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REVIEW

New concepts of the pathogenesis of cystic fibrosis lung disease

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New concepts of the pathogenesis of cystic fibrosis lung disease. R.C. Boucher. ©ERS Journals Ltd 2004.
ABSTRACT: Although there has been impressive progress in the elucidation of the genetic and molecular basis of cystic fibrosis (CF), the pathogenesis of CF lung disease remains obscure. The elucidation of the pathogenesis of CF lung disease requires both a full description of normal innate airway defence and how absent function of the cystic fibrosis transmembrane regulator protein (CFTR) adversely perturbs this activity.

Recent data have linked the abnormal ion transport properties of CF airway epithelia to depleted airway surface liquid (ASL) volume, reflecting the combined defects of accelerated Na^+ transport and the failure to secrete Cl^-. Depletion of a specific compartment of the ASL, *i.e.* the periciliary liquid (PCL), appears to abrogate both cilia-dependent and cough clearance.

Subsequent to PCL depletion, mucus adheres to airway surfaces and persistent mucin secretion generates the formation of "thickened" mucus plaques and plugs, which become the nidus for bacterial infection. The paucity of liquid in these plaques/plugs, and the hypoxia in this environment, appear to promote biofilm bacterial infection.

Therapeutic agents that restore airway surface liquid volume, *i.e.* blockers of Na^+ transport, initiators of Cl^- transport and osmolytes, are reviewed, as are strategies that may be required to use volume-restoring agents safely in patients with cystic fibrosis.
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There has been immense progress in the elucidation of the molecular and cellular pathophysiology of cystic fibrosis (CF) since the cloning of the CF gene in 1989 [1–3]. Despite this progress, the median life-span of CF patients at the turn of the century was ~32 yrs of age [4], and the great majority of CF patients die from lung disease. This review will touch briefly on the wealth of information on the genetics and cell biology of CF as it pertains to lung disease, and concentrate its focus on recent studies that have provided a more comprehensive delineation of the pathogenesis of CF airways disease and consequently opened new avenues for therapy. For more extensive reviews of the genetics and cell biology of CF, the reader is referred to previous reviews [5, 6].

The genetics of cystic fibrosis

The cystic fibrosis transmembrane regulator (CFTR) gene is a large, ~250 kb, gene that is located on the long arm of chromosome 7. To date, >1,000 candidate mutations in the CF gene have been identified and reported to the CF Gene Mutation Consortium in Toronto, ON, Canada [7]. The large number of different mutations has made population screening for CF problematic, but genetic diagnosis is now practical for counselling parents with one affected CF child.

An important area of CF research has focused on the topic of genotype-phenotype prediction [8, 9]. In brief, it has been possible to predict the severity of the CF organ-level phenotype from the genotype with high fidelity with respect to the sweat duct, pancreas, and the reproductive system. In contrast, it has been difficult to identify correlations between genotype and phenotype of lung disease, *i.e.* severity. This difficulty is perhaps best illustrated by the fact that patients who are homozygous for the ΔF508 mutation exhibit a wide spectrum in the rate of development and severity of lung disease [10, 11].

The failure to generate genotype-phenotype predictions in the lung has led to the notion that both environmental-lung interactions and the genetic background of the host contribute substantially to the severity of CF lung disease. With respect to the latter concept, a search has been initiated for "modifier genes", *i.e.* genes that modify the effect of CF mutations on lung dysfunction. At present, a number of modifier genes have been identified, based on "candidate selection" [12–17]. Thus far, these genes appear to include those that regulate aspects of innate lung defence and inflammatory cascades. The next level of studies designed to identify modifier genes will involve genome-wide searches, using single nucleotide polymorphisms, in an effort to identify novel genes that contribute to the severity of CF lung disease [18, 19]. Finally, it is likely that the genome-wide searches will soon be complemented by proteomic approaches designed to elucidate proteins that function as modifiers. A great hope is that these studies ultimately will identify key genes and proteins that may be rational therapeutic targets, *i.e.* their functions could be accelerated or decelerated as indicated.

The cell biology of cystic fibrosis

CF reflects the absence of functional CFTR protein at the proper cellular location [20]. Classifications of CFTR mutations have been developed that encompass the spectrum of genetic mutations, but the majority appear to involve

misfolding of CFTR protein. The most common CF mutation, ΔF508, was the first to be shown to exhibit a problem in polypeptide maturation and translocation to the appropriate cellular domain, *e.g.* the apical membrane [21]. Although the experiments in heterologous cell systems are elegant and demonstrate unequivocally a misfolding problem with the ΔF508 CFTR protein, the extent of misfolding and failure of translocation to the apical cell domain in patients homozygous for ΔF508 CFTR is still controversial. For example, whereas the evidence appears strong that virtually all ΔF508 CFTR fails to translocate to the apical membrane of the sweat duct [22], there are reports that a substantial fraction of the ΔF508 protein translocates to the apical membrane in the airways and colonic epithelium, as assessed by combinations of immunocytochemical, Western blot and biophysical studies [23, 24]. Others, however, have not been able to reproduce this result [25, 26]. As it is likely that ΔF508 protein exhibits ~30% of wildtype activity when fully stimulated [27, 28], and hence would be a logical therapeutic target, it is important to resolve the issue of whether ΔF508 is in the apical membranes of airway and colonic epithelia *in vivo* with newer high-affinity antibodies that may be suited to resolve this difficult question.

Organ-level pathogenesis of cystic fibrosis: a disease of abnormal innate lung defence

CF patients are born with apparently normal lungs, followed by the acquisition of chronic, unrelenting bacterial infections of the airways (bronchi) in the first few years of life. Thus, in the simplest view, CF lung disease reflects the failure of the innate defence mechanisms of the lung against inhaled bacterial organisms [29]. The recognition of this general problem has led to studies designed to elucidate the normal innate defences against inhaled bacteria and how these defences may be degraded by the absence of functional CFTR.

As described in figure 1a, two hypotheses have been developed to link epithelial ion transport to the innate defence of airways against inhaled bacterial pathogens. In perhaps the more classical schema, mechanical clearance has been viewed as the primary innate defence against inhaled bacterial species. Mucus clearance provides the mechanical clearance that removes bacteria from the airways in ⩽6 h under normal conditions [30]. It would appear that there are sufficient antimicrobial activities, provided by lactoferrin and lysozyme, to suppress bacterial growth over these time frames [31]. As described in detail below, the promotion of efficient mucus transport requires an elegant coordination of intertwined physiological events that, in the end, provide a well-defined periciliary liquid (PCL) layer that exhibits an optimal height (~7 µm, *i.e.* the height of extended cilia) and viscosity for effective ciliary beating and cell surface lubrication (fig. 1b). The capacity of the epithelium to maintain the PCL layer at the appropriate height requires the adjustment of airway surface liquid (ASL) volume, a process that is believed to be mediated by isotonic (100–150 mM NaCl) volume transport [32–34].

