Cytokines and aeroallergens in the pathogenesis of summer-pasture associated obstructive pulmonary disease: effects on endothelin production, neutrophil activation and chemotaxis

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CYTOKINES AND AEROALLERGENS IN THE PATHOGENESIS OF SUMMER-PASTURE ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE: EFFECTS ON ENDOTHELIN PRODUCTION, NEUTROPHIL ACTIVATION AND CHEMOTAXIS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences through the Department of Pathobiological Sciences

by

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LIST OF ABBREVIATIONS

ΔPpl = changes in pleural pressure
AB-PAS = alcian blue (pH 2.5)-periodic acid-Schiff
ALI = air-liquid interface
ALI = air-liquid interface
BALF = bronchoalveolar lavage fluid
big-ET = propeptide (39 amino acids), precursor of endothelin-1 peptide (21 amino acids)
CD18 = cluster of differentiation 18 (part of integrin adhesion molecule)
CHO = Chinese Hamster Ovary
CMF-PBS = calcium and magnesium-free physiological buffered saline
COPD = chronic obstructive pulmonary disease
cpm = counts per minute
CSRE = clinical score of respiratory effort
CSRE = clinical score of respiratory effort
DMEM = Dulbecco’s Modified Eagle’s Medium
DNA = deoxyribonucleic acid
Dnase = deoxyribonuclease
DPT_{delta} = delta dew-point temperature
DPT_{max} = maximum dew-point temperature
DPT_{min} = minimum dew-point temperature
EDTA = ethylene diaminotetra-acetate
EFTr = equine fetal tracheal
EGF = epithelial growth factor
EGF = epidermal growth factor
EGTA = ethylene glycol bis(β-aminoethyl ether)
ELISA = enzyme-linked immunosorbent assay
eqrIL-4 = equine inter leukin IL-4
FACS = fluorescence activation cell sorting
FCS = fetal calf serum
FITC = fluorescein isothiocyanate
fMet-Leu-Phe = N-formyl-L-methionyl-L-leucyl-L-phenylalanine
GLM = general linear model
GM-CSF = granulocyte-macrophage colony stimulating factor
H&E = hematoxylin and eosin
HI = heat-inactivated
IFN-β = interferon-β
IFN-γ = interferon-γ
Ig = immunoglobulin
IL-1β = interleukin-1β
IL-13 = interleukin-13
IL-4 = interleukin-4
IL-5 = interleukin-5
IL-8 = interleukin-8
IL-9 = interleukin-9
iNOS = inducible nitric oxide synthase
irET = immunoreactive ET-1
LPS = lipopolysaccharide
LPS = lipopolysaccharide
Mac-1 = integrin (αM:β2) surface adhesion molecule
MCPF = mean peak channel fluorescence
MIP-2 = macrophage inflammatory protein-2
mRNA = messenger ribonucleic acid
mRNA = messenger ribonucleic acid
NADPH = dnicotinamide adenine dinucleotide phosphate diaphorase
NDVI = normalized difference vegetation index
NO = nitric oxide
NT = nitrotyrosine
PBG = peripheral blood granulocytes
PBMC = peripheral blood mononuclear cells
PBS = phosphate buffered saline
PCR = polymerase chain reaction
PCR = polymerase chain reaction
PELF = pulmonary epithelial lining fluid
PHA = Phytohemaglutinin (mitogen)
PKC = protein kinase C
PMA = phorbol myristate acetate
PmB = polymixin B
PNU = protein nitrogen unit
PWM = pokweed mitogen
RAO = recurrent airway obstruction
RBCs = red blood cells
RNA = ribonucleic acid
RT-PCR = real-time polymerase reaction
RWV = rotating wall vessels
SI = stimulation index
SPAOPD = summer pasture-associated obstructive pulmonary disease
T20-PBS = Tween 20 in phosphate buffered saline
TBS = Tris-buffered saline
TH1 = T helper 1 lymphocytes
TH2 = T helper 2 lymphocytes type response
$T_{\text{max}}$ = maximum temperature
$T_{\text{mean}}$ = mean temperature
$T_{\text{min}}$ = minimum temperature
TNF-$\alpha$ = tumor necrosis factor-$\alpha$
ABSTRACT

Summer pasture-associated obstructive pulmonary disease (SPAOPD), a naturally occurring airway disease of horses, is characterized by clinical exacerbation associated with exposure to pasture environment during the summer. Aeroallergens are believed to trigger exacerbation of SPAOPD, cytokines are likely associated with the anamnestic response to aeroallergens, and endothelin (ET)-1 is a potential mediator of airway obstruction. The goal of this dissertation was to describe and explore the interaction of aeroallergens triggering inflammation and T lymphocytes cytokine profile with the recruitment and activation of neutrophils and synthesis of ET-1 by mononuclear leukocytes and airway epithelial cells.

The temporal pattern of clinical exacerbation was associated with hot and humid conditions and with increases in grass pollen and mold spore counts. Circulating concentrations of ET-1 were increased during clinical exacerbation of SPAOPD compared with remission and controls. Gene expression of ET-1 and cytokines, interleukin (IL)-8 and IL-4, but not interferon (IFN)-γ, tended to be greater in lungs of SPAOPD-affected than non-affected horses. The immunoreactive ET-1 distribution tended to be greater in airway tissues of affected horses.

The putative aeroallergens, grass pollen and mold spores, and ET-1 induced neutrophil activation and chemotaxis in vitro. Putative aeroallergens induced IL-4 and IFN-γ expression and up-regulation of ET-1 release in mononuclear leukocytes. Cultures of airway epithelial cells were established under air-liquid interface and microgravity conditions and evaluated for differentiation. Cytokines IL-4 and tumor necrosis factor (TNF)-α induced up-regulation and directional (basolateral) release of ET-1 by differentiated airway epithelial cells, grown under air-liquid interface.
CHAPTER 1. REVIEW OF THE LITERATURE IN SUMMER-PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE
1.1. Introduction

The first description of the respiratory syndrome of horses termed summer-pasture-associated obstructive pulmonary disease or SPAOPD was reported by Dr. Ralph E. Beadle in 1983. In this book chapter, Dr. Beadle described the disease as “a syndrome with signs similar to the equine chronic obstructive pulmonary disease or heaves, a condition observed in stabled horses exposed to moldy hay or bedding and dust, but occurring in horses grazing pasture land in the summer in the southeastern region of the U.S.A., especially Florida, Georgia, Louisiana and Mississippi” (Beadle 1983). Dr. Beadle had first observed the condition in the early 1970’s and performed a survey of horse owners listed in the Louisiana Diagnostic Laboratory as the source of information. He never published the results of this survey, but the information gathered suggested that 3%, and possibly as many as 5%, of the horses surveyed were affected with SPAOPD. Several years later, Dr. Thomas Seahorn also attempted to perform a survey on the incidence of the disease, but using veterinarians as the source of information. There was a poor response from the veterinarians, which limited the ability to estimate the incidence or prevalence of the disease. The study, however, provided important information with respect to the demographics, temporal trends and clinical signs of the disease (Seahorn and Beadle 1993).

Anecdotally, SPAOPD in the southeastern region of the U.S.A. is believed to be associated with a triad of environmental factors: hot, humid climate and high soil moisture (Beadle 1983). However, SPAOPD is not restricted to the southeastern region of the U.S.A. More recently, a pasture-associated seasonal respiratory disease has also been described in England and Scotland (Dixon and McGorum 1990; Mair 1996).
The condition in the United Kingdom appears to differ from the form observed in the southern U.S.A. in that exacerbation of the disease occurs during hot dry weather following exposure to dusts that originated from adjacent fields when crops are harvested or straw burned (McGorum and Dixon 1999). The term summer pasture-associated obstructive pulmonary disease or SPAOPD was used by Dr. Ralph Beadle in his first description of this syndrome, and is still used today, however, a number of other names have also been used.

In 2000, the first workshop to address the issue of terminology of equine lower airway disease clarified some of the confusion associated with the numerous names used to refer to these syndromes. The term once commonly used, equine chronic obstructive pulmonary disease or COPD, has then been identified as inappropriate to describe horses with lower airway inflammatory disease of varying etiologies and severity. The definition of heaves or recurrent airway obstruction (RAO) is “a syndrome observed in mature horses in which airway obstruction can be reversed by a change in environment and use of bronchodilators”. Therefore, in this dissertation the term barn-associated recurrent airway obstruction or barn-associated RAO will be used to refer to the classic equine lower airway obstructive disease occurring in horses kept in stables and exposed to organic dust of hay and bedding. However, the term summer pasture-associated obstructive pulmonary disease or SPAOPD will be used to refer to the condition observed in adult horses following exposure to pasture during the warm months of the year and which is the subject of this dissertation.
1.2. Respirable Particles and Inhaled Aeroallergens in Airway Disease

Airborne organic particles of sizes ranging between 2 to 60 µm constitute potential allergens; however, particles larger than 18 to 20 µm are deposited throughout the upper respiratory tract resulting in the relative exclusion of these particles from the subcarinal airways (Solomon and Platts-Mills 1998). Respirable particles, 5 to 7 µm in size, are small enough to reach and be deposited in the lower respiratory tract, often beyond the terminal bronchioles (Solomon and Platts-Mills 1998).

Most terrestrial fungi are composed of branching threads or hyphae of 3 to 10 µm in width. The sizes of the mold spores vary considerably; rusts tend to be larger spores of 28 to 45 µm in length and 10 to 15 µm in width; Zygomycetes (Rhizopus, Mucor, Absidia) are small unicellular spores of 4 to 8 µm in size (Solomon and Platts-Mills 1998). Most pollen grains range in diameter from 14 to 60 µm (Solomon and Platts-Mills 1998). Respirable particles of less than 7 µm may consist of intact mold spores, but most likely they are submicronic particles of pollen grains and molds.

The median diameter of particles that deposit predominantly in the pulmonary airways of horses were equal or less than 3 µm (Clarke 1987). The amount of respirable particles horses are exposed in the breathing zone while kept indoor is an important determining factor in airway disease. Airborne particles in stables (mean 0.41 mg/ml, ranging from 0.19 mg/ml to 0.91 mg/ml) were 10-fold greater than in the outdoor environment (mean 0.4 mg/ml) (Crichlow et al. 1980). A study comparing two different management systems, one with regular grass hay as diet and straw as bedding, and the other with complete pelleted diet and wood shavings as bedding. The total airborne dust in stables of hay/straw management was 3.6-fold greater than that of
the pellets/wood shavings management, and the concentration of respirable particles (less than 7 µm) in hay/straw management was 2.2-fold greater than in pellet/wood shavings management (Woods et al. 1993). Interestingly, the breathing zone had a much greater concentration of airborne dust. The total airborne dust particles and respirable particles were 33.6-fold and 31-fold greater in the hay/straw management than in the pellets/wood shavings management (Woods et al. 1993). The airborne dust particles in stables were variable and complex mixtures that include plant and grain fragments, fungal spores, insect parts and animal/fecal derived components (Derksen and Woods 1993). The predominant fungi in the hay were the thermotolerant actinomyces, especially Aspergillus fumigatus, Micropolyspora faeni and Thermoactinomyces vulgaris (Derksen and Woods 1993).

The presence of respirable airborne dust endotoxin was demonstrated in the breathing zone of horses kept in stables (McGorum et al. 1998). The airborne endotoxin concentrations in the breathing zone of horses kept in three conventional stables exceeded the concentrations that induce airway hyperresponsiveness in humans (McGorum et al. 1998). Inhalation of endotoxin was shown to induce dose-dependent airway inflammation in horses affected with RAO as well as control subjects (Pirie et al. 2001). Inhalation of endotoxin as respirable dust may play a role in airway inflammation, especially in horses while confined in stables. Whereas in the pasture environment inhaled respirable dust endotoxin is less likely to significantly contribute as a triggering agent of airway inflammation and hyperresponsiveness. However, the relevance of respirable airborne endotoxin dust in clinical exacerbation of SPAOPD has not been explored.
1.3. Epidemiological Facts in Summer Pasture-Associated Obstructive Pulmonary Disease

A temporal trend of the clinical exacerbation of SPAOPD was documented by Seahorn and Beadle in the veterinarian-based survey study (1993). The clinical signs of the disease were observed by the owners starting in July, peaking in September, and persisting until October (Seahorn and Beadle 1993).

The seasonal pattern of the disease and potential environmental factors has been suggested, however, the inciting agents have not been identified. Mold spores of several fungal species have been isolated from nasal passages of SPAOPD-affected and non-affected horses during the summer (Seahorn and Beadle 1994). The mold spore types isolated from affected horses with or without signs of clinical exacerbation included *Alternaria spp.*, *Curvularia spp.*, *Cladosporium spp.* and *Fusarium spp.* (Seahorn and Beadle 1994). During the same time of the year, mold spores of *Cladosporium spp.*, *Curvularia spp.*, *Fusarium spp.*, *Acremonium spp.* and *Choanephora spp.* were isolated from nasal passages of non-affected horses. When horses were kept confined in a barn, the mold spore types isolated from the nasal passages were *Cladosporium spp.*, *Acremonium spp.* and *Pullularia spp.* It was concluded that *Cladosporium spp.* and *Acremonium spp.* are ubiquitous in pasture and barn environments, whereas *Curvullaria spp.*, *Alternaria spp.* and *Fusarium spp.* are predominate in the pasture environment (Seahorn and Beadle 1994). This study provided evidence indicating that certain types of mold spores may be the triggering agent of clinical exacerbation of SPAOPD. Pollen grains were not considered in this study. It is possible that, similar to human asthma, the airway hyperresponsiveness in different individuals is triggered by different/several agents.
The limited demographic information of SPAOPD in the southeastern states of the U.S.A. comes from the study by Seahorn and Beadle (1993). In Louisiana, the parishes most frequently reporting the disease were located in Central and South Louisiana (Seahorn and Beadle 1993). The affected horses were mature horses, mean age of \(12 \pm 5.9\) years of age, predominantly of the Quarter Horse-type breeds (52\% Quarter Horses, 5.6\% Paints, 4.2\% Appaloosas), whereas only 5.6\% were Thoroughbreds, and 4.2\% Arabians. There was no indication of gender predilection; 51.4\% were females, 47.1\% castrated males and 1.4\% intact males (Seahorn and Beadle 1993).

Information concerning where the horses were kept and where they performed their primary activity indicated that 98.6\% of the affected horses were kept on pasture for more than half of the day and 60.6\% of the affected horses were kept on pasture at all times (Seahorn and Beadle 1993). Standing water after rains were reported in 47.6\%, whereas 33\% did not remain with standing water (Seahorn and Beadle 1993). Most of the horses (88.2\%) performed their primary activity in a grassy arena or pasture and 11.8\% worked mainly in dirt/sand arena (Seahorn and Beadle 1993). The level of exercise of SPAOPD-affected horses was reported as minimal (classified as broodmares, halter showing, walk/trot and pasture riding) in 60.6\% of the horses, moderate (classified as pleasure riding and pleasure trail riding) in 32.4\% of the horses and maximal (classified as track racing, barrel racing, roping and endurance riding) in 7\% of the horses (Seahorn and Beadle 1993).
1.4. Clinical Features of Summer Pasture-Associated Obstructive Pulmonary Disease

The clinical exacerbation of SPAOPD resembles those of equine barn-associated RAO and human asthma, which include airway hyperresponsiveness and airflow obstruction. The signs are manifested following exposure of affected horses to pasture during the warm months of the year, ranging from mid-spring to mid-fall (Beadle 1983). The clinical presentation ranges in severity. Horses may present with exercise intolerance or persistent and paroxysmal cough during the warmer months of the year. These signs may go unnoticed until the horses develop coughing and increase respiratory effort even at rest (Figure 1.1).

The survey study by Seahorn and Beadle described in detail the clinical signs observed by owners and veterinarians during clinical exacerbation of SPAOPD (1993). The clinical signs of disease were observed by the owners in the month of July, peaking in September, when most horses were examined by the veterinarians, and persisting until October (Seahorn and Beadle 1993). Labored expiratory effort and coughing were the most common signs reported, affecting 93% and 90% of the horses, respectively. In 60% of the horses, the coughing occurred regardless of whether the horse was exercising or eating. Flared nostrils were reported in 84.5% of the horses. Other signs such as lethargy and anorexia were reported in less than 25% of the horses. The vital signs, except the respiratory rate, were mostly within normal limits. The average respiratory rate at rest was $32 \pm 12$ breaths per minute. Auscultation of the thorax revealed abnormal respiratory sounds in 92.8% of the horses, of which 60% included both crackles and wheezes and 50% were observed throughout the respiratory cycle,
i.e., during expiration and inspiration whereas 28% were observed only during expiration (Seahorn and Beadle 1993).

Figure 1.1. Photograph of a horse with flared nostrils during clinical exacerbation of summer pasture-associated obstructive pulmonary disease. Note the fully abducted nostrils as a sign of increased respiratory effort. Photograph was taken after the animal had just been standing on pasture.

The clinical findings during exacerbation of SPAOPD have been previously described and correlated to the pathological abnormalities, the respiratory pattern evaluated as clinical scores of respiratory effort, based on the nostril flare and
abdominal lift, the changes in pleural pressure (ΔPpl) during tidal breathing, the cytology of the bronchoalveolar lavage fluid (BALF) and the pulmonary histopathology, collected via percutaneous biopsy and at postmortem, in eight horses affected with SPAOPD and six non-affected controls (Costa et al. 2000). In all SPAOPD-affected horses, auscultation of the lungs revealed crackles and wheezes throughout the lung fields and in some of them the expiratory wheezes were audible without a stethoscope (Costa et al. 2000). All SPAOPD-affected horses had spontaneous cough, pronounced nostril flaring and abdominal lift (Figure 1.2). The clinical score of respiratory effort for SPAOPD-affected horses (median 5.75; range 4.0 to 7.5) was significantly greater than in non-affected horses (median 2.0; range 2.0 to 3.0) (Costa et al. 2000). The ΔPpl of affected horses (median 33 cm H2O, range 23.6 to 58.0) was significantly greater than in non-affected horses (median 4.5 cm H2O, range 1.0 to 9.0) (Costa et al. 2000).

The passage of the endoscope for BALF collection elicited coughing of variable severity in most of the SPAOPD-affected horses, and most of the affected horses had white-to-yellow mucus randomly scattered through or extending the entire length of the trachea and accumulated at the thoracic inlet; three horses had a hyperemic appearance to the bronchial mucosa (Costa et al. 2000). Minimal coughing was elicited in the non-affected horses, and no mucus or hyperemia was evident within their airways. Cytologic evaluation of BALF from SPAOPD-affected horses yielded predominantly non-degenerate neutrophils, mean 52 ± 13 %, compared with non-affected controls, mean 10 ± 4 %. SPAOPD-affected horses had a significantly greater percentage of neutrophils and significantly lesser percentage of lymphocytes, macrophages and mast cells in BALF, compared with clinically normal horses (Costa et
al. 2000). None of the BALF samples from either group yielded a positive culture of bacteria under aerobic conditions.

Figure 1.2. Photograph of a horse showing signs of increased respiratory effort during clinical exacerbation of summer pasture-associated obstructive pulmonary disease. Note the fully stretched head and neck position, the flared nostrils and the abdominal lift or heave line demarcating the abdominal musculature. These signs of labored breathing are associated with obstruction to airflow. Photograph was taken while the animal had just been standing on pasture.

The lungs of all SPAOPD-affected horses appeared grossly over-inflated at postmortem, failing to collapse when the thoracic cavity was opened; many retained the impression made by pressure from the ribs. The lungs of the non-affected horses did collapse when the thoracic cavity was opened (Costa et al. 2000).
Figure 1.3. Photograph of the lungs obtained from a horse with summer pasture-associated obstructive pulmonary disease. Notice the over-inflated appearance of the lung.

Histopathologic lesions were identified throughout the lung lobes, and included marked accumulation of basophilic mucus and neutrophils within the small airways, bronchiolar goblet cell metaplasia and mild peribronchial inflammatory infiltrate, which was predominantly mononuclear (Costa et al. 2000). Histopathology of specimens collected via percutaneous biopsy was predictive of disease and corresponded to findings at postmortem (Costa et al. 2000). The clinical score of respiratory effort was highly correlated to $\Delta$Ppl in SPAOPD-affected horses. The clinical score of respiratory effort and $\Delta$Ppl were highly correlated with mucus accumulation in the airways in affected horses. Peribronchial inflammatory infiltrate correlated with percentage of neutrophils in BALF of affected horses (Costa et al. 2000).
Typically, the clinical signs of the disease disappear when the horses are taken away from the pasture environment and during the cooler parts of the year (Beadle 1983). As the horses enter clinical remission, the clinical signs of airflow obstruction and airway hyperresponsiveness often disappear without any medical treatment. Anecdotally, affected horses when taken to northern states of the U.S.A. will often not manifest the disease while in the northern states.

With repeated exposure, clinical signs become more obvious and the clinical exacerbation of SPAOPD include the increased respiratory effort, particularly at the end of expiration, bronchoconstriction and tracheo-bronchial accumulation of mucopurulent exudate. The disease may progress to a debilitating respiratory dyspnea, chronic hypoxemia and right-sided heart failure secondary to pulmonary hypertension.

Early clinical recognition of the disease can be difficult. As coughing often occurs while horses are out in pasture, observation of signs may not occur until the disease is more advanced. Similar to asthma, the recurrent episodes of reversible airway hyperresponsiveness and airflow obstruction may eventually culminate with non-specific bronchial hyperresponsiveness and advanced airway remodeling, resulting in irreversible airflow obstruction. Some degree of airway remodeling was observed in horses with reversible SPAOPD (Costa et al. 2000). Typically, these pathological findings include airway epithelium hyperplasia, mucus glands and goblet cell hyperplasia, goblet cell metaplasia in small airways and mononuclear peribronchial infiltrate (Costa et al. 2000).

Airway remodeling that culminates with irreversible airflow obstruction, which includes shedding of the epithelium, smooth muscle hypertrophy and hyperplasia,
thickening of the basement membrane, subepithelial and interstitial fibrosis and
disruption of the pulmonary architecture such as bronchiectasis and emphysema, i.e.,
loss of the alveolar architecture, were not commonly observed in SPAOPD (Costa et al.
2000). Airway hyperresponsiveness increases seasonally in asthmatic patients, and
allergen avoidance reduces airway responsiveness (Stewart, Tomlinson and Wilson
1993). Persistent exposure to allergens and the consequent chronic inflammatory
response are believed to result in airway remodeling, especially smooth muscle
hypertrophy and hyperplasia (Stewart, Tomlinson and Wilson 1993). This airway
remodeling is reported in chronic asthma, and is believed to be responsible for the non-
specific bronchial hyperresponsiveness b.

Epithelial damage is considered an important factor in airway remodeling.
Recurrent airway inflammation includes the release of a number of inflammatory
mediators, many of which have potential mitogenic activity (Stewart, Tomlinson and
Wilson 1993; Goldie 1999). These mediators include thromboxane A2, transforming
growth factor-α, epithelial growth factor, insulin-like growth factor, interleukin-1, platelet
activating factor and endothelin-1 (Stewart, Tomlinson and Wilson 1993; Goldie 1999,
Amishima et al. 1998).

1.5. Cytokines in the Pathogenesis of Equine Recurrent Airway Obstruction

Asthma is defined as a syndrome characterized by three major features: airway
hyperresponsiveness, airway inflammation, and intermittent and reversible airway
obstruction leading to recurrent episodes of breathlessness, wheezing and coughing
(Renauld 2001, Stewart, Tomlinson and Wilson 1993). By this definition, the two forms
of recurrent airway obstruction of horses, namely, the barn-associated recurrent airway obstruction and the summer-pasture-associated obstructive pulmonary disease are considered to resemble asthma. The cytokine profile in asthma is that of a CD4⁺ T helper 2 lymphocytes (TH2) type response, which includes interleukin (IL)-4, IL-5, IL-9 and IL-13 (Renauld 2001). The two T helper cells subtypes originally described are the TH2 described above, and the CD4⁺ T helper 1 lymphocytes (TH1). The latter is characterized by a cytokine profile including IL-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α (Mosmann et al. 1986 and Romagnani 1991).

Much of the cytokine research in equine recurrent airway diseases has attempted to characterize if the immunological basis of these diseases is of a TH2 type of response, however, there seems to be some discrepancy in the reported findings. Lavoie and co-workers (2001) compared the cytokine expression in BALF lymphocytes after a chronic exposure (i.e., more than three weeks) to dusty/moldy hay and straw using in situ hybridization and reported that horses with barn-associated RAO had a 3.5-fold greater IL-4 messenger ribonucleic acid (mRNA) expression, a two-fold greater IL-5 mRNA expression, and a 2.5-fold lesser IFN-γ mRNA expression than the non-affected controls. Cordeau and co-workers (2004) evaluated the temporal cytokine expression in BALF lymphocytes of horses with barn-associated RAO and non-affected controls after acute (24 hours) and chronic (9 days) exposures to dusty/moldy hay and straw. They reported a predominant TH2 cytokine mRNA expression, where IL-4 and IL-5 were increased 2.5-fold and 3-fold, respectively, in the acute response compared with baseline, and 5.5-fold and a 9-fold, respectively, in the chronic response compared with baseline (Cordeau et al. 2004). In contrast, IFN-γ mRNA expression was 1.5-fold
lesser than baseline in both acute and chronic responses (Cordeau et al. 2004). They also reported that the TH2 cytokine response and the development of airway obstruction were concurrent events, suggesting that the cytokines contributed to the airway dysfunction observed in RAO (Cordeau et al. 2004).

Giguere and co-workers (2002) employed a competitive polymerase chain reaction (PCR) to compare the mRNA expression of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNF-α in horses with barn-associated RAO and non-affected controls during exacerbation (exposure to moldy hay until horse developed signs of airway obstruction). Increased mRNA expression of IL-1β and TNF-α in BALF cells from RAO-affected horses coincided with development of signs of disease exacerbation (Guiguere et al. 2002). Moreover, the expression of IL-8 was greater in affected horses, compared with controls, regardless if affected horses were in clinical exacerbation or remission (Guiguere et al. 2002). Expression of IL-4 mRNA decreased and IFN-γ mRNA increased during exacerbation compared with remission, although these changes were reported as not statistically significant. There was a positive correlation between the BALF neutrophil percentage and expression of IL-1β and IL-8, but not TNF-α (Guiguere et al. 2002).

Ainsworth and co-workers (2003) reported no differences in the IL-4, IL-8, IL-13, IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF) mRNA expression using real-time PCR in BALF cells from RAO-affected horses during the acute phase (24 hrs) following exposure to moldy hay. The only significant change was a 3-fold and 2.5-fold increase in the expression of IL-8 and IFN-γ mRNA, respectively, in
BALF cells from RAO-affected horses during the chronic phase (five weeks) following exposure to moldy hay (Ainsworth et al. 2003).

Kleiber and co-workers (2005) reported the expression of cytokines IL-4, IL-5, IL-13 and IFN-γ in CD4+ and CD8+ T lymphocytes of peripheral blood or BALF using a different technique from the above mentioned studies for quantification of gene expression, the quantitative PCR. They reported no statistically significant difference in the cytokines IL-4, IL-5, IL-13 and IFN-γ expression in either CD4+ or CD8+ lymphocytes of peripheral blood and BALF from RAO-affected horses following 48 hours of exposure to moldy hay (Kleiber et al. 2005).

Some of the discrepancy between the various studies with barn-associated RAO may result from the techniques used to challenge of horses as well as the method to detect and report cytokine gene expression or the differences in research subjects, the latter suggesting heterogeneity of the cytokine profile despite the similarity of the clinical presentation of airway obstruction in all cases.

Fewer studies have reported the cytokine profiles from horses affected with SPAOPD. The first report suggested that during exacerbation horses affected with SPAOPD had a TH2 type of cytokine response, where there was a greater expression of IL-4 and IL-5 in peripheral blood mononuclear cells and BALF cells from affected horses compared with non-affected controls, although statistical analysis was not reported (Horohov 2000). The expression of IFN-γ was lesser in peripheral blood mononuclear cells and BALF cells from affected horses compared with the controls (Horohov 2000). Beadle and Horohov evaluated the gene expression of IL-4, IL-5 and IFN-γ in peripheral blood mononuclear cells and BALF cells obtained from SPAOPD-
affected and non-affected horses kept on pastures during the summer and winter, using a semi-automated quantitative PCR method (Beadle and Horohov 2002). During the summer, when the horses in the affected group had developed signs of airway obstruction, the expression of IL-4 in both peripheral blood mononuclear cells and BALF cells was greater compared with non-affected horses (Beadle and Horohov 2002). Interestingly, during the summer, the expression of IFN-γ in both peripheral blood mononuclear cells and BALF cells from affected horses was also greater than in non-affected horses (Beadle and Horohov 2002). In addition, there was a greater IFN-γ expression in peripheral blood mononuclear cells and BALF cells from affected horses during summer compared with winter (Beadle and Horohov 2002). No significant expression of IL-5 was detected in any of the samples (Beadle and Horohov 2002). The increased expression of IL-4 and IFN-γ, and no expression of IL-5 in SPAOPD-affected horses coinciding with signs of airway obstruction during the summer compared with winter suggested a mixed T helper cell response that differs from the classical TH1 and TH2 type of cytokine responses.

Cells of the innate immune system, such as macrophages and neutrophils, can also produce cytokines, which may have a tremendous impact on the inflammatory response. Alveolar macrophages are the most abundant cells in the normal airway lumen and are believed to be the major defender of the lung against infectious agents and other insults. They exert their function primarily by the release of mediators including nitric oxide, super oxide anion, lipid-derived mediators such as leukotrienes, metalloproteinases, chemokines and pro-inflammatory cytokines (Alam 1997, Gosset et al.1984, Dery and Bissonnette 1999, Tetley 2002). In horses with RAO, exposure to
moldy/dusty hay resulted in the synthesis of IL-8 and MIP-1 by alveolar macrophages (Franchini et al. 1998). In addition, alveolar macrophages are also responsible for the clearance of particulate matter, such as dust particles and microorganisms, in alveolar space (Fels and Cohn 1986). The alveolar clearance rate in horses affected with barn-associated RAO is more rapid during clinical crisis compared with remission and to non-affected controls (Votion et al. 1999). The rapid alveolar clearance may result in the quick exposure of macrophages to aeroallergens and the resulting secretion of mediators that culminate with airway hyperresponsiveness.

Equine neutrophils stimulated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe) and lipopolysaccharide (LPS) express mRNA for TNF-α, IL-1β, IL-6, IL-8 and macrophage inflammatory protein (MIP)-2 (Joubert et al. 2001). Considering the number of neutrophils that infiltrate the airways of horses during clinical exacerbation, the amount of pro-inflammatory cytokines produced is likely to trigger and perpetuate the airway inflammation.

1.6. Neutrophils and Cytokines in the Pathogenesis of Summer Pasture-Associated Obstructive Pulmonary Disease

Neutrophils are the predominant cell population in BALF of horses affected with SPAOPD and those affected with barn-associated RAO (Derksen et al. 1985, Fairbairn et al. 1993, Seahorn and Beadle 1993, Costa et al. 2000). Neutrophilic inflammatory influx into the airways is also a feature of human COPD (Hiemstra 1998). Similarly, neutrophils are the predominant cell, comprising 80% of the cells, in BALF fluid of asthmatic human patients during status asthmaticus (Lamblin et al. 1998).
Neutrophil accumulation in the airways is accompanied by mucus hypersecretion, goblet cell metaplasia in small airways and mucus gland hyperplasia in lungs of horses affected with SPAOPD (Costa et al. 2000). The triggering factors and mediators involved in neutrophilic chemoattraction and mucus hypersecretion have not been elucidated. Neutrophil-derived inflammatory mediators and proteases have been shown to trigger mucus hypersecretion by bronchial epithelial cells (Lundgren et al. 1994, Hiemstra 1998, Voynow et al. 1999, Nadel 2000). Pro-inflammatory cytokines, chemokines, endothelin, and reactive oxygen and nitrogen species are produced by bronchial epithelial cells upon stimulation with toxicants such as ozone, endotoxin and allergens like cockroach extracts and *Aspergillus fumigatus* protein extracts (Nakano et al. 1994; Jaspers et al. 1997; Hay et al. 1997; Rochelle et al. 1998, Borger et al. 1999; Bhat et al. 2003).

Several factors, including lipid-derived mediators, chemokines and cytokines have been implicated in the pathogenesis of recurrent airway disease and in neutrophil chemotaxis in several species. Some of those mediators, including leukotriene B₄, platelet activating factor, IL–8, TNF-α and MIP-2, are known neutrophil chemoattracts (Loike et al. 1995, Franchini et al. 1998, Brazil et al. 1998, Galligan & Coomber 2000). The chemokines IL-8 and MIP-2 have been shown to be up-regulated in equine COPD (Franchini et al. 2000).

1.7. Immunoglobulins in the Pathogenesis of Equine Recurrent Airway Obstruction

Immunoglobulins have been implicated in the pathogenesis of asthma and barn-associated RAO; however, the importance of immunoglobulins in the pathogenesis of
SPAOPD remains unclear. Immunoglobulin (Ig) E along with the TH2 cytokines, which include IL-4, IL-5 and IL-13, are the hallmarks of human asthma (Vercelli 2000). These cytokines are responsible for the sequential B cell isotype switch to $\gamma_4$ and finally $\varepsilon$ resulting in IgG$_4$ and IgE (Vercelli 2000). The cytokine profile in barn-associated RAO has been the subject of debate and so is the importance of the IgE in SPAOPD.

Isotype-specific antibodies to two mold spores, *Micropolyspora faeni* and *Aspergillus fumigatus*, which are considered to be putative allergens involved in barn-associated RAO, were quantified using an enzyme-linked immunosorbent assay (ELISA) (Halliwell et al. 1993). Evaluation of serum antibodies of RAO-affected horses were compared with non-affected controls, and revealed similarly low circulating levels of allergen-specific antibodies. However, antibodies of the IgE and IgA isotypes specific to both allergens were increased in BALF of RAO-affected horses compared with non-affected horses (Halliwell et al. 1993). Recombinant mold allergens were utilized to quantify allergen-specific IgE using an ELISA of serum and BALF from horses affected with RAO (Eder et al. 2000). RAO-affected horses had detectable levels of allergen-specific IgE; however, these values were not significantly greater than in non-affected controls (Eder et al. 2000).

Antigen-specific antibodies were quantified in tracheal lavage fluid of horses affected with SPAOPD and compared with non-affected controls (Seahorn et al. 1997). Antigens tested included: *Aspergillus fumigatus*, *Aspergillus nidulans*, *Alternaria tenuis*, *Faeni rectivirgula* (formerly called *Micropolyspora faeni*), *Penicillium expansum*, *Saccharonospora viridis*, *Thermoactinomyces thalophilus* (Seahorn et al. 1997).
SPAOPD-affected horses did not have an increased concentration of antigen-specific IgE or IgG in tracheal lavage fluids (Seahorn et al. 1997).

1.8. Inflammatory Mediators in the Pathogenesis of Summer Pasture-Associated Obstructive Pulmonary Disease

The airway obstruction observed in SPAOPD results mostly from bronchospasm as well as from mucus accumulation with massive neutrophilic infiltrate into the airway lumen (Beadle 1983, Costa et al. 2000).

Intracellular production of reactive nitrogen species and increased activity of inducible nitric oxide synthase (iNOS) have been shown in guinea-pig tracheal epithelia cells stimulated with LPS and TNF-α (Rochelle et al. 1998). Additionally, nitric oxide (NO) derived from iNOS in airway epithelial cells has been implicated as a mechanism of amplification and perpetuation of airway inflammation in human asthma (Barnes and Liew 1995). Large quantities of iNOS-derived NO are produced in bronchial epithelial cells of patients with asthma (Kacmarek 1997; Hamid et al. 1993). In large concentrations, NO derived from iNOS has as an inhibitory effect on TH1 lymphocytes and favors the proliferation of TH2 helper cells (Barnes and Liew 1995). Likewise, horses with SPAOPD are believed to have a bias toward a TH2 response as evidenced by the increased IL-4 expression in the BALF cells compared with non-affected horses (Horohov 2000).

The concentration of NO in plasma and bronchoalveolar lavage fluid and localization of nitric oxide synthesis was determined in lungs of seven adult horses affected with SPAOPD and compared with six non-affected adult horses that served as controls (Costa et al. 2001). All SPAOPD-affected horses had a history of recurrent
obstructive respiratory disease following exposure to summer pasture, and on examination, thoracic auscultation of those horses revealed crackles, wheezes and quiet areas whereas all non-affected horses had normal bronchovesicular sounds upon auscultation of the lung with a re-breathing bag (Costa et al. 2001). The clinical scores of respiratory effort (median 5.75; range 4.0 to 7.5) and the $\Delta P_{pl}$ (median 37.3 cm of H$_2$O; range 26.3 to 58.0 cm of H$_2$O) of SPAOPD-affected horses were greater than the clinical scores of respiratory effort (median 2.0; range 2.0 to 3.0) and $\Delta P_{pl}$ (median 5.0 cm of H$_2$O; range 1.0 to 9.0 cm of H$_2$O) of non-affected horses (Costa et al. 2001). Nitric oxide concentrations in plasma and BALF and pulmonary epithelial lining fluid (PELF) were determined via a chemiluminescent method. Plasma concentrations of NO in SPAOPD-affected horses (median 193 µM, range 16 to 351 µM) were greater, although not statistically significant, than the concentrations in non-affected horses (median 54 µM, range 12 to 162 µM) (Costa et al. 2001). Nitric oxide concentrations in BALF (median 6.05 µM and range 5 to 10.12 µM) from SPAOPD-affected horses did not differ significantly from that from non-affected horses (median 6.0 µM and range 5 to 9.9 µM) (Costa et al. 2001). Nitric oxide concentrations in PELF of SPAOPD-affected horses (median 50.2 µM and range 9.6 to 188 µM) did not differ significantly from that of non-affected horses (median 56.5 µM and range 29.3 to 142 µM).

Immunohistochemical staining of formalin-fixed pulmonary tissue specimens for iNOS and nitrotyrosine (NT), and histochemical staining of cryopreserved tissues for nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) activity were performed. The iNOS staining of bronchial epithelial cells was greater in affected than non-affected horses, whereas the NT staining and NADPHd activity of bronchial
epithelial cells, as well as the NT and iNOS staining and NADPHd activity of peribronchial leukocytes in the pulmonary tissue did not differ between SPAOPD-affected and non-affected horses (Costa et al. 2001). The increased immunoreactivity to iNOS in bronchial epithelial cells of SPAOPD-affected, compared with non-affected horses, was suggested to indicate that NO was likely to play a role in the amplification airway inflammation and hyperresponsiveness observed during clinical exacerbation of SPAOPD (Costa et al. 2001).

A number of inflammatory mediators have been evaluated in vitro with respect their ability to induce constrictive bronchial responses, including acetylcholine, histamine, 5-hydroxytryptamine and leukotriene D₄ (Venugopal et al. 2001). The bronchial response of bronchial rings obtained from SPAOPD-affected horses was significantly greater than those of non-affected control horses, suggesting that 5-hydroxytryptamine may be an important mediator of the airway of hyperresponsiveness. However, it was clear that other mediators must be equally or more important, especially if those mediators of inflammation are capable of inducing or contributing to several of the clinical and pathological features observed during clinical exacerbation of SPAOPD. Indeed, ET-1 has now been considered as a potential mediator in equine airway disease because in addition to the strong constrictive bronchial response, ET-1 can cause inflammatory cellular activation, induction of cytokines, mucus hypersecretion, cellular hyperplasia and airway remodeling.
1.9. Endothelin in the Pathogenesis of Summer Pasture-Associated Obstructive Pulmonary Disease

1.9.1. Endothelin Metabolism in the Lungs: Physiological and Pathological Effects

Endothelins are a class of mediators implicated in the pathogenesis of several respiratory diseases, including airway disease such as asthma. The endothelin family consists of three isoforms, ET-1, ET-2 and ET-3, encoded by three different genes (Rubanyi and Polokoff 1997). They resemble the cardiotoxic peptide sarafotoxin, present in snake venom (Turk 1998). Endothelins are the most potent smooth muscle constrictors known (Rubanyi and Polokoff 1997). All three isopeptides are synthesized as pre-propeptides of 212 amino acids, which are processed by endopeptidases to form the pro-peptide, also called big ETs containing 37 to 40 amino acids; the subsequent cleavage of the pro-peptide into the mature and active peptide results from the action of the metalloendoprotease endothelin converting enzyme (ECE) (Rubanyi and Polokoff 1997, Turk 1998). The pro-peptide big ET-1 has 1% of the biological activity of the ET-1, whereas the pre-propeptide is devoid of biological activity. All three endothelins can induce bronchoconstriction in intact normal airways, however ET-1 is the most potent bronchoconstrictor of the three (Fagan et al. 2001). Endothelin-1 is considered to exert an important role as physiological regulator of pulmonary circulation (Barnes and Liu 1995).

