Bleeding in Racehorses

Assessing Intrapulmonary Blood Accumulation in Racehorses with Exercise-Induced Pulmonary Haemorrhage (EIPH)

A report for the Rural Industries Research and Development Corporation

by Professor Ron Slocombe

January 2000

RIRDC Publication No 99/168
RIRDC Project No UM-36A
Exercise-induced pulmonary haemorrhage (EIPH) is a condition that occurs in almost all horses that compete. Although the basic mechanisms that lead to EIPH are now well understood, an understanding of these mechanisms has not led to new ways to prevent or treat EIPH. Large volumes of haemorrhage into lungs are known to severely affect performance but the minimum volume of blood that affects performance has not been determined. The effects of the presence of blood, especially at these minimum volumes, on lung structure was unknown.

This research identified the effects of small volumes of blood on lung structure and on performance, and explored ways of determining the amount of haemorrhage in live animals after episodes of EIPH. The results of these investigations show that small volumes of blood can affect performance, and that blood also induces long-lasting changes in lung structure.

Furthermore, this publication considered the important issue of whether lung injury could predispose to EIPH and confirmed under experimental conditions that it may, therefore indicating that EIPH may predispose to further episodes of haemorrhage.

This project was part of a continuing program which aims to develop improved preventative and treatment strategies in the management of EIPH. Projects currently funded at the University of Queensland and at the University of Melbourne explore the relationship between EIPH with other respiratory diseases, and assess ways of accelerating clearance of blood from the lungs, once EIPH develops.

This report, a new addition to RIRDC’s diverse range of over 450 research publications, forms part of our Equine R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of our publications are available for viewing, downloading or purchasing online through our website:

**Peter Core**  
Managing Director  
Rural Industries Research and Development Corporation
Acknowledgments

The authors acknowledge the generous support of Professor Warwick Bayly and Dr Russell Tucker in the conduct of treadmill exercise protocols and the scintigraphy studies at the College of Veterinary Medicine, Washington State University, Pullman, Washington, USA.

Abbreviations

RIRDC  Rural Industries Research and Development Corporation.
EIPH  exercise-induced pulmonary haemorrhage, known also as "bleeders".
segment  sublobar bronchus and all the lung parenchyma distal to, and supplied by this airway.
BAL  broncho-alveolar lavage
H&E  Haematoxylin and eosin stain for routine evaluation of tissues by light microscopy.

About the Authors

Professor RONALD F. SLOCOMBE grew up near Ballarat, and graduated with honours, Bachelor of Veterinary Science from the University of Melbourne in 1974. He worked in mixed practice in Alberta, Canada for 2 years prior to moving to Michigan State University, where he completed a residency of food animal medicine, Masters degree in respiratory physiology and PhD in pathology. After completing graduate work, he joined the faculty at Michigan State University prior to returning to Melbourne, to the Faculty of Veterinary Science in 1986. Professor Slocombe is a Diplomate of the American College of Veterinary Pathologists and a registered specialist in veterinary pathology. He is Chair of Veterinary Pathology at the University of Melbourne. His special interest is in respiratory disease, and he has published over 80 peer reviewed papers on a range of both infectious and non-infectious conditions affecting animals. His current research on EIPH is funded by grants from RIRDC and Racing Victoria.

Dr. SHAUN MCKANE grew up in Sydney and completed a Bachelor of Science degree in Veterinary Studies in 1992 prior to gaining his veterinary degree from the University of Sydney in 1993. He subsequently obtained a post-graduate teaching scholarship at the University of Melbourne in 1994, and over the last 4 years he has completed a PhD on the pathophysiology of EIPH. During this time he has had extensive undergraduate teaching responsibilities in the School of veterinary science at Melbourne and has also served as an equine clinician. He has published 8 research papers and abstracts from research conducted while at Sydney and Melbourne, and is a co-investigator on EIPH projects with Professor Slocombe.
## Contents

*Foreword*  
*Acknowledgements*  
*Abbreviations*  
*About the Authors*  
*Preface*  
*Executive Summary*  
*Overview*  

### 1. The Pathological Effects of Blood Within Lung Parenchyma
- 1.1 Introduction ........................................................................................................ 1  
- 1.2 Methods .............................................................................................................. 2  
- 1.3 Results ................................................................................................................ 4  
- 1.4 Discussion .......................................................................................................... 10  
- 1.5 Implications ....................................................................................................... 10  
- 1.6 Recommendations .......................................................................................... 11

### 2. Pulmonary Inflammation as a Predisposing Cause for EIPH
- 2.1 Introduction ........................................................................................................ 12  
- 2.2 Methods .............................................................................................................. 12  
- 2.3 Results ................................................................................................................ 16  
- 2.4 Discussion .......................................................................................................... 17  
- 2.5 Implications ....................................................................................................... 18

### 3. Estimation of Intrapulmonary Haemorrhage in Horses With Experimental EIPH Using 99mTc-Labelled Erythrocytes
- 3.1 Introduction ........................................................................................................ 19  
- 3.2 Methods .............................................................................................................. 19  
- 3.3 Results ................................................................................................................ 19  
- 3.4 Discussion .......................................................................................................... 21  
- 3.5 Implications ....................................................................................................... 21

### 4. General Discussion .............................................................................................. 22

### 5. References .......................................................................................................... 26

### 6. Publications ......................................................................................................... 30
Preface

This project, funded initially as a 1 year grant from RIRDC in 1996-97 and then subsequently as an additional grant for 1997-98, sought to develop a basic understanding of how much blood is present in the lungs of a typical subclinical "bleeder", and what effect that might have on the animal in terms of performance, pathology and the risk of further disease. This work has formed a central component of the research done by Dr Shaun McKane for his PhD, which was awarded by examination in March of this year (1999).

