STUDIES ON THE AETIOPATHOGENESIS OF EQUINE CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

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I declare that the contents of this thesis are my own work and that they have not been presented to any University other than the University of Edinburgh.

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INDEX

ABBREVIATIONS

AF . . . . . *Aspergillus fumigatus*

AIC . . . . . antigen inhalation challenge

BAL . . . . . bronchoalveolar lavage

BALF . . . . . bronchoalveolar lavage fluid

BASO . . . . . 'basophilic cell'

Cdyn . . . . . dynamic compliance

CONT . . . . . control horses

COPD . . . . . chronic obstructive pulmonary disease

CROSS . . . . . crossbred horse

dPtp . . . . . change in transpulmonary pressure

EDTA . . . . . ethylene diaminetetraacetic acid

ELISA . . . . . enzyme linked immunosorbent assay

EOS . . . . . eosinophil

EP . . . . . epithelial cell

ER . . . . . early response

F . . . . . female

FITC . . . . . fluorescein isothiocyanate

FSC . . . . . forward light scatter

HPRT . . . . . hypoxanthine phosphoryl transferase

HRF . . . . . histamine releasing factor

ID . . . . . internal diameter

Ig . . . . . immunoglobulin

LA . . . . . apical lobe of left lung

LD . . . . . diaphragmatic lobe of left lung

LPR . . . . . late phase response

lym . . . . . lymphocyte

M . . . . . .male

mAb . . . . . monoclonal antibody

MAC . . . . . .macrophage

MAST . . . . . mast cell

MCN . . . . . .mean channel number

MF . . . . . .*Micropolyspora faeni*

MN . . . . . .gelding

NC . . . . . 'natural challenge'

NEUT . . . . . neutrophil

NMS . . . . . .normal mouse serum

NRatS . . . . . normal rat serum

NRS . . . . . normal rabbit serum

OD . . . . . .outer diameter

OSR . . . . . oil seed rape

$PaCO_2$ . . . . . .arterial carbon dioxide tension

PaO_2 . . . . . .arterial oxygen tension

PB . . . . . peripheral blood

PBA . . . . . phosphate buffered saline containing
sodium azide and bovine serum albumen

PBL . . . . . peripheral blood leucocytes

PBS . . . . . .phosphate buffered saline

PELF . . . . . .pulmonary epithelial lining fluid

PF . . . . . paraformaldehyde

PFT . . . . . .pulmonary function test

PMT . . . . . pulmonary mechanics test

PNU . . . . . protein nitrogen units

RA . . . . . accessory lobe of right lung

RD . . . . . diaphragmatic lobe of right lung

RIA . . . . . .radioimmunoassay

RID . . . . . .radial immunodiffusion assay

R_L . . . . . average total pulmonary resistance

RR . . . . . respiration rate

SSC . . . . . .side light scatter

TB . . . . . Thoroughbred

TOTBAS . . . . . .total basophiloid cells

TV . . . . . *Thermoactinomyces vulgaris*

Vmin . . . . . .minute volume

V_T . . . . . tidal volume

v/v . . . . . volume for volume

w/v . . . . . .weight for volume

SUMMARY

This thesis describes an investigation into the aetiopathogenesis of equine chronic obstructive pulmonary disease (COPD).

To investigate the aetiology of equine COPD, control and asymptomatic COPD affected horses were given nebulised inhalation challenges with extracts of *Micropolyspora faeni* (MF), *Aspergillus fumigatus* (AF) and *Thermoactinomyces vulgaris* (TV). MF and AF challenges induced pulmonary disease, similar to naturally occurring COPD, only in the COPD affected horses, implicating MF and AF in the aetiology of equine COPD. The role of TV in the aetiology of equine COPD could, however, not be assessed as the TV challenges induced pulmonary inflammation in 2 control horses, which had been unaffected by hay and straw challenges i.e. 'natural challenges' (NC), indicating that the experimental TV challenge differed from the TV challenge which occurs during NC. The absence of pulmonary disease in control horses following MF, AF challenges and after NC suggests that equine COPD is a pulmonary hypersensitivity, rather than a non specific toxic response.

In this study, bronchoalveolar lavage fluid (BALF) cytology examinations proved to be more useful for detecting pulmonary disease than clinical, pulmonary mechanics, arterial blood gas tensions and arterial pH examinations.

The role of oil seed rape (OSR) (*Brassica spp.*) in the aetiology of equine pulmonary disease was investigated by exposing horses to a field of flowering *B.campestrus* and by experimental *B.napus* inhalation challenges. OSR had no detectable effect on control and asymptomatic COPD affected horses, suggesting that OSR is not a major cause of equine respiratory disease. However, the experimental *B.napus* inhalation challenges exacerbated pulmonary disease in some symptomatic COPD affected horses, presumably via non specific bronchial hyperresponsiveness/toxicity. Intradermal testing using a commercial *B.napus* pollen extract suggested that none of the horses investigated was hypersensitive to *B.napus* pollen antigens.

As BALF is comprised of lavage fluid and pulmonary epithelial lining fluid (PELF) in variable proportions, quantitative comparisons of the cellular and molecular components in different BALF samples are valid only if the proportions of PELF in the BALF samples are standardised. Two BALF standardisation techniques, namely the urea and albumen dilution techniques, were evaluated in the horse. While both techniques were found to be satisfactory, the urea dilution technique was considered to be the more accurate.

Comparison of the cellular and molecular components of BALF collected from 4 different lung segments of control and symptomatic COPD affected horses indicated that, in these horses, BALF components showed regional homogeneity. This suggests that the composition of PELF is uniform throughout the lungs of these horses and that a single BALF sample, collected from any lung lobe, is representative of the entire lung.

The role of mast cells/basophils in the pathogenesis of equine COPD was investigated by quantifying histamine, an indicator of mast cell/basophil degranulation, in plasma, BALF and PELF of control and COPD affected horses, before and at 0.5 and 5h after NC. The PELF histamine concentrations of COPD affected horses were significantly increased only at 5h after NC. NC had no significant effect on the PELF histamine concentrations of control horses nor on the plasma and BALF histamine concentrations of either group. As the histamine concentrations of whole BALF lysates were significantly correlated with the numbers of metachromatically staining BALF cells, presumed to be mast cells and/or basophils, these findings support involvement of a late phase, mast cell/basophil mediated, hypersensitivity reaction in the pathogenesis of equine COPD.

Quantification of tryptase, an inflammatory mediator which offers potential advantages over histamine as an indicator of mast cell degranulation, in equine serum and BALF, using a commercial radioimmunoassay kit for human tryptase, was unsuccessful.

The role of lymphocytes in the pathogenesis of equine COPD was investigated by determining the lymphocyte phenotype distributions of peripheral blood (PB) and BALF from control and COPD affected horses, before and after NC. Prior to NC, asymptomatic COPD affected horses had a significantly higher proportion of BALF B lymphocytes than control horses, suggesting that these cells have a role in the pathogenesis of equine COPD. NC significantly increased the ratios of CD4+, T helper/inducer lymphocytes and significantly reduced the ratios of CD8+, T suppressor/cytotoxic lymphocytes in BALF from COPD affected horses, suggesting that T lymphocytes have an important role in the pathogenesis of equine COPD.

Intradermal mould antigen testing was evaluated as a diagnostic technique for equine COPD. The intradermal endpoint titres of control horses for AF, MF and TV were not significantly different from those of COPD affected horses, suggesting that this technique is of limited value in the diagnosis of equine COPD. Furthermore, the lack of correlation between the intradermal endpoint titres for each antigen and the changes in pulmonary mechanics, arterial blood gas tensions and with BALF neutrophil ratios which had followed previous MF, AF and TV inhalation challenges and NC suggests divergence of equine dermal and pulmonary reactivities to these antigens.

Local transendoscopic endobronchial antigen challenge, which has proved to be a valuable clinical and research technique in the study of human pulmonary hypersensitivity, was evaluated in the horse. As local endobronchial challenges with phosphate buffered saline, MF extract and mouldy hay extract induced a non specific pulmonary neutrophilia in both control and asymptomatic COPD affected horses and elicited endoscopically visible responses in a proportion of horses from both these groups, this technique was considered to be of limited value as a clinical and research technique in the study of equine COPD.

CHAPTER 1

INTRODUCTION

While the domestication of the horse has benefited man in numerous ways, it has unfortunately resulted in the exposure of many horses to environments which are detrimental to their health. Frequently horses are maintained in poorly designed and poorly ventilated environments (Jones *et al* 1987), fed on poorly saved hay and bedded on poorly saved straw, and as a result are exposed to high levels of airborne pollutants including inorganic dusts, ammonia, endotoxins, bacteria, viruses, fungi, actinomycetes and mites. Consequently large numbers of housed horses develop environmental pulmonary diseases, which adversely affect both their welfare and athletic performance.

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Chronic obstructive pulmonary disease (COPD) is the most important environmental pulmonary disease to affect the horse. Unfortunately the literature regarding COPD and other equine environmental pulmonary diseases is confused by the plethora of terms used to name and define these conditions, many of which were erroneously adopted from human medicine e.g. heaves, chronic alveolar emphysema, chronic bronchiolitis and allergic small airway disease.

To enable valid comparisons of findings from different laboratories, it is important to fully define the disease under investigation. In this study COPD was defined as reversible pulmonary inflammation and airway obstruction, characterised by excessive numbers of tracheobronchial and bronchoalveolar neutrophils and by clinical and laboratory evidence of pulmonary dysfunction, which was consistently induced in susceptible horses by exposing

them to poorly saved hay and straw and fully reversed by transferring them to an environment free from hay and straw.

In this study, COPD affected horses with clinical or laboratory evidence of pulmonary disease i.e. those which were being maintained in an environment containing hay or straw or those which had not achieved complete remission following subsequent transfer to a hay and straw free environment were termed 'symptomatic'. Those horses which were in remission i.e. those which had been maintained in a hay and straw free environment until they showed no clinical or laboratory evidence of pulmonary inflammation were termed 'asymptomatic'. The populations of control and COPD affected horses used in this study and the clinical and laboratory techniques employed to detect pulmonary inflammation and dysfunction are further defined in Chapter 2.

COPD is the most commonly recognised equine environmental pulmonary disease and is considered to be one of the most common causes of premature loss due to disease in the United Kingdom and Western Europe (Gerber 1973; Cook 1976; McPherson and Thomson 1983).

COPD appears to occur world wide, although it is uncommon in Australia and New Zealand (Goulden, B. *pers comm* 1990).

While estimations of incidence vary from 54% in Switzerland (Bracher *et al* 1991) to up to 70% in riding stables in Holland (Sasse quoted by Dixon 1986), the apparent incidence of COPD in any population is likely to be influenced not only by the environments in which the horses are maintained but also by the sensitivity of the techniques employed to detect the disease. With the current expansion in the urban horse population in the United Kingdom, much of which is maintained predominantly in stables, the number of horses affected by this condition is likely to increase.

The incidence of COPD increases with age and there is no apparent sex or breed distribution (Alexander 1959; Lowell 1964; McPherson and Thomson 1983).

AETIOLOGY OF EQUINE COPD

COPD has long been recognised as a disease of domestication which is attributable to housing in poor environments containing poorly saved hay and/or straw and consequently does not affect horses maintained at pasture (Alexander 1959; Cook and Rossdale 1963; Lowell 1964; Thurlbeck and Lowell 1964), although a less common condition which shows many similarities to COPD, termed summer pasture associated obstructive pulmonary disease (SPAOPD), which may be a pulmonary hypersensitivity to inhaled moulds or pollens, has been recently reported in pastured horses (Beadle 1983; Dixon and McGorum 1990 A & B).

(1) INHALED THERMOPHILIC ACTINOMYCETES AND FUNGI

While the aetiology of equine COPD remains speculative, antigen inhalation challenges (reviewed in Chapter 2), intradermal antigen testing (reviewed in Chapter 3) and the detection of serum precipitins to putative allergens suggest that COPD is a pulmonary hypersensitivity to inhaled allergens. The major inhalant allergens are considered to be *Micropolyspora faeni*, a thermophilic actinomycete which causes extrinsic allergic alveolitis in man (Wenzel *et al* 1965) and cattle (Pirie *et al* 1971) and *A.fumigatus*, a fungus which causes bronchial asthma, allergic bronchopulmonary aspergillosis and allergic alveolitis in man (Vallery-Radot and Giroud 1928; Crompton 1990) (Eyre 1972; Halliwell *et al* 1979; McPherson *et al* 1979; McPherson and Thomson 1983). It is however possible that the aetiological agents may show geographical variation.

M.faeni and *A.fumigatus* aeroallergens originate from contaminated hay and straw. While hay and straw, when cut, is virtually free form these moulds and contains only small numbers of non pathogenic fungi, if it is baled with a moisture content above 20%, significant mould contamination is likely to occur during storage (Clarke 1987).

Stables which contain poorly saved hay and straw may contain high levels of airborne *M.faeni* and *A.fumigatus* antigens, exposing horses to large quantities of these aeroallergens.

Woods,P. *et al* (*pers comm* 1991) recorded levels of 1500ng/m^3 *M.faeni* and 1800ng/m^3 *A.fumigatus* aeroallergens in such stables, while hay and straw free stables contained approximately half of these levels. As thermophilic actinomycete and fungal spores are, respectively, 0.7-1.3u and <5u in diameter, if inhaled, many will be deposited in the small airways (Lacey and Lacey 1964) where they may induce local inflammation.

While the structural and chemical basis of the allergenicity of these agents is unknown, in addition to inducing specific immunological reactions, *M.faeni* has additional properties which may contribute to its pathogenic effects. It can activate complement by the alternative pathway (Marx and Flaherty 1976), induce lymphocyte mitogenesis (Smith *et al* 1980), has adjuvant activity (Bice *et al* 1977) and contains proteolytic enzymes (Roberts 1978). *A.fumigatus* may also activate complement by the alternate pathway (Marx and Flaherty 1976).

(2) FORAGE MITES

Large numbers of forage mites, belonging to several species, have been identified in stored hay (Halls and Gudmundsson 1985) and high concentrations of mite aeroallergens have been demonstrated in stables containing hay and straw (Robinson,N.E. *et al*, *pers comm* 1991). Forage mites depend on fungal spores for food and are thus always found in conjunction with mould contamination (Clarke and Madelin 1987). Forage mites excrete faeces containing semidigested mould spores and it is possible that this digestion may alter the antigenicity of the spores.

In man, there is a high incidence (30% of farmers in Scotland) of immediate type bronchial hypersensitivity to forage mites (Cuthbert *et al* 1984) and house dust mites are a major cause of human extrinsic allergic asthma in many regions of the world (Platts-Mills and de Weck 1989). However, the role of forage mites in the pathogenesis of equine COPD has received surprisingly little attention, and there is currently no evidence to implicate forage mites as

allergens in equine COPD. The findings of the limited number of studies which have performed intradermal testing with mite antigens are reviewed in Chapter 3. There are no reports of equine bronchial provocation challenges with mite antigens.

(3) BACTERIA AND ENDOTOXINS

Stables contain large numbers of airborne bacteria which may be inhaled and which are considered to be the major source of the pulmonary bacterial flora of housed animals (Jones 1922). The presence of hay and straw in a stable was shown to increase the number of bacteria within the lungs. There is however no good evidence that inhaled bacteria are causal agents of equine COPD.

Significant numbers of bacteria, mainly *Streptomyces* and *Bacillus* species, which produced elastases when cultured *in vitro*, were demonstrated in horse lungs (Grunig *et al* 1986). While it is possible that bacterial elastases could result in pulmonary damage, if produced *in vivo* , as elastase activity was not detected in equine tracheobronchial secretions (Grunig *et al* 1985) and pulmonary histopathological findings were not correlated with the numbers of elastase producing bacteria (Grunig *et al* 1986), the significance of this observation is not known. It seems unlikely that bacterial elastases have an important role in equine COPD as, if present, they would presumably induce emphysema (Janoff 1983), which is not major feature of equine COPD (Nicholls 1978)

Stables also contain airborne endotoxins (Robinson,N.E. *et al, pers comm* 1991), which are derived from bacterial cell walls. While endotoxins have been associated with occupational lung disease in humans and experimental lung disease in rabbits (Pernis *et al* 1967; Cavagna *et al* 1969), the role of endotoxins in equine COPD has not been investigated.

(4) POLLENS

McPherson *et al* (1979) demonstrated positive clinical, pulmonary mechanic and arterial blood gas responses to rye grass pollen dust inhalation challenges in 5 of 6 COPD affected horses but not in 6 controls. While this suggested that pollens may have a role in the aetiology of equine COPD, as some of the COPD affected horses used in their study were not fully asymptomatic prior to the challenges, the observed positive responses may have been due to non specific bronchial hyperresponsiveness (NSBHR) (Klein 1984; Derksen *et al* 1985A) rather than to true hypersensitivity. NSBHR is manifested by horses with symptomatic COPD as an exaggerated bronchospasm following inhalation with a wide range of inhaled agents including dusts. Furthermore, the absence of COPD in horses maintained at pasture, which are presumably exposed to large numbers of pollens, suggests that pollens are unlikely to be important in the aetiology of equine COPD.

(5) DIETARY TOXICITY

Breeze *et al* (1978) suggested that 3-methylindole, a derivative of ingested L-tryptophan, which is considered to be the cause of bovine fog fever (Carlson and Breeze 1983), could cause equine COPD. Experimental administration of 3-methylindole to horses induced an obstructive pulmonary toxicosis with necrotising bonchiolitis, small airway obstruction, acinar overinflation and ultrastructural changes in Clara cells, which had some pathological resemblance to COPD (Derksen *et al* 1982; Turk *et al* 1983; Breeze *et al* 1984). Recently, Kaup *et al* (1990B) demonstrated ultrastructural changes in Clara cells from horses with spontaneously occurring COPD which were similar to those reported in horses with experimental 3-methylindole toxicity (Turk *et al* 1983; Breeze *et al* 1984).

However, as COPD is not encountered in horses which are maintained at pasture, and as grass is an important source of dietary L-tryptophan, the precursor of 3-methylindole, it seems unlikely that 3-methylindole has a role in the pathogenesis of equine COPD.

(6) OIL SEED RAPE

The possible role of oil seed rape (*Brassica napus* and *B.campestrus*), an alleged cause of equine and human pulmonary disease, was investigated in this study and is reviewed in Chapter 10.

(7) INORGANIC DUSTS

While there is no evidence to suggest that inhaled inorganic dusts have an important role in the aetiology of equine COPD, they may exacerbate pulmonary dysfunction in horses with symptomatic COPD via NSBHR.

Thus the success of environmental management of equine COPD may not only reflect a reduction in exposure to the allergens which cause COPD but also the concomitant reduction in non specific irritants e.g. irritant gases and dusts, which may exacerbate the clinical signs via NSBHR.

(8) ANTIPROTEASE DEFICIENCY

The finding that one form of human emphysema is due to an inherited deficiency of alpha-1-antitrypsin, an enzyme which inhibits proteases, including trypsin, plasmin, cathepsin, collagenase, elastase, thrombin, kallikrein and chymotrypsin, which could otherwise induce proteolytic degradation of the pulmonary connective tissue elements (Kueppers and Black 1974), prompted the investigation of protease inhibitor function in horses with COPD.

While Littlejohn (1978) found reduced serum alpha-1-globulin levels in 12 horses with COPD and suggested that deficiency of antiproteases may be associated with equine COPD, further, and more detailed, studies have found no difference in serum antiprotease activity of controls and COPD affected horses (Gillespie and Tyler 1969; Breeze *et al* 1977; Matthews 1979).

Matthews (1979) demonstrated that the electrophoretically slower alleles of the equine homologue of human alpha-1-antitrypsin were present in increased frequency in horses with COPD. However, as the horses investigated were of mixed breeds and there was no significant correlation between these alleles and low serum trypsin inhibitory capacity, the significance of this finding is unclear. Further studies employing larger numbers of genetically similar horses may clarify this area.

Endogenous and exogenous, bacterially derived, proteases and protease inhibitors have been demonstrated in tracheobronchial secretions of horses with COPD, although their significance in the pathogenesis of equine COPD is unclear (Grunig 1985; Von Fellenberg 1987).

(9) RESPIRATORY INFECTIONS PREDISPOSING TO COPD

An association between the onset of COPD and previous bacterial or viral respiratory infections has long been recognised (Gerber 1973; McPherson and Lawson 1974; Thorsen *et al* 1983). Similarly, in man, severe respiratory infections often precede the onset of human asthma (Busse *et al* 1989).

While the mechanism underlying this association is unknown, pulmonary inflammation induced by the infectious agents may favour antigen contact with immunologically active tissues (McPherson and Lawson 1974), while virus induced reductions in mucociliary clearance may lead to the retention of antigens and thus result in prolonged and increased exposure to antigens (Willoughby and Ecker 1990). In man, viral infections have also been shown to induce NSBHR and increase the late phase airway obstruction which follows antigen challenge (Busse *et al* 1989).

PATHOGENESIS OF EQUINE COPD

The pathogenesis of equine COPD remains speculative. The putative causal agents, namely fungi and thermophilic actinomycetes, could potentially induce pulmonary inflammation

either by non specific irritation, e.g. by the action of proteases and/or by complement activation, or alternatively by hypersensitivity mechanisms (Pepys 1978; Halliwell *et al* 1979). Non specific irritation is, however, unlikely to be an important mechanism in the pathogenesis of equine COPD as all individuals exposed to a non specific irritant would be expected to develop pulmonary inflammation (Salvaggio and Hendrick 1989), in contrast to COPD, which affects only a proportion of horses.

A considerable body of evidence, including antigen inhalation challenge studies, intradermal antigen testing studies, serum precipitins studies (Eyre 1972; Lawson *et al* 1979; Mansmann *et al* 1975; Madelin *et al* 1991) and the demonstration of positive *in vitro* Schultz-Dale phenomena, active cutaneous anaphylaxis and Prausnitz-Kustner passive cutaneous anaphylaxis (Eyre 1972; Schatzmann *et al* 1973), suggests that COPD is a pulmonary hypersensitivity.

While one or more of the four types of hypersensitivity, as classified by Coombs and Gell (1968), could be involved in the pathogenesis of pulmonary hypersensitivity to moulds (Pepys 1978), most current evidence favours involvement of types I and III hypersensitivity (Gerber 1973; Schatzmann *et al* 1973; Halliwell *et al* 1979; McPherson and Thomson 1983).

TYPE I HYPERSENSITIVITY

Type I hypersensitivity is mediated by the interaction of allergen with allergen specific IgE or, less commonly, IgG antibodies, which are present on mast cells and basophils. The resultant degranulation of these cells releases numerous preformed and newly synthesised mediators capable of inducing pulmonary inflammation.

The role for type I hypersensitivity in equine COPD, proposed by Cook and Rossdale (1963), Lowell (1964), Eyre (1972), Halliwell *et al* (1979), McPherson *et al* (1979) and Thomson (1989), was further investigated in this study and is reviewed in Chapter 5.

TYPE II HYPERSENSITIVITY

Mould allergens may induce type II hypersensitivity, also termed antibody dependent cytotoxic hypersensitivity, by interacting with molecules on the surface of cells within the lungs, to produce haptens, against which IgG or IgM antibodies are produced (Pepys 1978). Subsequent interaction of the hapten and antibody induces activation of the complement cascade which results in lysis and/or phagocytosis of the cell.

There is currently no evidence to support involvement of this mechanism in the pathogenesis of equine COPD.

TYPE III HYPERSENSITIVITY

Type III, or immune complex hypersensitivity is considered to be the most important mechanism in the pathogenesis of hypersensitivity pneumonitis in man (Pepys 1978) and is thought to have a role in the pathogenesis of equine COPD (Schatzmann *et al* 1973; Mansmann *et al* 1975; Halliwell *et al* 1979; McPherson and Thomson 1983; Thomson 1989).

Immune complexes, formed by the interaction of specific antigen and IgG, or possibly IgM precipitating antibodies, activate the complement, coagulation and kinin systems and induce release of mediators from mast cells, platelets, neutrophils and eosinophils (Gorman and Halliwell 1989; Pelikan 1990), resulting in a pulmonary inflammatory response. Alternatively the precipitins may elicit a hypersensitivity response without forming immune complexes by directly interacting with IgG-Fc receptors on activated mast cells (Coble *et al* 1984), neutrophils and platelets (Pelikan 1990).

It has been suggested by Cochrane (quoted by Pepys 1978) that a preceding type I hypersensitivity response is a prerequisite to the development of a type III response.

The presence of complement fragment C3 and IgG deposits, within the alveolar septa of one horse with COPD supports involvement of this mechanism in the pathogenesis of COPD in that animal (Winder and Von Fellenberg 1986).

While serum precipitins to a wide range of environmental aeroallergens, including fungi, actinomycetes and chicken sera have been identified in the serum of horses with COPD (Lawson *et al* 1979; Mansmann *et al* 1975; Madelin *et al* 1991), their significance in the pathogenesis of COPD remains unclear. Their presence in horses which have no evidence of pulmonary disease indicates that they may merely reflect previous exposure to these antigens and need not be of pathogenic significance. Furthermore, some horses with COPD (Lawson *et al* 1979; Zeitler 1984) and some humans with hypersensitivity pneumonitis have no detectable precipitins (Edwards *et al* 1974), although this may reflect the limited number of antigens utilised to detect the precipitins in these studies.

TYPE IV HYPERSENSITIVITY

Type IV or delayed type hypersensitivity is mediated by a population of activated T lymphocytes, designated delayed type hypersensitivity cells (T_{DH} cells). Upon subsequent challenge with antigen, T_{DH} cells initiate an inflammatory reaction characterised by macrophage and lymphocyte infiltration and by granuloma formation (Gorman and Halliwell 1989).

While this mechanism has been implicated in the pathogenesis of human allergic alveolitis (Moore *et al* 1976; Pepys 1978), its proposed involvement in equine COPD (Gerber 1973; Schatzmann *et al* 1973) seems unlikely, as the classical hallmark of this reaction, namely granuloma formation, is not a histological feature of COPD (Nicholls 1978).

While Halliwell *et al* (1979) observed an occasional dermal type IV response in normal and COPD affected horses, at 48h after intradermal challenge with *Candida albicans*, this organism has not been associated with equine COPD.

EARLY AND LATE PHASE RESPONSES TO ANTIGEN

Horses with COPD may show early (maximal at 90min) or late (maximal at 4-6h) onset pulmonary or dermal inflammatory responses to experimental antigen inhalation challenges (AICs), 'natural (hay and straw) challenges' (NC) or intradermal antigen challenges (Mansmann *et al* 1975; Halliwell *et al* 1979; McPherson *et al* 1979; McPherson and Thomson 1983; Thomson 1989).

Similarly, natural and experimental allergen challenges of human allergic asthmatics may induce isolated early bronchial responses (ER) (reported incidence 7-50%), which occur within a few minutes of challenge and usually resolve by 30-60min, isolated late phase responses (LPRs) (18-84%), which commence 3-4h post challenge and resolve by 24-48h, or dual responses (9-53%) (reviewed by Lemanske and Kaliner 1988).

ERs, LPRs and dual responses have also been reported following bronchial challenges in rats (Lemanske and Kaliner 1981-1982), guinea pigs (Hutson *et al* 1990), rabbits (Metzger 1990), sheep (Russi 1990) and dogs (Griffin *et al* 1986), and following cutaneous and nasal allergen challenges in several species (Lemanske and Kaliner 1988).

EARLY RESPONSE (ER)

Numerous studies have focused on the ERs to experimental and natural allergen challenges in man and laboratory animals and have demonstrated that they are largely type I hypersensitivity responses (Kay 1988; Johnston and Holgate 1990; Pepys 1990).

In the horse, the early intradermal reactions to *M.faeni* and *A.fumigatus* antigens were shown histologically to be consistent with a type I hypersensitivity (Halliwell *et al* 1979; Beech and Gunson 1981) and it is possible that the early pulmonary response to these antigens and to NC may also be mediated by this mechanism (McPherson and Thomson 1983).

LATE PHASE RESPONSE (LPR)

It is apparent, however, that naturally occurring allergic disease does not result from a continuous series of immediate hypersensitivity responses (Gleich 1982) and considerable evidence now indicates that the LPR more closely resembles naturally occurring allergic disease than does the ER (reviewed by Iliopoulos *et al* 1990). Accordingly the LPR is now receiving greater attention.

While the pathogenesis of the LPR in man and animals is unclear, there is evidence to implicate a number of inflammatory cells and mediators.

(1) THE ROLE OF TYPE III HYPERSENSITIVITY IN LPRs

Classically, the LPR was considered to result from a type III hypersensitivity response to allergen (Pepys *et al* 1968). There is evidence to suggest that this mechanism is responsible, in man, for inducing the cutaneous and bronchial LPRs to *Aspergillus* antigens in allergic bronchopulmonary aspergillosis (Pepys *et al* 1968) and in subjects with non atopic extrinsic allergic alveolitis, which have specific precipitins but no IgE (Warren *et al* 1977).

However, alternative mechanisms are believed to induce LPRs to other antigens, and to cause LPRs in subjects which have no detectable precipitins (Booij-Noord *et al* 1971; Taylor and Shivalkar 1971; Robertson *et al* 1974). As a consequence, Pelikan (1990) emphasised that, where possible, a distinction should be made between a LPR and a Type III hypersensitivity response.

In horses the late phase dermal response to *M.faeni* and *A.fumigatus* antigens was shown histologically to be consistent with type III hypersensitivity (Halliwell *et al* 1979; McPherson *et al* 1979; Beech and Gunson 1981) and it is possible that the late phase pulmonary response

which follows antigen inhalation challenges and NC may also be mediated by this mechanism (McPherson and Thomson 1983).

(2) THE ROLE OF IgE, MAST CELLS AND BASOPHILS IN THE PATHOGENESIS OF LPRs

Considerable evidence indicates that IgE and mast cells and/or basophils are important in the pathogenesis of LPRs.

The release of mast cell and/or basophil mediators during the LPR to cutaneous (Shalit *et al* 1988), bronchial (Durham *et al* 1984) and nasal (Iliopolous *et al* 1990) allergen challenges and the ability of these mediators to recruit, prime and activate neutrophils, macrophages and eosinophils (reviewed by Johnston and Holgate 1990) is evidence for mast cell and/or basophil involvement in human LPRs.

Furthermore, LPRs may be induced solely by challenge with mast cell and basophil secretagogues, including $F(ab)_2$ anti-IgE and compound 48/80, or by isolated mast cell granules or granule contents (Dolovich *et al* 1973; Solley *et al* 1976; Umemoto *et al* 1976; Lemanske *et al* 1981-1982). The LPR which follows aeroallergen challenge in rabbits was also shown to be IgE and mast cell dependent (Behrens *et al* 1984).

Human asthmatics which showed LPRs following allergen inhalation challenges had higher levels of allergen specific IgE within their respiratory secretions than those which did not show LPRs (Crimi *et al* 1989), suggesting involvement of IgE in the LPR of human asthma. IgE molecules could induce LPRs by interacting with Fc_E receptors on macrophages, lymphocytes, eosinophils, mast cells or basophils (Rossi *et al* 1991).

An increased interest in the role of mast cells and basophils in LPRs has followed the discovery of histamine releasing factors (HRFs) and histamine releasing inhibitory factors

(HRIFs). These are a heterogeneous group of cytokines produced by inflammatory cells which interact with a special type of IgE molecule, designated IgE+, present on mast cells and basophils of atopic individuals, resulting in non cytotoxic degranulation of these cells (reviewed by Lichtenstein 1988, Kaliner 1989). These are further discussed in Chapter 5.

Evidence against significant mast cell involvement in the bronchial LPR of human asthma includes the failure of potent mast cell stabilisers, including B_2-adrenoceptors, to block the late phase pulmonary inflammatory response of human asthma (reviewed by Barnes 1989). Furthermore, corticosteroids, which have no detectable direct action on mediator release from human lung mast cells (Schleimer *et al* 1983) are highly effective in preventing LPRs in human asthmatics (Cockcroft and Murdock 1987; Dutoit *et al* 1987; Kerrebijn *et al* 1987). Corticosteroids also effectively alleviate the symptoms and reduce NSBHR in COPD affected horses (Klein and Deegen 1985), suggesting that, if corticosteroids have no direct effect on equine mast cells, mast cells do not have an important role in the LPR of equine COPD. However, since corticosteroids effectively inhibit mediator release from basophils (Lichtenstein and MacGlashan 1986), these findings do not preclude involvement of the basophil in bronchial LPRs to allergen.

The potential role of mast cells and basophils in the pathogenesis of equine COPD was further investigated in this study and is reviewed in Chapter 5.

(3) THE ROLE OF LYMPHOCYTES IN THE PATHOGENESIS OF LPRs

Increasing evidence suggests that activated T lymphocytes have an important role in inducing LPRs, in perpetuating the inflammatory response during ongoing chronic bronchial hypersensitivity and in inducing NSBHR in man and laboratory animals (Gonzalez *et al* 1987; Diaz *et al* 1989; Brusasco *et al* 1990; Kay 1991).

The role of lymphocytes in the pathogenesis of equine COPD was further investigated in this study and is reviewed in Chapter 4.

(4) THE ROLE OF NEUTROPHILS IN THE PATHOGENESIS OF LPRs

Neutrophils have been implicated in ozone induced bronchial hyperreactivity in dogs (Lee *et al* 1977) and in antigen induced bronchial hyperreactivity in rabbits (Chung *et al* 1985). The absence of a detectable bronchoalveolar lavage fluid (BALF) neutrophilia in some human asthmatics during the LPR suggests, however, that neutrophils do not necessarily contribute significantly to the development of the LPR in all human asthmatics (Rossi *et al* 1991).

Whilst marked pulmonary neutrophilia is a characteristic feature of equine COPD (Nicholls 1978; Derksen *et al* 1985B), the role of neutrophils in the pathogenesis of this condition remains unclear.

Winder and Von Fellenberg (1988) suggested that neutrophil derived enzymes, including elastase, collagenase and myeloperoxidase, may cause airway damage in horses with COPD. However, it seems unlikely that neutrophil proteases have a major role in the pathogenesis of equine COPD as pulmonary fibrosis and emphysema are not major features of this condition (Nicholls 1978).

While it was classically considered that extravasated neutrophils disintegrate and liberate proteases, causing pulmonary damage, before being phagocytosed (Hurley 1983), it is now clear that effete, intact neutrophils may undergo apoptosis and be removed by macrophages without resultant pulmonary damage (Haslett *et al* 1989). The state of neutrophil activation appears to be important in determining whether protease release occurs.

It seems likely that the large numbers of effete intrapulmonary neutrophils present in horses with COPD are removed by apoptosis without release of significant quantities of proteases.

(5) THE ROLE OF EOSINOPHILS IN THE PATHOGENESIS OF LPRs

There is considerable evidence to suggest that the eosinophil is perhaps the most important proinflammatory cell in human asthma (reviewed by Kay 1988).

The finding that human asthmatics which showed LPRs following AICs had an earlier onset bronchoalveolar lavage eosinophilia (Rossi *et al* 1991) and had increased BALF levels of eosinophilic derived cationic protein (De Monchy *et al* 1985), compared with those showing only ERs, was taken as evidence for involvement of eosinophils in the development of bronchial LPRs in human asthmatics.

While a small proportion of horses with COPD show pulmonary eosinophilia (Derksen *et al* 1985B; Grammel 1989; Kaup *et al* 1990A; Fogarty *pers comm* 1991), the significance of this finding is unclear. Grammel (1989) showed that COPD affected horses, given pretreatment with the platelet activating factor (PAF) antagonist WEB 2086 prior to NC, showed reduced BALF eosinophil ratios compared with untreated horses, while the clinical severity of their COPD and their BALF neutrophil ratios were unaffected. It was concluded that eosinophils do not have a major role in the pathogenesis of equine COPD.

INFLAMMATORY MEDIATOR STUDIES

It is unlikely that the entire pulmonary inflammatory response in horses with COPD could be attributed to the action of a single mediator, the combined effects of several inflammatory mediators and inflammatory cells being more likely.

Platelet activating factor (PAF), a mediator released from numerous cells including basophils, mast cells, eosinophils, neutrophils, platelets, macrophages, monocytes and endothelial cells (reviewed by Barnes 1991), may have a role in the pathogenesis of pulmonary inflammation in other species (Morley *et al* 1984; Page *et al* 1984). However, as pretreatment with a PAF antagonist did not prevent the development of COPD in asymptomatic COPD affected horses following NC, PAF was considered unlikely to have a major role in the pathogenesis of equine COPD (Grammel 1989).

Gray *et al* (1989) demonstrated that NC increased the concentration of the arachadonic acid metabolite, thromboxane B_2, in the plasma of COPD affected horses, while plasma PGI_2 and PGD_2 concentrations were unaffected by this challenge. However, as pretreatment of the horses with the cyclooxygenase inhibitor, flunixin meglumine, prevented this challenge induced increase in thromboxane B_2, but did not prevent airway obstruction and bronchial hyperreactivity it was concluded that thromboxane B_2 does not have an important role in the development of airway obstruction and bronchial hyperreactivity in horses with COPD.

Watson *et al* (1990) demonstrated that, while prostaglandins were not detectable in BALF from 3 control horses, BALF from all 3 COPD affected horses contained PGE_2 and BALF from one COPD affected horse contained immunoreactive PGF.

The potential role of histamine, which was further investigated in this study, in the pathogenesis of equine COPD is reviewed in Chapter 5.

PATHOLOGY OF EQUINE COPD

While the lungs of asymptomatic horses and those with short duration COPD may appear macroscopically normal, those of severely affected cases may fail to deflate following opening of the thorax (Nicholls 1978).

Various changes have been observed in the major airways of horses with COPD using light and electron microscopy, however these were considered to be focal and non specific (Kaup *et al* 1990A).

The primary histological lesion characterising equine COPD is a chronic diffuse bronchiolitis, with ciliary abnormalities, loss of ciliated epithelial cells, epithelial metaplasia and hyperplasia, goblet cell metaplasia, Clara cell degeneration and degranulation, peribronchial cellular infiltration with neutrophils, lymphocytes, mast cells, eosinophils and plasma cells, airway smooth muscle hypertrophy and the presence of inflammatory exudate

and excess mucus in the airway lumina (Thurlbeck and Lowell 1964; Gerber 1973; Nicholls 1978; Jubb and Kennedy 1985; Winder and Von Fellenberg 1986; Kaup *et al* 1990A & B).

Immunofluorescent studies demonstrate that horses with COPD have increased numbers of perivascular and peribronchiolar lymphocytes which bear either IgA, IgG or no ıg, with severely affected horses having large amounts of free intraepithelial IgA and IgG (Winder and Von Fellenberg 1968 & 1988).

The limited histological changes occurring in the alveolar region include necrosis of the type I epithelial cells, type II epithelial transformation, increased numbers of Kohn's pores and alveolar hyperinflation and emphysema (Nicholls 1978; Kaup *et al* 1990B). Emphysema, usually confined to the periphery of the lung, was considered to be a minor feature of COPD which occurred late in the development of the disease. As emphysema was observed predominantly in areas with obstructed bronchioles, it was considered to result from air trapping (Nicholls 1978; Kaup *et al* 1990B). While Nicholls (1978) and Viel (1983) found no pulmonary fibrosis in horses with COPD, Kaup *et al* 1990B found that all horses with severe disease had alveolar fibrosis which they considered commenced in the peribronchiolar area and extended to the alveolar septae.

The pathological features of equine COPD were considered to bear no resemblance to those of human chronic bronchitis or human emphysema (Nicholls 1978).

While Kaup *et al* 1990A demonstrated ultrastructural changes suggestive of an asthmoid reaction in one of 28 COPD affected horses, these changes were also present in one of 8 control horses. Furthermore, widening of the basement membrane, a characteristic feature of human asthma was not observed in any of the horses in this study, suggesting that the pathological features of equine COPD differ from those of human asthma.

A granulomatous interstitial pneumonia similar to human hypersensitivity pneumonitis has been reported in a number of horses with chronic pulmonary disease (Pauli *et al* 1972; Gerber 1973) but it seems likely that these cases represent a different disease process.

PATHOPHYSIOLOGY OF EQUINE COPD

The pulmonary dysfunction and impaired pulmonary gas exchange observed in horses with COPD are due to airway obstruction. The pathophysiology of airway obstruction in the horse was reviewed by Robinson and Sorenson (1978) and thus will be discussed only briefly.

Bronchial and bronchiolar obstruction may result from reflex neural bronchoconstriction and from structural airway changes including luminal mucus plugging, mural oedema, cellular infiltration and, in severe cases, intrathoracic airway collapse during expiration (Fischer 1980). Reflex neural bronchoconstriction is considered to be predominantly a consequence of increased parasympathetic activity mediated by the vagus nerve and/or hyperresponsiveness to acetylcholine (Armstrong *et al* 1986; Scott *et al* 1988). While horses with symptomatic COPD may have increased numbers of alpha receptors and/or increased alpha receptor activity compared with control horses, the contribution of alpha receptor activity to the bronchospasm was considered to be minimal (Armstrong *et al* 1986; Scott *et al* 1988).

The effects of airway obstruction on pulmonary mechanics and pulmonary gas exchange in horses with COPD are reviewed in Chapter 2.

In COPD affected horses, hypoxaemia resulting from pulmonary dysfunction results in pulmonary hypertension and increased right ventricular workload (Dixon 1978). However, while cor pulmonale has been reported in a small number of horses with COPD (Salutini 1959), this is an uncommon finding even in horses with chronic severe COPD (Dixon *et al* 1982).

CLINICAL MANIFESTATIONS OF EQUINE COPD

Depending on the severity of the pulmonary dysfunction, some horses with COPD show no detectable clinical signs, while others may show exercise intolerance, coughing, nasal discharge, tachypnoea, hyperpnoea, dyspnoea, exaggerated expiratory abdominal muscular contraction ('heaving'), hypertrophy of the external abdominal musculature ('heave line') and flaring of the nostrils (McPherson and Thomson 1983).

CLINICAL AND LABORATORY DIAGNOSIS OF COPD

The various clinical and laboratory methods employed in the diagnosis of equine COPD are reviewed in Chapter 2.

CHAPTER 2

THE RESPONSES OF CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AFFECTED HORSES TO ANTIGEN INHALATION CHALLENGES WITH *MICROPOLYSPORA FAENI, ASPERGILLUS FUMIGATUS* AND *THERMOACTINOMYCES VULGARIS.*

SUMMARY

To investigate the aetiology of equine chronic obstructive pulmonary disease (COPD), 8 control and 8 asymptomatic COPD affected horses were given nebulised inhalation challenges with extracts of *Micropolyspora faeni* (MF), *Aspergillus fumigatus* (AF) and *Thermoactinomyces vulgaris* (TV). Nebulised phosphate buffered saline challenges and exposure to hay and straw i.e. 'natural challenges' (NCs) were performed as negative and positive control challenges, respectively. Responses to the challenges were assessed by clinical, pulmonary mechanics, arterial blood gas tensions, arterial blood pH and bronchoalveolar lavage fluid (BALF) cytological examinations.

Pulmonary disease, similar to naturally occurring COPD, was induced in the COPD affected horses by MF and AF challenges, implicating MF and AF in the aetiology of equine COPD. Control horses were unaffected by these 2 challenges.

While TV challenge did not induce statistically significant changes in either group, it did elicit a neutrophilic pulmonary inflammatory response in 4 COPD affected horses. This was considered to represent non specific pulmonary toxicity, as 2 control horses, which were unaffected by NCs, also showed neutrophilic pulmonary inflammation in response to this

challenge. The experimental TV challenge was therefore considered to differ from the TV challenge which occurs naturally, during exposure to hay and straw.

The absence of pulmonary disease in control horses following MF, AF and NC challenges suggested that equine COPD is a pulmonary hypersensitivity, rather than a non specific toxic response.

Examination of BALF cytology was found to be the most sensitive technique for the detection of pulmonary disease induced by these challenges.

INTRODUCTION

An improved understanding of the aetiology of equine COPD, which is currently speculative (reviewed in Chapter 1), is a prerequisite to the selection of more rational managemental approaches for this condition.

ANTIGEN INHALATION CHALLENGE (AIC)

Antigen inhalation challenge (AIC) is considered to be the most useful technique for investigating the aetiology of pulmonary hypersensitivity, as it may yield more valuable information than other, less invasive investigative techniques, such as intradermal antigen testing and *in vitro* antigen induced basophil or mast cell degranulation (Spector and Farr 1974; McPherson *et al* 1979; Spector 1989).

FACTORS AFFECTING THE RESULT OF ANTIGEN INHALATION CHALLENGES

Several factors, besides the reactivity of the subject to the antigen, can influence the outcome of AICs. These include the quantity of antigen administered, the quantity and location of antigen deposited within the respiratory tract, and the rate and mechanism by which the deposited antigen is removed from the lungs.

(A) THE QUANTITY OF ANTIGEN ADMINISTERED

In human studies, allergens are commonly administered in incremental doses from 10^{-6} to 10^{-2} w/v (Anon. 1980; Salvaggio and Hendrick 1989). Administration of excessive quantities of antigen may induce clinically irrelevant, positive responses in normal humans (Townley *et al* 1965; Cavanaugh *et al* 1977).

(B) THE QUANTITY OF ANTIGEN DEPOSITED IN THE RESPIRATORY TRACT AND THE SITE OF PARTICLE DEPOSITION

Only a small fraction of the aerosol delivered to the subject enters the respiratory tact, due to losses into the conducting equipment and losses during exhalation.

On inhalation, particles are likely to be deposited at the site of initial contact with the moist respiratory epithelial lining fluid (Brain *et al* 1989). Physical mechanisms affecting particle motion within the tract include gravitational sedimentation, inertial forces, Brownian motion, electrostatic forces and interception (where particles become deposited as the airway diameter:particle diameter ratio gets progressively smaller) (Brain *et al* 1989).

Antigen is conveniently delivered to the human lung via a mouthpiece. However, as the horse is an obligate nasal breather, antigen is delivered via a facemask or a tracheostoma. If the former route is used, a large proportion of inhaled particles will deposit in the nasal cavities and only a small proportion will reach the small airways, the major anatomical site affected in equine COPD (Nicholls 1978) and the target site for particle deposition in investigations of this condition.

The aerosol particle size is the primary factor affecting the site of particle deposition, although the aerosol water and solute content and the temperature and relative humidity of the air in which the particles are delivered are further variables (Ferron and Gebhart 1988). Aerosol particle size is most usefully defined by the aerodynamic diameter, which takes into account the size, shape and the density of the particles, all of which have significant effects on their deposition patterns. Aerodynamic particle size may be determined using cascade impactors, aerosol centrifuges (Brain *et al* 1989) and laser particle velocimeters (Bouchikhi *et al* 1988). Accurate measurement of aerosol particle size distribution is, however, complex because particle size is frequently dynamic, due to evaporation, hygroscopic growth and agglomeration (Ferron and Gebhart 1988).

Human studies have demonstrated that most particles >8u and virtually all those >15u are deposited within the oropharynx by inertial impaction (Swift 1980), while particles of 5-10u deposit within the bronchi and bronchioles with a diameter greater than 2mm. Smaller particles i.e. those <5u reach the alveoli, where those of 2-5u are deposited by gravity. Particles <1u, being aerodynamically stable, are predominantly exhaled (Clarke 1990).

Aerosol particle size distribution is, in turn, influenced by the viscosity and surface tension of the antigen preparation and the type of nebuliser used (Brain *et al* 1989). An ultrasonic nebuliser, which is widely and effectively used for AICs, and which was used in this study, generates an aerosol when high frequency sound waves, produced by vibrations of a piezoelectric crystal, are focussed on the surface of the liquid (Mercer 1981). Aerosols produced by ultrasonic nebulisers are polydisperse, i.e. they are composed of particles of widely varying size, and produce comparatively lower pulmonary deposition than monodisperse aerosols (Brain *et al* 1989).

Breathing pattern, including tidal volume, lung volume, respiratory rate and flow rate, are additional factors affecting the deposition of inhaled aerosols (Heyder *et al* 1982). The effects of ventilation pattern on the deposition of inhaled particles in excised canine lungs showed that increased ventilatory frequency reduced the deposition of particles, while slow deep ventilation produced uniform deposition throughout the lungs, but with little deposition in the central airways. Rapid shallow ventilation resulted in a highly variable pattern with predominantly large airway deposition, while slow and shallow ventilation enhanced small airway deposition (Valberg *et al* 1982).

Species differences in airway morphology and/or breathing patterns may result in differences in the sites of deposition of inhaled antigens. Such differences may account for the finding that exposure to mouldy hay and straw causes predominantly an alveolitis (extrinsic allergic

alveolitis) in susceptible humans (Newman Taylor 1990) and cattle (Pirie *et al* 1971) but predominantly a bronchiolitis (COPD) in the horse (Nicholls 1978).

(C) REMOVAL OF ANTIGEN FROM THE RESPIRATORY TRACT

The rate and mechanism(s) by which deposited particles are cleared from the respiratory tract also affect the outcome of AICs.

Particle clearance is biphasic. The fast (half life of minutes to hours) and slow (half life of days to thousands of days) phases of clearance are considered to represent tracheobronchial mucociliary clearance and alveolar clearance, respectively (Morrow *et al* 1967; Brain *et al* 1989). Thus the site of particle deposition determines, in part, the mechanism and rate of particle clearance.

Mucociliary clearance may be affected by the number of cilia, the frequency and amplitude of ciliary strokes and mucus rheology (Brain *et al* 1989). In contrast to other species, equine ciliated epithelium, and hence mucociliary clearance mechanisms, extend distally to the terminal bronchioles and occasionally to the bronchoalveolar duct junction (Pirie *et al* 1990). Equine mucociliary clearance may be reduced by pulmonary diseases, including influenza (McChesney 1975; Willoughby and Ecker 1990) and COPD (Drommer *et al* 1986; Torgut and Sasse 1989).

Alveolar clearance involves particle translocation and/or catabolism by alveolar macrophages, movement of free particles into the interstitium and/or lymphatics, particle dissolution (Brain *et al* 1989) and mucociliary clearance.

Despite all of the aforementioned factors which could potentially influence the fate of particles and hence the outcome of AICs, it appears that the pulmonary function disturbances induced in susceptible subjects by inhalation challenges with polydisperse nebulised allergens

are relatively reproducible (Brain *et al* 1989). This suggests that the pulmonary function disturbances are mediated, in part, by reflex bronchoconstriction.

EVALUATING THE RESPONSE TO ANTIGEN INHALATION CHALLENGES

The response to AICs may be monitored using clinical, pulmonary function, arterial blood gas tensions, arterial pH, and respiratory cytology examinations.

(A) CLINICAL EXAMINATION

While clinical examination is a relatively simple, non invasive technique, it is unfortunately relatively insensitive in the detection of equine COPD, with only severely affected horses consistently showing clinical abnormalities (McPherson *et al* 1978; Viel 1983). The clinical signs manifested by horses with COPD were reviewed in Chapter 1.

(B) PULMONARY FUNCTION TESTING

Pulmonary function tests (PFT), first performed in the horse by Amoroso *et al* (1962), have been frequently utilised in the investigation of equine pulmonary dysfunction (Gillespie *et al* 1966; Muylle and Oyaert 1973; McPherson *et al* 1979; Willoughby and McDonell 1979; Derksen *et al* 1982). However, due to lack of patient cooperation, several of the PFTs which are of greatest value in man, including timed forced expiratory volume, closing volume, static compliance and vital capacity, cannot be conducted in animals.

The PFT parameters which have been measured in the horse include, respiratory rate (RR), tidal volume (V_T), minute volume (V_{min}), expiratory and inspiratory flow rates, inspiratory:expiratory ratios, dynamic (Cdyn) and static compliance, total pulmonary resistance (R_L), functional residual capacity, thoracic pressure changes, total lung capacity, nitrogen washout times, physiological dead space, and work and power of breathing (Willoughby and McDonell 1979).

EQUINE PULMONARY MECHANICS TESTING

Many of the PFTs used in the horse are pulmonary mechanics tests i.e. tests which assess the mechanical properties of the lung and the thoracic wall.

(1) Methodology for pulmonary mechanics testing in horses

Most equine pulmonary function studies have utilised integrated pneumotachography, with the horse breathing through a pneumotachograph on an airtight facemask. The pressure gradient across the pneumotachograph, which is linearly proportional to the flow through the pneumotachograph when airflow is laminar (Guyatt 1983), is measured using a differential pressure transducer, yielding expiratory and inspiratory flow data.

Intrathoracic pressure changes are measured using a rapid response manometer, directly via an intrapleural catheter or indirectly via an intraoesophageal balloon, the two techniques being highly correlated (Gillespie *et al* 1966; Willoughby and McDonell 1979). Indirect measurement is more suitable for repeated measurements, but is affected by the intrinsic oesophageal tone, swallowing, coughing, and by balloon location and volume, while pneumothorax, catheter tip blockage by blood or contact with the pleural surfaces and pleuritis are possible complications of the direct method (Derksen and Robinson 1980; Boerma *et al* 1986).

(2) Repeatibility of pulmonary mechanics testing in horses

Perhaps the greatest limitation of pulmonary mechanics testing in the horse is the marked variability in the results obtained, there being considerable variation in the normal values reported by different authors (Derksen *et al* 1982; Stadler and Deegen 1986). While part of this variation may be attributed to differences in methodology, considerable variation is observed with repeated examinations of the same horse, even if tests are conducted under apparently identical conditions.

In order to address this variability, Derksen *et al* (1982) determined the short term (hourly for 6h) and long term (at 2 month intervals on 4 occasions) repeatibility of pulmonary mechanics testing in conscious standing *sedated* ponies. Total pulmonary resistance showed minor short term variations, but varied considerably over the long term, possibly due to variation in functional residual capacity, upon which it is dependent. Respiratory rate, tidal volume, dynamic compliance and quasistatic compliance showed considerable variation over both short and long term periods. Variation in static compliance and pulmonary resistance and the possible effects of inertia, although assumed to be negligible in the calculation of dynamic compliance (Mead and Whittenburger 1957), may have contributed to the observed variation in dynamic compliance. Derksen *et al* (1982) concluded that, while this marked variability makes pulmonary mechanics testing unsuitable for detecting mild or moderate lung disease in individual horses, it is of value when assessing lung function in groups of horses.

(3) Pulmonary mechanics testing in COPD affected horses

Most studies of equine pulmonary mechanics have used horses with longstanding COPD rather than those with early COPD induced by relatively short duration AICs.

Horses with chronic COPD have V_T, RR and Vmin which do not differ consistently from those of controls, but have increased inspiratory and expiratory flow rates (Willoughby and McDonell 1979), enlarged pressure volume loops, increased non elastic work of breathing, increased pulmonary resistance, reduced dynamic compliance and increased mean maximum transpulmonary pressure change (dPtp) (Gillespie *et al* 1966; McPherson *et al* 1978; Willoughby and McDonell 1979).

Viel (1983) considered pulmonary mechanics testing was an insensitive technique, capable of detecting only those horses with severe COPD. Nuytten *et al* (1988) considered pulmonary mechanics testing was poorly tolerated by horses.

OTHER MEANS OF ASSESSING PULMONARY FUNCTION IN THE HORSE

Viel (1983) considered the nitrogen washout test, which determines the efficacy of clearance of nitrogen from the lungs during oxygen breathing, and which showed the best correlation with pulmonary histological changes, to be the most reliable pulmonary function test for the diagnosis of equine COPD.

Nuytten *et al* (1988) suggested that measurement of the pulmonary artery pressure and pulmonary wedge pressure and assessment of the pulmonary driving pressures, i.e. the difference between the pulmonary artery pressure and the pulmonary wedge pressure, was better tolerated than pulmonary mechanics testing and more suitable for the diagnosis and monitoring of COPD and for evaluating the severity of disease. However, as pulmonary wedge pressures were not significantly different in affected horses, measurement of pulmonary artery pressure alone would have provided the same information. Furthermore, as pulmonary artery pressure is largely inversely proportional to PaO_2 (Dixon 1978), measurement of PaO_2, which is more readily achieved, is probably as valuable.

(C) ARTERIAL BLOOD GAS TENSIONS AND ARTERIAL pH

Arterial blood oxygen (PaO_2) and carbon dioxide ($PaCO_2$) determinations provide a convenient and reliable measurement of overall pulmonary ventilation and gas exchange (Willoughby and McDonell 1979). In comparison with pulmonary mechanics measurements, blood gas determinations show little long or short term variation in individual horses (Derksen *et al* 1982).

Horses with COPD usually have normocapnic hypoxaemia and normal arterial pH (Willoughby and McDonell 1979; Nyman *et al* 1991). McPherson *et al* (1978) and Littlejohn (1978) suggested that COPD affected horses had PaO_2 values below 82 Torr and 76 Torr, respectively.

(D) EXAMINATION OF RESPIRATORY FLUID CYTOLOGY

Examination of bronchoalveolar lavage fluid (BALF) cytology is currently considered to be the most sensitive technique for the detection of equine COPD. This technique has made possible the detection of subclinical COPD, which is undetectable using pulmonary mechanics testing or clinical examination (Viel 1983). Furthermore equine BALF cytology and pulmonary histopathological findings are closely correlated (Viel 1983; Fogarty 1990). Bronchoalveolar lavage (BAL), however, induces a pulmonary neutrophilic response in horses which persists at least for 48h (Sweeney *et al* In Press). In man, BAL induces a pulmonary neutrophilia which resolves by 72h (Von Essen *et al* 1991). To avoid the BAL induced pulmonary inflammatory response influencing the outcome of an AIC, prechallenge BALs should be performed at least 72h prior to AIC.

Tracheal aspirate cytological examinations may be used to diagnose COPD. While collection of equine tracheal aspirates is relatively non invasive, examination of tracheal aspirate cytology is less valuable than BALF cytology examination, as it is poorly correlated with BALF cytology (Derksen *et al* 1989) and pulmonary histopathology in horses with pulmonary disease (Larson and Busch 1985).

To investigate the aetiology of equine COPD, control and asymptomatic COPD affected horses were given AICs with *Micropolyspora faeni*, *Aspergillus fumigatus* and *Thermoactinomyces vulgaris*.

MATERIALS AND METHODS

SUBJECTS

Eight control (median age 15.5 years, range 7-25 years; median body weight 577kg, range 212-652kg) and 8 COPD affected (median age 15.5 years, range 6-25 years; median body weight 482kg, range 371-546kg) geldings and mares of mixed breeds were used (Appendix 2.1).

'CONTROLLED ENVIRONMENT'

This refers to a hay and straw free environment which would be expected to contain minimal levels of the allergens implicated in the aetiology of COPD (McPherson *et al* 1979). When possible, it was achieved by keeping the horses continuously at pasture, 500m from the nearest hay and straw. Supplementary feeding, when necessary, was with ensiled grass (Horsehage, Mark Westaway, Paignton).

During winter, or when pasture was unavailable, horses were housed in a separate row of large (4.5m x 4.3m) stables, situated 50m from the nearest hay and straw. To maximise ventilation, the top half door and a back wall vent (40cm x 125cm) were kept permanently open. The airspace of each stable had no direct communication with adjacent stables. Horses were fed ensiled grass and concentrates and were bedded on dry wood shavings or dry shredded newspaper with damp bedding being removed daily.

'NATURAL CHALLENGE' (NC) ENVIRONMENT

Horses were housed in smaller (3.4m x 2.6m) stables which were, owing to the absence of back wall or roof vents, poorly ventilated. The horses were bedded on deep litter straw which had accumulated for several weeks, while other horses were being kept in the stables. They were fed poorly saved hay. The hay and straw used frequently had grossly visible fungal contamination and a mouldy odour.

Microscopic examination of airborne dust collected from NC environments, using a hand held particle sampler (Equigiene, Avon,) revealed large numbers of fungal spores, dust mites and dust mite faeces.

COPD AFFECTED HORSES

These horses had known histories of more than 2 years duration and all suffered from chronic pulmonary disease. Whilst in the NC environment they showed clinical signs of COPD and were thus termed 'symptomatic'. The time of onset, severity and range of clinical signs following NC differed among the horses but tended to be consistent for each individual subject. All COPD affected horses showed clinical disease within 5h of NC, including *some* of the following signs; coughing, tachypnoea, hyperpnoea, double expiratory lift ('heave'), bilateral mucopurulent nasal discharge and increased and abnormal tracheal and lung sounds. Bronchoscopic examination of horses with 'symptomatic' COPD consistently revealed abnormal accumulations of mucopurulent secretions, containing large numbers of neutrophils (usually >90%), in the horizontal trachea, and swelling and hyperaemia of the respiratory mucosa. In all cases, BALF contained greater than 5% neutrophils.

Previous investigations had also determined that these horses, when 'symptomatic', had increased dPtp and R_L, and decreased Cdyn and PaO_2.

All clinical and laboratory abnormalities reverted to normal when horses with symptomatic COPD were moved to a 'controlled environment' (*vide supra*). Horses tended to have their own individual rates of remission of usually between 2 and 6 weeks, the BALF cytological changes consistently being the last parameter to revert to normal. All COPD affected horses had received multiple alterations of NC and 'controlled', environments and had responded consistently as described above.

Prior to each AIC, all COPD affected horses were confirmed by clinical examination, arterial blood gas tensions and pH analyses and BALF cytology (< 5% neutrophils) to be in

remission. Horses in remission were termed 'asymptomatic'. BALF samples were collected at least 72h prior to AICs to minimise possible iatrogenic effects of prior lavage on the outcome of the AICs (Sweeney *et al* In Press).

CONTROL HORSES

These horses had shown no evidence of respiratory disease for 2 years and were found to be normal on clinical, bronchoscopic, arterial blood gas tensions and pH, pulmonary mechanics and BALF cytology examinations when maintained in either a controlled environment or in a 'natural challenge' environment. In all control horses neutrophils consistently accounted for less than 5% of BALF cells.

MAINTENANCE OF HORSES

All horses were maintained in a controlled environment prior to and during the challenges, except during the NC period. They were given anthelmintics monthly and given tetanus toxoid (Coopers Pitman Moore, Crewe) every 2 years.

ANTIGEN INHALATION CHALLENGES (AIC) AND 'NATURAL CHALLENGES' (NC)

Horses were given separate nebulised AICs with 20 000 protein nitrogen units (pnu) *Aspergillus fumigatus* (AF), 5mg *Thermoactinomyces vulgaris* (TV) or 5mg *Micropolyspora faeni* (MF) (Greer Laboratories, Lenoir, North Carolina, USA) in 20ml phosphate buffered saline (PBS) pH 7.4 (Sigma, Poole). Nebulised inhalation challenge with 20ml PBS acted as a diluent and a protocol control. Solutions were prepared immediately prior to use.

An ultrasonic nebuliser, with a reported average particle size output of 0.5-5u (UltraNeb 65, Devilbiss Health Care, Hounslow), was used to generate the challenge aerosols. The aerosol was delivered over a 5-15min period via a face mask incorporating a one way valve system and inspiratory reservoir bag, to minimise aerosol loss during expiration (Fig. 2.1).

NC, which represented a positive control challenge for the COPD affected horses, was performed by housing horses within a NC environment (*vide supra*). The top door of the box was closed 8h prior to and throughout the 5h duration of this challenge.

Fig. 2.1 Photograph of unsedated horse receiving antigen inhalation challenge, showing ulltrasonic nebuliser, delivery tubing, reservoir bag and airtight face mask with one way valves.

Fig. 2.2 Photograph of standing, unsedated horse during pulmonary mechanics testing, showing airtight facemask and heated pneumotachograph.

MONITORING THE RESPONSE TO CHALLENGES

Clinical examination of the respiratory system, arterial blood gas tensions and pH analyses and pulmonary mechanics testing were performed immediately prior to and at 1.5 and 5h after each challenge. After the 5h examinations, bronchoalveolar lavage (BAL) was performed (*vide infra*).

CLINICAL EXAMINATION

The horses were examined for coughing, nasal discharge, hyperpnoea and dyspnoea. The trachea and lung fields were auscultated, before and after inducing hyperpnoea by occluding the horse's nostrils for 30s to 1min.

ARTERIAL BLOOD COLLECTION AND PaO_2, $PaCO_2$ AND ARTERIAL pH DETERMINATIONS

Carotid arterial blood samples, collected using a 21g 4cm needle into precooled heparinised glass syringes, were stored on ice and analysed, always within 30min of collection, for PaO_2, $PaCO_2$ and arterial pH, using a Corning 168 blood gas analyser (Corning, Halstead), at an altitude of 180m. Blood gas tensions data were corrected to the rectal temperature of the horse.

PULMONARY MECHANICS TESTING

All horses were given 2 training periods prior to the collection of data to familiarise them with the experimental protocol and to minimise anxiety which may influence lung function (Deegen and Klein 1985). All measurements were performed on standing, unsedated horses restrained only with a headcollar. No sedation was administered to the horses prior to or during pulmonary mechanics testing to avoid any drug induced effects on pulmonary function (Reitmeyer *et al* 1986). Two horses that could not be safely restrained by headcollars were excluded from this study.

As some horses became restless during prolonged recording periods, recording was commenced immediately after the horses were connected to the apparatus, and continued until at least 5 consecutive representative, artifact free breaths were recorded.

