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Mucus and inflammation in equine heaves

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Summary

Accumulation of mucus within the airways is a consistent feature of the chronic inflammatory airway disease known as heaves or recurrent airway obstruction (RAO). Accumulations are greatest when heaves-affected horses are stabled and have neutrophilic inflammation in their airways but accumulations persist when horses are at pasture and inflammation is less severe. Mucus consists of an apoprotein core and oligosaccharide side chains that include a- 1,2 fucose. Persistence of mucus accumulation has been demonstrated by measurement of levels of a-1,2 fucose within the bronchoalveolar lavage fluid. When heaves-affected animals are stabled, mucus accumulation is due in part to decreased clearability (increased viscoelasticity) and to increased production as evidenced by increased expression of the gene eqMUC5AC. When horses return to pasture, clearability is not different from that of control animals and accumulation must therefore be due to persistently increased secretion that is facilitated by delayed apoptosis of mucous cells. The stimulus for increased mucus production and secretion in stabled horses is most likely neutrophil products including elastase and reactive oxygen species. Persistent secretion when horses are at pasture is probably due to persistent low–level inflammation within the epithelium as evidenced by continued expression of nuclear factor-kappaB.

Key words: mucus, inflammation, heaves, RAO, recurrent airway obstruction

Mukus und Entzündung bei der Chronisch Obstruktiven Bronchitis des Pferdes

Bei der chronisch entzündlichen Atemwegserkrankung COB (recurrent airway obstruction, RAO, heaves) kommt es in den Atemwegen üblicherweise zur Ansammlung von Mukus. Dies ist am ausgeprägtesten, wenn die Pferde aufgestallt sind und entzündliche Veränderungen aufweisen. Die Entzündung persistiert weniger ausgeprägt jedoch auch dann, wenn die Pferde auf die Weide gehen. Der Mukus besteht aus einem Apoproteinkern mit Oligosaccharid-Seitenketten, die a-1,2-Fucose enthalten. Durch deren Messung in bronchoalveolären Spülproben konnte die Persistenz der Mukus-Ansammlung nachgewiesen werden. Die Schleimproduktion in den Atemwegen aufgestallter COB-Pferde entsteht einerseits durch verminderte Clearance (erhöhte Viskoelastizität), andererseits durch erhöhte Produktion, was sich in vermehrter Expression des Gens eqMUC5AC ausdrückt. Erhalten die Pferde Weidegang, dann unterscheidet sich die Clearance-Kapazität nicht von der von Kontrollpferden. Die Schleimansammlung in den Atemwegen muss deshalb durch anhaltende erhöhte Sekretion verursacht werden, was durch verzögerte Apoptose der Mukosazellen erleichtert wird. Stimuli für die erhöhte Mukusproduktion und -sekretion aufgestallter COB-Pferde sind vermutlich Produkte neutrophiler Zellen wie Elastase und reaktive Oxydantien. Die anhaltende Sekretion unter Aufstallung erklärt sich möglicherweise durch eine persistierende geringgradige Entzündung innerhalb des Epithels, was sich in anhaltender Expression des Kernfaktors KappaB darstellt.

Schlüsselwörter: Mukus, Entzündung, Atemwege, COB, chronisch obstruktive Bronchitis, RAO, recurrent airway obstruction