From the outset, it was recognised by the RIRDC granting review process as well as by us that some aspects of the studies proposed would be very challenging. During the course of this research, we have developed a reliable system for inoculating, and then re-examining by lavage, multiple segments of lung with the same animal. These studies have allowed for the first time a clear understanding of the sequential physiologic and physical changes that occur when blood escapes into alveoli, and they have formed the basis for new studies on the clearance mechanisms for blood removal from the lungs.

The complexities involved in using radio labelled erythrocytes for scintigraphy studies were well understood from the outset but the changes in baseline radioactivity from normal lung prior to and after exercise remain an enigma. This same phenomenon has been seen by the group in Belgium (Votion and Lekeux, 1999) and it masks anticipated changes due to the effects of haemorrhage in the lungs. While this approach may ultimately offer the possibility of locating and accurately estimating the amount of bleeding after an episode of EIPH, the technical difficulties, lack of equipment and expertise in this country and expense lend us to believe continued research in this area would not be wise. Our future studies therefore emphasise research on blood clearance mechanisms and not in vivo estimations of the extent of haemorrhage with EIPH.
Executive Summary

Exercise-induced pulmonary haemorrhage (EIPH) is a widespread problem of horses involved in high-speed competitions, particularly Thoroughbred racing, pacing, steeple chase and three day events. It is now recognised that the prevalence of EIPH among Thoroughbred racehorses approaches 100%. The severity of EIPH within this population varies widely from large volume haemorrhage into the lung which presents a life threatening condition, to minute haemorrhages which appear clinically silent. The affects these extremes of haemorrhage volume have on performance are quite distinct and readily discernible, however the affects of intermediate volumes of pulmonary haemorrhage are less well understood. Recently it has been shown that volumes as small as 200 ml of pulmonary haemorrhage can adversely affect oxygen uptake and exercise tolerance in galloping horses.

This report details the findings of a number of studies focused on determining the responses of the lung to the presence of erythrocytes in the airways. The critical responses studied were: the period of time required for removal of erythrocytes from the airway; the development of inflammation in response to intrapulmonary blood; the role of subclinical pulmonary inflammation in predisposing the lung to EIPH; and the evaluation of possible techniques for quantifying the volume of pulmonary haemorrhage. The motivation for these studies came from a realisation that despite intensive study of EIPH for the past thirty years, the effects of EIPH and intrapulmonary blood on the lung were largely undetermined, as were the processes of blood removal from the lung.

To study the processes of erythrocyte removal from the lung and the changes in pulmonary cytology that may result from the presence of blood in the airways, 40 ml volumes of blood were inoculated into several segmental bronchi of the cranioventral regions of the caudal lobe. Each of these segments was then lavaged once at different times over a period of 21 days and changes in the erythrocyte and leucocyte numbers over this time span were noted. The results indicate that autologous blood is removed more slowly from the lung than has been previously reported. Erythrocytes from a relatively small simulated episode of EIPH were found to persist and were recoverable in bronchoalveolar lavages obtained 21 days following inoculation. Initial removal of erythrocytes from the lung was dependent upon mechanical processes such as mucociliary clearance, coughing and flow of material proximally along the trachea when the horse’s head is down for grazing. Mucociliary clearance of blood could not be detected endoscopically after 72 hours, and by this time it was estimated that more than half of the originally inoculated erythrocytes had been removed. Macrophage erythrophagocytic activity had commenced by 72 hours following inoculation and gradually increased until day 10 when it appeared to stabilise, although increased macrophage numbers persisted for the remainder of the 21 day monitoring period and presumably for some time after this. Haemosiderin was first observed within erythrophages at day 10 and was present in approximately 30% of macrophages at day 21. This is consistent with previous reports which indicate that the longevity of haemosiderin laden alveolar macrophages may by as much as 3 months (Step et al 1991).

The prolonged course of erythrocyte removal has a number of important implications for the current management and racing schedules of Thoroughbred horses. The first of these implications being that horses which race frequently may not be permitted sufficient time to remove all the erythrocytes present from a previous episode of the EIPH before they are expected to race again. This could result in the progressive accumulation of blood in the
peripheral lung, to a degree where a number of successive low volume haemorrhages may result in a volume of intrapulmonary blood which is sufficient to impair racing performance. Unfortunately at this time bronchoalveolar lavage provides only a semi-quantitative estimate of the severity of pulmonary haemorrhage within selected regions of the lung, however the results of the study demonstrate that bronchoalveolar cytology may be used to provide a general indication of the severity of haemorrhage within a region of the lung. Based on the findings of this study and on clinical experience it is suggested by the investigators that horses with lavage samples from regions of pulmonary haemorrhage in which greater than 40% of cells are erythrocytes are in danger of developing problems associated with EIPH. These problems include subsequent severe episodes of haemorrhage, loss of performance, and the development of interstitial pulmonary fibrosis.

The second important implication resulting from the prolonged course of erythrophagocytic activity is the persistent activation of macrophages. Macrophage activation is involved in both tissue destructive inflammatory processes and in the reparative processes of fibrosis and angiogenesis. Hence prolonged macrophage activation could potentially result in damage to the delicate structures of the alveolus and subsequently to the development of alveolar fibrosis. This alveolar fibrosis could be of great detriment to the lung because of the potential for regional changes in pulmonary compliance and interdependence to predispose the lung to EIPH (Robinson and Derksen 1980). If this is the case then the removal of blood via erythrophagocytic mechanisms over a prolonged period is not desirable, and methods to minimise this activity could form the basis for new treatments to reduce the increasing severity of EIPH seen in older racehorses (Burke 1973; Cook 1974; Pascoe et al 1981; Raphel and Soma 1982; Mason et al 1983; McKane et al 1993).

