The protease–antiprotease battle in the cystic fibrosis lung

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IMPORTANCE OF LUNG INFLAMMATION

It is well recognized that long-standing inflammation damages the lungs in children and adults with cystic fibrosis (CF), and it is no coincidence that most CF deaths are due to respiratory failure. The lung inflammation is primarily due to the host response to chronic bacterial infection, particularly with *Pseudomonas aeruginosa*, which calls into question the whole concept of colonization, with its implication of bacteria residing harmlessly in the body. However, it has also been suggested that inflammation may actually precede microbial infection in the CF lung, an idea based on the fact that significant inflammation has been detected in broncho-alveolar lavage (BAL) samples in which microbes (bacteria, viruses or fungi) were not isolated[1,2]. It is possible that in the young patients studied the infection had been eradicated but that the resultant inflammation simply persisted[3]. In older children, however, it is unlikely that the pathogenic bacteria are ever eradicated. In addition, using more sensitive molecular techniques for identifying bacteria, it may be that infection is going unrecognized when standard culture methods are employed[4]. More recently, it has been suggested that inflammation may be an integral part of the CF defect. Accumulation of faulty CFTR (CF transmembrane conductance regulator) protein in the cell endoplasmic reticulum may result in production of pro-inflammatory cytokines following activation of the transcription factor NFκB[5]. In conclusion, it seems most likely that the majority of lung inflammation is in fact secondary to recurrent bacterial or viral infections, but that there is a contribution from endogenous factors, and possibly even a reaction to the abnormal mucus lining the epithelium. It is still unexplained why the production of interleukin-10, an important anti-inflammatory cytokine, is reduced in CF epithelial cells[6,7].

Pivotal role of the neutrophil

Cytological examination of sputum or BAL fluid from a patient with CF reveals an abundance of neutrophils. It is a feature of CF that neutrophils are the predominant phagocytic cells in the lungs, rather than alveolar macrophages. An increased neutrophil cell count and raised levels of interleukin-8 (Il-8) in the CF lung is a constant feature which is not limited to older patients, infective exacerbations or to those with more severe disease[1,2,8,9]. Neutrophils are attracted to the lungs from the circulation by various chemo-attractants, including Il-8 whose production is up-regulated in CF[10,11], C5a complement component[12], and the lipoxygenase product leukotriene B_4[13]. During 'normal' inflammation, neutrophils migrate through the lung without causing much damage; this occurs when they are unprimed and secrete few of their contents[14]. However, when primed into a state of hyper-responsiveness (by a variety of inflammatory cytokines), there is an increase in cellular function leading to increased secretion, adhesion and mediator production. Neutrophils (and eosinophils) of CF patients have been shown to have an increased propensity to release their granule proteins[15], and it is thought that neutrophil function in CF may be affected by the underlying genetic defect, rather than just being secondary to chronic inflammation[16]. In this state, when they migrate into the lungs, tissue injury occurs due to the release of toxic mediators, including oxygen metabolites, cationic proteins and proteases[14].

WHAT ARE PROTEASES?

Neutrophil-derived proteases

Proteases are proteolytic enzymes that degrade proteins by hydrolyzing their peptide bonds. While some proteases function intracellularly, others are released by the cells, in which case they can modify the extracellular matrix and lung parenchyma[14]. Most are stored in a latent form in granules, but they are secreted by activated neutrophils during phagocytosis, particularly during the 'frustrated' phagocytosis associated with microcolonies of *P. aeruginosa*[17]. Proteases are also released by neutrophils at the time of their degeneration and death. At least six extracellular proteases have been identified in human neutrophils, which include serine proteases (elastase, cathepsin G and proteinase III), metallo-proteinases (collagenase and gelatinase), and plasminogen activator[14] (Table 1). Cathepsin G and proteinase III probably do contribute to CF lung inflammation, but their exact role is not clear. Collagenase is found in high levels in CF sputum and may be associated with disease severity; it is matrix degrading and inactivates antiproteases[18]. Gelatinase (Type IV collagenase) is also present in high levels in CF sputum and disrupts the basement membrane, leading to an increased permeability to

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inflammatory cells[19]. However, it is neutrophil elastase which predominates in CF—it occurs in both bound inactive and free active forms, and micromolar concentrations of active neutrophil elastase are found in sputum and the epithelial lining fluid of the respiratory tract (Figure 1)[20–22]. Concentrations found in the CF lung are higher than in any other pulmonary disease studied in humans[23]. Such elevated levels are mainly due to increased release of elastase, although there may also be a degree of proteolytic inactivation of the elastase inhibitors (antiproteases) by the excess neutrophil elastase itself[20].

