Adaptation of Three Different Apoptotic Methods in Equine Bronchoalveolar Cells and Comparison of Bronchoalveolar Lavage Cell Apoptosis in Normal and COPD Affected Horses Before and After Dexamethasone Administration

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Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science
In
Veterinary Medical Science

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June 21, 2001
Blacksburg, Virginia

Keywords: Lymphocyte, 7-AAD, Annexin-V, Propidium Iodide

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(ABSTRACT)

Recent studies suggest that lymphocyte apoptosis serves to regulate pulmonary inflammation. Equine COPD, an allergic disease of the lower airway, is likely due to dysregulation of the pulmonary immune response. In this study, the hypothesis tested was COPD affected horses would have less apoptotic airway lymphocytes than control horses during clinical disease. To achieve this, 3 methods of measuring apoptosis, Vindelov's propidium iodide with Triton-X (PI/Triton-X), 7-aminoactinomycin D (7-AAD), and Annexin V with propidium iodide (Annexin/PI) were evaluated in equine airway lymphocytes. A significant linear relationship was found for equine bronchoalveolar lavage (BAL) lymphocytes stained with 7-AAD and Annexin/PI . No relationship was identified with cells stained with PI/Triton-X and Annexin/PI, and 7-AAD and PI/Triton-X indicating that methods which preserve cell membrane characteristics are more comparable when measuring BAL lymphocytes apoptosis in a heterogeneous population of cells. Additionally, all stains appear to perform the same in COPD and normal horses in remission and disease.

Comparison of predominately BAL lymphocyte apoptosis using the above methods were performed at baseline, after natural challenge, and after dexamethasone administration in nine horses, five of which were affected with COPD. No differences in bronchoalveolar lavage lymphocyte apoptosis between COPD and control horses were detected either before or after dexamethasone administration, although numerical trends in COPD horses identified less

apoptosis after natural challenge indicating that defective apoptosis may play a role in equine COPD pathogenesis. Dexamethasone administration was associated with trends of improvement in the pulmonary gas exchange and increased apoptosis toward baseline in the COPD horses.

Funding for this research was granted from the American Association of Equine Practitioners, the Comparative Respiratory Society, and the Companion Animal Research Focus Unit at Virginia-Maryland Regional College of Veterinary Medicine.

DEDICATION

This work is dedicated to the gentle, patient, and magnificent horses,
Spot, Lazar, Chance, Shane, Cookie, Blazer, Scooter, Scotty, Buster and Myra,
for without them, this work could not have been done.
We have learnt so much from them.

ACKNOWLEDGEMENTS

Many grateful, heartfelt thanks go to my committee, Dr. Virginia Buechner-Maxwell, for your support, patience, and laughing with me, not at me (most of the time), Dr. Mark V. Crisman, for keeping everything in perspective, Dr. Francois Elvinger, for your great expertise and help with the many statistical evaluations, and Drs. Robert Gogal and S. Ansar Ahmed for tireless help and suggestions with the numerous manuscript revisions. I know I would still be at the starting line without all of you.

Many thanks to Dan Ward whom I shared laughter, friendship, and long hours with while he imparted his statistical wisdom. More thanks to Joan Kalnitsky for flow cytometric expertise and for trying to keep me on time. Additionally, muchas gracias to Shannon McCourt and Cristi Salmon whose tremendous help both with the horses and in the lab cannot be over stated.

I would also like to thank Dr. Kent Scarratt and Dr. John Dascanio for data collection help in times of need. Likewise, I would like to thank all those that have made my graduate studies and residency a time I will remember fondly.

TABLE OF CONTENTS

TABLE OF MULTIMEDIA OBJECTS

Chapter 1

Chapter 2

Chapter 3

Introduction:

Equine chronic obstructive pulmonary disease (COPD) is a disease characterized by wheezing, coughing, and bronchial hyper-reactivity when the horse is exposed to various antigens. The American Thoracic Society identified the horse as a possible natural model for studying human asthma as the two diseases share many clinical similarities. Additionally, human asthmatics have been found to have decreased bronchial lavage T lymphocyte apoptosis (cell death) when exposed to antigens as measured by different apoptotic methods.

Methods available to identify apoptosis in cells are numerous. Methodologies to identify DNA fragmentation, cell wall asymmetry, and apoptotic bodies are available. Many have been adapted to species other than humans (Chapter 2). Three methods, 7-aminoactinomycin-D (7-AAD), Annexin-V/Propidium Iodide (Annexin/PI), and Propidium Iodide with Triton-X (PI/Triton-X), were chosen for investigation of adaptation and comparison in detecting apoptosis in equine bronchoalveolar lavage retrieved lymphocytes because of their relative ease of application and previous history of adaptation in mice, fish and birds.

Treatment of equine COPD can include inhalant or parenteral administration of corticosteroids to suppress the pulmonary inflammation. Glucocorticoids like dexamethasone have been found to induce apoptosis in murine thymocytes and human peripheral blood lymphocytes. Apoptosis induction in lymphocytes might be one way that pulmonary inflammation is decreased in human asthmatics when corticosteroids are administered and might be one mechanism that helps to eliminate inflammation in equine COPD. Therefore, comparisons of apoptosis percentages using 7-AAD, Annexin/PI, and PI/Triton-X staining methods were performed at baseline, after natural challenge, and after dexamethasone therapy to investigate any differences (Chapter 3).

To understand the processes involved in apoptosis and regulation of an immune response, a review is provided of apoptotic mechanisms and attenuation of an immune response, focusing on equine COPD, human asthma and corticosteroids (Chapter1).

Chapter 1:

A. Pulmonary Immunology

The lung is an organ that is continually assaulted with foreign material that is encountered with every breath that an animal takes. The pulmonary system has evolved a defense structure that helps it to cope with the onslaught of particles while minimizing the amount of inflammation. The upper airways provide the first line of defense to large (>10 microns), inhaled particles. Larger particles are likely to be impeded in the hairs of the nasal passages and by collision on the airway walls (Twigg 1998). Mid-sized particles (5 – 10 microns) may make it past the upper airways and into the tracheobronchial tree. Here they are also likely to impact on the lower airway walls and be cleared by the mucociliary escalator mechanism. Coughing is also an effective mechanism that can help to clear the tracheobronchial tree of debris.

Inertial impaction is the predominent means of deposition for particles larger than 5 microns traveling at high velocities, as would occur in the upper airway and conducting bronchi (Buechner-Maxwell 1993). Other sources of eliminating small particles (< 5 microns) that may invade deeper into the airways include gravitational sedimentation and Brownian diffusion (Buechner-Maxwell 1993). Gravitational sedimentation involves gravitational forces 'pulling' the particle into the airway wall and occurs with near zero airflow velocities. Brownian diffusion occurs with very small (<0.5 microns) particles when random gas molecules displace the particle into the airway wall. Particles less than 5 microns, like bacteria, which are able to reach the alveolar space, are then cleared by immune mechanisms versus the aforementioned mechanical mechanisms of the airways.

Immune mechanisms that the pulmonary tree uses to rid itself of foreign material include both innate and acquired defenses. The pulmonary innate immune system involves phagocytic cells with other non-cellular mechanisms. The alveolar macrophage is very important in the homeostasis of the pulmonary immune system with it having phagocytic and secretory functions. The alveolar macrophage is also the most prominent cell in bronchoalveolar lavage fluid representing up to 60% of the cellular population from the lower respiratory tract of the horse (Rush-Moore 1996).

The three primary functions of the alveolar macrophage are intracellular killing, antibody dependent mediated cytotoxicity and antigen processing/presentation to T helper cells. The alveolar macrophage has many mechanisms in which to phagocytise foreign proteins. Sugar receptors like mannosyl-fucosyl receptor (MFR) and N-acetyl glucosamine bind surface sugars on foreign proteins, CD14 (lipopolysaccharide binding protein receptor) coats gram negative bacteria, and 3 distinct Fc receptors for IgG which can trigger extracellular killing and aid in phagocytosis of opsonized bacteria are present (Roitt 1998). The alveolar macrophage is able to generate toxic oxygen species like superoxide anion, hydrogen peroxide and hydroxyl radicals that function to destroy foreign proteins. Additionally, many preformed proteases within the alveolar macrophage can degrade the extracellular matrix of offending bacteria (Liew 1991). Alveolar macrophages also have receptors that aid in cellular adhesion (CR1) and activation (CD23, IgE receptor, IL receptors).

Products of activated alveolar macrophages include complement components (C5a), fibrinolytic fragments, interleukin-8 (IL-8), interleukin-1 (IL-1) and arachidonic acid metabolites like leukotriene B4. These cytokines not only attract other phagocytic cells like polymorphonuclear cells (PMN), but can stimulate lymphocytes too. PMN's are not normally found in human BAL fluid (Gratziou 1991) and comprise less than 5% of cells in horse BAL fluid (Mair 1987, Sweeney 1991). PMN's exert their killing effect by releasing lysosomes, granules that contain acid hydrolases, myeloperoxidases, and muramidase (lysozyme), into their surrounding areas. PMN's also contain lactoferrin, an iron chelator, that keeps this vital nutrient away from multiplying bacteria. Other less specific defense mechanisms used by PMN's to phagocytise and kill include defensins, seprocidins, cathelicidins, and bacterial permeable inducing protein (Roitt 1998).

Eosinophils are another granulocytic cell, much like the PMN, that are attracted by products of the alveolar macrophage. Eosinophils are believed to have an important role in parasitic infestations and allergic disease. Adherence to third stage larvae of Strongylus vulgaris (Klei 1992) has been documented in equine eosinophils. Like the PMN, eosinophils contain cytotoxic proteins that are used to destroy foreign antigen upon stimulation and resultant respiratory burst.

Equine eosinophils contain eosinophil peroxidase (Klebanoff 1989) and four basic proteins with one similar to human basic protein (Piller 1993) that are used in extracellular killing. Foster and coworkers (1998) identified a biologically active alkaline phosphatase that aids in generation of large amounts of superoxide anions used in the respiratory burst killing effect. Interleukin-5, a product of activated T helper 2 cells, attracts eosinophils to sites of inflammation. Degranulation of eosinophils results in damage to surrounding tissue, like respiratory epithelium. This may facilitate antigen entry and increase bronchial inflammation and bronchoconstriction.

Any residual foreign proteins that the innate immune defense mechanisms are not able to completely clear then activates the acquired pulmonary immunity. This system involves B lymphocytes and dendritic cells, which along with macrophages, are able to present processed antigen to T lymphocytes. Antigen processing by the antigen presenting cells (APC) involves cleavage of the antigen into smaller peptide fragments, synthesis of the major histocompatability class II (MHC) in the rough endoplasmic reticulum and binding of the peptide to the MHC class II molecule. The MHC class II – peptide molecule is then expressed on the surface of the cell for interaction with the T helper cells.

Dendritic cells are one of the more effective APC in the lung. Dendritic cells reside mainly in the bronchial walls and alveolar wall (Sertl 1986), although up to 0.4% of cells recovered from bronchoalveolar lavage fluid are dendritic cells (van Haarst 1994). Recently, 2 - 4% of peripheral leukocytes in humans were determined to be dendritic cells (Crawford 1999). Dendritic cells are perfectly situated to encounter and trap foreign antigen because of where they reside. Immature dendritic cells then traffic back to lymph nodes to induce allogenic T cell responses. They are potent producers of IL – 12; a cytokine capable of increasing B cells and Type 1 T cell response.

T cell Organization:

The population of T lymphocytes can be divided into cells expressing either $CD4^+$ or $CD8^+$ surface molecules. CD8+ cells are called cytotoxic T cells and CD4+ cells are called T helper cells. CD4+ T cells are further grouped into Type 1 (Th1) or Type 2 (Th2) cells depending on the cytokines that they elaborate. Th1 cells secrete interleukin – 2 (IL-2), interferon gamma (INFγ), and tumor necrosis factor – beta (TNFβ) while Th2 cells secrete interleukin – 4 (IL-4), IL-5, IL-10, and IL-13. IL-2 and IL-4 stimulate the B cell to enlarge and divide while IL-5 stimulates the B cell to transform into a antibody secreting plasma cell.

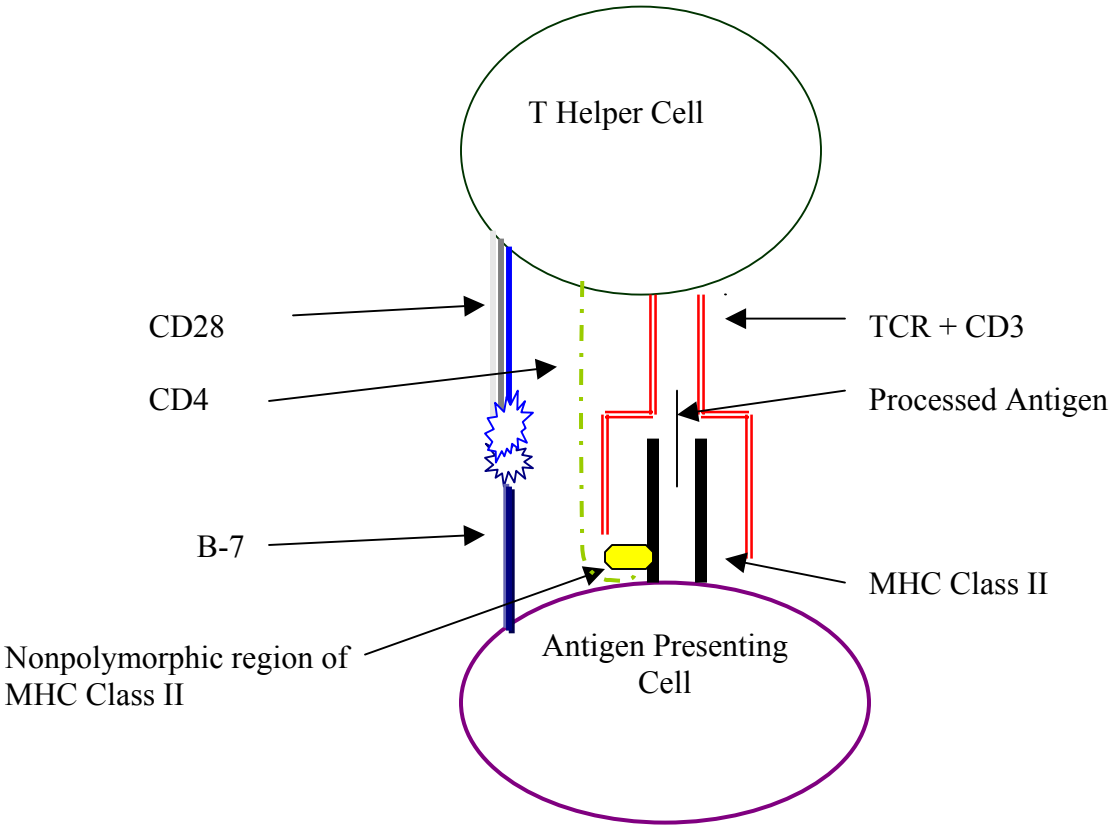
This pool of T helper and T cytotoxic cells can also be divided into naïve, effector and memory subsets. Maintenance of naïve $CD4^+$ and $CD8^+$ T cells depends on low level exposure to MHC class II and MHC class I, respectively (Tanchot 1997, Markiwicz 1998). Maintenance of memory T cells is as yet, unexplained. It has been proposed that memory T cell survival is dependent on cross reactive peptide recognition (Beverley 1990). Maintenance of the total population of T cells and their respective subsets are actively controlled (Tanchot 1997). Activation of effector T cells may cause a huge clonal expansion, followed by massive apoptosis once the need is gone (Maini 1999). The naïve and memory T cell subsets are maintained at a fairly constant size. The active control of these subsets allows diversity among the different pools. If one subset, i.e. memory cells, were to survive better and accumulate resulting in less naïve cells surviving, this could restrict immunologic diversity. Likewise, if naïve cells were to accumulate over memory cells, the immune system may lack versatility to respond to various antigens. This is solved at least in the peripheral pool of cytotoxic T cells by having two compartments of equal size. It is unknown if T helper cells follow this same strategy.

