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"Molecular mechanism of mucociliary transport: relevance to cystic fibrosis."

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Summary of Thesis

Summary

Cystic Fibrosis (CF) is an autosomal recessive disorder in which the genetic defect is the ΔF 508 deletion in 70% of patients (Rommens, 1989) . The ΔF 508 deletion gene predicts encodement of the cystic fibrosis transmembrane conductance regulator(CFTR), (Riordan, 1989). The mechanisms by which this abnormal regulatory protein leads to chronic airway infection, respiratory failure and death in CF are not clear.

This thesis examines one basic problem seen in patients with CF, namely the increased viscosity of their sputum. The increased viscosity leads to decreased mucociliary clearance and may be one of the factors leading to delayed bacterial clearance, chronic respiratory infection and respiratory failure.

The problem of increased mucus viscosity is examined by looking at two molecules which contribute to the increased viscosity of sputum: mucins and DNA. The fundamental physicochemistry of mucin rheology is explored, and the theoretical model of mucin expansion is developed further in an experimental intervention in patients with CF using an experimental new drug, aerosolized recombinant human DNase which fragments DNA and decreases sputum viscosity.

The models used to examine the questions of mucus rheology were the giant secretory granule of the slug, *Ariolimax columbianus*, and the goblet cell of the trachea of the New Zealand white rabbit. Normal subjects and patients with CF were used for the human studies.

Mucus is a polymer hydrogel that functions as a protective coat on the skin and mucosa of many species ranging from simple animals such as *coelenterates* to mammals. The polymer matrix of mucus is made out of long-chain glycoproteins called mucins that are tangled together to form a randomly woven, polyionic network. The swelling kinetics of both slug granules and the mucin granules of the goblet cell of the rabbit are similar to

those of artificial polyacrylamide gels. Mucins are condensed within granules and expand by hydration during or after exocytosis. The polyionic charges of mucins would prevent condensation unless they were shielded by a balancing cation. The experiments using the slug granule demonstrate the presence of shielding cations in the granule and the role of these cations at the time of secretion.

The rabbit studies show that mucin swelling is governed by a Donnan equilibrium and that the physico-chemical predictions of artificial gels also hold in naturally occurring gels such as mucus. The experiments reported here were designed to evaluate the effect of cations and the polyion, albumin on the swelling kinetics of mucin granules exocytosed from rabbit respiratory goblet cells in culture. The kinetics of swelling were monitored by video-microscopy. The diffusivity of newly released mucins, in the presence of different concentrations of cations, was evaluated using the expression: $D = r_f^2/\tau$ where τ is the characteristic time of the swelling, and r_f is the final equilibrium radius of the swollen granule.

The results indicated that diffusivity is decreased in the presence of cations and that serum albumin at concentrations of 10^{-7} M, which are equivalent to those found in the bronchial mucus of asthmatic or cystic fibrosis patients, can produce up to a 90% decrease in the diffusivity of newly released mucins.

Since swelling is a critical determinant of mucus rheology, the concentration of cations and plasma proteins on the mucosa could play an important role in the regulation of the rheological properties of mucus.

The justification of the use of the rabbit as a model for mucus exocytosis and the unequivocal identification of the 3 different epithelial cells found in the rabbit trachea using monoclonal antibodies is outlined. A complimentary epithelial cell preparative technique and the application of flow cytometry to enrich the identification of the airway epithelial cell sub-populations are described.

The basic defect of Cystic Fibrosis may be a defect in ion transport caused by the CFTR. We hypothesize that this defective ion transport on the mucosal surface alters the ion concentration in the periciliary fluid and prevents mucins from hydrating normally. The unhydrated mucin slows mucociliary transport and may be the initiating step to decreased bacterial clearance and bacterial infection. Once bacterial colonization has occurred leukocytes are attracted to the airway leading to purulent secretions. In this advanced diseased state, two molecules may then contribute to the increased viscosity of lung secretions in CF patients - mucins, and the leukocyte DNA. Like mucin, DNA is an extremely long, charged polymer. The charges of DNA are normally shielded by histone and by the cationic protein, spermine. The DNA content of the sputum of a CF patient can be as high as 25mg/g.

The viscosity of a patient's sputum could, theoretically be decreased by hydration, by addition of the necessary cationic charges, or by breaking DNA into smaller fragments thereby decreasing the intrinsic viscosity of the DNA polymer at the molecular level. It was with this last concept that we undertook the clinical trials of a new product of human recombinant DNase, which normally scavenges and breaks free DNA of dead cells *in vivo*.

The human studies reported here a phase 1 study of recombinant human deooxyribonuclease (rhDNase). Recombinant human DNase was aerosolized and inhaled by normal subjects and by adults CF patients. The objectives of these studies were to determine the safety of short term aerosol administration of rhDNase in adults with CF; to determine the systemic absorption of rhDNase in adults with CF following aerosol administration; and to obtain a preliminary indication of the pharmacologic effects of aerosolized rhDNase on sputum in adults with CF. There was little systemic absorption of the drug and no antibodies to rhDNase were detected. There was improvement in the spirometric values of the CF patients and the patients felt symptomatic better with improvement of their dyspnea score. Double blinded controlled studies are required to determine whether this improvement is due to rhDNase or a placebo effect.

Introduction

Introduction: Chapter 1

Genetic abnormality and clinical presentation of Cystic Fibrosis

Cystic Fibrosis is the most common lethal genetic disorder in the Caucasian population. It is an autosomal recessive disorder in which the genetic defect is located on the long arm of the 7th chromosome. In approximately 70% of Cystic Fibrosis patients, the defect is the DF508 deletion (Rommens et al, 1989). It is estimated that at least 60 other alleles account for the other 30% of the affected chromosomes. The ΔF508 deletion leads to the production of a defective protein, the cystic fibrosis transmembrane conductance regulator (CFTR) which, because of its theoretical conformation is believed to be important in the regulation of the transport of ions into the cell (Riordan 1989). Elegant patch clamping studies have demonstrated that in Cystic Fibrosis patients, chloride channels are present in the cell membranes, but it is their regulation that is defective (Schoumacher,Frizzell, 1987; Welsh,1989). The chloride channel will open when a voltage is applied across it, but not with normal physiological stimuli such as ATP (Frizzell,1987; Welch, 1989).

The clinical manifestations of Cystic Fibrosis appear to be primarily expressed in the epithelial cells, although the defective channels have been located in other cell types (Chen,1989). The Cystic Fibrosis patients symptoms involve the sinuses, the pulmonary epithelium, the gastrointestinal tract, the pancreas, the biliary tree, the sweat glands, the male and female reproductive tracts (Taussig, 1984). Cystic Fibrosis patients die at an average age of 27 years usually of chronic respiratory infections leading to progressive pulmonary failure (Corey, 1988).

This thesis examines some of the cellular defects that may be caused by the defective ion channels which consequently may lead to defective mucociliary transport seen in these patients.

Introduction : Chapter 2

Abnormalities of mucociliary transport in cystic fibrosis

Mucociliary transport is slowed in CF (Taussig,1984). Mucociliary transport is normally an efficient method of protecting the airways and the gas exchanging alveoli from environmental hazard of pollutants, dust, and bacteria. An abnormality in mucociliary transport may lead to retained bacteria causing the pathological airway inflammatory reaction seen in CF (Fick,1989).

As initially described by Lucas and Douglas (1934), mucociliary transport works like a conveyer belt where the cilia provide the driving force and the mucus functions as a fluidic belt (Figure 1). The cilia normally beat in a co-ordinated and synchronized fashion at about 15 Hertz toward the main carina (Pfaltz,1990). The cilia beat in the periciliary fluid, and the mucus and debris ride atop this periciliary layer. Once the mucus with the debris reaches the larger airways, it can be swallowed or expectorated.

Mucociliary transport in CF has been examined by a variety of methods (Pfaltz,1990) including radiolabelled albumin, radiolabelled erythrocytes and fibre-optically visualized teflon discs.

These studies all confirm that mucociliary transport is slowed in CF. The question is whether this is caused by a defect of the cilia or a defect of the mucus and/or periciliary fluid. This thesis addresses some of these questions.

We hypothesize that the basic ion channel defect may lead to abnormally thickened mucus. In addition to the thick unhydrated mucus, there may also be increased bacterial adhesion specifically to the CF mucus or to the CF epithelial cells (Franklin, 1987). By these, and possibly other mechanisms, the airways become bacterially colonized. The inflammatory reactions to infection with the influx of leukocytes become a confounding

factor in the increased viscosity of the secretions (Sruggs,1964), and ensuing lung fibrosis.

Patients in the later stages of their disease become infected with *Pseudomonas aeruginosa*. The gelatinous exopolysaccharide produced by the CF mucoid variants and the pili of the organism appear to be the adhesins to tracheal cells (Fick, 1989). Proteases are produced by neutrophils, pulmonary macrophages and *Pseudomonas*. These proteases, in addition to elastases produced by *Pseudomonas*, lead to proteolytic damage in the airway. The proteolytic enzymes cut immunoglobulins, specifically IgA and IgG within the airway, which compounds the problem of clearing airway infection.

The products of the prostaglandin cascade are produced in the presence of infection, and leukotrines and prostaglandins have been shown to increase mucus release. Moreover, supernatants of cultured *Pseudomonas* contain pyocyanin and 1-hydroxyphenazine (Wilson, 1987) which behave as ciliostatic factors, leading again to slowing of cilia and slowing of mucociliary transport. As will be outlined further in Chapter 7, the presence of DNA from the dead neutrophils will also add to the viscosity of the sputum and again decrease mucociliary transport time.

Thus, once the initiating factors of decreased mucociliary transport begin, a series of pathological sequences outlined above compound the primary problem and lead to increased mucus production, decreased cilia beat frequency (Hingley, 1986), interference of phagocytosis by breakage of immunoglobulins, and pulmonary fibrosis.

Introduction : Chapter 3

Identification of the different airway epithelial cell types.

In order to perform our *in vitro* mucin experiments we wished to have unequivocal identification of the different airway epithelial cell types.

The airway epithelium consists of several cell types each distinguishable *in vivo* by their distinct morphological appearance (Plopper, 1983). *In vivo* studies suggest that the stem cell of the airway epithelium appears to be dependent on the level within the airway and to be species dependent. In airway diseases such as bronchitis and asthma or cystic fibrosis, one cell type ie the secretory cell is more plentiful than in normal subjects (Reid,1960). Over the last decade, work has been performed that elucidated the morphological and biochemical characteristics of the many types of epithelial cells. The progress in this area has been achieved by applying the techniques of epithelial cell isolation and culture (Wu 1982; Whitcutt 1988), autoradiographic analysis of dividing cells *in vivo* (Donnelly,1982), monoclonal antibody production and immunocytochemistry (Basbaum1984,1986,1987; Singh1984,1985; St George 1984,1985).

Autoradiographic studies have examined the pattern of epithelial cell renewal, the timetable and the sequence of cell differentiation (Donnelly,1982; Plopper,1986). Data from several of these studies suggest that the basal cell is the epithelial stem cell. Donnelly proposed that both superficial goblet and ciliated cell differentiation is preceded by 2 cell divisions: a basal cell division followed by an intermediate cell division. Evans et al (1978) suggested that in the lower airways (the intrapulmonary airways and the bronchioli) of the rat, it is the Clara cell rather than the basal cell which is the stem cell or progenitor. Snider et al concluded that in the adult hamster, both the secretory and the basal cell contribute to cell renewal, but with the highest proliferative intensity in the basal cells. On the contrary,

studies of the fetal hamster trachea and rhesus monkey show that the basal cell produces basal, but not secretory or ciliated cells. Evans and Plopper suggested that the basal cell may have an additional role namely that of an adhesion of the columnar epithelium to the basement membrane(1988).

Rabbit cell suspensions containing 90% pure basal cells and inoculated into denuded tracheal grafts and transplanted to the backs of nude mice showed re-epithelialization of all cell types (Inayama, 1988,1989). When Clara cells were isolated and inoculated into the denuded tracheas, a low cuboidal epithelium was produced (Hook,1987).

It would appear that the differentiation patterns may be dependent on species, age of the animal, and environmental conditions. Resolution of these conflicting data require that epithelial cells be specifically identified and that the fates of their progeny be followed. Recently, 3 techniques have become available which permit analysis and identification of the various cell types beyond an *in vivo* morphological basis, namely: epithelial cell culture, monoclonal antibody production, and fractionation of the different cell types.

Although methods for epithelial cell growth and differentiation *in vitro* have been available for over a decade several improvements have been made recently. Wu described an improved method for culturing enzymatically dispersed tracheal epithelial cells (1982). The ability of the epithelial cells to differentiate both morphologically and biochemically *in vitro* depends on the growth factors and the hormones present (insulin, EGF, transferrin, hydrocortisone, cholera toxin, bovine hypothalamus extract, vitamin A). More recently Whittcutt, Alder and Wu described a bi-phasic chamber which maintained the polarity of differentiation in the guinea pig (1988).

Several workers have demonstrated the importance of vitamin A in differentiation of the airway epithelium (Jetten,1985, 1986,1987,1989; Wu,1982). Vitamin A deficiency changes the mucociliary epithelium into layers of squamous and stratified cells. *In vitro*, retinoids have been shown to exert regulatory effects on cellular proliferation and

differentiation in normal, preneoplastic and neoplastic cells. It is believed that the mechanism of retinoic acid is that it down regulates mRNA which encodes for transglutaminase type 1 (Jetten,1989). Type beta-transforming growth factor (Jetten,1986, Masui,1986)and platelet factors (Lecher,1983) have been shown to be responsible for inducing human epithelial cells to undergo squamous differentiation.

Other investigators have approached the question of secretory cell differentiation by looking at the ability of the cells to selectively bind the lectin Helix pomatia agglutinin (Wasano,1988) or by examining the biochemical characteristics of the glycoproteins secreted from the cultures (Kim,1985,1989).

Secondly, several investigators have produced monoclonal antibodies specific to airway epithelium and to specific cell types. Antibodies available include those to goblet cell granules, gland cell granules and the apical surface of the epithelium (Basbaum, 1984,1986,1987), Clara cell secretory proteins (Singh 1984,1985), submucous gland cells (Groele 1987) and transformed rat epithelial cells (Braslawsky,1984). Plopper and his group have prepared monoclonal antibodies both to the rabbit and the monkey epithelium (St George 1984,1985).

Thirdly, improved methods for fractionating cell populations have been developed. Sub-populations have been identified by a combination of centrifugation and elutriation procedures (Devereux,1981). Epithelial cell separation is a complex problem because of the heterogeneous cell population and because of cell death following isolation procedures. Devereux and Fouts (1981) examined 3 methods of separation namely centrifugal elutriation, metrizamide density gradients, and partition of cells in two-polymer aqueous phase. Nettesheim and his group are able to purify both a population of Clara cells and of basal cells by a combination of centrifugation and elutriation procedures (Hook,1987;Inayama,1988,1989).

The major problem in the production of an antibody to specific cell types other than the secretory cell has been the predominance of the mucus glycoprotein antigen dominating

the production of antibodies to other cells types.

In this thesis an antibody specific to the ciliated cell and its corresponding epitope are described. The preparation of an antibody to the ciliated cell was possible because of the new preparative methods used in the enzymatic micro-dissection of the airway epithelium which eliminates most of the mucus glycoprotein. This antibody allows unequivocal identification of the ciliated cell. In collaboration with Dr Carol Basbaum, we were also able to identify the goblet cell. We were able to identify goblet and ciliated cells in culture using these goblet and ciliated cell antibodies. The antibodies allowed identification of goblet and ciliated cells in culture without relying on phenotypic characteristics of each cell.

Introduction : Chapter 4

Ciliary structure and function in cystic fibrosis

Cilia are found throughout the animal world from paramecium to complex mammals. Cilia are found in several areas in human epithelium: the airways, the ear, and the reproductive system. Ciliary movements in man was first observed by Johannes Ham in 1677. However, it was not until the 1830's that the function of cilia within the airways was postulated (Pfaltz, 1990).

The ciliated cell of the human airway is one of the morphologically different cells which is part of the pseudostratified epithelium of the airway (Plopper,1983). The airway appears pseudostratified because all cells are attached to the basement membrane but not all the cells reach the lumenal surface. The pseudostratified epithelium of the tracheobronchial tree has several morphologically different cell types including secretory, non-secretory and endocrine. At least 10 cell types have been found in the tracheobronchial epithelium among several species (Wanner,1977). The cell types are categorized by their position within the epithelium, by the presence or absence of cilia and by the presence or absence of secretory granules. In most mammalian species the epithelium of the upper airway consists of 3 cell types namely the ciliated cell, the secretory cell and the basal cell (Plopper,1983). These 3 cell types are fundamentally different, and distinguishable from one another by their morphological appearance. Each is attached to the basement membrane. However, while the goblet and the ciliated cells extend to the lumenal surface, the basal cell does not extend, giving the epithelium of the trachea its pseudostratified appearance. These different cell types normally work together as the efficient mucociliary transport system described above, protecting the airways from environmental damage.

The ciliated cell of the airway is 8μm in height and 0.1-0.3μm in diameter. The

apical surface has 50-300 cilia. The ciliary shaft contains 9 peripheral double tubules and two central single tubules (Figure 4). The peripheral double tubules are arranged regularly and consist of two microtubules. The double tubules are connected to each other by nexin links in such a way that they are motile. An inner and outer dynein arm connect the microtubule doublet and the adjacent pair of microtubules. The two central tubules are surrounded by their own sheath. Nine protein spokes extend radially from the peripheral tubules to the central sheath. The radial spokes are important for the stabilization of cilia during movement. The main direction of beat of the cilia is characterized by the position of the two central tubules: it lies perpendicular to the connection between the two tubules. The ciliary tubule has multiple connections to the overlying membrane along the entire shaft and especially at the tip of the cilia. Small thorn-like structures are occasionally found on the cell membrane of the tip of the cilia. These possibly act like small claws to improve transit of the overlying mucus layer. The root of the cilia lies within the cell as a basal body. Ciliary movement occurs as the double tubules move relative to each other with the help of the formation of alternating attachments of the dynein arms. The cilia are upright and rigid during the effective beat and curved during the recovery phase (figure 5). Cilia produce a whip-like, relatively lateral movement. Adjacent cilia beat in time, but their movements are staggered and thus cause a continuous wave, termed metachrony. Cilia normally beat at 5-20 Hz. The frequency of cilia beat depends on temperature, and viscosity of the medium in which the cilia beats (figure 6). Transmission electron microscopy shows the ciliated cell to be be rich in mitochondria producing ATP (figure 2), the energy for ciliary movement .

Ciliary activity can be measured in a variety of ways *in vitro* as mentioned previously; reflected light by photodiodes (Villalon, 1990), stroboscopy, laser beam and high speed photography. *In vivo* techniques include marking techniques, the saccharin test, isotope and radiological methods and fibre-optic inspection of teflon discs .

The morphological appearance of the airways of the CF patient appears to be normal at birth. Transmission electron microscopy appearance of the cilia are normal. The

relative quantity of ciliated cells in the terminal patients with CF are less in number, just as is found in other inflammatory diseases of the airways such as chronic bronchitis and other causes of bronchiectasis. There is a relative abundance of secretory cells and squamous metaplasia (Reid,1967). The relative paucity of ciliated cells will contribute to slowing of mucociliary transport in the later stages of disease.

Preliminary studies of the cilia in CF suggest that they appear to function normally (Villalon, 1990). The ciliary beat frequency is the same as for normal humans, and the cilia beat frequency increases appropriately in the presence of ATP. Thus the evidence to date suggests that the ciliated cell in CF appears to function normally. The ciliated cells are less in number and whether the cells function in the normal physiological manner under certain pathological conditions such as increased viscous load is yet to be determined.

Thus, in CF the basic defect may lie with the ionic concentration of the periciliary fluid leading to thick unhydrated mucus (see chapter 5) rather than with the ciliated cell. However, the ciliated cell appears to demonstrate increased bacterial adhesion *in vitro* and may contribute to the airway infection.

Introduction : Chapter 5

Mucus abnormalities in cystic fibrosis.

The sputum of CF patients is copious, purulent and viscous. This thesis proposes that the cause of these purulent secretions are caused by 2 mechanisms which both follow a similar physico-chemical theory.

The initiating defect of the thick mucus may be related to the ionic content of the mucus and the ion concentration of the periciliary fluid rather than some structural defect of the mucus glycoprotein chains themselves. As will be outlined below, this ionic change leads to an increase in mucus viscosity. The increased viscosity slows mucociliary transport and may be the main factor, or one of the factors which lead to bacterial colonization. The second mechanism of increased viscosity in CF is caused by the highly viscous DNA of the dead leukocytes (Sruggs,1964).

Bronchial mucus is a heterogeneous mixture of secretions from a variety of cells including alveolar type II cells, clara cells, plasma cells and mucus and serous gland cells. The mucus gel consists of long glycoprotein chains which form a tangled network. The glycoprotein chains of the network are linked by low energy bonds rather than being covalently cross-linked disulphide bonds as was previously thought (Tam,1981). This theory was shown not to be the case because mucus is capable of hydrating and annealling (Tam,1981). The physical properties of a polymer gel depend on the elasticity of the polymer chains that make the network The density of polymer per unit volume of gel, the amount and nature of crosslinking between polymer chains, and the viscosity of the liquid in which the polymer is dissolved give the gel its physico-chemical properties.

The mucus gel is made of a high molecular weight glycoprotein. It has a protein backbone which is of variable length with short chain sugar moieties rich in sialic

terminals. The result is a highly negatively charged polyionic chain. The liquid or solvent of the mucus is water which also contains electrolytes and soluble proteins (Verdugo,1984,1990).

Analyzing the abnormalities in the secretions of patients is not a trivial problem. Expectorated sputum is contaminated by the upper airway and saliva and is not ideal for examination. The mucus of Cystic Fibrosis patients is infected with bacteria, and the changes seen in the mucus may reflect the infection rather than the basic Cystic Fibrosis defect. Furthermore, mucus is non-Newtonian and measurements which use mechanical force can be inaccurate and not reproducible (Verdugo,1984). Also, mucus dehydrates on contact with air, and thus *in vitro* measurements of mucus rheology and ion content can again be inaccurate. Despite these formidable barriers there has been significant research in the sputum of patients with Cystic Fibrosis. The expectorated sputum of patients with Cystic Fibrosis has increased concentrations of cations (Matthews 1963, Potter, 1967) and soluble proteins (Matthews,1963; Rose,1987). The sputum is rich in DNA, presumably from dead neutrophils (Smith,1988), and the spinnability is increased (Plotkowski,1989). Recent studies using electron probe microanalysis and the secretions from CF cells in culture have also suggested that the mucins are more sulfated (Roomans,1986; Cheng,1989).

In summary, the mucus of CF patients is more viscous because the mucin itself is more sulfated, the mucins may remain unhydrated, and the DNA from the dead neutrophils greatly contributes to the sputum viscosity.

Introduction : Chapter 6

Donnan mechanism of mucin swelling

Mucus is commonly found throughout the animal kingdom. It functions as a protective coat on the skin and on the mucosa of various organs in many species. Although secretory cells synthesize, store, and release the basic polymeric species that make up the mucus network, the final step in mucogenesis is the swelling and annealing of secretory products from various secretory cells to form a confluent hydrogel (Verdugo,1984). Mucus is a polymer gel composed of water and a polymer matrix. The polymer matrix consists of large linear polymers - mucins, which are coiled randomly to form a three dimensional network (Verdugo,1990). Water acts as a solvent, not only for the mucins but for the other soluble species found within the gel. Recently, Tanaka described a new theory of kinetics of the swelling or hydration of a gel (1979). He demonstrated, in artificial acrylamide polymer gels, the time of swelling of a gel is proportional to the diffusion coefficient of the gel network rather than to the diffusion coefficient of the solvent molecules (Tanaka,1981). The rheological properties of mucus are determined by the number of these glycoprotein tangles per unit volume of mucus (Tanaka,1981).

The mechanical properties of gels having a tangled polymer network depend upon tangle density. The rheological properties of mucus are for the large part determined by the water content of the mucin polymer network (Tam,1981). The rheological properties of tangled polymer gels like mucus are dependent upon tangle density (Lee, Verdugo, & Blandau, 1977; Verdugo, Tam, & Butler, 1983). As tangle density depends upon dilution, the mechanism of postexocytotic swelling of secretory products is particularly important to understand how the mucosa controls the rheological properties of the mucus gel (Wolf, Sokoloski, Khan, & Litt, 1977; Verdugo, 1984; Verdugo, Aitken, Langley, & Villalon,

1987).

The swelling of mucins is governed by a Donnan equilibrium (Donnan, 1924; Tam & Verdugo, 1981). Mucins are condensed inside secretory granules and undergo massive swelling upon release from the cell (Verdugo 1984). Due to the highly polyionic nature of the mucin chains, mucus swelling is governed by a Donnan Equilibrium (Donnan,1924). The mucin network behaves like a semi-permeable membrane because the twists and curves of the large molecular chains prevent the movement of polyions, generating a Donnan Equilibrium (Figure 8).

Thus the concentrations of ions and polyions in the liquid phase that wet the surface of the mucosa modulate the rate of swelling of the newly released mucins and thus the rheological properties of the mucus gel.

The presence of cations in the gel will balance the negatively changed mucin chains and thus allow condensation of the mucin network. Condensation of the network would lead to an increase in the viscosity of the mucin gel.

Soluble proteins, which are normally found on the surface of the mucosa, should act as large polyions that remain excluded from the newly released mucin polymer network and inhibit the postexocytotic swelling of mucin granules. The experiments reported here were designed to evaluate the effect of serum albumin on the swelling of newly released mucins from goblet cells grown in primary cultures of respiratory mucosa.

We examined the effect of the addition of increased concentrations of extracellular cations and proteins on newly exocytosed mucin granules. We hypothesized that when soluble proteins are present, they are unable to penetrate the glycoprotein network. The proteins, outside the gel, cause an osmotic effect. If these proteins are then charged, a Donnan potential is established outside the gel. Mucus hydration would be determined by a balance of these two Donnan potentials; the one inside and the one outside the glycoprotein network (See Figure 8 for explanation). We theorized that the presence of charged soluble proteins should limit the hydration of mucus.

The experiments outlined used newly exocytosed mucin granules from goblet cells *in vitro* to test this hypothesis.

Introduction : Chapter 7

Effect of leukocyte DNA on sputum viscosity

Improvements in the treatment of CF have resulted in a marked increase in life expectancy over the last few decades from less than 2 years to an average of 27 years (Corey 1988). However, the disease remains almost uniformly fatal. Lung infection is the major cause of morbidity and mortality in CF. The progressive deterioration in lung function in cystic fibrosis is primarily a result of persistent infection of the airways. Currently available mucolytic therapy for lung secretions are ineffective in reducing the viscosity of infected lung secretions in these patients. An agent which reduced the viscosity of infected secretions in the lungs should enable the patients to clear their secretions more easily. The high bacterial load found in these patients may be reduced, thereby reducing the chronic inflammatory reaction in the airways and slow the disease progression.

Two molecules contribute to the increased viscosity of lung secretions in CF patients; mucins, as previously discussed and DNA. Like mucin, DNA is an extremely long, charged polymer. As mentioned previously, its charges are normally shielded by histone and the cationic protein, spermine (Marquet,1985). The concentration of DNA is high in infected sputum because of the dead leukocytes which have been attracted to the area of inflammation (Smith, 1988). The DNA content of the sputum of a cystic fibrosis patient in an acute exacerbation of their disease can be as high as 25mg/g. It is believed that this is dead neutrophils rather than bacterial DNA because the ratio of A and T to G and C resembles that in the human genome and not that in the bacterial genome.

The viscosity of sputum could, theoretically be decreased by hydration (a concept which reminds the physician of the out moded mist tents), addition of the necessary cationic charges (by manipulating the ionic content of the sputum with such therapies as amiloride) (Knowles,1989), or by breaking the DNA into smaller fragments thereby

decreasing their intrinsic viscosity at the molecular level (Shak,1990). It was this last concept that we decided to undertake clinical trials of a new product of human recombinant DNase.