Prior to the development of significant macrophage activity in this study a brief acute phase neutrophil response was observed 24 and 72 hours after blood inoculation, during which the percentage of neutrophils in the leucocyte population rose from the normal 3.5 ± 0.6% to 10.5 ± 2.8%. This is representative of a moderate degree of acute pulmonary inflammation and may play an important role in the health of the lungs of horses involved in racing carnivals where they are required to race twice within a period of 48 - 72 hours. Over the years low grade pulmonary inflammatory disease has often been implicated in the development of EIPH (Cook 1974; O’Callaghan et al 1987; McKane et al 1993). In 1993 McKane et al noted a correlation between EIPH and increased numbers of neutrophils in bronchoalveolar lavages and following the same line of reasoning as the work by O’Callaghan et al (1987), concluded that bronchiolitis predisposed these horses to EIPH. In light of this new evidence it seems that this idea is in need of revision, as it appears that the presence of intrapulmonary blood itself is enough to evoke this inflammatory reaction and possibly also the fibrosis and angiogenesis observed by O’Callaghan et al (1987) in association with EIPH lesions.

To explore the issue of whether low grade inflammation could predispose to EIPH, a sterile lesion was produced that would elicit an acute inflammatory response of the same magnitude as that observed in response to intrapulmonary blood. To do this, 20 ml of dilute acetic acid was inoculated into the lung, which caused a rise in the neutrophil percentage to approximately 12%, 24 hours after inoculation. The horses were exercised 24 hours after inoculation and bronchoalveolar lavages obtained from the inoculated segment and corresponding uninoculated segment of the opposite lung. The neutrophil percentage in the acetic acid inoculated segments and the corresponding control segments were 12.3 ± 1.1% and 4.4 ± 0.3% respectively. Comparisons revealed that in the control segments 0.29 ± 0.29% of cells were erythrocytes, whereas in the inoculated segments 52.3 ± 15.0% of cells

viii
were erythrocytes. This indicated a much greater propensity for the segments with acute low grade pulmonary inflammation to haemorrhage during exercise. The results from these two studies indicate that it is possible that the moderate acute inflammatory reaction associated with episodes of EIPH is perhaps both a consequence of and a predisposing factor for further EIPH.

Furthermore, contrasting the cytology from segments inoculated with known volumes of blood compared to that from typical field cases of EIPH, there is indirect evidence to suggest that most subclinical episodes of EIPH involve less than 40 ml of blood per bronchial segment. This follows from the fact that lavages containing greater than 50% erythrocytes are rare, in survey samples (McKane et al 1993, Meyer et al 1998). However it is a common to find haemosiderophages comprising 30 - 40% of leucocytes in lavage fluid, suggesting that most horses with EIPH have multiple small bleeding episodes leading to gradual haemosiderin accumulation where 40 - 50% of macrophages may eventually become laden with haemosiderin.

If prodromal bouts of EIPH leads only to a loss of a few millilitres of blood into alveoli, it is not surprising that detection of these sites by scintigraphy is extremely difficult.

The scintigraphy studies were attractive because they potentially could overcome important limitations to all other studies estimating the severity of haemorrhage with EIPH, namely that the use of routine lavage cytology or fluorochrome -tagged erythrocytes as markers was totally reliant on adequate samples obtained by lavage, and this technique would not be. Haemorrhagic areas theoretically would be more radioactive than background, because blood cells are more densely packed in sites of haemorrhage than in normal lung.

At the Washington State University tri-state imaging facility we conducted a series of pilot studies to determine the feasibility of a more comprehensive study. When 5 ml of technecium labelled erythrocytes was deposited in the peripheral lung field of a horse, the inoculation site was readily detected by thoracic scintigraphy. For scintigraphy to be clinically useful, the tagged cells need to be injected IV and then located by scintigraphy if they accumulate in the lungs. We found that when 200 mCi of Technetium m99 linked to either DTPA or Ultratag was used to radiolabel erythrocytes, if 25 ml of autologous radiolabelled blood were injected into the lung after equilibrating in systemic blood for 30 minutes, the inoculation site was undetectable by scintigraphy. Additional studies where horses (n=8) were instrumental and injected with labelled erythrocytes during vigorous treadmill exercise, also failed to show scintographic changes on thoracic scans, despite the presence of radiolabelled erythrocytes in lavage fluids, proving that EIPH had occurred during treadmill exercise. Lavage/blood radioactivity ratios ranged from .0079 to .0001. Substantial background radiation diffusely from the lung fields after exercise interfered with the ability to detect subtle changes in regional radioactivity. A control group (n=4) were treated identically except for treadmill exercise, and in these the BAL/blood ratios were higher than the exercised group, a result that remains unexplained.

In conclusion, these studies were technically difficult to perform, in part due to the intense sources of radiation needed, and sites of localised intrapulmonary haemorrhage were not detectible by scintigraphy. It is our view that further studies using this methodology are not justifiable.
Overview

This project involved the conduct of several unrelated research studies, and this report has been divided into 3 sections to reflect each of the studies.

The first section deals with the structural changes associated with the inoculation of blood into horse lungs and describes sequential changes as monitored by lavage cytology and histopathology. This study extends and completes research that was initiated during the 1996-97 grant period.

The second section deals with the functional and physical effects mild acute pulmonary inflammation has on the risk of developing EIPH.

The third section reports on the studies using technetium 99 labelled erythrocytes and scintigraphy in an attempt to quantitate pulmonary haemorrhage in vivo.

The final section, the general discussion summarises all aspects of the project.
1 The Pathological Effects of Blood Within Lung Parenchyma

1.1 Introduction

No clinically useful prophylactic medication exists to completely prevent EIPH in racehorses (Pascoe et al. 1985; Sweeney et al. 1988; Erickson et al. 1992). A need remains to examine the effects of EIPH on the lung in order to identify a possible method of reducing the negative impact of EIPH on racehorse performance, once it has occurred. A study by Step et al. (1991) examined a number of qualitative changes which occur in tracheobronchial cytology following intrapulmonary autologous blood inoculation, but this was mainly concerned with examining the persistence of erythrocytes and haemosiderophages in the airways of horses, and did not examine the changes which occur in other cell populations of the lower respiratory tract. While tracheobronchial aspirates provide some information that is reflective of cellular changes occurring at the alveolar level, generally the cells recovered are more degenerate than those from BAL and little information is obtained from cell morphology. Step et al. (1991) were unable to determine from their work whether intrapulmonary blood evoked an inflammatory reaction within the airways.