T Helper Cell Activation:

Naïve CD4 T cells continuously migrate through the body and lymphoid organs. Lymphoid organs are where most naïve lymphocytes first encounter antigen (Westermann 1999). Activation of T cells involves coupling of the T cell receptor

(TCR), a heterodimer of two polypeptide chains with foreign antigen. The TCR does not bind free antigen, but rather recognizes foreign antigen along with the major histocompatibility complex (MHC) on an antigen presenting cell (APC). The TCR is closely associated with CD3, a collection of polypeptide chains that are expressed on all T cells and aid in signal transduction. Binding of the CD4 molecules on the T cells to the non-polymorphic region of the MHC on the APC, along with interactions between other costimulatory or adhesion molecules, triggers T cell activation.

Figure 1: T Cell activation and antigen presentation



T Cell Homing:

T cells first encounter antigen in lymphoid tissue like lymph nodes and Peyers patches. Immigration of T cells through the lymphoid tissue involves using high endothelial venules. Activated lymphocytes have been observed to accumulate preferentially in the original tissue where first activation took place (Bode 1997, Westermann 1999). Accumulation of activated T cell in tissues was speculated to be caused by preferential entry, increased proliferation and reduced death, reduced exit rate or a combination of the three. Preferential entry was determined not to be a factor when similar entry rates were observed in two different sources of activated T cells into a lymph node. These same two pools of activated T cells were found to have similar kinetics of movement into and out of lymph nodes negating decreased rate of exit as a possibility. Preferential proliferation and reduced apoptosis was identified as causes of accumulation of activated T cells in the tissue of origin (Bode 1997, Westermann 1999).

B. Regulation of the Pulmonary Immune Response

Apoptosis is a regulated and physiologic event. Apoptosis naturally occurs everyday in embryonic development, new tissue growth, hormone dependent involution, and immune system development. It is seen in varied amounts in many pathologic conditions like AIDS, Alzheimers, autoimmune disease, and cancer (Allen 1997).

Apoptosis is a complex event in cells that is triggered by internal mechanisms. Initiation of apoptosis results in redistribution of cell membrane phospholipids, cell shrinkage, endonuclease activation and DNA fragmentation (Squier 1995). Very early in apoptosis, plasma membrane changes occur that identify the apoptotic cell to phagocytes. The translocation of the phospholipid, phosphatidylserine, from the inner leaflet of the plasma membrane to the membrane surface is one such early recognition factor (Fadok 1992). Other cell surface molecules that may aid in early recognition have been identified

(Haslet 1994, Saville 1989,1990). Possibly concurrently or immediately after plasma membrane changes, DNA fragmentation begins. Initial cleavage of DNA into 50 to 300 kilobase pairs and then internucleosomal cleavage with formation of fragments containing 180 – 200 base pairs occurs (Oberhammer, 1993).

Morphologically, the earliest changes that can be visualized is nuclear chromatin condensation to form dark, crescentric masses (Wyllie, 1987). Concurrent cell shrinkage due to inhibition of the Na^{2+}–K^{1+}- Cl^{1-} cotransport system and net external fluid movement begins (Wilcock 1988). Blebbing of both nuclear and plasma membranes develops due to cell shrinkage. Nuclear fragments within cytoplasmic fragments are produced by the extensive shrinkage and blebbing of membranes and are termed 'apoptotic bodies'. Crosslinkage of cytoplasmic proteins by tissue transglutaminase (tTG) (Fesus 1991) helps to keep intracellular contents within the cell or apoptotic body thereby eliminating an inflammatory response due to spillage of proinflammatory mediators from the dying cell.

Apoptosis differs from other processes like oncosis and necrosis. Oncosis refers to initial swelling of the cell after death has occurred. Necrosis is the after effect of oncosis, occurring long after cell death. Necrosis is characterized by the many cells involved, cellular swelling, karyolysis, disrupted cytoplasmic organelles, blebbing and loss of cell membrane integrity resulting in inflammation. With the loss of the cell membrane, many pro-inflammatory mediators are released into the local and systemic environment. This in turn promotes cellular influx and possible amplification of the inflammation. Unlike necrosis, the shrinkage of the cell in the apoptosis process results in retention of the pro-inflammatory mediators. Apoptosis, therefore, terminates an inflammatory response.

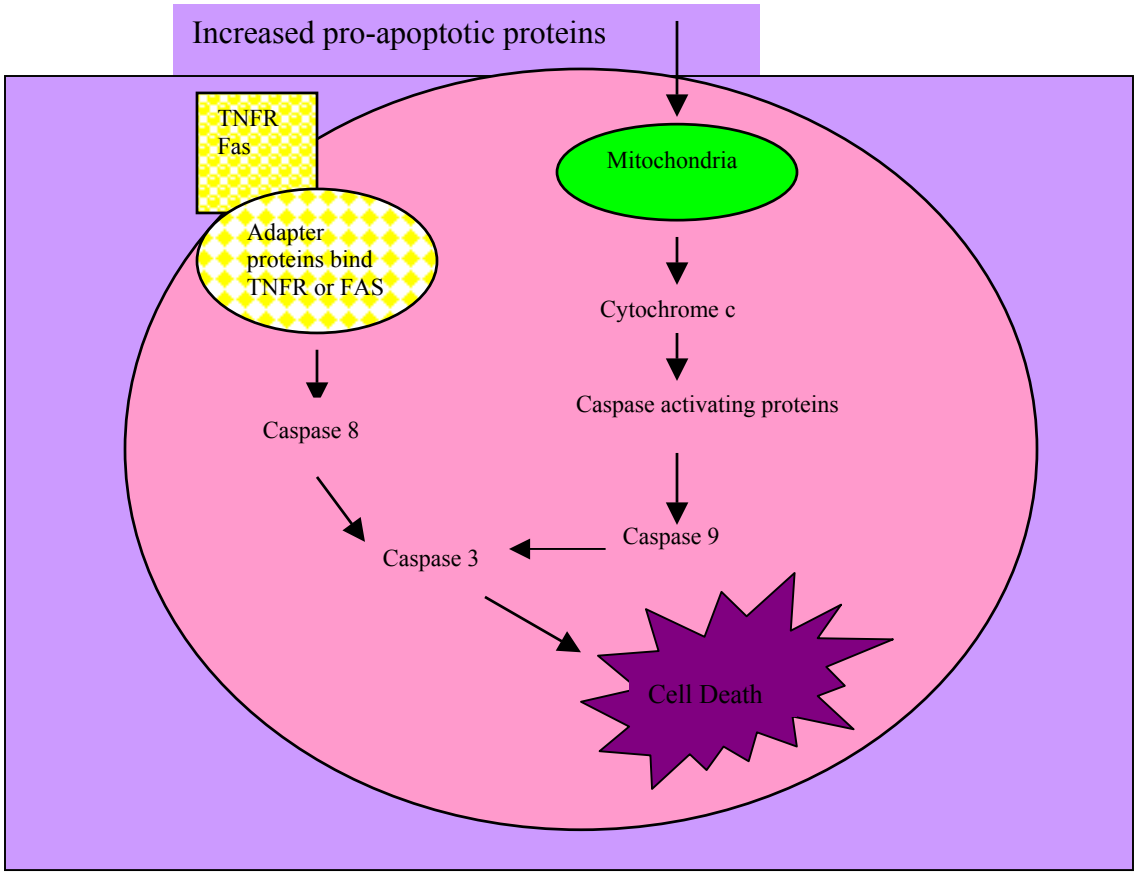
Apoptotic Signals:

The body uses many mechanisms to terminate an immune response. Tolerance is a mechanism where the body shows immunologic unresponsiveness to specific foreign proteins. Tolerance is induced by prior exposure to an antigen and is very important in areas like the gastrointestinal and respiratory tracts that continually encounter innocuous

antigens. It is also the mechanism that the body uses so that our own immune system does not destroy our body. Anergy, a result of lack of co-stimulatory signals, can occur with persistent tolerance in T helper cells. Apoptosis, another mechanism, results in cell suicide or a programmed cell death.

Apoptosis can result from an intrinsic and extrinsic pathway. The intrinsic pathway involves the release of cytochrome c from the mitochondria of the cell that is induced from various signals like elevations of pro-apoptotic proteins in the Bcl-2 family. Cytochrome c then stimulates a cascade that eventually induces caspase-9 activation and results in apoptosis. The extrinsic pathway can be induced by members of the tumor necrosis factor family receptors like Fas. These proteins then recruit other effector proteins which results in activation of caspase–8 and apoptosis through a final common pathway.

Figure 2: Extrinsic and Intrinsic Pathways for Apoptosis



Fas ligand (FasL) is a type II membrane protein in the tumor necrosis factor (TNF) family (Suda 1993, Nagata 1995) and is expressed predominately on activated T cells. Binding of FasL to its receptor, Fas, begins the process of apoptosis. The TNF family includes lymphotoxin, CD30 ligand, CD40 ligand, CD27 ligand, and TNF-related apoptosis-inducing ligand. FasL has its N-terminus in the cytoplasm while its C terminus extends into the extracellular space. This extracellular region consists of 150 amino acids that is well conserved among the TNF family members. The sequence and length of the cytoplasmic regions are significantly varied. Soluble TNF is produced by proteolysis of membrane associated TNF mediated by a metalloproteinase (Gearing 1994). Tanaka and coworkers (1996) found that membrane associated FasL goes through a similar proteolysis to become soluble. The soluble form of human FasL, but not murine FasL, is functional. FasL exists as a tertiary structure consisting of B-pleated sheets swirled in concentric circles similar to TNF (Jones 1989).

The cell surface molecule Fas (also known as APO1 or CD95) is a member of the tumor necrosis factor (TNF) receptor family and a type I membrane protein. Members of the TNF receptor family include two TNF receptors (TNF1, TNF2) lymphotoxin-B receptor, NGF receptor, CD40, CD27, CD30 (Nagata 1995), death receptor –3/Ws1 (Chinnaiyan 1996), human herpes virus early mediator (Montgomery 1996), and chicken cytopathic avian leucosis-sarcoma virus receptor (Brojatch 1996). Two to six repeats of a well-conserved cyteine-rich subdomain are found in the extracellular region of all the TNF receptor family members while the cytoplasmic regions have little similarity (Nagata 1997).

Both TNF1 and TNF2 receptors can transduce the apoptosis signal, although TNF1 is thought to do more (Vandenabeele 1995). Activation and costimulatory signals occur due to other receptors in this family, namely CD40 and CD30. Similarities between TNF1 and Fas have brought some to speculate that this area is responsible for apoptosis (death) signal transduction. This was verified in 1993 by two different research groups Itoh and Nagata, and Tartaglia and coworkers.

The first signal develops from ligation of Fas by FasL inducing conformational changes to the Fas receptor (Banner 1993). Recruitment and binding of caspase-8 to specific death domains in the Fas/FasL coupling can cause activation of additional protease domains. Cysteine proteases in the family of interleukin-1β converting enzymes (ICE) are activated by proteolytic cleavage from precursors during the ligation of Fas by FasL and induce some of the morphological changes seen in the cell. Homologues of ICE were identified and found to cause apoptosis also if expressed in high amounts (Alnemri 1996). These were determined to be cysteine proteases that are called caspases (cysteine aspases). Inhibitors of caspase-1 and -3 have been found to block Fas- and TNF- induced apoptosis suggesting that both are intimately involved in the process of apoptosis (Enari 1995, Enari 1996, Los 1995, Tewari 1995,). Increases in ceramide are also seen during fas mediated apoptosis. Ceramide itself can cause apoptosis. (Speigel 1996). Activation of ceramide likely occurs downstream of the ICE cascade, since ceramide is not found after ICE inhibitors are used (Gamen 1996).

Fas is expressed throughout the human body predominately in the thymus including lymphocytes, liver, heart, and kidney. Ligation of Fas induces apoptosis within hours. Expression of Fas and FasL in human airway epithelium, FasL in murine airway epithelium and in murine Clara cells is believed to modulate inflammation within the lung (Hamman 1998, Gochuico 1998). The close contact between inflammatory cells and respiratory epithelium allows for interactions and modulation of inflammation. Mice deficient in FasL exhibit prominent inflammatory reactions in the submucosa and peribronchial regions of the upper and lower airways (Gochuico 1998). Induction of excessive airway inflammation by lack of modulating surface proteins (Fas and FasL) in mice or the failure to resolve antigen driven immune responses in human atopic asthmatics may be directly related to dysfunctional Fas/FasL pathways. Abnormalities in Fas and/or FasL expression, and therefore apoptotic pathways, in the COPD horse may be one reason that affected horses are not able to develop tolerance to common barn allergens.

C. Immune Responses in Allergic Airway Diseases: COPD and Asthma

Chronic obstructive pulmonary disease (COPD) is an immune mediated allergic syndrome with a worldwide distribution that affects mature horses. The disease has been known by many similar names such as heaves, recurrent airway obstruction (Derkson 1985), chronic bronchiolitis, small airway disease and chronic bronchitis. The distribution of animals affected with COPD is predominately in the Northern Hemisphere where horses are routinely housed indoors for a portion of the year. Reports of the incidence of COPD has ranged from 12-54% (Larson 1985, Bracker 1991), but in the later report, the diagnostic criteria used could have included other inflammatory conditions of the equine lung. The syndrome has been defined as "a process characterized by chronic bronchiolitis… that may lead to the development of airway obstruction". The degree of obstruction may vary with the stage of the disease and is reversible either by medication or change of environment. Just stabling susceptible horses can exacerbate acute episodes of COPD. Hypersensitivity to common molds *Microsporum faeni* and *Aspergillus fumigatus* have been incriminated (McGorum 1993) as possible causes along with 3-Methylindole (Gerber 1973), previous infection with Equine Influenza virus (Thorsen 1983) and genetics (Marti 1991).

Hallmark signs of COPD include acute to chronic coughing, increased respiratory effort and rate, tracheal rattle, wheezes, crackles and exercise intolerance. Cytologic evaluation of tracheal aspirates from COPD affected horses with clinical disease show a higher proportion of well preserved segmented neutrophils than normal, non-affected horses (Rush-Moore 1995). Bronchoalveolar lavage fluid (BALF) obtained from asymptomatic horses contained mostly CD8+ T cells with lower proportions of CD4+ T cells and higher proportion of B cells. After natural challenge, recruitment of CD4+ T cells into the tracheobronchial tree predominates and decreases the CD4+/CD8+ ratio (McGorum 1993b). This is similar to findings in atopic asthmatics where there is a strong pulmonary recruitment of CD4+ T cells.