In the early to mid-1990s, a novel hypothesis for airways' defence emerged that focused on the role of antimicrobial peptides in ASL to provide a chemical shield on the airway surface as the primary innate defence [35–37]. For this shield to be effective, two processes must be normally regulated by the airway epithelium. First, there must be secretion of the appropriate quantities of antimicrobial, salt-sensitive peptides (defensins), and secondly, the ASL must be modified, *i.e.* made hypotonic (>50 mM NaCl), so antimicrobials are active.

Although there have been many studies designed to explore the relative merits of each hypothesis, particularly in model systems, perhaps the simplest approach is to test *in vivo* the concentrations of the relevant antimicrobial factors and their salt sensitivities, and most importantly, perhaps, the ionic composition of normal ASL. With respect to antimicrobial activities, the studies of COLE *et al.* [31] identified lactoferrin and lysozyme in airway surface liquids as the major antimicrobial substances, in concentrations that rendered them relatively salt-insensitive. In contrast, it has been difficult to identify small molecular weight defensin-like molecules in ASL, nor identify any defensin-like antimicrobial activity in the presence of human airway mucins.

With respect to ASL ionic composition, virtually all of the recent *in vivo* data have suggested that ASL in normal subjects is indeed isotonic. For example, human ASL has been measured in the upper and lower airways with a variety of techniques, including filter paper and ion-selective electrodes, and is isotonic [38, 39]. Cl^- ion-selective electrode measurements have shown that the ASL in primates is isotonic from the upper airways to the bronchioles [39]. These studies have been complemented by studies using other techniques, *e.g.*

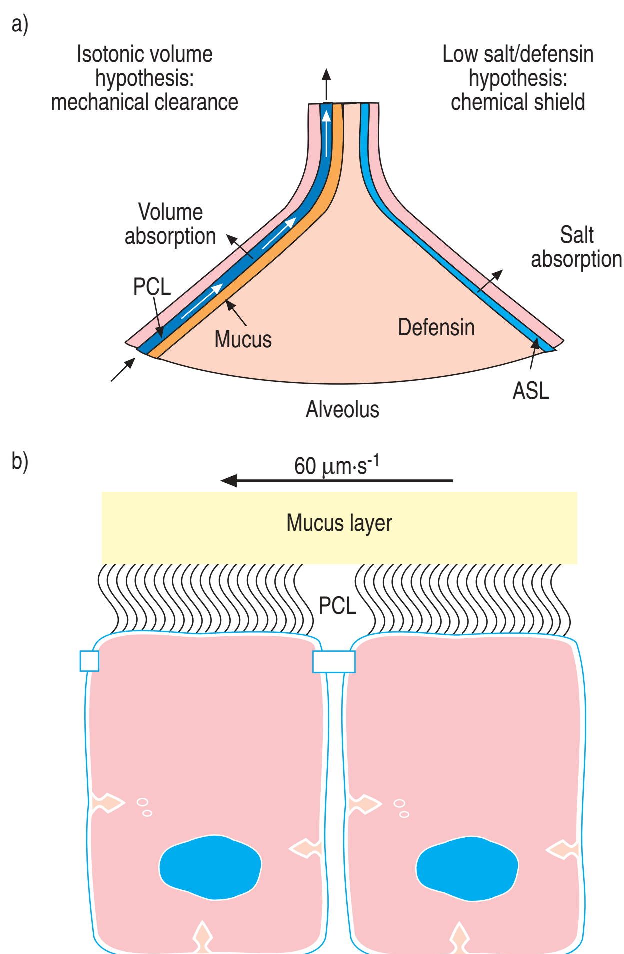

Fig. 1. – Models depicting elements of innate airways defense. a) Model depicting relative surface areas of distal and proximal airway regions. On the left is the depiction of the mechanical (mucus) clearance hypothesis, with the epithelium controlling the volume of the periciliary liquid (PCL) as the critical element mediating efficient mucus transport. On the right is shown the chemical shield hypothesis that predicts that the airways epithelium absorbs salt but not water from airway surface liquid (ASL) to form a "low salt" ASL to promote antimicrobial activities of defensins. b) Schema of airway surface liquid compartment depicting mucus layer, PCL and normal velocity of mucus transport.

fluorescent probes and *in vivo* microdialysis, in normal mice, which revealed an isotonic ASL. These studies add to a spectrum of older studies of ASL ion composition from a variety of normal mammals, including dog, sheep and pigs, which also revealed isotonic ASL [34]. Finally, despite the predictions of the defensin/low salt hypothesis, measurements of ASL ionic composition comparing uninfected CF and normal human subjects and normal and CF mice have failed to detect differences in ASL ion composition, *i.e.* both normal and CF ASL are isotonic [32, 38, 40–42]. Thus, it appears that the weight of the evidence would favour an isotonic liquid on normal airway surfaces, which strongly favours the mechanical clearance hypothesis [43–45].

The normal regulation of airway surface liquid clearance: processes designed to maintain an intact periciliary liquid layer

The ability to study the ~30 μm deep ASL, and study of the physiology of its component mucus and PCL layers, has been greatly buttressed by the availability of well-differentiated airway epithelial cultures. These cultures secrete mucins, transport salt and water, and organise ciliary beat direction so that the integrated function of surface (rotational) mucus transport is expressed *in vitro* [46] (fig. 2). The different compartments of these cultures can be labelled with fluorescent probes and studied as living cultures in the confocal microscope. This system thus offers unique opportunities to investigate the integrated physiology of epithelial salt and water transport, mucin secretion and ciliary beating. Indeed, studies with this system revealed that both components of ASL, *i.e.* the mucus and PCL layers, are transported at approximately equal rates along airway surfaces *via* the actions of cilia [46].

The mucus layer serves to trap inhaled material during the clearance process from the airways. The mucus layer uses two mechanisms to remove virtually all inhaled particles that deposit on airway surfaces: 1) mucus flow is "turbulent", so materials are mixed into the mucus layer and enmeshed/trapped during clearance [48]; and 2) mucin molecules exhibit a "combinatorial library" of carbohydrate epitopes to ensure low affinity binding to most particles [49]. However, a less recognised role of the mucus layer in mucus transport reflects its reservoir-like capacity to store and release liquid, *i.e.* swell and shrink [33]. This passive process is of enormous importance in maintaining PCL volume by donating to or accepting liquid from the PCL layer as needed. Thus, it appears that when there is a relative depletion of liquid on airway surfaces, the mucus layer donates liquid until its height/volume is reduced ~50% before its capacity to donate liquid is exceeded and detectable depletion of PCL height/volume occurs. Conversely, it appears that extra liquid added to the airway surface does not expand the PCL layer, but rather is selectively added to the mucus layer, "swelling" it. Interestingly, the addition of extra fluid to the mucus layer appears not only to maintain mucus clearance, but also to accelerate it [33].

