SERIES 'AIRWAY MUCUS'
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Experimental models for studying mucociliary clearance

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Experimental models for studying mucociliary clearance. M. King. ©ERS Journals Ltd 1998.
ABSTRACT: Respiratory tract mucus is a viscoelastic gel, the rheological properties of which are determined mainly by its content of mucous glycoproteins and water. The rheology and quantity of mucus, in concert with ciliary factors, are the major determinants of mucociliary clearance.

A wide range of animal models for studying the secretion and clearance of mucus are available. *Ex vivo* models, such as the frog palate or excised bovine trachea, provide direct, meaningful data regarding the clearability of mucus. Rodent models of chronic bronchitis, based on irritant gas or cigarette smoke exposure, show important features of the human condition in a relatively short time. The rheological characterization of mucus is made difficult by the small quantities obtainable, particularly from normal animals.

Large animal models, such as the dog or sheep, although more expensive, offer many advantages, such as the ability to carry out long-term serial measurements, and to make integrated measurements of the clearance of mucus, ciliary function, epithelial ion transport, and the rheology of mucus in the same preparation.
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The viscoelastic properties of mucus

Due to the cross-linking of glycoproteins, the rheological behaviour of mucus is described as viscoelastic, having characteristics of both a liquid and a solid [1, 2]. 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 stress applied. 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 the viscosity and elasticity of mucus 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, whilst some shear-thinning, termed "thixotropy", may be reversible.

The viscoelasticity of mucus can be effectively described by two relatively independent quantities, G^* and tangent (tan) δ, which vary with measurement frequency. The mechanical impedance (G^*), is the vector sum of viscosity and elasticity; it can be termed the "rigidity factor". Tan 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.

Rheological assessment of mucus

Collection of mucus

Tracheal mucus can be collected by a modification of the cytology brush technique or the endotracheal tube collection technique [2]. 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 mucous layer coating a freshly removed endotracheal tube. These techniques provide sufficient mucus for analysis even from very small animals [3].

Magnetic microrheometer

This instrument can be used to measure the bulk viscosity and elasticity of microlitre quantities of mucus

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[4]. 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 rheological properties can be used to predict the effectiveness of mucus in clearance, both by ciliary action and by airflow interaction [5]. The magnetic microrheometer is particularly suited to studies employing animal models because of the minimal sample requirement.

Filancemeter

Spinnability (also known as *Spinnbarkeit* or *filance*) is the thread-forming ability of mucus under the influence of low speed elastic deformation. Using a filancemeter [6], a [20–30 µL] sample of mucus is stretched at a retraction velocity of 10 mm·s⁻¹. An electric signal conducted through the sample of mucus is interrupted at the point where the mucous thread is broken; the length of this thread is known as the mucous spinnability (in mm). Spinnability has been correlated positively with mucociliary clearance [6], and negatively with cough clearance [7]. 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 cross-linking macromolecules, as evidenced by the response of cystic fibrosis sputum to deoxyribonuclease (DNase) and gelsolin [8].

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 both with mucociliary clearance and cough clearance [7, 9].

Solids content/collection rate

There is generally a positive relationship between the solid composition of mucus and the viscoelastic properties, although the relationship can change with disease state or source of mucus [10]. We use a microwave drying apparatus and microbalance to calculate the percentage solids for samples of mucus larger than 5 µL. The samples of mucus are also weighed to determine an index of secretion rate (collection rate of mucus in mg·min⁻¹). In dogs, knowing the linear velocity of mucus, the airway circumference and the collection rate, the *in vivo* depth of mucus can be estimated [11].

Transepithelial potential difference

The transepithelial potential difference (PD) 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 [12]. 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 [13–15].

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, radiolabelled particles). Cough or airflow clearance can be studied in 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⁻¹), *i.e.* the "linear" velocity of mucociliary clearance, can be determined by direct observation of marker particle movement with the aid of a fibreoptic bronchoscope [16]. 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 that it can be carried out at the same anatomical site where measurements of epithelial PD are performed, and from where mucus is collected for rheological and chemical analysis.

The viscoelasticity of the mucous 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 mucous simulant gels is directly related to the elasticity of mucus and the depth of the periciliary fluid, and is inversely related to the viscosity of mucus [5]. GIORDANO *et al.* [17] studied the TMV 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 of ciliated epithelium, *i.e.* ciliary transport rate decreases with increasing rigidity or "thickness" of the mucus, whatever quantitative measure is used [1, 18, 19]. A similar relationship between the viscoelasticity and clearance of mucus has also been demonstrated for rat nasal epithelium [20].