Respiratory flow was measured using a heated pneumotachograph (No.4, A.Fleisch, Bilthoven, Holland), mounted on an air tight facemask and connected to an electrospirometer (Mercury CS9, Mercury Electronics, Glasgow) which electronically integrated the flow signal to derive inspiratory and expiratory volume data. The electrospirometer incorporated an auto-zero circuit which automatically corrected the baseline at 10min intervals. The dead space of the mask, calculated by the technique of Art and Lekeux (1988) was approximately 0.75l, which was, for most recordings, less than that which significantly affects pulmonary function and gas exchange i.e. <25% tidal volume (Lekeux *et al* 1984). A flexible rubber seal attached to the exterior of the mask and a 65cm x 10cm x 1cm strip of closed cell foam rubber attached to its inner edge made the mask airtight. The face mask was positioned inside the halter, to which it was fastened (Fig. 2.2).

An oesophageal balloon catheter, consisting of a latex condom secured over the end of a polythene catheter (length 250cm, O.D.=6mm, I.D.=4mm, ARCO, Linlithgow) which had a series of spirally arranged holes distally, and a lateral mask catheter (OD=5mm, ID=3mm, ARCO), were connected to opposite ports of the electrospirometer to measure transpulmonary pressure (Ptp). The oesophageal catheter was positioned at the midpoint of the thoracic oesophagus, just behind the heart, at a length from the external nares predetermined by holding the tubing at the side of the horse (Art and Leukeux 1988). The catheter was passed to the same point for each individual horse. The catheter-balloon assembly was filled with 7ml air, this volume being within the range of high compliance of the balloon.

The electronic output from the electrospirometer was directed to a paper trace recorder (Both a M19, Devices, Letchworth Garden City and a Multitrace 4, Lectromed, Welwyn Garden

City, were used) to produce permanent records. The baseline drifts of these recorders were <1% per minute (M19) and <0.5% per minute (Multitrace 4) at the chart speed used in this study. As the frequency responses of these recorders (M19 100Hz, Multitrace 4 40Hz) were more than 10 times the fundamental frequency of equine respiratory patterns, they were considered suitable for this purpose (Guyatt 1983).

Flow, volume and pressure calibrations were made using, respectively, a rotating vane flow meter (Rotameter 2000, G.E.C. Elliot, Croydon), a 2 litre calibrated syringe, and a water manometer. All outputs were linear over the working ranges. The frequency response characteristics of the flow and pressure recording systems were phase matched up to 5Hz, using standard techniques (Macklem 1974).

Measurement was performed at an altitude of 170m above sea level. All electronic measuring and recording equipment was allowed to warm up overnight prior to use.

ANALYSIS OF PULMONARY MECHANICS DATA

Five representative breaths, devoid of artifacts, were analysed for each recording. Respiratory rate (RR), mean tidal volume (V_T), minute volume (Vmin), mean maximum change in transpulmonary pressure (dPtp), dynamic compliance (Cdyn) and 'average' pulmonary flow resistance (R_L) were determined. Cdyn and R_L were calculated by the methods of Amdur and Mead (1958). Pulmonary resistance calculated by this method represents the average inspiratory and expiratory resistance near peak inspiratory and peak expiratory flows, respectively.

COLLECTION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

Transendoscopic (CF-1T10L, Olympus Optical Co., Japan) bronchoalveolar lavage (BAL) was performed under sedation using intravenous 0.01mg/kg detomidine (Domosedan, SmithKline Beecham Animal Health, Tadworth) and 0.01mg/kg butorphanol (Torbugesic, C-

Vet, Bury St.Edmunds). Topical anaesthesia of the tracheal carina and major bronchi (10ml of 0.2% xylocaine, Astra, King's Langley) was used to minimise coughing during bronchoscopy. The horses were restrained using a nose twitch.

The 13mm diameter endoscope, introduced via the rhinopharyngeal route into the larynx and trachea, was passed distally until it 'wedged' in a third or fourth generation bronchus of the accessory lobe of the right lung. 300ml freshly prepared room temperature PBS was instilled via the biopsy channel of the endoscope into the occluded bronchus and immediately aspirated using 60ml plastic syringes. Instillation and recovery of the lavage fluid took less than 45s. The BALF was immediately filtered through a single layer of gauze into a precooled polythene measuring cylinder and maintained on ice until processed, always within 20min of collection.

PROCESSING OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

Total BALF cell counts were determined using a haemacytometer (Improved Neubaeur, Hawksley and Sons, London) and cell viabilities determined using trypan blue exclusion.

The degree of blood contamination in BALF samples was estimated by determining BALF haemoglobin concentrations using reagent strips (Ames Multiple Reagent Strips, Miles Laboratories, Stoke Poges). The strips had a reported sensitivity of 150-620ug/l haemoglobin, which is approximately equivalent to 5-20 intact erythrocytes per microlitre.

Cytospin preparations, prepared by centrifuging 100ul BALF at 400rpm for 5min (Shandon Elliott, Camberley), were air dried and fixed using a water soluble aerosol fixative (Smear fix, Vale Laboratories, London).

Differential counts of 300 BALF cells were made on Leishman's (Appendix 2.2) stained preparations. Several preparations were also stained with toluidine blue (Appendix 2.2), Giemsa (Appendix 2.2), Perl's stain (Perl 1867) and periodic acid Schiff (McManus 1946).

Absolute BALF cell counts were determined for each cell type by multiplying the cell ratio (%) by the total BALF cell count/100.

As total and absolute BALF cell counts are not only dependent on the cell count of the fluid lining the airways, termed the pulmonary epithelial lining fluid (PELF), but also on the variable volume of PELF recovered by BALs, comparison of total and absolute PELF cell counts is more valuable than comparison of total and absolute BALF cell counts. Total and absolute PELF cell counts were calculated from total and absolute BALF cell counts using the urea dilution technique reported by Rennard *et al* (1986). This technique is reviewed in Chapter 7. Plasma and BALF urea concentrations were determined as described in Chapter 7.

In addition to employing the urea dilution technique, the variable dilution which occurs during BAL was corrected for by determining the albumen adjusted BALF cell counts. This technique is reviewed in Chapter 7. Plasma and BALF albumen concentrations were determined as described in Chapter 7.

The remainder of the BALF was immediately centrifuged at 1500*g* at 4^{O}C for 5min (MSE Chilspin 2, Fisons, Crawley) and the supernatant stored at -20^{O}C.

STATISTICAL ANALYSES

Cell counts, cell ratios, BALF volumes and pulmonary mechanics data were non normally distributed. Paired and unpaired analyses of these data were performed using, respectively, the Wilcoxon Rank Sum test and the Mann Whitney test, while correlations were performed using the Spearman Rank Sign Test.

Arterial blood gas tensions and arterial pH data were normally distributed. Paired and unpaired analyses of these data were performed using the paired and the two sample T test respectively.

The effects of each AIC and of NC on arterial blood gas tensions and pH and on pulmonary mechanics were determined using both within and between group analyses.

Within group analyses were performed by comparing the changes which occurred in each parameter during the challenge with those occurring during the PBS (negative control) challenge. Thus the differences between measurements at 0h and 1.5h, between 0h and 5h and between 1.5 and 5h for each challenge were compared with the corresponding changes for the PBS challenge.

Within group analysis of the PBS challenge was achieved by direct comparison of the actual data recorded during this challenge, rather than by comparisons of their changes.

Between group analyses, performed for all challenges, involved comparison of the changes occurring in the control group with those occurring in the COPD affected group.

All statistical analyses was performed using Minitab (Minitab Inc., Pennsylvania, USA), assuming a significance level of 5%.

RESULTS

CLINICAL EXAMINATION FINDINGS

No detectable clinical pulmonary dysfunction was induced in the control group (Appendix 2.3) by any of the challenges, nor in the COPD affected group by the PBS challenge.

Two COPD affected horses became hyperpnoeic at 1.5 and 5h following AF and MF challenges, at 5h following TV challenge and at 1.5h after NC. At 5h following NC, 7 of the 8 COPD affected horses showed exaggerated expiratory effort ('heaving').

At 5h following NC, auscultation of the lung fields and distal cervical trachea, at rest and after forced breathing, revealed increased breath sounds in 3 COPD affected horses, although no adventitious sounds were evident.

EXAMINATION OF BALF

BAL was satisfactorily accomplished in all cases. All horses coughed several times as the endoscope tip passed from the trachea into the wedge site.

Most samples contained either a 'trace' or a 'small' amount of haemoglobin, and BALF cell viability always exceeded 90%.

RECOVERY OF BALF

BALF recovery was not significantly affected by any of the challenges and was not significantly different between the two groups (Table 2.1, Appendix 2.4).

Table 2.1 The percentage recovery of BALF from control (CONT) (n=8) and COPD affected (n=8) horses at 5h following AICs with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	CONTROL	COPD
PBS	32.7 (12.3-54.0)	19.2 (9.0-35.3)
AF	34.0 (13.7-45.0)	31.0 (13.3-53.3)
TV	39.7 (16.0-45.0)	30.5 (23.0-48.0)
MF	30.5 (17.7-42.3)	29.3 (18.0-37.7)
NC	45.0 (12.7-50.0)	38.3 (13.0-60.0)

BRONCHOALVEOLAR LAVAGE FLUID (BALF) CYTOLOGY

Examination of BALF revealed neutrophils, macrophages, lymphocytes, ciliated columnar epithelial cells, non ciliated cuboidal epithelial cells, Clara cells, lymphocytes, plasma cells, eosinophils, mast cells, 'basophiloid cells' and erythrocytes.

Neutrophils (Fig. 2.3), which exhibited varying maturity, as indicated by their varied lobulation, were usually readily identifiable. However, following NC, BALF from several horses with COPD contained numerous small, intensely basophilic, pyknotic neutrophils, which required careful differentiation from lymphocytes. Toxic degeneration of BALF neutrophils was not observed.

Macrophages (Fig. 2.3) were readily recognised when large and vacuolated, however differentiation of small macrophages and large lymphocytes was found difficult in some instances.

Macrophages were of varied size and morphology, with large multinucleate macrophages, mitotic forms and occasional haemosiderophages (macrophages containing haemosiderin) being identified in both control and COPD affected horses.

Many large macrophages had 'foamy cytoplasm'. Occasionally macrophages were found to contain phagocytosed lymphocytes, neutrophils, fungal spores and smaller, unidentified particles.

Lymphocytes (Fig. 2.3) were predominantly small cells with scant cytoplasm. Many lymphocytes contained approximately 5-15 small intracytoplasmic granules.

Cells resembling atypical lymphocytes, which have been described in other species (Haslam *et al* 1987), i.e. showing marked irregular invagination of the nucleus, were apparent in some BALF samples from COPD affected horses following NC (Fig. 2.3).

Occasional plasma cells were observed. They were, for the purposes of this study, counted as lymphocytes.

Epithelial cells (Fig. 2.3). Ciliated columnar and non ciliated columnar and cuboidal epithelial cells were commonly observed in clumps, when they were readily identified. Single non ciliated epithelial cells, however, were considered difficult to differentiate from macrophages.

Clara cells (Fig. 2.3) were frequently observed in epithelial clumps in association with ciliated epithelial cells. The prominent, large, variably sized basophilic intracytoplasmic granules made these cells easily recognisable.

No rounded clusters of ciliated epithelial cells similar to creola bodies (Johnston and Frable 1979) were observed.

Mast cells (Fig. 2.3) were more readily identified when stained with toluidine blue and Leishman's than with Giemsa.

Mast cells exhibited varying degrees of degranulation. Occasional cells were observed with eosinophilic cytoplasm, similar to that of mast cells, but without intracytoplasmic granules. These were possibly fully degranulated mast cells.

'Basophiloid cells' (Fig. 2.3). These uncommonly recognised cells showed some similarities to mast cells but had considerably larger and more densely staining cytoplasmic granules. In most instances the prominent granules prevented visualisation of the internal cell structure. The identity of these cells was not known.

'Total basophilic cells'. This was a classification which, for the purposes of this study, included both mast cells and 'basophiloid cells'.

Eosinophils were readily identified by their large eosinophilic cytoplasmic granules.

Most BALF preparations from COPD affected horses contained strings of material which appeared basophilic when stained with Leishman's, and which stained positively with periodic acid Schiff. This was considered to be mucus. Interestingly, a large proportion of the cells present within this material were neutrophils, while others (Fig. 2.3) resembled fully granulated mast cells.

Fig. 2.3 Photomicrographs of BALF cells.

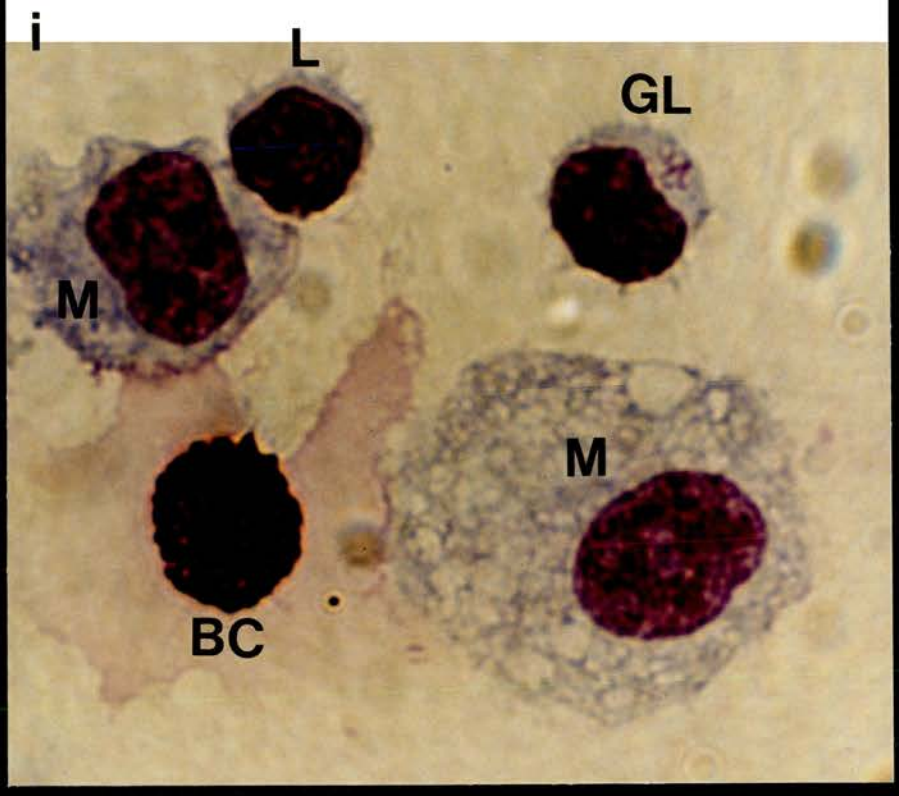
(i) Two macrophages (M), a lymphocyte (L), a granulated lymphocyte (GL) and a 'basophiloid cell' (BC), x3500.

(ii) Two macrophages (M), a neutrophil (N) and two mast cells (MA), x1400.

(iii) Bronchial epithelial cell clump, containing ciliated columnar epithelial cells (C) and a Clara cell (CL), x1400.

(iv) An 'atypical lymphocyte' (AL), showing marked invagination of the nuclear membrane, x1400.

(v) Granular cells (G) resembling 'basophiloid cells' or fully granulated mast cells, present in mucus, x1400.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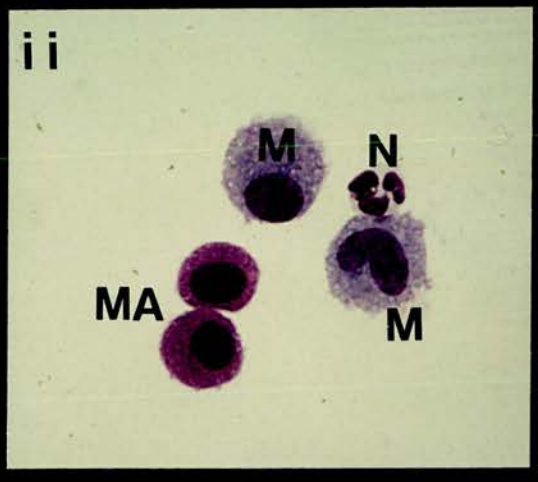
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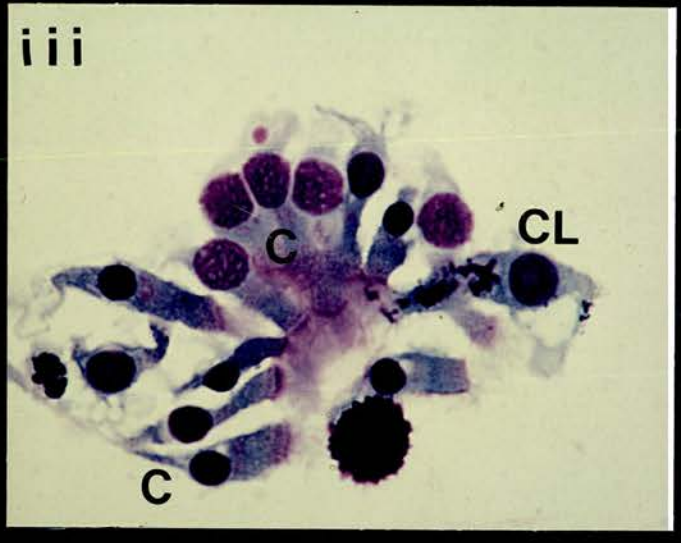
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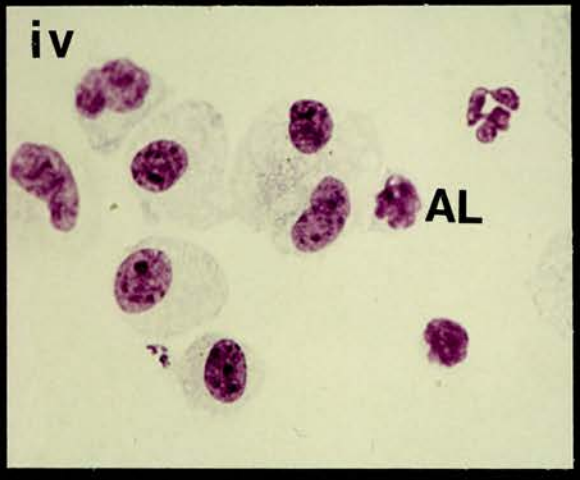
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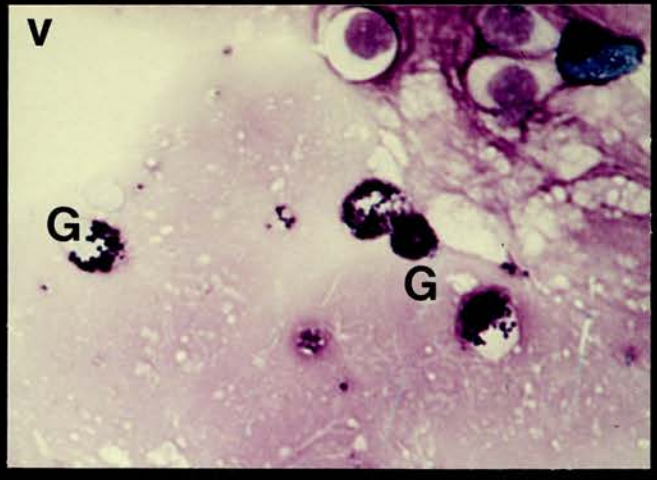
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TOTAL BALF CELL COUNTS

Coefficients of variation for total BALF cell counts, determined by performing 8 counts on 2 BALF samples, were 8.5% and 17.7%.

After PBS challenge, COPD affected horses had significantly lower total BALF cell counts than control horses ($p<0.05$) (Table 2.2, Appendix 2.5). Total BALF cell counts of COPD affected horses were significantly higher after NC than after PBS challenge ($p<0.05$).

Table 2.2 Total BALF cell counts (/ul) from control (n=8) and COPD affected (n=8) horses following inhalation challenges with PBS, AF, MF, TV and following NC (median and range).

CHALLENGE	CONTROL	COPD
PBS	237 (98-1125)	135 (45-200) [1]
AF	245 (60-387)	173 (73-360)
TV	241 (112-640)	159 (75-240)
MF	238 (120-400)	270 (113-680)
NC	335 (175-787)	372 (222-3737) [2]

[1] Significantly lower than for the control group ($p<0.05$).
[2] Significantly higher than after PBS challenge ($p<0.05$).

TOTAL PULMONARY EPITHELIAL LINING FLUID (PELF) CELL COUNTS AND TOTAL ALBUMEN ADJUSTED BALF CELL COUNTS

The plasma and BALF urea (Appendix 2.6) and albumen (Appendix 2.7) concentrations, which were used to determine PELF cell counts and albumen adjusted BALF cell counts, respectively, were not significantly affected by any of the challenges.

Within and between group statistical analyses of the total PELF cell counts yielded the same statistical results as did analyses of the albumen adjusted total BALF cell counts.

TABLE 2.3 Total PELF cell counts ($x10^3$/ul) for control (n=8) and COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC, determined using the urea dilution technique (median and range).

HORSE	PBS	AF	TV	MF	NC
CONTROL	80.2 (10.0-168.5)	33.3 (21.1-83.4)	67.7 (12.3-174.9)	39.7 (23.5-89.3)	51.9 (14.1-236.4)
COPD	31.8 (4.9-110.0)	38.2 (5.9-118.8)	43.2 (1.3-89.9)	64.3 (23.7-279.3)	118.6 * (21.3-637.2)

* Significantly higher than after PBS challenge ($p<0.05$).

TABLE 2.4 Albumen adjusted total BALF cell counts ($x10^3$/ul) for control (n=8) and COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC, determined using the albumen dilution technique (median and range).

HORSE	PBS	AF	TV	MF	NC
CONTROL	124.6 (36.0-621.9)	86.8 (32.4-152.8)	142.9 (47.5-449.3)	72.3 (25.6-157.5)	216.6 (44.9-650.3)
COPD	84.7 (23.7-219.3)	74.0 (19.7-597.2)	110.7 (6.5-319.6)	83.0 (69.6-285.2)	401.0 * (70.0-2125.0)

* Significantly higher than after PBS challenge ($p<0.05$).

NC elicited a significant increase in the total PELF cell counts (Table 2.3, Appendix 2.8) and the albumen adjusted total BALF cell counts (Table 2.4, Appendix 2.9) of COPD affected horses when compared with PBS challenge ($p<0.05$), although they were not significantly different from those of the control group.

DIFFERENTIAL BALF, ABSOLUTE PELF CELL COUNTS AND ALBUMEN ADJUSTED ABSOLUTE BALF CELL COUNTS

Within and between group statistical analyses of the absolute PELF cell counts yielded the same statistical results as did analyses of the albumen adjusted absolute BALF cell counts.

BALF cell ratios (Table 2.5, Fig. 2.4, Appendix 2.10), absolute PELF cell counts (Table 2.6, Appendix 2.11) and albumen adjusted absolute BALF cell counts (Table 2.7, Appendix 2.12) from control horses were not significantly affected by any of the challenges.

BALF cell ratios (Table 2.5, Fig. 2.4, Appendix 2.10), absolute PELF cell counts (Table 2.6, Appendix 2.11) and albumen adjusted absolute BALF cell counts (Table 2.7, Appendix 2.12) from COPD affected horses were not significantly affected by PBS and TV challenges.

When the responses of individual COPD affected horses were examined, 4 COPD affected horses and 2 control horses did, however, show increased BALF neutrophil ratios (i.e. >5%) following TV challenges (Fig. 2.4, Appendix 2.10).

AF challenge significantly increased BALF neutrophil ratios (Table 2.5, Fig. 2.4, Appendix 2.10), absolute PELF neutrophil counts (Table 2.6, Appendix 2.11) and albumen adjusted absolute BALF neutrophil counts (Table 2.7, Appendix 2.12) in COPD affected horses when compared both with PBS challenge and with control horses ($p<0.05$).

In COPD affected horses, MF challenge increased BALF neutrophil ratios (Table 2.5, Fig. 2.4, Appendix 2.10), absolute PELF neutrophil counts (Table 2.6, Appendix 2.11) and albumen adjusted absolute BALF neutrophil counts (Table 2.7, Appendix 2.12), when compared both with PBS challenge ($p<0.05$) and with control horses ($p<0.01$).

After MF challenge, BALF macrophage ratios of COPD affected horses were significantly lower than those of the control horses ($p<0.05$) (Table 2.5, Fig. 2.4, Appendix 2.10).

NC increased BALF neutrophil ratios (Table 2.5, Fig. 2.4, Appendix 2.10), absolute PELF neutrophil counts (Table 2.6, Appendix 2.11) and albumen adjusted absolute BALF neutrophil counts (Table 2.7, Appendix 2.12) in COPD affected horses, when compared with PBS challenge ($p<0.05$) and with the control horses ($p<0.01$).

After NC, absolute PELF macrophage counts, albumen adjusted absolute BALF macrophage counts and BALF macrophage ratios were lower in the COPD affected horses than in the controls ($p<0.05$).

After NC, BALF mast cell and 'total basophilic cell' ratios were lower in the COPD affected horses than in the controls ($p<0.05$).

COPD affected horses had significantly higher BALF neutrophil ratios after NC than after MF and AF ($p<0.05$).

The BALF neutrophil ratios of control and COPD affected horses after NC were significantly correlated with those after AF ($r_s=0.691$, $p<0.01$) and MF ($r_s=0.734$, $p<0.01$) challenges.

TABLE 2.5 BALF cell ratios for (A) control (n=8) and (B) COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC (median and range).

(A) Control horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	1.2 (0.0-4.3)	24.2 (15.3-62.3)	64.3 (37.7-71.7)	0.0 (0.0-1.3)	6.7 (0.0-14.7)	0.2 (0.0-4.0)	7.7 (0.0-15.0)	0.4 (0.0-2.0)
AF	2.5 (0.0-5.7)	35.6 (20.0-66.0)	52.7 (29.3-72.0)	0.0 (0.0-2.3)	5.2 (2.7-8.0)	0.0 (40.0-3.0)	5.8 (2.6-9.0)	0.0 (0.0-9.0)
TV	2.8 (0.3-33.7)	33.7 (15.3-59.7)	47.9 (27.3-77.0)	0.0 (0.0-5.0)	5.5 (1.7-21.0)	0.3 (0.0-1.7)	6.7 (1.7-21.3)	0.5 (0.0-2.3)
MF	1.0 (0.3-4.0)	30.3 (16.3-60.0)	54.4 (30.0-79.3)	0.0 (0.0-4.3)	5.5 (2.3-20.7)	0.3 (0.0-1.0)	5.7 (2.6-21.0)	0.9 (0.0-5.0)
NC	3.0 (0.3-4.0)	33.8 (17.0-49.0)	56.0 (40.7-71.3)	0.2 (0.0-12.3)	5.5 (2.3-8.7)	0.7 (0.0-1.3)	6.5 (2.3-9.3)	0.0 (0.0-1.0)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(TABLE 2.5 Continued)

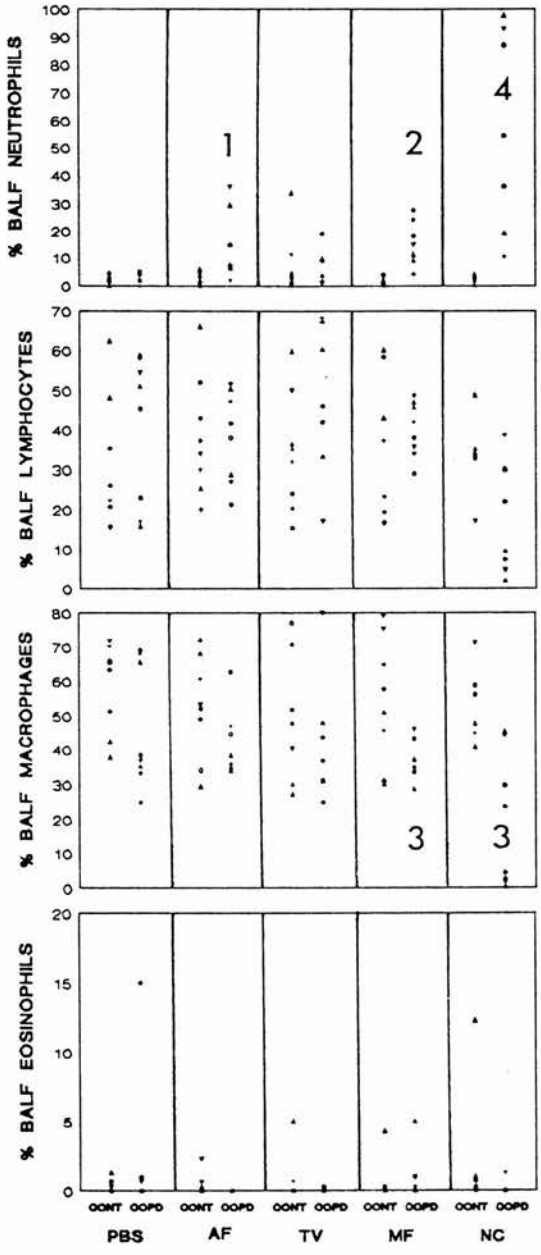
(B) COPD affected horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	4.0 (0.0-5.0)	48.2 (15.7-58.7)	37.5 (25.0-69.0)	0.0 (0.0-15.0)	4.4 (0.0-14.7)	0.0 (0.0-1.0)	4.5 (0.0-15.0)	1.0 (0.0-10.0)
AF	7.2 [1] (2.0-36.0)	39.9 (21.3-51.7)	37.0 (34.0-62.7)	0.0 (0.0-0.0)	4.7 (1.3-9.3)	0.0 (0.0-0.7)	4.7 (1.3-9.3)	0.0 (0.0-9.7)
TV	6.4 (1.0-19.0)	46.0 (17.0-68.3)	34.0 (25.0-80.0)	0.0 (0.0-0.3)	3.3 (0.0-9.0)	1.0 (0.0-2.0)	4.6 (2.0-9.0)	0.2 (0.0-2.3)
MF	12.9 [2] (4.0-21.7)	40.0 (29.0-48.7)	35.9 [3] (28.7-46.0)	0.7 (0.0-5.0)	3.2 (0.0-7.0)	0.2 (0.0-7.0)	5.4 (0.7-8.0)	1.2 (0.0-4.7)
NC	70.6 [4] (10.3-97.7)	15.7 (2.0-38.7)	13.9 [3] (0.3-45.3)	0.0 (0.0-1.3)	1.0 [3] (0.0-5.3)	0.0 (0.0-0.3)	1.0 [5] (0.0-5.3)	0.2 (0.0-1.7)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

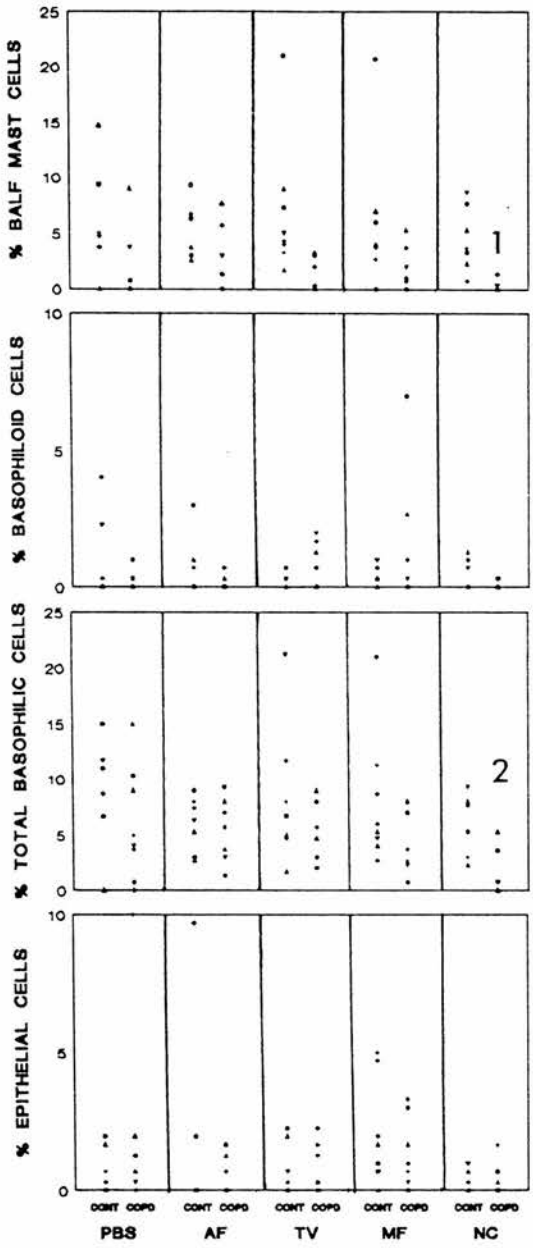
[1] Significantly higher than following PBS challenge and higher than controls ($p<0.05$).
[2] Significantly higher than following PBS challenge ($p<0.05$) and higher than controls ($p<0.01$).
[3] Significantly lower than controls ($p<0.05$).
[4] Significantly higher than following PBS challenge ($p<0.05$) and higher than controls ($p<0.001$).
[5] Significantly lower than controls ($p<0.01$).

Figure 2.4 BALF cell ratios for control (CONT) (n=8) and COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



1 Significantly higher than following PBS challenge and higher than controls ($p<0.05$).
2 Significantly higher than following PBS challenge ($p<0.05$) and higher than controls ($p<0.01$).
3 Significantly lower than controls ($p<0.05$).
4 Significantly higher than following PBS challenge ($p<0.05$) and higher than controls ($p<0.001$).

Figure 2.4 (Continued)



1 Significantly lower than controls ($p<0.05$).
2 Significantly lower than controls ($p<0.01$).

TABLE 2.6 Absolute PELF cell counts ($x10^3$/ul) for (A) control (n=8) and (B) COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC (median and range).

(A) Control horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	1.0 (0.0-4.2)	17.0 (4.8-85.7)	45.4 (4.2-111.2)	0.0 (0.0-0.7)	5.6 (0.0-11.3)	0.1 (0.0-2.8)	7.1 (0.0-11.3)	0.1 (0.0-1.4)
AF	0.8 (0.0-2.6)	14.0 (7.2-26.7)	17.3 (6.7-59.8)	0.0 (0.0-0.5)	2.4 (0.6-3.6)	0.0 (0.0-1.5)	2.5 (0.6-4.6)	0.0 (0.0-1.7)
TV	2.6 (0.1-15.6)	19.8 (3.0-87.5)	39.7 (6.3-70.5)	0.0 (0.0-2.0)	3.5 (0.8-8.8)	0.1 (0.0-3.0)	3.9 (0.8-11.7)	0.2 (0.0-1.2)
MF	0.4 (0.1-2.6)	16.0 (4.5-33.3)	22.3 (8.9-64.9)	0.0 (0.0-1.3)	2.7 (0.7-10.1)	0.1 (0.0-0.9)	3.1 (0.8-10.1)	0.5 (0.0-4.5)
NC	1.1 (0.1-6.4)	21.3 (4.8-66.2)	31.0 (5.7-126.9)	0.4 (0.0-1.7)	2.3 (0.9-33.8)	0.4 (0.0-1.7)	2.9 (1.0-35.5)	0.0 (0.0-0.7)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(TABLE 2.6 Continued)

(B) COPD affected horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	1.0 (0.0-3.4)	14.6 (5.7-27.4)	12.9 (1.8-71.8)	0.0 (0.0-7.1)	1.0 (0.0-16.2)	0.0 (0.0-0.4)	0.9 (0.0-16.5)	0.3 (0.0-2.2)
AF	2.1 [1] (0.8-34.8)	15.3 (1.6-34.1)	15.9 (2.0-45.5)	0.0 (0.0-0.0)	2.0 (0.2-4.4)	0.0 (0.0-0.3)	2.1 (0.2-4.4)	0.0 (0.0-4.4)
TV	1.4 (0.0-11.8)	23.7 (0.2-54.2)	10.9 (1.0-35.8)	0.0 (0.0-0.2)	1.3 (0.0-3.0)	0.1 (0.0-1.2)	1.4 (0.0-4.1)	0.0 (0.0-1.9)
MF	8.1 [2] (2.1-50.3)	20.7 (10.3-106.1)	25.4 (8.8-120.9)	0.4 (0.0-2.0)	1.8 (0.0-5.4)	0.1 (0.0-4.7)	2.3 (1.1-6.3)	0.7 (0.0-4.2)
NC	58.0 [2] (2.8-592.6)	9.3 (4.7-54.3)	9.1 [3] (1.2-53.7)	0.0 (0.0-0.4)	1.1 (0.0-6.0)	0.0 (0.0-1.9)	1.1 (0.0-6.5)	0.4 (0.0-1.3)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

[1] Significantly higher than following PBS challenge ($p<0.05$) and higher than control group ($p<0.05$).
[2] Significantly higher than following PBS challenge ($p<0.05$) and higher than control group ($p<0.01$).
[3] Significantly lower than the control group ($p<0.05$).

TABLE 2.7 Albumen adjusted absolute BALF cell counts ($x10^3$/ul) for (A) control (n=8) and (B) COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC (median and range).

(A) Control horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	3.0 (0.0-8.4)	35.5 (14.1-161.7)	78.0 (15.2-410.5)	0.0 (0.0-0.9)	11.0 (0.0-41.7)	0.1 (0.0-12.4)	15.0 (0.0-41.7)	3.0 (0.0-6.2)
AF	1.7 (0.0-7.6)	27.8 (12.1-79.5)	49.0 (15.4-102.8)	0.0 (0.0-2.0)	4.2 (1.4-9.2)	0.0 (0.0-4.6)	4.2 (1.4-13.8)	0.0 (0.0-2.0)
TV	2.9 (0.5-50.8)	37.5 (15.4-163.0)	76.1 (22.6-215.7)	0.0 (0.0-13.7)	9.0 (1.6-36.0)	0.3 (0.0-3.1)	0.9 (1.6-36.0)	0.5 (0.0-1.9)
MF	0.5 (0.1-4.8)	22.9 (4.9-94.5)	41.8 (14.8-83.6)	0.0 (0.0-6.8)	5.2 (1.4-9.0)	0.1 (0.0-1.1)	5.5 (1.6-9.0)	0.7 (0.0-4.0)
NC	4.0 (0.5-19.5)	62.5 (15.4-212.7)	104.0 (18.3-381.7)	0.4 (0.0-5.5)	14.0 (2.6-28.0)	0.8 (0.0-6.5)	15.0 (3.2-34.5)	0.0 (0.0-2.9)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(TABLE 2.7 Continued)

(B) COPD affected horses.

AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
PBS	2.4 (0.0-11.0)	35.7 (4.0-106.1)	36.3 (14.7-151.3)	0.0 (0.0-19.9)	3.0 (0.0-9.8)	0.0 (0.0-1.0)	3.0 (0.0-10.6)	0.9 (0.0-2.9)
AF	6.7 [1] (1.0-175.0)	31.0 (5.3-171.4)	35.7 (6.7-228.7)	0.0 (0.0-0.0)	5.4 (0.6-22.1)	0.0 (0.0-0.9)	5.5 (0.6-22.1)	0.0 (0.0-12.5)
TV	4.7 (0.1-28.8)	54.7 (1.1-147.0)	40.2 (5.2-117.3)	0.0 (0.0-0.5)	4.4 (0.0-23.3)	0.6 (0.0-2.8)	6.1 (0.1-25.6)	0.1 (0.0-1.7)
MF	13.6 [2] (7.8-30.5)	35.4 (20.2-119.8)	32.4 (22.4-122.6)	0.5 (0.0-3.9)	3.6 (0.0-11.8)	0.1 (0.0-4.9)	5.5 (0.7-13.8)	1.7 (0.0-9.3)
NC	264.0 [2] (8.0-2076.0)	39.7 (15.3-224.9)	32.3 [3] (6.4-240.2)	0.0 (0.0-1.0)	3.2 (0.0-13.3)	0.0 (0.0-6.2)	3.2 (0.0-14.6)	0.1 (0.0-2.9)

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

[1] Significantly higher than following PBS challenge ($p<0.05$) and higher than control group ($p<0.05$).
[2] Significantly higher than following PBS challenge ($p<0.05$) and higher than control group ($p<0.01$).
[3] Significantly lower than the control group ($p<0.05$).

PULMONARY MECHANICS TESTING

These were satisfactorily performed in all cases, without complications. The data are presented in Tables 2.8-2.13, in Figs. 2.5-2.8, and in Appendices 2.13-2.18.

The challenges had no significant effect on pulmonary mechanics of the control horses.

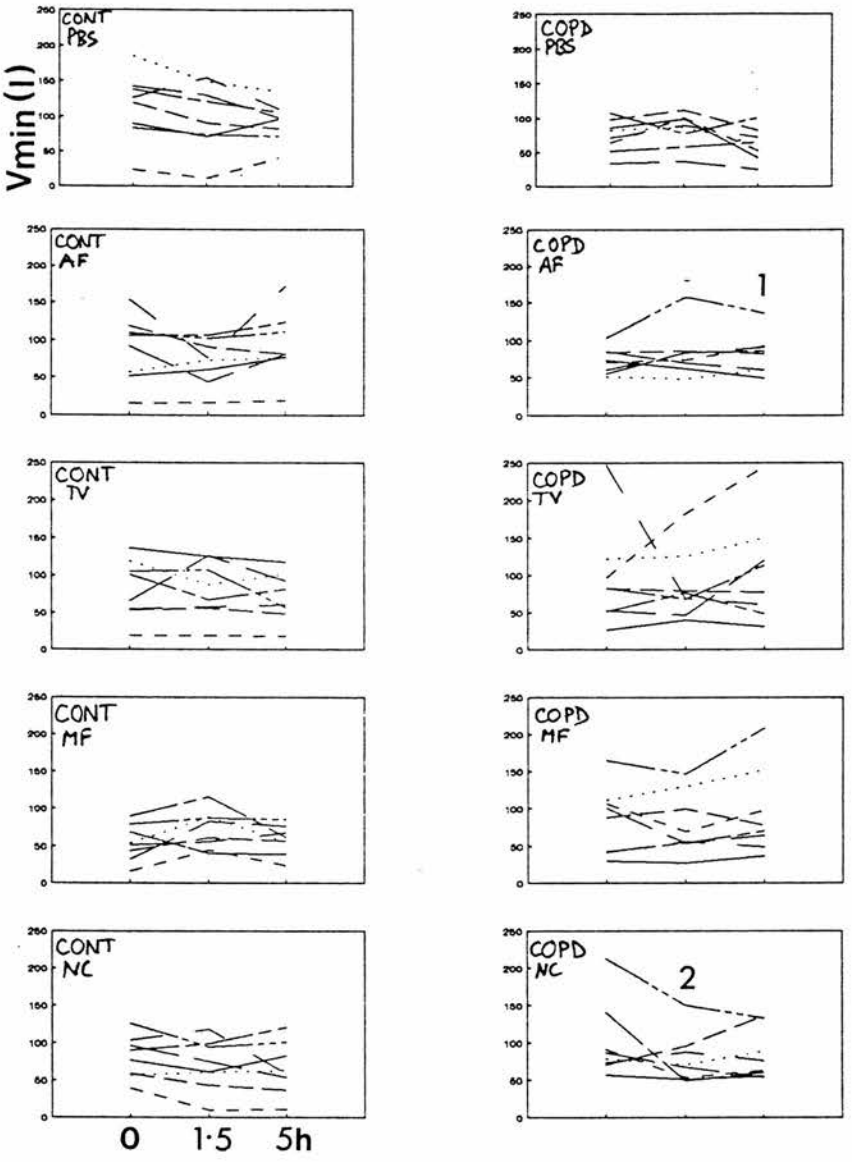
There were no significant differences between pulmonary mechanics of control and COPD affected horses prior to any of the challenges.

PBS, TV and MF challenges had no significant effect on the pulmonary mechanics of the COPD group.

At 5h following AF challenge, COPD affected horses showed increased Vmin ($p<0.05$) compared with PBS challenge although the values were not different from those of the control horses (Table 2.10, Fig. 2.5).

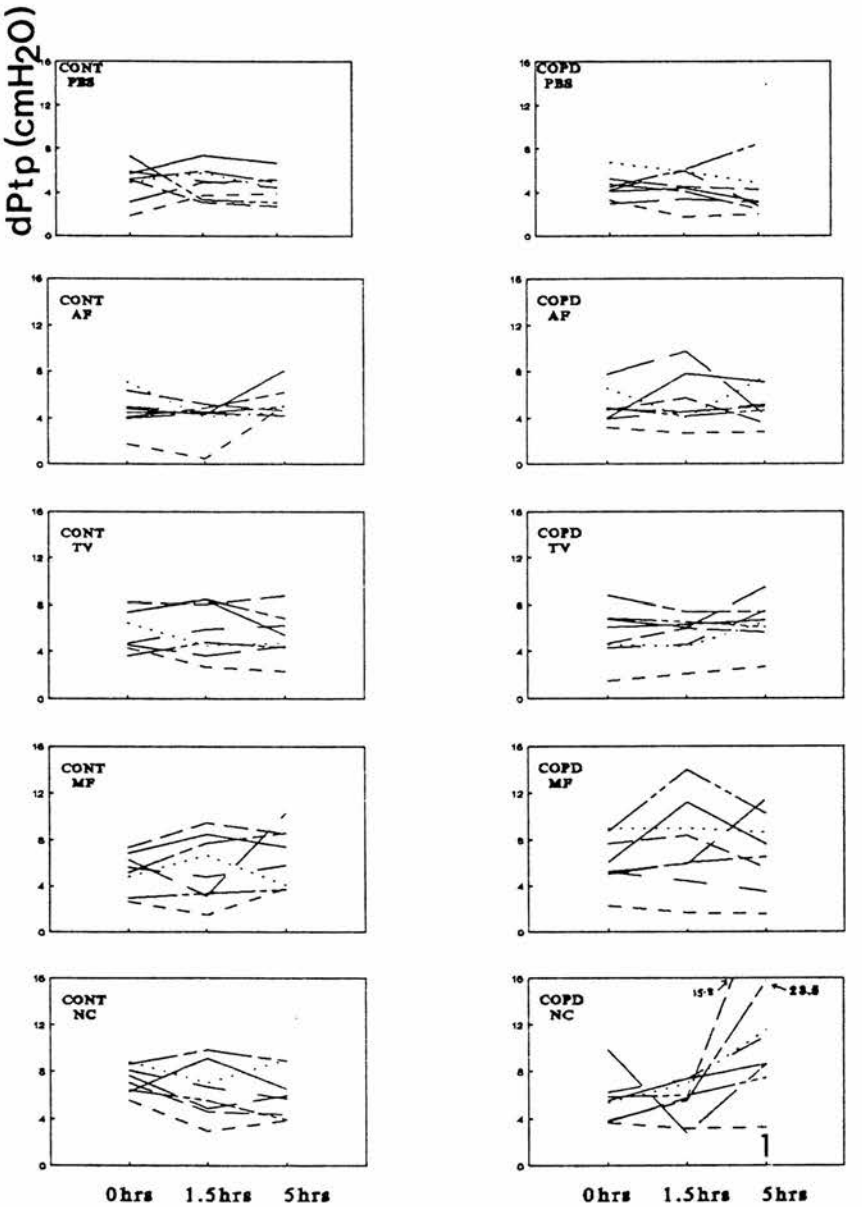
At 1.5h following NC, the COPD affected group had reduced Vmin ($p<0.05$) when compared with PBS challenge although the values were not significantly different from those of the control horses (Table 2.10, Fig. 2.5). At 5h following NC, COPD affected horses showed increased dPtp ($p<0.01$) compared with controls (Table 2.11, Fig. 2.6) and decreased Cdyn compared with PBS challenges ($p<0.05$) (Table 2.12, Fig. 2.7). COPD affected horses showed increased R_L at 1.5 and 5h ($p<0.05$) following NC when compared with PBS challenge, this parameter being also significantly increased when compared with the control group at 5h ($p<0.05$) (Table 2.13, Fig. 2.8).

Figure 2.5 Minute volumes (l) of control (CONT) (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



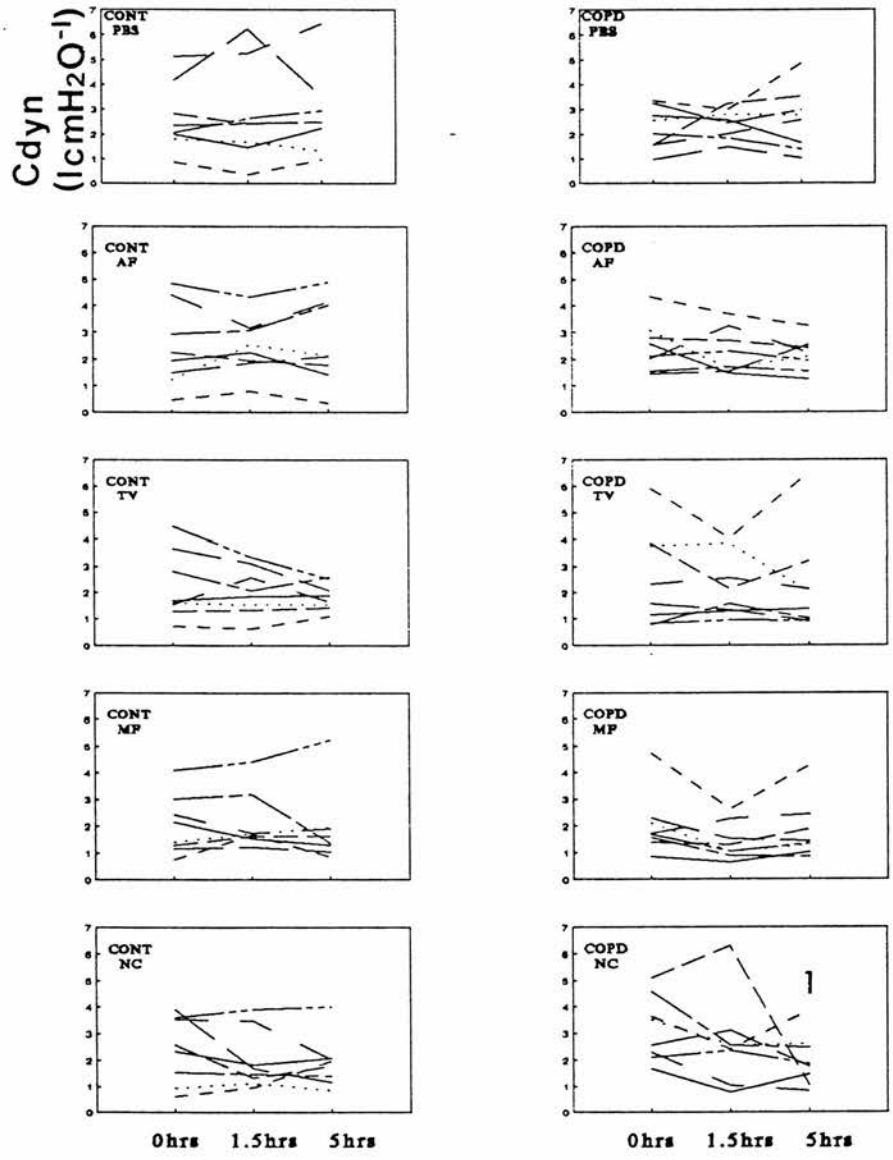
1 Significantly higher than for COPD affected horses at 5h after PBS challenge ($p<0.05$).
2 Significantly lower than for COPD affected horses at 1.5h after PBS challenge ($p<0.05$).

Figure 2.6 Mean maximum changes in transpulmonary pressure (cmH_2O) of control (CONT) (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



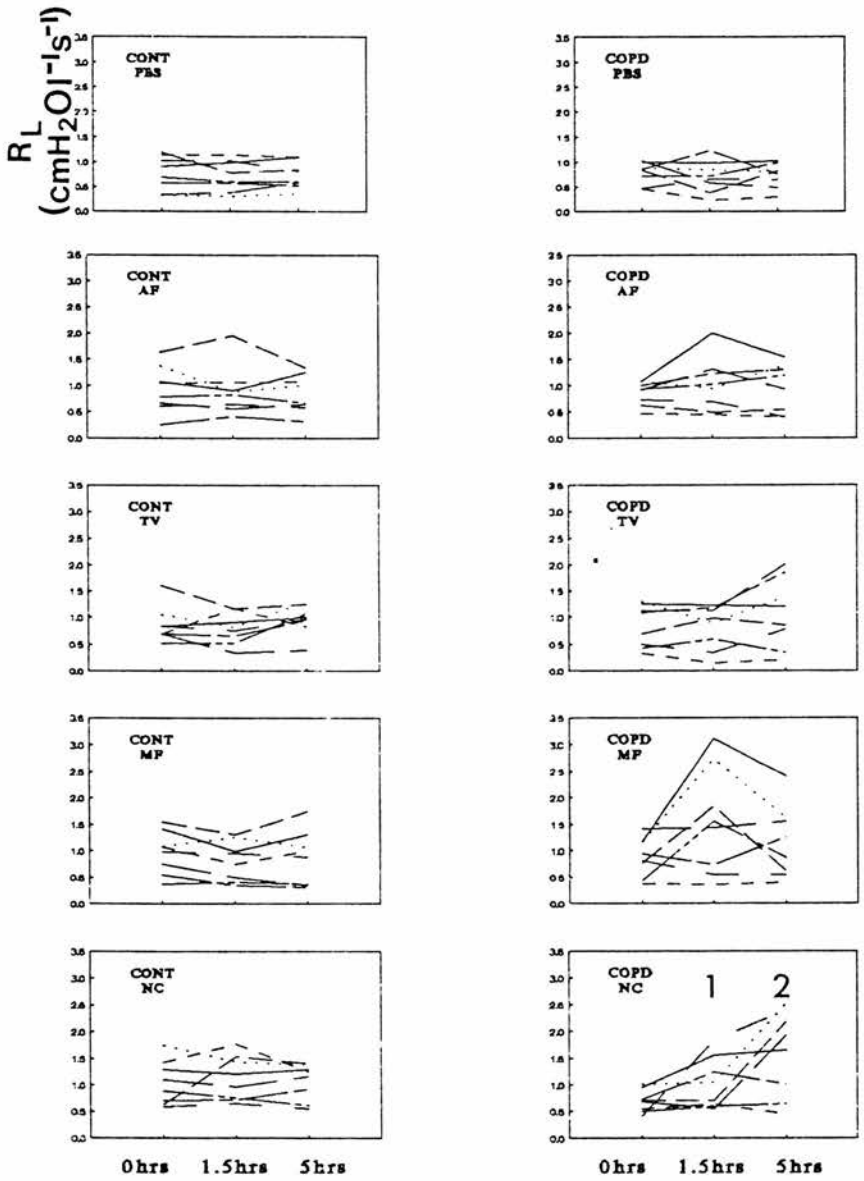
[1] Significantly higher than controls ($p<0.05$).

Figure 2.7 Dynamic compliance values (l/cmH_2O) of control (CONT) (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



[1] Significantly lower than at 5h after PBS challenge ($p<0.05$).

Figure 2.8 Average total pulmonary resistances ($cmH_2O/l/s$) of control (CONT) (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



1 Significantly higher than at 1.5h after PBS challenge ($p<0.05$).
2 Significantly higher than at 5h after PBS challenge and significantly higher than for controls ($p<0.05$).

Table 2.8 Respiratory rates (min^{-1}) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	RESPIRATORY RATE CONTROL	COPD
PBS	0	13.5 (10.7-23.2)	12.0 (7.5-15.0)
	1.5	16.8 (11.8-21.3)	12.2 (9.3-19.7)
	5	15.1 (11.9-21.4)	11.8 (7.2-19.0)
AF	0	12.4 (8.2-28.0)	10.5 (7.5-16.0)
	1.5	12.8 (8.6-19.0)	11.8 (7.9-20.1)
	5	11.8 (10.0-30.0)	13.4 (7.2-17.0)
TV	0	13.6 (6.2-26.0)	13.9 (5.1-33.0)
	1.5	15.0 (6.8-34.3)	13.3 (7.1-19.5)
	5	13.9 (5.1-22.0)	14.0 (5.3-28.4)
MF	0	10.0 (5.1-14.4)	12.0 (6.9-21.0)
	1.5	10.8 (6.9-33.3)	13.0 (4.5-43.0)
	5	10.6 (6.3-21.3)	14.4 (7.2-20.0)
NC	0	12.3 (8.2-26.7)	11.0 (9.0-21.4)
	1.5	10.0 (7.5-19.2)	10.3 (7.2-17.9)
	5	9.0 (6.1-23.3)	15.6 (5.9-33.3)

Table 2.9 Tidal volumes (l) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	TIDAL VOLUME CONTROL	COPD
PBS	0	7.32 (0.57-9.01)	6.95 (4.64-9.34)
	1.5	6.35 (0.46-9.92)	5.87 (3.61-9.38)
	5	5.40 (1.92-7.50)	5.75 (3.14-8.77)
AF	0	6.55 (1.11-10.91)	7.18 (5.15-9.72)
	1.5	6.19 (0.40-9.96)	7.17 (4.77-8.81)
	5	6.53 (0.78-11.03)	6.21 (3.98-8.32)
TV	0	6.97 (0.72-8.55)	5.86 (4.06-7.48)
	1.5	5.55 (0.89-10.15)	5.38 (3.64-7.30)
	5	5.55 (1.69-9.48)	5.68 (3.97-7.55)
MF	0	6.81 (1.33-9.43)	5.74 (4.18-9.55)
	1.5	7.28 (1.36-9.16)	5.89 (1.62-12.15)
	5	6.07 (1.12-8.88)	6.10 (4.25-11.54)
NC	0	6.99 (1.47-9.34)	8.13 (5.10-9.96)
	1.5	6.55 (1.28-9.63)	6.78 (4.75-8.77)
	5	6.84 (1.19-9.90)	5.57 (3.24-9.15)

Table 2.10 Minute volumes (l) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	MINUTE VOLUME CONTROL	COPD
PBS	0	123.1 (24.3-185.6)	77.2 (34.8-107.5)
	1.5	106.1 (11.6-154.5)	87.9 (36.9-111.3)
	5	97.2 (41.0-135.4)	63.4 (25.8-100.8)
AF	0	98.9 (15.3-153.3)	71.9 (50.7-103.6)
	1.5	74.9 (16.6-106.8)	78.6 (48.6-157.6)
	5	81.6 (19.0-172.0)	84.1 (49.3-135.8)[1]
TV	0	83.1 (18.8-136.0)	82.1 (26.6-246.8)
	1.5	77.1 (19.4-126.1)	72.1 (39.5-182.1)
	5	70.9 (17.9-117.0)	94.8 (31.2-244.3)
MF	0	52.9 (16.7-89.6)	94.1 (29.9-164.1)
	1.5	72.2 (40.6-115.7)	62.7 (28.1-146.5)
	5	59.6 (23.9-85.8)	74.3 (36.9-208.9)
NC	0	83.5 (39.2-126.0)	82.9 (56.6-213.1)
	1.5	67.8 (9.6-118.3)	68.9 (49.1-150.0)[2]
	5	61.0 (10.7-120.9)	69.3 (53.5-136.2)

[1] Significantly higher than for COPD affected horses at 5h after PBS challenge ($p<0.05$).
[2] Significantly lower than for COPD affected horses at 1.5h after PBS challenge ($p<0.05$).

Table 2.11 Mean maximum transpulmonary pressure changes (cmH_2O) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	MEAN MAX. TRANSPULMONARY PRESSURE CHANGE CONTROL	COPD
PBS	0	5.20 (1.85-7.33)	4.37 (3.00-6.72)
	1.5	4.97 (3.09-7.37)	4.45 (1.75-6.08)
	5	4.40 (2.71-6.67)	3.12 (2.00-5.47)
AF	0	4.68 (1.77-7.09)	4.77 (3.22-7.71)
	1.5	4.51 (1.51-5.22)	4.58 (2.70-9.68)
	5	4.86 (4.24-8.04)	4.85 (2.81-7.49)
TV	0	5.60 (3.67-8.21)	5.37 (1.51-8.78)
	1.5	5.35 (2.70-8.50)	6.00 (2.09-7.37)
	5	5.07 (2.30-8.75)	6.59 (2.72-9.50)
MF	0	5.40 (2.67-7.32)	5.68 (2.27-9.00)
	1.5	5.74 (1.52-9.46)	7.21 (1.67-14.00)
	5	6.60 (3.69-10.30)	7.07 (1.53-11.55)
NC	0	7.30 (5.55-8.80)	5.43 (3.65-9.84)
	1.5	6.10 (2.95-9.83)	5.87 (2.75-7.37)
	5	5.94 (3.89-9.00)	9.83 (6.26-23.50)[1]

[1] Significantly higher than controls ($p<0.05$).

Table 2.12 Dynamic compliance (l/cmH_2O) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after AICs with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	DYNAMIC COMPLIANCE CONTROL	COPD
PBS	0	2.22 (0.86-5.14)	2.29 (0.98-3.35)
	1.5	2.43 (0.34-6.24)	2.53 (1.48-3.26)
	5	2.50 (0.98-6.45)	2.68 (1.02-4.89)
AF	0	2.11 (0.45-4.85)	2.35 (1.44-4.33)
	1.5	2.41 (0.80-4.33)	2.01 (1.48-3.70)
	5	2.14 (0.35-4.90)	2.19 (1.26-3.26)
TV	0	1.64 (0.73-4.49)	1.95 (0.77-5.90)
	1.5	1.98 (0.62-3.36)	1.87 (0.94-4.04)
	5	1.77 (1.08-2.60)	1.70 (0.80-6.53)
MF	0	1.78 (0.74-4.11)	1.69 (0.84-4.73)
	1.5	1.69 (1.22-4.41)	1.17 (0.64-2.61)
	5	1.49 (0.85-5.23)	1.38 (0.84-4.27)
NC	0	2.47 (0.61-3.90)	3.03 (1.67-5.08)
	1.5	1.58 (0.96-3.91)	2.50 (0.78-6.30)
	5	1.89 (0.86-4.01)	1.82 (0.82-3.87)[1]

[1] Significantly lower than at 5h after PBS challenge ($p<0.05$).

Table 2.13 Average total pulmonary resistance ($cmH_2O/l/s$) of control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (median and range).

CHALLENGE	TIME (h)	PULMONARY RESISTANCE CONTROL	COPD
PBS	0	0.795 (0.330-1.192)	0.847 (0.452-1.020)
	1.5	0.687 (0.305-1.138)	0.684 (0.224-1.229)
	5	0.712 (0.355-1.107)	0.774 (0.288-1.024)
AF	0	0.917 (0.255-1.644)	0.924 (0.460-1.080)
	1.5	0.852 (0.409-1.955)	0.980 (0.448-2.001)
	5	0.837 (0.312-1.335)	1.061 (0.393-1.540)
TV	0	0.762 (0.518-1.600)	0.881 (0.323-1.284)
	1.5	0.779 (0.333-1.161)	0.890 (0.144-1.213)
	5	0.983 (0.393-1.293)	1.015 (0.209-2.010)
MF	0	1.028 (0.373-1.546)	0.876 (0.381-1.412)
	1.5	0.853 (0.350-1.305)	1.494 (0.363-3.114)
	5	0.945 (0.313-1.742)	1.065 (0.397-2.412)
NC	0	0.996 (0.577-1.755)	0.689 (0.416-1.017)
	1.5	1.086 (0.604-1.773)	0.879 (0.541-1.835)[1]
	5	1.206 (0.548-1.402)	1.795 (0.444-2.527)[2]

[1] Significantly higher than at 1.5h after PBS challenge ($p<0.05$).
[2] Significantly higher than at 5h after PBS challenge and significantly higher than for controls ($p<0.05$).

ARTERIAL BLOOD GAS TENSIONS AND pH

Arterial blood samples were obtained in all cases, without complications, with the exception of transient local, self resolving haematomas.

PBS, AF,TV and MF challenges had no significant effect on arterial blood gas tensions and arterial pH of either group (Tables 2.14-2.16, Fig. 2.9, Appendices 2.19-2.21).

COPD affected horses had significantly reduced PaO_2 ($p<0.01$) at 5h following NC when compared with the PBS challenge and with the control horses (Table 2.14, Fig. 2.11).