Physiology of the mucus apparatus in the horse

Mucus is an essential component of the mucociliary system that is part of the innate defense system of the respiratory tract (*Dixon* 1992; *Wanner* et al. 1996).The mucus blanket overlies the airway epithelium and is composed of a liquid sol layer that bathes the cilia overlain by a more viscous gel layer that is a complex mixture of water, electrolytes, and mucin glycoproteins. Mucins are high molecular weight glycoproteins composed of a core protein to which are attached numerous linear and branching oligosaccharide side chains. Mucus is produced by mucous secretory cells (MC) in the airway epithelium. In some species, such as man and the cat, there are also many secretory cells in submucosal glands but these are not common in horse airways (*Kaup* et al. 1990). Three gel-forming mucin apoproteins are known in human and rodent airways: MUC2, MUC5AC and MUC5B (*Rose* 1992; *Jeffery and Li* 1997). Based on reactivity with polyclonal antibodies raised against the human mucins, it has been proposed that homologues of MUC5AC and MUC5B are also present in horse airway secretions (*Kemmish* et al. 1998; *Walley* et al. 2001). Two equine homologues of mucin genes have been identified by cloning and sequencing fragments of eqMUC5AC and eqMUC2. In healthy horses, eqMUC5AC is expressed in all airway generations and in the stomach. In contrast, eqMUC2 is expressed in the colon but not in the airways (*Gerber* et al. 2002b (in press)). After the core mucin apoprotein is assembled, sugars are attached sequentially to the side chains as the mucin molecule moves through the Golgi apparatus of the goblet cell. As in other species, oligosaccharide side chains of equine mucins are composed of fucose, N-acetyl-galactosamine, sialic acid (N-acetylneuraminic acid) and galactose (*Rose* 1992; *Jefcoat* et al. 2001). The composition of these carbohydrate side chains imparts specific binding activity to certain bacterial adhesion molecules and can influence the viscoelasticity of the mucus layer (*Majima* et al. 1999; *Scharfman* et al. 1999). In healthy horses, mucus viscoelasticity is in the range found in healthy humans and dogs, but lower than in smaller species such as rabbits and rats (*Gerber* et al. 2000).

Quantifying mucus dynamics in horse airways

Improved understanding of the physiology of the mucus apparatus in the horse was made possible by the application of a broad array of techniques to measure mucus production, secretion, accumulation and clearance in health and disease. Tracheal mucus has been quantified by direct measurement. Less than 1 ml of mucus can be aspirated from normal horses but more than 3 ml can be aspirated from heaves-affected animals (*Schatzmann* et al. 1972). Subjective grading scales of mucus accumulation, such as the one developed by Dieckmann (*Dieckmann* 1987) in Hannover, has been used in several investigations (*Dixon* et al. 1995; *Holcombe* et al. 2001; *Gerber* et al. 2002a). Because no one mucus grading scale is generally accepted, comparison between studies is difficult. As with many subjective grading scales, it is easy to identify either the healthy animal with no mucus or only a few droplets or a horse with severe heaves that has confluent pools of mucus in the trachea. It is more difficult to use a subjective grading system to reach a conclusion about the presence or absence of airway disease when a horse has a history of poor performance and a questionable increase in the amount of mucus in the airways. For the latter reason, we have sought other, more quantitative and discriminating measures of airway mucus.

We developed an enzyme linked immunosorbent assay (ELISA) based on a monoclonal antibody 4E4 that stained MCs and surface mucins (*Jefcoat* et al. 2001). Further work with 4E4 revealed that it was identifying parts of the oligosaccharide side chains of mucus. We then used lectin assays to quantify the oligosaccharides in bronchoalveolar lavage fluid (BALF) of control horses and those affected with heaves. Of the various oligosaccharides tested, alpha-1,2 fucose discriminates best between control and heaves-affected horses (see below), is associated with mucus cells and can be measured in BALF by an enzyme linked lectin assay (ELLA) that uses Ulex europaeus agglutinin I. Therefore, it may be possible to use alpha-1,2 fucose levels as an indirect measure of mucin accumulation in BALF.

However, since mucus accumulation within the airways is a function of the rates of production, secretion and clearance, further measures are needed. To evaluate mucus production, we have developed gene probes for (eq)MUC5AC and (eq)MUC2 and used these to measure steady state mRNA levels in airway epithelium (*Gerber* et al. 2002b (in press)). Another aspect of mucus production can be assessed by use of morphometric techniques to quantify the amount of stored mucins within the airway mucus cells (Harkema and Hotchkiss 1992). Because the orientation of the airway epithelium must be maintained, this latter technique requires large biopsy or postmortem samples.

The physical properties of mucus determine its clearability. We have quantified mucus viscoelasticity by use of magnetic microrheometry (*Gerber* et al. 2000). This technique uses very small samples of mucus obtained by bronchial brushing so that it is possible to measure mucus rheology of mucus samples from airways of normal animals in which there is no obvious mucus accumulation (*King and Macklem* 1977).