The lung is the major site of ET synthesis and catabolism. Endothelin-1 is the most studied of all isoforms. It is not stored in the cells, instead ET-1 is synthesized and transported through the cell in vesicles resulting in directional secretion of ET-1 towards the interstitium and away from luminal surface of vessels or airways (Fagan et al. 2001). There are many cell types in the lung, including the endothelium, epithelium
and alveolar macrophages that express ECE and therefore produce and secrete ET-1. Directional secretion of ET-1 results in both paracrine and autocrine modes of action, whereas when ET-1 is secreted into the circulation it results in an endocrine manner of action (Fagan et al. 2001). The clearance of ET-1 from the circulation occurs primarily in the lung, kidney and liver (Battistini and Dussault 1998).

In the lungs, as in any other organs, ET can exert their effects through autocrine, paracrine modes of action when ET is released into the interstitial space; whereas if ET is secreted into the lumen of blood vessels, it results in endocrine mode of stimulation. The endocrine mode of stimulation results in systemically detectable concentrations of ET in the circulation. Airway epithelial cells were shown to preferentially secrete, about 80%, ET-1 through the basolateral surface of the cells, resulting in autocrine and paracrine modes of stimulation (Rubanyi and Polokoff 1997).

The effects of ET are mediated through the interaction with ET receptors, which are fairly ubiquitous. There are two well-described ET receptors, ETA and ETB, both of which belong to the guanidine-nucleotide-binding protein receptor (or G protein) family; they contain seven transmembrane motifs (Turk 1998). The signal transduction is mediated by activation of phospholipase C, formation of inositol triphosphate, resulting in increased cytosolic calcium from intracellular and extracellular sources that in turn initiates smooth muscle contraction, and diacylglycerol. The latter in conjunction with the increased cytosolic calcium activates protein kinase C (PKC), which mediated the mitogenic activity of ET-1 (Turk 1998, Salh 1998). Moreover, binding of ET-1 to the ET receptors results in activation of phospholipase D, phospholipase A2 and arachidonic acid metabolism (Rubanyi and Polokoff 1997, Turk 1998, Salh 1998).
In the airways, both $\text{ET}_A$ and $\text{ET}_B$ receptors are present. In horses, ET-1 was shown to be a potent endogenous spasmogen of vascular and airway smooth muscle, producing concentration-dependent contractions of equine pulmonary artery and bronchus (Benamou et al. 2003). The potency of ET-1 was 25 times greater in the equine artery than in equine bronchus (Benamou et al. 2003). The contractions of equine pulmonary artery were mediated by $\text{ET}_A$ receptors, and the contractions of equine bronchi were mediated by both the $\text{ET}_A$ and $\text{ET}_B$ receptors (Benamou et al. 2003).

The distribution of $\text{ET}_A$ and $\text{ET}_B$ receptors may vary among species. Generally, the $\text{ET}_A$ receptors are most abundant on the vascular and airway smooth muscle cells of normal healthy lungs (Battistini and Dussault 1998). The $\text{ET}_B$ receptors are most often found on endothelial cells, but also on airway smooth muscle cells (Battistini and Dussault 1998). Clearance of ET-1 from the circulation is mediated primarily by the $\text{ET}_B$ receptors (Battistini and Dussault 1998). Binding of ET to $\text{ET}_A$ and $\text{ET}_B$ receptors in normal lung was shown to mediate bronchoconstriction and vasoconstriction. Whereas, activation of $\text{ET}_B$ receptors in the airway results in bronchoconstriction, and in the endothelial cells results in release of nitric oxide, and therefore vasodilation (Battistini and Dussault 1998). The inflammatory and the mitogenic activities of ET-1 are mediated by $\text{ET}_A$ receptors (Battistini and Dussault 1998).

1.9.2. Relevance of Endothelin-1 in the Pathogenesis of Recurrent Airway Obstructive Diseases

Endothelin-1 was first shown to be produced by endothelial cells and to promote potent vasoconstriction upon its release (Yanagisawa et al. 1988). Later ET-1 was shown to be a potent bronchoconstrictor, although in humans and laboratory animals...
ET-1 is two orders of magnitude less potent as bronchoconstrictor as it is vasoconstrictor (Rubanyi and Polokoff 1994). Moreover, ET-1 may induce bronchoconstriction indirectly, because ET-1 was shown to promote the release of prostaglandins from isolated lungs (De Nucci et al. 1988). Therefore, the effects of ET-1 release in the airway tissues, such as bronchoconstriction and inflammation, it likely to be a result a combination of the ET itself as well as its interaction with other mediators.

Endothelin-1 has been implicated as an important mediator in human allergic respiratory diseases, including asthma because of its potent bronchoconstrictor, secretory, mitogenic and pro-inflammatory actions (Hay et al. 1996). Inhaled ET-1 promotes rapid-onset, dose-dependent bronchoconstriction that is 100 times more potent than methacoline in asthmatics, but it has no effect in non-asthmatic human subjects (Chalmers et al. 1997). Experimentally, ET-1 has been shown to induce bronchospasm, mucus hypersecretion, leukocyte accumulation and airway remodeling including smooth muscle hypertrophy/hyperplasia, using in vivo and in vitro models in several species, including mice, guinea pigs, rats and rabbits (Noveral et al. 1992; Helset et al. 1995; Sun 1997; Fernandes 1999; Hay 1999; Sampaio 2000). Plasma and bronchoalveolar lavage fluid ET-1 concentrations in human patients with asthma are increased and correlate with the severity of signs and degree of airflow obstruction (Sofia et al. 1991; Aoki et al. 1994; Redington et al. 1995). Likewise, ET-1 concentrations are significantly greater in plasma and pulmonary epithelial lining fluid of horses affected with the hay-associated RAO, compared with control horses (Benamou et al. 1998).
There is conflicting data concerning the relative expression of $\text{ET}_A$ and $\text{ET}_B$ receptors in asthma, and there is evidence that $\text{ET}_B$ receptor may have different subtypes. The expression of $\text{ET}_A$ and $\text{ET}_B$ receptors in horses affected with SPAOPD in comparison to non-affected horses is currently under investigation.

1.9.3. Airway Epithelial Cells and Synthesis of Epithelial-Derived ET

The first evidence that endothelin was produced by airway epithelial cells, indicating that epithelium-derived endothelin was an important mediator of bronchoconstriction, came from an *in vitro* study by Black and co-workers (1989). The release of ET by canine and porcine tracheal epithelial cells cultured in media containing fetal calf serum or serum-free media was quantified using a radioimmunoassay, and liquid chromatography was used to characterize the isoforms of ET (Black et al. 1989). These unstimulated cultures of tracheal epithelial cells produced and released into the media 0.25 to 0.35 pmol/ml of immunoreactive ET, which included both ET-1 and ET-3 isoforms, as the radioimmunoassay detected ET-1 but cross-reacted 30% with ET-3. There was no significant difference in the amounts of immunoreactive ET released by cells cultured in media with and without fetal calf serum (Black et al. 1989). Liquid chromatography revealed that 75% of the total ET was of the ET-1 isoform, and the remainder was of the ET-3 isoform (Black et al. 1989). After this initial report of cultured airway epithelial cells being able to produce endothelin, a large number of studies have reported both the basal as well as up-regulated secretion of ET by airway epithelial cultures from various species, as well as localization of immunohistochemical staining for ET in airway epithelium of healthy and diseased lungs.
Immunolabelling to ET-1 was shown to occur in normal airway epithelial cells in intact tissue as well as in cultured airway epithelial cells from rabbit (Rennick et al. 1992). Similarly, localization of mature ET-1 was shown to be present in airway epithelia and submucosal glands throughout the lung of healthy humans, suggesting that these are the principal sites of ET-1 synthesis (Marciniak et al. 1992). The localization and rank order for density of ET-1 binding site was lung parenchyma > airway smooth muscle > airway epithelia, suggesting that the airway is the principal site affected by ET-1 released in paracrine and autocrine manners (Marciniak et al. 1992). Localization of immunoreactive ET-1 in airway epithelium of rats and mice indicated that among the various types of epithelial cells, the mucus, serous and Clara cells stained intensely, whereas ciliated cells and alveolar pneumocytes type II stained occasionally, and basal cells did not stain (Rozengurt et al. 1990).

The secretion of ET-1 from cultured human bronchial epithelial cells in amounts ranging from 0.65 to 2.1 pmol/ml and the ET-1 binding to co-cultured autologous bronchial smooth muscle cells provided further evidence that epithelial-derived ET-1 mediated bronchoconstriction (Mattolli et al. 1990). This finding also supported the concept that airway diseases, which are characterized clinically by airflow obstruction and airway hyperresponsiveness, both of which involve bronchoconstriction, may be associated with ET dysfunction.

The evaluation of ET-1 immunoreactivity in bronchial biopsy specimens from 17 asthmatic patients and 11 non-asthmatic controls revealed significantly greater staining in of the airway epithelium of asthmatics (11/17), compared with non-asthmatics (1/11), supporting the concept that epithelial-derived ET may contribute to the
bronchoconstriction, airway inflammation and basement membrane thickening observed in asthma (Springall et al. 1991). More recently, the immunoreactive ET-1 was localized principally in the airway epithelium, which was greater in asthmatics (35.4% ± 3.8%) compared with control subjects (16.2% ± 1.9%) (Redington et al. 1997). Although this finding supported the importance of ET-1 in the pathogenesis of asthma, the authors reported that there was no significant correlation between ET-1 immunoreactivity of the biopsies and clinical indexes, i.e., airflow obstruction, bronchial reactivity and airway inflammation (Redington et al. 1997). Immunostaining is not a very sensitive or accurate method to quantify ET-1; therefore, the lack of correlation between immunoreactive staining of tissues and clinical evidence of disease severity does not exclude the importance of ET-1 in the pathogenesis of the disease.

The expression of ET-1 was reported in airway epithelial cells obtained by bronchial biopsy from 10/10 asthmatic patients showing signs of airflow obstruction and moderate airway hyperresponsiveness, whereas only 2/10 asthmatics during clinical remission (Ackerman et al. 1995). Moreover, stimulation of airway epithelial cell cultures obtained from these patients with IL-1 for 8 to 24 hours resulted in time-dependent and dose-dependent up-regulation of ET-1 mRNA quantified using in situ hybridization, as well as up-regulation of the ET-1 peptide release into the media and quantified by radioimmunoassay (Ackerman et al. 1995).

1.9.4. Regulation of ET-1 Synthesis

The regulation of ET-1 secretion is at the level of transcription (Michael and Markewitz 1996). The transcription of ET-1 mRNA and the secretion of the peptide are induced by stimuli as shear stress, hypoxia, endotoxin and by several cytokines,
including TNF-α, IL-1β, IL-4, IL-6, IFN-β and IFN-γ (Ninomiya et al. 1991; Nakano et al. 1994; Michael and Markewitz 1996; Yang et al. 1997; Kahaleh and Fan 1997; Skopal et al. 1998). Release of ET-1 was shown to increase by the adhesion of eosinophil to airway epithelial cells via integrin adhesion molecules (Endo et al. 1997).

Cultured endothelial and epithelial cells secrete both ET-1 and big ET-1, mostly directionally, towards the basal surface of the cell, into the media (Black et al. 1989, Kahaleh and Fan 1997, Mattoli et al. 1990, Rennick et al. 1992). Monocytes and macrophages are also an important source of ET-1 (Ehrenreich et al. 1990; Ehrenreich et al. 1993; Salh et al. 1998). Biosynthesis of ET-1 occurs by polymorphonuclear leukocytes and mast cells by the enzymatic conversion of big ET-1 into the active ET-1 (Sessa et al. 1991; Wypij et al. 1992).

1.9.4.1. Effects of Endotoxin on the Release of ET-1 by Airway Epithelial Cells

Gene expression and release of ET-1 by guinea-pig airway epithelial cells and normal human bronchial epithelial cells have been shown to be stimulated by endotoxin (Ninomiya et al. 1991, Nakano et al. 1994). The release of immunoreactive ET-1 in guinea-pig airway epithelial cells stimulated for 24 hours with 4 µg/ml was twice that of unstimulated cultures, and with 10 µg/ml and 40 µg/ml, was three-times that of unstimulated cultures (Ninomiya et al. 1991). Normal human bronchial epithelial cells stimulated with LPS with dose ranging from 0.1 µg/ml to 100 µg/ml for 6 to 96 hours showed a dose-dependent and time-dependent release of immunoreactive ET-1, which was measured using a sandwich ELISA (Nakano et al. 1994). The peak of ET-1 secretion (100 pg/ml) into the media occurred at 48 hours after stimulation with LPS at a
dose of 50 µg/ml; the same dose of LPS resulted in a 3.2-fold increase in ET-1 mRNA quantified by Northern blot (Nakano et al. 1994).

A time-dependent basal release of immunoreactive ET-1 from guinea-pig tracheal cells was inhibited by phosphoamiridon, a metalloprotease inhibitor, and by ethylene glycol bis(β-aminoethylether) or (EGTA), a calcium chelator (Hay et al. 1997). The release of ET-1 was 25 pg/ml in the first day after the cultures were confluent, and after 5 days the release of ET-1 increased 10-fold (Hay et al.1997). The media contained fetal calf serum and high concentration of epithelial growth factor (EGF) at 25 µg/ml (Hay et al.1997). This growth media differed from most other reports, which generally do not contain any sera and the EGF concentration of 25 ng/ml (Whitcut et al. 1988, Kaartinen et al. 1993,Gray et al. 1996). The stimulation of guinea-pig tracheal cell cultures with LPS at concentrations 0.3 ng/ml, 1 ng/ml, 3 ng/ml and 10 ng/ml for 24 hours resulted in 2.7-fold, 3.5-fold, 4-fold and 3-fold increases in ET-1 released, respectively (Yang et al. 1997).

1.9.4.2. Effects of Cytokines on the Release of ET-1 by Airway Epithelial Cells

Pro-inflammatory cytokines, including IL-1α, IL-1β and TNF-α were shown to up-regulate the release and gene expression of ET in airway epithelial cell cultures (Nakano et al. 1994, Hay et al. 1997). The ET-1 mRNA estimated using Northern blot was shown to increase almost three-fold compared to unstimulated cultures of normal human bronchial epithelial cells (Nakano et al. 1994).

Basal and cytokine-stimulated release of ET-1 was studied in guinea-pig tracheal epithelial cells (Yang et al. 1997). Immunoreactive ET-1 was released in a time-dependent and dose-dependent manner following stimulation with pro-inflammatory
cytokines, TNF-α and IL-1β (Yang et al. 1997). The release of ET-1 into the media 24 hours after stimulation with TNF-α at 1 ng/ml, 5 ng/ml or 10 ng/ml was 12.5%, 37.5% and 62.5% respectively. Whereas the increase in ET-1 release 24 hours after stimulation with IL-1β at 1 ng/ml, 5 ng/ml and 10 ng/ml was 100%, 111% and 6.7%, respectively (Yang et al. 1997). The basal concentration of ET-1, 90 pg/ml three days after confluency and 340 pg/ml seven days after confluency, was fairly high (Yang et al. 1997). Indeed, the culture conditions in this study differed from other studies, i.e., the media contained fetal calf serum and the EGF concentration was 1,000-fold greater than in other reports.

The effect of other cytokines such as IL-4 and IL-6 on the production and release of ET-1 by airway epithelial cell cultures have not been demonstrated. The production of ET-1 by human umbilical cord vein endothelial cells stimulated in culture with IL-4 and lymphotoxin did not significantly differ from basal production (Kahaleh and Fan 1997). Similar to airway cells, the stimulation of endothelial cells with TNF-α, IL-1 induced dose-dependent increase in ET-1 production (Kahaleh and Fan 1997). Moreover, IL-6 was even more potent than TNF-α, IL-1 in inducing ET-1 release by endothelial cells (Kahaleh and Fan 1997). Airway epithelia cell lines have been shown to express IL-4 receptors (Dabbagh et al. 1999). Stimulation of airway epithelial cell cultures with IL-4 was shown to affect airway epithelial cell surface expression of adhesion molecule CD54, to increase arachidonic acid metabolism by increasing the expression of 15-lipoxygenase, and to induce mucus hypersecretion and goblet cells metaplasia (Dabbagh et al. 1999; Jayawickreme et al. 1999; Striz et al. 1999). The effects of IL-4 stimulation on ET-1 release by airway epithelial cells have not been reported.
1.9.5. Endothelin-Induced Cytokine Production by Mononuclear Leukocytes and Bronchial Epithelial Cells

Endothelin-1 at concentrations of 0.05 nM to 100 nM was shown to stimulate the secretion of TNF-α, IL-1β, and IL-6 by human monocytes and monocyte-derived macrophages in a dose-dependent and time-dependent manner (Helset et al. 1993). The optimal ET-1 stimulation for each of the cytokines resulted in 350% increase in TNF-α, 200% increase in IL-1β, and 250% IL-6 (Helset et al. 1993).

In an airway epithelial cell line, ET-1 at concentrations ranging from 100 nM and 10,000 nM induced dose-dependent a time-dependent increase in the secretion of IL-6, IL-8, and granulocytes-macrophage-colony stimulating factor (GM-CSF) (Mullol et al. 1996). The release of IL-8 increased to 68% and IL-6 increased to 43% compared to unstimulated cultures four hours after stimulation, whereas GM-CSF increased 38% after 48 hours of stimulation (Mullol et al. 1996). It was postulated that in airway diseases, epithelial-derived ET-1 might act in an autocrine manner, resulting in cytokine production by airway epithelia, culminating with amplification of the inflammatory response.

In fact, airway epithelial cells have been shown to release a number of cytokines when subjected to an insult or stimulation. Airway epithelial cells from humans were shown to produce IL-5, which is a cytokine important in the pathogenesis of human asthma (Salve et al. 1999). Interleukin-8 was expressed by cultured respiratory epithelial cell line following exposure to ozone (Jasper et al. 1997). Primary human bronchial epithelia cells and a human bronchial epithelial cell line were shown to express IL-8 following exposure to German cockroach extract, which is a common
trigger aeroallergen for asthma (Bhat et al. 2003). The direct effect of Aspergillus fumigatus in airway epithelial cells was shown to induce the expression of IL-6 and IL-8 (Borger et al. 1999).

### 1.9.6. Endothelin Derived from Leukocytes

Human monocyte-derived macrophages were shown to produce and release ET-1 and ET-3 (Ehrenreich et al. 1990). Constitutive production of ET-1 resulted in release of small quantities of immunoreactive ET-1, i.e., basal ET-1 concentration of 11 pg/ml, into the media (Ehrenreich et al. 1990). Stimulation of macrophage cultures with LPS at 10 µg/ml and phorbol myristate acetate (PMA) at 10⁻⁷ M resulted in six-fold and a ten-fold increase in immunoreactive ET-1, respectively (Ehrenreich et al. 1990). Other stimuli like infectious agent-derived are potent stimulators of ET-1 gene expression and peptide release. The surface glycoprotein gp120 of human immunodeficiency virus was shown to induce expression of TNF-α, IL-6 and ET-1 in the same magnitude as LPS (Ehrenreich et al. 1993).

Production and release of ET-1 in human monocytes stimulated with LPS was further increased by the addition of IFN-γ, IL-3 and GM-CSF in a dose-dependent manner (Salh et al. 1998). The ET-1 production was blocked by the addition of a PKC inhibitor, cycloheximide, supporting the role pf PKC in the signaling pathway of ET production (Salh et al. 1998).

Polymorphonuclear leukocytes and mast cells appear to not express ET-1; however, these cells were shown to process extracellular less active pro-peptide bigET-1 into the active peptide ET-1 (Sessa et al. 1991; Wypij et al. 1992). BigET-1 is
secreted along with ET-1 at variable ratios. Neutral proteases from polymorphonuclear leukocytes and chymase from mast cells were shown to promote the extracellular processing of bigET-1 into ET-1.

1.9.7. Recruitment of Leukocytes by ET

*In vivo* studies showed that administration of exogenous ET-1 causes an inflammatory reaction in the lung of rats, resulting in adhesion of leukocytes to the vascular endothelium, sequestration of leukocytes into pulmonary capillaries and migration of the leukocytes into the alveolar space (Helset et al. 1995). Induction of allergic inflammatory reaction in the lung and pleural cavity of mice resulted in increased release of ET-1 and recruitment of eosinophils and lymphocytes (Sampaio et al. 2000). Whereas, the treatment with ET$_A$-receptor antagonist inhibited antigen-induced eosinophil and mononuclear cell migration (Sampaio et al. 2000). In a model of allergic airway disease in mice, the administration of ET$_A$, but not ET$_B$ receptor antagonists inhibited the eosinophil and neutrophil infiltration into the lung (Fujitani et al. 1997).

*An in vitro* study with neutrophils from rabbits demonstrated that ET-3 had a dose-dependent chemotactic activity towards fMet-Leu-Phe, which was blocked by an ET$_A$ receptor antagonist (Elferik and Koster 1995). Endothelin-1 had less chemotactic and more chemokinectic activity than ET-3 (Elferik and Koster 1995).

1.10. Goals of This Dissertation

It is believed that aeroallergens are likely triggering agents of clinical exacerbation of SPAOPD, cytokines are likely associated with the anamnestic response
to aeroallergens and ET-1 is a potential mediator of the cellular responses that are the hallmarks of SPAOPD, i.e., bronchoconstriction, mucus hypersecretion, epithelial hyperplasia and recruitment and activation of neutrophils. The overall goal of this dissertation was to describe and explore the interaction of aeroallergens triggering inflammation and the T lymphocytes cytokine profile with the recruitment and activation of neutrophils and synthesis of ET by mononuclear leukocytes and epithelial cells in horses affected with SPAOPD. The studies in this dissertation were designed as a number of questions that were answered like solving pieces of a puzzle (Figure 1.4).

This dissertation describes the temporal pattern of naturally-occurring episodes of clinical exacerbation of SPAOPD, characterizing its seasonal clinical exacerbation-remission cycle and the environmental factors including aeroallergen counts. This dissertation also reports the clinical findings and the concentrations of circulating and pulmonary ET-1 during clinical exacerbation and remission of SPAOPD in comparison to the same clinical variables of non-affected horses during the same times of the year. Considering the great variability with respect to severity of the signs of clinical exacerbation of SPAOPD among horses, it was possible to subgroup the SPAOPD-affected horses according to the severity of disease and evaluate the ET-1 concentration in light of the clinical spectrum of the disease, yielding a better understanding of how ET-1 may play a role in the disease process. This dissertation also evaluated the post-mortem lung tissue for the gene expression of cytokines IL-4, IL-8 and IFN-γ and ET-1 as well as the localization of immunoreactive ET-1.
Figure 1.4. Goals of this dissertation

- Do cytokines induce ET-1 release by bronchial epithelial cells?
- Do aeroallergens stimulate mononuclear leukocytes to release ET-1?
- Does ET-1 activate & attract granulocytes?
- Do extracts of grass pollen & mold spores induce granulocyte activation directly or via mononuclear leukocytes?
- Do aeroallergens stimulate cytokine production by mononuclear leukocytes?
- Evaluate gene expression of IL-8, IL-4, IFN-γ and ET-1 in lung tissues
- Evaluate circulating and pulmonary ET-1 during clinical exacerbation and remission of SPAOPD
- Relationship of clinical exacerbation, environmental factors and aeroallergens
- Quantify and localize immunoreactive ET-1 in lung tissues
With the information gathered from the naturally-occurring episodes of clinical exacerbation, putative aeroallergens were used as aqueous protein extracts of mold spores and pollen grains in *in vitro* assays with the goal of characterizing their role in ET-1 production and neutrophil activation and chemotaxis. Moreover, based on the cytokine profiles in lung tissue and BALF of naturally-occurring episodes of clinical exacerbation, selected cytokines were used *in vitro* to stimulate cultures of differentiated airway epithelial cells to evaluate directional secretion of ET-1.

### 1.11. Endnotes


**c** Clinical observation by the author.

**d** Drs. C. Venugopal and S. Polikepahad, Department of Comparative Biology, School of Veterinary Medicine, Louisiana State University.
CHAPTER 2. TEMPORAL DISTRIBUTION OF CLINICAL EXACERBATION OF EQUINE SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE AND ITS RELATIONSHIP WITH SELECTED ENVIRONMENTAL FACTORS/VARIABLES
2.1. Introduction

Summer pasture-associated obstructive pulmonary disease (SPAOPD) is a naturally occurring, recurrent condition that affects horses residing on pasture. The disease was first reported to occur in the southeastern region of United States (Beadle, 1983). Clinical signs of SPAOPD can vary from mild to life-threatening episodes of wheezing and paroxysmal cough, and chronic debilitating breathlessness, manifested most profoundly during the summer among horses maintained on pasture (Seahorn et al. 1996). The disease is considered to be an airway hyperresponsiveness to inhaled aeroallergens that differs from the barn-associated recurrent airway obstruction (RAO), primarily with respect to environmental conditions associated with the onset of clinical exacerbation.

Even though the etiology of SPAOPD is not completely understood, it appears that seasonal changes affect the exacerbation-remission cycle of the disease. Identification of environmental factors, particularly those that precede the onset of clinical signs, would prompt the removal of horses from the offending environment during high risk times even if the triggering agents or their mechanism are not completely understood. The principal goals of this study were to document the seasonal pattern of clinical exacerbation of SPAOPD, and to identify the environmental factors associated with exacerbation of the disease.

In equine barn-associated RAO, clinical exacerbation can be induced in asymptomatic RAO-affected horses and ponies, but not in unaffected control animals, following administration of aerosolized mold/fungal spores commonly present in hay fed to stabled animals (Derksen et al., 1988; McGorum et al., 1993). Moreover, mold/fungal spores and pollen have been associated with airway hyperresponsiveness in humans.
(Solomon and Platts-Mills, 1998). Additionally, environmental factors such as temperature and humidity affect the level of mold/fungal spores, and seasonal vegetative growth is associated with pollination in plants. Therefore, temporal evaluation of climatic factors associated with clinical exacerbation of the disease and environmental aeroallergens may give insight into the potential triggering agents. The second goal of this study was to evaluate the seasonal pattern of clinical exacerbation of SPAOPD in relation to aeroallergens counts to identify possible specific inciting or triggering agents of the airway hypersensitivity.

We hypothesized that environmental variables of heat and humidity are temporally related to clinical exacerbation of SPAOPD, and amongst the aeroallergens, mold/fungal spore counts will have a seasonal pattern that parallels clinical exacerbation of the disease.

2.2. Materials and Methods

2.2.1. Clinical Data

Twenty-nine horses, including 19 SPAOPD-affected and 10 non-affected horses, were acquired by donation and maintained on pastures at Louisiana State University in Baton Rouge, Louisiana, USA. Their age and breed distribution are depicted in Figure 2.1A and 2.1B. Affected animals were middle-aged to old horses (median age 17, ranging from 12 to 28 years), predominantly of the Quarter horse-type breed (i.e., Quarter Horse, Paint, Appaloosa). The affected horses were animals that originated from south and central Louisiana (Figure 2.1C) and developed clinical signs of SPAOPD during each summer, and had pulmonary function testing documenting recurrent airway obstruction
(data not shown). Non-affected horses had pulmonary function testing within normal limits (data not shown).

Evaluation of the breathing pattern was performed daily between 7 am to 8 am \(^a\), using a standardized clinical scoring system (Seahorn and Beadle, 1997; Costa et al., 2000). The daily assessment included the nostril flare and abdominal lift, which were assigned values from 0 to 4 as depicted in Table 2.1. The clinical score of respiratory effort (CSRE) was calculated according to the following algorithm:

\[
CSRE = \frac{\text{lateral aspect of nostrils} + \text{medial aspect of nostrils}}{2} + \text{abdominal lift}.
\]

A CSRE equal or greater than 4.5 was considered as clinical exacerbation of the disease. Clinically affected horses were removed from pastures for a period of time, and after clinical improvement, they were again placed on pastures. The clinical data evaluated in this study includes only that of horses while residing on pastures, and excludes the clinical scores of the horses while kept confined in stalls.

2.2.2. Source of Climate Data

Daily values of maximum temperature (\(T_{\text{max}}\)), minimum temperature (\(T_{\text{min}}\)), mean temperature (\(T_{\text{mean}}\)), maximum dew-point temperature (\(DPT_{\text{max}}\)), minimum dew-point temperature (\(DPT_{\text{min}}\)) and delta dew-point temperature (\(DPT_{\text{delta}} = DPT_{\text{max}} - DPT_{\text{min}}\)) were obtained from hourly recordings collected at Ryan Airport in Baton Rouge, Louisiana \(^b\). The location of the airport is approximately 9 miles from the pastures, which are located at approximately 30.4N latitude and 91.9W longitude.
Figure 2.1. Breed (A) and age (B) distributions of all horses included in the study, and place of origin (C) of the horses affected with summer-pasture associated obstructive pulmonary disease.
Table 2.1. Calculation of clinical score of respiratory effort. Numerical score of nostril flare and abdominal lift used for calculation of clinical score of respiratory effort (CSRE) as an estimate of clinical exacerbation of the disease. The formula used to calculate the CSRE: CSRE = (lateral aspect nostril + medial aspect of the nostril)/2 + abdominal lift. Horses were considered to have clinical exacerbation of summer-pasture associated obstructive pulmonary disease when CSRE ≥ 4.5.

<table>
<thead>
<tr>
<th>Numerical Score</th>
<th>Nostril Flare</th>
<th>Abdominal Lift</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No or very slight movement noted on inspiration</td>
<td>No or very little appreciable movement in the ventral flank</td>
</tr>
<tr>
<td>1</td>
<td>Aspect of the nostril flares only slightly during inspiration, returning to a normal position at the end of inspiration</td>
<td>Slight abdominal flattening with “heave line” just beginning to form in the cranial aspect of the ventral flank (not easily detectable)</td>
</tr>
<tr>
<td>2</td>
<td>Aspect of the nostril flares during inspiration, returning to a near normal position as it ends</td>
<td>Abdominal flattening is obvious and a “heave line” extending to a point halfway between the tuber coxae and the elbow (fairly easy to detect)</td>
</tr>
<tr>
<td>3</td>
<td>Aspect of the nostril is flared to a greater extent during inspiration and does not approach a normal position during exhalation</td>
<td>Abdominal flattening and a “heave line” extending beyond a point halfway between the tuber coxae and the elbow, but does not extend all the way to the elbow (easy to detect)</td>
</tr>
<tr>
<td>4</td>
<td>Aspect of the nostril is flared and remains maximally flared throughout the respiratory cycle</td>
<td>Abdominal flattening and a “heave line” extending cranially all the way to the elbow (very easy to detect)</td>
</tr>
</tbody>
</table>
2.2.3. Source of Vegetation Data

Earth-orbiting environmental satellites provide a system of measurements of earth surface conditions down-linked on a daily-weekly time lapse basis, which allows large area surveillance and detection of conditions favorable to the onset of SPAOPD clinical conditions. Remotely sensed measurements of visible and near infrared radiation by satellite-borne radiometers provide rapid, time-lapse estimates of the plant canopy-chlorophyll conditions and spectral vegetation indices (Yates et al., 1984; Norwine and Greegor, 1983; Tucker, Gatlin and Schneider, 1984; McGinnis and Tarpley, 1985). The spectral vegetation indices are the difference in absorption and reflectance of solar radiation in the visible/near infrared portion of the spectrum (Yates et al, 1984). The photosynthetically active radiation is strongly absorbed at visible wavelengths, whereas stressed, dead, or no vegetation strongly reflect all wavelengths of solar radiation (Asrar, 1989). Among the two possible operational indices, the simple vegetation index (VI) and the normalized difference vegetation index (NDVI), the latter is preferred for operational vegetation monitoring because it provides compensation for a number of factors, e.g., varied illumination conditions, surface slope, view angle/aspect and cloud shadow (Huh, 1991).

For this study, a weekly or biweekly NDVI was obtained for the period of three years (1992-1994) from USGS Eros data center, Sioux Falls, South Dakota. The NDVI was calculated from available suitable maps generated from satellite images recorded between 10:30 am and 2:30 pm by NOAA 11 (ID 19531) or NOAA 12 (ID 21263) satellites (Huh, 1991). Images with 90% or greater visibility of the Atchafalaya and Mississippi River valley were selected manually. NDVI was calculated from values for Channel 1 (CH1) and Channel 2 (CH2) obtained from a 250 square miles rectangular template
centered at 30.41N latitude and 91.9 W longitude according to the following formula: NDVI = CH2 - CH1 / CH2 + CH1.

2.2.4. Source of Aeroallergen Data

Qualitative allergen counts for the year 1994 were obtained from the Acadiana Allergy and Asthma Center, Louisiana Pollen and Mold/fungal Center, Lafayette, Louisiana. The average counts for a 24-hour period were collected (30 seconds every 10 minutes) and recorded. Daily reports of environmental pollen and mold/fungal spores included the total counts and the specific counts of 28 types of pollen and 20 types of mold/fungal spores. The allergen counts were grouped into four groups of allergens, i.e., tree pollen, grass pollen, herbaceous plant pollen (Table 2.2) and mold/fungal spores (Table 2.3).

2.2.5. Clinical and Climatic Evaluation of Seasonality

The temporal pattern of clinical exacerbation and the climatic variables (i.e., $T_{\text{max}}$, $T_{\text{min}}$, $T_{\text{mean}}$, $DPT_{\text{max}}$, $DPT_{\text{mean}}$ and $DPT_{\text{min}}$) was evaluated for three years. The daily frequency of CSRE equal or greater than 4.5 for the three years was graphed in conjunction with the variations in the environmental factors, including $T_{\text{max}}$, $T_{\text{min}}$, $T_{\text{mean}}$, $DPT_{\text{max}}$, $DPT_{\text{min}}$, $DPT_{\text{delta}}$ and the Julian date. In addition, the first episode of clinical exacerbation (CSRE $\geq 4.5$) was considered as the seasonal onset of clinical exacerbation for each given horse, and it was recorded for each of the three years. The calendar and Julian dates of the first episode were listed for each horse each year in order to highlight individual variations.
Table 2.2. Aerallergen types: categories of pollen grains. Information from the Acadiana Allergy and Asthma Center, Louisiana Pollen and Mold Center, Lafayette, Louisiana.

<table>
<thead>
<tr>
<th>Pollen Grains</th>
<th>Family or Latin name</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>Cyperaceae</td>
<td>Sedge family</td>
</tr>
<tr>
<td></td>
<td>Gramininae/ Poacea</td>
<td>Grass family</td>
</tr>
<tr>
<td>Tree</td>
<td>Acer</td>
<td>Maple, box elder</td>
</tr>
<tr>
<td></td>
<td>Alnus</td>
<td>Alder</td>
</tr>
<tr>
<td></td>
<td>Betulaceae</td>
<td>Birch family and alikes</td>
</tr>
<tr>
<td></td>
<td>Carya</td>
<td>Hickory, pecan</td>
</tr>
<tr>
<td></td>
<td>Cupressaceae</td>
<td>Juniper family, cedar</td>
</tr>
<tr>
<td></td>
<td>Fagus</td>
<td>Beech</td>
</tr>
<tr>
<td></td>
<td>Fraxinus</td>
<td>Ash</td>
</tr>
<tr>
<td></td>
<td>Juglans</td>
<td>Walnut, butternut</td>
</tr>
<tr>
<td></td>
<td>Liquidambar</td>
<td>Sweet gum</td>
</tr>
<tr>
<td></td>
<td>Morus</td>
<td>Mulberry</td>
</tr>
<tr>
<td></td>
<td>Oleaceae/Olea</td>
<td>Olive family</td>
</tr>
<tr>
<td></td>
<td>Pinaceae</td>
<td>Pine family and alikes</td>
</tr>
<tr>
<td></td>
<td>Platanus</td>
<td>Sycamore</td>
</tr>
<tr>
<td></td>
<td>Populus</td>
<td>Poplar, cottonwood</td>
</tr>
<tr>
<td></td>
<td>Quercus</td>
<td>Oak</td>
</tr>
<tr>
<td></td>
<td>Salix</td>
<td>Willow</td>
</tr>
<tr>
<td></td>
<td>Tilia</td>
<td>Basswood, linden</td>
</tr>
<tr>
<td></td>
<td>Ulmus</td>
<td>Elm</td>
</tr>
<tr>
<td>Herbaceous plants</td>
<td>Ambrosia/Franseria</td>
<td>Ragweed</td>
</tr>
<tr>
<td></td>
<td>Artemisia</td>
<td>Sage, wormwood</td>
</tr>
<tr>
<td></td>
<td>Chenopodiaceae/Amaranthaceae</td>
<td>Composites aster family except ragweed</td>
</tr>
<tr>
<td></td>
<td>Compositae/ other Asteraceae</td>
<td>Plantain</td>
</tr>
<tr>
<td></td>
<td>Plantago</td>
<td>Sheep sorrel, dock</td>
</tr>
<tr>
<td></td>
<td>Rumex</td>
<td>Cattail</td>
</tr>
<tr>
<td></td>
<td>Typha</td>
<td>Nettle, pellitory</td>
</tr>
</tbody>
</table>
Table 2.3. Aeroallergen types: categories of mold spores. Information from the Acadiana Allergy and Asthma Center, Louisiana Pollen and Mold Center, Lafayette, Louisiana.

<table>
<thead>
<tr>
<th>Mold/fungus spores</th>
<th>Types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alternaria</td>
</tr>
<tr>
<td></td>
<td>Ascospore</td>
</tr>
<tr>
<td></td>
<td>Basidiospore</td>
</tr>
<tr>
<td></td>
<td>Botrytis</td>
</tr>
<tr>
<td></td>
<td>Cercospora</td>
</tr>
<tr>
<td></td>
<td>Cladosporium</td>
</tr>
<tr>
<td></td>
<td>Curvularia</td>
</tr>
<tr>
<td></td>
<td>Drechslera/ Helminthosporium</td>
</tr>
<tr>
<td></td>
<td>Epicoccum</td>
</tr>
<tr>
<td></td>
<td>Erysiphe/Oidium</td>
</tr>
<tr>
<td></td>
<td>Fusarium</td>
</tr>
<tr>
<td></td>
<td>Nigrospora</td>
</tr>
<tr>
<td></td>
<td>Periconia</td>
</tr>
<tr>
<td></td>
<td>Peronospora</td>
</tr>
<tr>
<td></td>
<td>Pithomyces</td>
</tr>
<tr>
<td></td>
<td>Polythricium</td>
</tr>
<tr>
<td></td>
<td>Rusts</td>
</tr>
<tr>
<td></td>
<td>Smuts</td>
</tr>
<tr>
<td></td>
<td>Stemphylium</td>
</tr>
<tr>
<td></td>
<td>Torula</td>
</tr>
</tbody>
</table>

2.2.6. Association of Clinical Data with Measures of Temperature

The median and mode of CSRE from the group of affected horses on a given date (from January 1st, 1992 to December 31st, 1994) were calculated. These scores were then transformed to a binomial variable by setting the criterion that a median or mode score equal or greater than 4.5 was equivalent to clinical exacerbation of the disease. The binomial scores were then regressed on Julian date, $T_{max}$, $T_{min}$, $T_{mean}$, DPT$_{max}$, DPT$_{min}$, DPT$_{delta}$ using logistic regression (Proc LOGIT, SAS Institute 1990). The log odds ratios were deemed significant if the confidence limits did not include the value 1, where a value of 1 would indicate no significant effect. A backward elimination logistic regression analysis incorporating the environmental variables except NDVI ($T_{max}$, $T_{min}$, $T_{mean}$, DPT$_{max}$, DPT$_{min}$, DPT$_{delta}$).
DPT_{\text{min}} \text{ and } DPT_{\text{delta}} \) was then used to determine which of the environmental variables best explained the variations in clinical scores.