CORRELATIONS BETWEEN PULMONARY MECHANICS, ARTERIAL BLOOD GAS TENSIONS, ARTERIAL pH AND BALF NEUTROPHIL RATIOS

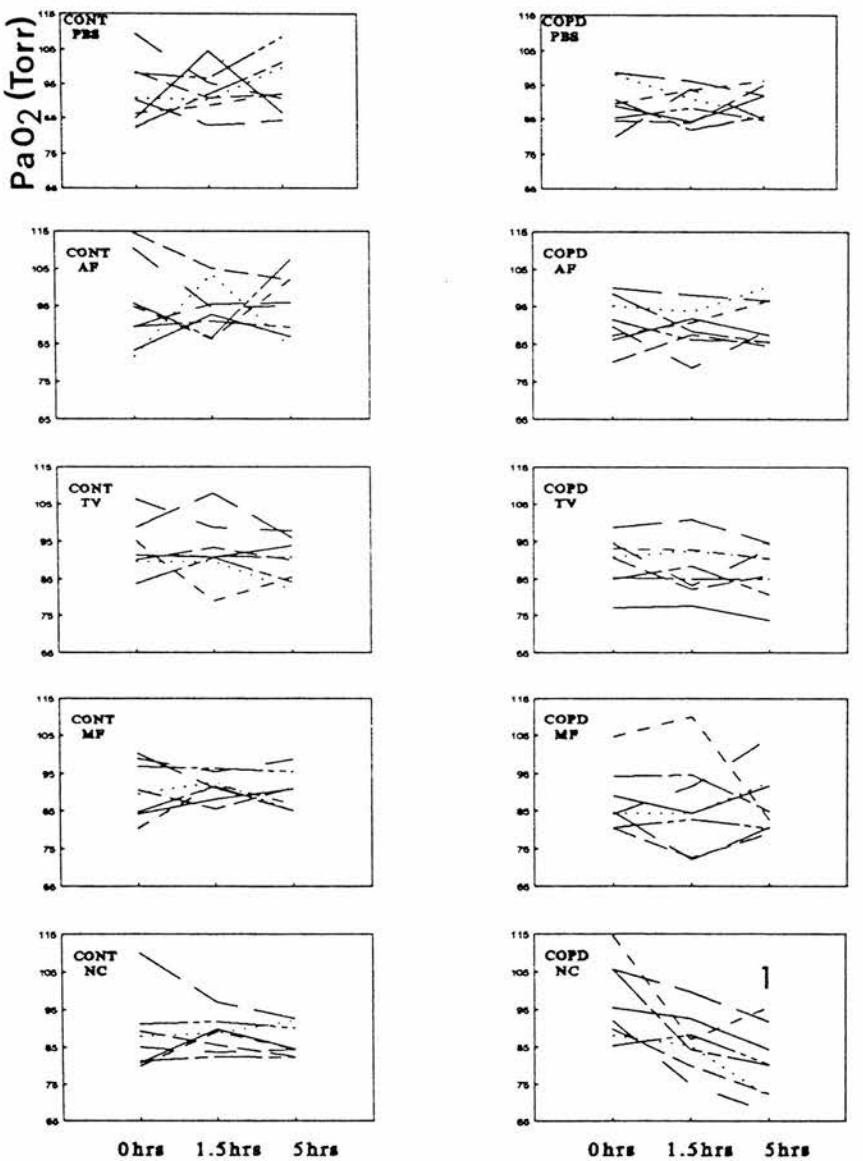
When the data for all the challenges were pooled and analysed, significant correlations were found between the changes in the following pairs of parameters; RR and Vmin, Vmin and V_T, V_T and Cdyn, Vmin and Cdyn, R_L and dPtp, Vmin and PaO_2, (all $p<0.01$) and Vmin and dPtp ($p<0.05$). BALF neutrophil ratios were not significantly correlated with any of these other parameters.

Table 2.14 Arterial O_2 tensions (Torr) for control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (mean±S.D.).

CHALLENGE	TIME (h)	ARTERIAL OXYGEN TENSION CONTROL	COPD
PBS	0	92.6 ±8.9	88.2 ±9.0
	1.5	92.7 ±6.4	89.2 ±5.4
	5	94.4 ±8.2	89.6 ±4.8
AF	0	96.2±14.6	91.0 ±6.6
	1.5	94.4 ±7.0	89.4 ±5.7
	5	95.3 ±8.2	90.7 ±6.2
TV	0	93.3 ±6.8	89.5 ±6.8
	1.5	92.6 ±8.2	87.8 ±7.4
	5	90.0 ±5.7	86.8 ±7.0
MF	0	90.6 ±7.4	87.7 ±8.2
	1.5	91.6 ±3.6	86.5±12.3
	5	89.8 ±5.2	87.1 ±8.7
NC	0	89.4±12.7	97.1±10.4
	1.5	88.5 ±4.7	86.6 ±7.5
	5	86.6 ±4.3	80.5 ±9.9 [1]

[1] Significantly lower than at 5h after PBS challenge and significantly lower than for controls ($p<0.05$).

Figure 2.9 Arterial oxygen tensions (Torr) of control (CONT) (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.



[1] Significantly lower than at 5h after PBS challenge and significantly lower than for controls ($p<0.05$).

Table 2.15 Arterial CO_2 (Torr) tensions for control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (mean±S.D.).

CHALLENGE	TIME (h)	ARTERIAL CARBON DIOXIDE TENSION CONTROL	COPD
PBS	0	38.3 ±3.1	42.0 ±2.3
	1.5	38.9 ±3.1	42.5 ±5.4
	5	39.0 ±1.9	42.1 ±4.8
AF	0	38.7 ±2.9	41.6 ±6.6
	1.5	39.2 ±3.6	42.2 ±5.7
	5	38.8 ±5.5	44.1 ±6.2
TV	0	38.4 ±2.4	41.8 ±6.8
	1.5	36.3 ±3.9	42.9 ±7.4
	5	39.0 ±3.6	40.7 ±7.0
MF	0	38.5 ±4.4	39.3 ±8.2
	1.5	39.8 ±4.3	39.4±12.3
	5	40.0 ±7.0	41.2 ±8.7
NC	0	38.1 ±3.7	42.5±10.4
	1.5	38.1 ±3.6	45.5 ±7.5
	5	37.7 ±4.9	44.1 ±9.9

Table 2.16 Arterial pH values for control (n=8) and COPD affected (n=8) horses immediately prior to (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, MF and TV and following NC (mean±S.D.).

CHALLENGE	TIME (h)	ARTERIAL pH CONTROL	COPD
PBS	0	7.391 ±0.035	7.394 ±0.039
	1.5	7.418 ±0.029	7.389 ±0.024
	5	7.408 ±0.021	7.402 ±0.031
AF	0	7.401 ±0.025	7.386 ±0.030
	1.5	7.395 ±0.023	7.393 ±0.028
	5	7.418 ±0.031	7.395 ±0.050
TV	0	7.422 ±0.045	7.391 ±0.020
	1.5	7.447 ±0.082	7.396 ±0.032
	5	7.399 ±0.024	7.398 ±0.027
MF	0	7.404 ±0.024	7.396 ±0.030
	1.5	7.403 ±0.028	7.403 ±0.030
	5	7.409 ±0.017	7.398 ±0.017
NC	0	7.390 ±0.027	7.378 ±0.026
	1.5	7.385 ±0.047	7.401 ±0.030
	5	7.389 ±0.020	7.403 ±0.025

DISCUSSION

To investigate the aetiology of equine chronic obstructive pulmonary disease (COPD), control and COPD affected horses were given antigen inhalation challenges (AICs) with extracts of *Micropolyspora faeni* (MF), *Aspergillus fumigatus* (AF) and *Thermoactinomyces vulgaris* (TV).

Previously, a similar inhalation challenge study performed by McPherson *et al* (1979) had suggested that MF and AF were common causes of respiratory hypersensitivity in horses affected with COPD, while an unidentified *Actinomycete* was a less common cause. However, *some* of the conclusions of this study may be questioned following the recent finding that horses with COPD, when symptomatic, may show an exaggerated bronchospasm in response to a wide range of inhaled agents such as cold air, histamine, methacholine, inorganic dusts and citric acid, a phenomenon termed non specific bronchial hyperresponsiveness (NSBHR) (Klein 1984; Derksen *et al* 1985A). As a number of the horses used by McPherson *et al* were partially symptomatic prior to antigen challenges, some of the reported positive clinical, pulmonary mechanics and arterial blood gas tensions responses may have been simply due to NSBHR rather than to specific pulmonary hypersensitivity reactions to these antigens.

To eliminate the problem of NSBHR, only asymptomatic COPD affected horses, which do not exhibit NSBHR (Derksen *et al* 1985A), were used in the present study. Thus all horses were shown to be asymptomatic, by clinical, arterial blood gas tensions, arterial pH and bronchoalveolar lavage fluid (BALF) cytological examinations, prior to all challenges.

PHOSPHATE BUFFERED SALINE (PBS) CHALLENGE

Inhalation challenge with nebulised isotonic PBS, which represented a negative diluent and protocol control challenge, did not elicit pulmonary disease in any control or COPD affected horses.

However, after PBS challenge, the control horses had unaccountably higher total BALF cell counts than the COPD affected horses. This finding is unlikely to indicate the presence of pulmonary inflammation in either group as the absolute and total pulmonary epithelial lining fluid (PELF) cell counts of the two groups were not significantly different after this challenge.

NATURAL CHALLENGE (NC)

NC, which was a positive control challenge for the COPD affected horses and a negative control challenge for the control horses, elicited pulmonary disease only in the COPD affected horses as evidenced by changes in clinical, pulmonary mechanic, arterial blood gas tension, arterial pH and BALF and PELF cytology parameters.

Clinical signs consistent with those of COPD (McPherson *et al* 1978) were detected in 7 of 8 COPD affected horses at 5h after NC. One COPD affected horse showed no clinical evidence of COPD after NC, despite having a markedly increased BALF neutrophil ratio (19%).

While clinical evidence of pulmonary dysfunction was most marked at the late phase (i.e. at 5h) after NC, 2 COPD affected horses developed clinically detectable early phase (i.e. at 1.5h) pulmonary dysfunction. This is consistent with previous findings that COPD affected horses may exhibit biphasic responses to NC (Mansmann *et al* 1975; Halliwell *et al* 1979; McPherson *et al* 1979; McPherson and Thomson 1983; Thomson 1989).

All COPD affected horses showed an, often marked, BALF and PELF neutrophilia after NC. This is consistent with the findings of previous BALF cytology studies (Viel 1983; Derksen *et al* 1985B and 1988), with the histopathological classification of COPD as a neutrophilic endobronchiolitis (Nicholls 1978) and with the recent observation that NC elicited pulmonary recruitment of peripheral blood neutrophils (Fairbairn,S. *pers comm* 1991). The BALF neutrophilia induced by NC was found to be the most useful technique for the detection of equine COPD, in agreement with Viel (1983).

NC also significantly increased the total BALF cell counts, albumen adjusted total BALF cell counts and total PELF cell counts of COPD affected horses. In contrast, Derksen *et al* (1985B) found that the total BALF cell count did not increase significantly after NC, but as they collected BALF at a different time after challenge than the present study, this could account for the discrepancy. PELF cell counts and albumen adjusted BALF cell counts were not determined by Derksen *et al* (1985B).

After NC, COPD affected horses showed unexplainable reductions in PELF and albumen adjusted BALF macrophage counts. Similar findings were observed in COPD affected horses after NC in a previous study (Derksen *et al* 1985B), in horses with bacterial pneumonia and pleuropneumonia (Rossier *et al* 1991) and in ozone exposed dogs (Fabbri *et al* 1984) and rabbits (Alpert *et al* 1971).

BALF, albumen adjusted BALF and PELF eosinophil counts were unaffected by NC. In contrast, Derksen *et al* (1985B) found increased BALF eosinophil counts in 3 of 6 ponies at 3-7days after NC. Some horses with longstanding COPD also show increased BALF eosinophil ratios (Grammel 1989; Fogarty,U. *pers comm* 1990). Eosinophilic pulmonary infiltrations were observed histologically in a proportion of COPD affected horses by Nicholls (1978) and by Kaup *et al* (1990A), who regarded them as exceptional findings.

The differences between the results of these studies may reflect heterogeneity of COPD affected horses with regard to their cellular responses, differences in the antigens to which the horses were exposed or differences in the timing of the BALF collection relative to the onset of the disease.

Similarly, human asthmatics have an unaccountably heterogeneous cellular response, with some showing a BALF eosinophilia while others show a BALF neutrophilia (Kay 1988).

NC did not alter PELF mast cell, basophiloid cell or total basophilic cell counts in either group. While BALF mast cell and total basophilic cell ratios were reduced in the COPD affected horses after NC, this was considered to be a result of the markedly increased neutrophil ratios, rather than an alteration in the absolute cell counts. Similarly, Derksen *et al* (1985B) found that absolute and differential BALF mast cell counts were unaffected by NC. In other studies, however, horses with longstanding COPD have shown increased BALF mast cell ratios compared with controls (Vrins *et al* 1989; Winder *et al* 1990). Again the differing findings of these studies may reflect heterogeneity of the pulmonary cellular response in COPD horses, differences in the allergens encountered or differences in the timing of BALF collection relative to the antigen challenge.

Albumen adjusted BALF and PELF lymphocyte counts were unaffected by NC. Thus while immunohistochemical studies suggest that lungs from horses with COPD have increased numbers of perivascular and peribronchiolar lymphocytes (Winder and Von Fellenberg 1986 & 1988), this lymphocytic infiltration appears not to extend to the pulmonary tissues sampled by bronchoalveolar lavage (BAL).

At 1.5h after NC the COPD affected group showed significantly reduced Vmin and increased R_L. This suggests that pulmonary ventilation was compromised due to central airway obstruction, probably, in view of the site and the rapid onset of the airway obstruction, by bronchospasm. While the resultant ventilation perfusion mismatching and/or hypoventilation did not statistically alter blood gas tensions, 7 of 8 COPD affected horses showed reduced PaO_2 and 6 showed increased $PaCO_2$.

At 5h after NC, COPD affected horses showed increased dPtp, R_L and reduced Cdyn and PaO_2, suggestive of central and peripheral airway obstruction possibly due to bronchospasm, mucosal swelling and accumulation of mucus, cells and inflammatory exudate (Nicholls 1978).

Comparison of the clinical, pulmonary mechanic, arterial blood gas tensions and pH and BALF cytology findings of this study with those from other studies should be made with caution, as most other studies have investigated horses with longstanding COPD. It is likely that there are important differences in the pathophysiological disturbances and, in particular, in the severity of the pulmonary inflammatory responses between horses given acute challenges, as performed in this study, and those given chronic challenges.

ASPERGILLUS FUMIGATUS CHALLENGE

AF challenge induced pulmonary disease, in 7 of 8 COPD affected horses, which was similar, but less severe, to that which followed NC.

With the exception of slightly elevated BALF neutrophil ratios in 2 control horses (5% and 5.7%), no evidence of pulmonary disease was observed in the control group after this challenge. While the marginal BALF neutrophilia in the 2 control horses could not be explained, administration of excessive antigen was unlikely, as the concentration used was ten fold lower than the upper limit recommended for human AIC studies (Spector 1989).

AF challenge significantly increased PELF and albumen adjusted BALF neutrophil counts, and BALF neutrophil ratios only in the COPD group. These BALF neutrophil counts were significantly correlated to those which followed NC, but were significantly lower.

Only 2 of the 7 COPD affected horses which showed increased BALF neutrophil ratios following AF challenge showed clinical signs consistent with COPD. This again emphasises the relative insensitivity of clinical examination in the diagnosis of equine COPD. Interestingly the 2 clinically affected horses showed both early and late phase clinical responses to this challenge.

In contrast to NC, AF challenge induced less marked pulmonary dysfunction in COPD affected horses, as determined by pulmonary mechanics and arterial blood gas tensions. The only significant change observed was an increase in the Vmin of COPD affected horses at 1.5h after challenge.

MICROPOLYSPORA FAENI (MF) CHALLENGE

MF challenge induced pulmonary disease in 7 of 8 COPD affected horses which was similar, but less severe, to that which followed NC.

MF challenge induced clinical signs consistent with COPD (McPherson *et al* 1978) in 3 COPD affected horses, one horse showing only an early response, one showing only a late response and the other showing a dual response.

MF challenge significantly increased PELF neutrophil counts, albumen adjusted BALF neutrophil counts and BALF neutrophil ratios of COPD affected horses. Increased BALF neutrophil ratios were considered to be the most useful indicator of pulmonary inflammation caused by this challenge.

BALF neutrophil ratios of control and COPD affected horses after MF challenges correlated significantly with those at 5h after NC, although the latter were significantly higher. The PELF and albumen adjusted BALF macrophage counts of the COPD affected horses were unaccountably reduced by MF challenge, as had occurred with NC.

MF challenge induced no significant changes in pulmonary mechanics or arterial blood gas tensions in either group.

In contrast to the findings of this study, Derksen *et al* (1987 and 1988) found that MF challenge elicited a BALF neutrophilia in both control and COPD affected ponies, but

induced significant pulmonary dysfunction, as indicated by increased RR, Vmin and R_L and reduced PaO_2, only in horses with COPD.

Four possible explanations could account for the contradictory results of the previous (Derksen *et al* 1987 & 1988) and present studies.

Firstly, Derksen *et al* (1987 & 1988) proposed that the controls used in their study could have been previously hypersensitised to MF and that a specific hypersensitivity response to MF could have induced the pulmonary neutrophilia which followed this challenge. This, however, is unlikely since the control ponies did not show a pulmonary neutrophilia after NC, despite this NC environment containing high levels of MF aeroallergen (Robinson,N.E. *et al pers comm* 1991).

Secondly, the use of different antigen preparations could account for the contradictory results, with soluble and particulate MF preparations being used in the present and previous studies, respectively. While MF shows no detectable antigenic variation between strains (Edwards 1972), considerable variation in the composition of antigenic extracts may follow the use of different culture, extraction and purification techniques (Pepys 1981). Particulate antigens may more readily elicit hypersensitivity pneumonitis and granulomata in laboratory animals than soluble components, possibly via macrophage activation (Salvaggio *et al* 1975; Salvaggio and Karr 1979).

It is possible that the neutrophilia observed in both the control and COPD affected groups by Derksen *et al* (1987 & 1988) was induced by a non specific toxic agent in the MF preparation. The use of commercial antigenic preparations, which are less likely to show antigenic variation than those prepared by individual laboratories, would facilitate more rational comparison of different antigen inhalation studies.

Thirdly, the contradictory results of the two studies could reflect differences in the quantity of antigen deposited in the horses' lungs. The present study administered 20ml $2.5x10^{-4}$ w/v MF via a facemask, while Derksen *et al* (1987 & 1988) administered a higher concentration (30ml 10^{-2} w/v) of MF via a tracheostoma, a route likely to yield greater pulmonary deposition of antigen. The concentration of antigen used by Derksen *et al* was higher than that recommended for AIC in man ($<10^{-2}$ w/v; Spector 1989). This antigen concentration has been shown to be induce clinically irrelevant false positive responses in humans which have no history of clinical disease attributable to the administered antigen (Townley *et al* 1965; Cavanaugh *et al* 1977). Administration of excessively high antigen concentrations by Derksen *et al* (1987 & 1988) may have induced the pulmonary neutrophilic responses in control horses and the severe pulmonary dysfunction in COPD affected horses, findings which contrast with those of the present study.

Fourthly, the BALF neutrophilia observed by Derksen *et al* (1988) in control ponies after MF challenge could have been induced by the repeated bronchoscopy and BAL performed during the short study period, consistent with previous findings for horses, humans, dogs and monkeys (Kazmierowski *et al* 1977; Cohen and Batra 1980; Von Essen *et al* 1991).

However, a sham protocol performed on 3 ponies by these authors, involving repeated BALs in the absence of MF challenges, did not elicit a neutrophilic pulmonary response, and an attempt was made to minimise this potential problem by performing consecutive BALs at different sites within the lungs.

Derksen *et al* (1987) demonstrated that, while MF inhalation challenge elicited pulmonary neutrophilia in both control and COPD affected horses, and pulmonary dysfunction in COPD affected horses, it did not induce NSBHR to histamine in horses which had been sensitised to MF by subcutaneous administration of MF in Freund's adjuvant. Since NSBHR is a feature of naturally occurring equine COPD, its absence was considered to indicate that the pulmonary inflammation elicited by MF challenge in their study differed from that of naturally occurring

COPD, and/or that the immunological responsiveness of the experimentally sensitised horses differed from those with naturally occurring COPD. Bronchial reactivities of COPD affected horses following MF challenges were not investigated in the present study.

It is thus apparent that the experimental MF challenge model used by Derksen *et al* (1987 & 1988) elicited pulmonary disease which differed in several important respects from naturally occurring COPD.

Both the MF and AF challenges used in this study elicited pulmonary disease, which closely resembled naturally occurring COPD, only in horses with a history of COPD. These findings further implicate MF and AF as important aetiological agents of equine COPD.

The pulmonary inflammatory response and the associated pathophysiological disturbances elicited by MF and AF challenges were, however, less severe than those elicited by NC. Four possible explanations may account for this finding.

Firstly, NC may have induced more severe pulmonary dysfunction because horses were exposed to antigen for longer or were exposed to higher concentrations of antigen during this challenge. While the relative magnitudes of the antigen burden encountered by horses during NC and AIC are unknown, the former involved continuous exposure of the horses to hay and straw for 5h, while AICs involved exposure to antigens for only 5-15min.

Secondly, as the duration of antigen challenge influences the release of inflammatory mediators during human cutaneous allergen challenges (Shalit *et al* 1988), the dynamics of the inflammatory mediators released during NC may have differed from those of the mediators released during the shorter duration AICs.

Thirdly, the pulmonary response induced by NC could have been caused or exacerbated by exposure to antigens other than AF and MF or to non specific toxic agents, including ammonia, endotoxins, bacteria, plant material and forage mites, which are present in stable environments containing hay and straw (Clarke 1987).

Fourthly, the alpha 2 agonist xylazine, used by Derksen *et al* (1987 & 1988) to sedate the ponies prior to MF challenge and bronchoalveolar lavage (BAL), may have altered the ponies' pulmonary reactivities to the inhaled MF, also possibly accounting for the differences in the two studies. Alpha agonists have been shown to enhance mediator release from human lung mast cells via interaction with cell surface alpha adrenergic receptors (Kaliner *et al* 1972).

However, the finding that alpha agonists reduced the allergen inhalation challenge induced microvascular leakage in guinea pig airways does not support this proposal (Boschetto *et al* 1989). Furthermore, alpha-2-agonists would be expected to antagonise cholinergic mediated bronchoconstriction via interaction with inhibitory alpha-2-receptors on cholinergic nerves (Andersson *et al* 1986) and reduce the vagally mediated bronchoconstriction induced in COPD affected horses by NC (Scott *et al* 1988).

Non specific pulmonary toxicity was unlikely to have caused the pulmonary disease observed in the COPD affected horses after AF and MF challenges and after NC, as all individuals exposed to a non specific toxic agent would have been equally affected (Salvaggio and Hendrick 1979), and the control horses in this study were unaffected by these challenges.

Differences in pulmonary deposition patterns, rather than differences in the immunological reactivity to the challenge antigens, could have determined whether or not horses responded to the challenges. However, as pulmonary deposition is influenced considerably by ventilation patterns (Heyder *et al* 1982; Valberg *et al* 1982), which vary considerably over long term periods (Derksen *et al* 1982) and are thus unlikely to remain consistently different between control and COPD affected horses, this is unlikely.

Ideally the breathing patterns of subjects undergoing AICs should be standardised to minimise variations in the pulmonary deposition of antigen, which can influence the outcome of challenges (Valberg *et al* 1982). Due to lack of subject cooperation this is not possible in equine studies, However, as all the horses in the present study breathed slowly and deeply during antigen inhalation, due to excessive resistance in the inspiratory valve of the facemask, pulmonary deposition patterns are likely to have been relatively constant. This slow, deep respiration is likely to have resulted in a uniform deposition of antigen throughout the lungs, with little deposition in the large airways (Valberg *et al* 1982).

Differences in the rates and/or mechanisms by which inhaled antigens are cleared from the lung are unlikely to have determined whether or not horses responded to the challenges, since

the antigen elicited pulmonary inflammation and dysfunction shortly after administered, i.e. before significant differences in the pulmonary clearance of antigen would have been expected.

In conclusion, COPD was induced in the COPD affected horses, and not in the control horses, by AF and MF inhalation challenges and by numerous NCs, performed over a 2 year period. This is clear evidence that equine COPD is a pulmonary hypersensitivity to inhaled allergens, including AF and MF.

TV CHALLENGE

TV challenge did not significantly affect any of the measured parameters in either group. However, after this challenge, 2 COPD affected horses showed clinical signs consistent with COPD (McPherson *et al* 1978), and 4 COPD affected and 2 control horses had increased BALF neutrophil ratios (i.e. >5%).

The 2 control horses which showed a BALF neutrophilia after TV challenge, had shown no clinical, arterial blood gas tension, pulmonary mechanic or BALF cytological evidence of pulmonary disease over a 2 year period, while being maintained in both NC and controlled environments. This suggests that the TV challenge employed in this study differed from the challenge encountered by horses when exposed to hay and straw. Thus, unfortunately, little valid information regarding the potential role of TV in the aetiology of equine COPD can be obtained from this study.

The TV challenge may have exposed horses to antigens which were not encountered in NC environments. Alternatively, while the concentration of TV antigen used ($2x10^{-4}$ w/v) was within the range recommended for human AICs (i.e. $<10^{-2}$ to 10^{-7} w/v; Spector 1989), it may have exceeded levels of TV aeroallergen present in the NC environment. Supportive of this possibility are the findings of a recent study which quantified the respirable aeroallergens in

North American horse barns by enzyme linked immunosorbent assay (ELISA) techniques and demonstrated that, in NC environments, TV is present at lower levels (approximately 500ng/m^3) than MF (approximately 1500ng/m^3) and AF (approximately 1800ng/m^3) (Woods,P. *et al, pers comm* 1991).

Further studies of aeroallergen concentrations in stables should help the selection of antigen concentrations to be used for future AICs, by ensuring that they are relevant to those encountered naturally.

BAL was accomplished without apparent complications in all cases. While most samples contained trace amounts of erythrocyte and free haemoglobin, it was considered unlikely that a significantly influx of cells and humoral components from the blood into the BALF had occurred. To minimise possible regional differences in cellular and humoral BALF components (see Chapter 8), BAL was always performed in the accessory lobe of the right lung.

The median recovery of BALF was slightly lower than that reported for other studies (Viel 1983; Derksen *et al* 1985B, 1987 & 1988), probably because aspiration of BALF was discontinued at 45s after instillation rather than after there was no further BALF recovery. BALF aspiration was discontinued at 45s after instillation to minimise the time dependent diffusion of urea and albumen, from the pulmonary tissues into the lavage fluid, which would have affected the accuracy of the PELF and albumen adjusted cell counts, which were calculated using the BALF urea and albumen concentrations, respectively (see Chapter 7).

As BALF represents a mixture of PELF and PBS, in variable proportions, the total and absolute PELF cell counts and the albumen adjusted total and absolute BALF counts were considered to be more useful than the total and absolute BALF cell counts.

Between and within group statistical analyses of PELF and albumen adjusted cell counts yielded identical results. Albumen adjusted BALF cell counts were, however, significantly higher than the PELF cell counts, consistent with the finding that the plasma:PELF albumen ratio is higher than that of urea (Rennard *et al* 1986). The albumen and urea dilution techniques were further evaluated in this study and are reviewed in Chapter 7.

Lymphocytes with marked irregular invaginations of their nuclear membrane were observed in BALF collected from some COPD affected horses after NC. While the identity of these cells was unknown, they resembled atypical lymphocytes which have been described in BALF from humans with allergic alveolitis (Haslam *et al* 1987) and in the lamina propria and submucosa of bronchial biopsies from symptomatic asthmatics (Jeffrey *et al* 1989). Haslam *et al* (1987) considered that these cells may represent activated delayed hypersensitivity T (T_{DH}) cells or mast cell precursors.

BALF from control and COPD affected horses contained small numbers of an unidentified cell type which were designated 'basophiloid cells'. These cells contained many large cytoplasmic granules, which were intensely basophilic when stained with Leishman's and which tended to obscure visualisation of the remainder of the cell. These cells were not identified on preparations stained with toluidine blue. They were easily differentiated from BALF mast cells and peripheral blood basophils, owing to their larger, intensely staining cytoplasmic granules.

Mast cells in BALF from both control and COPD affected horses showed varying degrees of degranulation. Occasional cells resembling fully degranulating mast cells were identified. Consistent with these findings, Fox *et al* (1981) and Lamb and Lumsden (1982) demonstrated mast cells in various stages of degranulation in human pulmonary epithelium.

Considerable variation was noted in pulmonary mechanic tests and arterial blood gas tensions, even in control horses which had no detectable pulmonary inflammation, consistent with previous findings (Derksen *et al* 1982; Stadler and Deegen 1986). Possible causes of this variation, which limit the value of these techniques in the detection of COPD, were reviewed in the introduction to Chapter 2.

CHAPTER 3

INTRADERMAL MOULD ANTIGEN TESTING IN CONTROL AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AFFECTED HORSES: RESULTS AND COMPARISON OF INTRADERMAL AND BRONCHIAL REACTIVITIES

SUMMARY

Intradermal endpoint titres for commercial extracts of *Micropolyspora faeni* (MF), *Thermoactinomyces vulgaris* (TV) and *Aspergillus fumigatus* (AF) were determined in control (n=8) and chronic obstructive pulmonary disease (COPD) affected (n=8) horses.

The intradermal endpoint titres of control and COPD affected horses were found to be not significantly different.

Comparison of the intradermal endpoint titres for each antigen with the changes in pulmonary mechanics, arterial blood gas tensions, arterial blood pH and with BALF neutrophil ratios which had followed previous MF, AF and TV inhalation challenges and 'natural (hay and straw) challenges' revealed no significant correlations. This suggests divergence of the dermal and pulmonary reactivities to these antigens in the horse.

This study provides further evidence that intradermal antigen testing is of limited value in the investigation of equine pulmonary hypersensitivity to moulds.

INTRODUCTION

Successful management of pulmonary hypersensitivity conditions often necessitates identification of the causal allergens. This may be attempted using antigen inhalation challenges (AICs) (see Chapter 2), endobronchial antigen challenges (see Chapter 9), intradermal antigen testing, antigen induced *in vitro* basophil or mast cell degranulation testing, or *in vitro* antigen challenges of airway smooth muscle preparations.

Intradermal antigen testing and antigen induced *in vitro* basophil or mast cell degranulation testing offer advantages over the other techniques as they facilitate testing of larger numbers of putative allergens, require less antigen, cause minimal suffering, are less likely to induce untoward complications and are relatively simple to perform.

Intradermal antigen testing is performed routinely at several veterinary centres as an additional diagnostic procedure for the investigation of suspected equine pulmonary hypersensitivity. However the value of this technique remains speculative.

Numerous intradermal antigen studies have been performed in the horse and positive intradermal responses to a wide variety of putative allergens including fungi, actinomycetes, pollens, mites, feedstuffs, beddings and chicken serum have been reported in control and chronic obstructive pulmonary disease (COPD) affected horses (Lowell 1964; Eyre 1972; Schatzmann and Gerber 1972; Mansmann *et al* 1975; McPherson *et al* 1979; Halliwell *et al* 1979; Beech and Gunson 1981).

Lowell (1964) found no difference in the dermal responsiveness of 6 control and 6 COPD affected horses to plant and tree pollens, hay dust extract and moulds. *Micropolyspora faeni* (MF), *Thermoactinomyces vulgaris* (TV) and *Aspergillus fumigatus* (AF) antigens were, however, not used in this study.

Schatzmann and Gerber (1972) found no correlation between intradermal responses to pollens, dusts and moulds and the presence of pulmonary disease in 300 horses.

Eyre (1972) observed marked dermal reactions to a range of mould antigens in horses with COPD, and weak responses to these antigens in most (60%) control horses, and concluded that intradermal testing was of limited value in the diagnosis of COPD.

McPherson *et al* (1979) showed that horses with COPD had a higher incidence of positive responses to MF and AF than controls, although many control horses also showed positive responses. These antigens elicited biphasic intradermal responses in many horses with COPD, with a weak immediate (maximum at 30-90min) reaction being followed by an Arthus-type late phase response at 4-8h.

Halliwell *et al* (1979) investigated the intradermal response to 24 moulds, 4 thermophilic actinomycetes, barn and hay dust, grain mix and soya dust. The early and late intradermal responses to these antigens were shown, by histological examination of skin biopsies, to be consistent with Type 1 and Type III hypersensitivity responses, respectively. Horses with COPD showed a higher incidence of positive early and late phase reactions to most antigens, especially to the moulds Rhizopus, Cephalosporium and MF. No correlation was found between intradermal responses of individual horses and the presence of moulds in their environments.

Beech and Gunson (1981) demonstrated that horses which suffered from chronic coughing showed a higher incidence of positive 30min and 4h intradermal reactions than control horses and concluded that allergen skin testing was a useful diagnostic procedure.

Hockenjos *et al* (1981) performed intradermal testing with forage mite extracts on 2 control and 7 COPD affected horses, but strong positive responses to control injections of mite culture medium precluded meaningful analysis.

Eriksen (1985) found that a large proportion of horses with COPD showed positive early and late intradermal responses to MF and to hay mites, however control horses were not included in this study.

Sasse *et al* (1985) found that horses showed a high incidence of late positive intradermal responses to MF, hay dust and meal mites, but few responded positively to hay dust mite. It was not reported whether control or COPD affected horses were used in this study.

IN VITRO ANTIGEN TESTING IN THE INVESTIGATION OF EQUINE PULMONARY HYPERSENSITIVITY

There exist only a few, conflicting, reports of antigen induced *in vitro* basophil degranulation testing in the investigation of equine pulmonary hypersensitivity.

Hockenjos *et al* (1981) showed that extracts of hay, aspergillus, candida and verticillium induced histamine release from peripheral blood leucocytes of horses with COPD. However, Dieckmann (1986) was unable to confirm this and suggested that the histamine release reported by Hockenjos and colleagues could have followed non specific basophil cytolysis induced by excessively high antigen concentrations.

Abdel-Salam (1989) investigated antigen induced histamine release from peripheral blood leucocytes and from cells in tracheobronchial washings from control horses and horses which they classified as having 'probable allergic COPD' or 'probable non allergic COPD', the latter two questionable categories being differentiated by the number of mast cells and eosinophils in the tracheobronchial secretions. Timothy pollen and house dust mite extracts were found to

be secretagogues only for tracheobronchial cells, with those from COPD affected horses with a 'probable allergic' aetiology releasing more histamine than those from 'non allergic' COPD affected horses. It is possible, however, that the tracheobronchial eosinophilia of the 'probable allergic' group may have been due to lungworm infection (MacKay and Urquhart 1979).

To evaluate intradermal mould antigen testing as a diagnostic technique for equine COPD, endpoint titres to AF, MF and TV extracts were determined for control and COPD affected horses and compared with the pulmonary reactivities to these antigens, as assessed by AICs.

MATERIALS AND METHODS

ANIMALS

Eight control (median age 15.5 years, range 7-25 years; median body weight 577kg, range 212-652kg) and 8 COPD affected (median age 15.5 years, range 6-25 years; median body weight 482kg, range 371-546kg) geldings and mares of mixed breeds, which had been given allergen inhalation challenges (Chapter 2) 3-6 months previously, were used (Appendix 2.1). The criteria used to define the two populations are described in Chapter 2.

INTRADERMAL TESTING TECHNIQUE

Animals were given intradermal antigen challenges with aqueous preparations of MF, TV and AF antigens (Greer Laboratories, Lenoir, North Carolina, USA), administered in ten fold dilutions within the range 10^{0} - 10^{-4} mg/ml for MF and TV and $4x10^{-3}$ - $4x10^{0}$ PNU/ml for AF. Sterile phosphate buffered saline (PBS) (Greer Laboratories, Lenoir, North Carolina, USA) was used as the diluent. The positive and negative controls were, respectively, 0.01% histamine phosphate in sterile PBS and PBS.

Intradermal injections of 0.05ml were given on a clipped neck site, using a 25g needle. The mean of the maximum wheal diameter and the diameter perpendicular to this was determined at 1.5 and 5h after injection. Positive reactions were those which elicited wheals with mean diameter exceeding the mean of the negative and positive control tests obtained at 1.5h for each individual horse. End point titres were the maximum dilution which elicited a positive response.

STATISTICAL ANALYSIS

Paired and unpaired endpoint data, which were not normally distributed, were compared by the Wilcoxon Rank Test and the Mann Whitney tests respectively.

Early (1.5h) and late (5h) phase endpoint titres were correlated, using Spearman's Rank Correlation, with the early (between 0 and 1.5h post challenge) and the late (between 0 and 5h post challenge) changes in respiratory rate (RR), minute volume (Vmin), tidal volume (V_T), mean maximum change in transthoracic pressure (dPtp), pulmonary resistance (R_L), dynamic compliance (Cdyn), arterial oxygen tension (PaO_2), arterial carbon dioxide tension ($PaCO_2$) and arterial pH which followed AICs with AF, MF and TV and followed NCs.

The early and late phase endpoint titres were also correlated with the neutrophil ratios of BALF collected at 5h after AICs and NCs.

Statistical analyses were performed using Minitab (Minitab, Pennsylvania, USA), assuming a significance level of 5%.

RESULTS

There were no differences between the endpoint titres of the control and COPD affected groups for any of the antigens (Table 3.1). The early and late phase endpoint titres were not significantly different.

The endpoint titres for the MF extract were significantly lower than those for the TV extract ($p<0.05$). Comparison with the endpoint titres for MF and TV with those for AF was not possible due to the antigen concentrations being expressed in different units.

The early and late phase intradermal endpoint titres for AF, TV and MF were not significantly correlated with the early and the late phase changes in RR, Vmin, V_T, dPtp, R_L, Cdyn, PaO_2, $PaCO_2$ and arterial pH which followed AICs with AF, MF and TV and which followed NC.

The early and late phase intradermal endpoint titres for AF, MF and TV were not correlated with the neutrophil ratios of BALF collected at 5h after AICs with each of these antigens and after NC.

Table 3.1 Early (1.5h) and late (5h) intradermal endpoint titres of (A) control (n=8) and (B) COPD affected horses (n=8) for extracts of *Micropolyspora faeni* (MF), *Thermoactinomyces vulgaris* (TV) and *Aspergillus fumigatus* (AF).

(A) Control horses

HORSE	AF (PNU/ml) EARLY	LATE	TV (mg/ml) EARLY	LATE	MF (mg/ml) EARLY	LATE
1	4000	4000	>1	1	>1	0.01
2	400	400	>1	>1	0.01	0.1
3	>4000	>4000	>1	1	>1	0.01
4	>4000	4000	>1	>1	>1	0.1
5	400	4000	>1	0.1	1	0.1
6	>4000	>4000	>1	>1	>1	>1
7	40	400	1	0.1	0.01	0.01
8	>4000	>4000	>1	>1	>1	1

(B) COPD affected horses

HORSE	AF (PNU/ml) EARLY	LATE	TV (mg/ml) EARLY	LATE	MF (mg/ml) EARLY	LATE
1	400	40	0.1	0.1	0.001	0.001
2	4000	>4000	0.1	0.01	0.1	0.01
3	400	4000	>1	>1	1	1
4	400	40	>1	0.1	0.1	0.001
5	>4000	400	>1	>1	0.001	0.001
6	>4000	4000	>1	>1	0.1	0.1
7	>4000	400	>1	0.1	>1	0.1
8	>4000	4000	>1	>1	0.01	0.001

DISCUSSION

The value of intradermal mould antigen testing in the diagnosis of equine COPD, which is currently speculative, was investigated.

There were no significant differences in the dermal reactivities of control and COPD affected horses to AF, MF and TV antigens.

Furthermore intradermal endpoint titres to these antigens were not significantly correlated with the changes in pulmonary mechanics, arterial blood gas tensions, arterial blood pH and BALF neutrophil ratios which had followed antigen inhalation challenges with these antigens.

These findings suggest that equine pulmonary and dermal reactivities differ and provide further evidence that intradermal testing is of limited value in the investigation of equine COPD.

Lack of correlation between bronchial and dermal reactivity, which has also been reported in man (Spector and Farr 1974; Aas 1975; Nelson 1989), may be attributed to local production of allergen specific IgE within the respiratory tract or skin (Huggins and Borstoff 1975) and/or to regional heterogeneity in mast cell responsiveness (Patterson *et al* 1972; Aas 1975; Warner *et al* 1989).

While a positive intradermal response usually indicates that previous exposure has induced dermal hypersensitivity to that antigen, it does not indicate whether this hypersensitivity has clinical significance. Similarly the presence of serum precipitins to the fungi and thermophilic actinomycetes found in stable dust may merely reflect previous exposure to these antigens and does not imply clinical significance (Lawson *et al* 1979; Madelin *et al* 1991) as these

precipitins have been demonstrated in COPD affected horses and in horses with no evidence of pulmonary disease.

The interpretation of intradermal antigen tests is also further complicated by the occurrence of false positive and false negative responses.

False positive responses, i.e. those occurring in individuals which are not hypersensitive to that antigen, may follow administration of excessive concentrations of antigen, placement of the intradermal injections too close to a large positive reaction, physical trauma to the test site, iatrogenic dermal haematoma formation or non specific irritant responses caused by contaminants in the extract.

False negative responses may occur with reduced allergen potency, fluctuation in the individual's sensitivity, suppression of sensitivity by medication or following a systemic reaction to that allergen (Beech 1983; Nelson 1989).

As excessively high concentrations of antigen may elicit false positive dermal responses, while excessively low concentrations may fail to demonstrate clinically relevant positive responses, selection of the appropriate antigen concentration for intradermal testing is essential. Determination of dermal endpoint titres, which involves testing with a wide range of antigen concentrations, effectively overcomes this problem.

CHAPTER 4

PHENOTYPIC ANALYSIS OF PERIPHERAL BLOOD AND BRONCHOALVEOLAR LAVAGE FLUID (BALF) LYMPHOCYTES IN CONTROL AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AFFECTED HORSES, BEFORE AND AFTER 'NATURAL (HAY AND STRAW) CHALLENGES'

SUMMARY

Phenotypic analyses of lymphocytes in peripheral blood (PB) and bronchoalveolar lavage fluid (BALF) of control and chronic obstructive pulmonary disease (COPD) affected horses, both before and after 'natural (hay and straw) challenges', were successfully performed using immunofluorescent labelling with monoclonal antibodies and flow cytometry. This technique has several advantages over other currently available techniques, and should prove to be a valuable technique for the investigation of pulmonary immune responses.

This is the first reported phenotypic analysis of equine BALF lymphocytes. BALF lymphocytes were shown to be predominantly EqCD5+ cells, approximately half of which were also EqCD8+, with a smaller proportion (<10% in control horses) of B cells. In comparison with PB, BALF contained higher proportions of EqCD5+ cells and EqCD8+ cells and a lower proportion of B cells.

Horses with asymptomatic COPD had a higher proportion of BALF B cells and a lower proportion of BALF EqCD5+CD8- cells (considered to be predominantly EqCD4+ cells) than controls. This suggests that B cells, via humoral immune mechanisms, have a role in the pathogenesis of equine COPD.

Hay and straw challenge increased the ratio of BALF EqCD5+CD8- cells and reduced the ratio of BALF EqCD8+ cells in COPD affected horses, but not in controls. This finding is similar to the pulmonary recruitment of CD4+ cells reported for human asthmatics following allergen challenges and suggests that T lymphocytes may have an important role in the pathogenesis of equine COPD.

INTRODUCTION

There is considerable evidence to suggest that B and T lymphocytes, via humoral and cellular immune mechanisms respectively, have a pivotal role in the pathogenesis of a number of pulmonary inflammatory conditions affecting man and some animals (Bernardo and Center 1981; Berman *et al* 1990; Crump *et al* 1991; Kay *et al* 1991). However, the role of the lymphocyte in the pathogenesis of equine chronic obstructive pulmonary disease (COPD) has not been determined.

Mair *et al* (1987) surveyed the extensive mucosal lymphoid tissue of the normal equine respiratory tract, which may comprise part of a common mucosal immune system. Solitary lymphocytes were located throughout the epithelium and lamina propria of the bronchi and bronchioles. Organised lymphoid patches and nodules were adjacent to bronchioles and less commonly to bronchi, and small lymphoid accumulations were present in the pulmonary interstitium.

Immunohistochemical studies have shown that most lymphocytes within equine peribronchiolar lymphocytic nodules and follicles do not express surface immunoglobulin (Ig) and are considered to be T lymphocytes (Winder and Von Fellenberg 1986 & 1988).

A role for the antibody producing B lymphocyte in the pathogenesis of equine COPD was implied by those who considered this condition to be a Type 1 and/or a Type III hypersensitivity response (Gerber 1973; Schatzmann *et al* 1973; Halliwell *et al* 1979; Wilkie 1982; McPherson and Thomson 1983). Such a role is supported by the observations that lungs from horses with COPD have increased numbers of perivascular and peribronchiolar lymphocytes which bear either IgA, IgG or no Ig, and that severely affected horses have large amounts of free intraepithelial IgA and IgG (Winder and Von Fellenberg 1986 & 1988).

Little attention has been given to the role of the T lymphocyte in equine COPD. The T lymphocyte is a major source of proinflammatory lymphokines, soluble mediators which are produced following antigen induced cell activation. Lymphokines could be responsible for inducing many of the immunopathological features associated with COPD. Furthermore, as the T lymphocyte has a central role in the regulation of humoral and cellular immune mechanisms (Moretta *et al* 1979; Berman *et al* 1990), this cell may determine the nature of the response, if any, mounted by an individual when exposed to inhaled antigens.

THE USE OF MONOCLONAL ANTIBODIES IN LYMPHOCYTE PHENOTYPIC ANALYSIS

The introduction of monoclonal antibodies (mAb) which delineate lymphocytes and lymphocyte subpopulations has revolutionised the investigation of lymphocyte biology. MAb technology, pioneered by Kohler and Milstein (1975), enables the production of antibodies, which are chemically, immunologically and physically homogeneous, in virtually unlimited quantities. The methodology employed in the production of mAbs has been extensively reviewed elsewhere (Mason *et al* 1983; Kipps and Herzenberg 1986) and thus will be discussed only briefly.

MAb production involves fusion of immunoglobulin producing B lymphocytes with cells which are capable of replicating continuously in tissue culture media. Fusion occurs in the presence of a fusion reagent, commonly polyethylene glycol (Pontecorvo 1976).

The B lymphocytes are usually splenocytes from a donor previously immunised with the antigen of interest, while their fusion partners are myeloma cells which are selected for lack of immunoglobulin production and their lack of hypoxanthine phosphoryl transferase (HPRT) activity. Following fusion, which is a relatively inefficient process, there exist three populations, namely splenocytes, myeloma cells and hybrids. As the splenocytes are incapable of prolonged survival in tissue culture they die spontaneously. The myeloma cells are unable to synthesise the purines and pyrimidines necessary for survival, due to the presence in the culture medium of aminopterin, which inhibits endogenous nucleic acid synthesis and their lack of HPRT activity, which prevents their utilisation of exogenous nucleic acids, and thus

also die. The hybrids in contrast, having a combined genome, survive and eventually extrude chromosomes until a diploid state is achieved.

The hybrids are then cloned by limiting dilution methods and the culture supernatant tested for presence of the mAb of required specificity. Suitable clones can then be used to produce mAb either by tissue culture methods or by intraperitoneal inoculation of the myeloma cells into laboratory animals with subsequent harvesting of mAb in the ascitic fluid.

The nature of mAbs affords them several advantages over conventional polyclonal antisera;

The range of antigens which may be detected using mAbs is theoretically greater, not being restricted to those antigens which can be purified to homogeneity.

MAbs are produced by cells derived from a single clone and thus are specific for a single antigen, indeed usually for a single epitope on an antigen. As a consequence, cross reactions and non specific reactions are rarely encountered when using monoclonal reagents, especially those obtained by tissue culture. A mAb may, however, react specifically with two different antigens if they share the epitope to which the mAb is specific.

In comparison with mAbs, only a limited proportion of the immunoglobulin present within polyclonal antisera, is specific for the immunising antigen. Furthermore, polyclonal antisera comprises antibodies which are specific to several epitopes on the immunising antigen. The remainder is non specific for the antigen and thus represents a major potential source of non specific staining.

Monoclonal antibodies are more homogeneous than polyclonal reagents, which may vary from batch to batch. MAb may be manufactured in relatively unlimited quantities and, following the initial development phase, are considerably cheaper to produce than polyclonal reagents.

THE USE OF FLOW CYTOMETRY IN LYMPHOCYTE PHENOTYPIC ANALYSIS

The introduction of the flow cytometer has proved invaluable in the phenotypic analysis of cells stained by immunofluorescent techniques, and has been successfully employed in the

phenotypic analysis of BALF cells from several species including man (Yamada *et al* 1986), mice (Curtis and Kaltreider 1989), rabbits (Shellito *et al* 1981), rats (Thrall and Barton 1984), pigs (Gehrke and Pabst 1990) and sheep (Cordier *et al* 1990). Phenotypic analysis of equine BALF cells has apparently not been reported.

Flow cytometry makes quantitative, correlated, multi parameter analyses of each individual cell (Parks *et al* 1986). Cells pass from the flow chamber of the fluorescent activated cell sorter (FACS) analyser into the centre of a laminar flow of sheath fluid where they become spatially separated. Each individual cell then passes through a focussed beam of laser light which excites the fluorescent molecules on the cell surface or in the cytoplasm of the cells. The resultant fluorescence emitted by individual cells is collected and quantified, at 90^{o} to the incident beam, by several detectors, each detecting different wavelength ranges. The FL1 detector is most suitable for the detection of the light emitted by the fluorophore fluorescein.

As each cell passes through the laser beam it scatters the laser light at various angles to the incident beam, with the direction of scatter providing very useful information regarding cell morphology. Scatter of light at small angles ($0\text{-}15^{o}$) to the incident beam, termed forward scatter (FSC), is most dependent on cell size, while cell granularity and internal structure more significantly affect the scatter detected at larger angles ($70\text{-}110^{o}$), termed side scatter (SSC) (Parks *et al* 1986). Quantification of FSC and SSC for each cell may enable discrimination of cell types which differ in cell size or internal structure, such as erythrocytes, lymphocytes, monocytes and granulocytes.

Furthermore, using this facility, it is possible to perform selective flow cytometric analysis of specified populations by analysing only those cells within an 'analysis gate', defined by the light scatter parameters of the cell type under investigation. This 'light scatter gating' enables selective analysis of chosen populations even in complex mixtures, without the necessity of preparatory population separation using biophysical techniques, such as density gradient centrifugation.

The quantitative fluorescence and FSC and SSC data obtained from the FACS analyser are displayed in two modes.

Firstly as a dot plot, which typically displays the FSC values for cells against their SSC values, to enable delineation of populations differing in cell morphology and permit light scatter gating prior to selective flow cytometry.

Secondly as a histogram, usually displaying cell frequency against fluorescence intensity. Computerised comparison of the fluorescence histograms from control and positively labelled samples enables definition of the limits of negative and positive staining and hence computation of the ratio of positively staining cells.

The use of flow cytometry for the phenotypic analysis of equine peripheral blood (PB) and BALF lymphocytes affords several potential advantages over methods employing fluorescent microscopic analysis;

1. While the fluorescence microscopy technique is simpler to perform than flow cytometry, its interpretation is subjective and necessitates considerable expertise (Loken and Herzenberg 1975).

2. The number of cells which can be reasonably assessed by the microscopy technique is limited (commonly 300-500), diminishing its suitability in studies requiring accuracy and those involving rare subpopulations. In comparison, flow cytometry enables rapid analysis of large numbers of cells (commonly 5000-10000).

3. Flow cytometry provides quantitative data which is essential for phenotypic analysis, since subpopulation differentiation is frequently based on the relative magnitudes of particular parameters rather than merely by their absence or presence. In contrast such quantification is unavoidably subjective when microscopy is employed.

4. As the FACS analyser performs simultaneous multi-parameter measurements on each individual cell, selective analysis of cell populations is possible using light scatter gating.

Phenotypic analyses of PB and bronchoalveolar lavage fluid (BALF) lymphocyte from control and COPD affected horses, both before and after 'natural challenges' (NCs), are described.

MATERIALS AND METHODS

SUBJECTS

Six control (median age 17 years, range 7-24; median body weight 604kg, range 212-652) and 6 COPD affected (median age 20 years, range 13-30; median weight 495kg, range 371-537) horses of mixed breeds were used (Appendix 4.1). The criteria used in the diagnosis of COPD were described in Chapter 2.

SAMPLE COLLECTION AND PROCESSING

Baseline PB and BALF samples were collected (Chapter 2), from the horses after they had been maintained in a 'controlled environment' (Chapter 2), in the case of the controls for at least one week, in the case of the COPD affected horses until they were demonstrated to be fully asymptomatic, according to criteria previously described (Chapter 2).

PB samples were collected into vacutainers containing potassium ethylene diamine tetra acetic acid (Becton Dickinson, Rutherford, USA).

Post challenge PB and BALF samples were collected 72h after moving the horses to a NC environment (Chapter 2).

ANTIBODIES USED IN THE STUDY

Two murine mAbs, HT23A (EqCD5) and HT14A (EqCD8) (VMRD, Pullman, Washington, USA) and three rat mAbs, MAC291 (Equine major histocompatability complex Class I), MAC284 (putative pan T cell marker) and MAC292 (B cell) (kindly provided by Dr.J.Kydd, Animal Health Trust, Newmarket) were used as primary stage reagents, the second stage conjugate being a $F(ab')_2$ fragment of rabbit anti mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC) (Dako, High Wycombe). An alternative rabbit anti mouse FITC conjugate (SAPU, Law Hospital, Carluke) was evaluated, but discarded because of high

non specific staining. The specificities of the primary mAbs used in this study have been previously reported (Crump *et al* 1988; Kydd 1990; Kydd and Antczak 1991).

All mAbs and both FITC conjugates were titrated to determine their optimal working dilutions. The mouse mAbs were used at 1:1000 dilution in PBA, the rat mAbs were used undiluted and the FITC conjugate was used at 1:50 dilution.

IMMUNOFLUORESCENT LABELLING TECHNIQUE

BALF cells were washed three times in 20ml of ice cold phosphate buffered saline (PBS) containing 1mg/ml bovine serum albumen (Sigma, Poole) and 1mg/ml sodium azide (Sigma, Poole) (PBA) and transferred in aliquots of $1x10^6$ cells into test tubes.

Equine peripheral blood leucocytes (PBL) were harvested from PB, following removal of erythrocytes by gravitational sedimentation and osmotic lysis using the technique of Watson *et al* (1987). Briefly, the PB samples were sedimented at 1g for 30min and the leucocyte rich supernatant collected and centrifuged at 2000g for 5min (MSE Chilspin 2, Fisons, Crawley). The pelleted cells were resuspended in 1ml Hank's balanced salt solution pH 7.2 (Sigma, Poole), containing 20mM HEPES (Flow Laboratories, Irvine). Residual erythrocytes were then lysed osmotically by the addition of 4.5ml distilled water, the isotonicity being restored after 30s by addition of 0.5ml 10x HBSS. Cells were washed three times in ice cold PBA and transferred in aliquots of $1x10^6$ cells into test tubes.

BALF and PBL cells were then centrifuged at 300g for 3min, the supernatant discarded and the cell pellet resuspended in 50ul mAb or control reagent and incubated at 4^oC overnight. Three negative primary stage controls, namely PBA, 1:500 normal mouse serum and 1:500 normal rat serum (Sigma, Poole), were used with each sample. The cells were then washed three times in PBA and incubated in 50ul FITC conjugate for 30min at 4^oC in the dark. Prior to use, the FITC conjugate was preadsorbed with 10% pooled normal equine serum for 30min at 4^oC and centrifuged at 11500g for 10min to remove immune complexes. PBA was used as a negative second stage control reagent for each sample.

Following three further washes, the cells were resuspended in 100ul PBS containing 1% w/v paraformaldehyde (BDH, Poole) and maintained at 4^{O}C in the dark pending analysis, which was always within 6 days of labelling.

MODIFICATIONS OF THE IMMUNOFLUORESCENT LABELLING TECHNIQUE

The possibility of reducing non specific labelling of lymphocytes was investigated. Five PBL and 5 BALF preparations were preincubated for 30min at 4^{O}C with 50ul 10% pooled normal rabbit serum (SAPU, Law Hospital, Carluke), which had been preadsorbed with an equal volume of 10% pooled normal equine serum in PBA for 30min at 4^{O}C, followed by centrifugation at 11500g for 10min to remove immune complexes. The ratios of non specifically labelled lymphocytes in these preparations were compared with those in untreated duplicate samples, after routine labelling using PBA as the first stage reagent.

The effect of hypotonic lysis on the immunofluorescent labelling of PB lymphocytes was also determined. The ratios of positively labelled lymphocytes, and their mean channel numbers, in 4 PBL samples prepared by hypotonic lysis and erythrocyte sedimentation, were compared with those of 4 duplicate samples prepared solely by erythrocyte sedimentation.

The effect of paraformaldehyde fixation and delayed flow cytometric analysis on the immunofluorescent labelling of lymphocytes was determined. The ratios of positively labelled lymphocytes in 4 PB and 4 BALF samples, which had been fixed using paraformaldehyde after routine labelling and analysed 6 days later, were compared with those of duplicate samples which had been analysed immediately after labelling and which had not been fixed.

The effect of preadsorbing the FITC conjugate with 10% pooled normal equine serum for 30min at 4^{O}C, followed by centrifugation at 11500g for 10min, on the non specific immunofluorescent labelling of lymphocytes was determined. The ratios of non specifically

labelled lymphocytes in 4 duplicate PB and BALF cell preparations, after incubation with either preadsorbed or untreated FITC, were compared.

FLOW CYTOMETRIC ANALYSIS

A fluorescent activated cell sorter (FACS IV, Becton Dickinson, Los Angeles, USA) was used to analyse light scatter gated BALF and PB lymphocytes by single colour flow cytometry. Lymphocyte gates were set for each BALF and PBL sample, based on correlated forward light scatter (FSC) and perpendicular light scatter (SSC) when a well circumscribed lymphocyte cluster was evident. In the absence of a well circumscribed lymphocyte cluster, the gates were defined by positive fluorescence gating of EqCD5+, EqCD8+ cells and B cells. A minimum of 5000, and in most cases 10000, gated cells were analysed.

STATISTICAL ANALYSIS

As data were not normally distributed, paired and unpaired sample comparisons were made using, respectively, the Wilcoxon Rank Test and the Mann-Whitney Test, with a significance level of 5%. Analyses were performed using Minitab (Minitab Inc, Pennsylvania, USA).

RESULTS

IMMUNOFLUORESCENT LABELLING TECHNIQUE

While preincubation of cells with preadsorbed normal rabbit serum generally reduced the non specific labelling of lymphocytes (Table 4.1), as this technique increased the non specific labelling in some samples, this treatment was not further employed.

Table 4.1 The effect of preincubation of PBL (n=5) and BALF cell preparations (n=5) with preadsorbed normal rabbit serum on the ratios of non specifically labelled gated cells, using either normal mouse (NMS) or rat sera (NRS) as the primary stage reagent (median and range).

CELL	PRIMARY REAGENT	% POSITIVE CELLS UNTREATED	TREATED
PBL	NMS	6.5 (1.3-9.1)	3.5 (1.7-12.6)
PBL	NRS	1.9 (1.2-4.7)	1.5 (1.0-12.6)
BALF	NMS	6.0 (1.2-11.2)	0.9 (0.2-9.4)
BALF	NRS	3.1 (1.9-9.5)	3.0 (1.9-9.9)

As preadsorption of the FITC conjugate with equine sera reduced non specific labelling to <1% (Table 4.2), all FITC conjugate was treated in this way prior to use.

Paraformaldehyde fixation and delayed flow cytometric analysis reduced the ratios of lymphocytes showing specific positive labelling and increased those showing non specific labelling and autofluorescence, although these effects were not statistically significant (Table 4.3).

TABLE 4.2 The effect of prior adsorption of the rabbit FITC conjugate with pooled normal equine serum on the ratios of non specifically labelled gated cells in PB samples (n=4).

HORSE	% POSITIVE CELLS	
	UNADSORBED FLUOROPHORE	ADSORBED FLUOROPHORE
1	4.5	0.7
2	3.2	0.4
3	2.8	0.1
4	2.6	0.5

Table 4.3 The ratios of positively fluorescent gated PBL and BALF cells from 4 horses following fixation in PBS containing 1% w/v paraformaldehyde (PF) for 6 days prior to analysis (PF-treated) and following analysis of unfixed cells within 4h of completion of the immunolabelling procedure (untreated) (median and range).

PRIMARY ANTIBODY	PBL		BALF	
	UNTREATED	PF-TREATED	UNTREATED	PF-TREATED
PBA *	0.7 (0.3-0.9)	3.3 (2.0-4.0)	0.8 (0.3-1.2)	4.1 (4.0-5.4)
NMS **	0.7 (0.6-0.9)	2.5 (1.9-3.4)	1.8 (0.9-2.1)	6.8 (4.5-7.2)
HT23A	70.8 (56.9-82.1)	63.3 (56.1-70.1)	88.4 (40.6-94.7)	82.7 (19.5-60.5)
HT14A	27.7 (23.6-32.7)	26.9 (24.3-29.4)	47.9 (24.0-61.6)	51.1 (19.5-60.5)
MAC291	98.5 (95.6-99.4)	97.5 (93.4-98.9)	87.1 (38.2-97.9)	83.7 (30.5-90.7)
MAC284	68.4 (63.8-85.3)	62.6 (52.8-77.5)	76.4 (25.0-95.3)	76.3 (17.3-82.3)
MAC292	40.3 (21.7-62.7)	24.8 (14.6-39.7)	5.2 (1.4-6.3)	5.3 (4.0-11.0)

* As no fluorophore was used with this control, the positively labelled cells were autofluorescent cells.
** As no primary mAb was used with this control, the positively labelled cells were non specifically labelled.

While hypotonic lysis did not affect the ratios of positively labelled lymphocytes, it did significantly increase their mean channel numbers ($p<0.05$) (Table 4.4).

TABLE 4.4 Ratios of positively labelled gated cells in 4 PBL samples, prepared with (LYS) or without (NON) hypotonic lysis. The mean channel numbers are displayed in brackets.

ANTIBODY	SAMPLE 1		SAMPLE 2		SAMPLE 3		SAMPLE 4	
	LYS	NON	LYS	NON	LYS	NON	LYS	NON
MAC291	99.9 (82)	97.9 (74)	99.5 (78)	96.4 (89)	99.8 (113)	97.5 (153)	99.9 (81)	96.1 (60)
HT23A	82.8 (95)	84.2 (163)	80.6 (120)	80.2 (47)	76.2 (123)	82.5 (86)	77.2 (199)	77.7 (44)
HT14A	28.4 (156)	21.3 (23)	19.2 (195)	17.7 (26)	20.9 (168)	21.8 (24)	20.1 (172)	21.1 (23)
MAC292	20.1 (26)	16.1 (27)	14.0 (43)	17.7 (75)	14.2 (24)	14.9 (39)	14.1 (52)	13.2 (33)

LIGHT SCATTER PROFILES OF PBL AND BALF CELLS

Four cell populations were readily identified on light scatter profiles of most PBL samples (Fig. 4.1). In contrast light scatter profiles of BALF cells (Fig. 4.2) were less easily delineated, with only three clusters being evident. The populations identified were;

(a) a variable density low FSC and low SSC cluster which was often more dense in BALF samples and which was considered to contain erythrocytes and dead cells.

(b) a low SSC and low to intermediate FSC cluster. This was distributed as a double cluster in many samples, with the higher and lower FSC peaks predominating in PBL and BALF samples, respectively. This population, which contained the vast majority of EqCD5+, EqCD8+ and B cells, was considered be predominantly lymphocytes.

(c) an often poorly delineated, low density cluster with high FSC and intermediate SSC, which was absent in BALF samples and which was considered to contain predominantly monocytes.

(d) a population with high SSC and intermediate to high FSC values, which was very extensive and dense in BALF samples and which was considered to contain granulocytes and macrophages.

While no consistent differences were observed in the scatter profiles of PBL samples from control and COPD affected horses, either before or after NC, 2 COPD affected horses had increased granulocyte/macrophage clusters after NC (Fig. 4.1).

Fig. 4.1 Light scatter profiles of ungated PBL from a control horse and a COPD affected horse, before (A and C, respectively) and at 72h after NC (B and D, respectively). The four cell populations identifiable on these profiles are labelled in (A) and were considered to represent (1) erythrocytes/dead cells, (2) lymphocytes, (3) monocytes and (4) granulocytes.