Like mucin, DNA is an extremely long, charged polymer and contributes to increased sputum viscosity (Sruggs,1964). The viscosity of sputum could be decreased by breaking DNA into smaller fragments. Evidence that DNA is a major factor responsible for the increased viscosity of infected sputum is derived from the data showing that DNase treatment decreases the viscosity of infected sputum *in vitro* (Shak,1990). Previously, bovine pancreatic DNase I was shown to reduce the viscosity of infected sputum and, in uncontrolled trials, was reported to be effective in clearing secretions when aerosolized in patients with lung infections (Armstrong,1950; Clifton,1961; Segal,1957; Solomon,1954; Spoer,1961; White,1950). This therapy was abandoned because of the high incidence of adverse reactions attributed to allergic reactions.

The studies reported here are a phase 1 study of recombinant human deoxyribonuclease (rhDNase). Since this enzyme is normally present in human plasma, it is not anticipated to induce allergic reactions. Recombinant human DNase was aerosolized and inhaled by normal subjects and adult CF patients. We sought to determine the safety of short term aerosol administration and the systemic absorption of rhDNase.

Material and Methods

Overview and justification of models:

The studies of this thesis were performed using the mucin granules of the slug *Ariolimax columbianus*, the airway epithelial cells of the New Zealand white rabbit, normal subjects and patients with CF.

The slug granules of *Ariolimax columbianus* were used as a method of examining the physicochemical properties of mucins because the granules can be extracted intact from the cell of the slug, and there are not the problems of cell interaction and mucin contamination using this model.

The New Zealand white rabbit was used as a model to examine epithelial secretory cells and mucin swelling in a mammalian model. The rabbit was used as the tissue is easier to obtain than the airway epithelium of humans and patients with Cystic Fibrosis, and the rabbit, like the human upper airway has 3 main morphologically different cell types, namely, the basal cell, the secretory cell, and the ciliated cell. There are no secretory glands in the rabbit and this has the advantage in making the different cell types easier to identify and hence recognize the cell under investigation. In addition, differentiation of the rabbit airway epithelium readily occurs from the tracheal explant outgrowth culture (Verdugo,1984).

The subjects studied were normal controls and patients with CF.

Material and methods : Chapter 8

Ariolimax columbianus studies

Preparation of slug granules

Slug granules are an excellent model to examine the questions of mucin rheology because the mucins are not contaminated by the factors outlined in Chapter 5 of the introduction.

The giant secretory granule of the terrestrial slug *Ariolimax columbianus* is a unique model for the study of mucin release in secretion because the granules can be removed with intact membranes from the skin of the slug (Deyrup-Olsen,1983). In brief, the posterior body wall of the slug foot is dissected ie the slug head or mantle is removed. The slug foot is attached to a glass tube in a vertical position and the foot is filled with Ringer's Solution. Pre-incubation of the preparation for 15 minutes with 10mM/L arginine vasotocin, followed by gentle mechanical or electrical stimulation to the skin of the foot yields a suspension of intact stable granules.

X-ray microanalysis

The granules underwent cryofixation before x-ray microanalysis. The granules, suspended in Ringer's Solution were adsorbed at the tip of a wedge of filter paper which was then plunged into liquid freon at -160^{o}C. The filter paper with the granules was cut at -100^{o}C using a Sorvall MT2B cryo-ultramicrotome. Thin cryosections were then mounted on grids and lyophylized. Elemental microanalysis was performed by means of a JOEL 100C electron microscope with a scanning attachment interfaced to a Kevex 7000 x-ray spectometer. Calibrations were made with binary salt and albumin standards, as described by Shuman (1976).

Intragranular charge shielding

To study calcium release, a drop of granule containing suspension was deposited between 2 coverslips separated by 25μm spacers, forming a tunnel chamber. After 5 minutes, the chamber was perfused with 1mL of a solution of 144 mmol/L NaCl, phosphate buffered at pH 7, 22°C, and containing 1mg of the calcium indicator arzenazo 111 (Palade and Vergara,1983). Following perfusion, the granules that became spontaneously bound to the glass surface remained in the chamber, while those in suspension were washed out. The bursting granules were monitored by phase-contrast microscopy and video-recorded in color at a sample rate of one frame every 33msec. Granules burst spontaneously or were induced to burst by gentle mechanical shearing.

Material and methods : Chapter 9

Rabbit goblet cell studies

Epithelial cell outgrowth from explants

New Zealand white adult female rabbits were sacrificed using an intravenous lethal injection of sodium pentothal and the tracheas were excised and rinsed in sterile Hank's medium. Small sections of tracheal wall were dissected into pieces approximately $10mm^2$. The mucosa was dissected and placed in 0.15% hyaluronidase (Cat # H3506 Sigma St Louis MO) for 40 minutes at 22^0C. Mucus was removed and the mucosa was further sectioned into $1mm^2$ explant pieces. The explants were set in groups of 6 on a gel coated glass coverslip of a Rose chamber and covered with a polycarbonate membrane (8 µm pores). The culture chambers (2ml) were filled with Eagle's medium (Steinberger modification) with 20% horse serum, pH 7.35 and incubated for 14-20 days at 37 ^oC. The cultures were fed thrice weekly. Monolayers of epithelial cells grew out from explants. After 5 days, ciliated cells were found 200µm from the explant. After 2 weeks, clusters of goblet cells were found scattered among a confluent monolayer of phenotypically squamous cells that expand 600-800µm from the explant.

Goblet cells in culture did not form a columnar epithelium: they were flat with irregular borders, and had a characteristic granular cytoplasm. Although they degranulated spontaneously in a fashion very similar to mast cells, their morphologic identification was confirmed both histochemically, using PAS and Alcian blue staining, and also immunocytochemically using anti-goblet cell monoclonal antibodies (Basbaum, Mann, Chow, & Finkbeiner, 1984, Figure 29).

Ciliated cells in culture were also flat but maintained their characteristic morphological appearance for up to 4 weeks.

Kinetics of mucus hydration

Exocytosis of mucin granules is driven by a large volumetric expansion of the granular content, very similar to the one observed in mast cells (Zimmerberg, Corran, Cohen, & Brodwick, 1987; Alvarez de Toledo & Fernandez, 1988). Upon release, the mucin network swells in about 5-10 seconds from approximately 1 μm radius while inside the goblet cells to 8-10 μm after exocytosis. The resulting microspheres of swollen mucin slowly detach from the surface of the cell, annealing to each other to form large aggregates of mucus that later become dispersed in the bathing medium.

Previous investigations have shown that the swelling of newly released mucin granules from goblet cells in culture is governed by the theory of the kinetics of swelling of polymer gels (Tanaka & Fillmore, 1979). For instance, the radial expansion r(t) of secretory material, observed during degranulation in goblet cells, follows a typical first order kinetics of the form:

$$r(t) = r_f - (r_f - r_i)e^{-t/\tau} \qquad (1)$$

where r_i and r_f are the initial and final radii of the swelling granule and τ is the characteristic time of swelling.

Also in agreement with Tanaka and Fillmore's theory is the finding that in newly released mucin granules the characteristic time of swelling (τ) is proportional to the square of the linear size of the gel and to the diffusion coefficient (D) of the gel's polymer network.

$$D = r_f^2/\tau \qquad (2)$$

In this expression D is the mobility of the mucin network in the extracellular fluid, and it can be independently estimated using laser light scattering spectroscopy (Tananka, Hocker, & Benedek, 1973; Lee et al., 1977; Verdugo et al., 1983). As shown previously D is a very sensitive parameter for evaluating the effect of ionic interactions on the swelling of the mucin gel (Verdugo, 1984; Verdugo et al., 1987).

In the experiments reported here spontaneous secretory activity and the swelling of

secreted material from goblet cells was visualized by phase microscopy at a magnification of 500x and video-recorded at 30 screens/second. The time course of the radial expansion of the swelling mucin gels (Figure 12) was evaluated every 200 msec, and then fitted to eq. (1). The characteristic time of swelling was calculated from eq. (1), while the diffusivity of the mucin polymer network was calculated from eq. (2) (Figure 13).

Swelling kinetics of exocytosed granules in the presence of calcium

The swelling kinetics of exocytosed granules in individual goblet cells is fairly consistent. However, it can vary broadly among cells. Thus, experiments were designed to conduct measurements in single cells, using the cell as its own control. Video-recordings were made for 3-4 minutes of spontaneous degranulation in a buffered solution containing 144mM NaCl, 1mM HEPES, 1 mM MOPS and 1mM $CaCl_2$. Observations were performed for 3-10 minutes in the same cells, this time equilibrated in a similar buffer, but containing either 2 or 4 mM $CaCl_2$. All the data were collected at 37^{o}C, pH7, and 288mOsm.

Swelling kinetics of exocytosed granules in the presence of albumin

Each goblet cells acted as its own control for the reasons given above. Secretory activity was videorecorded for 4 minutes, first during degranulation in a buffer containing 144 mM NaCl, 1 mM HEPES, 1 mM MOPS and 1 mM $CaCl_2$. Recordings were then performed for eight additional minutes in the same cell using a similar buffer, but this time containing 68 KDaltons mw serum albumin (Sigma) in concentrations of either 1.4×10^{-7} M (10 mg%), or 7.7×10^{-7} M (50 mg%). All data were obtained at 37^{o}C, pH 7, and 288 mOsm.

Identification of the cell types within the rabbit airway epithelium

Isolated epithelial cell preparation

New Zealand white adult female rabbits were sacrificed by the method described above and the tracheas were excised and rinsed in sterile Hank's medium. Small sections of tracheal wall were dissected into pieces approximately $10mm^2$. The mucosa was dissected and placed in 0.15% hyaluronidase (Cat # H3506 Sigma St Louis MO) for 40 minutes at 22^0C. Mucus is removed and the mucosa is then placed in 0.1% pronase at 37^0C for 30 minutes. With careful dissection the epithelium can be dissected from the mucosa in $10mm^2$ sheets. Sheets of epithelial cells were placed in 6 cc of an enzymatic solution containing collagenase 6mg of 163 units(Collagenase, Worthington Biochemical Co, Freehold NJ), bovine serum albumin 0.2% (cat# A4503 Sigma , St Louis, MO), soya bean trypsin inhibitor 0.04% (Cat# 57K104, Worthington Biochemical Co, Freehold NJ), 0.06cc 1M Hepes buffer for 3 hours at 37^oC. The cells were gently pipetted during the 3 hour period yielding a suspension of viable cells that are then loaded on to the flow cytometer.

In order to control their cellular composition, some epithelial sheets were fixed and embedded in paraffin, sectioned and stained with haematoxylin and eosin and examined morphologically by light microscopy.

Identification of airway epithelial sub-populations using flow cytometry

The cells were suspended in Hank's solution and 1% bovine serum albumin. Cells from the preparative method were loaded on to and sorted on an Orthocytoflurograph 50111 with the model 2150 computer (Ortho Diagnostic Systems, Westwood, MA). A model 164-01 krypton ion laser (Spectra Physics, CA) was used for excitation of cells at 488nm (180mW). Forward angle and 90^0 scatter were gated on the histogram. The morphologically different cells were sorted on the basis of variation of the forward and right angle scatter using a 100mm orifice and sorting rates of 1,500 cells per second. Cells were recovered by centrifugation (200g, 5 minutes) and resuspended in Hank's medium.

Bivariate data were displayed using the program CONTOUR kindly provided by Dr Peter S Rabinovitch (University of Washington, Seattle, WA.).

Cell viability following enzymatic digestion and following flow cytometry

Cell viability following dissection and enzymatic digestion was determined by the fluorescencent dye 4'-6'-diamidino-2-phenylindole (DAPI). DAPI is not taken up by live cells as energy dependent pumps produce rapid efflux of the dye.

Viability of cells following flow cytometry was determined using Hoeschst 33258 vital dye stain (Shapiro, 1988).

Cell morphology of the different sub-populations following flow cytometry

Aliquots of 500 ml of Hank's solution containing approximately 10^5 cells from each of the 3 different fractions obtained from the flow cytometer were placed on 1% poly-D-lysine (Cat # P6407, Sigma, St Louis, MO.) coated glass slides for 20 minutes. The cells attached to the slide were fixed by placing the slide in 2% paraformaldehyde. The slides were stained by haematoxylin and eosin. The relative percentage of each cell type present in the different fractions sorted by the flow cytometer were calculated from batches of at least 200 cells.

Aliquots of the 3 cell populations following flow cytometry were also prepared for transmission electron microscopy using standardized methods.

SDS Polyacrylamide Gel Electrophoresis

Approximately 10^5 cells of each of each cell type was combined with 2X sodium dodecyl sulphate (SDS) sample buffer and each placed on a 10% polyacrylamide gel. Following electrophoresis, the gel was silver stained (Heukeshoven,1985) and photographed.

Preparation of monoclonal antibodies to airway epithelial cells

The epithelium of the New Zealand white rabbit prepared by the techniques described above was injected intraperitoneally into BALB/c male adult mice with Freund's complete adjuvant (RIBI Immunochem Research Inc). The immunization procedure was repeated after 3 weeks and the mice sacrificed 5 days later. The spleens of the immunized mice were removed and homogenized. The homogenates were filtered, resuspended in MEM and fused with Fox NY cells (mouse myeloma cell line) (Taggart et al,1983) in the presence of 50% polyethylene glycol (mw 3,000-3,700, Sigma) . The pellet containing the fused cells was resuspended and plated into 96 well plates containing splenic feeder cells, RPMI, 20% fetal calf serum, thymidine, aminopterin and adenine (Kohler et al,1970; Kennett et al, 1980).

Immunofluorescence to identify the antibodies made against the airway epithelial cells.

Indirect immunofluorescence staining of the hybridoma supernatant was used both with tissue sections and with enzymatically dispersed cells.

Tissue sections for screening hybridoma supernatants were obtained by fixing pieces of trachea in 0.1M phosphate buffer/ 4% paraformaldehyde, pH 7.4 (2 hours, 4^0 C). The trachea sections were cryoprotected by an 18-hour incubation in 30% sucrose/0.1M phosphate buffer, pH 7.4. The trachea sections were then embedded in OCT compound (Miles Lab-Tek Division) and frozen. Sections were placed on to poly-D-lysine (Sigma) coated glass slides. Supernatants were applied to the sections overnight at 4^0C. Sections were rinsed with tris buffered saline/ Tween 20 and incubated with TBS/ NGS for 30 minutes. The sections were then incubated with goat anti-mouse IgG fluorescein isothiocyanate for 2 hours at room temperature. They were then rinsed with TBS/Tween 20, covered with glycerin/PPDA antifade and glass coverslips. Slides were viewed with a Zeiss fluorescence microscope.

Enzymatically dispersed single cells were fixed with 0.1M phosphate buffer/ 4% paraformaldehyde and attached to glass slides using poly-D-lysine. Supernatant was applied to the single cells for 2 hours at 22^0C, rinsed in TBS/Tween 20, blocked with TBS/NGS and incubated for 2 hours with goat anti-mouse IgG fluorescein. The cells were then covered with glycerin/PPDA antifade and a glass coverslip and viewed as before.

Confirmation of the specificity of the ciliated cell antibody using flow cytometry.

The single cells were suspended in the supernatant from the 5B4/H3 cell line for 2 hours. The cells were rinsed, blocked in TBS/NGS for 15 minutes and suspended in goat anti-mouse IgG for 30 minutes. Cells were then rinsed and resuspended in Hank's medium with 1% albumin. The cells from this preparative method were loaded on to and sorted on an Orthocytoflurograph 50111 with the model 2150 computer (Ortho Diagnostic Systems, Westwood, MA). A model 164-01 krypton ion laser (Spectra Physics,CA) was used for excitation of cells at 488nm (180mW). Green fluorescence emission was collected with a 525/30 dichroid filter. Forward angle and 90^0 light scatter were gated on the histogram. The morphologically different cells were sorted on the basis of variation of the green fluorescence using a 100µm orifice and sorting rates of 1,500 cells per second. Cells were recovered by centrifugation (200g, 5 minutes) and resuspended in Hank's medium. Bivariate data were displayed using the program CONTOUR.

Two dimensional gel electrophoresis to identify the epitope

Two dimensional gel electrophoresis was performed by a modified version as described by O'Farrell (1975). In the first dimension, the gel contained ampholytes pH 3-10. Prefocusing of the first dimension occurred at 200V overnight. Isoelectric focusing occurred at 500V for 15 hours. The first dimension gels were 9 cm in length. The second dimension was carried out in a Hoeffer SE 700 multigel unit according to Laemmli (1970) using 10% acrylamide gradient and 0.1% SDS. Electrophoresis was conducted at 45 milli-

Amps per slab gel until the bromophenol blue front emerged from the gels. Gels were then either Western blotted or fixed and stained with a solution containing 50% methanol, 7.5% acetic acid and 0.1% Coomassie Brilliant Blue. The gels were were then rinsed with 5% methanol and 7.5% acetic acid followed by several rinses with 30% ethanol. They were then silver stained according to the procedure of Heukeshoven and Dernick (1985). The double stain procedure enhances the detection level of proteins that were not easily seen by either method alone.

Western blot

The sodium dodecyl sulfate-polyacrylamide gels were transferred overnight on to nitrocellulose by the method described by Burnette(1981).

Immunostaining

The nitrocellulose with the transferred proteins was blocked overnight with 5% horse serum and 2% milk in tris buffered saline. The nitrocellulose was then placed in 5B4/H3 supernatant for 2 hours, washed in TBS/Tween 20 and placed in goat anti-mouse peroxidase for 2 hours. the nitrocellulose was then washed in TBS, then TB , then 3,3'-Diaminobenzidine tetrachloride (DAB) (Sigma).

Immunohistochemistry

Immunohistochemistry was performed by the method of Farr and Nakane (1981). The rabbit trachea mucosa was fixed in 4% phosphate buffered paraformaldehyde. It was then washed 3 times in TBS-5% sucrose and blocked overnight in TBS-NGS. The tissue was again washed 3 times in TBS-5% sucrose, the 2^{o} antibody (goat anti-mouse IgG/IgM peroxidase conjugate) (Caltag Lab, SSF, CA) was applied for 1 hour again washed 3 times with TBS-5% sucrose and DAB for applied for 15 minutes. The tissue was washed

and prepared for TEM.

Material and methods : Chapter 10

Human Studies

Patient selection

Normal volunteers

Twelve subjects were recruited by posting advertisements on the campus of the University of Washington. All subjects signed a consent form approved by the Institutional Review Board (IRB) of the University of Washington. Subjects were included if they were aged 18 to 65 years, had a normal chest X-ray and normal pulmonary function. Females of child bearing age had to be using contraception and have a negative serum pregnancy test. Subjects were excluded if they had had a recent (within 6 weeks) viral or bacterial lung infection defined by clinical diagnosis; if they were or had been smokers; if they had any chronic illness or were taking prescription medication.

The enrollment studies in the normal subjects were a medical history and physical exam, coagulation profile (PT,PTT), chemistry profile (albumin, alkaline phosphatase, total bilirubin, BUN, calcium, cholesterol, creatinine, glucose, LDH, potassium, SGOT, SGPT, sodium, triglycerides, uric acid), hematology profile (CBC with differential and platelet count), urinalysis, serum rhDNase level, anti-DNase antibody titers, chest x-ray, spirometry, serum pregnancy test in women.

Patients with Cystic Fibrosis.

Twelve subjects were recruited by mailing a description of the study to all adult patients with CF followed at the University of Washington CF clinic. All patients signed a consent form approved by the IRB of the University of Washington. CF subjects were included if they were aged 18 to 65 years. Females of child bearing age had to to be using contraception and have a negative serum pregnancy test. The patients had to have a forced vital capacity greater than 40% predicted at the time of screening, and daily expectoration of

green or yellow sputum. Subjects were excluded if they had a recent exacerbation (within 2 weeks) of their lung infection defined by a clinical diagnosis of an acute exacerbation of CF and which included the use of an antibiotic; if there was any change in dose or type of antibiotic, bronchodilator and/or corticosteroid therapy in the previous two weeks.

Pulmonary function testing

Forced expiratory volume in one second (FEV_1), and forced vital capacity (FVC) were measured using a Cybermedic Moose spirometer, Louisville, CO. Functional residual capacity (FRC) was measured using the nitrogen washout technique. Total lung capacity (TLC) and residual volume (RV)were calculated values. The single breath carbon monoxide diffusing capacity (DL_{CO}) was measured using the methods recommended by Jones and Mead (1960), Sensormedics 2100, Anaheim. The DL_{CO} measurement was standardized for hemoglobin concentration (Cotes, 1963). Lung volume and spirometric regression lines as reported by Crapo and associates were used as the predicted values (1981, 1982). The maximal value of at least two concordant spirometric measurements was retained on each subject (Crapo,1981).

Sputum sampling technique

Dental cotton was placed at the openings of the salivary ducts in the mouth in order to reduce saliva contamination of the sputum samples as described by Puchelle (1984).

Visual analogue scale

On days 1 and 12 of the study, the CF subjects were asked to score their dyspnea on a vertical visual analogue scale (Muza,1990).

Serum DNase levels

The concentration of rhDNase in serum was determined by a two-site enzyme-

linked immunosorbent assay (ELISA). An affinity purified goat anti-rhDNase was used as capture reagent. Affinity purified rabbit anti-rhDNase conjugated to horseradish peroxidase was used as the second antibody in the assay. rhDNase assay standards and controls were prepared in a diluent containing 10% normal human serum. All serum samples were diluted initially 10-fold in a serum free buffer. Subsequent dilutions of the samples were made in the same diluent that was used for the standards and controls, which contained 10% normal human serum, to maintain a uniform concentration of serum constituents throughout the assay. The assay quantitated accurately in the range of 0.2 to 12.5 ng rhDNase/ml of diluted sample. Therefore the limit of detection was 2ng rhDNase/ml undiluted serum. Inter-assay precision was good throughout the quantitative range with coefficients of variation less than 13%.

Serum antibody titers

Antibody titers were determined by a radioimmunoprecipitation assay similar to the procedure of Farr (1958) as modified by Chen (1986). Serum dilutions were incubated overnight with a fixed amount of ^{125}I-rhDNase and the immune complexes were precipitated with a goat anti-human IgG antiserum. After centrifugation and washing of the resulting pellets, immunoprecipitated radioactivity was determined in a gamma counter. The mean non specific binding (NSB) was determined by averaging the counts per minute (cpm) of four negative control tubes containing normal human serum at a final dilution of 1:100. Sera were screened at a final dilution of 1:100 and were determined to be antibody positive if the counts per minute in the pellets amounted to at least twice the non specific binding. Multiple two-fold serial dilutions of positive sera were re-analyzed to determine their titer. Titer was defined as the log of the reciprocal of the dilution having cpm equal to twice the NSB. Titers falling between dilutions points were calculated by linear interpolation. An anti-rhDNase serum produced in a cynomolgous monkey was used as a positive control. The inter-assay mean titer of the positive control was 4.97±0.2 (mean ±

SD) and the coefficient was 3.88%.

Normal volunteer protocol

The study was a repetitive dose escalation study of aerosolized rhDNase. All treatments were given under the supervision of the research personnel in the Clinical Research Center at the University of Washington Medical Center. The rhDNase concentration remained the same throughout the study, but the duration of inhalation increased. The drug was administered Monday through Friday on 2 consecutive weeks in escalating dosage with a rechallenge dose 3 weeks later. A pre and 30 minute post rhDNase dose spirometry was performed on each occasion the subjects inhaled the rhDNase. Three dose regimen were given, termed A, B or C. When safety was shown in the first four subjects (dose A), the initial exposure to rhDNase was increased in the next four patients, leading to a higher plateau dose. When safety was shown in the second four patients (dose B), the initial exposure was again increased for the final four patients (dose C). The dose levels employed are shown in table 2. Four normal subjects and four CF patients received each dose level.

On the first day, Monday, serum DNase at 0, 1, 2, 4, 8, 12, 16, 24 hours after the first rhDNase dose was measured. If the first day's dose was safely tolerated, the subject increased the dose as scheduled in table 2. On the second Friday of treatment, the subject again had serial blood samples drawn to determine systemic absorption at a presumed steady state. Blood was also obtained on the second Friday of treatment for coagulation profile (PT,PTT), chemistry profile (albumin, alkaline phosphatase, total bilirubin, BUN, calcium, cholesterol, creatinine, glucose, LDH, potassium, SGOT, SGPT, sodium, triglycerides, uric acid) and hematology (CBC with differential and platelet count). The medical history and physical was repeated. Repeat urinalysis and a chest X-ray was performed.

Three weeks after the last dose, a single rhDNase inhalation challenge of the final

dose was conducted to test for the delayed onset airways hyper-reactivity or hypersensitivity pneumonitis. Spirometry was performed prior to and 30 minutes following rhDNase aerosol treatment and an interval history, physical examination and oximetry were performed. Serum was obtained for measurement of anti-DNase titer.

The rhDNase was administered by an Acorn II jet nebulizer. The nebulizer was filled with 8.0mg or 16mg of rhDNase in 2ml or 4ml dependent on dose given. The dose was determined by varying the duration of aerosolization; the nominal output of the nebulizer is 0.25 mL/min. It is not possible to accurately deliver an exact nebulized dose as this varies with the subject's anatomy, breathing pattern, and minute ventilation (Montgomery, 1987) . These variables may cause the deposited aerosol dose to vary as much as five fold between subjects. Therefore dosage was expressed as the output from the nebulizer.

Patients with Cystic Fibrosis protocol

The protocol for the CF patients was the same as for the normal controls with these additional tests. Lung volumes were measured at days 1 and 12. A visual analogue dyspnea scale was used on days 1,12 and at the 3 week follow-up. The morning expectorated sputum was obtained in 8/12 CF patients on days 1 and 12 for bacterial density (Smith,1988). A morning expectorated sputum was also taken for measurement of rheological properties in 4/12 patients using a Brookfield cone-plate method (Lieberman, 1967). As sputum is non-Newtonian, using this methodology for measurement of viscosity can lead to gross inaccuracies. In addition, the cone-plate by its design breaks the molecular network of the sputum and repeated measurements of the same sample cannot be made. Moreover the sputum of CF patients is not a homogenous mixture and dependent on the part of the sputum taken for analysis can again vary the readings.

The last two subjects, both taking dose C, had a radionucleotide scan before and after the 10 day dose of rhDNase. Technetium Tc 99m albumin colloid was inhaled for 4

minutes and the patient was scanned over the subsequent hour (Agnew, 1984, Laube, 1988).

Adverse Respiratory Reaction

An adverse respiratory reaction was defined as the dose that caused a) a 10% or greater reduction in FVC or FEV1 in that morning's spirometry compared to day 1 FVC or FEV1, or in 30 minute post-dose spirometry, or a greater than 10% decrease in FVC compared to the previous days am FVC b) oxygen saturation less than 88% by oximeter c) a respiratory rate 200% of day 1 and greater than 20 breaths/minute d) acute bronchospasm detected clinically by new wheezing e) hemoptysis.

Chapter 11

Material and methods : statistical methods

Statistical analysis of the role of extracellular calcium

A paired student's t test was used to compare the regression lines of the granule rate of swelling at 1mM versus 2mM Ca^{2+}.

Statistics of the swelling kinetics of albumin

Measurements were performed from videorecordings of 110 exocytosed mucin secretory granules produced by 15 goblet cells. Paired Student's t-test was used to compare the diffusivity of individual cells between control conditions and 10 mg% albumin, and between control conditions and 50 mg% albumin.

rhDNase study

A paired student's t test was used to compare dyspnea scales, lung volumes, diffusion capacity and bacterial densities on days 1 and 12. Spirometric(FVC and FEV_1) and sputum viscosity changes were evaluated by plotting a regression analysis for the values of each patient's morning pre treatment over time (O' Brian,1988). The slope of the regression lines was compared to no change using an unpaired t test. The regression lines for days 1-12, days 1-5 and days 8-12 were calculated over time for both FVC and FEV_1.