### 2.2.7. Association of Clinical Data with Vegetation Data

The weekly NDVI obtained for the three years (1992 through 1994), and the mean weekly clinical scores for three years (1992 through 1994) were used for the association analysis. The binomial clinical scores were then regressed as mentioned above and the mean weekly clinical scores were analyzed against the weekly NDVI using logistic regression.

### 2.2.8. Temporal Patterns of Clinical Exacerbation, Climatic Data and Aeroallergen Counts

We evaluated the temporal distribution pattern of each of the aeroallergen categories (mold/fungal spore, grass pollen, tree pollen and herbaceous plant pollen) in relation to the pattern of clinical exacerbation of the disease for one year (1994). We also evaluated the temporal distribution pattern of each of the aeroallergen categories in relation to the climate variables (i.e., T_{\text{max}}, T_{\text{min}}, T_{\text{mean}}, \text{DPT}_{\text{max}}, \text{DPT}_{\text{delta}} \text{ and } \text{DPT}_{\text{min}}). \text{ In addition, counts of each aeroallergen type (i.e., 20 types of mold/fungal and 28 types of pollen) were evaluated individually in relation to clinical exacerbation and environmental temperature variables (i.e., T_{\text{max}}, T_{\text{min}}, T_{\text{mean}}, \text{DPT}_{\text{max}}, \text{DPT}_{\text{delta}} \text{ and } \text{DPT}_{\text{min}}) for the year 1994.}

### 2.3. Results

The SPAOPD-affected horses maintained on pastures had a clear seasonal onset of clinical exacerbation starting in late spring. Evaluation of each individual horse and the dates of the first episode of clinical exacerbation each year revealed a seasonal onset
varying from May to July (Table 2.4). Moreover, the dates of onset of clinical exacerbation for each given horse was fairly consistent from year to year, ranging from two to three weeks difference (Table 2.4).

Horses demonstrating severe signs of exacerbation of the disease were removed from pasture environment until clinical signs subsided. While on pastures, the number of horses showing signs of clinical exacerbation of the disease (CSRE ≥ 4.5) increased as the measures of environmental temperature (i.e., \( T_{\text{max}}, T_{\text{min}}, T_{\text{mean}}, DPT_{\text{max}} \) and \( DPT_{\text{min}} \)) increased (Figure 2.2). The data are displayed as the proportion of horses on pastures that were showing signs of clinical exacerbation. The clinical exacerbation of SPAOPD persisted until late fall, i.e., October and November. Non-affected horses kept at pasture conditions had no appreciable changes in clinical score, i.e., CSRE remained < 4.5, throughout the seasons of the year (data not shown).

For this group of horses in these pastures, the probability of onset of clinical exacerbation was closely associated with the calendar date across all three years, and the fifty percent probability of clinical exacerbation of the disease was day 161 (June 10\(^{th}\)) for all three years, 1992, 1993 and 1994 (Table 2.5). Regardless of whether the median or mode CSRE value was used in the logistic regression, the statistical model predicted similar values of Julian date, temperature and dew-point temperature variables associated with the probability of clinical exacerbation. The logistic model fits both mode and median CSRE similarly well (P> 0.5). The probability of significant CSRE was equally predicted by the temperature and dew-point temperature, whether either median or mode scores were used (t-test, P> 0.05).
Table 2.4. Date of onset of clinical exacerbation of summer pasture-associated obstructive pulmonary disease

<table>
<thead>
<tr>
<th>Onset of Clinical Exacerbation</th>
<th>Year 1992</th>
<th>Year 1993</th>
<th>Year 1994</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse # 1</td>
<td>6/15</td>
<td>7/1</td>
<td>6/21</td>
</tr>
<tr>
<td>Horse # 2</td>
<td>4/30</td>
<td>4/28</td>
<td>3/5</td>
</tr>
<tr>
<td>Horse # 3</td>
<td>6/15</td>
<td>7/2</td>
<td>7/1</td>
</tr>
<tr>
<td>Horse # 4</td>
<td>5/15</td>
<td>6/4</td>
<td>6/21</td>
</tr>
<tr>
<td>Horse # 5</td>
<td>Not in the study</td>
<td>7/15</td>
<td>8/2</td>
</tr>
<tr>
<td>Horse # 6</td>
<td>6/16</td>
<td>6/25</td>
<td>6/7</td>
</tr>
<tr>
<td>Horse # 7</td>
<td>5/3</td>
<td>5/8</td>
<td>Not in the study</td>
</tr>
<tr>
<td>Horse # 8</td>
<td>7/7</td>
<td>7/20</td>
<td>7/25</td>
</tr>
<tr>
<td>Horse # 9</td>
<td>6/9</td>
<td>7/2</td>
<td>6/21</td>
</tr>
<tr>
<td>Horse # 10</td>
<td>5/18</td>
<td>6/27</td>
<td>6/16</td>
</tr>
<tr>
<td>Horse # 11</td>
<td>5/13</td>
<td>5/11</td>
<td>No Remission</td>
</tr>
<tr>
<td>Horse # 12</td>
<td>5/6</td>
<td>5/7</td>
<td>5/13</td>
</tr>
<tr>
<td>Horse # 13</td>
<td>5/15</td>
<td>5/25</td>
<td>No Remission</td>
</tr>
<tr>
<td>Horse # 14</td>
<td>5/28</td>
<td>6/27</td>
<td>6/15</td>
</tr>
<tr>
<td>Horse # 15</td>
<td>5/5</td>
<td>5/7</td>
<td>3/24</td>
</tr>
<tr>
<td>Horse # 16</td>
<td>5/12</td>
<td>No Remission</td>
<td>No Remission</td>
</tr>
<tr>
<td>Horse # 17</td>
<td>7/4</td>
<td>7/8</td>
<td>Not in the study</td>
</tr>
<tr>
<td>Horse # 18</td>
<td>5/12</td>
<td>5/26</td>
<td>Not in the study</td>
</tr>
<tr>
<td>Horse # 19</td>
<td>5/15</td>
<td>5/31</td>
<td>2/16</td>
</tr>
</tbody>
</table>
Although there was no significant difference when the analysis was performed using median or mode CSRE, the median values were chosen for presentation because the median value is a better descriptor when quantification of levels of effect is of interest (Zar, 1996). The specific changes in each of the temperature variables were closely associated with the onset of clinical exacerbation (Table 2.6). There was a close correlation between the probability of disease and the temperature variables, $T_{\text{mean}}, T_{\text{min}},$ and $T_{\text{max}}$ (log odds ratios 1.09, 1.087, 1.08, respectively), as well as the dew-point temperature variables, $DPT_{\text{delta}}, DPT_{\text{min}},$ and $DPT_{\text{max}}$ (log odds ratios 0.861, 1.08, 1.085, respectively). Several of these variables appeared to be redundant in the sense that $T_{\text{min}}, T_{\text{max}}$ and $T_{\text{mean}}$ each provided similar information, and thus only one was selected from backward regression analysis. In the backward regression analysis, both a measure of one of the temperature variables alone, $T_{\text{max}},$ and a measure of one of the dew-point temperature variables, $DPT_{\text{min}},$ were included as the only variables in the explanatory model.

The NDVI provides a time-lapse estimate of plant canopy-chlorophyll conditions, indicating vegetation growth. Although NDVI appeared to have a strong correlation with CSRE (odds ratio = 1.32), the abundant variation in the data set led to a non-significant slope parameter (data not shown). The intent was to sample NDVI weekly or biweekly, however limitation on the number of usable satellite images, primarily due to cloud cover, resulted in incomplete data coverage. Only 10% (74/736) of the data attempted to be collected was usable. In addition, variation between consecutive measurements was often quite dramatic, which limited the usefulness of a single weekly value of this index.
Table 2.5. Probability of a significant clinical score of respiratory effort (CSRE ≥ 4.5) as evidence of clinical exacerbation of summer pasture-associated obstructive pulmonary disease. Median Julian date and environmental variables (T_{max}, T_{min}, T_{mean}, DPT_{max}, DPT_{min}, DPT_{delta}) predicted by logistic regression analysis.

<table>
<thead>
<tr>
<th>P</th>
<th>Julian Date</th>
<th>T_{max} (°C)</th>
<th>T_{min} (°C)</th>
<th>T_{mean} (°C)</th>
<th>DPT_{max} (°C)</th>
<th>DPT_{min} (°C)</th>
<th>DPT_{delta} (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>124</td>
<td>13.9</td>
<td>5.0</td>
<td>9.7</td>
<td>8.1</td>
<td>1.6</td>
<td>10.2</td>
</tr>
<tr>
<td>0.2</td>
<td>137</td>
<td>20.0</td>
<td>10.0</td>
<td>14.9</td>
<td>13.3</td>
<td>7.7</td>
<td>7.2</td>
</tr>
<tr>
<td>0.3</td>
<td>146</td>
<td>23.9</td>
<td>13.9</td>
<td>18.3</td>
<td>16.6</td>
<td>11.6</td>
<td>5.5</td>
</tr>
<tr>
<td>0.4</td>
<td>154</td>
<td>27.2</td>
<td>16.7</td>
<td>21.3</td>
<td>20.0</td>
<td>15.0</td>
<td>3.9</td>
</tr>
<tr>
<td>0.5</td>
<td>161</td>
<td>30.0</td>
<td>18.9</td>
<td>23.5</td>
<td>22.7</td>
<td>17.3</td>
<td>2.2</td>
</tr>
<tr>
<td>0.6</td>
<td>168</td>
<td>32.8</td>
<td>20.9</td>
<td>25.3</td>
<td>24.7</td>
<td>18.9</td>
<td>0.6</td>
</tr>
<tr>
<td>0.7</td>
<td>175</td>
<td>34.6</td>
<td>23.1</td>
<td>26.6</td>
<td>27.0</td>
<td>20.6</td>
<td>--</td>
</tr>
<tr>
<td>0.8</td>
<td>184</td>
<td>36.4</td>
<td>25.4</td>
<td>28.3</td>
<td>30.2</td>
<td>22.1</td>
<td>--</td>
</tr>
<tr>
<td>0.9</td>
<td>198</td>
<td>38.4</td>
<td>28.0</td>
<td>30.1</td>
<td>34.4</td>
<td>24.2</td>
<td>--</td>
</tr>
<tr>
<td>1.0</td>
<td>238</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

* P indicates probability of a significant CSRE.
Table 2.6. The effect of environmental variables on clinical score of respiratory effort. Log odds ratios and confidence intervals from separate logistic regression of the effects of environmental variables ($T_{\text{max}}$, $T_{\text{min}}$, $T_{\text{mean}}$, $DT_{\text{max}}$, $DT_{\text{min}}$, $DT_{\text{delta}}$) on clinical score of respiratory effort (CSRE).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dependent</th>
<th>Independent</th>
<th>Log odds ratio</th>
<th>Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSRE</td>
<td>$T_{\text{max}}$</td>
<td>1.080 $^s$</td>
<td>1.066—1.094</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_{\text{min}}$</td>
<td>1.087 $^s$</td>
<td>1.073—1.100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$T_{\text{mean}}$</td>
<td>1.090 $^s$</td>
<td>1.075—1.104</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$DT_{\text{max}}$</td>
<td>1.085 $^s$</td>
<td>1.070—1.100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$DT_{\text{min}}$</td>
<td>1.080 $^s$</td>
<td>1.068—1.093</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$DT_{\text{delta}}$</td>
<td>0.861 $^s$</td>
<td>0.834—0.889</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NDVI</td>
<td>1.320 ns</td>
<td>0.094—18.524</td>
<td></td>
</tr>
</tbody>
</table>

$s$ significant and $ns$ not significant.

The aeroallergen data, which included mold/fungal spore and pollen counts, were only available for the year 1994. Visual inspection of the aeroallergen data showed that the pattern of mold/fungal spore counts over the calendar year paralleled the proportion of significant CSRE and the changes in temperature, humidity (Figures 2.3 and 2.7). High mold/fungal spore counts (total counts) occurred during the same calendar periods that temperature and dew-point temperatures were high and during clinical exacerbation. Inspection of the pollen data showed an increase in grass pollen at the same time the increase in the proportion of horses with CSRE $\geq$ 4.5, continued throughout the summer and tapered off in the fall (Figures 2.3 and 2.4). The increase in grass pollen coincided with increases in the temperature and dew-point temperature.
Figure 2.2 Temporal distribution (1992 to 1994) of clinical exacerbation of SPAOPD in relation to environmental variables. Julian date is shown on the X-axis, temperature [maximum (Tmax), mean (Tmean), minimum (Tmin)] or dew-point temperature [maximum (DPTmax), minimum (DPTmin), delta (DPTdelta = DPTmax - DPTmin)] are shown on the Y1-axis (solid line), and the proportion of horses showing signs of clinical exacerbation of SPAOPD (CSRE ≥ 4.5) while kept on pasture is shown on the Y2-axis (grey fill area).
Figure 2.3. Temporal distribution of clinical exacerbation of SPAOPD in relation to aeroallergens counts in 1994. Julian date is shown on the X-axis, aeroallergen counts (grass pollen, tree pollen, herbaceous plant pollen and mold/fungal spore) are shown on the Y1-axis (solid line), and the proportion of horses showing signs of clinical exacerbation of SPAOPD (CSRE ≥ 4.5) while kept on pastures is shown on the Y2-axis (grey fill area).
Figure 2.4. Temporal distribution of grass pollen counts in 1994 in relation to the environmental variables. Julian date is shown on the X-axis, grass pollen counts are shown by the grey fill area (Y1-axis), and temperature [maximum (Tmax), mean (Tmean), minimum (Tmin)] and dew-point temperature [maximum (DPTmax), minimum (DPTmin), delta (DPTdelta = DPTmax - DPTmin)] are shown by the solid line (Y2-axis).
Figure 2.5. Temporal distribution of tree pollen counts for year 1994 in relation to the environmental variables. The X-axis indicates Julian date, tree pollen counts are shown on the Y1-axis (grey fill area), and measures of temperature (Tmax, Tmin, Tmean) and dew-point temperature (DPTmax, DPTmin, DPTdelta) are shown on the Y2-axis (solid line).
Figure 2.6. Temporal distribution of herbaceous plant pollen counts for year 1994 in relation to the environmental variables. Julian date is shown on the X-axis, herbaceous plant pollen counts are shown on the Y1-axis (filled grey area), and measures of temperature (Tmax, Tmin, Tmean) and dew-point temperature (DPTmax, DPTmin, DPTdelta) are shown on the Y2-axis (solid line).
Figure 2.7. Temporal distribution of mold/fungal spore counts for year 1994 in relation to the environmental variables. Julian date is shown on the X-axis, mold/fungal spore counts are shown on the Y1-axis (filled grey area) and measures of temperature (Tmax, Tmin, Tmean) and dew-point temperature (DPTmax, DPTmin, DPTdelta) are shown on the Y2-axis (solid line).
Figure 2.8. Temporal distribution of clinical exacerbation of SPAOPD in relation to specific types of mold/fungal spores in 1994. Julian date is shown on the X-axis, the counts of specific types of mold/fungal spore is shown by the solid line (Y1-axis), and the proportion of horses showing signs of clinical exacerbation of SPAOPD (CSRE ≥ 4.5) while kept on pastures is shown by the grey fill area (Y2-axis).
Figure 2.8 continued
Figure 2.8 continued
Figure 2.8 continued
Figure 2.8 continued
Figure 2.9. Temporal distribution of selected types of mold/fungal spore counts for year 1994 in relation to the environmental variables. Julian date is shown on the X-axis, counts of selected types of mold/fungal spores are shown on the Y1-axis (grey filled area), and measures of temperature (Tmax, Tmin, Tmean) and dew-point temperature (DPTmax, DPTmin, DPTdelta) are shown on the Y2-axis (solid line).
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
Figure 2.9. Continued
The temporal distribution of tree pollen counts and herbaceous plant pollen counts did not parallel the temporal distribution of horses with CSRE $\geq 4.5$ or the increases in the climatic variables (Figures 2.3, 2.5 and 2.6).

Evaluation of the temporal distribution of horses with CSRE $\geq 4.5$ and counts of specific types of mold/fungal spores revealed good similarity with Basidiospore, *Nigrospora* and *Curvularia*, followed by *Alternaria*, *Cladosporium*, *Drechslera/Helminthosporium*, *Cercospora*, *Periconia*, *Peronospora*, and Smuts; the increases in counts of other types of mold/fungal spores, i.e., Ascospore, *Botrytis*, *Epicoccum*, *Erysiphe/Oidium*, *Fusarium*, *Pithomyces*, *Polythrincium*, Rusts, *Stemphylium* and *Torula* did not coincide with the increase in the proportion of horses showing signs of clinical exacerbation (Figure 8). Moreover, comparison of the temporal distribution of specific types of mold/fungal spore and measures of heat and humidity indicated similarity with Basidiospore, *Nigrospora*, *Curvularia*, and to a lesser extent *Alternaria*, *Cercospora*, *Cladosporium*, *Drechslera/Helminthosporium*, *Periconia*, *Peronospora*, and Smuts (Figure 2.9).

2.4. Discussion

The temporal distribution of the clinical exacerbation of SPAOPD confirms the seasonal pattern of the disease during the warm and humid months of the year. Among the factors evaluated in this study, calendar date, climate variables of heat ($T_{\text{max}}$, $T_{\text{min}}$, $T_{\text{mean}}$) and humidity ($DPT_{\text{max}}$, $DPT_{\text{mean}}$, and $DPT_{\text{min}}$) temporally paralleled the onset and persistence of clinical exacerbations of SPAOPD in Louisiana. The tight clustering of Julian date of onset each year indicates that clinical exacerbation is fairly predictable with respect to calendar date in this region. Of course, in cases of significant climatic aberrations, clinical exacerbation may not occur around the same calendar date.
The seasonal occurrence of SPAOPD has a strong relationship with increases in temperature and dew-point temperature variables, the latter being an estimate of humidity. The similarity in temporal patterns could be either a direct effect or, most likely, due to the presence of environmental conditions that foster changes such as increased aeroallergens. The onset of clinical exacerbation of SPAOPD in the group of horses studied here was temporally associated with hot and humid weather conditions, and especially worsened after a rain (empirical observation). In contrast, clinical exacerbation of SPAOPD in the United Kingdom is observed in April and May, during hot dry weather or after exposure to dust generated during harvest or burns of nearby crops (Dixon and McGorum, 1990, Mair, 1996; McGorum et al., 1999).

Considering that the environmental temperature and dew-point temperature variables affect some of the aeroallergen counts evaluated in this study, one could speculate that these aeroallergens might be potential triggering agents of SPAOPD. Indeed, the seasonal pattern of clinical exacerbation of SPAOPD paralleled the temporal distribution of higher mold/fungal spore counts and grass pollen counts. Specifically, the distribution of mold/fungal spore counts of Curvularia, Basidiospore, Nigrospora, and, to a lesser extent, Alternaria, Cladosporium, Cercospora, Drechslera Helminthosporium, Periconia, Peronospora, and Smuts best matched the seasonal pattern of clinical exacerbation of SPAOPD.

If the onset of clinical signs of SPAOPD were related to the presence of particular pollens, then the stage of maturation of the vegetation associated with pollination would be a good predictor of disease exacerbation. Therefore, the NDVI, which is a measure of ‘greenness’ determined by comparing the absorption and reflection of different spectra of the light spectrum by surface structures on earth, would be strongly associated with onset
of clinical signs. Although a change in NDVI was well associated with the occurrence of clinical signs, the frequency with which we were able to obtain NDVI measurements was insufficient to make the index useful.

Our study suggests that SPAOPD results from seasonal exposure to the so-called natural, resource-related/outdoor aeroallergens, which include mold/fungal spores and grass pollens (Solomon and Platts-Mills, 1998; Kurup et al., 2000). A number of mold/fungal spores, such as Curvularia, Alternaria and Cladosporium species, have seasonal spore releasing patterns (Solomon Platts-Mills, 1998; Kurup et al., 2000) and are potential triggering agents in SPAOPD (Seahorn et al. 1997). Among the grasses growing in the southeastern coastal plains, Bermuda grass is the most common, followed by Johnson grass (Solomon and Platts-Mills, 1998). Pollen shedding of Bermuda grass occurs principally from March to September, and Johnson grass during summer and fall (Solomon and Platts-Mills, 1998; Harris and Madley 1961), both of which coincide with the time of clinical exacerbation of SPAOPD. The horses evaluated in this study were residing in a pastures containing predominantly Bermuda grass.

This is the first report evaluating seasonal conditions as they relate to the onset of clinical exacerbation of SPAOPD in the southeastern region of the United States. Although preliminary, the information learned from the present study will be useful for limiting the number of potential aeroallergens from among the many candidates for in vivo and in vitro study of immune responses. The study suggests that herbaceous plant and tree pollens are unlikely candidates whereas it more strongly supports mold/fungal spores and possibly grass pollen as areas for exploration. Nonetheless, considering the great daily fluctuations and the spatial gradients of aeroallergen levels, further studies are necessary to
specifically sample the aeroallergen particles that the horses are exposed to on pastures during the time preceding the onset of clinical exacerbation.

In conclusion, SPAOPD shows a distinct seasonal pattern of clinical exacerbation and remission cycle, the onset of exacerbation being strongly related to hot (high temperature variables) and humid (high dew-point temperature variables) weather. Changes in NDVI were weakly associated with the onset of clinical signs. Increases in mold/fungal spore counts and, to a lesser extent, grass pollen counts paralleled higher frequency of significant CSRE. Therefore, among the known aeroallergens groups, mold/fungal spores and potentially grass pollen, but not tree and herbaceous plant pollen, appear to be the most likely triggering agents of clinical exacerbation of SPAOPD.

2.6. Endnotes

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CHAPTER 3. QUANTIFICATION OF IMMUNOREACTIVE ENDOTHELIN IN PLASMA AND PULMONARY SECRETIONS DURING REMISSION AND CLINICAL EXACERBATION OF SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE IN HORSES
3.1. Introduction

Summer pasture-associated obstructive pulmonary disease (SPAOPD), first described in the early 1970’s, is a naturally-occurring recurrent airway obstructive disease of horses residing on pasture in the southeastern United States (Beadle1983). The disease is characterized by airway hyperresponsiveness and airflow obstruction following exposure to pasture during the late-spring to mid-fall (Seahorn and Beadle 1993).

Endothelins are potent bronchoconstrictors, mitogens, secretagogues and pro-inflammatory mediators (Rubanyi and Polokoff 1994). There are three isoforms of endothelin, ET-1, ET-2 and ET-3, encoded by three different genes. The lung is the major site of ET-1 and ET-3 expression, as well as ET clearance (Fagan et al. 2001). The most studied isoform, ET-1, has been implicated in the pathogenesis of airway hyperresponsive diseases such as human asthma and equine recurrent airway obstruction (RAO) formerly called equine chronic obstructive pulmonary disease (Aoki et al. 1994; Benamou et al. 1998).

The overall objective of this study was to evaluate the variations in circulating and pulmonary immunoreactive ET-1 concentrations during clinical exacerbation and remission of SPAOPD. We first describe the exacerbation-remission pattern of the disease. Then, we determine and compare the concentrations of ET-1 in pulmonary secretions, arterial and venous plasma samples obtained from non-affected control horses and horses affected with SPAOPD during different times of the year. We hypothesized that immunoreactive ET-1 concentrations would be increased during clinical exacerbation of the disease.
3.2. Methods and Materials

3.2.1. Maintenance and Monitoring of Animals and Experimental Design

The experimental protocol of this study was approved by the Institutional Animal Care and Use Committee of Louisiana State University. Twelve horses, including six SPAOPD-affected (one castrated male and five intact females; median age 15 years, ranging from 7 to 27) and six non-affected control horses (four castrated males and two intact females; median age 15 years, ranging from 14 to 17) were maintained on pastures at Louisiana State University in Baton Rouge, Louisiana, USA (Figure 3.1A and 3.1B).

The affected horses were acquired by donation. They were animals that originated from south Louisiana (Figure 3.2) and developed recurrent signs of obstructive airway disease during the summer. All animals, including the SPAOPD-affected and the non-affected horses were evaluated clinically, including pulmonary function testing and cytologic analysis of bronchoalveolar lavage fluid (BALF). All affected horses while showing signs of clinical exacerbation, had pleural pressure differences greater than 15 cm H$_2$O and neutrophilic inflammation on cytologic analysis of bronchoalveolar lavage fluid (BALF), therefore documenting recurrent airway obstruction (data not shown).

Evaluation of the breathing pattern was performed daily using a previously described clinical scoring system (Costa et al., 2000). Daily assessment included nostril flaring and abdominal lift subjectively scored by assigning values from 0 to 4 as depicted in Table 3.1, and the clinical score of respiratory effort (CSRE) was calculated. A CSRE equal to or greater than 4.5 was considered as clinical exacerbation of the disease.
Figure 3.1. Age and breed distributions of all horses included in the study. In the top graph, the box indicates the interquartile range of age, the line indicates the median age and the whiskers indicate the range of ages of horses.
Figure 3.2. Place of origin of the horses affected with summer-pasture associated obstructive pulmonary disease. All affected horses originated from parishes in south Louisiana.
Table 3.1. Numerical score of nostril flare and abdominal lift used for calculation of clinical score of respiratory effort (CSRE) as an estimate of clinical exacerbation of the disease. The following formula was used to calculate CSRE: \[ CSRE = \frac{\text{lateral aspect of nostrils} + \text{medial aspect of nostrils}}{2} + \text{abdominal lift}. \]

<table>
<thead>
<tr>
<th>Numerical Score</th>
<th>Nostril Flare</th>
<th>Abdominal Lift</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No or very slight movement noted on inspiration</td>
<td>No or very little appreciable movement in the ventral flank</td>
</tr>
<tr>
<td>1</td>
<td>Aspect of the nostril flares only slightly during inspiration, returning to a normal position at the end of inspiration</td>
<td>Slight abdominal flattening with “heave line” just beginning to form in the cranial aspect of the ventral flank (not easily detectable)</td>
</tr>
<tr>
<td>2</td>
<td>Aspect of the nostril flares during inspiration, returning to a near normal position as it ends</td>
<td>Abdominal flattening is obvious and a “heave line” extending to a point halfway between the tuber coxae and the elbow (fairly easy to detect)</td>
</tr>
<tr>
<td>3</td>
<td>Aspect of the nostril is flared to a greater extent during inspiration and does not approach a normal position during exhalation</td>
<td>Abdominal flattening and a “heave line” extending beyond a point halfway between the tuber coxae and the elbow, but does not extend all the way to the elbow (easy to detect)</td>
</tr>
<tr>
<td>4</td>
<td>Aspect of the nostril is flared and remains maximally flared throughout the respiratory cycle</td>
<td>Abdominal flattening and a “heave line” extending cranially all the way to the elbow (very easy to detect)</td>
</tr>
</tbody>
</table>
When affected horses showed prominent signs of clinical exacerbation (CSRE ≥ 5 and inspiratory wheezes), they were removed from pastures and samples were collected. Sample collection of affected horses during clinical remission of SPAOPD and control horses during summer and winter was performed at random times, with an interval of at least three weeks.

After every clinical data sample and collection, the horses were maintained in box-stalls with rubber mat flooring, without bedding, and they were fed a complete pelleted diet, with fiber content of 30% \(^{a}\) at 1.5 pounds of feed per 100 pounds of body weight, divided in two feedings. At times of clinical exacerbation, affected horses were kept in stalls for a period of at least four weeks after clinical improvement (i.e., CSRE = 4), and then they were again placed on pastures. Medical treatments were only administered when deemed necessary and for as short a period as possible, and included aerosolized bronchodilators (albuterol MDI 2\(\mu\)g/kg q 6h and ipatropium bromide MDI 2\(\mu\)g/kg q 12h) given via metered dose inhalers through an Aeromask \(^{b}\) or oral preparation of bronchodilator (albuterol oral syrup 8 \(\mu\)g/kg PO q 12h), and corticosteroid given systemically (dexamethasone 0.04 mg/kg to 0.01 mg/kg IV or PO q 24h). The treatments and the length of time until recovery from clinical exacerbation were recorded for each horse. The clinical data and samples evaluated in this study included only that of horses while on pastures, and excluded the clinical data of the horses while kept confined in box-stalls.

3.2.2. Clinical Data and Sample Collection

The clinical evaluations (physical examination including CSRE, cytologic analysis of BALF and pulmonary function testing determined by indirect measurement of pleural pressures difference using an esophageal balloon) were recorded and samples (arterial
and venous blood, and BALF) were obtained from each affected horse at least three times during the season of clinical exacerbation (mid-spring to mid-fall) and three times during clinical remission (winter). In addition, plasma samples and clinical evaluations of non-affected control horses were collected twice during summer and twice during winter (Figure 3.3). Clinical evaluation included recording breathing pattern and CSRE, vital signs, cardiac auscultation, auscultation of the lung fields during tidal breathing and auscultation with a re-breathing bag (except in affected horses during clinical exacerbation).

### 3.2.2.1. Pleural Pressure Measurement

Pleural pressure was measured indirectly, using an esophageal balloon secured over the end of a catheter that was connected to a pressure transducer interfaced with a physiograph (Willoughby and McDonnell 1979, Costa et al. 2000). A latex balloon, 10-cm in length and 1-cm diameter, was placed over the end of a 2-m long, 2-mm internal diameter cannula. The changes in esophageal pressure during tidal breathing measured with this system reflected the change in pleural pressure difference (PPD) or \( \Delta P_{pl} \) (Derksen and Robinson 1980). The balloon was inserted through a lubricated short nasogastric tube that was passed into the rostral esophagus. Once the balloon was located between the heart and the diaphragm, the nasogastric tube was removed. The balloon was inflated with 1.5 ml of water and five measurements (peak inspiratory pressure minus peak expiratory pressure) were collected and averaged. During tidal breathing, the normal changes in pleural pressure should be less than 10 cm of H\(_2\)O (Derksen and Robinson 1980).
3.2.2.2. Blood Sample Collection

Venous blood samples were obtained from the jugular vein and arterial blood samples were obtained from the transverse facial artery and placed into polypropylene tubes containing 1.375 mg of EDTA and 0.072 trypsin inhibition units of aprotinin per ml of blood. Blood samples collected from the jugular vein were also placed into tubes containing lithium heparin. Blood samples were centrifuged at 2,000 x g for 10 minutes. Plasma samples were frozen at – 70°C until assayed.

3.2.2.3. Bronchoalveolar Lavage Fluid Collection

Horses were sedated using a combination of xylazine hydrochloride (0.5 mg/kg, IV) and butorphanol tartarate (11 µg/kg, IV). Following placement of a nose twitch, a 244-cm (11-mm outer diameter, 3-mm inner diameter) flexible silicone tube was passed through the nasal passage into the trachea and wedged in the distal airway. As the silicone tube was advanced through the trachea and carina, 30 ml of lidocaine 1% was injected through the tube, followed by 30 ml of air. The cuff was inflated with 4 ml of air, and five 60-ml aliquots of warm sterile 0.9% saline were infused manually with 60-ml syringes (Sweeney and Beech 1991). Immediately following infusion, BALF was collected and pooled into a sterile flask. The volume of fluid infused and retrieved was recorded for each horse. A portion of the pooled aspirate was placed in vacuum tubes containing EDTA for cytologic evaluation and heparin for determination of urea concentration, and a portion was placed into polypropylene tubes containing 1.375 mg of EDTA and 0.072 trypsin inhibition units of aprotinin per ml of sample. The BALF samples were centrifuged at 2,000 x g for 10 minutes, and cell-free BALF samples were frozen at – 70°C until assayed.
3.2.2.4. Bronchoalveolar Lavage Fluid Cytologic Evaluation

The concentrated smears of the BALF were prepared by cytocentrifugation. Air-dried smears were stained with a modified Wright’s solution and 200 cells were classified using light microscopy under high magnification (1,000 x) as neutrophils, lymphocytes, alveolar macrophages, mast cells, eosinophils, or epithelial cells, and expressed as percentages.

3.2.3. Quantification of Immunoreactive ET in Plasma and BALF

The concentration of immunoreactive ET in plasma and cell-free BALF samples was determined using a commercially available capture enzyme-linked immunosorbent assay, which has been validated for use in the horse (Tetens et al. 2004; Stokes 2003). The diagram in Figure 3.4 depicts the ELISA method. The characteristics of the assay include reactivity of 100% to ET-1 (21 amino acids), 100% to ET-2 (21 amino acids), < 5% to ET-3 (21 amino acids), < 1% to big ET (38 amino acids), standard curve range from 0.625 to 10 fmol/ml, and the sensitivity of 0.5 fmol/ml or 0.125 pg/ml. The inter- and intra-assay variability for equine plasma is 15.4% and 6.4%, respectively.

The assay was performed following the manufacturer’s instructions. Briefly, the plasma samples were mixed with the precipitating agent at 1:1.5 ratio, cooled to 4°C and centrifuged at 3,000 x g for 20 minutes. The supernatants were transferred into polypropylene tubes, desiccated using nitrogen gas, and dissolved in 500 µl of assay buffer. For purification and concentration of ET in BALF, the samples were subjected to solid phase extraction using reverse phase sorbent C18 columns (Black et al. 1989, Sofia et al. 1993, Redington et al. 1995, Ramaswamy et al. 2002, de la Calle et al. 2003). The kit’s endothelin stock solution (lyophilized human ET) was diluted serially (ranging from 0 to 10 fmol/ml) to serve as standards. The enzyme immunoassay was performed in
duplicates using 200 µl of each serial dilution of 6 standards, positive and negative controls, processed plasma samples or extracted BALF samples, which were added to each well, followed by 50 µl of the detection antibody (mouse anti-ET monoclonal antibody) added to all wells except the blank well, mixed and incubated for 16 to 24 hrs at 20°C. The contents of the wells were discarded, and the wells were washed five times with washing buffer. Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody was added to each well and the plate was incubated for three hours at 37°C. The contents of the wells were discarded, the wells were washed again five times with washing buffer, and 200 µl the tetramethylbenzidine (TMB) substrate was added to all wells, incubated for 30 minutes at 20°C protected from light. The acid solution, called stop solution by the manufacturer, 50 µl, was added to each well and mixed. The optical densities at 405 nm and 620 nm were measured immediately with an ELISA plate reader. The concentration of immunoreactive ET in plasma or BALF was calculated as per manufacturer’s instructions and expressed as pg/ml.

3.2.4. Determination of Urea in Plasma and BALF

For the pulmonary secretion, the dilution of the pulmonary epithelial lining fluid (PELF) resulting from the lavage procedure was estimated using urea as a marker (Rennard et al. 1986; McGorum et al. 1993; Dargaville et al. 1999). Blood urea nitrogen concentrations were determined enzymatically. Urea concentrations in BALF samples were determined using a commercially available kit following the protocol modifications described by Rennard et al. 1986.
Figure 3.3. Experimental design of the study, including animals in each group and the sampling schedule. Animals were kept on pastures and monitored daily. The CSRE was recorded between the fall of 2001 and the winter of 2002. Clinical data and samples were collected from affected horses during clinical exacerbation, when CSRE $> 5.0$ and wheezes were ausculted, and during clinical remission, and from non-affected controls during summer and winter.
Figure 3.4. Quantification of immunoreactive endothelin-1 (ET-1) by a commercial capture ELISA kit. HRP = horse-radish peroxidase; TMP = tetramethylbenzidine.
For calculation of volume of PELF, the urea concentrations in plasma and BALF were used, such that we accounted for the dilution factor associated with the lavage. The volume of PELF was determined by the following formula (Dargaville et al. 1999; Conte et al. 2002): \( V_{PELF} (\text{ml}) = V_{BALF} \times \frac{[\text{Urea}]_{BALF} (\text{mg/ml})}{[\text{Urea}]_{Plasma} (\text{mg/ml})} \). The concentration of immunoreactive ET in PELF was calculated based on the urea concentrations in plasma and BALF and expressed as pg/ml of PELF: 
\[
[\text{ET}]_{PELF} \text{ pg/ml} = \frac{([\text{ET}]_{BALF} \times [\text{Urea}]_{Plasma})}{[\text{Urea}]_{BALF}} \quad (\text{van der Vliet et al. 1999; Conte et al. 2002}).
\]

### 3.2.5. Statistical Analyses

Data were tested for normality using of Shapiro-Wilk statistic, with normality determined by failure to reject the null hypothesis at \( p < 0.05 \). Data, including the vital signs, the percentage of inflammatory cells in BALF, and the concentrations of immunoreactive ET in arterial and venous plasma, BALF and PELF were considered continuous and compared among groups, i.e., affected horses during exacerbation and during remission, and non-affected horses during summer and during winter using a mixed effect linear model, in which the effects of sampling time and disease status were considered fixed, and the effect of horse was considered random. Continuous data were summarized by mean (± SEM). The data of clinical score of respiratory effort and pleural pressure difference were analyzed as non-parametric data using a Kruskal-Wallis test. All analyses were performed with the use of SAS v 8.2 n.

