AIRWAY MUCIN GLYCOPROTEINS IN INFLAMMATORY CONDITIONS: EMPHASIS ON EQUINE RECURRENT AIRWAY OBSTRUCTION, AND THE ROLES OF BACTERIAL ENDOTOXIN AND ELASTASE

By

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ABSTRACT

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Mucin glycoproteins are responsible for imparting viscoelastic and adhesive properties to mucus. Biochemical methods to characterize and quantify mucus in the airways of horses have not been described to date. Bacterial endotoxin, a component of Gram-negative bacterial cell walls and a powerful inflammagen, has been used in rodents to induce airway changes that include increased mucus production. Detailed information concerning secretion of specific mucin molecules form pulmonary airways of rats has not been reported, however.

The overall goals of this research project were to quantify mucus levels in the airways of animals with specific inflammatory conditions, and to better characterize the molecular composition for the mucous layer in these animals. Main hypotheses tested were *1)* increased airway mucus is a persistent phenomenon in recurrent airway obstruction (RAO)-affected horses; *2)* airway levels of the mucin-associated sialyl Lewis X (SLeX) tetrasaccharide increase during inflammatory conditions; and *3)* rats developing airway inflammation following bacterial endotoxin exposure produce and release specific mucins into airway lumens in a distinct temporal pattern. To test these hypotheses, research presented in this thesis details biochemical quantification and characterization of

specific mucin-associated structures in a) control and RAO-affected horses, both stabled and at pasture; and b) rats exposed to hay dust or endotoxin.

Increased airway mucus, as measured by level of α-1,2 fucose, a hexose sugar associated with mucin molecules produced by secretory mucous goblet cells, was detected in bronchoalveolar lavage fluid (BALF) of RAO-affected horses both during acute disease and in clinical remission. Persistent mucin increases in RAO horses indicated long-term airway epithelial changes in diseased horses, possibly related to prolonged inflammatory activity.

In rats, endotoxin and hay dust exposures resulted in increased amounts of stored and secreted mucosubstances as compared to saline controls. Levels of soluble mucins possessing the sialyl Lewis X tetrasaccharide structure, detected by immunohistochemistry to be cell surface-associated, were increased in rat BALF at 6 hours after exposure to endotoxin, whereas α-1,2 fucose-possessing glycoprotein levels in BALF did not increase until 24 hours post-exposure. These findings indicate that there is a temporal pattern to the composition of airway mucus that is produced following endotoxin exposure, with an early release of membrane-associated mucins into airway lumens, followed by release of secretory mucins from airway goblet cells.

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LIST OF ABBREVIATIONS

BALF	Bronchoalveolar lavage fluid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
EGF	Epidermal growth factor
EGFr	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ELLA	Enzyme linked lectin assay
Fuc	Fucose
Gal	Galactose
GalNAc	N-acetyl-galactosamine
GlcNAc	N-acetyl-glucosamine
GlyCAM-1	Glycosylated cell adhesion molecule 1
IL-1	Interleukin 1
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-13	Interleukin 13
LAD II	Leukocyte adhesion deficiency II
LPS	Lipopolysaccharide
Man	Mannose
MAPK	Mitogen activated protein kinase
MARCKS	Myristoylated alanine-rich C-Kinase substrate
MMP	Matrix metalloproteinase
NANA	N-acetyl-neuraminic acid
NE	Neutrophil elastase
NFκB	Nuclear factor kappa B
PAF	Platelet activating factor
PKC	Protein kinase C
RAO	Recurrent airway obstruction
ROS	Reactive oxygen species
SLeX	Sialyl Lewis X
TNFα	Tumor necrosis factor alpha
UEA 1	*Ulex europaeus* I

Chapter 1

Introduction and review of the literature

I. The mucociliary apparatus

At tidal breathing, an average adult human exchanges 6.5 liters of air per minute. Over 24 hours, this represents 9360 liters. For a fifty year time span, this comes to be 170,820,000 liters. A mature horse, of approximately 1000 pounds, has an average minute ventilation of around 40 liters. For a twenty year span, this equates to 420,480,000 liters.

This astounding volume of air that moves through the respiratory system of a human or horse means there is enormous opportunity for inhalation of a wide variety of irritants, allergens and pathogens. The respiratory system must therefore have an equally astounding method of responding to inhaled agents and protecting the whole organism from injurious exposure that can occur via the respiratory route. One aspect of the protective mechanism is the mucous layer, a vital component of the mucociliary apparatus.

The mucous layer itself is associated with respiratory epithelium, which extends from the nasal cavity to the terminal bronchioles. This epithelium is composed primarily of ciliated cells and mucous and serous secretory cells. Mucus overlies the airway epithelium, and serves as the initial barrier to inhaled substances. The mucus forms a physical barrier, trapping inhaled particulates and pathogens. The coordinated beating action of the cilia in the trachea and intrapulmonary airways pushes material proximally toward the pharynx, where

mucus and any trapped particles it contains can be swallowed or coughed out, thus ridding the respiratory tract of the inhaled agent (Sheehan et al., 1991; Lamblin and Roussel, 1993).

As shown in FIGURE 1, the mucus blanket that overlies the airway epithelium is composed of an aqueous liquid sol layer that surrounds the cilia, and a more viscous gel layer above the sol (Wanner et al., 1996). Cilia extend upward from the apical surface of ciliated cells through the sol layer. Each cilium possesses a clawed tip that catches the underside of the gel layer, enabling the mucus layer to be propelled in the direction of ciliary beating (Wanner et al., 1996). The gel layer is a complex mixture of water, electrolytes, lysozymes, cells and mucin glycoproteins (Lundgren and Shelhamer, 1990; Shak, 1997; Wanner et al., 1996). A gel is technically defined as a state intermediate between liquid and solid (Tanaka, 1981), and generation and maintenance of this physical property is of critical importance for the normal functioning of the mucous blanket.

Mucin glyocoproteins are very large molecules -- ranging in molecular weights from hundreds of thousands to over 1 million Daltons (Lamblin et al., 1991) -- that impart viscoelastic properties to mucus. Mucins are composed of a core protein (the mucin apoprotein) to which numerous linear and branching oligosaccharide side chains are attached, primarily by means of specific O-glycosidic linkages (Rose, 1992; Shak, 1997; Wanner et al., 1996). Both apoprotein glycosylation and mucin-mucin interactions (multimerization of

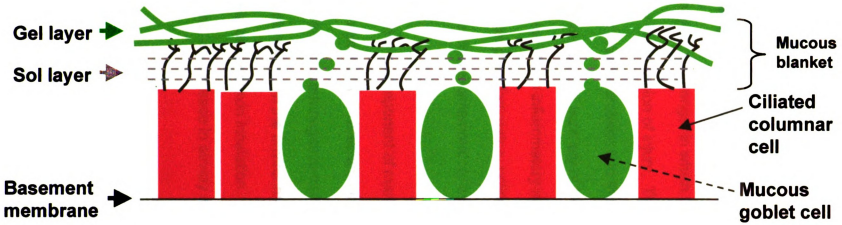

Figure 1. The mucociliary apparatus: respiratory epithelium and mucous blanket

mucins) are needed for realization of the appropriate physical characteristics of mucus.

II. Airway inflammatory diseases and mucus production: an overview

Inflammation is classically considered to be a protective response of vascularized tissue to any kind of injury. The central feature of any inflammatory response is a hemodynamic change, in which there is vasodilation and increased vascular permeability in the region of injury. These changes facilitate the entry of inflammatory cells and plasma proteins into the damaged tissues. Inflammatory cells are prodigious producers of a variety of bioactive compounds known as inflammatory mediators. These inflammatory mediators modulate a range of host biologic responses, from proinflammatory activity for maintenance of the overall inflammatory response, to anti-inflammatory activity for the eventual quelling of the response.

Airway inflammation is a component of many conditions, including asthma and cystic fibrosis in humans, and recurrent airway obstruction (RAO) of horses. Endotoxin, a component of Gram-negative bacterial cell walls, is a powerful inflammagen and is ubiquitous in the environment. Airway inflammation is also a characteristic sequela of endotoxin inhalation. In addition to a classic inflammatory response, an increase in airway mucus is a shared feature of all these conditions.

A. Asthma: bronchospasm, increased airway secretions, and edema and remodeling of airway walls are characteristics of asthma. Inflammation in the airways is a well-recognized feature of asthma, and asthma is considered to be a

chronic inflammatory airway disease (Global Initiative for Asthma, NIH, 1998). Persistent inflammation can be a feature of asthma even during clinical remission (Van den Toorn et al, 2001). Episodes of respiratory distress can be triggered by allergens, viruses, and indoor or outdoor pollutants (Bousquet et al, 2000). A complete understanding of the relationship between airway inflammation and altered mucus production has not been achieved, and as such requires further investigation.

B. Cystic fibrosis: cystic fibrosis is a primary genetic/metabolic disorder, arising from a defect in the gene that codes for the cystic fibrosis transmembrane conductance regulator protein, or CFTR. The CFTR is a cyclic AMP-responsive channel protein that has many functions, including the conduction of chloride ions and the negative regulation of a separate sodium channel (Krauss, 2001; Pitt, 2001). Cystic fibrosis is a complex disease characterized by many pathologic alterations that affect multiple body systems. In the respiratory tract, these include alterations in secretion and/or composition of airway mucus, impaired movement of the mucous blanket, excessive colonization and growth of endotoxin-bearing Gram-negative bacteria, and increased inflammatory cell presence in the airways (Krause, 2001; Tatterson et al., 2001). As with asthma, the association between inflammation and altered mucus production requires further study.

C. Recurrent airway obstruction: recurrent airway obstruction is an asthmalike condition of mature horses that is characterized by bronchoconstriction, airway wall thickening, and increased airway secretions

occurring as a result of exposure to a dust, allergen, and endotoxin-laden indoor (barn) environment (Robinson et al., 1996). A pronounced neutrophilic inflammation in the airways is also observed. Clinical signs, such as dyspnea, are episodic. Respiratory distress will diminish or be absent when animals are housed in a clean air environment. Clinical signs recur upon re-exposure to a precipitating environment, however.

D. Bacterial endotoxin: bacterial endotoxin is a component of the outer membrane of gram negative bacteria. A single endotoxin molecule is composed of two main subunits: a polysaccharide portion that is attached to a lipid portion, called lipid A. The polysaccharide is in turn composed of a terminal oligosaccharide chain that is attached to an invariable core section (Thorn, 2001). The precise composition of the terminal oligosaccharide can vary among bacterial species (Thorn, 2001). The core section links to the lipid A moiety, and this core/lipid A unit provides the immunomodulating effect of endotoxin (Rietschel et al., 1993; Wang and Hollingsworth, 1996). Endotoxin itself is not intrinsically harmful, but as a signal of gram negative bacterial presence, it provokes a powerful inflammatory response in host organisms. Endotoxin is ubiquitous in the environment, being present in locations such as cotton mills, wood mills, factories, and barns and stalls (Gordon and Harkema, 1995; Kullman et al., 1998; McGorum et al., 1998; Rylander, 1986; Schwarz et al., 1995). Inhalation of endotoxin results in a rapid influx of leukocytes -- primarily neutrophils -- into the airways (Thorn, 2001). Endotoxin exposure has also been implicated in the development and exacerbation of asthma (Park et al., 2001).

An increased mucus presence can be a significant pathologic feature of inflammatory airway diseases, and endotoxin and various inflammatory mediators have been linked to increased mucus production in the airways. Methods for quantifying mucus in the airways of horses have not been well developed, however, and rigorous investigation of the characteristics and cause(s) of altered mucus production in recurrent airway obstruction have not been conducted to date. Mucin glycoprotein molecules are the most significant component of the mucus layer. This thesis describes development of methods to quantify mucin levels in bronchoalveolar lavage fluid of horses, and details close associations between inflammation and specific changes in airway mucus presence. Additionally, detailed knowledge of the role of specific inflammatory cells and mediators on the carbohydrate structure of secreted mucin glycoproteins in mammalian lower airways is also lacking. This thesis also describes work that examines mucin glycoprotein structure in inflammatory airway disease.

III. Mucin apoproteins

In order to develop a better understanding of the effects of inflammation on the production, secretion and function of mucins, familiarity with the structural details of mucin glycoproteins and the cellular biology of their construction is essential.

Nineteen different genes that code for mucin apoproteins, called MUC genes, have been identified thus far in humans (Hannisch and Muller, 2000; Williams et al., 2001; Wu et al., 2001). These are referred to as MUC1 through MUC18.

Two similar forms of MUC5 exist, called MUC5AC and MUC5B. Mucins identified in humans have counterparts in other animal (Perez-Vilar and Hill, 1999). Some of these genes, such as MUC1, MUC3, MUC4 and MUC12, are transcribed and translated into membrane-bound mucins. Membrane bound mucins are postulated to help lubricate epithelial surfaces and trap particles and cellular debris (Hannisch and Muller, 2000), but there is evidence for more sophisticated functions, as well. MUC1 may be involved in cell signaling. Though MUC1 has no intrinsic kinase domain, it does possess potential tyrosine-phosphorylation sites on its intracellular domain (Pandey et al., 1995). These sites can be phosphorylated *in vitro*, and may serve as docking sites for SH2-containing signaling molecules such as Grb2. This may provide a signal transduction route through downstream MAP kinases (Gendler, 2001; Schroeder et al., 2001).

Major secreted mucins are products of MUC2, MUC5AC, MUC5B, MUC6 and MUC7 genes (Hannisch and Muller, 2000; Perez-Vilar and Hill, 1999). Major sites of mucin gene expression are MUC2 in small intestinal epithelium, MUC5B in salivary glands and the respiratory tract, MUC5AC in respiratory tract and stomach, and MUC6 in salivary glands and the gallbladder (Van Klinken et al., 1995). Several mucin genes can be expressed by the same or different cells within a single epithelium, however (Van Klinken et al., 1995). As an example of this, MUCs 1, 2, 4, 5AC, 5B, 7 and 8 are all expressed in the respiratory tract of humans (Wills-Karp, 2000). Some mucin genes may share a common ancestral gene. MUCs 2, 5AC, 5B and 6, for instance, map to the same chromosome

(chromosome 11p15.5) in humans (Buisine et al., 1998; Desseyn et al., 1997) and strong sequence similarity exists between MUC2 and MUC5AC (Li et al., 1998).

Mucin apoproteins are characterized by extensive sections of tandemly repeated amino acid sequences (Shak, 1997), which differ in number, length and sequence between mucin types (Perez-Vilar and Hill, 1999). These sequences are rich in threonine and serine, and also contain large numbers of proline and alanine residues (Rose, 1992; Shak, 1997). Presence of threonine and serine is critical for mucin structure, as these amino acids possess hydroxyl groups that are necessary for O-glycosidic attachment of oligosaccharide side chains to the mucin apoproteins (Voet and Voet, 1990). These linkages occur when a carbon atom of a sugar links to a hydroxylated amino acid via the latter's OH group. Central tandem repeat sections of apomucins exhibit little or no secondary structure, which indicates these regions likely serve as "scaffolds" for the O-glycosidic linking of oligosaccharide side chains (Eckhardt et al., 1987). Flanking regions on either side of the tandem repeat section consist of nonrepetitive sequences (Van Klinken et al., 1995). In the case of most secreted mucins, these regions contain large numbers of cysteine residues (Shak, 1997; Perez-Vilar and Hill, 1999). The amino terminal flanking region of most mucins, including MUC5AC, possess regions rich in the thiol (-SH) – containing amino acid cysteine that are referred to as D domains (Van Klinken et al., 1995; Perez-Vilar and Hill, 1999). It is common for secreted mucins to have three D domains, D1, D2 and D3. A fourth, D4, is present in some secreted mucins (Perez-Vilar

and Hill, 1999), including MUC2 and MUC5AC (Li et al., 1998). A partial D domain, termed D', is situated between D2 and D3 in all secreted mucins and in the glycoprotein von Willebrand's Factor [vWF] (Perez-Villar and Hill, 1999). The carboxy termini of mucins also possess cysteine-containing segments, regions that are homologous to the cystine knot (CK) superfamily of proteins (Perez-Vilar and Hill, 1999). FIGURE 2 is a detailed schematic diagram of two secreted mucin apoproteins, linked by a disulfide bond.

CK domains contain 6 conserved cysteine residues, cysteines 1 through 6. The knot-like structure arises from a ring-like structure formed by disulfide linkages between cysteine numbers 2 and 5 and between cysteines 3 and 6. A disulfide bond between cysteines 1 and 6 then passes through the center of this ring (Katsumi et al., 2000). CK domains are found in a superfamily of growth factor proteins such as TGFβ, nerve growth factor and platelet derived growth factor, as well as in mucins and vWF (Perez-Vilar and Hill, 1999), and are involved in formation of molecular oligomers (Katsumi et al., 2000).

The significance of these cysteine-rich D and CK domains is that the cysteine residues can become involved in disulfide linkages (Van Klinken et al., 1995; Perez-Vilar and Hill, 1999), thus creating a mechanism for covalent interaction of mucin molecules and the formation of mucin multimers. Molecular multimerization is important to the structure and function of the mucus layer, as the covalent linking of long chain molecules in a polymer gel is a critical aspect in the generation of the gel-like properties of the material (Tanaka, 1981). Supporting the idea that these domains are vital for mucin multimer formation,

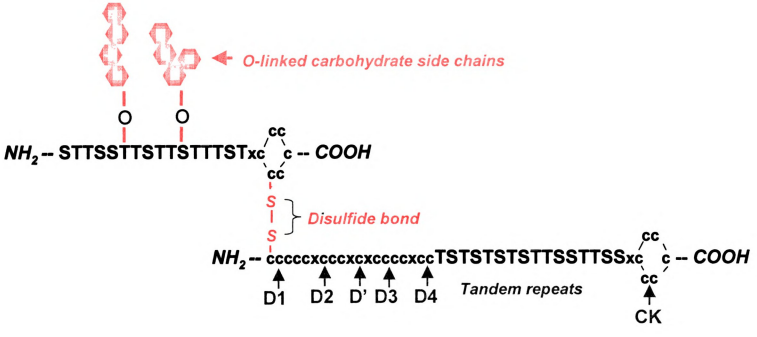

Figure 2. Structure of secreted mucin apoproteins. Majority of molecular weight is imparted by carbohydrate side chains. Size of tandem repeats is variable among different mucins.

MUC1 and MUC7, which exist as monomers, do not possess D or CK domains (Van Klinken et al., 1995). D domains were first discovered as a component of human von Willebrand Factor [vWF] (Sadler, 1998), and are necessary for multimerization of vWF. D domains have subsequently been found in many proteins (Cohen-Salmon, 1997; Gao, 1998; Mayadas, 1992; Perez-Vilar and Hill, 1999), indicating this motif is a conserved sequence and may be involved in many multimerization or adhesion processes. There is also a great deal of homology between the carboxy termini of various mucins and vWF. There is conservation of nearly all the carboxy terminus cysteine residues of MUCs 2, 5AC, 5B and vWF (Desseyn et al., 1997).

It is believed that CK domains contain N-linked oligosaccharide side chains at specific acceptor sites (Perez-Vilar et al.,1996). N-linkages occur between carbohydrate side chains and the amine group on an asparagine (Asn) residue that must be situated in a specific acceptor motif of Asn-X-Ser/Thr (Van den Steen et al., 1998). Presence of these N-linkages may be important in mucin dimer formation, potentially a critical early step in the final multimerization, packaging and transport of mucins such as MUC5AC (Asker et al., 1998a and b; Dekker and Strous, 1990; Sheehan et al., 1996; Perez-Vilar and Hill, 1999).

IV. Oligosaccharide side chains

A. Carbohydrate biochemistry

Mucins are glycoconjugates, molecules with one or more mono- or oligosaccharide units [referred to as the *glycone*] covalently linked to a non-carbohydrate moiety [the *aglycone*] (Freeze, 1999). Oligosaccharide side chains

form approximately 70 to 80 percent of the molecular weight of mucin molecules (Lamblin et al., 1991; Rose, 1992), and are essential for conferring gel-forming and viscoelastic properties to secreted mucins.

O-linked oligosaccharide side chains, as are found in mucin molecules, are typically composed of from 1 to around 20 individual monosaccharide molecules linked together by covalent bonds (Hanisch, 2001; Rose, 1992). Individual monosaccharides found in O-linked oligosaccharide side chains of mucins are N-acetyl galactosamine [GalNAc], N-acetyl glucosamine [GlcNAc], galactose [Gal], fucose [Fuc] and the sialic acid N-acetyl neuraminic acid [NANA] (Lamblin et al., 1991; Van den Steen et al., 1998). An additional monosaccharide, mannose [Man] is a major component of N-linked oligosaccharides (Drickamer and Taylor, 1998; Marth, 1999). Of these carbohydrates, galactose and mannose are hexose sugars, or 6 carbon cyclic neutral sugars. N-acetyl galactosamine and N-acetyl glucosamine are examples of hexosamines, or hexose sugars with an amine (-NH) group at the 2 position of the ring, which is N-acetylated. Fucose is an example of a deoxyhexose sugar, or a hexose lacking a hydroxyl group at the 6 postion. Sialic acids are a family of 9 carbon acidic sugars, of which N-acetyl neuraminic acid is the most common (Varki, 1999). Structural diagrams of these monosaccharides and glucose, the parent molecule for other monosaccharides, are given in FIGURE 3.

All carbohydrates are either polyhydroxy aldehydes or ketones. If visualized in chain form, a single carbohydrate possess an alcohol [ROH] end and a carbonyl [-CO-] end, which is either an aldehyde [R-CO-H] or ketone [R-C-R].

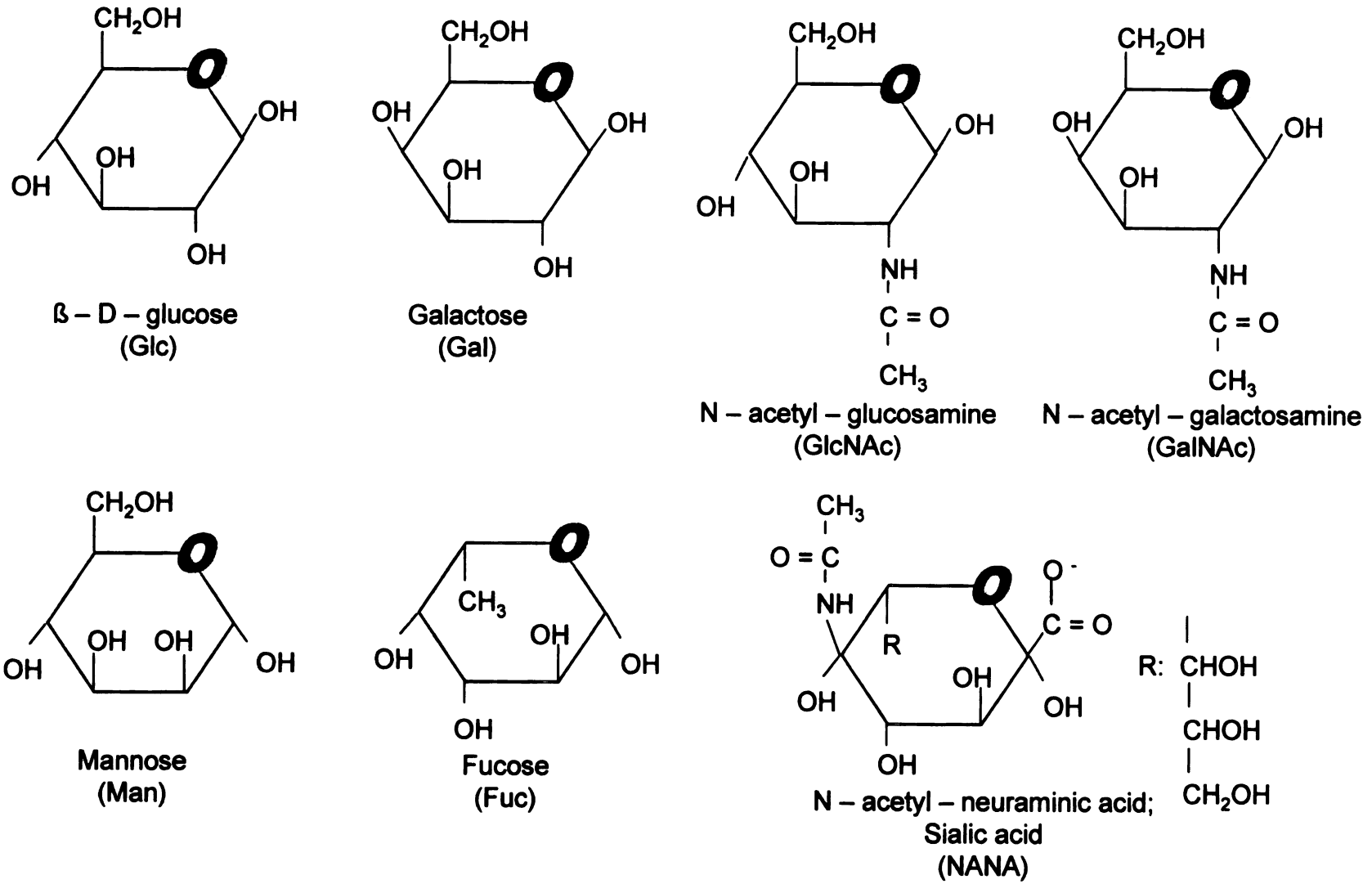

Figure 3. Hexose-ring structures of mucin-associated monosaccharides

In cyclic–form carbohydrates, the aldehyde or ketone end (referred to as the reducing end) reacts with the alcohol end to form ring structures (FIGURE 4). The carbonyl carbon in the ring is designated carbon 1, or the anomeric carbon, and other carbons in the ring are designated as 2, 3, 4, 5, and 6 (in the case of hexose rings).

Individual monosaccharides join together via glycosidic bonds, where a covalent bond forms between a hydroxyl group of one sugar and the anomeric carbon of another (Voet and Voet,1990). For example, a 1,4 glycosidic linkage would be a glycosidic bond between the anomeric carbon of one sugar (carbon #1) and carbon 4 of a second sugar. A 1,3 linkage is between a carbon 1 and a carbon 3.

All linkages can be either α or β anomers, as well. An α anomer is when the linkage at the anomeric carbon extends below the plane of the ring, whereas a β anomer is a linkage projecting above the amomeric carbon's ring plane (Voet and Voet, 1990).

Because linkages between carbons can vary according to specific carbon atoms involved and by different anomeric configurations, a tremendous number of linkage possibilities exist for cyclic sugars. For example, there are at least 1,056 unique trisaccharides that can form between 3 hexoses. In comparison, 3 amino acids can link together in only 6 distinct ways. It has been calculated that six hexoses can have more than 1 trillion possible combinations (Varki, 1999).

A practical significance to the large number of linkage possibilities is that each linkage can result in structural changes that result in specific functional results.

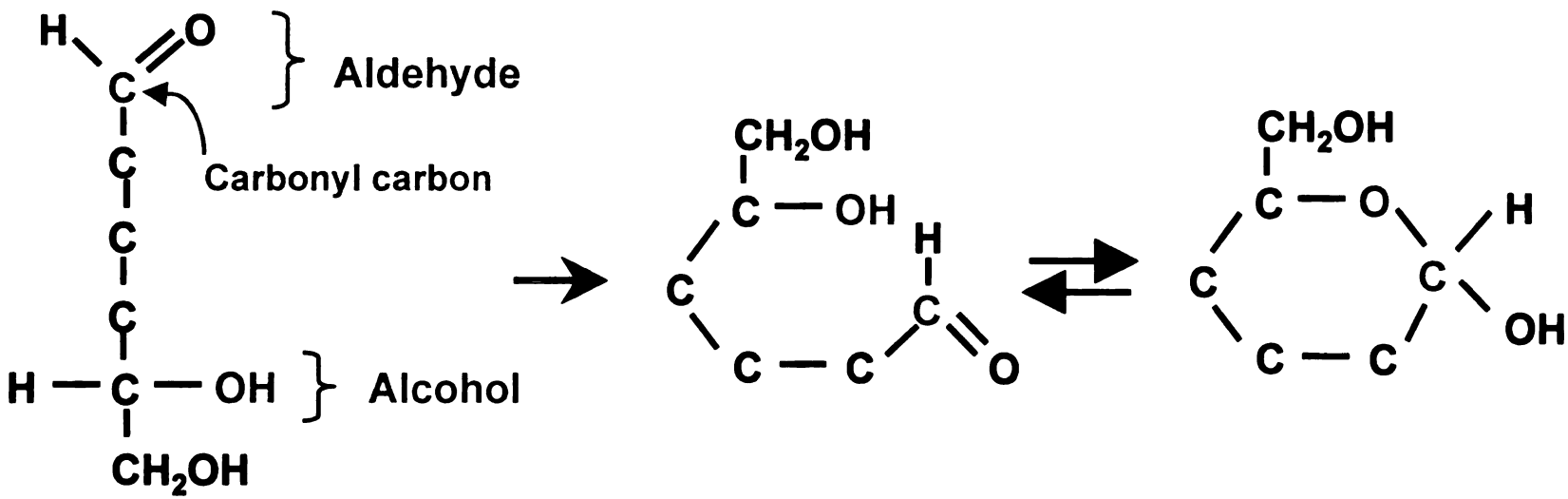

Figure 4. Formation of a cyclic hexose sugar from a polyhydroxy adlehyde

For example, as will be detailed later, a mucin monosaccharide of the same type linked to a preceding sugar in an α – 1,2 manner can result in different functional effects than if the sugar were linked in an α – 1,3 manner.

Though these monosaccharides are the molecules necessary for oligosaccharide side chain formation, construction of these chains involves more than simple interaction between individual, non-modified monosaccharide units. For chains to form, each monosaccharide must be in the form of a nucleotide sugar donor, where each individual monosaccharide is joined with a nucleotide, either uridine diphosphate [UDP], as in the cases of GalNAc, GlcNAc and Gal, guanidine diphosphate [GDP], for Fuc and Man, or cytidine monophosphate [CMP] for sialic acid (Dennis et al., 1999; Freeze,1999; Voet and Voet, 1990).

Glucose can serve as the parent molecule for all monosaccharides involved in mucin formation. Major enzymatic pathways involved in the conversion of glucose to sugar nucleotides are shown in FIGURE 5. Steps involved in nucleotide sugar donor formation all take place in the cytosol, with the exception of the last step in CMP-NANA formation (activation of CTP to create CMP-NANA), which occurs in the nucleus. CMP-NANA is subsequently exported to the cytosol, however (Freeze, 1999).

B. Oligosaccharide construction

Oligosaccharide construction occurs within intracellular compartments, specifically the Golgi complex and endoplasmic reticulum (Dennis et al., 1999; Van den Steen et al., 1998). Sugar nucleotides formed in the cytosol must therefore gain entry into these compartments. Simple diffusion into these

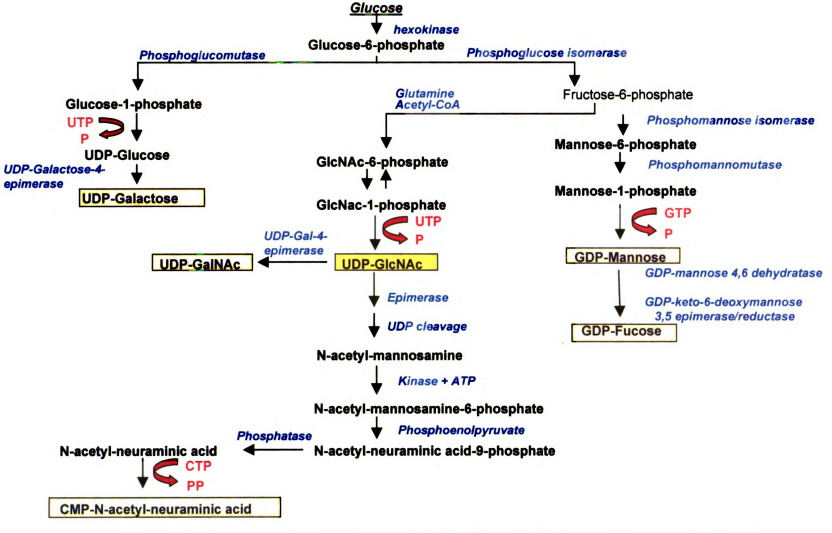

Figure 5: Enzymatic pathways for formation of sugar nucleotides

compartments is not possible, as sugar nucleotides carry an overall negative charge (Freeze, 1999). Movement into organelle lumens is facilitated by non energy-requiring sugar nucleotide transporters that are present in the membranes of the Golgi and endoplasmic reticulum (Abeijon et al., 1997; Freeze, 1999). These transporters are in fact specific nonenergy-requiring antiporters, which carry sugar nucleotides into the lumens of the Golgi and endoplasmic reticulum, and simultaneously carry nucleotide monophosphates out into the cytosol [FIGURE 6] (Freeze, 1999).

Oligosaccharide formation within the Golgi involves sugar nucleotides donating the sugar subunit for use in construction of carbohydrate chains. Donation of sugars results in nucleoside diphosphate by-products within the Golgi lumen. Nucleoside diphosphatase enzymes in the Golgi lumen then convert these molecules to nucleoside monophosphates, which can then be transported out by the antiporters [FIGURE 6] (Abeijon et al., 1997). The antiporter system therefore establishes a mechanism that can couple rate of sugar nucleotide utilization with import, as nucleotide monophosphates, by-products of oligosaccharide formation, are linked to the import of new sugar nucleotides. Additionally, regulation of transporter proteins can potentially be a method of controlling glycosylation of mucins and other glycoproteins by altering availability of sugars inside the Golgi compartments.

Though initial N-glycosylation and dimerization of mucin apoproteins such as MUC 5AC appear to occur in the ER (Perez-Vilar and Hill, 1999), the bulk of post-translational modification of MUC gene products occurs in the Golgi

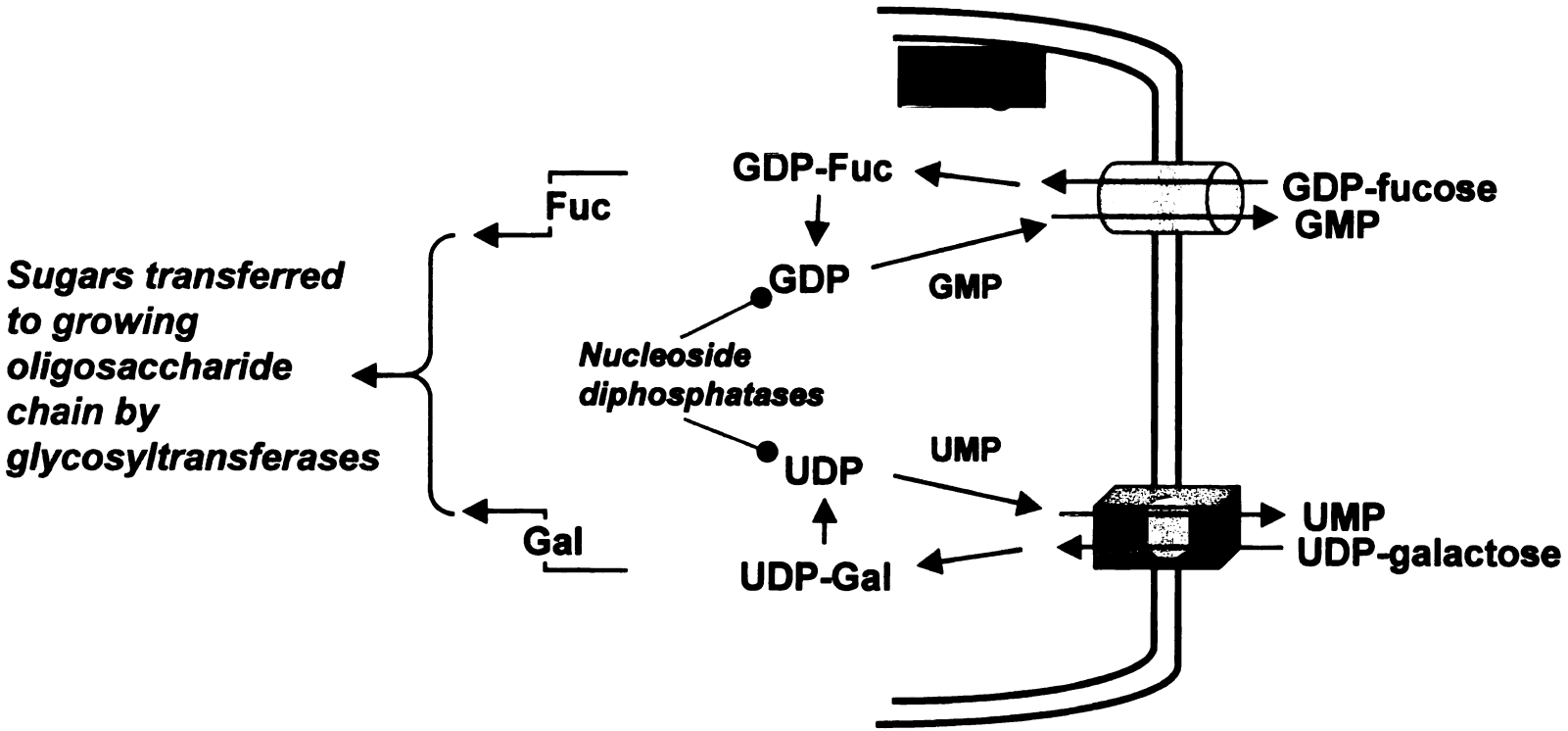

Figure 6. Action of Golgi membrane sugar nucleotide antiporters: transport of sugar nucleotides into Golgi apparatus is coupled with exit of reaction products from transfer of sugars to proteins.

complex. O-linked oligosaccharide side chain formation occurs as a stepwise process, where individual sugars are added one at a time, with addition of sugars catalyzed by glycosyltransferase enzymes (Baenziger et al., 1994; Lowe and Varki, 1999; Lamblin et al., 1991). The general scheme of a reaction resulting in formation of an O-glycosidic bond is illustrated in FIGURE 7, where a hexose from a sugar nucleotide is transferred to a hydroxyl group (-OH) on a precursor molecule referred to as an acceptor molecule.

In forming an oligosaccharide chain, glycosyltransferases act one at a time, in a stepwise or sequential fashion. In other words, sugar A is added to an original acceptor molecule by a specific glycosyltransferase. Sugar B is then added to sugar A by a second specific glycosyltransferase, Sugar C added to B, and so on. Glycosyltransferases act in a specific manner, not only in regard to the type of sugar involved in the transfer, but by the type of linkage (α or β anomeric) and location of the hydroxyl group on the acceptor, as well. As examples of this specificity, an α – 1,2 fucosyltransferase will transfer a fucose molecule from GDP-fucose to the #2 carbon of an acceptor hexose in an oligosaccharide chain, with the resultant linkage being in an α – anomeric configuration, whereas a separate α – 1,3 fucosyltransferase will transfer a fucose to the #3 carbon of an acceptor. A β– 1,4 galactosyl transferase will transfer a galactose to the #4 carbon of an acceptor hexose, creating a β – anomeric linkage. In addition, glycosyltransferases are specific in regard to the acceptor structural arrangement that they will recognize. In the glycosidic bond reaction sequence, the three-dimensional structure that is the unique product of a previous linkage reaction

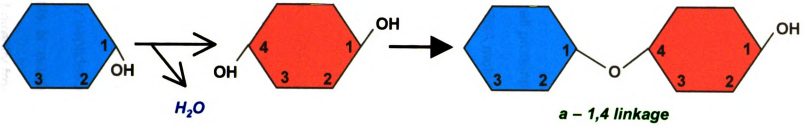

Figure 7: glycosidic bond formation

between monosaccharides serves as the substrate for the next linkage reaction (Baenziger et al., 1994).

Gycosyltransferase-catalyzed reactions typically require the presence of a divalent cation, such as Mg2+ or Mn2+, and the enzymes perform best in a pH range of 5.0 to 7.0 (Lowe and Varki, 1999). Divalent cation presence and these pH values can be found in the Golgi, obviously optimizing glycosyltransferase function in the principal organelle where oligosaccharide side chain formation takes place.