Human asthma is also characterized by episodic coughing, wheezing, chest tightness, or a combination of these signs (McFaddon 1992). Exascerbation of asthma can be provoked by inducers like allergens, viral infections and occupational agents which serve to enhance any

underlying inflammation. Additionally, exercise, irritants, emotions, and aspirin can promote bronchospasm (Brusse 1998). Bronchoalveolar lavage of asthmatic patients six hours after local end-bronchial single allergen challenge documented a decrease in overall T cell percentages and an increase in eosinophil percentages although absolute numbers showed a large standard error. These T cells recovered by BAL expressed a significant decrease in expression of adhesion molecules suggesting that there is a selective retention of T cells within the airway mucosa. No differences from saline challenge were seen in macrophage and neutrophil numbers (Gratziou 1996). Bronchial biopsies performed 6 hours after inhalation challenge have revealed an increased peribronchial recruitment of $CD4^+$ T lymphocytes (Montefort 1994). Yet another study looking at very early (10-15 minute) changes after local single allergen challenge reflecting an early allergic response, detected a marked decrease in $CD4^+$ T cells with moderate decreases in $CD8^+$ T cells. No change in the eosinophil numbers were detected (Gratziou 1992). Borgonovo and coworkers (1997) looked at allergen specific, peripheral $CD4^+$ T cell proliferation 10 minutes and 24 hours after inhalation allergen challenge. Allergen specific T cell clones were observed to diminish peripherally and expand in the lower respiratory tract. It appears that very early after allergen challenge there is an initial decrease in all T cells, but the late phase response is characterized by increased peribronchial $CD4^+$ cells. These studies suggest a role for T lymphocytes in allergen-induced inflammatory responses in the lung and that antigen specific T cell recruitment in asthma may contribute to the maintenance of a pathologic response.

Similarities between human atopic asthmatics and equine COPD have been recognized. Human asthmatics possess the same pulmonary recruitment of $CD4^+$ T lymphocytes in bronchoalveolar lavage fluid as COPD affected horses (Spinozzi 1998, Robinson 1993, McGorum 1993). Specific T helper 2 (Th2) subset of T lymphocytes accumulate within the peribronchial tissue of the lungs of asthmatics (Ricci 1994). These cells are IL-4 positive, interferon gamma negative (IFN-γ^-) CD4+ T lymphocytes that regulate IgE production. T cell derived IL-4 regulates IgE production and helps T helper precursor cells differentiate into Th2 cells. IFN-γ is one of many inhibitory signals for differentiation into Th2 cells. IgE production is also elevated in not only asymptomatic COPD horses, but in symptomatic animals (Halliwell 1993). This is consistent with the

shift from more $CD8^+$ T cells predominating in normal horses, to an increase in the proportion of $CD4^+$ T cells in COPD affected horses.

Th2 derived IL-4 and IL-5 have many important physiologic mechanisms that promote bronchial hyper-reactivity. IL-4 promotes mast cell proliferation, anti-immunoglobulin activated B cell proliferation along with T cell proliferation while IL-5 promotes B cell maturation into plasma cells and eosinophil maturation and activation. In bronchial biopsies and BAL fluid from normal and asthmatic patients, increased IL-4 and IL-5 mRNA was observed (Humbert 1996, Robinson 1993). Elevated levels have also been observed in BAL fluid and peripheral blood from asthmatic patients (Walker 1992, Del Prete 1993, Robinson 1992). Soluble IL-4 receptor administered to a murine asthma model significantly reduced the late phase inflammatory response of airway eosinophil infiltration, VCAM-1 expression, and mucus secretion (Henderson 2000). Additionally, administration of interleukin 12 (IL-12) and 18 (IL-18) before antigen challenge has been shown to inhibit airway hyper-responsiveness, bronchial inflammation, Th2 cytokine expression, serum IgE levels, and eosinophilia in mice (Hofstra 1998, Gavett 1995, Keane-Myers 1998). The use of IL-12 and –18 in inhibiting airway hyper-responsiveness suggests that it may in part be due to over stimulation of a Th2 response since both of these interleukins promote a Th1 response. Likewise, the use of soluble IL-4 receptors for controlling asthma symptoms distinguishes the Th2 response as well. Although specific subsets of $CD4^+$ T lymphocytes have not been determined in COPD horses, the possibility that they exist is good. The role of T cells, specifically Th2 cells, in the immunopathogenesis of asthma and COPD needs further exploration.

Likewise, the role of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) have been found to have important roles in mediating allergic pulmonary inflammation and airway hyper-responsiveness (Mark 2000). Efficient T cell activation requires antigen specific engagement of the TCR plus costimulatory molecules CD28 and cytotoxic T lymphocyte antigen – four (CTLA-4). These costimulatory molecules are predominately expressed on T cells and bind their receptors B7-1 or B7-2 on the APC (Green 2000). Interruption

of this pathway has been documented to produce anergy in T cells and thus block activation (Harding 1992, Lenschow 1992).

This activation pathway is also thought to play a role in T helper cell differentiation, although results have not been able to definitively determine whether B7-1 or B7-2 preferentially promote a Th1 or a Th2 response. In a study of in-vitro stimulated memory phenotype (CD45RA+) $CD4^+$ T cells, B7-2 induced an increase in IL-4 while B7-1 did not, thereby promoting a more Th2 phenotype (Freeman 1995). Other studies looking at diseases that are characterized as more of a Th1 disease (experimental allergic encephalitis, autoimmune diabetes) (Kuchroo 1995, Racke 1995, Lenschow 1995) have found conflicting results when using anti-B7-1 antibiodies, suggesting that there is more to discover before the exact role of these costimulatory molecules in Th1 and Th2 diseases is fully understood.

D. Corticosteroids and Attenuation of the Immune Response

Resolution of clinical signs of COPD can occur with environmental changes or immunomodulatory therapies like corticosteroids. Glucocorticoids like dexamethasone are a potent inhibitor of the arachidonic acid pathway thereby inhibiting the release of eicosanoids including interleukins, prostaglandins and several growth factors. Glucocorticoids readily cross the plasma membrane of cells. Induction of apoptosis in thymocytes and lymphocytes involves the binding of the glucocorticoid receptor with the steroid. Induction of lipomodulin by steroid receptor binding inhibits phospholipase A_2 (Cohn 1991). Phospholipase A_2 is able to convert cell membrane phospholipid into arachadonic acid and thereby promote inflammation. The absence of, deficiency of, or malfunction of this resceptor can abolish glucocorticoid induced apotosis (Harmon 1981, Rosenau 1972, Sibley 1974, Gehring 1974).

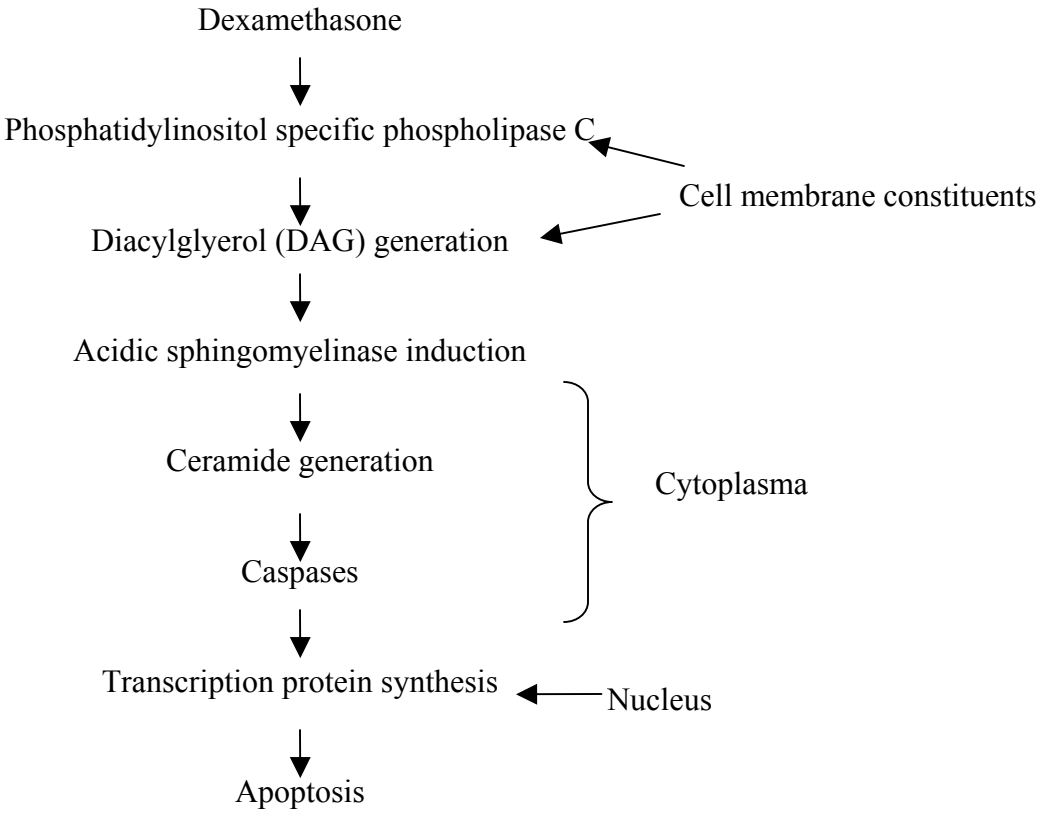
The mechanism that glucocorticoids like dexamethasone use to induce apoptosis has been investigated since the early 1980's. Glucocorticoid induced apoptosis was shown to require protein synthesis when actinomycin and cyclohexamide, RNA and protein synthesis blockers respectively, prevented steroid induced apoptosis in thymocytes (Wyllie 1984, Cohen 1984). Dexamethasone induced apoptosis is dependent on transcriptional regulation of specific cell death genes (Dieken 1992). Thirteen genes expressed specifically in apoptotic immune cells have been characterized (Harringan 1989, Baughman 1992); the majority show an increase in mRNA shortly (30 minutes to 1 hour) after dexamethasone exposure. Of these 13 genes, 2 have been found to repress apoptosis while 11 have been found to induce it (Baughman 1992).

Many researchers have investigated the induction of apoptosis by glucocorticoids and have battled with the complexity of it. Recently, a more complete understanding of dexamethasone induced lymphocyte apoptosis has been identified. Dexamethasone rapidly increases the generation of ceramide through a series of steps involving phosphatidylinositol-specific phospholipase C and acidic sphingomyelinase. This leads to caspase activation and eventual apoptosis of the cell (Cifone 1999). Interestingly, tumor necrosis factor-alpha and Fas induce rapid ceramide generation while promoting an apoptotic response.

Clinically, dexamethasone has been found to inhibit constitutive MHC class II expression on lymphocytes and IFN -γ inducible MHC class II expression on airway epithelial cells (Schweibert 1995). MHC class II cell surface molecules are expressed on all equine circulating T lymphocytes (Lunn 1993) and predominately on activated T lymphocytes in humans (Miyawaki 1992). Equine MHC class II cell surface molecules could not only differentiate memory from naïve T lymphocytes, but also be used to assess treatment efficacy. Induction of leukocyte apoptosis and phenotypic changes in pulmonary T cells by dexamethasone may therefore be responsible for resolution of the inflammatory response. Additionally, preferential apoptosis of human eosinophils with inhibition of neutrophil apoptosis when co-cultured has been identified (Meagher 1996). Dendritic cells when exposed to dexamethasone, do not undergo apoptosis, but have a decreased

antigen presenting capacity and therefore reduced ability to promote T cell proliferation (Matasic 1999). This illustrates the complex nature of dexamethasone-induced resolution of pulmonary inflammation.

Figure 3: Sequential signaling events in dexamethasone induced apoptosis



E. Equine COPD and Human Asthma:

The association between asthma, allergy and the immune system of affected individuals is not well understood. Asthmatic individuals display continued amplification of inflammatory cytokines from activated T helper cells. Research efforts currently are evaluating the role that programmed cell death or apoptosis plays in termination of the pulmonary inflammatory response. Additionally, why some individuals develop asthma while others do not may in fact be a direct result of failure in immune modulation of the inflammatory reaction incurred by these conditions.

Good animal models of human asthma have not been developed because spontaneous attacks of true asthma-like symptoms appear to be rare in animal species other than humans. Equine COPD represents an exception to this statement, and it is the only known naturally occurring disease in animals to fulfill the 1962 criteria of the American Thoracic Society definition of human asthma (Snapper 1986). Equine heaves occurs in response to exposure to specific allergens and resolves spontaneously when allergen exposure ceases (Rush, 1998). Human asthmatics and horses with COPD both experience bronchoconstriction, hypersecretion, and restricted airflow as clinical signs of disease. Activated T lymphocyte percentages from human asthmatic and equine COPD bronchoalveolar lavage (BAL) fluid increases with exposure to allergens (Owen-Shaub 1992, Spinnozi 1996, McGorum 1993). Furthermore, human asthmatics and COPD affected horses possess the same pulmonary recruitment of $CD4^+$ T lymphocytes in BAL fluid (McGorum 1993, Spinnozi 1998). These findings suggest that the cellular etiology of disease may be similar for both humans and horses.

Human atopic asthmatics show defective expression of Fas mRNA and Fas on pulmonary T lymphocytes (Spinnozzi 1998). T cells from asthmatics exposed to anti-Fas monoclonal antibodies (stimulating Fas/FasL activation) fail to die whereas over 50% of exposed T cells from normal humans undergo apoptosis. Continued activation is not due to a genetic deficiency in Fas expression on circulating T cells and lack of activation. Rather, asthmatic's peripheral blood T cells have greater surface expression of activation

markers CD25, HLA-DR, and CD45RO. This increase in activation marker expression is dependent on interleukins secreted by activated T cells and is reflective of activated memory cells. Flow cytometric analysis of asthmatics' pulmonary T lymphocytes reveals lack of Fas expression, whereas in the same patients, the percentage of peripheral T cells expressing Fas is similar to normal controls. The defective expression is therefore restricted to pulmonary T cells. COPD affected horses possess the same pulmonary cellular infiltrate of $CD4^+$ T lymphocytes as symptomatic atopic asthmatics. Therefore, apoptosis contributes to resolution of antigen induced airway inflammation in humans and mice and may be one mechanism by which lymphocytes can be programmed to tolerate nonpathogenic antigens.

The results of current research suggests that apoptosis plays a significant regulatory role in humoral and cellular immunity. For example, the response of activated B and T lymphocytes has been shown to be down regulated by the process of apoptosis (Nakayama 1995, Zhou 1998). Mice, in which lymphocyte apoptotic signaling has been incapacitated by mutation, develop autoimmune disease within the first year of life (Hagimoto 1997). Apoptosis also contributes to resolution of antigen induced airway inflammation in humans and mice (Hamman 1998, Goichico 1998). These findings suggest that apoptosis plays a role in allowing animals to develop tolerance to nonpathogenic antigens in their environment.

Chapter 2: Adaptation of Three Methods of Measuring Bronchoalveolar Lymphocyte Apoptosis in COPD and Normal Horses

Studies in humans and mice suggest that lymphocyte apoptosis serves to regulate pulmonary inflammation. The role of apoptosis in equine chronic obstructive pulmonary disease (COPD), a disease clinically similar to human asthma, has not been determined due to lack of applicable methods. Many techniques are available to detect apoptosis. Depending on what tissue or cell worked with, user familiarity with the techniques, and stage of apoptosis wished to be identified, can govern which techniques are employed. Currently, it is advised to use more than one method to identify apoptosis due to the inherent strengths and weaknesses of every technique. In this study, we chose three staining techniques, Vindelov's propidium iodide with Triton-X (PI/Triton-X), 7-aminoactinomycin D (7-AAD), and Annexin V with propidium iodide (Annexin/PI), that have already shown adaptability to other species including humans, mice, birds, and fish. These methods are user friendly, uncomplicated and readily adaptable to equine bronchoalveolar lymphocytes.

1). The first goal of this study was to adapt three different apoptotic detection methods, Annexin-V/Propidium Iodide, 7-aminoactinomycin-D, and Propidium Iodide with Triton-X, in equine bronchoalveolar cells.