### 3.3. Results

During remission, all SPAOPD-affected horses evaluated in this study had pleural pressure difference within normal range and CRSE less than or equal to 4 (Figures 3.5, A
through F), similar to values in the non-affected horses (Figures 3.6, A through F), indicating reversibility of the airway obstruction. The onset of clinical exacerbation varied among horses; the first clinical exacerbation episode of the year for each affected horses occurred in each May, June, July, August for four of the horses and September for two horses. Once the affected horses had recovered from their first episode of clinical exacerbation of the year, which meant 4 weeks of confinement in low dust environment after the CSRE dropped to 4, and the affected horses were placed back on pastures. Invariably, once the affected horses were again exposed to the pasture, the signs of clinical exacerbation returned within one or two days. The episodes of exacerbation, the time to recover, the treatments given and the mean ± SEM pleural pressure difference (PPD) and percentage of neutrophils are depicted for each individual horse (Figure 3.5 A through F). The severity of clinical signs during exacerbation episodes varied among affected horses. Two horses appeared mildly affected and did not require any medical treatment; their CSRE decreased within a few days of confinement in the dust-free environment (Figures 3.5, A and B). Nonetheless, during clinical exacerbation their PPD and percentage of neutrophils were as high as the other affected horses. Two horses were considered moderately affected. Although the moderately affected horses required a few days of medical treatment, they responded well to the treatment and their recovery was not prolonged (Figures 3.5, C and D). Two horses were considered severely affected, because they required prolonged medical treatments after exacerbation episodes despite the change into a dust-free environment. These two horses subjectively appeared to develop a gradual poorer response to treatment (Figures 3.5, E and F).
Figure 3.5. Clinical exacerbation episodes and remission periods of SPAOPD-affected horses. The periods of time horses were kept on pastures (green line) and those when horses were kept in box-stall (magenta line), and the episodes of clinical exacerbation (red arrows) between the fall of 2001 and the winter of 2002. The recovery time and medical treatment are listed in the table. The graphs indicate the mean ± SEM of pleural pressure difference (PPD in cm of H$_2$O) and the percentage of neutrophils in BALF during clinical exacerbation and remission episodes.
Clinical exacerbation episodes and remission periods of SPAOPD:

Horse #2, mildly affected

<table>
<thead>
<tr>
<th>Exacerbation episodes</th>
<th>Recovery time</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>4 d</td>
<td>None</td>
</tr>
<tr>
<td>E2</td>
<td>6 d</td>
<td>None</td>
</tr>
</tbody>
</table>

Figure 3.5. Continued
Clinical exacerbation episodes and remission periods of SPAOPD:
Horse # 3, moderately affected

<table>
<thead>
<tr>
<th>Exacerbation episodes</th>
<th>Recovery time</th>
<th>Dexamethasone</th>
<th>Albuterol/Ipatrop (MDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>4 d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E2</td>
<td>4 d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E3</td>
<td>2 d</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E4</td>
<td>2 d</td>
<td>1 d</td>
<td>2 d</td>
</tr>
<tr>
<td>E5</td>
<td>3 d</td>
<td>2 d</td>
<td>2 d</td>
</tr>
</tbody>
</table>

Figure 3.5. Continued
Clinical exacerbation episodes and remission periods of SPAOPD:

Horse #4, moderately affected

<table>
<thead>
<tr>
<th>Exacerbation episodes</th>
<th>Recovery time</th>
<th>Dexamethasone</th>
<th>Alb/Lpatr (MDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>4 d</td>
<td>2 d</td>
<td>3 d</td>
</tr>
<tr>
<td>E2</td>
<td>6 d</td>
<td>3d &amp; on d 5</td>
<td>5 d</td>
</tr>
<tr>
<td>E3</td>
<td>8 d</td>
<td>5 d &amp; on d 7</td>
<td>8 d</td>
</tr>
</tbody>
</table>

Figure 3.5. Continued
Clinical exacerbation episodes and remission periods of SPAOPD:

Horse # 5, moderately affected

<table>
<thead>
<tr>
<th>Exacerbation episodes</th>
<th>Recovery time</th>
<th>Dexamethasone</th>
<th>Alb/Ipatrop (MDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 *</td>
<td>4 d</td>
<td>-</td>
<td>7 d</td>
</tr>
<tr>
<td>E2</td>
<td>6 d</td>
<td>4 d</td>
<td>4 d</td>
</tr>
<tr>
<td>E3 *</td>
<td>6 d</td>
<td>2 d &amp; on d 4</td>
<td>3 d</td>
</tr>
<tr>
<td>E4 *</td>
<td>8 d</td>
<td>3 d &amp; on d 5</td>
<td>3 d</td>
</tr>
<tr>
<td>E5 *</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Not eating or drinking and showing signs of colic

Figure 3.5. Continued
Clinical exacerbation episodes and remission periods of SPAOPD:

Horse # 6, moderately affected

<table>
<thead>
<tr>
<th>Exacerbation episodes</th>
<th>Recovery time</th>
<th>Dexamethasone</th>
<th>Alb/Ipatrop (MDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>5 d</td>
<td>-</td>
<td>5 d</td>
</tr>
<tr>
<td>E2 *</td>
<td>6 d</td>
<td>-</td>
<td>4 d</td>
</tr>
<tr>
<td>E3</td>
<td>4 d</td>
<td>2 d &amp; on d 4</td>
<td>3 d</td>
</tr>
<tr>
<td>E4 *</td>
<td>6 d</td>
<td>2 d &amp; on d 4</td>
<td>5 d</td>
</tr>
<tr>
<td>E5 *</td>
<td>12 d</td>
<td>2 d &amp; on d 4</td>
<td>7 d</td>
</tr>
<tr>
<td>E6 *</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Not eating or drinking & Colic

Figure 3.5. Continued
In all cases, however, during clinical remission, which occurred in the winter, affected horses had PPD and percentage of neutrophils in BALF within the normal range, i.e., less than 10 cm of H₂O and 20%, respectively. During episodes of clinical exacerbation, thoracic auscultation of affected horses revealed wheezes, generally end-expiratory and in some cases inspiratory and expiratory; a re-breathing bag was not used. During clinical remission, affected horses had normal bronchovesicular sounds, no wheezes or crackles even with a re-breathing bag. In all instances, non-affected control horses had normal bronchovesicular sounds upon auscultation of the lung with a re-breathing bag. The median CSRE for affected horses during exacerbation (median 5.5; range 5 to 7) was significantly greater than the CSRE during clinical remission (median 3; range 1.5 to 4), and than the CSRE of non-affected controls during summer (median 2; range 1.5 to 3) and during winter (median 2; range 1 to 3) (Figures 3.6 and 3.7). The median change in pleural pressure (ΔPpl) for affected horses during exacerbation (median 38 cm of H₂O, range 17 to 67) and was significantly greater than during clinical remission (median 7 cm of H₂O, range 4 to 12.6), and non-affected controls during summer (median 5 cm of H₂O, range 2.5 to 5) and during winter (median 3 cm of H₂O, range 3 to 6) (Figures 3.6 and 3.8). Similar to the non-affected control horses, the affected horses during remission had clinical scores ≤ 4.0 and ΔPpl < 10.0 cm H₂O, with one exception (i.e., affected horse # 5, ΔPpl = 12.6 cm H₂O). The percentage of neutrophils in BALF for affected horses during exacerbation (mean ± SEM = 61 ± 6%) was significantly greater than the percentage of neutrophils in BALF during clinical remission (mean ± SEM = 14 ± 3%), and than that of non-affected controls during summer (mean ± SEM = 7 ± 1%) and winter (mean ± SEM = 4 ± 1%) (Table 3.2, Figure 3.9). The percentages of macrophages and lymphocytes in BALF were correspondingly decreased during exacerbation.
Figure 3.6. Pulmonary function (pleural pressure difference or PPD) and percentage of neutrophils in bronchoalveolar lavage (BAL) fluid from non-affected horses. The graphs indicate the mean ± SEM of pleural pressure difference (PPD in cm of H₂O) and percentage of neutrophils in BAL fluid during summer and winter of each of the six non-affected (A through F) control horses.
Figure 3.7. Clinical score of respiratory effort of affected horses during exacerbation and remission of SPAOPD, and non-affected horses during summer and winter. The box indicated 50% of the data, the whiskers represent the range and the horizontal line within the box represents the median values. Different letters indicate statistical significance (p > 0.05).
Figure 3.8. Pleural pressure difference (PPD in cm of H.0) of affected horses during exacerbation and remission, and non-affected horses during summer and winter. The box indicated 50% of the data, the whiskers represent the range and the horizontal line within the box represents the median values. Different letters indicate statistical significance (p > 0.05).
Figure 3.9. Percentage of neutrophils in BALF (mean ± sem) of affected horses during exacerbation and remission, and non-affected horses during summer and winter. Different letters indicate statistical significance (p > 0.05).
The clinical variables reflecting the severity of signs (CRSE, pleural pressure differences and percentage of neutrophils in BALF) in SPAOPD-affected horses during clinical exacerbation were significantly greater than during remission and greater than non-affected horses in either summer or winter sampling periods (Figures 3.7, 3.8 and 3.9). Other clinical data including vital signs did not differ significantly between groups (Table 3.2).

The immunoreactive ET-1 concentrations in both arterial and venous plasma samples were greater in SPAOPD-affected horses during clinical exacerbation, compared with those during clinical remission, and when compared with those from non-affected horses (Figure 3.10). There were no significant variations of ET concentrations in plasma samples from non-affected control horses during summer and winter. The fixed effect of sampling time was not found to be significant at p < 0.05. Hence, the data for all sampling times were pooled and considered replicates of each other within each group.

The concentrations of immunoreactive ET-1 in BALF samples and PELF were greater, although not statistically significant, in SPAOPD-affected horses during clinical exacerbation compared with those during clinical remission, and those from non-affected horses (Figure 3.10). There were no significant differences of ET-1 concentrations in BALF samples and PELF from non-affected control horses during summer and winter. Interestingly, if the ET-1 concentrations in BALF and PELF are evaluated for severely affected horses separately from moderately and mildly affected horses, differences between these subgroups became apparent (Table 3.3).
Figure 3.10. Circulating and Pulmonary Concentrations of Immunoreactive ET-1. Mean ± SEM endothelin-1 (ET) concentrations (pg/ml) in arterial blood (A), venous blood (B), bronchoalveolar lavage fluid, BALF (C), and pulmonary epithelial lining fluid, PELF (D). Different letters indicate statistical significance (p > 0.05).
Table 3.2. Summary of Mean ± SEM for vital signs and BALF cytologic findings.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Affected Horses during Exacerbation</th>
<th>Affected Horses during Remission</th>
<th>Non-affected Horses during Summer</th>
<th>Non-affected Horses during Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal temperature</td>
<td>101.3 ± 1.7 °F</td>
<td>99.8 ± 0.4 °F</td>
<td>100.2 ± 0.4 °F</td>
<td>99.5 ± 0.6 °F</td>
</tr>
<tr>
<td>HR</td>
<td>59 ± 13 bpm</td>
<td>50 ± 6 bpm</td>
<td>49 ± 7 bpm</td>
<td>50 ± 8 bpm</td>
</tr>
<tr>
<td>RR</td>
<td>32 ± 9 bpm</td>
<td>19 ± 7 bpm</td>
<td>20 ± 4 bpm</td>
<td>15 ± 3 bpm</td>
</tr>
<tr>
<td>BALF volume recovery</td>
<td>56 ± 2.5 %</td>
<td>49 ± 2.6 %</td>
<td>47.3 ± 3.8 %</td>
<td>49.3 ± 5.6 %</td>
</tr>
<tr>
<td>Neutrophils in BALF</td>
<td>61 ± 6 % *</td>
<td>14 ± 3 %</td>
<td>7 ± 1 %</td>
<td>4 ± 1 %</td>
</tr>
<tr>
<td>Macrophages in BALF</td>
<td>10 ± 2 % *</td>
<td>36 ± 3 %</td>
<td>36 ± 3 %</td>
<td>37 ± 5 %</td>
</tr>
<tr>
<td>Lymphocytes in BALF</td>
<td>21 ± 4 % *</td>
<td>36 ± 3 %</td>
<td>53 ± 3 %</td>
<td>51 ± 3 %</td>
</tr>
<tr>
<td>Epithelial cells in BALF</td>
<td>12 ± 4 %</td>
<td>20 ± 7 %</td>
<td>2 %</td>
<td>15 ± 5 %</td>
</tr>
<tr>
<td>Mast cells in BALF</td>
<td>2 ± 1 %</td>
<td>4 ± 1 %</td>
<td>2 %</td>
<td>2 %</td>
</tr>
</tbody>
</table>

* indicates statistical significance between groups, p > 0.05.
Table 3.3. Increase (percentage) in immunoreactive ET-1 concentration in SPAOPD-affected horses during clinical exacerbation compared with remission and non-affected controls.

<table>
<thead>
<tr>
<th></th>
<th>Exacerbation compared to Remission</th>
<th>Exacerbation compared to Non-affected Horses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arterial [ET-1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely affected</td>
<td>103 %</td>
<td>25 %</td>
</tr>
<tr>
<td>Moderately affected</td>
<td>128 %</td>
<td>75 %</td>
</tr>
<tr>
<td>Mildly affected</td>
<td>34 %</td>
<td>72 %</td>
</tr>
<tr>
<td>Venous [ET-1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely affected</td>
<td>46 %</td>
<td>65 %</td>
</tr>
<tr>
<td>Moderately affected</td>
<td>41 %</td>
<td>17 %</td>
</tr>
<tr>
<td>Mildly affected</td>
<td>87 %</td>
<td>68 %</td>
</tr>
<tr>
<td>[ET-1] in BALF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely affected</td>
<td>37 %</td>
<td>249 %</td>
</tr>
<tr>
<td>Moderately affected</td>
<td>69 %</td>
<td>317 %</td>
</tr>
<tr>
<td>Mildly affected</td>
<td>1133%</td>
<td>57 %</td>
</tr>
<tr>
<td>[ET-1] in PELF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severely affected</td>
<td>14 %</td>
<td>260 %</td>
</tr>
<tr>
<td>Moderately affected</td>
<td>39 %</td>
<td>392 %</td>
</tr>
<tr>
<td>Mildly affected</td>
<td>605 %</td>
<td>82 %</td>
</tr>
</tbody>
</table>
Mildly affected horses had very low pulmonary fluid ET-1 concentrations during remission, resulting in a great percentage increase when comparing exacerbation and remission. On the other hand, severely affected horses had a smaller difference between the ET-1 concentration during clinical exacerbation compared with remission, resulting in a much smaller percentage increase of ET-1 concentration during exacerbation compared with remission. Since there were only two horses in each subgroup, i.e., mildly, moderately and severely affected horses, no statistical analyses were performed.

3.4. Discussion

This is the first study reporting the clinical pattern of exacerbation and remission of SPAOPD during the seasons of the year in comparison to the same clinical variables of non-affected horses. The circulating concentrations of immunoreactive ET-1 in arterial and venous plasma were significantly greater in affected horses during exacerbation compared with remission and non-affected controls. Our results resemble the findings in barn-associated RAO and human asthma.

Considering the great variability with respect to severity of the signs of clinical exacerbation of SPAOPD among horses, it was possible to subgroup the SPAOPD-affected horses according to the severity of disease. Although, individual variation is one of the limitations of this type of study, it also provides information about the clinical spectrum of the disease and the stratification of how ET-1 concentrations change with the severity of the disease. Our results resemble those reported in human asthmatic patients (Aoki et al. 1994, Trakada et al. 2000). In contrast, Chalmers and co-workers reported no difference between ET-1 concentrations in plasma samples from asthmatics and control subjects (Chalmers et al. 1997). The circulating venous concentration of immunoreactive
ET-1 was greater in RAO-affected horses during crisis compared with remission and control samples (Benamou et al. 1998). However, the venous concentrations of immunoreactive ET-1 reported here are less than those reported for horses affected with barn-associated RAO (Benamou et al. 1998).

The concentrations of immunoreactive ET-1 were not significantly different in BALF and PELF from affected horses during exacerbation compared with remission, and non-affected controls. The lack of statistical significance may be a result of the small sample size. Chalmers and co-workers reported no difference between ET-1 concentrations in sputum samples from asthmatics and control subjects (Chalmers et al. 1997). Our results contrast with the finding in horses affected with barn-associated RAO, whereby, despite the great variation of ET-1 concentration in affected horses during crisis, the controls had significantly lower ET concentrations (Benamou et al. 1998). Similarly, our results also contrast with those reported in asthmatic patients where immunoreactive ET-1 concentrations were found to be greater in BALF samples of asthmatic patients compared to normal subjects (Sofia et al. 1993, Redington et al. 1995, Trakada et al. 2000). Similar to the present study, all the aforementioned studies reported that the BALF samples were subjected to extraction using SE-Pak C18 columns. Moreover, in the present study and the previously reported study, urea was utilized as a marker of dilution of the lavage. However, in contrast with the present study, the reported studies utilized a radioimmunoassay for determining ET-1 concentration (lowest detectable limit is 0.1 pg/ml), which is considered to be a more sensitive assay than the ELISA (lowest detectable limit is 0.125 pg/ml).

Although, the concentrations of immunoreactive ET-1 in pulmonary secretion, BALF and PELF, from affected horses during exacerbation had a wide variation between
individuals, there was a pattern of greater percentage increase of immunoreactive ET-1 in mildly affected horses during clinical exacerbation compared with remission. Severely affected horses had much smaller percentage increase of ET-1 concentration during clinical exacerbation compared with remission. It appears that severely affected horses have greater ET-1 concentrations in pulmonary secretion during both exacerbation and remission in contrast with mildly affected horses.

Endothelins are secreted and interact with the target receptors in an autocrine/paracrine manner. The increase in venous and arterial immunoreactive ET-1 concentration during clinical exacerbation of SPAOPD resembles the findings in human asthma and barn-associated equine RAO. This increase in circulating ET-1 may result from increased ET synthesis/release, delayed elimination, decreased degradation of ET, or a combination of these mechanisms. The overall increase in circulating ET-1 during clinical exacerbation of SPAOPD suggests that ET-1 is involved in the pathophysiology of this disease.

Increases in circulating immunoreactive ET-1 during clinical exacerbation of SPAOPD suggest that ET-1 contributes to the bronchoconstriction, mucus hypersecretion and inflammation observed in this disease. Intervention in ET-1 production and metabolism may prove to be an important therapeutic target for SPAOPD.

3.5. Endnotes

a  Horse Chow 200; Purina Mills, LLC; St. Louis MO, USA.
b  Trudell Medical International; London, Ontario, Canada.
c  Statham Model P50 pressure transducer, Statham Instruments, Hato Ray, Puerto Rico.
d  Model 7D polygraph, Grass Medical Instruments, Quincy MA, USA.
a Esophageal balloon made with latex penrose tubing, Sherwood Medical Company; St. Louis MO, USA.

f Cannula, PE 350 tubing, VWR Scientific Products, Willard OH, USA.

g Equine broncho-alveolar lavage catheter; Bivona, Inc., Gary ID, USA.

h Lidocaine 2% injectable, Butler Company, Columbus OH, USA.

i Cytospin 3, Shandon, Inc, Pittsburgh PA, USA.

j Biomedica Gruppe, Austria; Distributed by American Research Products, Inc., Belmont MA, USA.

k Sep-Pak C18 cartridge, Waters, Milipore Corporation, CA, USA.

l Olympus AU600 Chemistry Analyzer, Melville NY, USA.

m InfinityTM Urea Nitrogen, Thermo DMA, Louisville CO, USA.

n SAS Institute, Cary NC, USA.
CHAPTER 4. GENE EXPRESSION OF ENDOTHELIN-1 AND CYTOKINES, AND LOCALIZATION AND QUANTIFICATION OF IMMUNOREACTIVE ENDOTHELIN IN LUNGS OF HORSES AFFECTED WITH SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE
4.1. Introduction

The airway inflammation and hyperresponsiveness in equine recurrent airway obstruction (RAO) resemble those of asthma in people (Derksen 1993). Summer pasture-associated obstructive pulmonary disease (SPAOPD) is a form of RAO affecting horses residing on pasture during the summer. During clinical exacerbation of SPAOPD, the clinical signs of SPAOPD include respiratory wheezes associated with paroxysmal cough and excessive mucus production (Beadle 1983; Seahorn and Beadle 1993, Seahorn et al. 1996; Costa et al. 2000). Because of effects of ET-1 in the lung, i.e., potent bronchoconstrictor, pro-inflammatory and secretory actions, ET-1 has been implicated as an important mediator in human asthma as well as barn-associated RAO (Hay et al. 1996, Benamou et al. 1998).

The transcription of ET-1 mRNA and the secretion of the peptide are induced by hypoxia, endotoxin and by several cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-4, IL-6, interferon (IFN)-β and IFN-γ (Ninomiya et al. 1991; Nakano et al. 1994; Michael and Markewitz 1996; Yang et al. 1997; Kahaleh and Fan 1997; Skopal et al. 1998). ET -1 was shown to increase the secretion of IL-8 and IL-6 by airway epithelial cells in culture (Mullol et al. 1996). Moreover, the production and release of ET-1 by human monocytes stimulated with LPS was further increased by the addition of IFN-γ, IL-3 and GM-CSF in a dose-dependent manner (Salh et al. 1998).

Exposure to moldy/dusty hay resulted in the up-regulation of synthesis of IL-8 and MIP-1 by alveolar macrophages of horses with barn-associated RAO (Franchini et al.
Cultured human bronchial epithelial cells were shown to express IL-8 following exposure to common aeroallergens (Bhat et al. 2003, Borger et al. 1999). During clinical exacerbation of SPAOPD, the expression of IL-4 and IFN-γ in peripheral blood mononuclear cells and bronchoalveolar lavage cells was greater compared with non-affected horses (Beadle and Horohov 2002).

Pulmonary immunoreactivity to ET-1 has been shown to be greater in biopsy specimens from asthmatics than non-asthmatics (Springall et al. 1991, Redington et al. 1997). Moreover, ET-1 has been implicated in airway remodeling, especially because of its mitogenic action, inducing smooth muscle hyperplasia and fibrosis (Noveral et al. 1992; Sun 1997; Hay 1999; Goldie 1999). Expression of ET-1 as evidenced by detection of immunoreactive ET-1 in lung tissue has been shown to be greater in idiopathic pulmonary fibrosis, and cryptogenic fibrosing alveolitis compared to controls (Giaid et al. 1993; Uguccioni et al. 1995; Saleh et al. 1997).

We hypothesized that the expression of ET-1 is greater in airways of horses affected with SPAOPD, compared with non-affected horses. The purpose of this study was evaluate the gene expression of ET-1 and three cytokines, IL-4, IL-8 and IFN-γ, and to quantify and report the distribution of immunoreactive ET-1 in lungs of horses affected with SPAOPD, compared with normal controls. Gene expression was evaluated using real-time polymerase reaction (RT-PCR) and immunoreactive ET-1 was quantified using a commercial enzyme-linked immunosorbent assay (ELISA) kit. The distribution of immunoreactive ET-1 was evaluated by immunolabeling using anti-endothelin-1 antibody specific for human, bovine, porcine, canine, mouse and rat.
Figure 4.1. Outline of experiments: gene expression of equine endothelin (ET)-1, interleukin (IL)-4 and IL-8 and interferon (IFN)-γ in lung tissues from horses affected with summer pasture-associated obstructive pulmonary disease and non-affected horses. Data were expressed in units normalized for the housekeeping gene equine β-actin.
4.2. Materials and Methods

4.2.1. Animals and Sample Collection

All experimental procedures performed for this study were approved by the Animal Care and Use Committee, Louisiana State University. Twelve horses were included in this study, eight SPAOPD-affected horses (4 females, 4 geldings; 5 Quarter Horses, 1 Paint, 1 Thoroughbred, 1 Grade), ages ranging from 7 to 20 years, median 19 years, and four clinically normal, i.e., non-affected, horses (2 females, 2 geldings; 2 Quarter Horses, 1 American Saddlebred, 1 Thoroughbred), ages ranging from 3 to 11 years, median 8.5 years. The diagnosis of SPAOPD was based on the history of recurrent obstructive respiratory disease following exposure to summer pasture, physical examination revealing pronounced expiratory wheezes throughout the lung fields and tracheal rattle, cough, nostril flaring and prominent abdominal lift, assigned clinical score of respiratory effort (CSRE) and cytologic analysis of bronchoalveolar lavage fluid (BALF). The assigned CRSE ranged from 5 to 7.5 (median score 5.8), and cytologic evaluation of BALF from SPAOPD-affected horses revealed predominance of non-degenerate neutrophils, ranged from 10 to 90%, median 65%. All non-affected horses had normal bronchovesicular sounds upon auscultation of the lung with a re-breathing bag, assigned CSRE ranged from 2.0 to 3.0 (median score 2.0) and cytologic evaluation of BALF from SPAOPD-affected horses revealed neutrophils less than 20%.

All horses were euthanatized by intravenous administration of sodium pentobarbital (100 mg/kg, IV) 24 hours after BALF sample collection. Tissue samples from the each lung
lobes were collected in duplicates. One sample from each of the lobes were wrapped in foil, snap-frozen in liquid nitrogen and stored at –70°C. The other tissue samples from each lung lobe were submerged in aqueous buffered zinc formalin for 24 hours prior to embedding in paraffin, and were later sectioned for immunohistochemical staining (Figure 4.1).

4.2.2. Evaluation of Gene Expression of Endothelin-1 and Cytokines

The relative gene expression of ET-1 and cytokines was evaluated using real-time polymerase chain reaction (RT-PCR). Aliquots of the frozen lung samples were powdered using a mortar and pestle, placed into microcentrifuge conical tubes and homogenized in 300 µl of phenol/guanidinium thiocyanate monophase solution, a total ribonucleic acid (RNA)/mRNA isolation reagent. Samples were stored at –70°C until RNA extraction procedure. The isolation and extraction of total RNA was done according to the protocol provided by the manufacturer. Briefly, samples were thawed and incubated for 5 minutes at room temperature. The aqueous phase containing RNA was extracted using a standard chloroform extraction method, precipitation with 100% isopropanol, two washes with 75% ethanol and desiccation at room temperature.

The concentration of RNA for each sample was estimated by spectrophotometry, i.e., determining the absorbance at 260 nm and 280 nm. Total RNA was estimated to be 80% of the total nucleic acid concentration. All samples were subjected to deoxyribonuclease (DNase) I treatment using a commercial kit in order to eliminate genomic deoxyribonucleic acid (DNA) contamination. Total RNA was eluted from the RNA-binding column matrix with ribonuclease-free water. The DNA-free total RNA was
subjected to reverse transcriptase reaction using random oligohexamers as primers in the reverse transcription commercial kit \(^6\) to generate the cDNA templates for PCR amplification.

The complete and partial sequences of equine IL-4, IL-8, IFN-\(\gamma\) and \(\beta\)-actin mRNA have been published and are available \(^f\). The sequence for equine ET-1 mRNA was determined in our laboratory \(^g\). The TaqMan specific probes and primers were designed using specific software \(^h\) (Table 4.1). The fluorescent dye-labeled probes (oligonucleotide with a 5’ reporter dye FAM™ and 3’ quencher dye TAMRA™) were cleaved by the 5’ to 3’ nuclease activity of the AmpliTaq Gold DNA polymerase \(^i\) during the amplification steps of the polymerase chain reaction and the emitted fluorescence was detected and recorded by an automated fluorometer \(^l\).

The real-time PCR procedure was performed using the commercial kit, *TaqMan* Universal PCR Master Mix \(^i\) according to the manufacture’s recommended protocol. Briefly, the template (5 \(\mu\)l/ reaction) is mixed with *TaqMan* buffer (10 mmol/l Tris-HCl, pH 8.3, 50 mmol/l KCl, 5 mmol/l MgCl\(_2\), 2.5 mmol/l deoxynucleotide triphosphates), AmpliTaq Gold Polymerase (0.625 u/reaction) and the specific set of oligonucleotides, i.e., the *TaqMan* probe and reverse and forward primers. The 96-well plates \(^k\) were loaded into ABI Prism® 7700\(^a\) and subjected to activation and serial amplification cycles (50°C for 2 minutes, 95°C for 10 minutes for AmpiTaq Gold DNA polymerase activation, and 40 cycles of denaturation and annealing/extension, 15 seconds at 95°C and 1 minute at 60°C, respectively).
Three samples from each horse were assayed in duplicates, using a single reaction per well, i.e., singleplex where each replicate of each sample was assayed with the set of specific primers and probe for the cytokine gene and the set of primers and probe for the \( \beta \)-actin in separate wells (Leutenegger et al. 1999). The relative quantification of the IL-4, IFN-\( \gamma \) and \( \beta \)-actin PCR products was calculated based on a standard curve, which was constructed using serial dilutions of DNA plasmid constructs containing the IL-4, IFN-\( \gamma \) and \( \beta \)-actin cDNA sequences generated by Dr. Horohov’s laboratory (Beadle and Horohov 2002). The standard curves for IL-8 and ET-1 were constructed by serial dilutions of two samples of cDNA containing large amounts of the genes of interest. The ET-1, IL-4, IL-8 and IFN-\( \gamma \) gene expression were normalized in relation to the expression of the housekeeping gene, \( \beta \)-actin, which served as the internal control of the amplification reaction (Leutenegger et al. 1999).

Table 4.1. Summary information about the oligonucleotides primers and probes used in the TaqMan real-time PCR.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>PubMed: Nucleotide no</th>
<th>Amplicon length</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eq ( \beta )-actin*</td>
<td>AF035774</td>
<td>68 bp</td>
<td>616 to 634 (19 bases)</td>
<td>636 to 662 (27 bases)</td>
<td>664 to 683 (20 bases)</td>
</tr>
<tr>
<td>Eq IL-4*</td>
<td>AF305617</td>
<td>69 bp</td>
<td>150 to 169 (20 bases)</td>
<td>173 to 197 (25 bases)</td>
<td>198 to 218 (20 bases)</td>
</tr>
<tr>
<td>Eq IFN-( \gamma )</td>
<td>D28520</td>
<td>75 bp</td>
<td>326 to 334 (19 bases)</td>
<td>339 to 368 (30 bases)</td>
<td>382 to 390 (19 bases)</td>
</tr>
<tr>
<td>Eq IL-8*</td>
<td>AY184956</td>
<td>68 bp</td>
<td>100 to 119 (20 bases)</td>
<td>121 to 145 (25 bases)</td>
<td>149 to 168 (19 bases)</td>
</tr>
<tr>
<td>Eq ET-1**</td>
<td>AY730629***</td>
<td>70 bp</td>
<td>40 to 60 (20 bases)</td>
<td>62 to 86 (25 bases)</td>
<td>89 to 110 (21 bases)</td>
</tr>
</tbody>
</table>
4.2.3. Quantification of Immunoreactive ET-1

Aliquots of the frozen lung samples were weighed. Using a mortar and pestle, the frozen tissue samples were powdered, homogenized in 3 ml of protein extraction buffer (0.5% v/v Triton X-114, 0.2 mM of phenyl methyl sulfonyl fluoride, 5 µg/ml of aprotinin in 0.05 M Tris, 0.001M EDTA, 0.15 M NaCl) and incubated on ice for 1 hour. The homogenates were centrifuged at 14,000 x g for 30 minutes at 4°C. The supernatant was harvested, and total protein determination was performed using a protein determination assay. Tissue lysate supernatants were filtered using a centrifugal filter device at 3,000 x g for 90 minutes at 4°C. The filtrates were collected and diluted serially in assay buffer. Serial dilutions of the protein filtrates ranging from 1:2 to 1:200 were employed in the commercially available sandwich ELISA for quantitative determination of immunoreactive ET-1. The enzyme immunoassay was performed in duplicate using 200 µl of each dilution of protein filtrate according to the manufacturer's instructions. The characteristics of the assay were reactivity of 100% to ET-1 (1-21), 100% to ET-2 (1-21), < 5% to ET-3 (1-21), < 1% to big ET (1-38), standard range from 0.05 to 10 fmol/ml, and the sensitivity of 0.5 fmol/ml or 0.125 pg/ml. The amount of immunoreactive ET-1 was calculated in relation to the total amount of protein in each sample. Results were reported as nanogram of ET-1 per milligram of protein (ng/mg of protein) for each lung specimen lysate.
4.2.4. Methods and Evaluation of ET Immunohistochemistry

4.2.4.1. Histologic Methods and Evaluation Criteria

Zinc formalin-fixed paraffin-embedded sections of the lung were cut at 4-µm thickness, processed and stained with hematoxylin and eosin (HE) (Carson 1997). Two pathologists, HWT and MBG, both unaware of the group assignment of the horses, evaluated the sections under bright field microscopy. The microscopic abnormalities, including mucus production, peribronchial infiltrate and peribronchial fibrosis, were each assigned a score from 0 to 3 based on severity, i.e., 0 = normal, 1 = mild, 2 = moderate, 3 = severe, according to a previously described scoring system, which had been adapted from Naylor et al. 1992 (Costa et al. 2000).

4.2.5. Methods for Immunohistochemical Staining for ET-1 and Evaluation Criteria

The immunohistochemical staining was performed on formalin-fixed, paraffin-embedded sections according to the method established in our laboratory, using a commercially available rabbit anti-endothelin-1, that recognizes human, rat, mouse, porcine, canine and bovine ET-1 and an avidin-biotin complex method. Paraffin-embedded tissues were sectioned at 4 µm, placed on silanized slides and dried overnight at 37°C. Sections were de-paraffinized with xylene, dehydrated in grades of ethanol, and rinsed with phosphate buffered saline, pH 7.6, containing 0.25% Tween 20 (T20-PBS). All incubations were performed at room temperature (20 to 22°C). Endogenous peroxidase activity was eliminated by incubation with 0.3% hydrogen peroxide and methanol for 30 minutes. Slides were washed in T20-PBS and incubated with 2% normal goat-serum for 45 minutes. Slides were rinsed and incubated with the primary antibody, rabbit anti-
endothelin-1<sup>d</sup> diluted 1:700, for 60 minutes. Normal rabbit IgG was substituted for the primary antibody in negative controls. Slides were incubated with diluted 1:200 biotinylated goat anti-rabbit IgG<sup>s</sup> for 30 minutes, washed in T20-PBS and subsequently incubated with avidin-biotin complex<sup>r</sup> for 45 minutes. After application of the chromogen diaminobenzidine, the slides were counterstained with Mayer’s hematoxylin and mounted in resinous media. Positive control sections (including artery and vein from heart and lung of horses and dogs, all of which have a consistent and reproducible staining pattern for ET-1 and negative control sections (tissue sections incubated without primary antibody) were included.<sup>o,p</sup>

Slides of sections of lung were assigned a code number and then evaluated microscopically and scored by a pathologist (MBG) unaware of the group assignment of the horses. The distribution of ET-1 immunoreactivity was evaluated for airway and pulmonary parenchymal structures, including airway epithelium, airway smooth muscle, airway connective tissue, endothelium, vascular smooth muscle, vascular connective tissue and alveolar epithelium. The intensity of ET-1 immunoreactivity was scored for each tissue component from 0 to 4 (0 = absent, 1 = weak/focal, 2 = intermediate/ diffuse, 3 = moderate/diffuse and 4 = strong/diffuse brown staining) as described by Giaid et al. 1993. The histologic mucus score was assigned as previously described (Costa et al. 2000). The histologic mucus scores were plotted against the ET immunoreactivity scores in order to explore a possible relationship.

4.2.6. Statistical Analysis

The gene expression (units) and quantity of ET-1 (as nanograms of ET-1/mg of protein) were considered continuous data and found to follow a normal distribution using
Shapiro-Wilk statistic, with failure to reject the null hypothesis of normality at \( p \leq 0.05 \). The ET-1/protein measurement (mean ± SEM) was compared between groups using a mixed effect linear model, which included the random variance of horses nested within group. Each sample from each horse was considered a replicate from that horse. Significance was considered at \( p \leq 0.05 \). Analyses were performed using statistical software \(^1\). Immunostaining of ET-1 was not analyzed statistically, and results were only descriptive.

### 4.3. Results

The relative quantification of ET-1 gene expression in lung tissues of horses affected with SPAOPD was greater, although not statistically significant, than that of non-affected horses (\( p = 0.5632 \), Figure 4.2). Among the cytokines evaluated, the relative gene expression of IL-4 was greater, although not statistically significant, in lungs of SPAOPD-affected horses compared with non-affected horses (\( p = 0.0770 \), Figure 4.3). The relative gene expression of IL-8 was significantly greater in lungs of affected compared with non-affected horses (\( p = 0.0146 \), Figure 4.4). The relative gene expression of IFN-\( \gamma \) was lesser in lungs of SPAOPD-affected horses compared with non-affected (\( p = 0.5284 \), Figure 4.5).

The quantity of immunoreactive ET in lung tissue extracts from SPAOPD-affected horses (1.5225 ± 0.1901 ng/mg of protein) was greater than those from non-affected horses (1.0587 ± 0.1401 ng/mg of protein). However, the difference in the measured immunoreactive ET-1 between groups was not statistically significant (\( p = 0.19 \)). We chose to report the amount of ET-1 in relation to the amount of protein instead of the
weight of the tissue to ensure that we accounted for the variability in the efficiency of the protein extraction procedure.

The lungs of some of the affected horses appeared grossly overinflated. The histology of lung tissues from SPAOPD-affected horses revealed varying severity of mucus accumulation in the small airways, often obstructing the airways completely, goblet cell metaplasia, bronchial epithelial hyperplasia, peribronchiolar inflammatory infiltrate and smooth muscle hyperplasia (Figure 4.6). The mucus scores, which take into account the amount of mucus present in the airway as well as goblet cell hyperplasia/metaplasia, were greater in SPAOPD-affected than in non-affected horses (Figure 4.7). Peribronchial inflammatory infiltrate and fibrosis were not pronounced findings in SPAOPD (Figure 4.7).

Immunoreactivity to ET-1 scores ranged from weak (score 1) to moderate (score 2) in airway connective tissue, airway smooth muscle, vascular connective tissue and vascular smooth muscle in lung sections from non-affected horses (Figure 4.8). In this group, endothelium and alveolar epithelium had a focal/weak ET-1 immunoreactivity score. The airway epithelium from non-affected horses had minimal ET-1 immunoreactivity (Figure 4.9).

In lung sections from SPAOPD-affected horses, the ET-1 immunoreactivity was pronounced especially in the airway structures (Figures 4.10 and 4.11). The immunoreactivity scores in SPAOPD-affected horses were greater than in non-affected horses in the airway sites (Figures 4.9, 4.10 and 4.11), including airway connective tissue, airway smooth muscle and airway epithelium, and alveolar epithelium. The ET-1 immunoreactivity in vascular connective tissue, vascular smooth muscle and endothelium from SPAOPD-affected horses was slightly greater than those from non-affected horses.
Figure 4.2. Relative gene expression of equine endothelin (ET)-1 in lung tissues from horses affected with summer pasture-associated obstructive pulmonary disease and non-affected horses. Data were expressed in units normalized for the housekeeping gene equine β-actin. No significance at $p < 0.05$ ($p = 0.5632$).
Figure 4.3. Relative gene expression of equine interleukin-4 (IL-4) in lung tissues from horses affected with summer pasture-associated obstructive pulmonary disease and non-affected horses. Data were expressed in units normalized for the housekeeping gene equine $\beta$-actin. No significant difference at $p < 0.05$ ($p = 0.0770$).
Figure 4.4. Relative gene expression of equine interleukin-8 (IL-8) in lung tissues from horses affected with summer pasture-associated obstructive pulmonary disease and non-affected horses. Data were expressed in units normalized for the housekeeping gene equine β-actin. Different letter represent significant difference between affected and non-affected horses ($p = 0.0146$)
Figure 4.5. Relative gene expression of equine interferon (IFN)-γ in lung tissues from horses affected with summer pasture-associated obstructive pulmonary disease and non-affected horses. Data were expressed in units normalized for the housekeeping gene equine β-actin. No statistical difference at \( p < 0.05 \).
Figure 4.6. Photomicrograph of a section of lung obtained from a horse with summer pasture-associated obstructive pulmonary disease. Notice the mucus accumulation obstructing the small airways (purple). Stain = HE; Bar = 50 µm.
Figure 4.7. Proportional frequency of histopathologic scores: Mucus, Peribronchial Inflammatory Infiltrate (PbrInf), Peribronchial Fibrosis (PbrFibr). NA = non-affected horses; A = horses affected with summer pasture-associated obstructive pulmonary disease. Shaded area indicated the greater scores, ranging from no shade indicating least score to dark shade indicating strongest score.
Figure 4.8. Proportional frequency of ET immunoreactivity scores in the various sites of the in lung tissues. NA = non-affected horses; A = horses affected with summer pasture-associated obstructive pulmonary disease; Alv = alveolar epithelium; AwCon = airway connective tissue; AwEp = airway epithelium; AwSm = airway smooth muscle; VCon = vascular connective tissue; Vep = vascular epithelium; VSm = vascular smooth muscle. Shaded area indicated the greater scores, ranging from no shade indicating least score to dark shade indicating strongest score.
Figure 4.9. Photomicrograph of a section of lung obtained from a non-affected horse. The section was stained with avidin-biotin-peroxidase complex method, using rabbit anti-ET-1 and biotinylated goat anti-rabbit IgG, and counterstained with Mayer's hematoxylin. Notice the normal bronchial epithelia with no brown staining, the moderate brown staining (ET immunoreactivity) of the connective tissue and weak brown staining of the airway smooth muscle. Bar = 50 µm.
Figure 4.10. Photomicrograph of a section of lung obtained from a horse affected with summer pasture-associated obstructive pulmonary disease. The section was stained with avidin-biotin-peroxidase complex method, using rabbit anti-ET-1 and biotinylated goat anti-rabbit IgG, and counterstained with Mayer's hematoxylin. Notice the brown staining of the bronchial epithelial cells indicating ET immunoreactivity. Bar = 50 µm.
Figure 4.11. Photomicrograph of a section of lung obtained from another horse affected with summer pasture-associated obstructive pulmonary disease. The section was stained with avidin-biotin-peroxidase complex method, using rabbit anti-ET-1 and biotinylated goat anti-rabbit IgG, and counterstained with Mayer's hematoxylin. Notice the bronchial epithelial hyperplasia, and the moderate brown staining of the bronchial epithelial cells indicating ET immunoreactivity. Bar = 50 µm.
Figure 4.12. Proportional frequency of ET immunoreactivity scores (et) in relation to mucus scores. NA = non-affected horses; A = horses affected with summer pasture-associated obstructive pulmonary disease. Shaded area indicated the most frequent combination of ET immunoreactivity scores accompanying mucus scores.
There appears to be a positive relationship between airway epithelial ET immunoreactivity scores and mucus scores (Figure 4.12). The predominant frequency of the combination of ET immunoreactivity score: mucus score was 0:0 in non-affected, whereas it was 2:2 and 2:3 in SPAOPD-affected horses.