As with construction of all proteins, discrete MUC genes are transcribed, resulting in formation of mRNA molecules corresponding to the gene. Translation of the mRNA to a peptide chain, such as a MUC5AC core protein, occurs in ribosomes. Post-translational modification occurs in the endoplasmic reticulum and Golgi. Typically, the peptide moves through the endoplasmic reticulum then is transported to the Golgi. Extensive post-translational modification of mucin proteins takes place, specifically the complex glycosylation. Sulfation – addition of negatively charged SO_3^- groups by specific sulfotransferase enzymes to oligosaccharide chains – also takes place (Lamblin et al., 1991; Rose, 1992; Van Klinken et al., 1995). Like glycosyltransferases, individual sulfotransferases exhibit great specificity, catalyzing sulfate group addition to specific locations on specific sugars.

Import of most secretory peptides into the endoplasmic reticulum, including specific MUC gene products, is usually a co-translational event, where the peptide moves into the ER directly from ribosomes attached to the ER membrane

(Bhatia and Mukhopadhyay, 1999; Dell and Morris, 2001). Transit of a peptide in the cytosol can result in extensive folding of the peptide (Alberts et al., 1994), therefore co-translational importation is significant for mucin proteins, as minimizing protein folding maintains exposed tandem repeat sections on apoproteins, allowing for proper attachment of oligosaccharide side chains.

Secreted O-linked mucins follow a pathway referred to as the cellular biosynthetic pathway [BSP] (Alberts et al., 1994). In this pathway, molecules pass through multiple compartments where they are modified in a series of steps, stored, and eventually released by exocytosis.

In the BSP, a translated mucin peptide enters the ER. While O-glycosylation occurs in the Golgi, N-glycosylation is an early post-translational process that occurs within the ER (Bhatia and Mukhopadhyay, 1999). This early N-glycosylation may be a necessary step for the proper assembly and transport of secreted mucins such as MUC5AC, as it facilitates dimerization of mucins through CK-domains (Perez-Vilar and Hill, 1999). N-glycosylation differs from O-glycosylation in that a mannose-rich oligosaccharide precursor is formed first by addition of sugars (by glycosyltransferase activity) to a dolichol molecule, a long lipid chain with multiple isoprene [5 carbon] units (Marth, 1999). This mannose-rich precursor is then transferred from the dolichol molecule *en bloc* by an oligosaccharyltransferase enzyme which recognizes the asparagine (Asn) residue at the specific N-glycosylation acceptor site of – Asn – X – Ser/Thr – (Helinius and Aebi, 2002) . After transfer of the core, cleavage of or addition to

the N-linked chain can take place (Dell and Morris, 2001; Van den Steen et al., 1998).

Transport of partially completed mucin molecules to the Golgi must then occur in order for the molecules to be modified by O-glycosylation to attain final form. Vesicles containing the nascent mucin molecules moving from the ER to the Golgi bud from specialized regions of the ER called transitional elements, a region of rough ER membrane with no bound ribosomes. In a constitutive or default pathway, vesicles of the type containing mucins automatically move to the Golgi complex (Alberts et al., 1994).

A single Golgi complex is composed of multiple hollow flattened sac-like structures, with a unique CIS-face (in closest proximity to an ER structure), a TRANS-face oriented toward the plasma membrane, and multiple intervening cisternae. Vesicles transported from the ER to the Golgi fuse with the Golgi CIS-face. Proteins entering the CIS-face move forward through the complex, from the CIS-Golgi network to the medial cisterna to the TRANS-Golgi network, and after extensive intra-Golgi modification are released in secretory vesicles from the TRANS-face into the cytoplasm (Alberts et al., 1994).

Glycosyltransferases involved in mucin oligosaccharide formation are present in the Golgi. Glycosyltransferases associated with the Golgi all share a common structure of a transmembrane domain with a short extra-Golgi (cytoplasmic) amino terminus and a larger carboxy terminus that projects into the Golgi lumen. The catalytic domain of glycosyltransferases have been associated with the intraluminal carboxy terminus (Lowe and Varki, 1999).

Knowledge of the sequential construction of O-linked oligosaccharide side chains and glycosyltransferase structure and function has led to the development of the following scenario (FIGURE 8): glycosyltransferases associated with the early steps of side chain formation are anchored in CIS compartments, with glycosyltransferases involved in subsequent steps sequentially spaced through more distal Golgi sub-compartments. MUC proteins therefore are transported through the Golgi, encountering different transferase enzymes as they progress, with stepwise addition of new sugars to the chain occurring as the protein progresses through the complex. This scenario predisposes some method of specificity in regard to localization of glycosyltransferases to precise locations in the Golgi, though unequivocal proof of an enzyme localization mechanism is currently lacking.

V. Mucin exocytosis

Mucin molecules bud from the trans-face of the Golgi complex packaged in membrane-bound vesicles. These vesicles, also referred to as cytoplasmic granules, are stored in the cytoplasm. Inside the secretory granules, mucins are highly condensed and tightly packed. Negative charges that are associated with sialic acid molecules and sulfate groups give mucin oligosaccharide side chains a polyanionic character, however, and the repulsive interactions of these negative charges resist condensation. Divalent positively charged calcium ions are present within the secretory granules. By counteracting the negative charges of the mucins and creating a more electroneutral microenvironment within the Golgi, the calcium ions provide a counter-ion shielding force around the mucins,

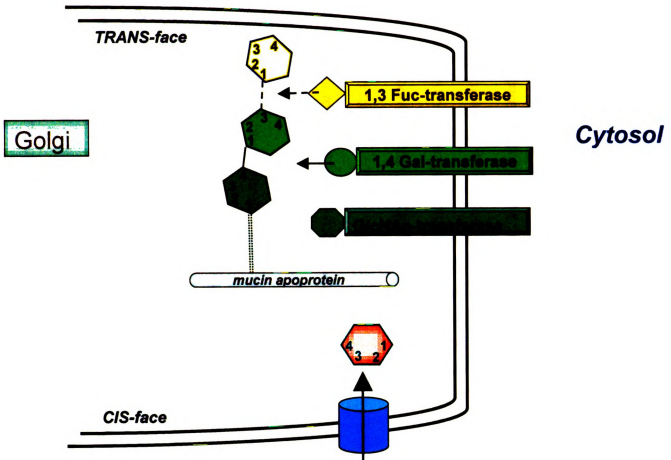

Figure 8. Construction of oligosaccharide side chains in the Golgi: specific glycosyltransferases add hexose sugars in a stepwise fashion

allowing for compression of the side chains and condensation of mucins (Verdugo, 1991). Upon stimulus for release, the cytoplasmic granules fuse with the plasma membrane and an opening, or fusion pore, forms where the two meet (Verdugo, 1991). The formation of a fusion pore allows intermingling of water and electrolytes between the extracellular space and the interior of the granule. This intermingling allows an ion exchange, where extracellular sodium exchanges with intragranular calcium (Verdugo, 1991). The electrical force supplied by monovalent cations for generation of counter-ion shielding is less than that supplied by the divalent calcium cations (Tanaka, 1981). When the sodium/calcium exchange occurs, then, the mucins explosively lose their condensed conformation, and are released into the extracellular environment in an expanded form.

Stimuli for release of mucin molecules from mucous goblet cells are varied, for example according to species and anatomic location, but can potentially include neuronal mechanisms, the action(s) of inflammatory mediators, and direct effect of bacterial endotoxin (Jackson, 2001). Whatever the stimulus for granule release, there is recent experimental data to support an intracellular pathway, converging on the MARCKS protein, that is common to all mucin granule release. Myristoylated alanine-rich C-Kinase substrate (MARCKS) protein is a major substrate for protein kinase C (PKC). Work by Adler et al, (2000) has demonstrated in humans that secretagogue activation of PKC leads to the phosphorylation and eventual activation of MARCKS. Activated MARCKS attaches to the outer granule membrane, and functionally interacts with

cytoskeletal elements, allowing granule movement to and fusion with the plasma membrane.

Fully expanded mucin molecules provide the gel layer of the mucous blanket with its viscoelastic properties. The interaction of mucins in the gel layer is not one of complex cell to cell crosslinkages, however. Rather, individual mucins link in long, end-to-end linear arrangements. Linear chains of mucins then freely intertwine and tangle amongst themselves in the gel layer (Verdugo, 1991).

VI. Conformation of O-linked mucin oligosaccharides

In mammals, O – glycosylation begins in the Golgi, almost always with addition of an N-acetyl galactosamine to a serine or threonine by a GalNAc-transferase enzyme (Hanisch, 2001). Though there can be an incredible amount of variation in precise structure of mucin oligosaccharides, each chain has a basic pattern. There is a core region, composed of the two or three sugars most proximal to the apomucin (of which GalNAc is the first), a backbone region that serves to elongate the chain, and a peripheral region, which displays much structural complexity and serves as a functional site in many instances (Hanisch, 2001; Lamblin et al., 1991). There are eight known core structures, cores 1 through 8. As an example of core structure, core 1 is composed of a GalNAc O-linked to a serine or threonine in an α anomeric configuration, and a single galactose linked to the GalNAc in a β – 1,3 fashion (Van den Steen et al., 1998). Core structures can be branched as well as linear, as can be exemplified by core 2, where the initial GalNAc has two sugars linked to it, a galactose [β – 1,3] and a GlcNAc [β – 1,6] (Van den Steen et al., 1998).

The backbone serves to elongate the chain, usually by the presence of repeating disaccharide units of galactose and N-acetyl glucosamine, in either a Gal β – 1,4GlcNAc linkage or a Gal β – 1,3GlcNAc linkage (Hanisch, 2001, Lamblin et al., 1991). This type of disaccharide unit is referred to as a lactosamine structure, with a β – 1,3 linkage referred to as a Type 1 lactosamine and a β– 1,4 as a Type 2 chain (Lamblin et al., 1991). Branched forms can be generated at the level of the backbone as well as the core by a lactosamine disaccharide linking to the C6 position of a preceding galactose while a second lactosamine links to the C3 or C4 (Hanisch, 2001). Polylactosamine structures of varying lengths and configuration can therefore significantly contribute to the overall length and shape of the completed oligosaccharide.

The degree of complexity of the oligosaccharide chain increases at the level of the periphery, as L-fucose and N-acetyl-neuraminic acid become potential components, as well as the GalNAc, GlcNAc and Gal sugars that comprise the core and backbone (Bhatia and Mukhopadhyay, 1999; Lamblin et al., 1991). Additionally, sulfation – the addition of an SO_3^- group catalyzed by a sulfotransferase – is a common peripheral modification of mucin sugar chains (Nieuw Amerongen et al., 1998). FIGURE 9 is a diagram of a representative oligosaccharide chain possessing a core 2 structure attached to the apomucin.

VII. Functional aspects of oligosaccharides

A. Oligosaccharides and leukocyte adhesion

Oligosaccharides are ubiquitous in organisms, serving as important contributors to cell and tissue structure as well being important structural

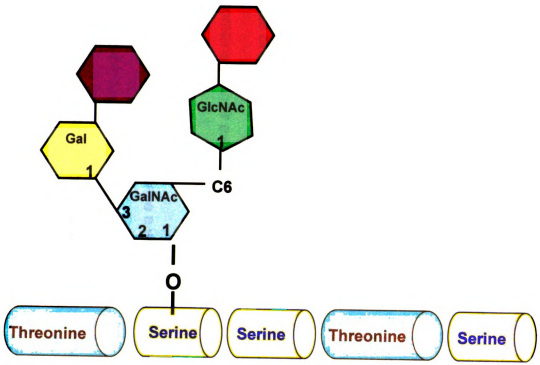

Figure 9. Representative mucin glycoprotein, with core 2 oligosaccharide side chain

components of secreted mucins. Specific oligosaccharide structures are of particular importance in cell adhesion activity, primarily in regard to leukocyte margination and adhesion.

Circulating leukocytes possess the capability to migrate from vessels to tissues in a multi-step process that begins with initial capture followed by rolling of the white blood cell along vessel walls (Wagner and Roth, 2000). This initial process of margination is followed by firm adhesion of white blood cells to a specific site on the endothelium, in turn followed by migration of the leukocyte from the vasculature into tissues.

Leukocyte capture and rolling events are dependent on selectin and selectin-ligand interactions, which ultimately depend on the structural and functional properties of oligosaccharides. Selectins are cell surface proteins that contain lectin domains on their extracellular amino-terminal projections.

B. Lectins

Lectin is a term used to describe a wide variety of calcium-dependent carbohydrate-binding proteins or glycoproteins. Lectins act in a specific manner, with a particular lectin domain binding to a specific sugar situated in a specific conformation. Lectins are widespread in nature. Not only are they found in mammalian cells, but also as component molecular structures of plants and fungi. For example, UEA 1 lectin is derived from the seeds of the plant *Ulex europaeus* (gorse), and exhibits binding specificity for α – 1,2 linked fucose molecules. In contrast, AAL lectin, derived from the fungus *Aleuria aurantia*,

binds to fucose but displays specificity to fucose molecules in an α– 1,3 configuration.

C. Oligosaccharide and leukocyte interactions: selectins and selectin ligands

Leukocyte selectins are essential for initial white blood cell capture and rolling (margination). Leukocyte selectin (L-selectin) was first discovered as an adhesion molecule on lymphocyte surfaces (Bochner, 1998), and is also constitutively expressed on neutrophils (Wagner and Roth, 2000). Ligands for L-selectin become expressed on the surface of endothelial cells in response to bacterial endotoxin and inflammatory cytokines (Wagner and Roth, 2000). When conditions that precipitate an inflammatory response call for leukocyte migration into tissues, a well characterized process of transient leukocyte rolling, firm adhesion, and migration occurs. The first step in this process, initial margination and rolling of circulating leukocytes along the endothelium, occurs when leukocyte selectins interact with their ligands. When the ligands are expressed, L-selectin/ligand interactions are responsible for initial capture and rolling of neutrophils along the vessel walls. Though selectin/ligand pairings are not powerfully adhesive, this first step is necessary in order for firm adhesion and subsequent leukocyte trafficking into the tissues to occur.

L-selectin ligands are glycoproteins, the best-characterized being GlyCAM-1 (glycosylated cell adhesion molecule) and CD34 (Hooper et al., 1996). The terminal configuration of GlyCAM-1 and CD34 is a fucosylated, sialylated (N-acetyl-neuraminic acid), sulfated tetrasaccharide unit, and this unit is the minimal

structure necessary in order for selectin binding to take place (Imai et al., 1993a; Hooper et al., 1996). The specific conformation of this tetrasaccharide has a sialic acid linked to a galactose which is linked to the C4 of an N-acetyl glucosamine. A fucose molecule is also linked to the N-acetyl glucosamine at the #3 C (NANA(β-2,3)Gal(β-1,4)GlcNAcFuc[α-1,3]). The GlcNAc is in turn linked to a preceding sugar in the oligosaccharide chain through a β-linkage. 6-O-sulfation of either the Gal or GlcNAc component of the tetrasaccharide is also necessary for ligand binding to occur (Green et al., 1992; Hooper et al., 1996; Imai et al. 1993b). This specific tetrasaccharide structure (FIGURE 10) is referred to as a sialylated Lewis X structure [SLeX] (Van den Steen et al., 1998).

Selectins can be endothelial cell-associated as well as leukocyte-associated. Platelet selectin and endothelial cell selectin (P-selectin and E-selectin, respectively) are molecules involved in leukocyte adhesion. Neither of these selectins are constitutively present on endothelial cell surfaces, and are expressed only following inflammatory stimuli (Wagner and Roth, 2000). Ligands for P- and E- selectin are present on leukocyte surfaces. P-selectin glycoprotein ligand (PSGL-1) is a glycoprotein dimer with terminal O-linked N-acetyl neuraminic acids, and is capable of binding two P-selectin ligands simultaneously (McEver and Cummings, 1997). As with L-selectin, the SLeX structure is the required terminal configuration for effective binding of P-selectin to PSGL-1 (Hooper et al., 1996). Though the E-selectin ligand has not been fully characterized, it contains vital fucose and sialic acid molecules as terminal modifications (Van den Steen et al., 1998).

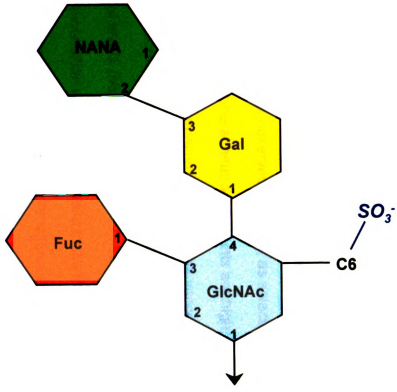

Figure 10. Sulfated sialyl Lewis X structure, a terminal modification of oligosaccharide side chains

The importance of the SLeX structure in normal leukocyte trafficking can be demonstrated by a study where exogenously administered SLeX tetrasaccharide diminished neutrophil-dependent lung damage following cobra-venom administration (Mulligan et al., 1993). In this instance, binding of L-selectins by the SLeX tetrasaccharide units decreased the initial margination activity of neutrophils, and subsequently decreased neutrophil migration to the lung tissues.

An additional significant observation regarding leukocyte adhesion involves a rare human inherited disorder termed leukocyte adhesion deficiency II (LAD II). LAD II is a defect in the *de-novo* synthesis of GDP-fucose (Lowe, 1999), with the end result being an inability to fucosylate oligosaccharide structures. Immunodeficiency is observed in LAD II, as selectin ligands cannot be properly synthesized. Peripheral neutrophil counts are high, as the neutrophils are unable to adhere to the endothelium and leave the vasculature. In a study with one LAD II patient, oral fucose treatment resulted in reduction of peripheral neutrophils to normal levels (Marquardt et al., 1999). LAD II and evidence of effective treatment for the condition with fucose speaks to the importance of oligosaccharide fucosylation in normal leukocyte function and behavior.

Evidence exists that secreted mucins expressing specific oligosaccharide structures can interact with leukocyte adhesion molecules. Mucins are components of saliva as well as mucus, and a study centered on MG2 -- a low molecular weight human salivary mucin encoded by the MUC7 gene (Bobek et al., 1993) -- showed that MG2 oligosaccharide termini carry SLeX determinants (Prakobphol et al., 1999). These SLeX structures demonstrated L-selectin ligand

activity, and MG2 molecules were found in association with neutrophils, suggesting specific neutrophil tethering activity by these mucins.

In summary, glycoprotein molecules that are not secreted mucins, but are composed of the same sub-units as secreted mucins and share common features with secreted mucins, are critical components of the complex system of leukocyte adhesion and subsequent migration into tissues. Additionally, it has been demonstrated that a SLeX determinant on a secreted salivary mucin can confer L-selectin ligand activity to the mucin. Collectively, this knowledge indicates that terminal oligosaccharide structure on secreted mucins can potentially interact in a functional manner with leukocytes that migrate into airway lumens.

D. Interactions between bacteria and mammalian mucins

i) Oligosaccharides. The mucus layer has long been recognized as an important defensive structure, serving as a protective layer on mucosal surfaces to trap foreign substances and facilitate their removal. The mucus layer can be thought of as more than a non-specific adhesive layer, however, as mucin oligosaccharide structures can impart specific functions. Structure/function relationships of mucin oligosaccharides are well-illustrated by bacterial/mucus interactions, as the peripheral regions of mucin oligosaccharide chains can serve as specific receptors for common bacterial pathogens (Gaillard and Plotkowski, 1996). These interactions occur between bacterial surface proteins, termed adhesins, and specific oligosaccharide structures that are components of either epithelial cell surfaces or molecules such as mucins and collagen that surround

epithelial surfaces (Gaillard and Plotkowski, 1996). Adhesins are typically protein molecules that are expressed on the surface of bacteria, or the pili of piliated strains (Lamblin and Roussel, 1993). Lamblin and Roussel have suggested that mosaics of oligosaccharide side chains on mucins constitute a "vast array" of potential binding sites for various bacteria.

Specific examples of bacterial organisms that are dependent on adhesin/host glycoprotein interactions are *Haemophilus influenzae, Helicobacter pylori*, *Candida albicans*, *streptococcal* species and *Pseudomonas aeruginosa*. *H. influenzae* is an important pathogen in chronic bronchitis and cystic fibrosis, and pilus-mediated adherence of the organism to human respiratory mucus takes place (Kubiet et al, 2000). *H. pylori* is a Gram negative organism found in the stomachs of a large percentage of the human population (Hooper and Gordon, 2001). *H. pylori* can interact directly with gastric epithelium that expresses the Lewis b tetrasaccharide structure (Fucα-1,2Galβ-1,3, GlcNAcβ[Fucα-1,4]) (Boren et al., 1993). Interestingly, the Lewis b tetrasaccharide displays structural similarity to the type O determinant on erythrocytes of humans of blood group O (Fucα-1,2Galβ-1,4GlcNAc), also referred to as the H antigen. Type A and B individuals express more complex oligosaccharide antigens on their erythrocyte surfaces, with additional terminal GalNAc and Gal sugars, respectively (Voet and Voet,1990). This difference has been proposed as a reason why there is an increased incidence of *H. pylori*-associated gastric ulcers in type O individuals (Hooper and Gordon, 2001), as type O individuals may not express glycosyltransferases that are involved in the additional modification of the Lewis

b and related structures, thus generating glycoproteins that can serve as more specific receptors for *H. pylori* adhesins.

A different example of how oligosaccharide-mediated adhesion processes have important and varied biological effects concerns a peptide produced by *H. pylori* organisms, the *H. pylori* neutrophil activating protein (HPNAP). This molecule recognizes and binds to specific surface carbohydrates of neutrophils and induces increased expression of adhesion molecules on the neutrophil surface, thus enhancing neutrophil adhesion to endothelial cells (Teneberg et al., 1997).

In a protocol examining the mechanisms of *C. albicans* adhesion to both buccal and vaginal epithelium, lectin and carbohydrate inhibition studies indicated that there are at least two types of adhesion mechanisms – one requiring presence of fucose and one requiring N-acetyl-glucosamine (Critchley and Douglas, 1987). Additionally, fucose and N-acetyl-neuraminic acid have been demonstrated to be of importance in the binding of oral viridans streptococci (a taxonomic grouping of commensal bacteria in the oro-pharyngeal cavity) to human epithelial cells (Vernier et al., 1996).

Of particular importance is *Pseudomonas aeruginosa*, a significant causative organism of respiratory tract infections (Adam et al., 1997a). *P. aureginosa* infection is also a major secondary complication in individuals with cystic fibrosis (Adam et al., 1997b). *P. aureginosa* expresses a variety of surface molecules that are involved in a range of bacterial adhesion processes with glycoconjugate molecules. One *P. aeruginosa* adhesion molecule is PA-II, a molecule that

preferentially binds to the L-fucose of multisaccharide structures referred to as H, A and B antigens (Gilboa-Garber et al., 1994). These structures possess a terminal fucose in an α – 1,2 linkage, a different linkage form than the fucose molecules found in the SLeX structure (α – 1,3). It has also been demonstrated that *P. aeruginosa* strains isolated from cystic fibrosis patients have a high binding affinity for mucin carbohydrates possessing the SLeX structure (Scharfman et al., 1999). Further investigation has shown that *P. aeruginosa* expresses a number of different adhesins, with flagellar cap protein, or FLiD, binding to the SLeX epitope (Scharfman et al., 2001). In this study, additional *P. aeruginosa* adhesins bound to different carbohydrate epitopes, such as the non-sialylated Lewis X epitope (Gal β – 1,4GlcNac β – 1[Fuc α -1,3]) further indicating that bacterial adhesion to host mucin molecules is a complex, multifactorial association.

Pseudomonas aeruginosa/mucin interactions extend beyond simple adhesion, as more complex functional consequences of this pairing have been reported. The PA-II lectin has ciliotoxic properties in addition to its role in specific binding. This ciliotoxic activity is believed to be a survival mechanism on the part of the bacteria, one that enables the bacteria to actively damage a component of the host organism's defense system and thus facilitate colonization of the respiratory system (Adam et al., 1997b).

The significance of bacterial adhesion/host glycoconjugate interaction is underscored by a study that used nasal polyps from control and cystic fibrosis patients to demonstrate that addition of exogenous fucose, to bind and cover the

P. aeruginosa adhesin molecules, resulted in cilioprotective effects (Adam et al., 1997b).

As mentioned, bacterial adhesion and host mucin oligosaccharide binding is an important interaction, although the primary beneficiary of this interaction is not necessarily clear. It can be argued that these specific interactions may be an evolutionary development on the part of the host, as targeted binding of xenobiotic organisms in the mucus layer to enhance their removal by the mucociliary apparatus can clearly be seen as protective. However, the case of the *P. aeruginosa* ciliotoxic adhesin PA-II offers evidence that pathogenic organisms can use these interactions to their own benefit. It is reasonable to view these prokaryotic/host-mucin interactions as an example of adaptive processes that occur continuously and simultaneously in evolutionary adversaries, where natural selection leading to an advantage for one party is countered by an adaptation on the part of the other.

ii) Bacteria and mucin apoproteins. The presence of bacteria in the airways can have direct effects on production to mucin apoproteins. *P. aeruginosa* can upregulate MUC 5AC and MUC 2 in human bronchial explants and bronchial epithelial cell cultures that lack inflammatory cells, and this upregulation can be mimicked by endotoxin molecules derived from *P. aeruginosa* (Li et al., 1997; Dohrman et al., 1998).

P. aeruginosa is not alone in its ability to initiate or provoke a specific response on the part of the host in regard to mucus production. *Bordetella pertussis*, the causative organism of whooping cough in humans, has been

demonstrated to upregulate MUC 2 and MUC 5 AC transcription in a human bronchial epithelial cells (Belcher et al., 2000), as have Gram-positive bacteria such as *Staph aureus* and *Strep pyogenes* (Dohrman et al., 1998). In the case of *B. pertussis*, it was also determined that the organisms preferentially bound to sialic acid-containing structures, which again speaks to important functional interactions between pathogenic respiratory tract organisms and host mucins.

VIII. Airway Inflammation

A. Inflammation and the airways: introduction

Increased and/or altered mucus production in airways is a feature of diseases such as asthma, chronic bronchitis and cystic fibrosis in humans, and recurrent airway obstruction (RAO) of horses (Lundgren and Shelhamer, 1990; Robinson et al., 1996; Shak, 1997). Common to all these conditions is some degree of airway inflammation. The inflammatory response in the airways involves an exquisitely complex interplay between inflammatory cells, inflammatory mediators and airway tissues. Though the complete role and mechanism of inflammation in these conditions is not fully elucidated, it is well established that substances associated with inflammation, such as leukocyte proteases and interleukins, can stimulate mucus production.

In the case of the respiratory system, the inflammatory response involves resident immune system cells such as macrophages, cells that migrate to the airways from blood or lymphatic vessels, and pulmonary tissues themselves -- specifically the airway epithelium.

B. Inflammatory cells and the respiratory system: an overview

Inflammatory cells that play important roles in the airway inflammation are not unique to the pulmonary vasculature or the respiratory system in general. These cells include macrophages, lymphocytes, neutrophils, eosinophils and mast cells.

i) Macrophages

Macrophages are normal residents of the lung, not only in the alveoli but in the airways as well, moving proximally via the mucociliary escalator.

Macrophages express MHC II and are antigen-presenting cells. They function in the lung as first-line defenders, phagocytizing inhaled bacteria and particulates (Gant et al., 1998).

Pulmonary macrophages produce a variety of bioactive molecules involved in the overall inflammatory response. These substances include reactive oxygen species, proteinases, and proinflammatory cytokines such as tumor necrosis factor α (TNFα), Interleukins 1 and 8, GM-CSF and the lipid mediator leukotriene B4 [LTB4] (Gant et al., 1998; Daftary et al., 1998; Holt and Williams, 1998). Additionally, macrophages express CD14, a glycosylphospatidylinositol-anchored protein, on their surfaces (Hailman et al., 1994). CD14 is an endotoxin-binding protein. Endotoxin/CD14 interactions result in intracellular signaling cascades that activate macrophages, resulting in production of proinflammatory cytokines (Su et al., 1995; Thorn, 2001). Macrophages therefore play a prominent role in host responses to endotoxin.

ii) Lymphocytes

Lymphocytes can be broadly classified as being either T lymphocytes (thymus- derived) or B lymphocytes (bone marrow-derived). T lymphocytes recognize antigens and can initiate cell mediated reactions, where various effecter cells of the immune system are activated. B lymphocytes produce antibodies (immunoglobulins), and are therefore principal cells of the humoral immune response. T cells are also important in the humoral response, however, as activation and proliferation of B cells is dependent on T cell function, for example via production of B cell-stimulating cytokines.

T lymphocytes become activated when they physically interact with antigen presenting cells that display antigenic peptides in concert with MHC II molecules. Once activated, different subsets of T cells -- either TH-1 or TH-2 subsets -- can produce a variety of cytokines that are important in airway inflammation. These include interferon gamma and TNFα by TH-1 cells, and interleukin 4 (IL-4), IL-13, IL-5 , IL-9, and IL-10 by TH-2 cells (Corrigan, 1998; Larsen and Holt, 2000).

iii) Neutrophils

Neutrophils are attracted from the vasculature to tissues of the airways by chemoattractant cytokines such as leukotriene B4 (LTB4), platelet activating factor (PAF) and interleukin 8 [IL-8] (O'Byrne, 1998; Holtzman et al., 1983; Lellouch-Tubiana et al., 1988; Richman-Eisenstat et al., 1993). Major sources of LTB4 are macrophages and neutrophils themselves (O'Byrne, 1998). Macrophages are producers of IL-8 and PAF, as are neutrophils themselves. Production of LTB4 and IL-8 by neutrophils indicates a positive feedback system

of neutrophil attraction during inflammatory processes. Additionally, injured airway tissues can independently recruit neutrophils, as the epithelium itself can produce IL-8 (Proud, 1998; Marshall et al., 2001; Nakamura et al., 1991; Park et al., 2000).

Once in the tissues, activated neutrophils can phagocytize bacteria and particulates, and produce a wide array of bioactive substances. These include reactive oxygen species (ROS), LTB4, PAF, IL-8, TNFα, IL-1β and proteinases such as neutrophil elastase (O'Byrne, 1998; Scapini et al., 2000).

iv) Eosinophils

Interleukin 5, produced primarily by the TH-2 subset of T lymphocytes, is a chemoattractant for eosinophils, and a powerful stimulator of eosinophil production and function (Broide, 2001). Like other inflammatory cells, eosinophils produce multiple substances. These include major basic protein, reactive oxygen species, proinflammatory cytokines such as TNFα and IL-1, and lipid mediators such as PAF and leukotriene C4 and C5 (Kita et al., 1998). These latter two compounds are also known as cysteinyl leukotrienes, and have been implicated in causing bronchoconstriction and increased mucus secretion in airway inflammatory disease (Bigby, 2000).

v) Mast cells

Mast cells are usually found within tissues such as the skin and mucosa of the respiratory and gastrointestinal tract -- that is, tissues that can act as potential entry portals for xenobiotic agents or foreign particles. Mast cell activation is a multi-step process. IgE, produced by properly activated B lymphocytes, must

bind to high-affinity IgE receptors (FCεR1) that are present on mast cell surfaces (Schwartz and Huff, 1998). Two separate IgE molecules must then be simultaneously bound be a single multivalent antigen (i.e. IgE cross linking). Once activated, mast cells can release large quantities of important inflammatory mediators such as LTC4, TNFα, histamine, and the neutral proteinases tryptase and chymase (Schwartz and Huff, 1998; Gurish and Austin, 2001).

C. The airway epithelium as active participant in inflammation

The airway epithelium is a complex functional structure, being much more than a mere physical barrier and the producer and transporter of a mucous blanket. Examples of metabolic function of the airway epithelium include synthesis of acetylcholine and metabolism of histamine and neuropeptides (Folkert and Nijkamp, 1998).

Airway epithelium can also be a proactive participant in the inflammatory process. Human bronchial epithelial cells have been shown to produce the neutrophil chemoattractant IL-8 (Marshall et al., 2001; Park et al., 2000) *in vitro*. Interleukin-8 has also been demonstrated to be produced by human epithelial cells *in vivo*, as Ryhoo et al (1999) have shown IL-8 gene expression in surgical biopsies of sinus mucosal tissue obtained from patients with rhinosinusitis. Epithelial cells have also been shown to produce proinflammatory reactive oxygen and nitrogen species in a study using guinea pig tracheal epithelial cells in culture (Rochell et al., 1998).

D. Airway inflammation and increased mucus production: specific diseases

i) Asthma

Asthma is a disease characterized primarily by bronchial hyperresponsiveness and reversible airway obstruction. Chronic inflammation is associated with the condition, as is an increase in mucus production. A pronounced eosinophilia has long been recognized as a prominent component of asthma. Other inflammatory components are present in the disease, however, and these components may have critical importance, as recent studies have brought the eosinophil's role in asthma into question. For example, Leckie et al. (2000) showed that depletion of circulating and airway eosinophils had no effect on clinical signs of asthma, and a study by Kips et al. (2000) demonstrated that reduction of eosinophil numbers by corticosteroids did not result in concomitant improvement in lung function. Inflammatory cell types other than eosinophils may play important roles in the development and/or maintenance of asthma, however. Lymphocytes of the TH-2 subset and the cytokines they produce, such as interleukins 9 and 13, are recognized to be associated with asthma (Robinson, 1992; Gavett et al., 1995; Bousqet et al, 2000), and neutrophils and the bioactive compounds they produce are now thought to contribute to clinical signs and symptoms of asthma (Jung and Park, 1999; Krawiec et al., 1999; Louis et al., 2000; Martin et al., 1991; Park et al., 1999). Additionally, a bronchial neutrophilia has been shown to be associated with severe, refractory prednisone-dependent asthma (Wenzel et al., 1997 and 1999), and with status asthmatacus,

or acute, severe asthma attacks (Lamblin et al., 1998). Ordonez et al, (2000) also showed an increased number of neutrophils and an increase in levels of the neutrophil chemoattractant interleukin 8 in airway secretions of patients with acute asthma attacks that required intubation and mechanical ventilation. In a study involving adults with persistent asthma and healthy controls, Gibson et al. (2001) also described a heterogeneity of airway inflammation in asthma, with an increase in IL-8 and a distinct role for neutrophils in noneosinophilic persistent asthma.

Excessive mucus production can be quite a significant problem in asthma, as extensive mucus plugging of the airways is a regular post-mortem finding in individuals who die during status asthmatacus (Sheehan et al., 1995; Tonnel et al., 2001).

Current therapy for asthma consists of regular, long term use of inhaled corticosteroids to reduce inflammation, coupled with bronchodilator agents such as long-acting β2 agonists or quaternary ammonium atropine compounds. Short acting β2 agonists agents are also used during bouts of acute respiratory distress (Global Initiative for Athma, NIH, 1998).

Asthma is a much-studied disease. And though much is known about this disease, knowledge is still lacking concerning the relationship between neutrophils and the increased presence of specific mucin structures in the airways. Further research in this area is warranted, and is detailed later in this thesis.

ii) Cystic fibrosis

Cystic fibrosis (CF) is a genetic condition that is not confined to the respiratory system, but is a body-wide condition that affects many exocrine tissues. The primary problem is a defect in one gene that is required for proper construction of a membrane protein, the CFTR [cystic fibrosis transmembrane conductance regulator] (Doull, 2001). This membrane protein has many functions, including conduction of chloride ions and the regulation of other types of membrane ion channel proteins. An example of the latter is exertion of a negative regulatory effect on an epithelial sodium channel (Krouse, 2001; Pitt, 2001). Though the CFTR defect is well known, much of the pathogenesis of CF remains a mystery, as the disease cannot be adequately and entirely explained by a defective apical epithelial chloride channel alone (Scanlin and Glick, 1999).

The principal respiratory complication of CF is one of chronic *P. aeruginosa* infection (Govan and Deretic; 1996). Chronic and permanent infection is by a strain of *P. aeruginosa* that is of a mucoid phenotype (Tatterson et al, 2001) where the bacteria themselves produce an exopolysaccharide called alginate. There is currently controversy concerning the reasons why *P. aeruginosa* infection occurs with CF (Krouse, 2001). One postulated reason is that abnormal ion composition of airway surface liquid (increased salt content) inhibits the activity of host antimicrobial peptides. A competing school of thought is that there is decreased volume of airway surface liquid due to increased reabsorption of sodium and water, secondary to loss of negative regulation of an epithelial sodium channel. The decreased volume allows collapse of the cilia, resulting in

cessation of normal mucus movement up the mucociliary escalator. Lack of proper mucociliary escalator function then allows bacterial colonization. Although recent studies have suggested that ion composition of the airway surface fluid is indeed normal (Verkman, 2001), supporting the notion that decreased volume is a critical aspect, the issue remains unresolved.

No matter the cause, bacterial colonization results in subsequent chronic inflammation, primarily of a neutrophilic nature (Kahn et al., 1995; Armstrong et al., 1997). It has also been reported that CF epithelium may have constitutively upregulated IL-8 production, which would contribute to neutrophilic airway inflammation (Doull, 2001; Tatterson, 2001).

No cure exists for CF. Current treatment in regard to airway pathology involves a) inhaled or systemic antibiotics to fight chronic *P. aeruginosa* infection; b) dornase alfa, an enzyme that fragments DNA, as intact DNA released from bacteria and inflammatory cells can thicken the mucus blanket; c) bronchodilators, such as β2 agonists; and d) mechanical disturbance and mobilization of airway mucus, for example by physical percussion of the chest (Mayo Foundation for Medical Education and Research, 2001).

There is no question as to the primary genetic defect in CF, and also no question that one of the main clinical signs of CF is the presence of thick, viscous mucus in the airways. Many reports have also demonstrated that mucins produced by CF airway epithelium are different from mucins produced by non-CF epithelium, with increased fucosylation and decreased sialylation of secreted mucins being characteristic of CF (Scanlin and Glick, 1999). Interestingly, the

fucose increase is of an α – 1,3 linkage (characteristic of Lewis antigens), and the sialic acid is of a 2,6 linkage (differing from the 2,3 linkage found in the SLeX structure). The precise reasons for the presence and characteristics of CF mucus remain unknown, however, despite a great deal of research into the disease. As Quinton points out in a historical overview of CF (1999), there is no clear evidence to determine what occurs first, increased (abnormal) mucus production and secretion in the airways, or infection and subsequent inflammation. If the latter eventually is found to be true, increased mucus presence in CF may be explained as a sequelae of infection by LPS-bearing bacteria and resultant chronic inflammation, and abnormal viscosity may be due to presence of cellular components such as DNA and actin.

Whatever the case in regard to CF, the fact remains that an excessive mucus presence exists, along with Gram-negative bacterial colonization and chronic inflammation. Some fraction of the altered mucus production may then be due to effects of endotoxin and/or endotoxin-induced inflammatory mediators. The mucus of CF, from mucin structure to gross characteristics, may therefore not be pathognomonic to the disease, but may be the same type of mucus that occurs in a variety of airway diseases.

With CF, as with asthma, the full relationship between inflammation and altered quantity and/or quality of mucus is unknown. Further research is required on many fronts before a thorough understanding of the connection between these two factors is realized.

iii) Recurrent airway obstruction of horses

Horses with recurrent airway obstruction (RAO) have a clinical presentation that can include coughing, increased respiratory secretions (i.e. mucus), a predominantly neutrophilic airways inflammation, and a forced expiratory abdominal effort that gives the disease the common name of heaves (Robinson et al., 1996). The labored breathing observed with RAO is due to an increase in airway resistance. This increase in resistance is due primarily to bronchospasm, or contraction of the airway smooth muscle (Robinson et al., 1996), although other factors such as increased mucus production and airway wall thickening may contribute.

The airways inflammation and clinical signs of respiratory distress follow exposures to the instigating environment of a barn or stable, where hay and grain dusts can be quite heavy in the air. Concentrations of dusts in the air of a horse stall can be as high as 20 mg/m^3 (Woods et al, 1993). As a comparison, OSHA guidelines for maximal dust exposure for humans are 10 mg/m^3 for an 8 hour period.

The principal pathologic feature of RAO is a bronchiolitis, where there is a peribronchiolar accumulation of lymphocytes. Increased mucus presence in the airways and infiltration of neutrophils into airway lumens are also characteristic components of the disease (Breeze, 1979; Kaup et al., 1990, Robinson et al., 1996, Robinson, 2001).

Neutrophil accumulation in the lung of RAO horses occurs at 3 to 5 hours after initiation of natural challenge (Fairbairn et al., 1993), and neutrophils are

present in increased numbers in the bronchoalveolar lavage fluid (BALF) by 5 hours (McGorum et al, 1993a).