2. The second goal was to compare and evaluate apoptotic staining relationships in all three methods.

Materials and Methods:

Animals:

Nine mixed breed horses ranging in age from 7-20 years (3 mares and 6 geldings) were used in the study. Five of the horses were affected with COPD and 4 were used as normal controls. Normal horses were chosen from the university herd and did not have a history of COPD. COPD horses at the time of donation to the university herd had a history of COPD so a complete respiratory evaluation including transtracheal wash cytology and culture, complete blood count, and thoracic radiographs were performed to confirm the diagnosis. All animals were maintained on pasture for at least 2 months prior to the beginning of the study. Each horse was dewormed and vaccinated against influenza and rhinopneumonitis two weeks prior to the first sample collection.

Housing and Treatment:

Each horse was sampled for baseline values while still on pasture and in clinical remission. Four days later, all horses were brought into stalls, bedded on straw and exposed to moldy hay. Stalls were cleaned once per day and each horse had ad libitum access to fresh water. All horses were maintained in this environment for four days, and then sampled again.

Bronchoalveolar Lavage Sample Collection:

Horses were restrained with a twitch and sedated by intravenous administration of acepromazine[1] (0.02 mg/kg), butorphenol tartate[2] (0.01 mg/kg) and xylazine hydrochloride[3] (0.05 mg/kg). Samples were obtained using a BAL tube[4] inserted into either the left or right main stem bronchi. The BAL tube was wedged into a caudal lobe and three-100 ml aliquots of phosphate buffered saline were infused and aspirated. Total volume of recovered saline and total cell numbers were recorded.

[1] Promace Fort Dodge, Fort Dodge, IA
[2] Torbugesic Fort Dodge, Fort Dodge, IA
[3] VedCo, St. Joseph, MO
[4] Bivona Gary, IN

Murine Thymocyte Collection and Processing:

Thymus's were removed from mice after CO_2 asphyxiation and washed twice in RPMI Media 1640. Thymocytes were separated through a 60 gauge wire screen and washed again in RPMI complete media[5] (with 10% fetal calf serum). Cells were spun down at 600 x g for 6 minutes, reconstituted in 10 ml complete RPMI media 1640 and counted. Cells were then brought up to a final concentration of 2×10^6. Thymocytes were incubated at either 4°C, 37°C, or 37°C with 4 μg/ml dexamethasone[6] added for 24 hours. Dexamethasone is known to induce apoptosis in murine thymocytes (Willie 1980, Migliorati 1992) and human peripheral blood lymphocytes (Dieken 1992, Migita 1997). Cells were then washed and prepared for staining with the apoptotic probes.

Quantifying Apoptosis in BAL and Lymphocytes:

Cells were washed twice in Ca^{++}, Mg^{++} free phosphate buffered saline[7] (CMF-PBS), counted and resuspended to a final concentration of 2×10^6 cells/ml. Aliquots of cells (200,000 cells) were cultured in complete RPMI media with 10% fetal calf serum added, for 30 minutes to decrease alveolar macrophage numbers, and 12 hours and 24 hours to evaluate apoptosis over time. Cells were then removed from culture, washed in CMF-PBS and immediately stained with three apoptotic markers described below. Cell samples were analyzed on a Coulter Epic XL flow cytometer[8]. Forward and side scatter characteristics of a purified population of equine peripheral blood lymphocytes, which were treated identically to the BAL samples, were used to define the region containing BAL lymphocytes. A minimum of 5000 events within this region were analyzed to determine the percentage of apoptotic cells in each BAL sample.

Method 1: Propidium Iodide (PI):

DNA fragmentation in individual lymphocytes will be demonstrated by detecting hypodiploid DNA content by propidium iodide[9] staining, using a modification of a model described by Nicoletti in 1991. Briefly, BAL cells were washed 2 times in CMF-PBS with centrifugation at 400 x g between washes. After the second wash, 2×10^5 BAL cells are aliquoted into 12 x 75

[5] Gibco BRL, Grand Island, NY
[6] Phoenix Pharmaceuticals St. Joeseph, MO
[7] Gibco BRL Grand Island, NY
[8] XL software ver 2.0, Hialeah FL
[9] PI; Sigma St. Louis, MO

mm polypropylene tubes and 200 µl of PI/Triton-X solution (1% Na Citrate, 0.01% Triton X-100, 0.1% PI qs to 50 ml diH_20), at 100 mg/ml concentration in CMF-PBS, were added to the cells.

Method 2: 7-Aminoactinomycin (7-AAD):
Identification of DNA fragmentation occurring in either intermediate or late apoptosis was demonstrated with vital dye 7-AAD[10]. The cells were prepared in the following manner using a modification of a model described by Schmid in 1994. A 100 µl aliquot containing 2 x 10^5 cells/100 µl was collected into 12 x 75 mm polypropylene tubes and washed in cold PBS at 400 x g for 5 minutes. Cells were incubated with 1.0 µg/100 µl of 7-AAD in PBS on ice in the dark for 15 minutes.

Method 3:Annexin V
Identification of early apoptotic cells via binding of the exposed phospholipid phosphatidylserine was demonstrated with Annexin V-FITC[11]. Double color analysis with vital dye PI allowed identification of plasma membrane integrity and early apoptotic versus dead cells. Briefly, washed cells were resuspended in 100 µl of kit binding buffer as per the manufacturer's protocol.[12] To each tube, 50 µl of Annexin V-FITC-binding buffer (0.5 µg/ml) and 50 µl PI (100µg/ml) was added and incubated in the dark for 15 minutes.

Statistical Evaluation:
The CORR procedure of the SAS system[13] was used to calculate Spearman partial correlation coefficients between each apoptotic staining method in equine BAL cells after partialing out day (baseline, after natural challenge) and disease status (COPD or normal). The CORR procedure of the SAS system was used to calculate Pearson correlation coefficients between each apoptotic staining method in murine thymocytes.

[10] Molecular Probes Eugene, OR
[11] Annexin V kit, PharMingen International San Diego, CA
[12] PharMingin International, Annexin V – FITC Technical Data Sheet, Catalog #65874H
[13] Sas System, version 8.01 Sas Institute Inc. Cary, NC 27513

Association between percentages of murine thymocytes stained with each method were subjected to and tested for significance. Pearson's correlation coefficients were calculated using the CORR procedure of SAS system.

Results:

Significant correlation was found between all staining procedures done with a homogeneous population of murine thymocytes (PI/Triton-X and Annexin/PI $r = 0.735$, $p<0.003$; PI/Triton-X and 7-AAD $r = 0.873$, $p<0.005$; Annexin/PI and 7-AAD $r = 0.952$, $p<0.0003$). Likewise, a significant linear relationship was identified between 7-AAD late and Annexin/PI late apoptotic staining at time 4 hours ($r = 0.69$, $p < 0.002$), time 12 hours ($r = 0.53$, $p < 0.03$) and time 24 hours ($r = 0.63$, $p < 0.007$) post BAL (Figure1). Annexin/PI late staining and 7-AAD early staining were significantly correlated at times 4 ($r = 0.59$, $p < 0.01$) and 24 ($r = 0.76$, $p < 0.0004$), but not at time 12 hours post BAL (Figure 2). Annexin/PI early staining and 7-AAD late staining were strongly correlated at time 12 ($r = 0.63$, $p < 0.007$), but not at times 4 and 24 hours post BAL (Figure 3). No significant staining relationships were identified between Annexin/PI early and 7-AAD early, PI/Triton-X and Annexin/PI and PI/Triton-X and 7-AAD total apoptotic staining. No differences in lymphocyte staining were identified by all three methods in COPD and normal horses at all time points.

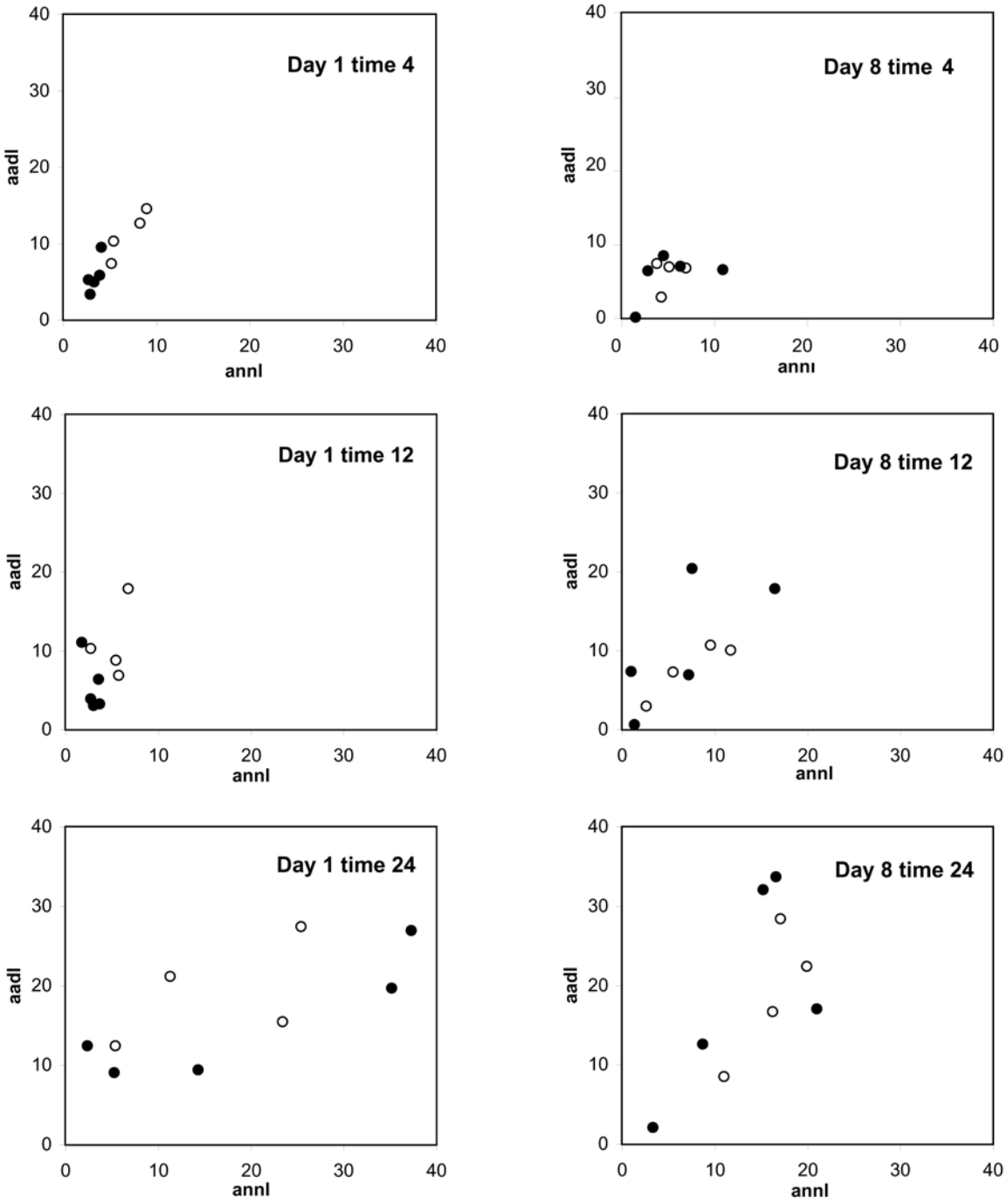


Figure 1: Scatter plots illustrating significant linear relationships at all time points between 7-AAD late (aadl) and Annexin/PI late (annl) apoptotic staining percentages in equine BAL lymphocytes. Solid circles represent COPD affected horses and open circles are control horses. Day 1 represents baseline values with all horses clinically normal. Day 8 represents apoptotic staining after natural challenge with moldy hay for 96 hours.

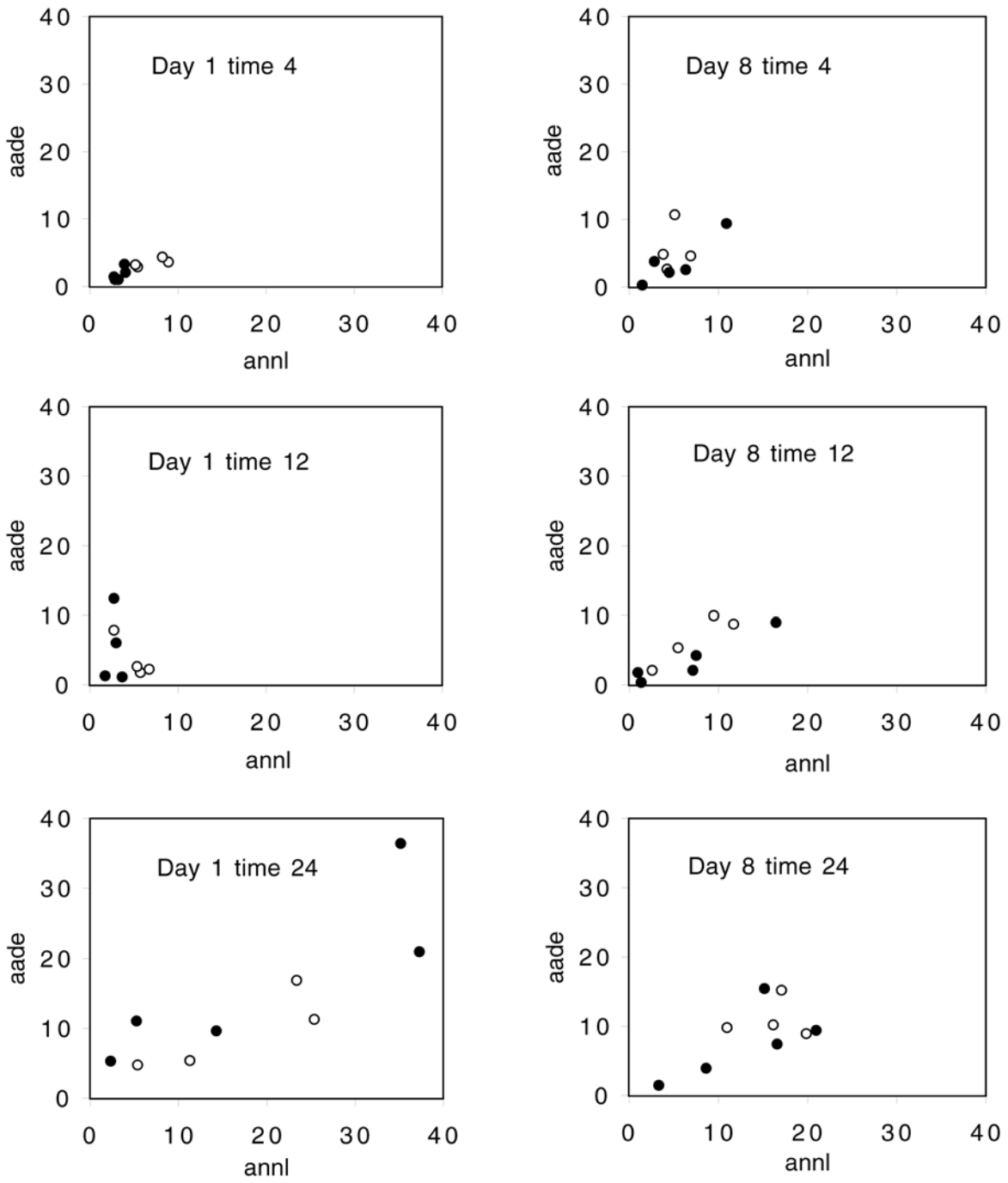


Figure 2: Scatter plots illustrating significant linear relationships between 7-AAD early (aade) and Annexin/PI late (annl) apoptotic staining percentages for time 4 and 24 hours. More variation and a lower correlation is seen at time 12 hours. Solid circles represent COPD affected horses and open circles are control horses. Day 1 represents baseline values with all horses clinically normal. Day 8 represents apoptotic staining after natural challenge with moldy hay for 96 hours.