Results : Chapter 12

Ariolimax columbianus

X-ray microanalysis of slug granule elemental composition

The averaged X-ray spectra illustrating the elemental composition inside and outside secretory granules of the slug *Ariolimax columbianus* are illustrated in Figure 11. The medium outside the granules is Ringer's solution. The large peak of intracellular calcium amounts in this case to 2.3moles/kg dry material. Intracellular calcium concentration obtained in 22 granules showed an average of 2.49 moles ± 0.94 SD.

Calcium indicator in the slug granules

Video-recordings of spontaneous and induced bursting show that the granules expand from their baseline state of 8μm in diameter to about 25 times their original volume in approximately 66msec. When the chamber was filled with the buffer solution containing arzenazo 111, the granules appeared as dark grey shadows in an intensely red background. Single frame analysis of color video-recordings showed that, 100msec before bursting, the granules became surrounded by a blue halo, suggesting that calcium release precedes bursting. The magnitude and rate of expansion and bursting of granules are not modified by arsenazo, and are similar in granules undergoing either spontaneous or induced bursting.

Significance of slug granule experiments

Mucins are composed of a highly charged polymer network. This highly charged network is condensed within granules. It had been hypothesized (Tam and Verdugo,1981) that these polyionic charges had to be shielded to allow condensation within the granules.

The finding of a remarkably high concentration of calcium inside mucin secretory granules is supportive evidence for this hypothesis.

These experiments were designed to examine the role of a shielding cations in the condensation of the charged mucin polymers within the slug granule.

The finding that calcium release precedes the bursting of the giant granules suggests a novel molecular mechanism of product release in mucin secretion, which may operate in other, mammalian, cells. The release of the secretory product from the granule may be driven by the molecular forces that are stored in the condensed polyionic network where the charges are normally screened by counterions. When these polyionic charges become unshielded, the negatively charged polymer network repel one another, rapidly unfold and expand the polyionic network. This hypothesized mechanism of mucin expansion is also supported by the fast rate of expansion of the gel. The rate of expansion of the gel demonstrated here is much faster than the predicted diffusion co-efficients expected for a polymer network composed of very high molecular weight mucin chains. Thus the rate at which the product contained within the granule is released is dependent on the polyionic charges and the concentration of the shielding cation present. This is an important concept not only for mucin secretion but other systems of polyions and shielding cations eg chromaffin granules shielded by H^+, ATP and catecholamines, or, relevant to the clinical studies presented here, DNA shielded by histones and the small cationic protein spermine (Marquet,1985).

Results : Chapter 13

The role of ions and polyions in mucin hydration in exocytosed secretory granules of the rabbit tracheal epithelium.

The role of extracellular calcium in mucin swelling in the rabbit goblet cell.

Because of the tangled nature of the mucin polymer network, mucin granules do not swell to a final volume, but to complete dispersion after a few hours of exposure to water. However, within the first 5 to 10 seconds of swelling the granule's radial expansion becomes asymptotic to a "final radius" (r_f). As shown earlier (Verdugo, 1984; Verdugo et al., 1987a), the time course of the increase of the granules' radii follows a typical first order kinetics (Figure 12). The linear relationship between τ and r_f^2 is illustrated in Figure 13 is a characteristic feature of the swelling of polymer gels (Tanaka & Fillmore, 1979), and is also in agreement with previous findings (Verdugo, 1984; Verdugo et al., 1987a). Variations in the slope of this line reflect changes in the diffusivity of the newly exocytosed mucin network.

Figure 15 shows the characteristic time swelling (τ) of secretory material released from exocytosed granules from goblet cells in culture as a function of the square of the final radius r_f^2. n is the number of granules. The shadowed area corresponds to the 95% confidence region of the linear regression lines.

Significance of extracellular ion concentration experiments.

These results are in agreement with the granule experiments above, that the swelling of the granules is governed by a Donnan effect rather than by simple diffusion. Thus, the expansion of the polymer network results principally from charge interaction due to the

polyionic nature of the mucin chains rather than from simple diffusional motions.

The final steps of product release in the goblet cell have not been characterized as they have been for another secretory cell- the mast cell. In the mast cell, the formation of a channel called a " fusion pore" precedes the release of the secretory product. However, the identity of these fusion pores as specific ionic channels has not yet been established.

Figure 14 represents a model for product release in secretion. Following the docking of the granule to the plasma membrane, excitation- release coupling is initiated by the gating of an ionic channel that either releases a shielding cation(Ca^{2+}) or possibly may allow the inflow of an anion. In either event, the result is that the negative charges in the condensed polyionic mucin become unshielded, driving a fast volumetric expansion and their release to the extracellular space. Once the channel is opened, the release of the shielding ions from the granule must be driven by a concentration gradient between the intragranular compartment and the extracellular space. Thus, if the concentration of calcium ions in the extracellular space increases, as in the experiments here, the release of Ca^{2+} from charged sites in the mucin chains should decrease, explaining the slowing down of the swelling of the polymer network.

The concept of a " jack in the box " mechanism, whereby the final release of secretory product is driven by potential energy stored in a condensed polyionic network may also be the explanation of product release in other secretory cells such as in the adrenal glands, the mast cell, the thyroid, the pancreatic islet cells, the sympathetic ganglia and the anterior pituitary.

Implications for cystic fibrosis

As outlined in the introduction, control of the rheological properties of mucus is a paramount issue in mucociliary transport. However, a cause-effect relationship between the control of the ionic environment on the surface of the mucosa and the rheological properties of mucus was first indicated by Tam and Verdugo when they demonstrated that mucus

hydration is governed by a Donnan equilibrium process. This observation prompted the idea that the ionic environment on the surface of the mucosa must be a key factor in the physiological regulation of mucus hydration, and hence mucus rheology. Thus, the rheological properties of mucus must be actively regulated not only by the neuro-hormonal mechanisms, that control the release of mucins, but also by those that control the movement of water and ions across the mucosa. Based on these data, it appears that a defect in the control of transepithelial hydroelectrolytic transport (as caused by the CFTR) may be the underlying common denominator in the pathophysiology of CF.

The role of extracellular albumin in mucin swelling in the rabbit goblet cell.

The effect of albumin on the diffusivity of the exocytosed mucin granules is shown in Figure 16. Notice that increasing the concentration of albumin to 1.4×10^{-7} M (10 mg%), and to 7.7×10^{-7} M (50 mg%) reduces the diffusivity of exocytosed mucins to about 80% and 90% of the control, respectively. Thus, small changes in extracellular concentration of albumin can drastically reduce the rate of swelling of exocytosed mucins.

Significance of the role of extracellular albumin in mucin swelling.

In these experiments, albumin was used as a probe to verify the predicted inhibitory effect of extracellular polyions on mucin swelling. Albumin was a convenient probe not only because of its size and polyionic properties, but also because it is a normal constituent of the surface of the mucosa of the airways, and is increased in concentration in inflammatory diseases of the airways. The studies show that the rate of swelling of exocytosed mucins can be dramatically reduced by relatively low concentrations of albumin. The results are significant for two reasons. First, they again validate the Donnan equilibrium as a critical governing principle in the control of mucin hydration; and secondly, they suggest that albumin and other polyions that are commonly found on the surface of the mucosa might have a physiological role in the control of mucin hydration and

mucin rheology.

The modulating effect of albumin on mucus hydration can provide an efficient way to control mucus rheology. A direct implication of this concept is that the concentration of serum proteins or other polyions should be under physiological control. A complete understanding of how proteins are translocated from the vascular space to the surface of the mucosa remains as yet not clear. However, the concentration of proteins does depend upon hormonal action, in the case of the cervix, and also increases in the presence of mucosal inflammation and infection.

Thus, the epithelial cells themselves may be able to regulate mucin hydration.

These experiments provide a theoretical explanation of how the abnormal transepithelial transport of ions, caused possibly by the CFTR, and proteins, found after inflammation, can effect the rheological properties of the mucus gel.

Results : Chapter 14

Identification of the rabbit tracheal epithelial cells.

Isolated epithelial cell preparation

Epithelium prepared by dissection followed by enzymatic digestion yields uncontaminated epithelial cells (Figure 17) The ciliated, goblet and basal cells can be identified clearly in this preparation. Few, if any, non- epithelial cells are identified. Approximately 10^7 epithelial cells are obtained from each trachea.

Identification of airway epithelial sub-populations using flow cytometry

Figure 19 illustrates the results obtained by flow cytometry. The left hand panel plots forward scatter on the y-axis against right angle scatter on the x-axis. The encircled regions labelled 1,2 and 3 indicate the 3 different cell types corresponding to the goblet, basal and ciliated cells respectively. The right hand panel illustrates the same data as the left hand panel. The y-axis again corresponds to the forward scatter and the x-axis to the right angle scatter. The contours perhaps more clearly illustrate the 3 cell groups.

Cell viability following enzymatic digestion and following flow cytometry

The viability of the cells was examined using 4'-6-diamidino-2-phenylindole (DAPI) stain and Hoechst 33258 stains. Using flow cytometry to assess fluorescence, the viability of the epithelial cells was greater than 98% before sorting and greater than 90% once the cells were sorted.

Cell morphology of the different sub-populations following flow cytometry

The cellular composition of the different fractions obtained from flow cytometry

was determined by light microscopy. Figure 20 illustrates the 3 different cell types obtained following flow cytometry. Panel a) shows the ciliated cells with their distinctive cilia. Panel b) shows the smaller (7μm diameter) basal cell with a large nucleus surrounded by a thin rim of cytoplasm. Panel c) shows the goblet cells some of which may appear to be secreting. The purity of the ciliated, the basal and the goblet cells is 90%, 97% and 94% respectively (table 1).

Transmission electron microscopy

Figure 21 shows the three morphologically different cell types are retrieved by light scatter analysis: ciliated cells (a), basal cells (b) and goblet cells (c). The ciliated cell is 12 by 14μm. It is polarized with the nucleus at its base and cilia with their corresponding basal bodies at the cell apex. Many mitochondria are seen at the apex of the cell. Panel b) shows the small basal cell, 4-5μm in diameter. A high nucleus to cytoplasmic ratio is seen. Mitochondria are present but are far fewer in number than either the secretory or the ciliated cell. Panel c) shows the secretory cell, 8 by 10μm in diameter. A translucent granule can be seen as can mitochondria. Rough endoplasmic reticulum and a Golgi apparatus is seen to the left of the nucleus.

SDS Polyacrylamide Gel Electrophoresis

Figure 22 shows a 10 % polyacrylamide gel which has been silver stained. The letters a,b and c correspond to panel a,b and c of Figures 20 and 21 indicating the ciliated, basal and goblet cells respectively. The arrows on the gel indicate unique proteins expressed by the morphologically different cell types.

Significance of epithelial cell identification

The unequivocal identification of the epithelial cells was an important step in the validification of our rabbit *in vitro* model. This thesis describes a new procedure for the

separation and sorting of epithelial cells. Enzymatic digestion and careful microdissection can produce uncontaminated populations of epithelial cells suitable for flow cytometry. Taking advantage of the different volume and light scatter patterns of the basal, goblet and ciliated cells, flow cytometry can successfully sort these different cell types. The purity of the cell populations obtained are identified both by their morphological characteristics and by the identification of unique proteins expressed by each morphologically different cell type.

The ideal goal of cell isolation and purification is to produce a high purity of the isolated sub-population. Using flow cytometry, we were able to find greater than 90% purity of each cell type. Our method of cell identification was to use light microscopy with histological staining and the expression of unique proteins by each sub-population. As shown in table 1, these techniques yield fractions of different cell types in high purity. In previous studies, other cell types, notably the Clara cell has been isolated in high purity, but only in one other study has the basal cell been purified and the ciliated cell enriched (Chilton,1981). In our study we were able to isolate all 3 cell types and in greater purity than previously described.

Preparation of monoclonal antibodies to airway epithelial cells

Fusions from 2 mice yielded 19 cell lines specific to epithelial cells. Of these one, 5B4/H3 was specific to the ciliated cell. Figure 23 shows the immunohistochemical appearance of a rabbit trachea section with 5B4/H3 antibody. Figure 24 shows the immunohistochemical appearance of enzymatically dispersed single cells with 5B4/H3 antibody. The morphologically distinct ciliated cells are fluorescent whereas the non-ciliated secretory and basal cells cells are not.

Figure 25 illustrates results of the flow cytometry of rabbit tracheal epithelial cells tagged with 5B4/H3 antibody. Forward scatter on the y-axis is plotted against green fluorescence on the x-axis. Three cell populations are identified as before. Only one of

these populations, the ciliated cells indicated by the arrow, are labelled with fluorescence demonstrating that the antibody is specific to the ciliated cells.

Figure 26 shows the 5B4/H3 antibody recognizing a ciliated cell in culture. Figure 27 shows the immunohistochemical staining of the 5B4/H3 antibody. The epitope is on the lumenal surface of the ciliated cell. It is found not only on the cilia, but also on the surface of the ciliated cell.

Figure 29 shows Dr Carol Basbaum's 4D4 antibody recognizing a rabbit tracheal goblet cell in culture.

Two dimensional gel electrophoresis to identify the epitope

Western blot: Immunostaining

Figure 28 shows a 2-D gel of the rabbit trachea epithelial cells and the corresponding Western blot of the 5B4/H3 antibody. The antigen has a molecular weight of 130 kD and a pI of 5.6.

Significance of cell identification using monoclonal antibodies.

In many *in vitro* conditions, the epithelial cells of the airway lose their characteristic morphology and de-differentiate. It is therefore essential to confirm the cell type being examined by markers which do not rely on cell morphology alone. To this end, we collaborated with Dr Carol Basbaum, University of California, San Francisco, who supplied us with some of her antibodies specific to some of the epithelial cell types. However, an antibody to the ciliated cell was not available.

This report describes for the first time a surface antibody specific to the ciliated cell. We also report the characteristics of the corresponding epitope with a molecular weight of 130kD and a pI of 5.6.

The applications of this antibody as a useful tool are multiple. Firstly, as shown in figure 25, it can be used to label ciliated cells with fluorescence and to isolate a pure

population of ciliated cells uncontaminated with either basal or secretory cells. Conversely, the basal and secretory cells can be isolated uncontaminated by ciliated cells.

Secondly, the antibody can be used to follow epithelial cells *in vitro*. Under most *in vitro* conditions, epithelial cells de-differentiate (Wu, 1982) and the ciliated cell marker which does not rely on cell morphology will allow the pattern of ciliogenesis to be followed without reliance on morphological characteristics. These *in vitro* studies will compliment the previous elegant autoradiographic studies of ciliogenesis *in vivo* which suggest that the ciliated cell is a terminally differentiated cell.

Results : Chapter 15

Recombinant DNase studies in normals

The demographic data of the normal subjects are given in Table 3. Thirteen normal subjects were recruited for the study. One subject failed entry criteria as his screening spirometry showed him to have airflow obstruction.

In the normal subjects, no adverse reactions were detected. There were no detectable adverse respiratory events. There were no significant changes in spirometric indices at any time during the two week trial or at the three week follow up interval ($p>0.10$). Serum DNase levels showed that in one normal subject receiving dose level A that serum DNase levels were above normal (52ng/ml: Normal 20-50ng/ml). No anti-rhDnase antibodies were detected.

Results : Chapter 16

Recombinant DNase studies in patients with Cystic Fibrosis

The agarose gel of a patients expectorated sputum is shown in figure 30. The left hand figure represent a patients sputum sample with increasing amounts of DNase against viscosity measured using a Brookfield cone and plate viscometer. With increasing amounts of rhDNase, the viscosity decreased. The right hand panel shows an agarose gel loading with sputum which had been previously incubated with increasing amounts of rhDNase.

The demographic data of the CF patients are given in Table 4. There was no difference in age or NIH score (Taussig, 1973) between the dose level groups of the CF patients. Pulmonary function tended to be higher in group C. Fourteen CF patients were recruited. One patient had an acute exacerbation of his CF on day 7 of the study: a second patient developed flu-like symptoms on day 9 and they were excluded from the study.both these patients had improvement in spirometry at the time of exclusion.

In the CF patients, there was both objective and subjective and improvement. As shown in Figures 31 and 32 the mean morning pre-dose FEV1 and FVC improved ($p=0.01, p=0.01$ respectively). The visual analogue scale improved between days 1 and 12 (18.8 ± 5.6 , day 1 to 7.3 ± 2.6 , day 12: mean $\pm$ SE, $p< 0.01$) (Figure 33). There was no significant change in TLC and DL_{CO} between days 1 and 12 ($p>0.9$ and $p>0.8$ respectively) 9 Table 5). Serum DNase levels were not increased above normal values in the CF patients. No antibodies to rhDNase were detected.

The results of the quantitative cultures on days 1 and 12 are shown in table 6. No significant change in the organisms or their sputum density were found.

The ventilation scans before and after treatment in two patients are shown on figure 34. In patient #12 (top panel), the scan before the medication was normal and remained

normal at the end of the study. In patient # 11 (lower panel), there were patchy defects in the ventilation scan before the study. Ventilation possibly improved in the left upper lobe following rhDNase, but the ventilation to her left lower lobe was less than it had been prior to treatment. One would have expected clearing of mucus plugs and possibly an improvement in the ventilation scan. The variability of lung scans in the CF population is unknown and it may be that this is the changing pattern seen in CF or it may be that the medication reduced ventilation to the left lower lobe.

Figure 35 shows the daily sputum viscosity measurements in 4/12 patients. In three of the patients the sputum viscosity tended to decrease with this measurement. The numbers used in this study are too small to determine whether the Brookfield viscometer is a useful tool to examine sputum viscosity. Moreover, the cone-plate method has intrinsic problems in the measurement of sputum viscosity as outlined in the methodology section above.

There were 15 adverse respiratory events. Three patients had five episodes of hemoptysis. This was not a new symptom in these three patients. Three patients had a greater than 10% decrease in their FEV_1 from day 1 FEV_1. Four patients had five episodes of a greater than 10% decrease in FVC from the previous days or that morning's FVC. Two patients on one occasion had a greater than 10% decrease in their FVC from the pre-aerosol measurement. These decrements were from -10.1% to - 27.9%. However the decrease of 27.9% from the previous days value was an 8.2% improvement from day 1. One patient developed a new small left upper infiltrate during the study: he was asymptomatic. This infiltrate resolved over the course of a month without treatment.

Significance of rhDNase studies

This phase 1 trial of recombinant human DNase showed it to be non-toxic in short term trials. Spirometric indices improved in the CF patients and they felt symptomatically better with improvement of their dyspnea scale. No clinically significant airway hyper-

reactivity was seen and there was no evidence of hypersensitivity pneumonitis. Systemic absorption may have occurred at very low levels in one normal subject. Antibodies to the rhDNase were not detected.

The study design did not randomly assign patients to treatment groups A,B or C. However, there was no difference in age or NIH score in the dose level groups of the CF patients. As can be seen in table 4, pulmonary function tended to be higher in group C. An improvement in spirometric indices was seen in each dose regimen. The improvement in FEV_1 between days 1 and 12 was 14%,9% and 9% for doses A,B and C respectively and FVC was 14%,10% and 6% respectively. The numbers in this study are too small to determine whether this reflects a greater improvement at a lower dose or whether a greater improvement is seen in those patients who have more obstructive airflow.

The variation in spirometry can be great for the CF population (Nickerson, 1980). The improvement in spirometry is encouraging but clinically small. However, FVC tended to improve in 11/12 CF patients in the first five days with no additional improvement thereafter. Double blinded controlled studies are required to determine whether the change in pulmonary function testing in the CF patients is due to the medication, a placebo effect, or secondary to performing repeated forced expiratory maneuvers three times daily.

Although there was a trend for sputum bacterial density to decrease, no significant changes were seen. If it is presumed that mucociliary clearance is improved by this aerosol treatment, then it was felt that the bacterial density may decrease. It may be that this requires a longer period of treatment before a change is seen, or that bacterial adherence to the mucins and epithelial cells (Fick ,1989) is uneffected by this medication.

Conclusions : Chapter 17

This thesis has examined the molecular basis of respiratory mucus swelling looking at 2 molecules namely mucin and the DNA in sputum.

We demonstrated that the presence of shielding cations was important for the highly negatively changed mucins to remained condensed within the secretory granule. When these cations are released from the network, the mucin chains can expand or hydrate. The rate of mucin chain expansion or hydration is dependent on the concentration of ions or polyions in the swelling medium. In diseased states such as CF, there is an ionic imbalance in the periciliary fluid and mucus hydration may be limited. In other airway diseases such as asthma, the mucosal barrier is not intact and there is leakage of proteins such as albumin into the airways. The presence of negatively charged albumin competes with mucus swelling and again causes the mucus to remain thick and unhydrated.

We used the rabbit epithelial cells as a model of mucin production. We wished to unequivocally identify the different cell types of the airway. We produced a monoclonal antibody to the ciliated cell and also used a monoclonal antibody to the rabbit goblet cell. These antibodies characterized the different cells that we grew in culture. We further went on to characterize the epitope of the ciliated cell antibody.

Finally we examined the effect of increased DNA content in the sputum of patients with CF in a pilot non-randomized non-controlled study. We used a new experimental drug, human recombinant aerosolized DNase which breaks DNA in random fashion. This caused the sputum to be thinner; it allowed patients to expectorate more easily; it decreased their dyspnea and it improved measurements of pulmonary function. A larger randomized study is required to evaluate if these changes were due to the medication, a placebo effect or as a result of repeated forced expiratory maneuvers throughout the study.

In CF, it would appear that the mucins are more sulfated (Cheng, 1989). This may

not allow such rapid hydration of the mucin network. The periciliary fluid ion content is probably also abnormal although it is difficult to measure this directly. A disturbance of the ion content in the periciliary fluid or "swelling medium" may also prevent mucin hydration, leaving the mucus abnormally viscous. Thick mucus slows mucociliary transport (Johnson, 1991). Whether the thick mucus and slower mucociliary transport leads to chronic bacterial colonization of the airways is not clear. Other diseases with slow mucociliary transport such as the immotile cilia syndrome do not have such a devastating course, but the mucus in these condition is presumably normal. Thus it may be the thickened mucus which is the initiating event or perhaps the epithelial cells themselves are abnormal and allow bacteria to become more easily attached. Whatever the mechanism, the airways in Cystic Fibrosis do become colonized with bacteria most notably *Haemophilus influenza, Staphylococcus aureus* and *Pseudomonas aeruginosa.* The bacterial products attract leukocytes to the airways. When the leukocytes die, they release DNA which further increases the viscosity of the airway secretions. By breaking the DNA into smaller fragments, the purulent secretions become less viscous. Whether smaller DNA fragments will improve mucociliary clearance and reduce the bacterial load in the airways and slow the rate of progression of airway inflammation and chronic respiratory failure are yet to be determined.

Mucociliary transport may be improved in CF by changing the sulfation of mucins. The presence of less negatively charged mucins will allow the mucins to hydrate more rapidly. Changing the ionic concentration of the periciliary fluid of the airways, by ion channel blockers such as amiloride, may also allow more rapid mucin hydration. Increased mucin hydration will alter its visco-elastic properties. Once CF sputum has become infected, and the DNA content, released by dead neutrophils is high. Breaking the extracellular DNA into small fragments will also change the visco-elastic properties of sputum.

Table 1

Purification of airway epithelial cells by different methods.

Author(Yr)	Animal	Method	% purified ciliated	basal	goblet	Clara	%viability
Aitken'89	rabbit	flow cytometer	90	97	94	NA	90Hoechst
Inayama'88	rabbit	centrifugal elutriation	NA	83-94	NA	NA	viable graft
Patton'86	rabbit	Percoll centrifugation Elutriation	NA	NA	NA	86	viable graft
Chilton'81	rabbit	unit gravity sedimentation	NA	2.5X	2X	0	93trypan blue
Devereux'80	rabbit	elutriation	NA	NA	NA	70	90trypan blue

NA= Not applicable to that study ie not sought in that study.
2.5X= purified 2.5 times
Hoechst=Hoechst 33258

Table 2

The doses of recombinant aerosolized DNase.

	Dose Level		
Tx day	**A**	**B**	**C**
1	6 seconds qd	18 seconds qd	36 seconds qd
2	6 seconds tid	18 seconds tid	36 seconds tid
3	18 seconds tid	36 seconds tid	2 minutes tid
4	36 seconds tid	2 minutes tid	6 minutes tid
5	2 minutes tid	6 minutes tid	10 minutes tid
6	-	-	-
7	-	-	-
8	2 minutes tid	6 minutes tid	10 minutes tid
9	2 minutes tid	6 minutes tid	10 minutes tid
10	2 minutes tid	6 minutes tid	10 minutes tid
11	2 minutes tid	6 minutes tid	10 minutes tid
12	2 minutes tid	6 minutes tid	10 minutes tid

Table 2 : Legend

The first four subjects and CF patients were given dose A, the second dose B and the third dose C. The nebulizer was filled with 8.0mg or 16 mg of rhDNase in 2ml or 4 ml dependent on dose given. The nominal output of the nebulizer is 0.25ml/min. See text for further details.

Table 3

Demographics of normal subjects

#	Age	Gender	Dose	FVC	FEV1
1	48	F	A	3.34(98%)	2.86(102%)
2	37	M	A	6.87(133%)	5.44(128%)
3	38	F	A	4.69(124%)	4.08(129%)
4	50	F	A	3.73(119%)	2.85(110%)
5	39	M	B	4.56(89%)	4.03(96%)
6	47	F	B	4.38(114%)	3.05(98%)
7	29	M	B	5.53(100%)	4.73(104%)
8	25	M	B	6.28(103%)	4.96(100%)
9	19	M	C	4.35(82%)	3.79(84%)
10	29	M	C	5.25(95%)	3.74(82%)
11	23	M	C	5.33(104%)	4.01(94%)
12	19	M	C	4.44(84%)	3.63(81%)
Mean	33.6±11.2			4.9±1.0 (104%)	3.9± 0.8 (101%)

Table 3: Legend

Table 3 shows the demographic information and spirometry on day 1 of the normal subjects. The mean ± SD are given.

Table 4

Demographics of CF patients

#	Age	Gender	NIH	Dose	FVC day 1	FEV1 day 1
1	20	M	70	A	3.09(50%)	1.93(38%)
2	27	M	61	A	3.98(69%)	1.67(35%)
3	27	M	57	A	4.12(87%)	3.21(80%)
4	30	F	57	A	1.58(48%)	0.82(28%)
5	49	M	90	B	4.96(89%)	3.31(75%)
6	28	M	55	B	1.96(35%)	0.97(21%)
7	19	F	78	B	3.47(71%)	1.78(43%)
8	18	M	74	B	2.91(59%)	1.91(46%)
9	24	M	88	C	4.24(76%)	2.95(64%)
10	21	M	59	C	2.59(56%)	1.57(40%)
11	28	F	82	C	2.92(91%)	1.96(68%)
12	25	F	78	C	3.38(109%)	2.71(96%)
Mean	26±8		73±12		3.22±0.98(69%±22)	2.07±0.0.78(53%±23)

Table 4: Legend
Table 4 shows the demographic information and spirometry on day 1 of the CF patients.The mean ± SD are given.

Table 5

Total lung capacity and diffusion capacity of CF patients before and after rhDNase

	TLC		DLCO	
#	pre	post	pre	post
1	6.62(86%)	6.88(90%)	25.6(54%)	26.5(56%)
2	7.72(106%)	7.48(103%)	24.5(56%)	27.3(62%)
3	6.37(108%)	6.38(109%)	28.3(78%)	26.6(73%)
4	3.31(72%)	3.18(69%)	10.4(39%)	11.6(43%)
5	6.86(88%)	7.31(94%)	39.6(96%)	31.8(77%)
6	5.65(80%)	5.43(77%)	23.2(55%)	23.1(54%)
7	6.33(104%)	6.56(107%)	26.8(76%)	27.6(79%)
8	5.54(83%)	5.48(82%)	25.5(60%)	25.7(60%)
9	7.29(106%)	7.33(107%)	25.9(61%)	31.1(73%)
10	6.29(114%)	6.18(113%)	20.3(57%)	17.4(49%)
11	5.04(117%)	4.75(110%)	21.1(81%)	20.9(81%)
12	4.27(103%)	4.33(104%)	20.2(78%)	19.7(77%)
Mean	9.93(97%)	5.94(97%)	24.3(66%)	24.1(65%)

Table 5: Legend

Total lung capacity and DLCO measurements of the Cystic Fibrosis patients before and after treatment DNase. There was no significant difference before and after 10 days of rhDNase.