In a study by Franchini et al, (2000) involving RAO and non-RAO horses, increased levels of the neutrophil chemokine IL-8 and neutrophils were present in RAO horses as compared to controls, and increases were also present in RAO horses exposed to dust as compared to non-exposed RAO horses. As well as underscoring the prominence of neutrophilic inflammation in RAO, the study suggested a mechanism for neutrophil recruitment via increased IL-8 presence, though roles for other neutrophil chemotactic compounds such as the leukotrienes and macrophage inflammatory protein – 2 were not ruled out.

There is also evidence that demonstrates persistent airway pathophysiologic changes to the airway epithelium in RAO-affected horses. Bureau et al (2000) showed a persistent granulocyte-dependent activation of NFκB in bronchial brushings from RAO horses removed from environmental challenge for 30 days.

Though neutrophils are the predominant cell type that can be recovered in BALF of RAO-affected horses, lymphocytes may also be important in the development and progression of the disease. A study by Lavoie et al (2001) demonstrated that lymphocytes of the TH-2 subtype may be associated with RAO. Using molecular techniques to assess expression of cytokines associated with either TH-1 or TH-2 lymphocytes, this study showed that the TH-2 – associated cytokines interleukin 4 and interleukin 5 (IL-4, IL-5) were increased in RAO horses as compared to control horses, and there was a concurrent

decrease in RAO horses of the TH-1 – associated cytokine interferon gamma (INFγ).

Similarities exist between RAO of horses and asthma. As well as phenotypical similarities such as a reversible bronchoconstriction and mucus accumulation in the airways, there are important parallels in regard to inflammatory patterns. Both diseases exhibit an increase in TH-2 cell activity, and activity of neutrophils may be important in the pathogenesis and progression of both conditions. Parallels exist between RAO and CF, as well – most notably the increased IL-8, neutrophil and mucus presence that are components of both diseases.

Similarities exist between asthma and RAO in terms of treatment approaches. As in asthma, treatment of RAO involves environmental control (i.e. separation from a precipitating environment), use of corticosteroids to reduce inflammation, and bronchodilators (Robinson et al., 2001).

Increased mucus presence in RAO has long been observed and noted, and subjective scoring systems to assess levels of mucus accumulation have been employed (Dixon et al, 1995; Mills et al, 1996). No objective methods of quantifying airway mucus in RAO have been developed, however, and no detailed examination of the cause(s) and significance of mucus in the disease has been performed. Work in this thesis project addresses these unknown areas.

iv) Etiologies: RAO and asthma.

Exact triggers of airway inflammation in these conditions are varied or not fully understood. In the case of asthma, for instance, most patients exhibit immediate hypersensitivity responses to inhaled allergens and display rapid IgE-dependent mast cell degranulation (Renauld, 2001), followed by development of signs and symptoms of acute asthma, including an inflammatory response. In contrast to this form of asthma (termed atopic asthma) is non-atopic asthma, a form seen in a large proportion of asthma patients. In non-atopic asthma, an asthma phenotype with airway inflammation develops without increases in IgE or indications of an allergic response (Renauld, 2001). In either case, there is nonetheless inflammatory cell influx into the airways, and generation of a cytokine profile indicative of a T-Helper cell type-2 (TH-2) response (Bousquet et al., 2000), a cytokine profile of great significance to an asthma phenotype.

Though a large body of evidence points to indoor housing and exposure to hay dust as being responsible for the signs and symptoms of equine RAO, the exact nature of the causative event is unknown. Some studies suggest an immunologic-based etiology. Hay dust contains many allergenic substances, including large amounts of respirable mold and actinomycete spores from organisms such as *Aspergillus fumigatus*, *Faeni rectivirgula* and *Thermoactinomyces vulgaris* (Clarke and Madelin, 1987; Robinson et al., 1996). McGorum et al. (1993a) demonstrated that natural challenge with dusty hay resulted in lung dysfunction and airway inflammatory cell infiltration in RAO but not control horses, and that specific antigenic challenge with *A. fumigatus* and *F.*

rectivirgula resulted in airway inflammatory cell influx but not in lung dysfunction. A reaction by RAO horses in both types of challenges that was not observed in control horses led the authors to conclude that RAO was a pulmonary hypersensitivity disease, one similar to atopic asthma. Other evidence of RAO being a hypersensitivity condition come from reports of increased numbers of CD4+ lymphocytes [i. e. T Helper cells], in BALF of RAO horses, (McGorum et al., 1993b), increased levels of TH-2 cytokines in BALF (Lavoie et al., 2001), and increased serum IgE concentrations (Halliwell et al., 1993; Eder et al., 2000). Controversy exists, however, as other investigators suggest that horses with RAO do not have an exaggerated immune response. Ainsworth et al., (2002) published a report demonstrating that RAO horses exposed to a nebulized antigen (keyhole limpet hemocyanin – KLH) did not have an elevated IgG response, and the IgG levels were actually lower than those of control horses.

Though the work of McGorum et al. (1993a) indicated that hay dust exposure resulted in lung function deficits and airway inflammation, specific antigen challenge resulted in inflammation but no pulmonary function deficits characteristic of RAO. As pointed out by Robinson et al. (1996), this suggests that factors other than hypersensitivity to specific antigens may be involved in the pathogenesis of RAO.

Hay dust is composed of many constituents, and though there is some evidence of RAO being a hypersensitivity condition, other, non-specific causes cannot be excluded. One important component of hay dust and barn environments is bacterial endotoxin, or lipopolysaccharide (LPS), a component of

Gram negative cell walls. LPS is ubiquitous in the environment, and has been implicated as being an important factor in respiratory diseases. McGorum and coworkers (1998) reported that total airborne endotoxin concentration in the breathing zone of stabled ponies exceeded levels which can induce airways inflammation and bronchial hyperresponsiveness in humans. Other studies and models have shown that LPS exposures result in increased airway mucus presence (Yanagihara et al., 2001, Vernooy et al., 2002), though it is not always clear if these changes can be stimulated by the direct effect of endotoxin, or if they are secondary to endotoxin-induced inflammation. Additionally, LPS has been proposed as a factor in exacerbation of asthma in humans (Park et al., 2001; Schwartz, 2001).

As no precise etiology of RAO has been discovered at this point, a possibility to be considered is that there may be no single cause in all cases, and that, like asthma, there may be multiple causes that converge on a single phenotype.

E. Specific cellular responses to inhaled agents

As mentioned, the totality of an airway inflammatory response is intricate and not fully elucidated. In regard to increased mucus production and inflammatory airway disease, important links have been established between mediators produced by TH cells of the TH-2 subtype and by neutrophils. It is therefore useful to more closely examine the biology of inflammation regarding these particular cell types.

i) Airway mucosal dendritic cells

Airway mucosal dendritic cells (AMDC) are motile, phagocytic cells of monocyte/macrophage lineage that are present in the airway epithelium (Sertl et al., 1986; McWilliam et al., 1995). Morphologically they possess many branching projections that interweave between epithelial cells and orient toward the airway luminal surface. Functionally, these projections serve as individual environment sampling stations, with many arising from a single cell body. AMDCs are important in the inflammatory process, as they express many MHC II surface markers and are the principal antigen-presenting cells in the airway wall (Lambrecht, 2001; Nicod et al., 2000). They are therefore a critical component in the activation of T cells.

As well as being resident cells in the airway mucosa, dendritic cells can be rapidly recruited to the airways. McWilliam et al (1996) demonstrated migration of dendritic cells to the airways in response to CC chemokines and the bacterial peptide fMLP (formyl-methionyl-leucyl-phenylalanine). Migration of dendritic cells to the airway mucosa in response to the presence of inhaled pathogens and inflammation can therefore be a mechanism for accelerating the airway immune response.

Inhaled foreign particles that deposit onto the mucosal surface of the airways can be detected by AMDCs, engulfed through endocytosis, phagocytized, and thus converted into peptides that can be presented to other cells of the immune system. Once a dendritic cell has taken in a foreign particle, it can migrate to

regional lymphatic tissues in what is known as the afferent phase of the of the T cell immune response (Lambrecht, 2001).

ii) Macrophages

Though macrophages may be involved in antigen presentation processes in regional lymph nodes, it is believed that macrophages act primarily as phagocytic cells in the airways and alveolar regions, serving as first line defense against inhaled particulates and bacteria.

When macrophages become activated in response to inhaled particulates, they can produce a wide array of bioactive agents, including eicosanoids, reactive oxygen species, and cytokines such as the proinflammatory mediator IL-1 and the neutrophil chemoattactant IL-8 (Gant et al., 1998).

Macrophages can also be involved in airway remodeling by the production of fibroblast growth factors, platelet derived growth facor, TGFβ and fibronectin (Bousqeut et al., 2000). Macropohages additionally produce matrix metalloproteinase 12 (MMP12), or macrophage metalloelastase. This enzyme is a member of the matrix metalloproteinase (MMP) family, a group with 23 known members (Parks and Shapiro, 2001). MMPs are zinc-dependent proteinases, that, as a group, can collectively degrade all connective tissue components of an organism. MMPs function in situations where connective tissue turnover is critical, such as during morphogenesis and wound repair (Dunsmore et al., 1998; Parks and Shapiro, 2001).

iii) Lymphocytes

Lymphocytes of the CD4+ (T Helper) family that reside in regional lymph tissues are referred to as TH-0 cells. These are naïve cells, capable of developing into either TH-1 or TH-2 cells. Antigenic presentation to TH-0 cells by airway mucosal dendritic cells (AMDC) acts to regulate further T cell development (Stumbles et al., 1999). The TH cell paradigm that has developed since a first description by Mosmann and Coffman (1989) involves antigen presentation to TH-0 cells by AMDCs, and development of the naïve lymphocyte into either a TH-1 or TH-2 lymphocyte. Antigens associated with a proliferative invasion of the airways, such as by pathogenic bacteria, direct TH-0 cells down a path toward development into TH-1 cells that then migrate to the site of invasion (i.e. the airway mucosa and luminal surface). Activated TH-1 cells then produce a variety of cytokines (a TH-1 cytokine profile) that stimulate a cell mediated immune response. Notable cytokines of the TH-1 profile are interleukin 2 (IL-2), interferon gamma (IFNγ) and tumor necrosis factor beta (TNFβ) (Corrigan,1998).

Antigens such as dusts, pollens and other allergens result in development of TH-0 cells into TH-2 lymphocytes, producers of a TH-2 cytokine profile that acts to promote an antibody mediated response. TH-2 cytokines are more prevalent in allergic reactions. Cytokines of TH-2 cell origin include IL-4, IL-5, IL-9, IL-10 and IL-13 (Corrigan, 1998). All play important roles in inflammatory airway disease, and selected TH-2 cytokines have been demonstrated to increase mucus production. The efferent phase of the T cell response is the migration of

activated TH-1 or TH-2 cells to the airways, where each type's particular battery of cytokines contributes to the immune/inflammatory reaction.

In a significant interplay between the two types of T cells, TH-1 cytokines, especially IFNγ, can have a negative regulatory effect on TH-2 cell development, and TH-2 cytokines such as IL-4, 10 and 13, have a corresponding downregulatory effect on TH-1 cells.

The basic premise of the TH-1/TH-2 paradigm is therefore that one TH pathway is generated depending on the type of invasion or injury to the airway, with a particular cytokine profile (1 or 2) being optimally designed to deal with the original insult. Though numerous studies support this premise, it is now generally accepted that there are no immune reactions with either complete TH-1 or 2 responses to the exclusion of the other sub-type, and that a particular response is skewed or polarized to one of the two T Helper types.

iv) Neutrophils

Cellular responses in inflammatory reactions of the airways are highly complex, and involve much more than T Helper cells. Of note in regard to mucus production in airway disease are neutrophils, as proteinases released from activated neutrophils are known to upregulate mucin production.

Neutrophils possess different receptor types for chemotactic stimuli, including platelet activating factor (PAF), leukotriene B4 (LTB4), and bacterial peptides such as fMLP (Wagner and Roth, 2000). Also of great importance are chemokines (cytokines that exhibit chemoattractant properties), specifically those of the CXC family. All chemokines have 4 cysteine residues at their amino-

termini that form disulfide linkages. In CXC chemokines, the 2 most distal cysteines are separated by an amino acid [X]. In contrast, in CC cytokines there are no intervening amino acids between the two distal cysteines. This relatively small structural difference translates into distinct and different abilities to attract leukocytes, with CXC chemokines attracting neutrophils and CC chemokines primarily attracting mononuclear cells such as lymphocytes (Wagner and Roth, 2000).

Interleukin 8 (IL-8) is a CXC cytokine and can be derived from multiple sources, including macrophages, epithelial and endothelial cells (Metzger and Page, 1998). Triggers for IL-8 release include the pro-inflammatory mediators TNFα and IL-1, as well as the powerful inflammagen LPS (Smart and Casale, 1994; Rot et al., 1996; Wagner and Roth, 2000). Though there are multiple sources of TNFα and IL-1, macrophages are major producers of these inflammatory mediators.

Cellular responses and inflammatory pathways that can lead to a response that includes increased airway mucus production can be summarized in a simplified manner as follows: A) inhalation of an initiating agent, such as allergenic peptides or bacterial LPS. B) macrophages respond to and phagocytize the agent and become activated, whereupon they release mediators such as TNFα, IL-1 and IL-8. These mediators in turn stimulate more IL-8 production from tissues such as airway epithelium, and IL-8 stimulates migration of neutrophils from the pulmonary vasculature into the airway epithelium and lumen. As neutrophils themselves are sources for neutrophil chemoattractants, a

temporary positive feedback loop can be initiated, with continued influx of neutrophils. C) simultaneously with neutrophil recruitment, airway mucosal dendritic cells detect and engulf initiating agents, setting off the afferent phase of T-helper cell activation. TH-0 cells in regional lymphatics are directed onto a TH-2 path, and the efferent phase progresses, where TH-2 cells migrate to the airway tissues and lumen. TH-2 cells produce cytokines of the TH-2 profile, which include IL-4, 5, 9, and 13.

F. Inflammatory mediators and mucus production

A variety of stimuli have been linked to increased mucus production, including PAF, interleukins 13 and 9, serine proteases such as neutrophil elastase, and reactive oxygen species (Henderson et al., 2000; Longphre et al., 1999; Shim et al., 2001; Takeyama et al., 2000; Voynow et al., 1999). As neutrophils are of importance in asthma, cystic fibrosis, equine RAO and endotoxin exposures, the effect of the serine protease neutrophil elastase and related compounds on mucin production and structure must be carefully considered.

i) Neutrophil elastase

Neutrophil elastase [NE] is a serine protease produced and secreted by neutrophils. Serine proteases are enzymes that possess a serine located at a critical point in the three-dimensional configuration of the enzyme, which allows for a nucleophilic attack on chemical bonds in substrate molecules (Voet and Voet, 1990). The specific evolutionary purpose of neutrophil elastase remains a subject of debate. As pointed out by Shapiro (2002), most knowledge concerning neutrophil elastase revolves around its ability to cause tissue

destruction. Neutrophil elastase may be principally designed to destroy pathogens, as NE has been shown to catalytically cleave outer wall proteins of Gram (-) bacteria (Belaaouaj et al., 1998). NE knockout mice have not been shown to be more susceptible to spontaneous infection, however (Shapiro, 2002). Though this indicates that NE is not an essential front line defense against pathogens, the proteolytic activity exhibited by NE may still be critical in host defense, as redundant effects of other, similar, neutrophil-derived proteinases such as cathepsin G may be essential. Controversy exists regarding the necessity of NE for local proteolysis, thus allowing migration of neutrophils through tissue barriers. One study has indicated that inhibition of NE can inhibit migration of neutrophils through a membrane (Delacourt et al., 2002), whereas others (Huber et al., 1989; Mackarel et al., 1999) have shown that elastase inhibition does not effect neutrophil migration.

Neutrophil elastase has distinct effects apart from catalytic destruction of pathogens or host tissue. For example, it can stimulate increased mucus production from the airway epithelium. NE is a well-known secretagogue for mucous goblet cells (Nadel et al., 1999), and several studies have demonstrated that NE can increase expression of MUC 5AC gene, increase MUC 5AC mRNA stability and increase MUC 5AC protein expression (Voynow et all, 1999; Martin et al., 2000).

Though there are many descriptions of the secretagogue effect of NE, the precise mechanisms of its effect is not fully known. Some light has recently been shed on its mode of action, however. DiCamillo and coworkers (2002) have

shown that soluble epidermal growth factor (EGF), proteolytically cleaved from cell surfaces, can transactivate EGFr in lung cells. In turn, work by Nadel (2000) and Takeyama et al. (2001a) has shown that EGFr activation can lead to increased mucus production. Additionally, Fischer and Voynow (2000) have demonstrated that NE increases Muc5AC mRNA expression through an oxidant-dependent mechanism. In this study, NE was shown to increase intracellular oxidant levels in human epithelial cell cultures, and NE's effect on Muc5AC expression was inhibited by addition of dimethylthiourea (DMTU), a scavenger of hydroxyl radicals and other hydroxylated products.

ii) Macrophage metalloelastase (MMP12; macrophage elastase)

Macrophage elastase is a powerful enzyme, with functional properties very similar to neutrophil elastase. Like NE, it contains a catalytic domain that can cleave elastin, a polymer that is normally highly resistant to proteolytic degradation (Parks and Shapiro, 2001).

As with all metalloproteinases (MMPs), macrophage elastase can be involved in tissue remodeling processes that require breakdown of connective tissue. These processes include morphogenesis and wound repair.

iii) Neutrophil-derived reactive oxygen species and the epidermal growth factor receptor

Reactive oxygen species (ROS) are molecules such as hydrogen peroxide, the hydroxyl radical and the superoxide anion. ROS can be produced by neutrophils and other granulocytes, and are released by activated neutrophils

during the inflammatory response (Larsen and Holt, 2000). ROS can have both cytotoxic and cell regulatory effects (Kehrer, 2000; Lander, 1997).

The epidermal growth factor receptor (EGFr) is a molecular dimer that is expressed on many cell types, either constitutively or induced, including the airway epithelium (Takeyama, 1999). Activation of the EGFr by ligands such as EGF or TGFα results in auto trans-phosphorylation of receptor intracellular domains. Auto phosphorylaton in turn activates a number of intracellular signal transduction events, for example signaling cascades involving the G protein-like molecule ras and its downstream partner raf, and mitogen-activated protein kinases [MAPKs] (Artaega, 2001). The phosphorylation event occurs because of inherent tyrosine kinase activity in the EGFr. Nadel (2000) showed that activation of EGFr can stimulate the growth of airway goblet cells, and Takeyama et al (2001b) demonstrated that EGFr activation can increase MUC5AC production at both the gene expression and protein levels, and that selective inhibition of EGFr tyrosine kinase activity can effectively block MUC5AC production by goblet cells.

Though EGFr ligands result in receptor and signal cascade activation, reactive oxygen species that can be produced by neutrophils, such as hydrogen peroxide, have been implicated in the ligand-free activation of EGFr. Wang et al (2000) showed that hydrogen peroxide can activate cell signaling *in vitro* through an EGFr-dependent mechanism. The study by Takeyama et al (2000) demonstrated that supernatant from stimulated neutrophils activated EGFr

cascades in the presence of neutralizing antibodies of EGFr ligands, and this effect was inhibited by antioxidants.

G. Leukocyte migration to the airways: a connection to glycoproteins

Presence of cells such as TH-2 lymphocytes and neutrophils in the airways is obviously a keystone marker of inflammation, and cytokines and other mediators they produce while in the airways are of great significance in terms of a protective response against inhaled agents and also in terms of effect on airway tissues (e.g. increased mucus production).

The process of migration of leukocytes from the pulmonary vasculature into the airways is therefore also of great significance. As this migration involves interactions between adhesion molecules and ligands that are mucin-type glycoproteins, implications exist regarding potential leukocyte/mucin interactions.

The first step in the migration of white blood cells from the bloodstream to the airways is the rolling of leukocytes along the endothelium, which involves transient interactions between selectins and selectin ligands. As previously detailed, neutrophils and lymphocytes express L-selectin, which interact with L-selectin ligands that are expressed on endothelial cells. Evidence suggests L-selectin ligands are both constitutively expressed and also induced by cytokines and LPS (Spertini, 91, Von Adrian, 91, Wagner and Roth, 2000, Walchek, 96).

In systemic tissues, interactions between L-selectin and L-selectin ligands leads to rolling behavior of white blood cells along the endothelium of post-capillary venules, an event which momentarily slows leukocyte movement

through the blood stream, thus allowing the subsequent steps of firm adhesion and transmigration to occur if the appropriate stimuli are present.

Activity in the pulmonary circulation differs, however, in that approximately 97% of neutrophils found in the pulmonary vasculature locate in capillary networks where vessel diameters are too small for rolling behavior (Doerschuk et al., 1993).

Though leukocyte rolling does not occur in the lungs in the same manner as within venules of the systemic circulation, there is evidence that demonstrates the importance of L-selectin in the lung. Kuebler et al. (1997) treated rabbits with fucoidan, a sulfated fucose polymer that adheres to selectin binding sites, and showed substantial decreases in neutrophil stopping in pulmonary capillaries. L-selectin's role was further illuminated by the work of Yamaguchi et al. (1997) as fucoidan treated rats had decreased leukocyte localization in alveolar capillaries. This same study showed that antibodies to P-selectin did not slow neutrophil transit in lungs, while rolling of neutrophils on systemic venule endothelium was decreased. E-selectin is not expressed in non-inflamed rat lungs, thus indicating all selectin-mediated binding in the lung was mediated by L-selectin.

Selectin/selectin ligand interactions are early steps in the transendothelial migration process, followed by firm adhesion involving integrins present on white blood cell surfaces, such as MAC-1 (macrophage antigen – 1), and their ligands, such as ICAM – 1 (intercellular adhesion molecule), which is induced on endothelial cells by inflammatory cytokines (Wagner and Roth, 2000). However, it is important to recognize L-selectin's presence on lymphocytes and neutrophils,

not only for the role in movement of the cells from the vasculature to the lung and airway tissues, but as potential interaction sites with terminal structures on secreted mucin molecules. As previously indicated, the L-selectin ligand is a terminally sulfated and fucosylated glycoprotein structure, similar in structure to oligosaccharide side chains of secreted mucin glycoproteins.

All L-selectin molecules do not necessarily survive intact as leukocytes move from the pulmonary vessels to the lung and airway tissue. L-selectin shedding from neutrophils certainly occurs (Wagner and Roth, 2000). However, it is not known to what degree, or to what degree shedding may occur from the surface of lymphocytes. The intriguing possibility of interaction between L-selectin molecules that may remain on the surface of leukocytes arriving at the airway epithelial surface and secreted airway mucins therefore exists. This interaction could be one of simple adhesion and anchoring, or could perhaps involve cellular activation, as signal transduction occurring downstream of L-selectin/selectin ligand, for example activation of ras and MAPKs, is known to occur (Brenner et al., 2002).

SUMMARY

An increased or abnormal mucus layer is a significant feature of many airway diseases. The purpose of this research project was to contribute to more complete understanding regarding the quantity, composition and function of mucus in the airways of animals with specific inflammatory airway conditions. To achieve this goal, the following hypotheses were developed: ***1)*** **increased mucus production from airway epithelium is a persistent phenomenon in RAO-affected horses;** ***2)*** **airway levels of the mucin-associated Sialyl Lewis X tetrasaccharide increase during inflammatory conditions; and** ***3)*** **rats developing airway inflammation following bacterial endotoxin exposure produce and release specific mucins into airway lumens in a distinct temporal pattern.** These hypotheses were tested, and this dissertation describes:

a) The development of methods to quantify and characterize secreted mucus (mucins) in bronchoalveolar lavage fluid of horses, application of these methods to quantify mucus in control and RAO horses both during housing and at pasture, and the effect of bronchodilation on these methods of measurement

b) Examination of the association of airway inflammation, as measured by inflammatory cell numbers and elastase level in bronchoalveolar lavage fluid, with alterations in mucin production and secretion in both recurrent airway obstruction of horses and a rodent model of inflammatory airway disease

c) The effect of both instilled and inhaled hay dust on mucus production and secretion in F344 rats

d) An increased presence of the mucin-associated sialyl Lewis X tetrasaccharide in horses and rodents with airway inflammation, and the positive correlation of SLeX levels with elastase levels

e) Temporal differences in entry of specific mucin structures into airway lumens of endotoxin-exposed rats

f) The possible functional consequences of specific mucin structures in regard to interactions between cells and the mucous layer

Chapter 2:

Development of methods to quantify mucus in bronchoalveolar lavage fluid of horses, and identification of persistent mucin glycoprotein alterations in RAO-affected horses

Jefcoat, A.M., Hotchkiss, J.A., Gerber, V., Harkema, J.R., Basbaum, C.B., Robinson, N.E. Persistent mucin glycoprotein alterations in equine recurrent airway obstruction. (2001). *Am J Physiol Lung Cell Mol Physiol* **281:** L704-L712.

Embarking on a graduate project to study airway secretion in recurrent airway obstruction (RAO)-affected horses first required development of methods to quantify recovered mucus (mucins) in bronchoalveolar lavage fluid (BALF). Chapter 2 of this thesis details development of these methods, using monoclonal antibodies and lectins directed against various mucin glycoprotein structures.

Early results in the development of techniques to measure airway mucus in BALF suggested that mucus levels in RAO-affected horses remained higher than those of control horses, even when the RAO horses were in clinical remission. These findings led me to hypothesize that increased mucus production was a persistent phenomenon in RAO-affected horses, continuing after separation from an instigating environment. Results of a study that tested this hypothesis are presented in Chapter 2.

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Persistent mucin glycoprotein alterations in equine recurrent airway obstruction

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[1]*Department of Large Animal Clinical Sciences and* [2]*Department of Veterinary Pathology, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan 48824; and* [3]*Department of Anatomy, School of Medicine, University of California, San Francisco, California 94143*

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Jefcoat, A. M., J. A. Hotchkiss, V. Gerber, J. R. Harkema, C. B. Basbaum, and N. E. Robinson. Persistent mucin glycoprotein alterations in equine recurrent airway obstruction. *Am J Physiol Lung Cell Mol Physiol* 281: L704–L712, 2001.—Horses with the episodic asthmalike condition of recurrent airway obstruction (RAO) have bouts of inflammation and bronchoconstriction associated with indoor housing. To assess the potential differences in airway secretions between RAO-affected and control horses, methods to quantify mucus secretions were developed and applied to bronchoalveolar lavage fluid. The relative difference in the amount of mucin glycoproteins between control and RAO-affected horses was assessed with a carbohydrate side chain-specific monoclonal antibody (4E4) in an enzyme-linked immunosorbent assay and by carbohydrate-specific enzyme-linked lectin assays. Significantly increased levels of 4E4-immunoreactive glycoprotein and the mucin-associated carbohydrates fucose (α-1,2 linkage) and *N*-acetylglucosamine were detected in RAO-affected horses in acute disease. RAO-affected horses in remission maintained significantly elevated levels of α-1,2-fucose and *N*-acetylglucosamine, whereas the 4E4-immunoreactive glycoprotein levels displayed a trend toward an increase over control levels. These results indicated that persistent changes in the quantity and/or quality of mucus glycoproteins occurred in the RAO-affected horses.

bronchoalveolar lavage fluid; O-linked mucin glycoproteins; oligosaccharide side chains; lectins

ACCUMULATION OF EXCESS MUCUS in airway lumens is a clinical feature of human airway diseases such as asthma, cystic fibrosis, and chronic bronchitis and of recurrent airway obstruction (RAO) in horses (10, 18, 22). RAO is an asthmalike condition of mature horses that is characterized by bronchoconstriction, airway wall thickening, and increased airway secretions occurring as a result of exposure to a dust, an allergen, and an endotoxin-laden indoor (barn) environment (18). Clinical signs are episodic, but the condition is progressive and permanent. Disease remission follows separation from the precipitating environment, but clinical signs recur on reexposure.

An increased mucus presence in airway lumens can be a direct cause of bronchial obstruction and can effectively increase resting airway wall thickness. This latter effect can amplify the lumen-narrowing effect of bronchoconstriction (15). Also, although RAO is not considered to be of infectious origin (16), it has been recognized in humans that mucus hypersecretion and/or decreased mucus clearance from the tracheobronchial tree can potentially predispose individuals to secondary bacterial colonization (10).

The mucus blanket that overlies the airway epithelium is composed of a liquid sol layer that surrounds the cilia and a more viscous gel layer above the sol (24). The gel layer is a complex mixture of water, electrolytes, lysozymes, cells, and mucin glycoproteins (10, 22, 24). Mucins are high molecular weight glycoproteins (154,000 to >7,000,000) that impart viscoelastic properties to mucus. They are composed of a core protein (the mucin apoprotein) to which numerous linear and branching oligosaccharide side chains are attached by means of specific O-glycosidic linkages (19, 20, 24). These side chains are composed of various combinations of five different individual sugar types: *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, sialic acid (*N*-acetylneuraminic acid), and fucose (4, 23). The addition of each sugar is dependent on the presence of a specific glycosyltransferase enzyme (23). Individual sugars in an oligosaccharide side chain can be linked to the preceding sugar molecule in a variety of ways; for example, a 1,2 linkage is a bond between the number 1 carbon of the distal sugar and the number 2 carbon of the proximal sugar, whereas a 1,3 linkage is between the distal carbon 1 and the proximal carbon 3.

The mucin oligosaccharide side chain structure can have functional importance, imparting specific binding activity toward structures such as bacterial adhesin molecules (20, 21) and potentially contributing to the degree of viscoelasticity of the mucous layer. As an example of the latter, fucose concentration has been positively correlated with mucus viscoelasticity (11). Individual mucin sugars such as fucose have also been

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used as markers of tracheobronchial mucus production in humans (9).

The purpose of this study was to develop methods for comparing mucus airway secretions in bronchoalveolar lavage fluid (BALF) of RAO-affected and control horses. This is a critical initial step in studying the mechanisms of altered mucus production during airway disease and accurately assessing the time course and functional significance of any accumulation. Although visual scoring systems of mucus quantity and quality have been previously employed (7, 14), a goal of this study was to use a more objective method to consistently analyze mucus secretion in the pulmonary airways. This report describes *1*) the development and use of an enzyme-linked immunosorbent assay (ELISA) as an immunochemical method to quantify airway mucus secretions in horses and *2*) the application of ELISAs and carbohydrate-specific lectin assays to the BALF from control and RAO-affected animals to identify possible quantitative and/or qualitative differences in mucins in these two populations of horses.

METHODS

Identification of RAO-Affected and Control Horses

In this study, horses from an RAO-affected herd that is maintained at Michigan State University (East Lansing) were used. To enter this herd, horses must meet the following criteria: *1*) clinical signs of RAO, including cough, increased respiratory sounds, and increased expiratory abdominal effort, are observed during housing and abate when the horses are kept at pasture where there is no exposure to dust found in hay and stables; *2*) horses develop changes in lung function compatible with airway obstruction when stabled and fed hay [maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing >15 cmH_2O]; and *3*) airway obstruction is reversible, in part, with atropine. Control horses, also maintained in university herds, had no known history of chronic airway disease and did not display any clinical signs characteristic of obstructive airway disease when stabled.

Development of Murine Antibodies

Previous work (3) resulted in the generation of a panel of murine monoclonal antibodies directed against sheep tracheal secretions. Fifteen of these antibodies were screened for immunoreactivity against stored and secreted equine mucosubstances.

Screening of Antibodies

Immunofluoresence studies. Frozen microdissected horse pulmonary airways were embedded in optimum cutting temperature compound (Miles, Elkhart, IN), snap-frozen in a liquid nitrogen-cooled isopentane slush, and stored at −80°C. Sections were fixed in zinc-formalin for 5 min, washed two times with phosphate-buffered saline (PBS; pH 7.4), and blocked with 2% normal horse serum-0.3% Triton X-100 (Sigma, St. Louis, MO) in PBS for 25 min to cover irrelevant binding sites. The primary antibodies were diluted in the 2% normal horse serum solution: 1:100 for ascites antibodies and 1:20 for antibodies harvested from the culture supernatant. The antibodies were applied to airway sections and incubated at 37°C for 90 min. After a wash with rinsing buffer (1:10 dilution of 10× automation buffer; Biomedia, Foster City, CA), biotinylated horse anti-mouse IgG (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) diluted 1:200 in 2% normal horse serum solution was added to each slide, and the slides were incubated for 30 min at 37°C followed by a wash with automation buffer. Fluorescein-conjugated avidin D (Vector Laboratories) was diluted 1:250 in automation buffer, applied to the tissue sections, and incubated for 30 min at 37°C. The sections were then washed with PBS and examined with an Olympus microscope equipped for epifluorescence studies. Each section was evaluated for location and intensity of immunofluorescent staining.

ELISA. Three ascites-derived and two culture supernatant-derived monoclonal antibodies with significant immunoreactivity to mucous goblet cells (based on immunofluorescence results) were screened for reactivity to mixed horse BALF in an ELISA. Serial dilutions from 1:100 to 1:1,280 were used for ascites antibodies, whereas dilutions for supernatant antibodies were 1:20 to 1:2,650. The diluent and blocking agent was 2% horse serum-0.3% Triton X-100 in PBS. The BALF was treated with 0.1% dithiothreitol (DTT; Sigma), shaken for 20 min to disperse the mucins, and then centrifuged at 1,500 rpm for 12 min to remove the cells. The supernatant was collected and dialyzed against distilled water overnight at 4°C to remove DTT. For the ELISA, 100 μl of cell-free supernatant were added to the wells of Immulon-4 HBX 96-well plates (Dynex Technologies, Chantilly, VA) in duplicate and incubated overnight at 40°C to thoroughly fix the antigen to the bottom of the wells. One hundred microliters of blocking agent (2% normal horse serum-0.3% Triton X-100 in PBS) was then added to each well to cover irrelevant binding sites and incubated at 37°C for 25 min. The plates were washed four times with 1× automation buffer, and then 100 μl of each primary antibody appropriately diluted in the blocking solution were added to the wells in duplicate followed by incubation at 37°C for 90 min. Washes with automation buffer were repeated, and 100 μl of biotinylated secondary antibody (VECTASTAIN Elite ABC kit) diluted 1:200 in 1× automation buffer was added to each well. The plates were incubated at 37°C for 30 min and then washed four times with automation buffer. One hundred microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes with automation buffer, 100 μl of chromagen (*o*-phenylenediamine in 0.05 M phosphate-citrate buffer at 0.4 mg/ml; Sigma) were applied to each well. Just before addition to the wells, 30% hydrogen peroxide was added to the chromagen at a dilution of 1:2,500. After 5 min, the plates were read with a spectrophotometer at 450 nm, which gave optical density (OD) readings that corresponded to antigen levels in the samples (colorimetric assay).

Antibody Immunoreactivity With Purified Standard Mucins

Purified mucins from porcine stomach and bovine salivary gland (Sigma) were diluted with carbonate binding buffer (pH 9.5) at 10 μg/100 μl, and ELISA was performed with identical methods as described in *ELISA*.

Sodium Periodate Incubation

Porcine gastric mucin in carbonate binding buffer (10 μg/100 μl) and lavage samples from four RAO-affected horses were compared by ELISA before and after sodium periodate treatment. Sodium periodate oxidation attacks vicinyl groups of the sugars of glycoproteins, cleaving O-glycosidic linkages to the protein (2). Samples were dried to the bottom of Immulon-4 HBX 96-well plates at 40°C and then incubated

with 2% horse serum blocking buffer (as described in *ELISA*) at 37°C for 90 min. One hundred microliters of 100 mM sodium periodate in 50 mM sodium acetate were added to the appropriate wells in duplicate. For comparison, untreated control wells (sample only) and wells with sample, sodium periodate, and glucose were utilized [glucose blocks the action of sodium periodate (2)]. For wells with added glucose, 100 μl of 100 mM sodium periodate-0.1 M glucose in 50 mM sodium acetate were used. The plates were then incubated at room temperature overnight (in the dark). After the overnight incubation, sodium periodate and sodium periodate-glucose wells were incubated for 30 min at room temperature with 10 mM sodium borohydride to prevent nonspecific cross-linking of antigen to antibody by Schiff base formation. The plates were washed four times with 1× automation buffer, and then ELISA was performed as described in *ELISA*.

Chondroitinase ABC and Heparinase Incubation

ELISA comparison of BALF before and after incubation with the proteoglycan-degrading enzymes chondroitinase ABC and heparinase was performed to differentiate antibody-mucin glycoprotein binding from antibody binding to O-linked glycosaminoglycan epitopes. The samples were the same as those used for the sodium periodate incubation. After the antigen was fixed to the wells and the nonspecific binding sites were blocked, the samples were incubated overnight with chondroitinase ABC (50 μU/well in 0.05 M Tris, pH 8.0) or heparinase (250 μU/well in 0.05 M Tris, pH 6.8, with 5 mM calcium chloride) at 37°C. After incubation, the plates were washed four times with 1× automation buffer in preparation for ELISA, and a colorimetric enzyme-linked assay was performed.

ELISA of Serum From Control and RAO-Affected Horses

Colorimetric ELISA was performed on the serum from 10 control and 10 RAO-affected horses to test for the presence of non-airway-specific immunoreactive antigen in both groups of horses.

ELISA of Native Tracheal Mucus

Mucus was harvested directly from the trachea of an RAO-affected horse at necropsy. The mucus was weighed, diluted to 10% in PBS, and then applied to microtiter plates for colorimetric 4E4-based ELISA.

High Molecular Weight Cutoff Dialysis

BALF was collected from four RAO-affected horses and prepared as described in *ELISA*. The BALF was then dialyzed overnight at 4°C against distilled milliQ water in 100,000 molecular weight cutoff (MWCO) dialysis tubing (Spectra/Por, Spectrum Medical Industries, Houston, TX). ELISA was then performed on milliQ water and nondialyzed BALF as controls and on all dialyzed samples and dialysates. The dialysate was tested in both native and 5× concentrated forms. Concentrations were made with 30,000 MWCO centrifugal concentrators (Centriprep, Amicon, Beverly, MA). For ELISAs, the samples were plated (100 μl/well in triplicate), incubated overnight, and blocked with an 8% purified casein solution (Roche Diagnostics, Indianapolis, IN). After secondary antibody and ABC reagent incubations, with dilutions and volumes identical to those described in *ELISA*, 100 μl of QuantaBlu fluorogenic peroxidase substrate (Pierce, Rockford, IL) were added to each well, and the plates were read at 5-min intervals for 20 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA), which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices) and is reported as V_{max} units per second. V_{max} units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

ELISA of BALF From Control and RAO-Affected Horses

BALF was collected from 6 control (5 being 7 yr of age or younger, 1 being 12 yr of age) and 6 RAO-affected horses (10+ yr of age). Control horses were sampled after 48 h of indoor housing (exposure). The BALF from RAO-affected horses was collected and tested after the horses were stabled for 48 h (exposure) and after pasture for 30 days (pasture). The BALF was collected by means of a 3-m lavage tube (8-mm external diameter; Bivona, Gary, IN) wedged in a peripheral bronchus. Three 100-ml aliquots of sterile PBS were infused and recovered by suction after each 100-ml infusion, and the samples were pooled. Total and differential white blood cell counts were performed on all BALF. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differential counts, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quik (Baxter Health Care, Dade Division, Miami, FL). With the exception of aliquots taken for cell counts, the samples were treated with 0.1% DTT and centrifuged at 1,500 rpm for 12 min to remove the cells. The supernatant was collected and dialyzed overnight at 4°C. ELISAs were performed with fluorogenic peroxidase substrate and a fluorescent plate reader as described in *ELISA*. Negative control well values (background) were subtracted from all sample readings.