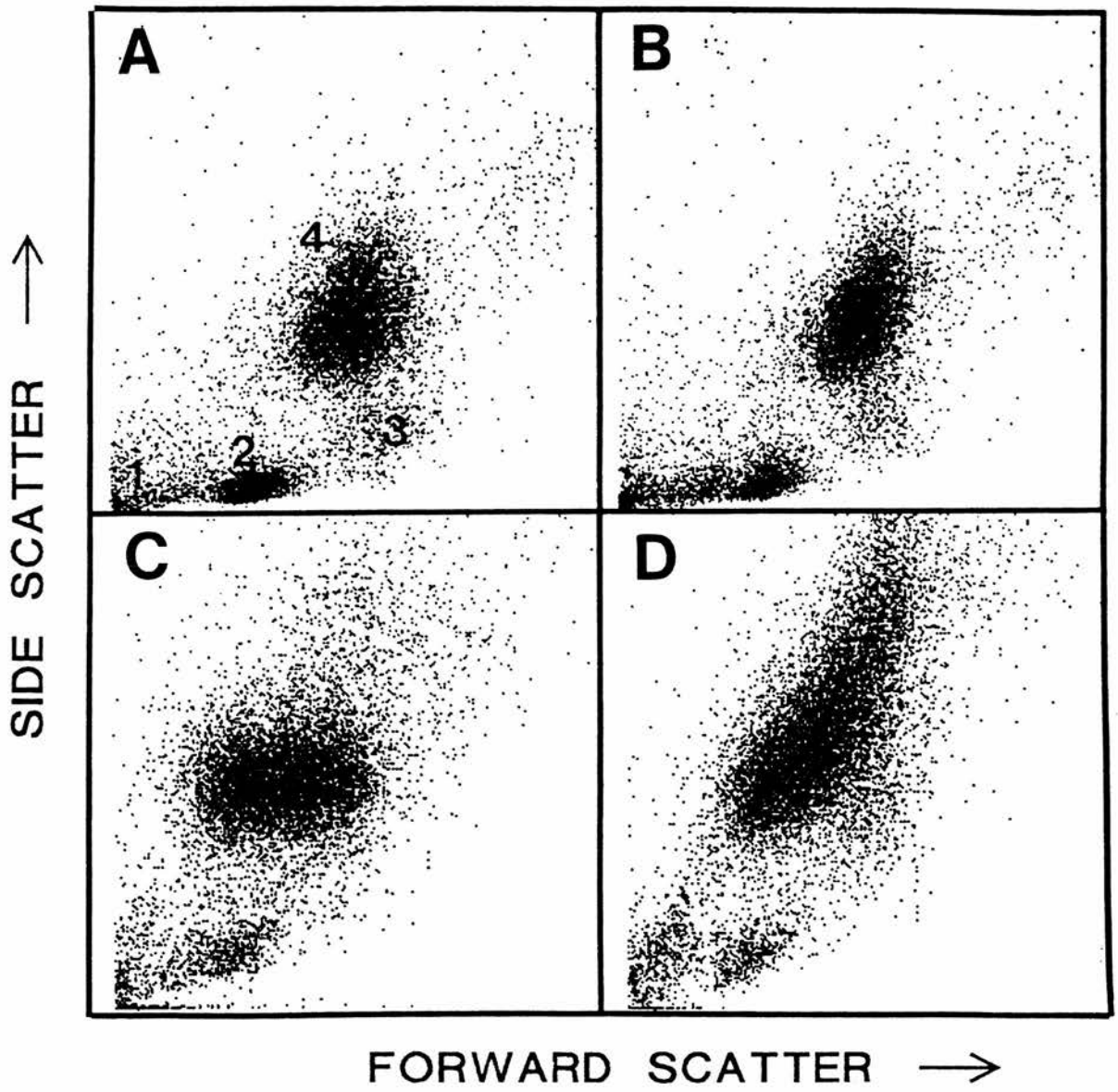
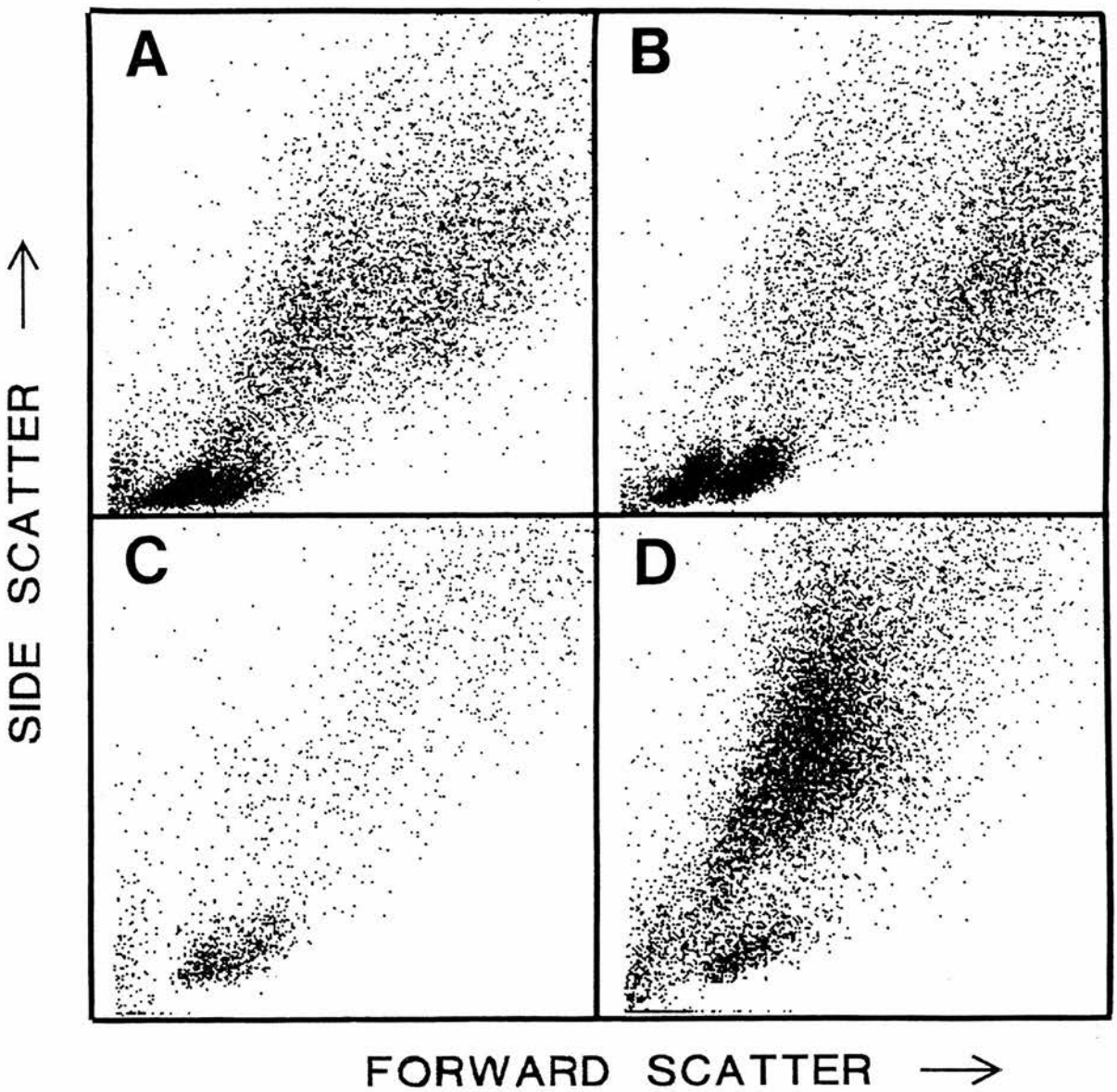


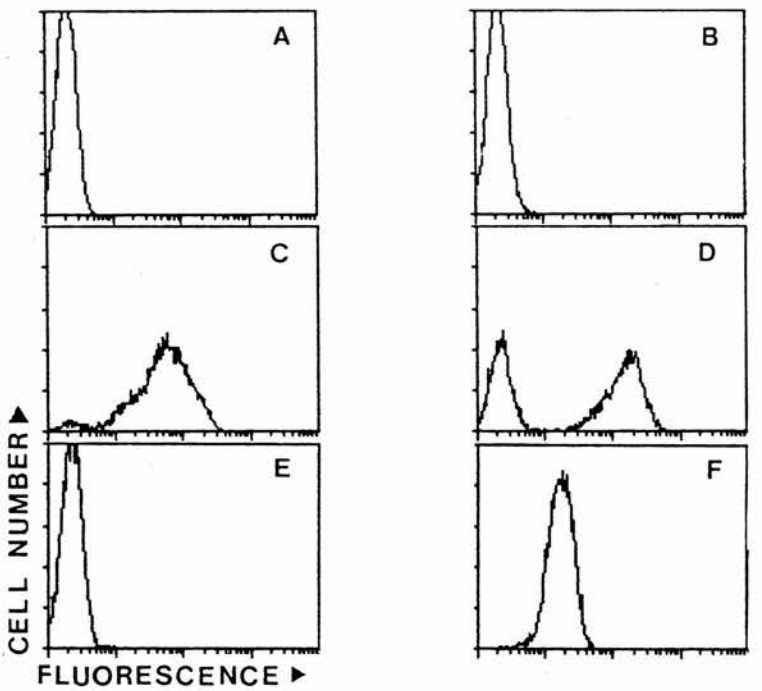
Fig. 4.2 Light scatter profiles of ungated BALF cells from a control horse and a COPD affected horse, before (A and C, respectively) and at 72h after NC (B and D, respectively).



THE FLUORESCENT (FL1) PROFILES OF LIGHT SCATTER GATED PBL AND BALF LYMPHOCYTES FOLLOWING LABELLING WITH INDIVIDUAL MONOCLONAL ANTIBODIES

Typical FL1 profiles of light scatter gated BALF cells from a control horse, at baseline, stained with the panel of mAbs are displayed in Fig. 4.3.

Fig. 4.3 Fluorescence (FL1) profiles of light scatter gated BALF cells from a control horse, at baseline, after using, as the primary stage reagent (a) PBA, (b) NMS, (c) HT23A, (d) HT14A, (e) MAC292 and (f) MAC284.



In samples free from autofluorescence, satisfactory negative controls were obtained using normal mouse or rat sera or PBA as the primary reagent. EqCD5+ cells frequently showed a biphasic FL1 profile. CD8+ cells were predominantly of high fluorescence, although a smaller population, which expressed fluorescence only slightly greater than that of the negative cells, appeared as a shoulder on the negatively stained cell peak. Class I+ cells showed fairly uniform, intermediate intensity staining, which was usually well delineated from the negatively stained cells. MAC284+ staining was found to be fairly variable, appearing either with the biphasic pattern similar to that of the EqCD5+ cells, or as a single broad peak, with poor definition between positively and negatively stained cells. While B cells showed fairly low intensity staining they were clearly delineated from the negatively stained population in all samples.

AUTOFLUORESCENT CELLS

Autofluorescent cells, i.e. cells exhibiting positive fluorescence in the absence of exogenous fluorophore, were identified in all ungated BALF samples and in 10 out of 24 gated BALF samples from COPD affected and control horses. In some samples a proportion of the cells were autofluorescent, appearing, on the FL1 profile, as a secondary shoulder adjoining the negative peak or as a separate peak. In other samples, all cells, including those within the analysis gate, were autofluorescent as indicated by a right shift in the entire FL1 profile.

Analysis of the FSC/SSC scatter profiles of autofluorescent BALF cells, following positive fluorescence gating, showed that they exhibited a wide range of cell sizes and complexities, with the majority exhibiting high FSC and SSC values. They were excluded from the analyses by light scatter gating in all but 10 samples. Three of these 10 samples were successfully analysed after altering the sensitivity of the FL1 detector. Attempts to analyse the remaining 7 samples using the FL2 detector, on the basis that this detector may preferentially detect the emission spectrum of the specific fluorescence, were unsuccessful, as both types of

fluorescence were detected with apparently similar sensitivity. These 7 samples were discarded, necessitating repetition of sample collection and analysis.

Fluorescent microscopic examination of 2 BALF cell preparations exhibiting both specific positive fluorescence and autofluorescence revealed that the autofluorescence was mainly a feature of large, macrophage-like cells. While autofluorescence manifested as a light yellow colour and the specific staining as greenish yellow, differentiation of specific staining and autofluorescence using fluorescent microscopy was considered subjective.

VALIDITY OF THE LYMPHOCYTE GATE

Valid flow cytometric analysis was only achieved when the analysis was restricted, by light scatter gating, to a population of cells within a lymphocyte gate. This technique generally eliminated autofluorescent and positively labelled non lymphocyte populations from the analyses.

For every sample, the validity of the analysis gate was confirmed by examining the FSC/SSC profiles of EqCD5+, EqCD8+ and B cells, which were generated by positive fluorescence gating.

Further validation of the analysis gate was sought by determining the ratios of total lymphocytes (i.e. the sum of the CD5+ ratio and the B cell ratio) within the gated population of PBL and BALF samples (Table 4.5).

TABLE 4.5 Total lymphocyte ratios (sum of CD5+ ratio and B cell ratio) within the analysis gates of PBL and BALF samples from control (N=6) and COPD affected (N=6) horses at 'baseline' (B) and at 72h post NC (median and range).

SAMPLE		RATIO TOTAL LYMPHOCYTES IN GATE	
		PBL	BALF
CONTROL	B	99.4 (77.2-110.9)	83.5 (63.0-95.1)
COPD	B	97.7 (88.1-114.7)	78.8 (66.0-89.3) *
CONTROL	NC	103.1 (90.8-107.0)	90.1 (70.0-93.8) *
COPD	NC	96.5 (94.6-102.0)	65.9 (32.9-88.1) *

* significantly lower than the values for the corresponding PBL samples ($p<0.05$).

Lymphocytes accounted for most (median ratios 96.5-103.1%) of the gated cells in the PBL samples, but were significantly lower ($p<0.05$) in the gated BALF populations in all cases except for the samples from the control horses at baseline.

NC had no significant effect on the ratio of total lymphocytes within the gated populations in control or COPD affected horses ($p>0.05$).

THE RATIOS OF CLASS I+ CELLS IN THE GATED POPULATION

In all PBL samples, most (median ratios 93.6-98.8%) of gated cells expressed Class I antigen (Table 4.6, Appendix 4.2). COPD affected horses had significantly reduced ratios of gated Class 1+ PBL cells compared with control horses at 72h after NC ($p<0.05$).

The proportion of gated Class I+ cells was significantly lower in the BALF samples than in PBL samples ($p<0.05$) (Table 4.6).

TABLE 4.6 The ratios of Class I+ gated PBL and BALF cells from control (CONT) (n=6) and COPD (n=6) affected horses at baseline (B) and at 72h following NC (median and range).

SAMPLE		RATIO OF GATED CLASS 1+ CELLS	
		PBL	BALF
CONTROL	B	93.6 (87.8-98.9)	76.9 (54.5-93.0)[2]
COPD	B	98.3 (95.6-98.7)	57.1 (47.6-94.7)[2]
CONTROL	NC	98.8 (94.6-99.9)	94.3 (30.3-97.6)[2]
COPD	NC	93.9 (86.2-99.1)[1]	60.0 (30.5-94.7)[2]

[1] significantly lower than control horses ($p<0.05$).
[2] significantly lower than PBL samples ($p<0.05$).

COMPARISON OF MAC284 AND HT23A LABELLING

In BALF, ratios of MAC284+ cells were significantly lower than those of EqCD5+ cells ($P<0.01$) (Table 4.7, Appendix 4.3). Positive fluorescent gating localised the majority of HT23A+ cells to the lymphocyte cluster, consistent with the reported specificity of this mAb, however, many MAC284+ cells had scatter profiles different from those of lymphocytes. As these findings cast doubt on the specificity of MAC284, the data obtained using this mAb was not used in further calculations and analyses.

Table 4.7 The ratios of gated CD5+ and MAC284+ cells in PBL (n=24) and BALF (n=24) samples from control and COPD affected horses (median and range).

SAMPLE	RATIO POSITIVE CELLS	
	PBL	BALF
CD5+	73.0 (55.0-89.0)	76.9 (29.3-91.4)
MAC284+	75.3 (43.8-96.9)	68.2 (17.3-93.6)*

* significantly lower than the CD5+ cell ratio ($p<0.01$).

COMPARISON OF THE LYMPHOCYTE PHENOTYPE DISTRIBUTIONS OF PB AND BALF SAMPLES FROM CONTROL AND COPD AFFECTED HORSES BEFORE AND AFTER NC

The ratios of B cells and EqCD5+, EqCD8+ and EqCD5+CD8- lymphocytes were expressed as ratios of the total lymphocytes present within the gated population (calculated as the sum of the EqCD5+ ratio and the B cell ratio). The ratio of EqCD5+CD8- lymphocytes were determined by subtracting the ratio of EqCD8+ lymphocytes from the ratio of EqCD5+ lymphocytes.

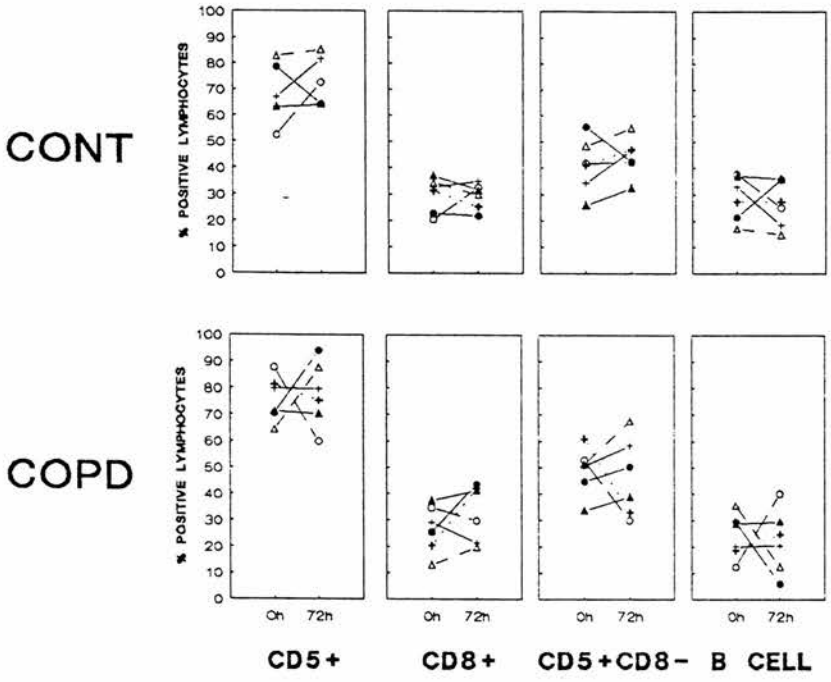
PB lymphocytes (Fig. 4.4, Table 4.8, Appendix 4.4) were predominantly EqCD5+ (median 65.1%, range 52.3-82.8 in control horses at baseline), with less than half of these (32.1%, 20.5-37.1 in control horses at baseline) being EqCD8+, and smaller proportions of B cells (30.1%, 17.2-37.7 in control horses at baseline).

PB lymphocyte phenotype ratios of control and COPD affected horses were not significantly different, either before or after NC (Fig. 4.4), and were not significantly affected by NC.

Table 4.8 The ratios of EqCD5+, EqCD8+, EqCD5+CD8- and B lymphocytes in PB from 6 control (CONT) and 6 COPD affected horses at baseline (B) and at 72h post NC (median and range).

GROUP	RATIO POSITIVE CELLS			
	EqCD5+	B cells	EqCD8+	EqCD5+CD8-
CONT B	65.1 (52.3-82.8)	30.1 (17.2-37.7)	32.1 (20.5-37.1)	41.5 (26.1-55.7)
CONT NC	73.7 (64.1-85.1)	26.4 (14.9-35.9)	30.7 (21.8-35.0)	44.4 (32.6-55.2)
COPD B	75.5 (64.3-87.6)	24.5 (12.4-35.8)	27.4 (12.9-37.5)	51.0 (33.7-70.0)
COPD NC	77.4 (59.8-94.1)	22.6 (5.9-40.2)	35.6 (19.8-43.7)	41.7 (29.0-67.8)

Fig. 4.4 The ratios of gated EqCD5+, EqCD8+, EqCD5+CD8- and B lymphocytes in PB from control (CONT) (n=6) and COPD affected (n=6) horses, before and at 72h after NC.



BALF (Fig. 4.5, Table 4.9, Appendix 4.5) had significantly higher ratios of EqCD5+ (median 95.3%, range 94.4-97.6 in control horses at baseline) and EqCD8+ (median 51.4%, range 48.0-63.0 in control horses at baseline) lymphocytes and a significantly lower ratio of B cells (median 4.7%, range 2.4-5.6 in control horses at baseline) than PB ($p<0.05$).

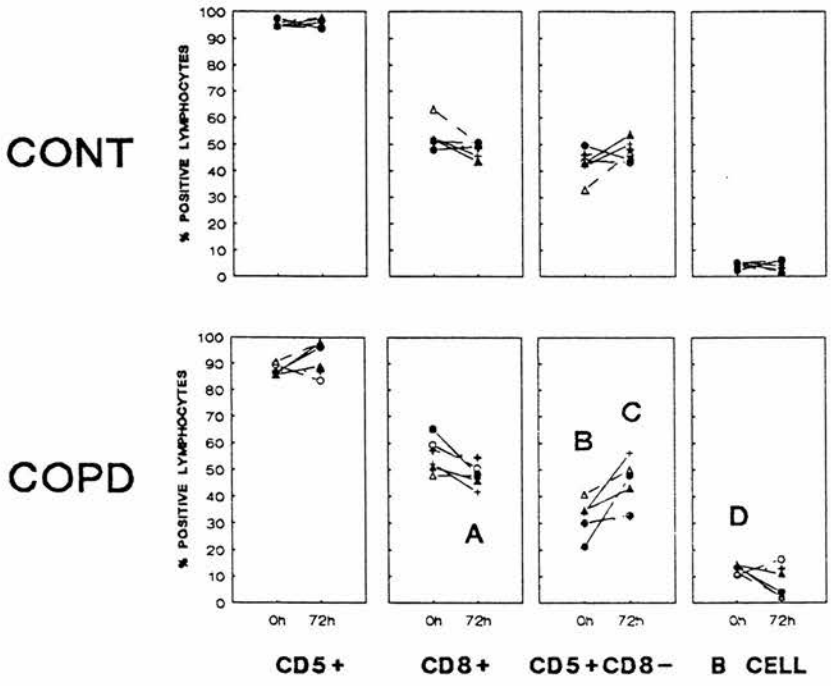
Before NC, the asymptomatic COPD affected horses had a significantly higher ratio of B cells ($p<0.01$) and a significantly lower ratio of EqCD5+CD8- cells ($p<0.05$) than the controls. NC significantly increased the ratio of EqCD5+CD8- lymphocytes and significantly decreased the ratio of EqCD8+ lymphocytes in the BALF of COPD affected horses ($p<0.05$).

Table 4.9 The ratios of CD5+, CD8+, CD5+CD8- and B lymphocytes in BALF from 6 control (CONT) and 6 COPD affected horses at baseline (B) and at 72h post NC (median and range).

GROUP	RATIO POSITIVE CELLS			
	EqCD5+	B cells	EqCD8+	EqCD5+CD8-
CONT B	95.3 **A** (94.4-97.6)	4.7 **B** (2.4-5.6)	51.4 **A** (48.0-63.0)	43.3 (32.8-49.7)
CONT NC	96.3 **A** (93.6-97.9)	3.7 **B** (2.1-6.4)	49.0 **A** (43.6-50.7)	47.7 (43.3-53.8)
COPD B	86.8 **A** (85.8-90.7)	13.2 **BD** (10.5-14.2)	54.7 **A** (47.9-65.3)	32.1 **C** (21.2-40.8)
COPD NC	92.6 **A** (83.6-97.9)	7.4 **B** (2.1-16.4)	48.1 **AE** (41.7-54.6)	45.5 **F** (32.6-56.2)

A Significantly higher than values for corresponding PBL samples ($p<0.05$)
B Significantly lower than values for corresponding PBL samples ($p<0.05$)
C Significantly lower than values for the control group ($p<0.05$)
D Significantly higher than values for the control group ($p<0.01$)
E Significantly lower than the baseline values ($p<0.05$)
F Significantly higher than the baseline values ($p<0.05$)

Fig. 4.5. The ratios of gated EqCD5+, EqCD8+, EqCD5+CD8- and B lymphocytes in BALF from control (CONT) (n=6) and COPD affected (n=6) horses, before and at 72h after NC.



A Significantly lower than before NC ($p<0.05$)
B Significantly lower than the control group ($p<0.05$)
C Significantly higher than before NC ($p<0.05$)
D Significantly higher than the control group ($p<0.01$).

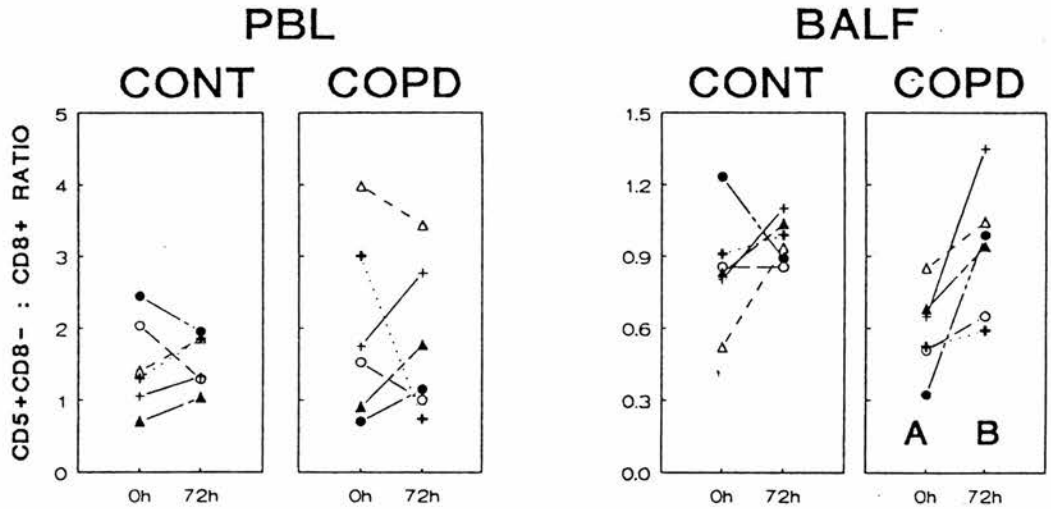
The EqCD5+CD8-:CD8+ ratios (Fig. 4.6, Table 4.10) were significantly ($p<0.05$) lower in the BALF of the COPD affected horses at baseline than in the PB. This ratio significantly increased in the BALF of COPD affected horses following NC ($p<0.05$).

Table 4.10 The EqCD5+CD8-:CD8+ ratios for gated PBL and BALF cells from 6 control (CONT) and 6 COPD affected horses at baseline (B) and at 72h after NC (median and range).

	EqCD5+CD8-:CD8+ RATIOS	
	PBL	BALF
CONT B	1.36 (0.70-2.44)	0.84 (0.52-1.04)
CONT NC	1.59 (1.04-1.95)	0.96 (0.85-1.23)
COPD B	1.75 (0.90-3.98)	0.59 (0.32-0.85)[B]
COPD NC	1.08 (0.70-3.43)	0.96 (0.60-1.35)[A]

[A] Significantly higher than baseline values ($p<0.05$).
[B] Significantly lower than values for corresponding PBL samples ($p<0.05$).

Fig. 4.6 The ratios of EqCD5+CD8-:CD8+ cells in PB and BALF from control (n=6) and COPD affected (n=6) horses before and at 72h after NC.



[A] Significantly lower than in PBL samples from the same horses ($p<0.05$)
[B] Significantly higher than before NC ($p<0.05$).

DISCUSSION

This is the first reported study to enumerate equine BALF lymphocyte phenotypes using immunofluorescent labelling with monoclonal antibodies (mAbs) and flow cytometry, a technique which should prove valuable for future investigations of equine pulmonary immune phenomena.

The recent development of monoclonal antibodies (mAbs) which identify equine leucocyte differentiation antigens has greatly facilitated detailed investigation of equine cellular immune responses. However, in comparison with other species, the range of mAbs which define equine leucocyte antigens is, as yet, limited and their specificities less well defined (Kydd and Antczak 1991).

In the absence of a mAb specific for equine CD3, which identifies T cells, a mAb specific for EqCD5, namely HT23A, was used. It is considered likely that HT23A is identical to the pan T cell marker EqT3, described by Wyatt *et al* (1988).

While HT23A defines most, if not all, T cells, a small proportion (2-5%) of B cells are also labelled (Davis *et al* 1984; Crump *et al* 1988). As B cells comprised only a small proportion of the gated cells (maximum ratio = 40.2% and 16.4% in PB and BALF, respectively), the cross reactivity of this mAb to B cells would have resulted in only a minor overestimation of the T cell ratios.

MAbs specific for EqCD4, which identifies T helper cells, became available only after completion of this study (Lunn *et al* 1991). The EqCD5+CD8-MAC292- population identified and enumerated in this study, however, was considered to be predominantly EqCD4+ cells, although a small proportion of these were probably the equine homologue of the CD3+CD4-CD8- cells identified in other species (Bank *et al* 1986).

Contrary to the findings of Kydd (1990), MAC284 was considered not to be a pan T lymphocyte marker. Many MAC284+ PB and BALF cells showed light scatter profiles inconsistent with those of lymphocytes, fluorescent microscopic examination revealed some MAC284+ BALF cells with a macrophage-like morphology and the ratios of EqCD5+ and MAC284+ BALF cells were found to be significantly different. Data derived using MAC284 was thus not included in the analyses.

Leucocytes were harvested from PB using gravitational sedimentation and hypotonic lysis. While hypotonic lysis did affect lymphocyte labelling, this effect was considered to be unimportant. All FITC conjugate was adsorbed with normal equine serum prior to use as this reduced non specific labelling, consistent with the findings of Kydd (1990). Paraformaldehyde fixation of cells, to permit storage of cells and prevent capping and shedding of surface proteins prior to analysis (Lanier and Warner 1981), did affect lymphocyte labelling, although this effect was considered be unimportant. In contrast, Lanier and Warner (1981) found that paraformaldehyde fixation did not significantly alter the volume, light scatter or fluorescent properties of a variety of cell types. The reason for this discrepancy is not known.

Analysis of forward (FSC) and perpendicular (SSC) scatter profiles of PBL cells enabled delineation of 4 populations, considered to represent dead cells/erythrocytes, lymphocytes, monocytes and granulocytes. While the scatter profiles of BALF cells were less well delineated, 3 populations, which were considered to represent dead cells/erythrocytes, lymphocytes and macrophage/granulocytes, were usually identified.

Lymphocytes frequently showed a biphasic light scatter distribution, with differing FSC values, suggesting that 2 sizes of lymphocytes were present. While CD5+, CD8+ and B lymphocytes were equally distributed between the two populations, the smaller and larger subpopulations predominated in PBL and BALF samples, respectively.

Light scatter gating enabled selective lymphocyte analysis. In most cases, this procedure eliminated intensely autofluorescent cells and positively labelled non lymphocytic cells from the analysis. This technique, previously used to analyse human (Wallace 1989) and murine (Curtis and Kaltreider 1989) BALF lymphocytes, eliminates the necessity for prior separation of lymphocytes by biophysical methods and hence eliminates the problems of incomplete population separation, population subselection or reduced cell labelling which are associated with these techniques.

The gating of a cell population prior to analysis, however, is not without potential problems. It is important that the gate selected is valid, optimally containing all the lymphocytes in the sample, while excluding all other cell types.

In this study, for every sample, analysis of the light scatter profiles of lymphocytes, identified by positive fluorescent gating of cells labelled with lymphocyte markers, confirmed that the vast majority of lymphocytes were included in the analysis gates.

Gated PBL cells were almost entirely positively labelled lymphocytes (the median sum of EqCD5+ and B cells always exceeded 96%), indicating that most non lymphocytic cells had been excluded from the analysis. However, compared with gated PB cells, gated BALF cells contained a significantly higher proportion of unlabelled cells, which could have been non-T, non-B lymphocytes (null cells), granulocytes, erythrocytes, macrophages or dead cells. Dead cells could have been identified and excluded from the flow cytometric analyses using propidium iodide staining (Parks *et al* 1986).

In previous studies, the identity of gated lymphocyte populations in human and murine BALF has been determined by (a) morphological, histochemical and immunochemical examination of gated cells after collection by fluorescent activated cell sorting, and (b) by enumeration of the macrophages and granulocytes within the gated population using single colour fluorescent analysis with population specific mAbs. Using these techniques, gated BALF cells were

shown to be predominantly lymphocytes (Curtis and Kaltreider 1989), with, in man, less than 10% (Yamada *et al* 1986) or less than 1% (Wallace 1989) macrophage contamination.

Autofluorescent cells were identified in all BALF samples, consistent with the findings of Edelson *et al* (1984) for human BALF.

While the cause of autofluorescence remains unclear, it may originate from normal cell constituents such as flavins and cytochromes (Benson *et al* 1979), or from intracellular 'ageing pigments' such as ceroid or lipofuschin (Elleder 1981), or reflect changes in the fluorescence of pyrimidine nucleotides following alterations in the intracellular redox state (Jongkind *et al* 1982; Edelson *et al* 1985).

Most autofluorescent BALF cells were shown, by positive fluorescence gating, to exhibit scatter profiles similar to macrophages/granulocytes in most instances. However, in 7 samples all BALF cells were intensely autofluorescent, precluding their analysis by flow cytometry.

The autofluorescence spectrum of alveolar macrophages has excitation maxima at 370nm and 490nm and an emission maximum at 541nm with a shoulder at 580nm (Edelson *et al* 1985). As the emission wavelength maxima are remarkably similar to those of fluorescein and rhodamine (Pesce *et al* 1971; Shapiro 1983), autofluorescence may prevent computerised subtraction of 'baseline' fluorescence and may preclude valid flow cytometric analysis (Edelson *et al* 1985).

Several strategies to overcome this problem have been used or postulated. Autofluorescence may be overcome by using a fluorophore which is not active in the region of the BALF cells' autofluorescence, such as Texas Red, which has an absorption maximum at 595nm and an emission maximum at 620nm (Parks *et al* 1986). Incubation of alveolar macrophages with 0.5mM cyanide reduced the intensity of their autofluorescence (Edelson *et al* 1985), although, as this technique resulted in cell death, it precluded FACS sorting of live cells for subsequent

functional studies. Alternatively, exposure of the cells, prior to staining, to light which excites the endogenous fluorophore, leading to attenuation of autofluorescence, may prove to be a useful technique (Edelson *et al* 1985).

Equine BALF lymphocytes were shown to be predominantly EqCD5+ T cells, approximately half of which are also EqCD8+, with a smaller proportion of B cells.

While the ratio of CD5+ cells in BALF from other species has not been reported, the ratios of CD3+ cells reported for man (63.0±11.4%, Yamada *et al* 1986; 75.6±2.2%, Wallace 1989) and for rats (>88%, Thrall and Barton 1984) are lower than the ratio of EqCD5+ gated cells in equine BALF (median 95.3%, range 94.4-97.6).

The ratio of EqCD8+ gated cells in equine BALF (median 51.4%, range 48.0-63.0) was similar to that reported for rats (approximately 50%, Thrall and Barton 1984), but higher than that reported for man (27.8±2.4%, Wallace 1989; 25.3±5.5%, Yamada *et al* 1986) and for mice (23±8%, Curtis and Kaltreider 1989).

The ratio of equine BALF EqCD5+CD8- cells (median 43.3%, range 32.8-49.7) approximated the ratios of BALF CD4+ cells reported for man (48.9±3.8%, Wallace 1989; 45.4±12.9%, Yamada *et al* 1986) and for rats (approximately 50%, Thrall and Barton 1984), but was higher than that reported for mice (23±8%, Curtis and Kaltreider 1989).

The ratio of EqCD5+CD8-:CD8+ cells in normal equine BALF (0.84, range 0.52-1.04) was lower than the CD4:CD8 ratio reported for human BALF (1.5-1.8, Reynolds 1987; 1.2±0.3, Meyer *et al* 1989; 1.99±0.34, Wallace 1989).

The ratio of gated B cells in equine BALF (median 4.7%, range 2.4-5.6) was similar to that reported for man (5-10% Reynolds 1987), rats (<12%, Thrall and Barton 1984) and mice (6±3% Curtis and Kaltreider 1989).

The apparent differences in the lymphocyte phenotype distributions reported for different species may reflect true species differences and/or differences in the methods used to enumerate the phenotype ratios, with, in the present study, null cells and gated non lymphocyte cells being excluded from the calculations.

While the presence of null cells could not be confirmed in this study, their presence in murine (Curtis and Kaltreider 1989) and human (Warr *et al* 1976; Daniele *et al* 1977) PB and BALF suggests their presence is likely. In man, null cells account for between 5% (Reynolds 1987) and 40% (Warr *et al* 1976; Daniele *et al* 1977) of BALF lymphocytes.

The lymphocyte phenotype distributions of equine BALF and PBL were significantly different, as was demonstrated in man (Yamada *et al* 1986). Equine BALF contained higher EqCD5+ and EqCD8+ lymphocyte ratios and significantly lower B cell and gated Class I+ cell ratios. While it is possible that BAL may selectively harvest certain lymphocyte phenotypes, it is considered more likely that the immune control mechanisms operating within the two sites differ (Yamada *et al* 1986).

BAL, being a safe and repeatable technique which yields large numbers of lymphocytes, offers advantages over alternative techniques for the collection of lower respiratory tract cells. As the BALF lymphocyte phenotype distribution has been shown to be identical to that of minced lung preparations (Curtis and Kaltreider 1989) and transbronchial lung biopsies (Tomichi *et al* 1989), BALF lymphocytes are considered to be representative of those throughout the lung.

Horses with asymptomatic COPD had increased ratios of BALF B cells and reduced ratios of BALF EqCD5+CD8- cells compared with control horses. As there was no overlap between the ratios of BALF B cells within the control (range 2.4-5.6%) and asymptomatic COPD affected horses (range 10.5-14.2%), quantification of BALF B cells permitted differentiation of the control and asymptomatic COPD affected horses.

These lymphocyte phenotype differences may be important in determining whether or not a horse exposed to poor quality hay and straw develops COPD. B cells, via humoral immune mechanisms, may have a role in the pathogenesis of equine COPD. The finding the BALF

from horses with COPD has significantly elevated levels of allergen specific IgG, IgA and IgE (Halliwell *et al, In preparation*) supports this proposal.

These differences in BALF lymphocyte phenotype distributions of control and asymptomatic COPD affected horses were unlikely to be a sequel to pulmonary inflammation, since, as the COPD affected horses were asymptomatic, they were considered to have no ongoing pulmonary inflammation. It is possible however, that the phenotype differences represent pulmonary inflammation which was undetected by the other investigative technique used to confirm that the horses were asymptomatic. The finding that abnormal accumulations of certain T lymphocyte subpopulations have been identified in BALF from humans which have no evidence of pulmonary disease detectable by other methods (Leatherman *et al* 1984; Wallace 1989) supports this hypothesis.

No significant differences were demonstrated between PB lymphocyte phenotype distributions of control and COPD affected horses, either before or after NC. Additionally, the PB lymphocyte phenotype distributions of either group were unaffected by NC.

While natural challenge (NC) had no effect on PB or BALF lymphocyte phenotype distributions of controls, it increased EqCD5+CD8- lymphocyte ratios and decreased EqCD8+ lymphocytes ratios in BALF from COPD affected horses, resulting in an increased EqCD5+CD8-:CD8+ ratio. This suggests that NC initiates a selective recruitment of EqCD5+CD8- lymphocytes from the PB to the pulmonary tissues and/or a redistribution of EqCD8+ lymphocytes from the pulmonary tissues to the PB. The former is consistent with the pulmonary recruitment of CD4+ lymphocytes demonstrated in human allergic asthmatics following allergen challenges (Gerblich *et al* 1984; Metzger *et al* 1987; Gerblich *et al* 1991), in humans with sarcoidosis (Berman *et al* 1990) and in humans with some types of hypersensitivity pneumonitis (Tomichi *et al* 1989; Ando *et al* 1991).

This selective pulmonary recruitment of lymphocyte phenotypes suggests that cellular immune mechanisms have a role in the pathogenesis of equine COPD. Activated T cells, by producing lymphokines, which have numerous effects including regulation of IgE production, regulation of mast cell differentiation, chemoattraction of neutrophils, eosinophils and basophils and activation of mast cells, neutrophils, monocytes and macrophages (Kay 1989), are potentially capable of inducing the pulmonary inflammation characteristic of equine COPD.

While the mechanism by which allergen induces pulmonary recruitment of lymphocyte subpopulations is unknown, human CD4 cells have been shown to respond to chemotactic stimuli including interleukins (Kornfeld *et al* 1985) and arachadonic metabolites (McCarty and Goetzl 1979; Bacon *et al* 1988) and C5a (El-Naggar *et al* 1981). In addition, certain mast cell mediators, notably histamine, which has been shown to increase within PELF during the late phase response of equine COPD (Chapter 5), may induce the elaboration by CD8+ cells of a CD4+ chemoattractant lymphokine termed lymphocyte chemoattractant factor (Center *et al* 1983; Berman *et al* 1984). Pulmonary lymphocyte recruitment has been recently reviewed by Berman *et al* (1990).

Antigen induced alterations in lymphocyte phenotype distributions are considered to be specific immunological responses as they were not elicited by challenge with equal amounts of inappropriate antigens (Gerblich *et al* 1984). Furthermore their absence in patients with severe hypoxaemia due to other pulmonary diseases indicates they are not simply a sequel to hypoxaemia (Corrigan *et al* 1988).

Alternatively, it is possible that the lymphocyte phenotype alterations observed in COPD affected horses after NC, rather than indicating selective phenotype recruitment to the lung, could reflect antigen induced alteration in the phenotypic expression of individual T cells. Lymphocyte phenotype alterations have been induced *in vitro* by histamine (Verhaegen *et al*

1977), cyclic nucleotides (Grieco *et al* 1976), various drugs (Limatibul *et al* 1978), cholinergic and alpha-adrenergic stimulation (Lang *et al* 1978) and the H2 agonist impromidine (Birch *et al* 1982). Such *in vitro* changes occurred within minutes, consistent with the rapid onset phenotype changes observed *in vivo* following bronchial allergen challenge in human asthmatics (Gerblich *et al* 1984).

While this study was unable to determine whether the state of activation of the lymphocytes was altered by challenge, there is considerable evidence that CD4+ cells, in addition to being recruited to the lungs of human asthmatics, are also activated by antigen inhalation challenges and that these cells have an important role in the pathogenesis of this condition (Gerblich *et al* 1984; Corrigan and Kay 1990). Investigation of the state of activation of CD4+ cells in horses with COPD is thus warranted.

Pharmacological modulation of the antigen induced lymphocyte phenotypic changes may prove to be an alternative strategy for the control of equine COPD. In man, inhaled albuterol, a B_2 sympathetic agonist, has been shown to abolish the antigen induced PB T cell phenotype changes in asthmatics (Varghese *et al* 1990). In addition, cyclophosphamide, a drug which is believed to exert its immunosuppressive actions primarily by inhibition of T lymphocyte activation (Kahan 1989), has been shown to, often dramatically, control the clinical symptoms in chronic human asthma (Alexander *et al* 1992).

CHAPTER 5

QUANTIFICATION OF HISTAMINE IN PLASMA AND PULMONARY FLUIDS FROM HORSES WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) BEFORE AND AFTER 'NATURAL (HAY AND STRAW) CHALLENGES'

SUMMARY

A commercial radioimmunoassay kit was used to quantify histamine in plasma and bronchoalveolar lavage fluid (BALF) of 8 control horses and 9 horses with chronic obstructive pulmonary disease (COPD), before and after 'natural (hay and straw) challenges' (NC). In addition, the pulmonary epithelial lining fluid (PELF) histamine and albumen adjusted BALF histamine concentrations were determined from the bronchoalveolar lavage fluid (BALF) histamine concentrations using the urea and albumen dilution techniques, respectively.

There were no significant changes in the concentrations of histamine in plasma or BALF at 0.5 or 5h after NC, but the PELF histamine concentration of COPD affected horses was significantly increased at 5h, but not at 0.5h, following NC.

As the histamine concentrations of whole BALF lysates were significantly correlated with the numbers of metachromatically staining cells, presumed to be mast cells and/or basophils, these findings support involvement of a late phase, mast cell/basophil mediated, hypersensitivity reaction in the pathogenesis of equine COPD.

INTRODUCTION

Mast cells and/or basophils are considered to have an important role in the pathogenesis of several human pulmonary inflammatory conditions including extrinsic allergic asthma (Holgate and Finnerty 1989), exercise induced asthma (Sheffer *et al* 1983), extrinsic allergic alveolitis (Haslam *et al* 1987) and hypersensitivity pneumonitis (Soler *et al* 1987). Increased concentrations of histamine, an inflammatory mediator released from degranulating mast cells and basophils, and its metabolites, have been identified in plasma and/or BALF from individuals suffering from these conditions.

BIOCHEMISTRY OF HISTAMINE

Histamine, 2-(4-imidazolyl) ethylamine or 5-beta-aminoethylimidazole, molecular weight 111, is synthesised in mast cells and basophils by the decarboxylation of the amino acid histidine by histidine decarboxylase (Plaut and Lichtenstein 1983), and is stored, closely associated with the anionic side chains of proteoglycans, in cytoplasmic granules.

Histamine, along with a wide spectrum of preformed and newly synthesised mediators, is liberated from mast cells and basophils in response to a wide variety of immunological and non immunological stimuli (*vide infra*). It is then rapidly deactivated (Keyzer 1984) by N-methyltransferase or monoamine oxidase (White *et al* 1987) mainly to N-methyl histamine, N-methylimidazolacetic acid and imidazoleacetic acid (Keyzer 1984), with only 2-3% of the histamine being excreted unchanged in the urine.

TECHNIQUES FOR QUANTIFICATION OF HISTAMINE IN BIOLOGICAL SAMPLES

The quantification of histamine, which is present at low levels within biological fluids, necessitates sensitive and specific techniques (Keyzer 1984) and has proved problematic until relatively recently.

There exist several different assay techniques, as outlined below;

1. **Bioassay** using guinea pig ileum has now been largely superseded (Cromwell *et al* 1986) since it is insensitive below 1ng/ml (Ind *et al* 1983).

2. **Radioenzymatic assay**, using either single or double isotope techniques may be employed. In the former the histamine specific enzyme, N-methyltransferase, isolated from rat kidney, transfers a tritiated methyl group from a substrate (s-[methyl-^{3}H]-adenosyl-L-methionine) to histamine, to produce labelled 1-methylhistamine. The tritiated histamine derivative is then extracted organically, isolated by thin layer chromatography and quantified radioactively (Dyer *et al* 1982; Ind *et al* 1983). The double isotope technique involves addition of a second tracer, which improves the sensitivity to 25 pg/ml (Kay 1987).

3. **Fluorimetric assay** is less expensive than the radioenzymatic assay and has also been fully automated (White *et al* 1987), but requires preliminary sample purification by ion exchange column chromatography and butanol extraction for adequate specificity and sensitivity (1-5ng/ml) (Lorenz and Doenicke 1978). Histamine, at an alkaline pH, is then reacted with *o*-phthalaldehyde to form a fluorescent compound which can then be quantified fluorimetrically.

4. **Gas chromatographic assay**, with nitrogen phosphorus detection, involve protracted sample preparation and require expensive equipment available only in specialist laboratories (Cromwell *et al* 1986). Standard quantities of synthetic homologues of histamine metabolites are added to the sample as internal standards. After extraction and derivatisation, the mixtures are separated on a capillary column and the nitrogen containing compounds selectively detected. The ratio of the natural metabolites and the internal standard is then related to the amount of histamine metabolite in the sample (Keyzer 1984).

5. For the **isotope dilution assay** a fixed amount of non radioactively labelled histamine, which is several mass units heavier than the naturally occurring histamine, is added to the sample. After extraction and derivatisation, gas chromatographic separation is performed, followed by mass spectrometric detection (Keyzer 1984).

6. **Radioimmunoassay** (RIA) techniques have revolutionised the assay of histamine in biological samples, being sensitive, specific, relatively inexpensive and comparatively simple to use. Consequently RIA was selected for this study.

COLLECTION AND PROCESSING OF SAMPLES FOR HISTAMINE ASSAY

While RIA has simplified the quantification of histamine in biological samples, accurate results are dependent on optimal sample collection and processing.

Guidelines for sample collection and preparation of human plasma for histamine determination were given by Lorenz *et al* (1982). Briefly, samples should not be collected within 10min of alcohol disinfection of the venupuncture site, within 30min after minor surgery, work, stress or anaesthesia or within 6h of a meal, although explanations for these recommendations were not given. Following collection, the samples should be placed on ice and processed within 30min, before changes in the histamine concentration occur. Ind *et al* (1983) advised that only the top two thirds of plasma should be aspirated when collecting plasma from centrifuged whole blood samples, to minimise basophil contamination.

PROBLEMS ASSOCIATED WITH QUANTIFICATION OF HISTAMINE IN BIOLOGICAL SAMPLES

'Leakage' of histamine from mast cells and basophils during sample collection or processing may lead to, possibly marked, overestimation of the histamine concentration of biological fluids (Ind *et al* 1983) but can be minimised by the use of calcium free solutions and by maintaining samples at low temperatures (Kalenderian *et al* 1988).

The quantification of histamine is further complicated by the short (approximately 1min, Keyzer *et al* 1984) half life of histamine in body tissues and body fluids. Consequently, transient increases in histamine concentrations, which occur prior to sampling, may not be detected.

ALTERNATIVES TO HISTAMINE QUANTIFICATION IN THE INVESTIGATION OF BASOPHIL/MAST CELL DEPENDENT HYPERSENSITIVITY PHENOMENA

Keyzer (1984) considered that quantification of the urinary histamine metabolites, N-methyl histamine and N-methylimidazolacetic acid, was more useful than quantification of histamine, since the metabolites have longer half lives. Furthermore these metabolite levels are less affected by inadvertent contamination of samples with basophils or mast cells. Both metabolites have been assayed in human urine and plasma by the isotope dilution technique, with mass spectrometric detection, and by gas chromatography (Keyzer 1984) and N-methyl histamine has been quantified by radioimmunoassay (Stephan *et al* 1988).

There are, however, potential problems associated with the quantification of histamine metabolites in the investigation of basophil/mast cell dependent hypersensitivity phenomena. Taylor *et al* (1990) found that urinary N-methyl histamine was only increased with persistent hyperhistaminaemia and that increased local histamine release in the lung or the upper airway did not cause a detectable change in its basal excretion. Furthermore, assay of urinary histamine metabolites may be affected by histamine produced by bacteria in the urogenital tract, although this problem may be minimised by collecting the urine by catheterisation (Keyzer 1984). As the concentration of N-methylimidazolacetic acid appears to be strongly dependent on the dietary histamine intake, exclusion of histamine containing foods from the diet is necessary prior to its assay (Keyzer 1984).

There appear to be no reports of the quantification of histamine metabolites in the horse.

HISTAMINE AND PULMONARY MAST CELLS IN THE HORSE - A REVIEW

While it has been proposed that equine chronic obstructive pulmonary disease (COPD) may involve Type I hypersensitivity (Cook and Rossdale 1963; Lowell 1964; Eyre 1972; Halliwell *et al* 1979; McPherson and Thomson 1983), little is known about the role of mast cells/basophils or histamine in the pathogenesis of this condition.

(A) HISTAMINE

A wide range of immunological and pharmacological agents may induce *in vitro* histamine release from equine peripheral blood leucocyte preparations. While the effect of anti equine IgE on basophils/mast cells has not been reported, anti rat IgE, anti human IgE, anti equine Fab (Magro *et al* 1988) and an anti-horse IgG antiserum (Kings and de Weck 1980) were shown to induce degranulation of equine basophils. Non IgE mediated equine basophil secretagogues include cocanavalin A (Kings and de Weck 1980), calcium ionophore and human C5a fragment (Abdel-Salam 1989). Kings and de Weck (1980) found that calcium ionophore A 23187, compound 48/80 and poly-*L*-lysine, which are well recognised secretagogues for basophils/mast cells of other species, had no effect on equine basophils/mast cells. Equine basophil degranulation is calcium, but not magnesium, dependent and is potentiated by deuterium oxide (Kings and de Weck 1980; Magro *et al* 1988).

Hockenjos *et al* (1981) found that extracts of hay, *aspergillus*, *candida* and *verticillium* could induce histamine release from peripheral blood leucocytes (PBL) collected from horses with COPD. However, Dieckmann (1986) was unable to demonstrate degranulation of equine peripheral blood basophils from control and COPD affected horses, as assessed by microscopic examination of toluidine blue preparations, following incubation with these antigen extracts, and proposed that the histamine release reported by Hockenjos and colleagues had been associated with basophil cytolysis induced by excessively high (100-500ug/ml) antigen concentrations. Dieckmann (1986) found that, while incubation of peripheral blood leucocytes with high concentrations of these antigens in the absence of

exogenous calcium elicited histamine release, there was no concomitant release of lactate dehydrogenase, an intracellular enzyme which is released during cytotoxic reactions. The significance of the findings of Hockenjos and colleagues thus remain unclear.

Abdel-Salam (1989) investigated histamine release from peripheral blood leucocytes and tracheobronchial cells from control horses and horses with 'probable allergic COPD' and with 'probable non allergic COPD', the 2 questionable categories being differentiated by the number of mast cells and eosinophils in their tracheobronchial secretions. Calcium ionophore and human C5a elicited histamine release from peripheral blood leucocytes and tracheobronchial cells, while extracts of timothy pollen and house dust mite were secretagogues only for tracheobronchial cells. Tracheobronchial cells released more histamine than peripheral blood leucocytes, and tracheobronchial cells from horses with 'probable allergic COPD' released more histamine than those from horses with 'probable non allergic COPD'.

Burka *et al* (1976) demonstrated that isolated lung segments from horses sensitised to bovine plasma in Freund's complete adjuvant liberated histamine and 5-hydroxytryptamine, but not slow-reacting substance of anaphylaxis, after subsequent challenge with bovine plasma. However, as the yield of histamine was low they suggested that histamine was less important in equine hypersensitivity than in man, guinea pigs and cattle, or that the antigen sensitisation was not optimal in their study. They also demonstrated that equine chopped lung preparations and sensitised PBL preparations released similar amounts of histamine.

During equine anaphylaxis, Lewis *et al* (1972) demonstrated that *whole blood* histamine and plasma 5-hydroxytryptamine were unaffected while whole blood kinins increased 4 fold. Eyre and Lewis (1973) found there was a 5-6 fold increase in plasma histamine approximately 2-4min after allergen induced anaphylaxis, with levels returning to normal by 20min.

Eyre (1972) found the level of *whole blood* histamine elevated in only 1 of 11 horses with COPD, while plasma 5-hydroxytryptamine was elevated in 5 of the horses. Unfortunately plasma histamine concentrations were not determined in this study.

Increased plasma histamine concentrations have been reported in horses with early grass sickness (Hodson *et al* 1989).

(B) PULMONARY MAST CELLS

The normal equine lung contains large numbers of mast cells, with approximately 20% being located within the bronchiolar walls, which is the primary site of histological changes in horses with COPD (Nicholls 1978; Mair *et al* 1988; Winder and Von Fellenberg 1990). Some pulmonary mast cells are located in the airway epithelium (Mair *et al* 1988) and in the airway and alveolar lumina (Kaup *et al* 1990A & B), where they are strategically located to interact with inhaled antigens.

The finding that sodium cromoglycate, which allegedly has mast cell stabilising properties, was prophylactically effective in equine COPD, in a proportion of studies, was considered as evidence that mast cell degranulation plays a central role in the pathogenesis of this condition (Murphy *et al* 1979; Thomson and McPherson 1983; Soma *et al* 1987). However, more recent studies in man indicate that the beneficial effect of sodium cromoglycate is more likely to be attributable to its many other antiinflammatory properties (Richards *et al* 1986; Kay *et al* 1987) rather than to mast cell stabilisation.

Winder and von Fellenberg (1990) demonstrated histological evidence of pulmonary mast cell hyperplasia in horses with undifferentiated chronic pulmonary disease. Some other studies (Yamashiro *et al* 1986; Vrins *et al* 1989; Winder *et al* 1990), have demonstrated increased numbers of mast cells in bronchoalveolar lavage fluid (BALF) from COPD affected

horses, although the findings of the present study (Chapter 2) and of Derksen *et al* (1985B) dispute this finding.

Yamashiro *et al* (1986) demonstrated that many BALF mast cells from a horse with COPD showed ultrastructural evidence of degranulation, while those from 3 control horses did not. While the authors considered this was evidence that mast cell degranulation is important in the pathogenesis of equine COPD, the small numbers of horses in this study and the presence of pulmonary inflammatory disease in the control group, as indicated by their excessively high BALF neutrophil ratios, casts doubt on the validity of this study.

In the present study, the role of pulmonary mast cells/basophils in the pathogenesis of equine COPD was investigated by quantifying histamine in plasma, BALF and pulmonary epithelial lining fluid (PELF) from control and COPD affected horses, before and at 0.5 and 5h after 'natural challenges' (NC) by exposure to hay and straw.

MATERIALS AND METHODS

SUBJECTS

Eight control horses (median age 15.5years, range 7-25; median body weight 577kg, range 212-652) and 9 COPD affected (median age 13.5years, range 6-25; median body weight 482kg, range 371-642) horses of various breeds were used (Appendix 5.1). All animals were maintained in a 'controlled', hay and straw free environment until they were shown to be fully asymptomatic, as described in Chapter 2.

NATURAL CHALLENGE

All horses received two separate NCs, as described in Chapter 2.

SAMPLE COLLECTION AND PROCESSING

The horses did not receive medication and no skin cleansing agent was used prior to venupuncture. Venous blood was collected immediately prior to and at 1.5 and 5h after one NC, by jugular venupuncture into precooled, sterile glass vacutainers containing potassium EDTA (Becton Dickinson, Meylan, France) and the samples placed immediately on ice. The blood was centrifuged, within 30min of collection, at 500*g* in a refrigerated centrifuge at 4^{o}C (MSE Chilspin 2, Fisons, Crawley) for 5min. The top two thirds of the plasma was aspirated without disturbing the cell pellet and was stored at -20^{o}C until assayed.

Baseline BALF samples were collected at least 72h prior to one NC, when horses were shown to be free from pulmonary disease by clinical, endoscopic and BALF cytological examinations, as described previously (Chapter 2). Post challenge BALF samples were collected 0.5h after one NC and 5h after the other. The baseline and the 2 post challenge BALF samples were collected on separate occasions to avoid possible interference caused by pulmonary inflammation induced by a recent (within 48h) lavage (Sweeney *et al*, In press B). BALF was processed and stored as described previously (Chapter 2).

HISTAMINE ASSAY

One commercial histamine RIA kit (Pharmacia Histamine RIA, Pharmacia, Milton Keynes), was found to be too insensitive, having a reported working range of 2-100ng/ml. Plasma and BALF histamine concentrations were, however, satisfactorily determined using a commercial competitive RIA kit developed by Morel and Delaage (1988) (Immunotech, Marseille, France). In this assay, histamine in the sample competes with an iodinated histamine tracer for binding sites on monoclonal antibody (mAb) coated tubes. A mAb specific for acylated histamine was employed to overcome problems associated with the production of a mAb specific for the small, and hence poorly immunogenic, histamine molecule. As a consequence histamine in the samples had to be acylated prior to assay. As sample pH can affect this assay (Morel and Delaage 1988), the pH of 10 BALF samples was determined using a SA520 pH meter (Orion Research, Cambridge, Mass. USA) and found to be (median 7.31, range 7.14-7.44) within the range in which acylation of histamine occurs almost instantaneously, but lower than that required for optimal binding of histamine to the histamine tracer (pH 8.2). The kit was used according to the manufacturers' instructions, total and bound radioactivity being determined using a gamma counter (NE1600, Nuclear Enterprises, Edinburgh).

As BALF histamine concentrations are not only dependent on the concentration of the histamine in the PELF, but also on the variable volume of PELF recovered by bronchoalveolar lavages (BALs), comparison of PELF histamine concentration is more rational than comparison of BALF histamine concentrations. PELF histamine concentrations were calculated from BALF histamine concentrations using the urea dilution technique reported by Rennard *et al* (1986). This technique involves concomitant quantification of plasma and BALF urea, with the assumption that urea, a small and freely diffusible molecule is present in equal concentrations in plasma and in PELF.

In addition to using the urea dilution technique, the variable dilution which occurs during BAL was also corrected for by determining the albumen adjusted BALF histamine

concentrations i.e. albumen adjusted BALF histamine concentration = BALF histamine concentration x plasma albumen concentration / BALF albumen concentration. Plasma and BALF albumen concentrations were determined as described in Chapter 7. These 2 dilution techniques are discussed fully in Chapter 7.

BALF metachromatic cell ratios were determined using 2 techniques, namely by performing differential counts of 1000 BALF cells on toluidine blue (Appendix 2.2) stained cytospin preparations and of 300 cells on Leishman's (Appendix 2.2) stained cytospin preparations. The ratios of these cells in 51 BALF samples, collected from 8 control and 9 COPD affected horses, before, and at 0.5 and 5h after NC, were compared.

The histamine content of metachromatic cells in BALF from 6 control and 5 COPD affected horses was determined by quantification of histamine in BALF supernatants and in 'whole BALF' which had been subjected to 3 freeze/thaw cycles (from room temperature to -20^{o}C) to lyse cells and liberate intracellular histamine. The BALF supernatant histamine concentration was subtracted from the 'whole BALF' histamine concentration, to yield the cellular histamine concentration and then divided by the absolute metachromatic cell count to yield the histamine content of BALF metachromatic cells.

STATISTICAL ANALYSES

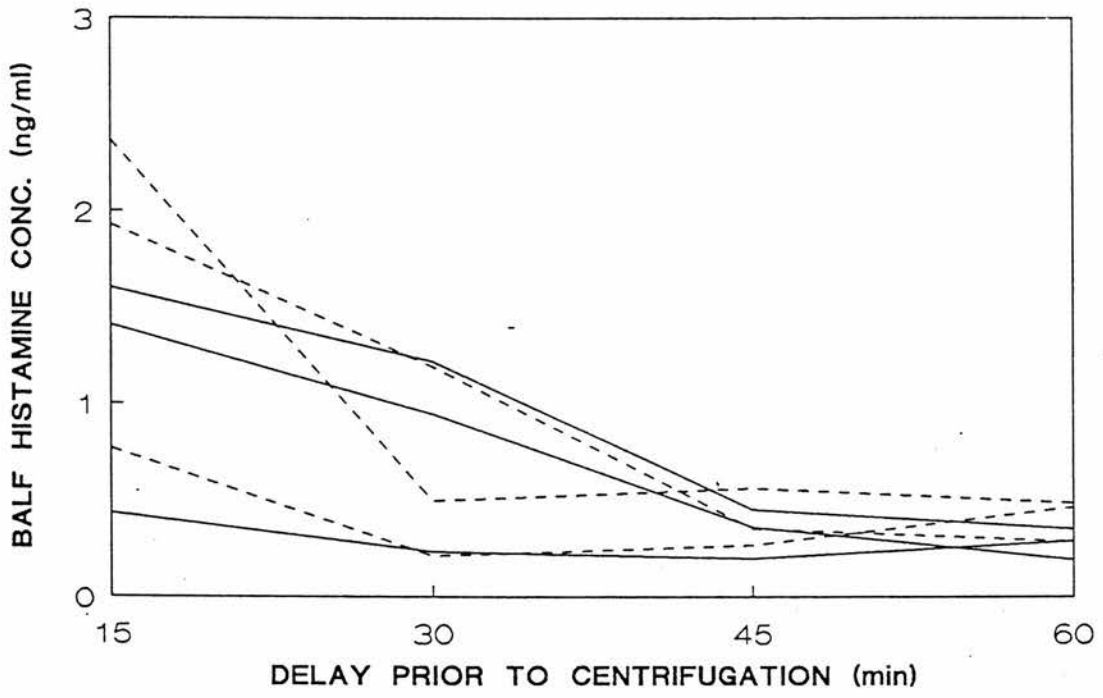
Paired and unpaired data, which were shown to be non normally distributed, were compared using the Wilcoxon Rank and Mann Whitney tests, respectively. Correlation was performed using the Spearman Rank Correlation. All analyses were performed using Minitab (Minitab Inc., Pennsylvania, USA), assuming a significance level of 5%.

RESULTS

Intraassay coefficients of variation for histamine assay, calculated from 4 repeated duplicate determinations on 2 plasma and 4 BALF samples with high and low histamine concentrations were 10.5% and 13.9% for the plasma and 8.7%, 13.7%, 18.0% and 30.7% for BALF. Inter-assay coefficients of variation, calculated from 4 duplicate repetitions using 2 plasma and 4 BALF samples, were 5.0 and 13.6% for plasma, and 1.0, 22.4, 25.4 and 30.4% for BALF.

Storage of whole BALF samples, from 3 control and 3 COPD affected horses, on ice for 15, 30, 45 or 60 min prior to collection of the supernatant resulted in a progressive decline in the histamine concentrations of the supernatants (Fig. 5.1). The half life of histamine in these samples varied from 15-50min.

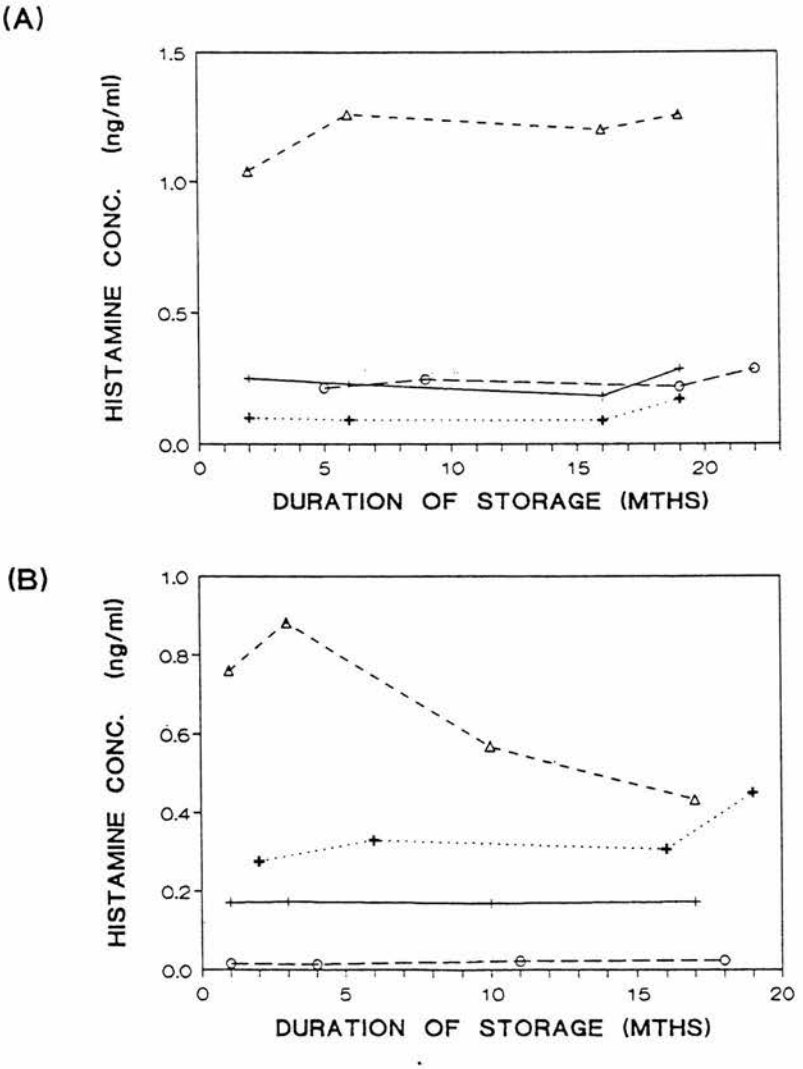
Fig. 5.1 The effect of delayed sample processing on the concentration of histamine (ng/ml) in the supernatant of BALF from control (n=3) (——) and COPD affected (n=3) horses (- - -).



