Experimental Models for Studying Mucociliary Clearance

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The Viscoelastic Properties of Mucus:

Due to the crosslinking of glycoproteins, mucus rheologic behavior is described as viscoelastic, having characteristics of both a liquid and a solid (King 1989, King & Rubin 1994). Viscosity is the resistance to flow and represents the capacity of a material to absorb energy as it moves. Elasticity is the capacity of a material to store the energy used to move or deform it. With ideal fluids, viscosity is independent of the applied stress. With viscoelastic liquids such as mucus, viscosity decreases with increasing stress or rate of strain (shear rate). Mucus responds to stress with an initial solid-like deformation followed by a viscoelastic deformation and finally by a period of steady flow in which the rate of deformation is constant. Only partial recovery of the strain follows removal of the stress, indicating a permanent deformation of its gel structure. Changes in mucus viscosity and elasticity are generally interrelated.

Mucus exhibits shear thinning following exposure to high shear forces, exhibiting a decreased viscosity at low shear rates. Some shear thinning may be permanent, indicating altered molecular structure, while some shear thinning, termed *thixotropy*, may be reversible.

Mucus viscoelasticity can be effectively described by two relatively independent quantities, G* and tan δ, which vary with the measurement frequency. G*, the mechanical impedance, is the vector sum of viscosity and elasticity; it can be termed the *rigidity factor*. Tangent δ is the ratio of viscosity to elasticity; it is also known as the loss tangent and can be considered as a *recoil factor*. The relative proportions of elasticity and viscosity are important in describing how a material such as mucus behaves when it is subjected to external forces.

Rheologic Assessment of Mucus:

Mucus collection: Tracheal mucus can be collected by a modification of the cytology brush technique or the endotracheal tube collection technique (King & Rubin 1994). Cytology brush collection involves placing a soft-bristled cytology brush against the pulmonary airway and removing the brush once it is covered with sufficient mucus for analysis. Endotracheal tube collection involves the removal of the mucus layer coating a freshly removed endotracheal tube. These techniques provide sufficient mucus for analysis even from very small animals (App & King 1990).

Magnetic microrheometer (King 1988): This instrument can be used to measure the bulk viscosity and elasticity of microliter quantities of mucus. A 100 μm steel ball is positioned in a 1-10 μL sample of mucus and oscillated by means of an electromagnetic field gradient. The motion of this sphere is tracked with the aid of a photocell. Plots of ball displacement versus magnetic force are used to determine the viscosity and elasticity of the mucus as a function of applied frequency. These rheologic properties can be used to predict the effectiveness of mucus in clearance, both by ciliary action and for clearance by airflow interaction (King 1987). The magnetic microrheometer is particularly suited to animal model studies because of the minimal sample requirement.

Filancemeter (Puchelle *et al.* 1983): *Spinnability* (*Spinnbarkeit, filance*) is the thread forming ability of mucus under the influence of low speed elastic deformation. Using a filancemeter, a 20 to 30 μL mucus sample is stretched at a distraction velocity of 10 mm/s. An electric signal conducted through the mucus sample is interrupted at the point where the mucus thread is broken; the length of this thread is known as the mucus spinnability (in mm). Spinnability has been correlated positively

with mucociliary clearance (Puchelle *et al.* 1983) and negatively with cough clearance (King *et al.* 1989). Although the filancemeter requires greater volumes of mucus than the magnetic microrheometer, it has two main advantages--its ease of use, and the fact that the measurements appear to be more sensitive to alterations in molecular weight of crosslinking macromolecules, as evidenced by the response of cystic fibrosis sputum to DNase and gelsolin (King *et al.* 1995a).

Adhesivity is the ability of mucus to bond to a solid surface measured as the force of separation between one or more solid surfaces and the adhesive material. This is dependent on mucus surface tension, hydration, wettability, and contact (dwell) time. Adhesivity has been found to correlate inversely with both mucociliary clearance and cough clearance (Puchelle *et al.* 1987, King *et al.* 1989a).