Enzyme-Linked Lectin Assay

Lectins are well recognized as specific carbohydrate-binding molecules. Lectins for each of the five sugar types found in O-linked mucins (Table 1) were used in enzyme-linked lectin assays (ELLAs). BALF samples from RAO-affected horses at pasture and after 48 h of housing were compared with BALF samples from control horses after 48 h of exposure. BALF samples were incubated overnight in the same manner as described for ELISAs. The wells were blocked for 35 min with 8% casein blocking reagent. After being blocked, the wells were washed three times with 1× automation buffer. One hundred microliters of biotinylated lectins specific for α-1,2-fucose [*Ulex europaeus* agglutinin (UEA) I; 0.5 μg/ml], *N*-acetylglucosamine [succinylated wheat germ agglutinin (WGA); 0.5 μg/ml], *N*-acetylgalactosamine [soybean agglutinin (SBA; *Glycine max*); 1.0 μg/ml], sialic acid [*Maackia amurensis* lectin (MAL) II; 0.75 μg/ml PBS], and

Table 1. *Lectins used in enzyme-linked assays*

Lectin	Preferred Binding Sugar
Glycine max (soybean agglutinin)	*N*-acetylgalactosamine
Succinylated *Triticum vulgaris* (wheat germ agglutinin)	*N*-acetylglucosamine
Ricinus communis agglutinin I	Galactose
Ulex europaeus agglutinin I	Fucose (α-1,2)
Maackia amurensis lectin II	*N*-acetylneuraminic acid (sialic acid)

galactose [*Ricinus communis* agglutinin (RCA) I; 0.5 μg/ml PBS] were then added to each well and incubated for 45 min at 37°C (all lectins from Vector Laboratories). Diluent for each lectin was per supplier recommendation. The plates were then washed four times with 1× automation buffer. ABC reagent and QuantaBlu were added as described in *High Molecular Weight Cutoff Dialysis*, and fluorescence (in V_{max} units/s) was measured. As with the ELISA, negative control well values (background) were subtracted from all sample readings.

Lectin assays with UEA 1, succinylated WGA, and MAL II were also performed on serum collected from two control horses.

BALF Protein Content

BALF samples were analyzed for protein content with the bicinchoninic acid method. Ten microliters of bovine serum albumin standard (serially diluted 1:2 from 1,000 to 8.44 μg/ml) and each sample were plated in duplicate on a 96-well microtiter plate. Two hundred microliters of bicinchoninic acid working reagent (Pierce, Rockford, IL) were added to each well. The plate was shaken for 30 s and incubated at 37°C for 30 min. The plate was then cooled to room temperature, and absorbance was measured at 550 nm.

Lung Function Testing

For confirmation of chronic RAO in disease-affected animals, ΔPpl_{max} was recorded during the indoor housing period and after time at pasture. The change in pleural pressure (ΔPpl) was measured by use of an esophageal balloon connected to a pressure transducer (Validyne model DP45-22) system. Calculations were performed with a lung function computer (Buxco, Sharon, CT) (1) from 30 breaths (17).

ELLA and High MWCO Dialysis

BALF samples from four RAO-affected horses were dialyzed in the same manner as for high MWCO ELISA. ELLA procedures with UEA I, succinylated WGA, and MAL II were performed on samples before and after dialysis.

Mucin Standard Serial Dilutions

All ELISA and ELLA plates contained a serial dilution of porcine gastric and bovine salivary gland mucins (from 100 to 0.001 μg/100 μl) diluted in PBS as a means to quantify the amount of mucin in samples and standardize multiple plates. Mucin standards were treated in an identical manner as BALF samples.

Statistical Analyses

For direct comparisons of one factor between two groups, means of groups were compared with Student's *t*-test. For comparisons of one factor within a group, a paired *t*-test was employed. Multiple comparisons between groups were performed with one-way ANOVA. Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

RESULTS

Confirmation of RAO-Affected Population

All RAO-affected horses displayed clinical signs of obstructive pulmonary disease during housing, with ΔPpl exceeding 15 cmH_2O (mean ± SE = 59.4 ± 9.8 cmH_2O). The mean value on *day 30* (pasture) was 15.1 ± 2.7 (SE) cmH_2O. The control horses did not display any signs of obstructive pulmonary disease during 48 h of indoor housing. BALF cytology from the horses used for the ELISA and ELLA experiments is presented in Table 2. Both total cell counts and percent neutrophils were increased in RAO-affected horses after the housing period compared with pasture and control values.

Screening of Antibodies

Immunofluoresence. Five of fifteen antibodies bound strongly to secretory cells in the surface epithelium and submucosal glands of airway sections. These antibodies, 4E4, 5C7C6, IG5C11F10, 3F11 and 3C10, were selected for use in pilot ELISA studies. Figure 1 is a photomicrograph of the binding activity of antibodies 5C7C6, 3F11, and 4E4.

ELISA on mixed BALF. Only antibodies 4E4, 5C7C6, and 3F11 displayed noticeable immunoreactivity with BALF (Fig. 2).

As shown in Fig. 2, ELISA-negative control values were near zero, and the highest value generated at the greatest antibody concentration was an OD of 2.0. Therefore, an OD of 1.0 at 450 nm was arbitrarily chosen as a standard reference value to quantify secretory product in BALF with a colorimetric assay. Dilutions of antibodies 4E4, 5C7C6, and 3F11 that corresponded with an approximate OD of 1.0 (1:400, 1:100 and 1:80, respectively) were then chosen for future studies.

ELISA on Standard Mucins

Antibodies 4E4, 5C7C6, and 3F11 all generated OD values of >1.0 when applied to beef salivary gland mucin. 5C7C6 and 3F11 displayed similar low levels of reactivity toward porcine gastric mucin as toward bovine mucin, but 4E4 exhibited strong immunoreactivity to porcine mucin (Fig. 3).

Sodium Periodate Incubation

With 4E4 as the primary antibody, a mean post-sodium periodate incubation OD value of 0.44 ± 0.08 (SD) between duplicate wells was significantly differ-

Table 2. ***Bronchoalveolar lavage cytology from control and RAO-affected horses***

	Control Horses	RAO-Affected Horses	
	Indoor housing	Indoor exposure	Pasture
Cell count, $\times 10^3$ cells/ml	99.2 ± 21	1,367 ± 416*	112.5 ± 30
Neutrophils, %	18.5 ± 8	87 ± 3.5*	28.75 ± 11
Macrophages, %	37.5 ± 8.5†	6 ± 1.4	41 ± 11†
Lymphocytes, %	42 ± 4‡	6 ± 3	25 ± 5.5†
Mast cells, %	2 ± 1.6	0.5 ± 0.2	4 ± 1.5
Average volume returned, ml	135 ± 5‡	47 ± 9	41 ± 8

Values are means ± SE. RAO, recurrent airway obstruction. Three hundred milliliters were infused. *Significantly elevated over control and RAO pasture. †Significantly elevated over RAO indoor exposure. ‡Significantly elevated over RAO indoor exposure and RAO pasture.

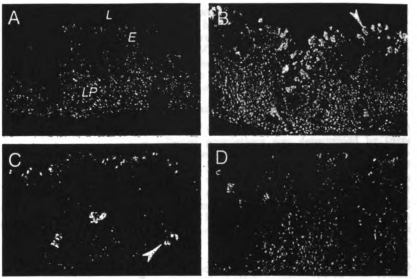

Fig. 1. Photomicrographs of monoclonal antibody binding to horse airway epithelium (immunofluorescence). *A*: negative control (no antibody). *B*: antibody 3F11. *C*: antibody 5C7C6. *D*: antibody 4E4. Yellow-green fluorescence indicates binding of monoclonal antibodies. Sections were also stained with propidium homodimer to label nuclei. L, airway lumen; E, airway epithelium; LP, lamina propria. Arrowheads, mucosubstance in surface epithelium and submucosal glands.

ent from the mean preincubation OD value of 1.19 ± 0.15 (SD). The mean negative control well value (no antibody) was 0.132. Sodium periodate effects were blocked by the presence of 0.1 M glucose in glucose-added wells.

Chondroitinase ABC and Heparinase Incubation

Neither chondroitinase nor heparinase treatment affected 4E4 binding. The mean postincubation OD values of 1.33 ± 0.37 (SD) between duplicate wells for chondroitinase and 1.26 ± 0.35 (SD) for heparinase were not significantly different from the mean preincubation value of 1.24 ± 0.32 (SD).

Serum ELISA

4E4-immunoreactive antigen was not detected in the serum of either control or RAO-affected horses. The mean OD for 10 control horses was 0.197 ± 0.025 (SD), whereas the mean OD for 10 disease-affected horses was 0.191 ± 0.006 (SD). No significant differences existed between the two groups. Negative control values were 0.220 ± 0.009 (SD).

Tracheal Mucus ELISA

4E4-immunoreactive glycoprotein was detected in diluted directly harvested tracheal mucus. The amount of 4E4 binding in 10% tracheal mucus was similar to that of the porcine mucin standard at 10 μg/100 μl (OD values of 1.418 and 1.366, respectively).

High MWCO Dialysis

4E4-based ELISA showed no decrease in signal after BALF samples had been dialyzed in 100,000 MWCO tubing. The mean value for predialysis samples was 3.1074 ± 0.866 (SE) V_{max} units/s, whereas the postdialysis value was 2.746 ± 0.464 (SE) V_{max} units/s.

BALF ELISA for Control and RAO-Affected Horses

4E4-based ELISA showed a significantly elevated mean V_{max} units per second value in the RAO-affected horses during exposure compared with the control value (Fig. 4*A*). The mean ELISA reading for RAO-affected horses in remission decreased from that in acute disease but remained greater than the control level, which was the near background level. The average volume of BALF returned and the total and differential cell counts for each group of horses are given in Table 2.

ELLAs

Significant elevations were noted in α-1,2-fucose and *N*-acetylglucosamine levels in RAO-affected horses during exposure and at pasture compared with those in control horses (Fig. 4, *B* and *C*). There were also increased sialic acid levels in RAO-affected horses during exposure and at pasture compared with control levels, with a trend toward significant differences between groups (P = 0.074; Fig. 4*D*). Although the porcine mucin standard responded to soybean agglutinin, the detected level of *N*-acetylgalactosamine in all groups of horses was not distinguishable from the background level, and no significant differences existed. No significant differences in the levels of galactose existed between groups.

UEA I, succinylated WGA, and MAL II did not respond to serum elements. Mean V_{max} values for the lectins were 0.319 ± 0.02 (SD), 0.336 ± 0.06, and 0.277 ± 0.10 V_{max} units/s, respectively. None of these were elevated over background V_{max} units per second values.

Protein Assay

Total protein in BALF from RAO-affected horses in acute disease was significantly greater than that in

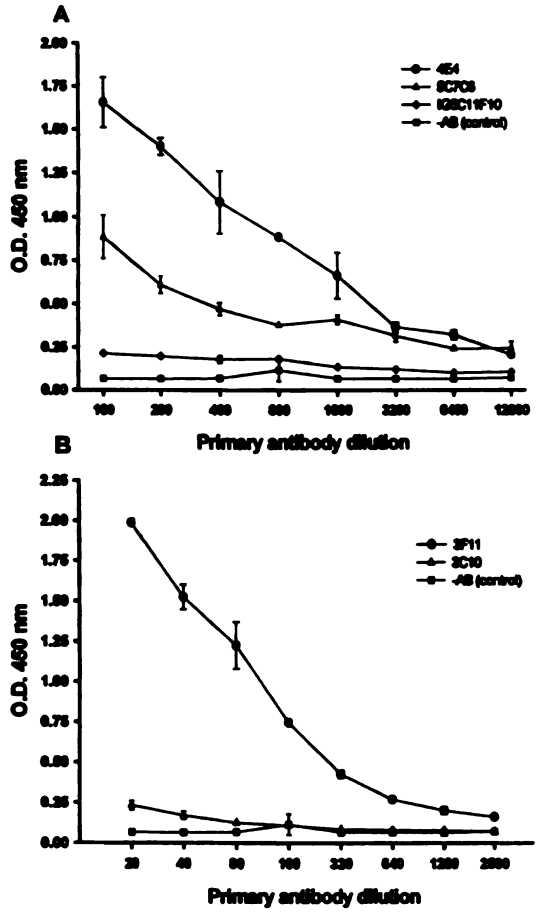

Fig. 2. Binding of indicated monoclonal antibodies (AB) to epitopes in horse bronchoalveolar lavage fluid. *A*: ascites-derived antibodies. *B*: culture supernatant-derived antibodies. OD, optical density. Values are means ± SD of duplicate wells. Bars not seen are within symbol.

control horses. Protein levels in BALF from RAO-affected horses in remission remained elevated over control levels, but there was no significant difference. The mean absorbance value for RAO horses in acute disease was 0.394 ± 0.038 (SE), which corresponded to 800 μg/ml of protein. Absorbance values for control and RAO-affected horses in remission were 0.198 ± 0.025 and 0.290 ± 0.047, respectively, corresponding to 250 and 500 μg/ml of protein.

High MWCO Dialysis ELLAs

No decrease in signal compared with that in the predialyzed sample was noted in BALF after 100,000 MWCO dialysis for any of the tested lectins (Fig. 5).

DISCUSSION

The experimental findings detailed in this study are the results of the first study, to our knowledge, to utilize monoclonal antibodies and lectins to examine disease-associated changes in glycoprotein secretion in RAO-affected horses during exacerbation and remission. Novel investigation regarding airway secretion in RAO can be of benefit on two fronts because RAO is both a significant disease of the horse population and a condition with similarities to chronic human airway diseases such as asthma.

Confirmation of Study Populations

Inclusion of individual horses into either control or RAO-affected populations was determined by three factors accepted as criteria for establishing a phenotype of RAO (16): presence of clinical signs, BALF cytology data, and lung function testing. Clinical signs of obstructive airway disease were observed in RAO-affected but not in control horses after indoor housing. RAO-affected horses showed significant increases in both total cell counts and percent neutrophils in BALF during indoor housing compared with pasture or to control horses. This pattern is characteristic of RAO (6, 12). Lung function test results showed that all RAO-affected horses had ΔPpl_{max} values of >15 cmH_2O after 48 h of indoor housing, paralleling development of clinical signs of obstructive disease and increases in total cells and percent neutrophils in BALF. This pattern of an elevated ΔPpl in response to natural challenge is typical of RAO (13).

Monoclonal Antibody 4E4 as a Tool for Identification and Quantification of Secreted Mucous Cell Product

4E4 was chosen from a panel of monoclonal antibodies as a primary antibody for use in an ELISA that can be applied to BALF from horses to quantify mucus secretions. The recognition by 4E4 of a mucin or mucinlike molecule was evidenced by its binding to mucous cells in airway epithelium and its immunoreactivity to a large (>100,000) glycoprotein present in BALF. Antibody binding was not affected by chondroitinase or

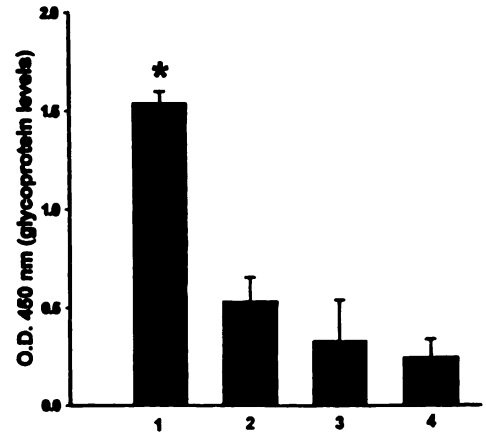

Fig. 3. Monoclonal antibody binding to purified porcine gastric mucin. *1*, 4E4; *2*, 3F11; *3*, 5C7C6; *4*, negative control (no antibody). Values are means ± SD of 4 replicates. *4E4 binding was significantly elevated over negative control, $P < 0.05$.

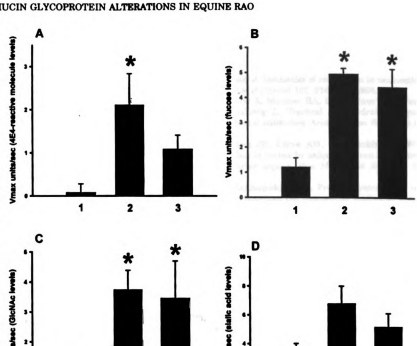

Fig. 4. Results of 4E4-based ELISAs and enzyme-linked lectin assays (ELLAs) for control and recurrent airway obstruction (RAO)-affected horses (n = 6/group). *1*, control horses; *2*, RAO exposure; *3*, RAO pasture. *A*: 4E4 ELISA. RAO-affected horses had significantly elevated 4E4-immunoreactive glycoprotein levels in bronchoalveolar lavage fluid (BALF) during exposure (acute disease) compared with control horses. *B*: *Ulex europaeus* agglutinin I (α-1,2-fucose-specific) ELLA. RAO-affected horses had significantly elevated α-1,2-fucose levels in BALF during pasture (remission) and exposure (acute disease) compared with control horses. *C*: succinylated wheat germ agglutinin [*N*-acetylglucosamine (GlcNAc)-specific] ELLA. RAO-affected horses had significantly elevated GlcNAc levels in BALF during exposure and at pasture compared with control horses. *D*: *Maackia amurensis* lectin II (sialic acid-specific) ELLA. Values are means ± SE. *P < 0.05.

heparinase treatment, further indicating its specificity for a mucin or mucins. Additionally, a 4E4-recognized epitope was present on purified porcine gastric mucin molecules. As well as serving to help identify the target of the antibody, 4E4 immunoreactivity to a commercially available mucin allowed development of a standard ELISA reference curve.

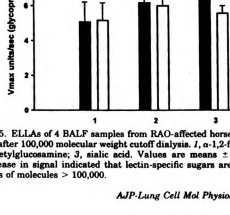

Fig. 5. ELLAs of 4 BALF samples from RAO-affected horses before and after 100,000 molecular weight cutoff dialysis. *1*, α-1,2-fucose; *2*, *N*-acetylglucosamine; *3*, sialic acid. Values are means ± SE. No decrease in signal indicated that lectin-specific sugars are components of molecules > 100,000.

4E4-Based ELISAs and ELLAs of BALF

Levels of a 4E4-immunoreactive molecule were significantly increased in the RAO-affected horses during indoor exposure compared with those in the control horses. Levels in RAO-affected horses decreased when the horses were in remission but remained elevated compared with the control levels, which were near background readings. Levels of sialic acid followed the same pattern as 4E4, although significant differences were not present (P = 0.074). These results indicated that 4E4 and, to a lesser degree, sialic acid measured an increase in mucus production in RAO-affected horses during acute disease and suggested that there is a persistent nature to altered mucus production in the disease state.

Results of enzyme-linked assays with α-1,2-fucose- and *N*-acetylglucosamine-specific lectins demonstrated disease-dependent increases in these sugars that persisted 30 days after the horses were removed from environmental challenge. Although mean values at 30 days were less than those observed during acute disease, a significant elevation remained. The pronounced neutrophilic inflammation in RAO-affected horses in acute disease markedly abated during clinical remission, but elevations in total protein and percent neutrophils did remain in diseased horses after 30 days at pasture compared with control levels. Inflammatory

mediators such as neutrophil elastase have long been known to be mucus secretagogues (8). It is therefore possible that the persistently increased levels of mucin glycoproteins detected in BALF may be secondary to sustained, although attenuated, inflammation. Supporting this hypothesis is a recent study (5) describing persistent granulocyte-dependent activation of nuclear factor-κB in bronchial brushing samples from RAO-affected horses even after removal from challenge for 21 days. Although a link between sustained inflammation and increased mucin secretion can be hypothesized, the exact mechanisms of persistent glycosylation changes, such as upregulation of specific glycosyltransferases and mucin apoproteins, increased glycosyltransferase and apoprotein mRNA stability, or altered availability of mucin sugar molecules, have not been identified.

High MWCO dialysis experiments with multiple lectins yielded similar results as with the 4E4-based assay, where no loss of signal was detected in samples after dialysis in 100,000 MWCO tubing. This demonstrated that lectin-based differences detected in BALF were due to glycosylation patterns that are integral parts of larger molecules (i.e., mucin glycoproteins) and are not due to individual sugar or small soluble sugar-bearing molecules in airway fluids. In addition, lack of target molecules in serum indicated that serum leakage into the airways, as expected with airway inflammation, did not influence the test results.

The mean ΔPpl value in the RAO-affected horses in remission, although significantly lower than the mean value in acute disease, remained >15 cmH_2O, the minimum value accepted as an indicator of airway obstruction in RAO-affected horses postexposure (16). Although other factors such as airway wall thickening may be involved, maintenance of airway obstruction while at pasture coupled with persistent mucin alterations suggested that an increased or abnormal mucus presence in remission may have contributed to long-term low-level airway obstruction in the RAO-affected horses.

Although not measures of the absolute amounts of mucin glycoproteins or mucin carbohydrates in the airways, the ELISAs and ELLAs used in this study characterized mucin glycoprotein components and demonstrated significant relative differences in the levels of the target molecules in BALF from RAO-affected and control horses, highlighting disease and exposure effects on airway secretory products.

Collectively, these results indicated that disease-dependent alterations in the quantity and/or quality of mucin oligosaccharide side chains occurred in the horses with the asthmalike condition of RAO. Most significantly, persistent elevations in secreted mucin glycoproteins were evident in the airways of RAO-affected horses, perhaps secondary to sustained low-level inflammation.

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Chapter 3:

Quantification of mucin glycoproteins and inflammatory cells in BALF of horses is unaffected by bronchodilation

Results of the initial foray into studying mucus production and secretion in control and RAO-affected horses showed persistent increases in mucin glycoproteins in bronchoalveolar lavage fluid (BALF) of RAO horses (detailed in Chapter 2). These results were most evident in levels of α-1,2 fucose, a deoxyhexose sugar that is a common terminal component of mucin oligosaccharide side chains.

Bronchospasm is a powerful component of RAO. As the airways in disease-affected horses are hyperreactive, this raised the concern that the physical process of bronchoalveolar lavage resulted in localized bronchospasm in RAO horses. This bronchospasm could in turn affect the nature of the mucin (mucus) return, for example due to prolonged lavage fluid contact with a limited airway surface area. To address this concern, I first developed the hypothesis that bronchodilation of horses would result in decreased levels of measured airway mucins in BALF. Then, to test this hypothesis, a protocol was developed to bronchodilate control and RAO horses, followed by collection of bronchoalveolar lavage fluid to quantify mucins by methods detailed in Chapter 2.

Abstract

Accurate analysis of inflammatory cells and mucin glycoproteins in bronchoalveolar lavage fluid (BALF) is critical in the study of secreted mucin glycoproteins in inflammatory airway disease. By widening airways and minimizing constriction, bronchodilator medications can potentially increase volume of return of lavage fluid and/or allow lavage fluid access to more peripheral airway regions, thereby altering the nature of BALF returns. To test the hypothesis that bronchodilation affects the return of mucins and inflammatory cells, bronchoalveolar lavage was performed on control and recurrent airway obstruction-affected (RAO) horses both before and after bronchodilation with intravenous atropine followed by an inhaled β-2 agonist. Bronchoalveolar lavage was performed on horses both prior to indoor housing and after housing in stables with straw bedding for 48 hours. BALF total and differential cell counts were performed, and levels of the mucin-associated sugar α-1,2 fucose were measured. No significant effect of bronchodilation was seen in cell counts or in levels of either elastase or fucose. A group effect was observed, however, with a persistent increase of α-1,2 fucose detected in BALF of RAO-affected horses. This latter finding confirms earlier results obtained by our laboratory. For quantification of inflammatory cells and specific mucin markers, these results indicated that equivalent results could be obtained from bronchoalveolar lavage fluid collected both before and after bronchodilation.

Introduction

The airway mucus blanket is a heterogeneous layer containing a variety of molecules, but it is high molecular weight mucin glycoproteins secreted from goblet cells that give mucus its viscoelastic properties. Mucins are composed of a central protein to which are attached linear and branching oligosaccharide side chains. Mucin oligosaccharides are typically 2 to 20 sugars in length (Hanisch, 2001; Rose, 1992), and the deoxyhexose sugar fucose (Fuc) can be a terminal component of these chains (Bhatia and Mukhopadhyay, 1998; Lamblin, et al., 1991).

An increased or altered mucus presence in the airways is a feature of recurrent airway obstruction (RAO; heaves) of horses, and is also characteristic of the significant human diseases asthma and cystic fibrosis (Bousquet, et al., 2000; Kaup, et al., 1990; Quinton, 1999; Robinson, 2001). Neutrophilic airway inflammation is a component of RAO (Robinison, et al., 1996), asthma (Krawiec, et al., 1999; Louis, et al., 2000, Park, et al., 1999) and cystic fibrosis (Armstrong, et al., 1997; Kahn, et al., 1995), and the inflammatory mediator neutrophil elastase has been demonstrated to increase mucin glycoprotein production (Fischer and Voynow, 2000; Martin et al., 2000; Voynow, et al., 1999).

RAO is a significant disease of the horse population in the northern hemisphere, and can potentially serve as a useful model of inflammatory airway disease in humans. Increasing understanding of RAO can therefore be of benefit to horse and human populations. As such, studying altered mucin production in RAO is an important advance in understanding the overall pathophysiology of

this disease. To this end, we have previously demonstrated persistent mucin glycoprotein alterations in RAO-affected horses as compared to controls (Jefcoat, et al., 2000) by analyzing bronchoalveolar lavage fluid (BALF).

In order to fully study RAO, it is essential to be able to consistently analyze the molecular components of BALF. It is therefore critical to identify circumstance or treatments that may alter the nature of collected lavage fluid. One treatment that can potentially alter lavage fluid return is bronchodilation. Bronchodilation can potentially alter mucin content in lavage fluid by allowing increased access of lavage fluid to terminal areas of the airways (alveolar ducts and sacs, where mucus-producing cells are absent), or by widening and stretching airways, which in turn may stretch the mucus layer and make washing and retrieval of mucins less likely (Dixon, 1992). This study tested the hypothesis that bronchodilation would alter (decrease) measured amounts of both inflammatory cells and the mucin-associated sugar fucose in lavage fluid of control and RAO-affected horses.

Material and methods

Animals. Control and recurrent airway obstruction (RAO)-affected horses from herds maintained at Michigan State University (East Lansing) were used. To be classified as an RAO-affected horse, animals had to meet the following criteria: *1)* clinical signs of RAO, including cough, increased respiratory sounds, and increased expiratory abdominal effort, were observed during housing and abated when the horses were kept at pasture where there was no exposure to

dust found in hay and stables; *2)* horses developed changes in lung function compatible with airway obstruction [maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing > 15 cm H_2O] when stabled and fed hay; and *3)* airway obstruction was reversible, in part, with atropine. Control horses had no known history of chronic airway disease and did not display any clinical signs characteristic of obstructive airway disease when stabled.

Protocol. Four control and four RAO-affected horses were used for this protocol. Bronchoalveolar lavage was performed on RAO-affected horses and control horses before housing in a barn environment (time 0). Horses were kept in individual stalls with straw bedding, and were fed hay. Prior to the first lavage, one half of these animals were bronchodilated with intravenous atropine (0.02 mg/Kg), followed in 20 minutes by 8 puffs of inhaled β2 agonist (perbuterol), administered by use of an AeroMask® device, (Trudell Medical, London, Ont., Canada). Lavage was repeated on horses after indoor housing for 48 hours, with time 0 bronchodilated horses once again receiving bronchodilator in identical fashion. After the 48 hour lavage, horses were returned to pasture for at least three weeks. The protocol was then repeated. In a crossover design, horses not receiving bronchodilator for the initial round of the protocol were bronchodilated, and vice versa.

Bronchoalveolar lavage. Bronchoalveolar lavage was collected by means of a 3-m endoscope wedged in a peripheral bronchus, with alternate lungs used at time 0 and 48 hours. Three 100 ml aliquots of sterile phosphate buffered saline (PBS) were infused and recovered by suction after each 100-ml infusion, and

samples were pooled. Samples were treated with 0.1% dithiothreitol [DTT] (Sigma, St. Louis, MO) and shaken for 30 minutes to disperse mucins. With the exception of aliqouts taken for total and differential cell counts, samples were centrifuged at 1500 rpm for 10 minutes to remove cells. Supernatant for immunochemistry and lectin chemistry was collected and stored at -20°C.

Analysis of bronchoalveolar lavage fluid

1) Cell counts. Total and differential white blood cell counts were performed on all bronchoalveolar lavage fluid (BALF) samples. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differential cell counts, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quick (Baxter Health Care, Dade Division, Miami FL), and 200 cells were examined.

2) Enzyme-linked lectin assay (ELLA) for α – 1,2 fucose

ELLA procedure: one hundred µl of BALF supernatant from each animal was applied in triplicate to wells of an Immulon-4 HBX 96-well plate (Dynex Technologies, Chantilly, VA) and incubated overnight at 40°C to thoroughly fix protein to the bottom of wells. One hundred µl of 6.5% casein blocking reagent (Roche Diagnostics, Indianapolis, IN) was then added to each well to cover potential irrelevant binding sites and incubated for 30 minures at 37°C. The plate was washed four times with 1X automation buffer (Biomedia Corp., Foster City, CA), and Biotinylated *Ulex europaeus I* (UEAI) lectin (Vector Laboratories, Burlingame,CA), at .5 µg/ml in diluent as recommended by the supplier, was applied for 1 hour at 37°C. UEA1 is specific for α – 1,2 fucose, a mucin-

associated deoxyhexose sugar. Lectin histochemistry by our laboratory has localized fucose of this linkage to secretory (goblet) cells (data not shown). Washes with automation buffer were repeated, and 100 microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes with automation buffer, 100 ml of QuantaBlu® fluorogenic peroxidase substrate (Pierce, Rockford, IL) were added to each well, and the plate were read at 3-min intervals for 21 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA) which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). Excitation and emission wavelengths were 318 nm and 410 nm, respectively. The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and reported as Vmax units per second. Vmax units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

Statistical analysis. Multiple comparisons between groups, time and treatment were performed by three-way ANOVA (SigmaStat statistical software, Jandel Scientific Software, San Rafael, CA). Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

Results

Cell counts. No significant treatment (bronchodilator) effect was observed in regard to total cell counts (P = .667) or neutrophil counts (P = .431) in horses at any of the time points. Significant time and group effects existed regarding total cell counts and numbers of neutrophils, with increases seen at 48 hours, and in RAO-affected horses. Data are shown in Figures 1 and 2.

ELLA for α-1,2 fucose. No significant bronchodilator effect on level of the mucin-associated sugar α-1,2 fucose was observed in lavage fluid of control or RAO-affected horses [treatment effect P = .231] (Fig. 3). A group effect was observed, however, showing an elevated level of fucose in RAO-affected horses (P = .004). Mean Vmax units per second values for RAO horses at time 0 and 48 hours were 34.4 and 33.3, respectively, whereas corresponding control horse values were 26.3 and 21.4.

Discussion

Though I hypothesized that bronchodilation would alter cell counts and measurements of fucose in bronchoalveolar lavage fluid, this demonstrated that bronchodilation of horses had no significant effect on measurement of either cells or fucose-possessing mucin molecules in lavage fluid. Fucose levels in lavage fluid from bronchodilated control or recurrent airway obstruction-affected horses, as measured by enzyme-linked assays, were not significantly altered from levels in these same horses under identical environmental conditions without bronchodilation. Total cell counts and numbers of neutrophils in lavage fluid

likewise showed no treatment effect, though group and time effects were present. These latter effects were an increase in inflammatory cells in RAO horses after housing, a well-reported characteristic of this disease. Of interest, a group effect regarding fucose was noted, where RAO-affected horses had persistently increased levels of this mucin-associated sugar. This observation is identical to that reported in the previous chapter (Jefcoat, et. al., 2001).

Analysis of lavage fluid cannot be a sole mechanism for arriving at a complete understanding of the pathophysiology of altered mucus production in inflammatory airway disease. Yet it remains a necessary tool, and as such it is critical to understand how analysis of lavage returns can be affected by different conditions of lavage fluid collection. The findings from this study indicate that bronchodilation is neither necessary for obtaining useful lavage samples from RAO-affected horses, nor is it detrimental to the recovery of useful samples.

Figure 11. Total cell counts per ml bronchoalveolar lavage fluid (BALF) from non-bronchodilated and bronchodilated control and RAO-affected horses. Bronchoalveloar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of indoor housing (48 hours), with and without bronchodilator treatment. Cells in BALF were quantified as described in Methods. Results are expressed as mean ± SEM. Three way ANOVA, with time, group and treatment as factors, was performed. Tukey's test was used for post-hoc analysis. No significant treatment (bronchodilation) effect was present (P = .667). Significant time (48 hr greater than time 0 in RAO horses) **[a]** and group (RAO 48 hr greater than control 8 hr) **[b]** effects were present (P = 0.027 and 0.027, respectively).

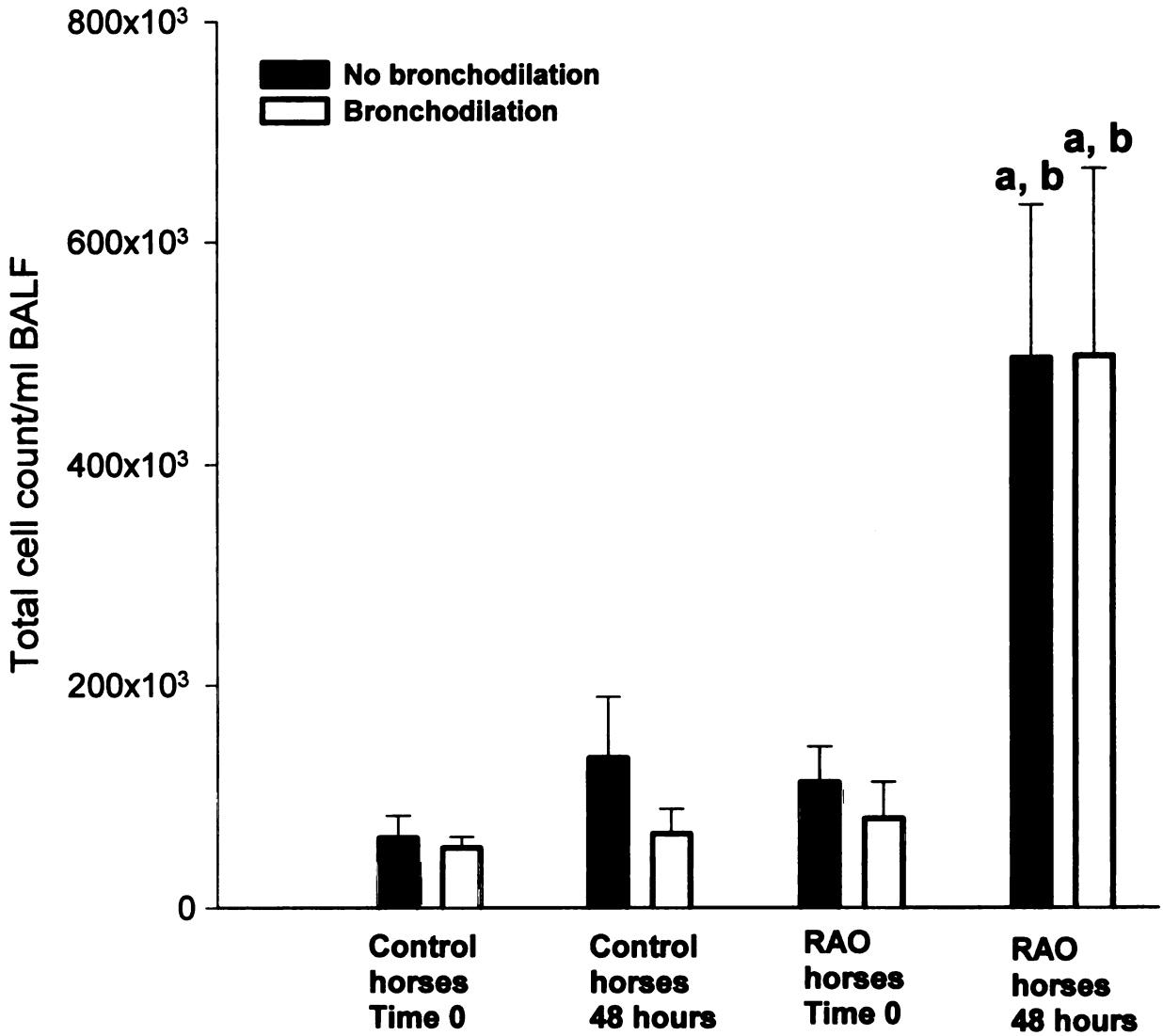

800x10³
600x10³
400x10³
200x10³
0
Total cell count/ml BALF
No bronchodilation
Bronchodilation
a, b
a, b
Control horses Time 0
Control horses 48 hours
RAO horses Time 0
RAO horses 48 hours

Figure 12. Number of neutrophils per ml bronchoalveolar lavage fluid (BALF) from non-bronchodilated and bronchodilated control and RAO-affected horses. Bronchoalveloar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of indoor housing (48 hours), with and without bronchodilator treatment. Cells in BALF were quantified as described in Methods. Results are expressed as mean ± SEM. Three way ANOVA, with time, group and treatment as factors, was performed. Tukey's test was used for post-hoc analysis. No significant treatment (bronchodilation) effect was present ($P = .458$). Significant time (48 hr greater than time 0 in RAO horses) **[a]** and group (RAO 48hr greater than control 48 hr) **[b]** effects were present ($P < 0.001$ and < 0.001, respectively).

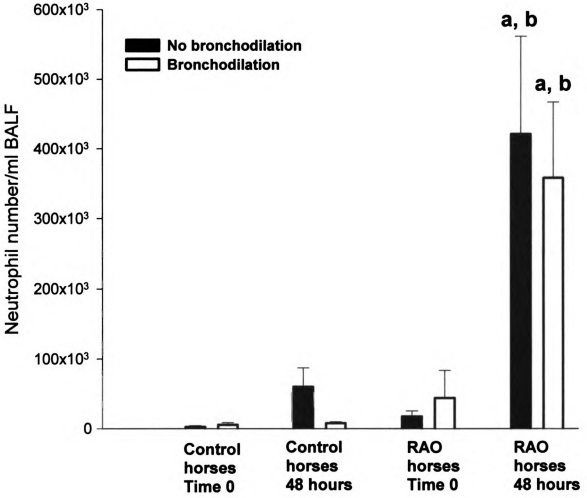
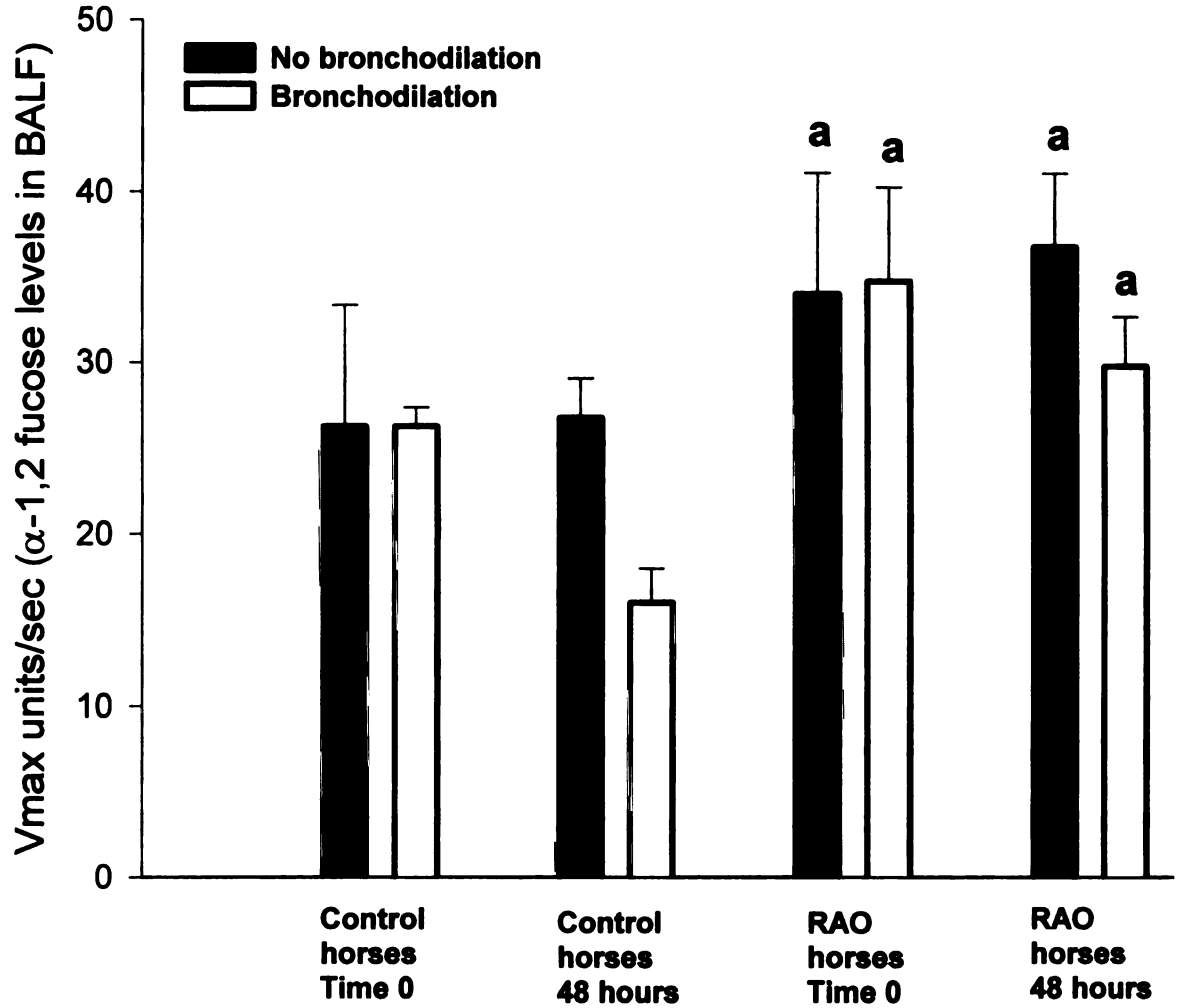

600x10³
500x10³
400x10³
300x10³
200x10³
100x10³
0
Neutrophil number/ml BALF
No bronchodilation
Bronchodilation
a, b
a, b
Control horses Time 0
Control horses 48 hours
RAO horses Time 0
RAO horses 48 hours

Figure 13. α-1,2 fucose levels in bronchoalveolar lavage fluid (BALF) from non-bronchodilated and bronchodilated control and RAO-affected horses. Bronchoalveloar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of indoor housing (48 hours), with and without bronchodilator treatment. α-1,2 fucose in BALF was quantified by enzyme-linked lectin assay as described in Methods. Results are expressed as mean ± SEM. Three way ANOVA, with time, group and treatment as factors, was performed. Tukey's test was used for post-hoc analysis. No significant treatment (bronchodilation) or time effects were present (P = .231 and .406, respectively). A significant group effect (RAO greater than control) **[a]** was evident (P = 0.004).