Meyer et al. (1998) used BAL sampling techniques in an attempt to quantify changes occurring in cell populations of the lower respiratory tract following EIPH, but their results do not indicate changes in cell numbers per microlitre of ELF because they did not calculate the dilution factor of the ELF by the lavage fluid. The qualitative results of BAL agree with those found using tracheobronchial lavages in that the persistence of erythrocytes following EIPH was found to be less than one week, although haemosiderophages persist for more than one month (Step et al. 1991; Meyer et al. 1998).

O'Callaghan et al. (1987) suggested that the bronchiolitis observed in association with areas of pulmonary haemorrhage was a predisposing cause of EIPH. However, a more recent theory proposed that the aetiology of EIPH was related to the stress failure of pulmonary capillaries and that this did not require the presence of pre-existing pulmonary inflammation (West et al. 1991). This has left doubt as to whether the bronchiolitis observed in association with chronic lesions of EIPH by O'Callaghan et al. (1987e) was a cause or an effect of pulmonary haemorrhage. This study examines the changes that occur in bronchoalveolar cell populations in the first 21 days following inoculation of the equine bronchial tree with autologous blood. The results indicate that intrapulmonary blood is capable of inducing pulmonary inflammation, and that inflammation can be an effect of EIPH. Previously we investigated the functional effects of blood inoculation (RIRDC 1996-7) and this study complements the previous one, by investigating the changes in lung cytology in response to the presence of blood.

Objective: to determine the sequential changes that occur in lung segment cytology over time following a single inoculum of autologous blood.
1.2 Methods

Horses
Six horses with a mean age of 4.3 years (range 3 to 6 years) were rested for 4 months to ensure that their lungs were free of recent effects of pulmonary haemorrhage. All the horses were in good health and remained so for the duration of the experimental period. On two occasions each of the horses underwent bronchoscopy and inoculation of 4 ventrolaterally located segmental bronchi with 40 ml of autologous blood (Figure 1). A different inoculated segmental bronchus was lavaged on each of 8 occasions over a 21 day period following autologous blood inoculation. The horses also had a single lavage taken from a random caudal bronchus 2 months before blood inoculation to provide normal (control) values.

![Figure 1: Schematic diagram of the equine bronchial tree indicating the 8 segmental bronchi (A-H) inoculated with 40ml of autologous blood.](image)

Inoculation Protocol
Prior to bronchoscopy each horse was sedated with 300mg of xylazine\(^1\) administered intravenously. A 200 cm optical endoscope\(^2\) was passed via the ventral nasal meatus into the trachea until the tracheal bifurcation could be visualised. To reduce the coughing reflex, the bifurcation of the trachea and the junctions of the first 3 ventrolateral segmental bronchi of the caudal lobe of one lung were sprayed with 2 ml of 2% mepivicaine\(^3\), injected through a 2.5 m long, 1.9 mm diameter catheter\(^4\) passed via the biopsy channel of the endoscope. Blood was then drawn from the left jugular vein of the horse and 40 ml inoculated into each of the 4 segmental bronchi, through the 2.5 m catheter without addition of anticoagulant. The first 4 segmental bronchi of the other lung of each horse were inoculated with 40 ml of autologous blood 7 days later. The lung inoculated on the first occasion was selected at random, and the horses were observed daily following the inoculations for clinical signs of respiratory disease or pyrexia.

\(^{1}\) Xylazil-100, Troy Laboratories, Smithfield, N.S.W. 2164, Australia.
\(^{2}\) Colonoscope LB3R-CF, Olympus, New Hyde Park, N.Y. 11042, U.S.A.
\(^{3}\) Mepivicaine, Nature Vet, Agnes Banks, N.S.W. 2753, Australia.
\(^{4}\) Polyethylene tubing, Cat. No. 112102, Critchley Electrical Products, Auburn, N.S.W. 2144, Australia
**Lavage Protocol**

The horses each had a different bronchial segment lavaged on days 0 (15 min after inoculation), 1, 3, 5, 7, 10, 14, and 21 days following autologous intrapulmonary blood inoculation (Figure 1). The order of lavage of each segmental bronchus within a lung was randomised and the use of the endoscope to obtain the lavages enabled the desired bronchi to be positively identified and lavaged on every occasion. A lavage was considered successful if more than 50 ml of fluid was returned and the fluid contained a white froth, indicating that it contained alveolar surfactant. All 48 lavages obtained during the course of the experiment were successful based on these criteria.

To obtain lavages the endoscope was inserted to the level of the tracheal bifurcation, the bronchial segment anaesthetised with 2% mepivicaine spray, the catheter then removed and the tip of the endoscope advanced into the desired segmental bronchus until a silicon cuff on the endoscope tip wedged in the segmental bronchus. Sterile, 5% glucose solution (180 ml) was injected through the 3 mm biopsy channel and then approximately 2-3 kpa continuous suction applied to draw the fluid out of the bronchial segment until approximately 50 to 100 ml of lavage fluid had been returned.

**Sample Processing**

At the same time as lavages were obtained, venous blood samples were collected from each horse into tubes containing lithium heparin for determination of the plasma urea nitrogen concentration. A 10 ml aliquot of each lavage sample was centrifuged at 600 g for 10 minutes to remove cells and mucus before the supernatant was analysed using a modified urea nitrogen assay kit\(^5\) to enable accurate measurement of low concentrations in the range of 20 to 600 µmol.l\(^{-1}\). The plasma/lavage urea nitrogen ratio was used to calculate the dilution of the epithelial lining fluid (ELF), by the 5% glucose lavage solution.