The stability of histamine was evaluated by performing BALs on 2 horses using, as the lavaging fluid, PBS containing a known concentration of added histamine, and determining the proportion of exogenous histamine in the recovered BALF. The data and calculations are given in Appendix 5.2. The exogenous histamine was found to be stable during the collection, processing and storage of the BALF, the percentage recoveries for the two horses being 99% and 115%.

There were only minor changes in histamine concentrations of 4 plasma and 4 BALF supernatant samples during prolonged storage at -20^{0}C (Fig. 5.2).

Fig. 5.2 The effect of prolonged storage, at -20^{0}C, on the concentrations of histamine (ng/ml) in (A) equine plasma (n=4) and (B) equine BALF (n=4) samples.



NC had no significant effect on the concentrations of histamine in plasma (median = 0.24, range 0.07-0.57 for control horses at baseline) (Table 5.1, Appendix 5.3) and BALF (median = 1.78ng/ml, range 0.11-2.66 for control horses at baseline) (Table 5.2, Appendix 5.4) from control and COPD affected horses.

Table 5.1. Plasma histamine concentrations (ng/ml) of control (n=8) and COPD (n=8) affected horses before and at 1.5h and 5h after NC (median and range).

Time after NC (h)	Plasma histamine concentration	
	Control horses	COPD affected horses
0	0.24 (0.07-0.57)	0.16 (0.19-0.48)
1.5	0.36 (0.15-0.64)	0.18 (0.12-0.48)
5.0	0.23 (0.07-0.38)	0.17 (0.06-0.54)

Table 5.2. BALF histamine concentrations (ng/ml) of control (n=8) and COPD (n=9) affected horses at baseline and at 0.5h and 5h after NC (median and range).

Time after NC (h)	BALF histamine concentration	
	Control horses	COPD affected horses
0	1.78 (0.11-2.66)	0.28 (0.01-3.93)
0.5	1.92 (0.00-3.05)	1.01 (0.10-2.37)
5.0	1.36 (0.13-2.37)	1.10 (0.02-6.38)

PELF histamine concentrations and albumen adjusted BALF histamine concentrations were significantly higher in the COPD affected horses at 5h after NC (median PELF histamine concentration = 246.0ng/ml, range 5.0-1087.0) than at baseline (35.2, 4.6-430.0) ($p<0.05$),

although the values for control and COPD affected horses were not significantly different (Table 5.3 & 5.4, Figs. 5.3 & 5.4, Appendices 5.5 - 5.8).

Table 5.3. PELF histamine concentrations (ng/ml), derived using the urea dilution technique, for control (n=8) and COPD (n=9) affected horses before and at 0.5 and 5h after NC (median and range).

Time after NC (h)	PELF histamine concentration	
	Control horses	COPD affected horses
0	189.3 (31.3-435.9)	35.2 (4.6-430.0)
0.5	243.9 (1.1-651.4)	174.6 (11.1-777.3)
5.0	146.2 (64.9-658.2)	* 246.0 (5.0-1087.0)

* significantly higher than for the COPD affected horses at baseline ($p<0.05$).

Table 5.4. Albumen adjusted BALF histamine concentrations (ng/ml) for control (n=8) and COPD (n=9) affected horses before and at 0.5 and 5h after NC (median and range).

Time after NC (h)	Albumen adjusted BALF histamine concentration	
	Control horses	COPD affected horses
0	314.0 (45.0-1178.0)	125.1 (16.4-925.3)
0.5	784.0 (8.0-1120.0)	400.3 (38.1-779.6)
5.0	706.0 (49.0-1824.0)	* 898.0 (93.0-2278.0)

* significantly higher than for the COPD horses at baseline ($p<0.05$).

Fig. 5.3 Concentrations of histamine (ng/ml) in PELF of (A) control (n=8) and (B) COPD affected (n=9) horses, before and at 0.5 and 5h after NC, as determined by the urea dilution technique.

* Significantly higher than the prechallenge values, ($p<0.05$).

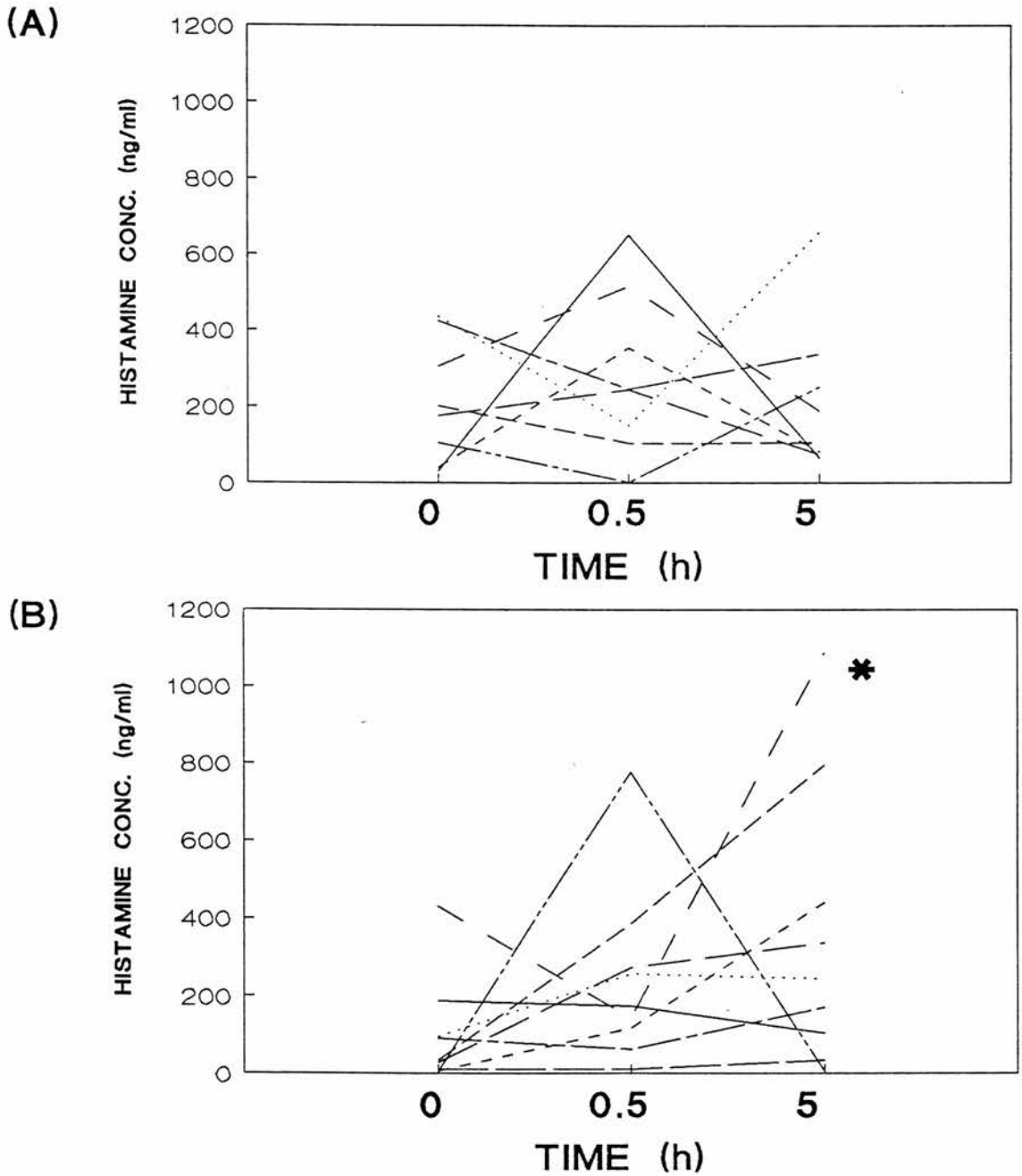
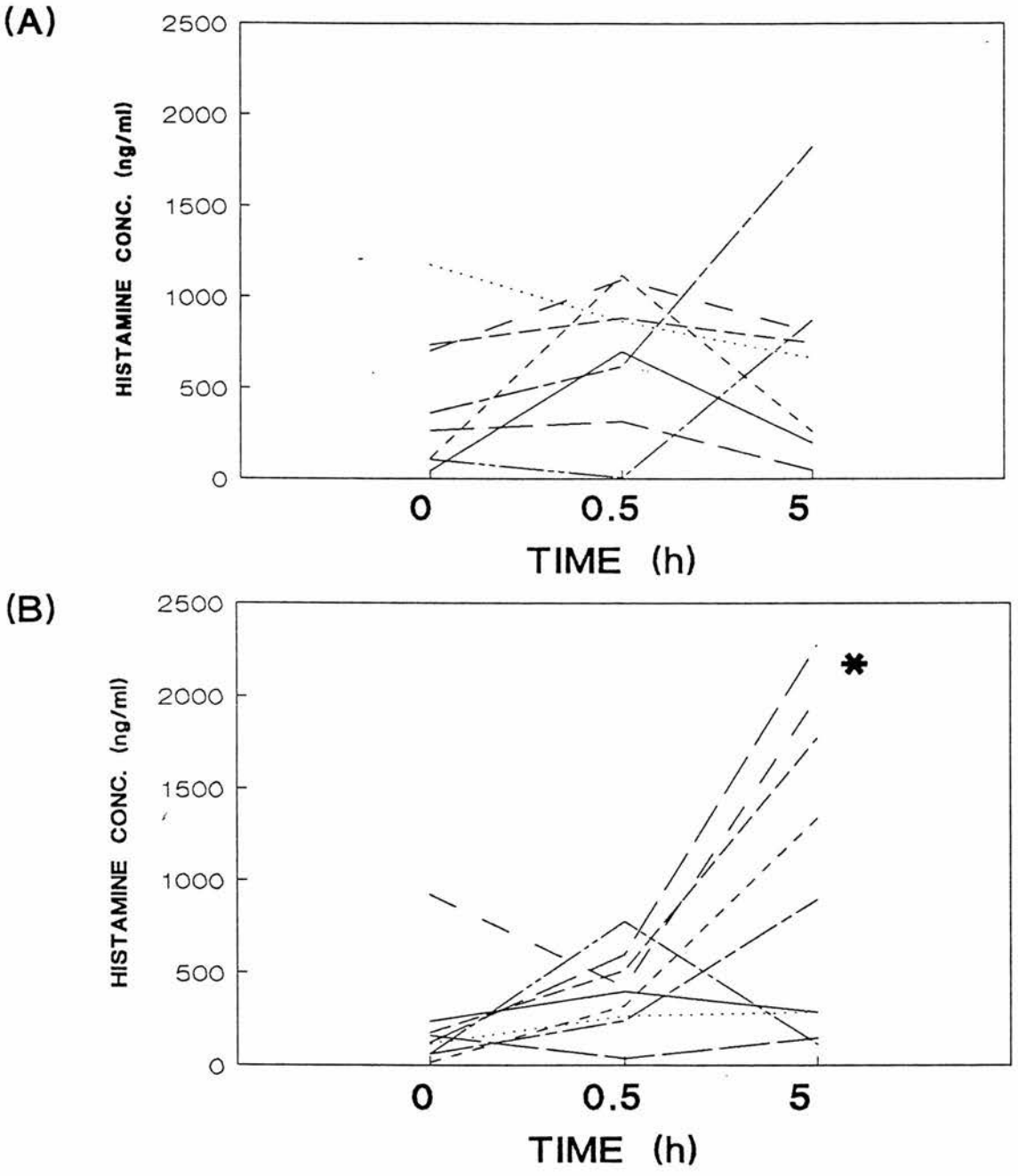


Fig. 5.4 Albumen adjusted BALF histamine concentrations (ng/ml) for (A) control (n=8) and (B) COPD affected (n=9) horses, before and at 0.5 and 5h after NC, as determined by the albumen dilution technique.

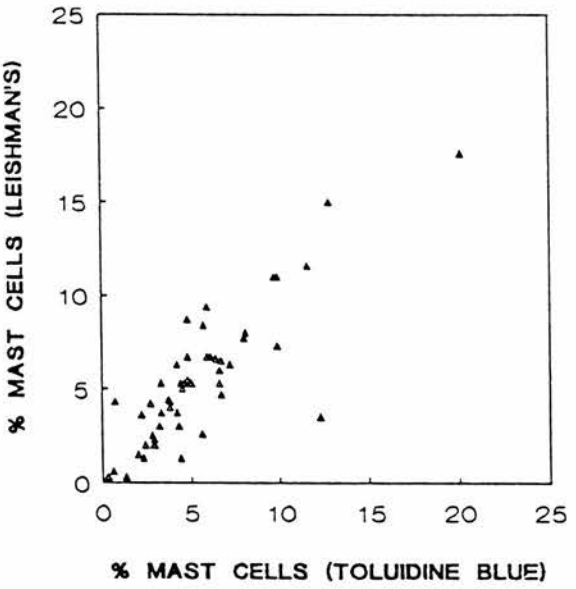
* Significantly higher than the prechallenge values, ($p<0.05$).



The concentration of histamine in PELF was on average 215 fold higher than that of BALF, which in turn was on average 7 fold higher than that of plasma.

The BALF mast cell ratios, when determined using toluidine blue and Leishman's staining techniques were not significantly different ($p>0.05$), the median difference between the counts obtained using the two techniques being 0.0 (Q1=-0.9, Q3=0.9, range -8.8 to 3.9) (Fig. 5.5, Appendix 5.4).

Fig. 5.5 Correlation between the BALF mast cell ratios obtained by performing differential counts of 1000 BALF cells on toluidine blue stained preparations and of 300 BALF cells on Leishman's stained preparations. The median difference of the ratios obtained using the 2 techniques was 0.0 (Q1=-0.9, Q3=0.9, range -8.8 to 3.9).



The median histamine content of metachromatic cells was calculated to be 2.79 pg/cell (range 0.63-4.60). There was no significant difference in the histamine content of metachromatic cells from control (median 2.86, range 1.54-4.60 pg/cell) and COPD affected (2.79, 0.63-3.12 pg/cell) horses (Appendix 5.9).

Three rapid freeze thaw cycles did not affect the concentration of histamine within 4 BALF supernatants (Table 5.5) and thus, by extrapolation, is unlikely to have affected the histamine concentration of the 'whole BALF' samples, which were subjected to this procedure in order to liberate the intracellular histamine.

Table 5.5 Histamine concentrations (ng/ml) of BALF supernatants (n=4) before and after being subjected to 3 rapid freeze thaw cycles.

HORSE	STATUS	BEFORE FREEZE THAW	AFTER FREEZE THAW
1	COPD	0.64	0.64
2	COPD	0.43	0.56
3	CONTROL	0.47 [1]	0.42
4	CONTROL	2.42 [1]	2.42

[1] Mean of 4 paired determinations.

Histamine concentrations of plasma were not significantly correlated with those of BALF or PELF ($p>0.05$). The BALF supernatant histamine concentrations were significantly correlated with the absolute BALF metachromatic cell counts ($r_S=0.328$, $p<0.05$) and the total BALF cell counts ($r_S=0.873$, $p<0.01$), but were not significantly correlated with the BALF metachromatic cell ratios. The absolute BALF metachromatic cell counts were significantly correlated with the whole BALF histamine concentrations ($r_S=0.675$, $p<0.05$). The PELF histamine concentrations were significantly correlated with the absolute BALF metachromatic cell counts ($r_S=0.356$ $p<0.05$), but not with the BALF metachromatic cell ratios.

There was no significant difference in the ratios of whole BALF histamine concentration:BALF supernatant histamine concentration for control (median 43.6, range 6.4-99.7) and COPD affected (23.3, 8.8-60.0) horses.

DISCUSSION

Histamine concentrations of equine plasma and bronchoalveolar lavage fluid (BALF) were satisfactorily determined using a commercial radioimmunoassay (RIA) kit which, being relatively inexpensive, of suitable sensitivity and precision and not requiring the use of specialist equipment, offers advantages over other histamine assay techniques.

The intraassay and interassay precisions for plasma histamine were, in most cases, higher than those previously reported for man (Morel and Delaage 1988), possibly due to the smaller number of repetitions performed in this study. BALF histamine concentrations showed more variation than those of plasma. There are no previously reported precision data for histamine determinations of BALF from any species using this RIA kit.

Exogenous histamine, instilled into the equine lung, was recovered by BAL without loss, indicating that the techniques used to collect, process and store the BALF samples, in this study, were unlikely to have affected the BALF histamine concentrations.

It was necessary to harvest the BALF supernatant immediately after collecting the BALF, as the histamine concentrations of BALF supernatants were found to decrease significantly when their separation from whole BALF was delayed. The half life of histamine in the supernatant of whole BALF samples ranged from 15-50min. This was greater than the half life of 1min for histamine in human plasma (Keyzer *et al* 1984), possibly because the whole BALF samples were stored on ice immediately after collection, thereby reducing BALF histaminase activity. In contrast, Soler *et al* (1987) found that histamine concentrations of whole BALF *increased* when separation of the supernatant was delayed, and concluded that this was due to leakage of histamine from BALF mast cells. Differences in the conditions used to store whole BALF samples prior to collection of the supernatant may account for these contradictory findings, as the conditions employed by Soler *et al* (1987) were not reported.

Histamine in plasma and BALF was stable during prolonged storage at -20°C, the minor changes observed being considered to be within the limits of interassay variation. Similarly, Rankin *et al* (1987) reported the coefficient of variance for human BALF histamine assay to be only ±8% and ±10% in samples stored at -70°C for more than 8 weeks.

Plasma histamine concentrations of control horses prior to NC (median = 0.24ng/ml, range = 0.07-0.57), were similar to those reported in a previous study for horses (0.313 ± 0.062ng/ml, range 0.078-0.733, Hodson *et al* 1989), and for monkeys (Lorenz *et al* 1974) and humans (Dyer *et al* 1982; Lorenz *et al* 1982; White *et al* 1987). The plasma histamine concentrations reported for the dog show marked variation (from 0.2 ± 0.3, Lorenz *et al* 1974 to 14.3 ± 4.1ng/ml, Nimmo Wilkie *et al* 1990), making comparison difficult.

This is the first study to quantify histamine in equine BALF. The values for control horses at baseline (median 1.78ng/ml, range 0.11-2.66) were similar to those reported for the dog (Casolaro *et al* 1989). The wide range of BALF histamine concentrations reported for normal humans (from 0.026 ± 0.02ng/ml, Casale *et al* 1987 to 1.16 ± 0.5, Haslam *et al* 1981) makes comparison difficult, but in general values for man appear to be lower than for the other species. Unexplainably high BALF histamine concentrations (6.4 ± 2.4ng/ml, Ingenito *et al* 1991) have been reported for the guinea pig.

As expected equine PELF histamine concentrations were considerably higher than those of BALF, which in turn were higher than those of plasma. While there are apparently no reports of PELF histamine determinations for other species, as human BALF and plasma histamine concentrations are similar (Rankin *et al* 1987) and normal human PELF constitutes 1.0 ± 0.1% of BALF (Rennard *et al* 1986), it can be estimated that the human PELF histamine concentration is approximately 100 fold higher than that of plasma.

Four possible explanations may account for the finding that PELF histamine concentrations are markedly higher than those of plasma.

Firstly, PELF histamine concentrations may be markedly higher than those of plasma as a result of local histamine release, i.e. from epithelial or luminal mast cells/basophils into the PELF. The presence of mast cells in various stages of degranulation in normal human pulmonary epithelia (Fox *et al* 1981; Lamb and Lumsden 1982) supports this theory.

Secondly, the high human PELF histamine concentrations may arise from the diffusion of histamine from pulmonary tissues across the alveolar capillary membrane into the BALF during BAL (Rankin *et al* 1987), as has been demonstrated for potassium (Davis *et al* 1982), urea, albumen and glucose (Rennard *et al* 1986). However, this is unlikely in the present study since equine BALF histamine concentrations were higher than those of plasma, and therefore net diffusion of histamine from BALF into plasma would have been expected.

Thirdly, as equine whole BALF lysates contained, on average, 21.4 (range 6.4-99.7) fold more histamine than BALF supernatants, artefactual release of histamine from mast cells/basophils during BALF collection or processing could cause an overestimation of BALF and PELF histamine concentrations. However there is considerable evidence from human studies to suggest that this does not occur (Kaliner,M. *pers obs.* quoted Rankin *et al* 1987).

(a) artefactual histamine release was not induced by reaspiration of BALF through a bronchoscope (Casale *et al* 1987),

(b) the fluid usually used in this study for lavage contained no calcium and was maintained between 4-25^{o}C making physiological release of histamine, which is calcium and temperature dependent (Magro *et al* 1988), unlikely (Kalenderian *et al* 1988),

(c) in man, BALF tryptase levels were on average 20 fold higher than those of histamine, rather than being comparable to those of histamine, as would be expected with artefactual mast cell degranulation (Kalenderian *et al* 1988).

Fourthly, histamine produced by bacteria within the lung could account for the higher histamine concentration of equine BALF compared with plasma. To minimise the errors arising from the presence of bacterial derived histamine, Kalenderian *et al* (1988) suggested discarding the first BALF aliquot, which was considered to contain bacterially derived histamine. However, as Rankin *et al* (1987) found no significant difference in histamine content of the first and pooled BALF samples, bacterially derived histamine would seem to have little effect on BALF histamine concentrations.

The absolute BALF metachromatic cell counts were significantly correlated with the concentrations of histamine in whole BALF lysates, consistent with the findings for man (Rankin *et al* 1987) and dogs (Sommerhoff *et al* 1989). This finding suggests that BALF histamine is derived from metachromatic cells which are recovered by BAL i.e. mast cell or basophils within the epithelium, airway lumen or alveoli. In man, mast cells rather than basophils were considered to be the source of BALF histamine, as the concentrations of tryptase, which is abundant in human mast granules but is virtually absent from basophils (Schwartz 1985), were considerably higher in human BALF than those of histamine (Kalenderian *et al* 1988).

Equine BALF metachromatic cells were found to contain more histamine (median 2.86ug/cell range 1.54-4.60) than was reported for dogs (1.80 $\pm$ 0.1, Sommerhoff 1989) and monkeys (1.68 $\pm$ 0.26, Patterson *et al* 1977). The values reported for normal human BALF mast cells (from 7.1 $\pm$ 3.8, Miura *et al* 1989 to 15.3 $\pm$ 10.8, Tomioka *et al* 1984) show considerable variation, possibly due to the different methods used to fix and stain mast cells (Overveld *et al* 1989).

While the validity of human BALF mast cell counts, based on examination of Wright's stained cytospin preparations, has been questioned due to the small numbers of mast cells

present in human BALF (Tomioka *et al* 1984; Rankin *et al* 1987), the accuracy of equine BALF mast cell counts may be greater due to the comparatively higher ratios of BALF mast cells in this species. It is possible that the absolute and differential BALF mast cell counts reported for all species may underestimate the true counts, due to the presence of partially or completely degranulated mast cells, which are not readily identified with light microscopy (Fox *et al* 1981; Lamb and Lumsden 1982).

The histamine concentration of PELF from COPD affected horses was significantly increased at 5h but not at 0.5h following NC, while that of controls was unaffected. This suggests that mast cell and/or basophil degranulation occurs only during the antigen induced late phase response (LPR) of COPD and that these cells may have a role in the pathogenesis of this condition. Plasma and BALF histamine concentrations were unaffected by NC, the former suggesting that peripheral blood basophils had not degranulated and that there was no significant transfer of histamine from the PELF to the plasma.

In man, increased concentrations of histamine in BALF (Diaz *et al* 1989) and plasma (Durham *et al* 1984) have been reported during LPRs to bronchial allergen challenges, and in skin blister sites (Charlesworth *et al* 1989) and nasal lavage fluids (Naclerio 1990) during LPRs to local allergen challenges.

The factors which induce histamine release during the LPR of equine COPD are not known, but could include specific antigen, complement proteins, histamine releasing factors, interleukins (reviewed by White 1990) or an antigen specific lymphokine which is produced by T lymphocytes (Askenase and Van Loveren 1983; Ezeamuzie and Assem 1983).

Histamine releasing factors (HRF) and histamine releasing inhibitory factors (HRIF) are recently described heterogeneous groups of cytokines which are believed to be involved in the

local control of mast cell activation (Alam *et al* 1990) and which may be important in the pathogenesis of antigen induced LPRs.

HRFs induce non cytotoxic release of histamine from mast cells and basophils *in vitro*, by IgE dependent and independent mechanisms. They may be responsible for recruiting mast cell or basophil participation in LPRs and possibly other chronic inflammatory conditions (Kaliner 1989; White 1990) and for inducing basophil degranulation during LPRs (Lichtenstein 1988).

These apparently low molecular weight (<15kDa) cytokines are produced largely by cells with low affinity IgE receptors i.e. by mononuclear cells (Theuson *et al* 1979), T cells (Sedgwick *et al* 1981), B cells (Alam *et al* 1989), neutrophils (White and Kaliner 1987) and platelets (Orchard *et al* 1986).

As HRF and HRIF have been identified in BALF of normal humans (Alam *et al* 1989), it appears that HRF production occurs in all individuals, including non atopics. However, only mast cells and basophils from atopic individuals, which have a special form of IgE molecule, designated IgE+, appear to be able to respond to HRF (Lichtenstein 1988). Alternatively, the increased amounts of HRF demonstrated in lavage fluids from humans with idiopathic pulmonary fibrosis (Broide *et al* 1990), asthma and allergic rhinitis (Wasserman 1990), may account for the increased BALF histamine noted in patients suffering from these conditions.

It is possible that HRFs, so far unidentified in the horse, could induce the antigen induced LPR histamine release demonstrated in horses with COPD.

Recently the duration of the allergen challenge has been shown to have an important effect on the pattern of allergen induced histamine release.

Classically, *in vivo* allergic responses to single or short term antigenic challenges, and *in vitro* stimulation of mast cells/basophils with immunological or non immunological agents suggested that allergens induced a very intense and rapid mast cell/basophil degranulation,

with almost complete release of preformed mediators within 30min (Lichtenstein and Oster 1964). These short term antigen challenges rarely induce LPRs (Shalit *et al* 1988) .

In contrast, prolonged intradermal allergen challenges elicited release of histamine which peaked at 1h and which continued at a constant level for at least 9h (Shalit *et al* 1988).

It is possible that prolonged antigen challenge, as would occur during naturally occurring equine COPD and during NCs, may have induced a prolonged pattern of histamine release which differed from that induced by the short duration AF, TV and MF antigen inhalation challenges described in Chapter 2.

In addition, the pattern of histamine release appears to be affected by the concentration of allergen (Peters *et al* 1982).

While it is unclear whether histamine released during the LPR in equine COPD arises from basophils or mast cells, there is increasing evidence to implicate basophils in human nasal, bronchial and dermal late phase responses to allergen (Okuda *et al* 1978; Nagy *et al* 1982; Mitchell *et al* 1986; Bascom *et al* 1988; Shalit *et al* 1988; Naclerio 1990; Kay *et al* 1991).

Histamine may have numerous roles in the pathogenesis of equine COPD, as it can induce bronchoconstriction, airway mucosal oedema and mucus secretion and increase pulmonary vascular permeability in several species (reviewed by White *et al* 1987). Histamine may also induce production of a CD4+ T cell chemoattractant lymphokine, termed lymphocyte chemoattractant factor (Center *et al* 1983; Berman *et al* 1984), which may induce pulmonary recruitment of selective lymphocyte phenotypes, as has been shown to occur in horses with COPD (Chapter 4). Other mediators which are released from mast cells and basophils concomitantly with histamine may also have roles in the pathogenesis of equine COPD.

In conclusion, the findings of this study are consistent with the hypothesis that a late phase, mast cell/basophil mediated, hypersensitivity reaction is involved in the pathogenesis of equine COPD.

CHAPTER 6

ATTEMPTED QUANTIFICATION OF TRYPTASE IN EQUINE SERUM AND BRONCHOALVEOLAR LAVAGE FLUID

SUMMARY

A commercial radioimmunoassay kit for human tryptase was found to be unsuitable for the quantification of tryptase in equine serum and bronchoalveolar lavage fluid (BALF) samples.

INTRODUCTION

Tryptase is a neutral protease, comprised of four 35-37Kd chains, which constitutes approximately 30% of the protein content of human mast cells (Schwartz *et al* 1981).

In man, tryptase, which is secreted from degranulating mast cells, has been used as a specific indicator of mast cell involvement in anaphylaxis (Schwartz *et al* 1987B), allergen induced early and late phase nasal responses (Castells and Schwartz 1988), allergen induced early cutaneous responses (Shalit *et al* 1988) and in local endobronchial allergen challenge responses (Wenzel *et al* 1988).

Tryptase offers several advantages over histamine as an indicator of mast cell degranulation.

(a) Tryptase has a half life in body tissues and body fluids of approximately 2h, which is considerably longer the 1 minute half life of histamine (Keyzer *et al* 1984).

(b) Tryptase is a more specific indicator of mast cell degranulation than histamine, as its concentration is 250-900 fold higher in human mast cells than in basophils, while mast cell histamine concentration is only 1-2 fold higher than that of basophils (MacGlashan and Lichtenstein 1980; Schwartz *et al* 1987A). No other cell type, including those from normal lung, skin, peripheral blood and small intestine contain appreciable amounts of tryptase (Schwartz 1985; Craig *et al* 1986; Castells *et al* 1987).

(c) Tryptase may be assayed at lower concentrations than histamine (Wenzel *et al* 1988).

There are apparently no reports describing or quantifying tryptase in the horse.

A commercial radioimmunoassay (RIA) kit for quantification of human tryptase which employs anti human tryptase monoclonal antibodies, was used in an attempt to quantify equine tryptase.

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION

Sera and bronchoalveolar lavage fluid (BALF) samples were collected from control and COPD affected horses as described in Chapter 2. The samples were processed and stored according to the recommendations of the manufacturers of the RIA kit (Pharmacia, Milton Keynes).

Tryptase was assayed in 5 samples, as follows;

1. Serum collected from a COPD affected horse at 0.5h after 'natural challenge' (NC).
2. Serum collected from a control horse at 0.5h after NC.
3. BALF supernatant collected from a COPD affected horse at 0.5h after NC.
4. A whole BALF lysate. Whole BALF, with a high mast cell ratio (22%), which had been collected from a control horse, was subjected to three rapid freeze thaw cycles (from -20^{o}C to room temperature) to rupture the cells and liberate intracellular enzymes.
5. A BALF cell pellet lysate. 100ml BALF, with a high mast cell ratio (22%), which had been collected from a control horse, was centrifuged at 500g for 5min at 4^{o}C and the resultant pellet subjected to three freeze thaw cycles to liberate intracellular enzymes.

TRYPTASE ASSAY

A commercial human tryptase RIA kit, capable of measuring tryptase within the range 2-50U/l (Pharmacia Tryptase RIACT, Pharmacia, Uppsala, Sweden), was used according to the manufacturers' instructions. The 5 samples were assayed in duplicate.

Total and bound radioactivity were measured in a gamma counter (NE1600, Nuclear Enterprises, Edinburgh).

RESULTS

While a satisfactory standard curve was obtained using the human tryptase standards provided in the RIA kit, assay of the equine samples yielded negative results.

DISCUSSION

Attempted assay of human tryptase using a commercial RIA kit was unsuccessful. This was likely to be due to lack of antigenic cross reactivity between equine tryptase and the 2 anti human tryptase monoclonal antibodies employed in the kit. Alternatively the negative results could indicate that the horse does not possess a homologue of human tryptase.

CHAPTER 7

EVALUATION OF UREA AND ALBUMEN AS ENDOGENOUS MARKERS OF DILUTION OF EQUINE BRONCHOALVEOLAR LAVAGE FLUID

SUMMARY

Bronchoalveolar lavage fluid (BALF) is comprised of lavage fluid and pulmonary epithelial lining fluid (PELF) in variable proportions. Quantitative comparisons of the cellular and molecular components in different BALF samples are thus valid only if the proportions of PELF in the BALF samples are standardised. Two BALF standardisation techniques, namely the urea and albumen dilution techniques, were evaluated in the horse.

Four BALF samples, from the left and right diaphragmatic lobes, the accessory lobe of the right lung and the apical lobe of the left lung, and plasma were collected from 7 control horses. The concentrations of urea and albumen in these samples were determined and the plasma:BALF urea and albumen ratios calculated. The products of these ratios and the BALF cell counts yielded the urea adjusted BALF cell counts, which were taken to be the PELF cell counts, and the albumen adjusted BALF cell counts, respectively.

The PELF cell counts calculated for the 4 lung lobes of individual horse showed less variation than the albumen adjusted BALF cell total counts. Assuming that PELF cell counts are uniform throughout the lung, the urea dilution technique appears to be the more accurate BALF standardisation technique.

BALF contained 0.4% PELF (range 0.1-1.0%), as determined by the urea dilution technique.

Albumen adjusted BALF cell counts were significantly higher than PELF cell counts.

INTRODUCTION

Bronchoalveolar lavage (BAL), a simple and safe technique for collecting cellular and molecular components from the lung, facilitates qualitative studies of these components. However, quantitative comparison of cellular and molecular components in different bronchoalveolar lavage (BALF) samples may not be valid, as the concentration of these components in BALF depends not only on their concentrations in pulmonary epithelial lining fluid (PELF), but also on the proportion of PELF in each BALF sample. The latter is variable and may be affected by the composition and volume of the lavage fluid, the dwell time (i.e. the time the BAL fluid is in contact with the pulmonary epithelium), the site of the lavage and the presence of disease (Brain and Frank 1973; Pinsker *et al* 1980: Davis *et al* 1982; Pingleton *et al* 1983; Dohn and Baughman 1985; Yam *et al* 1985; Rennard *et al* 1986; Pringle *et al* 1988; Sweeney *et al* In Press A).

To overcome the problem of variable PELF recovery which occurs during BAL, the concentrations of BALF components are commonly compared to endogenous reference substances, usually urea or albumen (Rennard *et al* 1986). Assuming that the plasma:PELF ratios of urea and albumen are uniform throughout the lung and are identical in different individuals, simultaneous determinations of either of these markers in plasma and in BALF permit standardisation of the concentrations of PELF within BALF samples.

There are important differences between the 'urea and albumen dilution techniques'. Urea, being a small molecule which diffuses readily throughout body fluids, is in equilibrium in PELF and plasma (Rennard *et al* 1986). Thus, in addition to permitting standardisation of the variable concentrations of PELF in BALF, the urea dilution technique can also be used to determine the concentration of PELF in BALF samples, and hence to determine the concentration of cells and molecules in PELF. As the alveolar membrane has reduced permeability to the large albumen molecules, plasma albumen concentrations exceed those of PELF (Rennard *et al* 1986). Thus, while the albumen dilution technique can be used to

standardise the variable concentrations of PELF in BALF samples, it cannot be used to determine the concentration of PELF in BALF samples.

In the present study both the 'urea and albumen dilution techniques' were evaluated in the horse.

MATERIALS AND METHODS

SUBJECTS

Seven control (median age 16years, range 10-25; median body weight 593kg, range 500-652) horses were used (Appendix 7.1).

SAMPLE COLLECTION AND PROCESSING

BALF samples, from the left and right diaphragmatic lobes, the accessory lobe of the right lung and the apical lobe of the left lung and plasma samples were collected during a single bronchoscopy session.

The samples were collected, processed and stored prior to assay as described in Chapter 2. The total and differential BALF cell counts were determined as described in Chapter 2. The plasma and BALF albumen and urea concentrations were determined as described below.

PLASMA AND BALF ALBUMEN DETERMINATIONS

The albumen concentrations of plasma and BALF supernatants were kindly determined, using a radioimmunodiffusion (RID) assay, by Ms.P.Irving, Department of Veterinary Clinical Studies, R(D)SVS.

Ten ul sample or purified equine albumen standard (Cohn Fraction V, Sigma, Poole, which had been purified through Sephacryl S300, Pharmacia, Milton Keynes) was placed in 4mm diameter wells in 3% agar (Difco, Detroit, USA) containing 0.06M barbital buffer pH 8.6 (Appendix 7.2) and the optimised concentration of anti-equine albumen (purified rabbit anti equine albumen, prepared by Prof.R.E.W. Halliwell) and incubated at room temperature for 48h. The agar plates were then washed in phosphate buffered saline (pH 7.2) (PBS) for 48h and in distilled water for 48h. After drying, the agar plates were stained with amido blue-black (Appendix 7.2) until dark blue, then decolourised with 4 washes in 2% glacial acetic acid. The diameters of the precipitin rings were determined using a magnifying lens and graticule (Graticules Ltd., Tonbridge). The albumen concentrations of samples were determined using a

standard curve, plotted for each plate, which related the square of the precipitin diameter to the albumen concentration of the standards. Samples were assayed in duplicate, using different plates for each assay. Duplicates which varied by >10% were repeated.

PLASMA AND BALF UREA DETERMINATIONS

BALF supernatant urea concentrations were determined using a commercial enzymatic assay kit (BUN Endpoint, Sigma, Poole), employing a modification of the technique of Rennard *et al* (1986). Samples were processed and assayed immediately after thawing to room temperature, to minimise bacterial degradation of urea. Prior to assay, BALF supernatants were ultracentrifuged at 12000g for 5min (Maxifuge, Whiteleafe Scientific, Dulverton) to remove precipitated matter which could affect optical density measurements. Reaction mixtures of 2.08ml freshly prepared reagent and 0.42ml BALF supernatant or urea standard were mixed by gentle inversion of the cuvette (Semi micro, BDH, Poole) and incubated at room temperature for 5min. The absorbance was determined spectrophotometrically (CE 292, Cecil Instruments, Cambridge) at 340nm using a 0.5cm light path. The mean absorbances of duplicate samples were subtracted from the sum of the absorbance of a reagent blank (2.08ml reagent and 0.42ml PBS) and that of a BALF supernatant blank (2.08ml PBS and 0.42ml BALF supernatant) to yield the change in absorbance.

The urea concentration was determined from a standard curve prepared using a glucose/urea nitrogen standard solution (Sigma, Poole).

Plasma urea concentrations were kindly determined by the Clinical Diagnostic Laboratory, R(D)SVS using a Technicon RAXT (Technicon, Dublin).

DETERMINATION OF PELF CONCENTRATION OF BALF SAMPLES

Assuming that urea, being a small freely diffusible molecule, is present in equal concentrations in plasma and in PELF, the concentration of PELF in BALF samples can be determined by the 'urea dilution technique' (Rennard *et al* 1986);

PELF concentration of BALF (%) = 100 x BALF urea concentration (mg/dl) / plasma urea concentration (mg/dl)

DETERMINATION OF TOTAL AND ABSOLUTE PELF CELL COUNTS

These were calculated by the urea dilution technique;

Total PELF cell count ($x10^3$/ul) = Total BALF cell count ($x10^3$/ul) x plasma urea concentration / BALF urea concentration.

Absolute PELF cell count ($x10^3$/ul) = Absolute BALF cell count ($x10^3$/ul) x plasma urea concentration / BALF urea concentration.

DETERMINATION OF ALBUMEN ADJUSTED TOTAL AND ABSOLUTE BALF CELL COUNTS

As the PELF and plasma albumen concentrations are not equal (Rennard *et al* 1986), the albumen dilution technique cannot be used directly to determine PELF cell counts. However the variable dilution of BALF samples may be standardised by determining the 'albumen adjusted BALF cell count', using the albumen dilution technique;

Albumen adjusted total BALF cell count ($x10^3$/ul) = Total BALF cell count ($x10^3$/ul) x plasma albumen concentration / BALF albumen concentration.

Albumen adjusted absolute BALF cell count ($x10^3$/ul) = Absolute BALF cell count ($x10^3$/ul) x plasma albumen concentration / BALF albumen concentration.

STATISTICAL ANALYSIS

Plasma and BALF urea and albumen concentrations, which were not normally distributed, were analysed by non parametric techniques. Paired and unpaired data were compared using the Wilcoxon Rank and Mann Whitney tests respectively.

RESULTS

Plasma urea (median 23.6mg/dl, range 18.2-31.8; Table 7.1, Appendix 7.3) and albumen concentrations (median 22.8mg/ml, range 20.4-25.7; Table 7.1, Appendix 7.4) were within the working ranges of the assays.

Table 7.1. Concentrations of albumen (mg/ml) and urea (mg/dl) in plasma and in BALF, recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA), of 7 control horses (median and range).

	UREA	ALBUMEN
PLASMA	23.6 (18.2-31.8)	22.8 (20.4-25.7)
RD	0.105 (0.042-0.147)	0.0675 (0.0429-0.1047)
RA	0.170 (0.058-0.246)	0.0970 (0.0747-0.1620)
LD	0.089 (0.056-0.333)	0.0834 (0.0562-0.1216)
LA	0.106 (0.024-0.330)	0.0803 (0.0029-0.0097)

All BALF samples contained urea concentrations (median 0.109mg/dl, range 0.024-0.333; Table 7.1, Appendix 7.3) within the linear range of the assay (0.04-1mg/dl). Interassay coefficients of variation for urea determination of BALF samples with low (median 0.079 mg/dl) and high (median 0.581 mg/dl) urea concentrations, based on 5 repeated duplicate assays, were 6.1% and 12.8% respectively. Intraassay coefficient of variation for urea determination in BALF, based on 10 repeated duplicate assays, was 1.6%.

All BALF samples contained albumen concentrations (median 82.3ug/ml, range 29.0-162.0; Table 7.1, Appendix 7.4) within the working range of the assay. Duplicate determinations did not vary by >10%.

BALF samples were shown, by the urea dilution technique, to contain on average 0.4% PELF (range 0.1-1.0%).

The total and differential BALF cell counts, which were used to calculate the total and absolute PELF cell counts and the albumen adjusted total and absolute BALF cell counts, are given in Table 7.2 and Appendices 7.5 and 7.6.

The total PELF cell counts (Table 7.3, Appendix 7.7) were significantly lower ($p<0.01$) than the albumen adjusted total BALF cell counts (Table 7.4, Appendix 7.8), on average by 1.2 fold (range 0.6-2.7).

The total and absolute (Appendix 7.9) PELF cell counts calculated for the 4 lung segments of individual horses showed less variation than the albumen adjusted total and absolute (Appendix 7.10) BALF cell total counts.

Table 7.2 Total BALF cell counts (/ul) and BALF cell ratios for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses (median and range).

CELL	RD	RA	LD	LA
TOTAL	90 (50-140)	150 (65-280)	80 (35-200)	105 (25-130)
NEUT	1.0 (0.7-4.0)	1.3 (0.7-4.0)	1.3 (0.0-2.3)	3.0 (0.7-4.3)
LYM	39.7 (20.0-51.3)	23.0 (16.0-47.0)	24.3 (17.0-56.7)	28.7 (16.3-52.7)
MAC	49.7 (36.0-74.3)	63.3 (42.7-73.7)	61.7 (29.3-75.7)	60.7 (35.7-78.0)
EOS	0.0 (0.0-0.7)	0.0 (0.0-0.7)	0.0 (0.0-0.7)	0.0 (0.0-2.3)
MAST	9.3 (0.3-10.7)	6.3 (2.0-18.7)	8.0 (3.3-13.7)	4.0 (1.0-13.7)
BAS	0.3 (0.0-2.3)	0.3 (0.0-1.3)	0.0 (0.0-1.0)	0.3 (0.0-4.0)
TOTBAS	9.3 (0.7-12.3)	6.3 (2.3-19.0)	8.0 (4.3-13.7)	5.7 (2.0-14.0)
EP	0.3 (0.0-1.7)	0.7 (0.0-2.7)	1.0 (0.0-3.3)	0.7 (0.0-2.0)

Table 7.3 Total and absolute PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses (median and range).

CELL	RD	RA	LD	LA
TOTAL	18.9 (14.2-36.8)	26.5 (12.7-36.2)	19.1 (14.0-27.1)	24.9 (12.5-30.3)
NEUT	0.2 (0.1-1.5)	0.4 (0.1-1.0)	0.2 (0.0-0.5)	0.4 (0.1-1.3)
LYM	7.3 (4.3-12.9)	5.8 (3.5-12.4)	6.3 (3.1-13.5)	7.6 (2.0-13.2)
MAC	12.8 (5.4-27.3)	13.9 (6.5-26.5)	13.8 (5.1-16.7)	9.8 (5.7-20.0)
EOS	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.1)	0.0 (0.0-0.6)
MAST	1.5 (0.1-3.0)	1.3 (0.4-4.1)	1.1 (0.7-3.3)	0.7 (0.1-2.1)
BAS	0.1 (0.0-0.3)	0.1 (0.0-0.3)	0.0 (0.0-0.2)	0.0 (0.0-1.1)
TOTBAS	1.8 (0.3-3.0)	1.7 (0.4-4.2)	1.1 (1.0-3.3)	1.7 (0.3-2.2)
EP	0.1 (0.0-0.3)	0.2 (0.0-0.8)	0.2 (0.0-0.8)	0.1 (0.0-0.6)

Table 7.4 Albumen adjusted total and absolute BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

CELL	RD	RA	LD	LA
TOTAL	26.6 (21.7-38.0)	34.3 (19.0-51.8)	23.5 (15.7-52.4)	39.5 (17.6-35.8)
NEUT	0.3 (0.2-1.5)	0.5 (0.2-1.4)	0.3 (0.0-1.2)	0.9 (0.2-1.5)
LYM	10.8 (4.4-13.6)	8.3 (6.1-10.3)	7.0 (2.7-17.5)	8.3 (4.8-18.5)
MAC	13.8 (7.8-28.2)	23.3 (8.1-32.8)	11.9 (8.2-38.1)	16.2 (7.9-23.6)
EOS	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.8)
MAST	2.1 (0.1-3.4)	1.6 (0.7-9.7)	1.9 (0.7-4.2)	1.3 (0.3-3.1)
BAS	0.1 (0.0-0.5)	0.1 (0.0-0.2)	0.0 (0.0-0.4)	0.7 (0.0-0.7)
TOTBAS	2.1 (0.3-3.9)	1.6 (0.8-9.8)	1.9 (0.9-4.2)	1.3 (0.6-0.7)
EP	0.1 (0.0-0.5)	0.3 (0.0-0.9)	0.2 (0.0-0.9)	0.2 (0.0-0.4)

DISCUSSION

As BALF comprises variable proportions of lavage fluid and PELF, comparison of the concentrations of molecules and cells in different BALF samples is only valid if the concentrations of PELF in the samples are standardised. Two techniques to standardise the concentration of PELF within BALF samples, namely the urea and albumen dilution techniques (Rennard *et al* 1986), were evaluated in the horse.

Both the urea and albumen dilution techniques were shown to be suitable for standardising the concentration of PELF within equine BALF samples. These techniques assume that the plasma:PELF ratios of urea and albumen are uniform throughout the lung and are identical in different horses.

Both techniques were employed to standardise the concentration of PELF within equine BALF samples in other aspects of this study (Chapters 2, 5, 8 and 9). In all cases, within and between group statistical comparisons of the BALF cellular and molecular components yielded identical results, irregardless of whether the data were standardised using the urea or albumen dilution techniques.

However, assuming that there is no regional inhomogeneity in the PELF cell counts of individual horses, as the PELF cell counts calculated for the 4 lung lobes of individual horse showed less variation than the albumen adjusted BALF cell total counts, the urea dilution technique appears to be the more accurate technique for standardising the variable PELF recovery in BALF samples.

The urea dilution technique was used to calculate the proportion of PELF in BALF samples from control horses. This technique assumes that urea, being a small molecule (60 Daltons), which diffuses readily throughout body fluids, is in equilibrium in equine PELF and plasma.

This assumption has been confirmed by direct measurements of PELF urea concentrations in foetal sheep (Adams *et al* 1963; Strang 1968). Equine BALF was shown to comprise 0.4% PELF (range 0.1-1.0%). The proportions of PELF in human BALF have been determined using the urea dilution technique (1.0±0.1%; Rennard *et al* 1986), using methylene blue as an exogenous (*vide infra*) marker of dilution (10.7±6.8, range 2.3-21.1%; Baughman *et al* 1983) and by a technique combining the use of urea as an endogenous marker of dilution and methylene blue and technetium as exogenous markers of dilution (2.2%, range 0.7-2.5%; Kelly *et al* 1988). The reason for the variation in these reported values is not known.

The albumen dilution technique may be used to standardise the variable concentration of PELF in BALF, however, as plasma and PELF albumen concentrations are not in equilibrium (Rennard *et al* 1986), it cannot be used to determine the concentrations of molecules and cells in PELF. The alveolar membrane is considered to have properties similar to cell membranes (Taylor *et al* 1965), with reduced permeability to larger molecules such as albumen (60kDaltons) and plasma albumen concentrations exceed those of PELF. Albumen concentrations of PELF obtained by direct collection from foetal lambs (Strang 1968) and from rats (Reifenrath 1973) were, respectively, 3% and 13% that of plasma albumen concentrations. In man the albumen concentration of PELF was calculated to be 8.8% that of the plasma albumen concentrations. In contrast, in this study, the albumen adjusted total BALF cell counts were on average 1.2 fold higher than the total PELF cell counts, suggesting that in the horse the concentration of albumen in plasma is, only, on average 1.2 fold higher than that in PELF. As it is unlikely that the permeability of the alveolar membrane of normal horses to albumen differs considerably from that reported for man (Rennard *et al* 1986), foetal lambs (Strang 1968) and rats (Reifenrath 1973), it is likely that the BALF albumen concentrations reported in the present study are overestimations. The finding that the median BALF albumen concentration of control horses (82.3ug/ml, range 29.0-162.0) was higher than those reported previously for the horse (64.5± 8.3ug/ml; Robinson *et al* 1986) and for man (34±2ug/ml; Rennard *et al* 1986) is consistent with this possibility. If this is the case, the albumen adjusted total and absolute BALF cell counts reported are underestimations of their actual values, although the magnitude of this error is likely to be uniform throughout the study.

Unfortunately, potential problems limit the accuracy of both the albumen and the urea dilution techniques.

During BAL, large volumes of fluid rapidly cross the alveolar membrane, both from the lavage fluid into the pulmonary interstitium and *vice versa* (Kelly *et al* 1988), with a net movement of fluid from the pulmonary interstitium into the BALF. During human BAL, approximately 60ml of the 180ml instilled fluid passed into the pulmonary circulation, while approximately 105ml fluid passed from the circulation into BALF (Kelly *et al* 1988).

The rate of fluid transfer is related to the transmembrane differences in hydrostatic pressure, which vary markedly during the injection and suction phases of BAL, and to the transmembrane diffusion gradient, which is likely to be minimal when isotonic lavage fluids are used (Kelly *et al* 1988).

Similarly, both urea and albumen diffuse passively from the pulmonary interstitium into BALF during the BAL procedure (Rennard *et al* 1986; Marcy *et al* 1987). Consequently, BALF contains urea and albumen derived from PELF and from the pulmonary interstitium, the latter leading to overestimation of the volume of PELF recovered. The magnitude of this error is greater for urea than for albumen, as the rate of diffusion is inversely proportional to the molecular size. The magnitude of this error is also proportional to the duration the BALF remains in contact with the pulmonary tissues, termed the 'dwell time'. In man, dwell times lasting several minutes may result in equilibration of interstitial and BALF urea concentrations (Sietsema *et al* 1986), while equilibration of the larger albumen molecules would take considerably longer.

In this study, to minimise the diffusion of urea and albumen from the pulmonary interstitium into the BALF, BALF was aspirated immediately after instillation, thus the dwell time was always less than 45s. In man, BALF which was recovered immediately after instillation contained urea which was derived mainly (>80%) from the PELF, rather than from the pulmonary interstitium (Rennard *et al* 1986).

Further inaccuracies in the albumen dilution technique may follow alterations in the plasma:PELF albumen ratio, a feature of lung diseases which are associated with increased lung permeability (Rennard *et al* 1986).

Other endogenous markers of BALF dilution, including potassium and calcium, have been used in man, but are generally considered to be less useful than the albumen and urea dilution techniques (Davis *et al* 1982; Lam *et al* 1984).

Several exogenous markers of dilution, i.e. markers which are added to the lavage fluid prior to BAL, have been used, but are not considered to be as useful as the urea and albumen dilution techniques (Rennard *et al* 1986). The most commonly employed exogenous marker technique, the methylene blue dilution technique, results in a greater overestimation in PELF recovery than the urea technique, possibly due to loss of the dye by diffusion into the pulmonary interstitium or by binding to cells within the lung (Baughman *et al* 1983).

Recently, a new 'rewash' lavage procedure, incorporating a radioactive tracer, was reported to give more accurate estimations of PELF volume than the urea dilution technique (Peterson *et al* 1990). However, as this technique utilises radioactive materials it was considered unsuitable for the present study.

The median equine BALF urea concentration of 0.109mg/dl (range 0.024-0.333) was similar to that reported for man (0.120$\pm$ 10mg/dl) (Rennard *et al* 1986). The median BALF albumen concentration of control horses (82.3ug/ml, range 29.0-162.0) was unaccountably higher than those reported previously for the horse (64.5$\pm$ 8.3ug/ml; Robinson *et al* 1986) and for man (34$\pm$2ug/ml; Rennard *et al* 1986).

CHAPTER 8

COMPARISON OF CELLULAR AND MOLECULAR COMPONENTS OF BRONCHOALVEOLAR LAVAGE FLUID (BALF) HARVESTED FROM DIFFERENT SEGMENTS OF THE EQUINE LUNG

SUMMARY

A comparison was made of the cellular and molecular components of bronchoalveolar lavage fluid (BALF) harvested from four different lung segments, namely the left and right diaphragmatic lobes, the accessory lobe of the right lung and the apical lobe of the left lung, of 7 control horses and 6 horses with symptomatic chronic obstructive pulmonary disease (COPD).

Neither control nor symptomatic COPD affected horses showed significant regional differences in BALF recovery volumes, total and differential BALF cell counts, albumen adjusted total and absolute BALF cell counts, total and absolute pulmonary epithelial lining fluid (PELF) cell counts, and BALF albumen and urea concentrations. This suggests that the composition of PELF is uniform throughout the lungs of normal horses and horses with symptomatic COPD and that a single BALF sample is representative of the entire lung in these horses.

INTRODUCTION

Bronchoalveolar lavage (BAL) is a safe and relatively simple technique for harvesting molecules and cells from the distal airways and alveoli. It has proved invaluable in the investigation of equine pulmonary disease.

However, numerous different BAL techniques are currently being used in the horse, and variations in methodology may affect the cellular and molecular components of the recovered bronchoalveolar lavage fluid (BALF) (Sweeney and Beech 1991). Identification and standardisation of the factors which affect BALF composition are thus prerequisites to accurate comparison of BALF data from different laboratories.

In other species, BALF composition is affected by the type, pH and volume of the lavage fluid (Brain and Frank 1973; Pinsker *et al* 1980: Davis *et al* 1982; Pingleton *et al* 1983; Dohn and Baughman 1985; Yam *et al* 1985), the diameter of the collection instrument (Pringle *et al* 1988), the processing technique (Mordelet-Dambrine *et al* 1984) and the duration of storage of BALF prior to processing (Costabel *et al* 1988).

The site of BAL also affected the BALF volume and total BALF cell counts of healthy humans (Pingleton *et al* 1983) and dogs (Carre *et al* 1985), although no regional differences in their BALF cell ratios were demonstrated. Furthermore, humans with interstitial pulmonary disease showed marked interlobar variation in their BALF cell counts (Nugent *et al* 1984; Garcia *et al* 1986). These findings cast doubt on the common assumption that the cellular and molecular components of a single BALF sample are always representative of those throughout the lung.

In healthy calves, no significant difference was demonstrated in the cellular and protein components of BALF harvested from the cranial and caudal lobes, although there was

considerable variation in the concentrations of these parameters in BALF collected from the 2 lobes of individual calves (Pringle *et al* 1988).

In the normal horse, the volume of fluid used for BALs affected the total, absolute and differential BALF cell counts (Sweeney *et al* In Press A). A large lavage volume (300ml) yielded lower total BALF cell counts and lower BALF neutrophil, macrophage and lymphocyte ratios than a small lavage volume (50ml).

No difference was demonstrated in the cell populations in BALF collected from the right and left lungs of normal horses, with the exception of an unaccountably higher number of mast cells in BALF collected from the left lung (Sweeney *et al* In Press A). In contrast, horses with localised pulmonary diseases, including pneumonia and pleuropneumonia, may show regional variations in BALF cytology (Rossier *et al* 1991). This regional variation may have clinical diagnostic significance, as 'normal' BALF may be harvested for these horses if BAL is inadvertently performed in a lung segment unaffected by the inflammatory process. Consequently, BALF examination is of limited value in the diagnosis of localised pulmonary conditions.

This study investigated whether there are regional differences in the cellular and molecular components of BALF harvested from 4 lung segments of normal and symptomatic COPD affected horses.

MATERIALS AND METHODS

SUBJECTS

Seven control (median age 16years, range 10-25; median body weight 593kg, range 500-652) and 6 symptomatic COPD affected (median age 18.5years, range 6-25; median body weight 482kg, range 371-546) horses were used (Appendix 8.1). The criteria used to define control and symptomatic COPD affected horses are described in Chapter 2.

SAMPLE COLLECTION AND PROCESSING

Venous blood was collected by jugular venupuncture, and the plasma harvested as described in Chapter 2. BALF samples were collected, at a single bronchoscopy session, from the diaphragmatic lobes of the right and left lungs, the accessory lobe of the right lung and the apical lobe of the left lung, as described in Chapter 2.

Blood and BALF samples were processed, and the plasma and BALF urea and albumen concentrations determined, as described in Chapters 2 and 7. Total and differential BALF cell counts, albumen adjusted total and absolute BALF cell counts and total and absolute pulmonary epithelial lining fluid (PELF) cell counts were determined as described in Chapters 2 and 7.

STATISTICAL ANALYSIS

The data, for the 4 lung segments, which were not normally distributed, were compared using the Kruskal-Wallis test, performed using Minitab (Minitab Inc., Pennsylvania, USA). The data for the control and the COPD affected horses were analysed separately.

RESULTS

There were no significant regional differences in volume recovery (Table 8.1, Appendix 8.2), total (Table 8.2, Appendix 8.3) and differential BALF cell counts (Table 8.3, Appendix 8.4), albumen adjusted total (Table 8.4, Appendix 8.5) and absolute BALF cell counts (Table 8.5, Appendix 8.6), total (Table 8.6, Appendix 8.7) and absolute PELF cell counts (Table 8.7, Appendix 8.8), and albumen (Table 8.8, Appendix 8.9) and urea (Table 8.9, Appendix 8.10) concentrations of BALF collected from the 4 different lung segments of either control or COPD affected horses ($p<0.05$).

PELF total cell counts of control horses were significantly lower than albumen adjusted total BALF cell counts ($p<0.01$), by on average by 1.2 fold (range 0.6-2.7). The 4 PELF cell counts of individual horses showed less variation than the albumen adjusted total BALF cell counts.

Table 8.1. Volume (ml) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses (median and range).

LUNG LOBE	CONTROL	COPD
RD	109 (61-130)	101 (79-155)
RA	79 (56-113)	99 (24-139)
LD	89 (65-142)	97 (45-145)
LA	98 (10-112)	95 (26-182)

Table 8.2. Total cell counts (/ul) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses (median and range).

LUNG LOBE	CONTROL	COPD
RD	90 (50-140)	105 (70-240)
RA	150 (65-280)	115 (95-250)
LD	80 (35-200)	127 (65-300)
LA	105 (25-130)	175 (45-250)

Table 8.3 Cell ratios of BALF collected from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of (A) control (n=7) and (B) COPD affected (n=6) horses (median and range).

(A) Control horses.

CELL	RD	RA	LD	LA
NEUT	1.0 (0.7-4.0)	1.3 (0.7-4.0)	1.3 (0.0-2.3)	3.0 (0.7-4.3)
LYM	39.7 (20.0-51.3)	23.0 (16.0-47.0)	24.3 (17.0-56.7)	28.7 (16.3-52.7)
MAC	49.7 (36.0-74.3)	63.3 (42.7-73.7)	61.7 (29.3-75.7)	60.7 (35.7-78.0)
EOS	0.0 (0.0-0.7)	0.0 (0.0-0.7)	0.0 (0.0-0.7)	0.0 (0.0-2.3)
MAST	9.3 (0.3-10.7)	6.3 (2.0-18.7)	8.0 (3.3-13.7)	4.0 (1.0-13.7)
BAS	0.3 (0.0-2.3)	0.3 (0.0-1.3)	0.0 (0.0-1.0)	0.3 (0.0-4.0)
TOTBAS	9.3 (0.7-12.3)	6.3 (2.3-19.0)	8.0 (4.3-13.7)	5.7 (2.0-14.0)
EP	0.3 (0.0-1.7)	0.7 (0.0-2.7)	1.0 (0.0-3.3)	0.7 (0.0-2.0)

Table 8.3 (B) COPD affected horses.

CELL	RD	RA	LD	LA
NEUT	64.2 (5.7-94.3)	59.0 (5.3-94.0)	62.2 (5.0-89.3)	64.5 (4.7-95.3)
LYM	22.2 (3.7-36.3)	15.9 (5.3-41.3)	6.9 (4.0-40.7)	14.0 (2.7-26.7)
MAC	11.9 (1.7-57.3)	14.5 (0.3-60.7)	12.7 (3.0-82.3)	15.0 (1.7-71.0)
EOS	0.0 (0.0-0.7)	0.3 (0.0-0.7)	0.0 (0.0-1.0)	0.0 (0.0-1.0)
MAST	1.8 (0.0-4.3)	0.9 (0.0-4.0)	2.0 (0.3-4.0)	1.7 (0.3-5.0)
BAS	0.0 (0.0-0.7)	0.0 90.0-0.3)	0.0 (0.0-0.7)	0.0 (0.0-1.3)
TOTBAS	2.2 (0.0-4.3)	1.0 (0.0-4.0)	8.0 (4.3-13.7)	1.7 (0.3-5.0)
EP	0.0 (0.0-1.0)	0.0 (0.0-1.3)	1.0 (0.0-3.3)	0.2 (0.0-1.3)

Table 8.4 Albumen adjusted total BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses.

LUNG LOBE	CONTROL	COPD
RD	26.6 (21.7-38.0)	35.9 (18.6-53.2)
RA	34.3 (19.0-51.8)	43.0 (15.9-64.2)
LD	23.5 (15.7-52.4)	43.3 (33.0-62.9)
LA	29.5 (17.6-35.8)	59.1 (22.3-95.3)

Table 8.5 Albumen adjusted absolute BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of (A) control (n=7) and (B) COPD affected (n=6) horses.

(A) Control horses.

CELL	RD	RA	LD	LA
NEUT	0.3	0.5	0.3	0.9
	(0.2-1.5)	(0.2-1.4)	(0.0-1.2)	(0.2-1.5)
LYM	10.8	8.3	7.0	8.3
	(4.4-13.6)	(6.1-10.3)	(2.7-17.5)	(4.8-18.5)
MAC	13.8	23.3	11.9	16.2
	(7.8-28.2)	(8.1-32.8)	(8.2-38.1)	(7.9-23.6)
EOS	0.0	0.0	0.0	0.0
	(0.0-0.2)	(0.0-0.2)	(0.0-0.2)	(0.0-0.8)
MAST	2.1	1.6	1.9	1.3
	(0.1-3.4)	(0.7-9.7)	(0.7-4.2)	(0.3-3.1)
BAS	0.1	0.1	0.0	0.7
	(0.0-0.5)	(0.0-0.2)	(0.0-0.4)	(0.0-0.7)
TOTBAS	2.1	1.6	1.9	1.3
	(0.3-3.9)	(0.8-9.8)	(0.9-4.2)	(0.6-0.7)
EP	0.1	0.3	0.2	0.2
	(0.0-0.5)	(0.0-0.9)	(0.0-0.9)	(0.0-0.4)

Table 8.5 (B) COPD affected horses.

CELL	RD	RA	LD	LA
NEUT	18.8	16.3	24.2	22.6
	(3.0-40.0)	(3.4-41.9)	(1.7-52.0)	(3.0-81.6)
LYM	6.4	5.4	4.0	9.8
	(1.3-19.3)	(1.5-23.6)	(1.5-1919)	(0.6-24.4)
MAC	2.9	3.5	5.8	9.6
	(0.6-28.3)	(0.1-36.1)	(0.1-51.8)	(0.4-44.6)
EOS	0.0	0.1	0.0	0.0
	(0.0-0.1)	(0.0-0.3)	(0.2-0.3)	(0.0-1.0)
MAST	0.4	0.4	0.7	0.9
	(0.0-2.0)	(0.0-0.8)	(0.0-2.5)	(0.1-3.0)
BAS	0.0	0.0	0.0	0.0
	(0.0-0.4)	(0.0-0.1)	(0.2-0.3)	(0.0-1.2)
TOTBAS	0.5	0.5	0.8	1.5
	(0.0-2.0)	(0.0-0.8)	(0.2-2.8)	(0.1-4.0)
EP	0.0	0.0	0.0	0.1
	(0.0-0.5)	(0.0-0.6)	(0.0-0.2)	(0.0-0.7)

Table 8.6 Total PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses (median and range).