No bronchodilation
Bronchodilation
Vmax units/sec (α-1,2 fucose levels in BALF)
50
40
30
20
10
0
a
a
a
Control horses Time 0
Control horses 48 hours
RAO horses Time 0
RAO horses 48 hours

Chapter 4:

Effect of intranasal endotoxin instillation on mucus production in F344 rats

Recurrent airway obstruction of horses is characterized in part by a pronounced neutrophilic airway inflammation that follows exposure to a dust- and endotoxin-laden environment. As detailed in Chapters 2 and 3, increased airway mucus production, as measured by fucose-bearing mucin glycoproteins, was detected in RAO horses over controls during both acute disease and clinical remission. Additionally, it was postulated in Chapter 2 that persistent increases in mucus production may be secondary to sustained inflammation and/or inflammatory effects that are present in RAO horses while at pasture.

Increased α-1,2 fucose levels were detected in the airways of RAO-affected horses (Chapters 2 and 3). RAO is a disease characterized in part by neutrophilic airways inflammation, and acute bouts are associated with exposure to an allergen and endotoxin-rich environment. Exposure to bacterial endotoxin causes a neutrophilic airways inflammation, and is known to result in increased stored and secreted mucosubstances in rats. I therefore hypothesized that increased airway α-1,2 fucose levels would be associated with a neutrophilic inflammation in endotoxin-exposed rats, similar to the finding in RAO-affected horses.

Chapter 4 of this thesis describes a study protocol that tests this hypothesis.

Some of the work detailed in this chapter was conducted by Dr. James Wagner, who worked with me on this project.

Abstract

F344 rats were exposed to doses of 0, 2, 20 or 200 μg of bacterial endotoxin by intranasal instillation to test the hypothesis that intranasal administration of endotoxin would increase airway mucus production in a dose-dependent manner. Rats exposed to 20 or 200 μg endotoxin had elevations over both control and 2 μg animals in amount of stored intraepithelial mucosubstances in intrapulmonary airways and secreted mucus as measured by MUC5AC protein and the mucin-associated sugar α-1,2 fucose in bronchoalveolar lavage fluid. No significant differences were found between rats receiving 20 or 200 μg endotoxin, or between control rats and rats exposed to 2 μg. These results demonstrated that endotoxin administration via intranasal administration can cause mucous cell metaplasia and increased mucus secretion in intrapulmonary airways in F344 rats, and that 20 μg total dose of endotoxin is a sufficient dose to cause significant pathologic changes. Additionally, this study demonstrated that α-1,2 fucose is associated with molecules produced in mucous goblet cells, and that fucose of the α-1,2 linkage is an important marker of secreted mucin glycoproteins.

Introduction.

Bacterial endotoxin, a ubiquitous environmental contaminant, causes a neutrophilic airway inflammation and causes or exacerbates a variety of respiratory disorders, including asthma (Park, et al., 2001; Schwartz, 2001; Thorn, 2001). Intratracheal administration of endotoxin (1 mg total dose) increases stored mucosubstances and levels of secreted mucin-like molecules in rats (Steiger, et al., 1995). Additionally, airway level of mucin glycoprotein-associated fucose molecules increases in horses with recurrent airway obstruction, an asthmalike condition that is exacerbated by dust- and endotoxin-laden environments (Jefcoat, et al., 2001).

Mucus is a heterogeneous substance, with mucin glycoproteins being the most functionally significant component. Mucins are composed of a protein core, the mucin apoprotein, to which are attached numerous O-linked carbohydrate side chains. Nineteen different genes that code for mucin apoproteins, called MUC genes, have thus far been identified. The product of the MUC5AC gene is a secreted mucin that is produced by airway epithelium (Van Klinken, et al., 1995). Several different hexose sugars serve as components of mucin carbohydrate chains. One of these sugars, fucose, is an important terminal component of these chains (Bhatia and Mukhopadhyay, 1998), and increased airway fucose levels have been detected in horses with the asthmalike condition recurrent airway obstruction (Jefcoat, et al, 2001).

In the study detailed in this chapter, F344 rats were instilled intranasally with increasing doses of bacterial endotoxin to test the hypotheses that level of the

mucin-associated sugar fucose increases in the airways of endotoxin-exposed rats, and that intranasal endotoxin exposure results in dose-dependent increases in stored and secreted airway mucosubstances.

Material and methods.

Animals. Sixteen male F344 rats (Harlan Sprague-Dawley, Indianapolis, IN), 10-12 weeks of age, were randomly assigned to one of 4 experimental groups (n = 4/group). Rats were used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University.

Protocol. Rats were instilled with either sterile pyrogen-free saline, or 2, 20 or 200 μg total dose of endotoxin suspended in sterile pyrogen-free saline. Volume of instillation for each animal was 150 μL/naris. For instillations, rats were anesthetized with 4% halothane in oxygen.

Necropsy and tissue preparation. Rats were sacrificed 48 hours after a single dose of saline or endotoxin. Rats were anesthetized with sodium pentobarbital, then killed by exsanguination via the abdominal aorta. The chest cavity was opened and trachea exposed. The trachea was nicked just below the larynx, and a cannula (teat infusion cannula, Jorgensen Laboratories, Inc., Loveland, CO) was inserted and tied tightly in place with suture material. Lungs were removed, and the left primary bronchus was gently clamped off with an alligator clamp. Bronchoalveolar lavage was performed on the right lung with 10 ml of sterile saline. With the exception of aliquots taken for total and differential cell counts, samples were centrifuged at 1500 rpm for 10 minutes to remove

cells. Supernatant for immunochemistry was collected and stored at -20°C. After lavage, the lung was perfused with zinc formalin under 30 cm pressure and processed for light microscopy.

Quantification of stored intraepithelial mucosubstances. Pulmonary airways were collected and stained with Alcian blue (pH 2.5)/periodic acid-Schiff (AB/PAS) sequence to highlight stored acidic and neutral mucosubstances. To estimate the amount of intraepithelial mucosubstances at the level of 5^{th} and 11^{th} generation airways (G5 & G11), the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstance was calculated from the automatically circumscribed perimeter of stained material on a Power Macintosh 7100/66 computer using the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health, and available on the internet at http://rsb.info.nih.gov/nih-image/). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was determined as described previously (Harkema, et al., 1997). It was expressed as nl of intraepithelial mucosubstances per mm^2 of basal lamina (i.e., volume density).

Lectin histochemistry. Fifth generation airways were used for lectin histochemistry. Tissues from rats instilled with both saline and endotoxin were used. Lung sections containing fifth generation airways were embedded in

paraffin and mounted on Probe-on® plus glass slides. Slides were deparaffinized with xylene and hydrated with decreasing concentrations of ethanol in water, then washed with PBS. Endogenous peroxidases were blocked with a mix of 3% hydrogen peroxide in deionized water, then rinsed with PBS. Slides were nonspecifically blocked with a mixture of 2% horse serum in PBS, rinsed with PBS, and incubated overnight at room temperature with biotinylated *Ulex europaeus* 1 lectin (UEA 1) [Vector Laboratories, Burlingame, CA] diluted in PBS. UEA 1 is a specific binding lectin for α-1,2 fucose, a mucin-associated deoxyhexose sugar (Van den Steen, et al., 1998). Slides were then washed in PBS. ABC-AP reagent (VECTASTAIN Elite kit) was next applied for 30 minutes and slides incubated at 37°C. Slides were washed in PBS, and Vector Red® (Vector Laboratories, Burlingame, CA) was applied for 30 min at 37°C. After a final wash in PBS and rinse in deionized water, slides were dehydrated in ethanol, cleared with xylene, and a coverslip was applied.

Analysis of bronchoalveolar lavage fluid

1) Cell counts. Total and differential white blood cell counts were performed on all bronchoalveolar lavage fluid (BALF) samples. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differentials, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quick (Baxter Health Care, Dade Division, Miami FL), and 200 cells were examined to obtain a differential count. Following removal of aliquots for the purpose of cell counts, BALF was centrifuged at 1500 rpm for 12 minutes to remove cells, and supernatant was collected.

2) Enzyme linked immunosorbent assay (ELISA) for MUC5AC.

a) *Primary antibody:* anti-MUC5AC was obtained from Neomarkers (Fremont, CA) and diluted 1:500 in PBS.

b) *ELISA procedure:* one hundred µl of BALF supernatant from each animal was applied in duplicate to wells of Immulon-4 HBX 96-well plates (Dynex Technologies, Chantilly, VA) and incubated overnight at 40°C to thoroughly fix protein to the bottom of wells. One hundred µl of 6.5% casein blocking reagent (Roche Diagnostics, Indianapolis, IN) was then added to each well to cover potential irrelevant binding sites and incubated at 37°C for 30 minutes. Plates were washed four times with 1X automation buffer (Biomedia Corp., Foster City, CA), and then 100 µl of primary antibody in appropriate dilutions was added to the wells, followed by incubation at 37°C for 90 minutes. Washes with automation buffer were repeated, and 100 µl of biotinylated secondary antibody (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) was added to each well. Secondary antibody was diluted 1:200 in 1X automation buffer. Two hundred ul of rat serum was added to 10 ml of secondary antibody to eliminate any non-specific binding. The plates were incubated at 37°C for 30 minutes, then washed four times with automation buffer. One hundred microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes with automation buffer, 100 ml of QuantaBlu® fluorogenic peroxidase substrate (Pierce, Rockford, IL) were added to each well, and the plates were read at 3-min intervals for 21 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices,

Sunnyvale, CA) which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). Excitation and emission wavelengths were 325 nm and 420 nm, respectively. The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and reported as Vmax units per second. Vmax units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

3) Enzyme-linked lectin assay (ELLA) for α – 1,2 fucose

Samples were plated, blocked and washed as for ELISAs. Biotinylated *Ulex europaeus I* (UEAI) lectin (Vector Laboratories, Burlingame,CA), at 0.5 µg/ml in diluent as recommended by the supplier, was then applied for 1 hour at 37°C. Plates were then treated with ABC, QuantaBlu® and read as for ELISAs.

Statistical analysis. Multiple comparisons between groups were performed with one-way ANOVA (SigmaStat statistical software, Jandel Scientific Software, San Rafael, CA). Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

Results.

Stored intraepithelial acidic and neutral mucosubstances. Rats exposed to 20 and 200 µg endotoxin had significantly increased levels of stored intraepithelial mucosubstances (Vs) at the level of fifth generation (G5) intrapulmonary airways (Fig. 1 and 2) as compared to controls and animals

exposed to 2 μg endotoxin. No significant differences in Vs existed between the 20 and 200 μg groups.

UEA 1 histochemistry for α-1,2 fucose. Lectin histochemistry with UEA 1 lectin localized α-1,2 fucose to the interiors of secretory mucous goblet cells (Fig. 2) in rat airway epithelium.

BALF cytology. Significant increases in number of neutrophils per ml of lavage fluid were noted in 20 and 200 μg groups as compared to controls and 2 μg (Fig. 3).

MUC5AC ELISA. MUC5AC protein was significantly elevated in BALF of rats exposed to 200 μg endotoxin over the control and 2 μg groups [P = 0.003 and 0.003 for both groups] (Fig. 4). A trend toward increased levels existed in the 20 μg group as compared to controls and 2 μg rats (P = 0.062 and 0.069, respectively). No significant differences were noted between the 20 and 200 μg groups, or between the control and 2 μg groups.

UEA 1 ELLA. α-1,2 fucose levels detected by UEA1 ELLA were significantly elevated in BALF of rats exposed to 20 and 200 μg endotoxin over controls and 2 μg (Fig. 5). No significant differences were noted between the 20 and 200 μg groups, or between the control and 2 μg groups.

Discussion.

Quantification of intraepithelial acidic and neutral mucosubstances demonstrated significant increases in stored airway mucus in rats exposed to 20 and 200 μg total dose of bacterial endotoxin as compared to saline controls or

rats exposed to 2 μg endotoxin. Increased levels of MUC5AC protein and α-1,2 fucose in bronchoalveolar lavage fluid were also detected in the 20 and 200 μg endotoxin groups as compared to control and 2 μg endotoxin rats. These results indicated that intranasal instillation of endotoxin resulted in intrapulmonary changes in mucus production and secretion, and that a single instillation of 20 μg total dose endotoxin is sufficient to cause pathologic changes to the airway mucus-producing apparatus in F344 rats.

No significant differences were observed between the 20 and 200 μg groups with respect to stored or secreted mucosubstances, and no differences were detected between the control and 2 μg groups. A classic dose-response curve was therefore not generated with the endotoxin doses used for this study. A trend toward a dose-related response to endotoxin existed, however, as the 200 μg exposure group had the highest values for stored intraepithelial mucosubstances and for secreted MUC5AC and α-1,2 fucose.

Intranasal instillation also resulted in significant elevations in numbers of neutrophils per ml of lavage fluid in rats treated with 20 and 200 μg of endotoxin. Neutrophil elastase has been demonstrated to cause increased mucus production (Fischer and Voynow, 2000; Martin et al., 2000; Voynow et al., 1999). Increases in stored and secreted mucosubstances detected in this study may therefore be due at least in part to airway neutrophilic inflammation.

Lectin histochemistry with the fucose-specific lectin UEA-1 localized α-1,2 fucose to secretory cells in the airway epithelium, and enzyme-linked lectin assay showed an increase in fucose in lavage fluid of endoxin-treated rats. These

results strongly support the hypothesis that production and secretion of mucin-associated α-1,2 fucose molecules increase in airways of rats following endotoxin exposure.

Presence of increased fucose levels in bronchoalveolar lavage fluid has been demonstrated in recurrent airway obstruction-affected horses (Jefcoat, et al., 2001; Chapters 2 and 3 of this thesis), and results of this study showed increased fucose in the airways of endotoxin-exposed rats. There may be functional significance to these increased fucose levels. Fucose is typically a terminal component of mucin carbohydrate side chains (Bhatia and Mukhopadhyay, 1998; Lamblin, et al, 1991), and terminal mucin carbohydrate structures can interact with a variety of cell adhesion molecules, including many expressed on bacterial surfaces (Gaillard and Plotkowski, 1996; Scharfman, et al, 2001). Fucose-bearing mucins may therefore be involved in specific binding activities that occur in the airway mucous blanket, such as in adhesive interactions between bacteria and mucin glycoproteins.

Figure 14. Stored intraepithelial acidic and neutral mucosubstances in 5th generation intrapulmonary airways of F344 rats exposed to increasing doses of bacterial endotoxin. Rats were intranasally instilled with sterile saline or 2, 20 or 200 µg total dose of endotoxin (n = 6/group). The amount of stored intraepithelial mucosubstances at the level of 5th generation airways was quantified as described in Methods. Results are expressed as means ± SEM. Stored mucosubstances in rats receiving 20 and 200 µg were significantly elevated over saline controls ($P < 0.05$) **[a]**.

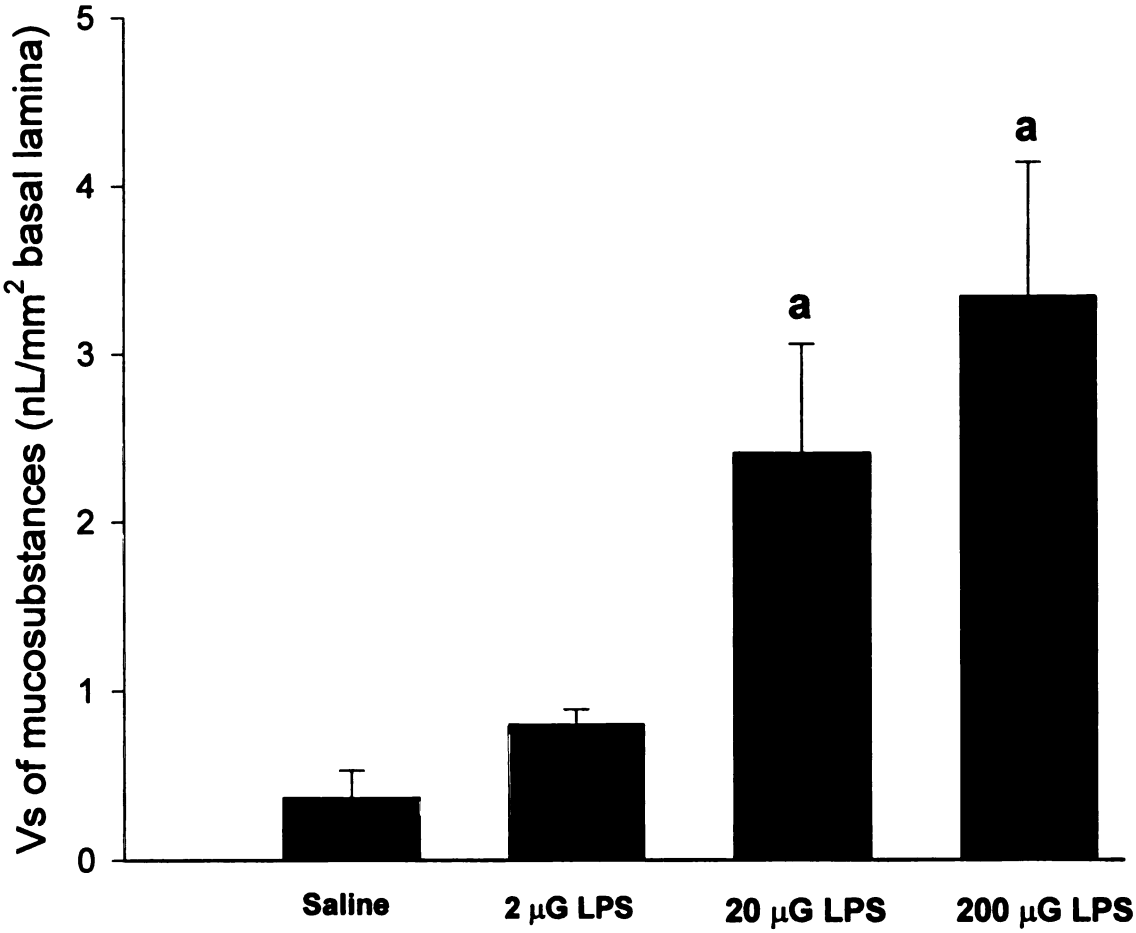
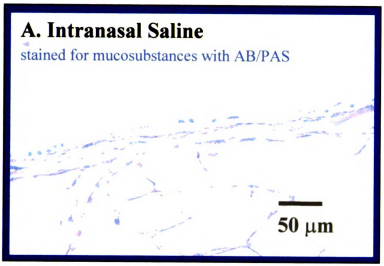
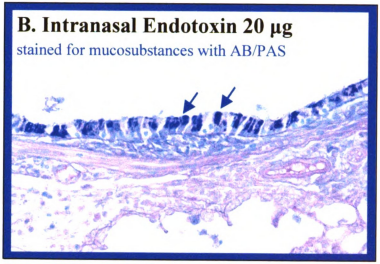
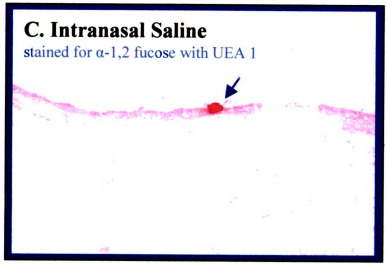

Vs of mucosubstances (nL/mm2 basal lamina)
5
4
3
2
1
0
a
a
Saline
2 μG LPS
20 μG LPS
200 μG LPS

Figure 15. Effect of intranasal endotoxin on stored intraepithelial mucosubstances in 5th generation intrapulmonary airways of F344 rats, and localization of α-1,2 fucose to airway secretory cells. Rats intransally instilled with 20 μg total dose of endotoxin displayed increases over saline-instilled controls in acidic and neutral mucosubstances, as identified by alcian blue/periodic acid Schiff's sequence [AB/PAS] (A and B). Lectin histochemistry with *Ulex europaeus* 1 lectin (UEA1), specific for α-1,2 fucose, localized fucose to secretory cells in the airway epithelium (C and D). Increased fucose was observed in the airways of endotoxin-instilled rats (D) as compared to those instilled with saline (C).


A. Intranasal Saline
stained for mucosubstances with AB/PAS
50 μm


B. Intranasal Endotoxin 20 μg
stained for mucosubstances with AB/PAS


C. Intranasal Saline
stained for α-1,2 fucose with UEA 1

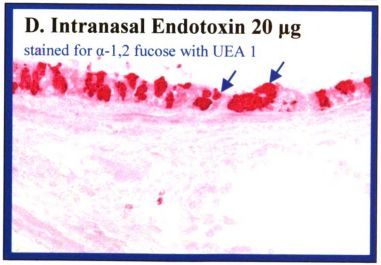

D. Intranasal Endotoxin 20 μg
stained for α-1,2 fucose with UEA 1

Figure 16. Neutrophil numbers in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to increasing doses of bacterial endotoxin. Rats were intranasally instilled with sterile saline or 2, 20 or 200 µg total dose of endotoxin (n = 6/group). Total and differential cell counts were performed on BALF as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid neutrophil numbers were significantly elevated in the 20 and 200 ug endotoxin groups compared to saline controls ($P < 0.001$) **[a]**..

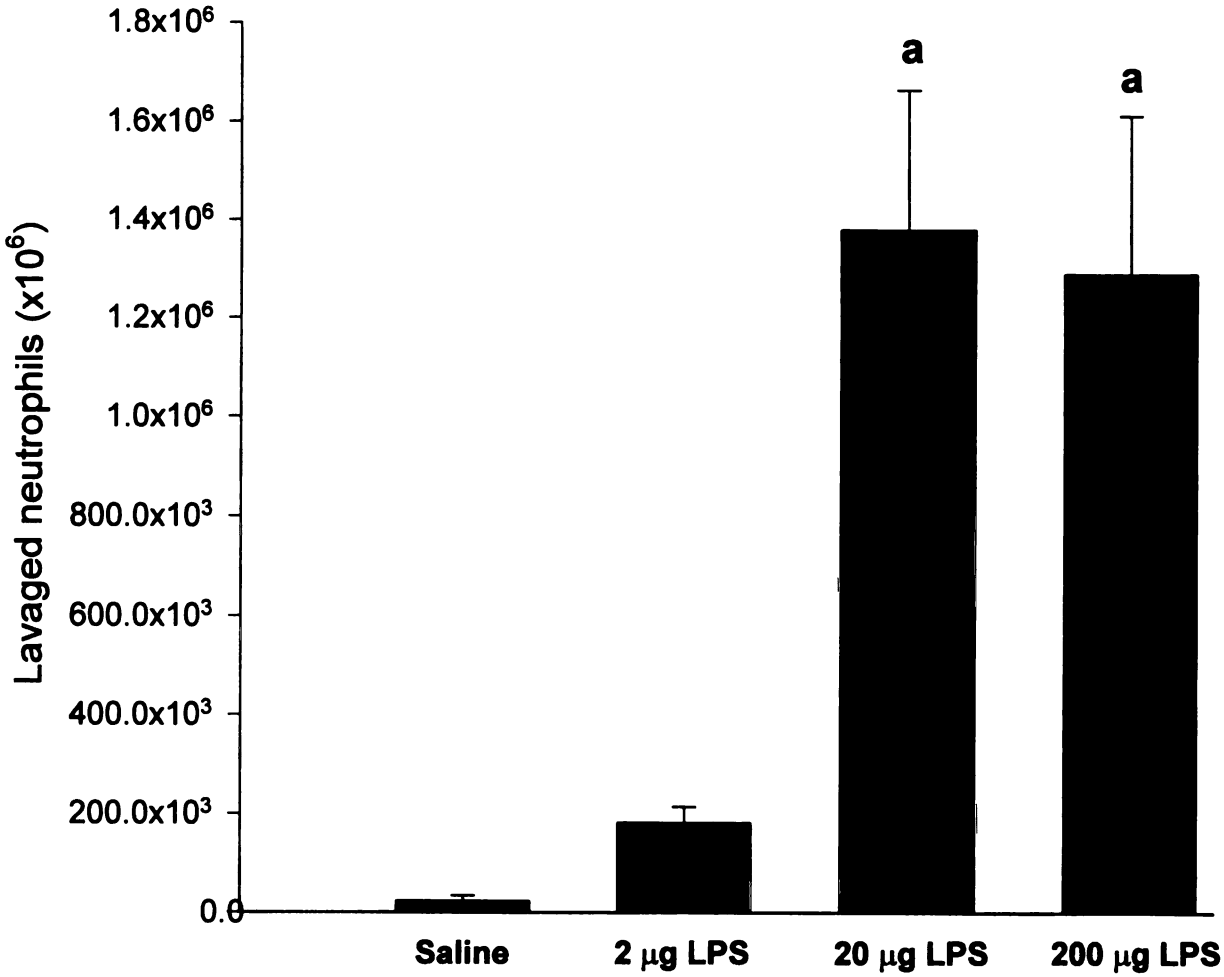

1.8x10^6
1.6x10^6
1.4x10^6
1.2x10^6
1.0x10^6
800.0x10^3
600.0x10^3
400.0x10^3
200.0x10^3
0.0
Lavaged neutrophils (x10^6)
a
a
Saline
2 μg LPS
20 μg LPS
200 μg LPS

Figure 17. MUC5AC protein levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to increasing doses of bacterial endotoxin. Rats were intranasally instilled with sterile saline or 2, 20 or 200 µg total dose of endotoxin (n = 6/group). Enzyme-linked immunosorbent assay for MUC5AC levels in BALF was performed as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid MUC5AC levels were significantly elevated in the 200 ug endotoxin group compared to saline controls and rats exposed to 2 µg endotoxin (P = 0.003 and 0.003 for both groups) **[a]**. A trend toward increase in the 20 µg group over controls and 2 µg rats existed (P = 0.062 and P = 0.069, respectively)

ge fluid (BALF) o
toxin. Rats were
dose of endotoxin
AC levels in BALF
essed as means ±
ficantly elevated in
rats exposed to 2
d toward increase
62 and P = 0.069.

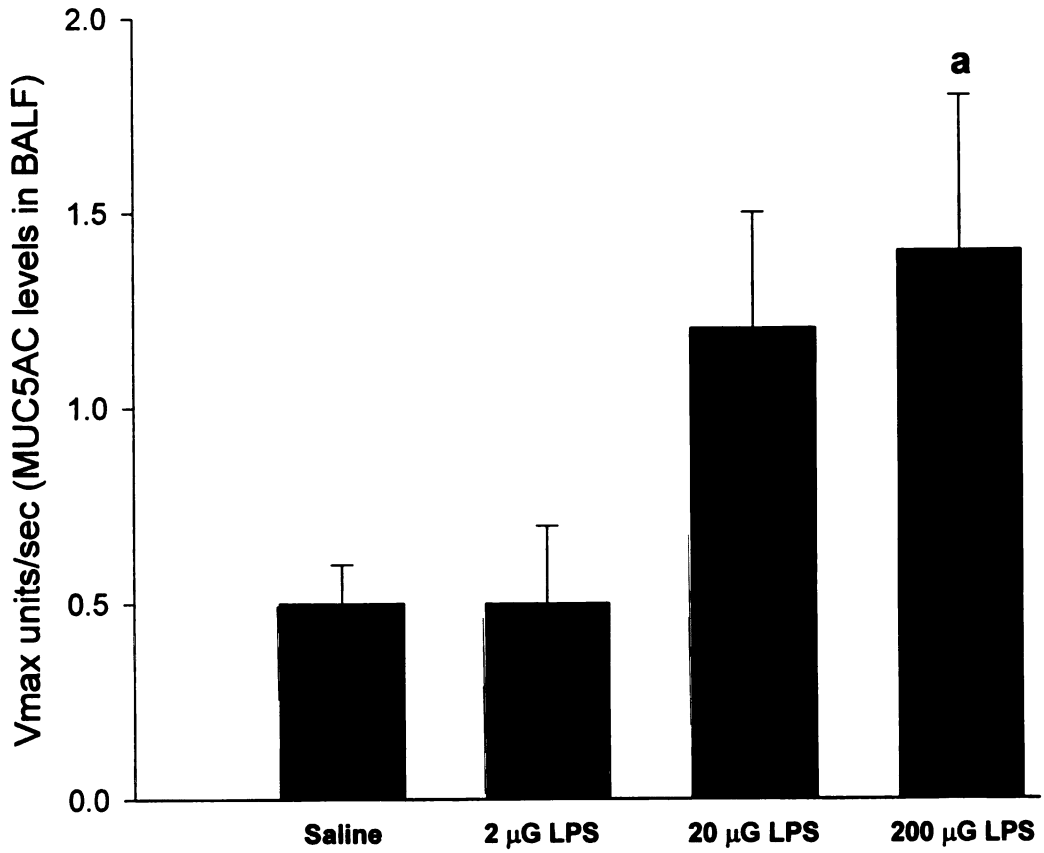


Figure 17. MUC5AC protein levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to increasing doses of bacterial endotoxin. Rats were intranasally instilled with sterile saline or 2, 20 or 200 μg total dose of endotoxin (n = 6/group). Enzyme-linked immunosorbent assay for MUC5AC levels in BALF was performed as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid MUC5AC levels were significantly elevated in the 200 ug endotoxin group compared to saline controls and rats exposed to 2 μg endotoxin (P = 0.003 and 0.003 for both groups) **[a]**. A trend toward increase in the 20 μg group over controls and 2 μg rats existed (P = 0.062 and P = 0.069, respectively)

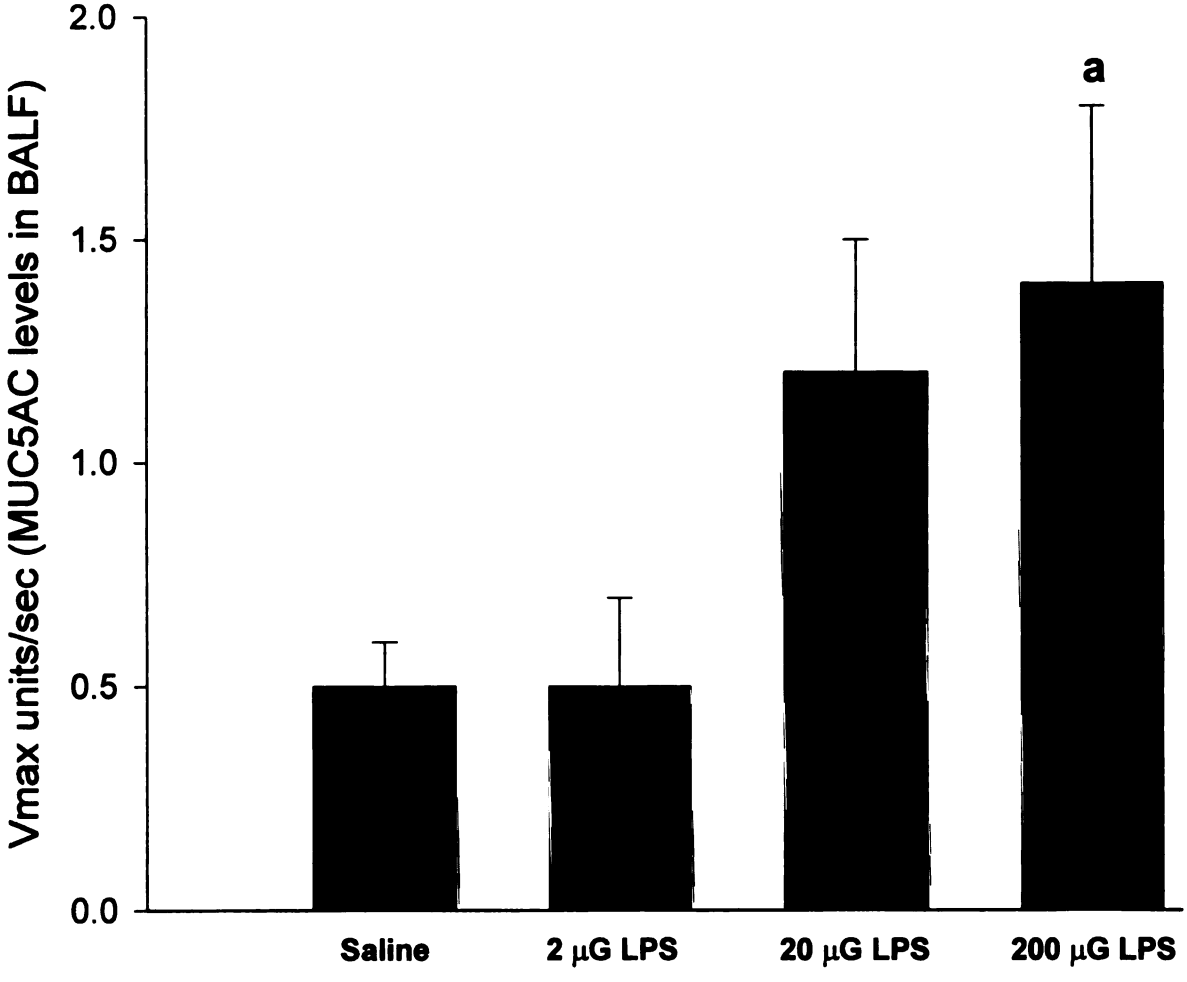
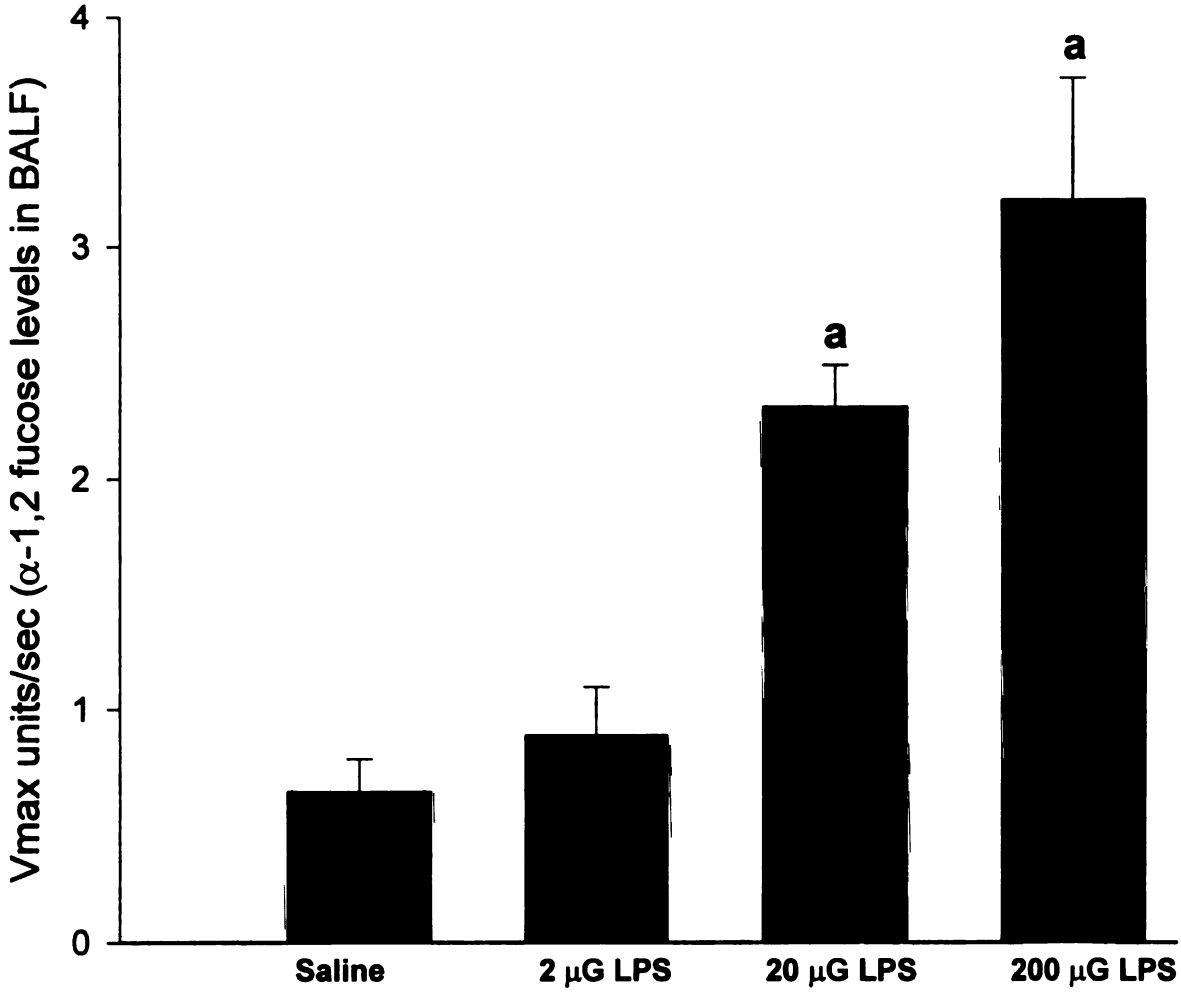

2.0
1.5
1.0
0.5
0.0
Vmax units/sec (MUC5AC levels in BALF)
a
Saline
2 μG LPS
20 μG LPS
200 μG LPS

Figure 18. α-1,2 fucose levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to increasing doses of bacterial endotoxin. Rats were intranasally instilled with sterile saline or 2, 20 or 200 μg total dose of endotoxin (n = 6/group). Enzyme-linked lectin assay for fucose levels in BALF was performed as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid α-1,2 fucose levels were significantly elevated in the 20 and 200 ug endotoxin groups compared to saline controls and rats exposed to 2 μg endotoxin ($P < 0.05$) **[a]**.


Vmax units/sec (α-1,2 fucose levels in BALF)
4
3
2
1
0
a
a
Saline
2 μG LPS
20 μG LPS
200 μG LPS

Chapter 5:

Instillation and inhalation of hay dust increases stored and secreted mucosubstances in airways of F344 rats

Dusty hay, rife with allergens and endotoxins, is recognized to be the main instigator of acute attacks of respiratory distress in RAO-affected horses. Endotoxin, as detailed in Chapter 4, was shown to increase stored and secreted mucosubstances in F344 rats. Data presented in Chapters 2, 3 and 4 showed that α-1,2 fucose can serve as a marker of secreted mucus production in both rats and horses, and that increased airway levels of this mucin-associated sugar is a shared characteristic of RAO-affected horses and endotoxin-exposed rats. Chapter 5 tests the hypothesis that hay dust challenge to rats results in similar changes in the airway apparatus to those observed in endotoxin-challenged rats and RAO-affected horses. As nasal instillation of endotoxin resulted in increased airway mucus production in F344 rats, this method of exposure was repeated in rats using a hay dust suspension. The effect of inhalation of hay dust in rats was also explored, to test the hypothesis that this system would serve as an effective model of natural inhalation of agricultural dusts, mimicking exposures experienced by both horses and humans in barn and stable settings.