Cytological analysis involved determination of the total leucocyte count per µl of lavage fluid using a standard haemocytometer and adjusted for dilution to calculate the total leucocyte count of the ELF. Cytocentrifuge\(^6\) smears were made using 200 µl of lavage fluid and these smears then fixed and stained using a rapid Geimsa like stain\(^7\). Differential cell counts were determined by counting the cells contained in 5, randomly chosen, 40x magnification, fields on each slide, and from these, the percentages and absolute cell numbers per µl of ELF were determined.

**Statistical Analysis**

The mean ± sem of the differential and absolute cell counts for the lavaged segments of the 6 horses were calculated and compared between each of the 8 inoculated samples and control lavage using a one way analysis of variance using the Minitab\(^8\) statistical package. Where the F-value returned was significant (p<0.05) then a Fisher's pairwise comparison, post hoc, analysis was conducted to identify means which differed. Log transformation of the absolute cell count data facilitated analysis because of the large variability between horses.

### 1.3 Results

\(^5\) Kinetic urea kit, Sigma Diagnostics, St Louis, Mo 63178, U.S.A.
\(^6\) Cytospin 2, Shandon Southern Products, Cheshire, England.
\(^7\) Diff Quik, Lab-Aids, Narrabeen, N.S.W. 2101, Australia.
\(^8\) Minitab 8.21, Minitab Inc, State College, PA 16801-3008, U.S.A.
Cell counts for all cell types are shown in tables 1 and 2. Eosinophil and mast cell percentages and absolute cell counts did not vary significantly between the 9 sample times. The mean percentages for eosinophils ranged from $1.0 \pm 0.1$ to $1.5 \pm 0.3\%$ and the absolute count from $11.5 \pm 2.5$ to $92.4 \pm 23.3$ cells.$\mu l^{-1}$ over the experimental period. Mast cell numbers were similarly variable with ranges of $0.3 \pm 0.1$ to $1.0 \pm 0.1\%$ and $9.2 \pm 2.1$ to $92.4 \pm 23.3$ cells.$\mu l^{-1}$ for the study.

**Erythrocyte Removal**

Gross, bronchoscopic evidence of blood removal by the mucociliary clearance system was evident 1 day after blood inoculation in all 6 horses, but only evident at day 3 in 2 horses and not evident in any horse by day 5. Initially erythrocyte numbers in lavage samples were very high and declined rapidly in the first 3 days following blood inoculation, despite the fact that erythrophagocytosis was observed to be minimal prior to day 3. The number of erythrocytes was observed to increase rapidly from day 3 to 10 and the development of haemosiderin within macrophages was observed from day 10 onwards. The number of erythrocytes remaining after day 10 was low, although some erythrocytes persisted in the alveolar space for the 21 days of the sampling period.

**Morphologic Cell Changes**

The most noticeable change in cell morphology occurred in the macrophages which were observed to increase in size and vacuole content within the first 3 days of blood inoculation (Figure 2), prior to substantial erythrophagocytosis occurring. Between days 3 and 10 alveolar macrophage size was obviously larger than at day 0 or 1 and many macrophages were seen to contain one or more entire erythrocytes (Figure 3). At about day 10 a number of macrophages were noted to develop golden brown granules of various sizes, indicating the early stages of haemosiderin production. In samples from day 14 and 21, macrophages could be observed which contained either erythrocytes, early haemosiderin or the darker blue staining, consistent with more mature forms of haemosiderin (Figure 4 and 5). Small numbers of macrophages were observed to have undergone transformation to epithelioid and giant cell forms, consistent with high levels of macrophage activation. The morphology of all other cell types were not visibly altered during the period of study, except for minimal toxic changes observed in neutrophils at days 3 and 5.

**Differential Cell Count Changes**

Neutrophil percentages were observed to more than double within the first 24 hours from 3.5% to 10.5% of all leucocytes present (Table 1). After day 3 the neutrophil percentage decreased toward pre-inoculation levels and remained there for the rest of the sampling period. The percentage of macrophages containing either whole erythrocytes or haemosiderin (siderophages) rose steadily from less than 1.0% on day 0 to 45.7% by day 14. The percentage of erythrocytes in the samples declined rapidly between day 3 and day 14. Leucocyte percentages, other than neutrophils, remained stable over the experimental period.