Neutrophil elastase has several functions detrimental to the lungs (reviewed by Birrer *et al.*[22] and Döring[17]) (Table 2). It degrades elastin, fibronectin and other structural proteins, thus damaging the architecture of the lung and airways. Increased turnover of elastin has been demonstrated in CF by the finding of increased urinary degradation products such as desmosine and isodesmosine[24]. Epithelial cell damage, and in particular cleavage of cell surface fibronectin, enhances adhesion of *P. aeruginosa* to the cells[25]. Neutrophil elastase is a potent secretogogue[26], which when combined with the fact it also impairs ciliary function, leads to a further accumulation of airway secretions in the lungs. Neutrophil elastase also harms the lungs' host defence, by fragmenting the immunoglobulins IgG and IgA, as well as complement. It also impairs phagocytosis and killing of bacteria such as

Table 1 **Neutrophil-derived extracellular proteases**

Serine proteases	Elastase
	Cathepsin G
	Proteinase III
Metalloproteinases	Collagenase
	Gelatinase
Plasminogen activator	

Table 2 **Principle functions of neutrophil elastase**

Effect	*Mechanism*
Structural damage	Degrades elastin, fibronectin and other structural proteins
Accumulation of airway secretions	Secretogogue Impairs ciliary function
Impaired immunity	Fragments IgG, IgA and complement Impairs phagocytosis and killing of *Pseudomonas aeruginosa*
Promotion of inflammation	Stimulates release of Il-8 Degrades antiproteases