The contribution of active epithelial ion transport to ASL and PCL volume regulation has been studied in similar systems [33]. As shown in figure 3a, the human airway ciliated epithelial cell expresses the epithelial Na^+ channels (ENaC) and pumps (Na^+/K^+-ATPase) to mediate transcellular Na^+ absorption. The ciliated cell also has the capacity, *via* expression of apical Cl^- channels (CFTR and Ca^{2+}-activated Cl^- channels) and the basolateral Na^+-K^+-$2Cl^-$ cotransporter, to secrete Cl^- when ENaC is blocked and the appropriate Cl^- secretory driving forces are generated. The importance of the capacity to both absorb and secrete is revealed in the processes that maintain PCL height (volume). As shown in figure 3b, it appears that "excess" ASL is absorbed until a steady state is achieved at approximately the height of the extended cilium, *i.e.* 7 μm, the normal PCL layer height. This regulation is complex and involves both sensors that detect the volume of ASL and effectors that control the rates of volume absorption and perhaps secretion. Unfortunately, relatively little is known about the sensors of ASL volume and how these sensors transmit information to the various effectors (ion channels) in the apical cell membrane. It may be that there are redundant sensor systems, some sensing chemical information encoded in the ASL, and others sensing mechanical properties (*e.g.* viscosity) of the ASL.

It is perhaps more clear what are the effectors that regulate the volume of ASL in response to the surface environment. For example, the volume absorptive process that removes excess liquid from airway surfaces is mediated by transepithelial Na^+ transport [50, 51] (fig. 3c). It appears that ENaC is highly active when ASL volume is large, whereas ENaC is inhibited when ASL volume approaches that of the normal PCL volume. When ENaC is inhibited, the electrical driving forces for initiating Cl^- secretion are developed. Thus, the steady state PCL is likely maintained by a balance between Na^+ absorption and Cl^- secretion. Recent studies have shown that the Cl^- secretion induced by ENaC inhibition is mediated by CFTR, and that the level of CFTR activity is governed by signals in the lumenal bath, *e.g.* adenosine, interacting with compartmentalised adenosine receptors A_{2b}, G proteins (G_s), adenylate cyclase, and cyclic adenosine monophosphate (cAMP)-dependent protein kinases [52]. Thus, it is speculated that the signal transduction pathways and effectors that control on a minute-to-minute basis the volume of ASL may be located in the apical domain of the cell.

Cystic fibrosis airway surface liquid abnormalities: failure to "defend" the periciliary liquid layer

Since the original detection of a raised potential difference (PD) across CF airway epithelia *in vivo* [53], there has been the notion that abnormal electrolyte transport is a key component of CF lung pathogenesis. Early PD studies detected an increase in the amiloride-sensitive component of the PD, which suggested an accelerated rate of Na^+ transport. However, the PD measurements also detected a failure to secrete Cl^- ions, under basal conditions and β-adrenergic stimulation, which predicted a second defect [54].

Subsequent studies with freshly excised tissues and cultured cells established evidence for both Na^+ transport (upregulated) and Cl^- transport (downregulated) defects in CF airway epithelia compared with normals. For example, radioisotope studies of active ion transport revealed accelerated Na^+ transport under short-circuit and the more physiological open-circuit conditions [55–57]. The same technique revealed the absence of Cl^- secretion under conditions when ENaC was blocked with amiloride under basal or β-agonist-stimulated conditions. Subsequent double-barrelled microelectrode studies of freshly excised and cultured normal and CF cells detected a raised apical membrane Na^+ conductance in CF cells and a reduced or absent Cl^- conductance [58–61]. Ultimately, studies of the apical membrane channels with patch clamp techniques revealed an increased activity of ENaC (increased open probability) in CF compared with normal cells [62]. In summary, these studies suggested that the CFTR protein had dual functions in airway epithelia, *i.e.* to conduct Cl^- ions and to regulate ENaC. The absence of CFTR thus produces upregulation of Na^+ absorption and a failure of cAMP-regulated Cl^- secretion (fig. 4a).

Studies designed to describe the mechanisms of CFTR

a) Mucus layer PCL

b)

c)

d)

e)

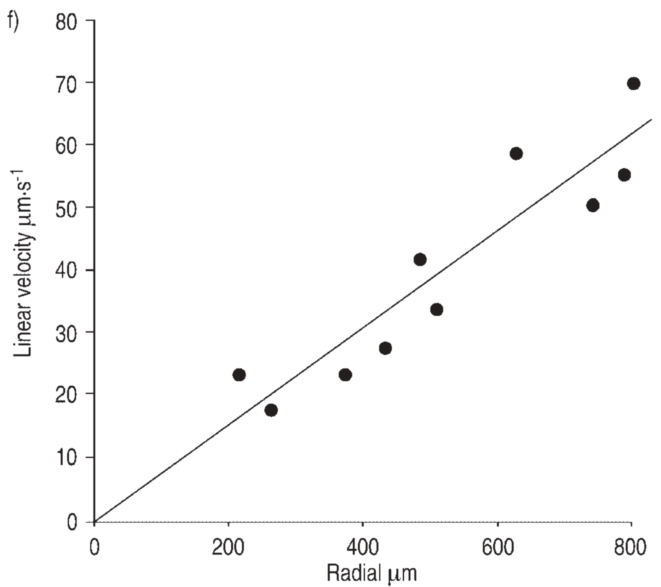

Fig. 2. – Cell culture system designed to study integrated activities required for mucus transport *in vitro*. a) Light micrograph of osmium-perfluorocarbon-fixed 6-week-old human bronchial air-liquid interface culture revealing distinct mucus and periciliary liquid layers (PCL). b) X-z confocal micrograph of columnar cells (green) and airway surface liquid (red) labelled with fluorophors. c) Fluorescent micrograph of mucus stained with fluorescent (1 µm) beads, "looking down" at culture surface. d) 5-s time-lapse fluorescent micrograph of mucus rotating on surface of culture. e) X-z confocal micrograph showing bead-containing (light) and bead-free zones. f) Mucus rotation velocity as a function of the distance from the centre of rotation (0 µm). Scale bars=10 µm. Adapted from [46, 47].