**Absolute Cell Count Changes**

The mean total leucocyte concentration ($1146 \pm 252$ cells.$\mu l^{-1}$) determined from samples taken within 15 minutes of blood inoculation (day 0) was significantly lower than both control and other post inoculation values (Table 2). Values calculated for day 1, post inoculation, are similar to control values for the various leucocyte populations within the alveolus. A moderate rise in the bronchoalveolar leucocyte population was evident from day
3 and persisted throughout the 21 days of sampling. This change appears to relate to increases in the concentration of macrophages and to a lesser degree lymphocytes in the pulmonary lining fluid of the lower airways. The haemosiderophage concentration over the 21 days rose from 7.0 ± 1.3 to 2255 ± 736 cells.µl⁻¹ of ELF, while the increase in the neutrophil percentage noted on day 3 resulted in a maximum neutrophil concentration of 920 ± 342 cells.µl⁻¹ ELF.
Table 1: Differential cell percentage (mean ± sem) in bronchoalveolar lavages obtained sequentially over 21 days following intrapulmonary inoculation of autologous blood.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>%</th>
<th>Control</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage1</td>
<td></td>
<td>64.0±1.3</td>
<td>63.5±2.9</td>
<td>61.8±3.5</td>
<td>57.7±1.5</td>
<td>64.7±2.8</td>
<td>64.3±1.6</td>
<td>63.2±1.0</td>
<td>66.5±1.9</td>
<td>67.8±2.7</td>
</tr>
<tr>
<td>Siderophage2</td>
<td></td>
<td>0.7±0.2</td>
<td>1.0±0.1</td>
<td>10.5±2.7</td>
<td>11.3±1.9</td>
<td>15.8±1.9</td>
<td>19.2±3.3</td>
<td>25.3±3.7</td>
<td>45.7±7.4</td>
<td>36.8±7.7</td>
</tr>
<tr>
<td>Lymphocyte1</td>
<td></td>
<td>30.3±1.6</td>
<td>32.0±3.0</td>
<td>25.5±1.1</td>
<td>31.2±2.1</td>
<td>29.2±2.3</td>
<td>29.8±1.4</td>
<td>29.2±0.8</td>
<td>27.5±1.7</td>
<td>27.5±2.6</td>
</tr>
<tr>
<td>Neutrophil1</td>
<td></td>
<td>3.7±0.6</td>
<td>3.5±2.8</td>
<td>10.5±1.9</td>
<td>9.8±0.8</td>
<td>5.0±0.7</td>
<td>4.0±0.5</td>
<td>6.2±1.1</td>
<td>5.0±0.5</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>Erythrocyte3</td>
<td></td>
<td>0.7±0.3</td>
<td>97.3±0.3</td>
<td>97.6±0.5</td>
<td>84.7±3.6</td>
<td>72.8±11.6</td>
<td>56.2±2.4</td>
<td>38.3±11.5</td>
<td>9.5±2.4</td>
<td>3.5±1.4</td>
</tr>
<tr>
<td>Eosinophil1</td>
<td></td>
<td>1.2±0.2</td>
<td>1.0±0.3</td>
<td>1.5±0.1</td>
<td>1.0±0.1</td>
<td>1.3±0.2</td>
<td>1.2±0.1</td>
<td>1.2±0.2</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Mast cell1</td>
<td></td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
<td>&lt;1±0.1</td>
</tr>
</tbody>
</table>

1Percentage of leucocytes present in sample
2Percentage of macrophages containing erythrocytes or haemosiderin
3Percentage of all cell types present
Means with same superscript are equivalent at 95% confidence level.
### Table 2: Absolute cell count (mean ± sem) in bronchoalveolar lavage samples collected over 21 days following intrapulmonary autologous blood inoculation.

<table>
<thead>
<tr>
<th>Cells per µl ELF</th>
<th>Control</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
<th>Day 7</th>
<th>Day 10</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Leukocyte</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3982b</td>
<td>1146a</td>
<td>3520b</td>
<td>8123c</td>
<td>8137c</td>
<td>5708c</td>
<td>8385b</td>
<td>5888c</td>
<td>9238c</td>
</tr>
<tr>
<td></td>
<td>± 885</td>
<td>± 252</td>
<td>± 1308</td>
<td>± 2754</td>
<td>± 2714</td>
<td>± 1814</td>
<td>± 1525</td>
<td>± 2072</td>
<td>± 2327</td>
</tr>
<tr>
<td><strong>Macrophage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2544b</td>
<td>703a</td>
<td>2246b</td>
<td>4724c</td>
<td>5119c</td>
<td>3650c</td>
<td>5282c</td>
<td>3939c</td>
<td>6413c</td>
</tr>
<tr>
<td></td>
<td>± 559</td>
<td>± 131</td>
<td>± 852</td>
<td>± 1660</td>
<td>± 1551</td>
<td>± 1113</td>
<td>± 959</td>
<td>± 1347</td>
<td>± 1743</td>
</tr>
<tr>
<td><strong>Siderophage</strong></td>
<td>18.3b</td>
<td>± 5.8</td>
<td>7.0a</td>
<td>131c</td>
<td>430d</td>
<td>706def</td>
<td>672de</td>
<td>1350efg</td>
<td>1875fgg</td>
</tr>
<tr>
<td></td>
<td>± 5.8</td>
<td>± 1.3</td>
<td>± 36</td>
<td>± 104</td>
<td>± 161</td>
<td>± 207</td>
<td>± 161</td>
<td>± 1347</td>
<td>± 1743</td>
</tr>
<tr>
<td><strong>Lymphocyte</strong></td>
<td>1225bc</td>
<td>391a</td>
<td>872b</td>
<td>2374c</td>
<td>2499bc</td>
<td>1709bc</td>
<td>2433c</td>
<td>1680bc</td>
<td>2406c</td>
</tr>
<tr>
<td></td>
<td>± 288</td>
<td>± 117</td>
<td>± 310</td>
<td>± 758</td>
<td>± 1031</td>
<td>± 596</td>
<td>± 441</td>
<td>± 695</td>
<td>± 505</td>
</tr>
<tr>
<td><strong>Neutrophil</strong></td>
<td>132a</td>
<td>± 26</td>
<td>37.3a</td>
<td>347a</td>
<td>920b</td>
<td>457a</td>
<td>262a</td>
<td>349a</td>
<td>235a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 7.9</td>
<td>± 150</td>
<td>± 342</td>
<td>± 226</td>
<td>± 109</td>
<td>± 127</td>
<td>± 57</td>
<td>± 108</td>
</tr>
<tr>
<td><strong>Erythrocyte</strong></td>
<td>29.4a</td>
<td>± 7.0</td>
<td>14438</td>
<td>± 47479</td>
<td>112569</td>
<td>47788d</td>
<td>31893d</td>
<td>20328c</td>
<td>10734c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 17.0</td>
<td>± 23547</td>
<td>± 10569</td>
<td>± 13577</td>
<td>± 11685</td>
<td>± 7969</td>
<td>± 160</td>
<td>± 63</td>
</tr>
<tr>
<td><strong>Eosinophil</strong></td>
<td>51.3</td>
<td>± 12.5</td>
<td>11.5</td>
<td>42.7</td>
<td>81.3</td>
<td>81.4</td>
<td>69.2</td>
<td>91.0</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 2.5</td>
<td>± 11.6</td>
<td>± 27.6</td>
<td>± 27.2</td>
<td>± 19.9</td>
<td>± 12.9</td>
<td>± 19.7</td>
<td>± 23.3</td>
</tr>
<tr>
<td><strong>Mast cell</strong></td>
<td>39.9</td>
<td>± 8.9</td>
<td>11.5</td>
<td>35.2</td>
<td>81.3</td>
<td>81.4</td>
<td>57.1</td>
<td>83.9</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 2.5</td>
<td>± 13.1</td>
<td>± 27.6</td>
<td>± 27.2</td>
<td>± 18.1</td>
<td>± 15.3</td>
<td>± 20.7</td>
<td>± 23.3</td>
</tr>
</tbody>
</table>