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 [19]. Although this phenomenon has not been observed for intact mucus from healthy animals, it has been seen in pathological human material [21]. 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 the velocity of

mucus 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 the elasticity or viscosity of mucus from normal almost invariably results in a decrease in clearance rate. However, it has been demonstrated that as the elasticity of mucus decreases from the normal range, the ciliary transport rate eventually passes through a maximum, and further decreases in the elasticity of mucus resulting in a reduction in transport rate [22]. The range of optimum mucociliary clearance of airway mucus is located at the low end of the normal range of viscoelasticity [18, 21, 23]. 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 defence taking over, in the case of mucous overload or when mucociliary clearance becomes inadequate. The relationship between cough clearance and the rheology of mucus has been studied *in vitro* by means of a cough simulator [24]. The viscosity of mucus, *i.e.* resistance to flow, is the major rheological variable affecting cough clearance. Elasticity is involved in terms of the recoil effect, *i.e.* a high degree of spinnability or a low viscosity/elasticity ratio inhibits cough clearance. Adhesivity or surface tension inhibits cough clearance through the suppression of mucus/airflow interaction, which manifests itself as wave formation in the mucous layer during the cough [7]. ZAHM *et al.* [25] demonstrated that mucous 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 mucous clearability (by cough and by ciliary action) can be successfully predicted on the basis of the measurement of the viscoelastic properties of mucus. Mucus that is elastic rather than viscous is transported well by ciliary action, but less well by coughing [5]. 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

The frog palate can be used to assay mucociliary clearability. The epithelium of frog palate is ciliated and mucus-secreting, similar to that found in human conductive airways. Leopard frogs (*Rana pipiens*) or bullfrogs (*Rana catesbiana*) are prepared by double pithing, *i.e.* 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 oesoph-

agus out to the skin of the back. The excised palate is placed on gauze saturated with "amphibian" Ringer's solution (2/3 mammalian Ringer's solution and 1/3 distilled water; 207 mOsm·L^{-1}). The preparation is loosely covered with plastic wrap and allowed to rest in a refrigerator at 4–6°C for 12–24 h to allow depletion of mucus. The palate is then rewarmed to room temperature and placed in an acrylic chamber, where humidity is maintained by Ringer's 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 the transport rate of mucus are taken to minimize measurement variability. The average transport rate of a sample is normalized to the transport rate for collected endogenous frog mucus [26, 27]. This technique has been widely used as a means of defining the inherent "transportability" of mucus, independent of systemic ciliary function.

WILLS *et al.* [28] 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 depletion of mucus, WILLS *et al.* [29] have used this preparation to advantage in monitoring the effects of *in vitro* treatment of sputum with salt as a potential "mucolytic" therapy.

Although the frog palate assay requires only microlitre 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 [30]. In fact, by observing the clearance of collected frog mucus or a standard preparation of mucus, 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 a fresh condition, before depletion of the endogenous mucous layer (within 3 h 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 secretion rate of mucus, the rheology of mucus, the ciliary beat frequency, the transepithelial PD, and the linear velocity of mucus. We have used the freshly-excised frog palate model in several recent studies, involving alteration of epithelial ion and water transport with amiloride and uridine triphosphate (UTP) [15, 31], and the effects of artificial pulmonary surfactant [32]. Freshly excised mammalian tracheas can also be used as integrated model systems for mucociliary transport. GERBER *et al.* [33] have recently carried out such a study with horse trachea, where concurrent measurements of particle transport, ciliary beat frequency, and grading of mucus were made. Even in animals as small as mice, it is possible to carry out integrated studies of mucociliary function [34].

Animal models for hypersecretion

Chronic bronchitis is primarily an airway disease, involving mucous glands and goblet cell hypertrophy,

leading to inflammation and infection, which further amplifies the elevated production of mucus [35, 36]. Alterations in the chemical nature of the mucous glycoproteins [37] and impaired mucociliary clearance [38] 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 aetiological factor in the development of chronic bronchitis is cigarette smoking, whilst 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 the airway production of mucus, combined with slowed mucociliary clearance leading to retention of secretions. Such a model would be useful in designing and testing new therapy for clearance disorders of mucus.