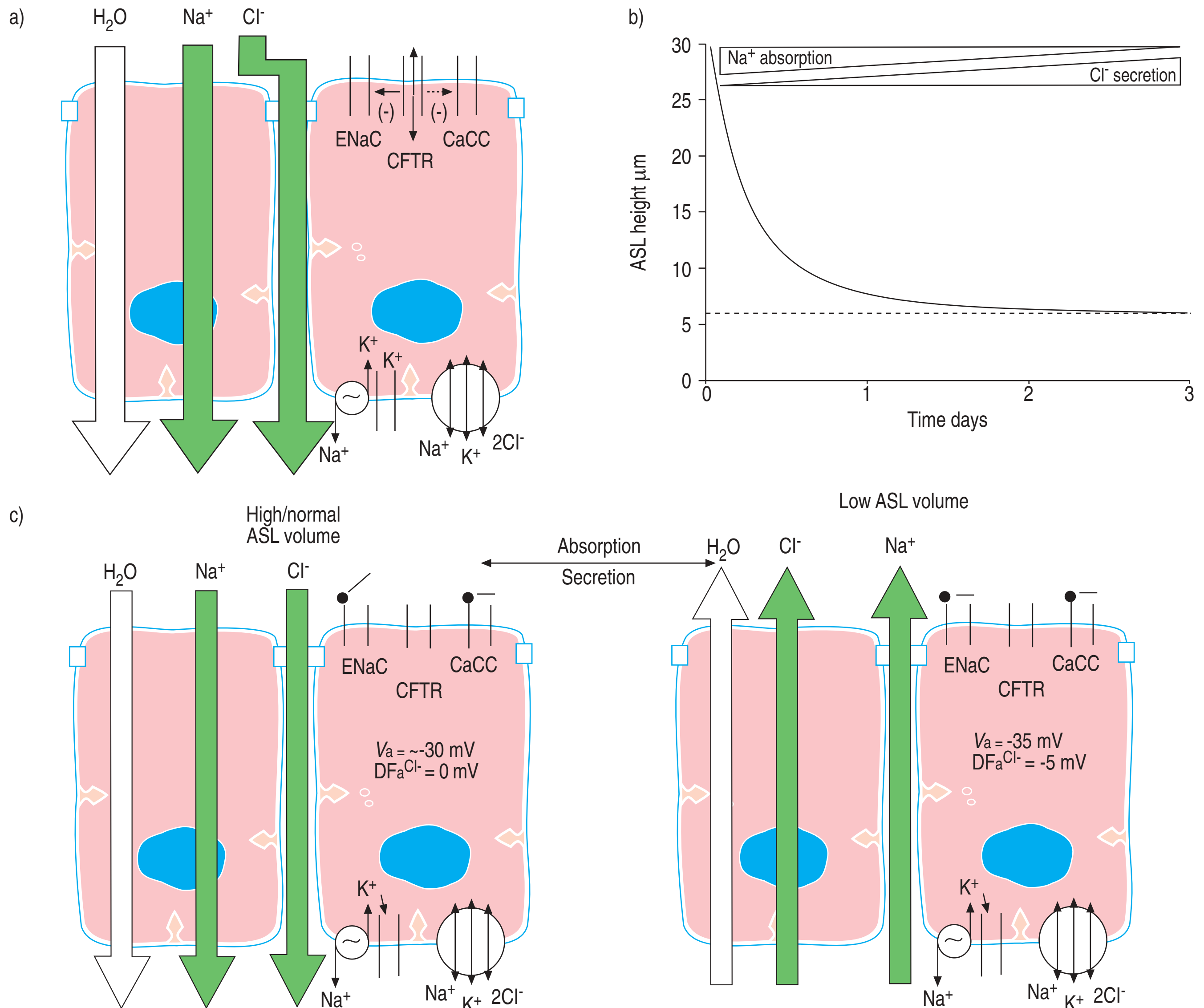

Fig. 3. – Regulation of the volume of periciliary liquid (PCL) layers by active ion transport. a) Schema describing routes of Na^+, Cl^-, and H_2O transport and ion transport elements that mediate these flows. At the lumen are an epithelial Na^+ channel (ENaC) and two Cl^- channels: cystic fibrosis transmembrane regulator (CFTR) and the Ca^{2+}-activated "alternative" Cl^- channel (CaCC). CFTR is depicted as both a regulator of channels and as a Cl^- channel itself. On the basolateral surface are the Na^+/K^+ pump, the K^+ channels, and the loop diuretic sensitive Na^+-K^+-$2Cl^-$ cotransporter. b) Regulation of "excess" PCL volume by Na^+ absorption and maintenance of PCL at functionally relevant height (7 µm as defined by height of extended cilium), by a mix of the Na^+ absorption and Cl^- secretion. c) Interconversion of normal human airway epithelia between absorptive and secretory ion transport modes. When excess airway surface liquid (ASL) is present, Na^+ absorption mediated *via* ENaC is dominant (left panel). Cl^- is projected to be absorbed passively *via* the paracellular path due to the fact that there is no electrochemical driving force (DFa^{Cl-}) favoring Cl^- exit from the cell. In contrast, both the negative apical membrane potential (Va) and low intracellular Na^+ activity (~20 mM) favour Na^+ entry into the cell. When ASL volume is low (right panel), ENaC is inhibited, which makes the apical membrane potential more negative and generates a driving force for Cl^- secretion. Information regarding ASL volume is postulated to be "encoded" within the ASL.

regulatory activities emerged with the availability of the CFTR and the ENaC genes. Thus, in a variety of heterologous systems, it has been possible to show that CFTR functions as a regulator of ENaC [63–85]. However, it has not yet been been elucidated how the molecular interaction between CFTR and ENaC may occur. Theories ranging from CFTR controlling the Cl^- concentration in the local membrane domain containing ENaC [66] to ones that involve a series of protein-protein interactions and positioning of various regulator molecules, *e.g.* kinases, have been proposed. Thus, this remains an important and unresolved area of research.

What has recently become more clear is the importance of both the accelerated Na^+ absorption and the failure to initiate Cl^- secretion to the abnormal ASL volume homeostasis in CF. As shown schematically in figure 4b, abnormalities in both processes ultimately lead to depletion of the PCL layer and formation of thickened ("concentrated") mucus plaques and plugs adherent to CF airway surfaces. For example, studies of the well-differentiated cell culture system interfaced to the confocal microscope have provided direct evidence that CF airway epithelia excessively absorb ASL, deplete the PCL and lose ciliary-dependent mucus transport [47] (fig. 4). These studies have been buttressed by recent *in vivo* studies in CF mice that directly demonstrated depletion of the ASL volume (but not a difference in the ion composition) that was associated with a spontaneous airways inflammatory (goblet