LUNG LOBE	CONTROL	COPD
RD	18.9 (14.1-36.8)	14.2 (10.3-29.2)
RA	26.5 (12.7-36.2)	15.6 (11.1-29.8)
LD	19.1 (14.0-27.1)	17.6 (11.6-28.7)
LA	24.9 (12.5-30.3)	20.3 (11.3-26.1)

Table 8.7 PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of (A) control (n=7) and (B) COPD affected (n=6) horses (median and range).

(A) Control horses.

CELL	RD	RA	LD	LA
NEUT	0.2 (0.1-1.5)	0.4 (0.1-1.0)	0.2 (0.0-0.5)	0.4 (0.1-1.3)
LYM	7.3 (4.3-12.9)	5.8 (3.5-12.4)	6.3 (3.1-13.5)	7.6 (2.0-13.2)
MAC	12.8 (5.4-27.3)	13.9 (6.5-26.5)	13.8 (5.1-16.7)	9.8 (5.7-20.0)
EOS	0.0 (0.0-0.2)	0.0 (0.0-0.2)	0.0 (0.0-0.1)	0.0 (0.0-0.6)
MAST	1.5 (0.1-3.0)	1.3 (0.4-4.1)	1.1 (0.7-3.3)	0.7 (0.1-2.1)
BAS	0.1 (0.0-0.3)	0.1 (0.0-0.3)	0.0 (0.0-0.2)	0.0 (0.0-1.1)
TOTBAS	1.8 (0.3-3.0)	1.7 (0.4-4.2)	1.1 (1.0-3.3)	1.7 (0.3-2.2)
EP	0.1 (0.0-0.3)	0.2 (0.0-0.8)	0.2 (0.0-0.8)	0.1 (0.0-0.6)

Table 8.7 (B) COPD affected horses.

CELL	RD	RA	LD	LA
NEUT	7.7 (1.7-16.4)	7.2 (1.6-18.3)	8.6 (1.0-15.0)	8.3 (1.2-19.1)
LYM	3.5 (0.5-10.6)	2.5 (0.6-11.0)	1.6 (0.5-7.4)	0.1 (0.3-5.7)
MAC	1.7 (0.3-15.6)	2.0 (0.0-16.8)	2.1 (0.5-23.6)	2.0 (0.2-18.5)
EOS	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.0 (0.0-0.2)
MAST	0.2 (0.0-1.1)	0.2 (0.0-0.4)	0.3 (0.0-1.1)	0.4 (0.0-0.7)
BAS	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.3)
TOTBAS	0.3 (0.0-1.1)	0.2 (0.0-0.4)	0.4 (0.0-1.1)	0.4 (0.0-0.9)
EP	0.0 (0.0-0.3)	0.0 (0.0-0.3)	0.0 (0.0-0.1)	0.0 (0.0-0.2)

Table 8.8 Albumen concentrations (ug/ml) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses (median and range).

LUNG LOBE	CONTROL	COPD
RD	67.5 (42.9-104.7)	86.7 (37.0-120.0)
RA	97.0 (74.7-162.0)	74.9 (45.0-146.0)
LD	83.4 (56.2-121.6)	66.5 (37.0-134.0)
LA	80.3 (29.0-97.0)	93.1 (8.9-122.8)

Table 8.9 Urea concentrations (ug/dl) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses (median and range).

LUNG LOBE	CONTROL	COPD
RD	105 (42-147)	252 (68-349)
RA	170 (58-246)	231 (126-284)
LD	89 (56-333)	223 (127-307)
LA	106 (24-330)	238 (49-581)

DISCUSSION

This study tested the assumptions that (a) cellular and molecular components of PELF are uniformly distributed throughout the lungs of healthy and symptomatic COPD affected horses and (b) that a single BALF sample, collected from any lung segment, is representative of the entire lung in these horses.

No significant regional variation was demonstrated in BALF and PELF cytology, or in BALF albumen and urea concentrations of control and symptomatic COPD affected horses. Thus a single BALF sample is representative of the entire lung of normal and symptomatic COPD affected horses, and analysis of a single BALF sample, harvested from any lung segment, appears to be adequate for the diagnosis and monitoring of equine COPD. These findings are consistent with the observation that COPD is a generalised pulmonary condition (Nicholls 1978).

However, while no significant regional variation was demonstrated in the BALF and PELF cytology of symptomatic COPD affected horses, considerable variation was noted in the neutrophil ratios of the 4 BALF samples from individual symptomatic COPD affected horses. Indeed, in 3 of the 6 symptomatic COPD affected horses, the maximum and minimum BALF neutrophil ratios differed by more than 10%. This variation should be taken into consideration when performing quantitative studies of BALF cytology in horses with symptomatic COPD.

The 4 total PELF cell counts of individual horses showed less variation than their 4 albumen adjusted BALF total cell counts. Thus, assuming that the total PELF cell counts of individual horses show regional homogeneity, the urea dilution technique would appear to be more accurate than the albumen dilution technique for correcting for the variable PELF recovery which occurs during BAL.

PELF cell counts were significantly lower than albumen adjusted BALF cell counts, consistent with the finding that the plasma:PELF ratio for albumen is greater than that for urea (Rennard *et al* 1986). This topic is discussed fully in Chapter 7.

Total and absolute PELF cell counts of control horses were similar to those reported previously for normal horses (Robinson *et al* 1986) and for healthy humans (Rennard *et al* 1986) (Table 8.10). However, the PELF neutrophil ratio reported for 'normal horses' by Robinson *et al* (1986), of approximately 12%, appears to be excessively high when compared those reported for normal horses by the present study (median BALF neutrophil ratio 1.5%, range 0.0-4.3%) and previous equine studies (4.7±1.16%, Viel, 1983; 6.2±5.0%, Mair *et al* 1987; 8.9±1.2%, Derksen *et al* 1989; 1.2±1.2% - 5.4±3.2%, Sweeney *et al* In Press A). It is possible that some of the horses studied by Robinson *et al* (1986) suffered from COPD.

Table 8.10 PELF cell counts ($x10^3$/ul) reported for normal horses and humans (median and range or mean±SE).

CELL	EQUINE ROBINSON *ET AL* (1986)	EQUINE PRESENT STUDY	HUMAN RENNARD *ET AL* (1986)
TOTAL	15.5±2.7	21.7 (12.5-36.8)	21.0±0.3
NEUT	1.8±1.4	0.3 (0.0-1.5)	0.2±0.0
MAC	6.8±0.9	12.7 (5.1-27.3)	17.0±0.3
LYM	5.0±1.1	6.9 (2.0-13.5)	4.0±0.4
EOS	0.5±0.5	0.0 (0.0-0.6)	NR
MAST	0.7±0.3	1.2 (0.1-4.1)	NR
BASO	NR	0.1 (0.0-1.1)	NR
TOTBAS	NR	1.6 (0.3-4.2)	NR
EP	0.8±0.2	0.1 (0.0-0.8)	NR

NR = Not reported.

CHAPTER 9

EVALUATION OF LOCAL ENDOBRONCHIAL ANTIGEN CHALLENGES IN THE INVESTIGATION OF EQUINE CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

SUMMARY

Local transendoscopic endobronchial antigen challenge, which has proved to be a valuable clinical and research technique in the study of human pulmonary hypersensitivity, was evaluated in normal and asymptomatic chronic obstructive pulmonary disease (COPD) affected horses.

Transendoscopic endobronchial challenges with phosphate buffered saline (PBS), 60ug/ml and 600ug/ml *Micropolyspora faeni* extract (MF) and mouldy hay extract elicited neutrophilic airway inflammatory responses in both control and asymptomatic COPD affected horses, as determined by cytological examinations of bronchoalveolar lavage fluid (BALF) harvested from the challenged lung segments.

Endobronchial challenges with 600ug/ml MF induced a significant BALF neutrophilia only in horses with asymptomatic COPD, when compared with PBS challenges. However, as the BALF neutrophil ratios of COPD affected horses after this MF challenge did not differ significantly from those of control horses, this finding has little clinical diagnostic value. The BALF neutrophilia induced in control and asymptomatic COPD affected horses by 60ug/ml MF and mouldy hay extract challenges was not significantly different from that induced by PBS challenge.

Endoscopically visible bronchial changes were observed in a proportion of both control and COPD affected horses within 5min and at 5h after PBS, MF and mouldy hay extract challenges.

As local endobronchial challenges induced a non specific pulmonary neutrophilia in both control and COPD affected horses and elicited endoscopically visible responses in a proportion of horses from both groups, this technique is considered to be of limited value in the investigation of equine COPD.

INTRODUCTION

Local endobronchial antigen challenge, a technique introduced by Metzger *et al* (1985A), has yielded valuable information on the pathogenesis of human asthma (Metzger *et al* 1985B; Metzger *et al* 1985C; Fick *et al* 1987; Metzger *et al* 1987; Casale *et al* 1987; Wenzel *et al* 1988). Transendoscopic instillation of minute quantities of appropriate allergens onto the bronchial mucosa of human asthmatics was shown to induce, within several minutes, endoscopically visible bronchial pallor, followed by hyperaemia, oedema and bronchial narrowing (Casale *et al* 1987). Despite using 100 fold higher allergen concentrations, no visible response was elicited in control subjects. Bronchoalveolar lavage (BAL) was then performed at the challenge sites at various times post challenge, to harvest cellular and molecular components of the inflammatory response for further characterisation.

This technique offers three potential advantages over conventional antigen inhalation challenges, namely (a) a rapid visual response is obtained, (b) the presence of numerous challenge sites within the lung permits challenge with several antigens or different concentrations of antigen and the inclusion of simultaneous control challenges during a single bronchoscopy, and (c) minute quantities of antigen, approximating those required for intradermal antigen testing, are required, which is advantageous when challenge substances are scarce, expensive or potentially toxic.

We report here an evaluation of local endobronchial antigen challenge as a tool for the diagnosis and investigation of equine chronic obstructive pulmonary disease.

MATERIALS AND METHODS

SUBJECTS

Seven control (median age 10years, range 6-20; median body weight 531kg, range 500-652) and 7 fully asymptomatic COPD affected (median age 20years, range 13-30; median body weight 497kg, range 371-546) horses (Appendix 9.1), which had no evidence of pulmonary disease according to criteria previously described (Chapter 2), were used. All horses were maintained in a 'controlled environment' (Chapter 2), prior to and during all challenges.

ENDOBRONCHIAL CHALLENGE WITH HISTAMINE

One control horse and one asymptomatic COPD horse were given endobronchial challenges with histamine under general anaesthesia which was induced by 10 mg/kg intravenous thiopentone sodium (Intraval, RMB Animal Health, Dagenham) and maintained with a halothane (Halothane M&B, RMB Animal Health, Dagenham) and oxygen mixture. During bronchoscopy, gaseous anaesthesia was administered via a permanent tracheostomy in one horse and via a nasal catheter in the other.

Horses were given incremental challenges with 5ml of solutions containing 10^{-6} to 10^{-1} mg/ml histamine acid phosphate (BDH, Poole) in PBS pH 7.4 (Sigma, Poole) at $37^{o}C$, administered transendoscopically (CF-1T10L, Olympus Optical Co., Japan) via an endoscopic catheter (OD=2mm, ID=1mm; Lectrocath, Vygon, Ecouen, France), each challenge being performed in a different second or third generation bronchus. The challenge solution was instilled with the catheter approximately 15cm beyond the tip of the endoscope. Care was taken to avoid touching the challenge site with the endoscope or catheter, although this was occasionally unavoidable. The bronchial response was visualised endoscopically for 10min and sequential photographs taken for later comparisons. Direct visualisation of the bronchi via the eyepiece of the endoscope was found to be more useful than using the videoendoscope monitor. Comparison of the subtle colour changes of bronchi at the challenge site with those of unchallenged sites was useful.

ENDOBRONCHIAL CHALLENGE WITH PBS AND M.FAENI EXTRACT

Control (n=7) and asymptomatic COPD affected (n=7) horses were given endobronchial challenges under standing sedation (10ug/kg i/v detomidine, Domosedan, SmithKline Beecham Animal Health, Tadworth and 10ug/kg i/v butorphanol, Torbugesic, C-Vet, Bury St.Edmunds). 10ml 0.2% xylocaine (Astra, King's Langley) was sprayed onto the tracheal carina and major bronchi to minimise coughing.

Control endobronchial challenges, with 5ml PBS at 37^{O}C, were performed in the right diaphragmatic lobe bronchus and the challenge site was examined endoscopically for visible changes for 10min. A control bronchoalveolar lavage was then performed in the accessory lobe of the right lung, as previously described (Chapter 2). Five hours later the horses were sedated and the PBS challenge site was examined endoscopically, photographed, and transendoscopically lavaged.

Three days later, under sedation, the horses were given endobronchial antigen challenges with 5ml PBS containing 60ug/ml and 600ug/ml *M.faeni* extract (Greer Laboratories, Lenoir, North Carolina, USA), instilled into the left diaphragmatic lobe bronchus and left apical lobe bronchus respectively. Five hours later the horses were sedated and the challenge sites were examined endoscopically, photographed and lavaged.

BALF was processed as described previously (Chapter 2) and the total and differential BALF cell counts determined.

LOCAL ENDOBRONCHIAL CHALLENGE WITH MOULDY HAY EXTRACT

The hay used to prepare the mouldy hay extract had visible mould contamination, a mouldy smell and contained large quantities of mould spores, forage mites and mite faeces, as assessed microscopically. The hay was from a batch which had induced COPD in asymptomatic COPD affected horses (Chapter 2). Thirteen grammes hay was incubated in 300ml PBS at room temperature for 12h with constant stirring, the resultant suspension then being filtered through a 5um membrane (Metricel GA-1, Gelman Instruments, Ann Arbor,

USA) and stored at -20^{o}C. The protein nitrogen concentration of the extract, kindly determined by Ms.R.Muirhead, R(D)SVS, using the Kjeldahl technique (Ministry of Agriculture, Fisheries and Food 1973), was 1.5x10^{5} PNU/ml.

Five control and fully 5 asymptomatic COPD affected horses, sedated with 200mg i/v xylazine (Rompun, Bayer UK, Bury St.Edmunds), were given endobronchial challenges with 1ml hay extract at 37^{o}C, in a third generation bronchus in the accessory lobe of the right lung. The challenge site was visualised endoscopically for 5min post challenge and photographed. Five hours later the horses were sedated and the challenge site was examined endoscopically and BALs were performed in the left diaphragmatic lobe bronchus and at the challenged site. The total and differential BALF cell counts were determined.

STATISTICAL ANALYSES

As data were not normally distributed, paired and unpaired data were analysed using the Wilcoxon Rank and the Mann Whitney tests respectively, with a 5% significance level.

RESULTS

ENDOBRONCHIAL CHALLENGE WITH HISTAMINE

Local endobronchial challenge with 10^{-2} mg/ml histamine acid phosphate induced endoscopically visible erythema and mild bronchial narrowing within 3min in both horses. One minute after challenge with 10^{-1} mg/ml both horses had visible bronchial narrowing, by 2min the bronchi were diffusely erythematous, with obvious annular rugae and had froth bubbling through their almost totally occluded lumina. These changes were localised to the challenge sites. No visible responses were elicited by challenge with more dilute histamine solutions.

Attempts to quantify the degree of bronchial constriction using a transendoscopic pressure balloon and by measuring bronchial diameter using a transendoscopic telescopic umbrella device were unsuccessful.

ENDOBRONCHIAL CHALLENGES WITH PBS AND M.FAENI EXTRACT

PBS challenge significantly increased ($p<0.05$) BALF neutrophil ratios from the prechallenge values, in both control and COPD affected horses (median values 1.0% and 3.3% respectively, to 11.0% and 8.0%, respectively) (Table 9.1, Appendix 9.2). In the COPD affected group, BALF neutrophil ratios were significantly increased by challenge with 600ug/ml MF (median 36.0%), when compared with the PBS challenge (median 8.0%) ($p<0.05$). The BALF cell ratios and total BALF cell counts of controls were not significantly different from those of COPD affected horses before or after the challenges (Table 9.1, Appendix 9.3).

TABLE 9.1 Total BALF cell counts (/ul) and BALF cell ratios for control (CONT) (n=7) and COPD affected (n=7) horses before and after local endobronchial challenges with PBS, 60ug/ml MF and 600ug/ml *Micropolyspora faeni* (MF) (median and range).

CELL TYPE	GROUP	BASELINE	PBS	60ug/ml MF	600ug/ml MF
TOTAL CELLS	CONT	400 (100-625)	270 (155-1005)	330 (95-770)	395 (175-645)
	COPD	170 (95-555)	100 (60-375)	190 (40-760)	410 (40-695)
NEUTROPHILS	CONT	1.0 (0.3-4.7)	11.0 (0.3-28.0) [1]	10.0 (1.7-27.0) [1]	25.0 (0.7-75.0) [1]
	COPD	3.3 (0.0-4.3)	8.0 (2.0-52.0) [1]	16.0 (0.7-68.0) [1]	36.0 (2.0-65.7) [2]
LYMPHOCYTES	CONT	35.7 (5.3-53.0)	35.3 (18.0-44.7)	37.7 (13.0-51.3)	21.7 (5.0-49.7)
	COPD	39.3 (11.3-63.7)	35.0 (3.0-51.3)	36.0 (20.-48.7)	15.0 (0.0-35.0)
MACROPHAGES	CONT	60.3 (38.7-86.7)	49.0 (41.7-61.0)	50.7 (39.0-68.0)	45.7 (16.7-61.0)
	COPD	49.0 (25.0-82.0)	48.0 (37.0-57.7)	43.0 (23.0-50.0)	38.0 (18.0-87.0)
EOSINOPHILS	CONT	0.0 (0.0-11.0)	0.0 (0.0-28.0)	0.0 (0.0-4.3)	0.0 (0.0-3.7)
	COPD	1.0 (0.0-3.7)	0.3 (0.0-2.0)	0.7 (0.0-6.0)	0.3 (0.0-4.0)
MAST CELLS	CONT	3.0 (2.0-4.0)	2.0 (0.0-5.7)	2.3 (1.0-5.0)	2.0 (0.3-7.0)
	COPD	3.0 (0.7-6.0)	3.0 (0.0-9.3)	4.0 (0.0-6.3)	4.0 (0.3-5.3)
BASOPHILS	CONT	1.0 (0.0-1.7)	0.0 (0.0-1.0)	0.3 (0.0-0.7)	0.0 (0.0-2.3)
	COPD	0.3 (0.0-1.0)	0.3 (0.0-1.0)	0.7 (0.0-2.0)	0.3 (0.0-2.0)
TOTAL BASOS	CONT	4.0 (2.0-5.0)	2.7 (0.0-6.3)	3.0 (1.0-5.7)	2.0 (0.3-9.3)
	COPD	3.7 (1.0-6.7)	4.0 (0.0-10.3)	4.3 (0.0-8.3)	4.0 (0.0-7.0)
EPITHELIAL	CONT	1.0 (0.0-3.3)	0.3 (0.0-1.3)	0.3 (0.0-1.0)	0.3 (0.0-3.0)
	COPD	0.3 (0.0-3.0)	0.7 (0.0-6.3)	1.3 (0.0-11.0)	2.0 (0.0-4.3)

[1] Significantly higher than baseline values ($p<0.05$).
[2] Significantly higher than baseline values ($p<0.05$) and significantly higher than values for PBS challenge ($p<0.05$).

Local hyperaemia was observed in one control horse at 4min after PBS challenge and in another at 5min after challenges with 60ug/ml and 600ug/ml MF. Three COPD affected horses had immediate local bronchial blanching followed by hyperaemia after 60ug/ml MF challenge, with one of these also having visible luminal frothing within 4min of the 600ug/ml MF challenge (Fig 9.1).

While no endoscopically visible bronchial changes were apparent at the sites of PBS or MF challenges at 5h post challenge, all horses had marked local inflammation of the mucosa which had been in contact with the endoscope.

ENDOBRONCHIAL CHALLENGE WITH MOULDY HAY EXTRACT

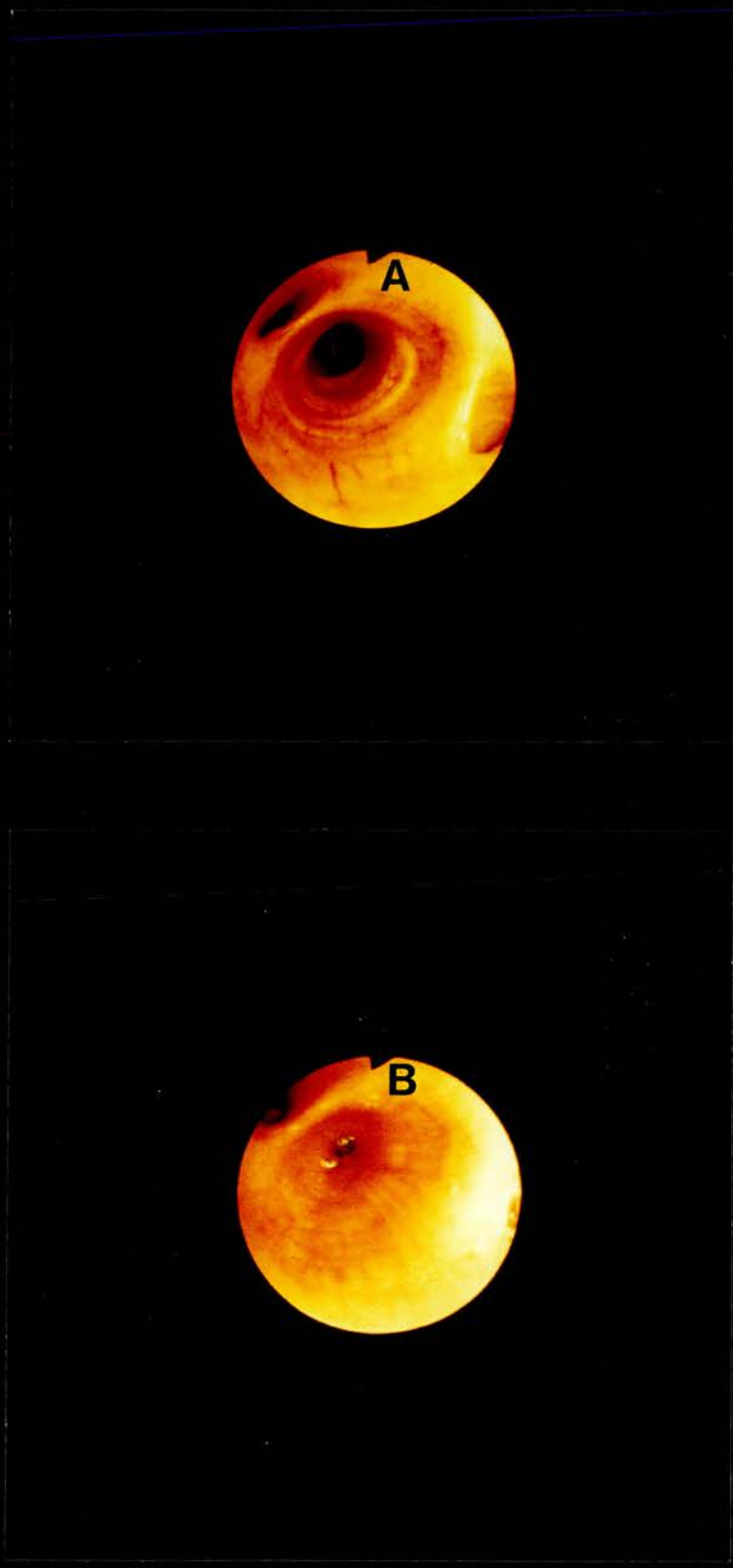
Five hours after mouldy hay challenge all control and COPD affected horses showed non significantly increased BALF neutrophil ratios in the challenged segment (median 19.7% and 30.7%, respectively) when compared with the control segment in the contralateral lung (median 7.7% and 8.3%, respectively) (Table 9.2, Appendix 9.4). BALF neutrophil ratios were also non significantly higher in both the control and challenged sites when compared with the prechallenge ratios (0.7% and 2.3%, respectively). The total BALF cell counts were not significantly affected by the mouldy hay challenge (Table 9.2, Appendix 9.5).

While no endoscopically visible changes were observed within 5min of the challenges, 1 control and 3 COPD affected horses showed obvious inflammatory changes at 5h post challenge, including bronchial narrowing, hyperaemia and accumulation of froth on the bronchial walls. In one horse marked bronchial narrowing prevented passage of the endoscope during the postchallenge BAL, which was consequently performed in an adjacent bronchus.

TABLE 9.2 Total BALF cell count (/ul) and BALF cell ratios for control (CONT) (n=5) and COPD affected (n=5) horses before challenge (baseline) and for control and challenge sites at 5h after local endobronchial challenges with mouldy hay extract (median and range).

CELL TYPE	GROUP	BASELINE	CONTROL SITE	CHALLENGE SITE
TOTAL CELLS	CONT	130 (95-275)	210 (95-400)	240 (90-130)
	COPD	215 (85-420)	230 (100-315)	215 (100-400)
NEUTROPHILS	CONT	0.7 (0.0-3.3)	7.7 (0.3-13.7)	19.7 (8.7-30.7)
	COPD	2.3 (1.3-4.0)	8.3 (4.0-17.3)	30.7 (9.0-70.3)
LYMPHOCYTES	CONT	32.3 (24.3-40.7)	25.0 (13.0-45.7)	37.3 (10.3-51.7)
	COPD	45.0 (28.7-56.0)	44.7 (41.0-70.3)	31.0 (16.3-59.3)
MACROPHAGES	CONT	59.7 (48.3-70.3)	58.3 (28.0-80.7)	44.0 (19.3-58.3)
	COPD	48.3 (37.7-62.7)	38.0 (9.3-53.0)	24.0 (3.7-44.3)
EOSINOPHILS	CONT	0.0 (0.0-0.3)	0.0 (0.0-0.3)	0.3 (0.0-0.3)
	COPD	0.0 (0.0-0.3)	0.0 (0.0-2.0)	0.3 (0.0-1.0)
MAST CELLS	CONT	5.7 (3.7-9.0)	6.7 (3.3-11.0)	4.0 (1.3-11.7)
	COPD	4.3 (2.3-4.7)	3.3 (0.0-7.7)	4.7 (2.0-5.3)
EPITHELIAL CELLS	CONT	0.3 (0.3-2.3)	1.0 (0.0-3.7)	0.7 (0.0-1.3)
	COPD	0.3 (0.0-1.0)	0.0 (0.0-2.3)	0.3 (0.3-0.7)

Fig 9.1 Endoscopic photograph of third generation bronchus of an asymptomatic COPD affected horse (A) before and (B) at 4min after endobronchial challenge with 5ml 600ug/ml MF antigen extract. After challenge, the bronchus is markedly narrowed and blanched and has froth within its almost totally occluded lumen.


A
B

DISCUSSION

Endobronchial challenges with PBS, MF extract and mouldy hay extract elicited a neutrophilic pulmonary inflammatory response in both control and COPD affected horses, as determined by cytological examination of BALF collected from the challenged segments. The mouldy hay extract also induced a neutrophilic inflammatory response in the contralateral lung.

Only horses with asymptomatic COPD showed a significant BALF neutrophilia after challenges with 600ug/ml MF, when compared with PBS challenges. However, as the BALF neutrophil ratios of COPD affected horses after this MF challenge were not significantly different from those of control horses, this finding has little clinical diagnostic value.

These findings contrast with the nebulised PBS *inhalation* challenges, which were shown to have no significant effect on the BALF cytology of control or COPD affected horses (Chapter 2). The findings also differ from the nebulised MF extract *inhalation* challenges and natural exposure to hay and straw, which induced a statistically significant BALF neutrophilia in horses with asymptomatic COPD, but not in controls (Chapter 2). Endobronchial challenges with 5ml 600ug/ml MF ($6x10^{-4}$ w/v) would have produced higher local bronchial antigen concentrations than the previous MF *inhalation* challenges with 20ml $2.5x10^{-4}$ w/v MF. Antigen administered during endobronchial challenges is likely to have remained at the challenge site or be transported proximally within the tracheobronchial tree by the mucociliary clearance mechanisms, while the inhalation challenges, performed by administering nebulised MF antigen via a face mask, would have produced a more uniform pattern of antigen deposition throughout the entire respiratory tract.

As antigen inhalation challenges with excessive antigen concentrations (i.e. $>10^{-2}$ w/v; Spector 1989) induce false positive bronchial responses in normal humans (Townley *et al*

1965; Cavanaugh *et al* 1977), endobronchial challenge with concentrations of MF greater than $6x10^{-4}$ w/v were not performed in this study.

Metzger *et al* (1987) elicited positive bronchial inflammatory responses in asthmatic humans by endobronchial challenges with approximately 100 fold more dilute antigen preparations than were required to elicit positive intradermal challenge responses. In the present study endobronchial antigen challenges were performed using $6x10^{-4}$ and $6x10^{-5}$ w/v MF, while the median minimal intradermal end point titres for MF for these horses were $1x10^{-4}$ w/v (range $1/10^{-6}$ - $1x10^{-3}$) and $1x10^{-6}$ w/v (range $1x10^{-6}$ w/v - $1x10^{-3}$), for early and late phase responses, respectively (Chapter 3).

Bacterial growth and subsequent endotoxin production, which could have occurred during the preparation of the mouldy hay extract, may have affected the results of the mouldy hay extract challenges, as endotoxins have been shown to elicit pulmonary disease in man and animals (Pernis *et al* 1967; Cavagna *et al* 1969). However, it is likely that the mouldy hay contained endotoxin prior to the preparation of the extract, due to bacterial growth which occurs after baling and during storage (Lacey 1971). Endotoxins have been demonstrated in airborne dust in stable environments which contain poorly saved hay and straw (Robinson,N.E., *pers comm*) and it is possible that they may also have a role in the aetiology of equine COPD.

It was concluded that, in the horse, local endobronchial challenges, even with PBS, induce a non specific pulmonary neutrophilia. This is consistent with the finding that BAL, and in some instances bronchoscopy alone, may elicit a transient pulmonary neutrophil influx in horses (Sweeney *et al* In Press B), humans (von Essen *et al* 1991), monkeys (Kazmierowski *et al* 1977; Krombach *et al* 1985; Haley *et al* 1989), dogs (Cohen and Batra 1980; Damiano *et al* 1980) and sheep (Weiss *et al* 1983). The extent of pulmonary neutrophilia observed in these previous studies varied, being either diffuse or localised to the lavaged segment. In contrast to these findings, Derksen *et al* (1987) demonstrated that three repeated BALs, performed in

different segments within a 29h period, did not significantly affect BALF cytology of 3 ponies.

The pulmonary neutrophil influx induced in control and COPD affected horses by endobronchial challenges greatly limits the use of this technique in the study of equine COPD, a disease which is also characterised by pulmonary neutrophilia (Nicholls 1978; Derksen *et al* 1985B).

Endoscopically visible local bronchial changes were observed in a proportion of control and COPD affected horses within 5min of challenge with PBS and MF and at 5h after challenge with hay extract. Initial pallor, followed by hyperaemia, bronchial narrowing and an accumulation of froth on the bronchial walls were noted. However, as these changes were not observed consistently in COPD affected horses and were observed in a proportion of control horses, the visible bronchial response appears to have little clinical diagnostic value.

Most subjects had endoscopically visible hyperaemia, petechiation and fibrin like exudation in the trachea and major bronchi at 5h post challenge. As this was confined to the larger airways which had been in contact with the endoscope, iatrogenic trauma was the likely cause. Similar tracheal inflammation has been reported as a sequel to routine endotracheal intubation in normal horses (Heath *et al* 1989).

While sedation and local anaesthesia of the carina and major bronchi minimised the marked cough response, which would otherwise have been elicited by bronchoscopy and which would have precluded the use of this technique, occasional coughing did occur during the bronchoscopy.

In conclusion, local endobronchial challenges, as performed in this study, were associated with several problems and appear to offer no advantage over conventional antigen inhalation challenges in the investigation of equine pulmonary hypersensitivity.

CHAPTER 10

PRELIMINARY OBSERVATIONS ON INHALATION AND INTRADERMAL CHALLENGES OF NORMAL AND CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) AFFECTED HORSES WITH OIL SEED RAPE

SUMMARY

Three different pulmonary challenges with oil seed rape (OSR), namely (a) exposure to a field of flowering *Brassica campestrus* for 6 days, (b) inhalation challenge with freshly collected *Brassica napus* pollens and (c) nebulised inhalation challenge with a commercial *B.napus* pollen extract, were performed on asymptomatic and symptomatic chronic obstructive pulmonary disease (COPD) affected horses and on control horses.

The challenges did not induce detectable pulmonary disease in control or asymptomatic COPD affected horses, as determined by clinical and bronchoscopic examinations, and did not significantly affect their pulmonary mechanics, arterial blood gas tensions, arterial pH and bronchoalveolar lavage fluid (BALF) cytology.

However, inhalation challenges with fresh *B.napus* pollen and commercial *B.napus* pollen extract exacerbated the pulmonary disease in some symptomatic COPD affected horses, possibly due to non specific toxicity/bronchial hyperresponsiveness.

Intradermal testing using a commercial *B.napus* pollen extract suggested that none of the horses was hypersensitive to this agent.

INTRODUCTION

Over the last two decades there has been a massive EEC supported increase in the growing of oil seed rape (OSR) in Britain, with the acreage increasing from 6,500 ha in 1968 to 300,000 ha in 1989. In Scotland the acreage has increased from 1,600 ha in 1982 to 42,000 ha in 1988 and subsequently remained at that level. Approximately 90% is winter sown *Brassica napus* which flowers in the spring while the remainder is *B. campestrus*, sown in spring and flowering in mid-summer.

Simultaneously there have been numerous reports in all branches of the media alleging a relationship between the growing of OSR and diseases affecting man, including hay fever, asthma, allergic rhinitis, headaches and dermatitis and affecting the horse, including pulmonary dysfunction, dermatitis, parotid salivary gland swelling and the so called 'poor performance syndrome'.

Mair and Lane (1990) also suggested that seasonal headshaking in horses, which is probably a rhinitis, may be associated with OSR exposure. In addition, some clinical cases of summer pasture associated obstructive pulmonary disease (SPAOPD) in Northern England and Scotland have been found in proximity to OSR fields (Dixon and McGorum 1990A and 1990B). However, in view of the very widespread distribution of OSR throughout these areas, i.e. covering 6% of arable land in Scotland, this observation is perhaps not surprising.

A large increase in the incidence of human asthma has been recorded in the last 15 years, with inhalation of newly introduced pollens being one of the causal factors implicated (Hide 1990; Layzell 1991).

Colldahl (1954) reported the first case of human OSR hypersensitivity, manifested as conjunctivitis, rhinitis and asthma, with positive responses to intradermal and nasal

provocation testing with an OSR pollen extract. Satisfactory control was achieved with hyposensitisation.

Bucur and Arner (1978) showed that 23% of 366 patients in southern Sweden suffering bronchial asthma and other allergies had positive intradermal tests to OSR pollens, with 81% of 54 of these patients showing positive conjunctival provocation tests with OSR pollens.

Meding (1985) reported a case of human contact dermatitis to mustard seed (*B.nigra*) which also reacted to prick testing with *B.napus*.

Parrat *et al* (1990) demonstrated IgE antibodies to OSR in human sera. While 26.3% of sera from patients, suffering mainly from atopy, in a rural area of central Scotland (Tayside) were positive, only 7.8% of sera from patients in an urban area (Strathclyde) were positive. These authors concluded that OSR was a new antigenic hazard to man and that it was one of the most potent antigens described to date. However, the relationship between the noted OSR sensitisation and clinical disease was not determined.

In contrast, Ninian *et al* (1990) studied 81 children from Northern Scotland with hay fever, allergic rhinitis or asthma suspected by the referring general practitioners to be due to OSR, and found that only 9% were mildly positive on intradermal testing to OSR antigen, whilst over 50% were positive to the usual antigens implicated in these diseases, i.e. grass and tree pollens, house dust, house dust mite and cat and dog hair. These authors concluded that OSR was not a potent antigen in man, causing only mild and infrequent sensitisation.

OSR related disease could potentially arise from hypersensitivity and/or non specific toxic responses to OSR pollens (pollenosis), volatile substances released from the crop, fungi growing on the crop or to some of the numerous chemical insecticides and fungicides that are sprayed on these crops.

It is generally considered that OSR pollenosis is unlikely to account for the alleged OSR related symptoms (Hannant 1988) as OSR is primarily a self pollinating plant producing small numbers of large sticky pollens (Olsson 1960), which would be expected to travel only short distances. Seaton, A. *et al* (*pers comm* 1991) found only small numbers of airborne OSR pollens adjacent to fields of OSR, in contrast to large numbers of fungal spores. Furthermore the OSR pollen season is relatively short and does not appear to coincide with all the alleged OSR related disease in the horse. However under certain circumstances the distribution of OSR pollens may be more widespread. Parrat, D. *et al* (*pers comm* 1990), using portable continuous pollens monitors worn by human volunteers, demonstrated that OSR pollens and OSR pollen fragments were frequently isolated up to 1.7 km from OSR fields. Likewise OSR pollens have been detected in pollen traps 15 km from the nearest known OSR fields, (Caulton, E., *pers comm* 1990).

It is possible that inhalation of smaller airborne grass pollens or fungal spores, which have a more widespread distribution and are produced over a longer period of time than OSR pollens, may have caused the alleged OSR diseases in the horse and man.

Despite repeated fungicide applications, OSR plants are subject to fungal infection during the flowering season, particularly by *Alternaria brassicae* and *Botrytis cinerea* and these fungi have also been suggested as a possible cause of OSR related disease (Wilson, R., *pers comm* 1990).

It is possible that symptoms may result from exposure to one of the many volatile organic compounds released from OSR (Seaton, A. *et al, pers comm* 1991). Tollsen and Bergstrom (1988) identified, respectively, 21 and 16 volatile compounds in the headspaces of *B.napus* and *B.campestrus* plants.

Despite the anecdotal evidence, it is unclear if OSR causes any disease in the equine, either by non specific toxic or hypersensitivity mechanisms. This study was performed to determine whether OSR respiratory challenge, using a variety of antigens and challenge techniques, caused respiratory changes in normal and in asymptomatic and symptomatic chronic obstructive pulmonary disease (COPD) affected horses, as assessed by clinical, bronchoscopic, pulmonary function and bronchoalveolar lavage fluid (BALF) cytological examinations.

Symptomatic COPD affected horses were utilised to determine whether OSR could exacerbate respiratory disease in horses with preexisting pulmonary inflammation.

Additionally, intradermal tests with a commercial OSR pollen extract were performed on 20 horses.

MATERIALS AND METHODS

ANIMALS

Seven control (median age 15years, range 7-25; median body weight 593kg, range 212-652) and 6 COPD affected (median age 18.5years, range 13-25; median body weight 469kg, range 371-546) horses were used (Appendix 10.1). The criteria used to define control and symptomatic COPD affected horses are described in Chapter 2.

CHALLENGE PROCEDURE

All horses were maintained in a 'controlled environment' throughout the duration of the challenges.

Horses were given three different OSR inhalation challenges according to the protocol outlined in Fig. 10.1.

OSR FIELD CHALLENGE

The horses were confined in a 4 ha paddock directly adjacent to a 9 ha flowering *B. campestrus* field for 6 days.

OSR POLLEN INHALATION CHALLENGE

B.napus pollens, laboriously collected within the previous 24h at the Scottish Crop Research Institute, Invergowrie, were found to be too large to nebulise as a saline suspension. However 0.1g of pollen was effectively administered using a modified 'spinhaler' (Fisons, Loughborough) inserted into one, partially occluded nostril, while occluding the other nostril.

Fig. 10.1 Experimental protocol.

CHALLENGE	N	SUBJECTS	TIME	EXAMINATIONS
FIELD	2	CONTROL	day 0	CLIN, BG, PMT, BRONCH, BALF
	2	ASYMPTOMATIC COPD	days 1-5	CLIN
	3	SYMPTOMATIC COPD	day 6	CLIN, BG, PMT, BRONCH, BALF
FRESH POLLEN	2	CONTROL	day -4	BALF
	3	ASYMPTOMATIC COPD	day 0	CLIN, BG, PMT, BRONCH
	3	SYMPTOMATIC COPD	+ 5h	CLIN, BG, PMT, BRONCH, BALF
NEBULISED POLLEN EXTRACT	2	CONTROL		
	3	ASYMPTOMATIC COPD		
	3	SYMPTOMATIC COPD		

CLIN=clinical examination, BG=arterial blood gas and pH examination, PMT=pulmonary mechanics testing, BRONCH=bronchoscopy, BALF=bronchoalveolar lavage cytological examination.

NEBULISED B.NAPUS POLLEN EXTRACT INHALATION CHALLENGE

Horses received nebulised (Ultra Neb 99, DeVilbliss Co., Somerset, USA) inhalation challenges with 20ml phosphate buffered saline (PBS), containing 10mg commercial *B.napus* pollen extract (Solupric, ALK, Denmark) which had been dialysed (dialysis tubing, Medicell, London) twice, in 50ml and 100ml PBS, for 24h at $4^{o}C$ and sterile filtered through a 0.8um filter (Millipore, Buc, France). The aerosol was delivered via a face mask incorporating a one way valve system to minimise aerosol loss during expiration (Fig. 2.1).

Previous control challenges with nebulised PBS had not induced clinical, bronchoscopic, pulmonary mechanic, arterial blood gas or BALF cytological changes in these horses (Chapter 2).

ARTERIAL BLOOD GAS and pH ANALYSES

Carotid arterial blood samples were collected and analysed as previously described (Chapter 2).

PULMONARY MECHANICS TESTING

Respiratory rate, tidal volume, minute volume, mean maximum transthoracic pressure change, dynamic compliance and total pulmonary resistance were determined immediately before and after each challenge, as described in Chapter 2.

All horses were familiar with the pulmonary mechanics testing procedure.

COLLECTION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

BALF was collected from all horses before and after the challenges, according to the protocol outlined in Fig 10.1. Bronchoalveolar lavage (BAL) and BALF processing were performed as previously described (Chapter 2). Differential cell count of 300 cells were performed on Leishman's stained cytospin preparations (Chapter 2).

INTRADERMAL TESTING

Twenty horses, including 8 which had been given experimental OSR inhalation challenges, as described above, and 12 which had no known exposure to OSR, were intradermally tested with a commercial extract of *B.napus* pollens (10mg dry matter/ml in a solvent containing 50% glycerol, 2.5% NaCl and 1.25% $NaHCO_3$; Solupric, ALK, Denmark), undiluted and diluted to 10^{-1}, 10^{-2} and 10^{-3} in sterile PBS diluent (Greer Laboratories, Lenoir, North Carolina, USA). The positive and negative controls were, respectively, 0.01% histamine phosphate in sterile PBS and PBS. Intradermal injections were administered and interpreted as described previously (Chapter 3).

Ten horses, all of which had no known exposure to OSR, were also intradermally tested with undiluted and 10^{-1}, 10^{-2} and 10^{-3} dilutions of the same *B.napus* pollen extract, which had been dialysed, as previously described, to remove low molecular weight irritants including glycerol.

STATISTICAL ANALYSIS

The data for all three challenges was pooled for each group and the prechallenge and postchallenge data for each group were compared. Arterial blood gas tensions and pH data were normally distributed and were analysed by the paired T-test, while the other data, which were not normally distributed, were analysed using the Wilcoxon Rank Test, with a significance level of 5%.

RESULTS

The challenges had no significant effect on any of the groups (Table 10.1, Appendix 10.2) and none of the horses showed any other clinical signs such as headshaking, dermatitis or parotid swelling.

No individual control or asymptomatic COPD affected horses developed any evidence of respiratory disease following the challenges (Appendix 10.2).

Following the field challenge two symptomatic COPD affected horses showed improvements in all parameters examined, while the other horse showed no improvement in BALF neutrophil ratio (Fig. 10.2) or pulmonary mechanics.

With the fresh pollen challenge, while two of the three symptomatic COPD affected horses showed reduced BALF neutrophil ratios (Fig. 10.2), one horse showed a markedly increased BALF neutrophil ratio without concomitant deterioration in its pulmonary function.

Following *B.napus* pollen extract inhalation challenge all three symptomatic horses showed increased BALF neutrophil ratios (Fig. 10.2) without detectable deterioration of their pulmonary function.

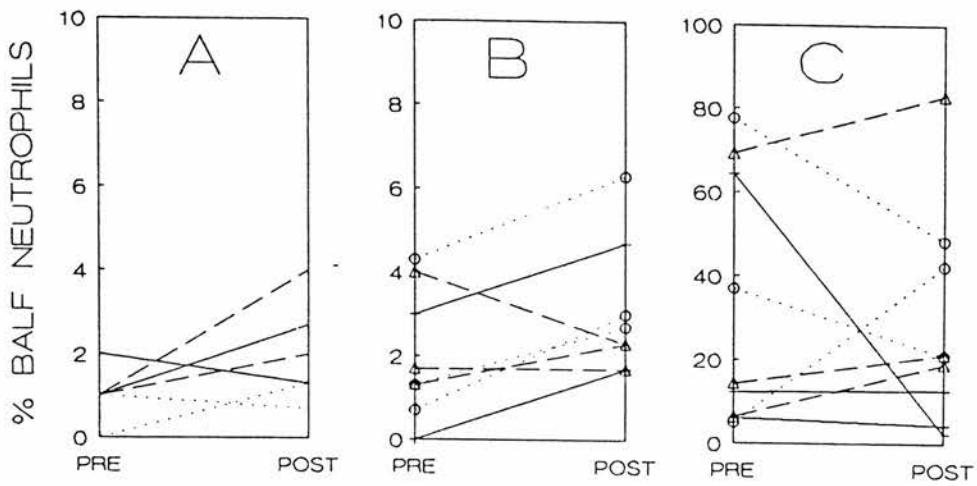
Table 10.1 Arterial blood gas tensions, arterial pH, pulmonary mechanics parameters and BALF neutrophil ratios for control horses (n=6), and horses with asymptomatic (n=8) and symptomatic (n=9) COPD, before and after challenges with OSR. (mean±SD, or median and range).

GROUP		$Pa0_2$ (Torr)	$PaC0_2$ (Torr)	ART pH	dPtp (cmH_2O)	Cdyn (l/cmH_2O)	R_L ($cmH_2O/l/s$)	BALF NEUT %
CONT	PRE	90.7±4.4	39.4±1.7	7.41±0.03	7.0 (4.3-13.1)	1.16 (0.69-2.06)	1.24 (0.85-1.62)	1.0 (0.0-2.0)
CONT	POST	91.5±7.3	40.5±2.1	7.40±0.01	5.6 (2.5-15.7)	1.33 (0.76-1.98)	1.08 0.63-1.74)	1.6 (0.7-4.0)
ASY	PRE	92.6±6.6	40.4±2.0	7.39±0.03	5.0 (2.9-12.8)	1.67 (1.32-2.12)	1.07 (0.56-1.69)	1.5 (0.0-4.3)
ASY	POST	94.8±7.5	39.7±1.2	7.40±0.03	5.5 (2.7-9.5)	1.33 (1.24-2.44)	0.90 (0.58-1.70)	2.5 (1.7-6.3)
SYMP	PRE	86.8±6.9	42.9±2.2	7.39±0.03	10.1 (6.1-32.8)	0.76 (0.37-1.05)	1.85 (1.24-9.98)	14.3 (6.0-77.6)
SYMP	POST	91.8±8.8	41.6±2.1	7.40±0.02	10.7 (4.6-20.3)	0.96 (0.51-1.77)	1.48 (0.87-3.22)	21.0 (2.3-83.0)

$Pa0_2$=arterial oxygen tension, $PaC0_2$=arterial carbon dioxide tension, ART pH=arterial pH, dPtp=mean maximum transpulmonary pressure change, Cdyn=dynamic compliance, R_L=average pulmonary resistance, BALF NEUT=bronchoalveolar lavage fluid neutrophil ratio.

Fig. 10.2 BALF neutrophil ratios of (A) control horses, (B) asymptomatic COPD horses and (C) symptomatic COPD affected horses, before (PRE) and after (POST) OSR challenges.

——— = OSR field challenge.
····· = fresh *B.napus* pollen challenge.
– – – = *B.napus* pollen extract challenge.



INTRADERMAL TESTS

The intradermal test results obtained are presented in Table 10.2 and Appendix 10.3.

TABLE 10.2 The percentages of horses showing positive dermal reactions at 1.5 and 5h following intradermal testing with dialysed and undialysed *B.napus* pollen extracts at 10^0, 10^{-1}, 10^{-2} and 10^{-3} dilutions.

OSR EXTRACT	n	1.5 HOURS				5 HOURS			
		10^0	10^{-1}	10^{-2}	10^{-3}	10^0	10^{-1}	10^{-2}	10^{-3}
undialysed	20	85	80	10	0	90	75	10	0
dialysed	10	0	0	0	0	80	10	0	0

DISCUSSION

The field challenge was performed to reproduce exposure to OSR as occurs naturally. The field used, however, contained *B.campestrus* which has been shown to release a slightly different range of volatile substances than the more commonly encountered *B.napus* (Tollsten and Bergstrom 1988). The significance of this difference on the outcome of the field challenge is not known.

Freshly harvested *B.napus* pollens were used for the pollen inhalation challenge to minimise potential losses of any volatile or unstable compounds associated with the pollens. Deposition of pollen in the nasal cavity was observed, but the extent and depth of pulmonary deposition was not determined. While the vast majority of particles of this size are deposited by impaction within the nasal cavities and upper respiratory tract (Swift 1980), it is probable that small numbers of whole pollens, pollen fragments or volatile or soluble OSR components penetrated to the distal airways (Particle dynamics are discussed further in Chapter 2).

It is likely that the field and fresh pollen inhalation challenges exposed the horses to the volatile compounds released from OSR, while it is possible that they were absent from the commercial OSR preparation. No details of its production were available.

For the *B.napus* pollen extract inhalation challenge $5x10^{-4}$ w/v antigen was used. In human studies allergens are commonly administered in incremental doses from 10^{-6} to 10^{-2} w/v (Anon. 1980), challenge with the higher concentration being usually required to exclude hypersensitivity (Salvaggio and Hendrick 1989). In this study the possibility of positive reactions being elicited by higher antigen concentrations cannot be ruled out.

The OSR challenges did not elicit detectable non specific toxic or hypersensitivity pulmonary responses in any control or asymptomatic COPD affected horses, suggesting that OSR is unlikely to be a major cause of equine pulmonary disease.

As antigen challenges will only induce hypersensitivity phenomena in individuals previously sensitised to that antigen, the absence of OSR hypersensitivity in these horses could indicate either that they had no prior exposure to OSR, or that prior exposure had not induced sensitisation. While the horses used had no known close contact with OSR fields during the two years before the field challenge, exposure prior to this period could not be ruled out. Indeed, as OSR cultivation is so widespread in central Scotland, all of these animals may have been previously exposed to OSR antigens. The horses had their first *known* exposure to OSR antigens during the field challenge and consequently may have become sensitised prior to the two subsequent inhalation challenges.

While the challenges had no significant effect on symptomatic COPD affected horses, some individual horses did show responses.

Exposure to a field of flowering *B.campestrus* for 6 days did not induce any detectable exacerbation of pulmonary disease in the symptomatic COPD affected horses. Indeed partial improvement in all clinical and laboratory parameters were noted in 2 of these 3 horses, consistent with the improvement shown by symptomatic COPD affected horses when moved to a hay and straw free environment (Thomson and McPherson 1983). Whilst the absence of expected improvement in BALF cytology and pulmonary mechanics noted in the other horse suggests it may have undergone pulmonary toxicity, individual variations in the rate of remission could provide an alternative explanation.

Following fresh *B.napus* pollen inhalation, one symptomatic COPD affected horse showed a marked increase in its BALF neutrophil ratio without a concomitant deterioration of its pulmonary function. This response was considered to be due to non specific pulmonary

toxicity/bronchial hyperresponsiveness rather than to hypersensitivity as this horse showed no response to this challenge when asymptomatic. The other two symptomatic COPD affected horses showed improvements which would be expected following housing in a hay and straw free environment for 4 days prior to the challenge.

Following the nebulised *B.napus* extract challenge, all three symptomatic COPD affected horses showed mildly increased BALF neutrophil ratios, without concomitant increased pulmonary dysfunction. As two of these horses were unaffected by this challenge when performed while they were asymptomatic, this response was considered to be due to non specific pulmonary toxicity/bronchial hyperresponsiveness rather than hypersensitivity.

Undialysed commercial *B.napus* pollen extract, undiluted and at 10^{-1}, induced numerous positive early and late phase intradermal responses. These were considered to be false positive responses due to non specific toxicity by glycerol (Slavin 1974) as they were markedly reduced by double dialysis of the pollen extract.

The *B.napus* pollen extract used was formulated for human epicutaneous prick testing (Nelson 1981), which is the preferred method of skin testing in man and which involves making a needle prick or scratch in the skin to permit passive diffusion of topically applied antigen into the superficial dermis. However, differences in the sensitivity of epicutaneous and intradermal skin testing techniques render this OSR extract, which contains 50% glycerol to stabilise the antigens, unsuitable for equine intradermal testing without prior dialysis. While extracts containing 50% glycerol can be employed for epicutaneous testing in man, even 6% glycerol causes a high incidence of false positive reactions when used intradermally (Lindblad and Farr 1961).

Intradermal tests are more sensitive than epicutaneous tests, but studies in man show that the two techniques produce similar skin reactions only if the intradermal preparation is 1000 times more dilute (Voorhorst and van Krieken 1973). Thus intradermal testing using preparations formulated for epicutaneous testing which have been diluted to less than 10^{-3}

have been associated with positive reactions in humans with no history of allergic disease (Lindblad and Farr 1961). It would thus be prudent to consider only the results obtained following intradermal testing with the 10^{-3} dilution of the dialysed preparation. At this concentration no positive responses were obtained, suggesting the horses were not hypersensitive to the antigens present in this extract. Furthermore, as immediate cutaneous responses have been shown in man to be correlated with *in vitro* measurement of allergen specific IgE (Norman *et al* 1973; Lessof *et al* 1980), it could be concluded that sera from these horses does not contain *B.napus* specific IgE.

These findings suggest that, while OSR exposure does not appear to be a major cause of equine respiratory disease, it appears to exacerbate pulmonary disease in some horses with preexisting pulmonary disease such as COPD. No evidence of pulmonary or dermal hypersensitivity to OSR was demonstrated, although as only a limited number of horses, without histories suggestive of OSR related disease, were investigated in relatively short term studies, the existence of individual horses with pulmonary hypersensitivity to OSR cannot be ruled out. Performing similar challenges on horses with a history suggestive of alleged OSR related diseases e.g. SPAOPD is thus warranted.

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APPENDICES

APPENDIX 2.1 Description of horses used for antigen inhalation challenge study.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	CROSS	MN	15	593
2	"	SHETLAND	F	7	212
3	"	TB	MN	19	643
4	"	CROSS	F	16	561
5	"	CROSS	F	10	515
6	"	TB	MN	13	652
7	"	TB	MN	25	609
8	"	CROSS	MN	20	500
9	COPD	CROSS	F	20	371
10	"	CROSS	F	20	488
11	"	CROSS	MN	13	501
12	"	CROSS	MN	25	422
13	"	CROSS	F	17	546
14	"	TB	MN	13	537
15	"	TB	MN	6	475
16	"	CROSS	MN	14	450

F=female, MN= gelding, cross=crossbred, TB=Thoroughbred.

APPENDIX 2.2 Histochemical staining techniques employed.

TOLUIDINE BLUE STAIN

Cytospin preparations, prepared and fixed as described previously (Chapter 2), were stained overnight in 0.5% toluidine blue (Sigma, Poole) in 0.7N HCl at room temperature, washed quickly in distilled water and air dried.

LEISHMAN'S STAIN

Cytospin preparations, prepared and fixed as described previously (Chapter 2), were covered with one volume of Leishman's stain (Leishman's staining solution, BDH, Poole) and incubated at room temperature for 4min. Two volumes of phosphate buffered saline (pH 6.8) were then added and left for 12min. The preparations were washed well with tap water and air dried.

GIEMSA STAIN

Cytospin preparations, prepared and fixed as described previously (Chapter 2), were stained in 1:10 Giemsa stain (BDH, Poole), differentiated in distilled water for 5min, dehydrated in isopropyl alcohol, cleared and mounted in neutral mountant.

APPENDIX 2.3 Findings of clinical examinations performed at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) CONTROL HORSES

No clinical evidence of pulmonary dysfunction was detected in the control horses after any of the challenges.

(B) COPD AFFECTED HORSES

CHALLENGE	1.5 HOURS	5 HOURS
PBS	NAD	NAD
AF	Hyperpnoea - horses 6 & 7	Hyperpnoea - horses 6 & 7
TV	NAD	Hyperpnoea - horses 6 & 8
MF	Hyperpnoea - horses 2 & 6	Hyperpnoea - horses 5 & 6
NC	Hyperpnoea - horses 3 & 8	Hyperpnoea and expiratory dyspnoea - horses 1,3,4,5,6,7 & 8 Increased breath sounds detected by thoracic auscultation - horses 3,4 & 8

NAD = No abnormality detected.

APPENDIX 2.4 Volume (ml) of BALF recovered by BAL from control (n=8) and COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NCs.

	CONTROL					COPD				
	PBS	AF	TV	MF	NC	PBS	AF	TV	MF	NC
1	114	130	48	75	145	52	78	92	68	120
2	67	72	135	80	122	63	98	130	113	180
3	105	100	114	53	129	44	88	84	76	43
4	83	104	124	106	79	71	40	73	54	44
5	37	135	125	85	38	29	112	144	91	140
6	162	41	64	103	150	103	71	69	85	39
7	91	95	89	98	141	27	160	132	109	132
8	147	104	135	127	150	106	102	91	102	110

APPENDIX 2.5 Total BALF cell counts (/ul) for control (n=8) and COPD affected (n=8) horses at 5h following inhalation challenges with PBS, AF, TV and MF and after NC.

	CONTROL					COPD				
	PBS	AF	TV	MF	NC	PBS	AF	TV	MF	NC
1	300	250	287	200	675	45	212	187	305	222
2	98	240	200	312	175	200	360	130	113	280
3	362	250	640	320	362	132	73	75	120	251
4	1125	212	112	200	212	175	80	220	250	520
5	237	387	262	400	787	75	275	240	290	2750
6	220	60	147	120	308	190	240	95	680	3737
8	168	270	220	143	425	138	133	230	123	463

APPENDIX 2.6 Urea concentrations of plasma (P) (mg/dl) and BALF (B) (ug/d) from (a) control (n=8) and (b) COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(a) Control horses

	PBS		AF		TV		MF		NC	
	P	B	P	B	P	B	P	B	P	B
1	26.6	144	34.4	272	25.3	73	32.6	73	29.9	223
2	26.5	260	21.9	63	25.6	131	19.4	203	33.6	418
3	38.4	115	19.9	236	28.7	105	26.4	98	23.4	210
4	33.7	225	27.8	71	31.9	47	22.9	144	22.5	81
5	28.1	68	26.4	199	28.7	101	23.6	202	30.3	199
6	27.5	44	25.0	66	24.5	78	28.7	65	33.9	232
7	26.4	88	31.8	205	28.1	272	23.6	216	29.7	320
8	20.1	64	18.8	179	21.7	389	25.1	153	35.6	64

(b) COPD affected horses.

	PBS		AF		TV		MF		NC	
	P	B	P	B	P	B	P	B	P	B
1	33.0	72	27.5	145	21.5	145	21.1	68	26.1	214
2	26.4	48	24.1	73	23.7	283	23.7	113	23.1	281
3	27.8	97	23.8	48	27.8	108	25.8	46	31.0	43
4	19.5	148	28.9	51	26.4	99	28.9	80	30.9	90
5	32.9	96	25.6	223	28.1	75	32.5	241	26.5	180
6	26.7	75	20.5	88	27.6	32	30.8	75	29.5	198
7	28.9	294	20.9	132	20.0	390	34.1	167	34.1	200
8	21.9	64	26.7	125	32.6	121	25.5	104	27.1	214

APPENDIX 2.7 Albumen concentrations of plasma (P) (mg/ml) and BALF (B) (ug/ml) from (a) control (n=8) and (b) COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(a) Control horses

	PBS		AF		TV		MF		NC	
	P	B	P	B	P	B	P	B	P	B
1	28.1	103.9	28.3	81.0	29.9	19.1	23.5	59.0	32.8	80.0
2	24.8	67.6	18.7	29.7	24.3	17.8	27.0	53.5	22.4	87.3
3	38.0	44.5	28.8	83.5	30.1	104.0	32.4	93.4	27.2	34.3
4	40.3	72.9	23.3	50.5	22.3	52.6	20.5	68.7	21.0	75.0
5	21.2	74.0	30.8	78.0	27.3	71.1	25.8	159.0	27.6	33.4
6	41.8	94.1	38.5	43.9	25.0	38.9	26.8	78.7	28.9	56.9
7	30.8	23.6	26.8	186.0	31.3	90.0	29.8	69.0	30.2	25.5
8	23.0	25.5	28.8	150.5	27.7	88.6	23.3	130.0	23.0	62.9

(b) COPD affected horses.

	PBS		AF		TV		MF		NC	
	P	B	P	B	P	B	P	B	P	B
1	29.0	55.1	33.1	134.0	34.6	35.9	28.8	30.8	26.0	77.8
2	29.7	89.4	28.2	17.0	24.3	33.4	28.6	37.5	26.2	104.9
3	36.8	47.2	25.7	21.3	29.4	6.9	31.5	54.3	32.2	20.0
4	19.1	18.5	28.1	17.5	29.4	39.8	29.0	36.8	29.1	72.0
5	25.6	39.4	25.3	70.2	30.3	86.1	30.4	112.8	33.0	42.7
6	27.7	24.0	25.5	102.0	27.0	36.5	21.7	160.0	38.6	5.4
7	32.3	40.2	42.0	78.9	25.6	98.6	30.5	28.0	40.0	72.6
8	32.0	33.3	39.6	95.7	30.4	55.1	32.6	50.2	31.4	36.6

APPENDIX 2.8 Total PELF cell counts ($x10^3$/ul) for control (CONT) (n=8) and COPD affected (n=8) horses after inhalation challenges with PBS, AF, TV and MF and after NC.

HORSE		PBS	AF	TV	MF	NC
1	CONT	55.4	31.6	99.5	89.3	90.5
2	"	10.0	83.4	39.1	29.8	14.1
3	"	89.4	21.1	174.9	86.2	40.3
4	"	168.5	83.0	76.0	31.8	58.9
5	"	97.9	51.3	74.4	46.7	119.8
6	"	137.5	22.7	46.2	53.0	45.0
7	"	71.1	34.9	61.0	30.0	22.0
8	"	52.8	28.4	12.3	23.5	236.4
1	COPD	20.6	40.2	27.7	94.6	27.1
2	"	110.0	118.8	10.9	23.7	23.0
3	"	37.8	36.2	19.3	67.3	181.0
4	"	23.1	45.3	58.7	90.3	178.5
5	"	25.7	31.6	89.9	39.1	404.9
6	"	67.6	55.9	81.9	279.3	21.3
7	"	4.9	5.9	1.3	61.3	637.2
8	"	47.2	28.4	62.0	30.2	58.6

APPENDIX 2.9 Albumen adjusted total BALF cell counts ($x10^3$/ul) for control (CONT) (n=8) and COPD affected (n=8) horses after inhalation challenges with PBS, AF, TV and MF and after NC.