Table 6

Bacterial density in sputum in 8 CF patients before and after rhDNase.

	P. aeruginosa n=5	*S. aureus* n=6	*P. cepacia* n=1	*S. epidermidis* n=1
Day 1	6.49±0.87	5.62±1.7	5.04	3.04
Day 12	5.97±0.44	5.01±1.56	4.76	5.00

Table 6: Legend

All bacterial densities are expressed as the $\log_{10}$ CFU/g sputum. The standard deviations are shown in parentheses. No significant differences were found following 10 days of treatment. n is the number of patients with the organism.

igure 1
ucas and Douglas model of the pseudostratified airway epithelium. All the epithelial cells re in contact with the basement membrane of the airway, but only the secretory and ciliated ells reach the lumenal surface. The cilia beat in the periciliary fluid and the mucus layer ides atop of the periciliary fluid.

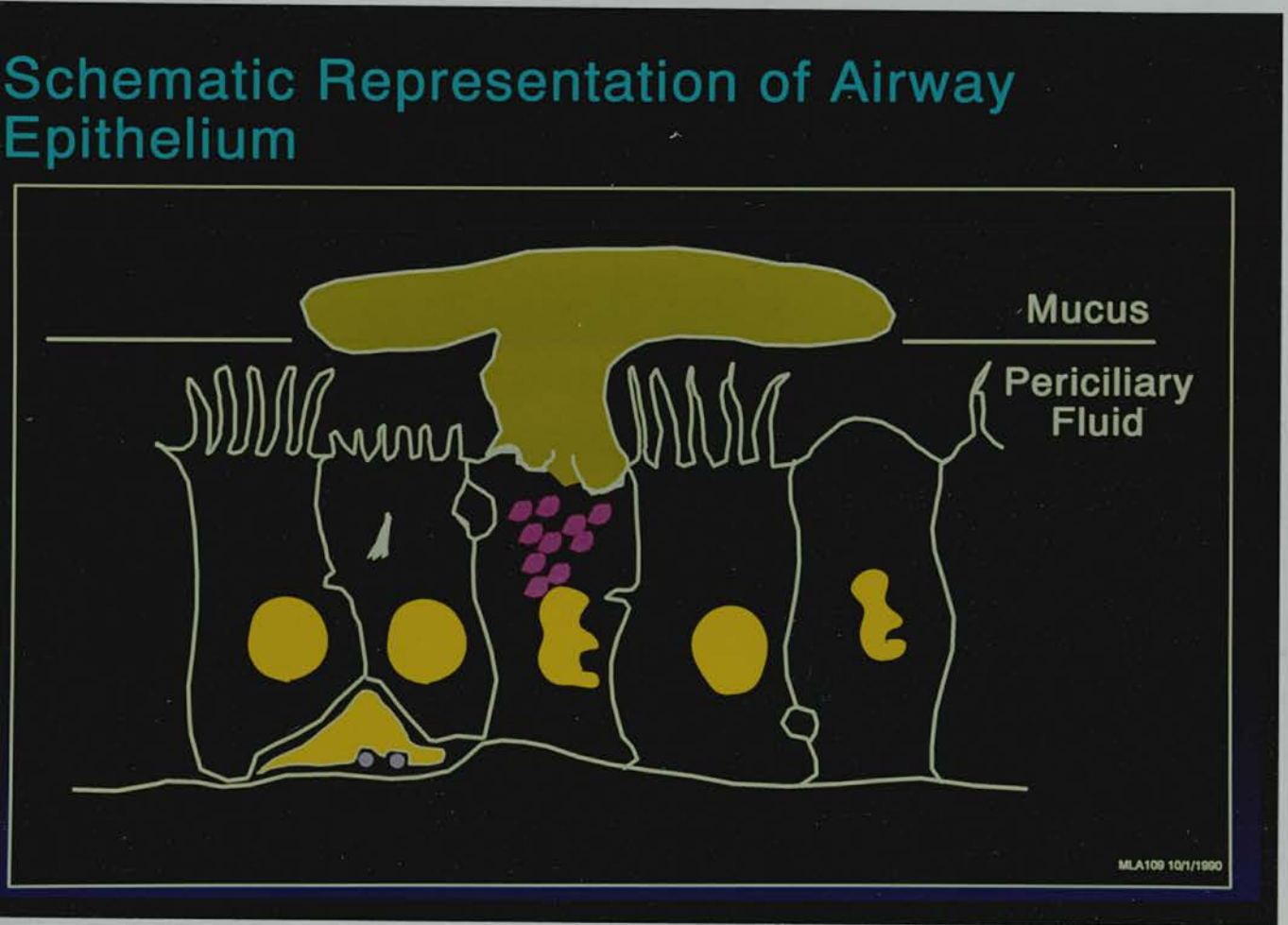


Figure 2
Transmission electron microscopy of the tracheal epithelium of the New Zealand rabbit. The small basal cell with its large nucleus, a goblet cell and a ciliated cell with its distinctive cilia can be clearly seen.

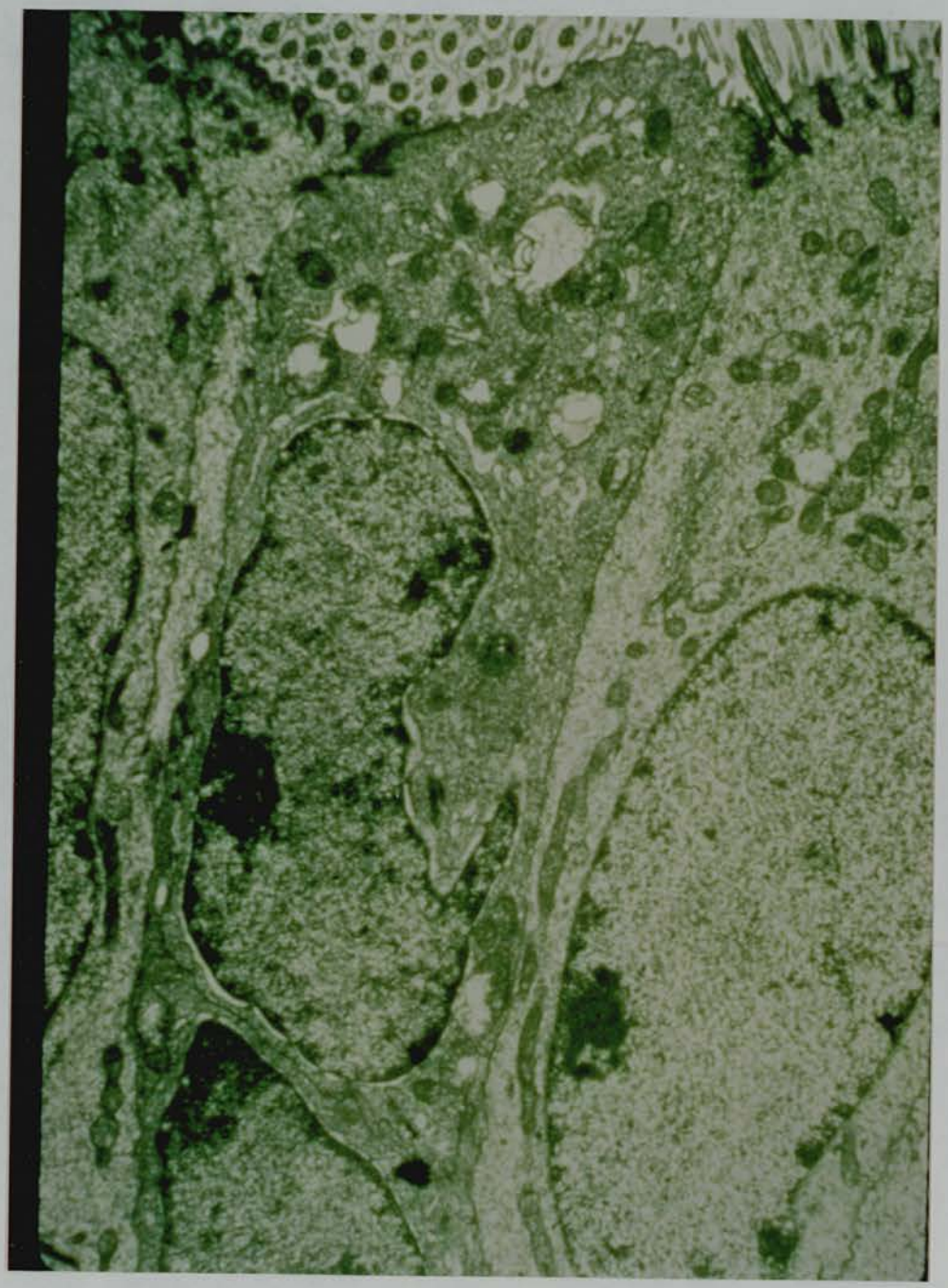


Figure 3
Scanning electron microscopy of the tracheal epithelium of the New Zealand rabbit. Numerous ciliated cells with the occasional secretory cells can be seen.

Figure 4
Schematic cross section of the ultrastructure of a cilium. 1 : B microtubule, 2 : A microtubule, 3 : spoke head, 5 : central sheath, 6 : central microtubule, 7 : external dynein arm, 8 : inner dynein arm, 9 : nexin link.

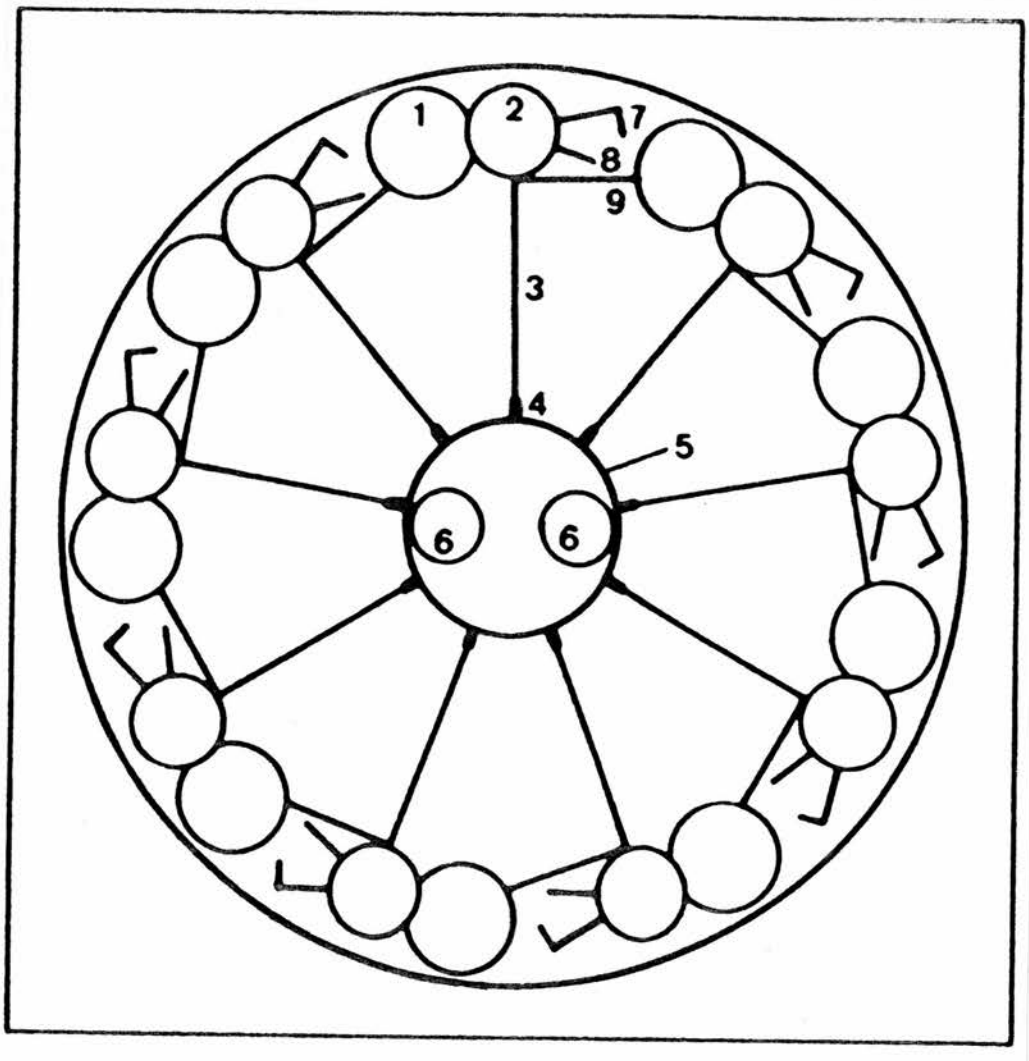


Figure 5
Diagram of the ciliary beat and the mechanism of the sliding filament.

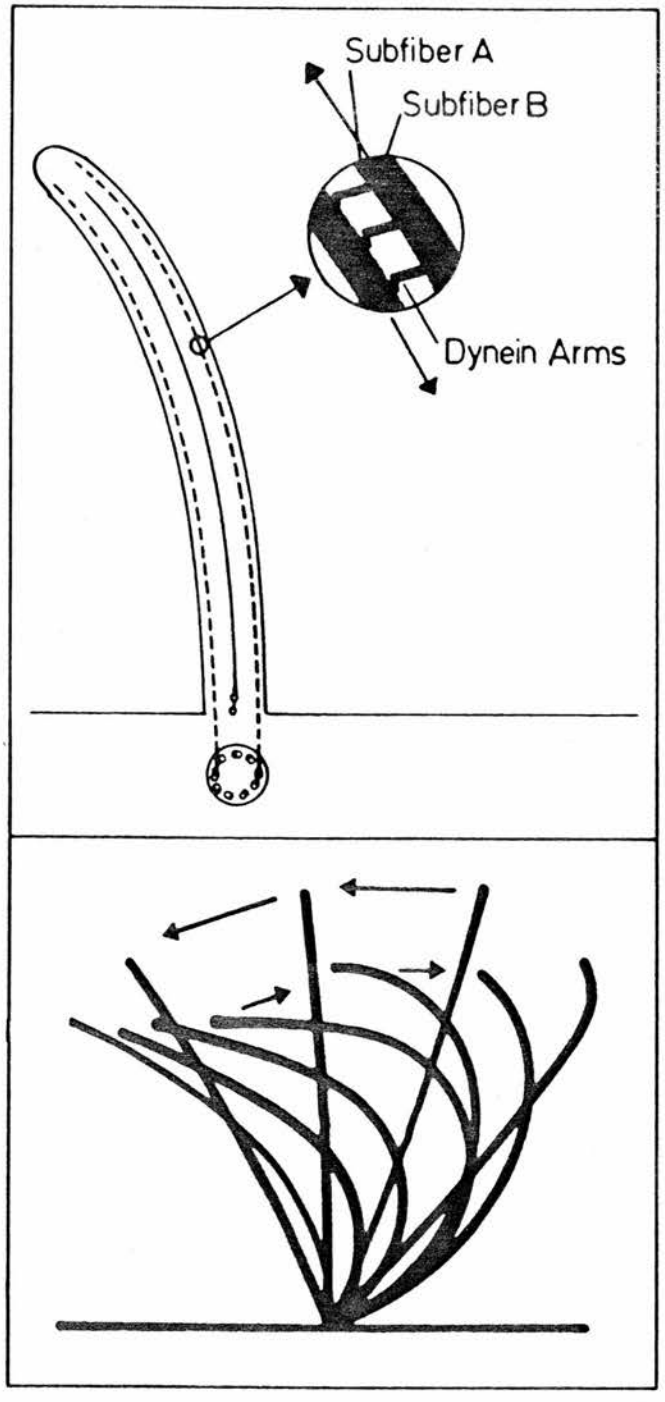


Figure 6
Measurement of change in the frequency of ciliary beat (CBF) plotted over time for 6 different artificial mucus (dextrans) ranging in viscosity from 1.5 (white) to 200 (red) centipoise. When viscous load is added, there is an immediate fall in the CBF. The fall in CBF is greater in the higher viscous loads. Over time there is some recovery of CBF. This shows that CBF is slowed with higher viscous loads, and that the ciliated cell does seem to have some autoregulation as some recovery is seen.

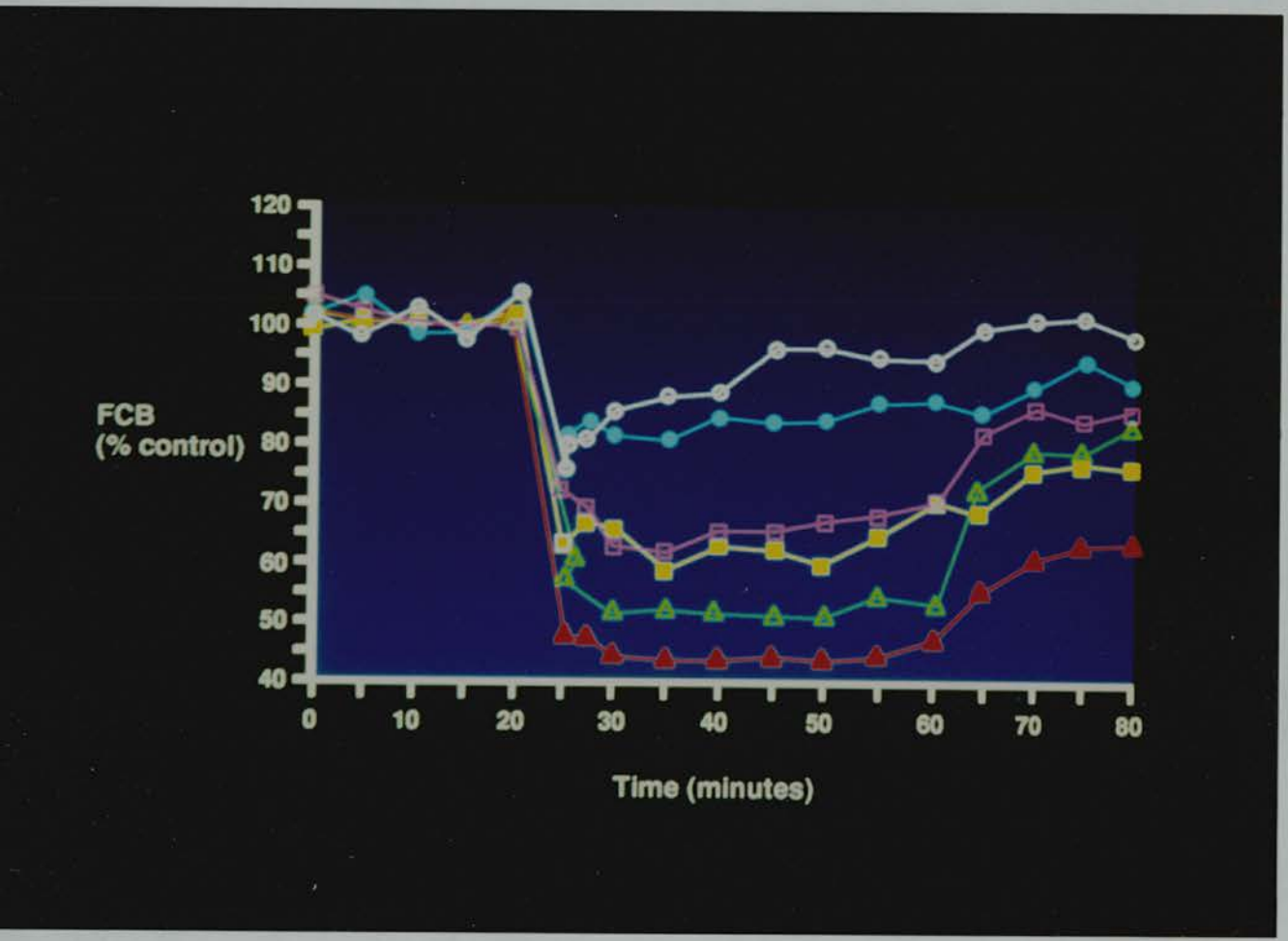


Figure 7
Cartoon illustrating hydrated vs unhydrated mucin chains. Mucins are high molecular weight molecules with a protein backbone and with charged carbohydrate side-chains. As described in chapter 6, it is the tangle density that governs the rheological properties of the mucus.

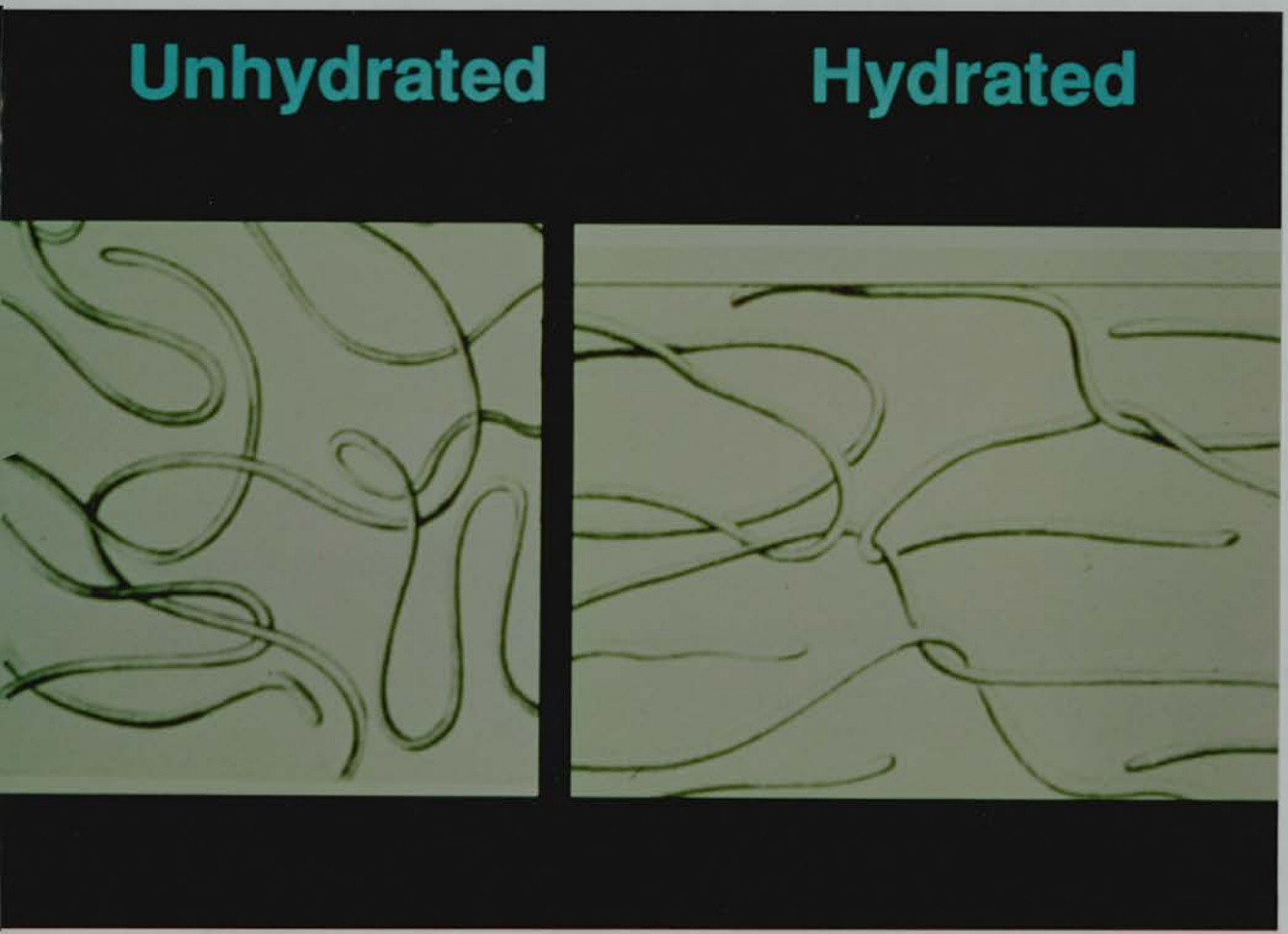


Figure 8
A tangled high molecular weight protein has an osmotic force. The same protein with a charge has, in addition, a Donnan potential. If a smaller molecule is placed on the other side of a semi-permeable membrane, it will also have an osmotic force competing for expansion or hydration of the larger molecule. If the smaller molecule is charged, then there will be a Donnan equilibrium between these molecules. In the mucin chains, the twists and curves of the mucin network act like a semi-permeable membrane.

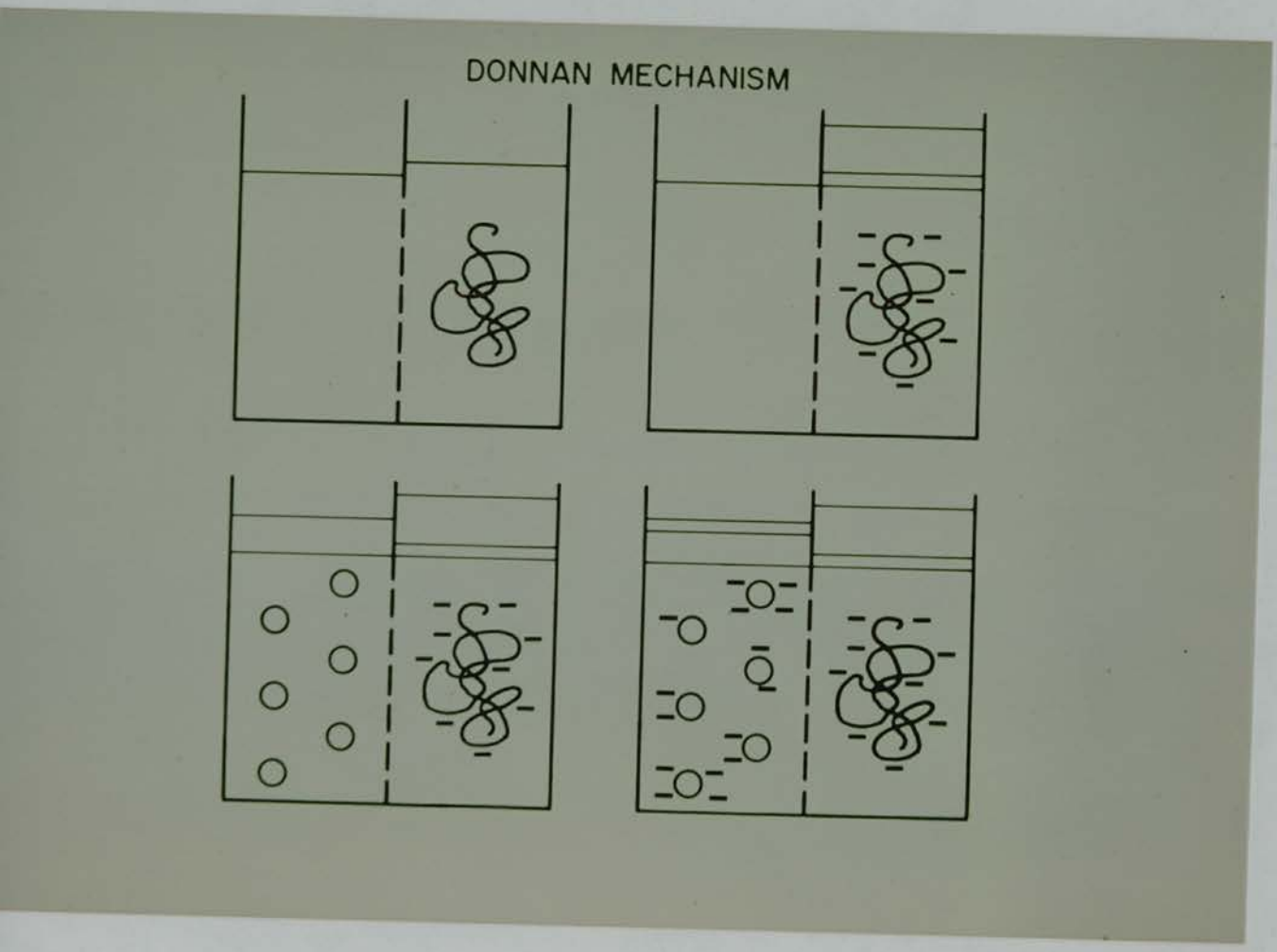


Figure 9
Phase contrast of a rabbit tracheal goblet cell in culture. The arrow shows an individual mucin granule expanding over time. The equation is the the calculation of the time of swelling or diffusivity.