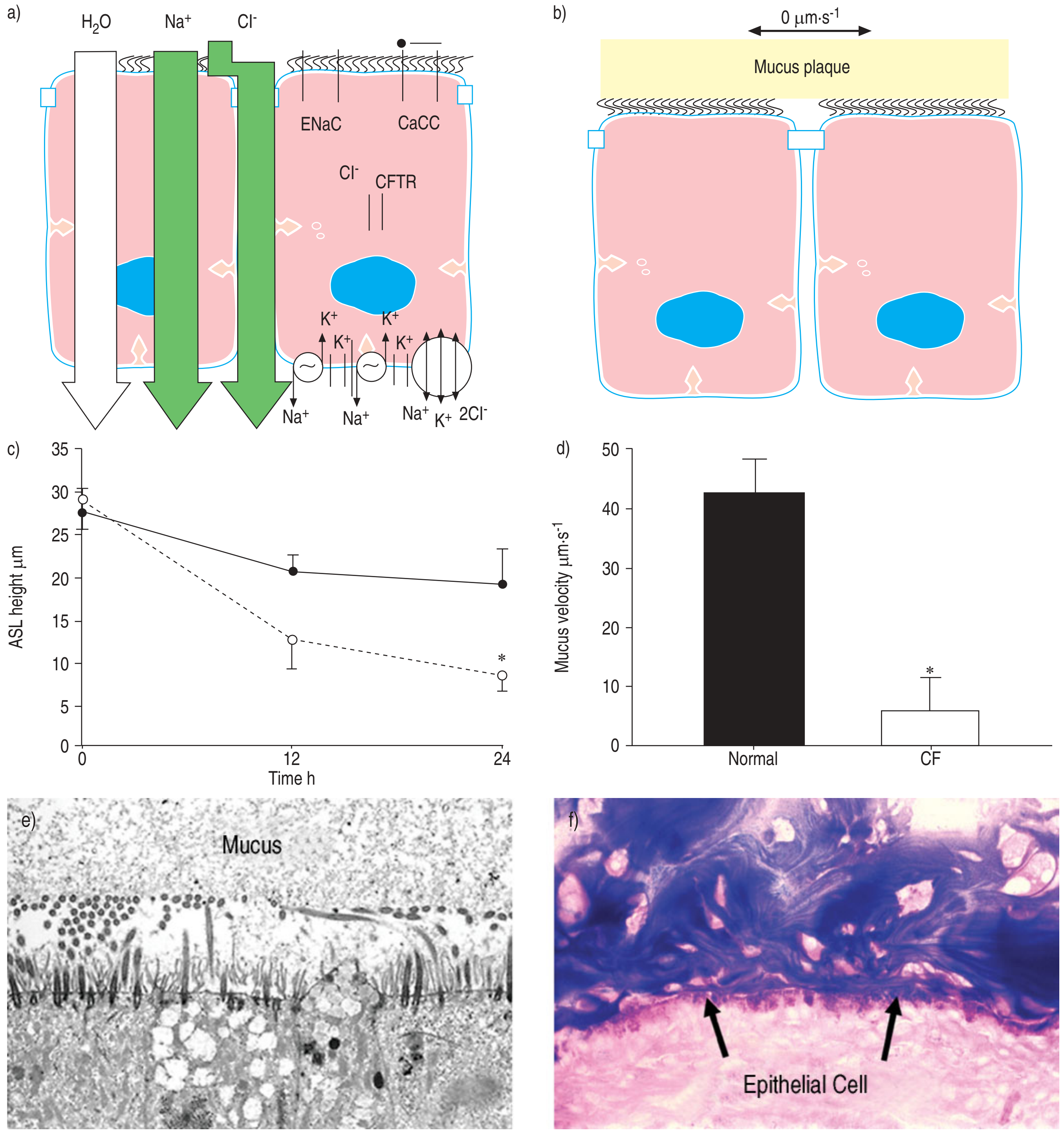

Fig. 4. – Links between abnormal epithelial ion transport and mucus stasis in cystic fibrosis (CF) airways. a) Schema showing routes for raised Na^+, Cl^-, and H_2O absorption and cellular mechanisms for raised Na^+ transport. The absence of cystic fibrosis transmembrane regulator (CFTR) from the apical membrane both limits Cl^- secretory capacity and releases the epithelial Na^+ channel (ENaC) from tonic inhibition. CaCC: Ca^{2+}-activated "alternative" Cl^- channel. b) Schema depicting the absence of periciliary liquid (PCL) layers with formation of adherent mucus plaque on CF airway epithelial cells. c) Volume absorption as measured by airway surface liquid (ASL) height with confocal microscopy. CF airway epithelia (○) absorb ASL more rapidly than normal airway epithelia (●). d) Effects of excessive volume absorption on rotational mucus transport 24 h after ASL challenge. Normal cells maintain mucus transport, whereas on CF cells mucus transport is abolished. e) Low power electron micrograph of osmium-perfluorocarbon-fixed CF culture showing PCL depletion with "bent-over" cilia and thickened mucus adhering to the glycocalyx coating ciliary shafts. f) Light micrograph of freshly excised CF airway stained with alcian blue/period acid-Schiff for mucins. Arrows point to cell surface. Area above arrows is thickened mucus that is adherent to cell surface. Adapted from [47].

cell hyperplasia) phenotype [32] and histological studies of freshly excised CF airways (fig. 4f). Thus, it appears that it is the combination of accelerated Na^+ transport and the failure to initiate cAMP-dependent Cl^- secretion that leads to depletion of the PCL and failure of mechanical mucus clearance in CF.

The sequence of disease that follows periciliary liquid depletion in cystic fibrosis

Mucus stasis

The depletion of PCL prevents the cilia from extending normally (fig. 5), abolishing the efficiency of ciliary-dependent mucus clearance. A reduction in ciliary-dependent clearance may also result from the concentration (thickening) of the mucus layer, which renders its viscoelastic properties less favourable for transport. However, perhaps more problematic is the fact that PCL depletion allows the mucus layer to come into contact with the cell surface glycocalyx. It seems highly likely, but not yet proven, that adhesive interactions occur between the mucus layer and the cell surface glycocalyx that effectively "glue" the mucus layer to airway surfaces [47]. The adhesive interactions between these two layers may be further strengthened by the low pH that appears to characterise CF airway epithelial ASL [86]. It remains to be elucidated what the strength of these interactions may be, whether the interactions are dominated by carbohydrate-carbohydrate interactions or protein-protein interactions, and