HORSE		PBS	AF	TV	MF	NC
1	CONT	81.1	87.3	449.3	79.7	276.7
2	"	36.0	151.1	273.0	157.5	44.9
3	"	309.1	86.2	185.2	111.0	287.1
4	"	621.9	97.8	47.5	59.7	59.4
5	"	67.9	152.8	100.6	64.9	650.3
6	"	97.7	52.6	94.5	40.9	156.4
7	"	309.3	32.4	205.2	118.8	280.7
8	"	151.5	51.7	68.8	25.6	155.4
1	COPD	23.7	52.4	180.2	285.2	74.2
2	"	66.4	597.2	94.6	86.2	69.9
3	"	102.9	88.1	319.6	69.6	404.1
4	"	180.7	128.5	162.5	197.0	210.2
5	"	48.7	99.1	84.5	78.2	2125.3
6	"	219.3	60.0	70.3	92.2	1022.2
7	"	40.2	19.7	6.5	71.5	2059.0
8	"	132.6	55.0	126.9	79.9	397.2

APPENDIX 2.10 BALF cell ratios for control horses (n=8) and COPD affected (n=8) horses at 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	22.3	70.3	0.0	6.3	0.3	6.7	0.7
2	"	0.0	48.0	42.3	1.3	6.7	0.0	6.7	1.7
3	"	1.0	15.3	71.7	0.3	9.3	2.3	11.7	0.0
4	"	1.3	26.0	66.0	0.0	6.7	0.0	6.7	0.0
5	"	4.3	20.7	63.3	0.7	11.0	0.0	11.0	0.0
6	"	0.0	62.3	37.7	0.0	0.0	0.0	0.0	0.0
7	"	2.7	35.3	51.3	0.0	4.7	4.0	8.7	2.0
8	"	2.0	15.7	65.3	0.0	14.7	0.3	15.0	2.0
1	AF	1.3	30.0	60.7	0.0	8.0	0.0	8.0	0.0
2	"	1.0	25.3	68.0	0.3	4.3	1.0	5.3	0.0
3	"	4.0	34.0	53.3	2.3	6.3	0.0	6.3	0.0
4	"	3.0	20.0	72.0	0.0	3.0	0.0	3.0	2.0
5	"	5.0	52.0	34.0	0.0	6.0	3.0	9.0	0.0
6	"	2.0	66.0	29.3	0.0	2.7	0.0	2.7	0.0
7	"	5.7	37.3	49.0	0.7	6.7	0.7	7.3	0.0
8	"	0.0	43.0	52.0	0.0	3.0	0.0	3.0	2.0
1	TV	11.3	32.0	48.0	0.7	8.0	0.0	8.0	0.0
2	"	0.3	59.7	30.0	5.0	5.0	0.0	5.0	0.0
3	"	2.3	50.0	40.3	0.0	5.0	1.7	6.7	0.7
4	"	3.3	36.3	47.7	0.0	11.0	0.7	11.7	1.0
5	"	1.0	15.3	77.0	0.0	6.0	0.7	6.7	0.0
6	"	33.7	35.3	27.3	0.0	1.7	0.0	1.7	2.0
7	"	4.3	20.3	70.7	0.0	4.3	0.3	4.7	0.3
8	"	0.7	24.0	51.7	0.0	21.0	0.3	21.3	2.3
1	MF	0.7	37.3	45.7	0.0	11.3	0.0	11.3	5.0
2	"	0.3	60.0	30.0	4.3	5.0	0.3	5.3	0.0
3	"	3.0	16.3	75.3	0.0	3.7	1.0	4.7	0.7
4	"	1.5	16.7	79.3	0.0	2.3	0.3	2.7	0.0
5	"	0.7	58.3	31.0	0.3	8.0	0.7	8.7	1.0
6	"	0.3	43.0	51.0	0.0	4.0	0.0	4.0	1.7
7	"	4.0	23.3	64.7	0.0	6.0	0.0	6.0	2.0
8	"	1.3	19.3	57.7	0.0	20.7	0.3	21.0	0.7
1	NC	3.0	49.0	45.0	0.0	2.3	0.7	3.0	0.0
2	"	4.0	34.3	40.7	12.3	6.7	1.3	8.0	0.7
3	"	1.3	17.0	71.3	0.0	8.7	0.7	9.3	1.0
4	"	2.7	35.0	56.3	0.7	4.3	1.0	5.3	0.0
5	"	3.0	32.7	58.7	0.3	4.3	1.0	5.3	0.0
6	"	0.3	48.7	47.7	1.0	2.3	0.0	2.3	0.0
7	"	3.0	33.3	56.0	0.0	7.7	0.0	7.7	0.0
8	"	2.7	28.0	53.7	0.3	14.3	0.7	15.0	0.3

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(**APPENDIX 2.10** Continued).

(B) COPD affected horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	17.0	68.0	0.0	5.0	0.0	5.0	10.0
2	"	2.0	15.7	65.3	0.0	14.7	0.3	15.0	2.0
3	"	4.7	45.3	38.3	0.0	9.3	1.0	10.3	1.3
4	"	4.0	58.7	33.3	0.0	3.7	0.0	3.7	0.3
5	"	4.3	51.0	35.0	0.0	9.0	0.0	9.0	0.7
6	"	5.0	23.0	69.0	1.0	0.7	0.0	0.7	1.3
7	"	4.0	54.3	36.7	0.7	3.7	0.3	4.0	0.3
8	"	2.0	58.0	25.0	15.0	0.0	0.0	0.0	0.0
1	AF	2.0	47.3	47.0	0.0	3.7	0.0	3.7	0.0
2	"	29.3	28.7	38.3	0.0	3.7	0.0	3.7	0.0
3	"	6.3	38.0	44.7	0.0	9.3	0.0	9.3	1.7
4	"	7.3	41.7	34.3	0.0	6.3	0.7	7.0	9.7
5	"	6.3	50.3	34.0	0.0	7.7	0.3	8.0	1.3
6	"	14.7	21.3	62.7	0.0	1.3	0.0	1.3	0.0
7	"	36.0	27.0	34.0	0.0	3.0	0.0	3.0	0.0
8	"	7.0	51.7	35.7	0.0	5.7	0.0	5.7	0.0
1	TV	1.7	68.3	25.3	0.0	3.3	1.3	4.7	0.0
2	"	9.7	33.3	48.0	0.0	9.0	0.0	9.0	0.0
3	"	9.0	46.0	36.7	0.0	7.3	0.7	8.0	0.3
4	"	1.7	67.3	25.0	0.3	4.0	1.7	5.7	0.7
5	"	3.7	60.3	31.0	0.0	3.3	1.3	4.7	0.3
6	"	9.0	42.0	43.7	0.0	3.0	0.0	3.0	2.3
7	"	1.0	17.0	80.0	0.0	0.0	2.0	2.0	0.0
8	"	19.0	46.0	31.3	0.3	2.0	0.0	2.0	1.3
1	MF	10.7	42.0	43.0	1.0	2.7	0.0	2.7	0.7
2	"	9.0	47.0	37.0	0.0	7.0	0.0	7.0	0.0
3	"	27.7	29.0	33.3	0.0	0.0	7.0	7.0	3.0
4	"	4.0	48.7	34.7	1.0	6.0	1.0	7.0	4.7
5	"	11.0	45.7	28.7	5.0	5.3	2.7	8.0	1.7
6	"	18.0	38.0	43.3	0.0	0.7	0.0	0.7	0.0
7	"	14.7	35.7	46.0	1.0	2.0	0.3	2.3	0.3
8	"	24.0	34.0	34.7	0.3	3.7	0.0	3.7	3.3
1	NC	10.3	38.7	44.3	1.3	3.7	0.0	3.7	1.7
2	"	19.0	30.0	45.3	0.0	5.3	0.0	5.3	0.3
3	"	36.0	30.0	29.7	0.0	3.3	0.3	3.7	0.7
4	"	87.3	7.3	4.3	0.0	0.7	0.0	0.7	0.3
5	"	97.7	2.0	0.3	0.0	0.0	0.0	0.0	0.0
6	"	54.7	22.0	23.3	0.0	0.0	0.0	0.0	0.0
7	"	93.0	4.7	1.7	0.0	0.3	0.3	0.7	0.0
8	"	86.7	9.3	2.7	0.0	1.3	0.0	0.3	0.0

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

APPENDIX 2.11 Absolute PELF cell counts (x10^3/ul) for (A) control horses (n=8) and (B) COPD affected (n=8) horses after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	12.4	39.0	0.0	3.5	0.2	3.7	0.4
2	"	0.0	4.8	4.2	0.1	0.7	0.0	0.7	0.2
3	"	0.9	13.7	64.1	0.3	8.3	2.1	10.5	0.0
4	"	2.2	43.8	111.2	0.0	11.3	0.0	11.3	0.0
5	"	4.2	20.3	62.0	0.7	10.8	0.0	10.8	0.0
6	"	0.0	85.7	51.8	0.0	0.0	0.0	0.0	0.0
7	"	1.9	25.1	36.5	0.0	3.3	2.8	6.2	1.4
8	"	1.1	8.3	34.5	0.0	7.8	0.2	7.9	1.1
1	AF	0.4	9.5	19.2	0.0	2.5	0.0	2.5	0.0
2	"	0.8	21.1	56.7	0.3	3.6	0.8	4.4	0.0
3	"	0.8	7.2	11.2	0.5	1.3	0.0	1.3	0.0
4	"	2.5	16.6	59.8	0.0	2.5	0.0	2.5	1.7
5	"	2.6	26.7	17.5	0.0	3.1	1.5	4.6	0.0
6	"	0.5	15.0	6.7	0.0	0.6	0.0	0.6	0.0
7	"	2.0	13.0	17.1	0.2	2.3	0.2	2.6	0.0
8	"	0.0	12.2	14.7	0.0	0.9	0.0	0.9	0.6
1	TV	11.2	31.8	47.7	0.7	8.0	0.0	8.0	0.0
2	"	0.1	23.3	11.7	2.0	2.0	0.0	2.0	0.0
3	"	4.0	87.5	70.5	0.0	8.7	3.0	11.7	1.2
4	"	2.5	27.6	36.3	0.0	8.4	0.5	8.9	0.8
5	"	0.7	11.4	57.3	0.0	4.5	0.5	5.0	0.0
6	"	15.6	16.3	12.6	0.0	0.8	0.0	0.8	0.9
7	"	2.6	12.4	43.1	0.0	2.6	0.2	2.8	0.2
8	"	0.1	2.9	6.3	0.0	2.6	0.0	2.6	0.3
1	MF	0.6	33.3	40.8	0.0	10.1	0.0	10.1	4.5
2	"	0.1	17.9	8.9	1.3	1.5	0.1	1.6	0.0
3	"	2.6	14.1	64.9	0.0	3.2	0.9	4.1	0.6
4	"	0.5	5.3	25.2	0.0	0.7	0.1	0.8	0.0
5	"	0.3	27.2	14.5	0.1	3.7	0.3	4.1	0.5
6	"	0.2	22.8	27.0	0.0	2.1	0.0	2.1	0.9
7	"	1.2	7.0	19.4	0.0	1.8	0.0	1.8	0.6
8	"	0.3	4.5	13.5	0.0	4.9	0.1	4.9	0.2
1	NC	2.7	44.3	40.7	0.0	2.1	0.6	2.7	0.0
2	"	0.6	4.8	5.7	1.7	0.9	0.2	1.1	0.1
3	"	0.5	6.9	28.8	0.0	3.5	0.3	3.8	0.4
4	"	1.6	20.6	33.2	0.4	2.5	0.6	3.1	0.0
5	"	3.6	39.2	70.3	0.4	5.2	1.2	6.4	0.0
6	"	0.1	21.9	21.5	0.5	1.0	0.0	1.0	0.0
7	"	0.7	7.3	12.3	0.0	1.7	0.0	1.7	0.0
8	"	6.4	66.2	127.0	0.7	33.8	1.7	35.5	0.7

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(**APPENDIX 2.11** Continued)

(B) COPD affected horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	3.5	14.0	0.0	1.0	0.0	1.0	2.1
2	"	2.2	17.3	71.8	0.0	16.2	0.3	16.5	2.2
3	"	1.8	17.1	14.5	0.0	3.5	0.4	3.9	0.5
4	"	0.9	13.5	7.7	0.0	0.9	0.0	0.9	0.1
5	"	1.1	13.1	9.0	0.0	2.3	0.0	2.3	0.2
6	"	3.4	15.6	46.7	0.7	0.5	0.0	0.5	0.9
7	"	0.2	2.7	1.8	0.0	0.2	0.0	0.2	0.0
8	"	0.9	27.4	11.8	7.1	0.0	0.0	0.0	0.0
1	AF	0.8	19.0	18.9	0.0	1.5	0.0	1.5	0.0
2	"	34.8	34.1	45.5	0.0	4.4	0.0	4.4	0.0
3	"	2.3	13.8	16.2	0.0	3.4	0.0	3.4	0.6
4	"	3.3	18.9	15.5	0.0	2.9	0.3	3.2	4.4
5	"	2.0	15.9	10.7	0.0	2.4	0.1	2.5	0.4
6	"	8.2	11.9	35.1	0.0	0.7	0.0	0.7	0.0
7	"	2.1	1.6	2.0	0.0	0.2	0.0	0.2	0.0
8	"	2.0	14.7	10.1	0.0	1.6	0.0	1.6	0.0
1	TV	0.5	18.9	7.0	0.0	0.9	0.4	1.3	0.0
2	"	1.1	3.6	5.2	0.0	1.0	0.0	1.0	0.0
3	"	1.7	8.9	7.1	0.0	1.4	0.1	1.5	0.1
4	"	1.0	39.5	14.7	0.2	2.3	1.0	3.3	0.0
5	"	3.3	54.2	27.9	0.0	3.0	1.2	4.1	0.3
6	"	7.4	34.4	35.8	0.0	2.5	0.0	2.5	1.9
7	"	0.0	0.2	1.0	0.0	0.0	0.0	0.0	0.0
8	"	11.8	28.5	19.4	0.2	1.2	0.0	1.2	0.8
1	MF	10.1	39.7	40.7	0.9	2.6	0.0	2.6	0.7
2	"	2.1	11.1	8.8	0.0	1.7	0.0	1.7	0.0
3	"	18.5	19.5	22.5	0.0	0.0	4.7	4.7	2.0
4	"	3.6	44.0	31.3	0.9	5.4	0.9	6.3	4.2
5	"	4.3	17.9	11.2	2.0	2.1	1.1	3.1	0.7
6	"	50.3	106.1	120.9	0.0	2.0	0.0	2.0	0.0
7	"	9.0	21.9	28.2	0.6	1.2	0.2	1.4	0.2
8	"	7.2	10.3	10.5	0.1	1.1	0.0	1.1	1.0
1	NC	2.8	10.5	12.0	0.4	1.0	0.0	1.0	0.5
2	"	4.4	6.9	10.4	0.0	1.2	0.0	1.2	0.1
3	"	65.1	54.3	53.7	0.0	6.0	0.5	6.5	1.3
4	"	155.9	13.0	7.7	0.0	1.2	0.0	1.2	0.5
5	"	395.5	8.1	1.2	0.0	0.0	0.0	0.0	0.0
6	"	11.6	4.7	5.0	0.0	0.0	0.0	0.0	0.0
7	"	592.6	29.9	10.8	0.0	1.9	1.9	3.8	0.0
8	"	50.8	5.5	1.6	0.0	0.8	0.0	0.8	0.0

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

APPENDIX 2.12 Albumen adjusted absolute BALF cell counts (x10^3/ul) for (A) control horses (n=8) and (B) COPD affected (n=8) horses after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	18.1	57.0	0.0	5.1	0.2	5.4	0.6
2	"	0.0	17.3	15.2	0.5	2.4	0.0	2.4	0.6
3	"	3.1	47.3	221.6	0.9	28.7	7.1	36.2	0.0
4	"	8.1	161.7	410.5	0.0	41.7	0.0	41.7	0.0
5	"	2.9	14.1	43.0	0.5	7.5	0.0	7.5	0.0
6	"	0.0	60.9	36.8	0.0	0.0	0.0	0.0	0.0
7	"	8.4	109.2	158.7	0.0	14.5	12.4	26.9	6.2
8	"	3.0	23.8	98.9	0.0	22.3	0.5	22.7	3.0
1	AF	1.1	26.2	53.0	0.0	7.0	0.0	7.0	0.0
2	"	1.5	38.2	102.8	0.5	6.5	1.5	8.0	0.0
3	"	3.4	29.3	46.0	2.0	5.4	0.0	5.4	0.0
4	"	2.9	19.6	70.4	0.0	2.9	0.0	2.9	0.0
5	"	7.6	79.5	52.0	0.0	9.2	4.6	13.8	0.0
6	"	1.1	34.7	15.4	0.0	1.4	0.0	1.4	0.0
7	"	1.8	12.1	15.9	0.2	2.2	0.2	2.4	0.0
8	"	0.0	22.2	26.9	0.0	1.6	0.0	1.5	1.0
1	TV	50.8	143.8	215.7	3.1	35.9	0.0	35.9	0.0
2	"	0.8	163.0	81.9	13.7	13.7	0.0	13.7	0.0
3	"	4.3	92.6	74.6	0.0	9.3	3.1	12.4	1.3
4	"	1.6	17.2	22.6	0.0	5.2	0.3	5.6	0.5
5	"	1.0	15.4	77.5	0.0	6.0	0.7	6.7	0.0
6	"	31.8	33.3	25.8	0.0	1.6	0.0	1.6	1.9
7	"	8.8	41.7	145.1	0.0	8.8	0.6	9.4	0.6
8	"	0.5	16.5	35.6	0.0	14.4	0.2	14.7	1.6
1	MF	0.6	29.7	36.4	0.0	9.0	0.0	9.0	4.0
2	"	0.5	94.5	47.2	6.8	7.9	0.5	8.3	0.0
3	"	3.3	18.1	83.6	0.0	4.1	1.1	4.2	0.8
4	"	0.9	10.0	47.3	0.0	1.4	0.2	1.6	0.0
5	"	0.5	37.8	20.1	0.2	5.2	0.5	5.6	0.6
6	"	0.1	17.6	20.8	0.0	1.6	0.0	1.6	0.7
7	"	4.8	27.7	76.8	0.0	7.1	0.0	7.1	2.4
8	"	0.3	4.9	14.8	0.0	5.3	0.1	5.4	0.2
1	NC	8.3	135.6	124.5	0.0	6.4	1.9	8.3	0.0
2	"	1.8	15.4	18.3	5.5	3.0	0.6	3.6	0.4
3	"	3.7	48.8	204.7	0.0	25.0	2.0	26.7	2.9
4	"	1.6	20.8	33.4	0.4	2.6	0.6	3.1	0.0
5	"	19.5	212.7	381.7	2.0	28.0	6.5	34.5	0.0
6	"	0.5	76.2	74.6	1.6	3.6	0.0	3.6	0.0
7	"	8.4	93.5	157.2	0.0	21.6	0.0	21.6	0.0
8	"	4.2	43.5	83.5	0.5	22.2	1.1	23.3	0.5

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

(**APPENDIX 2.12** Continued)

(B) COPD affected horses.

	AIC	NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PBS	0.0	4.0	16.1	0.0	1.2	0.0	1.2	2.4
2	"	1.3	10.4	43.4	0.0	9.8	0.2	10.0	1.3
3	"	1.8	46.6	39.4	0.0	9.6	1.0	10.6	1.3
4	"	7.2	106.1	60.2	0.0	6.7	0.0	6.7	0.5
5	"	2.1	24.9	17.1	0.0	4.4	0.0	4.4	0.3
6	"	11.0	50.4	151.3	2.2	1.5	0.0	1.5	2.9
7	"	1.6	21.8	14.7	0.3	1.5	0.1	1.6	0.1
8	"	2.7	76.9	33.2	19.9	0.0	0.0	0.0	0.0
1	AF	1.0	24.8	24.6	0.0	1.9	0.0	1.9	0.0
2	"	175.0	171.4	228.7	0.0	22.1	0.0	22.1	0.0
3	"	5.5	33.5	39.4	0.0	8.2	0.0	8.2	1.5
4	"	9.4	53.6	44.1	0.0	8.1	0.9	9.0	12.5
5	"	6.2	49.9	33.7	0.0	7.6	0.3	7.9	1.3
6	"	8.8	12.8	37.6	0.0	0.8	0.0	0.8	0.0
7	"	7.1	5.3	6.7	0.0	0.6	0.0	0.6	0.8
8	"	3.9	28.5	19.6	0.0	3.1	0.0	3.1	0.0
1	TV	3.1	123.1	45.6	0.0	5.9	2.3	8.3	0.0
2	"	9.2	31.5	45.4	0.0	8.5	0.0	8.5	0.0
3	"	8.8	147.0	117.3	0.0	23.3	2.2	25.6	1.0
4	"	2.8	109.4	40.6	0.5	6.5	2.8	9.3	0.0
5	"	3.1	50.9	26.2	0.0	2.8	1.1	3.9	0.3
6	"	6.3	29.5	30.7	0.0	2.1	0.0	2.1	1.6
7	"	0.1	1.1	5.2	0.0	0.0	0.1	0.1	0.0
8	"	24.1	58.4	39.7	0.4	2.5	0.0	2.5	1.6
1	MF	30.5	119.8	122.6	2.9	7.7	0.0	7.7	2.0
2	"	7.8	40.5	31.9	0.0	6.0	0.0	6.0	0.0
3	"	19.1	20.2	23.3	0.0	0.0	4.9	4.9	2.1
4	"	7.9	95.9	68.4	2.0	1.8	2.0	13.8	9.3
5	"	8.6	35.7	22.4	3.9	4.1	2.1	6.3	1.3
6	"	16.6	35.0	39.9	0.0	0.6	0.0	0.6	0.0
7	"	10.5	25.5	32.9	0.7	1.4	0.2	1.6	0.2
8	"	19.2	27.2	27.7	0.2	3.0	0.0	3.0	2.6
1	NC	7.6	28.7	32.9	1.0	2.7	0.0	2.7	1.3
2	"	13.3	21.0	31.7	0.0	3.7	0.0	3.7	0.2
3	"	145.5	121.2	120.0	0.0	13.3	1.2	14.5	2.8
4	"	183.5	15.3	9.0	0.0	1.5	0.0	1.5	0.6
5	"	2076.4	42.5	6.4	0.0	0.0	0.0	0.0	0.0
6	"	557.1	224.9	240.2	0.0	0.0	0.0	0.0	0.0
7	"	1914.8	96.8	35.0	0.0	6.2	6.2	12.4	0.0
8	"	344.4	36.9	10.7	0.0	5.2	0.0	5.2	0.0

NEUT = neutrophils, LYM = lymphocytes, MAC = macrophages, EOS = eosinophils, MAST = mast cells, BASO = basophiloid cells, TOTBAS = total basophilic cells, EP = epithelial cells.

APPENDIX 2.13 Respiratory rates (min^{-1}) of (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	10.7	12.6	16.0	8.4	9.5	12.0	17.0	19.0	17.0	7.5	8.5	6.3	8.2	10.0	8.4
2	23.2	22.9	21.4	13.8	16.2	11.5	26.0	21.8	10.6	12.6	33.3	21.3	26.7	7.5	9.0
3	13.3	14.5	12.5	13.1	15.5	12.5	6.2	6.8	5.1	5.1	6.9	6.4	9.9	8.3	6.1
4	18.0	21.1	28.2	8.2	8.6	10.0	14.0	16.0	18.0	9.0	11.0	9.6	9.2	8.6	9.0
5	13.6	12.2	14.1	12.2	12.1	16.7	17.0	14.0	16.5	9.5	12.6	13.7	11.6	12.6	23.3
6	13.0	11.8	13.0	10.0	10.3	10.1	13.0	10.5	7.6	10.5	10.5	11.2	13.5	9.9	10.5
7	15.8	19.0	21.0	28.0	19.0	30.0	10.6	10.2	11.2	12.6	9.4	9.9	12.9	14.2	6.8
8	12.3	21.3	11.9	12.6	13.5	11.0	13.1	34.3	22.0	14.4	24.0	16.2	16.2	19.2	13.8

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	15.0	18.0	13.5	8.2	8.1	7.5	5.1	7.1	5.3	6.9	5.5	7.2	11.1	10.7	10.0
2	8.4	16.5	7.8	10.5	11.4	16.5	14.3	19.5	16.2	16.7	43.0	15.0	10.5	7.4	8.3
3	12.5	19.7	13.1	16.0	18.0	17.0	13.5	11.9	11.8	21.0	17.0	13.8	11.7	17.9	33.3
4	10.5	10.1	12.0	7.5	12.2	7.5	21.5	17.1	20.0	13.3	20.0	20.0	9.0	9.2	24.3
5	13.1	11.5	19.0	10.4	15.5	14.7	10.1	14.6	9.4	8.4	12.8	16.7	17.0	7.2	18.8
6	11.5	8.3	11.5	12.4	20.1	16.4	20.2	18.5	28.4	17.2	13.2	18.1	21.4	17.1	21.0
7	15.0	12.9	11.4	11.6	10.4	2.1	33.0	12.0	11.0	9.2	9.4	8.6	9.8	10.5	12.9
8	7.5	10.2	7.2	8.8	7.9	7.2	8.7	9.0	24.8	10.6	4.5	9.5	10.9	10.0	5.9

APPENDIX 2.14 Tidal volumes (l) for (A) control (n=8) and (B) COPD affected horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	8.4	6.2	6.0	6.1	6.3	6.4	8.0	6.6	6.9	9.3	6.5	6.3	9.3	6.1	9.9
2	0.6	0.5	1.9	1.1	0.4	0.8	0.7	0.9	1.7	1.3	1.4	1.1	1.5	1.3	1.2
3	9.0	6.4	6.6	9.1	5.9	6.5	8.6	8.3	9.5	8.7	8.9	8.9	5.9	5.2	5.9
4	6.6	7.1	4.8	7.0	8.5	7.7	8.5	5.4	5.7	6.0	8.0	5.8	6.2	7.0	7.0
5	7.2	9.9	7.5	8.6	8.8	7.7	5.9	4.8	4.9	9.4	9.2	4.6	7.8	7.9	6.7
6	6.4	6.3	5.5	10.9	10.0	11.0	8.1	10.1	7.5	7.6	8.3	7.7	9.3	9.6	9.6
7	8.0	8.1	5.3	5.5	4.1	5.7	5.1	5.7	5.4	4.1	5.9	6.9	8.1	8.3	8.7
8	4.5	4.2	4.6	3.5	6.1	6.5	5.0	4.2	4.2	2.2	3.5	4.7	6.0	3.9	3.9

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	5.7	5.5	3.1	8.9	7.6	6.6	5.2	5.6	5.9	4.3	5.1	5.1	5.1	4.7	5.5
2	7.6	6.1	6.7	6.8	6.5	5.7	6.8	4.7	7.6	6.3	1.6	6.5	8.7	7.3	7.7
3	7.8	5.7	6.3	5.3	4.8	4.9	6.1	6.6	6.5	4.2	5.8	5.6	6.0	5.4	4.1
4	7.9	7.8	5.2	7.6	6.8	4.0	5.7	7.3	7.5	8.4	6.4	7.6	8.7	7.7	3.6
5	6.3	5.1	3.4	5.3	5.4	5.8	5.1	5.2	5.1	5.2	4.3	4.2	8.3	6.8	3.2
6	9.3	9.4	8.8	8.4	7.8	8.3	4.1	3.6	4.0	9.5	11.1	11.5	10.0	8.8	6.3
7	4.8	6.9	6.3	5.2	8.0	7.5	7.5	5.7	5.5	5.0	5.9	5.7	7.5	5.4	5.6
8	4.6	3.6	3.6	9.7	8.8	8.3	6.1	5.1	4.8	9.6	12.1	6.8	8.0	6.7	9.1

APPENDIX 2.15 Minute volumes (l) for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	90.0	72.1	96.0	51.6	60.3	77.2	136.0	124.9	117.0	69.5	40.6	39.5	76.6	61.0	83.2
2	24.3	11.6	41.0	15.3	16.6	19.0	18.8	19.4	17.9	16.7	45.4	23.9	39.2	9.6	10.7
3	119.8	91.1	83.0	118.9	91.5	81.6	53.0	56.1	48.4	44.3	61.6	56.8	58.6	43.4	36.6
4	185.6	149.4	135.4	57.2	72.9	76.9	119.0	87.0	102.1	54.2	88.4	56.0	57.1	60.2	63.0
5	138.1	121.0	105.8	105.6	106.8	123.6	100.8	67.2	81.2	89.6	115.7	62.3	90.4	99.4	120.9
6	83.6	74.4	71.8	109.1	102.6	111.5	104.7	106.6	57.1	79.7	86.9	85.8	126.0	95.3	101.3
7	126.4	154.5	110.6	153.3	76.9	172.0	54.8	57.5	60.7	51.5	55.8	68.1	103.9	118.3	59.1
8	141.8	130.2	98.4	92.2	44.1	81.7	65.3	126.1	92.2	32.6	82.7	76.6	97.0	74.7	54.0

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	86.0	98.5	42.4	72.9	61.5	49.3	26.6	39.5	31.2	29.9	28.1	36.9	56.6	50.7	55.0
2	64.3	100.3	52.4	70.9	73.9	93.2	96.4	182.1	244.3	105.6	69.7	97.8	91.4	53.9	63.1
3	97.6	111.3	82.6	84.2	85.8	82.6	82.2	78.1	76.8	87.8	98.8	77.7	71.0	96.0	136.2
4	82.7	86.5	62.2	50.7	48.6	60.8	121.9	124.8	150.0	111.3	128.9	152.0	78.7	70.5	88.6
5	52.3	58.8	64.6	54.9	84.2	85.6	51.6	75.7	48.2	43.2	54.7	70.9	140.4	49.1	60.8
6	107.5	77.4	100.8	103.6	157.6	135.8	82.0	67.4	112.8	164.1	146.5	208.9	213.1	150.0	132.6
7	71.6	89.2	72.4	59.8	83.2	90.8	246.8	68.6	60.2	42.3	55.7	48.8	73.2	88.1	75.6
8	34.8	36.9	25.8	85.5	69.6	59.9	52.5	46.2	119.4	100.3	54.7	64.4	87.1	67.4	53.5

APPENDIX 2.16 Mean maximum transpulmonary pressure changes (cmH_2O) for (A) control (n=8) and (B) COPD (n=8) affected horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	5.8	7.4	6.7	4.8	4.4	8.0	7.4	8.4	5.4	6.8	8.5	7.4	6.3	9.1	6.5
2	1.9	3.7	3.9	1.8	1.5	5.1	4.3	2.7	2.3	2.7	1.5	3.8	5.6	3.0	3.9
3	5.2	3.1	2.7	6.4	5.2	4.7	8.2	8.0	8.7	7.3	9.5	8.6	7.0	4.6	4.4
4	4.9	5.8	4.4	7.1	4.2	4.6	6.5	4.5	4.8	4.8	6.7	4.1	8.8	7.0	9.0
5	5.2	6.0	4.9	4.1	4.9	6.2	7.4	8.5	6.8	5.2	7.7	8.6	8.6	9.8	8.9
6	7.3	3.3	3.1	4.5	4.5	5.0	3.7	4.8	4.4	3.0	3.4	3.7	6.4	5.5	5.9
7	5.9	5.0	4.4	5.0	4.6	4.2	4.7	5.9	6.2	5.6	4.8	5.8	8.1	6.7	5.7
8	3.1	4.9	5.1	4.0	4.5	4.3	4.6	3.7	4.5	6.3	3.2	10.3	7.6	4.8	6.0

(B) COPD affected horses

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	4.1	4.4	3.1	4.0	7.8	7.1	6.1	6.3	6.7	6.1	11.2	7.6	5.5	7.3	8.6
2	3.3	1.7	2.0	3.2	2.7	2.8	1.5	2.1	2.7	2.3	1.7	1.5	3.7	3.2	6.3
3	5.2	4.5	4.2	4.7	5.8	3.6	4.7	6.0	5.6	7.7	8.4	5.5	3.7	5.8	23.5
4	6.7	5.9	4.9	6.6	4.1	7.5	4.6	4.4	6.5	9.0	9.0	8.7	5.4	6.9	11.6
5	4.7	4.1	2.4	4.8	4.6	5.0	8.8	7.4	7.4	5.0	6.0	6.5	3.9	5.5	15.8
6	4.5	6.1	8.5	4.9	4.2	4.7	6.9	6.5	6.1	8.7	14.0	10.3	5.8	6.0	7.4
7	4.2	6.0	2.8	4.0	4.6	5.2	4.3	4.6	7.5	5.2	4.4	3.5	6.2	7.4	11.0
8	3.0	3.4	3.1	7.7	9.7	4.3	6.7	6.0	9.5	5.3	5.9	11.6	9.8	2.8	8.6

APPENDIX 2.17 Dynamic compliances (l/cmH_2O) for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	1.99	1.45	2.25	1.95	2.27	1.43	1.68	1.87	1.89	2.15	1.51	1.28	2.35	1.83	2.09
2	0.86	0.34	0.98	0.45	0.80	0.35	0.73	0.62	1.08	0.74	1.66	0.85	0.61	0.96	1.98
3	2.82	2.43	2.48	2.27	1.96	1.79	1.28	1.32	1.42	1.29	1.63	1.62	2.59	1.33	1.79
4	1.80	1.68	1.33	1.24	2.55	2.14	1.59	1.55	1.55	1.41	1.72	1.95	0.95	1.13	0.86
5	2.35	2.43	2.52	2.95	3.08	4.02	2.82	2.08	2.60	3.02	3.18	1.35	1.55	1.47	1.41
6	2.08	2.64	2.96	4.85	4.33	4.90	4.49	3.36	2.54	4.11	4.41	5.23	3.59	3.91	4.01
7	5.14	5.27	6.45	4.42	3.17	4.14	1.54	2.57	1.64	2.41	1.74	1.90	3.53	3.47	2.03
8	4.19	6.24	3.55	1.50	1.85	2.13	3.66	3.11	2.07	1.17	1.22	1.05	3.90	1.69	1.16

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	2.75	2.59	1.64	2.58	1.48	1.26	1.13	1.28	1.37	0.84	0.64	1.02	1.67	0.78	1.47
2	3.35	3.00	4.89	4.33	3.70	3.26	5.90	4.04	6.53	4.73	2.61	4.27	3.61	2.43	3.87
3	1.55	3.26	3.55	2.80	2.71	2.43	3.87	2.15	3.20	1.39	1.29	1.89	5.08	6.30	1.05
4	2.55	2.79	2.76	3.09	1.64	2.12	3.75	3.87	2.03	2.09	1.05	1.28	3.50	2.56	2.61
5	3.25	2.47	2.97	1.54	1.70	1.55	0.77	1.58	0.99	1.55	0.87	0.84	4.59	2.57	2.49
6	2.02	1.85	1.37	2.11	2.32	1.96	0.82	0.94	0.92	1.66	1.04	1.34	2.11	2.37	1.86
7	1.57	2.02	2.59	2.02	3.26	2.25	2.32	2.55	2.13	1.71	2.25	2.42	2.29	1.04	0.82
8	0.98	1.48	1.02	1.44	1.54	2.56	1.57	1.33	0.80	2.28	1.49	1.42	2.56	3.11	1.77

APPENDIX 2.18 Total pulmonary resistances ($cmH_2O/l/s$) for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	0.90	0.99	1.11	1.08	0.91	1.26	0.84	0.91	1.00	1.41	0.99	1.31	1.30	1.21	1.29
2	1.14	1.14	1.10	1.05	1.06	1.07	0.69	1.16	0.82	1.08	0.75	1.01	1.42	1.77	1.25
3	1.19	0.78	0.84	1.64	1.96	1.34	1.60	1.16	1.24	1.55	1.30	1.74	0.63	1.53	1.40
4	0.33	0.31	0.35	1.38	0.88	1.01	1.05	0.81	1.29	1.08	1.25	1.06	1.75	1.43	1.38
5	0.57	0.57	0.52	0.25	0.41	0.31	0.69	0.65	0.94	0.55	0.35	0.31	0.70	0.71	0.92
6	0.69	0.59	0.60	0.78	0.82	0.66	0.52	0.52	1.08	0.37	0.41	0.37	0.89	0.75	0.61
7	1.02	1.02	0.82	0.60	0.64	0.58	0.83	0.75	0.97	0.98	0.96	0.88	0.58	0.60	0.55
8	0.35	0.38	0.58	0.66	0.55	0.64	0.69	0.33	0.39	0.74	0.49	0.35	1.10	0.96	1.16

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	1.00	0.98	1.02	1.07	2.00	1.54	1.24	1.21	1.19	1.17	3.11	2.41	0.95	1.56	1.66
2	0.45	0.22	0.29	0.46	0.45	0.39	0.32	0.14	0.21	0.38	0.36	0.40	0.55	0.63	0.44
3	0.83	0.38	0.82	0.62	0.50	0.52	0.69	0.90	0.84	0.77	1.83	0.61	0.70	0.70	2.19
4	0.87	0.85	0.79	1.08	0.94	1.39	1.28	0.88	1.38	1.16	2.73	1.61	1.02	1.06	2.53
5	0.86	1.23	0.75	1.00	1.23	1.30	1.07	1.17	1.86	0.94	0.74	1.26	0.72	1.24	1.01
6	0.71	0.72	0.99	0.93	1.02	1.20	0.42	0.58	0.34	0.44	1.55	0.87	0.50	0.60	0.64
7	0.47	0.65	0.64	0.73	0.69	0.40	0.49	0.34	0.77	0.81	0.55	0.55	0.42	1.84	2.40
8	1.02	0.57	0.49	0.92	1.32	0.92	1.12	1.11	2.01	1.41	1.43	1.55	0.67	0.54	1.93

APPENDIX 2.19 Arterial blood O_2 tensions (Torr) for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	84.9	104.6	86.6	83.2	92.5	86.8	91.4	90.8	93.9	84.2	88.1	90.9	80.8	89.8	84.5
2	86.4	88.7	92.4	94.8	86.1	102.0	95.2	79.0	85.5	80.3	92.1	86.8	80.0	89.2	84.3
3	98.5	90.9	91.9	124.6	105.1	101.9	90.1	93.5	89.9	90.5	85.5	91.2	89.4	85.5	82.4
4	90.9	90.2	99.9	81.6	103.5	84.1	89.6	89.6	82.0	89.4	92.7	85.1	88.0	88.8	92.1
5	82.4	91.9	101.3	89.5	90.9	89.2	83.8	90.6	84.2	84.6	91.5	85.1	81.4	82.5	82.2
6	98.2	96.8	108.5	95.7	86.6	107.4	91.3	91.0	90.8	96.8	96.3	95.5	91.2	91.8	90.2
7	109.4	95.5	90.6	110.4	94.7	94.9	106.3	98.5	97.8	99.0	95.5	98.7	85.0	83.6	84.3
8	90.3	82.9	84.2	89.4	95.5	95.8	98.8	97.9	95.8	100.3	91.1	85.1	119.0	97.0	92.6

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	88.9	84.3	91.8	86.2	91.7	87.4	77.0	77.6	73.7	88.9	84.3	91.8	95.5	92.6	84.4
2	89.5	93.5	96.4	87.4	90.6	96.7	93.3	92.9	90.5	104.7	109.9	82.6	114.6	87.0	95.7
3	84.4	83.8	95.1	80.3	87.5	84.5	90.7	82.2	86.1	80.3	72.7	79.2	89.8	80.0	72.5
4	98.2	91.1	85.9	95.2	93.5	100.4	91.0	92.5	90.5	84.3	84.4	92.8	88.1	85.4	71.6
5	90.6	82.0	85.7	98.4	88.4	85.4	84.9	88.4	80.7	94.2	94.7	84.9	105.6	84.4	80.1
6	85.4	88.2	84.6	91.3	86.2	85.7	85.3	84.9	85.0	80.6	82.5	80.4	85.3	88.3	80.3
7	70.0	94.0	84.9	89.6	78.8	88.9	94.6	83.3	93.3	84.2	91.5	104.5	92.1	75.1	67.4
8	98.7	96.3	92.0	99.9	98.1	96.5	98.8	100.9	94.5	84.6	72.1	80.7	105.7	99.7	91.7

APPENDIX 2.20 Arterial blood CO_2 tensions (Torr) for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	42.0	46.3	44.2	49.5	47.8	55.0	37.5	42.8	38.8	40.9	37.5	39.8	45.7	40.2	41.4
2	43.2	41.4	39.1	43.1	42.9	50.0	46.6	40.9	39.1	38.6	38.5	48.5	37.0	48.4	44.5
3	46.1	49.3	40.6	46.1	48.8	47.5	39.6	42.5	40.7	37.7	43.0	44.6	44.6	55.9	50.5
4	44.0	44.1	39.0	42.7	47.0	49.0	50.9	49.9	47.7	41.8	43.8	42.3	49.9	51.0	53.1
5	41.0	36.8	49.0	37.9	33.3	32.5	39.1	38.7	39.8	38.0	37.3	38.7	41.5	41.7	42.6
6	40.2	40.0	41.2	36.4	40.1	39.4	46.1	45.3	40.2	40.9	38.8	39.1	46.1	38.8	34.4
7	39.5	42.6	41.2	39.5	40.7	40.1	33.8	41.5	39.3	36.1	36.6	41.1	39.8	43.8	41.0
8	40.1	39.1	42.2	37.9	36.6	39.5	40.8	41.3	39.7	40.7	39.8	35.8	35.6	44.0	45.2

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	42.5	38.6	42.4	38.7	40.7	41.7	37.0	36.0	39.9	36.1	40.3	37.7	39.8	41.4	42.4
2	40.7	42.2	40.0	37.6	40.2	44.2	38.8	31.5	41.8	43.8	40.7	40.3	41.1	39.9	40.2
3	36.9	37.8	39.1	37.4	35.5	31.3	37.9	35.4	33.5	35.2	32.4	33.1	32.3	36.8	32.5
4	35.4	37.2	39.4	35.7	37.4	33.5	39.5	38.0	37.5	36.6	38.8	37.0	37.7	36.7	33.8
5	36.5	35.1	35.9	35.5	34.1	35.5	39.6	34.2	36.1	36.1	36.6	37.0	35.2	32.3	33.3
6	42.6	44.4	39.1	43.6	45.3	43.4	43.0	44.0	45.3	47.0	46.3	45.8	44.2	40.3	40.1
7	35.1	36.2	37.3	39.1	38.8	35.0	36.5	32.9	38.7	36.8	39.1	34.5	36.8	34.5	33.9
8	36.9	39.6	38.9	42.2	41.8	45.4	35.0	38.3	39.2	36.2	44.2	54.3	37.7	42.5	45.2

APPENDIX 2.21 Arterial pH values for (A) control (n=8) and (B) COPD affected (n=8) horses, before (0h) and at 1.5 and 5h after inhalation challenges with PBS, AF, TV and MF and after NC.

(A) Control horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	7.409	7.465	7.434	7.433	7.423	7.439	7.501	7.572	7.406	7.430	7.409	7.424	7.436	7.419	7.394
2	7.389	7.438	7.380	7.430	7.391	7.418	7.442	7.390	7.407	7.405	7.399	7.436	7.386	7.380	7.399
3	7.354	7.399	7.402	7.392	7.350	7.420	7.381	7.362	7.390	7.355	7.390	7.410	7.397	7.351	7.381
4	7.434	7.442	7.392	7.411	7.417	7.449	7.373	7.471	7.351	7.407	7.434	7.406	7.413	7.414	7.421
5	7.403	7.422	7.403	7.407	7.407	7.444	7.464	7.569	7.430	7.430	7.449	7.406	7.355	7.449	7.406
6	7.367	7.390	7.393	7.362	7.398	7.400	7.393	7.401	7.387	7.412	7.397	7.411	7.389	7.342	7.377
7	7.338	7.401	7.443	7.373	7.379	7.350	7.399	7.398	7.421	7.387	7.368	7.403	7.359	7.310	7.356
8	7.430	7.385	7.413	7.400	7.396	7.423	7.421	7.410	7.401	7.409	7.374	7.378	7.386	7.412	7.377

(B) COPD affected horses.

	PBS			AF			TV			MF			NC		
	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5	0	1.5	5
1	7.373	7.406	7.401	7.412	7.412	7.452	7.401	7.386	7.372	7.435	7.419	7.374	7.403	7.431	7.434
2	7.420	7.390	7.426	7.385	7.396	7.401	7.375	7.380	7.444	7.401	7.402	7.414	7.396	7.430	7.397
3	7.341	7.346	7.400	7.357	7.341	7.352	7.376	7.350	7.407	7.371	7.385	7.395	7.345	7.354	7.380
4	7.342	7.372	7.361	7.370	7.362	7.378	7.360	7.368	7.360	7.342	7.347	7.377	7.351	7.364	7.372
5	7.409	7.415	7.421	7.354	7.387	7.301	7.399	7.407	7.382	7.400	7.396	7.388	7.393	7.401	7.381
6	7.390	7.377	7.389	7.441	7.402	7.406	7.389	7.405	7.396	7.390	7.410	7.402	7.389	7.405	7.432
7	7.433	7.414	7.363	7.373	7.415	7.427	7.418	7.425	7.409	7.430	7.449	7.414	7.348	7.392	7.396
8	7.440	7.389	7.452	7.399	7.425	7.442	7.409	7.450	7.414	7.402	7.419	7.419	7.401	7.427	7.428

APPENDIX 4.1 Description of horses used for lymphocyte study.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	TB	MN	24	615
2	"	CROSS	MN	15	593
3	"	SHETLAND	F	7	212
4	"	TB	MN	19	643
5	"	TB	MN	13	652
6	"	CROSS	F	20	500
1	COPD	CROSS	F	20	371
2	"	CROSS	F	20	488
3	"	CROSS	MN	13	501
4	"	CROSS	MN	25	422
5	"	TB	MN	13	537
6	"	TB	M	30	504

F=female, M=male, MN= gelding, Cross=crossbred, TB=Thoroughbred.

APPENDIX 4.2 Ratios of positively fluorescent gated PBL cells from (a) control (n=6) and (b) COPD affected (n=6) horses, after control labelling with phosphate buffered saline (PBS), normal mouse serum (NMS) and normal rat serum (NratS) and ratios of CD5+, CD8+, equine class I+ (CL1), MAC284+ (284+) and B cells, before (PRE) and at 72h after (POST) NC.

(a) Control horses.

		PBA	NMS	CD5+	CD8+	NratS	CL1+	284+	B
1	PRE	2.0	2.9	69.3	33.8	2.3	90.5	68.8	34.0
2	"	2.9	2.5	70.1	29.1	3.2	96.6	77.5	14.6
3	"	1.8	4.3	59.5	19.6	1.0	87.8	43.8	36.0
4	"	2.0	1.9	56.1	24.3	2.3	98.9	53.7	21.1
5	"	1.3	1.6	70.0	41.1	1.7	91.3	85.8	40.9
6	"	0.9	2.2	81.8	23.8	0.7	94.6	68.0	22.4
1	POST	0.8	2.3	87.2	37.5	1.1	99.7	96.3	19.8
2	"	0.4	0.5	86.8	30.5	3.4	99.9	96.9	15.2
3	"	0.7	1.3	67.9	29.6	2.4	99.4	89.4	22.9
4	"	1.1	2.4	72.8	25.5	1.8	98.1	89.2	27.6
5	"	0.5	2.5	68.5	33.7	2.7	98.0	67.9	38.4
6	"	2.1	3.1	67.2	22.8	2.4	95.9	60.7	37.5

(b) COPD affected horses.

		PBA	NMS	CD5+	CD8+	NratS	CL1+	284+	B
1	PRE	0.6	4.2	73.7	26.9	1.1	97.0	69.8	18.7
2	"	3.3	3.1	73.7	14.8	2.3	95.6	78.0	41.0
3	"	2.0	3.5	77.2	30.6	2.5	98.6	80.8	10.9
4	"	1.5	2.7	80.7	20.2	1.5	98.3	80.3	18.5
5	"	0.9	3.1	73.1	38.5	2.9	98.7	87.5	29.5
6	"	4.0	3.4	67.7	24.6	4.4	98.3	71.4	28.4
1	POST	0.2	0.9	76.3	20.3	1.2	95.7	58.9	19.6
2	"	0.4	1.6	82.9	18.7	3.8	94.3	77.1	11.8
3	"	3.7	2.5	59.0	29.4	3.6	93.4	52.8	39.7
4	"	0.4	0.7	76.7	43.0	0.8	99.1	95.9	25.3
5	"	0.5	3.1	68.2	40.1	4.7	90.3	77.7	28.8
6	"	0.9	2.3	89.0	41.3	3.9	86.2	73.5	5.6

APPENDIX 4.3 Ratios of positively fluorescent gated BALF cells from (a) control (n=6) and (b) COPD affected (n=6) horses after control labelling with phosphate buffered saline (PBS), normal mouse serum (NMS) and normal rat serum (NratS) and ratios of CD5+, CD8+, equine class I+ (CL1), MAC284+ (284+) and B cells, before (PRE) and at 72h after (POST) NC.

(a) Control horses.

		PBA	NMS	CD5+	CD8+	NratS	CL1+	284+	B
1	PRE	0.4	5.1	59.5	33.0	1.3	64.7	59.3	3.5
2	"	4.0	4.5	91.1	59.9	5.1	90.7	82.3	4.0
3	"	3.1	5.0	74.6	40.2	1.9	54.5	46.3	4.2
4	"	0.6	4.7	83.0	43.5	0.9	93.0	86.9	2.5
5	"	4.1	4.7	77.2	42.2	6.1	81.1	78.1	4.2
6	"	0.6	2.6	91.0	44.7	5.2	72.6	65.3	2.2
1	POST	1.1	1.8	89.2	42.4	1.9	96.7	91.5	3.9
2	"	1.2	1.1	88.0	45.5	5.1	96.7	93.6	1.9
3	"	0.2	1.1	83.8	45.2	3.5	97.6	91.4	5.3
4	"	0.8	2.5	87.3	43.9	2.4	90.3	80.8	2.9
5	"	0.9	1.8	91.4	40.9	2.8	92.0	74.4	2.4
6	"	1.8	2.2	65.5	34.5	1.9	30.3	25.0	4.5

(b) COPD affected horses.

		PBA	NMS	CD5+	CD8+	NratS	CL1+	284+	B
1	PRE	0.2	4.8	70.0	42.4	0.9	67.9	71.0	11.1
2	"	1.5	4.7	78.1	42.2	2.4	65.6	56.3	10.0
3	"	1.2	0.1	62.3	41.3	1.6	47.6	56.3	7.3
4	"	1.8	3.4	66.6	43.7	3.7	48.5	32.0	9.8
5	"	2.8	0.6	76.6	45.6	4.6	94.7	83.4	12.7
6	"	3.6	4.5	57.1	43.1	5.1	47.6	47.5	8.9
1	POST	3.4	5.7	65.0	27.7	1.9	71.4	64.7	1.4
2	"	1.7	4.7	64.0	31.3	1.7	45.7	42.2	1.4
3	"	4.0	7.2	32.2	19.5	4.1	30.5	17.3	6.3
4	"	1.8	3.4	66.6	41.7	2.8	48.5	32.0	9.8
5	"	1.5	5.7	29.3	15.1	3.3	48.3	48.0	3.6
6	"	1.3	1.2	84.7	42.6	1.9	87.5	72.3	3.4

APPENDIX 4.4 Ratios of CD5+, CD8+, CD5+CD8- and B lymphocytes, expressed as ratios of the total gated lymphocytes, in PB of control (CONT) (n=6) and COPD affected (n=6) horses before (PRE) and at 72h post (POST) NC.

	mAb	CONT PRE	CONT POST	COPD PRE	COPD POST
1	CD5+	67.1	81.5	79.8	79.6
2	"	82.8	85.1	64.3	87.5
3	"	62.3	74.8	87.6	59.8
4	"	52.3	72.5	81.4	75.2
5	"	63.1	64.1	71.2	70.3
6	"	78.5	64.2	70.4	94.1
1	CD8+	32.7	35.0	29.1	21.2
2	"	34.4	29.9	12.9	19.7
3	"	20.5	32.6	34.7	29.8
4	"	31.5	25.4	20.4	42.2
5	"	37.1	31.5	37.5	41.3
6	"	22.8	21.8	25.6	43.7
1	CD5+CD8-	34.4	46.4	50.6	58.4
2	"	48.4	55.2	51.4	67.8
3	"	41.8	42.2	52.9	30.0
4	"	41.2	47.1	61.0	33.0
5	"	26.1	32.6	33.7	29.0
6	"	55.7	42.4	44.8	50.4
1	B CELL	32.9	18.5	20.2	20.4
2	"	17.2	14.9	35.7	12.5
3	"	37.7	25.2	12.4	40.2
4	"	27.3	27.5	18.6	24.8
5	"	36.9	35.9	28.8	29.7
6	"	21.5	35.8	29.6	5.9

APPENDIX 4.5 Ratios of CD5+, CD8+, CD5+CD8- and B lymphocytes, expressed as ratios of the total gated lymphocytes, in BALF of control (CONT) (n=6) and COPD affected (n=6) horses, before (PRE) and at 72h post (POST) NC.

	mAb	CONT PRE	CONT POST	COPD PRE	COPD POST
1	CD5+	94.4	95.8	86.3	97.9
2	"	95.8	97.9	90.7	97.9
3	"	94.7	94.1	89.5	83.6
4	"	97.1	96.8	87.2	87.2
5	"	94.8	97.4	85.8	89.1
6	"	97.6	93.6	86.5	96.1
1	CD8+	52.4	45.5	52.3	41.7
2	"	63.0	50.6	47.9	47.9
3	"	51.0	50.7	59.3	50.6
4	"	50.9	48.7	57.2	54.6
5	"	51.8	43.6	51.1	45.9
6	"	48.0	49.3	65.3	48.4
1	CD5+CD8-	42.1	50.3	34.0	56.2
2	"	32.8	47.3	40.7	50.0
3	"	43.7	43.3	30.2	33.0
4	"	46.2	48.1	30.0	32.6
5	"	43.0	53.8	34.7	43.2
6	"	49.7	44.3	21.2	47.8
1	B CELL	5.6	4.2	13.7	2.1
2	"	4.2	2.1	11.4	2.1
3	"	5.3	5.9	10.5	16.4
4	"	2.9	3.2	12.8	12.8
5	"	5.2	2.6	14.2	10.9
6	"	2.4	6.4	13.5	3.9

APPENDIX 5.1 Description of horses used for histamine study.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	CROSS	MN	15	593
2	"	SHETLAND	F	7	212
3	"	TB	MN	19	643
4	"	CROSS	F	16	561
5	"	CROSS	F	10	515
6	"	TB	MN	13	652
7	"	TB	MN	25	609
8	"	CROSS	MN	20	500
9	COPD	CROSS	F	20	371
10	"	CROSS	F	20	488
11	"	CROSS	MN	13	501
12	"	CROSS	MN	25	422
13	"	CROSS	F	17	546
14	"	TB	MN	13	537
15	"	TB	MN	6	475
16	"	CROSS	MN	14	450
17	"	DUTCH	MN	8	642

F=female, MN= gelding, cross=crossbred, TB=Thoroughbred, DUTCH= Dutch Warmblood.

APPENDIX 5.2 INVESTIGATION OF THE STABILITY OF EXOGENOUS HISTAMINE DURING THE COLLECTION, PROCESSING AND STORAGE OF BALF

The stability of exogenous histamine during the collection, processing and storage of BALF was investigated. Exogenous histamine, which had been instilled into the apical lobes of the left lungs of 2 horses, was recovered by BAL, and the proportions remaining after collection, processing and storage were determined.

BALF, recovered from the apical lobe following instillation of the exogenous histamine, contained both the exogenous histamine which was instilled into the lung and 'endogenous, PELF derived' histamine. Thus, to determine the proportion of exogenous histamine recovered by BAL, the concentration of 'endogenous, PELF derived' histamine within the BALF had to be calculated.

Assuming that the PELF histamine concentration in the left and right equine lungs are identical, as has been reported for man (Rankin *et al* 1987), the 'endogenous, PELF derived' histamine concentration of BALF recovered from the 'test site' can be determined by quantifying histamine in BALF harvested from the opposite lung ('control site');

$$[\text{Endogenous histamine}] = \frac{[\text{BALF urea (test site)}] \times [\text{BALF histamine (control site)}]}{[\text{BALF urea (control site)}]}$$

Subtraction of the 'endogenous, PELF derived histamine' concentration from the BALF histamine concentration yielded the exogenous histamine concentration. This value was then compared with the concentration of exogenous histamine present within the fluid which was instilled into the lung to determine the proportion of histamine remaining after the collection, processing and storage of BALF.

The calculation steps are shown on page 323.

REFERENCE

RANKIN,J.A., KALINER,M. and REYNOLDS,H.Y. (1987) Histamine levels in bronchoalveolar lavage from patients with asthma, sarcoidosis and idiopathic pulmonary fibrosis. J.Allergy Clin.Immunol. *79* 371-377.

(APPENDIX 5.2 Continued)

The data and calculation steps are indicated in the table below.

	HORSE 1		HORSE 2	
	CONTROL SITE	TEST SITE	CONTROL SITE	TEST SITE
Concentration histamine instilled into lung (ng/ml)	-	0.96	-	0.40
Histamine concentration of recovered BALF (ng/ml)	0.07	1.13	1.30	0.76
BALF urea concentration (mg/dl)	0.071	0.186	0.438	0.100
Calculated endogenous histamine concentration (ng/ml) of recovered BALF (using urea dilution technique)	-	0.18	-	0.30
Calculated exogenous BALF histamine concentration (ng/ml)		0.95		0.46
Proportion of exogenous histamine recovered		99%		115%

APPENDIX 5.3 Plasma histamine concentrations (ng/ml) for control (n=8) and COPD affected (n=8) horses at 0, 1.5 and 5h after NC.

	CONTROL			COPD AFFECTED		
HORSE	0	1.5	5	0	1.5	5
1	0.27	0.38	0.29	0.26	0.39	0.54
2	0.11	0.15	0.27	0.23	0.48	0.26
3	0.24	0.48	0.18	0.11	0.13	0.10
4	0.34	0.27	0.35	0.16	0.38	0.26
5	0.57	0.64	0.07	0.18	0.16	0.16
6	0.21	0.33	0.38	0.10	0.20	0.19
7	0.07	0.19	0.08	0.17	0.17	0.12
8	0.24	0.48	0.18	0.11	0.12	0.06

APPENDIX 5.4 Differential and absolute metachromatic cell (MET) counts in BALF samples from (A) 8 control and (B) 9 COPD affected horses at 0, 0.5 and 5h after NC. Ratios were derived by two techniques, namely by counting 300 cells on a Leishman's stained cytospin preparation (LEISH) and by counting 1000 cells on a toluidine blue stained cytospin preparation (TOL BLUE).

(A) CONTROL HORSES.

HORSE	GROUP	TIME	% MET (TOL BLUE)	% MET (LEISH)	BALF CELL COUNT (/ul)	MET COUNT (TOL BLUE) (/ul)	MET COUNT (LEISH) (/ul)	BALF SUPERNATANT HISTAMINE (ng/ml)
1	CONTROL	0h	3.3	5.3	150	4.95	7.95	0.121
2	"	"	4.5	5.0	120	5.40	6.00	0.110
3	"	"	4.2	6.3	640	26.88	40.32	1.640
4	"	"	2.3	1.3	190	4.37	2.47	2.659
5	"	"	4.6	5.3	230	10.58	12.19	1.598
6	"	"	2.8	2.5	300	8.40	7.50	0.359
7	"	"	5.7	8.4	590	33.63	49.56	1.926
8	"	"	9.9	7.3	240	23.76	17.52	2.330
1	CONTROL	0.5h	6.4	6.6	300	19.20	19.80	2.598
2	"	"	4.8	6.7	98	4.70	6.57	3.052
3	"	"	11.6	11.6	250	29.00	29.00	0.450
4	"	"	6.1	6.7	1125	68.63	75.38	1.566
5	"	"	9.9	11.0	237	23.46	26.07	2.161
6	"	"	7.2	6.3	50	3.60	3.15	0.003
7	"	"	4.8	8.7	225	10.80	19.58	2.112
8	"	"	20.1	17.6	50	10.05	8.80	1.721
1	CONTROL	5h	3.2	3.0	650	20.80	19.50	0.484
2	"	"	8.1	8.0	175	14.18	14.00	1.011
3	"	"	6.6	5.3	362	23.89	19.19	0.943
4	"	"	4.4	5.3	212	9.33	11.24	2.369
5	"	"	9.7	11.0	50	4.85	5.50	2.207
6	"	"	2.9	2.3	308	8.93	7.08	1.716
7	"	"	5.9	9.4	237	13.98	22.28	2.017
8	"	"	12.8	15.0	168	21.50	25.20	0.133

APPENDIX 5.4 (B) COPD AFFECTED HORSES.

HORSE	GROUP	TIME	% MET (TOL BLUE)	% MET (LEISH)	BALF CELL COUNT (/ul)	MET COUNT (TOL BLUE) (/ul)	MET COUNT (LEISH) (/ul)	BALF SUPERNATANT HISTAMINE (ng/ml)
1	COPD	0h	3.2	3.0	362	11.58	10.86	1.677
2	"	"	6.6	6.0	200	13.20	12.00	0.012
3	"	"	4.8	5.5	90	4.32	4.95	0.364
4	"	"	2.9	2.0	252	7.31	5.04	0.609
5	"	"	12.3	3.5	312	38.38	10.92	0.184
6	"	"	2.4	2.0	180	4.32	3.60	0.019
7	"	"	6.7	4.7	185	12.39	8.69	3.932
8	"	"	5.9	6.7	85	5.01	5.69	0.101
9	"	"	4.3	3.0	110	4.73	3.30	0.276
1	COPD	0.5h	0.7	4.3	240	1.68	10.32	0.860
2	"	"	3.3	3.7	300	9.90	11.10	0.640
3	"	"	8.0	7.7	210	16.80	16.17	1.721
4	"	"	3.8	4.3	270	10.26	11.61	1.011
5	"	"	2.7	4.2	250	6.75	10.50	0.638
6	"	"	6.7	6.5	230	15.41	14.95	2.370
7	"	"	3.7	4.4	250	9.25	11.00	1.011
8	"	"	5.6	2.6	400	22.40	10.40	1.604
9	"	"	3.8	4.0	245	9.31	9.80	0.100
1	COPD	5h	4.2	3.7	222	9.31	8.21	0.860
2	"	"	5.0	5.3	480	14.03	14.84	5.365
3	"	"	2.2	3.6	251	5.55	9.04	1.103
4	"	"	4.4	1.3	520	22.88	6.76	0.715
5	"	"	1.3	0.3	2750	35.75	8.25	1.162
6	"	"	0.3	0.3	143	0.43	0.43	0.016
7	"	"	0.6	0.6	3737	22.42	22.42	6.377
8	"	"	2.3	1.3	463	10.65	6.02	2.655
9	"	"	2.0	1.5	75	1.50	1.13	0.146

APPENDIX 5.5 PELF histamine concentrations (ng/ml) for control (n=8) and COPD affected (n=9) horses at 0, 0.5 and 5h after NC.

	CONTROL			COPD AFFECTED		
HORSE	0	0.5	5	0	0.5	5
1	31.27	651.35	64.87	187.73	174.63	104.93
2	40.42	353.36	81.25	7.00	118.12	441.03
3	202.18	103.23	105.10	35.16	385.63	794.97
4	435.86	150.61	658.16	95.48	256.31	245.59
5	424.08	244.53	336.07	90.44	62.84	171.10
6	105.96	1.14	250.78	4.63	777.27	4.88
7	305.36	515.93	187.21	430.05	143.52	1087.36
8	176.51	243.30	74.16	29.10	273.21	336.21
9	-	-	-	11.50	11.10	34.15

APPENDIX 5.6 Albumen adjusted BALF histamine concentrations (ng/ml) for control (n=8) and COPD affected (n=9) horses at 0, 0.5 and 5h after NC.

	CONTROL			COPD AFFECTED		
HORSE	0	0.5	5	0	0.5	5
1	45.48	702.69	198.35	238.56	400.32	287.52
2	112.11	1119.76	259.36	16.44	322.29	1339.94
3	737.35	886.90	747.99	178.39	514.32	1775.35
4	1177.65	865.58	663.42	118.27	265.77	289.11
5	362.18	619.17	1823.92	64.64	241.04	898.16
6	107.65	7.52	871.68	62.73	779.60	115.94
7	703.02	1095.38	806.85	925.28	423.84	1996.08
8	265.21	315.35	48.75	125.07	598.75	2277.74
9	-	-	-	161.20	38.10	92.81

APPENDIX 5.7 Concentrations of urea (mg/dl) in plasma and BALF from (A) 8 control horses and (B) 9 COPD affected horses at 0, 0.5 and 5h after NC.

(A) CONTROL HORSES

	PLASMA UREA			BALF UREA		
HORSE	0	0.5	5	0	0.5	5
1	24.8	36.1	29.9	0.076	0.144	0.223
2	25.9	30.1	33.6	0.071	0.260	0.418
3	29.1	22.0	23.4	0.236	0.096	0.210
4	24.1	30.3	22.5	0.147	0.315	0.081
5	26.8	19.8	30.3	0.101	0.175	0.199
6	19.8	18.6	33.9	0.067	0.044	0.232
7	32.5	21.5	29.7	0.205	0.088	0.320
8	42.2	30.4	35.6	0.557	0.215	0.064

(B) COPD AFFECTED HORSES

	PLASMA UREA			BALF UREA		
HORSE	0	0.5	5	0	0.5	5
1	35.7	20.5	26.1	0.319	0.101	0.214
2	28.7	27.7	23.1	0.048	0.150	0.281
3	19.9	25.1	31.0	0.206	0.112	0.043
4	31.2	35.5	30.9	0.199	0.140	0.090
5	37.4	19.9	26.5	0.076	0.202	0.180
6	24.7	26.9	29.5	0.101	0.082	0.098
7	22.2	30.1	34.1	0.203	0.212	0.200
8	19.9	18.4	27.1	0.069	0.108	0.214
9	22.2	8.1	23.4	0.532	0.163	0.100

APPENDIX 5.8 Concentrations of albumen in plasma (mg/ml) and BALF (ug/ml) from (A) 8 control and (B) 9 COPD affected horses at 0, 0.5 and 5h after NC.

(A) CONTROL HORSES

	PLASMA ALBUMEN			BALF ALBUMEN		
HORSE	0	0.5	5	0	0.5	5
1	22.1	28.1	32.8	59.1	103.9	80.0
2	25.8	24.8	22.4	25.5	67.6	87.3
3	38.0	38.0	27.2	84.5	19.3	34.3
4	23.3	40.3	21.0	52.6	72.9	75.0
5	23.5	21.2	27.6	103.7	74.0	33.4
6	27.8	41.8	28.9	92.6	15.0	56.9
7	26.9	38.8	30.2	73.7	74.8	75.5
8	25.5	23.0	23.0	224.0	125.5	62.9

(B) COPD AFFECTED HORSES

	PLASMA ALBUMEN			BALF ALBUMEN		
HORSE	0	0.5	5	0	0.5	5
1	15.8	18.1	26.0	111.1	38.9	77.8
2	30.6	26.1	26.2	21.8	51.8	104.9
3	29.8	21.7	32.2	60.8	72.6	20.0
4	20.1	23.9	29.1	103.5	90.9	72.0
5	19.1	19.5	33.0	54.3	51.6	42.7
6	18.9	20.4	38.6	5.7	62.0	5.4
7	30.4	30.4	40.0	129.2	72.5	127.8
8	20.7	23.0	31.4	16.7	61.6	36.6
9	34.5	36.5	37.2	59.0	95.8	58.5

APPENDIX 5.9 BALF mast cell ratios, absolute BALF mast cell counts (/ml), total BALF cell counts (/ul) , whole BALF histamine concentrations (ng/ml), BALF supernatant histamine concentrations (ng/ml) and mast cell histamine contents (pg/cell) for 11 horses after PBS challenges or after NCs.

