IgA was detected in mucous cells compared to serous cells. In ciliated cells, IgA was not identified, whereas the secretory component was present in the cell membrane. The role of secretory IgA in bronchial secretions is thought to prevent entry of antigens beyond the mucosal barrier by its antiviral properties and capacity to agglutinate and impair the adhesion of bacteria to the mucosal surface and, consequently, increase the antigen elimination by phagocytosis and clearance mechanisms. DANIELE [70] has recently reported that the combination of sIgA, lysozyme and, perhaps, components of complement in bronchial secretions may promote phagocytosis by alveolar macrophages of IgA-coated particles and bacteria.

Other immunoglobulins such as IgG and IgM are found in airway secretions. In cystic fibrosis (CF) patients, IgM concentrations in sputum are strongly associated with the degree of bronchial superinfection, evaluated by a quantitative cytobacteriology in sputa [74]. The IgM concentration in CF sputa was shown to significantly increase with the severity of the disease assessed by the Schwachman score. Among the other main proteins with potential antibacterial activity (IgA, IgG, lysozyme and lactoferrin) measured in CF sputa, a significant negative correlation was observed between the Schwachman score and the concentration of lactoferrin [74]. Lactoferrin is an iron-binding glycoprotein (75–80 kDa) of the transferrin family that is common to exocrine secretions of mammals [75]. In human tracheobronchial tissue, BOWES *et al.* [76] showed that lactoferrin was localized in the secretory granules of submucosal gland serous cells. The biological functions of lactoferrin are still unclear, but it has been suggested that it is part of the primary system of nonspecific local secretory immunity with sIgA, myeloperoxidase and lysozyme against bacterial

infection [77] and, perhaps, as a regulator of human granulopoeisis [78]. Lactoferrin and transferrin exhibit very similar structure and biochemical properties, but differ by their affinity for ferric ions and their delivery process of iron to the cell. Transferrin transports ferric ions into cells by receptor mediated endocytosis, a unique process by which transferrin and its receptor are reutilized repeatedly in iron delivery. On the other hand, it has recently been shown that the lactoferrin mediates transfer of iron to HT 29-D4 cells, not by receptor-mediated endocytosis, but by releasing iron at the plasma membrane without itself being internalized [79].

Since the fundamental work of FLEMING [80], lysozyme (a 14.4 kDa protein) has been shown to exhibit a broad spectrum of antibacterial properties, serving in concert with IgA and lactoferrin to protect the mucous membrane. Both lysozyme, lactoferrin and IgA are detected in high concentrations in exocrine secretions such as saliva, tears, colostrum, gastroduodenal, middle ear and tracheobronchial secretions and only in small amounts in internal secretions, such as serum and pleural fluids [71]. In human tracheobronchial tissue, HINNRASKY *et al.* [81] recently demonstrated quantitative differences in the distribution of lysozyme in human airway secretory granule phenotypes. At the tracheal level, the density of lysozyme, evaluated by post embedding immunogold technique, did not vary significantly within the different secretory granule phenotypes, whereas, at the bronchial level, the differences were significant. Moreover, the lysozyme labelling density was much higher in the bronchial than in the tracheal secretory granules. In human airway secretions, KONSTAN *et al.* [82] estimated that 10–20 mg of lysozyme was secreted per day by the tracheobronchial tract, lysozyme being mainly synthesized in the submucosal layer containing glandular cells. Whether human tracheal surface epithelial cells participate in the synthesis of lysozyme has not yet been clearly demonstrated.