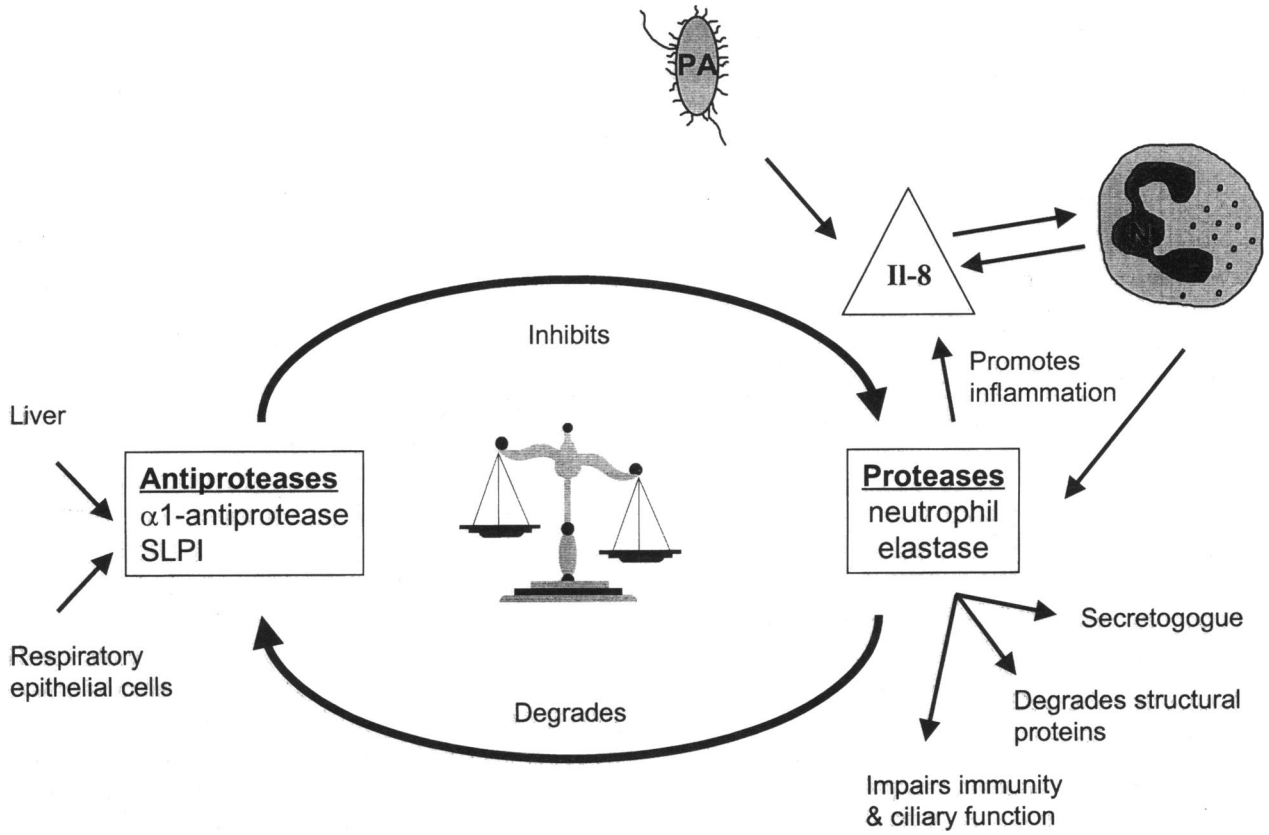

Figure 1 **The interaction between neutrophil elastase and the antiproteases.** PA=*Pseudomonas aeruginosa*, N=neutrophil, Il-8=interleukin-8, SLPI=secretory leukoprotease inhibitor

P. aeruginosa. Finally, it stimulates release of Il-8, thus recruiting even more neutrophils into the lung to perpetuate the cycle of inflammation by further release of neutrophil elastase[27]. The level of neutrophil elastase measured in sputum or BAL fluid is often used as a marker of inflammation in CF lung disease, and to a degree sputum levels correlate with disease severity, as judged by lung function and chest X-ray scores[20]. Plasma levels, however, are unreliable as a marker of lung inflammation, as there is no correlation with either sputum levels or severity of lung disease[20]. This is understandable since proteases released outside the lung probably never reach the lung due to the abundance of antiproteases in the plasma[14].

Degraded neutrophil DNA makes a significant contribution to sputum viscosity in CF, hence the use of DNase to reduce viscosity of airway secretions. Being a cationic molecule, some of the neutrophil elastase is bound and inhibited by the negatively charged polyanionic DNA and mucin, and is thus inactivated[28]. There were initial concerns that treatment with DNase would lead to a freeing of some of this bound neutrophil elastase and thus worsen the inflammation. However, studies have shown that although there is a transient increase in sputum free neutrophil elastase after initial doses of DNase, over time neutrophil elastase levels reduce if anything, and Il-8 levels are unchanged[29,30].

Bacterial proteases

The bacteria commonly found in the CF lung secrete a variety of proteases, for example *P. aeruginosa* produces elastase and alkaline protease while *Staphylococcus aureus* and *Haemophilus influenzae* produce several others[31]. Their effect is similar to neutrophil-derived proteases: they have the potential to damage the respiratory epithelium, cleave immunoglobins and impair ciliary function[31]. However, it is thought that this is limited to the early infectious process and that bacterial proteases are of minor importance (relative to neutrophil-derived proteases) in the CF lung[32]. Their effect is diminished since pseudomonal proteinases are neutralized by specific host antibodies in immune complexes[33]. In addition, although about two-thirds of *P. aeruginosa* isolated from patients with CF actively secrete proteases, there is reduced production by mucoid strains (the predominant form in chronic CF infection) compared to non-mucoid strains[34]. Furthermore, there appears to be a down-regulation of genes encoding for *P. aeruginosa* elastase production in CF[35]. Interestingly, attention has recently focused on the use of macrolide antibiotics as anti-inflammatory agents in CF, and erythromycin has been shown to inhibit elastase production by *P. aeruginosa*[36]. However, a recent *in vitro* study, reported as an abstract, has shown that erythromycin and colomycin both increased elastase activity in sputum collected from adolescents with CF, whilst gentamicin, tobramycin and ceftazidime had no effect[37].

WHAT ARE ANTIPROTEASES?

In normal individuals, the epithelial surface of the lung is protected from neutrophil elastase by antiproteases[22], the main two of which have several names (Box 1).