Solids content/ Collection rate: There is generally a positive relationship between the solids composition of mucus and the viscoelastic properties, although the relationship can change with disease state or source of mucus (Tomkiewicz *et al.* 1993). We use a microwave drying apparatus and microbalance to calculate the percent solids for samples of mucus larger than 5 μL. The mucus samples are also weighed to determine an index of secretion rate (mucus collection rate, mg/min). In dogs, knowing the mucus linear velocity, the airway circumference and the collection rate, we can estimate the *in vivo* mucus depth (King 1985a).

Transepithelial potential difference (PD): The transepithelial potential difference represents an integral of the ion fluxes across an epithelial membrane. PD measurements are useful in mucociliary clearance studies since variations in PD relate to changes in the ion and water content of mucus (Tomkiewicz *et al.* 1996); they also help to assess the integrity of the epithelium, since alterations of cellular and paracellular pathways contribute to PD. PD is measured by using two flexible microelectrodes connected with KCl-saturated agar bridges to calomel half-cells. The reference electrode is placed subcutaneously, and the test electrode is placed at various locations on the epithelial surface. Our laboratory has made extensive use of this technique in rats, dogs, and frogs (De Sanctis *et al.* 1990, App *et al.* 1993, Festa *et al.* 1995).

Mucus and Mucociliary Clearance:

There are two major mechanisms for clearing mucus from the airways – by ciliary action, the primary mechanism, and when this fails or is overloaded, by coughing or other forms of airflow interaction. Methods for studying mucociliary clearance range from *in vitro* direct observation (e.g. frog palate) to *in vivo* tracer methods (e.g. inhaled, radiolabeled particles). Cough or airflow clearance can be studied in both mechanical models, as well as *in vivo*, with the use of appropriate tracers.

In dogs and other large animals, tracheal mucus velocity (TMV, mm/min), i.e. the *linear* velocity of mucociliary clearance, can be determined by direct observation of marker particle movement with the aid of a fiberoptic bronchoscope (King *et al.* 1983). Mainstem bronchial mucus velocities can also be measured under bronchoscopic control; these are useful in monitoring the effects of local or unilateral interventions. The main advantages of measuring TMV is that it is straightforward, and can be carried out at the same anatomical site where measurements of epithelial potential difference are performed, and from where mucus is collected for rheological and chemical analysis.

The viscoelasticity of the mucus layer contributes to the effectiveness of the mucociliary interaction, but the surface interaction between mucus and cilia also play a critical role. The transport velocity of mucus simulant gels is directly related to mucus elasticity and the depth of the periciliary fluid, and is inversely related to mucus viscosity (King 1989). Giordano *et al.* (1978) studied the tracheal mucous velocity of dogs prepared with tracheal pouches and found a clear negative correlation between the *in vivo* tracheal clearance rate and the elasticity of the mucus secreted by the pouch.

Results generally similar to this have been obtained in studies employing the frog palate as a model ciliated epithelium; i.e., ciliary transport rate decreases with increasing rigidity or "thickness" of the mucus, whatever quantitative measure of this is used (King 1979, 1989). A similar relationship between mucus viscoelasticity and clearance has also been demonstrated for rat nasal epithelium (Lorenzi *et al.* 1992).

The *ratio* of viscosity to elasticity is also an important determinant of mucociliary clearance. Increasing viscosity at constant elasticity in a model system caused a pronounced decrease in the mucociliary transport rate (King 1980). Although this phenomenon has not been observed for intact mucus from healthy animals, it has been seen in pathological human material (Puchelle *et al.* 1980). The viscosity/elasticity ratio (tan δ) represents the ratio of mechanical energy dissipated as friction per cycle versus that stored as kinetic energy. A decrease in mucus velocity with increasing tan δ is consistent with increased dissipation of ciliary energy by the mucus. It thus appears that decreasing either the elasticity or the viscosity/elasticity ratio of mucus would be of benefit in enhancing the clearance of secretions.

Increasing mucus elasticity or viscosity from normal almost invariably results in a decrease in clearance rate. However, it has been demonstrated that as the mucus elasticity decreases from the normal range, the ciliary transport rate eventually passes through a maximum, and further decreases in mucus elasticity then result in a reduction in transport rate (Shih *et al.* 1975). The range of optimum mucociliary clearance of airway mucus is located at the low end of the normal range of viscoelasticity (Dulfano & Adler 1974, King 1979, Puchelle *et al.* 1980). Overliquification of mucus by mucolytic treatments represents a potential hazard in any therapeutic trial, and consideration should be given to defining baseline rheological properties and the *in vitro* effect of any potential treatment before initiating its use in patients.