ABSTRACT

Organic dusts rich in bacterial endotoxins are ubiquitous in barn environments. These dusts are associated with occupational respiratory diseases in humans, and with the asthma-like condition recurrent airway obstruction (RAO) of horses. Previous work in our laboratory has demonstrated increased stored mucosubstances and levels of the mucin-associated sugar α-1,2 fucose in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to endotoxin, and that RAO-affected horses have a persistent elevation of α-1,2 fucose in BALF. I therefore hypothesized that hay dust challenge to rats will result in similar changes in the airway apparatus to those observed in endotoxin-challenged rats and RAO-affected horses. Hay representative of that found in barn environments was used as bedding material for F344 rats. Rats were housed on hay for 3 or 10 days. A single dose of dust suspension prepared from barn hay in sterile saline was nasally instilled into a 3rd group (200 μL/naris, 5 mg dust total exposure). 50,000 endotoxin units/ml of suspension was measured by Limulus amoebocyte assay. BALF was recovered at necropsy, and large diameter airways were collected and stained to highlight stored epithelial mucosubstances. Necropsy of rats housed on hay occurred immediately after end of the housing period, while necropsy of instilled rats took place 72 hours post exposure. Nasally instilled rats and rats housed on hay for 10 days showed a significant increase in stored mucosubstances in 5th generation (G5) airways compared to controls (12x and 4x, respectively). Level of α-1,2 fucose in BALF of nasally-instilled rats was significantly elevated over controls (P = .002). A

trend toward elevated MUC5AC apomucin levels in BALF of instilled rats was also present (P = .079). Numbers of neutrophils and lymphocytes per ml of lavage fluid were significantly increased in nasally instilled rats as compared to controls. Conclusion: hay dust components, i.e. endotoxin or other factors, increased stored and secreted airway mucosubstances in F344 rats.

Introduction

Organic dusts rich in endotoxins are common in barns and stalls in agricultural settings, and respirable endotoxin (lipopolysaccharide – LPS) concentration in barns can exceed minimum threshold exposure levels for respiratory health effects (Kullman, et al., 1998). LPS-rich dusts have been associated with occupational respiratory diseases of humans (Schwartz, et al., 1995; Thorn, 2001) and declines in lung function (Post, 1998, Thorn, 2001). Endotoxin inhalation is known to cause a powerful neutrophil inflammation in the airways (Thorn, 2001), and it has been implicated in the development and exacerbation of asthma (Park, et al., 2001; Schwartz, 2001).

Increased airway mucus is a feature of a number of airway diseases, including asthma and cystic fibrosis (Bousquet, et al., 2000; Quinton, 1999), and it is also a sequela to endotoxin exposure (Gordon, et al., 1996; Harkema and Hotchkiss, 1991; Steiger et. al., 1995). High molecular weight mucin glycoproteins are a major component of the mucus layer, and these molecules provide mucus with the majority of its viscoelastic properties. Mucin glycoproteins are composed of a protein core to which are attached linear and

branching oligosaccharide side chains. The protein core is a product of a family of genes called MUC genes, and the specific product of the MUC5AC gene is a component of secreted mucus (Hannisch and Muller, 2000; Perez-Vilar and Hill, 1999). MUC5AC is known to be expressed in airway epithelium (Wills-Karp, 2000). Oligosaccharide side chains are composed of 2 to 20 individual sugars linked together by covalent bonds (Rose, 1992). The deoxyhexose sugar fucose is a common terminal component to these mucin carbohydrate chains (Bhatia and Mukhopadhyay, 1998), and increased levels of fucose in the airways have been demonstrated in inflammatory airway conditions (Jefcoat, et al., 2001; Chapter 4).

Earlier work presented in this thesis (Chapter 4) has demonstrated that nasal instillation of endotoxin causes increased stored and secreted mucosubstances in airways of F344 rats, including increased airway fucose levels. Steiger and coworkers (1995) have also shown increased release of mucin-like molecules by rat epithelial cells in response to endotoxin. Additionally, I have demonstrated increases in fucose-bearing mucins in the airways of recurrent airway obstruction-affected horses (Chapters 2 and 3). For this study, I hypothesized that a) nasal instillation of hay dust extract in F344 rats would cause mucus secretory changes similar to those observed following endotoxin instillation, including increases in airway fucose levels; and b) that housing of F344 rats on hay bedding would result in sufficient dust exposure to cause significant airway pathology.

Materials and Methods

Animals. Twenty male F344 rats (Harlan Sprague-Dawley, Indianapolis, IN), 10-12 weeks of age, were randomly assigned to one of 4 experimental groups (n = 5/group). Rats were used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University.

Protocol. Control group rats were kept on Cell-Sorb Plus® bedding (A & W Products, Cincinnati, OH). Two additional groups of rats were housed in polycarbonate boxes, with hay collected from university farms and representative of that used in barn environments used as bedding material. One group was housed for 3 days, and a second group housed for 10 days. The hay bedding was changed daily. Animals in a third treatment group were intranasally instilled with a single dose of hay dust extract. For preparation of the extract, dust was collected under suction from a flake of hay obtained from a stable setting. Vacuum suction was used on a bagged (with an inlet to allow air flow) flake of hay for collection of both fine and coarse dust. The collected dust was suspended in pyrogen-free sterile saline. Limulus amoebocyte assay (Kinetic-QCL, BioWhittaker, Walkerville, MD) of the suspension indicated an endotoxin concentration of 50,000 endotoxin units (EU)/ml. By definition, 10 EU is the equivalent of 1 nanogram endotoxin. For instillations, rats were anesthetized with 4% halothane in oxygen, and 200 µl of hay dust extract was instilled into each nasal passage. The total volume instilled in each rat was representative of 5 mg dust (original dry weight), and the 400 µl of suspension administered to

each rat was the equivalent of 20,000 EU (2000 ng). Instilled rats were housed on traditional bedding. Control group rats were housed on traditional bedding and were not intranasally instilled.

Necropsy and tissue preparation. Treated animals were sacrificed either immediately after the housing period, or 72 hours after intranasal instillation of hay dust extract. Seventy two hours was chosen as an end point to correspond with the 3 day group of hay-bedded rats. Rats were anesthetized with sodium pentobarbital, then killed by exsanguination via the abdominal aorta. The chest cavity was opened and trachea exposed. The trachea was incised just below the larynx, and a cannula (teat infusion cannula, Jorgensen Laboratories, Inc., Loveland, CO) was inserted and tied tightly in place with suture material. Lungs were removed, and the left primary bronchus was gently clamped with an alligator clamp. The right lung was lavaged with 10ml of sterile saline. With the exception of aliquots taken for total and differential cell counts, samples were centrifuged at 1500 rpm for 10 minutes to remove cells. Supernatant for immunochemistry was collected and stored at -20°C. After lavage, the lung was perfused with zinc formalin under 30 cm pressure, and processed for light microscopy.

Quantification of stored intraepithelial mucosubstances. Pulmonary airways were collected and stained with Alcian blue (pH 2.5)/periodic acid-Schiff (AB/PAS) sequence to highlight stored acidic and neutral mucosubstances. To estimate the amount of intraepithelial mucosubstances at the level of 5^{th} and 11^{th} generation airways (G5 & G11), the volume density (Vs) of AB/PAS-stained

mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of AB/PAS-stained mucosubstance was calculated from the automatically circumscribed perimeter of stained material on a Power Macintosh 7100/66 computer using the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health, and available on the internet at http://rsb.info.nih.gov/nih-image/). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was determined as described previously (Harkema, et al., 1997). It was expressed as nl of intraepithelial mucosubstances per mm^2 of basal lamina (i.e., volume density).

Analysis of bronchoalveolar lavage fluid

1) Cell counts. Total and differential white blood cell counts were performed on all bronchoalveolar lavage fluid (BALF) samples. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differential cell counts, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quick (Baxter Health Care, Dade Division, Miami FL), and 200 cells were examined to obtain a differential count. Following removal of aliquots for the purpose of cell counts, BALF was centrifuged at 1500 rpm for 12 minutes to remove cells, and supernatant was collected.

2) Enzyme linked immunosorbent assay (ELISA) for MUC5AC.

a) *Primary antibody:* anti-MUC5AC (MUC5AC) was obtained from Neomarkers (Fremont, CA), and diluted 1:500 in PBS.

b) *ELISA procedure:* one hundred µl of BALF supernatant from each animal was applied in duplicate to wells of Immulon-4 HBX 96-well plates (Dynex Technologies, Chantilly, VA) and incubated overnight at 40°C to thoroughly fix protein to the bottom of wells. One hundred µl of 6.5% casein blocking reagent (Roche Diagnostics, Indianapolis, IN) was then added to each well to cover potential irrelevant binding sites and incubated at 37°C for 30 minutes. Plates were washed four times with 1X automation buffer (Biomedia Corp., Foster City, CA), and then 100 µl of primary antibody in appropriate dilutions was added to the wells, followed by incubation at 37°C for 90 minutes. Washes with automation buffer were repeated, and 100 µl of biotinylated secondary antibody (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) was added to each well. Secondary antibody was diluted 1:200 in 1X automation buffer. Two hundred ul of rat serum was added to 10 ml of secondary antibody to eliminate any non-specific binding. The plates were incubated at 37°C for 30 minutes, then washed four times with automation buffer. One hundred microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes with automation buffer, 100 ml of QuantaBlu® fluorogenic peroxidase substrate (Pierce, Rockford, IL) were added to each well, and the plates were read at 3-min intervals for 21 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices,

Sunnyvale, CA) which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). Excitation and emission wavelengths were 325 nm and 420 nm, respectively. The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and reported as Vmax units per second. Vmax units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

3) Enzyme-linked lectin assay (ELLA) for α – 1,2 fucose

Samples were plated, blocked and washed as for ELISAs. Biotinylated *Ulex europaeus I* (UEAI) lectin (Vector Laboratories, Burlingame,CA), at 0.5 µg/ml in diluent as recommended by the supplier, was then applied for 1 hour at 37°C. UEA1 is specific for α – 1,2 fucose, a mucin-associated deoxyhexose sugar. Lectin histochemistry by our laboratory has localized fucose of this linkage to secretory (goblet) cells (data not shown). Plates were then treated with ABC, QuantaBlu® and read as for ELISAs.

Statistical analysis. Comparisons between groups were performed with one-way ANOVA (SigmaStat statistical software, Jandel Scientific Software, San Rafael, CA). Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

Results

Intraepithelial mucosubstances

The volume density (Vs) of stored intraepithelial mucosubstances at the G5 airway level of nasally-instilled rats was significantly elevated over control and 3 day hay-bedded rats. A trend toward increased Vs of stored mucosubstances at the G5 level existed in 10 day rats compared to controls [P = 0.062] (Figs. 1 and 2). A trend toward increased Vs of stored mucosubstances at the G11 level of nasally instilled rats was also present (P = 0.070). Figure 3 is a photomicrograph of epithelia from the G5 airway of a rat from the control group, the 10-day housed on hay group, and the nasally-instilled group, showing mild increases in stored mucosubstances in the 10-day group, and substantial increases in the nasally

Total and differential cell counts

Bronchoalveolar lavage fluid total cell counts of treated rats were not statistically different from controls (Fig. 4). Significant elevations in neutrophil number per ml of lavage fluid existed in nasally instilled rats compared to controls and 10 day hay-bedded rats, but not in comparison to the 3 day hay-bedded rats. Significant elevations in neutrophil number per ml BALF existed in 3 day hay-bedded rats compared to control rats (Fig. 5). Significant elevations in lymphocyte number per ml lavage fluid existed in nasally-instilled rats compared to control and 3 and 10 day hay-bedded groups (Fig. 5).

MUC5AC ELISA

Elevated levels of MUC5AC detected in nasally-instilled rats were statistically significant over 3 day hay-bedded rats only [P = .004] (Fig. 6).

α-1,2 fucose ELLA

α-1,2 fucose level in BALF of nasally-instilled rats was significantly elevated over control and 3 and 10 day hay-bedded groups (Fig. 7).

Discussion

Agricultural dusts known to contain high concentrations of bacterial endotoxin can cause or exacerbate a variety of respiratory disorders. This report details the first study to our knowledge to explore a) the effect of intranasal instillation of hay dust extract on production of specific markers of airway mucus, and b) the effect of natural inhalation of hay dust on laboratory rodents. For this study, rats were either intranasally instilled with a hay dust suspension that was rich in endotoxin (50,000 endotoxin units/ml), or were housed on hay bedding for 3 or 10 days.

Effect of intranasal instillation of hay dust extract. The hay dust suspension instilled in the nares of F344 rats, equivalent to 5 mg of hay dust and 20,000 EU (2000 ng) endotoxin, resulted in an airway inflammatory response as measured by an increase in lavage fluid total cell count, and number of neutrophils and lymphocytes per ml BALF. Other studies by our laboratory have shown that intranasal instillation of 20 μg endotoxin in F344 rats resulted in significant airway inflammation at 48 hours post instillation (Chapter 4). As lavage fluid was collected at 72 hours post instillation, it is likely that an even more robust inflammatory cell influx into the airways occurred at earlier time points.

Instillation of hay dust resulted in significant increases in the amount of stored intraepithelial mucosubstances in both fifth and eleventh generation airways

compared to amounts in control rats and rats housed on hay, and also resulted in a significant increase in levels of the mucin-associated sugar α-1,2 fucose in bronchoalveolar lavage fluid. A trend toward elevated MUC5AC levels in the nasally-instilled group was also present.

Effect of natural exposure to hay dust. Regarding mucus production, the only difference detected in rats that inhaled dust from hay bedding was a trend toward increase in stored intraepithelial mucosubstances in fifth generation airways of animals housed on hay for 10 days. No effects of housing on hay in regard to secreted airway mucins were detected. Rats housed on hay for 3 days showed a significant elevation over control rats in number of neutrophils per milliliter of lavage fluid. These results suggest that natural inhalation of hay dust from housing on hay initiated a mild early inflammation that abated by 10 days.

Though early airway inflammatory cell increases were observed in rats housed on hay, the relatively mild nature of these changes indicated that housing of rats on dusty hay as a sole means of exposure is insufficient to cause significant airway pathology in F344 rats. Significant inflammation and increases in stored and secreted mucosubstances were detected in rats nasally-instilled with hay dust suspension, however, indicating that this method of exposure can be used in modeling the effects of agricultural dusts on airway disease. Changes in stored and secreted mucosubstances may have been secondary to the airway inflammation instigated by instillation of hay dust suspension, as it has been demonstrated that neutrophil elastase can increase production of airway mucins (Fischer and Voynow, 2000; Martin, et al., 2000; Voynow, et al., 1999). Direct

effects of endotoxin or other hay dust components on airway epithelium cannot be ruled out, however.

Collectively, the results of this study indicate that hay dust components, i.e. bacterial endotoxin and/or other factors, can cause airway inflammation and increase both stored airway mucosubstances and secreted mucins in F344 rats.

Figure 19. Stored intraepithelial acidic and neutral mucosubstances in 5th generation intrapulmonary airways of F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. Stored intraepithelial mucosubstances, as detected by alcian blue/periodic acid Schiff's sequence, at the level of 5th generation airways was quantified as described in Methods. Results are expressed as means ± SEM. A trend toward increased intraepithelial mucosubstances existed in 10 day rats compared to controls (P = 0.062). Stored mucosubstances in nasally instilled rats were significantly elevated over controls and 3 day rats. (P < 0.001 and P = 0.015, respectively) **[a]**.

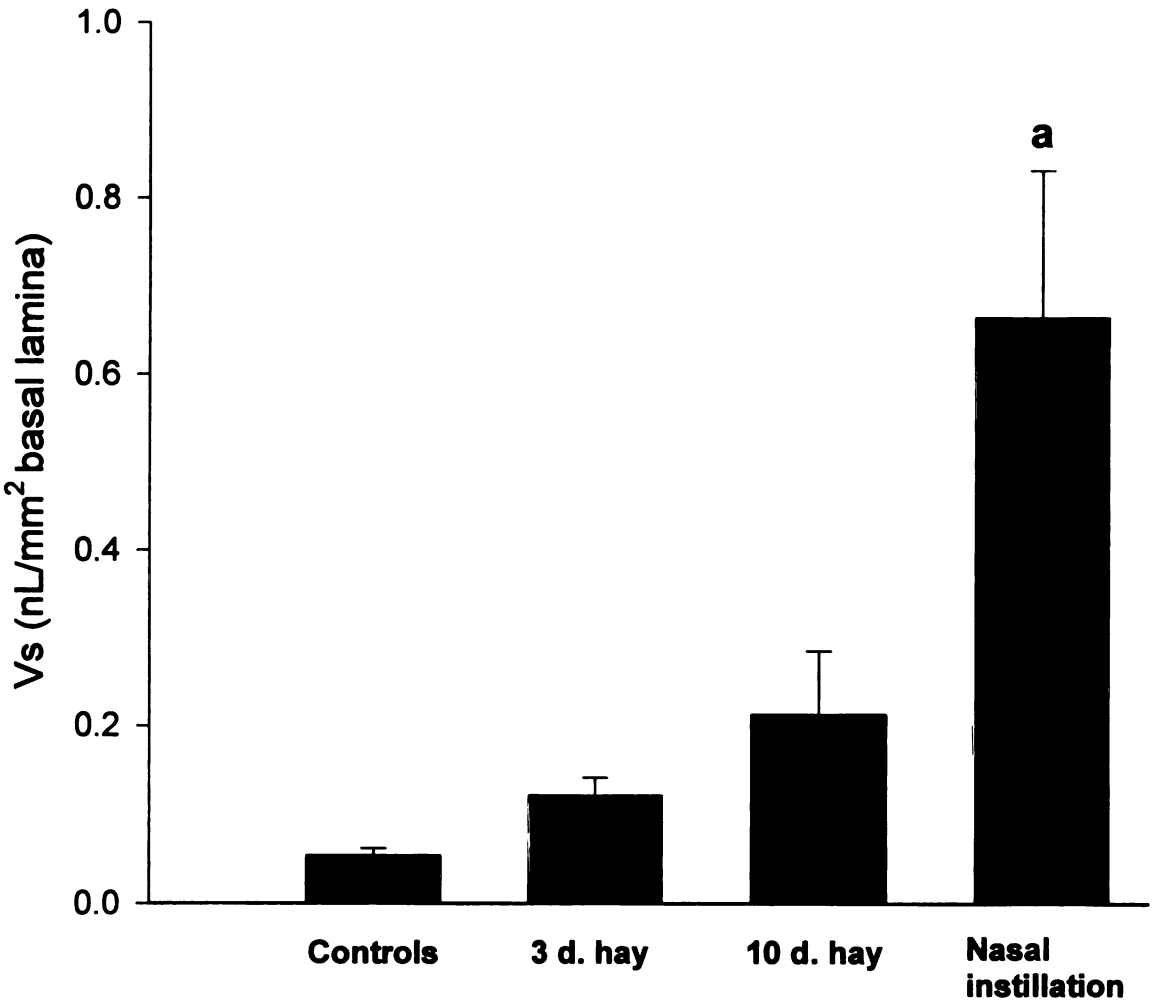

1.0
0.8
0.6
0.4
0.2
0.0
Vs (nL/mm2 basal lamina)
a
Controls
3 d. hay
10 d. hay
Nasal instillation

Figure 20. Stored intraepithelial acidic and neutral mucosubstances in 11th generation intrapulmonary airways of F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. Stored intraepithelial mucosubstances, as detected by alcian blue/periodic acid Schiff's sequence, at the level of 11th generation airways was quantified as described in Methods. Results are expressed as means ± SEM. A trend toward increased intraepithelial mucosubstances existed in nasally-instilled rats. (P = 0.070).

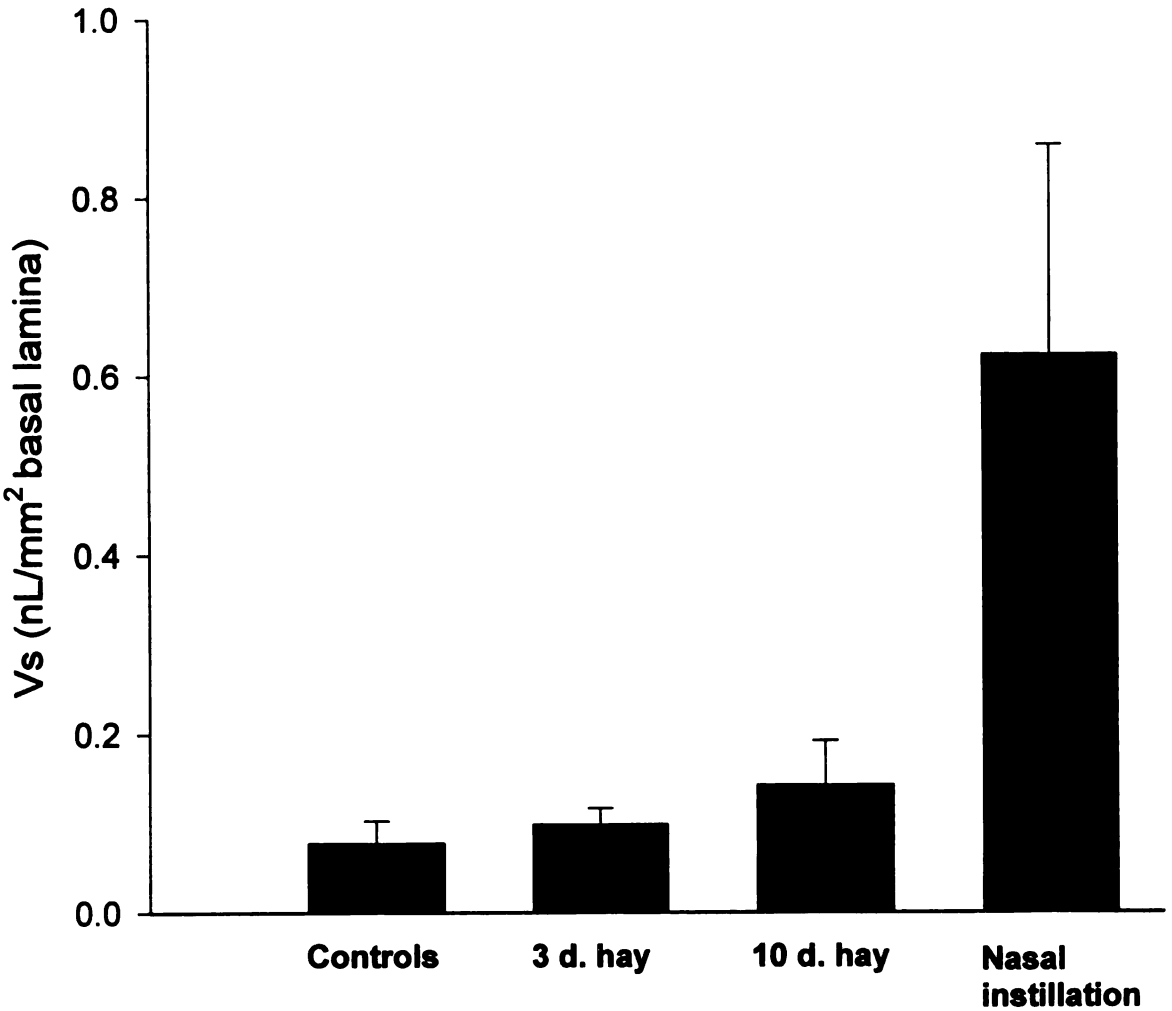

1.0
0.8
0.6
0.4
0.2
0.0
Vs (nL/mm2 basal lamina)
Controls
3 d. hay
10 d. hay
Nasal instillation

Figure 21. Photomicrographs of 5th generation intrapulmonary airway epithelium from F344 rats exposed to hay dust by inhalation or intranasal intstillation, stained with alcian blue/periodic acid Schiff's sequence to highlight stored acidic and neutral mucosubstances. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed 72 hours after a single instillation. Stored intraepithelial mucosubstances, as detected by alcian blue/periodic acid Schiff's sequence (AB/PAS), at the level of 5th generation airways was quantified as described in Methods. Arrows point to stored intraepithelial mucosubstances. Note that the epithelium in 10 day and intranasally instilled rats is thickened as compared to control rats. A = control rat. B = 10 day housed on hay rat. C = intranasally instilled rat. 20X magnification.

A
B
C

Figure 22. Total cell count per ml bronchoalveolar lavage fluid (BALF) in F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. Total and differential cell counts were performed on BALF as described in Methods. Results are expressed as means ± SEM. Though elevated total cell counts per ml BALF were noted in 3 day hay rats and nasally-instilled rats, increases were not significant.

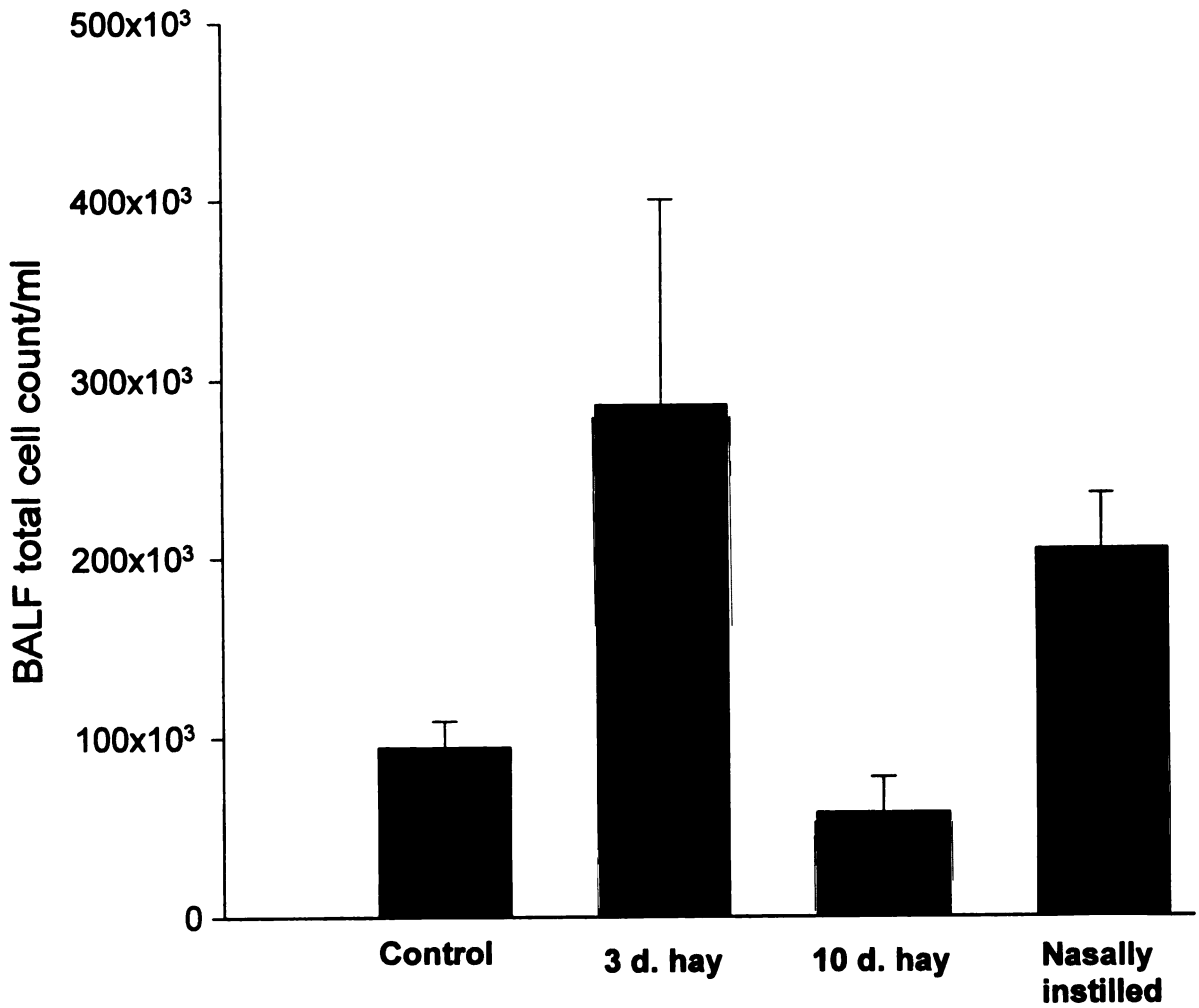

500x10³
400x10³
300x10³
200x10³
100x10³
0
BALF total cell count/ml
Control
3 d. hay
10 d. hay
Nasally instilled

Figure 23. Number of neutrophils and lymphocytes per ml bronchoalveolar lavage fluid (BALF) of F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. Total and differential cell counts were performed on BALF as described in Methods. Results are expressed as means ± SEM. Number of neutrophils in nasally-instilled rats was significantly elevated over controls and rats housed on hay for 10 days ($P < 0.001$ and $P = 0.011$, respectively) **[a]**. Number of neutrophils in rats housed on hay for 3 days was significantly elevated over control rats ($P = 0.036$) **[b]**. Number of lymphocytes in nasally instilled rats was significantly elevated over all groups ($P < 0.05$) **[c]**.

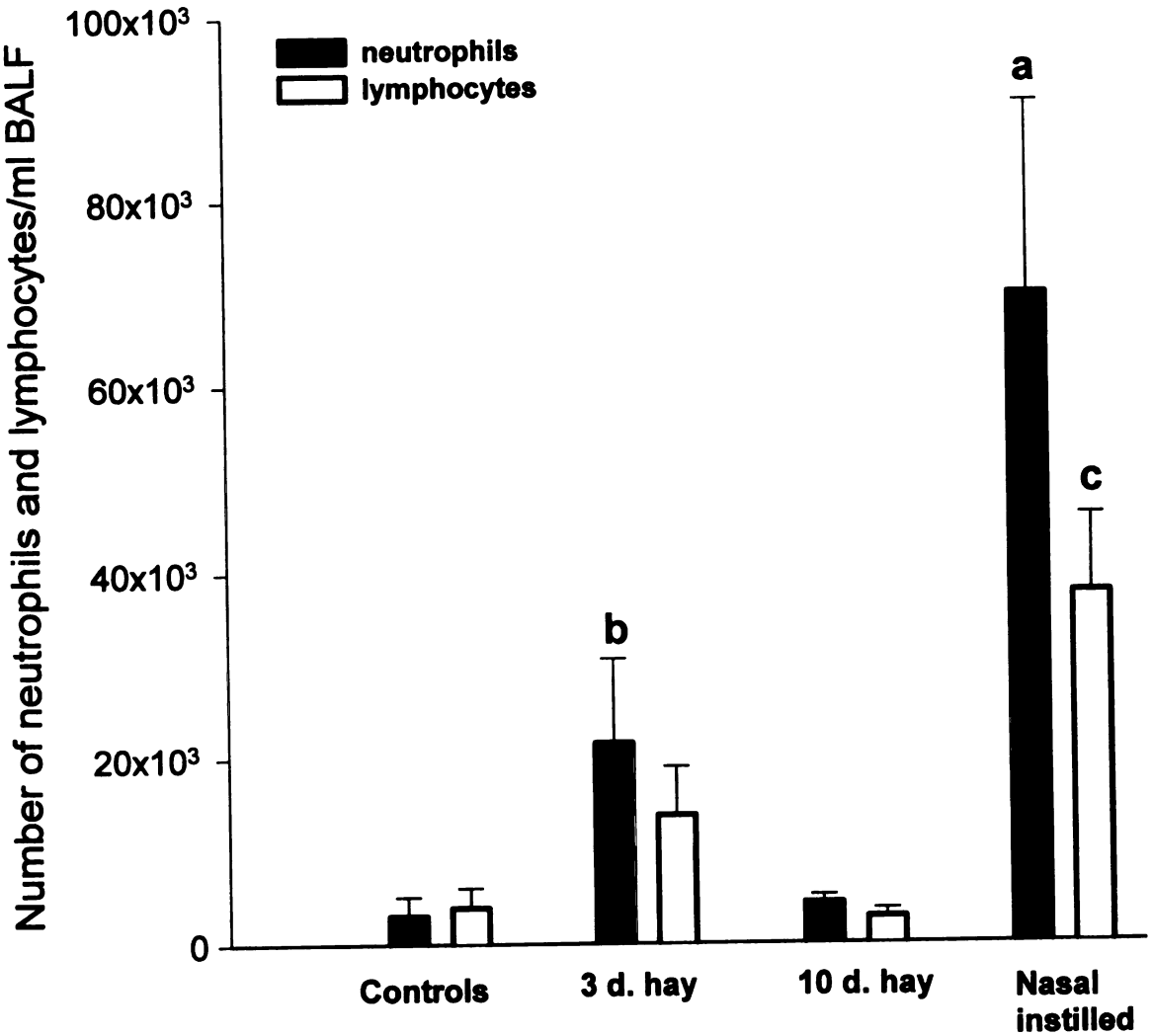

Number of neutrophils and lymphocytes/ml BALF
100x10³
80x10³
60x10³
40x10³
20x10³
0
neutrophils
lymphocytes
a
b
c
Controls
3 d. hay
10 d. hay
Nasal instilled

Figure 24. MUC5AC protein levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. MUC5AC level in BALF was detected by enzyme-linked immunosorbent assay as described in Methods. Results are expressed as means ± SEM. Elevated levels in nasally-instilled rats were statistically increased over 3 day hay rats only (P = 0.004).

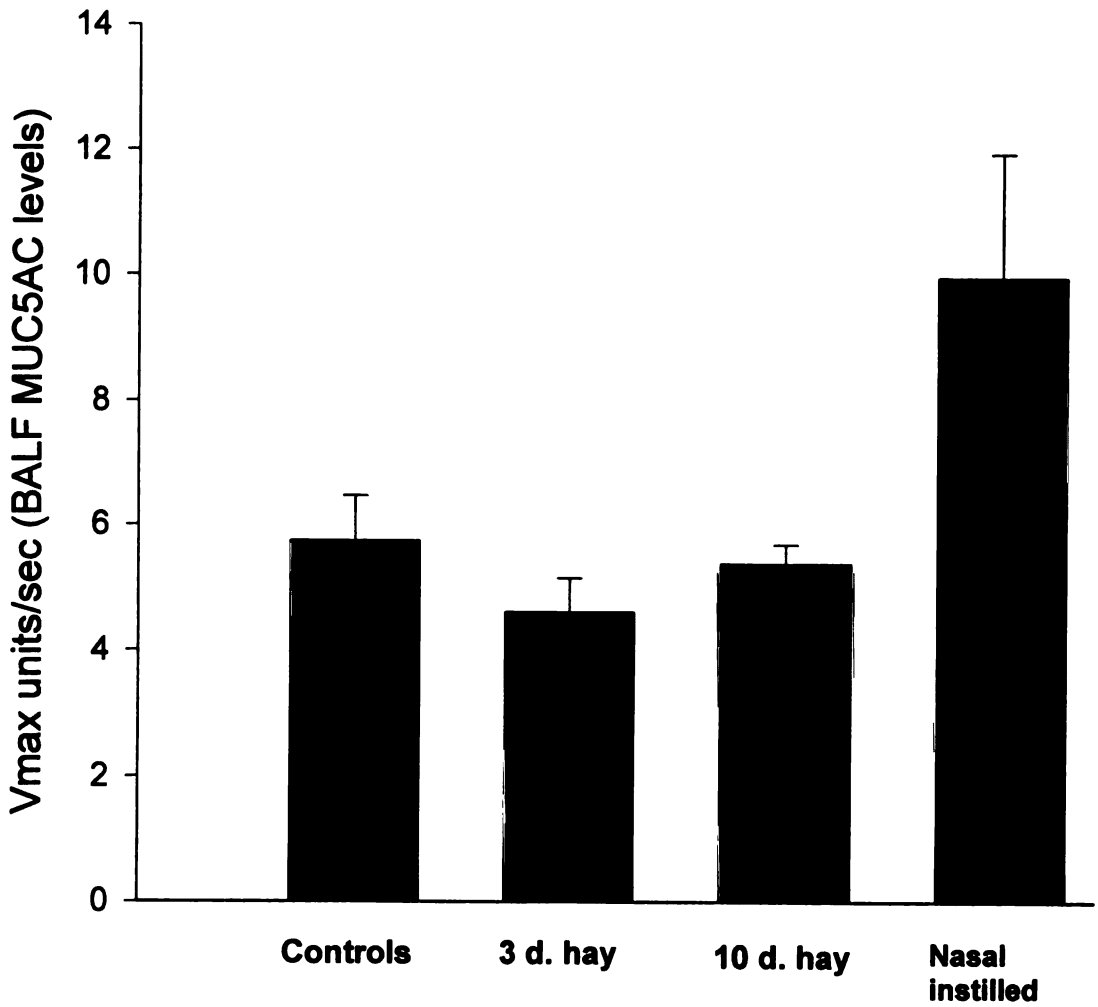
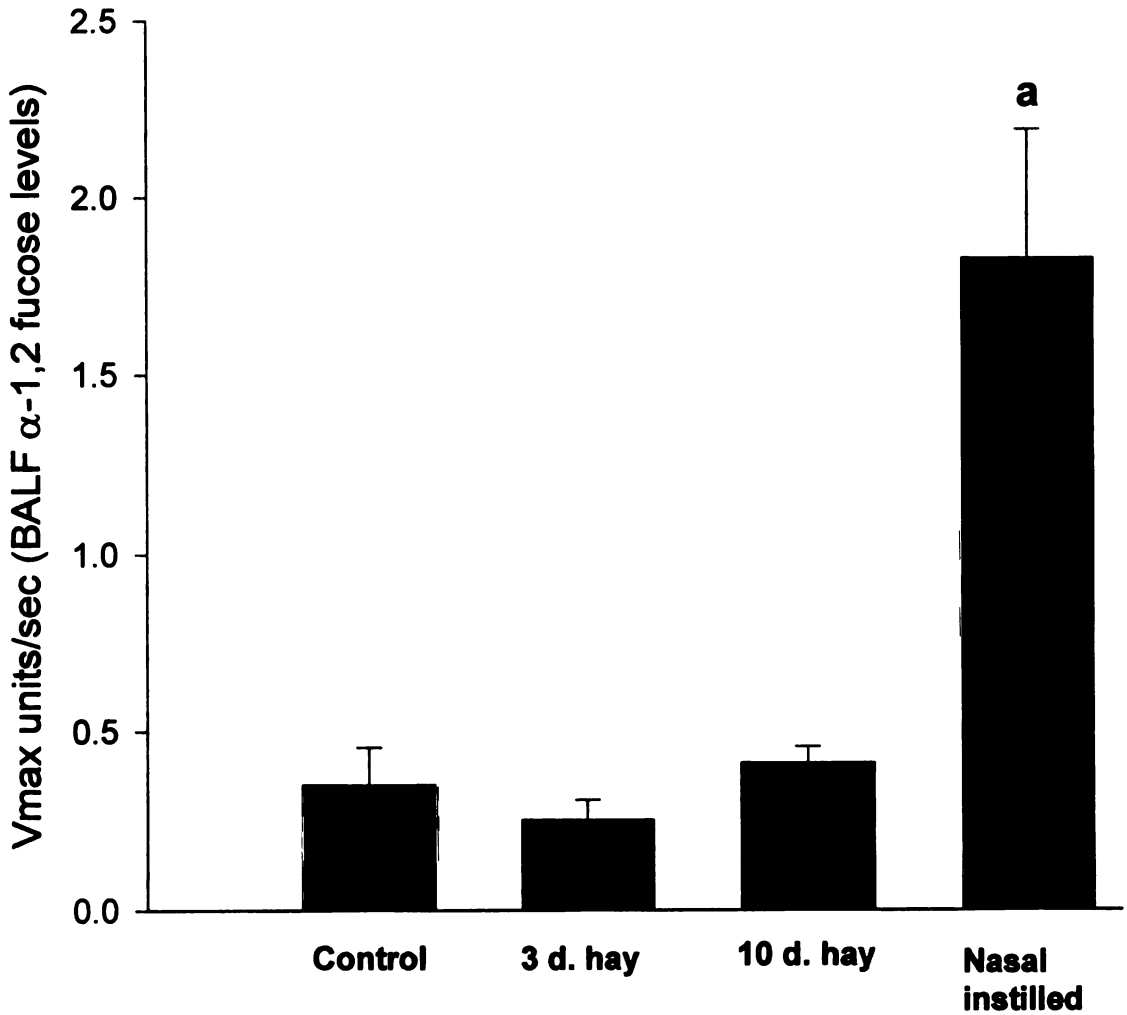

Vmax units/sec (BALF MUC5AC levels)
14
12
10
8
6
4
2
0
Controls
3 d. hay
10 d. hay
Nasal instilled

Figure 25. α-1,2 fucose levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to hay dust by inhalation or intranasal instillation. Rats were housed on hay for 3 or 10 days, or intranasally instilled with hay dust suspension (n = 5/group). Rats housed on hay were sacrificed at the end of the housing period. Instilled rats were sacrificed 72 hours after a single instillation. α-1,2 fucose level in BALF was detected by enzyme-linked lectin assay as described in Methods. Results are expressed as means ± SEM. Fucose levels in nasally instilled rats were significantly elevated over all other groups ($P < 0.002$) **[a]**.