Small animal models of chronic bronchitis

Considerable work has been performed using the rat as a model for chronic bronchitis. The major approaches have been to use either exposure to SO_2 gas [39, 40], or to air-diluted cigarette smoke [41, 42]. With either agent, 1–3 weeks exposure is 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 (fig. 1), the quantity of tracheal mucus increases markedly with 1–3 weeks exposure, and the viscoelasticity of mucus is altered in the direction of less rigidity [43]. Tobacco smoke models are particularly relevant to human chronic bronchitis, since the main aetiological factor is the
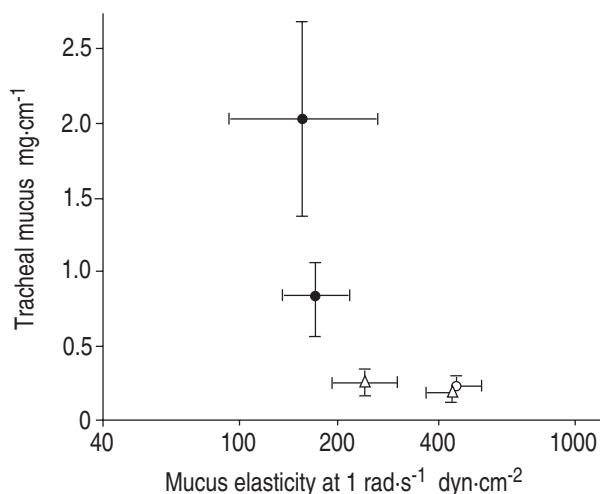

Fig. 1. – The influence of tobacco smoke on the quantity and physical quality of tracheal mucus in Sprague-Dawley rats. Rats were exposed to cigarette smoke diluted 30:1 by air daily for 1–3 weeks. Mucus was obtained by sacrificing the rats 3–4 days after the last exposure and scraping the exposed trachea with a scalpel blade. The elasticity of the mucus was determined by magnetic rheometry [4], after which the samples were weighed. Values are presented as mean±SEM. Each point represents the data from six rats. ● : 48 cigarettes·day^{-1}; Δ : 36 cigarettes·day^{-1}; ○ : control. (From [43]).

same; however, such experiments have become increasingly difficult to carry out because of ethical and social considerations.

In more recent years, a variety of models for chronic bronchitis have been developed. Endotoxin exposure has been used successfully to induces chronic bronchitis type changes in rats with as little as 3 days of exposure [44]. This model showed 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 [45, 46]. In this case, the lesion develops much more slowly, in 3–6 months, but otherwise shares many of the features expected for a successful model for 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 metabisulphite (MBS), which releases sulphur dioxide *in situ*, has been used to produce similar lesions to the classic SO_2-induced bronchitis. Pon *et al*. [47] have found that 3 weeks of exposure to MBS aerosol in rats causes an increase in epithelial goblet cell number, and a shift to a more acidic mucin. Because of the lower risk to laboratory personnel, this model may provide a suitable alternative to SO_2 gas or tobacco smoke.

A wide variety of agents applied *in vitro* or in culture systems have been shown to produce hypersecretion of mucus or upregulation of the production of mucus. These include cholinergic agonists, histamine, neuropeptides, adenosine triphosphate (ATP), platelet-activating factor (PAF), tumour necrosis factor-α (TNF-α), interleukins, elastase, and ozone. Adler *et al*. [48] 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 hypersecretion of mucus as well as exfoliation of tracheal cilia [49]. Ferrets show a brisk tracheal response to methacholine and substance P as secretagogues [50], but an even more intense response to neutrophil elastase [51].

Large animal models for mucus hypersecretion

Perhaps the most useful large animal model of mucus hypersecretion has been the dog. Lengthy periods of cigarette smoke exposure in the dog, up to 1 yr, produce well-developed airway changes consistent with the pattern of chronic bronchitis: goblet cell hyperplasia and metaplasia, increased mass of submucosal glands, and a more acidic mucin [52, 53]. Long-term smoke exposure also results in significant slowing of tracheal mucociliary clearance [54]. Our own studies on dogs exposed to whole cigarette smoke through a tracheostomy (fig. 2) showed that hypersecretion developed in most dogs
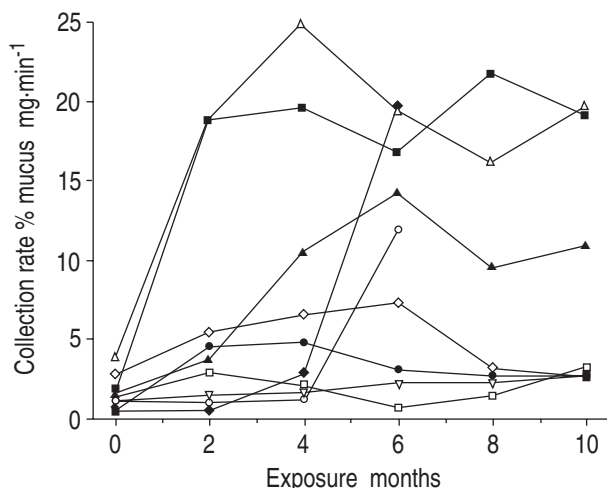
Fig. 2. – Collection rates of tracheal mucus in nine beagle dogs before and after exposure to cigarette smoke for periods of 6–10 months. The dogs were exposed for 5 days·week^{-1} to whole smoke from 10 cigarettes·day^{-1} delivered *via* a tracheostomy tube. The mucus was collected on a cytology brush inserted through the tracheostomy and placed in contact with the posterior tracheal membrane. Each data point represents the mean of up to 16 values determined over a 2 month interval. Each dog is represented by a different symbol. (From [55]).