Mucociliary *versus* Cough Clearance:

Cough clearance represents the second line of airway defense, taking over in the case of mucus overload or when mucociliary clearance becomes inadequate. The phenomenology relating cough clearance with mucus rheology has been studied *in vitro* by means of a cough simulator (King *et al.* 1985b). Mucus viscosity, i.e. resistance to flow, turns out to be the major rheological variable affecting cough clearance. Elasticity enters the picture in terms of the recoil effect, i.e. a high degree of spinnability or a low viscosity/elasticity ratio being inhibitive of cough clearance. Adhesivity or surface tension is inhibitive of cough clearance through the suppression of mucus airflow interaction, which manifests itself as wave formation in the mucus layer during the cough (King *et al.* 1989a). Zahm *et al.* (1991) demonstrated that mucus thixotropy and shear-thinning were important in describing the movement of mucus in multiple rapid coughs, and by extension high frequency oscillation.

Both forms of mucus clearability (by cough and by ciliary action) can be successfully predicted on the basis of the measured mucus viscoelastic properties. Mucus that is elastic rather than viscous is transported well by ciliary action, but less well by coughing (King 1987). The existence of an optimal range of viscoelastic properties, and the fact that in some cases both mucociliary and cough clearance should be optimized, suggests that therapeutic measures designed to modify the rheology of secretions should consider the initial state of the mucus, and that the monitoring of the viscoelastic properties of the mucus should be an essential part of any potential mucotropic therapy.

Ex vivo Ciliary Transportability:

Frog palate assay of mucociliary clearability: The epithelium of frog palate is ciliated and mucus-

secreting, similar to the situation found in human conductive airways. Leopard frogs (*Rana pipiens*) or bullfrogs (*Rana catesbiana*) are prepared by double pithing--bending the head forward and inserting an 18-gauge needle into the brain and the spinal cord. The jaw is disarticulated and the palate removed by cutting through from the junction of the posterior pharynx and esophagus out to the skin of the back. The excised palate is placed on gauze saturated with "amphibian" Ringers (2/3 mammalian Ringers, 1/3 distilled water, 207 mOsm/L). The preparation is loosely covered with plastic wrap and allowed to rest in a refrigerator at 4-6°C for 12-24 hours to deplete of mucus. The palate is then re-warmed to room temperature and placed in an acrylic chamber where humidity is maintained by Ringer aerosol. The palate is focused under a dissecting microscope fitted with a micrometer scale, and the movement of a 2-5 μL aliquot of mucus is timed; 3-5 measurements of mucus transport rate are taken to minimize measurement variability. The average transport rate of a sample is normalized to the transport rate for collected endogenous frog mucus. (King *et al.* 1974; Rubin *et al.* 1990). This technique has been widely used as a means of defining the inherent "transportability" of mucus, independent of systemic ciliary function.

Wills and colleagues (1994) have recently developed a mammalian alternative to the frog palate assay, using excised bovine trachea depleted of endogenous mucus by repeated passage of mucus. Although the bovine preparation appears to be more cumbersome than the traditional frog palate, which requires no active treatment to effect mucus depletion, these authors have used this preparation to advantage in monitoring the effects of *in vitro* treatment of sputum with salt as a potential "mucolytic" therapy (Wills & Cole 1995).

Although the frog palate assay requires only microliter volumes of mucus, this test should be interpreted with caution in experiments where residual mediators in the mucus could cause alterations in the frog palate ciliary activity and invalidate the basic assumption that ciliary activity is normalized (King 1984). In fact, by observing the clearance of collected frog mucus or a standard mucus preparation, one can monitor the effects of cilioactive drugs delivered to the frog palate, since variations in clearance rate can in this case be attributed to changes in ciliary activity.