Mucus accumulation in horse airway disease

Accumulation of airway mucus is a feature of several types of equine lung disease (*Dixon* et al. 1995). In horses with heaves (*Robinson* et al. 1996; *Jefcoat* et al. 2001) there is a massive influx of neutrophils into the airway lumen when susceptible animals are stabled in an environment where they inhale organic dusts (*Woods* et al. 1993). Within 4 to 8 hours of exposure, neutrophilic airway inflammation accompanied by airway obstruction is observed (*Fairbairn* et al. 1993). The latter is due in large part to bronchospasm, but accumulation of mucoid airway secretions is also a prominent clinical feature (*Robinson* et al. 1996). Endoscopically visible accumulation of mucus within the large airways increases within 24 hours of exposure (*Gerber* et al. 2001). Descriptive studies also have reported MC hyperplasia of the bronchioles in such horses (*Kaup* et al. 1990; *Kaup* et al. 1990). Organic dust in stables contains antigens (*Woods* et al. 1993) and endotoxin (McGorum et al. 1998). The role of each of these in equine airway inflammation is unknown but allergic responses (*Tesfaigzi* et al. 2000) and endotoxin (*Harkema and Hotchkiss* 1992) can all induce mucus production. When heaves-affected horses are returned to pasture so that their exposure to dusts is reduced, the number of neutrophils within their airways decreases, yet, in many of these horses, visible mucus accumulations can persist.

Endoscopically visible accumulation of mucus is also a hallmark of the syndrome known as inflammatory airway disease (IAD) (*Moore* et al. 1995) in which mucus accumulations occur despite low numbers of neutrophils in the bronchoalveolar lavage fluid (BALF). Formerly, heaves and IAD were grouped as chronic obstructive pulmonary disease (COPD), but a recent workshop in which Professor Deegen was a participant recommended that the term COPD no longer be used because it is poorly defined and bears little similarity to the human syndrome of the same name (*Robinson* 2001). It is important to note that mild to moderate mucus accumulations can frequently be observed in asymptomatic well-performing sport-horses (*Gerber* et al. 2002a). Therefore, the functional significance of mild to moderate mucus accumulations is unclear, and a diagnosis of IAD based on endoscopically visible mucus alone is inadequate.

What is the functional significance of airway mucus accumulation in heaves?

The airway obstruction of heaves is to a large degree due to bronchospasm but mucus accumulation and airway wall remodeling may also play a role (*Robinson* 2001). There have been no critical studies, however, to determine just how much effect mucus has on the mechanics of breathing or on gas exchange. In order to address this question, it will be necessary to maximally bronchodilate heaves-affected horses and then to measure lung function and examine regional ventilation. In a preliminary investigation (*Robinson* et al. 2001), we administered atropine (0.02 mg/kg IV) and then aerosol pirbuterol (10 puffs) to a group of heaves-affected and control horses at pasture and then after 1 and 7 days of stabling. Pirbuterol administration was repeated until no further change in lung function was observed. At this point, it was assumed that horses were maximally bronchodilated. Stabling caused airway obstruction in the heaves-affected horses but not in controls. After maximal bronchodilation, there was still significant airway obstruction in heaves-affected animals at 1 and 7 days of stabling. Furthermore, this residual

obstruction was worse on Day 7 than on Day 1 (Fig. 1). It appears therefore that factors other than bronchospasm do actually cause some obstruction of the airways that becomes worse the longer horses are stabled. It is likely that accumulated mucus is one of the factors responsible for obstruction.

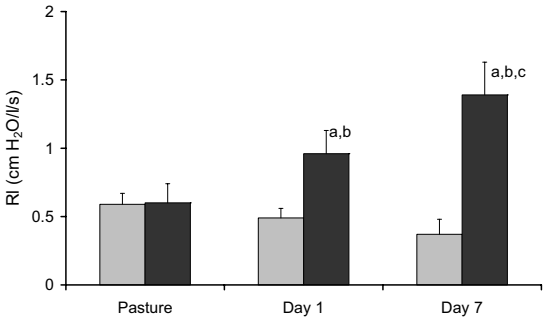

Fig 1 Pulmonary resistance after maximal bronchodilation in control horses (gray bars) and heaves-affected horses (black bars). Horses were studied at pasture and after 1 and 7 days in the stable. a = Significant difference ($P<0.05$) from controls at same time period; b = significant difference from heaves-affected horses at pasture; c = significant difference from heaves-affected horses on Day 1. Data are means +/- SEM.