Means with same superscript are equivalent at 95% confidence level.
**Figure 2:** BAL cytology 3 days after inoculation with blood. E phagocytosed erythrocytes; N neutrophils; F foamy cytoplasm of activated macrophages

**Figure 3:** BAL cytology 7 days after inoculation with blood. Note the rosetting of erythrocytes around macrophages R; Early erythrophage E; and the modest macrophage response
**Figure 4**: BAL cytology 14 days after inoculation with blood. Note the golden colour of the early haemosiderin inside activated macrophages $H$; Insert of multinucleated giant cell $G$.

**Figure 5**: BAL cytology 21 days after inoculation with blood. Note the few remaining erythrocytes; Early haemosiderophage $H$; and mature haemosiderin inside a macrophage $MH$. 
1.4 Discussion

Most of the findings observed agreed with impressions gained from interpreting clinical lavage samples, however there are a number of findings which are of important note. Firstly, there was a surprisingly slow removal of erythrocytes from the lung, with erythrocytes evident in the lavages of all horses at both 14 and 21 days following inoculation. Previous observations of tracheal wash aspirates and BAL samples have found erythrocytes to be a rare finding in the respiratory tract of horses 1 week after blood inoculation (Step et al 1991; Meyer et al 1998). This has major implications for the management of racehorses which are known to suffer adversely from episodes of EIPH, particularly under racing conditions where horses are raced as frequently as once a week for 3 or more months during a racing campaign or season. The results of this study suggest that this may be too frequent to allow sufficient removal of erythrocytes and resolution of respiratory inflammatory damage between races and training gallops, causing an accumulation of erythrocytes within the airways of the lung and cumulative adverse effects on the health of the lung and performance of the horse.

The value obtained for the total leucocyte count on day 0 is approximately 70% lower than the control value. This dilution effect may be partly due to the added volume of fluid when blood is inoculated. The added volume of fluid would also dilute the alveolar surfactant to low concentrations which may be ineffective as a stabilising agent within the inoculated regions. Oedema and atelectasis have been observed to occur when surfactant activity is impaired (Holm and Notter 1987), and the development of regional pulmonary oedema within the inoculated alveoli would further dilute ELF. Regardless of the cause of the low leucocyte concentration on day 0, the results demonstrate that after day 3 there is a modest but persistent inflammatory response in the airways of the lung associated with the presence of autologous intrapulmonary blood. Initially this response is dominated by a rapid increase in the neutrophil population, the duration of which is brief before a more chronic increase in macrophage and to a lesser extent lymphocyte numbers occurs. The histological effects of this inflammatory response can only be speculated at this time, however it is conceivable that the inflammatory response to intrapulmonary blood is the cause of the bronchiolitis and subsequent bronchial neovascularisation and fibrosis observed by O'Callaghan et al (1987c and 1987e) in association with areas of gross EIPH. This does not mean that the observed pulmonary inflammation plays no role in the aetiogenesis of EIPH, since it is possible that pulmonary inflammation may alter the mechanics of the local region and predispose it to further more severe haemorrhage.

1.5 Implications

Intrapulmonary blood may cause adverse changes in the health of the lung in several ways. The most obvious is that the presence of substantial volumes of haemorrhage can impair pulmonary gas exchange and therefore negatively impact on racing performance, whether the blood is haemorrhaged in one episode or a succession of episodes of EIPH. Another possible impact of intrapulmonary blood on the health of the lung is the development of infectious pulmonary disease. Normally bacteria inhaled into the small bronchi and alveoli are removed by macrophage phagocytosis and the mucociliary clearance system before infection can be established, however blood may interfere with these processes and provide an excellent medium for the growth of bacteria within the lower airways, perhaps predisposing racehorses to infectious respiratory disease. A study by Adamson and Slocombe (1995) reported a reduction in macrophage activity after exercise and erythropagocytosis has been
demonstrated in vitro to also suppress macrophage bacteriocidal abilities (Hand and King-Thompson 1983).

Blood itself causes a modest but long standing inflammatory reaction within the alveoli and small bronchi, which could alter the response of the alveoli to the stresses of high frequency ventilation. This may occur via the development of increased pulmonary resistance in areas of haemorrhage (Aguilera-Tejero et al 1995), presumably because of bronchoconstriction in response to bronchiolitis, or potentially through bronchial neovascularisation and possible fibrosis of the alveolar walls. All these changes may predispose areas of the lung which have haemorrhaged to haemorrhage again, perhaps more severely. This would establish a cyclic self propagating phenomenon of gradually worsening EIPH in the lungs of horses as they continue to race, which has been observed through the increased prevalence and severity of EIPH in older racehorses (Mason et al 1983).