When used in fresh condition before depletion of the endogenous mucus layer (within three hours of excision at room temperature), the frog palate provides an excellent integrated model system for studying all of the relevant variables for mucociliary clearance, namely the mucus secretion rate, the mucus rheology, the cilia beat frequency, the transepithelial potential difference, and the mucus linear velocity itself. We have used the freshly-excised frog palate model in several recent studies, involving alteration of epithelial ion and water transport with amiloride and UTP, and the effects of artificial pulmonary surfactant (Festa *et al.* 1995, 1996). Freshly excised mammalian tracheas can also be used as integrated model systems for mucociliary transport. Gerber *et al.* (1995) have recently carried out such a study with horse trachea, where concurrent measurements of particle transport, CBF, and mucus grading were made. Even in animals as small as mice, it is possible to carry out integrated studies of mucociliary function (Kurosawa *et al.* 1995).

Animal Models for Hypersecretion:

Chronic bronchitis is primarily an airway disease, involving mucous gland and goblet cell hypertrophy, leading to inflammation and infection, which further amplify the elevated mucus production (Reid 1967, Wanner 1990). Alterations in the chemical nature of the mucous glycoproteins (Lopata *et al.* 1974) and impaired mucociliary clearance (Goodman *et al.* 1978) are important hallmarks of the disease. Chronic bronchitis is not necessarily associated with smooth muscle or parenchymal dysfunction, although clearly these often co-exist with the airway disease. The principal etiologic factor in the development of chronic bronchitis is cigarette smoking, while atmospheric air pollution

and environmental tobacco smoke also play important roles in the development of this condition.

In an animal model, we are primarily looking for enhancement of airway mucus production, combined with slowed mucociliary clearance leading to retention of secretions. Such a model would be useful in designing and testing of new therapy for mucus clearance disorders.

Small Animal Models of Chronic Bronchitis:

Considerable work has been done using the rat as a model for chronic bronchitis. The major approaches have been to use either SO2 gas exposure (Reid 1963, Lamb & Reid 1968) or whole cigarette smoke (Lamb & Reid 1969, Hayaski *et al.* 1979). With either agent, 1-3 weeks exposure are generally sufficient to produce pathological changes related to human bronchitis. These include an increase in goblet cell numbers as well as in the size of submucosal glands. Proliferation of goblet cells to more peripheral airways and a histochemical shift towards acid mucin are also seen. In our experience with cigarette smoke exposed rats, tracheal mucus quantity increases markedly with 1-3 weeks exposure, and mucus viscoelasticity is altered in the direction of less rigid mucus (King & Angus 1980). Tobacco smoke models are particularly relevant to human chronic bronchitis, since the main etiologic factor is the same; however, such experiments have become increasingly difficult to carry out because of ethical and social considerations.

In more recent years, a variety of other models of chronic bronchitis have been developed. Endotoxin exposure has been used successfully to induced chronic bronchitis type changes in rats with as little as three days of exposure (Harkema & Hotchkiss 1992). This model evoked the appearance of mucus-containing cells in peripheral airways 3-10 days following the last instillation of the endotoxin. Although the lesion develops perhaps too rapidly, the exposure may be biologically relevant in lower doses, particularly as it relates to endotoxin exposure in agricultural workers.

Urban air pollution in a heavily industrialized city (São Paulo, Brazil) has been used to produce experimental bronchitis in rats (Saldiva *et al.* 1992, Lemos *et al.* 1994). In this case, the lesion develops much more slowly--in 3 to 6 months, but otherwise shares many of the expected features for a successful model of bronchitis. The value of this model is its biological and social relevance to a real problem affecting many of the largest cities. The disadvantages are the length of time required to develop the lesion, and the variability of the environmental insult over time and location.

Recently, sodium metabisulfite (MBS), which releases sulfur dioxide *in situ*, has been used to produce similar lesions to the classic SO2-induced bronchitis. Pon and associates (1994) have found that 3 weeks of exposure to MBS aerosol in rats causes an increase in epithelial goblet cell number, and a shift to more acid mucin. Because of the lower risk to laboratory personnel, this model may provide a suitable alternative to SO2 gas or tobacco smoke.