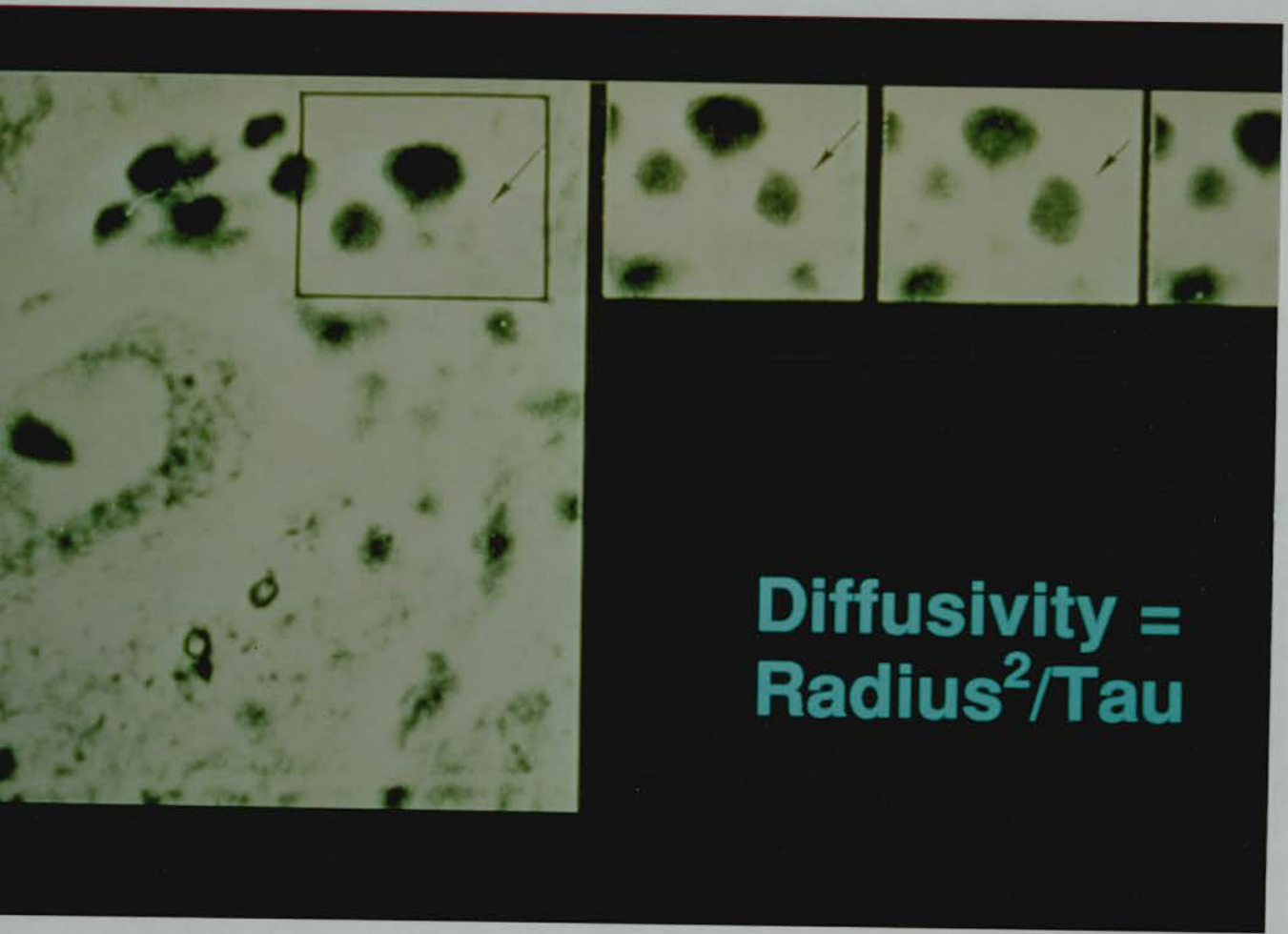


Figure 10
Photograph of the slug, *Ariolimax columbianus*

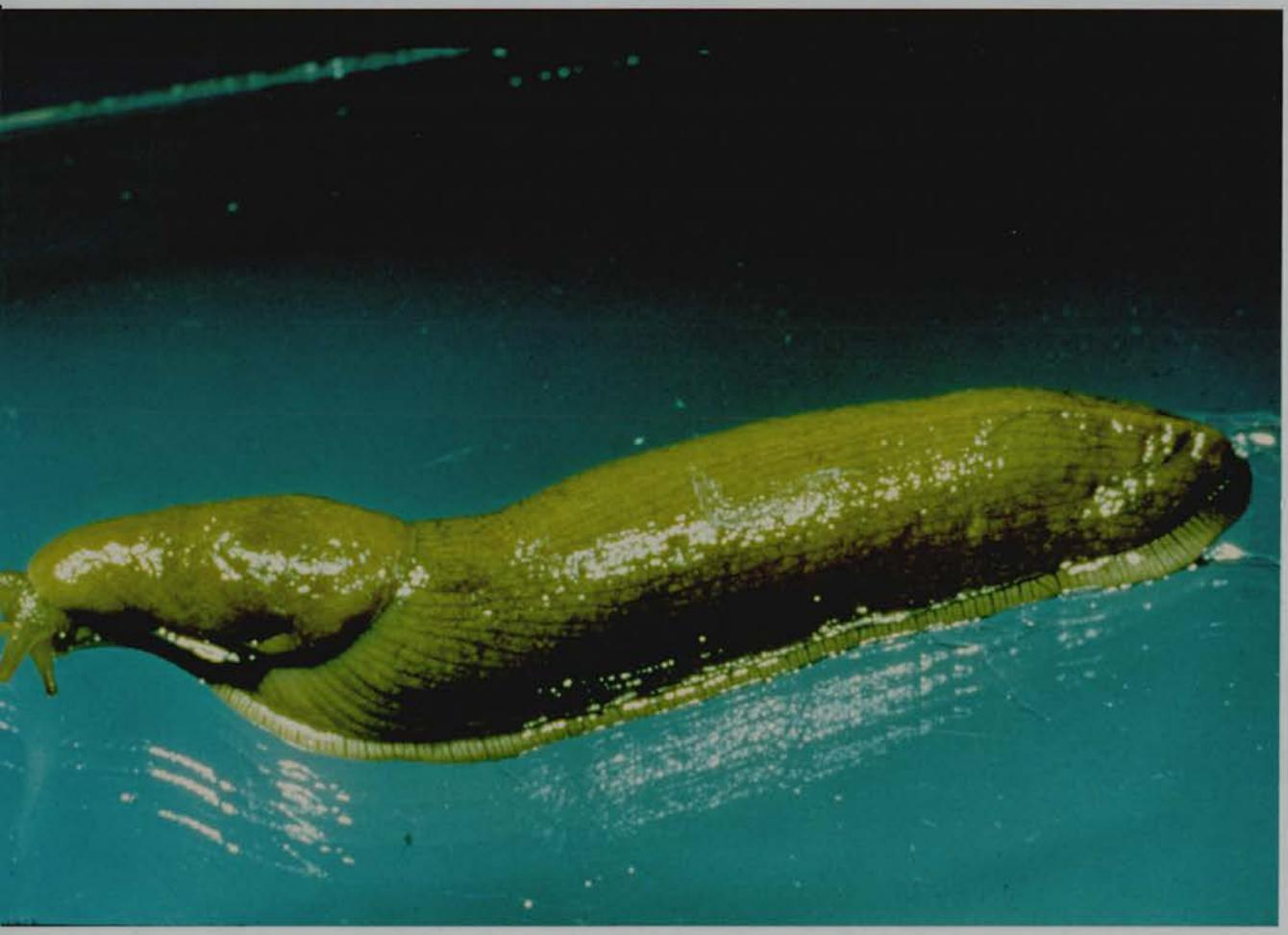
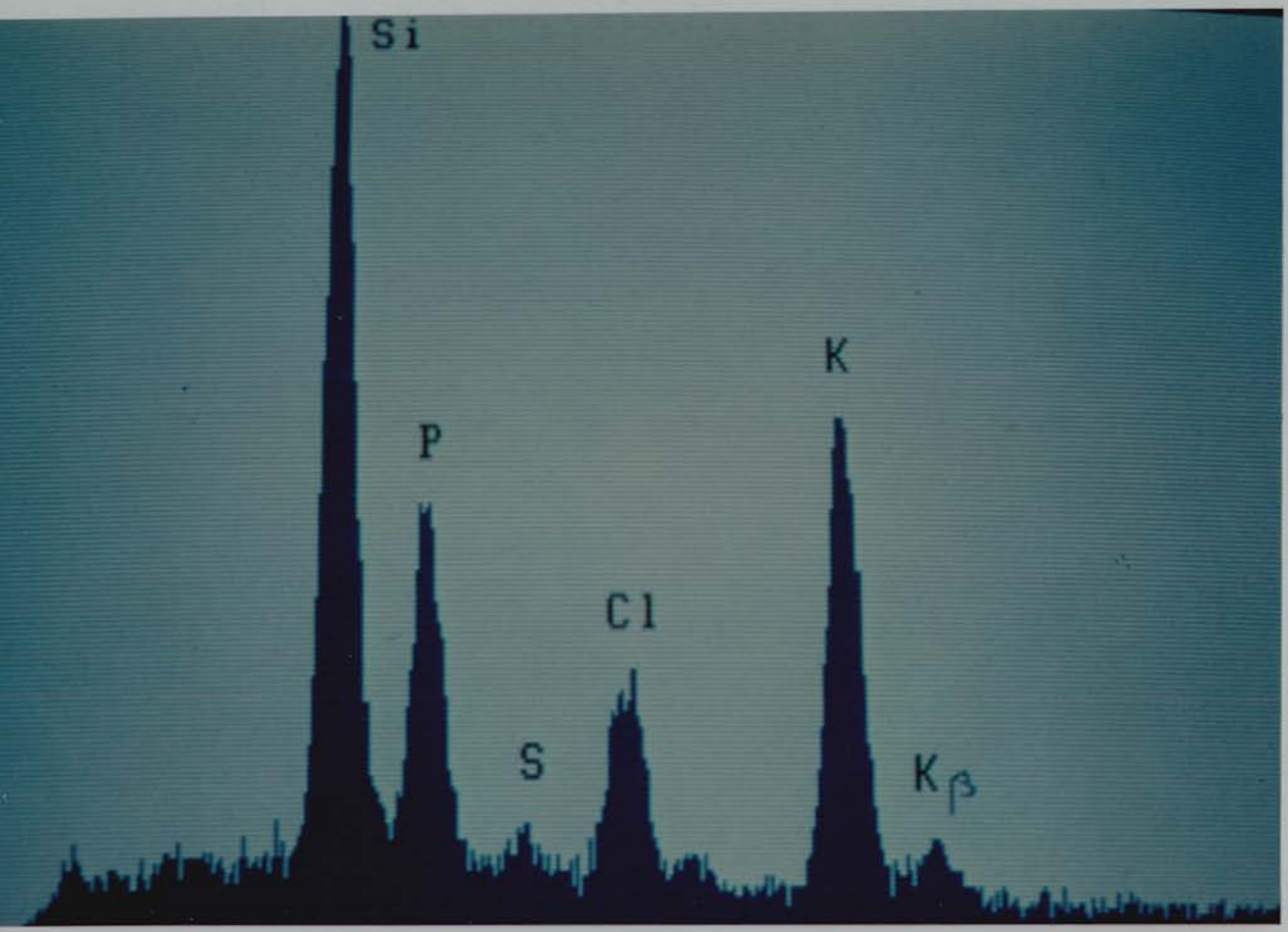


Figure 11
Averaged x-ray spectra illustrating the elemental composition inside(b) and outside (a) a giant secretory granule of the slug *Ariolimax columbianus*. The medium outside the granule is Ringer's solution. The large peak of intragranular calcium amounts in this case to 2.3 moles/kg of dry material. Statistical analysis of intragranular calcium concentration obtained in 22 granules showed an average of 2.49±0.935SD. The peak at 1.76 KeV in (b) corresponds to silicon contamination.


Si
P
K
Cl
S
Kβ

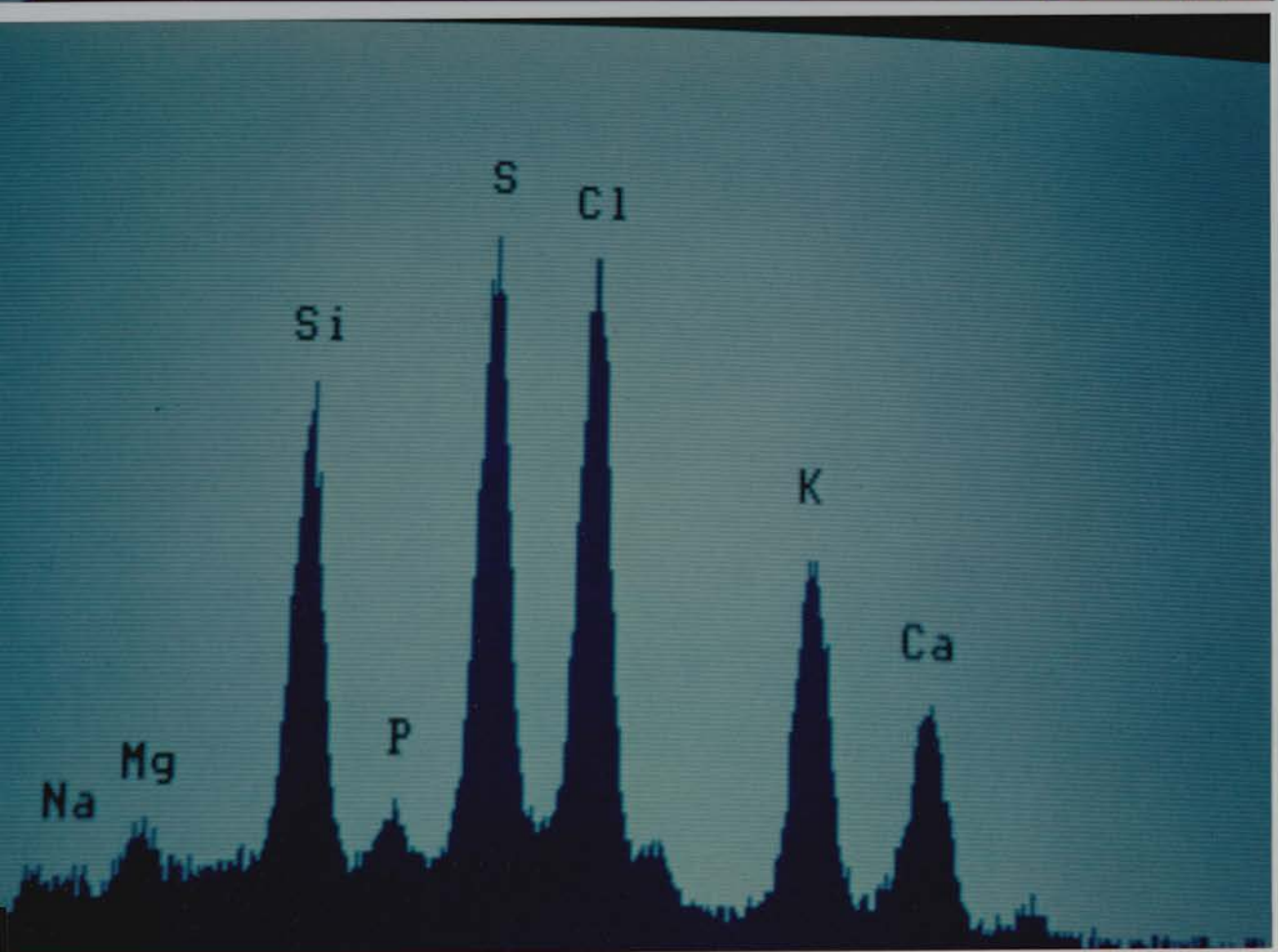

S
Cl
Si
K
Ca
P
Mg
Na

Figure 12
Radial expansion of a typical exocytosed mucin granule of a goblet cell in culture. The time course of swelling follows a typical first order kinetics. The continuous line is a transformed least-squares fit of the data points to eq. (1), $r(t)= rf-(rf-ri)e^{-t/\tau}$

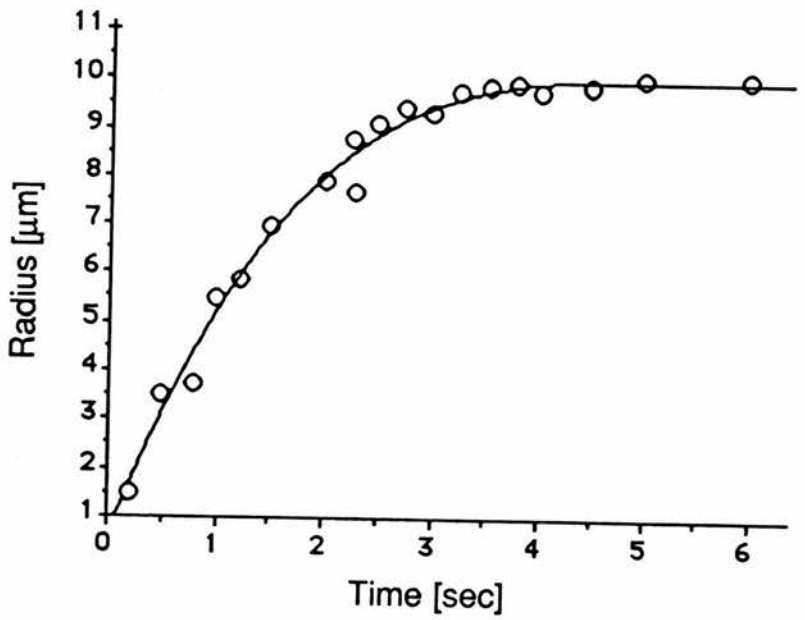


Figure 13
Characteristic time of swelling (τ) of exocytosed granules from a respiratory goblet cell equilibrated in a solution containing 1.4×10^{-7} M serum albumin, as a function of the final swelling radius (r_f^2). The line is a least-squares fitting to the data points.

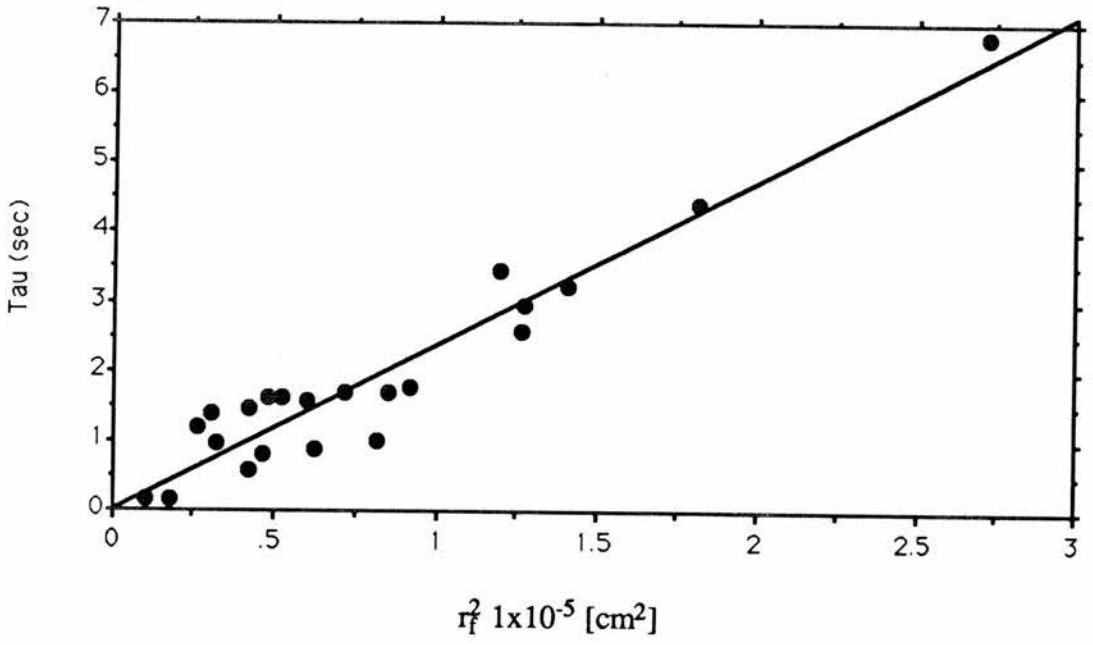


Figure 14
Schematic representation of product release in secretion. The upper panel shows the sequence of events in a typical exocytotic cell. After membrane fusion (a), the excitation-release coupling is initiated by the gating of an ionic channel that either releases a shielding cation or permits the inflow of an ion. The net result is that condensed polyions inside the granule become unshielded and rapidly de-condense, driving a large volumetric expansion and the release of the granular content(c).

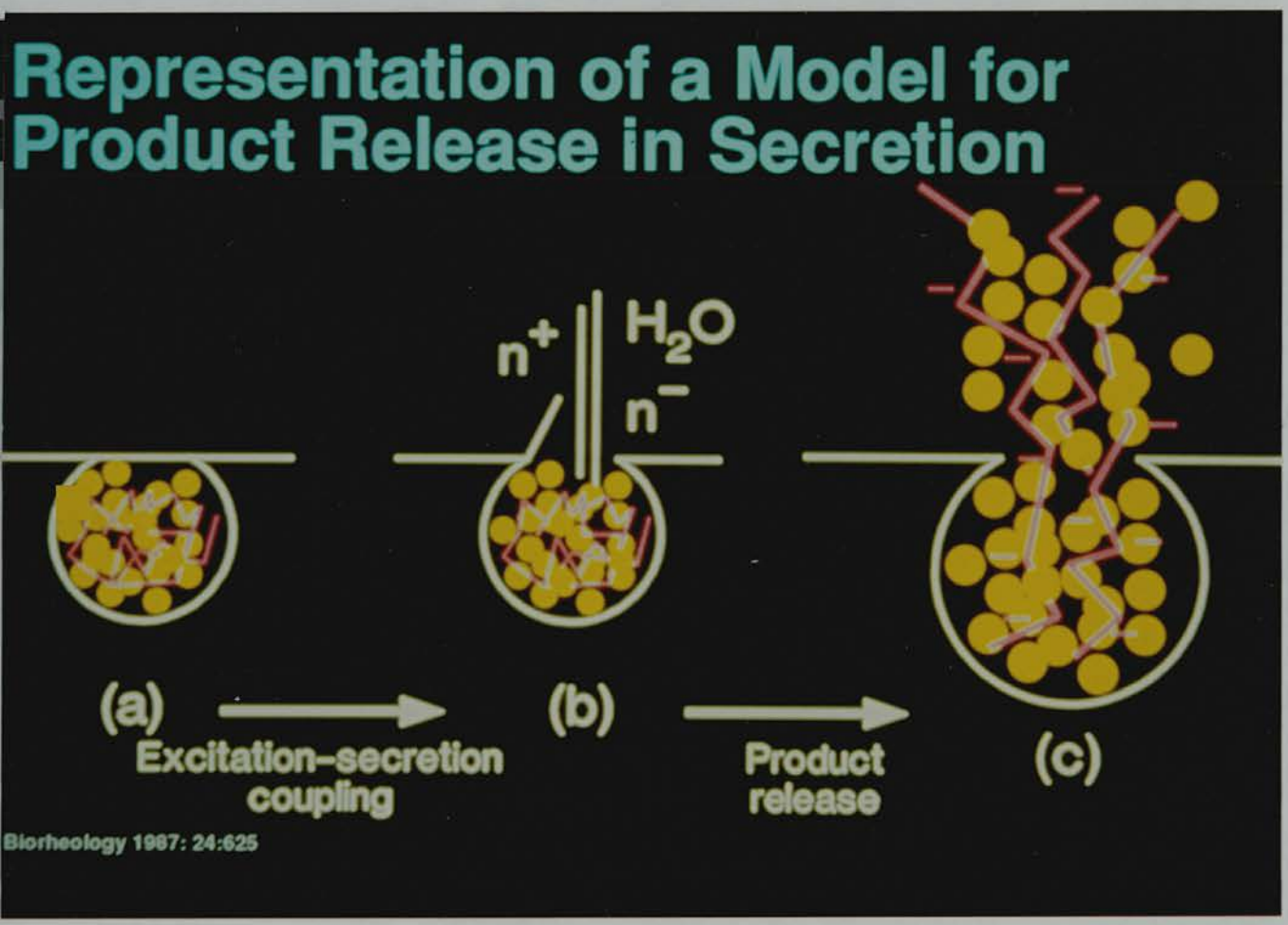


Figure 15

Characteristic time of swelling (τ) of secretory material released from exocytosed granules from a typical goblet cell in culture, as a function of the square of the final radius rf^2. n is the number of granules. Paired student t test was used to compare the regression lines. The shadowed area corresponds to the 95% confidence region of the linear regression lines. n is the number of observations. With increasing calcium concentration, the mucins swelled less.

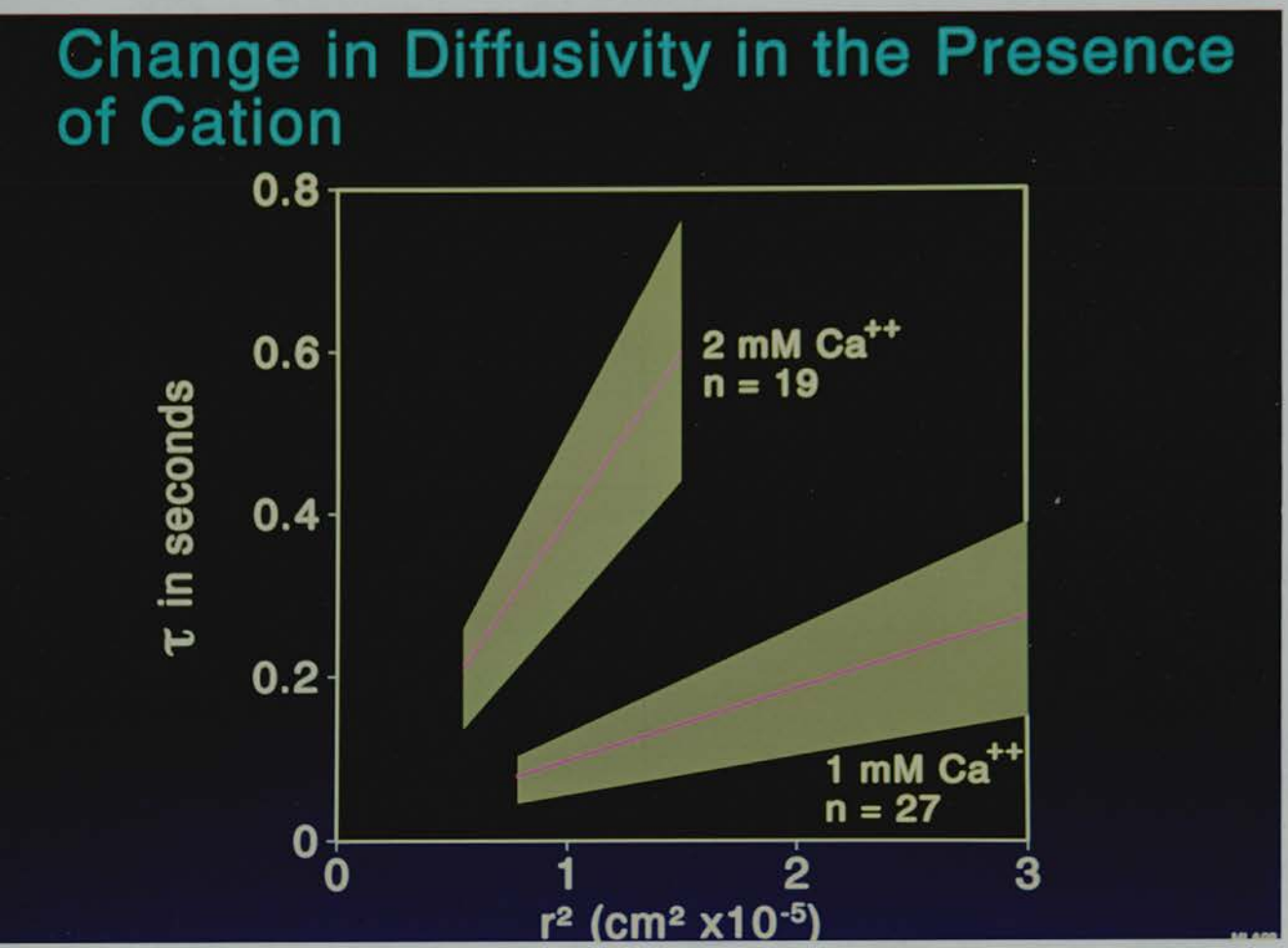


Figure 16

Mean % change in diffusivity ($\bar{D}$) ± SD of exocytosed mucins from respiratory goblet cells equilibrated in solutions containing different concentrations of serum albumin. n is the number of observations.