α_1-antitrypsin/α_1-antiprotease

α_1-antitrypsin (α_1-AT) is probably better referred to as α_1-antiprotease (α_1-AP), but since the former term has a disease named after it, this older term is still used. It is an acute phase protein that is produced in the liver (by hepatocytes) and reaches the lungs via the plasma, where it coats the respiratory bronchioles and alveoli[38]. It is the main inhibitor of neutrophil elastase within the lower respiratory tract, providing more than 90% of the anti-neutrophil elastase defence[39]. In normal individuals, the molar quantities of the antiproteases in the respiratory epithelial lining fluid far outweigh that of neutrophil elastase, hence damage to the epithelium and local host defence is prevented[22]. Unfortunately, this is not the case in CF, where huge quantities of neutrophil elastase simply overwhelm the antiproteases, with molar levels of immunoreactive neutrophil elastase being 12 times higher than that of α_1-antiprotease in the sputum[20]. It is not a production problem, as α_1-antiprotease is present in normal amounts and fully active in the serum[40]. Not only is α_1-antiprotease outnumbered, but it is also inactivated in the CF airways by the proteases themselves[41], since α_1-antiprotease is proteolytically degraded by neutrophil elastase as soon as the molar ratio exceeds 1:1 in favour of neutrophil elastase[42]. In addition, α_1-antiprotease is inactivated by bacterial proteases, with *P. aeruginosa* elastase inactivating the antiprotease more rapidly than *S. aureus* proteinase[38].

Box 1 **Synonyms for the two major antiproteases**

α_1-antitrypsin (α_1-AT)
α_1-antiprotease (α_1-AP)
α_1-proteinase inhibitor (α_1-PI)
Secretory leukoprotease inhibitor (SLPI)
Secretory leukocyte protease inhibitor
Serine leukocyte protease inhibitor
Secretory leukocyte proteinase
Antileukoprotease (ALP)
Bronchial mucosal inhibitor

Low serum levels of α_1-antitrypsin can occur as a result of mutations of the α_1-antitrypsin gene, and more than 75 variants have been identified. In a Danish study, carriage of deficient α_1-antitrypsin variants in patients with CF was shown to be associated with earlier onset of *P. aeruginosa* infection and higher serum IgG levels[43] (both of which are poor prognostic signs). However, lung function did not seem to be affected, although this may have been due to the Danish policy of using regular anti-pseudomonal antibiotics. In a UK study, 20/147 unrelated CF patients were found to have α_1-antitrypsin deficiency phenotypes (MS, S and MZ) but this was in fact associated with significantly better lung function[44]. Of course, in patients with α_1-antitrypsin deficiency alone, the lungs are not usually affected in childhood, emphysematous changes tend to begin in early adulthood, and are more common in smokers.

Secretory leukoprotease inhibitor

Secretory leukoprotease inhibitor (SLPI) is produced by the respiratory epithelium in bronchi and bronchioles[45]. However, proportionately it is the main antiprotease of the upper respiratory tract and the large airways only[46]. Although it has been shown to be an excellent inhibitor of neutrophil elastase, the majority (about two-thirds) of SLPI in the epithelial lining fluid is inactive[47] (Table 3). This means it is unlikely to play a significant role, compared to α_1-antiprotease, in protecting the respiratory epithelium except in the upper airways where it is at its highest concentration[47]. The reason for this high degree of inactivation in normal individuals is not clear, the molecule is intact, not bound in a complex, and has not been exposed to neutrophil elastase or oxidants, factors that may be more relevant in CF[47]. Certainly bacterial proteases can inactivate SLPI, although it is much less susceptible (20–50 000-fold less) to this type of degradation than α_1-antiprotease[38]. Nevertheless, SLPI probably acts in conjunction with α_1-antiprotease, but since it is a smaller molecule with a higher isoelectric point, close to that of neutrophil elastase, it might compartmentalize in a similar fashion to neutrophil elastase, thus increasing its relevance in the lungs' defence against proteases[47].

Table 3 **Relative activities of the two main antiproteases in respiratory epithelial lining fluid**

	α_1-AP	SLPI
Molar ratios (quantitative)	1	0.56
% functionally active	95	33
Molar ratios (functional)	1	0.16

(Source: Vogelmeier *et al.*, Ref 47)

SLPI has other useful properties besides its antiprotease activity. Work with recombinant SLPI (rSLPI) has shown that it increases glutathione levels in lung secretions within hours of administration[48]. The resultant increase in lung antioxidant capacity might well prove to be important in CF, and will also help protect α_1-antiprotease from oxidative inactivation. In addition, SLPI may have a role as a naturally-occurring broad-spectrum antibiotic, with antiretroviral, bacteriocidal and antifungal activity[49]. Finally, an *in vitro* study has shown that when the corticosteriod fluticasone propionate was added to airway epithelial cells, there was an increase in mRNA transcript levels of SLPI within 12 h, which may be one of the anti-inflammatory mechanisms of this steroid[50].