Lysozyme can also originate from leucocytes, present in high concentration when airways are infected by pathogenic bacteria [74]. Four enzymatically active lysozyme forms have been isolated and purified depending on whether lysozyme was purified from purulent secretions, non-purulent secretions from chronic bronchitic patients, or from normal tracheobronchial explants in organotypic culture [83]. Additional studies are needed to characterize and define the possible sources for these multiple forms of human airway lysozyme. The physiological role of human lysozyme remains unclear and several functions have been reported in recent years. It is generally claimed that lysozyme is an antibacterial agent, but there is no clear evidence to show whether this enzyme plays *in vivo* a direct or indirect role against pathogens present in airway secretions. It has been reported that most pathogenic bacteria are insensitive to lysozyme in the absence of antibodies, complement or other enzymes [84]. In fact, the major *in vitro* measurements of antibacterial activities of lysozyme have essentially been conducted using hen egg-white lysozyme on oral or intestinal bacteria [85]. Nevertheless, it has been shown that exposure of pneumococci to purified human airway lysozyme results in a higher bactericidal activity than that obtained with hen egg-white lysozyme [86]. Apart from the restructuring effects of lysozyme in the gel network formation and, consequently, in the rheological and transport properties of airway mucus [87], GORDON *et al.* [88] have postulated that human lysozyme may also function in a negative feedback system to modulate the inflammatory response. They reported that *in vitro* human lysozyme inhibited the leucocyte chemotactic motility and the production of toxic oxygen radicals by stimulated leucocytes. Recently, PRIOR *et al.* [89] reported that serum lysozyme levels appear to be a useful marker both to assess disease activity in pulmonary sarcoidosis and to correlate clinical impairment and response to steroid therapy.

The cellular regulation of mucus production by the human airways and the mechanisms involved in mucus hypersecretion in chronic obstructive respiratory diseases are not completely elucidated. In the same way, whether the cell mechanisms involved in the excessive secretion are similar in all patients with obstructive airway diseases is not yet defined. Although the hypersecretion in patients with chronic bronchitis and cystic fibrosis follows a period of glandular hypertrophy, BHASKAR *et al.* [90], recently reported that in acute quadriplegic patients, the onset of mucus hypersecretion is sudden and due to disturbed neuronal control of bronchial mucous gland secretion. In humans, glandular secretions from tracheal and nasal airways are stimulated by exogenous acetylcholine, cholinergic and peptidergic analogues, leading to the secretion of mucous cell products such as glycoconjugates (mucins) and serous cell products such as lactoferrin, lysozyme and sIgA [91, 92]. Among airway neuropeptides, vasoactive intestinal peptide (VIP, a 28 amino acid peptide) is the most potent endogenous airway relaxant and has been demonstrated to have a protective effect against certain bronchoconstricting stimuli. VIP is released with acetylcholine by parasympathetic nerves surrounding the submucosal glands and may contribute to the physiological regulation of airway mucus secretion [92–95]. COLES *et al.* [92] have shown that in *in vitro* human bronchial cultures, VIP (10 ng to 1 $mg \cdot ml^{-1}$) causes a dose-dependent inhibition of baseline and methacholine-stimulated release of both glycoconjugates and lysozyme. On the other hand, VIP does not inhibit glycoconjugates and lysozyme in bronchial explants from chronic bronchitic patients. These authors suggest that this absence of sensitivity to VIP inhibition is one possible cause in the pathogenesis of the airway mucus hypersecretion. Recently, OLLERENSHAW *et al.* [94] showed that in airway tissues from patients with asthma, no immunoreactive VIP was found, compared to the abundance of VIP found in normal airways. Whether this loss of VIP and/or the absence of VIP sensitivity is a cause or a result of asthma and chronic bronchitis is unclear, but it is a matter for further investigation. BARANIUK *et al.* [95]

recently demonstrated that in normal human nasal mucosa, VIP-immunoreactive nerve fibres were found to be most concentrated in submucosal glands adjacent to serous and mucous cells. They also showed that VIP stimulated lactoferrin release from serous cells but did not affect glycoconjugate secretion from short-term nasal explant cultures.

Lipids. It is well known that carbohydrates can be specific receptors for bacterial lectins and can mediate the adhesion of bacteria to host tissue. The carbohydrate chains of glycosphingolipids have been shown to be specific adhesion receptors for different microorganisms in humans. This could be an important mechanism of bacterial colonization or, conversely, of bacterial clearance in the respiratory tract [96]. KRIVAN *et al.* [97], demonstrated that two pathogenic bacteria frequently isolated in CF respiratory secretions, *Pseudomonas aeruginosa* and *Pseudomonas cepacia*, specifically bind to gangliotetraosylceramide and gangliotriaosylceramide. These results suggest that the glycolipids associated to respiratory secretions as well as the carbohydrate chains of mucins are potential sites of binding for microorganisms. At the surface of the epithelia, as suggested by RAMPHAL and PYLE [98], the glycolipids present at the apical cell membrane could also represent receptors for bacterial adhesion. Gangliosides could also interact in bacterial adhesion by their ability to bind to fibronectin [99]. In some reports, high contents of free fatty acids have been observed in tracheobronchial secretions. It has been demonstrated by COONROD [100] that in alveolar surfactant, free fatty acids may have an antimicrobial activity. The same mechanism could be evoked in the upper respiratory tract.