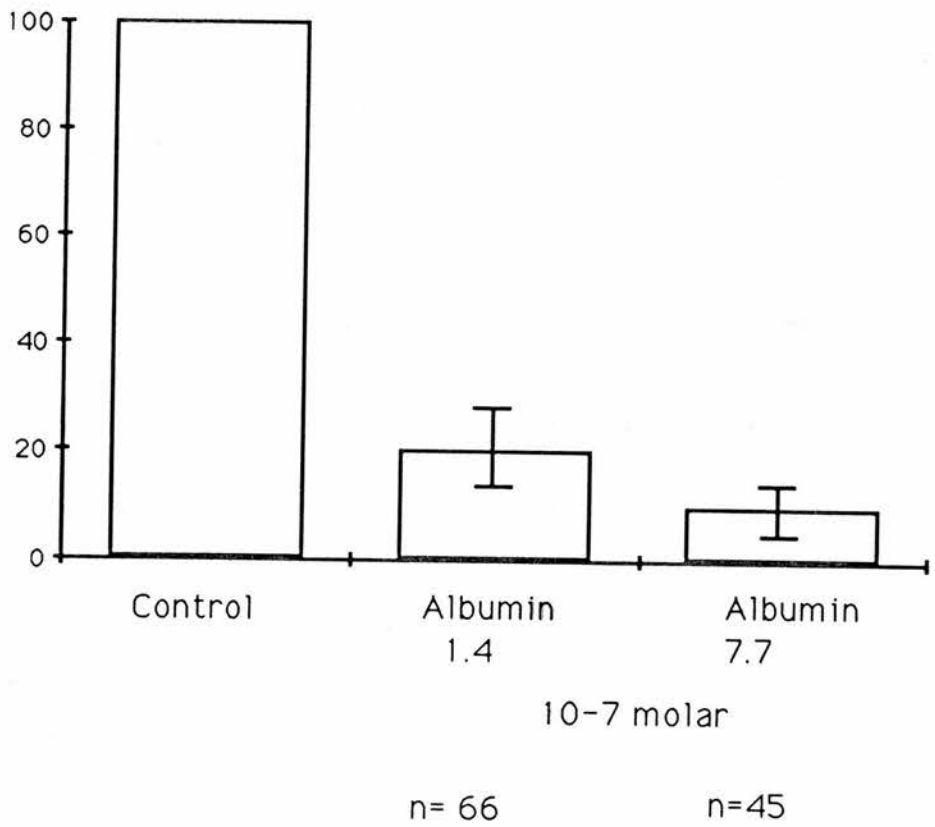


Figure 17
Cross-section of rabbit trachea epithelium (400X) , hematoxylin and eosin stain. The epithelium is removed by enzymatic digestion with pronase 0.1% at 37^0C for 30minutes followed by careful micro-dissection. The ciliated, goblet and basal cells can be identified in this preparation.

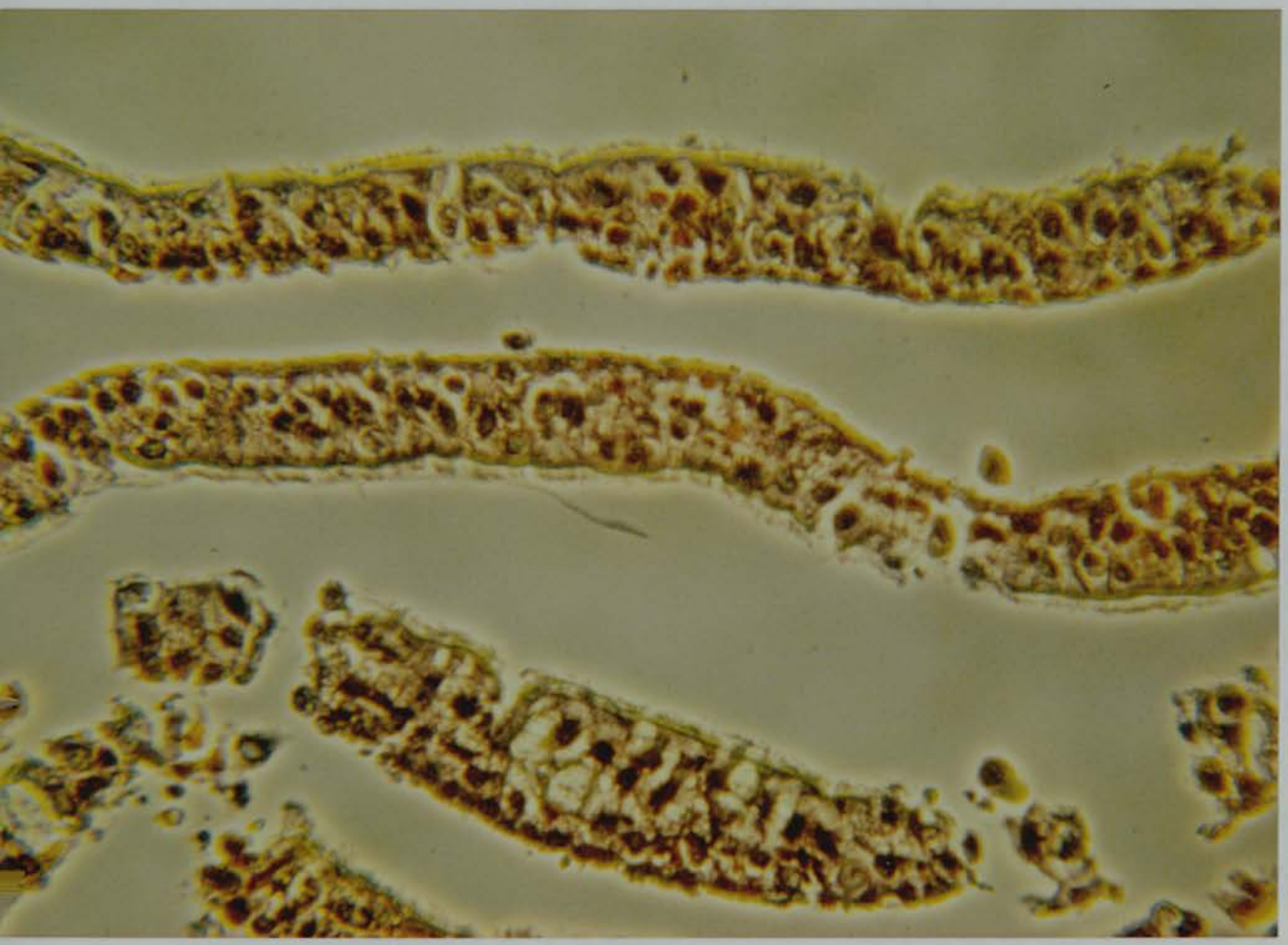


Figure 18
Schematic representation of flow cytometry. A krypton ion laser is used to excite the cells at 488nm. The morphologically different cells are sorted on the basis of variation of the forward and right angle scatter using a 100μm orifice and sorting rates of 1,500 cells per second.

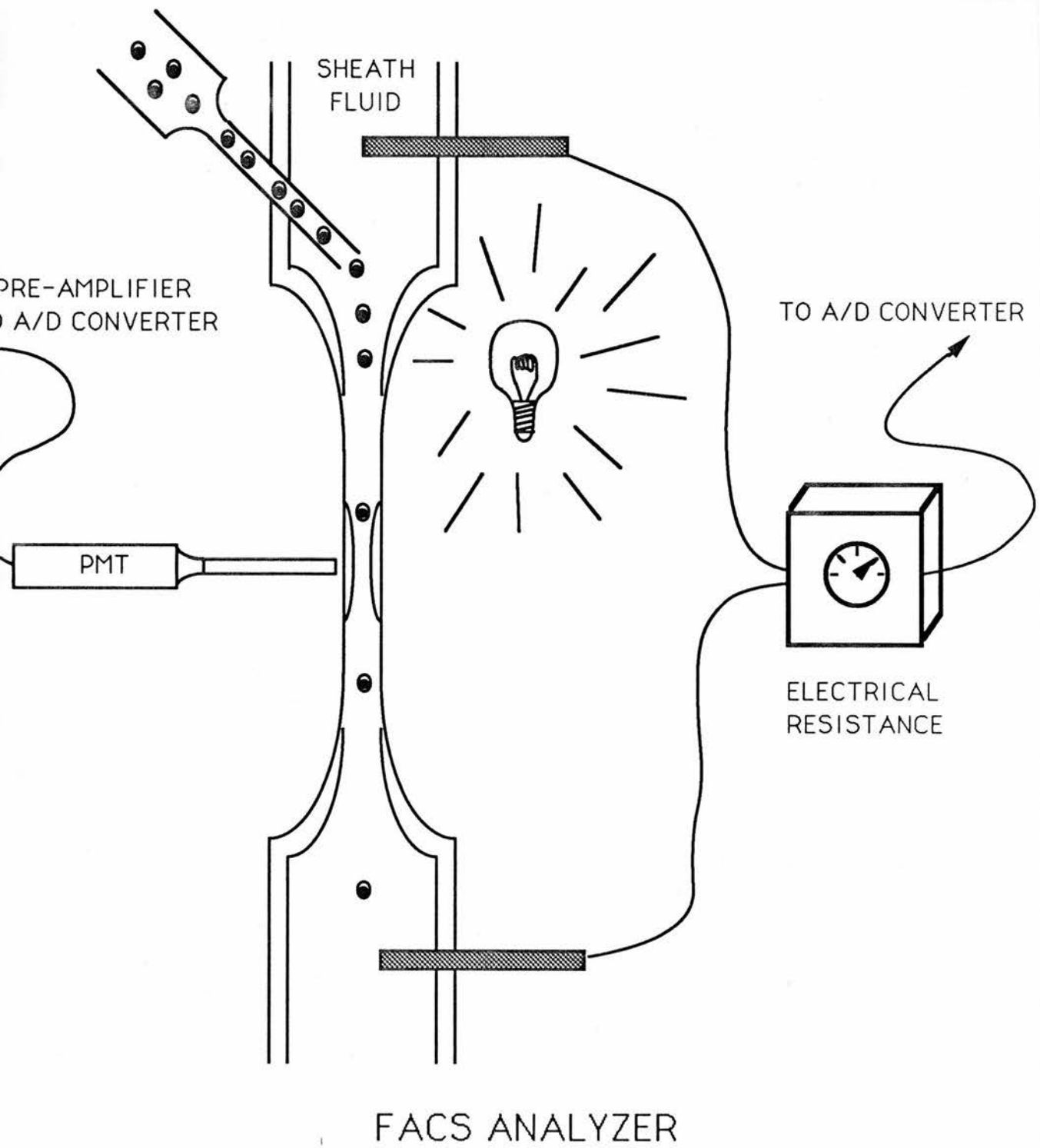


Figure 19
Light scatter analysis of rabbit tracheal epithelial cells. The left hand panel plots bivariate forward angle scatter versus 90^0 scatter. The encircled numbered areas outline the 3 cell populations. The right hand panel is the same experiment as in the left hand panel with the same axes and illustrating the the 3 populations as a contour plot. The encircled regions indicate the 3 different cell types corresponding to the goblet, basal and ciliated cells respectively.

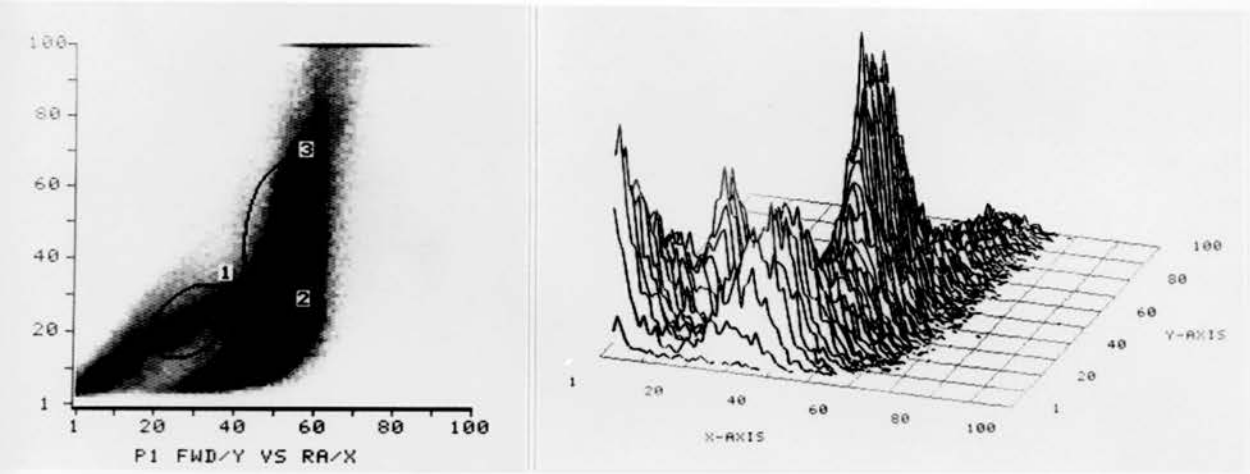


Figure 20
Three morphologically different cell types are retrieved by light scatter analysis: ciliated cells (a), basal cells (b) and goblet cells (c). Cilia can clearly be seen in panel a), large nuclei with a high nuclear/ cytoplasmic ratio in panel b), and cells showing secretory activity in panel c).

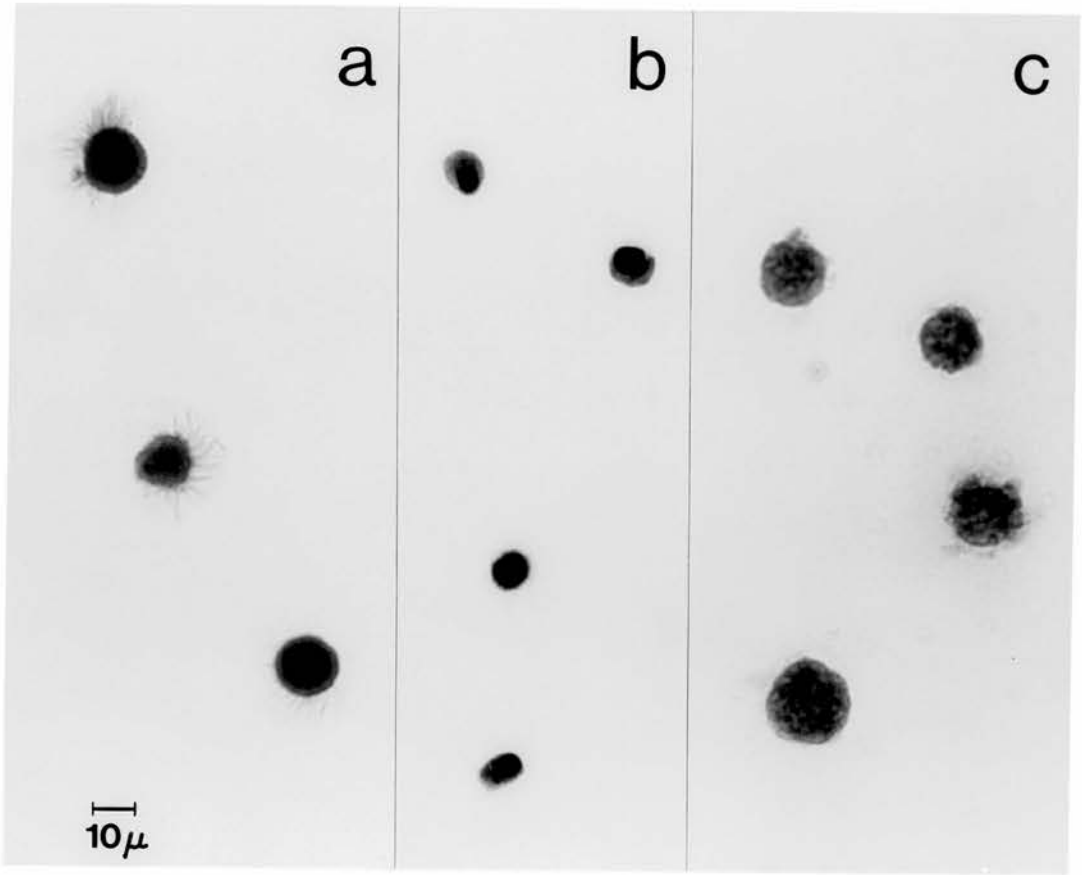
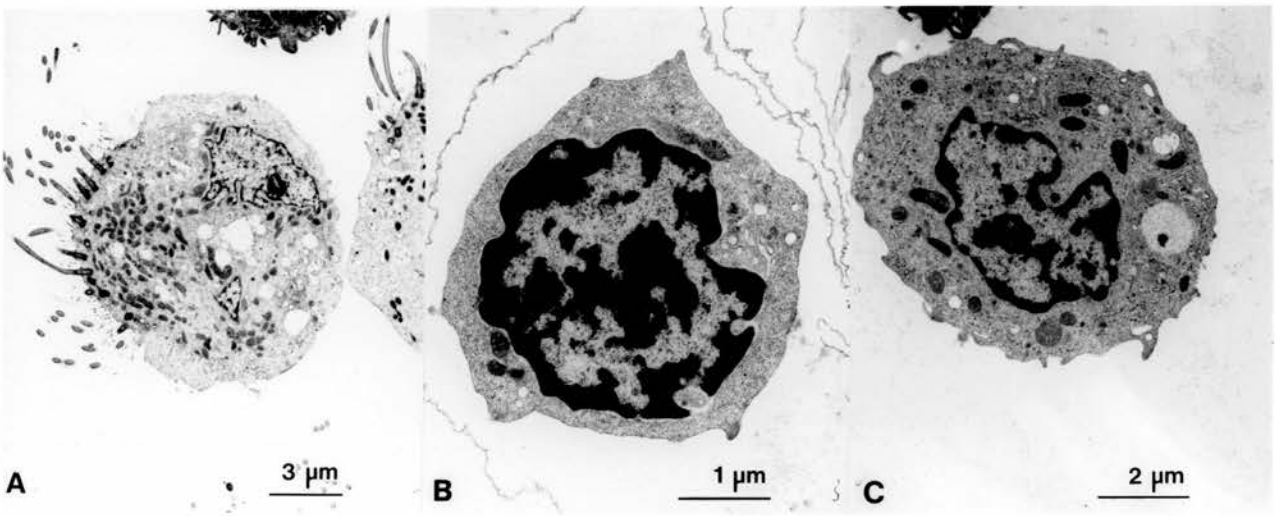


Figure 21
TEM of the 3 cell populations harvested from the flow cytometer. Three morphologically different cell types are retrieved by light scatter analysis: ciliated cells (a), basal cells (b) and goblet cells (c). The ciliated cell is 12 by 14µm. It is polarized with the nucleus at its base and cilia with their corresponding basal bodies at the cell apex. Many mitochondria are seen at the apex of the cell. Panel b) shows the small basal cell, 4-5µm in diameter. A high nucleus to cytoplasmic ratio is seen. Mitochondria are present but are far fewer in number than either the secretory or the ciliated cell. Panel c) shows the secretory cell, 8 by 10µm in diameter. A translucent granule can be seen as can mitochondria. Rough endoplasmic reticulum and a Golgi apparatus is seen to the left of the nucleus.



'igure 22
:DS polyacrylamide gel with silver stain of the 3 populations of cells found after light
catter analysis. As in Figures 20 and 21, lane a) corresponds to the ciliated cells, lane b) to
-ıe basal cells and lane c) to the goblet cells. The arrows indicate unique proteins expressed
'y each cell type.

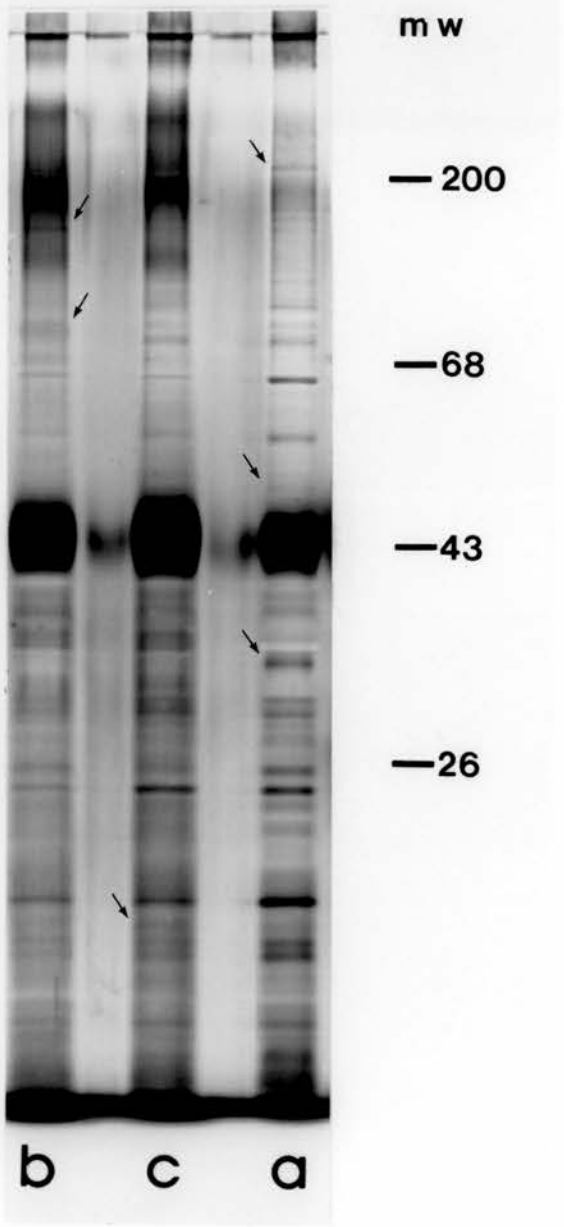
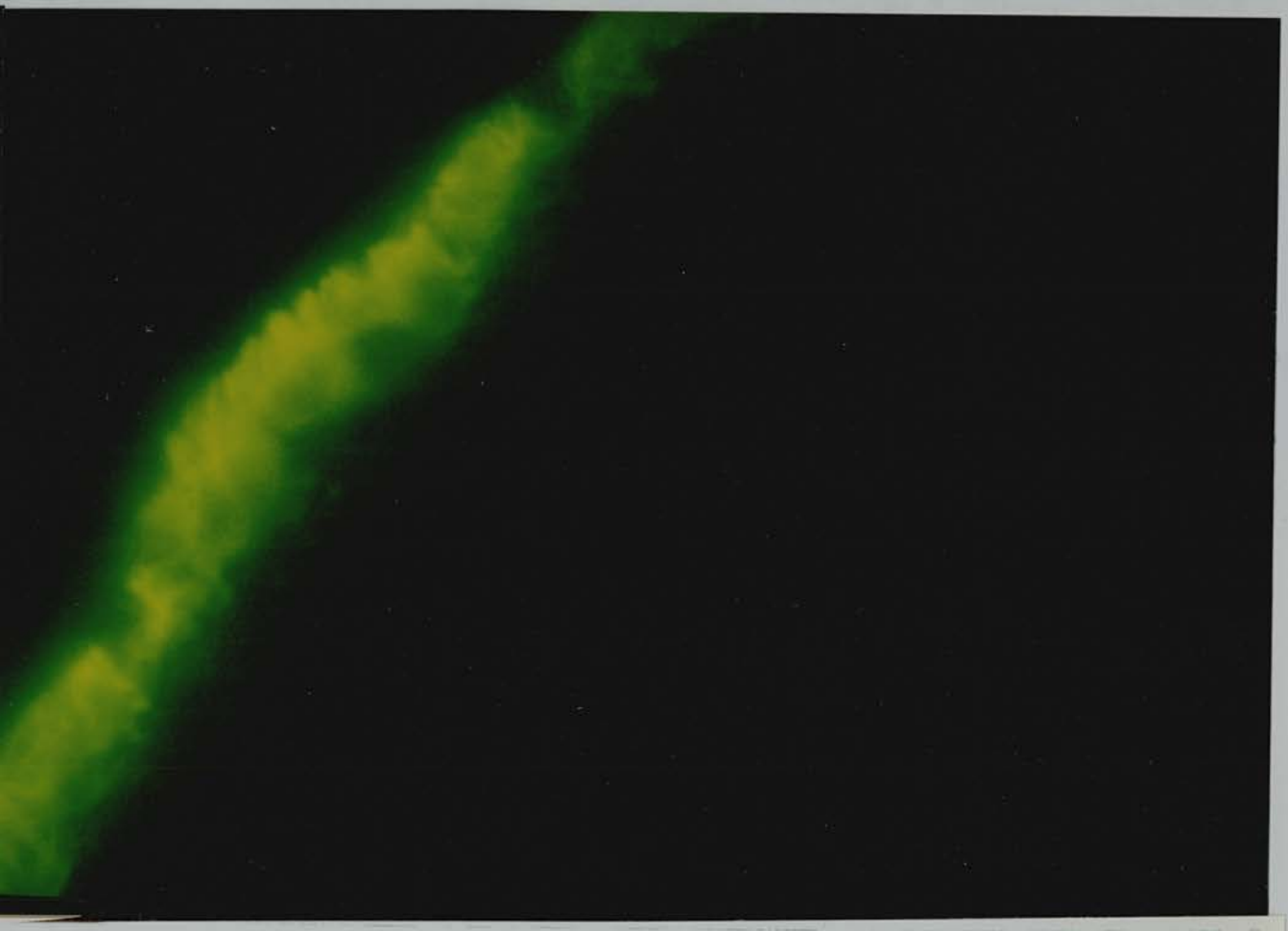


Figure 23
Shows a rabbit trachea section with and without immunofluorescent staining with 5B4/H3 antibody. The epitope appears to be on the lumenal surface of the epithelium (X400)