4.4. Discussion

This is the first report of the expression of ET-1 transcripts as well as the immunoreactive ET-1 peptide in the lungs of SPAOPD-affected horses and non-affected horses. There was a trend of greater amounts of ET-1 transcripts and immunoreactive ET-1 peptide in lungs of affected compared to non-affected horses. Because the lung is an important organ in ET-1 metabolism, the expression of ET-1 is constitutively high, and therefore the up-regulation of ET-1 expression in the lungs of SPAOPD-affected horses may not be easy to demonstrate.

The term trend was used to indicate differences that did not represent statistical significance at a type I error level of 5%. The lack of statistical significance in the ET-1 and IL-4 gene expression between affected and non-affected horses is likely to result from variation within each group (affected and non-affected) being as large as the variation between groups. Therefore, a large number of horses should be evaluated to elucidate if the trends noted in the present study are indeed real differences between affected and non-affected horses. Moreover, the individual variation between animals is a feature of naturally-occurring diseases in out bred species, and a large group of individuals is often necessary to demonstrate differences between affected and non-affected animals. Taking into account the change in the distribution of ET-1 within the lung tissues of SPAOPD-
affected compared to non-affected horses, the greater expression of ET-1 in airway structures, especially the epithelium may have significance in the pathogenesis of the disease.

The ET-1 immunoreactivity is present at low intensity in airway and vascular structures of lungs of normal horses. Similarly, immunolabeling to ET-1 was shown to occur in intact airway epithelium from normal rabbits and healthy humans, suggesting that airway epithelia and submucosal glands are normally sites of ET-1 synthesis (Marciniak et al. 1992; Rennick et al. 1992). The ET-1 immunoreactivity in airway epithelium, airway connective tissue and vascular smooth muscle in horses affected with SPAOPD was greater than in non-affected horses. Although only a relatively small number of horses were evaluated, the ET-1 immunoreactivity of various structures in lungs from SPAOPD-affected horses suggests an altered expression of ET-1, especially in airway epithelium. These results resemble those reported in human asthma Springall et al. 1991; Vittori et al. 1992; Redington et al. 1997). It remains unclear how the greater ET-1 immunoreactivity is associated with morphologic abnormalities observed in SPAOPD, such as airway epithelial hyperplasia or dysfunction, smooth muscle hyperplasia and peribronchial fibrosis.

In healthy horses, the rank order for intensity of ET immunoreactivity resembles the distribution in the human lung (Marciniak 1992). In lungs of healthy horses, the ET immunoreactivity was greatest in connective tissue (vascular > airway), followed by smooth muscle (vascular > airway), with minimal staining of airway epithelium, endothelium and alveolar epithelium. In horses with SPAOPD, there was an overall increase in the intensity of staining and change in the distribution of ET immunoreactivity even though the quantification of immunoreactive ET in lung lysates was not statistically
significantly different between affected and non-affected horses. The ET immunoreactivity in all airway structures from horses affected with SPAOPD was greater than those from non-affected horses. The greater ET immunoreactivity in airway epithelium of SPAOPD-affected horses resembles the findings reported in human patients with asthma, in which there is increased expression of ET by airway epithelial cells (Springall et al. 1991; Vittori et al. 1992; Redington et al. 1997). Increased immunoreactivity to ET-1 in asthmatics, compared with normal subjects, has been reported in several studies to be principally localized in the airway epithelium (Springall et al. 1991; Vittori et al. 1992; Redington et al. 1997). This is the first report of distribution of ET immunoreactivity in lung sections of horses.

The association between ET-1 immunoreactivity in the lungs and severity of airway remodeling in asthmatic patients has not been clearly demonstrated. In the present study, there was a relationship between the mucus score and the intensity level of ET-1 immunoreactivity in lungs of SPAOPD–affected horses, with high mucus scores accompanying greater immunoreactivity to ET-1. The relationship between increased ET-1 immunoreactivity in the airway connective tissue and degree of peribronchial fibrosis remains to be determined. These findings suggest that increased expression of ET in the airways of SPAOPD-affected horses may be related to the mucus hypersecretion seen with this disease.

Increased expression of ET-1 in tissues has been identified in asthma, and also in other pulmonary inflammatory conditions, including idiopathic pulmonary fibrosis, cryptogenic fibrosing alveolitis and post respiratory viral infection (Druml, 1993; Giaid, 1993; Langleben et al. 1993; Ugguccioni et al. 1995, Carr et al. 1998). Endothelin-1 has
biologic effects that mimic several features of human asthma including bronchospasm, airway remodeling, inflammatory cell recruitment and activation, edema, mucus hypersecretion, dysfunction in neuronal input and airway hyperresponsiveness (Hay et al. 1996). Likewise, these are hallmark features of equine RAO, a disease in which the amounts of immunoreactive ET-1 in systemic circulation and BALF have been shown to be greater than in normal control subjects (Benamou et al. 1998).

A number of cell types in addition to endothelial cells can synthesize ET-1. In the lung, airway epithelial cells, smooth muscle cells and macrophages are important sources of ET-1. The up-regulation of ET-1 synthesis has been shown to occur in response to a number of stimuli such as hypoxia, endotoxin, tumor necrosis factor (TNF-α), INF-γ, and granulocyte-macrophage-colony stimulating factor (GM-CSF) (Xu, 1997; Salh, 1998; Xu, 1999). Moreover, ET-1 induced up-regulation of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 in human macrophages (Helset et al. 1993). Endothelin-1 has also been shown to stimulate the expression of IL-6, IL-8 and GM-CSF in airway epithelial cells (Mullol et al. 1996). We found the expression of IL-8 and IL-4 in lungs of SPAOPD-affected horses to be greater than that of non-affected horse. We did not evaluate the pro-inflammatory cytokines. It is possible that ET-1 played a role in the upregulation of IL-8 in lungs of horses affected with SPAOPD. The relationship between the expression of ET-1 and cytokine in lungs of horses affected with SPAOPD remains to be determined.

The airway inflammation and hypersensitivity that occurs in horses with SPAOPD undoubtedly involves a multitude of mediators. We identified an increased immunoreactive ET in airway epithelium, airway connective tissue and vascular smooth muscle of SPAOPD-affected horses.
4.5. Endnotes

a ABI Prism™ 7700 Sequence Detection System, PE Applied Biosystems, a division of Perkin Elmer, Foster City, CA, USA.

b RNA STAT-60, TEL-TEST, Inc., Frienswood, TX, USA.

c DNA-FREE RNA™ Kit, Zymo Research, Orange, CA, USA.

d Zymo-spin Ic column, RNA binding capacity of 5µg, Zymo Research, Orange, CA, USA.

e TaqMan Reverse Transcription Reagents, Applied Biosystems manufactured by Roche Molecular Systems, Inc., Branchburg, NJ, USA.

f PubMed, Nucleotide, NCBI

g Stokes AM, Chirgwin SR and Moore RM: 1-270 bp, Equus caballus mRNA, similar to preproendothelin-1, partial sequence; submitted to GenBank, Accession AY730629.

h Primer Express™, version 1.5, Applied Biosystems, Foster City CA, USA.

i TaqMan Universal PCR Master Mix; Applied Biosystems manufactured by Roche Molecular Systems, Inc., Branchburg NJ, USA

j Sequence Detector version 1.7 ABI Prism, Mac App® software, PE Applied Biosystems, a division Perkin Elmer, Foster City CA, USA.

k MicroAmp Optical 96-well reaction plate and caps, Applied Biosystems, manufactured by Roche Molecular Systems, Inc., Branchburg NJ, USA

l BioRad Protein Assay; BioRad, Hercules, CA, USA

m Centricon YM 30 centrifugal filter device; Millipore Corporation; Distributed by Fisher Scientific.
n ELISA for ET-1, Biomedica Gruppe, Austria; Distributed by American Research Products, Inc., Belmont MA, USA.

o Rammaswamy C. M. “Role of endothelin-1 in gastrointestinal tract of horses during health and disease” PhD dissertation, Department of Comparative Biological Sciences, Louisiana State University, Baton Rouge LA, USA.

p A. M. Stokes “Role of endothelin-1 in the pathogenesis of acute laminitis in horses” PhD dissertation, Department of Comparative Biological Sciences, Louisiana State University, Baton Rouge LA, USA.

q Rabbit anti-endothelin-1, Peninsula Laboratories; Belmont CA, USA.

r Avidin-biotin complex, Vectastain Elite ABC-Rabbit IgG Kit; Vector Laboratories, Burlingame CA, USA.

s Biotinylated goat anti-rabbit IgG Vector Laboratories, Burlingame CA, USA.

CHAPTER 5. *IN VITRO* EVALUATION OF AQUEOUS PROTEIN EXTRACTS OF SELECTED MOLD SPORES AS POTENTIAL AEROALLERGENS INVOLVED IN INDUCING MONONUCLEAR LEUKOCYTE PROLIFERATION AND GENE EXPRESSION OF INTERLEUKIN-4 AND INTERFERON-γ
5.1. Introduction

Summer pasture-associated obstructive pulmonary disease (SPAOPD), a naturally-occurring, recurrent obstructive pulmonary disease is characterized by airway hyperresponsiveness following exposure of affected horses to pasture environment during late spring until mid autumn (Seahorn et al. 1996). The seasonal pattern of the disease and potential environmental factors have been suggested, however, the inciting agents have not yet been identified. Mold spores of several fungal species have been isolated from nasal passages of affected horses during time of clinical exacerbation, and those include Alternaria spp., Curvularia spp., Cladosporium spp. and Fusarium spp. (Seahorn and Beadle 1994). Moreover, the temporal distribution of the mold spore counts of Alternaria spp., Curvularia spp., Cladosporium spp. are related to the temporal distribution of clinical exacerbation of SPAOPD (Chapter 2).

Accumulation of mucopurulent secretion in the airways accompanied by goblet cell metaplasia in small airways and peribronchial mononuclear inflammatory cell infiltrates are common pathological features in lungs of horses affected with SPAOPD (Costa et al. 2000). Several factors, including peptides, lipid-derived mediators, cytokines and chemokines, have been implicated as mediators of airway hyperresponsiveness, mucus hypersecretion and inflammatory cellular infiltration in the pathogenesis of recurrent airway disease in several species. Some of those mediators include leukotriene B4, endothelin, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL) -4 and IL-8 (Loike et al. 1995, Franchini et al. 1998,
The overall goal of this study was to evaluate the *in vitro* response of mononuclear leukocytes to stimulation with selected mold spore extracts. The specific objectives were to culture and stimulate *in vitro* mononuclear leukocytes from SPAOPD-affected and non-affected horses and evaluate cell proliferation and cytokine, IFN-γ and IL-4, expression.

We hypothesized that mononuclear leukocytes from SPAOPD-affected horses stimulated *in vitro* by selected mold spore extracts would proliferate and up-regulate the expression of the gene for IL-4 and not interferon (IFN)-γ, and such a response could not be seen on stimulated mononuclear leukocytes from non-affected horses.

### 5.2. Materials and Methods

The experimental procedures included in this study were approved by the Institutional Animal Care and Use Committee of Louisiana State University. Twelve horses, including six SPAOPD-affected (four castrated males and two intact females; median age 19 years, ranging from 14 to 25; three Quarter Horses, one Appaloosa and two Quarter Horse mixed breeds) and six non-affected control horses (four castrated males and two intact females; median age 17 years, ranging from 12 to 22; five Thoroughbred and one Quarter Horse) were maintained on pastures at Louisiana State University in Baton Rouge, Louisiana, USA. The affected horses were animals that originated from south and central Louisiana, and during every summer developed clinical signs of SPAOPD that was documented by pulmonary function testing and cytologic analysis of bronchoalveolar lavage fluid (BALF) demonstrating recurrent airway obstruction and neutrophilic inflammation, respectively (data not
shown) (Costa et al. 2000). Non-affected horses had no appreciable changes in pulmonary function and cytologic analysis of BALF throughout the year (data not shown). Samples were collected from the six SPAOPD-affected horses during clinical remission and the six non-affected control horses at the same time. The SPAOPD-affected horses were considered to be in clinical remission when there were no signs of respiratory disease. The outline of the experimental design and methods in this study are depicted in Figure 5.1.

5.2.1. Blood Collection and Preparation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells were isolated using Ficoll gradient sedimentation. Briefly, venous blood samples were obtained from the jugular vein into evacuated glass tubes containing preservative free heparin, at 10 U/ml of blood, and centrifuged at 800 x g for 10 minutes. Most of the plasma was removed and discarded. The buffy coat cells were carefully removed from the top of the red blood cells and transferred to a sterile 50-ml conical polypropylene tube containing three parts of calcium and magnesium-free physiological buffered saline (CMF-PBS), and mixed. A 30-ml aliquot of the cell suspension was placed into 50-ml conical polypropylene tubes, underlayed with ten ml Ficoll-sodium metrizoate solution and centrifuged at 800 x g for 30 minutes at 20°C, with no brake. The top layer (CMF-PBS and plasma) was discarded and the interface (Ficoll-sodium metrizoate layer, containing mononuclear cells) was aspirated and placed into a new sterile 50 ml-conical polypropylene tube.
Figure 5.1. Outline of method for cell proliferation and cytokine production by equine peripheral blood mononuclear cells. DNA = deoxyribonucleic acid; RNA = ribonucleic acid; DNase = deoxyribonuclease; IL-4 = interleukin-4; IFN-γ = interferon-γ; RT-PCR = real-time polymerase chain reaction; cpm = counts per minute.
The cell suspension was diluted to 40 ml with CMF-PBS, mixed and centrifuged at 800 x g for 10 minutes without brake. The supernatant fluid was removed by decanting. The cells were mixed and diluted to the same volume (40 ml) with CMF-PBS and centrifuged at 150 x g for 10 minutes without brake. This wash was repeated three times. After decanting the supernatant, the cells were resuspended in RPM1-1640 supplemented with Na₂HCO₃, 25mM HEPES, L-glutamine b (2 mM), penicillin G / streptomycin b (50 U/ml and 50 µg/ml, respectively), 2-mercaptoethanol c (5.5 x 10⁻⁵ M), and heat inactivated fetal calf serum d (FCS, 5 %). The cell number and viability was determined using the 0.04% trypan-blue vital stain, and cells were counted using a hemocytometer. For the proliferation assay, the final cell concentrations were adjusted to 2 x 10⁶ cells/ml (200 µl/ well), and for the gene expression assay, cell concentrations were adjusted to 6 x 10⁶ cells/ml (1,000 µl/ well).

5.2.2. Stimulation of Equine Mononuclear Leukocytes

Aqueous mold spore extracts of Alternaria alternata, Curvularia spicifera, or Cladosporium herbarum (stock concentration 40,000 PNU/ml) e were added to the cultures at three different concentrations (final dilutions 1:25, 1:50 and 1:100, final concentrations of 1,600 PNU/ml, 800 PNU/ml and 400 PNU/ml). Mitogens phytohemagglutinin c (PHA, 2µg/ml) and pokweed mitogen c (PWM, 0.5µg/ml) were included as positive controls. Unstimulated control cultures contained media only. The final volumes for the proliferation assay cultures were 200 µl per well (96-well microtiter plate), and for the gene expression assay cultures were 1,000 µl (24-well plate). Plates were incubated in a 5% CO₂ humidified atmosphere at 38° C for 3 or 5 days.
5.2.3. Quantification of Lymphocyte Proliferation

On the third and fifth days, the cultures were pulsed with 1 µCi of radiolabeled thymidine per well for four hours. The plates were frozen –20°C, until DNA was harvested onto glass fiber mats, using a 96-well plate harvester, and 10 ml of liquid scintillation solution was added to each filter mat for liquid scintillation counting. The lymphocyte proliferative responses were expressed as a stimulation index (SI), which was calculated by dividing the counts per minute (cpm) of stimulated by the unstimulated cultures.

5.2.4. Evaluation of Gene Expression of Cytokines IL-4 and IFN-γ

The relative gene expression of equine cytokines IL-4 and IFN-γ, and the house-keeping gene β-actin, was evaluated using real-time polymerase chain reaction (RT-PCR). The cells were harvested from each well of the 24-well plates into microcentrifuge tubes and centrifuged at 12,000 x g for 5 minutes. The supernatant was discarded and the cell pellets were resuspended and homogenized in 300 µl of phenol/guanidinium thiocyanate monophase solution, a total ribonucleic acid (RNA)/mRNA isolation reagent. Samples were stored at –70°C until RNA extraction procedure. The isolation and extraction of total RNA was done according to the protocol provided by the manufacturer. Briefly, samples were thawed and incubated for 5 minutes at room temperature. The aqueous phase containing RNA was extracted using a standard chloroform extraction method, precipitation with 100% isopropanol, two washes with 75% ethanol and desiccation at room temperature.

The concentration of RNA for each sample was estimated by spectrophotometry, i.e., determining the absorbance at 260 nm and 280 nm. Total RNA was estimated to be
80% of the nucleic acid concentration. All samples were subjected to deoxyribonuclease (DNase) I treatment using a commercial kit in order to eliminate genomic deoxyribonucleic acid (DNA) contamination. Total RNA was eluted from the RNA-binding column matrix with ribonuclease-free water. The DNA-free total RNA was subjected to reverse transcriptase reaction using random oligohexamers as primers in the reverse transcription commercial kit to generate the cDNA templates for PCR amplification.

The complete or partial sequence of equine IL-4, IFN-γ and β-actin mRNA have been published and are available, and were used for the design of TaqMan specific probes and primers using specific software (Table 5.1). The fluorescent dye-labeled probes (oligonucleotide with a 5’ reporter dye FAM™ and 3’ quencher dye TAMRA™) are cleaved by the 5’ to 3’ nuclease activity of the AmpliTaq Gold DNA polymerase during the amplification steps of the polymerase chain reaction and the emitted fluorescence was detected and recorded by an automated fluorometer.

<table>
<thead>
<tr>
<th>Gene product</th>
<th>PubMed: Nucleotide no.</th>
<th>Amplicon length</th>
<th>Forward primer</th>
<th>Probe</th>
<th>Reverse primer</th>
</tr>
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<tbody>
<tr>
<td>Eq β-actin*</td>
<td>AF035774</td>
<td>68 bp</td>
<td>616 to 634 (19 bases)</td>
<td>636 to 662 (27 bases)</td>
<td>664 to 683 (20 bases)</td>
</tr>
<tr>
<td>Eq IL-4*</td>
<td>AF305617</td>
<td>69 bp</td>
<td>150 to 169 (20 bases)</td>
<td>173 to 197 (25 bases)</td>
<td>198 to 218 (20 bases)</td>
</tr>
<tr>
<td>Eq IFN-γ</td>
<td>D28520</td>
<td>75 bp</td>
<td>326 to 334 (19 bases)</td>
<td>339 to 368 (30 bases)</td>
<td>382 to 390 (19 bases)</td>
</tr>
<tr>
<td>Eq IL-8*</td>
<td>AY184956</td>
<td>68 bp</td>
<td>100 to 119 (20 bases)</td>
<td>121 to 145 (25 bases)</td>
<td>149 to 168 (19 bases)</td>
</tr>
</tbody>
</table>

* Designed by Dr. D. Horohov.
The real-time PCR procedure was performed using the commercial kit, TaqMan Universal PCR Master Mix according to the manufacture’s recommended protocol. Briefly, the template (5 µl/ reaction) is mixed with TaqMan buffer (10mmol/l Tris-HCl, pH 8.3, 50mmol/l KCl, 5 mmol/l MgCl₂, 2.5 mmol/l deoxynucleotide triphosphates), AmpliTaq Gold Polymerase (0.625 u/reaction) and the specific set of oligonucleotides, i.e., the TaqMan probe and reverse and forward primers. The 96-well plates were loaded into ABI Prism® 7700 and subjected to activation and serial amplification cycles (50°C for 2 minutes, 95°C for 10 minutes for AmpiTaq Gold DNA polymerase activation, and 40 cycles of denaturation and annealing/extension, 15 seconds at 95°C and 1 minute at 60°C, respectively).

Samples from each culture stimulant of cells from SPAOPD-affected and non-affected horses were assayed in duplicates, using a single reaction per well, i.e., singleplex where each replicate of each sample was assayed with the set of specific primers and probe for the cytokine gene and the set of primers and probe for the β-actin in separate wells (Leutenegger et al. 1999). The relative quantification of the PCR products was calculated based on a standard curve, which was constructed using serial dilutions of DNA plasmid constructs containing the IL-4, IFN-γ and β-actin cDNA sequences generated by Dr. Horohov’s laboratory (Beadle and Horohov 2002). The IL-4 and IFN-γ gene expression were normalized in relation to the expression of the housekeeping gene, β-actin, which served as the internal control of the amplification reaction (Leutenegger et al. 1999).
5.2.5. Statistical Analysis

The data were determined to follow a normal distribution using the Shapiro-Wilk statistic. The stimulation index (SI) and the IL-4 and IFN-γ gene expression and the IL-4/IFN-γ ratio in cultures of PBMC from SPAOPD-affected and non-affected horses were compared using a two-way ANOVA. Post-hoc comparisons were performed where appropriate, using Tukey’s test. A $p \leq 0.05$ was considered significant for all tests. Analyses were performed using statistical software.

5.3. Results

The lymphocyte proliferation response to aqueous mold spore extracts showed a dose-response to *Alternaria alternata* and *Curvularia spicifera* at day 5 of culture (Figure 5.2). Stimulation with *Cladosporium herbarum* extract resulted in a very low proliferative response. The proliferative response to mitogen stimulation with both phytohemaglutinin and pokweed mitogen were as expected. The overall effect of the stimulant was statistically significant ($p < 0.05$, Figure 5.3). The proliferative responses to different concentrations of the specific aqueous mold spore extracts, for all horses combined, were not statistically significant ($p = 0.060$, Figure 5.4). There was an overall a trend of lower proliferative response of lymphocytes from SPAOPD-affected horses compared with non-affected horses ($p = 0.0691$, Figure 5.5). The difference in the proliferative response between SPAOPD-affected horses and non-affected controls to specific aqueous mold spore extracts and mitogens was not statistically significant ($p = 0.4318$). Based on the proliferative response the culture time comparing three and five days of culture, the five days of culture was chosen for the cytokine gene expression assay (data not shown).
Figure 5.2. Lymphocyte proliferation following stimulation of mononuclear leukocytes from horses affected with summer pasture-associated obstructive pulmonary diseases and non-affected controls with selected aqueous protein extracts of mold spores for five days. Stimulation index (SI) = counts per minute (cpm) of stimulated cultures divided by the cpm of unstimulated cultures; PHA = phytohemagglutinin, PWM = pokweed mitogen.
Figure 5.3. The effect of the stimulants on cell proliferation of mononuclear leukocytes from horses affected with summer pasture-associated obstructive pulmonary diseases and non-affected controls stimulated with selected aqueous protein extracts of mold spores for five days. Stimulation index (SI) = counts per minute (cpm) of stimulated cultures divided by the cpm of unstimulated cultures. Different letters denote statistically significant differences were noted ($p < 0.05$).
Figure 5.4. The effect of animal group on mononuclear leukocytes proliferation. Data from all aqueous protein extracts of mold spores were combined for horses affected with summer pasture-associated obstructive pulmonary diseases and for non-affected controls for five days. Stimulation index (SI) was calculated by dividing the counts per minute (cpm) of stimulated cultures by the cpm of unstimulated cultures. No statistically significant differences were noted at $p < 0.05$ ($p = 0.0691$).
Figure 5.5. The effect of the stimulant concentrations on the proliferation of equine mononuclear leukocytes after five days of culture. Data from horses affected with summer pasture-associated obstructive pulmonary diseases and non-affected controls were combined. Stimulation index (SI) calculated by subtracting the counts per minute (cpm) of unstimulated cultures from the subtracted from the cpm of stimulated cultures. No statistically significant differences were noted at $p < 0.05$ ($p = 0.0600$).
Figure 5.6. Gene expression of interleukin (IL)-4 in cultures of mononuclear leukocytes following stimulation with selected aqueous protein extracts of mold spores for 5 days. Different letter denote statistically significant differences ($p < 0.05$).
Figure 5.7. Gene expression of interferon (IFN-γ) in cultures of mononuclear leukocytes following stimulation with selected aqueous protein extracts of mold spores for 5 days. No statistically significant differences were noted at $p < 0.05$ ($p = 0.3503$).
Figure 5.8. Ratio of interleukin (IL)-4 and interferon (IFN)-γ gene expression in mononuclear leukocytes following stimulation with selected aqueous protein extracts of mold spores for 5 days. Different letters denote statistically significant differences noted at $p < 0.05$. 

Figure: A bar graph showing the ratio of IL-4/IFN-γ for different treatments. The treatments include Media, Alternaria, Curvularia, Cladosporium, and PHA. The bars for Affected and Non-Affected are labeled with letters indicating statistical significance.
After five days of culture, the gene expression of cytokines IL-4 and IFN-\(\gamma\) in mononuclear leukocytes from SPAOPD-affected horses differed from non-affected controls, although the differences were not statistically significant, except for *Cladosporium spp.* (Figures 5.6 and 5.7). The trends in the cytokine profile of stimulated cultures were interesting. Mononuclear leukocytes from SPAOPD-affected horses had an overall greater gene expression of these two cytokines than the non-affected controls, with the exception of IL-4 expression following stimulation with *Alternaria alternata* extract (Figure 5.6).

The unstimulated cells (i.e., media) from SPAOPD-affected horses had a high cytokine expression for both IL-4 and IFN-\(\gamma\), greater than the stimulated cultures for the same animals, despite the presumed absence of antigenic stimulation (Figure 5.6). Moreover, the unstimulated cells from SPAOPD-affected horses had an overall greater cytokine expression than non-affected control horses. Mitogen stimulation induced a greater IL-4 expression and lower IFN-\(\gamma\) expression in cells from SPAOPD-affected than non-affected horses.

Aqueous extracts of *Curvularia spicifera* and *Cladosporium herbarum* but not *Alternaria alternata* induced greater IL-4 expression in SPAOPD-affected horses than in non-affected horses (Figure 5.6). Extracts of *Alternaria alternata* and *Curvularia spicifera* induced greater IFN-\(\gamma\) expression in SPAOPD-affected horses than in non-affected horses (Figure 5.7). The extract of *Cladosporium herbarum* induced an overall lower cytokine production by lymphocytes from both affected and non-affected horses compared with the other stimulants (Figures 5.6 and 5.7). In lymphocytes from SPAOPD-affected horses, the extract of *Cladosporium herbarum* induced a relatively greater production of IL-4 and lower production of IFN-\(\gamma\), compared with the response in non-affected horses, resulting in a high IL-4/IFN-\(\gamma\) ratio (Figure 5.8).
The ratio of expression of IL-4/IFN-γ in cultures from SPAOPD-affected horses was greater for unstimulated cultures and any stimuli except the mitogen PHA compared with non-affected controls. Although *Cladosporium herbarum* extract induced an extremely small cytokine expression, the IL-4/IFN-γ ratio was greater in cells from SPAOPD-affected horses than non-affected controls (Figure 5.8). In fact, the IL-4/IFN-γ ratio for *Cladosporium herbarum* extract was the greatest of all the stimulants tested (Figure 5.8).

### 5.4. Discussion

The *in vitro* stimulation of equine mononuclear leukocytes from SPAOPD-affected and non-affected horses with aqueous mold spore extracts induced lymphocyte proliferative responses that differed between the groups, affected horses having a lower response than non-affected. With respect to the proliferative response, *Cladosporium herbarum* differed from all the other stimulants, and *Alternaria alternata* and *Curvularia spicifera* did not differ from each other.

The term trend was used to indicate differences that did not represent statistical significance at a type I error level of 5%. The lack of statistical significance in the proliferation and the cytokines gene expression between affected and non-affected horses is likely to result from variation within each group (affected and non-affected) was as large as the variation between groups. Therefore, a large number of horses should be evaluated to elucidate if the trends noted in the present study are indeed real differences between affected and non-affected horses. Moreover, the individual variation between animals is a feature of naturally-occurring diseases in out bred species, and a large group of individuals is often necessary to demonstrate differences between affected and non-affected animals.
The cytokine expression differed between groups; SPAOPD-affected horses had a trend of a greater expression of IL-4 and IFN-γ than non-affected horses. These results suggest that the antigenic stimulation with selected protein extracts of mold spores did not induce either a polarized T helper 1 lymphocytes (TH1) response or a T helper 2 lymphocytes (TH2) response. The TH1 response is characterized by a cytokine profile of IFN-γ, IL-2 and tumor necrosis factor (TNF)-α, whereas the TH2 response includes a cytokine profile of IL-4, IL-5, IL-9 and IL-13 (Mosmann et al. 1986 and Romagnani 1991). Interestingly, the cytokine profile reported in peripheral blood mononuclear cells and BALF cells from SPAOPD-affected horses during disease exacerbation had a similar cytokine profile (Beadle and Horohov 2002). The clinical exacerbation of the disease was characterized by a mixed cytokine profile, where both IL-4 and IFN-γ, where greater than those during clinical remission of the disease (Beadle and Horohov 2002).

In unstimulated cultures, cells were cultured in media that contained FCS but no additional antigenic stimulant. The unstimulated cultures from SPAOPD-affected horses had much greater cytokine expression than those from non-affected control horses. This high background in cultures from SPAOPD-affected horses suggests that the cells from these horses might have been primed in vivo prior to the in vitro stimulation assay, or that these horses may have a more pronounced overall cytokine response to any antigenic stimuli when compared with non-affected horses. Mitogen stimulation induced a greater IL-4 expression but lower IFN-γ expression in cells from SPAOPD-affected than non-affected horses, suggesting that SPAOPD-affected horses may have a TH2 lymphocyte response bias.

The unstimulated cells had an overall high cytokine expression in cells from horses affected with summer pasture associated obstructive pulmonary disease, but not in cells
from non-affected horses despite the presumed absence of antigenic stimulation. This may be a result of the unexpected and undesirable stimulation caused by the FCS itself. It may be indicated to culture the equine mononuclear leukocytes with autologous serum rather than with the FCS, in an attempt to decrease this high background stimulation of the cells. Alternatively, the cells from SPAOPD-affected horses were primed or had a greater basal production of these cytokines. Another possibility is that all the stimulants had a suppressive effect on cytokine gene expression of SPAOPD-affected horses, but not in cells from non-affected horses. Suppressive cytokine such as IL-10 and transforming growth factor (TGF)-β may have been induced upon stimulation with extracts of mold spores, resulting in decreased response compared with unstimulated cultures.

To our knowledge, no other study has reported the in vitro stimulation of peripheral blood mononuclear cells the obtained from SPAOPD-affected and non-affected horses using protein extracts of putative mold spores to evaluate cytokine gene expression. Although this was a small study, it suggested that the SPAOPD-horses have a mixed cytokine expression profile in response to specific mold spore stimulation, with both IL-4 and IFN-γ being greater than that of non-affected horses. Moreover, this study highlights a different response of the affected horses to Cladosporium herbarum. In fact, stimulation with Cladosporium herbarum concurs with the original hypothesis of SPAOPD horses having an allergens-induced polarized TH2 lymphocyte response, with high IL-4/IFN-γ ratio. The significance of the findings from this in vitro study, especially with respect to the in vivo response to aeroallergens requires further investigation.

5.5. Product Information

a Ficoll-Paque Plus, Amersham Biosciences Corporation, Piscataway NJ, USA.
L-glutamine, Gentamicin, Penicillin/Streptomycin, Gibco-BRL, Grand Island NY, USA.

2-mercaptoethanol, Phytohemaglutinin, Pokweed mitogen; Sigma-Aldrich, Inc., Saint Louis, MO USA.

Fetal Calf Serum, HyClone Sera, Logan UT, USA.

Greer Laboratories; Lenoir NC, USA.

Methyl-3H Thymidine, Life Science Products Inc., Boston MA, USA.

Glass fibre filtermats, Wallac, Oy Turku, Finland.

Tomtec Harvester, 96-well, Mach II, Orange, Connecticut, USA.

BetaPlate Scint Fluor, Wallac, Oy Turku, Finland.

Liquid Scintillation Counter LKB 1205 Betaplate, Wallac, Oy Turku, Finland.

ABI Prism TM 7700 Sequence Detection System, PE Applied Biosystems, a division of Perkin Elmer, Foster City CA, USA.

RNA STAT-60, TEL-TEST, Inc., Frienswood TX, USA.

DNA-FREE RNA TM Kit, Zymo Research, Orange CA, USA.

Zymo-spin Ic column, RNA binding capacity of 5µg, Zymo Research, Orange CA, USA.

TaqMan Reverse Transcription Reagents, Applied Biosystems manufactured by Roche Molecular Systems, Inc., Branchburg NJ, USA.

PubMed, Nucleotide, NCBI.

Primer Express TM, version 1.5, Applied Biosystems, Foster City CA, USA.

TaqMan Universal PCR Master Mix; Applied Biosystems manufactured by Roche Molecular Systems, Inc., Branchburg NJ, USA.

Sequence Detector version 1.7 ABI Prism, Mac App® software, PE Applied Biosystems, a division Perkin Elmer, Foster City CA, USA.

MicroAmp Optical 96-well reaction plate and caps, Applied Biosystems, manufactured by Roche Molecular Systems, Inc., Branchburg NJ, USA.

SAS version 8.0; SAS Institute, Cary, NC, USA.
CHAPTER 6. *IN VITRO* EVALUATION OF ENDOTHELIN-1 AND AQUEOUS PROTEIN EXTRACTS OF POLLEN GRAINS AND MOLD SPORES OF POTENTIAL AEROALLERGENS INVOLVED IN ACTIVATION AND CHEMOTAXIS OF GRANULOCYTES FROM HORSES AFFECTED WITH SUMMER PASTURE-ASSOCIATED OBSTRUCTIVE PULMONARY DISEASE
6.1. Introduction

The inciting causes and the early events involved in the pathogenesis of clinical exacerbation of summer pasture-associated obstructive pulmonary disease (SPAOPD) remain unknown. Specific types of mold spores have been isolated from nasal passages of affected horses during clinical exacerbation of SPAOPD, including \textit{Alternaria spp.}, \textit{Curvularia spp.}, \textit{Cladosporium spp.} and \textit{Fusarium spp.}, and those have been suggested as triggering agents of the airway inflammation (Seahorn and Beadle 1994). We have identified a temporal pattern of certain types of mold spores and of grass pollen grains to coincide with the seasonal pattern of clinical exacerbation of SPAOPD (Chapter 2). Although, mold spores and grass pollen are suspected, their role in the disease has not been demonstrated.

Because neutrophils are the predominant cell population in bronchalveolar lavage fluid (BALF) of horses affected with SPAOPD and barn-associated RAO, their role in the amplification and propagation of airway inflammation has been suggested (Grunig et a. 1991). Several inflammatory mediators, including lipid-derived mediators, peptides, chemokines and cytokines are believed to induce neutrophil chemotaxis and have been implicated in the pathogenesis of RAO (Franchini et al. 1998). Some of those mediators including leukotriene B$_4$, platelet activating factor, interleukin (IL)–8, tumor necrosis factor (TNF)-$\alpha$ and macrophage inflammatory protein (MIP)-2 are known neutrophil chemoattracts (Loike et al. 1995; Franchini et al. 1998; Brazil et al. 1998; Franchini et al. 2000; Galligan & Coomber 2000). Moreover, endothelin-1 has been suggested to be involved in the recruitment and activation of inflammatory cells. Endothelin (ET)-1 resulted in intravascular sequestration of neutrophils in guinea pigs (Filep et al. 1995). Endothelin
antagonism prevented antigen-induced airway influx of neutrophils and eosinophils in mice (Fujitani et al. 1997). We investigated the neutrophil activation and chemoattraction following in vitro stimulation of peripheral blood granulocytes and peripheral blood mononuclear cells with putative mold spores and grass pollen extracts. Elucidation of the mechanism involved in the neutrophil activation and chemotaxis will help our understanding of the pathogenesis of SPAOPD. Evaluation of neutrophil activation and chemotaxis following stimulation of mononuclear leukocytes from SPAOPD-affected horses with implicated putative aeroallergens to our knowledge has not been reported.

The overall goal of this study was to evaluate possible early events in the pathogenesis of SPAOPD involving mononuclear leukocytes and neutrophils. The specific objectives were to evaluate granulocyte activation directly and indirectly by aqueous protein extracts of selected pollen grains and mold spores of potential aeroallergens. First, peripheral blood granulocytes were stimulated with aqueous protein extracts of selected pollen grains and mold spores, and cell activation was evaluated by measuring the up-regulation of surface marker CD18 adhesion molecule using quantitative flow cytometry. Secondly, peripheral blood mononuclear cells from SPAOPD-affected and non-affected horses were stimulated in vitro by the same aqueous protein extracts of pollen grains and mold spores, in the presence of either fetal calf or autologous serum, and the supernatants from these cultures were used to evaluate the up-regulation of surface marker CD18 on granulocytes by quantitative flow cytometry. Finally, the aqueous mold spore and pollen protein extracts and ET-1 were evaluated for their ability to chemoattract equine granulocytes using a microtiter chemotaxis system.

The first hypothesis of this study was that antigens in mold spore or pollen protein extracts are able to promote neutrophil/ granulocyte activation (up-regulation of surface
marker CD18) and induce neutrophil/ granulocyte migration in a concentration-dependent manner. The second hypothesis of this study was that mononuclear leukocytes stimulated in vitro by selected mold spore or pollen antigens release mediators into the media that promote activation of neutrophils/ granulocytes (up-regulation of surface marker CD18).

The third hypothesis of this study was that ET-1 is able to activate neutrophils/ granulocytes (up-regulate expression of surface marker CD18) in a concentration-dependent manner, and that ET-1 can induce concentration-dependent migration of neutrophils/ granulocytes. In addition the types of serum, either fetal calf serum or autologous serum heat-inactivated or not, were evaluated for their ability of synergistically interact with the antigens in mold spore or pollen protein extracts and promote neutrophil/ granulocyte activation (up-regulation of surface marker CD18).

6.2. Materials and Methods

6.2.1. Collection of Sera, Peripheral Blood Mononuclear Cells and Granulocytes

All experimental procedures performed for this study were approved by the Animal Care and Use Committee of Louisiana State University. Jugular venous blood samples were collected into evacuated glass tubes without anticoagulant from six SPAOPD-affected horses during clinical exacerbation and six non-affected control horses at the same time. Blood was allowed to clot and tubes were centrifuged at 800 x g, 4°C for 10 minutes. Serum was harvested and serum samples were stored at –70°C. Jugular venous blood samples were collected into evacuated glass tubes without anticoagulant and containing preservative free heparin (10 U/ml of blood) from six SPAOPD-affected horses during clinical remission and six non-affected control horses at the same time. All 12 horses, six SPAOPD-affected (one castrated male and five intact females; one
Thoroughbred and two Quarter Horse, one Paint, one Appaloosa/Quarter Horse and one Arabian/Quarter Horse median age 15 years, ranging from 7 to 27) and six non-affected control horses (four castrated males and two intact females; five Thoroughbred and one Quarter Horse median age 15 years, ranging from 14 to 17) were kept on pastures at Louisiana State University in Baton Rouge, Louisiana, USA. The affected horses were originated from south Louisiana and developed clinical signs of SPAOPD during every summer; they had pulmonary function testing and cytologic analysis of bronchoalveolar lavage fluid (BALF) documenting reversible recurrent airway obstruction. The SPAOPD-affected horses were considered to be in clinical remission when there were no signs of respiratory disease, the intrapleural pressure difference was less than 10 cm of water, and percentage of neutrophil in bronchoalveolar lavage fluid was less than 15%, similar to non-affected horses (Lavoie et al. 2001).