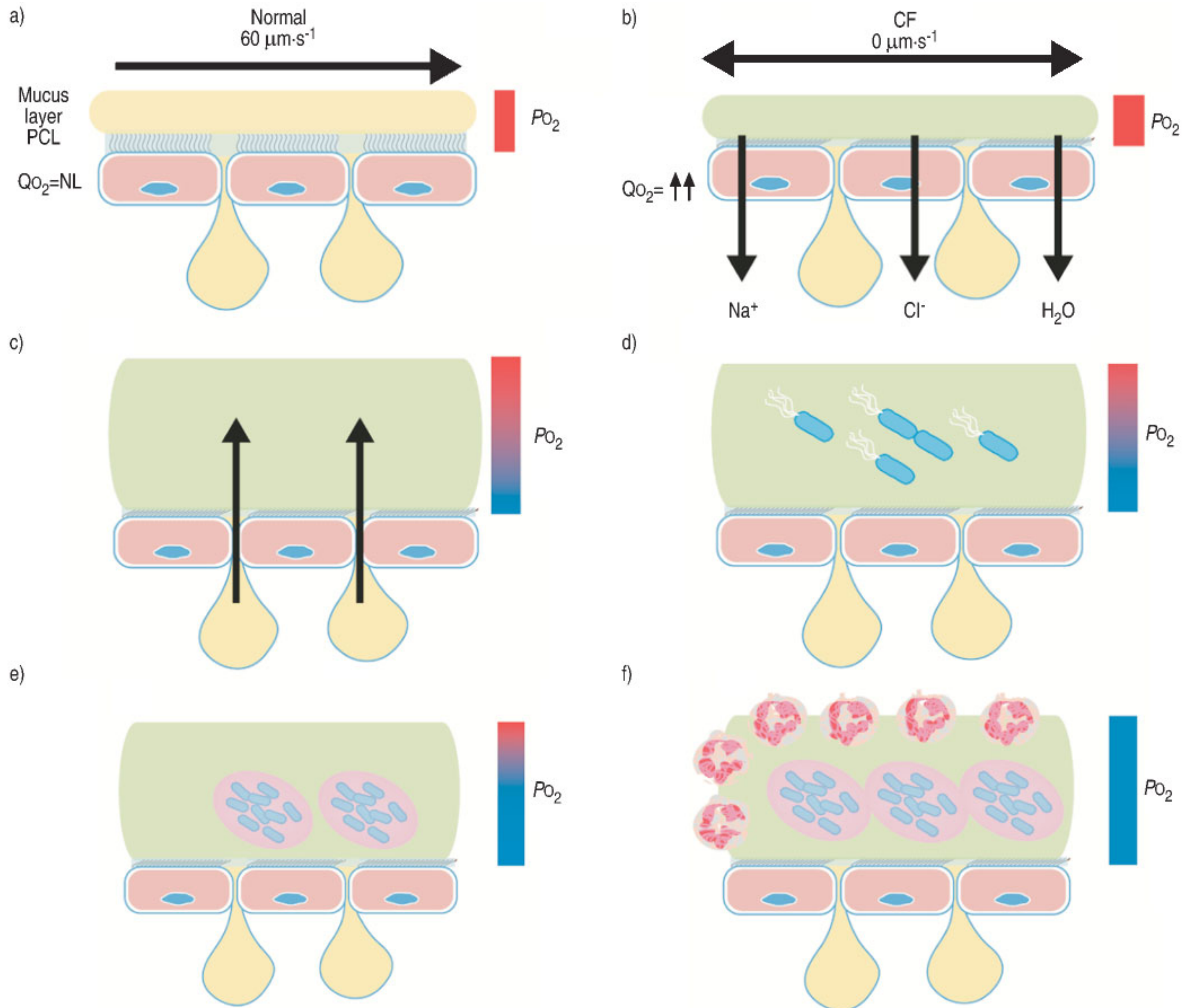

Fig. 5. – Schematic model of the pathogenic events hypothesised to lead to chronic *Pseudomonas aeruginosa* infection in airways of cystic fibrosis (CF) patients. a) On normal airway epithelia, a thin mucus layer resides atop the periciliary liquid (PCL) layer. The presence of the low-viscosity PCL layers facilitates efficient mucociliary clearance (denoted by vector). A normal rate of epithelial O_2 consumption (QO_2; left) produces no O_2 gradients (PO_2) within this thin airway surface liquid (ASL; denoted by light-stippled bar). b–f) CF airway epithelia. b) Excessive CF volume depletion (denoted by vertical arrows) removes the PCL layers, mucus adheres to epithelial surfaces, and mucus transport slows/stops (bidirectional vector). The raised O_2 consumption (left) associated with accelerated CF ion transport does not generate gradients in thin films of ASL. c) Persistent mucus hypersecretion (denoted as mucus secretory gland/goblet cell elements) with time produces luminal mucus masses/plugs. The raised CF epithelial QO_2 generates steep hypoxic gradients (light to dark stippling in bar) in thickened mucus masses. d) *P. aeruginosa* bacteria deposited on mucus surfaces penetrate by virtue of their flagellar activity into hypoxic zones within the mucus masses. e) *P. aeruginosa* adapts to hypoxic niches within mucus masses with increased alginate formation and the creation of macrocolonies. f) Macrocolonies resist secondary defences, including neutrophils, setting the stage for chronic infection. The presence of increased macrocolony density and, to a lesser extent, neutrophils, render the now mucopurulent mass hypoxic (dark stippled bar). Adapted from [48].

most importantly, how to "unglue" the mucus layer from airway surfaces. However, the functional consequence of this adhesive interaction is easily predicted, *i.e.* a greatly reduced efficiency of cough clearance [87]. Thus, it has been speculated that the critically important effects of PCL depletion in CF reflect the fact that both ciliary-dependent and cough clearance are abolished [47].

It should be noted that critical measurements of mucociliary clearance and cough clearance have not been made in CF patients, especially CF patients prior to infection. In part, this absence of data reflects the inability to expose control (normal) children to the radioisotopes typically used to make these measurements. Perhaps the most instructive studies available are those reported by REGNIS *et al.* [88] that showed reduced mucociliary clearance in CF patients with normal lung function compared with age-matched normal subjects.

Formation of mucus plaques and mucus hypoxia

Despite the failure to transport mucus from airway surfaces, it appears that mucin secretion persists from goblet cells and glands (fig. 5c). The continued secretion of mucins into an immobilised mucus layer will eventually lead to the formation of thick mucus plaques and plugs on airway surfaces. As these plaques approach heights of $\geqslant 100$ μm, there appears to be a relative depletion of O_2 in areas of these plaques near the CF cell surface [48]. The generation of mucus hypoxia reflects a combination of both the thickened plaques, providing for an increased diffusion path for O_2, and accelerated epithelial O_2 consumption that appears to be a unique feature of CF airway epithelia to fuel (*via* O_2-consuming mitochondrial production of adenosine triphosphate (ATP)) accelerated Na^+ transport.

Infection of thickened hypoxic mucus plaques

As reviewed elsewhere, when the mucus layer is being transported normally, there is turbulence within the layer that allows for efficient trapping of particulate material deposited within the mucus layer during clearance from the lung [29, 48]. This turbulence within the mucus layer appears to cease when mucus (horizontal) transport is abolished [47]. However, recent studies have suggested that motile bacteria, including environmental strains of Pseudomonas, can penetrate into thickened mucus plaques and migrate into the hypoxic mucus zones that are just above the epithelial cell layer [48] (fig. 5d). Further, these studies demonstrated that Pseudomonas, when exposed to hypoxia, will grow in a nitrate-dependent fashion (ASL nitrate=20 μM), with growth rates somewhat slower than under normoxic conditions (fig. 5e). However, this environment appears to be stressful to Pseudomonas, since an early phenotypic response to growth in hypoxic ASL is the formation of alginate. It is speculated that increased alginate formation reflects the conversion of planktonic Pseudomonas growth to biofilm growth under anaerobic conditions. Thus, mucus hypoxia may, in part, select for organisms that can adapt to this environment, *e.g. Pseudomonas aeruginosa*, and exert pressures on bacteria that promote biofilm formation.

Establishment of chronic infection

With slow proliferation of bacterial microorganisms and macrocolony/biofilm formation, the stage for persistent infection of adherent mucus is set. The growth of biofilms in thickened mucus plaques affords potential niche advantages to the bacteria (fig. 4f). It may be difficult for migratory neutrophils to penetrate into the thickened mucus plaques to engulf Pseudomonads, and the diffusion of antimicrobial activities into the thickened mucus plaques may be limited. This evasion of secondary defence mechanisms, coupled with the competitive advantages for bacteria in the biofilm form of growth, lead to the scenario that the infection of adherent mucus becomes persistent. An important prediction of these studies was that bacterial growth in densities sufficient to generate biofilms would deplete the mucus plaques of virtually all O_2, rendering the infected material on airway surfaces anaerobic. This prediction was confirmed by direct *in vivo* measurements in CF airways [48].

The likelihood that CF airway infections reflect an anaerobic mucus/mucopurulent surface infection has broad implications for the therapy of CF infectious lung disease. For example, it has been recently shown that the sensitivity of many antibiotics is very different when bacteria are grown under aerobic *versus* anaerobic conditions. Perhaps the most relevant to CF have been the data of DE KIEVIT *et al.* [89] who showed that the sensitivity of Pseudomonas to macrolides shifted one to two logs to the left under anaerobic compared with aerobic conditions. Conversely, other commonly used antibiotics are less effective under anaerobic *versus* aerobic conditions. It appears that future studies should mimic the anaerobic conditions of CF airways to identify new antibiotics for therapy of persistent lung infections and in-hospital testing for *in vitro* sensitivities should include anaerobic conditions.