Vmax units/sec (BALF α-1,2 fucose levels)
2.5
2.0
1.5
1.0
0.5
0.0
a
Control
3 d. hay
10 d. hay
Nasal instilled

Chapter 6:

Temporal differences in airway mucus composition in F344 rats exposed to endotoxin

Increased airway levels of the mucin-associated sugar fucose in RAO-affected horses and in rats exposed to endotoxin and hay dust suspension have been described in preceding chapters. These findings led me to more detailed background research into biochemical and functional aspects of the carbohydrate side chains of mucin glycoproteins. This research revealed important functional roles of a specific fucose-containing tetrasaccharide structure referred to as the sialyl Lewis X (SLeX) structure. The SLeX tetrasaccharide is a significant intercellular adhesion structure, capable of binding with selectins expressed on leukocyte surfaces and with adhesion molecules on bacterial surfaces. The fucose sugar in the SLeX structure is connected by a 1,3 glycosidic linkage, rather than a 1,2 linkage as is detected by the UEA 1 lectin used in previous studies detailed in this thesis. Disease-associated increases in one of many possible forms of fucose linkages (1,2), and increases found in other sugar types such as sialic acid and N-acetyl-glucosamine (Chapter 2), suggested that multiple sugar linkages and structures may be increased in inflammatory airway diseases.

Endotoxin and hay dust instillation studies presented thus far in this thesis have examined animals at a single time point, either 48 or 72 hours post exposure. As the mucous layer in the airways is highly heterogeneous, I speculated that the production and secretion of airway mucus in inflammatory

conditions may be a dynamic event, with qualitative differences in composition evident at different time points of the inflammatory process.

I therefore hypothesized that the SLeX tetrasaccharide is a component of the airway mucous blanket and that levels of SLeX would increase in rats exposed to bacterial endotoxin. Additionally, I hypothesized that there are temporal differences in the qualitative composition of the airway mucus layer during airway inflammation. The study presented in Chapter 6 tests these hypotheses, and offers speculations regarding the functional significance of SLeX presence in the airways during inflammatory disease.

Abstract

I hypothesized that the presence of sialyl Lewis X tetrasaccharide molecules increases in airway mucus during inflammation, and that there are temporal differences in airway mucin production during inflammatory airway disease. To test these hypotheses, F344 rats were exposed to endotoxin, sacrificed at 6, 24, 48 and 72 hours post exposure, and bronchoalveolar lavage fluid (BALF) was assayed for multiple mucin glycoprotein markers. Bronchoalveolar lavage fluid levels of the sialyl Lewis x (SLeX) tetrasaccharide were measured by enzyme-linked immunosorbent assay, and levels were significantly elevated over saline controls at 6 hours. High molecular weight cut-off dialysis and sodium periodate incubation indicated the SLeX epitope was located on a high molecular weight O-linked glycoprotein characteristic of mucins. Levels of the membrane-associated mucin MUC 1 were also significantly increased in BALF at 6 hours. Levels of the goblet cell-associated molecules MUC5AC and α – 1,2 linked fucose were not significantly increased in treated animals at 6 hours, but were elevated by 24 hours and remained elevated at 72 hours. These results demonstrated *1)* that the SLeX tetrasaccharide is a component of airway mucus in F344 rats, and levels of SLeX increase following endotoxin exposure; and *2)* that there are temporal differences in the production and/or release of specific mucins during the inflammatory process that follows endotoxin exposure.

Introduction

The mucus blanket that overlies the airway epithelium is composed of a liquid sol layer that surrounds the cilia and a more viscous gel layer above the sol (Wanner, et al., 1996). Mucins are high molecular weight glycoproteins that impart viscoelastic properties to the gel layer. They are composed of a core protein (the mucin apoprotein), coded for by one of a number of MUC genes, to which numerous linear and branching oligosaccharide side chains are attached by means of specific O-glycosidic linkages (Rose, 1992; Roussel, et al., 1996; Wanner, et al., 1996). A number of MUC genes are expressed in airway epithelium, including the secreted mucin MUC5AC and the membrane-bound mucin MUC1 (Wills-Karp, 2000). Secreted mucins, produced by mucous goblet cells, form the bulk of the mucus layer. Transmembrane mucins such as MUC 1 may serve as components of the mucus blanket, however, being released into the gel layer via proteolytic cleavage from cell surfaces (Gendler, 2001).

The mucus layer has long been recognized as an important defensive structure, serving as a protective layer on mucosal surfaces to trap foreign substances and facilitate their removal. The mucus blanket can be thought of as more than a non-specific adhesive layer however, as mucin oligosaccharide side chains can impart specific functions. Functional aspects of mucin oligosaccharides can be well-illustrated by interactions between microorganisms and mucus, as the peripheral regions of mucin oligosaccharide chains can serve as receptors for common bacterial pathogens (Gaillard and Plotkowski, 1996).

Mucin oligosaccharides are composed of several different hexose sugars, including fucose and N-acetyl-neuraminic acid (sialic acid). Both of these sugars are necessary subcomponents of the sialyl Lewis X (SLeX) tetrasaccharide. The SLeX unit is an important binding structure, functioning as an adhesion site for L-selectins expressed by leukocytes, and serving as a docking site for bacterial adhesion molecules, including surface adhesins expressed by *Pseudomonas aeruginosa* (Hooper, et al, 1996; Sharfman, et al, 2001). The fucose component of the SLeX structure is attached by a 1,3 glycosidic linkage, where the #1 carbon of the fucose molecule is linked to the #3 carbon of the preceding hexose sugar.

Bacterial endotoxin has been shown to increase the presence of α-1,2 linked fucose in the airways of F344 rats, and that fucose of this linkage is associated with mucins produced by secretory cells (Chapter 4). Endotoxin has additionally been shown to cause a pronounced neutrophilic airway inflammation and leads to increased mucus production (Gordon et al., 1996; Harkema and Hotchkiss, 1991; Thorn, 2001; Steiger, et al., 1995; Yanagihara, 2001). These studies have not quantified different specific markers of airway mucus production at a variety of time points after endotoxin exposures, however.

This study was designed to test the hypotheses that *1)* the SLeX tetrasaccharide is a component of the airway mucous blanket and that levels of SLeX would increase in rats exposed to bacterial endotoxin; and *2)* there are temporal differences in release of mucin glycoproteins into the airway mucus layer during airway inflammation.

Materials and Methods

Animals. Forty-eight male F344 rats (Harlan Sprague-Dawley, Indianapolis, IN), 10-12 weeks of age, were randomly assigned to one of 8 experimental groups (n = 6/group). Rats were used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University. Animals were housed two per cage in polycarbonate boxes, on Cell-Sorb Plus bedding (A&W Products, Cincinnati, OH), covered with filter lids, and had free access to tap water and food (Tek Lad 1640, Harlan Sprague Dawley, Indianapolis, IN). Room lights were set on a 12-h light/dark cycle beginning at 6:00 am, and temperature and relative humidity were maintained between 21-24 °C and 40-55%, respectively.

Protocol. Treated rats were intranasally instilled with a single dose of 20 µg *Pseudomonas aeruginosa*-derived endotoxin in 300 µl of saline (150 µl/naris), and sacrificed at 6, 24, 48 and 72 hours post instillation (n = 6 for each time point). Chapter 4 of this thesis showed that this dose of endotoxin causes a robust mucous metaplasia in airways of F344 rats. Control group rats were intranasally instilled with an equal volume of saline, and sacrificed at identical time points.

Animals were intranasally instilled with endotoxin by methods described in Harkema and Hotchkiss, 1991. Briefly, rats were anesthetized with 4% halothane in oxygen, and 150 µl of endotoxin (lipoplysaccharide from *Pseudomonas aeruginosa*, Sigma Chemical Co., St. Louis, MO) in pyrogen-free

saline was instilled into each nasal passage (total dose 20 μg). Control rats were instilled with an equivalent volume of pyrogen-free saline.

Necropsy and tissue preparation. Rats were anesthetized with sodium pentobarbital, then killed by exsanguination via the abdominal aorta. The chest cavity was opened and trachea exposed. The trachea was incised just below the larynx, and a cannula (teat infusion cannula, Jorgensen Laboratories, Inc., Loveland, CO) was inserted and tied tightly in place with suture material. Lungs were removed, and the left primary bronchus was gently clamped off with an alligator clamp. The right lung was lavaged with 10ml of sterile saline, With the exception of aliqouts taken for total and differential cell counts, samples were centrifuged at 1500 rpm for 10 minutes to remove cells. Supernatant for immunochemistry was collected and stored at -20°C. After lavage, the lung was perfused with glutaraldehyde/paraformaldehyde under 30 cm pressure, and processed for light microscopy.

B. Sialyl Lewis X immunohistochemistry

Lung tissues sections containing fifth generation airways were used for immunohistochemistry. Tissues from rats instilled with both saline and endotoxin were used. Fifth generation airway sections were embedded in paraffin and mounted on Probe-on® plus glass slides. Slides were deparaffinized with xylene, hydrated with decreasing concentrations of ethanol in water, then washed with PBS. Endogenous peroxidases were blocked with a mix of 3% hydrogen peroxide in deionized water, then rinsed with PBS. Slides were non-specifically blocked with a mixture of 2% horse serum in PBS, rinsed

with PBS, and incubated overnight at room temperature with primary antibody [mouse anti-SLeX, KM93 clone] (Chemicon, Temecula, CA) diluted 1:40 in PBS. Slides were washed in PBS and incubated with biotinylated anti-mouse secondary antibody (VECTASTAIN Elite ABC kit, Vector laboratories, Burlingame, CA) for 30 minutes at 37°C followed by a wash with PBS. ABC-AP reagent (VECTASTAIN Elite kit) was applied for 30 minutes and slides incubated at 37°C. Slides were washed in PBS, and Vector Red® alkaline phosphatase substrate (Vector laboratories, Burlingame, CA) was applied for 20 min in the dark at room temperature. After a final wash in PBS and rinse in deionized water, slides were dehydrated in ethanol, cleared with xylene, and coverslips were applied.

C. Analysis of bronchoalveolar lavage fluid

1) Cell counts. Total and differential white blood cell counts were performed on all bronchoalveolar lavage fluid (BALF) samples. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differential cell counts, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quick (Baxter Health Care, Dade Division, Miami FL), and 200 cells were examined to obtain a differential count. Following removal of aliquots for the purpose of cell counts, BALF was centrifuged at 1500 rpm for 12 minutes to remove cells, and supernatant was collected.

2) Enzyme linked immunosorbent assay (ELISA) for elastase, sialyl Lewis X, MUC1 and MUC5AC.

a) *Primary antibodies:* monoclonal antibody directed against neutrophil elastase (rabbit anti-human) was obtained from Calbiochem (La Jolla, CA), monoclonal antibodies against the sialyl Lewis X antigen (KM93 clone) and MUC1 were obtained from Chemicon (Temecula, CA). Anti-MUC5AC was obtained from Neomarkers (Fremont, CA). Anti-elastase antibody was diluted 1:1000 in sterile PBS. Twenty µl of 100µg/ml Anti-SLeX antibody was added to 10 ml of sterile PBS. Anti-MUC1 was diluted 1:200 in PBS. Anti-MUC5AC was diluted 1:500 in PBS.

b) *ELISA procedure:* one hundred µl of BALF supernatant from each animal was applied in triplicate to wells of Immulon-4 HBX 96-well plates (Dynex Technologies, Chantilly, VA) and incubated overnight at 40°C to thoroughly fix protein to the bottom of wells. One hundred µl of 6.5% casein blocking reagent (Roche Diagnostics, Indianapolis, IN) was then added to each well to cover potential irrelevant binding sites and incubated at 37°C for 30 minutes. Plates were washed four times with 1X automation buffer (Biomedia Corp., Foster City, CA), and then 100 µl of primary antibody in appropriate dilutions was added to the wells, followed by incubation at 37°C for 90 minutes. Washes with automation buffer were repeated, and 100 µl of biotinylated secondary antibody (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) was added to each well. Secondary antibody was diluted 1:200 in 1X automation buffer. For SLeX, MUC1 and MUC5AC ELISA, 200 µl of rat serum was added to 10 ml of

secondary antibody to eliminate any non-specific binding. The plates were incubated at 37°C for 30 minutes, then washed four times with automation buffer. One hundred microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes with automation buffer, 100 ml of QuantaBlu® fluorogenic peroxidase substrate (Pirece, Rockford, IL) were added to each well, and the plates were read at 3-min intervals for 21 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA) which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). Excitation and emission wavelengths were 318 nm and 410 nm, respectively. The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and reported as Vmax units per second. Vmax units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

3) Enzyme-linked lectin assay for α – 1,2 fucose

Samples were plated, blocked and washed as for ELISAs. Biotinylated *Ulex europaeus I* (UEAI) lectin (Vector Laboratories, Burlingame,CA), at .5 µg/ml in diluent as recommended by the supplier, was then applied for 1 hour at 37°C. UEA1 is specific for α – 1,2 fucose, a mucin-associated deoxyhexose sugar. Plates were then treated with ABC, QuantaBlu® and read as for ELISAs.

4) Localization of SLeX to mucin-like molecules: High molecular weight cutoff dialysis and sodium periodate incubation of lavage fluid was performed to

confirm that the SLeX-immunoreactive molecule was of a molecular weight characteristic of mucins, and was associated with an O-linked glycoprotein.

High molecular weight cutoff dialysis: fractions of rat BALF samples were pooled and dialyzed for 6 hours at 4°C against distilled milliQ water in 300,000 molecular weight cutoff (MWCO) dialysis tubing (Spectra/Por, Spectrum medical laboratories, Houston, TX). SLeX ELISA as described above was performed on pooled samples before and after dialysis.

Sodium periodate incubation: sodium periodate oxidation is a common procedure to cleave O-glycosidic linkages from glycoproteins. Two individual rat lavage samples were dried to the bottom of Immulon-4 HBX 96 well plates and blocked as described above. One hundred microliters of 100 mM sodium periodate in 50 mM sodium acetate were added to the appropriate wells. For comparison, untreated control wells (sample only) and wells with sample, sodium periodate, and glucose were utilized [glucose blocks the action of sodium periodate]. For wells with glucose added, 100 µl of 100 mM sodium periodate-0.1 M glucose in 50 mM sodium acetate were used. Plates were then incubated at room temperature overnight (in the dark). After overnight incubation, sodium periodate and sodium periodate-glucose wells were incubated for 30 min at room temperature with 10 mM sodium borohydride (100 µl/well) to prevent nonspecific crosslinking of antigen to antibody by Schiff base formation. The plates were washed four times with 1X automation buffer, and then ELISA was performed as described above.

Statistical analysis. Linear regressions and correlation analyses were performed using SigmaStat® statistical software (Jandel Scientific Software, San Rafael, CA). For direct comparisons of one factor between two groups, means of groups were compared with Student's *t*-test. Multiple comparisons between groups were performed with one-way ANOVA. Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

Results

SLeX Immunohistochemistry

The SLeX primary antibody bound strongly to apical surfaces of airway epithelial cells. Antibody binding did not take place in the interiors of secretory cells. Figure 1 is a photomicrograph of SLeX binding activity on airway epithelium.

BALF Cytology

Total cell counts and number of neutrophils per milliliter of lavage fluid were significantly elevated in endotoxin-treated rats over respective saline control groups at all time points ($P < 0.05$). Mean number of neutrophils/ml for each group and time are shown in Figure 2.

BALF Elastase Levels

Bronchoalveolar lavage fluid elastase levels in endotoxin-treated rats were significantly elevated at all time points over respective saline control values [$P < 0.05$] (Figure 3). No significant time differences existed in endotoxin-treated rats.

BALF SLeX levels

Bronchoalveolar lavage fluid SLeX levels in endotoxin-treated rats were significantly elevated at all time points over respective saline control values [$P < 0.05$] (Figure 4). No significant time differences existed in endotoxin-treated rats.

BALF MUC1 levels

Soluble MUC1 levels in bronchoalveolar lavage fluid were significantly elevated over respective saline control at 6 and 24 hours [$P = 0.003$ and 0.024, respectively] (Figure 5). Increases noted over saline controls at all other time points were not statistically significant. No significant time differences existed in endotoxin-treated rats.

BALF MUC5AC levels

MUC5AC levels in BALF were significantly elevated over respective saline controls at 24, 48 and 72 hours (Figure 6). MUC5AC levels of endotoxin-treated rats at 24 hours were significantly elevated over endotoxin-treated rats at 6, 48 and 72 hours.

BALF α-1,2 fucose levels in rats

α-1,2 fucose levels in BALF were significantly elevated over respective saline controls at 24, 48 and 72 hours (Figure 7). α-1,2 fucose levels in endotoxin-treated rats at 24 and 48 hours were significantly elevated over endotoxin-treated rats at 6 hours. Lectin histochemistry described in Chapter 4 has localized fucose of this linkage to secretory (goblet) cells (Figure 8).

Elastase and SLeX correlations

Linear regression analysis of BALF elastase levels (independent variable) and BALF SLeX (dependent variable) is shown in Figure 9. Statistical analysis of the correlation of BALF elastase levels to BALF SLeX levels in all animals resulted in a correlation coefficient (R value) of .717 (regression P value < 0.001)

Elastase and MUC1 correlations in rats

An R value of .657 (regression P value < 0.001) was determined in linear regression analysis of BALF elastase and BALF MUC1 levels.

SLeX and MUC1 correlations in rats

An R value of .710 (regression P value < 0.001) was determined in linear regression analysis of BALF SLeX and BALF MUC1 levels.

High MWCO dialysis.

Dialysis did not result in loss of signal detected by ELISA, indicating that target molecules were greater in size than 300,000 daltons. Pre-dialysis Vmax units/sec values of pooled rat BALF was 6. Post-dialysis Vmax units/sec values were 6.5.

Sodium periodate incubation

Sodium periodate oxidation resulted in decrease in signal detected by ELISA, indicating that epitopes for the SLeX antibody were associated with O-linked glycoproteins. Pre-incubation Vmax units/sec values for two rats were 16 and 15. Post-incubation values were 7 and 8.

Discussion

This study reports *1)* the identification of the sialyl Lewis X (SLeX) tetrasaccharide, associated with high molecular weight O-linked glycoproteins characteristic of mucins, in the airways of F344 rats; *2)* increased SLeX levels in bronchoalveolar lavage fluid of rats following exposure to bacterial endotoxin; and *3)* temporal differences in entry of different mucin glycoproteins into the airway mucus layer of F344 rats with neutrophilic airways inflammation following endotoxin exposure.

The sialyl Lewis X epitope was identified in BALF using a monoclonal primary antibody directed against SLeX. High molecular weight cut-off dialysis did not result in passage of molecules bearing the SLeX epitope through 300,000 molecular weight tubing. Sodium periodate incubation of BALF to cleave O-linked oligosaccharides resulted in loss of antibody reactivity. These results indicated that SLeX detected in BALF was associated with high molecular weight O-linked molecules, both characteristic features of mucin glycoproteins.

At 6 hours post exposure to 20 μg endotoxin, increased levels of SLeX and the cell surface-associated mucin MUC1 were present in BALF as compared to saline controls, whereas no statistically significant differences existed with respect to MUC5AC and α-1,2 fucose. Strong correlations existed between SLeX and MUC1. Strong correlations were also noted between elastase and both SLeX and MUC1. Additionally, immunohistochemistry localized SLeX to the surfaces of airway epithelial cells, and not to the interiors of mucous goblet cells. MUC5AC and α-1,2 fucose levels, goblet cell-associated molecules and markers

of secreted mucus production, were not elevated in BALF at 6 hours post exposure. Significant elevations of these molecules over saline controls were present by 24 hours, however, and levels remained elevated at 72 hours.

The SLeX antigen has previously been reported to be a component of MUC1 carbohydrate side chains (Ho, et al., 1995; Hey and Aplin, 1996; Gendler, 2000). Neutrophil elastase has been demonstrated to cleave MUC1 molecules from cell surfaces (Lillehoj, et al., 2001), and to increase MUC5AC production (Fischer and Voynow, 2000; Martin, et al., 2000). Additionally, although neutrophil elastase is present in intracytoplasmic granules in resting neutrophils, it has been demonstrated that activated neutrophils express elastase on cell surfaces, and this surface expression results in cleavage of elastase-specific substrates (Owen, et al., 1997; Nadel et al., 1999). This information, together with the findings described in this report, suggest the following scenario: the presence of bacterial endotoxin in the airways, a signal to the body of Gram negative bacterial infection, results in a powerful neutrophilic inflammatory response. Neutrophils migrating through the airway epithelial layer come in close contact with epithelial cells. Production of the enzyme elastase, expressed on the surfaces of activated neutrophils, results in proteolytic cleavage of cell surface-associated molecules such as MUC1 that possess the SLeX epitope. Glycoproteins which possess SLeX therefore enter the mucus blanket as an early event in host response to endotoxin. A later increase (at 24 hours) in production and release of secreted mucins from goblet cells, indicated by increased levels of α-1,2 fucose and MUC5AC, stimulated at least in part by neutrophil elastase, then occurs. This

stimulation of production and release of secreted mucins may be instigated during the early phases of inflammation, such as during neutrophil/goblet cell contact during neutrophil migration, but a time lag takes place between initial stimulation and gene transcription and translation.

The early entry of MUC1 into the mucus blanket supports the concept that transmembrane MUC1 molecules form a pre-constructed and readily releasable pool of functional mucins. This method of quickly adding a specific mucin type to the mucus layer, by neutrophil-directed proteolytic cleavage of pre-existing surface mucins, may be more efficient than construction, packaging and release of secreted mucins from secretory cells.

Entry of mucins that possess the SLeX epitope into airway lumens may serve an important functional role. The presence of SLeX in the airway mucus layer during the airway inflammatory process that follows endotoxin exposure may be to specifically bind adhesion molecules present on the surfaces of Gram (-) bacteria, facilitating their entrapment and removal by the mucociliary escalator. SLeX-bearing molecules in the mucous blanket may also bind with selectins expressed on leukocytes in the airways. Selectin/selectin-ligand interactions are relatively weak, as evidenced by their involvement in the transient adhesive processes of initial leukocyte margination in the vasculature (Wagner and Roth, 2001), and rather than serving to fix leukocytes to the mucus layer, these interactions may be to aid leukocyte movement through the airway mucus during inflammation.

Figure 26. Anti-SLeX immunohistochemistry on rat airway epithelial section. Immunohistochemistry, performed as described in Methods, on 5th generation rat airway section. Primary antibody was directed against the SLeX epitope. Staining for SLeX was present on apical surfaces of airway epithelial cells. Some anti-SLeX binding also occurred in the periciliary region. No binding occurred in the interiors of secretory cells. A = SLeX. B = basement membrane. Top figure is negative control (no primary antibody).

A
B
20 micron

Figure 27. Neutrophil numbers in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 µg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). Total and differential cell counts were performed on BALF as described in Methods, and neutrophil numbers per ml BALF were calculated. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid neutrophil numbers were significantly elevated in endotoxin-exposed animals over respective saline controls at all time points ($P < 0.05$).

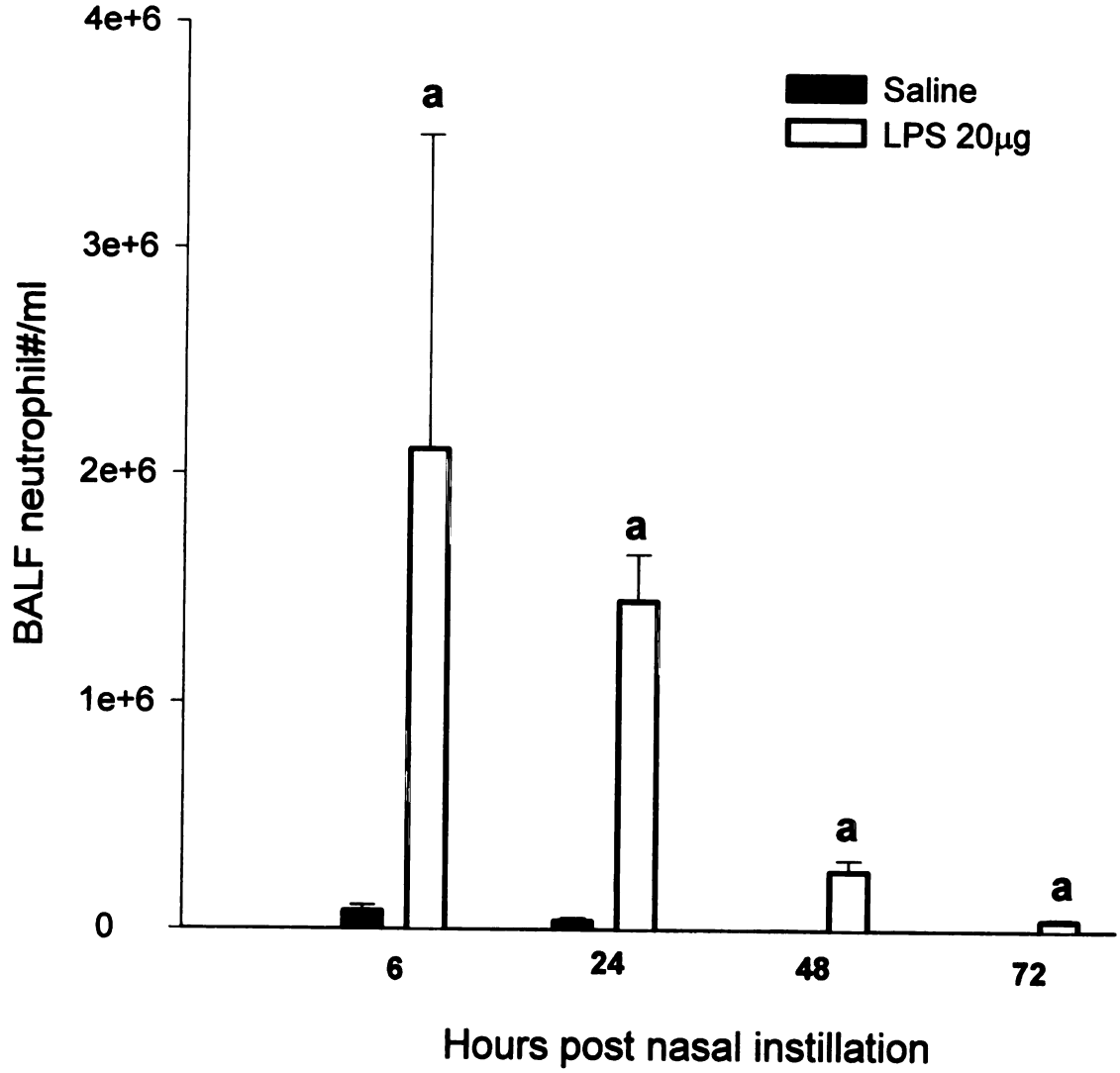

4e+6
3e+6
2e+6
1e+6
0
BALF neutrophil#/ml
Saline
LPS 20μg
a
a
a
a
6
24
48
72
Hours post nasal instillation

Figure 28. Elastase levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 μg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). Elastase level in BALF was measured by enzyme-linked immunosorbent assay as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid elastase levels were significantly elevated in endotoxin-exposed animals over respective saline controls at all time points ($P < 0.05$).

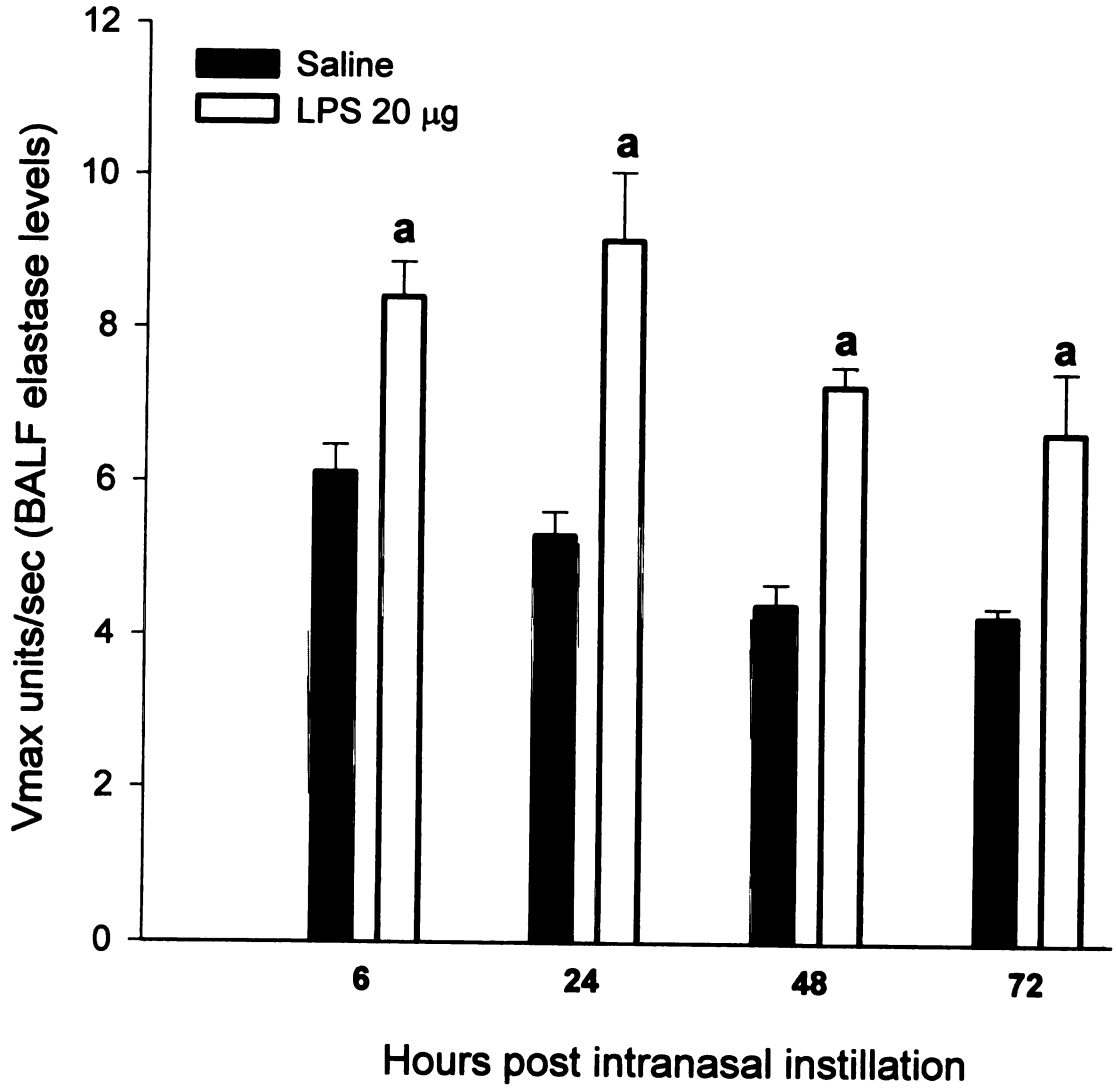

12
10
8
6
4
2
0
Saline
LPS 20 μg
a
a
a
a
Vmax units/sec (BALF elastase levels)
6
24
48
72
Hours post intranasal instillation

Figure 29. SLeX levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 μg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). SLeX level in BALF was measured by enzyme-linked immunosorbent assay as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid SLeX levels were significantly elevated in endotoxin-exposed animals over respective saline controls at all time points ($P < 0.05$).

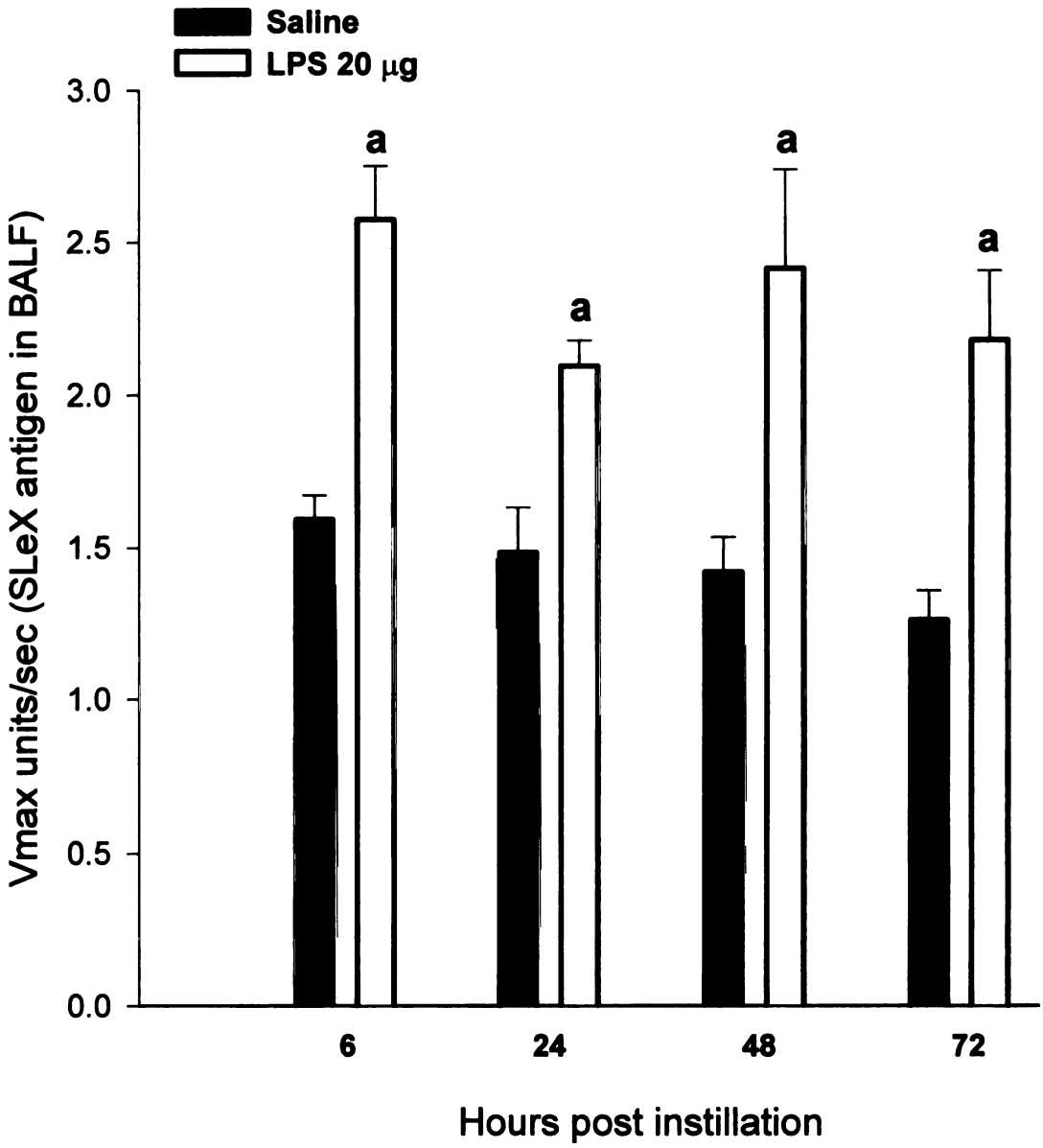

Saline
LPS 20 μg
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Vmax units/sec (SLeX antigen in BALF)
a
a
a
a
6
24
48
72
Hours post instillation

Figure 30. MUC1 levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 µg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). MUC1 level in BALF was measured by enzyme-linked immunosorbent assay as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid MUC1 levels were significantly elevated in endotoxin-exposed animals over respective saline controls at 6 ($P = 0.003$) and 24 hours ($P = 0.024$) post-exposure.

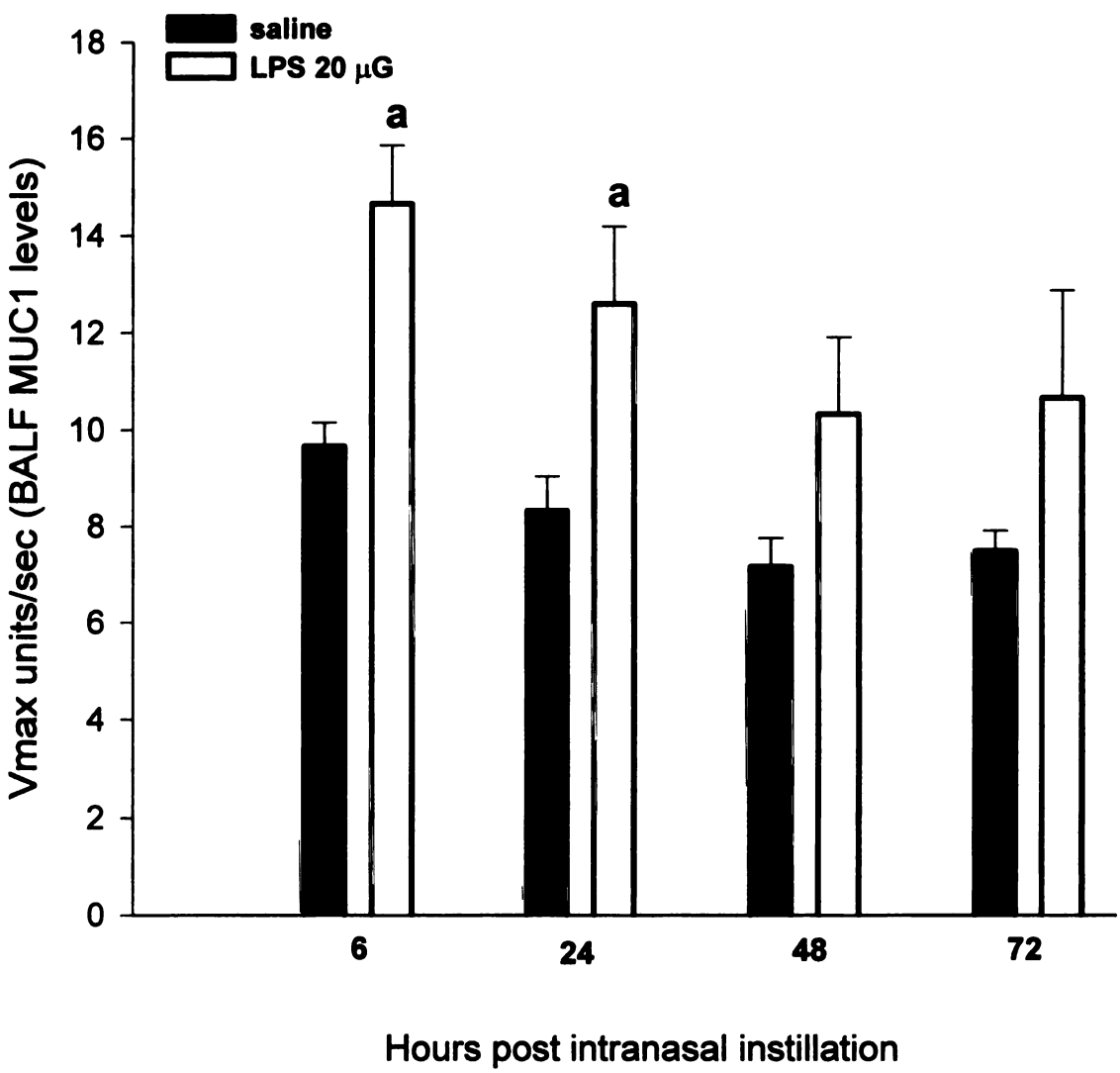

saline
LPS 20 μG
a
a
18
16
14
12
10
8
6
4
2
0
Vmax units/sec (BALF MUC1 levels)
6
24
48
72
Hours post intranasal instillation

Figure 31. MUC5AC levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 μg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). MUC5AC level in BALF was measured by enzyme-linked immunosorbent assay as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid MUC5AC levels were significantly elevated in endotoxin-exposed animals over respective saline controls at 24 ($P < 0.001$), 48 ($P = 0.004$) and 72 hours ($P = 0.038$) post-exposure.