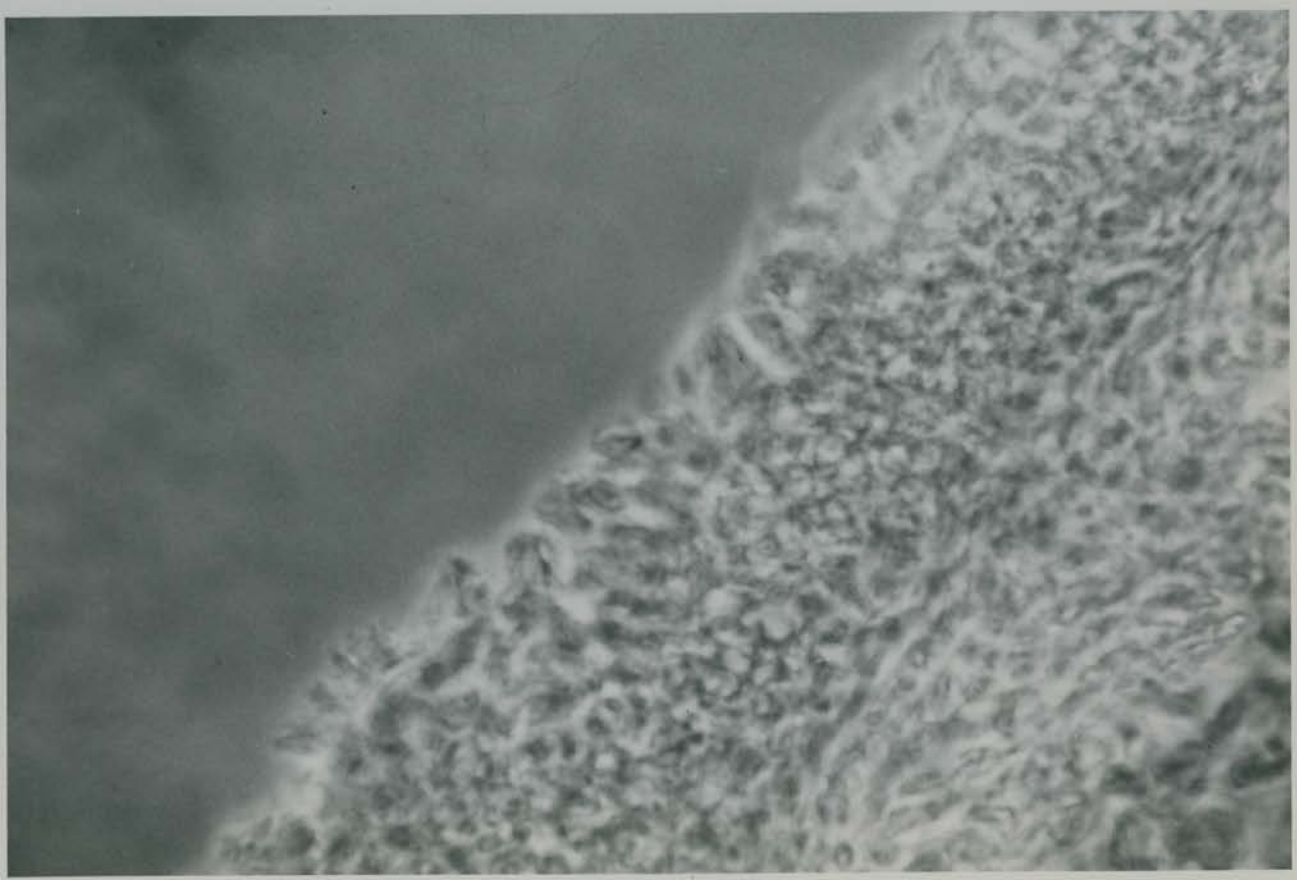
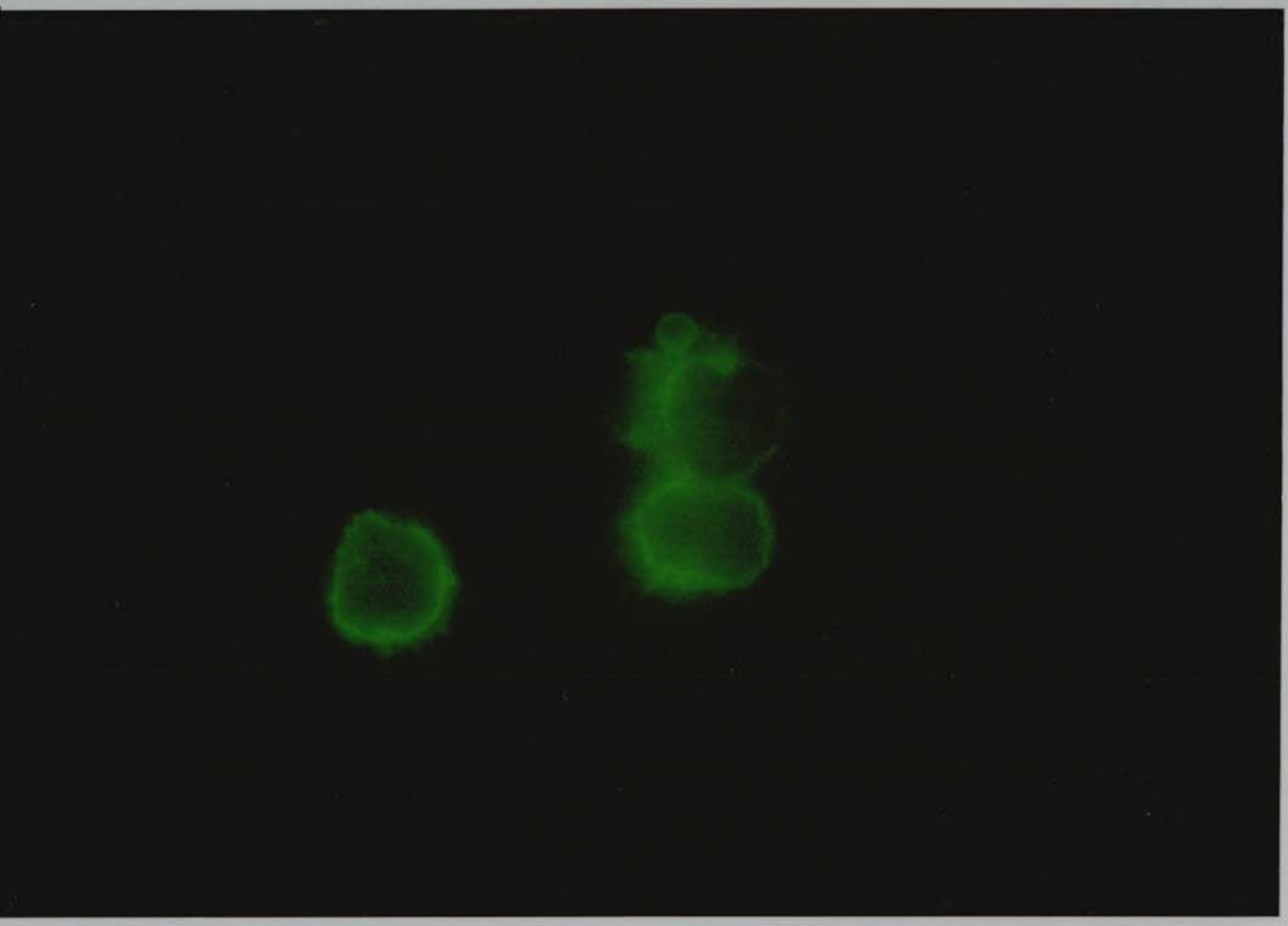
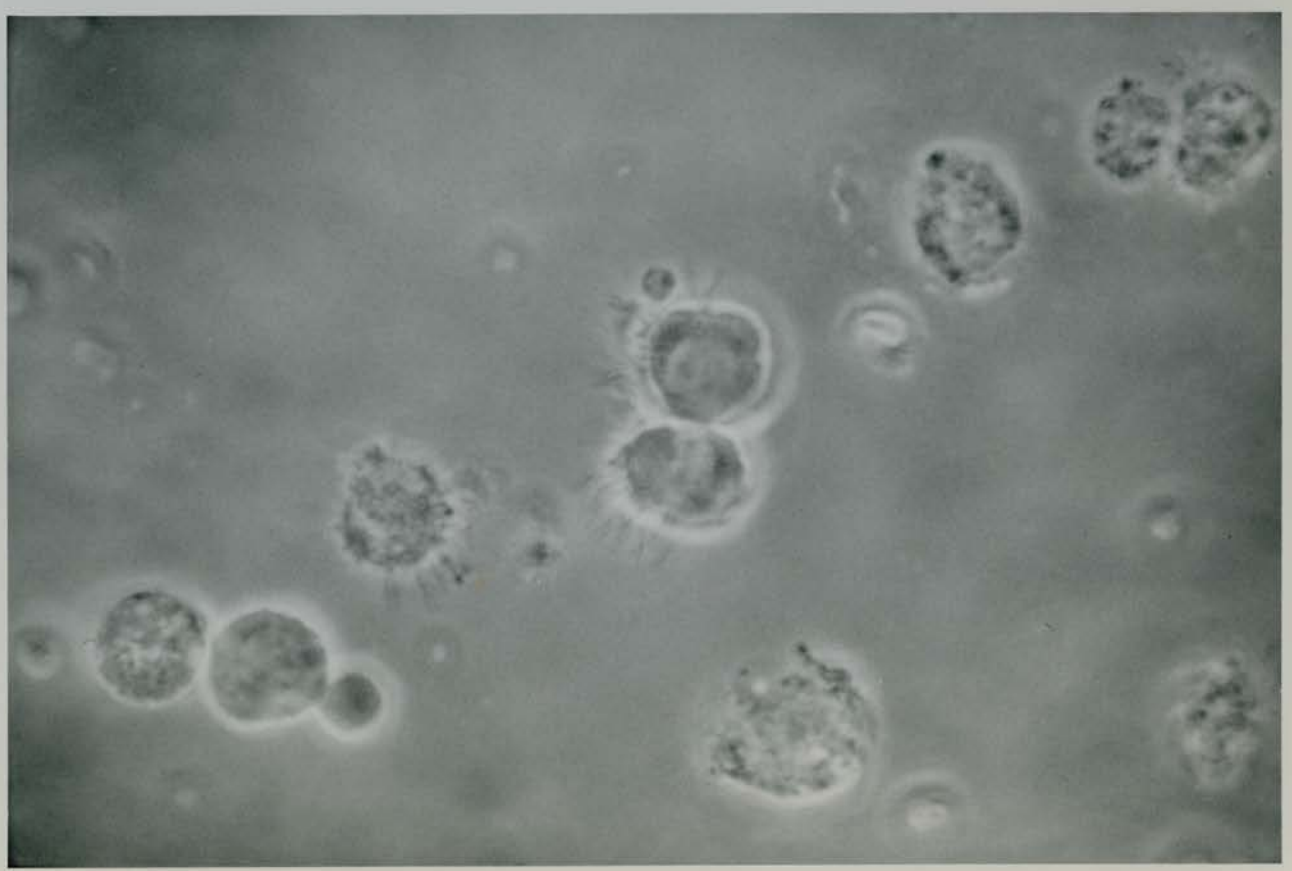
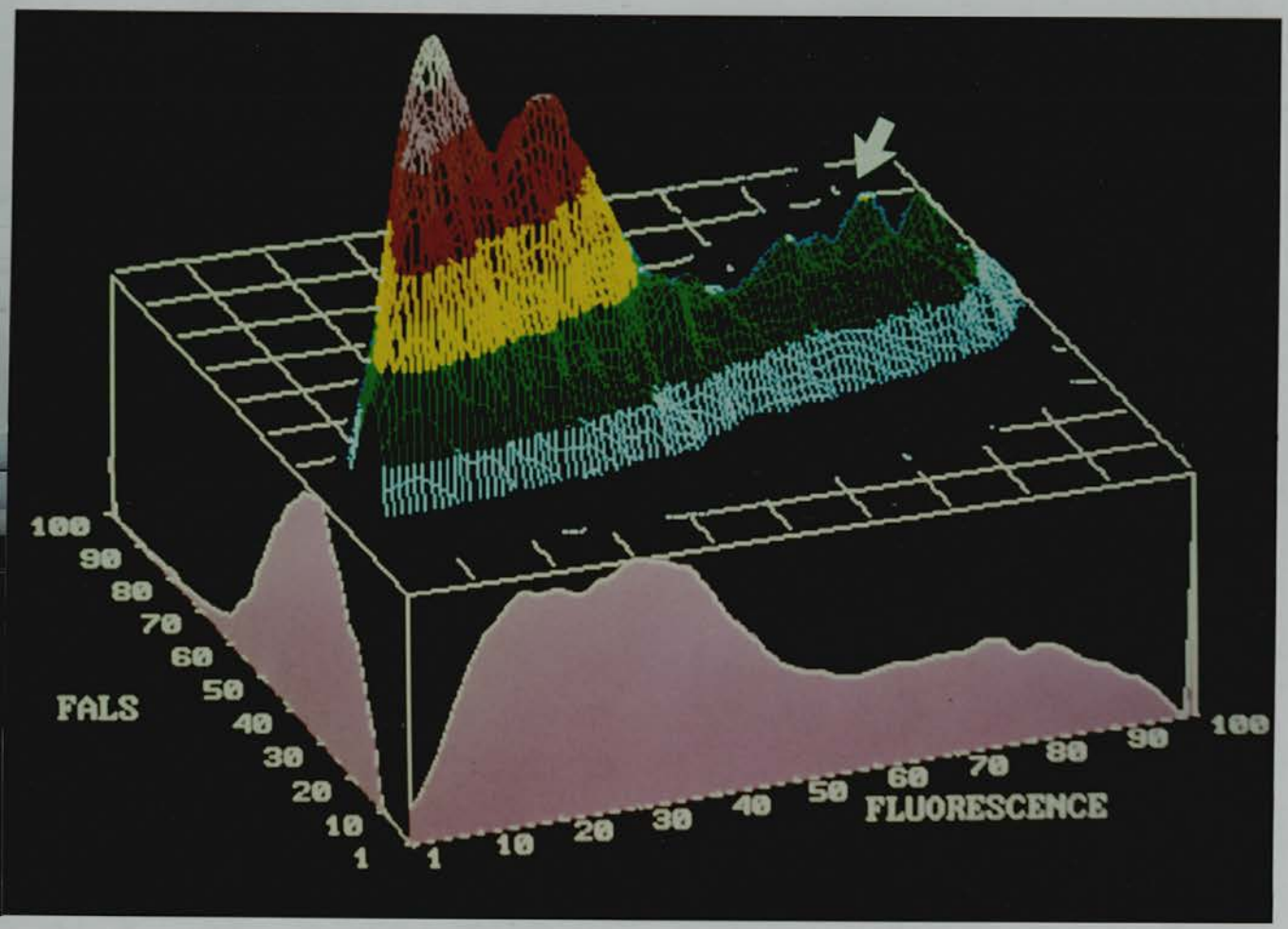


Figure 24
Shows the immunohistochemical appearance of enzymatically dispersed single cells with 5B4/H3 antibody. The morphologically distinct ciliated cells are florescent whereas the non-ciliated secretory and basal cells cells are not (X1000).





'igure 25
llustrates results of the flow cytometry of rabbit tracheal epithelial cells tagged with B4/H3 antibody. Forward scatter on the y-axis is plotted against green fluorescence on the -axis. 3 cell populations are identified as before. Only one of these populations, the iliated cells indicated by the arrow, are labelled with fluorescence ie the antibody is pecific to the ciliated cells. The purity of ciliated cells in this population was 99%.



'igure 26
'hows that the 5B4/H3 antibody is able to recognize the rabbit ciliated cell *in vitro*. The ntibody again appears to recognize a surface antigen of the ciliated cell. In this field non iliated cells can be recognized and are not stained with the antibody.

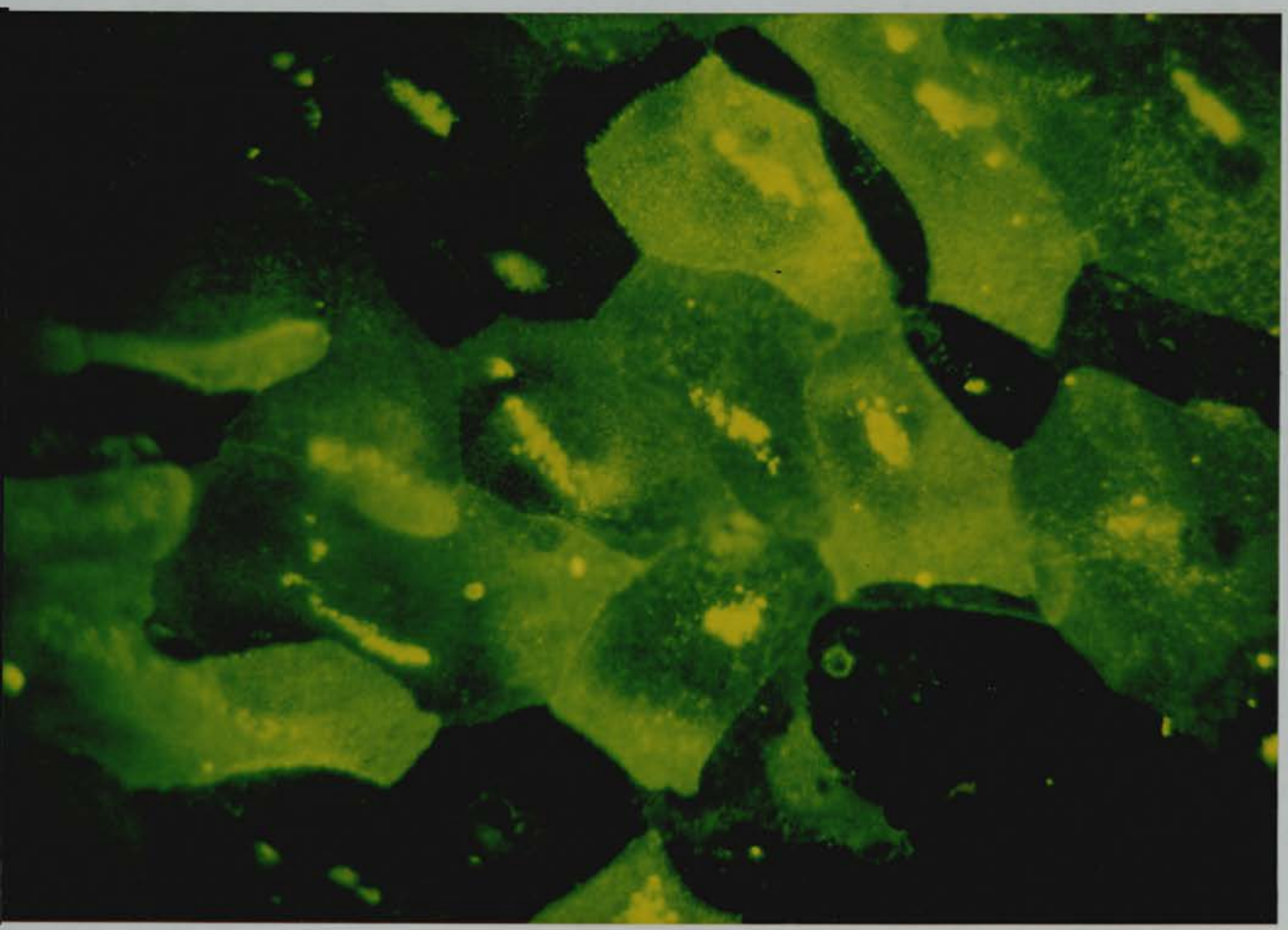


Figure 27
This figure shoes the immunohistochemical appearance of the 5B4/H3 antibody. The epitope is on the lumenal surface of the ciliated cell. It is found not only on the cilia but also on the surface of the ciliated cell itself.

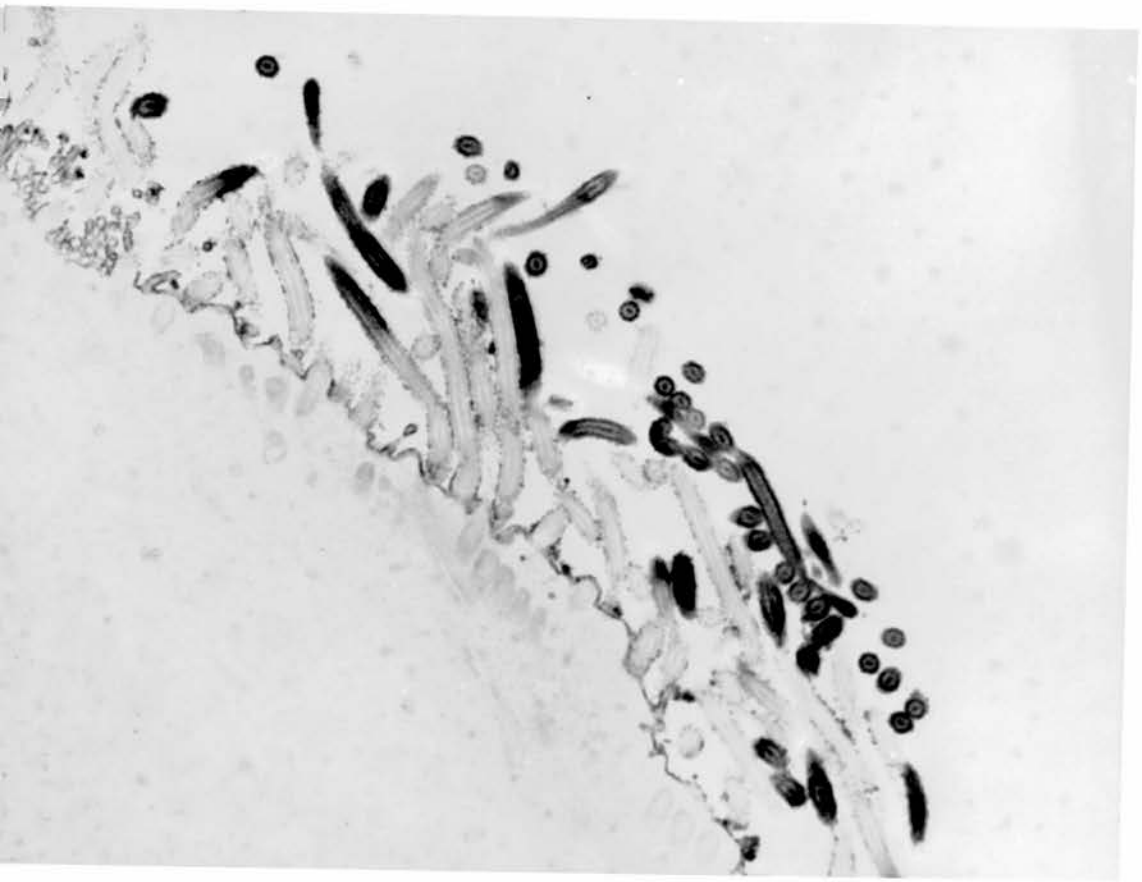


Figure 28
Shows the two dimensional gel of the rabbit tracheal epithelial cells, and the corresponding immunoblot of the 5B4/H3 antigen. The antigenic protein of the ciliated cell has a molecular weight of 130 kiloDaltons and a pI of 5.6.

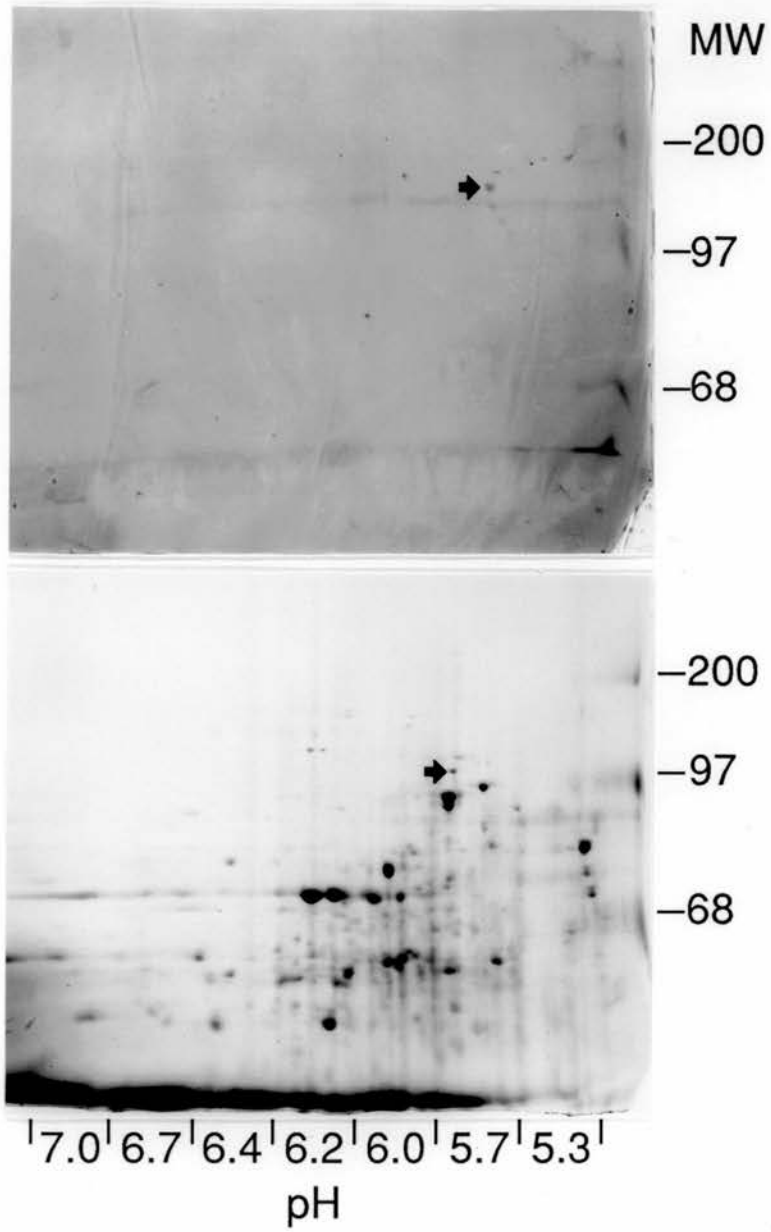
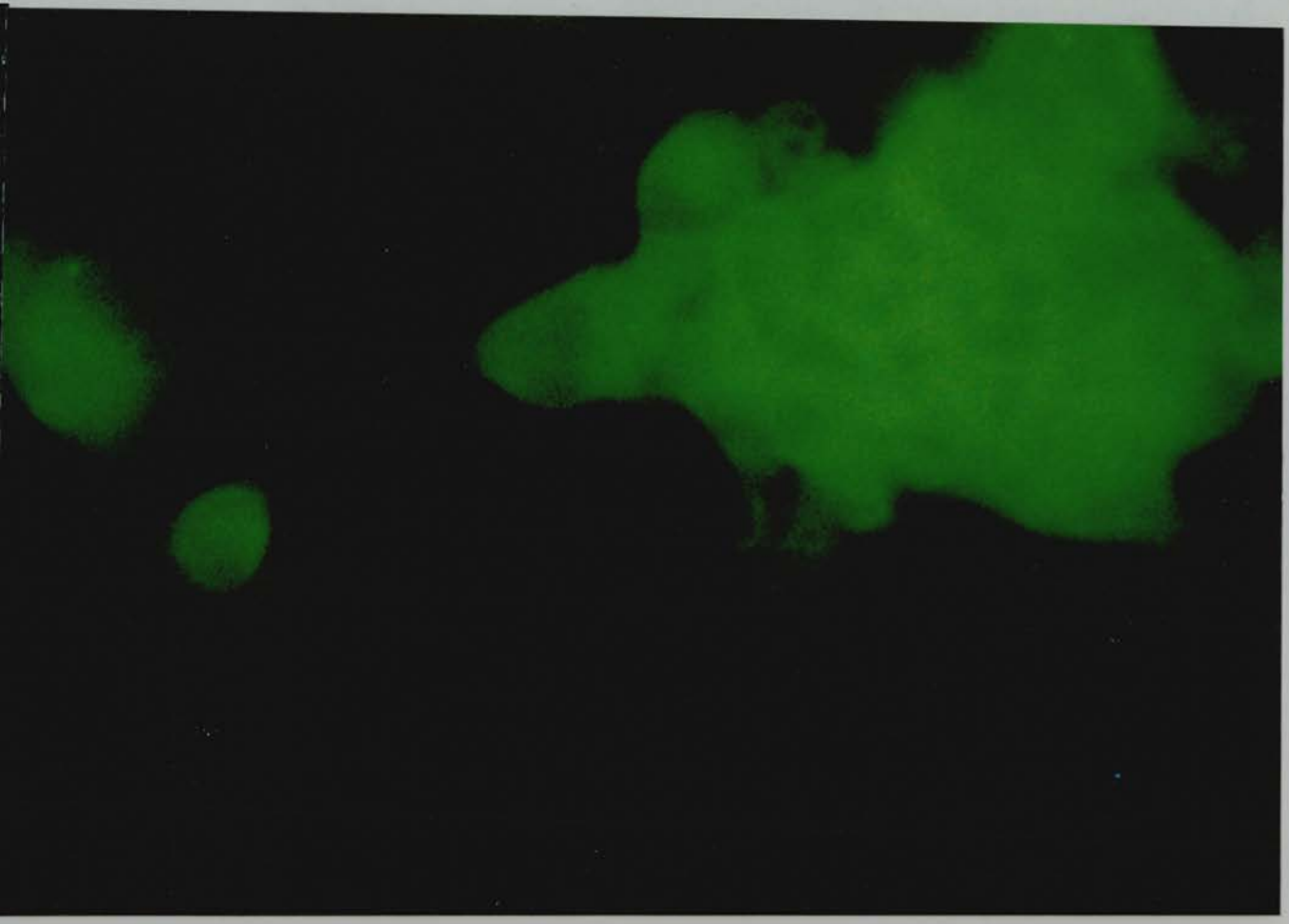


Figure 29
This shows a goblet cell *in vitro* identified by the 4D4 antibody of Dr Carol Basbaum.