Are there compensations for accelerated airway surface liquid volume absorption/periciliary liquid depletion?

It is clear that CF infants are born with relatively normal lungs [90], and that it takes many months to years for chronic infections to become a feature of the CF airways. If persistent excessive volume absorption were to generate mucus plaques from birth, CF lung disease may be expected to have a more rapid onset. In parallel, the inability to predict the severity of lung disease based on genotype suggests that there are other compensatory or modifier activities in the lung.

At present, as reviewed above, there is a search for modifier genes. With respect to potential compensatory mechanisms, it is possible that nucleotide (ATP) release secondary to persistent cough may provide sufficient ATP concentrations on airway surfaces to modify electrolyte transport and ciliary beat and thus maintain some mucus transport [91]. Alternatively, it is possible that as mucus plaques build up on airway surfaces, the increased diffusion paths for O_2 restrict O_2 availability to airway epithelial cells, *i.e.* make them hypoxic, and hence slows electrolyte transport. This scenario may be unlikely due to the large capillary circulation under the airway epithelial cells. Finally, once early infection starts, it is attractive to speculate that cytokines may modify electrolyte transport rates. For example, it is has been shown recently that inteleukin-1β slows the rate of Na^+ absorption in normal tissues, initiates Cl^- and ASL secretion, alkalinises ASL to neutralise potential acidic inflammatory products on airway surfaces, and promotes secretion of rather lesser amounts of mucin, in what appears to be an integrated response to "flush" toxins off airway surfaces [92]. It has not yet been reported whether CF cells respond similarly to cytokines, but mechanisms to modify accelerated Na^+ transport may prove to be buffers to the rapid onset of diffuse, persistent infectious CF lung disease.

Novel therapeutic approaches

Currently, there are a large numbers of new drugs being tested for efficacy in CF. Many of these efforts are focused on the anti-infective and the anti-inflammatory classes of drugs. In this review, the focus will be on drugs and strategies to treat the primary volume depletion defect in CF, and the clinical ramifications of such therapies will be explored.

Therapies directed at volume restoration

As ASL is isoosmotic/isotonic, volume depletion reflects the removal of osmotically active salt and secondarily, water from airway surfaces. The recognition that a volume deficit is important in CF pathogenesis has led to strategies to restore osmotically active agents to airway surfaces as a simple, direct approach for adding liquid to CF airway surfaces. The most studied agent of this class has been inhaled hypertonic saline. The concept is that the inhaled hypertonic/hyperosmolar salt will draw water to the airway surface, enhancing the capacity of aerosol solutions deposited on the airway surface to liquefy secretions on CF airway surfaces. Several acute studies of hypertonic saline tested with a surrogate marker of efficacy for CF, *i.e.* mucus clearance, have shown acute acceleration of mucus clearance [93, 94]. However, a feature of these *in vivo* studies has been the very short duration of action of hypertonic saline on mucus clearance. This feature has been mimicked in *in vitro* studies in which hypertonic saline was added to the surfaces of well-differentiated cultures interfaced to a confocal microscope to measure ASL height, ion composition and PD responses to such manoeuvres [32]. These *in vitro* studies revealed that the mechanism for the short duration of action reflected an upregulation of airway epithelial ion transport mechanisms to rapidly clear added NaCl (and H_2O) from airway surfaces. Thus, these studies would predict that long-term chronic therapeutic studies of hypertonic saline will have difficulty in demonstrating efficacy because of the short duration of active therapy. Preliminary long-term studies bear this prediction out [95], although most studies have small numbers and the largest, an Australian study, has yet to be completed.

An alternative approach is to deliver to airway surfaces osmolytes that are not actively transported and poorly absorbed. The nonelectrolyte mannitol has been one such agent tried previously. *In vitro* studies have demonstrated that mannitol (or, as an alternative, raffinose) added to CF airway surfaces can restore ASL volume for many hours, and by dilution of ASL Na^+ concentrations, slow Na^+ transport [94]. However, acute *in vivo* studies monitoring mucus clearance again reveal a very short duration of action (20 min) for inhaled mannitol [96]. It is not yet clear whether the short duration of action *in vivo* reflects the relative inefficiency of delivering the large mass of mannitol required to produce an effective osmotic load on airway surfaces, or other factors. Based on *in vitro* studies, other possible poorly absorbed osmolytes that may be used in the future could be comprised of K^+ ions, since they are not absorbed *via* ENaC channels, and poorly absorbed anions, *e.g.* gluconate [32]. Recent data suggest that HCO_3^- is poorly absorbed through the paracellular path in CF, and this feature, combined with the possible acidification of CF ASL, could make this anion an attractive component of an inhaled osmolyte therapy [86].

An alternative approach is to rebalance the abnormal ion transport properties of CF airway epithelia. Compounds that appear to possess actions that inhibit excessive Na^+ transport and trigger Cl^- secretion are the triphosphate nucleotide molecules (*e.g.* ATP or uridine triphosphate). Triphosphate nucleotides interact with apical membrane $P2Y_2$ nucleotide receptors that are coupled to activation of phospholipase C-β. A variety of studies in cultured cells, and, most compellingly, freshly excised human airway epithelia have demonstrated that luminally applied UTP both inhibits ENaC-mediated Na^+ absorption and triggers Ca^{2+}-activated Cl^- secretion in CF as well as normal airway epithelia [97–102]. Further, studies of ASL volume responses to UTP with confocal microscopy have revealed that the net effect of inhibition of Na^+ transport and activation of Cl^- transport is that volume is secreted onto the surface of CF airway epithelia and, as predicted from previous electrophysiological studies, the volume secretory response to UTP is greater in CF than normal cultures [32]. Finally, acute administration of UTP will restore the PCL and rotational mucus transport in well-differentiated cultures of CF airway epithelia [32].

These data have set the stage for development of purinoceptor agonists for CF therapy. Early candidates, *e.g.* UTP, were shown to be poor drug candidates due to rapid hydrolysis (~45 s half-life) on airway surfaces [32]. These observations led to the search for stabilised nucleotide analogues that were active at the luminal $P2Y_2$ receptor. INS37217 is a candidate nucleotide analogue that is both active at $P2Y_2$-R and resistant to hydrolysis by airway cell surface nucleotidases and hydrolysis by nucleotidases contained in mucus of CF patients [103]. Initial Phase I safety studies of INS37217 in CF adults have been completed and INS37217 was found to be safe. currently, INS37217′s efficacy in improving lung function and increasing muscle clearance, as assessed by CT scanning, is being tested in phase-II studies through the Cystic Fibrosis Foundation Therapeutic Development Network.

A complementary approach is to directly inhibit the ENaC that mediates volume hyperabsorption. This concept originated from studies performed many years ago that demonstrated that the raised nasal PD in CF airway epithelia was inhibited by topically applied amiloride [53]. Subsequent studies employing surrogate markers, *e.g.* mucus clearance, showed that aerosolised amiloride was effective acutely in CF patients [104]. Further, inhaled amiloride appeared to preserve forced expiratory volume in one second, in a small, long-term (6-month) crossover study in which most other therapies for CF were eliminated [105]. However, studies that have evaluated amiloride in the context of usual therapies failed to detect clinical benefit [106].