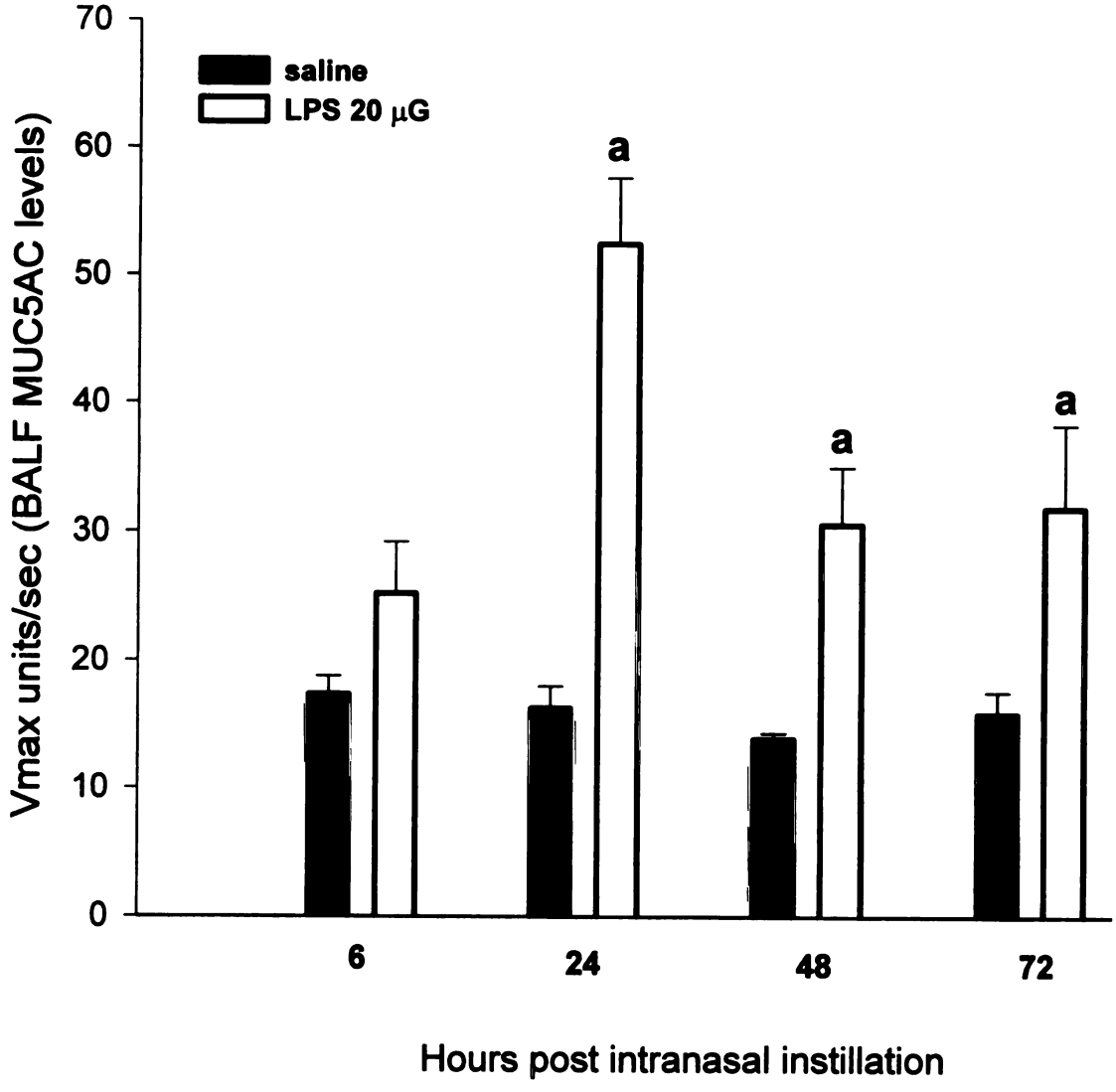

70
60
50
40
30
20
10
0
saline
LPS 20 μG
a
a
a
Vmax units/sec (BALF MUC5AC levels)
6
24
48
72
Hours post intranasal instillation

Figure 32. α-1,2 fucose levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 μg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). α-1,2 fucose level in BALF was measured by enzyme-linked lectin assay as described in Methods. Results are expressed as means ± SEM. Bronchoalveolar lavage fluid α-1,2 fucose levels were significantly elevated in endotoxin-exposed animals over respective saline controls at 24 ($P < 0.001$), 48 ($P < 0.001$), and 72 hours ($P = 0.032$) post-exposure.

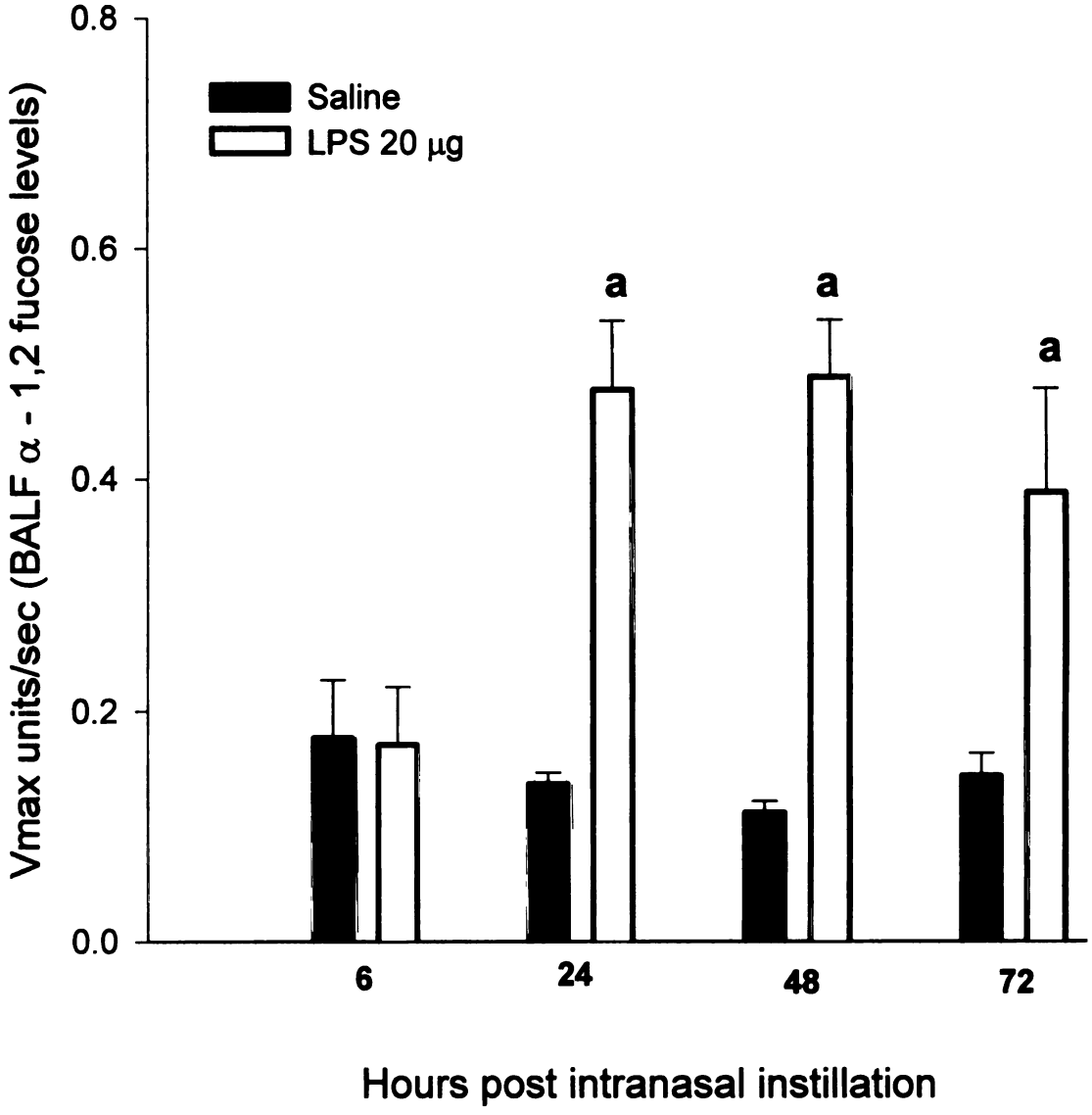
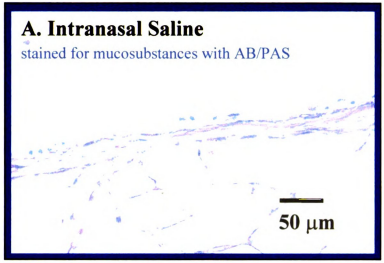
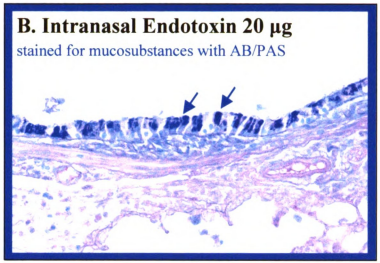
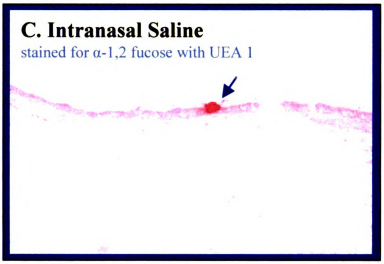

0.8
0.6
0.4
0.2
0.0
Saline
LPS 20 μg
Vmax units/sec (BALF α - 1,2 fucose levels)
a
a
a
6
24
48
72
Hours post intranasal instillation

Figure 33. UEA1 lectin histochemistry on rat airway epithelial section. F344 rats intranasally-instilled with either saline or 20 μg endotoxin. Intraepithelial acidic and neutral mucosubstances, stained by alcian blue/periodic acid Schiff's sequence (AB/PAS), are shown in C and D. Mucins possessing α-1,2 fucose, stained by UEA1 lectin, are shown in C and D. Fucose-bearing mucins are localized to the interiors of secretory cells.


A. Intranasal Saline
stained for mucosubstances with AB/PAS
50 μm


B. Intranasal Endotoxin 20 μg
stained for mucosubstances with AB/PAS


C. Intranasal Saline
stained for α-1,2 fucose with UEA 1

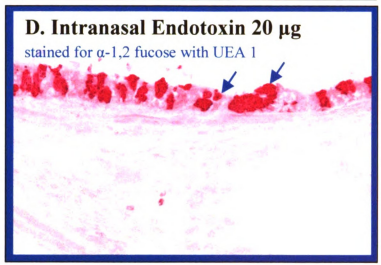
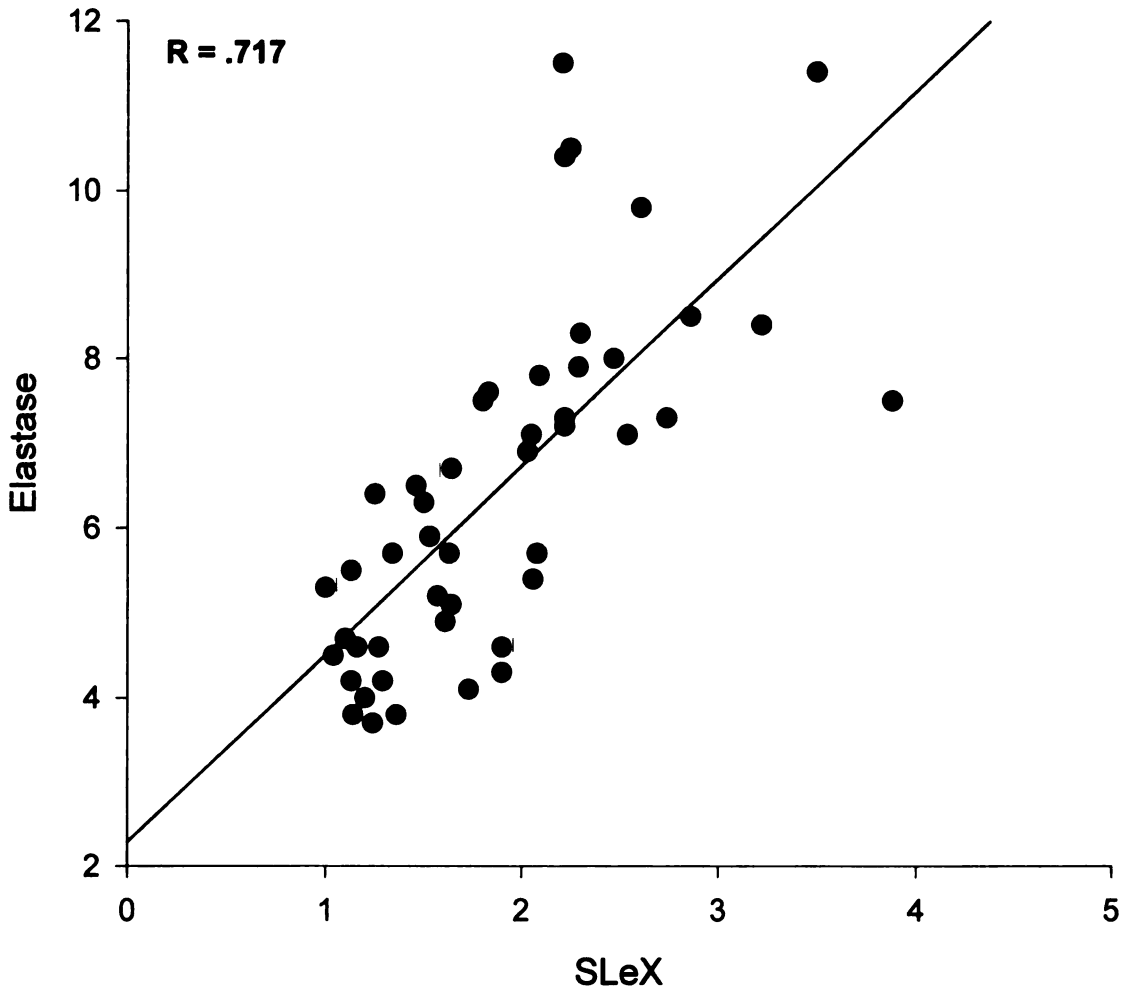

D. Intranasal Endotoxin 20 μg
stained for α-1,2 fucose with UEA 1

Figure 34. Positive correlation between elastase and SLeX levels in bronchoalveolar lavage fluid (BALF) of F344 rats exposed to saline and bacterial endotoxin. Rats were intranasally instilled with sterile saline or 20 μg total dose of endotoxin, and sacrificed at 6, 24, 48 and 72 hours post-exposure (n = 6/group). Elastase and SLeX levels in BALF were measured by enzyme-linked immunosorbent assay as described in Methods. Statistical analysis of the correlation of BALF elastase levels to BALF SLeX levels in all animals resulted in a correlation coefficient (R value) of .717. An R value of 0 indicates no relationship between 2 variable, while an R value of 1 indicates a perfect positive relationship between 2 variables, with both always increasing together. R values closer to 1 indicate increasing positive relationships between 2 variables.


R = .717
Elastase
SLeX
12
10
8
6
4
2
0
1
2
3
4
5

Chapter 7:
The amount of sialyl Lewis X-bearing mucin molecules in bronchoalveolar lavage fluid, originating from cell surfaces, is correlated with elastase levels in airways of horses

ABSTRACT

Mucin molecules that possess the Sialyl Lewis X (SLeX) tetrasaccharide have been demonstrated to be present in bronchoalveolar lavage fluid of rats exposed to endotoxin, and I have shown that levels of SLeX-bearing mucins are correlated with inflammation. I therefore hypothesized that mucins possessing the SLeX epitope would also be present in the airways of horses, and that levels would be associated with airway inflammation. Recurrent airway obstruction (RAO) of horses is an asthmalike condition characterized by neutrophilic inflammation, airway hyperreactivity and increased mucus production, with acute episodes instigated by housing in dust-laden indoor (barn or stable) environments. Bronchoalveolar lavage was performed on control and (RAO)-affected horses both at pasture and after housing in an exacerbating indoor environment. The SLeX epitope was determined to be associated with surfaces of ciliated epithelial cells, and levels of SLeX-bearing mucins were correlated with elastase levels. These findings suggest that proteinases released during airway inflammation can cleave SLeX-possessing molecules from cell surfaces, and these molecules enter the airway lumens to become components of the mucus blanket.

Introduction

A correlation between airway inflammation in rats and the presence of mucins that possess the sialyl Lewis X (SLeX) tetrasaccharide has been demonstrated as part of this thesis project (Chapter 6). The SLeX structure is present across animal species, and is a functional unit that has specific intercellular binding activity. I therefore hypothesized that the SLeX structure would be present in airways of horses, and that levels in the airways would correlate with the degree of airway inflammation. This protocol tests the hypothesis that SLeX-bearing mucin molecules are present in airway lining fluids of horses, and that levels in airway lumens increase with increasing inflammation in pulmonary airways.

Methods

Animals. Control horses and recurrent airway obstruction (RAO)-affected horses from herds maintained at Michigan State University (East Lansing, MI) were used. To be classified as an RAO-affected horse, animals must meet the following criteria: *1)* clinical signs of RAO, including cough, increased respiratory sounds, and increased expiratory abdominal effort, must be observed during housing and must abate when the horses are kept at pasture where there is no exposure to dust found in hay and stables; *2)* horses must develop changes in lung function compatible with airway obstruction [maximal change in pleural pressure (ΔPpl_{max}) during tidal breathing > 15 cm H_2O] when stabled and fed hay; and *3)* airway obstruction must be reversible, in part, with atropine. Control horses used in this study had no known history of chronic airway disease and did

not display any clinical signs characteristic of obstructive airway disease when stabled.

Protocol. Bronchoalveolar lavage fluid was obtained from RAO horses and control horses (n = 4/group, 8 total) before housing in a barn environment (time 0), and after indoor housing for 48 hours. After housing, horses were returned to pasture for at least three weeks, and additional BALF samples were then obtained from the same horses both before and after housing. This resulted in a total of 16 time 0 lavages and 16 48 hour lavages, performed on 8 different horses.

Bronchoalveolar lavage. Bronchoalveolar lavage was collected by means of a 3-m tube wedged in a peripheral bronchus. Three 100 ml aliquots of sterile phosphate buffered saline (PBS) were infused and recovered by suction after each 100-ml infusion, and samples were pooled. Samples were treated with 0.1% dithiothreitol [DTT] (Sigma, St. Louis, MO) and shaken for 30 minutes to disperse mucins. With the exception of aliqouts taken for total and differential cell counts, samples were centrifuged at 1500 rpm for 10 minutes to remove cells. Supernatant for immunochemistry and lectin chemistry was collected and stored at -20°C.

Sialyl Lewis X immunohistochemistry

Tissue sections of tracheal mucosa from control and RAO-affected horses had been collected from earlier necropsies and stored. Tissues were embedded in paraffin and mounted on Probe-on® plus glass slides. Slides were deparaffinized with xylene and hydrated with decreasing concentrations of

ethanol and water, then washed with PBS. Endogenous peroxidases were blocked with a mix of 3% hydrogen peroxide in deionized water, then rinsed with PBS. Slides were non-specifically blocked with a mixture of 2% horse serum in PBS, rinsed with PBS, and incubated overnight at room temperature with primary antibody [mouse anti-SLeX, KM93 clone] (Chemicon, Temecula, CA) diluted 1:40 in PBS. Slides were washed in PBS and incubated with biotinylated anti-mouse secondary antibody (VECTASTAIN Elite ABC kit, Vector laboratories, Burlingame, CA) for 30 minutes at 37°C followed by a wash with PBS. ABC reagent (VECTASTAIN Elite kit) was applied for 30 minutes and slides incubated at 37°C. Slides were washed in PBS, and a mix of DAB/NiCL2 + 30% hydrogen peroxide was applied for 30 min at 37°C. After a final wash in PBS and rinse in deionized water, slides were dehydrated in ethanol, cleared with xylene, and coverslips were applied.

Analysis of bronchoalveolar lavage fluid

1) Cell counts. Total and differential white blood cell counts were performed on all bronchoalveolar lavage fluid (BALF) samples. Total cell counts were performed by use of a hemacytometer within 2 h of collection. For differentials, slides prepared with a Cytospin 3 centrifuge (Shandon, Pittsburgh, PA) were stained with Diff-Quick (Baxter Health Care, Dade Division, Miami FL), and 200 cells were examined to obtain a differential count. Following removal of aliquots for the purpose of cell counts, BALF was centrifuged at 1500 rpm for 12 minutes to remove cells, and supernatant was collected.

2) Enzyme linked immunosorbent assay (ELISA) for neutrophil elastase and sialyl Lewis X.

a) *Primary antibodies:* monoclonal antibody directed against neutrophil elastase (rabbit anti-human) was obtained from Calbiochem (La Jolla, CA), and monoclonal antibody against the sialyl Lewis X antigen (KM93 clone) was obtained from Chemicon (Temecula, CA). Anti-elastase antibody was diluted 1:1000 in sterile PBS. Twenty µl of 100µg/ml Anti-SLeX antibody was added to 10 ml of sterile PBS.

b) *ELISA procedure:* one hundred µl of BALF supernatant from each animal was applied in triplicate to wells of Immulon-4 HBX 96-well plates (Dynex Technologies, Chantilly, VA) and incubated overnight at 40°C to thoroughly fix protein to the bottom of wells. One hundred µl of 6.5% casein blocking reagent (Roche Diagnostics, Indianapolis, IN) was then added to each well to cover potential irrelevant binding sites and incubated at 37°C for 30 minutes. Plates were washed four times with 1X automation buffer (Biomedia Corp., Foster City, CA), and then 100 µl of primary antibody in appropriate dilutions was added to the wells, followed by incubation at 37°C for 90 minutes. Washes with automation buffer were repeated, and 100 µl of biotinylated secondary antibody (VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) was added to each well. Secondary antibody was diluted 1:200 in 1X automation buffer. The plates were incubated at 37°C for 30 minutes, then washed four times with automation buffer. One hundred microliters of VECTASTAIN ABC reagent were added to each well followed by 30 min of incubation at 37°C. After four washes

with automation buffer, 100 ml of QuantaBlu® fluorogenic peroxidase substrate (Pirece, Rockford, IL) were added to each well, and the plates were read at 3-min intervals for 21 min (kinetic runs) with a SpectraMax Gemini fluorescent plate reader (Molecular Devices, Sunnyvale, CA) which detected the amount of fluorescence emitted from the reaction (in relative fluorescence units). Excitation and emission wavelengths were 318 nm and 410 nm, respectively. The maximum slope of the kinetic display of relative fluorescence units versus time was calculated with SOFTmax PRO software (Molecular Devices, Sunnyvale, CA) and reported as Vmax units per second. Vmax units per second values were then used as end points for sample comparisons (fluorogenic assay), with higher values corresponding to increased presence of target molecule.

Localization of SLeX to mucin-like molecules

High molecular weight cutoff dialysis and sodium periodate incubation of lavage fluid was performed to confirm that the SLeX-immunoreactive molecule was of a molecular weight characteristic of mucins, and was associated with an O-linked glycoprotein.

High molecular weight cutoff dialysis: fractions of BALF samples were pooled and dialyzed for 6 hours at 4°C against distilled milliQ water in 100,000 and 300,000 molecular weight cutoff (MWCO) dialysis tubing (Spectra/Por, Spectrum medical laboratories, Houston, TX). SLeX ELISA as described above was performed on pooled samples before and after dialysis.

Sodium periodate incubation: sodium periodate oxidation is a common procedure to cleave O-glycosidic linkages from glycoproteins. Two individual

horse lavage samples were dried to the bottom of Immulon-4 HBX 96 well plates and blocked as described above. One hundred microliters of 100 mM sodium periodate in 50 mM sodium acetate were added to the appropriate wells. For comparison, untreated control wells (sample only) and wells with sample, sodium periodate, and glucose were utilized [glucose blocks the action of sodium periodate]. For wells with glucose added, 100 µl of 100 mM sodium periodate-0.1 M glucose in 50 mM sodium acetate were used. Plates were then incubated at room temperature overnight (in the dark). After overnight incubation, sodium periodate and sodium periodate-glucose wells were incubated for 30 min at room temperature with 10 mM sodium borohydride to prevent nonspecific crosslinking of antigen to antibody by Schiff base formation. The plates were washed four times with 1X automation buffer, and then ELISA was performed as described above.

Statistical analysis. Linear regressions and correlation analyses were performed using SigmaStat® statistical software (Jandel Scientific Software, San Rafael, CA). For correlation analysis, each bronchoalveolar lavage sample collected, regardless of group or time, was considered to be a separate, individual sample. Comparisons between groups, factoring group and time, were performed with two-way ANOVA (SigmaStat®). Tukey's test was used for post hoc analysis. $P < 0.05$ was considered significant.

Results

SLeX Immunohistochemistry

The SLeX primary antibody bound strongly to apical surfaces of ciliated cells in airway epithelium. Figure 1 is a photomicrograph of SLeX binding activity in horse airway epithelium.

BALF Cytology

Significant group and time effects for total cell counts and number of neutrophils per milliliter of BALF were evident for RAO-affected horses at 48 hours (P = 0.024 and 0.025, respectively). Means of neutrophils/ml for each group and time are shown in Figure 2.

BALF Elastase Levels

No statistically significant differences in mean elastase values in bronchoalveolar lavage fluid were detected between control or RAO-affected horses at any time point (Figure 3). Elastase levels in control horses were sporadically quite high (Table 1).

BALF SLeX levels

No statistically significant differences in mean SLeX values in bronchoalveolar lavage fluid were detected between control or RAO-affected horses at any time point (Figure 4).

Elastase and SLeX correlations

Linear regression analysis of BALF elastase levels (independent variable) and BALF SLeX (dependent variable) in all horses (control and RAO-affected) is shown in Figure 5. Statistical analysis of the correlation of BALF elastase levels

to BALF SLeX levels in all samples resulted in a correlation coefficient (R value) of .750 (regression P value < 0.001).

High MWCO dialysis.

Dialysis did not result in loss of signal detected by ELISA, indicating that target molecules were greater in size than 300,000 daltons. Pre-dialysis Vmax units/sec values of pooled BALF was 12. Post-dialysis Vmax units/sec values was 10.8.

Sodium periodate incubation

Sodium periodate oxidation resulted in decrease in signal detected by ELISA, indicating that epitopes for the SLeX antibody were associated with O-linked glycoproteins. Pre-incubation Vmax units/sec values for two horses were 32 and 35. Post-incubation values were 10 and 10.

Discussion

This study demonstrated that the sialyl Lewis X (SLeX) structure was present on cell surface-associated mucin-like molecules in horses, and that the level of this molecule in airway lumens was highly correlated with elastase levels in bronchoalveolar lavage fluid. The correlation of SLeX with elastase levels was irrespective of disease status, as individual horses from the control group, though having no clinical signs of recurrent airway obstruction, had episodes of greatly increased elastase levels in the airways during the course of this protocol. High elastase levels in BALF of control horses resulted in no significant differences in mean elastase levels between the RAO-affected and control groups. Episodes

of greatly increased elastase level were accompanied by an increased SLeX level in individual control horses, however. These results, coupled with similar findings in F344 rats exposed to endotoxin, suggest that it is an increased elastase presence in the airways, and not a specific disease, that leads to increased SLeX presence in the airway mucus blanket. As with rats (detailed in Chapter 6), I therefore hypothesize that the proteolytic enzyme elastase cleaves SLeX-bearing mucins from airway cell surfaces of horses, and these molecules subsequently enter the airway mucus layer.

Table 1

Control horse	BALF elastase (Vmax units/sec)
#1 Time 0	46
#1 48 hour	34
#2 Time 0	23
#2 48 hour	63
#3 Time 0	34
#3 48 hour	23
#4 Time 0	9
#4 48 hour	18
RAO-affected horse	
#1 Time 0	42
#1 48 hour	33
#2 Time 0	17
#2 48 hour	23
#3 Time 0	30
#3 48 hour	21
#4 Time 0	16
#4 48 hour	35

Table 1: Elastase values in bronchoalveolar lavage fluid (BALF) of control and RAO-affected horses before indoor housing (time 0) and after 48 hours of indoor housing (48 hours). Values are Vmax units/sec (from kinetic enzyme-linked immunosorbent assay, as described in methods).

Figure 35. Anti-SLeX immunohistochemistry on horse airway epithelial section. Immunohistochemistry, performed as described in Methods, on horse tracheal sections. Primary antibody was directed against the SLeX epitope. Negative control was without primary antibody. The SLeX epitope was identified as a cell membrane-associated structure. G = goblet cell. Ci = ciliated cell.

Negative Control

SLeX

Figure 36. Neutrophil numbers per ml in bronchoalveolar lavage fluid (BALF) of control and RAO-affected horses. Bronchoalveolar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of housing in a dusty environment (48 hr). Total and differential cell counts were performed on BALF as described in Methods, and neutrophil numbers per ml BALF were calculated. Results are expressed as means ± SEM. Significant group and time effects for 48 hr RAO-affected horses existed (P = 0.024 and 0.025, respectively).

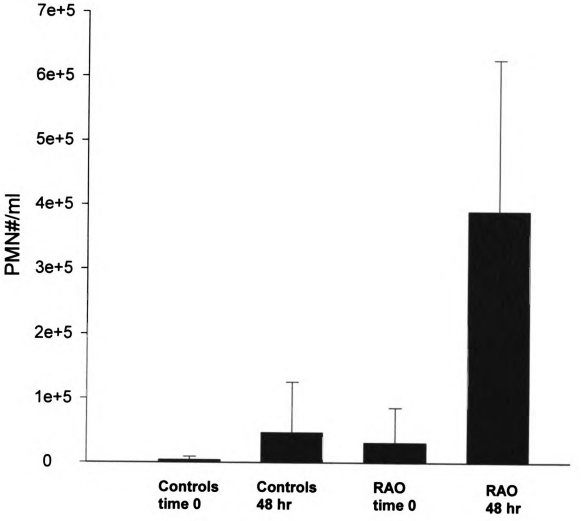

7e+5
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
PMN#/ml
Controls time 0
Controls 48 hr
RAO time 0
RAO 48 hr

Figure 37. Elastase levels in bronchoalveolar lavage fluid (BALF) of control and RAO-affected horses. Bronchoalveolar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of housing in a dusty environment (48 hr). BALF elastase levels were determined by enzyme-linked immunosorbant assay as described in Methods. Results are expressed as means ± SEM. No significant group or time effects existed.

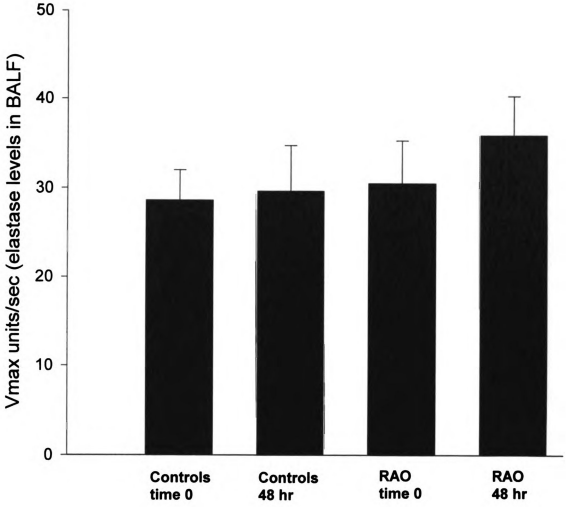

Vmax units/sec (elastase levels in BALF)
50
40
30
20
10
0
Controls time 0
Controls 48 hr
RAO time 0
RAO 48 hr

Figure 38. Sialyl Lewis X (SLeX) levels in bronchoalveolar lavage fluid (BALF) of control and RAO-affected horses. Bronchoalveolar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of housing in a dusty environment (48 hr). BALF SLeX levels were determined by enzyme-linked immunosorbant assay as described in Methods. Results are expressed as means ± SEM. No significant group or time effects existed.

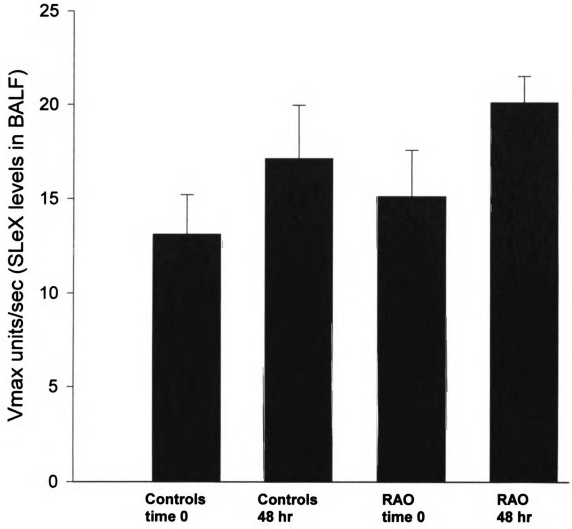
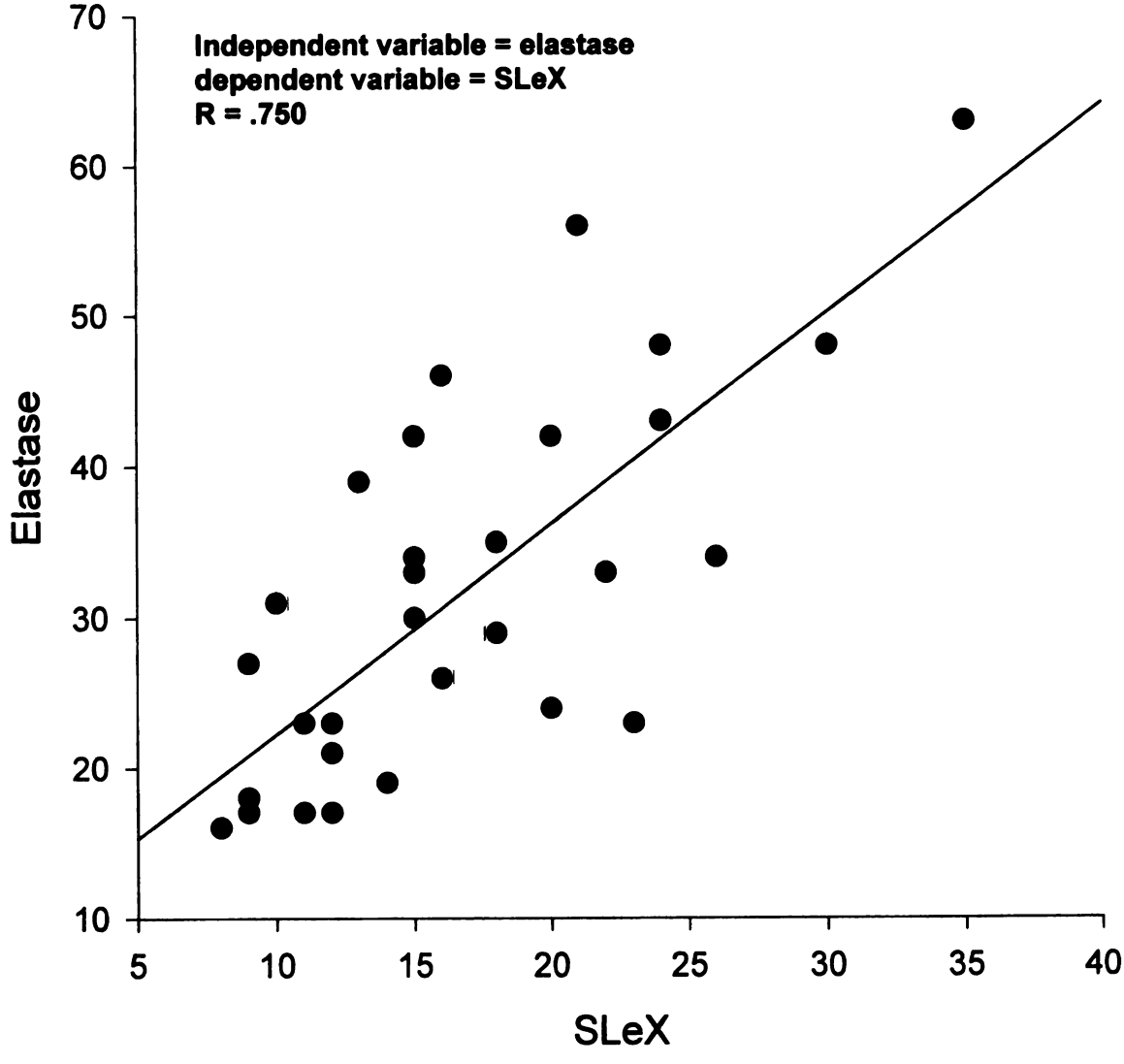

Vmax units/sec (SLeX levels in BALF)
25
20
15
10
5
0
Controls time 0
Controls 48 hr
RAO time 0
RAO 48 hr

Figure 39. Correlation analysis of elastase and SLeX levels in bronchoalveolar lavage fluid (BALF) of control and RAO-affected horses. Bronchoalveolar lavage was performed on control and RAO-affected horses before indoor housing (time 0) and after 48 hours of housing in a dusty environment (48 hr). BALF elastase and SLeX levels were determined by enzyme-linked immunosorbant assay as described in Methods. Statistical analysis of the correlation of BALF elastase levels to BALF SLeX levels in all animals resulted in a correlation coefficient (R value) of .750. An R value of 0 indicates no relationship between 2 variable, while an R value of 1 indicates a perfect positive relationship between 2 variables, with both always increasing together. R values closer to 1 indicate increasing positive relationships between 2 variables.


Independent variable = elastase
dependent variable = SLeX
R = .750
Elastase
SLeX
70
60
50
40
30
20
10
5
10
15
20
25
30
35
40

SUMMARY

Three major hypotheses were developed and tested during this thesis project:

1. Recurrent airway obstruction-affected horses have increased amounts of airway mucus as compared to control horses, and this increase is of a persistent nature.

My results showed that RAO-affected horses have increased levels of airway mucus compared to control horses, and this alteration is of a persistent nature, evident even during clinical remission. Alterations detected were in the form of increased levels of the mucin glycoprotein-associated sugars α-1,2 fucose, sialic acid, and N-acetyl-glucosamine.

This information contributes to the greater understanding of RAO by demonstrating that increased mucus production in RAO horses can be quantified by biochemical means, and that this increased mucus production is not a short term phenomenon that is associated only with acute episodes of the disease. The identification of long-term changes is significant, as this prolonged mucus production may be an indicator of the wide-ranging effects of sustained airway inflammation, which may be driving persistent alterations in mucin glycoprotein production. Prolonged increases in mucus production may also have long-term pathophysiological effects, contributing to airway narrowing or enhancing the binding of bacteria or leukocytes to the mucus blanket.

Additionally, the findings derived from testing this hypothesis can be of practical importance in that the biochemical identification of persistently

increased mucus production can provide a marker for the identification of RAO or other chronic inflammatory airway condition in the absence of obvious clinical signs.

2. The Sialyl Lewis X tetrasaccharide is a component of airway mucus in horses and rats, and levels of SLeX increase during airway inflammation.

The sialyl Lewis X (SLeX) tetrasaccharide has well known functional properties, including serving as a binding site for prokaryotic and eukaryotic adhesion molecules. SLeX, associated with high molecular weight O-linked glycoproteins characteristic of mucins, was identified in bronchoalveolar lavage fluid of both horses and rats. Levels of SLex were increased following endotoxin administration in rats, and SLeX levels were positively correlated with elevated elastase levels in both rats and horses.

3. Temporal differences exist in the composition of airway mucus during inflammation.

Using bacterial endotoxin as a mechanism for inducing airway inflammation, I have shown in this thesis that there are temporal differences in the qualitative composition of mucus that enters the airway lumen during inflammation. That is, mucins released into the mucous blanket early in inflammation are not the same types of mucins that are produced and released at later time points. Specifically, SLeX-bearing mucins, released from epithelial cell surfaces, enter the mucous blanket first (SLeX presence can be detected by 6 hours post-challenge). Mucins produced and secreted from mucous goblet cells (as measured by

MUC5AC and α-1,2 fucose) then follow, and are present in the mucous layer by 24 hours post-challenge.

This finding is significant, in that it contributes to a better understanding of the dynamic nature of mucin release through the course of an inflammatory airway condition. This understanding is a vital addition to the overall concept that mucus production is not a highly generalized and non-specific host response to injury and inflammation, but a precise and directed event, with airway mucins providing specific functions at specific time points as part of their protective role in host defense.

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