6.2.2. Isolation of Equine Peripheral Blood Granulocytes

Peripheral blood granulocytes (PBG) were isolated using Ficoll centrifugation and dextran sedimentation. Briefly, peripheral blood samples were collected from the jugular veins into evacuated tubes containing preservative-free heparin (10 U/ml). Blood was mixed with three parts of 0.9% saline at room temperature (20 to 22°C). A 30-ml aliquot of the cell suspension was placed into each sterile 50 ml-polypropylene conical tubes, carefully underlayed with 10 ml Ficoll-sodium metrizoate solution and centrifuged at 400 x g, for 40 minutes, 20°C with no brake. The top layer (plasma and saline) and the interface (Ficoll-sodium metrizoate solution layer containing mononuclear cells) were aspirated and discarded, leaving the granulocyte/red blood cells (RBCs) pellet. The granulocyte/RBCs pellet was resuspended in 20 ml phosphate buffered saline (PBS), and an equal volume of 3% dextran/0.9% saline solution was added, mixed and incubated in upright position for
approximately 20 minutes at room temperature. The upper layer (neutrophil-rich) was aspirated, placed into a fresh 50-ml polypropylene conical tube, and pelleted by centrifugation at 250 x g for 10 minutes, 5°C with no brake. The supernatant was discarded and the pellet was immediately resuspended in a volume of 0.9% saline equal to the starting volume of blood. The residual RBCs were removed by subjecting the cells to hypotonic lysis, i.e., resuspending the pellet in 20 ml of cold 0.2% saline for 30 seconds; at the end of 30 seconds isotonicity was restored by adding 20 ml of ice-cold 1.6% saline. Cell suspension was centrifuged at 250 x g for 6 minutes, 5°C, and the supernatant was discarded. Hypotonic lysis was repeated until pellet was visibly free of RBCs. Cell pellet was resuspended in ice-cold serum-free RPMI. The total cell number and viability were determined using the 0.04% trypan-blue vital stain, cells were counted using a hemocytometer, and cell concentrations were adjusted to 10^7 cells/ml. Cell suspension was kept in ice bath until used for the assays. For cytologic evaluation, the concentrated smears of the cell suspensions were prepared by cytocentrifugation. Air-dried smears were stained with a modified Wright’s solution and 200 cells were classified using light microscopy as neutrophils, eosinophils, basophils, lymphocytes, monocytes and expressed as percentages.

### 6.2.3. Preparation of Peripheral Blood Mononuclear Cells

Venous blood samples collected in evacuated glass tubes containing preservative free heparin, at 10 U/ml of blood, were centrifuged at 800 x g for 10 minutes. Most of the plasma was removed and discarded. The buffy coat cells were carefully removed from the top of the red blood cells and transferred to a sterile 50-ml polypropylene conical tube containing three parts of calcium and magnesium-free phosphate buffered saline (CMF-PBS), and mixed. A 30-ml aliquot of the cell suspension was placed into each 50-ml
polypropylene conical tubes, underlayed with 10 ml Ficoll-sodium metrizoate solution and centrifuged at 800 x g, for 30 minutes at 20°C, with no brake. The top layer (CMF-PBS and plasma) was discarded and the interface (Ficoll- sodium metrizoate layer containing mononuclear cells) was aspirated and placed into a new sterile 50-ml polypropylene conical tube. The cell suspension was diluted to 40 ml with CMF-PBS, mixed and centrifuged at 800 x g 10 minutes without brake. The supernatant fluid was removed by decanting. The cells were mixed and diluted to the same volume (40 ml) with CMF-PBS and centrifuged at 150 x g for 10 minutes without brake. This wash was repeated three times. After decanting the supernatant, the cells were resuspended in RPM1-1640 supplemented with Na₂HCO₃, 25mM HEPES, L-glutamine (2 mM), penicillin G / streptomycin (50 U/ml and 50 µg/ml, respectively). The cell number and viability was determined using the 0.04% trypan-blue vital stain, cells were counted using a hemocytometer, and cell concentrations were adjusted to 6 x 10⁶ cells/ml.

6.2.4. Stimulation of Equine Peripheral Blood Granulocytes

Aqueous protein extracts of pollen grains and mold spores were selected based on the relationship with onset of clinical exacerbation of SPAOPD (Chapter 2). The selected aqueous protein extracts of mold spores: *Alternaria alternata*, *Curvularia spicifera*, *Cladosporium herbarum* and *Aspergillus fumigatus*, and selected aqueous protein extracts of grass pollen grains: Bahia grass (*Paspalum notatum*), Bermuda grass (*Cynodon dactylon*) and Johnson Grass (*Sorghum halepense*), stock concentration 40,000 PNU/ml, were added to the PBG cell suspensions (100 µl at 10⁷ cells/ml) at three different concentrations (final dilutions 1:10, 1:100 and 1:1,000, and final concentrations in the tubes of 4,000 PNU/ml, 400 PNU/ml and 40 PNU/ml). Serial dilutions of endothelin-1.
(final concentrations: 500 nM, 100 nM, 50 nM), hr IL-8 (final concentrations: 50 ng/ml, 10 ng/ml), lipopolysaccharide (LPS; final concentration 5 µg/ml), and LPS plus polymixin B (5 µg/ml + 100 µg/ml) were use as stimulants. Alternatively, 400 µl of the supernatants from the mononuclear leukocyte cultures were added to 100 µl of granulocyte suspension (10^7 cells/ml). Unstimulated control cultures contained media only. The final volume per tube of PBG stimulation assay was 500 µl and the total number of cells was 10^6 cells. Tubes were incubated in a 5% CO₂ humidified atmosphere at 38°C for 1 hour and then the cells were stained for flow cytometry analysis.

6.2.5. Stimulation of Equine Peripheral Blood Mononuclear Cells

Aqueous protein extracts of mold spores: *Alternaria alternata*, *Curvularia spicifera*, *Cladosporium herbarum* and *Aspergillus fumigatus*, and selected aqueous protein extracts of grass pollen grains: Bahia grass (*Paspalum notatum*), Bermuda grass (*Cynodon dactylon*) and Johnson Grass (*Sorghum halepense*), were added to the cultures at final concentrations of 100 PNU/ml. A positive control of LPS at two final concentrations 1 ng/ml and 10 ng/mg were included. For each stimulant, media containing 10% of either one of the three serum types: heat inactivated fetal calf serum (FCS), equine sera from each of the 12 horses either non-heat-inactivated or heat-inactivated. Unstimulated control cultures contained media only. The final volume for PBMC stimulation cultures was 2 ml (12-well plate), of which 1 ml was cell suspension and 1 ml was the stimulant dilution. The resulting cell concentration was 3 x 10^6 cells/ml, or 6 x 10^6 cells/well. The plates were incubated in a 5% CO₂ humidified atmosphere at 38°C for 1 hour, centrifuged and the supernatants containing the stimulant were removed, cells were resuspended in fresh complete RPMI in a 5% CO₂ humidified atmosphere at 38°C. The wash was repeated
twice and then plates were incubated for 48 hours. An aliquot of the culture was collected at 12 hours after incubation, and then at the end of the 48 hours. Culture media were collected into microfuge conical tubes, centrifuged at 12,000 x g, and the cell-free supernatants were stored at −70°C until PBG stimulation assay and flow cytometric measure of CD18 surface marker was performed. All supernatant samples for 12 hours and 48 hours were assayed at once using PBG from a healthy horse. Therefore, the only variables were the supernatants themselves, thus allowing a comparison between the stimulatory effects of the supernatants.

Table 6.1. Median and range percentage of cells after Ficoll gradient/dextran sedimentation procedure.

<table>
<thead>
<tr>
<th></th>
<th>Neutrophils</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPAOPD-affected</td>
<td>84.5%</td>
<td>11.6%</td>
<td>0.5%</td>
<td>2.8%</td>
<td>1.4%</td>
</tr>
<tr>
<td></td>
<td>(82 to 88%)</td>
<td>(6 to 15%)</td>
<td>(0 to 0.7%)</td>
<td>(0.7 to 4.5%)</td>
<td>(0.4 to 1.9%)</td>
</tr>
<tr>
<td>Non-affected</td>
<td>81.7%</td>
<td>13.4%</td>
<td>0.8%</td>
<td>2.9%</td>
<td>1.0%</td>
</tr>
<tr>
<td></td>
<td>(80 to 82%)</td>
<td>(12 to 15%)</td>
<td>(0.6 to 0.9%)</td>
<td>(2.7 to 4.1%)</td>
<td>(0.9 to 1.2%)</td>
</tr>
</tbody>
</table>

6.2.6. Quantitative Flow Cytometry

The expression of surface adhesion molecule CD18 on equine granulocytes was evaluated using flow cytometry. Peripheral blood granulocytes harvested using a Ficoll gradient/dextran sedimentation procedure yielded an average of 81 to 85% neutrophils and 11 to 13% eosinophils (Table 6.1). After the incubation of the equine granulocytes with various stimulants for one hour, the cells were then stained using a single fluorochromes, i.e., fluorescein isothiocyanate (FITC), which is excited with 488 nm lasers,
and has the peak emission wavelength at 530 nm. The stimulation of granulocytes was conducted in polystyrene tubes with 100 µl of cell suspension (concentration of 10^7 cells/ml) and 400 µl of stimulant, so the final cell concentration of 2 x 10^6 cells/ml. After one hour of incubation, the tubes were centrifuged at 200 x g for 8 minutes. The supernatants were decanted and 50 µl of mouse anti-equine CD18 (diluted to 20 µg/ml in PBS) was added to the appropriate tubes, so the final volume was approximately 100 µl. To the isotype control tube, 50 µl of mouse IgG1 (diluted to 20 µg/ml) was added instead of the anti-CD18, and two tubes did not receive any primary antibody; these were the two other control tubes, i.e., autofluorescence and FITC control tubes. The samples were mixed by vortexing the tubes and they were incubated for 20 minutes on ice. Cells were washed by adding 2 ml of PBS per tube and centrifuged at 200 x g for 8 minutes. The supernatants were decanted, and the wash was repeated twice, for a total of three washes. All tubes except the autofluorescence control then received 50 µl of goat anti-mouse IgG1-FITC, the autofluorescence control tube received 50 µl of PBS. The samples were mixed by vortexing, and were incubated for 15 minutes in the dark (wrapped in foil) at room temperature. Cells were washed by adding 2 ml of PBS, and centrifuged at 200 x g for 8 minutes. The supernatants were decanted, and the wash was repeated once. The cell pellets in remaining residual wash fluid were mixed by vortexing, and 200 µl of cold 1.5% formaldehyde-PBS fixative was added to each tube. The fixative was added in order to preserve the staining until flow cytometric analysis. The tubes were mixed again using a vortex, wrapped in aluminum foil and stored at 2^o to 6^oC overnight until FACS analysis. The autofluorescence control was included because of the natural inherent fluorescence of the cells due to the presence of pyridine and flavin nucleotides. In addition, the secondary antibody/FITC control was also included because some cells, especially
monocytes and granulocytes, carry immunoglobulin Fc receptors, which can bind antibody reagents nonspecifically.

Data analysis of granulocyte populations (neutrophils and eosinophils) consisted of setting a polygon gate based on forward angle light scatter (cell size) and 90° light scatter (internal granularity) on a dot plot display using the WinMDI software package. The percentages of cells stained for the activation marker, i.e., the CD18 adhesion molecule detected by the anti-CD18 monoclonal antibody, were determined by analysis of one-parameter histogram of fluorescence intensity. The measures of mean and median peak channel fluorescence (MPCF) for either gated and ungated populations were obtained for each test sample; the control samples, i.e., unstained, isotype control and FITC-labeled secondary antibody, were used in order to determine any autofluorescence or non-specific staining. The results were reported net fluorescence intensity, which is the mean peak channel fluorescence from stimulated granulocytes minus the mean peak channel fluorescence from unstimulated granulocytes. The unstimulated granulocytes were cells incubated with media only (media containing serum of different types, if applicable).

6.2.7. Chemotaxis of Equine Granulocytes

Equine PBG were obtained as described above. The granulocytes were stained using a general cell membrane labeling kit. Approximately 2 x 10^7 cells resuspended in serum-free media were placed in the bottom of a polypropylene 15-ml conical tube and centrifuged at 400 x g for 5 minutes. The supernatant was discarded leaving only approximately 25 µl of supernatant on the pellet. One ml of specific diluent was added, and cells were resuspended by pipetting up and down. Immediately prior to staining, the PKH26 dye was prepared to a concentration of 4 x 10^-6 M by diluting 8 µl of PKH26 dye
into 1,992 µl of diluent C. One ml of the diluted dye was added to each ml of cell suspension (1 x 10⁷ cells/ml), immediately mixed, and incubated at 25°C for 5 minutes. The tube was periodically inverted to mix and assure homogeneous staining. The staining reaction was stopped by adding an equal volume of fetal calf serum and incubated for one minute. The sample was then diluted with an equal volume of complete RPMI media which contained 10% FCS. The cell suspension was centrifuged at 400 x g for 10 minutes at 25°C, the supernatant was removed and cells were transferred to a new tube for further washing. A total of three washes were performed using complete RPMI media. Cells were resuspended to a final concentration of 2 x 10⁶ cells/ml. Cells were examined using fluorescence microscopy, checking for recovery and fluorescence intensity; the staining was reasonably uniform and much brighter than the background autofluorescence.

The chemotaxis assay was carried out using a disposable chemotaxis system⁹. The chemotaxis system consisted of a 96-well format microtiter tissue culture plate with bottom chambers holding a volume of 30 µl, the membrane with pore size of 5 µm and 4 x 10³ pores/mm². A volume of 30 µl of each chemoattractants or negative controls were added to the bottom chambers of the 96-well format microtiter plate. After the bottom chambers of the microtiter plate were loaded, the framed filter was placed on top by aligning the holes in the corner of the frame with the four pins on the microtiter plate. Thirty µl of fluorescent-stained cell suspension was pipetted onto each site of the 96-well format framed filter to a final concentration of 64 x 10³ cells/well (i.e., the final ratio of 2 cells per pore) and the cover lid was placed on top of the microtiter plate. The optimal cell/pore ratio had been determined in previous experiments. The disposable chemotaxis plates were incubated for 60 minutes at 38°C, 5% CO₂ humidified incubator. The optimal incubation time had been determined in previous experiments by evaluating serial
incubation periods of equine granulocytes chemotaxis toward a positive control, i.e., serial dilutions of human recombinant IL-8. After the incubation, the disposable chemotaxis plate was carefully dismantled, and the bottom microtiter plate was centrifuged at 250 x g for 5 minutes. Cells that had transmigrated into the lower chamber were at the bottom of the wells, and ten µl of supernatant was removed from the top part of each well, then 20 µl of 4% paraformaldehyde fixative was added to each well in order to preserve the staining until capture of field using fluorescent microscopy. Plates were covered with foil in a moist chamber and stored protected from light at 4°C until cell counts were performed under fluorescent microscopy. The counts of the cells that had transmigrated into the lower chamber were obtained by capturing 7 fields per well (Figure 6.2) using an Axiovert (Zeiss) fluorescent microscope, filter P12x = 101-2 TRITC, at 20 x magnification. After the images were captured the fluorescent-labeled granulocytes of each field were counted using computer software. Cells not counted by the computer were manually counted and a total count per field was recorded. The total count per well was obtained by adding the counts from the seven fields. Results of the chemotactic activity of the substances were reported as the migration index, which represents a ratio between number of cells that transmigrated to the lower chamber in the presence of chemotactic substance and the cells transmigrated in response to media alone.

6.2.8. Statistical Analysis

The data were determined to follow a normal distribution using the Shapiro-Wilk statistic. The up-regulation of granulocyte surface adhesion molecule CD18 was displayed as the net fluorescence intensity and compared between groups (SPAOPD-affected horses and non-affected controls), between stimulants (types of aqueous protein extracts of mold spores and pollen grains) at various concentrations.
Figure 6.1. Outline of procedures for evaluation of activation of peripheral blood granulocytes directly by protein extracts of pollen and mold spores and by mononuclear leukocytes-derived supernatants.
The effect of PBMC-induced up-regulation of granulocyte surface adhesion molecule CD18 was displayed as the net fluorescence intensity and compared between groups (SPAOPD-affected horses and non-affected controls), between stimulants (types of aqueous protein extracts of mold spores and pollen grains), and between the types of serum (FCS, autologous serum and heat-inactivated autologous serum). All comparisons were performed using a factorial ANOVA. Post-hoc comparisons were performed where appropriate, using Tukey’s test; a $p \leq 0.05$ was considered significant. All analyses were performed with the use of the GLM procedure, SAS system, v 8.2.

6.3. Results

The overall increase in the expression of the adhesion marker CD18 on the surface of granulocytes from SPAOPD-affected horses following incubation with aqueous protein extracts of mold spores and pollen grains did not differ significantly from that of non-affected horses (Figure 6.3 to 6.5). Although individual stimulants at different concentrations induced different degrees of expression of the CD18 on the surface of granulocytes, none of the mold spore or pollen grain extracts induced a statistically significant increase in the expression of CD18 on granulocytes. Therefore, neither the status of the horses (SPAOPD-affected or non-affected), the type of stimulant (i.e., specific protein extracts of mold spores and pollen grains) or the concentration of the stimulant (40, 400, or 4,000 PNU/ml) had a significant effect on granulocyte activation. Among the three concentrations of protein extracts of mold spores and pollen grains, a concentration between the lowest (40 PNU/ml) and the middle (400 PNU/ml) produced the highest up-regulation of CD18 on the surface of granulocyte, thus a concentration of 100 PNU/ml was chosen for the experiments of mononuclear leukocyte stimulation assay.
The positive controls, IL-8 and LPS, induced approximately 60% increase in the expression of CD18 adhesion marker (Figure 6.6, Table 6.2). Polymixin B at 100 µg/ml was able to abolish the LPS-induced up-regulation of the CD18 expression on granulocyte of affected and non-affected horses (Figure 6.6, Table 6.2). Endothelin-1 was much less potent than LPS and IL-8 at the concentrations tested in inducing up-regulation of the CD18 expression on granulocyte of affected and non-affected horses (Figure 6.6). Nonetheless, ET-1 at 50 nM, 100 nM and 500 nM induced granulocyte activation in a dose-dependent pattern, i.e., 11%, 21% and 33% (Table 6.2). There was no significant difference between the expression of CD18 on granulocytes from SPAOPD-affected compared with non-affected horses following stimulation with IL-8, LPS or ET-1.

When the supernatants from stimulated mononuclear leukocyte cultures were evaluated for induction of CD18 expression, the variables time of supernatant collection, i.e., 12 hours and 48 hours, according to the group of horses, i.e., SPAOPD-affected and non-affected significantly affected the net fluorescence intensity of CD18 expression on the surface of granulocytes ($p < 0.05$, $p$ value = 0.0001, Figure 6.7). The type of stimulant, i.e., the different aqueous extracts of mold spores and pollen grains, significantly affected the net fluorescence intensity of CD18 expression on the surface of granulocytes ($p < 0.05$, $p$ value = 0.0001, Figure 6.8). None of the other variables (i.e., status and serum) evaluated individually or in combination significantly affected the net fluorescence intensity of CD18 expression on the surface of granulocytes.

The overall increase of the net fluorescence intensity of CD18 expression on the surface of granulocytes following incubation with the supernatants from stimulated mononuclear leukocytes from non-affected horses collected at 12 hours was greater than the from SPAOPD-affected horses (Figures 6.9 to 6.11), whereas the net fluorescence
intensity of CD18 expression on the surface of granulocytes following incubation with supernatants collected after the 48 hours of PBMC stimulation was greater in SPAOPD-affected horses than non-affected horses (Figures 6.14 to 6.16). The three types of serum, or the combination of serum and stimulant after 12 hours or 48 hours did not significantly affect the expression of CD18 on the surface of granulocytes from affected or non-affected horses (Figures 6.12, 6.13, 6.17 and 6.18). There was, however, a significant difference in the granulocytes expression of CD18 induced by PBMC cultures following incubation with different stimulants (Figure 6.8). Supernatants from cultures stimulated with LPS at 10 ng/ml induced significantly greater expression of CD18 than the cultures stimulated with protein extracts of *Cladosporium herbarum*, *Curvularia spicifera*, *Aspergillus fumigatus* and unstimulated (media). Supernatants from cultures stimulated with protein extracts of *Alternaria alternata*, Johnson grass, Bermuda grass and Bahia grass and LPS at 1 ng/ml induced an intermediate expression of CD18 on granulocytes that did not differ significantly from the others. Therefore, the status of the horses (affected or non-affected) and time (12 hours and 48 hours) and the type of stimulant (i.e., specific protein extracts of mold spores and pollen grains) had a significant effect on the mononuclear leukocyte-induced granulocyte activation, whereas the type of serum within the media (FCS, autologous and heat-inactivated autologous serum) did not.

The chemotactic activity of ET-1 was dose-dependent in both SPAOPD-affected in non-affected horses. In the SPAOPD-affected horse the ET-1 induced transmigration of granulocytes that were comparable to the chemotactic activity of LPS at 5 µg/ml (Figure 6.19). The chemotactic activity of protein extracts of selected mold spores (Figure 6.20) and selected pollen grains (Figure 6.21) was also dose-dependent. The chemoattraction of granulocytes obtained from the SPAOPD-affected horse (during clinical remission) was
pronounced and appeared to be greater than the non-affected horse, especially to LPS (5 µg/ml), IL-8 (25 and 50 ng/ml), *Alternaria alternata* (1,000 PNU/ml) and ET-1 (1,000 nM, 500 nM and 100 nM) (Figure 6.22). It is unclear if this difference constitutes an individual variation, or if SPAOPD-affected horses have a greater chemoattraction toward these substances. Alternatively, it is possible that although the SPAOPD-affected horse was not showing clinical signs of airway obstruction (clinical remission) antigenic stimulation was occurring by environmental allergens and the peripheral blood granulocytes were already primed.

The term trend was used to indicate differences that did not represent statistical significance at a type I error level of 5%. The lack of statistical significance in the granulocyte activation between affected and non-affected horses is likely to result from variation within each group (affected and non-affected) was as large as the variation between groups. Therefore, a large number of horses should be evaluated to elucidate if the trends noted in the present study are indeed real differences between affected and non-affected horses. Moreover, the individual variation between animals is a feature of naturally-occurring diseases in out bred species, and a large group of individuals is often necessary to demonstrate differences between affected and non-affected animals.

### 6.4. Discussion

The up-regulation of the surface expression of integrins (CD11/CD18) on neutrophils and monocytes is a well-established parameter of leukocyte activation (Bokoch 1995). Aqueous protein extracts of mold spores and pollen grains induced activation of granulocytes from both SPAOPD-affected horses as well as non-affected horses.
Figure 6.2. Diagram of the seven fields captured per well of the chemotaxis microtiter plate. The images were collected and the fluorescent-labeled granulocytes were counted using Image Pro software; cells not counted by the computer were manually counted and a total count per well was recorded.
Figure 6.3. Activation of granulocytes by protein extracts of mold spores and pollen grains at a concentration of 40 PNU/ml. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of stimulated peripheral blood granulocytes (PBG) minus MPCF of PBG incubated with media only. No significant differences were noted at p < 0.05.
Figure 6.4. Activation of granulocytes by protein extracts of mold spores and pollen grains at concentration of 400 PNU/ml. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of stimulated peripheral blood granulocytes (PBG) minus MPCF of PBG incubated with media only. No significant differences were noted at p < 0.05.
Figure 6.5. Activation of granulocytes by protein extracts of mold spores and pollen grains at concentration of 4,000 PNU/ml. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of stimulated peripheral blood granulocytes (PBG) minus MPCF of PBG incubated with media only. No significant differences were noted at p < 0.05.
Figure 6.6. Activation of granulocytes by lipopolysaccharide (LPS, 5 (g/ml), LPS + polymixin B (LPS/PmB, 5 (g/ml/, 100 (g/ml ), human recombinant IL-8 (IL-8 at 10 and 50 ng/ml) and endothelin-1 (ET at 50 nM, 100 nM and 500 nM). Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of stimulated PBG minus MPCF of PBG incubated with media only.
Figure 6.7. The effect of time (12 hours and 48 hours) and group of horses (affected with summer pasture associated obstructive pulmonary disease and non-affected) on activation of granulocytes by mononuclear leukocytes-derived supernatants. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of peripheral blood granulocytes (PBG) stimulated with supernatants of peripheral blood mononuclear cell (PBMC) cultures minus the MPCF of PBG incubated with media only.
Figure 6.8. The effect of type of stimulant on activation of granulocytes by mononuclear leukocytes-derived supernatants. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of peripheral blood granulocytes (PBG) stimulated with supernatants of peripheral blood mononuclear cell (PBMC) cultures minus the MPCF of PBG incubated with media only.
Figure 6.9. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + fetal calf serum, and collected 12 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MCF) of peripheral blood granulocytes (PBG) stimulated with supernatants of peripheral blood mononuclear cell (PBMC) cultures minus the MCF of PBG incubated with media only.
Figure 6.10. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + autologous serum, and collected 12 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.11. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + heat-inactivated autologous serum, and collected 12 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.12. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen grains and either fetal calf serum (FCS), autologous serum or heat-inactivated (HI) autologous serum, and collected 12 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.13. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of mold spores and either fetal calf serum (FCS), autologous serum or heat-inactivated (HI) autologous serum, and collected 12 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.14. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + fetal calf serum, and collected 48 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MCF) of PBG stimulated with PBMC culture supernatants minus the MCF of PBG incubated with media only.
Figure 6.15. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + autologous serum, and collected 48 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.16. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen or mold spores + heat-inactivated autologous serum, and collected 48 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.17. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of pollen grains and either fetal calf serum (FCS), autologous serum, heat-inactivated (HI) autologous serum and collected 48 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.18. Activation of granulocytes by mononuclear leukocytes-derived supernatants stimulated with protein extracts of mold spores and either fetal calf serum (FCS), autologous serum, heat-inactivated (HI) autologous serum and collected 48 hours after stimulation. Y-axis = net fluorescence intensity, i.e., mean peak channel fluorescence (MPCF) of PBG stimulated with PBMC culture supernatants minus the MPCF of PBG incubated with media only.
Figure 6.19. Chemotactic activity of serial dilutions of ET-1 (1,000 nM, 500 nM, 100 nM and 50 nM), and the positive controls LPS (5 µg/ml) and IL-8 (50 ng/ml). Results are reported as migration index = the number of PBG that transmigrate toward stimuli divided by the number of PBG that transmigrate toward media only.
Figure 6.20. Chemoattraction of granulocytes by protein extracts of pollen grains. Results are reported as migration index = number of PBG that transmigrate toward stimuli divided by number of PBG that transmigrate towards media only.
Figure 6.21. Chemoattraction of granulocytes by protein extracts of mold spores. Results are reported as migration index = number of PBG that transmigrate toward stimuli divided by number of PBG that transmigrate towards media only.
Figure 6.22. Chemotactic activity of all stimuli to equine granulocytes. Chemoattractants: LPS (5μg/ml) and IL-8 (25 ng/ml and 50 ng/ml), serial dilutions of protein extracts of mold spores and serial dilutions of ET-1 ET-1 (1,000 nM, 500 nM and 100 nM). Results are reported as migration index = number of PBG that transmigrate toward stimuli divided by number of PBG that transmigrate towards media only.
The findings in this study are in agreement with the report that aqueous pollen extracts induced activation of polymorphonuclear cells from human patients with allergic rhinitis and healthy human subjects as documented by the up-regulation of the surface adhesion molecule CD 11b (Traidl-Hoffman et al. 2002). The polymorphonuclear cells activation was independent of the donor sensitization, similar to our findings.

Equine mononuclear leukocytes from SPAOPD-affected horses showed a delayed (48 hours) but greater response to aqueous protein extracts of putative aeroallergens by producing and releasing in the media factors that induced granulocyte activation. All stimulants induced mononuclear-derived granulocyte activation, although some stimulants induced a greater response. The different types of protein extracts of mold spore and grass pollen grains induced a significantly different response, suggesting an antigenic recognition. The various serum types made no difference; autologous serum would contain immunoglobulins of all isotypes, whereas heat-inactivated would contain immunoglobulins of all isotypes except IgE (heat-labile), and FCS does not contain any immunoglobulins. Our results suggest that immunoglobulins may not play an important role in this differential response to aeroallergen extracts. Alternatively, as the cultures contained peripheral blood mononuclear cells, among which T lymphocytes constituted a substantial percentage, the antigenic recognition may have resulted from antigen presentation by monocyte-derived macrophages to T lymphocytes resulting in production of factors/ or larger amounts of factors that had a greater ability to activate granulocyte.

The present study demonstrated the chemotactic activity of mold spores and pollen grains to equine granulocytes. Only two horses, one SPAOPD-affected and one non-affected were evaluated. There was greater chemotactic response to the protein extract of *Alternaria alternata* by granulocytes from the SPAOPD-affected horses compared with the
non-affected, whereas the protein extracts from other mold spores or grass pollen grains did not appear to differ. Polymorphonuclear cells from both human patients with allergic rhinitis and healthy human subjects displayed equally significant migration to aqueous pollen extracts (Traidl-Hoffman et al. 2002). It is unclear whether horses, like the human patients, have no difference in granulocyte migration to aqueous pollen and mold extracts between SPAOPD-affected and non-affected. A comparison of at least six SPAOPD-affected horses and six non-affected controls would have been interesting to perform.

Endothelin-1 induced granulocyte activation and chemotactic activity in a dose-dependent manner. The final concentration of LPS of 5 µg/ml to stimulate granulocytes and 10 ng/ml to stimulate mononuclear leukocytes were chosen based on studies reported by others (Parviainen et al. 2001; Rodgerson et al. 2001; Weiss and Evanson 2002). The LPS at a final concentration of 5 µg/ml induced granulocytes activation and chemoattraction as described (Weiss and Evanson 2002). The human recombinant IL-8 served as a good positive control for granulocyte activation and chemotaxis.

The present study demonstrated that ET-1 displays chemotactic activity to equine granulocytes, which has not been previously demonstrated. Although only two horses were evaluated and no quantitative or comparative analysis of the chemotactic activity of the substances was performed, the dose-response of the chemotactic activity indicated that this is a satisfactory technique to be employed in the future. A standard checkerboard analysis must be performed to demonstrate chemotactic versus chemokinetic activity of the substances (Rot 2000).

Finally the chemoattraction of equine granulocytes from a non-affected horse and a SPAOPD-affected horse was pronounced, especially to LPS, IL-8, and ET-1. It is unclear if this great difference constituted an individual variation, or if SPAOPD-affected horses
have a greater chemoattraction toward these substances. Further studies are warranted to clarify this question.

Aeroallergens including mold spores and grass pollen grains appear to induce granulocyte activation regardless of the sensitization status of the horse. Airways diseases characterized by mucus hypersecretion and bronchoconstriction are associated with poor pulmonary clearance (Lundgren and Shelhamer 1990; Moreno et al. 1986). The findings that aqueous extracts of mold spores and grass pollen grains can induce equine granulocyte activation suggests that the inadequate pulmonary clearance and persistence of these aeroallergens in the tracheobronchial tree, especially the small airways, is likely to induce neutrophil recruitment and activation, which in turn can further amplify the airway inflammation. Mononuclear leukocytes from SPAOPD-affected horses displayed a delayed but greater ability to induce granulocyte activation than those from non-affected horses. AND the mononuclear leukocyte response differs according to aeroallergen types. The lack of effect from the serum type suggests that immunoglobulins do not play an important role in this differential response to aeroallergen extracts.

6.5. Product Information

a Current Protocols in Immunology section 7.23.1.
b Ficoll-Paque Plus; Amersham Biosciences Corporation, Piscataway, NJ, USA.
c Dextran T-500; Pharmacia Chemicals, Uppsala, Sweden.
d L-glutamine, Gentamicin, Penicillin/Streptomycin; GIBCO-BRL, Grand Island, NY, USA.
e Greer Laboratories; Lenoir, NC, USA.
f Endothelin-1; American Peptide Company, Sunnyvale CA, USA
g Human recombinant monocyte IL-8 (hrIL-8); Intergen Company, Purchase NY, USA.

h Lipopolysaccharide (E.coli O55:B5 LPS); Sigma-Aldrich, Inc., Saint Louis, MO, USA.

i Fetal Calf Serum; HyClone Sera, Logan UT, USA.

j 5-ml polystyrene clear, round-bottom 12 x 75 mm tubes, 352054 Falcon; Becton Dickson Biosciences, Bedford, MA, USA.

k Mouse IgG1 monoclonal anti-equine CD18, cell line H20A; VMRD,Inc. Pullman, WA, USA.

l Mouse IgG1 isotype control, clone 15H6; Southern Biothechnology Associates,Inc.Birmingham, AL, USA.

m Goat anti-mouse IgG1-fluorescein (FITC) conjugate; Southern Biothechnology Associates,Inc.Birmingham, AL, USA.


o FACS and Software

p PKH26 Red Fluorescent Cell Linker and Diluent C; Sigma-Aldrich, Inc., Saint Louis, MO, USA.

q ChemoTx, disposable chemotaxis system: 30 µl bottom chamber of the 96-well format microtiter tissue culture grade transparent polystyrene plates, proprietary membrane with 5 µm pore size and 4 x 10³ pores/mm²; Neuro Probe, Inc., Gaithersburg, MD 20877, USA.

r Image Pro software, USA.

s SAS Institute, Cary, NC, USA.
CHAPTER 7. DEVELOPMENT OF A SYSTEM TO STUDY AIRWAY EPITHELIAL CELLS OF EQUINE ORIGIN: CULTURE OF PRIMARY EPITHELIAL AND TRANSFORMED EQUINE AIRWAY CELLS UNDER AIR-LIQUID INTERFACE OR MICROGRAVITY CONDITIONS
7.1. Introduction

Airway epithelial cells serve as a barrier interface between the environment and the host, therefore representing an important site of injury by a number of damaging agents including pollutants, allergens, and infectious agents such as viruses, bacteria and fungi. The injurious stimuli can trigger the production of inflammatory mediators, cytokines and growth factors that result in alterations in airway function as well as airway remodeling (Adler et al. 1992, Hay et al. 1997, Rochelle et al. 1998, Jayawickreme et al. 1999, Antony et al. 2002, Bhat et al. 2003,). Moreover, several respiratory diseases are characterized by epithelial hyperplasia, changes in mucus production and in cellular differentiation.

The ciliated columnar epithelium that lines the lower respiratory airways is composed of seven epithelial cell types: ciliated cells, goblet cells, basal cells, brush cells, serous cells, Clara cells and neuroendocrine cells (Plopper 1993). The ability to culture differentiated equine bronchial epithelial cells would allow the evaluation of a variety of cellular mechanisms involved in the pathogenesis of equine respiratory diseases.

Culture of airway epithelial cells can be challenging due to the loss of differentiation. When airway epithelial cells are cultured immersed in media, even if attached to a biomatrix, the cells tend to loose their normal differentiated mucociliary features (Gray et al. 1996, Kaartinen et al. 1993). The culture of airway epithelial cells with morphological and functional characteristic that resemble the epithelium in vivo permits the evaluation of cellular functions and the cellular response to injury. Two culture systems were evaluated to grow primary equine airway epithelial cells and a spontaneously transformed fetal equine airway cells.
The first culture system studied consisted of the use of air-liquid interface (ALI) culture conditions, which have been reported to be a successful technique for the establishment of differentiated airway epithelial culture in various species, including laboratory animals (e.g., guinea pig, hamster, rat, mouse), human, canine, bovine and porcine (Whitcutt et al. 1988, Kaartinen et al. 1993, Kondo et al. 1993, Adler et al. 1995, Gray et al. 1996, Bernacki et al. 1999, Kim et al. 1985, Davidson et al. 2000). There are no reports of culture of equine airway epithelial cells and their growth condition requirements. Moreover, it is not known if equine bronchial epithelial cells require an air-liquid interface in order to retain their normal morphological and functional characteristics.

The second culture system studied used rotating wall vessels (RWV) or bioreactor chambers that produce low shear and low turbulence conditions that allow the cells to divide and differentiate as three-dimensional assemblies. Unlike traditional cell culture techniques that are limited to growing cells two-dimensionally in monolayers, cells grown in a RWV are in constant fall resulting in microgravity conditions (Goodwin et al. 1993; Unsworth and Lelkes 1998; Nickerson et al. 2001; Hammond and Hammond 2001). The RWV promotes three-dimensional growth of the cells, allowing them to form bridges between the microcarrier beads, which are used as the substrate for the cells, resulting in aggregates of high-density cell assemblies that simulate many aspects of the tissue of origin (Goodwin et al. 1993; Unsworth and Lelkes 1998; Nickerson et al. 2001; Hammond and Hammond 2001). A number of cell types have been successfully grown under these conditions; however, equine airway cells have not been reported.

The overall purpose of this study was to establish and to evaluate culture techniques of equine cells of respiratory origin by light and electron microscopy. The specific goals were to (1) establish and evaluate cultures of primary equine bronchial
epithelial cells grown submerged, under air-liquid interface and microgravity conditions and, (2) evaluate a spontaneously transformed fetal equine tracheal cell line grown under air-liquid interface and microgravity conditions.

7.2. Materials and Methods

7.2.1. Isolation and Culture of Primary Equine Bronchial Epithelial Cells

Primary cultures were established from fresh post-mortem lung tissues obtained from eight adult horses without signs of respiratory disease and three full-term newborn foals. To obtain these foals, parturition was induced based on acute elevation of milk calcium levels indicating readiness of the fetus for birth. The newborn foals were euthanized immediately after delivery. The bronchial epithelium was dissected from adjacent tissues and bronchial epithelial strips were placed in conical tubes and washed with calcium-magnesium-free phosphate-buffered saline (CMF-PBS). Bronchial mucosa specimens were maintained in Dulbecco's Modified Eagle's Medium (DMEM) until processed. The bronchial epithelium sections were stirred in protease solution, containing 2.5 mg/ml of trypsin and 1 mg/ml of deoxyribonuclease in DMEM: Ham’s F12 (1:1 v/v) with 15mM Hepes, for 16 to 24 hours at 4°C. The dispersed cells were centrifuged at 800 x g for 10 minutes. Pelleted cells were resuspended in supplemented serum-containing media, composed of DMEM: Ham’s F12 (1:1 v/v) with Hepes (15mM), fetal calf serum (FCS, 10%) a, L-glutamine b (4 mM), penicillin G b (50 U/ml), streptomycin b (50 µg/ml), amphotericin c (50 ng/ml), gentamicin b (50 ng/ml), insulin c (5µl/ml), transferrin c (10µg/ml), and epidermal growth factor c (EGF, 25 ng/ml), and placed in collagen-coated petri dishes d or 25 cm² tissue culture flasks coated with fibronectin-collagen coating mix e. One ml of fibronectin-collagen coating mix was placed and spread evenly over the bottom wall of the
cell culture flask, and after 10 minutes the excess was removed prior to adding the cells. Cell suspension was added carefully to the side wall of the flask in order to prevent disturbing the fibronectin-collagen coating, then the flask was rocked gently to spread the cell suspension evenly over the bottom wall of the flask. Cultures were maintained in 38°C humidified 5%CO2 incubator. Media was changed every other day. Figure 8.1 depicts the methodology utilized for collection and culture of the primary equine bronchial epithelial cells.

7.2.2. Initial Culture of Transformed Equine Fetal Tracheal Cell Line

An equine airway cell line that originated by spontaneous transformation of equine fetal tracheal (EFTr) cell was used. These cells have been used in indirect immunofluorescent antibody assay for equine herpesvirus f. Figure 8.2 depicts the methodology utilized to culture and evaluate spontaneously transformed EFTr.

These cells were evaluated for equine origin using karyotype and microsatellite analysis. Chromosomal analysis of the 38th and 40th passages of this cell line revealed an abnormal karyotype not typical of equine species with highly variable size and number of chromosomes, the modal chromosome number of 53 to 56 g. The amplified polymerase chain reaction (PCR) products using 13 equine-specific microsatellite pairs of primers were tested on ABI 377 with Genotyper software. The sizes of the fragments were compared with the normal allele size ranges of each microsatellite. All 13 pairs of primers yielded PCR products, and all the products were within the expected size ranges h, thus, confirming that the cells were of equine origin.