CLINICAL TRIALS OF ANTIPROTEASE THERAPY

The imbalance of the neutrophil elastase–anti-neutrophil elastase system is well established by 1 year of age, allowing for many years of lung destruction[22]. It is likely that there would be a major impact on lung morbidity and even CF mortality if it were possible to reduce the amount of free neutrophil elastase in the lungs. Although intravenous (anti-pseudomonal) antibiotics do significantly reduce levels of neutrophil elastase, the effect is short-lived and a considerable amount of neutrophil elastase activity remains in the lower respiratory tract secretions[21]. The alternative and more logical approach is to supplement the over-whelmed antiproteases to combat the proteolytic destruction in the lungs[51]. In contrast to α_1-antitrypsin deficiency, where intravenous replacement therapy of human plasma-derived α_1-antitrypsin may have some role[32], this is not an option in CF[40]. Aside from the fact that serum activity is normal anyway in CF, frequent massive doses would be required to free the lung of elastolytic activity. It has been estimated that only 2% of infused α_1-antitrypsin reaches the lungs[52]. Furthermore, an abstract report in which CF patients were given an intravenous infusion of α_1-antitrypsin showed elastase activity persisted for 12 h only in the respiratory epithelial lining fluid, compared to 1 week in patients with α_1-antitrypsin deficiency[53].

α_1-antitrypsin

A commercial preparation of α_1-antiprotease is now available, derived from pooled human plasma, called Prolastin (Bayer Corporation, Pittsburgh, USA). The problem with this form of therapy is first the limited availability, and secondly the safety concerns of using a plasma product over many years. Indeed, since 1994, Bayer Corporation has withdrawn several batches of Prolastin due to fears over possible transmission of Creutzfeldt–Jakob

disease. It is probably for these reasons that a larger trial has not been carried out after the initial work.

Pharmacokinetic studies in normal volunteers showed that aerosolized Prolastin gave no substantial side-effects and 36 h after a single dose, α_1-antiprotease concentrations and anti-neutrophil elastase activity were still double that at baseline[54]. Surprisingly, this study was performed 6 years after the first trial in CF was reported[55]. In this initial promising study, 12 adults with CF were given nebulized Prolastin (1.5–3.0 mg/kg) twice daily for 1 week[55]. It suppressed neutrophil elastase in the respiratory epithelial lining fluid and reversed the inhibitory effect of epithelial lining fluid on *P. aeruginosa* killing by neutrophils. The treatment was well tolerated. Following on from this, a phase I study in five centres across the USA was carried out[56]. Twenty-two adult patients were given 100–350 mg nebulized Prolastin twice daily for 4 weeks. In all patients but one, there was a decrease in elastase activity and an increase in the capacity to inhibit added elastase in secretions recovered from the lungs. There was no change in neutrophil count nor Il-8 level in epithelial lining fluid. Five patients had moderate adverse effects possibly related to the drug (four had chest symptoms, one had joint symptoms), while one patient had severe respiratory symptoms which were probably related to the drug.

Secretory leukoprotease inhibitor

There are reasons why using SLPI may have some advantages over exogenous α_1-antiprotease, and these are mainly related to its physical properties[39], but recombinant SLPI (rSLPI) is also far less susceptible to degradation by bacterial proteases than α_1-antiprotease[38]. rSLPI is produced by *Escherichia coli* using a synthetic SLPI gene and is identical in terms of structure and function to the naturally occurring human SLPI[39]. Intravenous SLPI is of no use as, being a small molecule, it is quickly excreted in the urine[57]. Pharmacokinetic studies in normal individuals showed that rSLPI retained its form and function after nebulization, but did not accumulate on the respiratory epithelial surface, needing to be given every 12 h[58]. There was a marked increase in SLPI levels and anti-neutrophil elastase capacity in airway epithelial lining fluid in both normal adults and adults with CF[58]. Following inhalation, the rSLPI moves from the epithelium into the interstitium of the lung[39]. In a pilot study (done in conjunction with the pharmacokinetic study described above), 100 mg rSLPI was given twice daily for 1 week to 17 adults with CF[59]. The increase in epithelial lining fluid SLPI levels was accompanied by a reduction in active neutrophil elastase levels in almost all individuals. There was also a significant reduction in neutrophil numbers and Il-8 levels in the epithelial lining fluid, which implied that the rSLPI also broke the cycle of inflammation in the airways. The treatment was well tolerated. A further study using radiolabelled rSLPI has shown that the aerosol is only deposited in well-ventilated areas of the lung in patients with CF[57]. This probably applies to all inhaled drugs in CF but is important, as it is the poorly ventilated areas of the lungs that need the therapy most.