Fig. 3. – Quantity, elasticity and ciliary transportability of tracheal mucus in seven beagle dogs exposed to sulphur dioxide (SO_2) gas *via* nose and mouth. The dogs were exposed for 2 h·day^{-1}, 3 days·week^{-1} for 10–13 months. The concentration of SO_2 was increased from 350 to 650 ppm over the exposure period. Tracheal mucus was collected on a cytology brush passed through an endotracheal tube inserted under xylazine analgesia. The elasticity of the mucus was determined by magnetic rheometry, and ciliary transportability by means of the frog palate technique [26]. The results illustrated compare the chronic effect, 3 days after the last exposure *versus* the acute effect, immediately after 2 h exposure to SO_2 gas during the first month of the study. Values are presented as mean±SEM. ▨ : acute exposure; ▢ : chronic exposure, *i.e.* 10–13 months. (From [59]).

within 2–4 months of exposure [55]. 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.

SO_2 exposure in dogs (50–200 parts per million (ppm) by tracheostomy for 3–6 months, or up to 650 ppm by nose and mouth) appears to produce similar histological changes to the airway epithelium as those due to cigarette smoke, *i.e.* epithelial thickening and an increase in the size of mucous glands [56, 57], and the expression of mucous glycoprotein typical of chronic bronchitis in humans [58]. Our findings on the rheology and clearability of mucus with SO_2 exposure in dogs (fig. 3) showed similarities with the response to cigarette smoke, namely an increased quantity of less rigid, more easily clearable mucus [59]. The upregulation of the production of mucus 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 [60]. However, this apparent "advantage" to smoking disappears with continued exposure, over the course of 20–40 pack-years, and abnormalities in the elastic recoil of mucus predictive of poor cough clearance eventually develop [61]. The decrease in lung clearance due to cigarette smoking has been well-correlated with pack-years of exposure [62].

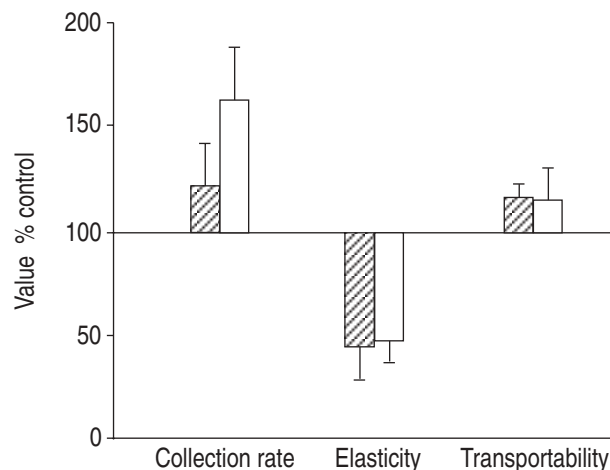Acute exposures in dogs have been used in a variety of situations to produce hypersecretion. Some examples include methacholine, by infusion or aerosol, which induces acute hypersecretion, as well as bronchoconstriction [11, 63]. Similarly, antigen challenge, *e.g.* with *Ascaris suum* cross-reactivity, can be employed [64]. Human neutrophil elastase (HNE) produces an acute, intense hypersecretion, accompanied by a reduced clearance rate and an elevated viscoelasticity [65], but repeated exposures would probably result in upregulation of natural inhibitors, such as secretory leucocyte protease inhibitor (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 [66]. Mucociliary clearance can be studied in a variety of large animal species, including primates, cows and horses. Intraspecies differences in the rheology and clearance of mucus have recently been reviewed by TOMKIEWICZ *et al*. [67]. The choice of species ultimately depends on which features of human chronic bronchitis one desires to emulate.

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