HORSE	GROUP	CHALLENGE	BALF MAST CELL RATIO	TOTAL BALF CELL COUNT (/ul)	BALF MAST CELL COUNT (/ml)	WHOLE BALF HISTAMINE (ng/ml)	HISTAMINE CONTENT OF MAST CELL (pg)	BALF SUPERNATANT HISTAMINE (ng/ml)
1	CONTROL	NC +5h	7.7	237	18249	86.04	4.71	2.02
2	"	"	9.5	362	34390	94.32	2.74	0.95
3	"	"	20.1	50	10050	35.05	3.49	2.37
4	"	NC +0.5h	11.6	75	8700	26.67	3.07	0.45
5	"	PBS +5h	8.7	85	7395	13.51	1.83	2.11
6	"	"	3.2	245	7840	19.28	2.46	0.43
7	COPD	NC +0.5h	4.6	270	12420	8.82	0.71	1.01
8	"	"	5.2	210	10920	31.98	2.93	1.50
9	"	"	0.9	700	6300	19.28	3.06	1.60
10	"	"	4.3	190	8170	25.95	3.18	0.43
11	"	"	7.0	245	17150	27.21	1.59	1.93

APPENDIX 7.1 Description of horses used in evaluation of urea and albumen as endogenous markers of dilution for equine BALF.

HORSE	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CROSS	MN	15	593
2	TB	MN	19	643
3	CROSS	F	16	561
4	CROSS	F	10	515
5	TB	MN	13	652
6	TB	MN	25	609
7	CROSS	MN	20	500

F=female, MN= gelding, Cross=crossbred, TB=Thoroughbred.

APPENDIX 7.2 Solutions used in the albumen radial immunodiffusion assay.

Amido blue-black stain

500mg . . . . . Amido blue-black stain
225ml . . . . . 0.1M Na acetate
225ml . . . . . 1M acetic acid

Barbital buffer (pH 8.6)

Na barbitone 10.3g
Barbituric acid 1.84g
HCl . . . . . to pH 8.6
NaN_3 . . . . . 0.2g

APPENDIX 7.3 Urea concentrations (mg/dl) of plasma and of BALF, recovered from the diaphragmatic lobes of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA), of control (n=7) horses.

HORSE	PLASMA	RD	RA	LD	LA
1	30.1	0.125	0.108	0.089	0.106
2	22.1	0.105	0.120	0.172	0.162
3	23.6	0.086	0.058	0.071	0.109
4	27.3	0.147	0.204	0.274	0.024
5	31.8	0.121	0.246	0.333	0.330
6	20.7	0.073	0.176	0.056	0.075
7	18.2	0.042	0.170	0.080	0.063

APPENDIX 7.4 Albumen concentrations of plasma (mg/ml) and of BALF (ug/ml), recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA), of control (n=7) horses.

HORSE	PLASMA	RD	RA	LD	LA
1	21.4	87.4	74.7	56.7	84.0
2	25.2	104.7	101.9	56.2	80.3
3	25.7	65.0	88.1	89.4	84.2
4	20.4	58.2	75.0	121.6	29.0
5	22.0	81.1	62.0	84.0	97.0
6	22.8	42.9	99.7	63.2	67.6
7	24.5	67.5	97.0	83.4	59.7

APPENDIX 7.5 Total cell counts (/ul) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

	RD	RA	LD	LA
1	90	95	80	105
2	90	165	35	100
3	55	65	55	115
4	90	95	140	25
5	140	280	200	130
6	50	150	60	110
7	75	205	105	55

APPENDIX 7.6 BALF cell ratios for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

N^{o}	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.7	20.0	68.7	0.7	9.7	0.0	9.7	0.3
2	"	1.0	25.3	67.3	0.0	5.3	0.0	5.3	1.0
3	"	1.7	50.3	36.0	0.0	9.7	2.3	12.0	0.0
4	"	1.7	40.3	43.7	0.0	10.7	1.7	12.3	1.7
5	"	4.0	20.7	74.3	0.0	0.3	0.3	0.7	0.3
6	"	1.0	51.3	42.3	0.0	4.0	1.3	5.3	0.0
7	"	0.7	39.7	49.7	0.3	9.3	0.0	9.3	0.3
1	RA	0.7	26.3	63.3	0.7	8.3	0.7	9.0	0.0
2	"	1.3	22.7	73.7	0.0	2.3	0.0	2.3	0.0
3	"	4.0	47.0	42.7	0.0	5.0	1.3	6.3	0.0
4	"	1.0	40.0	51.0	0.3	6.3	0.0	6.3	1.3
5	"	1.7	16.0	73.3	0.0	6.7	0.0	6.7	2.3
6	"	4.0	23.0	68.0	0.0	2.0	0.3	2.3	2.7
7	"	0.7	16.0	63.3	0.3	18.7	0.3	19.0	0.7
1	LD	1.7	23.3	61.7	0.0	10.0	0.3	10.3	3.0
2	"	0.7	17.0	75.7	0.0	6.0	0.0	6.0	0.7
3	"	1.0	38.3	51.7	0.0	8.0	0.0	8.0	1.0
4	"	1.3	52.0	36.3	0.7	8.0	0.0	8.0	1.7
5	"	2.3	19.7	72.7	0.0	4.3	0.7	5.0	0.3
6	"	2.0	24.3	65.7	0.3	3.3	1.0	4.3	3.3
7	"	0.0	56.7	29.3	0.3	13.7	0.0	13.7	0.0
1	LA	2.0	28.7	60.7	0.3	6.3	1.0	7.3	1.0
2	"	3.0	26.3	66.3	0.0	4.0	0.0	4.0	0.3
3	"	3.7	52.7	38.3	2.3	2.7	0.0	2.7	0.3
4	"	1.0	46.3	44.7	0.0	2.0	4.0	6.0	2.0
5	"	3.0	16.3	78.0	0.0	1.0	1.0	2.0	0.7
6	"	4.3	24.0	66.0	0.0	5.7	0.0	5.7	0.0
7	"	0.7	47.7	35.7	0.3	13.7	0.3	14.0	1.7

APPENDIX 7.7 Total PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

	RD	RA	LD	LA
1	21.7	26.5	27.1	29.8
2	18.9	30.4	18.2	13.6
3	15.1	26.4	18.3	24.9
4	16.7	12.7	13.9	28.4
5	36.8	36.2	19.1	12.5
6	14.2	17.6	22.2	30.3
7	32.5	21.9	23.9	15.9

APPENDIX 7.8 Albumen adjusted total BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

	RD	RA	LD	LA
1	22.0	27.2	30.2	26.8
2	21.7	40.4	15.7	31.4
3	21.7	19.0	15.8	35.1
4	31.5	25.8	23.5	17.6
5	38.0	38.0	52.4	29.5
6	26.6	34.3	21.6	35.8
7	27.2	51.8	30.8	22.6

APPENDIX 7.9 PELF cell counts (x10^3/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) horses.

N^o	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.2	4.3	14.9	0.2	2.1	0.0	2.1	0.1
2	"	0.2	4.8	12.7	0.0	1.0	0.0	1.0	0.2
3	"	0.3	7.6	5.4	0.0	1.5	0.3	1.8	0.0
4	"	0.3	6.7	7.3	0.0	1.8	0.3	2.1	0.3
5	"	1.5	7.6	27.3	0.0	0.1	0.1	0.3	0.1
6	"	0.1	7.3	6.0	0.0	0.6	0.2	0.8	0.0
7	"	0.2	12.9	16.2	0.1	3.0	0.0	3.0	0.1
1	RA	0.2	7.0	16.8	0.2	2.2	0.2	2.4	0.0
2	"	0.4	6.9	22.4	0.0	0.7	0.0	0.7	0.0
3	"	1.1	12.4	11.3	0.0	1.3	0.3	1.7	0.0
4	"	0.1	5.1	6.5	0.0	0.8	0.0	0.8	0.2
5	"	0.6	5.8	26.5	0.0	2.4	0.0	2.4	0.8
6	"	0.7	4.1	12.0	0.0	0.4	0.1	0.4	0.5
7	"	0.2	3.5	13.9	0.1	4.1	0.1	4.2	0.2
1	LD	0.5	6.3	16.7	0.0	2.7	0.1	2.8	0.8
2	"	0.1	3.1	13.8	0.0	1.1	0.0	1.1	0.1
3	"	0.2	7.0	9.5	0.0	1.5	0.0	1.5	0.2
4	"	0.2	7.3	5.1	0.1	1.1	0.0	1.1	0.2
5	"	0.4	3.8	13.9	0.0	0.8	0.1	1.0	0.1
6	"	0.4	5.4	14.6	0.1	0.7	0.2	1.0	0.7
7	"	0.0	13.5	7.0	0.1	3.3	0.0	3.3	0.0
1	LA	0.6	8.6	18.1	0.1	1.9	0.3	2.2	0.3
2	"	0.4	3.6	9.0	0.0	0.5	0.0	0.5	0.0
3	"	0.9	13.1	9.5	0.6	0.7	0.0	0.7	0.1
4	"	0.3	13.2	12.7	0.0	0.6	1.1	1.7	0.6
5	"	0.4	2.0	9.8	0.0	0.1	0.1	0.3	0.1
6	"	1.3	7.3	20.0	0.0	1.7	0.0	1.7	0.3
7	"	0.1	7.6	5.7	0.0	2.2	0.0	2.2	0.3

APPENDIX 7.10 Albumen adjusted absolute BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung of control (n=7) horses.

N°	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.2	4.4	15.1	0.2	2.1	0.0	2.1	0.1
2	"	0.2	5.5	14.6	0.0	1.1	0.0	1.1	0.2
3	"	0.4	10.9	7.8	0.0	2.1	0.5	2.6	0.0
4	"	0.5	12.7	13.8	0.0	3.4	0.5	3.9	0.5
5	"	1.5	7.9	28.2	0.0	0.1	0.1	0.3	0.1
6	"	0.3	13.6	11.2	0.0	1.1	0.3	1.4	0.0
7	"	0.2	10.8	13.5	0.1	2.5	0.0	2.5	0.1
1	RA	0.2	7.2	17.2	0.2	2.3	0.2	2.4	0.0
2	"	0.5	9.3	30.1	0.0	0.9	0.0	0.9	0.0
3	"	0.8	8.9	8.1	0.0	0.9	0.2	1.2	0.0
4	"	0.3	10.3	13.2	0.1	1.6	0.0	1.6	0.3
5	"	0.6	6.1	27.9	0.0	2.5	0.0	2.5	0.9
6	"	1.4	7.9	23.3	0.0	0.7	0.1	0.8	0.9
7	"	0.4	8.3	32.8	0.2	9.7	0.2	9.8	0.4
1	LD	0.5	7.0	18.6	0.0	3.0	0.1	3.1	0.9
2	"	0.1	2.7	11.9	0.0	0.9	0.0	0.9	0.1
3	"	0.2	6.1	8.2	0.0	1.3	0.0	1.3	0.2
4	"	0.3	12.2	8.5	0.2	1.9	0.0	1.9	0.4
5	"	1.2	10.3	38.1	0.0	2.3	0.4	2.6	0.2
6	"	0.4	5.3	14.2	0.1	0.7	0.2	0.9	0.7
7	"	0.0	17.5	9.0	0.1	4.2	0.0	4.2	0.0
1	LA	0.5	7.7	16.2	0.1	1.7	0.3	2.0	0.3
2	"	0.9	8.3	20.8	0.0	1.3	0.0	1.3	0.1
3	"	1.3	18.5	13.4	0.8	0.9	0.0	0.9	0.1
4	"	0.2	8.1	7.9	0.0	0.4	0.7	1.1	0.4
5	"	0.9	4.8	23.0	0.0	0.3	0.3	0.6	0.2
6	"	1.5	8.6	23.6	0.0	2.0	0.0	2.0	0.0
7	"	0.2	10.8	8.1	0.1	3.1	0.1	3.2	0.4

APPENDIX 8.1 Description of horses used in the investigation of regional variation in the cellular and molecular components of equine BALF.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	CROSS	MN	15	593
2	"	TB	MN	19	643
3	"	CROSS	F	16	561
4	"	CROSS	F	10	515
5	"	TB	MN	13	652
6	"	TB	MN	25	609
7	"	CROSS	MN	20	500
8	COPD	CROSS	F	20	371
9	"	CROSS	F	20	488
10	"	CROSS	MN	13	501
11	"	CROSS	MN	25	422
12	"	CROSS	F	17	546
13	"	TB	MN	6	475

MN=gelding, F=female, Cross=crossbred, TB=Thoroughbred.

APPENDIX 8.2 Volume (ml) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses.

	CONTROL				COPD			
	RD	RA	LD	LA	RD	RA	LD	LA
1	116	113	89	98	95	85	45	26
2	105	94	67	112	103	124	98	84
3	61	70	142	90	79	139	145	182
4	130	76	122	10	103	86	67	105
5	111	79	65	101	98	111	98	123
6	65	56	104	86	155	24	95	27
7	109	91	67	104	–	–	–	–

APPENDIX 8.3 Total cell counts (/ul) of BALF recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses.

	CONTROL				COPD			
	RD	RA	LD	LA	RD	RA	LD	LA
1	90	95	80	105	90	95	140	250
2	90	165	35	100	110	155	65	160
3	55	65	55	115	70	250	300	200
4	90	95	140	25	240	130	169	190
5	140	280	200	130	180	95	115	45
6	50	150	60	110	100	100	110	110
7	75	205	105	55	–	–	–	–

APPENDIX 8.4 BALF cell ratios for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of (A) control horses (n=7) and (B) COPD affected (n=6) horses.

(A) Control horses.

N^{o}	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.7	20.0	68.7	0.7	9.7	0.0	9.7	0.3
2	"	1.0	25.3	67.3	0.0	5.3	0.0	5.3	1.0
3	"	1.7	50.3	36.0	0.0	9.7	2.3	12.0	0.0
4	"	1.7	40.3	43.7	0.0	10.7	1.7	12.3	1.7
5	"	4.0	20.7	74.3	0.0	0.3	0.3	0.7	0.3
6	"	1.0	51.3	42.3	0.0	4.0	1.3	5.3	0.0
7	"	0.7	39.7	49.7	0.3	9.3	0.0	9.3	0.3
1	RA	0.7	26.3	63.3	0.7	8.3	0.7	9.0	0.0
2	"	1.3	22.7	73.7	0.0	2.3	0.0	2.3	0.0
3	"	4.0	47.0	42.7	0.0	5.0	1.3	6.3	0.0
4	"	1.0	40.0	51.0	0.3	6.3	0.0	6.3	1.3
5	"	1.7	16.0	73.3	0.0	6.7	0.0	6.7	2.3
6	"	4.0	23.0	68.0	0.0	2.0	0.3	2.3	2.7
7	"	0.7	16.0	63.3	0.3	18.7	0.3	19.0	0.7
1	LD	1.7	23.3	61.7	0.0	10.0	0.3	10.3	3.0
2	"	0.7	17.0	75.7	0.0	6.0	0.0	6.0	0.7
3	"	1.0	38.3	51.7	0.0	8.0	0.0	8.0	1.0
4	"	1.3	52.0	36.3	0.7	8.0	0.0	8.0	1.7
5	"	2.3	19.7	72.7	0.0	4.3	0.7	5.0	0.3
6	"	2.0	24.3	65.7	0.3	3.3	1.0	4.3	3.3
7	"	0.0	56.7	29.3	0.3	13.7	0.0	13.7	0.0
1	LA	2.0	28.7	60.7	0.3	6.3	1.0	7.3	1.0
2	"	3.0	26.3	66.3	0.0	4.0	0.0	4.0	0.3
3	"	3.7	52.7	38.3	2.3	2.7	0.0	2.7	0.3
4	"	1.0	46.3	44.7	0.0	2.0	4.0	6.0	2.0
5	"	3.0	16.3	78.0	0.0	1.0	1.0	2.0	0.7
6	"	4.3	24.0	66.0	0.0	5.7	0.0	5.7	0.0
7	"	0.7	47.7	35.7	0.3	13.7	0.3	14.0	1.7

APPENDIX 8.4 (B) COPD affected horses.

N°	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	49.0	30.0	17.0	0.7	2.3	0.7	3.0	0.3
2	"	86.3	5.7	6.7	0.0	1.3	0.0	1.3	0.0
3	"	5.7	36.3	53.3	0.0	3.7	0.0	3.7	1.0
4	"	79.3	14.3	5.3	0.0	0.3	0.7	1.0	0.0
5	"	16.3	35.7	43.7	0.0	4.3	0.0	4.3	0.0
6	"	94.3	3.7	1.7	0.3	0.0	0.0	0.0	0.0
1	RA	33.0	41.3	21.7	0.0	4.0	0.0	4.0	0.0
2	"	85.0	5.3	7.3	0.0	1.0	0.0	1.0	1.3
3	"	5.3	36.7	56.3	0.3	1.3	0.0	1.3	0.0
4	"	85.0	9.0	3.3	0.3	0.7	0.3	1.0	1.3
5	"	15.0	22.7	60.7	0.7	0.7	0.3	1.0	0.0
6	"	94.0	5.3	0.3	0.3	0.0	0.0	0.0	0.0
1	LD	37.7	40.7	17.7	0.0	3.0	0.7	3.7	0.0
2	"	86.7	4.0	7.7	0.0	1.7	0.0	1.7	0.0
3	"	6.3	7.0	82.3	0.0	4.0	0.0	4.0	0.3
4	"	88.0	6.7	3.0	1.0	1.3	0.0	1.3	0.0
5	"	5.0	28.3	63.7	0.3	2.3	0.3	2.7	0.0
6	"	89.3	6.3	4.0	0.0	0.3	0.0	0.3	0.0
1	LA	43.3	25.7	24.3	0.3	5.0	0.0	5.0	1.3
2	"	85.7	6.0	6.0	0.0	0.7	1.3	2.0	0.3
3	"	4.7	22.0	71.0	0.0	1.7	0.0	1.7	0.7
4	"	93.7	3.0	1.7	0.0	1.7	0.0	1.7	0.0
5	"	19.7	26.7	48.7	1.0	3.3	1.0	4.3	0.0
6	"	95.3	2.7	1.7	0.0	0.3	0.0	0.3	0.0

APPENDIX 8.5 Albumen adjusted total BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses.

	CONTROL				COPD			
	RD	RA	LD	LA	RD	RA	LD	LA
1	22.0	27.2	30.2	26.8	18.6	15.9	48.8	55.5
2	21.7	40.4	15.7	31.4	32.9	49.3	37.8	95.3
3	21.7	19.0	15.8	35.1	53.2	64.2	62.9	62.8
4	31.5	25.8	23.5	17.6	50.0	47.8	34.4	38.7
5	38.0	38.0	52.4	29.5	37.4	38.7	33.0	91.5
6	26.6	34.3	21.6	35.8	34.3	28.7	58.3	22.3
7	27.2	51.8	30.8	22.6	-	-	-	-

APPENDIX 8.6 Albumen adjusted absolute BALF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung of (A) control (CONT) (n=7) and (B) COPD affected (n=6) horses.

(A) Control horses.

N^0	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.2	4.4	15.1	0.2	2.1	0.0	2.1	0.1
2	"	0.2	5.5	14.6	0.0	1.1	0.0	1.1	0.2
3	"	0.4	10.9	7.8	0.0	2.1	0.5	2.6	0.0
4	"	0.5	12.7	13.8	0.0	3.4	0.5	3.9	0.5
5	"	1.5	7.9	28.2	0.0	0.1	0.1	0.3	0.1
6	"	0.3	13.6	11.2	0.0	1.1	0.3	1.4	0.0
7	"	0.2	10.8	13.5	0.1	2.5	0.0	2.5	0.1
1	RA	0.2	7.2	17.2	0.2	2.3	0.2	2.4	0.0
2	"	0.5	9.3	30.1	0.0	0.9	0.0	0.9	0.0
3	"	0.8	8.9	8.1	0.0	0.9	0.2	1.2	0.0
4	"	0.3	10.3	13.2	0.1	1.6	0.0	1.6	0.3
5	"	0.6	6.1	27.9	0.0	2.5	0.0	2.5	0.9
6	"	1.4	7.9	23.3	0.0	0.7	0.1	0.8	0.9
7	"	0.4	8.3	32.8	0.2	9.7	0.2	9.8	0.4
1	LD	0.5	7.0	18.6	0.0	3.0	0.1	3.1	0.9
2	"	0.1	2.7	11.9	0.0	0.9	0.0	0.9	0.1
3	"	0.2	6.1	8.2	0.0	1.3	0.0	1.3	0.2
4	"	0.3	12.2	8.5	0.2	1.9	0.0	1.9	0.4
5	"	1.2	10.3	38.1	0.0	2.3	0.4	2.6	0.2
6	"	0.4	5.3	14.2	0.1	0.7	0.2	0.9	0.7
7	"	0.0	17.5	9.0	0.1	4.2	0.0	4.2	0.0
1	LA	0.5	7.7	16.2	0.1	1.7	0.3	2.0	0.3
2	"	0.9	8.3	20.8	0.0	1.3	0.0	1.3	0.1
3	"	1.3	18.5	13.4	0.8	0.9	0.0	0.9	0.1
4	"	0.2	8.1	7.9	0.0	0.4	0.7	1.1	0.4
5	"	0.9	4.8	23.0	0.0	0.3	0.3	0.6	0.2
6	"	1.5	8.6	23.6	0.0	2.0	0.0	2.0	0.0
7	"	0.2	10.8	8.1	0.1	3.1	0.1	3.2	0.4

APPENDIX 8.6(B) COPD affected horses.

Nº	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	9.1	5.6	3.2	0.1	0.4	0.1	0.6	0.1
2	"	28.4	1.9	2.2	0.0	0.4	0.0	0.4	0.0
3	"	3.0	19.3	28.3	0.0	2.0	0.0	2.0	0.5
4	"	39.7	7.2	2.7	0.0	0.2	0.3	0.5	0.0
5	"	6.1	13.4	16.4	0.0	1.6	0.0	1.6	0.0
6	"	32.3	1.3	0.6	0.1	0.0	0.0	0.0	0.0
1	RA	5.2	6.6	3.4	0.0	0.6	0.0	0.6	0.0
2	"	41.9	2.6	3.6	0.0	0.5	0.0	0.5	0.6
3	"	3.4	23.5	36.1	0.2	0.8	0.0	0.8	0.0
4	"	40.6	4.3	1.6	0.1	0.3	0.1	0.5	0.6
5	"	5.7	8.7	23.2	0.3	0.3	0.1	0.4	0.0
6	"	26.9	1.5	0.1	0.1	0.0	0.0	0.0	0.0
1	LD	18.4	19.9	8.6	0.0	1.5	0.3	1.8	0.0
2	"	32.8	1.5	2.9	0.0	0.6	0.0	0.6	0.0
3	"	4.0	4.4	51.8	0.0	2.5	0.0	2.5	0.2
4	"	30.3	2.3	1.0	0.3	0.4	0.0	0.4	0.0
5	"	1.7	9.4	21.0	0.1	0.8	0.1	0.9	0.0
6	"	52.0	3.7	2.3	0.0	0.2	0.0	0.2	0.0
1	LA	24.0	14.3	13.5	0.2	2.8	0.0	2.8	0.7
2	"	81.6	5.7	5.7	0.0	0.7	1.2	1.9	0.3
3	"	3.0	13.8	44.6	0.0	1.1	0.0	0.7	0.0
4	"	36.2	1.2	0.7	0.0	0.7	0.0	0.7	0.0
5	"	18.0	24.4	44.6	0.9	3.0	0.9	3.9	0.0
6	"	21.3	0.6	0.4	0.0	0.1	0.0	0.1	0.0

APPENDIX 8.7 Total PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of control (n=7) and COPD affected (n=6) horses.

	CONTROL				COPD			
	RD	RA	LD	LA	RD	RA	LD	LA
1	21.7	26.5	27.1	29.8	13.5	11.1	18.1	11.3
2	18.9	30.4	18.2	13.6	10.3	21.5	13.4	21.2
3	15.1	26.4	18.3	24.9	29.2	29.8	28.7	26.1
4	16.7	12.7	13.9	28.4	20.7	14.7	17.0	20.3
5	36.8	36.2	19.1	12.5	13.7	16.6	19.9	20.2
6	14.2	17.6	22.2	30.3	14.7	11.4	11.6	12.2
7	32.5	21.9	23.9	15.9	-	-	-	-

APPENDIX 8.8 Absolute PELF cell counts ($x10^3$/ul) for the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA) of (A) control (CONT) (n=7) and (B) COPD affected (n=6) horses.

(A) Control horses.

N^o	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	0.2	4.3	14.9	0.2	2.1	0.0	2.1	0.1
2	"	0.2	4.8	12.7	0.0	1.0	0.0	1.0	0.2
3	"	0.3	7.6	5.4	0.0	1.5	0.3	1.8	0.0
4	"	0.3	6.7	7.3	0.0	1.8	0.3	2.1	0.3
5	"	1.5	7.6	27.3	0.0	0.1	0.1	0.3	0.1
6	"	0.1	7.3	6.0	0.0	0.6	0.2	0.8	0.0
7	"	0.2	12.9	16.2	0.1	3.0	0.0	3.0	0.1
1	RA	0.2	7.0	16.8	0.2	2.2	0.2	2.4	0.0
2	"	0.4	6.9	22.4	0.0	0.7	0.0	0.7	0.0
3	"	1.1	12.4	11.3	0.0	1.3	0.3	1.7	0.0
4	"	0.1	5.1	6.5	0.0	0.8	0.0	0.8	0.2
5	"	0.6	5.8	26.5	0.0	2.4	0.0	2.4	0.8
6	"	0.7	4.1	12.0	0.0	0.4	0.1	0.4	0.5
7	"	0.2	3.5	13.9	0.1	4.1	0.1	4.2	0.2
1	LD	0.5	6.3	16.7	0.0	2.7	0.1	2.8	0.8
2	"	0.1	3.1	13.8	0.0	1.1	0.0	1.1	0.1
3	"	0.2	7.0	9.5	0.0	1.5	0.0	1.5	0.2
4	"	0.2	7.3	5.1	0.1	1.1	0.0	1.1	0.2
5	"	0.4	3.8	13.9	0.0	0.8	0.1	1.0	0.1
6	"	0.4	5.4	14.6	0.1	0.7	0.2	1.0	0.7
7	"	0.0	13.5	7.0	0.1	3.3	0.0	3.3	0.0
1	LA	0.6	8.6	18.1	0.1	1.9	0.3	2.2	0.3
2	"	0.4	3.6	9.0	0.0	0.5	0.0	0.5	0.0
3	"	0.9	13.1	9.5	0.6	0.7	0.0	0.7	0.1
4	"	0.3	13.2	12.7	0.0	0.6	1.1	1.7	0.6
5	"	0.4	2.0	9.8	0.0	0.1	0.1	0.3	0.1
6	"	1.3	7.3	20.0	0.0	1.7	0.0	1.7	0.3
7	"	0.1	7.6	5.7	0.0	2.2	0.0	2.2	0.3

APPENDIX 8.8(B) COPD affected horses.

N^{o}	SITE	NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	RD	6.6	4.1	2.3	0.1	0.3	0.1	0.4	0.0
2	"	8.9	0.6	0.7	0.0	0.1	0.0	0.1	0.0
3	"	1.7	10.6	15.6	0.0	1.1	0.0	1.1	0.3
4	"	16.4	3.0	1.1	0.0	0.1	0.1	0.2	0.0
5	"	2.2	4.9	6.0	0.0	0.6	0.0	0.6	0.0
6	"	13.9	0.5	0.3	0.0	0.0	0.0	0.0	0.0
1	RA	3.7	4.6	2.4	0.0	0.4	0.0	0.4	0.0
2	"	18.3	1.1	1.6	0.0	0.2	0.0	0.2	0.3
3	"	1.6	10.9	16.8	0.1	0.4	0.0	0.4	0.0
4	"	12.5	1.3	0.5	0.0	0.1	0.0	0.1	0.2
5	"	2.5	3.8	10.1	0.1	0.1	0.0	0.2	0.0
6	"	10.7	0.6	0.0	0.0	0.0	0.0	0.0	0.0
1	LD	6.8	7.4	3.2	0.0	0.5	0.1	0.7	0.0
2	"	11.6	0.5	1.0	0.0	0.2	0.0	0.2	0.0
3	"	1.8	2.0	23.6	0.0	1.1	0.0	1.1	0.1
4	"	15.0	1.1	0.5	0.2	0.2	0.0	0.2	0.0
5	"	1.0	5.6	12.7	0.1	0.5	0.1	0.5	0.0
6	"	10.4	0.7	0.5	0.0	0.0	0.0	0.0	0.0
1	LA	4.9	2.9	2.8	0.0	0.6	0.0	0.6	0.1
2	"	18.1	1.3	1.3	0.0	0.1	0.3	0.4	0.1
3	"	1.2	5.7	18.5	0.0	0.3	0.0	0.3	0.0
4	"	19.1	0.6	0.3	0.0	0.3	0.0	0.3	0.0
5	"	4.0	5.4	9.8	0.2	0.7	0.2	0.9	0.0
6	"	11.6	0.3	0.2	0.0	0.0	0.0	0.0	0.0

APPENDIX 8.9 Albumen concentrations of plasma (mg/ml) and of BALF (ug/ml), recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA), of control (CONT) (n=7) and COPD affected (n=6) horses.

HORSE	GROUP	PLASMA	RD	RA	LD	LA
1	CONT	21.4	87.4	74.7	56.7	84.0
2	"	25.2	104.7	101.9	56.2	80.3
3	"	25.7	65.0	88.1	89.4	84.2
4	"	20.4	58.2	75.0	121.6	29.0
5	"	22.0	81.1	62.0	84.0	97.0
6	"	22.8	42.9	99.7	63.2	67.6
7	"	24.5	67.5	97.0	83.4	59.7
1	COPD	24.4	118.0	146.0	70.0	110.0
2	"	25.9	86.5	81.5	44.5	43.5
3	"	28.1	37.0	109.5	34.0	89.5
4	"	25.0	120.0	68.0	122.8	122.8
5	"	18.1	87.0	45.0	63.0	8.9
6	"	19.6	57.2	68.4	37.0	96.6

APPENDIX 8.10 Urea concentrations of plasma (mg/dl) and of BALF (mg/dl), recovered from the diaphragmatic lobe of the right (RD) and left (LD) lungs, the accessory lobe of the right lung (RA) and the apical lobe of the left lung (LA), of control (CONT) (n=7) and COPD affected (n=6) horses.

HORSE	GROUP	PLASMA	RD	RA	LD	LA
1	CONT	30.1	0.125	0.108	0.089	0.106
2	"	22.1	0.105	0.120	0.172	0.162
3	"	23.6	0.086	0.058	0.071	0.109
4	"	27.3	0.147	0.204	0.274	0.024
5	"	31.8	0.121	0.246	0.333	0.330
6	"	20.7	0.073	0.176	0.056	0.075
7	"	18.2	0.042	0.170	0.080	0.063
1	COPD	26.3	0.175	0.225	0.203	0.581
2	"	32.8	0.349	0.236	0.159	0.248
3	"	28.4	0.068	0.238	0.297	0.218
4	"	24.4	0.283	0.216	0.242	0.228
5	"	22.0	0.289	0.126	0.127	0.049
6	"	32.4	0.220	0.284	0.307	0.292

APPENDIX 9.1 Description of horses used in the evaluation of local endobronchial antigen challenges in the horse.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	CROSS	MN	15	593
2	"	TB	MN	6	542
3	"	CROSS	MN	10	515
4	"	TB	MN	13	652
5	"	TB	MN	6	525
6	"	CROSS	F	20	500
7	"	TB	MN	8	531
8	COPD	CROSS	F	20	371
9	"	CROSS	F	20	488
10	"	CROSS	MN	13	501
11	"	CROSS	F	20	497
12	"	CROSS	F	17	546
13	"	TB	M	30	504
14	"	CROSS	MN	14	450

CROSS=crossbred, F=female, M=male, MN= gelding, TB=Thoroughbred.

APPENDIX 9.2 BALF cell ratios for (A) control (n=7) and (B) COPD affected (n=7) horses before (NIL) and at 5h after endobronchial challenges with PBS, 60ug MF and 600ug MF.

(A) Control horses.

HORSE		NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	NIL	3.3	19.0	74.3	1.0	2.0	0.3	2.3	0.0
2	"	1.0	6.0	76.7	11.3	4.0	1.0	5.0	0.0
3	"	0.3	22.0	72.7	0.0	2.7	1.3	4.0	1.0
4	"	1.0	53.0	38.7	0.0	3.3	0.7	4.0	3.3
5	"	0.7	35.7	60.3	0.3	2.0	0.0	2.0	1.0
6	"	4.7	42.0	50.7	0.3	2.0	0.3	2.3	0.0
7	"	2.0	5.3	86.7	0.0	3.0	1.3	4.3	1.7
8	"	1.7	39.7	52.7	0.0	3.0	1.7	4.7	1.3
1	PBS	0.3	37.7	51.3	4.7	5.7	0.7	6.3	0.3
2	"	11.0	18.0	41.7	28.0	1.0	0.0	1.0	0.3
3	"	5.0	44.7	44.0	0.0	4.0	1.0	5.0	1.3
4	"	3.7	35.3	61.0	0.0	0.0	0.0	0.0	0.0
5	"	12.7	44.0	43.3	0.0	0.0	0.0	0.0	0.0
6	"	11.0	29.3	56.3	0.0	2.0	1.0	3.0	0.3
7	"	28.0	20.0	49.0	0.3	2.7	0.0	2.7	0.0
1	60ug MF	4.3	37.7	50.7	4.3	2.3	0.7	3.0	0.0
2	"	10.7	45.7	40.3	0.0	3.3	0.0	3.3	0.0
3	"	1.7	51.3	41.0	0.0	0.0	0.7	5.7	0.3
4	"	5.0	25.0	68.0	0.0	0.0	0.0	1.0	1.0
5	"	10.0	49.0	39.0	0.0	1.3	0.0	1.3	0.7
6	"	15.0	26.7	55.3	0.0	2.7	0.3	3.0	0.0
7	"	27.0	13.0	57.7	0.0	1.0	0.3	1.3	1.0
1	600ug MF	5.3	37.3	51.0	3.7	2.0	0.7	2.7	0.0
2	"	25.0	17.7	45.7	0.0	7.0	2.3	9.3	2.3
3	"	54.7	21.7	21.7	0.0	2.0	0.0	2.0	0.0
4	"	40.7	38.0	61.0	0.0	1.0	0.0	1.0	0.3
5	"	7.7	49.7	41.7	0.0	1.0	0.0	1.0	0.0
6	"	75.0	5.0	16.7	0.0	0.3	0.0	0.3	3.0
7	"	33.0	18.0	46.3	0.0	2.0	0.3	2.3	0.3

APPENDIX 9.2 (B) COPD affected horses.

HORSE		NEUT	LYM	MAC	EOS	MAST	BAS	TOTBAS	EP
1	NIL	3.3	19.0	74.3	1.0	2.0	0.3	2.3	0.0
2	"	1.7	63.7	25.0	0.0	6.0	0.7	6.7	3.0
3	"	3.0	32.0	59.0	0.3	4.7	1.0	5.7	0.0
4	"	0.0	63.7	33.0	1.0	2.0	0.0	2.0	0.3
5	"	3.0	11.3	82.0	1.3	0.7	0.0	1.0	1.3
6	"	4.0	39.3	49.0	2.0	3.0	0.7	3.7	2.0
7	"	4.3	43.7	44.0	3.7	4.0	0.3	4.3	0.0
1	PBS	8.0	35.0	51.0	1.0	4.0	0.3	4.3	0.7
2	"	5.3	51.3	38.0	0.0	3.0	1.0	4.0	1.3
3	"	9.7	39.0	48.0	0.0	3.0	0.3	3.3	0.0
4	"	2.0	28.0	57.7	2.0	9.3	1.0	10.3	0.0
5	"	52.0	3.0	43.0	2.0	0.0	0.3	0.0	0.0
6	"	48.0	8.0	37.0	0.0	0.7	0.0	0.7	6.3
7	"	3.3	35.7	52.0	0.3	4.0	0.7	4.7	4.0
1	60ug MF	9.7	33.3	50.0	1.0	4.3	0.7	5.0	0.3
2	"	17.3	36.0	40.0	0.0	5.0	1.0	6.0	0.7
3	"	16.0	48.7	23.0	6.0	4.0	0.3	4.3	2.0
4	"	0.7	45.0	45.3	0.7	6.3	2.0	8.3	0.0
5	"	68.0	2.0	28.7	0.0	0.0	0.0	0.0	1.3
6	"	43.0	3.0	43.0	0.0	1.0	0.0	1.0	11.0
7	"	6.0	41.0	46.0	1.0	4.0	0.7	1.7	1.3
1	600ug MF	30.0	24.0	38.3	2.3	5.0	0.3	5.3	0.0
2	"	38.3	35.0	18.0	0.0	5.0	2.0	7.0	1.7
3	"	19.0	33.0	38.0	4.0	4.0	0.0	4.0	2.0
4	"	2.0	0.0	87.0	1.0	5.3	0.7	6.0	4.0
5	"	65.7	7.7	26.0	0.3	0.3	0.0	0.3	0.0
6	"	51.3	14.7	28.0	0.0	1.3	0.3	1.7	4.3
7	"	36.0	15.0	43.7	0.0	1.3	0.3	1.7	3.7

APPENDIX 9.3 Total BALF cell counts (/ul) for control (CONT) (n=7) and COPD affected horses (n=7) before (BASE) and at 5h after endobronchial challenges with PBS, 60ug MF and 600ug MF.

	CONTROL				COPD			
HORSE	BASE	PBS	60ugMF	600ugMF	BASE	PBS	60ugMF	600ugMF
1	455	630	280	345	95	95	190	410
2	400	155	240	215	135	100	100	195
3	135	270	680	645	515	375	760	515
4	250	245	95	175	555	365	440	40
5	435	265	460	575	425	70	175	695
6	100	330	330	435	155	60	40	210
7	625	1005	770	395	170	185	325	635

APPENDIX 9.4 BALF cell ratios for (A) control and (n=5) (B) COPD affected horses (n=5) before challenge (PRE) and for control sites (CONT) and challenge sites (CHAL) at 5h after endobronchial hay extract challenge.

(A) Control horses.

HORSE		NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PRE	0.0	30.7	63.3	0.3	5.3	0.3	0.3	5.7
2	"	0.3	32.3	59.7	0.0	5.3	0.0	2.3	5.3
3	"	0.7	24.3	70.3	0.0	3.3	0.3	1.0	3.7
4	"	2.0	34.7	53.7	0.3	6.7	2.3	0.3	9.0
5	"	3.3	40.7	48.3	0.0	5.3	2.0	0.3	7.3
1	CONT	0.3	20.0	73.0	0.0	6.7	0.0	0.0	6.7
2	"	7.7	25.0	58.3	0.3	4.7	0.3	3.7	5.0
3	"	1.7	13.0	80.7	0.0	3.3	0.0	1.0	3.3
4	"	13.7	44.3	28.0	0.3	9.0	2.0	3.0	11.0
5	"	9.3	45.7	36.0	0.0	6.7	2.3	0.0	9.0
1	CHAL	14.7	37.3	44.0	0.0	4.0	0.0	0.0	4.0
2	"	30.7	10.3	56.0	0.3	1.7	0.0	0.7	1.7
3	"	8.7	31.7	58.3	0.0	1.3	0.0	1.0	1.3
4	"	21.0	40.0	25.7	0.3	11.3	0.3	1.3	11.7
5	"	19.7	51.7	19.3	0.3	8.7	0.3	0.0	9.0

(B) COPD affected horses.

HORSE		NEUT	LYM	MAC	EOS	MAST	BASO	TOTBAS	EP
1	PRE	2.3	48.3	48.7	0.0	2.3	0.3	0.0	2.7
2	"	1.7	44.3	48.3	0.0	3.7	1.0	1.0	4.7
3	"	3.3	28.7	62.7	0.3	4.3	0.3	0.3	4.7
4	"	4.0	56.0	37.7	0.0	2.0	0.3	0.0	2.3
5	"	1.3	45.0	48.4	0.0	4.3	0.0	1.0	4.3
1	CONT	4.0	52.7	41.0	0.7	1.3	0.3	0.0	1.7
2	"	8.3	44.7	37.0	0.0	7.0	0.7	2.3	7.7
3	"	17.3	41.3	38.0	0.0	2.3	1.0	0.0	3.3
4	"	15.0	70.3	9.3	0.0	4.7	0.3	0.7	5.0
5	"	4.0	41.0	53.0	2.0	0.0	0.0	0.0	0.0
1	CHAL	70.3	16.3	8.7	0.7	1.0	3.7	0.3	4.7
2	"	19.0	31.0	44.3	0.0	4.3	1.0	0.3	5.3
3	"	30.7	42.3	24.0	0.3	2.3	0.0	0.3	2.7
4	"	63.0	27.0	3.7	1.0	4.3	0.3	0.7	4.7
5	"	9.0	59.3	29.3	0.0	1.3	0.7	0.3	2.0

APPENDIX 9.5 Total BALF cell counts (/ul) for control (n=5) and COPD affected horses (n=5) before challenge (PRE) and for control sites (CONT) and challenge sites (CHAL) at 5h after endobronchial mouldy hay extract challenge.

	CONTROL			COPD		
HORSE	PRE	CONT	CHAL	PRE	CONT	CHAL
1	95	300	90	230	187	400
2	115	400	300	420	230	215
3	130	210	240	100	315	320
4	265	140	310	85	275	100
5	275	95	175	215	100	100

APPENDIX 10.1 Description of horses used for oil seed rape challenge study.

HORSE	GROUP	TYPE	SEX	AGE (y)	WEIGHT (kg)
1	CONTROL	CROSS	MN	15	593
2	"	SHETLAND	F	7	212
3	"	TB	MN	19	643
4	"	CROSS	F	16	561
5	"	CROSS	F	10	515
6	"	TB	MN	13	652
7	"	TB	MN	25	609
8	COPD	CROSS	F	20	371
9	"	CROSS	F	20	488
10	"	CROSS	MN	13	501
11	"	CROSS	MN	25	422
12	"	CROSS	F	17	546
13	"	CROSS	MN	14	450

F=female, MN= gelding, Cross=crossbred, TB=Thoroughbred.

APPENDIX 10.2 Arterial blood gas tensions, arterial pH, pulmonary mechanics parameters and BALF cell ratios for control horses (C) (n=6), and horses with asymptomatic (A) (n=8) and symptomatic (S) (n=9) COPD, before and after (a) *B.campestrus* 'field challenge', (b) fresh *B.napus* pollen challenge, and (c) nebulised *B.napus* pollen extract inhalation challenge.

(a) *B.campestrus* 'FIELD CHALLENGE'

HORSE	GROUP	PaO_2	$PaCO_2$	Art pH	RR	V_T	Vmin	dPtp	Cdyn	R_L	NEUT	LYM	MAC	EOS	MAST	BAS	TB	EP
1	C	93.2	39.6	7.430	14.0	1.6	21.7	7.9	0.725	1.242	1.0	42.0	47.7	5.0	4.3	0.0	4.3	0.0
		95.1	39.5	7.391	18.0	1.6	28.4	6.3	0.779	1.740	2.7	33.3	52.0	4.0	7.7	0.0	7.7	0.3
2	C	94.1	38.3	7.414	14.4	5.3	75.8	4.3	2.061	1.509	2.0	40.7	50.0	0.0	7.3	0.0	7.3	0.0
		93.0	39.9	7.392	14.2	3.3	47.0	5.6	1.982	1.678	1.3	29.0	43.7	0.0	15.3	9.0	24.3	1.0
3	A	85.3	38.6	7.433	7.6	9.4	71.9	4.3	1.543	0.558	0.0	38.0	54.0	0.5	6.3	0.0	6.3	1.0
		92.0	38.6	7.434	17.0	6.4	108.9	6.0	1.244	0.589	1.7	41.0	47.0	0.0	8.0	1.3	9.3	1.0
4	A	92.0	42.7	7.398	9.5	5.6	53.6	5.4	1.320	0.831	3.0	57.7	37.0	0.3	2.0	0.0	2.0	0.0
		91.2	40.9	7.410	17.5	6.0	104.7	5.4	1.263	0.960	4.7	52.0	39.3	0.0	1.7	0.0	1.7	0.3
5	S	89.6	44.6	7.434	12.5	3.9	48.6	8.1	0.593	1.963	6.0	50.3	25.7	0.7	17.0	0.0	17.0	0.3
		89.8	40.6	7.383	8.7	4.6	40.1	4.6	1.770	1.484	4.3	69.3	15.7	0.3	9.0	0.3	9.3	1.0
6	S	87.3	46.5	7.398	12.5	5.6	69.5	32.8	0.488	9.891	64.3	16.3	15.3	1.7	2.0	0.3	2.3	0.0
		92.7	43.1	7.376	9.2	6.1	56.4	18.1	0.777	1.621	2.3	49.0	41.7	3.7	2.0	1.0	3.0	0.3
7	S	87.8	42.2	7.443	4.8	7.2	34.5	8.7	0.992	1.283	12.3	31.0	51.0	0.0	4.7	0.0	4.7	1.0
		101.6	38.8	7.411	7.2	6.3	45.2	10.7	1.108	1.143	12.7	40.7	40.3	0.0	4.7	0.0	4.7	1.7

APPENDIX 10.2 (B) FRESH *B.napus* POLLEN INHALATION CHALLENGE

HORSE	GROUP	PaO_2	$PaCO_2$	Art pH	RR	V_T	Vmin	dPtp	Cdyn	R_L	NEUT	LYM	MAC	EOS	MAST	BAS	TB	EP
1	C	93.6	40.1	7.403	8.0	3.1	55.7	10.0	1.349	0.981	1.0	18.3	70.7	0.0	8.3	0.3	8.6	1.3
		97.6	43.5	7.401	15.6	3.4	53.8	8.7	1.404	0.855	0.7	22.0	68.0	0.0	6.7	1.3	8.0	1.3
2	C	93.2	41.8	7.408	5.5	3.5	19.3	5.8	1.314	1.619	0.0	49.0	27.3	13.7	9.3	0.0	9.3	0.7
		98.5	40.3	7.410	9.4	5.6	52.4	5.4	1.533	1.173	1.3	38.3	45.7	0.3	14.3	0.0	14.3	0.0
3	A	91.2	42.3	7.349	10.0	2.4	23.6	12.8	1.691	1.689	1.3	41.3	50.3	0.0	13.3	1.7	15.0	2.0
		98.5	40.7	7.345	7.7	6.9	52.7	9.5	1.845	1.198	2.7	57.0	27.7	0.0	8.0	4.7	12.7	0.0
4	A	107.5	38.1	7.379	13.3	6.0	79.4	6.8	1.751	0.839	4.3	57.7	25.0	0.0	3.3	0.7	4.0	9.0
		108.9	40.4	7.416	10.3	5.2	53.2	5.6	1.359	0.847	6.3	47.7	38.7	0.0	5.0	1.7	6.7	0.0
5	A	94.0	42.4	7.398	8.5	6.5	55.6	4.6	1.962	1.307	0.7	36.3	59.0	0.0	4.0	0.0	0.0	0.0
		95.9	41.2	7.443	7.0	5.2	36.3	3.2	1.415	0.953	3.0	19.3	73.3	0.0	3.7	0.0	0.0	0.7
4	S	94.7	44.5	7.382	5.4	3.9	21.2	6.1	1.051	1.851	5.0	24.7	63.7	0.0	0.7	0.3	1.0	5.0
		92.9	45.2	7.389	7.6	3.5	26.7	7.0	0.959	2.322	42.3	25.0	21.3	0.3	2.7	0.3	3.0	1.0
6	S	80.5	39.8	7.406	21.2	5.91	25.1	12.3	0.836	1.648	77.6	16.0	4.3	0.0	1.6	0.3	1.9	0.0
		86.9	43.3	7.375	17.1	4.6	77.5	14.5	0.656	1.438	48.3	42.6	6.6	0.0	1.3	0.3	1.6	0.6
7	S	95.3	42.4	7.368	11.8	4.9	58.3	8.5	0.784	1.242	37.0	39.6	21.0	0.3	3.6	0.3	3.9	5.6
		98.9	40.4	7.412	8.5	4.7	40.0	7.1	1.186	0.867	21.0	32.0	35.6	0.0	2.6	0.0	2.6	1.0

APPENDIX 10.2 (C) NEBULISED *B.napus* POLLEN EXTRACT INHALATION CHALLENGE

HORSE	GROUP	PaO_2	$PaCO_2$	Art pH	RR	V_T	Vmin	dPtp	Cdyn	R_L	NEUT	LYM	MAC	EOS	MAST	BAS	TB	EP
1	C	84.5	39.4	7.363	18.6	2.3	43.5	6.0	0.690	0.850	1.0	51.0	33.0	0.7	12.3	0.0	12.3	2.0
		83.1	42.4	7.390	14.1	1.4	20.1	2.5	0.763	0.982	4.0	36.0	49.0	0.3	9.0	0.3	9.3	1.3
2	C	85.5	36.5	7.397	18.0	3.9	69.3	3.1	0.995	1.237	1.0	42.3	36.7	0.0	14.7	3.2	17.0	3.0
		81.9	37.6	7.410	9.9	4.5	44.1	5.7	1.250	0.629	2.0	47.7	30.3	0.0	18.7	0.3	19.0	1.0
4	A	90.2	38.6	7.334	9.3	7.8	72.4	6.7	1.463	1.372	4.0	41.3	48.0	0.0	3.0	2.0	5.0	1.7
		91.9	38.5	7.384	10.5	5.2	54.3	8.7	1.260	1.697	2.3	35.0	51.0	0.0	5.3	0.0	5.3	6.3
5	A	92.2	40.9	7.402	9.0	8.4	75.6	2.9	2.121	0.753	1.3	38.0	50.0	0.0	9.0	0.0	9.0	1.7
		96.9	38.8	7.403	9.0	4.9	44.1	2.7	2.441	0.579	2.3	24.0	62.7	0.0	7.7	0.0	7.7	3.3
6	A	88.6	39.8	7.382	5.4	3.1	22.2	3.4	1.624	1.292	1.7	26.7	64.7	0.7	5.3	1.0	6.3	0.0
		82.9	38.4	7.413	6.2	2.5	15.7	5.3	1.291	0.778	1.7	49.7	42.7	0.3	3.3	0.0	3.3	2.3
3	S	72.7	43.6	7.375	12.0	4.9	58.3	21.3	0.373	3.224	14.3	58.0	25.0	2.0	0.6	0.0	0.6	0.0
		76.5	42.9	7.385	8.6	5.6	48.3	20.3	0.507	3.909	21.3	52.6	23.0	0.3	1.3	0.0	1.3	1.3
4	S	87.4	39.8	7.424	5.4	4.8	25.8	10.1	0.76	1.753	6.3	12.3	67.0	2.0	6.0	0.3	6.3	6.0
		93.8	40.7	7.433	7.6	5.2	39.8	9.4	1.311	1.194	19.0	31.7	42.0	0.0	5.7	0.7	6.4	1.0
6	S	85.6	42.6	7.368	12.8	4.1	53.0	13.8	0.712	2.964	69.3	12.6	14.0	2.3	0.3	0.6	0.9	0.6
		82.8	39.5	7.398	8.4	5.0	41.8	11.0	0.623	2.938	83.0	10.3	5.3	0.3	0.3	0.3	0.6	0.6

APPENDIX 10.3 Mean intradermal wheal size (mm) at 1.5 and 5h following testing with 10^{-0}, 10^{-1}, 10^{-2} and 10^{-3} dilutions of *B.napus* pollen extract and following negative (-CONT) and positive (+CONT) controls. The mean of the negative and positive controls ($\bar{x}$CONT), considered as the lowest wheal diameter of the positive responses is given.

(A) CONTROL HORSES

		1.5 HOURS							5 HOURS						
HORSE	N	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}
1	3	10	0	5	9	10	3	0	10	0	5	10	10	6	0
2	3	12	0	6	13	16	0	0	11	0	5.5	13	8	0	0
10	2	13	0	6.5	12	10	0	0	9	0	4.5	10	9	0	0
11	1	12	0	6	0	0	0	0	17	0	9.5	8	0	0	0
12	1	15	0	7.5	0	0	0	0	11	0	5.5	10	0	0	0
13	1	15	0	7.5	12	9	8	0	11	0	5.5	13	9	0	0
14	1	18	0	9	18	18	11	0	15	0	7.5	43	26	0	0
15	1	15	0	7.5	18	11	0	0	23	0	11.5	30	0	0	0
16	1	14	0	7	19	11	0	0	14	0	7	31	12	0	0
17	1	13	0	6.5	15	10	0	0	16	0	8	22	10	0	0
18	1	12	0	6	11	10	0	0	10	0	5	11	9	0	0
19	1	23	0	10	20	14	0	0	20	0	10	27	17	8	0
20	1	16	0	8	22	16	0	0	15	0	7.5	25	15	0	0
21	1	13	0	6.5	16	11	0	0	15	0	7.5	41	24	9	6

APPENDIX 10.3 (CONTINUED)

(B) COPD AFFECTED HORSES

		1.5 HOURS							5 HOURS						
HORSE	N	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}
3	3	21	0	10.5	17	9	0	0	21	0	10.5	13	6	0	0
4	3	16	0	8	17	12	0	0	11	0	5.5	16	9	4	4
6	2	17	0	8.5	17	15	0	0	16	0	8	24	25	0	0
7	1	16	0	8	0	0	0	0	15	0	7.5	0	0	0	0
8	2	12	0	6	11	9	0	0	11	0	5.5	18	9	0	0
9	1	14	0	7	25	16	0	0	14	0	7	38	25	0	0

(C) DIALYSED EXTRACT

		1.5 HOURS							5 HOURS						
HORSE	N	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}	+CONT	-CONT	$\bar{x}$CONT	10^{-0}	10^{-1}	10^{-2}	10^{-3}
9	1	30	4	17	10	0	0	0	23	3	13	15	5	7	7
10	1	17	0	8.5	7	3	3	3	12	0	6	11	7	3	3
14	1	23	3	13	8	6	5	6	17	3	10	12	6	8	6
15	1	15	3	9	7	6	6	6	12	0	6	7	5	4	3
16	1	18	0	9	9	5	6	5	13	3	8	10	7	0	6
17	1	12	4	8	5	4	5	4	15	0	7.5	15	6	4	4
18	1	26	3	14.5	7	6	5	5	17	3	10	10	6	5	6
20	1	23	3	13	10	0	0	0	20	0	10	15	0	0	5
21	1	22	0	11	7	0	0	5	13	0	6.5	12	6	5	3
22	1	22	0	11	8	0	0	0	15	0	7.5	10	7	0	0

CONCLUDING ADDENDUM

The aim of this concluding addendum is to provide an overview of the possible aetiopathogenesis of equine COPD, in light of the findings of the present study.

AETIOLOGY OF EQUINE COPD

This study and previous studies (reviewed in Chapter 1) indicate that inhaled moulds, including *Micropolyspora faeni* (MF) and *Aspergillus fumigatus* (AF), are aetiological agents of equine COPD in Northern Britain. Geographical variations in the aetiology of this disease may, however, exist.

The potential role of other airborne agents, including forage mites and endotoxins, which have been demonstrated in stables containing hay and straw (Woods,P. *et al, pers comm*) and which have been shown to cause pulmonary disease in other species (reviewed in Chapter 1), warrants further study.

PATHOGENESIS OF EQUINE COPD

The present study and previously published studies (reviewed in Chapter 1) provide considerable evidence to suggest that equine COPD is a pulmonary hypersensitivity, rather than a pulmonary toxicity.

Inhaled antigens may interact with the pulmonary immune system to induce a hypersensitivity response in a number of possible ways;

1. Inhaled antigen may interact with pulmonary dendritic cells, which function as antigen presenting cells, and be presented, in association with Class II major histocompatability antigens, to antigen specific CD4+ T helper/inducer cells. Subsequent lymphokine production by the activated T cells may then attract and activate other inflammatory cells (reviewed in Chapter 4) and induce the pulmonary inflammatory response of equine COPD. The natural

challenge (NC) induced pulmonary recruitment of CD4+ T cells, which was demonstrated in COPD affected horses in the present study (Chapter 4), may be fundamental to the development of such an inflammatory response.

2. Inhaled antigen may interact with antigen specific IgG or IgA antibody, present in pulmonary epithelial lining fluid (PELF) or in the pulmonary parenchyma, to form immune complexes. These complexes may induce a type III hypersensitivity response via activation of the complement, coagulation and kinin systems or by stimulating release of inflammatory mediators from activated mast cells, neutrophils, macrophages or platelets.

Generation of the complement fragments C3a and C5a, which are chemoattractants for equine neutrophils (Camp and Leid 1982) and human CD4+ T helper cells (El Naggar *et al* 1981), may account for the pulmonary recruitment of neutrophils and CD4+ T cells observed in equine COPD. Alternatively the pulmonary neutrophilia characteristic of equine COPD may be induced by interleukin-8, a recently described potent human neutrophil chemoactivator which is produced predominantly by alveolar macrophages, possibly in response to immune complexes (Lynch *et al* 1992).

Immune complex or anaphylatoxin mediated mast cell/basophil degranulation (Abdel-Salam 1989; Peters 1990) may account for the NC induced late phase increase in PELF histamine which was demonstrated in COPD affected horses in the present study (Chapter 5).

Consistent with a role for type III hypersensitivity in the pathogenesis of equine COPD are the increased levels of MF and AF specific IgG and IgA in bronchoalveolar lavage fluid (BALF) from asymptomatic and symptomatic COPD affected horses (Halliwell *et al* In Press), the increased ratios of B cells in BALF from asymptomatic COPD affected horses (Chapter 4, present study), the presence of C3 and IgG deposits in the alveolar septae of a COPD affected horse (Winder and Von Fellenberg 1986) and the finding that intradermal mould antigen testing of horses induced a late phase intradermal response which was histologically consistent with an Arthus type response (Halliwell *et al* 1979; McPherson *et al* 1979).

3. Inhaled antigen may complex with homocytotropic IgE antibody present on pulmonary mast cells/basophils, inducing an early and/or late phase type I hypersensitivity response. A late phase, allergen mediated type I hypersensitivity response could account for the increased PELF histamine concentrations observed in COPD affected horses at 5h after NC in the present study (Chapter 5).

Histamine releasing factors and histamine releasing inhibitory factors may play an important role in the pathogenesis of equine COPD by controlling immunological and non immunological mast cell/basophil degranulation (discussed in Chapter 5).

The finding that BALF from asymptomatic and symptomatic COPD affected horses has increased levels of MF and AF specific IgE (Halliwell *et al* In Press) is consistent with involvement of an early or late phase type I hypersensitivity response in the pathogenesis of COPD, although IgE may also induce pulmonary inflammation via interaction with Fc_E receptors on macrophages, lymphocytes and eosinophils (Rossi *et al* 1991).

While the present study did not yield evidence to support involvement of an early phase, type I hypersensitivity response in the pathogenesis of equine COPD, its involvement cannot be ruled out. Indeed, the dependence of human late phase bronchial and cutaneous responses on a preceding early phase response (Cochrane, quoted by Pepys 1978) suggests that such a response is indeed likely to have a role in the pathogenesis of equine COPD. Thus, while histamine was not increased in plasma or pulmonary fluids from COPD affected horses at 0.5h after NC (Chapter 5), it may have increased transiently prior to this time.

The role of early type I hypersensitivity responses in the pathogenesis of equine COPD warrants further investigation. This may be achieved by quantifying histamine in plasma and in pulmonary fluids collected sooner after NC than in the present study, i.e. at 5-15min after NC. Alternatively, ultrastructural examination of mast cells/basophils in BALF or bronchial biopsies, collected from COPD affected horses at 5-15min after NC, for evidence of degranulation, may prove useful. Mast cells, in bronchial biopsies from humans with atopic asthma (Beasley *et al* 1989), and in BALF from human asthmatics during the early phase

response to allergen challenge (Metzger *et al* 1986) showed ultrastructural evidence of degranulation.

If mast cell/basophil degranulation does, indeed, occur in the early phase of COPD, mediators released from these cells may be responsible for recruiting CD4+ T helper cells to the lungs of COPD affected horses, as demonstrated in the present study (Chapter 4) (Center *et al* 1983; Berman *et al* 1984).

4. Inhaled MF and AF may activate the complement system directly, via the alternate pathway (Marx and Flaherty 1976), inducing a type III hypersensitivity response independent of specific precipitating antibody. As previously discussed, complement fragments may induce mast cell/basophil degranulation (Abdel-Salam 1989) and initiate the pulmonary recruitment of CD4+ T helper cells (El Naggar *et al* 1981) and neutrophils (Camp and Leid 1982), which was demonstrated in this study.

However, as complement activation by the alternate pathway would be expected to occur equally in all individuals exposed to appropriate concentrations of AF and MF antigens, this mechanism does not account for the development of pulmonary disease in COPD affected horses but not in control horses following NC. It is thus unlikely that alternate complement activation by AF and MF has an *important* role in the pathogenesis of equine COPD. Complement fragments produced by this mechanism may, however, potentiate the effects of complement fragments generated, in horses with specific precipitins, via the direct pathway, as is thought to occur in human hypersensitivity pneumonitis (Edwards 1976).

5. Inhaled antigen may induce an inflammatory response by interacting directly with pulmonary epithelial cells. Epithelial cells from other species can convert arachidonic acid to metabolites of the 5-lipoxygenase pathway *in vitro* and yield metabolites which are potent neutrophil chemotaxins (Bigby *et al* 1985)

As the previous discussion has highlighted several possible mechanisms by which complement fragments may have an important role in the pathogenesis of equine COPD, the role of complement in this disease warrants further investigation.

Complement activation is thought to have an important role in the pathogenesis of Goodpasture's syndrome, adult respiratory distress syndrome and hypersensitivity penumonitis in man and of experimental lung diseases in laboratory animals, while its role in the pathogenesis of human chronic bronchitis, bronchial asthma, sarcoidosis and 'heroin lung' is less well defined (Johnson and Ward 1974; Yoshizawa *et al* 1982; Langlois and Gawryl 1988; Morgan 1990).

The role of complement in equine COPD may be investigated by quantifying individual complement fragments in plasma and/or pulmonary fluids from COPD affected horses before and after NC. However, immunoassay of equine complement is currently hampered by the lack of specific monoclonal antibodies to individual complement fragments. Furthermore, quantification of complement fragments in pulmonary fluids from human asthmatics has proved problematic and has yielded conflicting results (reviewed by Barnes *et al* 1991). Perhaps a more useful strategy would be to determine the protective effect of specific inhibitors of the complement pathway, such as N-acetyl aspartyl glutanic acid (NAAGA), on the response of COPD affected horses to NC. In this respect, preliminary studies have suggested that NAAGA may ameliorate human allergic rhinitis, implicating complement in the pathogenesis of this condition (Ghaem 1987).

Despite the neutrophil being, by far, the most predominant cell to infiltrate the airways of COPD affected horses, the role of the neutrophil in the pathogeneis of this condition has received scant attention.

While it is unlikely that neutrophils release significant quantities of mediators in the lungs of COPD affected horses (discussed in Chapter 1), the neutrophil has a well established role in several pulmonary diseases in man including adult respiratory distress syndrome, idiopathic pulmonary fibrosis, emphysema and hypersensitivity pneumonitis (reviewed by Sibille and Reynolds 1990), and the role of this cell in equine COPD warrants further investigation.

The role of the neutrophil in the pathogenesis of equine COPD may be elucidated by quantifying neutrophil derived mediators, in plasma and/or pulmonary fluids before and after NC, biochemically (Bieth *et al* 1974) or, when suitable monoclonal antibodies become available, immunologically. Alternatively, ultrastructural analyses of neutrophil granule morphology and numbers (Yamada *et al* 1982) and cytofluorographic assay of neutrophil granule contents (Abrams *et al* 1983) may be used to determine whether neutrophils have degranulated.

From the preceding discussion and from the review presented in Chapter 1, it will be evident that there is considerable potential interaction between the many different immune effector mechanisms which may be involved in the pathogenesis of equine COPD. This interaction limits the value of classification of different types of hypersensitivity effector responses. Additionally, it is unlikely that the inflammatory response characteristic of equine COPD is induced by a single mediator or a single effector cell type, and more likely that it represents the culmination of a complex interaction of many different cells and mediators. Furthermore, the present study has demonstrated that COPD is a dynamic inflammatory response, with different cell types and inflammatory mediators playing different roles at different stages in the disease. Unfortunately, most of the techniques currently used to dissect the inflammatory response of equine COPD provide data which reflect the inflammatory response at single time points. This limitation is likely to explain, in part, the different results of other studies. Fortunately, as equine COPD is a naturally occurring, reversible inflammatory response, which may be induced and reversed in affected horses, simply and reproducibly by environmental alteration, it is a useful disease model to investigate pulmonary hypersensitivity responses.

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