What have quantitative measures told us about mucins in horses with heaves?

We have used the ELLA assay for alpha-1,2 fucose as a measure of mucus in BALF. Levels are greater in heaves-affected than in control horses. This difference is present when horses are stabled so that the heaves-affected animals have a severe neutrophilic inflammation of the airways. Interestingly, the difference persists when both groups of animals are grazing pasture and the number of neutrophils in the airways of the heaves-affected animals has decreased (Fig 2) (*Jefcoat* et al. 2001). This observation of persistent mucus accumulation is in agreement with data we have obtained using a subjective mucus score (*Gerber* et al. 2001). In a recent unpublished study, we have confirmed the persistent elevation of alpha-1,2 fucose in horses with heaves and have demonstrated that alpha-1,2 fucose is associated with mucus secreting cells (Fig. 3).

To determine whether the accumulated mucus was the result of decreased clearance or increased production or both, the viscoelasticity of mucus from both heaves-affected and control animals was measured by magnetic microrheometry

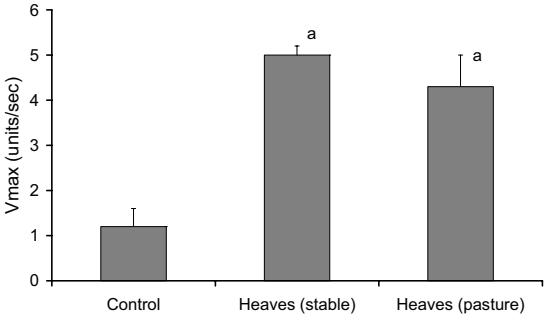

Fig 2 Results of ELLA for a-1,2 fucose in bronchoalveolar lavage fluid of control horses at pasture and heaves-affected horses in the stable and at pasture. a = significant difference ($P<0.05$) from controls. Data are means +/- SEM. (Data are from *Jefcoat* et al. 2001)

(*Gerber* et al. 2000). When both groups of animals were being kept pasture, there was no difference in the viscoelasticity or calculated clearability of the mucus in the two groups of animals. Six hours after stabling, at the time that neutrophils are migrating through the epithelium (*Fairbairn* et al. 1993), viscoelasticity and clearability were still not different. Twenty-four hours after stabling however, the viscoelasticity was dramatically increased in heaves-affected animals and this led to a marked decrease in mucus clearability (Fig. 4). These measurements clearly show that mucus accumulation in the stabled animals could be explained in part by the

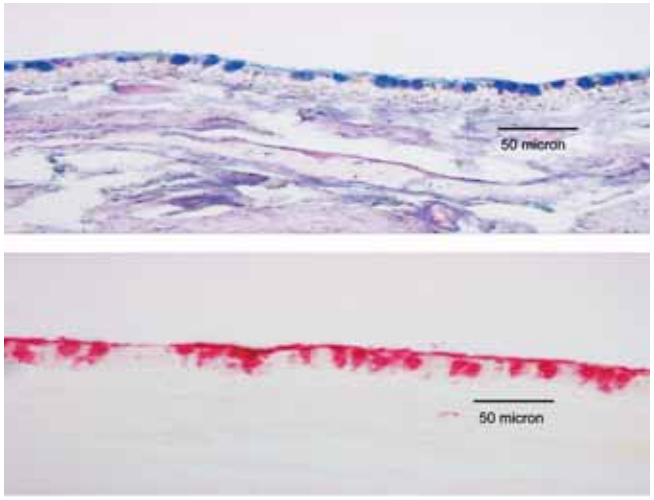

Fig 3 Airway epithelium stained for mucosubstances with Alcian blue/PAS (upper) and for a-1,2 fucose with UEA-1 lectin (lower). Note that a-1,2 fucose is located in mucous cells and on the airway surface in the mucus layer.