Enzymes. Recent studies have clarified the previously described antibacterial properties of some neutrophil proteases [101], which will be described in a later chapter. Other antimicrobial polypeptides of human neutrophils (defensins, azurocidin, bactericidal permeability increasing factor (BPI) and cationic antimicrobial proteins (CAP)) possess the capacity to kill bacteria by a mechanism independent of their specific enzymatic activities [102, 103]. The most potent neutrophil protease possessing an antimicrobial activity is cathepsin G. The antimicrobial domain within the molecule is constituted by a heptapeptide [104] located at the surface of the molecule.

Antioxidant and anti-inflammatory components

Antioxidant enzymes. Three enzymes provide the main defence against oxygen-mediated tissue injury: catalase, superoxide dismutase and glutathione peroxidase [105].

Data on the "oxidant-antioxidant imbalance" in airway secretions can be obtained in the literature [105–110]. PEDEN *et al.* [111] recently reported that uric acid secreted by human nasal submucosal glands may play an important role in airway antioxidant physiology. Because cigarette smoking is a most pertinent example of chronic oxidant stress of the lower respiratory tract, most measurements of these antioxidant enzymes were carried out in bronchoalveolar lavages from smokers: alveolar macrophages from smokers contain increased activities of catalase and superoxide dismutase [112]. Catalase present in peroxisomes is one of the enzymes which cleaves H_2O_2. In rats, catalase was found to be fundamental in protecting alveolar epithelial cells [113]. CANTIN *et al.* [114] recently demonstrated that, among antioxidant macromolecules in the epithelial lining fluid of the normal human lower respiratory tract, catalase played a major role in protecting lung parenchymal cells against oxidants present in the extracellular milieu. Superoxide dismutase is a metalloenzyme, with Cu and Zn, the gene of which is located on chromosome 21 [115]: unfortunately, no studies in humans have documented a clinical association between trisomy 21 and a possible protection from toxic effects of oxygen [116]. Superoxide dismutase is lacking in sputum samples from patients with cystic fibrosis [117].

Glutathione peroxidase has been indirectly evaluated by measuring the ratio of oxidized glutathione to total glutathione [118]. Epithelial lining fluids from patients with idiopathic pulmonary fibrosis are deficient in glutathione [119]. On the contrary, bronchoalveolar lavage fluids from smokers contain a higher glutathione concentration than those from nonsmokers [120].

Anti-inflammatory agents. Strategies to reduce inflammation in the airways may be considered for the treatment of mucus hypersecretion in patients with asthma, chronic bronchitis and cystic fibrosis. There is evidence that endogenous neuropeptides such as substance P and other tachykinins released from sensory nerves in human airway mucosa [121] are involved in the genesis of bronchial hyperresponsiveness and airway inflammation. It has been shown that tachykinins produce a series of effects, referred to as neurogenic inflammation, including increased vascular permeability [122], neutrophil adhesion and chemotaxis [123], submucosal gland secretion [124], and cough and smooth muscle contraction [125]. Studies have demonstrated that neutral endopeptidase (encephalinase, EC 3.4.24.11), a cell membrane binding peptidase found in many organs and tissues including human lung [126] can prevent the neurogenic inflammation effects [127–129]. Recently, it has been proposed that therapy using recombinant human encephalinase may be useful in treating cough or bronchial narrowing associated with airway diseases such as asthma, chronic bronchitis and cystic fibrosis [129].

Glucocorticoids are effective drugs used for the treatment of chronic inflammatory diseases. The mode of action of glucocorticoids is very complex [130], as almost all known pro-inflammatory mediators are modulated in their synthesis or degradation [131, 132]. The anti-inflammatory actions of glucocorticoids include the inhibition of the release of arachidonic acid derivatives and the increase of lipocortin levels in

airways associated with a significant reduction in respiratory glycoconjugate secretion [133]. Lipocortins, also called annexins, belong to a family of calcium and phospholipid binding proteins, which have been proposed as mediators of the anti-inflammatory actions of glucocorticoids, possibly *via* inhibition of phospholipase A2 activity [134, 135].