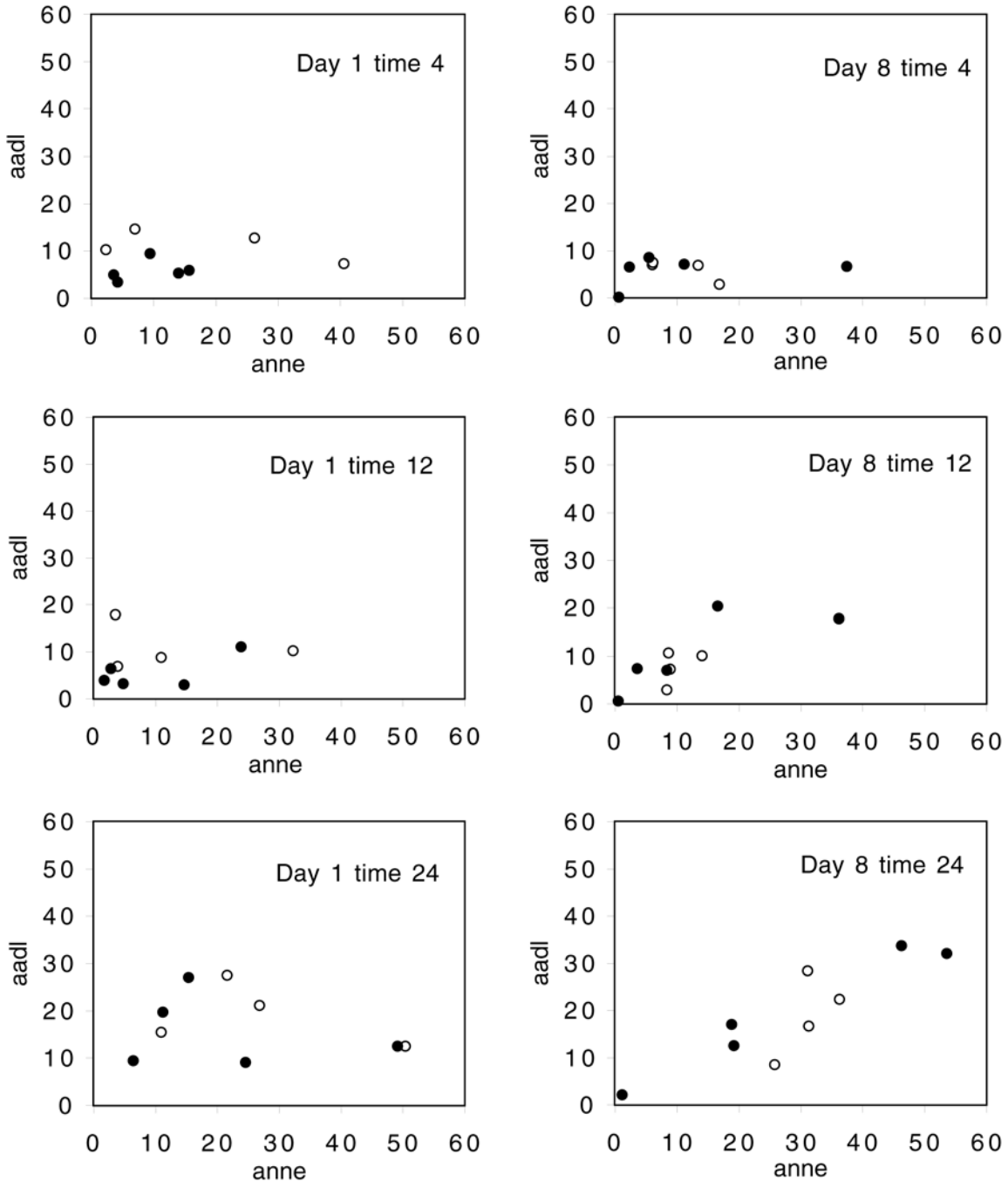


Figure 3: Scatter plots illustrating significant linear relationships between 7-AAD late (aadl) and Annexin/PI early (anne) apoptotic staining percentages for time 12 and 24 hours. Solid circles represent COPD affected horses and open circles are control horses. Day 1 represents baseline values with all horses clinically normal. Day 8 represents apoptotic staining after natural challenge with moldy hay for 96 hours.

Table 1: Spearman's correlation coefficient for each apoptotic staining relationship in equine BAL lymphocytes

	Time in Culture		
Apoptotic Stain	4	12	24
Annexin early x 7-AAD early	0.4	0.23	-0.15
Annexin early x 7-AAD late	0.38	0.63*	0.48*
Annexin late x 7-AAD late	0.69*	0.53*	0.63*
Annexin late x 7-AAD early	0.59*	0.39*	0.76*
PI x Annexin/PI total staining	-0.12	0.23	0.39
PI x 7-AAD total staining	-0.11	0.21	0.39

An * denotes significant ($p < 0.05$) linear relationship between the two stains. Values are the result of 18 observations per stain.

Table 2: Individual staining medians and range of each apoptotic probe for each time point in equine BAL lymphocytes[a] .

	Time in Culture		
Apoptotic Stain	4	12	24
Annexin early	8.2 (0.64 – 40.6)	8.6 (0.56 – 36.2)	25.2 (1.18 – 53.6)
Annexin late	4.4 (1.48 – 10.9)	4.5 (0.98 – 16.5)	15.7 (2.34 – 37.3)
7-AAD early	3.1 (0.30 – 10.7)	3.5 (0.40 – 57.0)	9.7 (1.47 – 36.5)
7-AAD late	6.9 (0.16 – 14.6)	7.3 (0.63 – 20.4)	16.9 (2.09 – 33.7)
Propidium Iodide	2.7 (1.10 – 38.6)	3.3 (1.10 – 24.3)	11.6 (4.48 – 38.6)

[a] Each value is the median of nine horses with COPD and control horses together

Discussion

The first method that was examined was propidium iodine (PI) in a buffer containing 0.01 percentage Triton X. PI is a vital stain that intercalates into DNA, fluoresces in the orange range of the spectrum, and can be detected by flow cytometry using a 562-588 nm band pass filter. Most commonly, this stain is used to detect live verses dead cells; the intact cell membrane of live cells excludes the stain, while the cell membrane of dead cells fails to act as an effective barrier to PI. Since loss of cell membrane integrity occurs only in necrotic and late apoptotic cells, PI staining alone is not considered a sensitive marker of apoptosis. The use of PI in combination with Triton X permits the stain access to DNA at a much earlier time in the apoptotic process. Triton X, acting as a detergent, eliminates the membrane from all of the cells in the population. The size of the exposed DNA may then be determined based on the amount of fluorescence that the particle emits as it passes though a flow cytometer. The larger the DNA fragment, the greater amount of PI contained within that fragment, and the greater the fluorescence. DNA from cells that are alive produces the greatest amount of fluorescence, while DNA from apoptotic cells have a characteristic hypodiploid subG_o peak, and DNA fragments from necrotic cells give off very little fluorescence (Refer to appendix B - E).

One significant advantage of this method is that it is very simple; addition of PI/Triton-X to the cell population is a one step process, limiting time between collection and staining, and minimizing the effects of sample processing. Cell populations stained with PI/Triton-X can also be stored (4°C in the dark) for at least 24 hours without affecting the results. The disadvantage to this method is that removal of the cell membrane eliminates the possibility of using forward and side scatter characteristics to differentiate between cell populations within a sample. As shown this study, the results obtained using the PI/Triton-X method only correlate well with methods when the cell population being evaluated is homogeneous. In heterogeneous populations, like those obtained by BAL, the percent of apoptosis present in the whole population does not consistently correlate well with results obtained using methods that permit evaluation of specific populations, like lymphocytes, within the group.

7-amino-actinomycin (7-AAD), a nucleic acid binding dye, was the second method adapted to measure apoptosis in equine BAL lymphocytes. Like PI, 7-AAD is a vital, fluorescent dye that binds to DNA. 7-AAD fluoresces in the far-red range of the spectrum and can be detected using a 650 nm long-pass filter. Unlike PI, 7-AAD is able to traverse an intact plasma membrane and intercalate in between cytosine and guanine bases in DNA. It is thought that 7-AAD crosses the plasma membrane of apoptotic cells and achieve higher intercellular concentrations than PI (Schmid 1994), but the exact mechanism of membrane transfer of 7-AAD is unknown. Using lower concentrations of 7-AAD (1 μg/ml concentration), as was done in this experiment, minimizes staining and background florescence of live cells. For the purpose of this study, cells with dull fluorescence were considered alive, intermediate fluorescent cells were considered early to mid apoptotic (aade), and bright fluorescent cells were considered late apoptotic (aadl). This categorization has previously been published by Schmidt and coworkes (1994).

7-AAD is a sensitive indicator of DNA fragmentation associated with apoptosis that does not require the removal of the cell membrane to facilitate DNA staining. Since the cell membrane remains intact, forward and side characteristics can be used to differentiate between cell population within a heterogeneous sample. Double staining methods may also be applied by counterstaining with markers labeled with fluorescent dyes that emit light in a wavelength that differs from 7-AAD. 7-AAD staining is also rapid and requires limited manipulation of the cell population. The disadvantage to this method is that cells cannot be stored overnight and analyzed the following day. Methods have been reported for fixing cells in paraformaldehyde to permit analysis later. Results of preliminary experiments performed in this lab, however indicate that twelve hour fixation of cells stained with 7-AAD significantly increases the amount of apoptosis observed in the samples (data not shown).

Annexin-V with PI was the third method that was utilized for this project. Annexin-V is a calcium dependent binding dye used to identify phospholipid changes in the cell membrane. For the purpose of this study, Annexin V was labeled with fluorescein isothiocyanate (FITC), which fluoresces, in the blue-green range of the spectrum.

Staining with only Annexin-V identifies early apoptotic cells with their phosphatylserine molecules exposed. Double staining with PI permits identification of cells that are later in the apoptotic process, since substantial changes in cell membrane integrity must occur before PI can gain access to the DNA. By applying both stains, live cells have minimal fluorescence, early apoptotic cells (anne) stain with Annexin V FITC only, late apoptotic cells (annl) stain both with the Annexin V FITC and PI, and necrotic cells stain with PI only. In this experiment, Annexin/PI total (anntot) was considered the sum of values measured for Annexin early and Annexin late in each sample. For the purpose of this study, the total number of cells undergoing apoptosis was used for comparison with the PI/Triton-X and 7-AAD results, since staining with Annexin/PI was the only method that could distinguish cell changes associated with stages of apoptosis.

While staining with Annexin/PI is not complex, it does require more time and preparation that the first two methods described. If Annexin-V FITC staining is used alone, it may be possible (though not recommended) for cells to be fixed and analyzed the next day. In the absence of PI staining, early and late apoptosis cannot be detected, but the total number of cells undergoing apoptosis can be identified. If double staining with PI is performed, cells cannot be fixed, since paraformaldehyde fixation will result in all of the cells taking up PI stain. If the cells are not fixed, the samples must be analyzed immediately to obtain accurate data.

The results obtained when analyzing murine thymocytes demonstrate that all three methods correlate well when a homogeneous population of cells is being analyzed. This finding is in agreement with previously published apoptotic results in murine thymocytes (Donner 1999). Likewise, studies using homogeneous populations of peripheral lymphocytes in humans (Lecoeur 1997), fish (Gogal 2000), and birds (Gogal, submitted for publication 2001) have identified significant staining relationships between 7-AAD and Annexin-V/PI. In contrast, results do not correlate well when evaluating a heterogeneous cell populations, like equine BAL lymphocytes. Since morphologic characteristics of the cell membrane are lost when using the PI\Triton-X method, specific cells population within the BAL samples can not be analyzed individually. In contrast,

both 7-AAD and Annexin V/PI are methods which preserve the cell membrane characters and permit gating on lymphocytes with the BAL population, based on predetermined forward and side scatter characteristics. It is therefore likely that the percent of lymphocytes undergoing apoptosis in BAL samples differs from the percentage of total cells within the population that are apoptotic. Values obtained from all three methods may more accurately detect lymphocyte apoptosis if steps were taken to isolate BAL lymphocytes from the rest of the cells in BAL samples. Phagocytosis of apoptotic BAL lymphocytes by alveolar macrophages may have erroneously lowered the percentage of apoptosis detected by each method and affected correlation values. However, isolation procedures require additional manipulation of the sample, and may alter apoptotic percentages also. Strong correlation values obtained with 7-AAD late and Annexin/PI late apoptotic staining for all time points measured suggests that these stains are identifying similar apoptotic time points. More variation in correlation values between 7-AAD early and Annexin late, and 7-AAD late and Annexin early staining, suggests that these stains are likely to have some staining overlap of the apoptotic cycle, but that it might not be consistent in a heterogeneous population. Apoptotic staining with 7-AAD has been shown to be altered in the presence of granulocytes and red blood cells (Philpott 1996) which may have altered correlation values in this study. Additionally, at different time points in culture, a greater proportion of cells may be either early or late apoptotic, thereby affecting correlation values when evaluating this type of relationship. Lastly, the involvement of the alveolar macrophage to identify and phagocytose apoptotic cells can not be underestimated. Although culture of BAL cells for 30 minutes reduced alveolar macrophage numbers by approximately 30% (data not shown), complete elimination was not possible in this present study. Based on these finding, 7-AAD and AnnexinV/PI methods are the most comparable methods for evaluating lymphocyte apoptosis in equine BAL lymphocytes, although the PI\Triton-X method is the simplest and most convenient of the three tested. When evaluating apoptosis in a homogeneous population of cells, the PI\Triton-X method should be considered.

Chapter 3: Comparison of Equine Bronchoalveolar Lavage Lymphocyte Apoptosis in Normal and COPD Affected Horses Before and After Dexamethasone Administration

An introduction for this section was provided in Chapter 1.

Aims of Study:

1). The first goal of this study was to compare bronchoalveolar lavage retrieved lymphocyte apoptosis in control and COPD affected horses during remission and after natural challenge.

2). The second goal of this study was to compare bronchoalveolar lavage retrieved lymphocyte apoptosis in control and COPD affected horses after four days of dexamethasone administration.

I. Materials and Methods:

Animals:

Nine mixed breed horses ranging in age from 7-20 years (3 mares and 6 geldings) were used in the study. Five of the horses were affected with COPD and 4 were used as normal controls. Normal horses were chosen from the university herd and did not have a history of COPD. COPD horses at the time of donation to the university herd had a complete respiratory evaluation including transtracheal wash cytology and culture, complete blood count, and thoracic radiographs to diagnose COPD. All animals were maintained on pasture for at least 2 months prior to the beginning of the study. Each horse was dewormed with ivermectin[14] (200μg/kg PO) and vaccinated against influenza and rhinopneumonitis two weeks prior to the first sample collection.

Housing and Treatment:

Each horse was sampled for baseline values while still on pasture and in clinical remission. Four days later, all horses were brought into stalls, bedded on straw and exposed to moldy hay. Stalls were cleaned once per day and each horse had ad libitum access to fresh water. All horses were maintained in this environment for four days, and then sampled again. Horses were then administered dexamethasone sodium phosphate[15] (0.1 mg/kg IV,) for four days, and sampled a final time.