decreased clearability of the mucus. However, when animals are at pasture and mucus clearability is normal, there still is increased mucus accumulation in the trachea, visible endoscopically, and in the lower airways, as judged by levels of alpha-1,2 fucose. These observations indicate that the mucus accumulation in heaves-affected animals on pasture must be due to increased mucus production. This increased production continues even when they are removed from the contact with organic dust and their inflammation has waned. To evaluate mucus production, we examined the expression of the mucin gene (eq)MUC5AC (*Gerber* et al. 2002b (in press)) and measured the amount of stored mucus in epithelial cells. The expression of (eq)MUC5AC was compared to that of ZO-

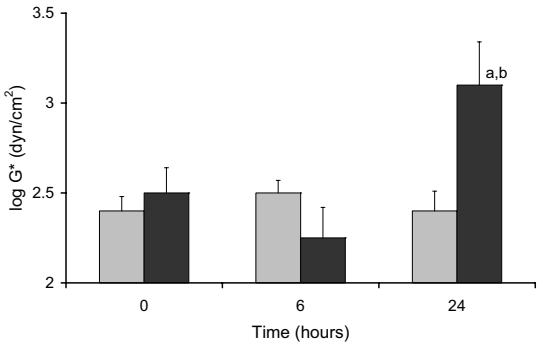

Fig 4 Viscoelasticity at 10 radian/s on a logarithmic scale (log G*; dyn/cm2) of mucus samples in heaves-affected (black bars) and control horses (gray bars) before (0) and 6 and 24 hours after stabling. a = Significant differences between control and heaves-affected animals. b = Significant difference from time 0. Data are means +/- SEM. (Data are from *Gerber* et al. 2000)

1, a tight junction protein. The (eq)MUC5AC/ZO-1 ratios were higher in heaves-affected compared to control horses at all airway generations. In contrast, initial measurements of the volume of stored mucins within the epithelium have shown no difference between control and heaves-affected animals (unpublished data). This observation has been very surprising given the descriptions of mucus cell metaplasia in the bronchioles of heaves-affected animals (*Thurlbeck and Lowell* 1964; *Kaup* et al. 1990). However, our samples were taken soon after the heaves-affected animals were placed in the stable, that is, during the period of neutrophil migration into lung. It is possible, therefore, that the activated neutrophils initiated concomitantly both production of mucin (MUC gene up-regulation) and secretion of stored product in the heaves-affected animals. The increase in rate of secretion would be responsible for the lack of difference of stored mucus product between the two groups. A decrease in the volume of stored mucus during acute neutrophilic inflammation has been observed in response to endotoxin challenge (*Steiger* et al. 1995). The studies of stored mucus were only conducted after horses were stabled but they indicate that at this time, (eq)MUC5AC up-regulation may be a primary mechanism responsible for mucus hypersecretion and accumulation in heaves. It will be interesting to make similar measurements when heaves-affected animals are at pasture.
During the period of acute neutrophilic inflammation, cytokines are being produced and elastase is being secreted so that it is easy to understand why there is increased mucus production and secretion. However, when heaves-affected animals are at pasture, their neutrophil numbers are decreased and frequently do not differ significantly from those of control animals. However, the prolonged activity of NF-kappaB and intracellular adhesion molecule)-1 (ICAM-1) expression (*Bureau* et al. 2000; *Bureau* et al. 2000; *Sandersen* et al. 2001) after removal of horses from organic dust exposure indicate persistence of inflammation within the epithelium that may explain the persistent secretion of mucus.