Studies of the pharmacodynamic properties of amiloride revealed that the half-life of amiloride on airway surfaces was ~20–30 min, suggesting its duration of action was insufficient to treat CF lung disease chronically even when administered four times per day [32, 107]. Since amiloride is of relatively low potency and insoluble in solution, its duration of action could not be extended simply by increasing the inhaled dose. Recent studies in patients with congenital loss of function of airway ENaC, *i.e.* pseudohypoaldosteronism (PHA), showed that these patients had increased volumes of liquid on airway surfaces and compensated for this defect in Na^+-dependent liquid absorption by greatly accelerating mucus (ASL) clearance [108]. These observations suggest that high potency, long-acting Na^+ channel blockers, mimicking the completeness of PHA ENaC block, may have sufficient activity to restore the ASL volume deficit and restore mucus clearance in CF.

Clinical lessons from the use of volume-restoring agents

From studies of hypertonic saline and first- and second-generation purinoceptor agonists, it appears that several themes are emerging with respect to clinical use of agents that

add volume to airway surfaces. First, as shown in figure 6a, adding volume to dehydrated mucus plugs is predicted to make them "swell". As these plugs move from distal smaller airways to more proximal airways, the increased size of the plug may lead to transient obstruction of larger airways, with periods of transient volume/perfusion mismatch and hypoxaemia. It appears from studies of both purinoceptor agonists and hypertonic saline that this phenomenon does occur, but is transient, *i.e.* $\leqslant$30 min [109, 110].

Secondly, mucus that is mobilised has to be coughed from the lung to be cleared. Thus, use of hypertonic saline and/or pharmacological agents of the volume-restoring class may be associated with increases in cough post-therapy in keeping with their expectorant action. Presumably, therefore, a "productive" cough following inhalation of drug or osmolytes is an index of efficacy rather than an adverse event.

Thirdly, the CF lung in young adults may contain up to 150 mL of thickened, concentrated mucopurulent material (fig. 6b). For example, if the percentage solids ("concentration") of normal mucus is 1.5%, and CF mucus 15%, then to "thin" CF mucus to a normal level so that it can be cleared requires that ~1,350 mL of liquid be added to airway surfaces. Since the volume of the conducting airways in a young adult is ~300 mL, if this thinning process were performed acutely, the patient would, in effect "drown". Thus, because it has taken CF patients many months or years to accumulate this volume of thickened mucus, it would appear sensible to take a "low-dose medication/go slow" approach to removing these inspissated materials. This latter admonition may have effects on clinical trials, *i.e.* the ability to capture the efficacy

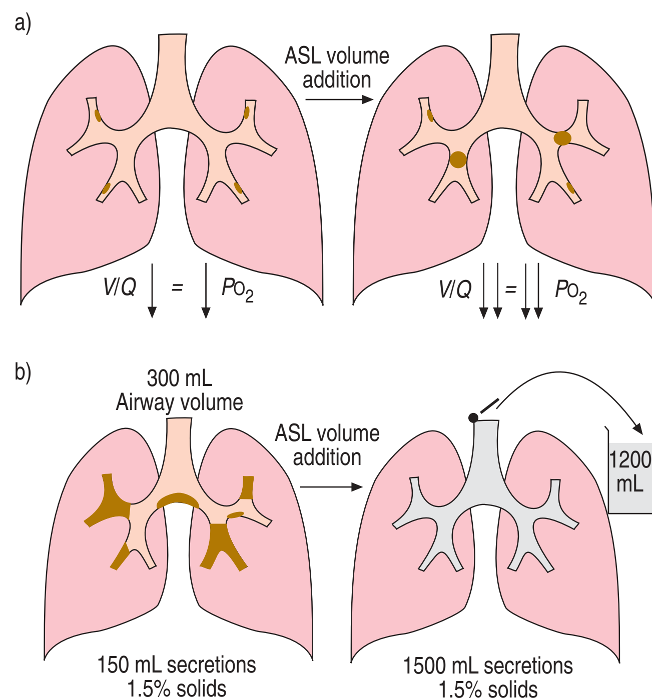

Fig. 6. – Potential clinically relevant outcomes of "volume addition" therapies. a) Scenario by which dehydrated mucus plugs could cause transient worsening of hypoxia as they are moved from distal to proximal airway after rehydration and expansion. b) Scenario depicting "volume debt" burden of cystic fibrosis airways with markedly dehydrated (15% solids) mucopurulent material occupying ~50% of the airway luminal volume. Rapid normalisation of mucus hydration (~1.5% solids) would lead to filling of airway luminal volume (drowning), plus a large volume (1200 mL) of expectorated material. ASL: airway surface liquid; *V*/*Q*: volume/perfusion; P_{O_2}: oxygen tension.

of these compounds *versus* potential adverse events due to cough and transient hypoxaemia, would appear better in trials of low doses of compounds for prolonged periods.

Finally, it is not clear that initiation of volume-restoring therapies by aerosol after CF lung disease is established, with poor airflow, and hence, limited delivery to mucus-obstructed regions will effectively "chip away" obstructing plugs. Several solutions to this problem are apparent. The simplest is to start therapy early in the life of CF patients before obstruction occurs. For patients with substantial obstruction, delivery of drugs parenterally is rational, but no volume-restoring drugs are available for use by this route. A final thought, borrowed from cancer trials, is that perhaps therapy with volume-restoring agents should include both "induction" and "maintenance" phases. Thus, it may be reasonable to consider "debulking" CF patients of retained mucus with intensive inhalational therapy with multiple complementary agents, *e.g.*, volume restoring agents, mucolytics, and deoxyribonuclease, and vigorous physical therapy. This phase would be followed by maintenance therapy with inhaled ion transport modulators.

Conclusion

The processes that initiate and perpetuate CF lung disease have perhaps become more clear. If volume depletion on CF airway surfaces, particularly the PCL, is the initiating lesion, then all efforts should be made to redress this defect. In health, the PCL may equal ~3.5 mL, suggesting that restoring this volume early in life should not be difficult. However, the problem is to achieve this result chronically, *i.e.* 24 h·day^{-1}. The half-life of small molecular weight osmolytes (~500) and hydrophilic drugs may be only ~1.5 h on airway surfaces, likely a reflection of the relatively permeable paracellular path that characterises airway surfaces. Thus, for osmolytes to be effective, they may have to be given continually. For drugs to be effective, they will have to be given safely in concentrations far in excess of their half-maximal activity level and/or have extended pharmacodynamic effects.

For treatment of the infectious components of mucus stasis, the ramifications of cystic fibrosis airway lumen anaerobiasis must be explored. These studies should focus on novel antimicrobial targets based on processes rate-limiting for bacterial adaptation to anaerobic environments and evaluate the utility of routine hospital testing of cystic fibrosis isolates for antimicrobial sensitivities under anaerobic conditions. Despite these technical obstacles, the path to novel and specific therapy for cystic fibrosis lung disease seems clear. The goal of the cystic fibrosis community is to move down this path with a broad variety of approaches as rapidly and safely as possible.

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