Clinical Disease Assessment:

Horses were deemed to be experiencing an acute exacerbation of COPD if they were exhibiting increased respiratory rate, increased respiratory effort, coughing, and/or if auscultable wheezes were heard. None of the COPD horses were experiencing clinical disease at the start of the study, but all developed varied degrees of clinical severity by Day 8 (4 days after natural challenge with moldy hay). None of the control horses exhibited any of the above clinical signs.

[14] Eqvalan liquid, MSD-Agvet
[15] American Regent Laboratories, Inc. Shirley, NY

Pulmonary Function Evaluation:

Horses were restrained in stocks and methods previously described by Davis in 1998 was used. Determination of gas exchange indices that measure respiratory function including physiologic shunt fraction (Qs/Qt), alveolar dead space ventilation fraction (Vd/Vt), and alveolar to arterial oxygen tension difference ($p_{(A-a)}O_2$) were performed. Sampling procedures for determination of Qs/Qt and Vd/Vt included arterial (carotid artery) and venous (jugular vein) puncture, end-tidal CO_2 ($ETCO_2$) measured from an 18-gauge needle placed percutaneously (with infiltration of a local anesthetic) into the trachea, rectal temperature, and atmospheric barometric pressure. Qs/Qt, Vd/Vt and $p_{(A-a)}O_2$ were calculated using standard formulas (Davis 1998).

Bronchoalveolar Lavage Sample Collection:

Horses were restrained with a twitch and provided sedation by intravenous administration of acepromazine[16] (0.02 mg/kg), butorphenol tartate[17] (0.01 mg/kg) and xylazine hydrochloride[18] (0.05 mg/kg). Samples were obtained using a BAL tube[19] inserted into either the left or right main stem bronchi. The BAL tube was wedged into a caudal lobe and three-100 ml aliquots of phosphate buffered saline were infused and aspirated. Endoscopic evaluation of BAL tube placement precluded repeated sampling of one lung more than once in 96 hours. The cells were kept in phosphate buffered saline and on ice until processed. Cytologic evaluation of a minimum of 300 cells was also performed.

Quantifying Apoptosis in BAL and Lymphocytes:

Cells were washed twice in Ca^{++}, Mg^{++} free phosphate buffered saline[20] (CMF-PBS), counted and resuspended to a final concentration of 2×10^6 cells/ml. Aliquots of cells (200,000 cells) were cultured in complete RPMI media with 10% fetal calf serum added, for 30 minutes, 12 hours and 24 hours on each day. Cells were then removed from culture, washed in CMF-PBS and immediately stained with three apoptotic markers described below. Cell samples were analyzed on a Coulter Epic XL flow cytometer[21]. Forward and side scatter characteristics of a

[16] Promazine Fort Dodge, Fort Dodge, IA
[17] Torbugesic Fort Dodge, Fort Dodge, IA
[18] VedCo, St. Joseph, MO
[19] Bivona Gary, IN
[20] Gibco BRL Grand Island, NY
[21] XL software ver. 2.0, Hialeah FL

purified population of equine peripheral blood lymphocytes, which were treated identically to the BAL samples, were used to define the region containing BAL lymphocytes. A minimum of 5000 events within this region was analyzed to determine the percentage of apoptotic cells in each BAL sample.

Apoptotic Staining Procedures:
Refer to materials and methods Chapter 2.

Statistical Evaluation:
To evaluate effects on lymphocyte apoptotic staining, repeated measures analysis of variance was performed using the MIXED Procedure of the SAS System[22]. The analysis of variance tested for the effects of disease status (COPD, normal), time in culture, day, and their interactions. Tukey's HSD test was performed when significant differences were detected.

To verify development of clinical disease, pulmonary function values were subjected to repeated measures analysis of variance using the MIXED Procedure of the SAS System. The analysis of variance tested for the effects of disease status (COPD, normal), day, and their interactions.

[22] ver. 8.01 SAS Institute Inc. Cary, NC 27513

II. Results:

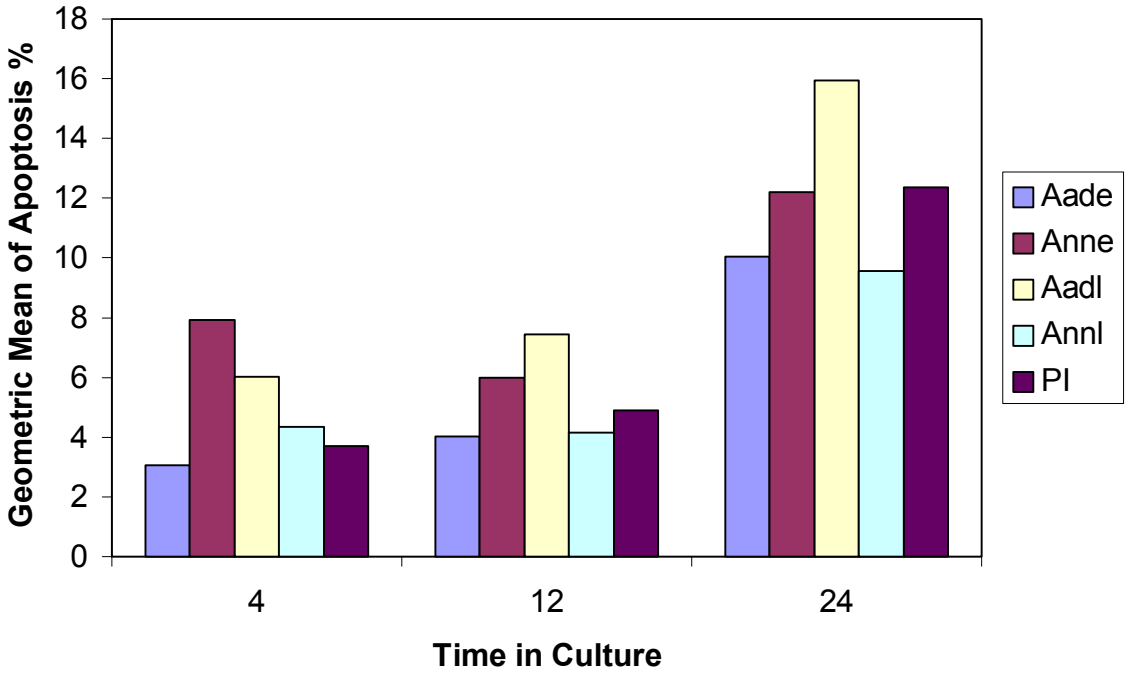
No significant differences were observed between groups (COPD, control) for percentages of apoptotic lymphocytes for all three sampling days with all three apoptotic staining probes. A significant time in culture effect with increased lymphocyte apoptosis identified by all stains at time 24 hours post BAL detected (figure 1). Annexin early (Anne) staining identified a significant time by day effect with decreased early apoptosis seen on Day 12 (after dexamethasone administration) at time 12 and 24 hours post BAL (figure 2). Numerical trends were observed in the COPD group for less apoptosis on Day 8 (after natural challenge) and increases in apoptosis by Day 12 (after dexamethasone administration) more toward initial baseline values (table 3).

Before treatment and natural challenge with moldy hay, mean baseline responses +/- standard error for control and COPD horses on Day 1 were within the normal reported ranges for physiologic shunt fraction (Qs/Qt), alveolar dead space fraction (Vd/Vt), and alveolar to arterial oxygen tension difference ($p_{(A-a)}O_2$). No differences in baseline values were observed between normal and COPD horses on Day 1 for Qs/Qt, although significant differences were seen after natural challenge on Day 8 and after dexamethasone administration on Day 12. Significant differences were observed on all days for Vd/Vt between normal and COPD horses. Additionally, for both groups of horses, significant differences were observed in Vd/Vt between baseline values on Day 1 and after natural challenge on Day 8, and from Day 1 and after dexamethasone administration on Day 12 (Table 1) substantiating the observed clinical signs of airway obstruction in the COPD horses. COPD horses had significantly elevated $p_{(A-a)}O_2$ values above the normal horses for all days although the elevations were above the reported normal range for Days 8 and 12 only. Dexamethasone administration was associated with trends of decreasing Qs/Qt values in both groups of horses with similar trends in Vd/Vt values in the COPD group.

Bronchoalveolar lavage retrieved macrophage percentages were significantly lower in the COPD horses than conrol horses at all days. COPD horses also had significantly less observed macrophages on Days 8 after natural challenge and 12 after dexamethasone administration when

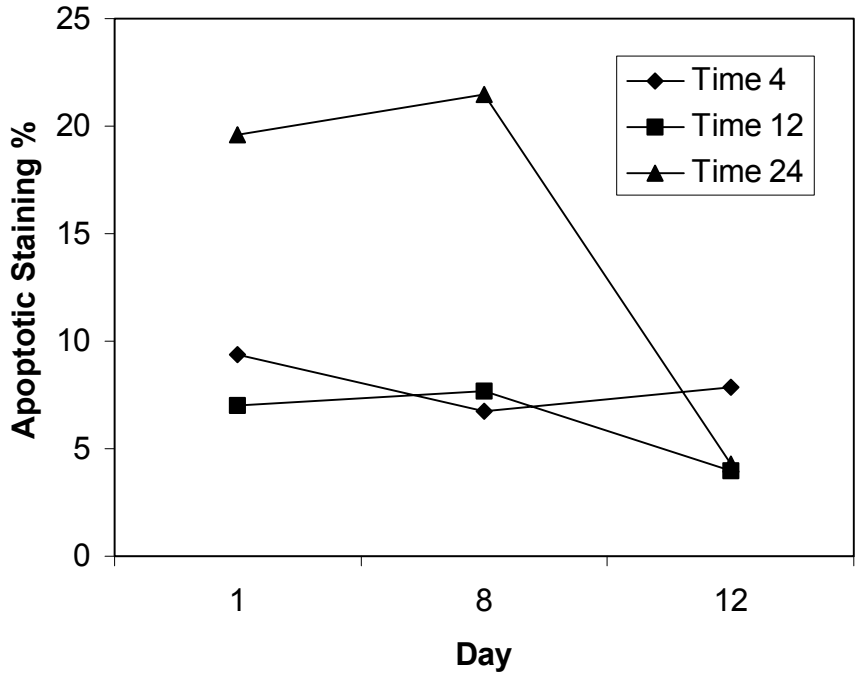
compared to baseline values on Day 1. Lymphocyte percentages retrieved from BAL fluid were significantly greater on Day 1 than on Days 8 or 12 for both groups of horses. Likewise, BAL retrieved neutrophil percentages were significantly less on Day 1 than Day 8 for both groups of horses (Table 2). Dexamethasone administration was associated with trends of improvement toward normal reported values for BAL cytology in the COPD horses.

Figure 1: Geometric means of predominately BAL lymphocyte apoptosis for each stain plotted by time in culture[a]



[a]Plot illustrates significant increases in apoptosis percentages at time 24 ($p < 0.001$) hours in culture versus times 4 or 12 hours regardless of disease status. Geometric means are calculated from the percentages of nine horses including both COPD and controls.

Figure 2: Annexin early (Anne) staining time by day effects[a]



[a] Graph illustrates significant differences in apoptotic staining lymphocyte percentages on Day 1 and Day 8 at time 24 hours culture when compared to time 4 and 12 hours culture. Day 12 time 4 hour culture illustrates significant differences from time 12 and 24 hours culture. All points represent 18 observations each from 4 control horses and 5 COPD horses.

Table 1: Mean pulmonary function testing responses +/- standard error at baseline (Day 1), after natural challenge (Day 8), and after dexamethasone administration (Day 12) for COPD and control horses[a].

Group	Day	Qs/Qt %	Vd/Vt %	$p_{(A-a)}O_2$ mmHg
Control	1	4.21 +/- 1.23	-14.78 +/- 7.48	12.29 +/- 3.22
COPD	1	6.30 +/- 1.27	-3.10 +/- 5.35	23.03 +/- 3.81
Control	8	5.24 +/- 0.53*	-5.80 +/- 5.05	10.86 +/- 3.79
COPD	8	19.41 +/- 5.73*	17.44 +/- 8.21	28.10 +/- 5.18
Control	12	1.45 +/- 0.65*	-5.75 +/- 4.68	8.87 +/- 6.67
COPD	12	12.10 +/- 3.85*	6.48 +/- 7.06	29.23 +/-3.89

[a]Each value is the mean of 4 control horses or 5 COPD horses. * Qs/Qt values illustrates significant differences between COPD horses and normal horses on Days 8 and 12. Vd/Vt values illustrates significant differences between COPD horses and normal horses on all days measured, and for both groups, Day 1 is significantly different than Days 8 and 12. $P_{(A-a)}O_2$ values are all significantly higher in the COPD horse group than the control group for all days measured.

Table 2: Mean cytologic responses +/- SE at baseline (Day 1), after natural challenge (Day 8), and after dexamethasone administration (Day 12) in COPD and control horses[b].

Group	Day	Macrophage %	Lymphocyte %	Neutrophil %	Eosinophil %	Mast Cell %
Normal	1	45.18 +/-3.20	49.95 +/-3.47	1.22 +/-0.46	0.05 +/-0.03	3.60 +/-1.54
COPD	1	35.42 +/-4.92	53.18 +/-6.52	6.60 +/-2.96	0.22 +/-0.20	4.22 +/-2.20
Normal	8	42.88 +/-4.25	40.53 +/-8.08	13.18 +/-8.09	0.20 +/-0.12	3.15 +/-0.93
COPD	8	26.46 +/-4.48*	35.74 +/-8.02*	35.50 +/-9.33*	0.00 +/- 0.0	2.30 +/-0.34
Normal	12	57.00 +/-8.22	34.58 +/-8.18	5.38 +/-4.73	0.30 +/-0.18	2.58 +/-0.71
COPD	12	33.18 +/2.91	41.78 +/-5.58	22.10 +/- 6.60	0.10 +/-0.06	2.92 +/-2.35

[b]Each value is the mean of 4 control horses or 5 COPD horses. COPD horses had significantly less BAL macrophages on all days compared to control horses and Day 1 macrophage percentages were different than Days 8 and 12 for both groups. A day effect was observed in both groups of horses for BAL lymphocyte percentages with Day 1 significantly different than Days 8 and 12 values. Both groups had a significant day effect for BAL neutrophil percentages on Day 1 compared to Day 8. No significant changes were observed in the eosinophil or mast cell percentages.

Table 3: Numerical apoptotic cell percentage means for each stain and horse group for all time points in culture[a]

Group	Day 1	Day 8	Day 12	Stain
Control	4.49	6.77	5.05	Aade
COPD	5.05	3.10	6.63	Aade
Control	13.16	14.27	5.71	Anne
COPD	9.34	8.03	4.71	Anne
Control	12.72	8.80	14.47	Aadl
COPD	7.23	6.68	7.68	Aadl
Control	7.69	7.87	5.32	Annl
COPD	4.83	5.73	3.84	Annl
Control	2.84	5.85	4.64	PI
COPD	6.71	9.61	8.16	PI

[a] Illustrates numerical trends seen in time in culture by day of disease (baseline, after natural challenge, and after dexamethasone administration) staining effects for each stain and horse grouping. Means are the results from 4 control or 5 COPD horses.

III. Discussion

Apoptosis is one of the mechanisms that the body uses to terminate an immune response once that response is not needed anymore (Nakayama 1995, Zhou 1998). Alterations in physiologic apoptosis of lymphocytes has been shown to contribute to pathologic conditions in mice in which lymphocyte apoptotic signaling has been incapacitated by mutation (Hagimoto 1997). Additionally, apoptosis also contributes to resolution of antigen induced airway inflammation in humans and mice (Hamman 1998, Goichico 1998).