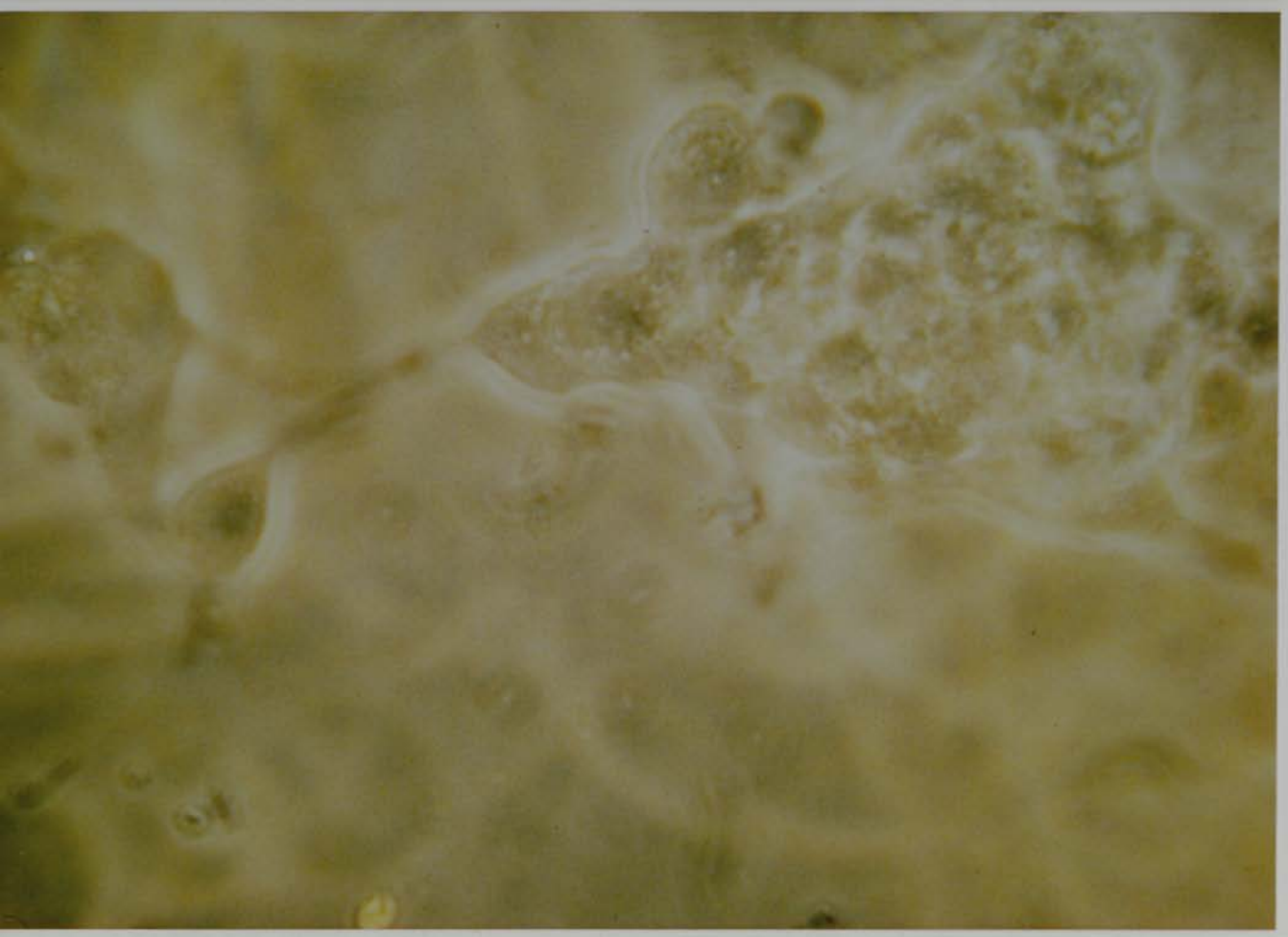
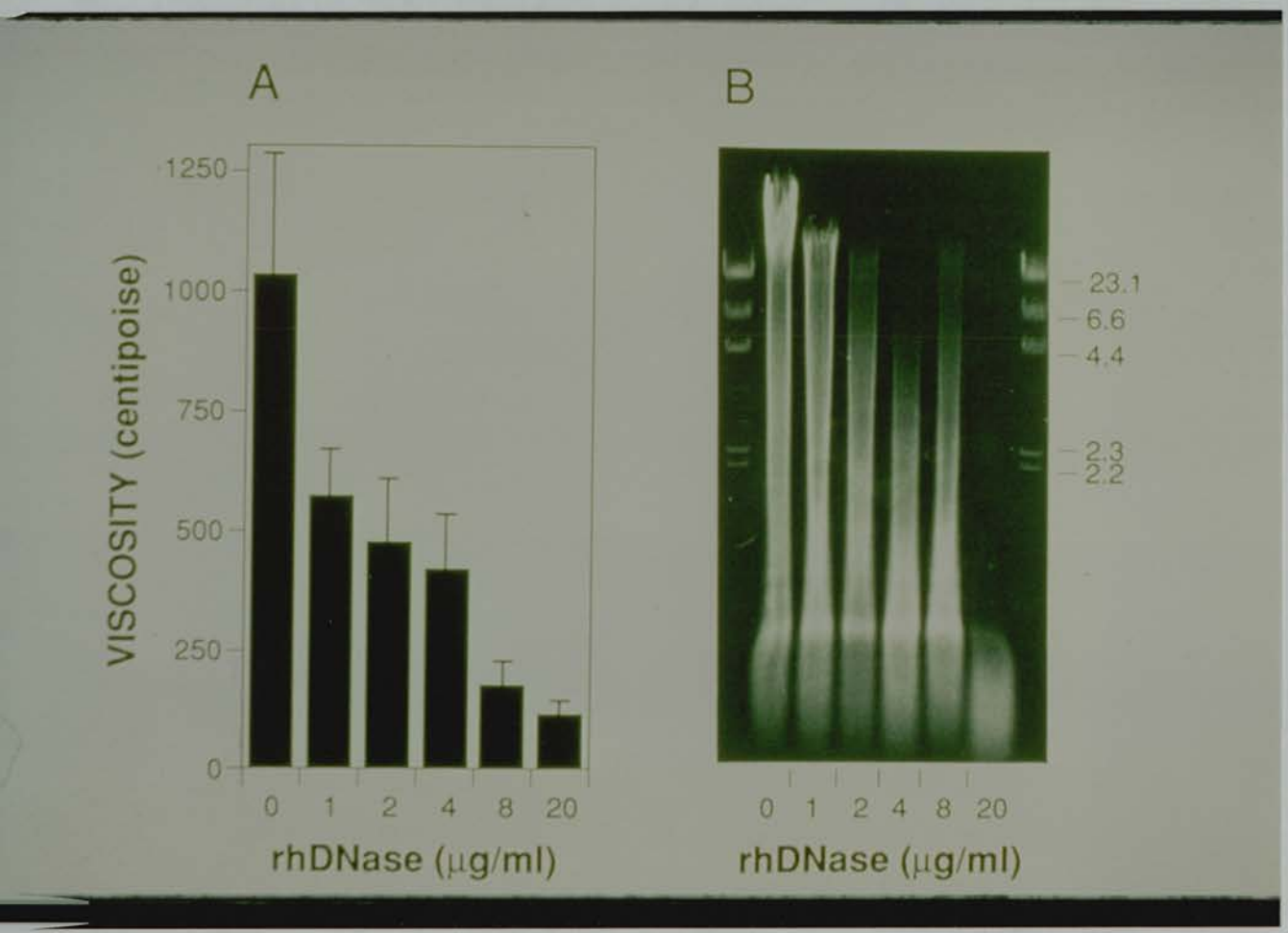


Figure 30
The left hand figure represent a patients sputum sample with increasing amounts of DNase against viscosity measured using a Brookfield cone and plate viscometer. With increasing amounts of DNase, the viscosity decreased. The right hand panel represents an agarose gel with DNA of shorter lengths with increasing DNase.



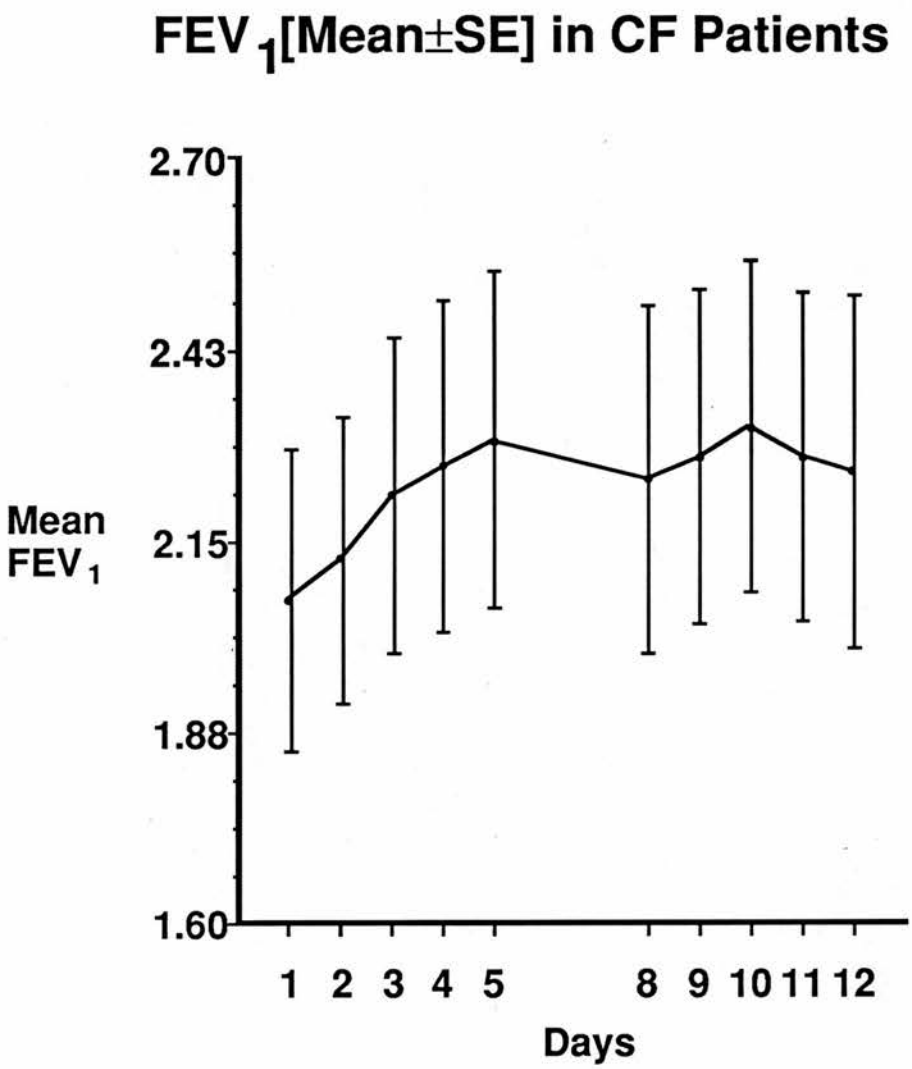


Figure 31: Legend

The FEV_1 (mean±SE) of each morning prior to dosing in 12 CF patients. The FEV_1 improved over the 12 days of treatment ($p=0.01$). The improvement occurred in the first five days of treatment ($p<0.05$) with no additional change during days 8-12 ($p=0.88$). See text for statistical methods.

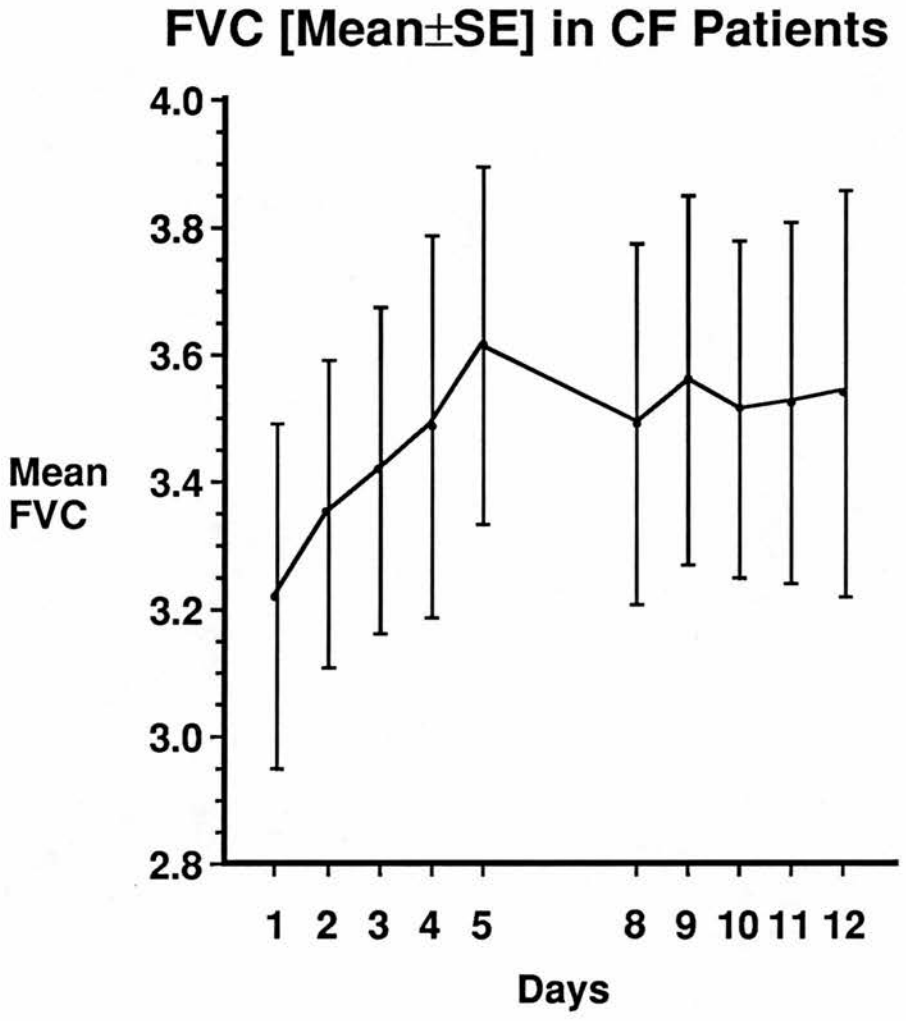


Figure 32: Legend

The FVC (mean±SE) of each morning prior to dosing in 12 CF patients. The FVC improved over the 12 days of treatment (p=0.01). The improvement occurred in the first five days of treatment (p=0.001) with no additional change during days 8-12 (p=0.77). See text for statistical methods.

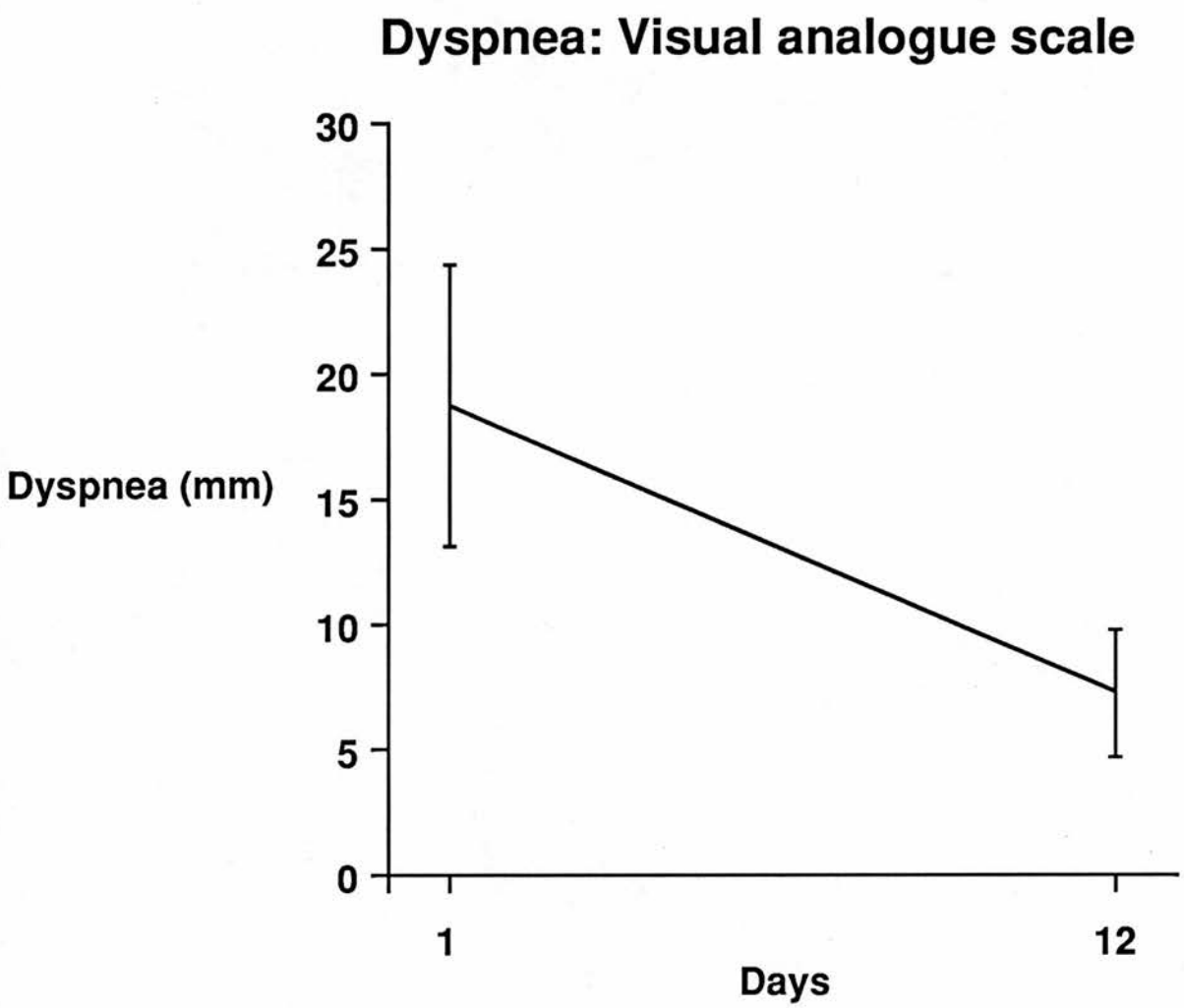
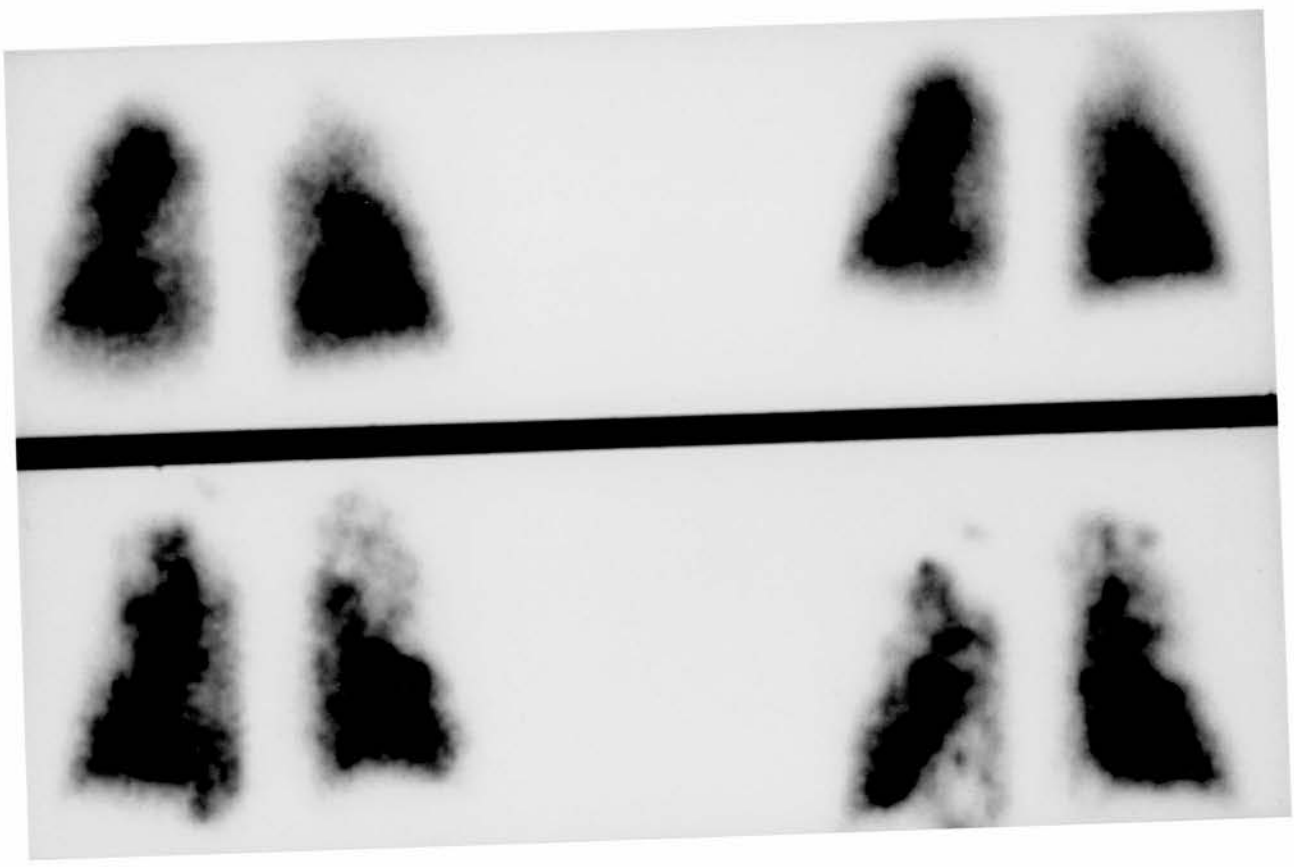


Figure 33: Legend

The visual analogue scale (mean ± SE) in the 12 CF patients. The score improved in 10/12 patients ($p < 0.01$). Two patients scored zero both on day 1 and day 12.

Figure 34
Ventilation scans with radiolabelled albumin in 2 patients before and after aerosolized DNase. These are posterior views of the chest with the pre- dose scans on the left. The upper panel shows an essentially normal ventilation scan in CF patient #12. This remained unchanged after 10 days of rhDNase. The lower panel of CF patient #11 shows patchy abnormalities especially in the right upper lobe and to a lesser extent in the left upper lobe before rhDNase. Following rhDNase, ventilation to the left lower lobe has decreased. See text for details.



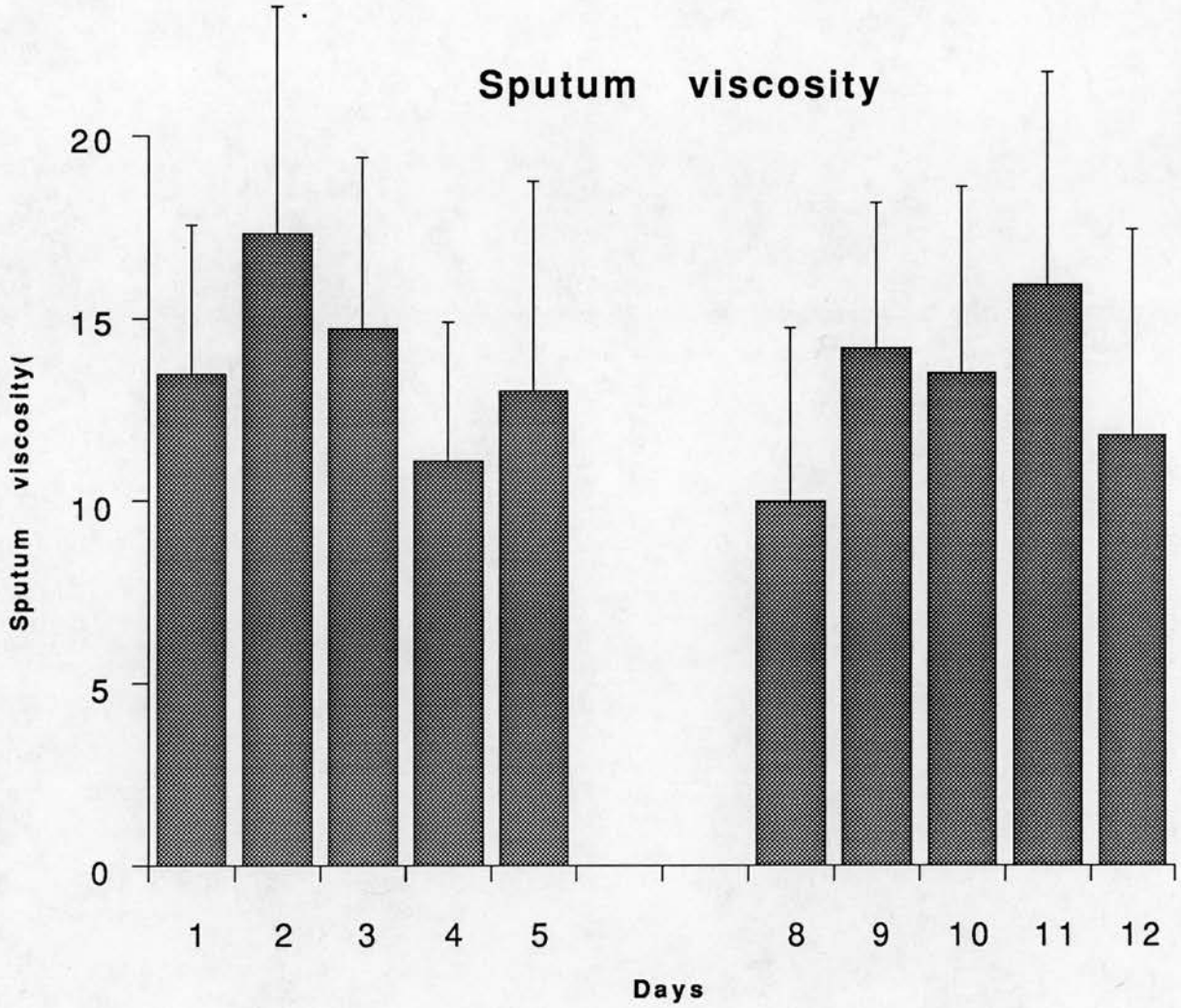


Figure 35
Sputum viscosity as measured by a Brookfield cone-plate viscometer in 4 subjects. Sputum viscosity decreased in 3/4 patients ($p>0.05$). See text for details.

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