Association of inflammation with mucus production and secretion

In human chronic bronchitis, bronchiectasis, and cystic fibrosis (*Rogers* 1994) and in experimental airway diseases (*Harkema* et al. 1987; *Harkema and Hotchkiss* 1993; Rogers 1994; *Steiger* et al. 1995), MC hyperplasia is accompanied by neutrophilic inflammation. Neutrophil elastase is a potent mucus secretagogue (*Sommerhoff* et al. 1990). To initiate secretion of mucus, neutrophils must have intimate contact with MC (*Takeyama* et al. 1998) so that the secretagogue elastase (*Niles* et al. 1986; *Sommerhoff* et al. 1990; *Schuster* et al. 1992), which is expressed on the surface of the neutrophil (*Takeyama* et al. 1998), can act directly on the MC. This close contact between the two cell types is brought about by expression of adhesion molecules under the control of chemoattractants such as IL-8 (*Takeyama* et al. 1998). Conditions for mucus secretion exist in heaves-affected horses because IL-8 and ICAM-1 are expressed (*Bureau* et al. 2000; *Franchini* et al. 2000) and BALF elastase levels and activity are elevated even in remission (unpublished data). Neutrophils and the oxidative stress that they produce are also involved in the up-regulation of mucin synthesis (*Fischer and Voynow* 2000; *Takeyama* et al. 2000) via up-regulation and activation of epidermal growth factor (EGF) receptors on MCs (*Takeyama* et al. 1999; *Takeyama* et al. 2000). The latter play a central role in mucin synthesis in response to a variety of stimuli (*Lee* et al. 2000; *Shim* et al. 2001; *Takeyama* et al. 2001). Hydrogen peroxide and neutrophils activated by IL-8 or TNF-alpha increase expression of EGF-R which, when activated by by EGF or transforming growth factor alpha (TGF-alpha) and other ligands, leads to up-regulation of the mucin gene MUC5AC (*Takeyama* et al. 1999; *Takeyama* et al. 2000). Conditions favorable to this sequence of events exist in the heaves-affected horse. Neutrophil influx (Fairbairn et al. 1993), oxidative stress (*Art* et al. 1999) and increased levels of TNF-alpha (Bureau et al. 2000) and IL-8 (Franchini et al. 2000) have all been documented. Furthermore, eqMUC5AC is up-regulated in heaves-affected animals (*Gerber* et al. 2002b in press).

The upregulation of mucin genes by the presence of airway inflammation indicates an increased rate of mucus production in individual mucus cells. In addition, inflammation increases the number of mucus secreting cells by the process of mucus cell metaplasia (MCM) (*Wells* 1970; *Basbaum and Jany* 1990). The MCM is a result of differentiation of existing and proliferating epithelial cells into MC. In addition, delaying apoptosis maintains the increased number of MCs associated with inflammation (*Rogers* 1994). In rats challenged with ozone, which induces neutrophilic airway inflammation and MCM, the appearance and regression of MCs is associated with the expression of Bcl-2 protein, an inhibitor of apoptosis (*Tesfaigzi* et al. 1998; *Tesfaigzi* et al. 2000). Furthermore, Bcl-2 protein expression is increased in hyperplastic mucosa of rats when neutrophilic inflammation is induced by ozone and when eosinophilic inflammation is induced in allergic rats (*Tesfaigzi* et al. 2000). MCM and the regulation of apoptosis are due to an interplay of cytokines. The TH2 family of cytokines cause MCM in humans and in mice (*Wills-Karp* et al. 1998; *Longphre* et al. 1999; *Townsend* et al. 2000; *Zuhdi Alimam* et al. 2000). Recent evidence suggests that heaves is a TH-2-mediated disease (*Lavoie* et al. 2001) and so it is possible that some of these same cytokines may play a role in MCM of heaves-affected animals. The interplay between TH-1 and TH-2-mediated responses is well known and it also applies to the mucous system. IFN-g, a TH-1 cytokine is capable of reducing the number of MC in allergen exposed rats by inducing apoptosis (Shi et al. 2002). Due to a TH-2 bias and relative lack of IFN-g, delayed apoptosis of MC may be important in the mucus hypersecretion of heaves-affected animals. In fact, we have observed an increased number of epithelial cells expressing Bcl-2 protein in 2 heaves-affected horses in clinical remission compared to 2 control animals (unpublished data).

Conclusions

In the secretion of airway mucus, as in most of its other activities, the horse is a mammal. The accumulation of mucus within its airways is, to a large extent, the consequence of inflammation that intiates mucus secretion, stimulates mucus synthesis, causes differentiation of epithelia into MCM, prolongs life of MCM, and leads to changes in mucus viscoelasticity that can delay mucus clearance. If veterinarians are to advise their clients wisely about airway disease, it will be

important to know the functional significance of mucus accumulation in relation to the use of the horse. By using the tools now at our disposal to quantify various aspects of the mucus apparatus, it may be possible to evaluate not only the causes of mucus accumulation but also to quantitatively assess the relationship between mucus, lung dysfunction, and performance.

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