The lack of any significant differences between the COPD and control horse groups in percentage of apoptotic BAL lymphocytes from baseline to after antigenic challenge and after dexamethasone administration may be due, in part, to the small (nine horses) sample population. Additionally, individual horse to horse variation within each group may have contributed. There was a large variation in disease severity of the COPD horses ranging from mild clinical signs to severe. This can be appreciated in the lack of significant changes between groups for baseline to natural challenge and after dexamethasone administration in both the pulmonary function data (Qs/Qt, Vd/Vt, ($p_{(A-a)}O_2$)) and BAL cytologic evaluation. Although there were changes from day to day within the COPD group that were significant, the magnitude of the change when compared to the normal horses was not.

The possibility that there are no real differences between COPD and control horse BAL lymphocyte apoptosis exists, but this study can not draw that conclusion definitively. Additional manipulation of the BAL cells to purify the population or inactivation of phagocytic cells may be required to further investigate this phenomenon. In vitro inactivation of macrophages has recently been reported (Callahan 2000). Additionally, pre-incubation of lymphocytes with Annexin-V, has been shown to retard macrophage phagocytosis (Krahling 1999). This may be a viable alternative to purification of the BAL cell population, as purification (and added manipulation of the cells) may in itself, alter the percentage of apoptotic cells thereby skewing the results.

Dexamethasone has been shown to induce apoptosis in leukocytes (Donner1999, Schmid 1994), but these results are from in-vitro studies. This study dealt with dexamethasone in an in-vivo model and our ability to quantitate apoptosis in BAL cells. Lymphocytes exposed to dexamethasone in-vitro can exhibit early apoptosis as early as 1 – 2 hours after exposure (Donner 1999, Schmid 1994), while in this present study, we did not perform BAL until 96 hours after the first exposure. Therefore, the possibility that we may have missed a major apoptotic event may exist. Additionally, activated lymphocytes have been shown to be resistant to Fas-mediated apoptosis (Inaba 1999) and rescued from apoptosis in the tissue of origin from where they first encountered the antigen (Bode 1997). It is therefore possible that BAL lymphocytes may be predetermined to enter the bronchial or alveolar lumen and die. Peribronchial lymphocytes that infiltrate the walls of the bronchi during inflammatory states may therefore be the cells that need to be investigated more thoroughly. More studies involving more horses are needed to identify this.

This study did identify increased lymphocyte apoptosis as measured by all stains at time 24 hours post BAL. This is in agreement with previously published studies with the kinetics of lymphocyte cell death in culture (Donner 1999, Schmid 1994). Early lymphocyte apoptosis as measured by Annexin-V^+/PI^- staining had a significant Day by Time effect magnified by Day 12. Annexin/PI staining is the earliest method of determining apoptosis that this study employed. Therefore, decreased early apoptotic staining on Day 12 at times 12 and 24 hours post BAL is indication that a greater proportion of cells were dying earlier than on previous days. This indirectly supports our hypothesis that dexamethasone increases lymphocyte apoptosis.

The use of pulmonary function testing in horses is to help determine the degree of gas exchange compromise that exists with both upper and lower airway conditions. Most techniques have been verified in the exercised horse with the use of a treadmill. Davis (1998) developed methods to measure physiologic shunt fraction and alveolar dead space fractions that can be used in the standing horse. Friday (2000) further modified these

methods for use in the standing horse. These methods are generally considered more sensitive in determining subtle gas exchange inefficiencies as compared to classic techniques (Votion 1999).

Pulmonary ventilation in the horse is used to deliver oxygen to the pulmonary venous blood resulting in continuous oxygen renewal. Areas of gas exchange in the lungs include the alveoli, alveolar sacs, alveolar ducts and respiratory bronchioles. The rate at which air reaches these areas is termed 'alveolar ventilation'. During normal quiet breathing, only a small amount of inspired air actually reaches the alveoli; most inspired air moves by diffusion into the terminal airways (Guyton 1999).

The air in the larger airways, nasal passage, pharynx and trachea, make up what is termed 'dead air space'. All respiratory passages except the alveoli is termed the 'anatomic dead space'; an area that does not participate in gas exchange. When alveoli are not participating in gas exchange due to inadequate ventilation or hypoperfusion, and are included in the measurement of dead space, the resultant term is 'physiologic dead space'.

Important determinates of arterial blood oxygenation is ventilation-perfusion (V/Q) matching. Mild mismatching occurs normally in the standing horse with increased perfusion (decreased V/Q) to the ventral lung lobes and increased ventilation (increased V/Q) to the dorsal lobes (Amis 1984). This is also the most important and common gas exchange problem in horses with lower airway disease (O'Callaghan 1991). Indices that are used to determine any amount of V/Q mismatching are the physiologic shunt fraction (Qs/Qt), dead space fraction (Vd/Vt), and the alveolar to arterial gradient ($P_{(A-a)}O_2$). Qs/Qt is used to identify areas of oxygen deficits with a V/Q <1 as with extrapulmonary shunting and diffusion impairments. Vd/Vt is used to identify areas of oxygen deficits with a V/Q >1. $P_{(A-a)}O_2$ is another method to determine relative gas exchange efficiency and the amount of physiologic shunting of blood away from low V/Q or nonventilated areas (McDonell 1996). With $P_{(A-a)}O_2$ elevations above normal, the oxygen diffusion barrier is increased. In clinically affected COPD horses, elevations in the Qs/Qt and $P_{(A-}$

$_{a)}O_2$ have been observed (Nyman 1991, Votion 1999, Littlejohn 1982) likely due to increased bronchoconstriction resulting in lower oxygen tensions in some alveoli and/or underperfusion of some alveoli. Hypersecretion of mucus and bronchiol plugging could contribute also. Increased Vd/Vt has also been observed in COPD horses associated with hypercapnea although the reported values are quite varied (0.49% +/- 0.05, -18.2% +/- 3.1, and 6.1%, mean +/- s.d.) (Davis 1998, Gallivan 1989, Littlejohn 1982). Additionally, normal horses have been found to have a wide variation is what is considered normal for Qs/Qt and $P_{(A-a)}O_2$ values. Qs/Qt values range from 0.7% +/- 0.31 (mean +/- s.d.) to 8.8 +/- 3.9 while $P_{(A-a)}O_2$ has been observed to range from 4-25 mmHg (Aguilera – Tejero 1998, Mauderly 1974).

Significant mean alterations in the physiologic shunt fraction in the COPD horses from baseline (Day1) to after natural challenge (Day 8) and after dexamethasone administration when compared to normal horses substantiates the observed clinical signs of COPD. Additionally, alveolar dead space fractions and the alveolar to arterial difference are significantly different in the COPD horses compared to the normal horses at all days suggesting that the COPD horses had possible bronchial remodeling from chronic COPD. Although the alveolar dead space value is elevated from baseline, it is still within reported normal ranges (-17.5% - 57.04%), but the physiologic shunt fraction is elevated above normal reference ranges (0.7% – 8.8%). Likewise, elevations in $P_{(A-a)}O_2$ in the COPD group above normal ranges after natural challenge is additional support that theses horses were experiencing gas exchange impairments. Elevations in the physiologic shunt fraction indicates venous admixture of blood from poorly ventilated but adequately perfused alveoli with end-pulmonary capillary blood from normally functioning alveoli (McDonell 1996). Bronchoconstriction and hyper-secretion of mucus, classic clinical signs of COPD, will result in inadequately ventilated and/or underperfused alveoli and thus elevations in the percent venous admixture (Qs/Qt) and alveolar-arterial oxygen difference ($P_{(A-a)}O_2$) in horses with COPD. Elevations of Vd/Vt within the COPD group and thus increased areas of hypoventilation within the lung, still help confirm that these horses were indeed experiencing an acute exacerbation of inflammatory airway disease. Dexamethasone administration in the COPD horses was observed to cause a downward trend in Qs/Qt and Vd/Vt percentages bringing the values closer to their initial baseline values on Day 1.

Elevations in the neutrophil percent of BAL and transtracheal wash preparations are well documented in clinical cases of COPD. This study is in support of this fact as the number of neutrophils increased dramatically from baseline to after antigenic challenge in the COPD group of horses. A downward trend in neutrophil percent was observed with dexamethasone administration in both groups. Decreases in macrophage and lymphocyte percentages in the COPD horses from baseline to after natural challenge were most likely a function of the increase in neutrophil percentages. Increased circulating catecholamines from stress of the disease could have contributed to the lower lymphocyte percentage. Additionally, peribronchial recruitment of both lymphocytes and possibly macrophages may have lowered the percentage recovered.

Conclusion:

Adaptation of the apoptotic methods Vindelov's propidium iodide with Triton-X, 7-aminoactinomycin-D, and Annexin-V/Propidium iodide indicated that all three methods were able to be successfully used in the horse. Evaluation of the staining relationships between each stain for different time points in culture revealed that only the methods that preserve cell membrane integrity, 7-aminoactinomycin-D and Annexin-V/Propidium iodide, were comparable over time. Vindelov's propidium iodide with Triton-X destroyed the cell membrane and thus included all apoptotic cells with DNA fragmentation in the total apoptotic percent. Alterations in the apoptotic staining ability of each stain was observed to be unaffected by disease state indicating that these stains can be used in COPD horses during all stages of disease and remission. Therefore, when evaluating apoptosis in a heterogeneous population of cells, like those found in equine bronchoalveolar fluid, the 7-aminoactinomycin-D and Annexin/Propidiun iodide methods should be considered.

Although no differences were seen in mean lymphocyte apoptosis between COPD and control horses before and after dexamethasone administration, this study is unable to definitively state there are no differences. Numerical trends in the COPD horses did note a decrease in lymphocyte apoptosis after natural challenge when compared to the normal horses. Small study size, wide horse to horse variability, and sampling of a heterogeneous population including phagocytic cells could have contributed to any lack of differences. Potentially, we should be targeting our efforts at the T lymphocytes that infiltrate the peribronchial tissues instead of the bronchoalveolar area. Additional work to identify the most appropriate population of cells and methods to purify them are needed at this time.

Dexamethasone administration was associated with significant improvements in the physiologic shunt fraction and trends of improvement in alveolar dead space fractions indicating improved pulmonary gas exchange due to decreased shunting of blood away from underperfused alveoli. Additionally BAL cytologic trends of improvements were seen with decreasing numbers of neutrophils with more normal numbers of macrophages and lymphocytes. Increasing trends in lymphocyte apoptosis in the COPD group was observed after dexamethasone administration. These trends paralleled clinical signs of recovery in the COPD horses. The trends observed in

the COPD horses including the decreased apoptosis after natural challenge and improvements more toward baseline apoptosis after dexamethasone needs further exploration.

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APPENDIX A:

Calculation of Physiologic Shunt Fraction

$$\frac{Qs}{Qt} = \frac{(Cc'O_2 - CaO_2)}{(Cc'O_2 - Cv\ O_2)}$$

($Cc'O_2$: Alveolar oxygen content; CaO_2 : arterial blood oxygen content; CvO_2 : venous blood oxygen content)

Calculation of the Alveolar Dead Space Fraction

$$\frac{Vd}{Vt} = \frac{(p_aCO_2 - ETCO_2)}{p_aCO_2}$$

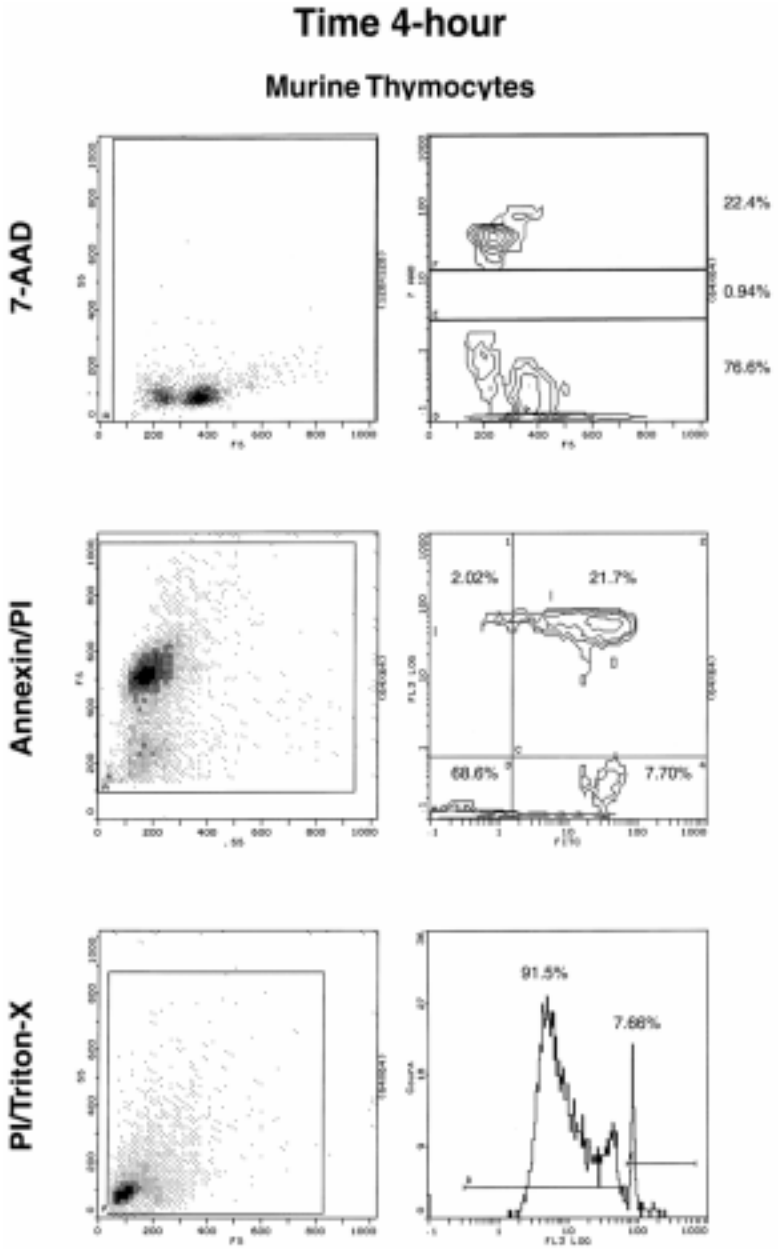
(p_aCO_2 : arterial carbon dioxide tension; $ETCO_2$: end tidal CO_2 tension of expired gas)