In human lungs, lipocortin 1 (a 35–37 kDa protein) has been detected in bronchoalveolar lavage fluids [136] and has also been reported to be synthesized and released by human airway submucosal gland cells in culture [137]. Using cultured rat alveolar epithelial cells, it has been shown that corticosteroids increase the amounts of lipocortin 1 produced by these cells [138]. Human recombinant lipocortin 1 impairs the release of thromboxane A_2 and prostacyclin *in vivo* in perfused lung and isolated cells [139]. Two other reports have also described anti-inflammatory actions of recombinant lipocortins *in vivo* [140, 141], although their physiological importance has not been demonstrated directly. Lipocortin 1 also causes inhibition of superoxide anion release from activated guinea-pig alveolar macrophages [142] and decreases platelet-activating factor (PAF) production from stimulated human polymorphonuclear neutrophils (PMN) [143]. Since reports have demonstrated that messenger ribonucleic acid (mRNA) and protein amounts of lipocortin 1 in other cell types [144–146] were not altered by dexamethasone, lipocortins do not seem, to date, to have a central function in the mediation of glucocorticoid effects. It has also been proposed that lipocortins are involved in phospholipid cytoskeleton interaction, exocytosis and that some of them (annexins I and II) are able to promote aggregation of phospholipid vesicles in mediating contact between vesicle membranes [147].

Protease and antiprotease components

Proteases: major destructive agents. The proteolytic enzymes which have been identified in lung secretions seem to be mainly neutrophil-derived [148] and many papers have dealt with proteases from purulent sputum. In fact, some proteases have been described originating from cells in culture, either normal [149], transformed [150], or cancerous [151]. Testing for the presence of an elastolytic activity in bronchial secretions has been, for a long time, the major investigation carried out to determine a possible destruction of lung parenchyma. Up to now, it seems that neutrophil elastase could be mainly in charge of proteolytic events in lungs.

Neutrophil elastase is a serine protease found in azurophil granules of the mature neutrophil. In blood neutrophils, the content is about 0.5–3 pg·cell^{-1}. Since neutrophils enter the lungs through the pulmonary vasculature [152], it is likely that lung neutrophils are identical to blood neutrophils in that way. However, some recent data have shown different amounts of neutrophil elastase in blood neutrophils from patients with moderate to severe emphysema [153]. The gene has been described and is located in chromosome 11 at q14 [154]. The primary structure of the molecule has been determined before by sequencing at the protein level [155] and corresponds to that derived from the nucleotide sequence.

Neutrophil elastase presents a broad substrate specificity against extracellular matrix components: several lines of evidence have shown its *in vitro* destructive ability not only against elastin but also against type III collagen [156, 157], type IV collagen [158], adhesion molecules such as fibronectin [159] or laminin [160] and proteoglycans [161]; its role *in vivo* was extrapolated from these results. If neutrophil elastase has a role to play in some bronchopulmonary pathologies ("protease-antiprotease imbalance hypothesis" [162]), evidence for uncontrolled elastolysis would have to be given. Different amounts of active neutrophil elastase have been noted between mucoïd, mucopurulent or purulent sputum samples [117, 163–165]. The results of BRUCE *et al.* [166] showed a significant correlation between the excretion of elastolysis products and the severity of lung disease in cystic fibrosis patients. Localization of neutrophil elastase was investigated in normal and emphysematous lungs: contradictory results have been obtained [167, 168] and it is not clear whether or not neutrophil elastase is associated to elastic tissue in emphysematous lung. Moreover, when the lungs are free of inflammatory reaction, neutrophils do not seem to contribute significantly to the normal turnover of pulmonary elastin in normal subjects [169].

Cathepsin G accompanies elastase in the azurophil granules of neutrophils. It is also a serine protease of interest in bronchial pathology because it possesses an *in vitro* elastolytic activity [170–173]. It is also able to stimulate bovine airway gland secretion [174]. Free cathepsin G was identified in cystic fibrosis sputum [117], in which it can enhance the activity of leucocyte elastase towards elastin, and is able to cleave fibronectin [175], thus favouring *Pseudomonas aeruginosa* colonization of the upper respiratory tract.