FUTURE DIRECTIONS

Transgenic human α_1-antitrypsin

Given the problems with availability and the concerns over safety, an alternative approach to α_1-antiprotease replacement is being explored. PPL Therapeutics in Scotland has developed transgenic sheep that produce human α_1-antitrypsin in their milk (tg-hAAT)[60,61]. The human protein is produced only in the lactating mammary gland and secreted into the sheep's milk. Comparative analyses of this protein have been performed for bioactivity, glycosylation state, amino acid terminal sequencing, isoelectric point and molecular weight. With the exception of some of the side-chain sugars, it was found to be identical to the human plasma derived protein and is now being studied in safety trials.

One theoretical concern has been whether tg-hAAT would be antigenic in humans, with possible epitopes being sheep protein, sheep α_1-antitrypsin, side-chain sugars and M phenotype. However, so far, in safety studies of more than 100 patients with CF exposed to multiple doses of nebulized tg-hAAT, no Type I allergic reactions have occurred, no skin-prick tests have been positive and fewer than 10 patients have developed non-neutralizing IgG antibodies (personal communication, Dr H Colquhoun, 1998). This latter phenomenon has been associated with all recombinant drugs marketed to date. Further safety trials are still ongoing in both adults and children with CF. The largest trial is multicentre and tg-hAAT is being given for 6 months to CF patients over 12 years-of-age. A safety trial will be starting shortly in younger children, and it is hoped that a major clinical trial can start soon, once all the safety work is complete.

Synthetic oral anti-elastase drugs

Several pharmaceutical companies have been developing oral agents to combat excess elastase activity. DMP777, manufactured by Dupont Pharmaceuticals USA, is a human neutrophil elastase inhibitor which is highly selective and potent. It has an intracellular action within azurophilic granules of viable neutrophils, as well as extracellular activity at sites of neutrophil elastase release from activated or degenerating neutrophils[23]. There is a phase II trial in progress and a phase III clinical trial is planned for 1999. Other agents at early stages of development include L-658,758 and ICI 200,355. L-658,758 is a cephalosporin-based anti-elastase

which blocks neutrophil elastase and proteinase III, and was shown to block >97% elastinolytic activity in CF sputum[62]. ICI 200,355 has been shown to inhibit the protease-induced secretory response in CF sputum, and reduced destruction of insoluble elastin[63]. Clearly these drugs are all at an early stage of development, but if proved effective an oral preparation may well have advantages in terms of patient compliance.

Synthetic inhaled anti-elastase drugs

Another agent that has been developed is FK706. In an open-labelled pilot study, it was nebulized once or twice per day for 10 days in 16 patients[64]. It led to a significant reduction in sputum neutrophil elastase activity, as well as a significant reduction in plasma Il-8 and E-selectin. However, four patients withdrew, with a decrease in lung function parameters in three of them. It may simply be that a bronchodilator is required before the drug is administered, but it is not yet clear whether the pharmaceutical company will continue with its development.

Gene therapy

Finally, an alternative approach to the problem has been reported utilizing the principles of gene therapy[65]. Plasmid-cationic liposome-mediated human α_1-antitrypsin gene transfer to a CF bronchial epithelial cell line has been achieved. It succeeded in protecting the cells from the toxic effects of elastase and inhibited elastase-stimulated release of neutrophil chemotactic activity from the epithelial cells. Whether this *in vitro* work can be translated into a clinical therapy remains to be seen.

CONCLUSIONS

Neutrophil-dominated inflammation is a major factor in CF lung disease. Much of the damage is due to the huge quantity of proteases, and in particular neutrophil elastase, which is released into the lungs. These proteolytic enzymes overwhelm the lungs' own antiprotease defences, α_1-antiprotease and SLPI. Correcting this imbalance may well lead to a reduction in the consequences of chronic lung inflammation. Plasma-derived α_1-antiprotease may be effective, but there are safety issues; the transgenic (sheep-derived) human α_1-antitrypsin is undergoing safety trials and may prove to be of great benefit. Recombinant SLPI has also been shown to be effective in an early study and further trials are needed. Finally, a range of synthetic oral agents is also being developed.

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