Calculation of the Partial Pressure of Oxygen in the Alveolus

$$p_AO_2 : (BP - P_{H20}) \times F_iO_2 - \frac{p_aCO_2}{R}$$

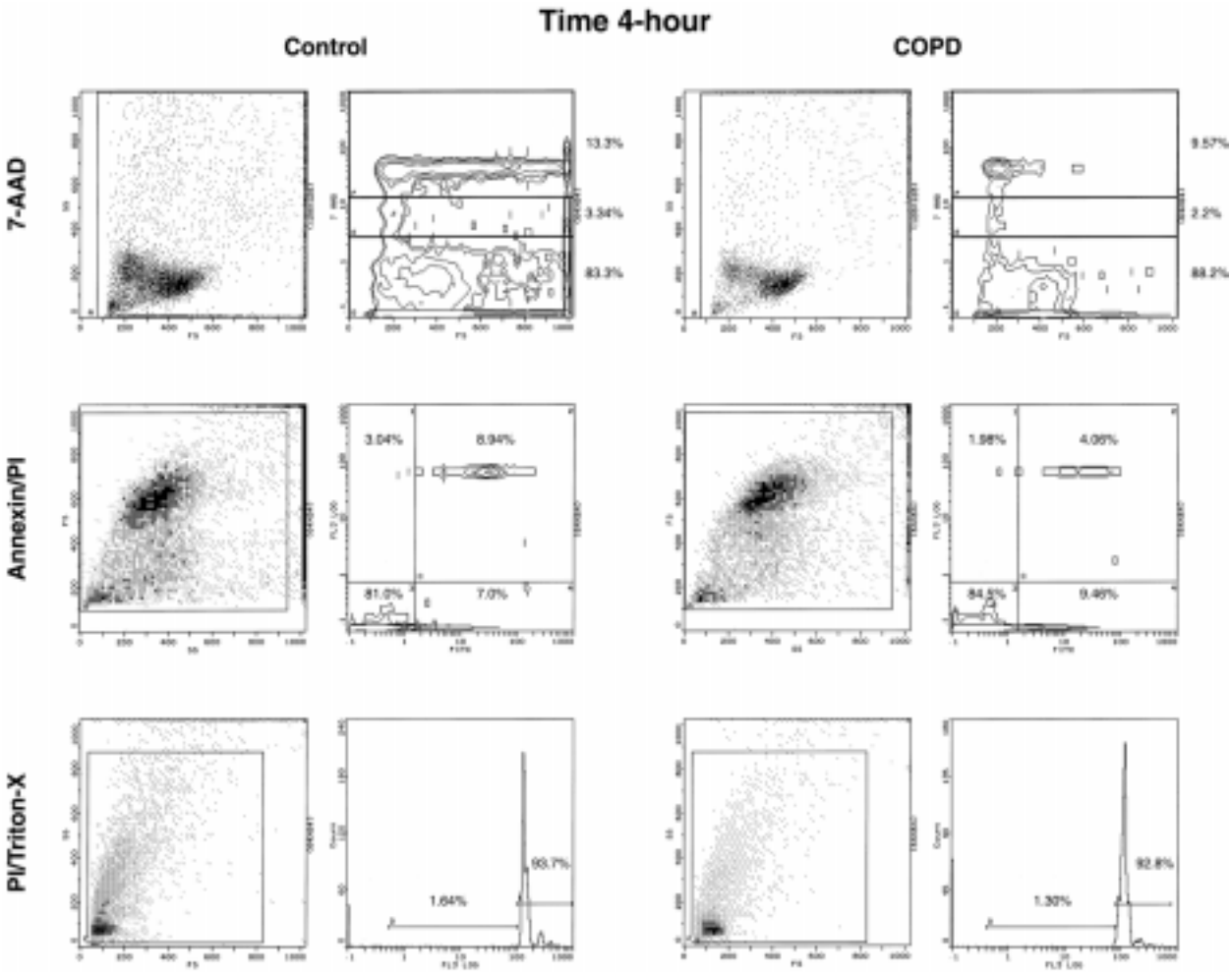
(p_AO_2 :Alveolar oxygen tension; BP: barometric pressure; P_{H20} : partial pressure of water vapor at body temperature; F_iO_2 : inspired O_2 fraction; R: respiratory exchange ration assumed to be 0.85)

Appendix B: Example of murine thymocyte apoptosis at time 4 hours



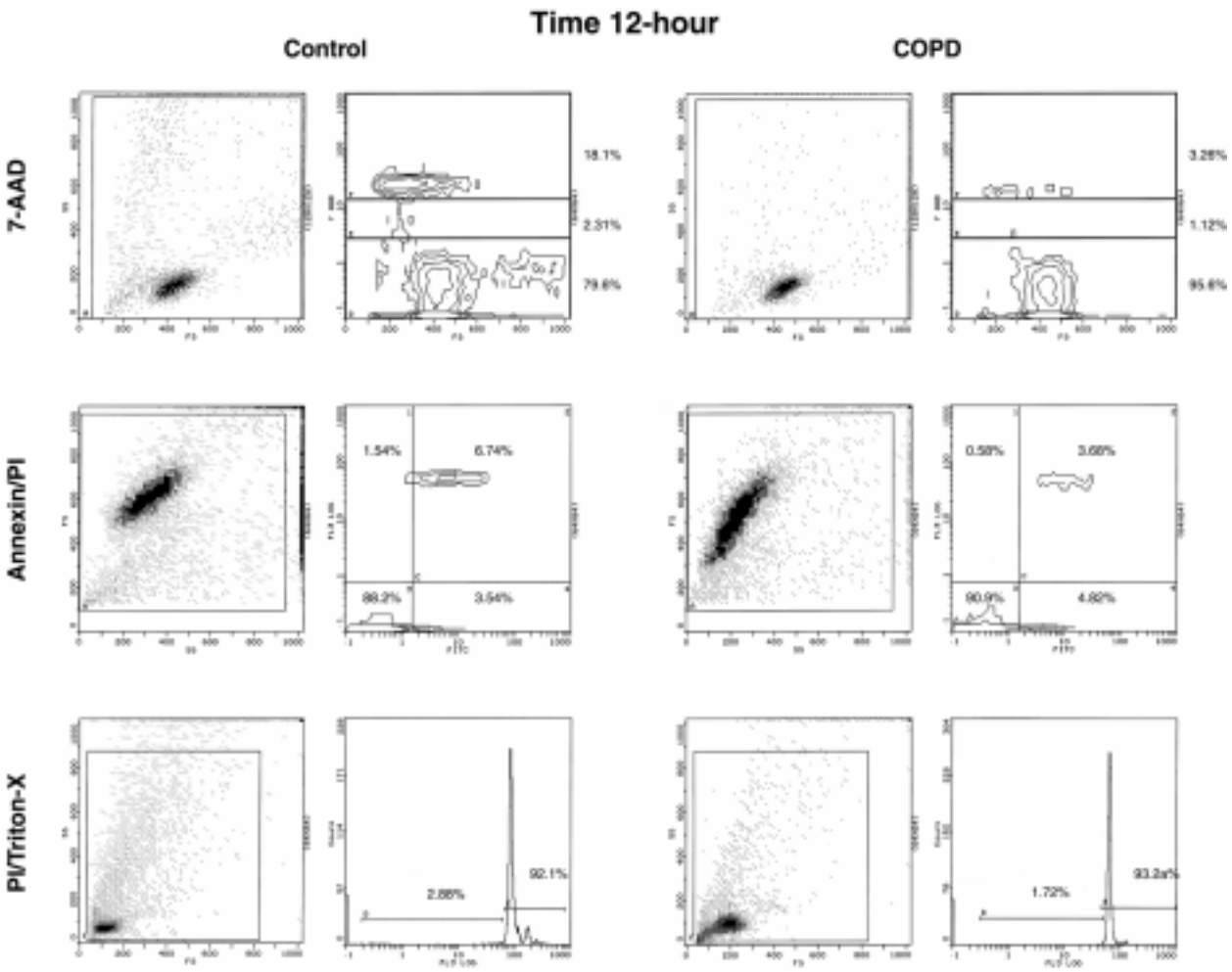
Representative histograms of murine thymocytes were analyzed by 7-AAD, Annexin/PI and PI/ triton-X. For 7-AAD, three fluorescent peaks were determined: dull (live), intermediate (early apoptosis), and bright (late apoptosis/necrosis) staining forward (FSC) versus side scatter (SSC) histograms. For Annexin/PI, four distinct populations were identified based on $Annexin^{-}/PI^{-}$ (live), $Annexin^{+}/PI^{-}$ (early apoptosis), $Annexin^{+}/PI^{+}$ (late apoptosis), and $Annexin^{-}/PI^{+}$ (necrotic) staining. For Propidium iodide with Triton-X, dull fluorescence identified late apoptosis and bright fluorescence identified live cells. The FSC versus SSC setting were standardized for all analysis. The percentage of fluorescence on the gated population is reported for each stain. All gates were standardized for all analysis.

Appendix C: Equine BAL lymphocyte apoptosis at time 4 hours:



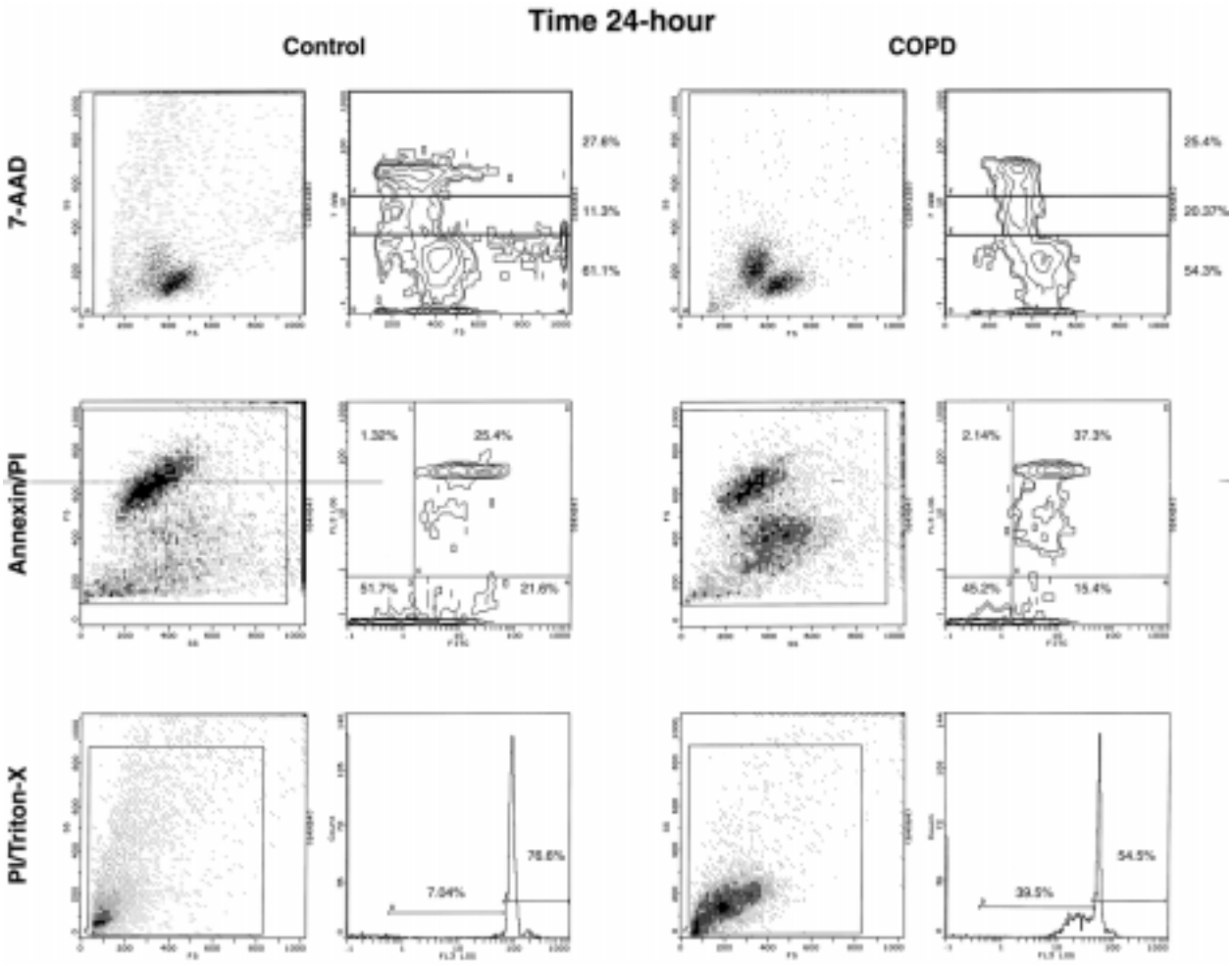
Representative histograms of equine BAL lymphocytes were analyzed by 7-AAD, Annexin/PI and PI/triton-X. For 7-AAD, three fluorescent peaks were determined: dull (live), intermediate (early apoptosis), and bright (late apoptosis/necrosis) staining forward (FSC) versus side scatter (SSC) histograms. For Annexin/PI, four distinct populations were identified based on $Annexin^{-}/PI^{-}$ (live), $Annexin^{+}/PI^{-}$ (early apoptosis), $Annexin^{+}/PI^{+}$ (late apoptosis), and $Annexin^{-}/PI^{+}$ (necrotic) staining. For Propidium iodide with Triton-X, dull fluorescence identified late apoptosis and bright fluorescence identified live cells. The FSC versus SSC setting were standardized for all analysis. The percentage of fluorescence on the gated population is reported for each stain. All gates were standardized for all analysis.

Appendix D: Equine BAL lymphocyte apoptosis at time 12 hours:



Representative histograms of equine BAL lymphocytes were analyzed by 7-AAD, Annexin/PI and PI/triton-X. For 7-AAD, three fluorescent peaks were determined: dull (live), intermediate (early apoptosis), and bright (late apoptosis/necrosis) staining forward (FSC) versus side scatter (SSC) histograms. For Annexin/PI, four distinct populations were identified based on $Annexin^{-}/PI^{-}$ (live), $Annexin^{+}/PI^{-}$ (early apoptosis), $Annexin^{+}/PI^{+}$ (late apoptosis), and $Annexin^{-}/PI^{+}$ (necrotic) staining. For Propidium iodide with Triton-X, dull fluorescence identified late apoptosis and bright fluorescence identified live cells. The FSC versus SSC setting were standardized for all analysis. The percentage of fluorescence on the gated population is reported for each stain. All gates were standardized for all analysis.

Appendix E: Equine BAL lymphocyte apoptosis at time 24 hours:



Representative histograms of equine BAL lymphocytes were analyzed by 7-AAD, Annexin/PI and PI/triton-X. For 7-AAD, three fluorescent peaks were determined: dull (live), intermediate (early apoptosis), and bright (late apoptosis/necrosis) staining forward (FSC) versus side scatter (SSC) histograms. For Annexin/PI, four distinct populations were identified based on $Annexin^{-}/PI^{-}$ (live), $Annexin^{+}/PI^{-}$ (early apoptosis), $Annexin^{+}/PI^{+}$ (late apoptosis), and $Annexin^{-}/PI^{+}$ (necrotic) staining. For Propidium iodide with Triton-X, dull fluorescence identified late apoptosis and bright fluorescence identified live cells. The FSC versus SSC setting were standardized for all analysis. The percentage of fluorescence on the gated population is reported for each stain. All gates were standardized for all analysis.

APPENDIX F: Table of physical exam parameter means and ranges for control and COPD horses at baseline, after natural challenge and after dexamethasone therapy.

Group	Day	Heart Rate	Respiratory Rate	Temperature
Control	1	44 (40 –52)	17 (12 – 20)	99.2 (97.5 – 100.3)
COPD	1	42 (36 – 48)	18 (16 – 24)	99.5 (99 – 100.2)
Control	8	40 (36 – 44)	21 (12 –32)	99.3 (98.7 – 99.9)
COPD	8	44.8 (36 – 72)	29 (12 – 48)	100.4 (99.3 – 102.7)
Control	12	40 (36 – 44)	18 (8 – 28)	99 (98.2 – 100)
COPD	12	44.8 (40 – 48)	25 (16 – 36)	99 (98.5 – 99.5)

Means are calculated from 4 control horses and 5 COPD horses.

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Comparison of Pulmonary Lymphocyte Apoptosis in Normal and Chronic Obstructive Pulmonary Disease Affected Horses Before and After Dexamethasone Administration. $4875.00, 2000.