Very little information is available about neutrophil collagenase in bronchial secretions. Presence of neutrophil collagenase, a metallo-protease which is a component of the specific granules [176], was noticed in purulent sputum [177]. It has been considered as an important destructive factor in lung pathologies in which a high turnover of collagen was involved [178].

Cathepsin B, which is a thiol-protease, can also degrade *in vitro* native collagen [179]. Its presence has been reported in bronchial secretions and bronchoalveolar lavage fluids obtained from patients with chronic obstructive lung diseases [180], bronchiectasis [165] or lung damage associated with cigarette smoking [181]. The enzyme purified from purulent sputum [182] is slightly different from that of liver. It could originate from bronchial epithelium and serous cells [183], or alveolar macrophages.

Although it does not share proteolytic properties, myeloperoxidase can be classified among the destructive lung enzymes: it is now well known that protein and

tissue damage in the lung may occur by an oxidative attack caused by the very effective system composed of myeloperoxidase, halide and hydrogen peroxide [184]. Myeloperoxidase is synthesized by promyelocytes and stored in azurophil granules of the neutrophils (localization of the gene at locus 17q11). Its presence in bronchoalveolar lavage fluids was considered as being a marker of local neutrophil activity [185] and an additional factor in the development of emphysema in smokers [105, 186]. In sputum samples from patients with cystic fibrosis, high myeloperoxidase amounts were correlated with high amounts of neutrophil elastase [117].

In addition to neutrophil proteases, bacterial proteases could also be involved in proteolytic lung injury. It is well known that *Pseudomonas aeruginosa* is the major pathogen (with *Staphylococcus aureus*) associated with the bronchial infections of cystic fibrosis patients. Therefore, in their bronchial secretions, *Pseudomonas aeruginosa* elastase has been considered as the main deleterious enzyme. *Pseudomonas aeruginosa* elastase (PsE) was first prepared and studied by MORIHARA [187] in 1965. It has a typical metallo-enzyme inhibition profile with one Zn atom per molecule. The gene has now been studied [188, 189]. *In vitro* PsE is able to degrade lung elastin [190], basement membranes [191], complement C_3 [192], and some components of bronchial secretions: immunoglobulins [193] and lysozyme [194]. Some controversy still exists about the degradation of leucocyte elastase inhibitors: α_1-proteinase inhibitor (α_1PI) is effectively cleaved at a single peptide bond located just before the active site [195], but recent studies have demonstrated that this phenomenon does not occur in the presence of leucocyte elastase [196], which is largely represented in cystic fibrosis sputum samples. Nor is the bronchial mucous inhibitor inactivated in the presence of leucocyte elastase [197], in contrast to some previous findings [198]. Moreover, PsE would represent only 2% of sputum elastolytic activity [197]. However, the occurrence of this enzyme in cystic fibrosis bronchial secretions is not controversial [199]; in fact PsE induces production of antibodies present in bronchial secretions, responsible for a neutralization of the enzyme. In bronchoalveolar fluids, an excess of antigenic PsE compared to elastolytic activity was demonstrated [200].

A thiol-protease from *Staphylococcus aureus* was shown to be elastolytic *in vitro* [201]; its presence has not been reported in bronchial secretions, but this enzyme could be a candidate for lung injury in cystic fibrosis patients infected by *Staphylococcus aureus*. Another very specific protease might be involved in a lowering of lung defences: it is immunoglobulin A_1 protease which cleaves the IgA molecule into Fab and Fc fragments. These IgA proteases originate from several bacterial species: among them *Haemophilus influenzae* and *Streptococcus pneumoniae*, which are often detected in bronchial infections. The exact cleavage site is dependent on the bacterial strain, but always follows one of the proline residues in the hinge region. The gene of the protease from *Haemophilus influenzae* has been cloned [202]. Such enzymes may promote perturbations in mucosal immune defence mechanisms [203].

Antiproteases. Inasmuch as neutrophil elastase is the major aggressive enzyme in bronchial secretions, the protective role of neutrophil elastase inhibitors against proteolytic lung injury has been widely explored.

These studies were initiated by the observation of the link between α_1PI deficiency and emphysema by LAURELL and ERIKSSON [204]. Human airway secretions obtained either by cough (sputum samples) or by lavage (bronchial and bronchoalveolar washings) were analysed to define the presence of inhibitors and their properties [162].