The 38th and 40th passages of the transformed EFTr cell line were thawed at 37°C and initially cultured in 25 cm² tissue culture flasks without collagen coating. The cell line cultures were maintained in the serum-containing media, i.e., DMEM supplemented with
15mM Hepes, 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin G (50 U/ml), streptomycin (50 µg/ml), amphotericin (50 ng/ml) and gentamicin (50 ng/ml). Cultures were maintained in a 38°C humidified 5%CO₂ incubator. Media was changed every two days.

7.2.3. Culture of Primary Equine Bronchial Epithelial Cells or Transformed EFTr Cell Line under Air-Liquid Interface

The monolayer of primary equine bronchial epithelial cells or transformed EFTr were culture until they became nearly confluent, and then they were detached by trypsin digestion and counted using trypan blue exclusion dye. Two hundred µl of fibronectin-collagen coating mix was placed and spread evenly over the surface of each polyester membrane insert, and after 10 minutes the excess was removed prior to adding the cells. Bronchial epithelial cells were seeded onto the apical surface of membranes of either collagen-coated Transwell or Transwell culture inserts coated with fibronectin-collagen coating mix. Cells were seeded at low density (50, 000 cells/cm²) or high density (150,000 to 200,000 cells/cm²). Transformed EFTr cells were plated onto the apical surface of Transwell culture inserts coated with fibronectin-collagen coating mix at 50,000 cells/cm². Serum-containing media was placed in the bottom compartment, and cells were seeded onto the top (apical) compartment using the serum-containing media. Transwell plates were incubated for 36 to 48 hrs at 38°C humidified 5%CO₂ atmosphere to allow the cells to attach to the matrix. Unattached cells were removed when media was changed, and media was changed in both compartments every other day thereafter. The concentration of serum in the media was decreased from 10% to 5% when the cells were 70% confluent. Once cell monolayers on the inserts became almost confluent, the air-liquid interface conditions were established by removal of the media in the top compartment, and the cultures were provided serum-free media in the bottom compartment (Gray et al. 1996,
Two wells per plate were maintained submerged, i.e., media was provided on the top compartment (apical surface) as well as the bottom compartment (basolateral surfaces). The supplemented serum-free culture media consisted of: DMEM: Ham’s F12 (1:1v/v) with 15mM Hepes, L-glutamine (4 mM), penicillin G (50 U/ml), streptomycin (50 µg/ml), amphotericin (50 ng/ml), gentamicin (50 ng/ml), insulin (5µl/ml), transferrin (10µg/ml), hydrocortisone (0.5 µg/ml), EGF (0.5 ng/ml), epinephrine (0.5 µg/ml), triiodothyronine (6.5 ng/ml), trans-retinoic acid (5x10^{-8} M), bovine serum albumin (1.5 µg/ml) and bovine pituitary extract (1% v/v). After the beginning of ALI media was changed once daily. Plates were incubated at 38°C humidified 5%CO2 atmosphere.

7.2.4. Culture of Primary Equine Bronchial Epithelial Cells or Transformed EFTr Cell Line under Microgravity Conditions

7.2.4.1. Preparation of Microcarrier Beads

The collagen-coated microcarrier beads were prepared two days prior to use. One gram of dry beads was soaked in 50 ml of CMF-PBS for three hours. After hydration of the beads, they were rinsed in 50 ml of CMF-PBS and then soaked again in fresh CMF-PBS for another hour. The beads were resuspended in 25 ml of 70% ethanol overnight. The following day, the beads were washed with CMF-PBS three times. Beads were allowed to settle and CMF-PBS was removed and the beads were resuspended in 50 ml of supplemented media without FCS (resulting in a concentration of 20 mg of beads/ml). Beads were stored in media at 4°C. The day prior to placing the cells into the RWV, the chamber was filled with media and incubated overnight in order to equilibrate the membrane.
Figure 7.1. Methodology for collecting/harvesting and culturing primary equine bronchial epithelial cells.

- Cold trypsinization
- Removal of airway epithelia
- Rotating wall vessel (low shear)
- Fibronectin-collagen coated Transwells
- Air-liquid interface
- DMEM: Ham F12 + 10% FBS w/ EGF
- DMEM: Ham F12 + 5% FBS w/ EGF
- Serum-free media w/ EGF & RA
**EFTr** (38th and 40th passage of spontaneously transformed cell line)

- Serum-containing media
- Transwells
- Serum-free media
- Karyotype & Microsatellite analysis
- RWV

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**Figure 7.2. Methodology for evaluation of equine fetal tracheal (EFTr) cell line**
7.2.4.2. Initiating and Maintaining the Cell Cultures under Microgravity Conditions

After the initial growth, the primary bronchial epithelial cells or the transformed EFTr were trypsinized, placed into each 50-ml RWV, and maintained with a supplemented serum-containing media. A total of $10^7$ cells and 250 mg of microcarrier beads (i.e., 12.5 ml of 20 mg/ml) were added to each RWV. The RWV was emptied, the beads and the cell suspension were placed into the RWV, and the remainder of the volume in the RWV was filled with the serum-containing media. The resulting final volume of 50 ml contained a bead concentration of 5 mg/ml, a ratio of 10 cells per bead and cell concentration of $2 \times 10^5$ cells/ml. Two 5-ml syringes were attached to the luer ports of the RWV, one of them was filled with media and the other left empty. The chamber was manipulated such that the air bubbles were removed into one syringe while media was replaced from the other syringe. The RWV was mounted on the rotator within the incubator, at 37°C humidified 5%CO₂ atmosphere, and the rotating speed was set to 20 rpm. The primary equine bronchial epithelial cells in RWV were maintained in a serum-containing media composed of DMEM: Ham's F12 (1:1 v/v) with 15mM Hepes, 10% fetal calf serum (FCS), L-glutamine (4 mM), penicillin G (50 U/ml), streptomycin (50 µg/ml), amphotericin (50 ng/ml), gentamicin (50 ng/ml), insulin (5µl/ml), transferrin (10µg/ml), and EGF (25 ng/ml).

Whereas, the EFTr cell line cultures in RWV were maintained in the serum-containing media consisting of: DMEM supplemented with 15mM Hepes, 10% FCS, L-glutamine (2 mM), penicillin G (50 U/ml), streptomycin (50 µg/ml), amphotericin (50 ng/ml) and gentamicin (50 ng/ml). Media was changed after five days, and every day thereafter. Photographs of samples of beads under phase contrast microscopy.
7.2.5. Light Microscopy and Transmission Electron Microscopy

7.2.5.1. Preparation and Evaluation of the Cultures Grown under Air-Liquid Interface Conditions

For evaluation of cell morphology, samples from Transwell culture inserts were collected on days 6, 9, 12, 14, 21 and 28 days after starting ALI, and prepared for microscopy as described below. For light microscopy, the Transwell culture inserts were washed three times with CMF-PBS and fixed with 4% paraformaldehyde for 30 minutes. The polyester membranes were cut out of the Transwell inserts, the membranes were embedded in LR White or paraffin. Cross-sections of the polyester membranes were prepared at one-micron thickness and mounted. Cross-sections of the membrane were stained with hematoxylin and eosin (H&E) toluene blue, methylene blue and alcian blue (pH 2.5)-periodic acid-Schiff (AB-PAS). Whole-mounts of the membrane were stained with AB-PAS. Additionally, cross-sections of polyester membranes were stained for cytokeratin. The immunohistochemistry procedure for cytokeratin was done using a cocktail of two monoclonal antibodies. The formalin-fixed, paraffin-embedded sections were pretreated for epitope enhancement with proteinase K prior to loading on the Autostainer. The automated staining procedure was performed following manufacturer’s recommendations.

For transmission electron microscopy, the Transwell culture inserts were washed three times with 0.1 M sodium cacodylate buffer, pH 7.39, then they were fixed for 30 minutes in fixative containing 2% paraformaldehyde and 1.25% glutaraldehyde. The fixed Transwell culture membranes were cut out of the insert. The fixed polyester membranes were again washed three times in 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide. The fixed Transwell culture membranes were cut out of the insert, and dehydrated in a graded ethanol series to the final 100% ethanol, embedded in epoxy resin.
q, sectioned with an ultramicrotome (MT-XL), stained with uranyl acetate and lead citrate, and examined in a Zeiss EM-10C transmission electron microscope.

7.2.5.2. Preparation and Evaluation of the Cultures Grown under Microgravity Conditions

For evaluation of cell morphology, samples from aggregates of beads/cells from the RWV were collected on day 20 after the beginning of the low shear/ low turbulence culture, and prepared for microscopy as described below. For light microscopy, the aggregates of beads/cells were harvested and allowed to sediment, washed three times with CMF-PBS and fixed with 4% paraformaldehyde for 30 minutes. The aggregates of beads/cells were embedded in LR White or paraffin. Sections of the aggregates of beads/cells were obtained at one-micron thickness and mounted. Sections of the aggregates of beads/cells were stained with H&E, and stained for cytokeratin and vimentin. The immunohistochemistry procedure for cytokeratin and vimentin were performed using an automated staining procedure following manufacturer's recommendations. For cytokeratin, the formalin-fixed, paraffin-embedded sections were prepared as described in the previous section, using a pretreatment with proteinase K, and immunolabeling was done using a cocktail of two mouse monoclonal antibodies at a dilution of 1:50. For the vimentin, the formalin-fixed, paraffin-embedded sections were pretreated with heat-induced epitope retrieval in a citrate buffer retrieval solution and staining was performed using a mouse monoclonal antibody at a dilution 1:50. The pretreated for epitope enhancement was performed prior to loading the slides on the Autostainer. The aggregates of beads/cells from cultures grown in RWV were not evaluated by transmission electron microscopy.
7.3. Results

7.3.1. Isolation and Culture of Primary Equine Bronchial Epithelial Cells

The dissection procedure for obtaining bronchial epithelium strips and the use of cold trypsinization with constant, gentle stirring during enzymatic digestion yielded epithelial cell cultures that grew better than those generated by explants or by scrapping of the mucosa (data not shown). Cell viability was greater than 95% and there were very few contaminating fibroblasts (data not shown). Excessive cell clumping was successfully avoided by adding deoxyribonuclease to the protease solution. The high cell densities for seeding the tissue culture flasks resulted in very good cell attachment, such that these cultures became confluent within five days of culture. Cells attached to the fibronectin-collagen matrix very well, similar to the collagen-coated surfaces. Although there was some variation between animals, the cultures grew well and became nearly confluent (75 to 90% confluence) within five to ten days.

When trypsinization was harsh or it had to be repeated, there were more clumped cells and the cell viability dropped. When passaged into Transwell inserts these cultures took longer to attach than the others. Harsh manipulation and re-trypsinization damaged the cells, resulting in more clumping, decreased viability and less attachment to the matrix.

Evaluation of the cultures on Transwell inserts were overall difficult because of the presence of the dotted/porous membrane, especially when the cells were submerged. Cells were much easier to visualize after the beginning of air-liquid interface (Figure 7.3B). Cells from all eight adult horses and three newborn foals grew well initially although not very rapidly, however, after several days many cultures failed to thrive.

The primary bronchial epithelial cell cultures were successfully established from bronchial epithelium harvested in strips from all eight adult horses and two newborn foals.
Only three of the adult horses and two of the newborn foals were successfully subcultured. The bronchial epithelial cell cultures from the remaining five adult horses and one of the foals grew well initially, but as the monolayer on the transwell membranes approached confluency, areas of cellular degeneration were observed, and progressed to coalesce resulting in the complete detachment and loss of the monolayer.

Only four of the eight adult horse primary cell cultures and one of the three foal cell cultures were seeded into the RWV. The cells of adult horses did not attach to the beads, they remained in suspension for several days and appeared to proliferate forming slightly larger aggregates of cells without beads. However, after five to seven days, the clumps started to dispersed into single cells that were mostly dead. As a result, none of the adult horse cell cultures grew successfully in this culture system. The cultures from the foal, while in monolayers had several small areas with long, spindle-shaped cells scattered throughout the flask, indicating fibroblast growth, and therefore were not pure epithelial cell cultures (Figure 7.4). This culture after seeded into the RWV grew very well.

7.3.2. Morphologic Evaluation of Primary Equine Bronchial Epithelial Cells Grown under ALI and Submerged Conditions

The equine bronchial epithelial cells grown under ALI and submerged had similar differentiation (i.e., apical location of vesicles and apical microvilli) initially; however, cells grown under ALI had a tight juxtaposed and dense appearance when compared with the cells grown submerged (Figure 7.5). After the ALI cultures became completely confluent, there was very little, and later no, media leaking onto the apical surface of the cultures, suggesting the formation of an “impermeable barrier”. The apical mucus secretion was evident in ALI cultures on the apical surface of the live cell monolayer (Figure 7.6), using
light microscopy of the whole-mounts of the Transwell membrane and by AB-PAS staining of the apical vesicles in cross-section of the cell monolayer (Figure 7.7).

Polygonal cells and the presence of apical cytoplasmic vesicles (mucus vesicles), apical microvilli, interdigitating projections with tight junctions and desmosomes were observed by transmission electron microscopy of primary cultures from adult horses (Figure 7.8) and newborn foal (Figure 7.9). Ciliated cells were not seen on transmission electron microscopy in any of the cultures, even those maintained for up to 28 days under ALI conditions.

Incidentally, one of the three primary cultures that was successfully subcultured and two that did not grow well after subculturing when evaluated by transmission electron microscopy were determined to be infected by virus. The viral particles were noted in the nuclei, in cytoplasmic vesicles and in the intercellular spaces (Figure 7.10). The viral capsids in the nuclei appeared to lack the outer layer, whereas the particles present in the cytoplasmic vesicles and intercellular spaces appeared to have an envelope, thus resembling a herpevirus.

7.3.3. Morphologic Evaluation of Primary Equine Bronchial Epithelial Cells Grown under Microgravity Conditions

Only one of the primary equine epithelial cell culture, which was from a newborn foal, grew in the RWV system (Figure 7.11). These cells had a distinct polygonal shape and grew forming several layers around the microcarrier beads, joining them together to form aggregates of beads bridged by the cells (Figure 7.12). Abundant keratin filaments throughout the cytoplasm were observed by immunohistologic staining for cytokeratin, indicating epithelial differentiation (Figure 7.12). In contrast, vimentin immunostaining was scarcely distributed, especially in cells closest to the beads, suggesting a mixed population
of cells. Most cells appeared to be of epithelial origin, however, a smaller number of contaminating fibroblasts were present.

**7.3.4. Growth and Morphologic Evaluation of Transformed Equine Fetal Tracheal Cell Line Grown under Air Liquid Interface and Microgravity Conditions**

The EFTr cell line grew successfully under ALI conditions. These cells tended to overgrow, often pulling away from the edges of the Transwell inserts and quickly repopulating the recently uncovered areas. After the ALI cultures reached complete confluency, some media leaked onto the apical surface of the cultures. Under light microscopy, EFTr cultures formed multiple layers of poorly differentiated cells on the polyester membrane with squamous to spindloid morphology, not tightly juxtaposed. Moreover, mucus differentiation was not evident. The cells stained weakly for cytokeratin, indicating minimal presence of epithelial-specific keratin filaments in the cytoplasm (Figure 7.13).

The EFTr cell line was cultured under microgravity conditions for more than 35 days in RWV. The cells proliferated well forming large aggregates that were clearly visible. Under contrast phase microscopy the cells of EFTr cell line formed a thin layers of cells coating the beads (Figure 7.14). Within days, as the cells continued to proliferate, the cells started forming multiple layers of cells bridging beads to form aggregates of beads covered by cells as seen under contrast microscopy (Figure 7.14). Aggregates of cells involving more then two beads, often involving several beads became prevalent.

Under light microscopy, the EFTr cells exhibited the same squamous to spindloid morphology with indistinct borders (Figure 7.15). The nuclei were spindloid to oval. The cells within the solid tissue area between the beads were larger and more polygonal with larger, oval nuclei.
Figure 7.3. Contrast phase microscopy of primary equine bronchial epithelial cells. A. Cells after enzymatic digestion (Note the clumps of cells) and B. Cell culture grown on the apical surface of Transwell insert under air-liquid interface.
Figure 7.4. Contrast phase microscopy of primary equine bronchial epithelial cells from a newbon foal. A. Monolayer, illustrating the polygonal-shaped cells; and B. Monolayer of cell culture form the same foal, note the mixed cell shapes, most are spindle-shape intermixed with polygonal cells.
Figure 7.5. Whole-mount of primary equine bronchial epithelial cells stained with AB-PAS. A. and B. Cells grown submerged C. and D. Cells grown under air-liquid interface.
Figure 7.6. Primary equine bronchial epithelial cells grown under air-liquid interface. A. Twelve-well plate of Tranwells inserts. B. Tranwells inserts where cells were grown onto the apical surface of polyester membrane and C. Apical surface of cells grown under air-liquid interface, note the mucus accumulation.
Figure 7.7. Cross-section of primary equine bronchial epithelial cells. A. and B. Cells from adult horses cultured onto Transwells for 6 days under air-liquid interface; PAS. C. Cells maintained for 9 days under air-liquid interface; Toluene Blue.
Figure 7.8. Transmission electron microscopy of primary equine bronchial epithelial cells from adult horses. A. Cells grown submerged at nine days after confluency. B. Cells grown under air-liquid interface six days after confluency. C. and D. Cell cultured for 21 days after beginning air-liquid interface; Note the tightly juxtaposed cells with the apical microvilli and the interdigitating projections and desmosomes.
Figure 7.9. Transmission electron microscopy of primary equine bronchial epithelial cells from newborn foal. A. and B. Twenty-eight days after beginning air-liquid interface; Note the interdigitating projections and desmosomes between cells and the microvilli on the apical surface. C. and D. Twenty-eight days after beginning air-liquid interface and stained with ruthenium red; Note the tightly juxtaposed interdigitating projections between cells.
Figure 7.10. Transmission electron microscopy of primary equine bronchial epithelial cells from adult horses. Viral particles: in the nucleus (A), intracytoplasmic vesicles (B) and intercellular space (C).
Figure 7.11. Contrast phase microscopy of primary equine bronchial epithelial cells grown in RWV. A. Lower magnification, cells covering some of the Cytodex microcarrier beads; Note an aggregate of three beads joined by cells (arrow). B. and C. Higher magnification, polygonal cells joining two microcarrier beads.
Figure 7.12. Light microscopy of primary equine bronchial epithelial cells grown in RWV. A. and B. Beads and cell aggregates stained with H&E; and C. Beads and cell aggregates stained for cytokeratin. D. Beads and cell aggregates stained for vimentin.
Figure 7.13. Equine Fetal Tracheal cell line grown under air-liquid interface. A. Cross-section of polyester membrane and cell grown onto the apical surface of Transwells stained with Toluene Blue and B. Cross-section of polyester membrane and cells stained for cytokeratin.
Figure 7.14. Contrast Phase microscopy of equine fetal tracheal cell line grown under microgravity  

A. Microcarrier beads with a thin layer of cells around them and B. Microcarrier beads with a thicker layer of cells joining beads to form aggregates.
Figure 7.15. Bright field light microscopy of equine fetal tracheal cell line grown under microgravity

A. Microcarrier beads and cell aggregates stained with H&E and B. Microcarrier beads and cell aggregates stained for cytokeratin.
The solid areas frequently had central necrosis. Moderate amounts of cytoplasm were present which had variable amounts of PAS positive material (Figure 7.15). The cells stained poorly for cytokeratin, and only scattered cells, mostly adjacent to the beads, were weakly positive for cytokeratin (Figure 7.15). EFTr cells stained strongly for vimentin (Figure 7.15).

7.4. Discussion

The goal of this study was to establish cultures of differentiated normal equine bronchial epithelial cells. Establishment of these cultures has been a problem in other species as well as the horse due to contamination with other cell types, excessive clumping of cells during harvest and failure to attach to culture vessels. We were able to establish primary cultures of normal equine bronchial epithelial cells and in a few instances subculture them, and we were able to grow and maintain the equine bronchial epithelial cells under air-liquid interface conditions. We have developed a novel method to harvest equine bronchial epithelial cells, which avoids excessive clumping. The strips of bronchial epithelia were easily peeled off from the adjacent structures, yielded good epithelial cell recovery, no fibroblasts, and very little contaminating red blood cells. One of the main disadvantages of cell clumping is that cell counting is difficult and inaccurate, and results in variability in the number of cells seeding the culture vessels, as a consequence different vessels reach confluency at different times. Addition of EDTA has been recommended in an attempt to decrease cell clumping. EDTA was added in a previous experiment and it did not significantly diminished cell clumping (data not shown). As previously described, the addition of deoxyribonuclease much reduced the cell clumping associated with protease treatment (Kaartinen et al. 1993).
Bronchial epithelial cells do not adhere to vessels without the presence of matrix. Fibronectin-collagen coating mix allowed the cells to attach as described by others \(^8\). Fibronectin-collagen coating was omitted accidentally in one of the flasks and cells did not attach well (data not shown). Most reports recommend the use of collagen coated vessels, which did provide excellent attachment conditions for equine bronchial epithelial cells; however, the fibronectin-collagen mix gave as good attachment as collagen alone. Moreover, the polyester membrane Transwells coated with fibronectin-collagen coating mix was more economical than the collagen-coated Transwells.

Cell density was clearly very important for cell attachment and growth. The greater the cell densities, the better the cells grew in culture. Epithelial cells attached as groups of cells appeared to grow to confluency more rapidly and have a healthier appearance than at lower densities. The higher cell density promotes better attachment and growth of epithelial cells (Kondo et al. 1993).

Ciliated cells were not evident after 28 days of ALI culture. This was an unexpected finding, since in other species ciliated cells appear in culture around 21 days after ALI (Gray et al. 1996, Kondo et al. 1993). Retinoic acid is important for ciliary differentiation, and its absence results in squamous differentiation of the mucociliary epithelium ( Kaartinen et al. 1993, Gray et al. 1996). Retinoic acid requirements vary from species to species \(^1\). Higher concentrations of retinoic acid may be needed for differentiation of ciliated cells in equine primary airway epithelial cultures.

Primary equine bronchial epithelial cells did not grow well under microgravity conditions. One reason may be that the harvest of primary equine bronchial epithelial cells from adult horses was devoid of fibroblasts. The primary equine bronchial epithelial cells did not attach to microcarrier beads, eventually dying shortly after seeded into RWV.
Bronchial epithelial cells harvested from newborn foal contained fibroblasts, and did grow well in RWV. Fibroblasts may be necessary to provide attachment of the epithelial cells to the microcarrier beads. Therefore, unlike most culture systems, the microgravity culture system requires a mixed population of cells, rather that a single cell type culture.

Although, the EFTr cell line was successfully grown under ALI and microgravity conditions, the latter yielding three-dimensional assemblies, the cell morphology under either of these conditions did not resemble that of differentiated airway epithelial cells. These cells exhibit some features of epithelia cells including the presence of PAS positive staining and microvilli, however, the spindloid shape of the cells and the minimal cytokeratin staining suggested that this spontaneously transformed cell line might have undergone considerable genetic alteration, as suggested by the highly variable chromosome number, resulting extreme loss of differentiation. This cell line may be of mesenchymal rather than epithelial origin.

In conclusion, culture of primary equine airway epithelial cells under ALI conditions yielded cells with differentiation that resembles those of the airway epithelial cells in vivo. Plating the primary equine airway epithelial cells directly onto fibronectin-collagen coated polyester-membrane Transwells and subjecting them to ALI conditions appears to allow for well-differentiated epithelial culture resembling the airway epithelium. The subculture of equine primary airway epithelial cells was not reliable, possibly due in some cases to latent viral infection by an equine herpesvirus. It appears that the equine primary airway epithelial cells grown under ALI are suitable for studies of airway physiology and pathology. The spontaneously transformed EFTr cell line did not develop characteristics of differentiated cells and does not appear suitable for in vitro studies.
7.5. Endnotes

a Fetal Calf Serum, HyClone Sera, Logan UT, USA.

b L-glutamine, Gentamicin, Penicillin/Streptomycin, GIBCO-BRL, Grand Island, NY, USA.

c Transferrin, Insulin, Human Recombinant Epidermal Growth Factor Expressed in E. Coli, Hydrocortisone, Epinephrine, Tri-iodo-thyronine, Trans-Retinoic Acid, Bovine Serum Albumin and Bovine Pituitary Extract; Sigma-Aldrich, INC., Saint Louis, MO, USA.

d Cellcoat Collagen type I, TC Petri Dish 100/20 mm; Greiner Labortechnik, Germany.

e Fibronectin-Collagen Coating Mix, Biological Research Faculty Facility, Inc., Ijamsville, MD, USA.

f Dr. R.E. Corstvet, Louisiana State University, unpublished.

g Dr. Lance Buoen, Cytogenetics Laboratory, Minnesota Veterinary Diagnostic Laboratory.

h Microsatellite analysis included a panel of 13 equine-specific microsatellite markers. Dr. E. G. Cothran, University of Kentucky.

i Transwell-COL, collagen-coated 12 mm diameter, 0.4 \( \mu \)m pore size tissue culture treated polytetrafluoroethylene, membrane polystyrene plates; Corning Costar Corporation, Cambridge, MA, USA.

j Transwell, 12 mm diameter, 0.4 \( \mu \)m pore size tissue culture treated polyester, membrane polystyrene plates; Corning Costar Corporation, Cambridge, MA, USA.

k Rotary cell culture vessel, 50 ml; Synthecon, Houston TX, USA.

l Microcarrier beads Citodex™, Sigma-Aldrich, INC., Saint Louis, MO, USA.

m Synthecon, Bioreactor Rotator; Synthecon, Houston TX, USA.

n LR White, Electron Microscopy Sciences, Fort Washington, PA, US.

o Monoclonal Mouse Anti-Human Cytokeratin, Isotype IgG1/ kappa, cocktail of two monoclonal antibodies AE1 and AE3; DAKOCytomation, Denmark.

p DAKO Autostainer; DAKOCytomation, Denmark.

q Epon-Araldite, Electron Microscopy Sciences, Fort Washington, PA, USA.

r DAKO Target Retrieval Solution; DAKOCytomation, Denmark.

s Monoclonal mouse anti-vimentin Clone V9, Isotype IgG1, kappa; DAKOCytomation, Denmark.
Personal communication Dr. Nickolaus Osterrieder, School of Medicine, Cornell University, Ithaca, NY.

Swiderek MS and Mannuza FJ “Attachment of primary rat airway epithelium to a fibrillar collagen/fibronectin matrix: effect of fibronectin concentration” Becton Dickson Labware, Two Oak Park, Bedford MA, USA

Personal communication Dr. Ilona Jaspers, School of Medicine, University of North Carolina, Chapel Hill, NC.
CHAPTER 8. ENDOTHELIN PRODUCTION BY EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS STIMULATED WITH AQUEOUS MOLD AND POLLEN EXTRACTS AND BY EQUINE BRONCHIAL EPITHELIAL CELLS STIMULATED WITH CYTOKINES
8.1. Introduction

An important feature in the pathogenesis of airway disease is that injurious stimuli can trigger the production of mediators by inflammatory cells as well as by airway epithelial cells. Among the many mediators implicated in the pathogenesis of airway inflammation, endothelins are thought to contribute to airway hyperresponsiveness, mucus hypersecretion, bronchoconstriction and mitogenesis (Hay et al. 1996; Hay and Goldie 1996, Goldie et al. 1999). The production of endothelin (ET)-1 was shown to be greater in human asthma and in equine recurrent airway obstruction (RAO) compared with normal subjects (Aoki et al. 1994; Benamou et al. 1998).

Because the lung is the major site of ET synthesis and catabolism, there are many cell types responsible for ET-1 synthesis in the lung. In the airways, the epithelial cells, including epithelial cells as well as specialized Clara cells and neuroendocrine cells are the most important site, followed by smooth muscle cells (Goldie et al. 1999; Fagan et al. 2001). Parenchymal pulmonary macrophages and endothelial cells are also important sites. Neutrophils have been shown to play a role in ET metabolism by converting the poorly active pro-endothelin-1, also known as bigET-1, into the potent biologically active ET-1 (Sessa et al. 1991). Moreover, macrophages and peripheral monocytes have also been shown to produce ET-1 (Ehrenreich, Anderson et al. 1990, Salh et al. 1998).

The overall objective of this study was to evaluate the production of ET-1 by equine mononuclear leukocyte and equine airway epithelial cell cultures. We first established cultures of peripheral blood mononuclear cells, and then cultures of differentiated equine bronchial epithelial cells. The latter was evaluated morphologically by light, confocal and transmission electron microscopy. Then, we analyzed the production of ET-1 secreted by
mononuclear leukocytes following stimulation with aqueous protein extracts of mold spores and pollen grains and by the bronchial epithelial cells following stimulation with lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α and interleukin (IL)-4 using a commercial capture ELISA for ET-1.

8.2. Materials and Methods

8.2.1. Collection of Peripheral Blood Mononuclear Cells and Epithelial Cells

The experimental procedures included in this study were approved by the Institutional Animal Care and Use Committee, Louisiana State University. The methods for establishing and stimulating cultures of peripheral blood mononuclear cells and of differentiated equine bronchial epithelial cells are depicted in Figures 8.1 and 8.2, respectively. Twelve horses, six SPAOPD-affected (one castrated male and five intact females; one Thoroughbred and two Quarter Horse, one Paint, one Appaloosa/Quarter Horse and one Arabian/Quarter Horse median age 15 years, ranging from 7 to 27) and six non-affected control horses (four castrated males and two intact females; five Thoroughbred and one Quarter Horse median age 15 years, ranging from 14 to 17) were kept on pastures at Louisiana State University in Baton Rouge, Louisiana, USA. The affected horses originated from south Louisiana and developed clinical signs of SPAOPD during each summer; they had pulmonary function testing and cytologic analysis of bronchoalveolar lavage fluid (BALF) documenting recurrent airway obstruction (Costa et al. 2000).

The SPAOPD-affected horses were considered to be in clinical remission when there were no signs of respiratory disease, the intrapleural pressure difference was less
Figure 8.1. Outline of method for determination of basal and up-regulated release of endothelin (ET)-1 by equine mononuclear leukocytes stimulated in culture.
Figure 8.2. Outline of method for determination of endothelin-1 (ET-1) production by differentiated equine bronchial epithelial cells.
Figure 8.3. Quantification of immunoreactive endothelin-1 (ET-1) by a commercial capture ELISA kit. HRP = horse-radish peroxidase; TMB = tetramethylbenzidine.
Figure 8.4. Differentiated equine bronchial epithelial cell culture. A. Twelve-well plate with ten Tranwell inserts where cells were grown onto the apical surface of polyester membrane. B. Apical surface of cells grown under air-liquid interface. Note the mucus accumulation.
than 10 cm of water, and percentage of neutrophil in bronchoalveolar lavage fluid was less than 15%, similar to non-affected horses (Lavoie 2003). Jugular venous blood samples were collected into evacuated glass tubes without anticoagulant from six SPAOPD-affected horses during clinical exacerbation and six non-affected control horses at the same time. Blood was allowed to clot and tubes were centrifuged at 800 x g, 4°C for 10 minutes. Serum was harvested and serum samples were stored at −70°C. Blood samples were obtained by jugular venipuncture into evacuated glass tubes containing preservative free heparin from the six SPAOPD-affected horses during clinical remission and the six non-affected control horses at the same time.

Fresh post-mortem lung tissues were obtained from three of the SPAOPD-affected adult horses during clinical remission, from three adult horses with no signs of respiratory disease that were euthanized for orthopedic reasons, and from two newborn foals. The birth of the foals was induced and the foals were euthanatized immediately after birth.

8.2.2. Preparation of Peripheral Blood Mononuclear Cells

Venous blood samples collected in evacuated glass tubes containing preservative free heparin, at 10 U/ml of blood, were centrifuged at 800 x g for 10 minutes. Most of the plasma was removed and discarded. The buffy coat cells were carefully removed from the top of the red blood cells and transferred to a sterile 50-ml polypropylene conical tube containing three parts of calcium and magnesium-free physiological buffered saline (CMF-PBS), and mixed. A 30-ml aliquot of the cell suspension was placed into each 50-ml polypropylene conical tubes, underlayed with 10 ml Ficoll-sodium metrizoate solution and centrifuged at 800 x g, for 30 minutes at 20°C, with no brake. The top layer (CMF-PBS and plasma) was discarded and the interface (Ficoll-sodium metrizoate layer containing
mononuclear cells) was aspirated and placed into a new sterile 50-ml polypropylene conical tube. The cell suspension was diluted to 40 ml with CMF-PBS, mixed and centrifuged at 800 x g for 10 minutes without brake. The supernatant fluid was removed by decanting. The cells were mixed and diluted to the same volume (40 ml) with CMF-PBS and centrifuged at 150 x g for 10 minutes without brake. This wash was repeated three times. After decanting the supernatant, the cells were resuspended in RPM1-1640 supplemented with Na₂HCO₃, 25mM HEPES, L-glutamine b (2 mM), penicillin G / streptomycin b (50 U/ml and 50 µg/ml, respectively). The cell number and viability was determined using the 0.04% trypan-blue vital stain, cells were counted using a hemocytometer, and cell concentrations were adjusted to 6 x 10⁶ cells/ml.

8.2.3. Stimulation of Equine Peripheral Blood Mononuclear Cells

Aqueous protein extracts of pollen grains and mold spores were selected based on the relationship with onset of clinical exacerbation of SPAOPD (Chapter 2). The selected aqueous protein extracts of mold spores: *Alternaria alternata*, *Curvularia spicifera*, *Cladosporium herbarum* and *Aspergillus fumigatus*, and selected aqueous protein extracts of grass pollen grains: Bahia grass (*Paspalum notatum*), Bermuda grass (*Cynodon dactylon*) and Johnson Grass (*Sorghum halepense*), stock concentration 40,000 PNU/ml c, were added to the cultures at final concentrations of 100 PNU/ml. For each stimulant, media containing 10% of either one of the three sera: heat inactivated fetal calf serum d (FCS), equine autologous sera from each of the 12 horses either non-heat-inactivated or heat-inactivated. Unstimulated control cultures contained media with either of the three types of sera. The final volume for PBMC stimulation cultures was 2 ml (12-well plate), of which 1 ml was cell suspension and 1 ml was the stimulant dilution. The resulting cell concentration was 3 x 10⁶ cells/ml, or 6 x 10⁶ cells/well. The plates were incubated in a
5% CO₂ humidified atmosphere at 38°C for 1 hour, centrifuged and the supernatants containing the stimulant were removed, cells were resuspended in fresh complete RPMI in a 5% CO₂ humidified atmosphere at 38°C. The wash was repeated twice and then plates were incubated for 48 hours. At the end of the 48 hours, culture media were collected into microfuge conical tubes, centrifuged at 12,000 x g, and the cell-free supernatants were stored at −70°C until assay for ET-1 determination was performed.

8.2.4. Culture of Primary Equine Bronchial Epithelial Cells under ALI Conditions

Fresh post-mortem lung tissues were collected and brought to be dissected under sterile conditions. The bronchial epithelium was dissected from adjacent tissues and bronchial epithelial strips were subjected to cold trypsinization, i.e., digestion with Dulbecco’s Modified Eagle’s Medium (DMEM): Ham’s F12 (1:1 v/v) media containing 2.5 mg/ml trypsin and 1 mg/ml of deoxyribonuclease for 16 to 24 hours at 4°C. Cells were plated directly onto the apical surface of membranes of Transwell culture inserts coated with fibronectin-collagen coating mix. Cells were seeded at 150,000 to 200,000 cells/cm². Cultures were initially fed a serum-containing media, composed of DMEM: Ham’s F12 (1:1 v/v) with 15mM Hepes, 10% fetal calf serum (FCS), L-glutamine (4 mM), penicillin G / streptomycin (50 U/ml and 50 µg/ml, respectively), amphotericin (50 ng/ml), gentamicin (50 ng/ml), insulin (5 µl/ml), transferrin (10 µg/ml), and epidermal growth factor (EGF, 25 ng/ml). Cultures were maintained in 38°C humidified 5%CO₂ incubator. The concentration of FCS in the media was decreased from 10% to 5% when the cells were 70% confluent. Once confluent, the air-liquid interface was established by removal of the media in the top compartment, and the cultures were provided the serum-free media in the bottom compartment. The supplemented serum-free culture media
consisted of: DMEM: Ham’s F12 (1:1v/v) with 15mM Hepes, L-glutamine (4 mM), penicillin G (50 U/ml), streptomycin (50 µg/ml), amphotericin (50 ng/ml), gentamicin (50 ng/ml), insulin (5µl/ml), transferrin (10µg/ml), hydrocortisone \(^e\) (0.5 µg/ml), EGF (0.5 ng/ml), epinephrine \(^e\) (0.5 µg/ml), triiodothyronine \(^e\) (6.5 ng/ml), trans-retinoic acid \(^e\) (5x10\(^{-8}\) M), bovine serum albumin \(^e\) (1.5 µg/ml) and bovine pituitary extract \(^e\) (1% v/v). After the beginning of ALI media was changed once daily. Plates were incubated at 38°C humidified 5% CO\(_2\) atmosphere.

8.2.5. Light, Confocal and Transmission Electron Microscopy of Epithelial Cells

On the 9th, 21st and 28th days after ALI, the unstimulated Transwell culture inserts were prepared for light, confocal or transmission electron microscopy using standard techniques. For light microscopy, the Transwell culture inserts were washed three times with CMF-PBS and fixed with 4% paraformaldehyde for 30 minutes. The polyester membranes were cut out of the Transwell inserts, and the membranes were embedded in LR White h. Cross-sections of the polyester membranes were sectioned at one-micron thickness and stained with toluene blue or methylene blue.

For transmission electron microscopy, the Transwell culture inserts were washed three times with 0.1M sodium cacodylate buffer, then fixed for 30 minutes in fixative containing 2% paraformaldehyde and 1.25% glutaraldehyde. The fixed culture inserts were again washed three times in 0.1M sodium cacodylate buffer, pH 7.4. The fixed culture membranes were cut out of the insert, and dehydrated in a graded ethanol series, embedded in epoxy resin \(^i\), sectioned with an ultramicrotome (MT-XL), stained with uranyl acetate and lead citrate, and examined in a Zeiss EM-10C transmission electron microscope.
For confocal microscopy, after 28 days in ALI culture membranes were cut out of the insert, washed with Tris-buffered saline (TBS; 1.38 M NaCl, 0.05 M KCl, 0.248 M Tris-base, 0.135 M CaCl₂ and 0.11 M MgCl₂, pH 7.5), fixed with 8% paraformaldehyde for 10 minutes, washed again in TBS once and in 0.2% Triton-X-100/TBS for 5 minutes. A blocking step to prevent non-specific binding was performed by incubating the membranes in 10% bovine serum albumin in TBS. The membranes were incubated with anti-cytokeratin monoclonal antibody (4% v/v in TBS) at 4°C overnight. The membranes were then washed and a secondary antibody, i.e., goat anti-mouse IgG labeled with 488 nm (diluted 1:750 in TBS), for 20 minutes at 4°C. The actin staining was performed using Phalloidin 597 nm (0.7% v/v). Stained membranes were mounted with prolong anti-fade media. Material was evaluated in confocal microscope 48 hours after mounting and analyzed using specialized software.

8.2.6. Basolateral Stimulation of Airway Epithelial Cell Cultures

After 9 days in ALI, the cultures were stimulated basolaterally with LPS at 5 µg/ml, human recombinant TNF-α at 5 and 20 ng/ml, or equine recombinant IL-4 (eqrIL-4) at 1%, 10% and 50% v/v. Supernatants from the bottom compartment of the Transwells were harvested at 24, 48 and 72 hours and the cell-free supernatants were stored at –70°C until assay for ET-1 determination was performed.