A wide variety of agents applied *in vitro* or in culture systems have been shown to produce mucus hypersecretion or upregulation of mucus production. These include cholinergic agonists, histamine, neuropeptides, ATP, PAF, TNFα, interleukins, elastase, and ozone. Adler and colleagues (1995) have recently shown that several of these agents share a common pathway to hypersecretion, namely a dependence on nitric oxide synthase.

Other small animals have also been used for studies of mucus hypersecretion. Guinea pigs exhibit an intense response to cigarette smoke that includes both mucus hypersecretion as well as exfoliation of tracheal cilia (Hulbert *et al.* 1981). Ferrets show a brisk tracheal response to methacholine and substance P as secretagogues (De Sanctis *et al.* 1993), but an even more intense response to neutrophil elastase (Schuster *et al.* 1992).

Large Animal Models for Mucus Hypersecretion:

Perhaps the most useful large animal model for mucus hypersecretion has been the dog. Lengthy periods of cigarette smoke exposure in the dog, up to one year, produce well developed airway changes consistent with the chronic bronchitis pattern--goblet cell hyperplasia and metaplasia, increase mass of submucosal glands, and more acid mucin (Auerbach *et al.* 1967, Park *et al.* 1977). Long term smoke exposure also results in significant slowing of tracheal mucociliary clearance (Wanner *et al.* 1977). Our own studies on dogs exposed to whole cigarette smoke through a tracheostomy, 10 cigarettes per day, showed the development of hypersecretion occurring in most dogs within 2-4 months of exposure (King *et al.* 1989b). The initial hypersecretion was associated with decreased viscosity and elasticity, which gradually recovered towards normal during 6-10 months of continued exposure. However, the mucin appeared to change character, assaying for considerably less neutral mucin than early in the exposure period.

SO2 exposure in dogs, 50-200 ppm by tracheostomy for 3-6 months, or up to 650 ppm by nose and mouth, appears to produce similar histologic changes to airway epithelium to those due to cigarette smoke, i.e. epithelial thickening and an increase in the size of mucous glands (Angus *et al.* 1976, Scanlon *et al.* 1987), and the expression of mucous glycoprotein typical of human chronic bronchitis (Bhaskar *et al.* 1988). Our findings on mucus rheology and clearability with SO2 exposure in dogs showed similarities with the response to cigarette smoke, namely an increased quantity of less rigid, more easily clearable mucus (King *et al.* 1980). The upregulation of mucus production and hypersecretion of watery, more easily clearable mucus appears to be a natural and common response to airway injury involving loss or damage to the cilia.

The animal model findings have their parallels in human chronic bronchitis, although over a much longer time frame of exposure. We found, for example, that respiratory mucus from asymptomatic smokers is better hydrated and more easily cleared by mucociliary action than mucus from nonsmokers (Rubin *et al.* 1992). However, this apparent "advantage" to smoking disappears with continued exposure, over the course of 20-40 pack-years, and abnormalities in mucus elastic recoil predictive of poor cough clearance eventually develop (Zayas *et al.* 1990). The decrease in lung clearance due to cigarette smoking has been well-correlated with pack years of exposure (Vastag *et al.* 1986).

Acute exposures in dogs have been used in a variety of situations to produce hypersecretion. Some examples include methacholine, by infusion or aerosol, which induces acute hypersecretion, but also bronchoconstriction (King & Viires 1979, King *et al.* 1985a). Similarly, antigen challenge, such as with *Ascaris suum* cross-reactivity can be employed (King *et al.* 1985c). HNE (human neutrophil elastase) produces an acute, intense hypersecretion, accompanied by reduced clearance rate and elevated viscoelasticity (King *et al.* 1995b), but repeated exposures would likely result in upregulation of natural inhibitors such as SLPI, limiting the potential of this approach as a model for chronic bronchitis.

Other large animal models for mucociliary function have been used with success. Sheep sensitized to *Ascaris suum* antigen show prolonged impairment of mucociliary clearance, along with bronchoconstriction, and represent a useful model for altered airway clearance in asthma (Allegra *et al.* 1983). Mucociliary clearance can be studied in a variety of other large animal species, including primates, cows and horses. Intraspecies differences in mucus rheology and clearance have recently been reviewed by Tomkiewicz *et al.* (1995). The choice of species ultimately depends on the features of human chronic bronchitis one desires to emulate.

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