Isolation methods, properties [205] and genetic data [206] concerning α_1PI have been reviewed. Moreover, important data have recently been obtained concerning the expression of α_1PI gene in alveolar macrophages [207–209]. Therefore, α_1PI in bronchial secretions may originate from the blood by passive diffusion and from alveolar macrophages by active secretion (at least in alveolar lining fluid). Its elimination is mediated by the serpin-enzyme complex (SEC) receptor localized on the surface of human hepatoma cells and mononuclear phagocytes [210]. According to kinetic constants, α_1PI is a very rapid and, therefore, effective inhibitor of neutrophil elastase [211]. The inhibition process of this enzyme leads to the formation of an equimolar complex, of which the size, isoelectric point and antigenicity are different from those of both elastase and α_1PI. In addition to this complex, which showed the presence of elastase in the lung before the moment that airway secretions had been collected, one can find the so-called α_1PI* which represents a proteolysed form of α_1PI, due to either degradation of the complex or proteolytic cleavage by proteases non-inhibited by α_1PI or proteolytic cleavage of oxidated α_1PI (for example by myeloperoxidase) [212, 213].

In bronchial secretions, many studies have focused on the relative importance of these three forms (native, complexed, proteolysed) because neither the complex nor α_1PI* are able to inhibit some newly released neutrophil elastase or cathepsin G. This was generally achieved by electrophoretic methods followed by an immunological reaction [214–217], but the results are strictly dependent on the greater or lesser specificity of the antibodies for the different α_1PI forms [218, 219].

Presence of the complex α_1PI-elastase in bronchial secretions may constitute evidence of the protective role of α_1PI. In bronchoalveolar lavage fluids, it appears to be a marker of the severity of emphysema in non-α_1PI-deficient subjects [220]. It has been shown that α_1PI displays a chemotactic activity [221], and the newly recruited neutrophils contribute to the continuous delivery of elastase in the lungs.

Alpha$_1$-proteinase inhibitor is not the only inhibitor involved in the defence of the bronchial tree against leucocyte elastase damage. It is now well known that mucus proteinase inhibitor (MPI) is present in the bronchial tree, its physiological role as a serine

proteinase inhibitor being to protect the larger airways [222]. MPI is the common name given to the low-molecular mass (12 kDa) inhibitors present in several mucous secretions [223], which are encoded by a single gene [224]. This molecule is synthesized in the respiratory tract, mainly in the serous cells of the submucosal glands of the bronchial epithelium, together with lactoferrin [76] and lysozyme [82]. MPI consists of 107 amino acid residues [225, 226], which are organized into two domains. Recently, it was demonstrated that the proteinase inhibitory activities are only located in the C-terminal domain [227–229]. This molecule has inhibitory properties against different serine proteinases including leucocyte elastase and cathepsin G. However, its target enzyme is still unknown. This inhibitory activity is suppressed by oxidants from cigarette smoke or phagocyte-derived oxidants [230]. In bronchial secretions, it is partly bound to mucins [231]. Recent studies have demonstrated that α_1PI and MPI act differently on elastin-bound elastase [232, 233]: MPI is able to efficiently inhibit *in situ* elastin-bound elastase, while α_1PI dissociates elastase from elastin for a further inhibition. Methods for the determination of the concentration of immunoreactive MPI in bronchial secretions have been described [234, 235]: they both give results for total MPI (free and complexed with enzymes). It seems that: 1) MPI concentration is higher in bronchial secretions than in bronchoalveolar lavage fluids; and 2) in the upper respiratory tract, the molar ratio MPI/α_1PI is always in favour of MPI, contrary to the situation observed in the lower respiratory tract of all subjects, except in α_1PI deficient subjects [236]. A genetic defect in MPI has not so far been described. On the other hand [237], it was demonstrated that α_1PI gene expression in human alveolar macrophages is directly regulated by the presence of elastase. Therefore, the question is: has all released elastase to be inhibited in the bronchial tree?