8.2.7. Determination of ET-1 Concentration in Media of Stimulated Cells

The concentrations of immunoreactive ET-1 (irET) in cell-free supernatants were determined using a commercially available capture enzyme-linked immunosorbent assay (Figure 8.3). The amount of cell-free supernatant from cultures was determined in a preliminary LPS stimulation assay of equine epithelial cells and PBMC, volumes ranging
from 25 to 200 µl were tested. A volume of 25 µl was determined to be within the middle of the range of the standard curve for the assay. The extraction step was omitted and the cell culture supernatants were added directly to the wells as described by the manufacturer. Briefly, the standard curve was constructed using the kit’s endothelin stock solution (lyophilized human ET) diluted serially in cell culture media (standards ranged from 0 to 10 fmol/ml). The enzyme immunoassay was performed in duplicates using 200 µl of each serial dilution of six standards, the positive and negative controls, or the cell culture supernatants (25 µl), which were added to each separate well, followed by 50µl of the detection antibody, i.e., mouse anti-ET monoclonal antibody, which was added to all wells except the blank well. The plates were gently shaken to mix the contents in the well, and plates were incubated for 16 to 24 hrs at 20°C. The contents of the wells were discarded, the wells were washed five times with washing buffer, and the horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody was added to each well and incubated for three hours at 37°C. The content of the wells were discarded, the wells were washed again five times with washing buffer, and 200 µl the TMB substrate was added to all wells, incubated for 30 minutes at 20°C protected from light. The stop solution, 50 µl, was added to each well and mixed. The optical densities at 405 nm and 620 nm were measured immediately with an ELISA plate reader. The concentration of irET in cell culture supernatants was calculated as per manufacturer’s instructions and expressed as pg/ml. The ET-1 concentration results of the bronchial epithelial cell cultures were reported as folds increases of ET-1 compared to the unstimulated cultures. The ET-1 concentration results of the mononuclear leukocyte cultures were reported pg/ml, which was equal to pg/3 x 10^6 cells, and compared to the unstimulated cultures.
8.2.8. Statistical Analysis

The distribution of the data was evaluated for normality using the Shapiro-Wilk test statistic. The irET concentrations in supernatants from mononuclear leukocyte cultures (pg/ml, i.e., pg/3 x 10^6 cells) were compared between groups (SPAOPD-affected horses and non-affected controls), between stimulants (types of aqueous protein extracts of mold spores and pollen grains), and between the types of sera (FCS, autologous serum and heat-inactivated autologous serum). All comparisons were performed using a multifactorial ANOVA. Post-hoc comparisons was performed where appropriate, using Tukey’s test; a p ≤ 0.05 was considered significant. All analyses were performed with the use of the GLM procedure, SAS system, v 8.2 s. The ET-1 concentration results of the bronchial epithelial cell cultures were not compared statistically between groups because there were only two animals per group (two SPAOPD-affected adult horses and two newborn foals).

8.3. Results

Epithelial cells from two of the three SPAOPD-affected horses and both of newborn foals successfully grew and differentiated as expected, whereas none of the three adult non-affected horses grew. The epithelial cells from one of the three SPAOPD-affected horses and from three of the non-affected adult horses grew well initially to the point of confluency, however, within 24 to 48 hours that they had reached confluency, areas of degeneration started to appear; the cells started to pull away from the membrane forming centers denuded of cells, and within 48 hours the cultures had failed.

The equine airway epithelial cells cultures that grew successfully had evident apical mucus secretion by 10 to 14 days after air-liquid interface (Figure 8.4). At 28 days, bright field microscopy revealed 3 or 4 layers of polygonal cells with a basal layer of small
cuboidal cell with small cytoplasm along the polyester membrane (basiloid layer), and a upper continuous layer of cells ranging from low to moderately tall columnar cells with basally located nuclei (Figure 8.5). Transmission electron microscopy revealed the presence of apical microvilli, prominent intercellular bridges of interdigitation with tight junctions and desmosomes (Figure 8.6 and 8.7). No viral particles were noted in any of the sections on the 9th, 21st or 28th days. Confocal microscopy for actin and cytokeratin revealed an apical layer consisting of columnar epithelial cells, and middle layers of polygonal cells all of which stained moderately to strongly for cytokeratin, whereas the basal layer composed of smaller cells stained only slightly for cytokeratin (Figure 8.8 and 8.9).

Evaluation of irET released by equine cell cultures indicated that both the mononuclear leukocytes and bronchial epithelial cells were sources of ET-1. Concentration of irET in unstimulated cultures revealed that ET-1 was produced constitutively in both equine cell types, and those concentrations were considered as the basal release of ET-1, as illustrated in the graphs as “media” in the X-axis (Figures 8.10, 8.11, 8.12 and 8.18). For SPAOPD-affected horses, the basal release of ET in mononuclear leukocyte cultures was 1.5-fold greater in cultures containing FCS than those containing autologous sera (Figures 8.10, 8.11, 8.12, 8.16 and Table 8.1). The unstimulated mononuclear leukocyte cultures maintained in media containing heat-inactivated and non-heat-inactivated autologous sera had similar basal ET-1 concentration (Figures 8.10, 8.11, 8.12, 8.16 and Table 8.1). The basal production of ET-1 in bronchial epithelial cell cultures from newborn foals (mean ET-1 concentration 49.6 pg/ml) was four-fold greater than that in cultures from SPAOPD-affected horses (12.1 pg/ml) (Table 8.2).
Table 8.1. Summary of immunoreactive ET-1 concentration in supernatants from mononuclear leukocyte cultures stimulated with lipopolysaccharide (LPS). Immunoreactive ET-1 concentrations (pg/ml) and the corresponding percentage increase compared with unstimulated cultures. Data include all horses.

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Fetal calf serum</th>
<th>Autologous serum</th>
<th>Heat-inactivated autologous serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>37 pg/ml</td>
<td>24.2 pg/ml</td>
<td>25 pg/ml</td>
</tr>
<tr>
<td>LPS 1 ng/ml</td>
<td>43.3 pg/ml</td>
<td>34.2 pg/ml</td>
<td>31.1 pg/ml</td>
</tr>
<tr>
<td>% increase</td>
<td>17 %</td>
<td>41.4 %</td>
<td>24.5 %</td>
</tr>
<tr>
<td>LPS 10 ng/ml</td>
<td>49.4 pg/ml</td>
<td>37.5 pg/ml</td>
<td>38.4 pg/ml</td>
</tr>
<tr>
<td>% increase</td>
<td>33.5 %</td>
<td>55 %</td>
<td>54 %</td>
</tr>
</tbody>
</table>

In mononuclear leukocyte cultures, the groups of horses, i.e., SPAOPD-affected horses and non-affected controls (p < 0.0001), and the types of sera, i.e., FCS, autologous serum and heat-inactivated autologous serum (p = 0.0001) had a significant effect on the ET-1 concentration. Although important, the effects of the stimulants, i.e., the types of aqueous protein extracts of mold spores and pollen grains, were not significant (p = 0.0513, Figure 8.15). Affected horses had an overall greater mean ET concentration of 43.57 pg/3 x 10^6 cells than the non-affected horses, 29.82 pg/3 x 10^6 cells (Figure 8.13). The mononuclear leukocyte cultures from SPAOPD-affected horses stimulated with media containing FCS had a significantly greater ET-1 concentration than the cultures stimulated with media containing autologous serum and heat-inactivated autologous serum (Figures 8.14, 8.16 and 8.17).
Table 8.2. Summary of immunoreactive ET-1 concentration in supernatants from differentiated bronchial epithelial cell cultures stimulated with lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α and equine recombinant interleukin (eqrIL)-4. Immunoreactive ET-1 (irET-1) concentrations (pg/ml) and the corresponding percentage increase compared to unstimulated cultures (the optimal incubation period).

<table>
<thead>
<tr>
<th></th>
<th>IrET in cells from SPAOPD-affected horses (incubation time)</th>
<th>IrET in cells from newborn foals (incubation time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>17.2 pg/ml</td>
<td>47.07 pg/ml</td>
</tr>
<tr>
<td>LPS 5 µg/ml</td>
<td>34 pg/ml (48 hours)</td>
<td>55.3 pg/ml (24 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>97 %</td>
<td>17.5 %</td>
</tr>
<tr>
<td>TNF-α 5 µg/ml</td>
<td>44.56 pg/ml (48 hours)</td>
<td>55.2 pg/ml (24 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>157%</td>
<td>17.3 %</td>
</tr>
<tr>
<td>TNF-α 20 µg/ml</td>
<td>51.3 pg/ml (48 hours)</td>
<td>59.8 pg/ml (24 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>197 %</td>
<td>27 %</td>
</tr>
<tr>
<td>eqrIL-4 1 v/v</td>
<td>42.5 pg/ml (72 hours)</td>
<td>75.6 pg/ml (72 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>145 %</td>
<td>61 %</td>
</tr>
<tr>
<td>eqrIL-4 10 v/v</td>
<td>58.4 pg/ml (24 hours)</td>
<td>130.2 pg/ml (48 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>237 %</td>
<td>177 %</td>
</tr>
<tr>
<td>eqrIL-4 50 v/v</td>
<td>56.3 pg/ml (48 hours)</td>
<td>139.7 pg/ml (48 hours)</td>
</tr>
<tr>
<td>% increase</td>
<td>225 %</td>
<td>197 %</td>
</tr>
</tbody>
</table>
Table 8.3. Summary of the increase in immunoreactive endothelin (irET) in supernatants from mononuclear leukocyte cultures stimulated with mold spore and pollen grain extracts. Data included all horses. Percentage increase of irET concentrations compared to unstimulated cultures.

<table>
<thead>
<tr>
<th>Extracts of mold spore and pollen grains</th>
<th>IrET-1 in cells grown w/ fetal calf serum</th>
<th>IrET-1 (cells grown w/ autologous serum)</th>
<th>IrET-1 (cells grown w/ heat-inactivated autologous serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alternaria</td>
<td>6.2 %</td>
<td>32.6 %</td>
<td>21.6 %</td>
</tr>
<tr>
<td>Aspergillus</td>
<td>52.8 %</td>
<td>41.7 %</td>
<td>83.2 %</td>
</tr>
<tr>
<td>Cladosporium</td>
<td>17.7 %</td>
<td>29.2 %</td>
<td>40 %</td>
</tr>
<tr>
<td>Curvularia</td>
<td>14 %</td>
<td>30.8 %</td>
<td>8 %</td>
</tr>
<tr>
<td>Bahia grass</td>
<td>27.2 %</td>
<td>45 %</td>
<td>61 %</td>
</tr>
<tr>
<td>Bermuda grass</td>
<td>42.8 %</td>
<td>59.7 %</td>
<td>42.8 %</td>
</tr>
<tr>
<td>Johnson grass</td>
<td>0</td>
<td>38 %</td>
<td>25 %</td>
</tr>
</tbody>
</table>
Figure 8.5. Bright field photomicrograph of a cross-section of differentiated equine bronchial epithelial cells. Cells cultured onto Transwells for 9 days (A) and 28 days (B) after air-liquid interface, and stained with methylene blue. Note the layers of polygonal cells, the low to moderate columnar cells on the top layers and the small basiloid cells on the bottom layer.
Figure 8.6. Transmission electron photomicrographs of differentiated equine bronchial epithelial cells from adult horses. Cells grown for 28 days after beginning of air-liquid interface. Note the layers of polygonal cells and the columnar top layer (A); and the interdigitating projections and desmosomes between cells (B).
Figure 8.7. Transmission electron photomicrograph of differentiated equine bronchial epithelial cells from newborn foal. Twenty-one days after beginning air-liquid interface. Note the microvilli on the apical surface of the cells and the tight interaction between adjacent cells (A), and the interdigitating projections and desmosomes between cells (B).
Figure 8.8. Confocal photomicrographs of differentiated equine bronchial epithelial cells from adult horses. Cells grown submerged for 28 days after beginning of air-liquid interface. Cells stained for cytokeratin (A) and actin (B).
Figure 8.9. Three-dimensional confocal photomicrographs of differentiated equine bronchial epithelial cells from adult horses. Cells grown for 28 days after beginning of air-liquid interface. Top panel shows individual and overlay views of differential interference contrast (DIC), actin and cytokeratin. Bottom panel shown the three dimensional view of the culture stain for actin (red) and cytokeratin (green).
Figure 8.10. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with protein extracts of mold spores or grass pollen grains plus fetal calf serum. ET-1 concentrations reported in pg/ml, which corresponds to pg/3 x106 cells. LPS = lipopolysaccharide.
Figure 8.11. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with protein extracts of mold spores or grass pollen grains plus autologous serum. ET-1 concentrations reported in pg/ml, which corresponds to pg/3 x 10^6 cells. LPS = lipopolysaccharide.
Figure 8.12. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with protein extracts of mold spores or grass pollen grains plus heat-inactivated autologous serum. ET-1 concentrations reported in pg/ml, which corresponds to pg/3 x10^6 cells. LPS = lipopolysaccharide.
Figure 8.13. ET-1 released by cultured equine mononuclear leukocytes from SPAOPD-affected and non-affected horses 48 hours after stimulation. The data of the groups (affected and non-affected) were combined for all stimulants and sera types in order to illustrate the effect of the status of sensitization of the horses on the concentrations of ET-1 released into the media; significance was considered at p < 0.05. ET-1 concentrations reported in pg/ml, which means pg/3 x106 cells. Different letters (a, b) represent significant difference (p < 0.05).
Figure 8.14. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation various stimulants plus either fetal calf serum (FCS), autologous serum, heat-inactivated (HI) autologous serum. ET-1 concentrations reported in pg/ml or pg/3 x 10^6 cells. Note the effect of sera type; Cultures from affected horses containing FCS (a) had significantly greater ET-1 release than those containing other types of sera (b). Different letters (a, b) represent significant difference (p < 0.05).
Figure 8.15. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with lipopolysaccharide (LPS) or protein extracts of mold spores or grass pollen grains. ET-1 concentrations reported in pg/ml = pg/3 x10^6 cells. The data combined the data of affected and non-affected horses and the sera types. Note the effect of stimulant; media = unstimulated cultures. Different letters (a, b) denote significant differences (p < 0.05).
Figure 8.16. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with protein extracts of grass pollen grains plus either fetal calf serum (FCS), autologous serum or heat-inactivated (HI) autologous serum. Note the effect of sera in each individual stimulant in both affected and non-affected horses. ET-1 concentrations reported in pg/ml or pg/3 x10^6 cells.
Figure 8.17. ET-1 released by cultured equine mononuclear leukocytes 48 hours after stimulation with protein extracts of mold spores plus either fetal calf serum (FCS), autologous serum or heat-inactivated (HI) autologous serum. Note the effect of sera in each individual stimulant in both affected and non-affected horses. ET-1 concentrations reported in pg/ml or pg/3 x10^6 cells.
Figure 8.18. ET-1 released basolaterally by differentiated equine bronchial epithelial cells. Top graph: ET-1 concentrations in culture media from two SPAOPD-affected horses; Bottom graph: ET-1 concentrations in culture media from two newborn foals. LPS = lipopolysaccharide, TNF = tumor necrosis factor, eq rIL-4 = equine recombinant interleukin-4.
The *in vitro* stimulation of mononuclear leukocyte cultures with aqueous protein extracts of mold spores and grass pollen grains resulted in up-regulation of ET-1 released (Figures 8.10, 8.11, 8.12 and 8.15). The ET concentration in stimulated cultures was greater than that in unstimulated (Figure 8.15). When analyzed individually, only stimulation with *Aspergillus fumigatus* was significantly different from the stimulation with other extracts. Stimulation with LPS induced a percentage increase in the irET released into the media of mononuclear leukocyte cultures that varied considerably if cells were cultured with media containing the three types of sera (Table 8.1).

The stimulation of bronchial epithelial cell cultures from SPAOPD-affected (adult) horses with LPS, hrTNF-α, or eqrIL-4 for 24 and 48 hours, and eqrIL-4 for 72 hours resulted in increased release of irET, ranging from 2- to 3.5-fold increases, whereas stimulation with supernatants from non-transfected CHO cells had no effect (Figure 8.18). Stimulation with LPS for 24 and 48 hours resulted in a similar 2-fold increase in ET-1 concentration (Figure 8.18, Table 8.2). Stimulation with hrTNF-α resulted in a dose-dependent (i.e., with 5 ng/ml the increase in ET-1 concentration was lower than with 20 ng/ml), and time-dependent (i.e., with stimulation for 24 hours the increase in ET-1 concentration was lower than with 48 hours) increase in the basolateral secretion of ET-1 (Figure 8.18). Stimulation of bronchial epithelial culture from adult SPAOPD-affected horses with eqrIL-4 resulted in a dose-dependent increase in the basolateral secretion of ET-1 (i.e., with 1% v/v being the lowest increase in ET-1 concentration, the 10% v/v the optimal and the 50% v/v was intermediate).

In contrast, stimulation with LPS and TNF-α and supernatants from non-transfected CHO cells had no to little effect in bronchial epithelial cell cultures from newborn foals (Figure 8.18). Stimulation of bronchial epithelial culture from foals with eqrIL-4 resulted in a
dose-dependent increase in the basolateral release of ET (i.e., with 1% v/v being about 1.5-fold increase in ET concentration, the 10% v/v a 2.8-fold increase in ET concentration and the 50% v/v a 3-fold increase in ET concentration).

8.4. Discussion

Equine mononuclear leukocytes and differentiated equine bronchial epithelial cells displayed a basal release of ET-1, and stimulation with LPS resulted in up-regulation of ET-1 release into the media. Basal release of ET-1 by monocytes has been reported in cells from humans (Ehrenreich et al. 1990). Basal release of ET by airway epithelial cells has been reported in cells from dogs, pigs, humans and guinea-pigs (Black et al. 1989, Mattolli et al. 1990, Yang et al. 1997).

The concentrations of ET released in unstimulated bronchial epithelial cell cultures were comparable to those reports for other species (Black et al. 1989, Mattolli et al. 1990, Yang et al. 1997). The equine mononuclear leukocytes displayed a basal release of ET-1 (33 pg/ml), which was greater than those reported of human monocytes (11 pg/ml) (Ehrenreich et al. 1990).

The stimulation of equine mononuclear cells with LPS at 5 µg/ml induced an increase in ET-1 release that was 10-fold less than that reported for human monocytes (Ehrenreich et al. 1990). It is unclear the reason for this difference. The stimulation of differentiated equine bronchial epithelial cells from adult SPAOPD-affected horses with 5 µg/ml of LPS induced an increase in ET release (97%) after 24 hours that was greater than that reported for guinea-pig tracheal epithelial cells (66%) for the same dose and time (Yang et al. 1997). Whereas differentiated bronchial epithelial cells from newborn foals had a much lesser response (17.5%) after 24 hours of stimulation with the same dose of
LPS. One of the striking differences is that the cells from newborn foals had a much
greater basal release of ET-1, around 4-fold greater than the SPAOPD-affected horses. It
is unclear if this difference is a result of the age difference or the disease status. Ideally,
aged-matched control horses should have been used, however, none of the cultures of
non-affected adult horses could be maintained.

The effect of type of serum present in the media during stimulation of equine
mononuclear cell cultures was a significant finding. All of the studies ET-1 release by
monocyte/macrophage cultures reported the use of FCS, and none of them describe the
use of autologous sera. The high ET release in unstimulated cultures (i.e., media) when
FCS was present suggests that the FCS itself exerted a stimulatory effect on the ET-1
production by the mononuclear leukocytes of SPAOPD-affected, but not the non-affected.
Moreover, this stimulatory effect appeared to distort the evaluation of the ET-1 release in
response to the mold spore and pollen grain extracts. Evaluation of ET-1 release from
cells cultured in the presence of autologous sera suggests little difference between
SPAOPD-affected and non-affected horses with respect to the stimulatory agent. These
differences are subtle and difficult to interpret. Some extracts of mold spore or grass
pollen seemed to have a slightly greater stimulatory effect or slightly lesser than others,
however, it is difficult to extrapolated the relevance of this difference. It appears that
aqueous protein extracts of mold spores and grass pollens can stimulate mononuclear
leukocytes and induce an increase in the basal release of ET-1. The persistence of these
aeroallergens in the lumen of the airway of SPAOPD-affected horse, which are believed to
have an inadequate pulmonary clearance, may result in induction of up-regulation of ET-1.

Cytokine stimulation, specifically TNF-α and IL-4, resulted in increased production
and basolateral release of ET-1 by equine bronchial epithelial cells. These results suggest
that equine airway epithelial cells represent a potentially important source of ET-1 in response to cytokines that are likely to be secreted during the inflammatory response associated with airway disease, including TNF-\(\alpha\) and IL-4. Airway inflammation associated with increased synthesis of these cytokines is likely to result in up-regulation of epithelial-derived ET-1.

Lastly, LPS clearly causes up-regulation of ET-1 released by both equine mononuclear cells as well as equine airway epithelial cells. Although the LPS-induced up-regulation of ET-1 release appears to be lesser in magnitude than the cytokine-induced ET-1 release, the presence of endotoxin in the airways is likely to augment ET-1 release and possibly amplify the inflammatory response. The presence of respirable airborne dust endotoxin was demonstrated in the breathing zone of horses, and inhalation of endotoxin was shown to induce dose-dependent airway inflammation in horses affected with RAO as well as control subjects (McGorum et al. 1998, Pirie et al. 2001). It is possible that inhaled endotoxin induces up-regulation of ET-1 among other mediators which than culminate with airway inflammation and airflow obstruction.

Because ET-1 appears to be involved in the pathogenesis of recurrent airway obstruction, it is likely that mononuclear cells as well as epithelial cells contribute appreciably as a source of this inflammatory mediator. The interactions of ET-1 with other mediators may play a role in the pathogenesis of SPAOPD.

8.5. Endnotes

\(^a\) Ficoll-Paque Plus, Amersham Biosciences Corporation, Piscataway, NJ, USA.

\(^b\) L-glutamine, Gentamicin, Penicillin/Streptomycin, GIBCO-BRL, Grand Island, NY, USA.

\(^c\) Greer Laboratories; Lenoir, NC, USA.
d Fetal Calf Serum, HyClone Sera, Logan UT, USA.

e Trypsin, Deoxyribonuclease, 2-mercaptoethanol, Transferrin, Insulin, Human Recombinant Epidermal Growth Factor Expressed in E. Coli, Amphotericin B, Hydrocortisone, Epinephrine, Tri-iodo-thyronine, Trans-Retinoic Acid, Bovine Serum Albumin and Bovine Pituitary Extract; Sigma-Aldrich, INC., Saint Louis, MO, USA.

f Transwell, 12 mm diameter, 0.4 µm pore size tissue culture treated polyester, membrane polystyrene plates; Corning Costar Corporation, Cambridge, MA, USA.

g Fibronectin-Collagen Coating Mix, Biological Research Faculty Facility, Inc., Ijamsville, MD, USA.

h LRWhite, Electron Microscopy Sciences, Fort Washington, PA, USA.

i Epon-Araldite; Electron Microscopy Sciences, Fort Washington, PA, USA.

j Mouse anti-human cytokeratin 7 monoclonal antibody, isotype IgG1/ kappa; Biomeda

k Goat anti-mouse IgG labeled with 488nm-green; Molecular Probes, Invitrogen detection technology, Eugene, OR, USA.

l Phalloidin 597 nm-red Molecular Probes, ; Molecular Probes, Invitrogen detection technology, Eugene, OR, USA.

m Prolong anti-fade media, Molecular Probes, ; Molecular Probes, Invitrogen detection technology, Eugene, OR, USA.

n Leica TCS 5P2 and Confocal Laser Scanning Microscopy Integrated Leica Confocal Software; Leica Microsystems, Heideberg Gmbh, Germany.

o Lypopolysaccharide, Sigma-Aldrich, INC., Saint Louis, MO, USA.

p Human recombinant TNF-alpha; Sigma-Aldrich, INC., Saint Louis, MO, USA.

q The eq rIL-4 was kindly provided by Dr. D. Horohov. The eq rIL-4 had been generated by sequencing the cDNA for equine IL-4, cloning it in the mammalian expression vector pcDNA3.1/Myc-His(-) (Invitrogen, San Diego, CA) and transfecting the construct into Chinese Hamster Ovary (CHO) cells (Vandergifft et al. 1994). The cell culture supernatant of IL4-expressing CHO cells were harvested and purified by HPLC.

f enzyme-linked immunosorbent assay, Biomedica Gruppe, Austria; Distributed by American Research Products, Inc., Belmont, MA, USA.
CHAPTER 9. SUMMARY AND CONCLUDING REMARKS
9.1. Summary

The overall goal of this dissertation was to identify potential aeroallergens and cytokines involved in the pathogenesis of summer pasture-associated obstructive pulmonary disease (SPAOPD) and evaluate their role in endothelin (ET)-1 production, neutrophil activation and chemotaxis. The hypotheses of this dissertation were that (1) mold spores are potential aeroallergens involved in triggering clinical exacerbation of SPAOPD; (2) aeroallergens induce granulocyte activation; (3) aeroallergens induce mononuclear leukocytes to produce inflammatory mediators, including ET-1, that promote granulocyte activation and migration; (4) ET-1 induces granulocyte activation and migration; (5) cytokines induce up-regulation of ET-1 by bronchial epithelial cells; and (6) aeroallergens induce up-regulation of ET-1 by mononuclear leukocytes.

Three in vivo studies were designed to evaluate the seasonal pattern of clinical exacerbation and relationship with environmental factors and aeroallergens, to evaluate circulating and pulmonary ET-1 concentrations during clinical exacerbation and remission, and to evaluate expression of ET-1 and cytokines, interleukin (IL)-4 interferon (IFN) -γ and IL-8 expression in lungs of SPAOPD-affected horses compared with non-affected horses. The putative aeroallergens and cytokines identified from the in vivo studies were employed in vitro studies designed to assess their effect on cells of the immune system and airway epithelial cells. The in vitro studies included the evaluation of the effects of aeroallergens on mononuclear leukocytes-derived cytokines, on activation and migration of granulocytes and on mononuclear leukocytes-derived granulocyte activation; the effects of ET-1 on granulocytes activation and chemotaxis, and the basal production & up-regulation of ET-1
in mononuclear leukocytes following stimulation with aeroallergens and in bronchial epithelial cells following stimulation with cytokines.

The first in vivo study was designed to describe the seasonal pattern of the clinical exacerbation–remission cycle of SPAOPD in relation to environmental factors and aeroallergen counts. The seasonal pattern of the clinical exacerbation–remission of SPAOPD was evaluated temporally and in parallel with the variations of the environmental factors, and the aeroallergen counts, including 20 specific types of mold/fungal spores and 28 types of pollen, were evaluated in relation to the onset of clinical exacerbation. We demonstrated the seasonal pattern of the clinical exacerbation of SPAOPD, and its relationship to increases in environmental variables of temperature (heat) and dew-point temperature (humidity). The seasonal pattern of clinical exacerbation paralleled increases in mold/fungal spore counts and grass pollen counts. Specific types of molds were identified.

The second in vivo study was designed to evaluate the circulating and pulmonary concentration of ET-1 during clinical exacerbation and remission of SPAOPD. The specific aim was to determine and compare the concentrations of ET-1 in arterial and venous plasma, bronchoalveolar lavage and pulmonary epithelial lining fluid samples obtained from SPAOPD-affected and non-affected horses, during different times of the year. The ET-1 concentrations in arterial and venous plasma samples were greater in SPAOPD-affected horses during clinical exacerbation (mid-spring to mid-fall) than those during remission (winter). There were no significant variations of ET-1 concentrations in plasma samples from non-affected control horses during summer and winter. During remission, all SPAOPD-affected horses evaluated in this study had pleural pressure differences within the normal range (less than 10 cm of H2O), similar to those seen in the non-affected
horses, which indicated complete reversibility of the airway resistance and obstruction. The increase in venous and arterial ET-1 concentration during clinical exacerbation of SPAOPD resembles the findings in human asthma and equine RAO. The overall increase in circulating ET-1 during clinical exacerbation of SPAOPD suggests that ET-1 contributes to the bronchoconstriction, hypersecretion and inflammation observed in this disease. Intervention with ET-1 production, metabolism or use of ET-1 antagonists may prove to be an important therapeutic target.

The objective of the third in vivo study was to evaluate the gene expression of ET-1 and three cytokines, IL-4, IL-8 and IFN-γ, and to quantify and report the distribution of immunoreactive ET-1 in lungs of horses affected with SPAOPD, compared with normal controls. We hypothesized that the expression of ET-1 was greater in airways of horses affected with SPAOPD, compared with non-affected horses. We demonstrated that the amounts of ET-1 transcripts and immunoreactive ET-1 peptide were greater in lungs of affected compared to non-affected horses, although the difference was not statistically significant. We demonstrated that ET-1 immunoreactivity was present at low intensity in airway and vascular structures of lungs of normal horses. The intensity of ET-1 immunoreactivity in airway epithelium, airway connective tissue and vascular smooth muscle in horses affected with SPAOPD was greater than in non-affected horses. The trend of greater gene expression of ET-1 in the lung, and of greater ET-1 immunoreactivity in airway structures of SPAOPD-affected horses compared to non-affected controls supports the concept that ET-1 may contribute to the clinical and pathologic finding in SPAOPD. The trend of greater expression of IL-4, but not IFN-γ, suggests a bias towards a TH2 response. The greater gene expression of IL-8, a chemokine that activates and attracts neutrophil, supports the clinical findings in clinical exacerbation of SPAOPD.
The purpose of the first *in vitro* study was to evaluate the proliferation of mononuclear leukocytes and gene expression of IL-4, a TH2 cytokine, and IFN-γ, a TH1 cytokine, following stimulation with aqueous mold spore extracts. Lymphocyte proliferation response to aqueous mold spore extracts showed a dose-response to Alternaria and Curvularia, whereas the stimulation with Cladosporium extract resulted in negligible proliferative response. Cells from SPAOPD-affected horses had an overall greater gene expression of these two cytokines than the non-affected controls. The ratio of expression of IL-4/ IFN-γ tended to be greater for any stimuli as well as for unstimulated cells, from SPAOPD-affected horses compared with non-affected controls, and stimulation with Cladosporium extract resulted in a significantly greater IL-4/ IFN-γ. The greater IL-4/ IFN-γ in affected horses compared with non-affected suggests a trend towards TH2 type of response.

The second *in vitro* study was designed to investigate putative aeroallergens, mold spores and grass pollen grains, as stimulants of neutrophil activation and chemoattraction. All of the stimulants tested induced activation of granulocytes, and there was no difference between affected and non-affected horses, suggesting that regardless of the type of stimulant, neutrophils may be activated and attracted toward the airways. As the pulmonary clearance in affected horses is decreased in SPAOPD-affected horses, the presence/persistence of these aeroallergens in the airways may exert an important stimulation for neutrophil accumulation within the airways. Moreover the stimulation of mononuclear leukocytes with extracts of potential aeroallergens after 48 hours induced greater granulocyte activation than in non-affected controls, suggesting that mononuclear leukocytes of affected horse may have a greater intrinsic inflammatory response than cells from non-affected horses.
In order to evaluate the effects of ET-1 on airway epithelial cells, a culture of differentiated bronchial epithelia cells had to be established, such that it resembled the normal airway epithelium. Therefore, a study was designed to evaluate primary and transformed airway cells in two culture systems and identify the best system to be used for the cytokine stimulation and evaluation of ET-1 release. The overall purpose of the study was to establish and to evaluate culture techniques of equine cells of respiratory origin by light and electron microscopy, specifically to establish and evaluate cultures of primary equine bronchial epithelial cells grown submerged, under air-liquid interface and microgravity conditions and, evaluate a spontaneously transformed fetal equine tracheal cell line grown under air-liquid interface and microgravity conditions. We identified that primary bronchial epithelial cells grown under the air-liquid interface conditions and serum-free media differentiated well, resembling the morphology of normal airway epithelium, with the formation if tight intercellulary interactions, i.e., numerous interdigitating projections between cells containing desmosomes.

The last in vitro study was designed to evaluate the release of ET-1 by airway epithelium and mononuclear leukocytes from horses affected with SPAOPD and non-affected horses. As a number of stimuli, especially cytokines have been incriminated in the induction of ET-1 synthesis. The overall goal of this study was to stimulate cultures of differentiated equine bronchial epithelial cells with TNF-α and IL-4 and measure the basal release as well the up-regulation of ET-1 basolaterally. Based on the previous study, differentiated airway epithelial cultures were established from primary bronchial epithelial cells cultured under air-liquid interface conditions. We successfully cultured cells from two affected horses and two, term-foals, however we were unable to establish the cultures from non-affected adult horses. Stimulation of cultures from SPAOPD-affected horses with
hrTNF-α and eqrIL-4 for 24 and 48 hours, and eqrIL-4 for 72 hours resulted in increased production of ET-1 (ranging from 1.5 to 4 fold). Whereas, cultures from term foals had a much higher basal production, which may be age-related phenomena and the stimulation with hrTNF-α had minimal effect on up-regulation of ET-1. In contrast, eqrIL-4 induced up-regulation of ET-1 in cultures from SPAOPD-affected horses and term foals to a similar degree. These results suggest that bronchial epithelial cells represent a potentially important source of ET-1 in response to cytokine, i.e., TNF-α and IL-4, stimulation. The interactions of cytokines and ET-1 appear to be important in the pathogenesis of SPAOPD.

In addition, the evaluation of release of ET-1 by mononuclear leukocytes from horses affected with SPAOPD and non-affected horses stimulated with various extracts of potential aeroallergens revealed a difference between affected and non-affected horses. Mononuclear leukocytes from affected horses respond to all stimulants with a greater up-regulation of ET-1 than non-affected controls, suggesting that affected horses may have an intrinsic greater ET-1 production in response to stimulation compared with non-affected controls.

9.2. Concluding Remarks

Overall goal was to identify potential aeroallergens and cytokines involved in the pathogenesis of SPAOPD and evaluate their role in vitro by assessing neutrophil activation and chemotaxis and ET-1 production.
Figure 9.1. Conclusions of this dissertation

- Extracts from grass pollen & mold spores up-regulated release ET-1 by mononuclear leukocytes
- Clinical exacerbation in hot & humid conditions; grass pollen grains & mold spores potential aeroallergens
- Cytokines IL-4 & TNF-α up-regulated ET-1 production by bronchial epithelial cells
- ET-1 activated & attracted granulocytes
- Extracts from grass pollen & mold spores stimulated mononuclear leukocytes to induce granulocyte activation
- Trend of greater expression of ET-1, IL-3, IL-4, but not IFN-γ, in lungs of affected horses
- Extracts from mold spores induced gene expression of IL-4 and IFN-γ in mononuclear leukocytes
- irET-1 localized to airway structures in affected horses compared with controls
- Circulating concentration of ET-1 increased during clinical exacerbation of SPAOPD
The hypotheses of this dissertation included: (1) mold spores are potential aeroallergens; (2) aeroallergens induce granulocyte activation; (3) aeroallergens induce mononuclear leukocytes to produce inflammatory mediators, including ET-1, that promote granulocyte activation and migration; (4) ET-1 induces granulocyte activation and migration; (5) cytokines induce up-regulation of ET-1 by bronchial epithelial cells; and (6) aeroallergens induce up-regulation of ET-1 by mononuclear leukocytes.

In the first part the in vivo studies were designed to evaluate the seasonal pattern of clinical exacerbation and relationship with environmental factors and aeroallergens, evaluate ET-1 and cytokine expression in lungs of affected horses, and to evaluate circulating and pulmonary ET-1 concentrations during clinical exacerbation and remission.

The putative aeroallergens and cytokines identified from the in vivo studies were employed in in vitro studies designed to evaluate the effects of aeroallergens on mononuclear leukocytes-derived cytokines, activation and migration of granulocytes, and mononuclear leukocytes-derived granulocyte activation; the effects of ET-1 on granulocytes activation and chemotaxis, and the basal production & up-regulation of ET-1 in mononuclear leukocytes following stimulation with aeroallergens and in bronchial epithelial cells following stimulation with cytokines.

We have identified that clinical exacerbation is temporally associated with hot & humid conditions, and with increases in exposure to grass pollen grains and mold spores, suggesting that these are potential aeroallergens triggering clinical exacerbation of the disease. We have identified a trend of greater expression of ET-1, IL-8, IL-4, but not IFN-γ, in lungs of affected horses compared to nor-affected. The lack of statistical significance is likely a result of the small sample size. A greater sample size of 12 to 15 horses would better define if there was truly a difference between affected and non-affected horses.
That is true for other studies within this dissertation, when evaluating the overall variance of the entire population; the larger sample size is likely to reveal significant differences that were only determined to be trends with the small sample size used in the studies. The immunoreactive ET-1 localized to airway structures in affected horses compared with controls was not a quantitative evaluation, but it did suggest that along with the morphologic changes in lungs of affected horses, a shift in the source of ET-1 may play an important role in the effects of ET-1 in the airway, regardless if there is or not an overall increase in the total amount of ET-1 produced in the lungs of affected horses. The increased circulating concentration of ET-1 during clinical exacerbation of SPAOPD, suggests that ET-1 may in fact mediate the inflammatory, secretory and constrictive effects associated with clinical signs of disease, as these were reversible; and during clinical remission when these signs were not present the concentration of ET-1 is similar to non-affected controls. The extracts of mold spores induced gene expression of IL-4 and IFN-γ in mononuclear leukocytes, suggesting a TH2 bias in affected horses compared to non-affected. Aqueous extracts of grass pollen and mold spores induced granulocyte activation directly. Moreover, these extracts of grass pollen and mold spores stimulated mononuclear leukocytes to induce granulocyte activation, to a greater extent in affected horses than in non-affected controls. Another important finding is that ET-1 activated and attracted granulocytes. This is an important finding, especially if bronchial epithelial cells are important sources of ET-1 release. Lastly, the cytokines IL-4 & TNF-α, which are likely cytokines being produced in inflammatory response of the airways were able to up-regulate the production and release of ET-1 by bronchial epithelial cells, suggesting that during clinical exacerbation the production of ET-1 by bronchial epithelial cells is likely to play an important role in the clinical hallmarks of the disease. Moreover, the extracts of
grass pollen and mold spore up-regulated release ET-1 by mononuclear leukocytes to a greater extent in affected than non-affected horses, suggesting that the exposure to aeroallergens is likely to induce greater ET-1 production in airway of affected horses that in airway of non-affected horses. Evaluation of alveolar macrophages and their response to these stimulants, rather than peripheral blood-derived mononuclear cells should be performed.
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VITA

Lais Rosa Rodrigues Costa was born and raised in the city of Sao Paulo, State of Sao Paulo, Brazil. She entered the School of Veterinary Medicine, FMVZ, Sao Paulo State University, UNESP-Botucatu and completed the five-years program earning the Award “Oracio Passos” for the Best Student in the Class 1987. She graduated from Veterinary School in December 1987. After almost 2 years working as a Large Animal Clinician at UNESP-Botucatu, in Brazil, Dr. Costa came to the School of Veterinary Medicine at Louisiana State University for her Internship in Equine Medicine and Surgery. She then moved to Lexington where she obtained her Master of Science degree at University of Kentucky in May 1994 studying the immune response to Equine Infectious Anemia Virus, under Dr. C. Issel. While at the Maxwell Gluck Equine Research Center, she worked with Dr. G. Allen characterizing the immune response to Equine Herpesvirus 1. For the following 2 years Dr. Costa worked for the Veterinary Medical Teaching Hospital - Equine Medicine Service, at University of California-Davis, and obtained a grant to study genetic variability of Corynebacterium pseudotuberculosis isolates affecting horses, cows, sheep and goats. In 1996, Dr. Costa returned to LSU for the three-year Residency Program in Equine Medicine, she became Board Certified in Large Animal Internal Medicine in 1999, and she was hired as clinical instructor in Equine Medicine at LSU for six months and at University of Georgia for one month. Dr. Costa initiated her Ph.D. Program in the spring of 2000. While pursuing her Ph.D. in the Department of Pathobiological Sciences, Dr. Costa worked as a Clinical Fellow in Equine Medicine, through the Department of Veterinary Clinical
Sciences. She obtained funding for several clinical studies and five research projects involving equine airway disease. Dr. Costa has also been involved in several activities concerning neonatology and intensive care.