Alpha$_1$-antichymotrypsin is a member of the serpin superfamily; its sequence has been established comparative as with that of α_1PI [238]. It is a potent inhibitor of all chymotrypsin-like enzymes, including leucocyte cathepsin G [239]. Its presence in bronchial secretions was reported by different groups [240–242]. It enters the lung by passive diffusion, but can be synthesized and secreted by alveolar macrophages [243]. The role of α_1-antichymotrypsin in lung secretions should be the inhibition of leucocyte cathepsin G, but α_1-antichymotrypsin in lung secretions was shown not to be an effective inhibitor of cathepsin G [244]. Recent studies may cast new lights on possible roles of α_1-antichymotrypsin in lungs: 1) α_1-antichymotrypsin-cathepsin G complexes have been shown to stimulate the synthesis of interleukin-6 by fibroblasts in culture [245]; 2) α_1-antichymotrypsin inhibits neutrophil superoxide anion generation [246], this property being independent of the protease inhibitory activity; however, it has no significant effect on degranulation.

In plasma, α_2-macroglobulin and α_1-proteinase inhibitor are the major inhibitors of leucocyte elastase. Alpha$_2$-macroglobulin is a glycoprotein of 728 kDa and, therefore, its movements from plasma to lung interstitium are somewhat restricted by its size. However, alveolar macrophages are able to secrete α_2-macroglobulin [247]. The presence of immunoreactive α_2-macroglobulin has been noted in sputum samples from chronic bronchitis [241, 248] and cystic fibrosis patients [117], although its contribution to the anti-elastase defence is very low [249]. In adult respiratory distress syndrome, α_2-macroglobulin in bronchoalveolar fluids is complexed with elastase [250] and may be involved in the inhibition of metalloproteases such as collagenase or *Pseudomonas aeruginosa* elastase.

A low-molecular weight inhibitor called BSI-E I, isolated from bronchial mucus, was shown to be specific for porcine pancreatic and leucocyte elastases [251]. In bronchial secretions from healthy subjects, it is present in an active form, and mainly as a complex with elastase in sputum from chronic bronchitis patients [252].

Two other elastase inhibitors have been isolated from bronchial secretions [253, 254]. Their origin and functions are as yet unknown. Cystatin C and cystatin S (from saliva) appeared as constituents of bronchial secretions [255, 256] without any knowledge about their effective inhibitory activity. Tissue inhibitor of metalloproteinases (TIMP) has been identified in sputum and bronchoalveolar lavage fluid [257]; it is assumed that it forms the major defence against metalloproteinases, but *in vitro* studies have demonstrated the preferential binding of collagenase to α_2-macroglobulin in the presence of TIMP [258].

It is clear that, in healthy nonsmoking subjects, the antiprotease screen is largely sufficient to prevent proteolysis of the lung matrix. In contrast, it is very evident that the emphysematous ongoing process in α_1PI-deficient subjects can be stopped (or slowed down) by locally increasing the anti-elastase screen. In contrast, in patients suffering from bronchiectasis, cystic fibrosis or adult respiratory distress syndrome, active (hence, uncontrolled) proteases are present, without giving rise to the development of emphysematous lesions. Therefore, except in emphysema of α_1PI-deficient subjects, a pathological role for proteases as destructive enzymes in human lung diseases is so far not proven. Hence, have antiproteases a role to play in the defence of the lung? Are they essential by their other properties, only some of them being known? Are proteases only implicated by their non-proteolytic properties (for example inducing cell metaplasia and gland secretion). To our current knowledge, these questions remain unanswered.

Conclusion and future directions

Respiratory mucus actively participates in the airway epithelial protection. In physiological situations, the biochemical components taking place in the antibacterial and antioxidant protection as well as in the antiprotease screen appear to be efficient in preventing infection, inflammation, hypersecretion and, in some cases, the proteolysis of lung matrix occurring in human

respiratory diseases. In physiopathological conditions, following epithelium injury, biochemical modifications of the airway secretions may induce a dysregulation in the natural protective function of the airway mucosa. The cellular mechanisms inducing changes in the intrinsic programme of synthesis and regulation of secretory products are not completely elucidated. Future studies are needed to gain better insights into the understanding of the molecular and cellular processes which shift the normal secretion pattern to abnormal airway secretions. In order to study these processes (*i.e.* synthesis and regulation of secretory products from individual airway cell types, control of airway cell growth and differentiation, intracellular mechanisms regulating airway secretion, specific gene expression, ...), systems for culturing airway epithelial cells from human and animal sources have been developed. *In vitro* cell culture could represent an interesting approach to the understanding of the specific cellular events that control mucus secretion in health and diseases.

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