There appeared to be a delay in the development of significant erythrophagocytosis which may be related to development of a recognition signal for macrophage activity. It is impossible to say at this time, exactly what this signal could be and whether it is a change that occurs in the erythrocyte membrane or a cytokine release by lymphocytes which triggers erythrophagocytosis. A degree of rosetting of erythrocytes also occurred in several samples, indicating the possibility of opsonisation of erythrocytes and then phagocytosis by macrophages. Surfactant protein-A (SP-A) has been demonstrated to opsonise gram-negative bacteria by adhering to their lipopolysacharide walls and then attaching to a receptor of the macrophage, which appears remote from the normal Fc receptor site for immunoglobulin mediated opsonisation (Pison et al 1992; Binford and Palm 1994; Pikaar et al 1995).

Macrophage activation was seen to develop rather slowly but persisted for a prolonged period. During this time there is potential for the release of 100 different secretory products from the macrophage including superoxide ions, elastases, prostaglandins, interleukins, cytokines, coagulation factors and growth factors (Fels and Cohn 1986). Transforming growth factor-beta (TGF-β) is a product often secreted by activated macrophages and its function is to attract and stimulate the activity of fibroblasts (Zhang and Phan 1996). It is very possible that the prolonged stimulation of macrophages and the respiratory burst associated with phagocytosis damages the alveolar epithelium to a degree, and that the chronic stimulation of macrophages owing to the persistence of erythrocytes in the alveoli causes the release of several cellular signals including TGF-β. Once released TGF-β will stimulate fibroblasts to produce alveolar fibrosis and angiogenesis (Chegini 1997), consistent with the histological description of Mason et al (1983) and O’Callaghan et al (1987e).

1.6 Recommendations

These data suggest that blood is cleared slowly from the lungs and incites inflammation. Based on this study, horses at risk of severe EIPH should not be raced as frequently as is the current practice. Secondly, research into mechanisms to facilitate clearance of blood from lungs affected with EIPH is indicated, and is the subject of our next research proposal for 1999-2000.
2 Pulmonary Inflammation as a Predisposing Cause for EIPH

2.1 Introduction

The role of inflammation in the aetiogenesis of EIPH has long been suspected, but never proven and recently placed in doubt. Cook (1974) suggested that the 2% prevalence of epistaxis he observed was a result of continuing to race horses which had suffered previously from pulmonary inflammatory disease. This theory was supported by the extensive study of O’Callaghan et al (1987a-h) which concluded that localised areas of bronchiolitis and fibrosis, predisposed areas of the lung to EIPH because of changes and damage caused by inflammatory lesions. However their study did not prove a causative relationship between the inflammatory lesions and EIPH, and the authors could not explain the localisation of EIPH to the caudodorsal regions of the lung.

More recent studies have suggested that stress failure of capillary walls without the necessity for predisposing pulmonary inflammation is the cause of EIPH (West et al 1991). This theory has received wide acceptance because it is able to account for the high prevalence of EIPH amongst racehorses, although it still fails to fully explain the distribution of lesions. If pulmonary inflammation is not a prerequisite for the development of EIPH then it must be concluded that the inflammatory lesions, so consistently observed in association with areas of grossly evident EIPH are largely the result of the inflammatory response of the lung to the presence of blood. However what consequences, if any, does this resultant pulmonary inflammation have on further episodes of EIPH?

It is possible that the normal exercise physiology of the horse, which causes EIPH (West et al 1991 and 1993) may act synergistically with pulmonary inflammation to increase the severity of bleeding from vessels which have altered blood flow and vascular wall permeability. If this were to be the case then pulmonary inflammation would not only be a result of an episode of EIPH but it may be the cause of the next episode, if the horse is not permitted to convalesce for an appropriate period. This study examines whether a mild localised inflammatory lesion, similar in intensity to that observed after intrapulmonary blood inoculation, predisposes an area of the lung to develop EIPH more severely than would otherwise be expected. The results reveal that mild pulmonary inflammation did predispose the lung to EIPH in 5 of 7 horses studied, and further that this intensity of inflammatory disease was sufficient to provoke EIPH within a cranoventral bronchial segment, an area not previously reported to suffer EIPH.

Objective: to assess whether local inflammation in bronchopulmonary segments predisposes to EIPH.

2.2 Methods

Pilot Study Protocol

Following a 3 month rest from exercise, 2 Standardbred horses each had one segmental bronchus inoculated with 20 ml of sterile, 0.01% acetic acid solution (pH = 4.31) and a second segmental bronchus inoculated with 20 ml of sterile, 0.05% acetic acid solution (pH =
These dilute acetic acid solutions were inoculated in order to provoke localised pulmonary inflammation within the lower airways of the respiratory tract. The inoculations were performed using a fibre-optic endoscope\textsuperscript{9} which had a 250 cm long, catheter\textsuperscript{10} passed down the biopsy channel, to permit positive identification of the sites inoculated. As the endoscope was passed into the lungs, the tracheal bifurcation and junctions of the first lateral segmental bronchus of the caudal lobe of each lung were sprayed with 2 ml of 2% mepivicaine\textsuperscript{11} to reduce the coughing reflex during bronchoscopy. The sites inoculated in each horse were the caudal branch of the first lateral segmental bronchus of the caudal lobe of each lung (Figure 1).

Twenty four hours later the horses were sedated with 300 mg of xylazine\textsuperscript{12} administered intravenously and again had the tracheal bifurcation and junctions of the first lateral bronchi, as well as the second lateral bronchus of one lung (Figure 1), anaesthetised with 2 ml of 2% mepivicaine. These 3 bronchial segments were then each lavaged with 180 ml of sterile, 5% glucose solution infused via the biopsy channel of the endoscope and aspirated using 3-5 kpa continuous suction, until approximately 50 to 100 ml of fluid was recovered. Smears were prepared by cytocentrifugation\textsuperscript{13} of 200\,µl of each of the three samples from each horse and stained using a rapid Geimsa like stain\textsuperscript{14}, before performing differential cell counts to determine the effects of inoculation with 0.01% and 0.05% acetic acid solutions on bronchoalveolar cell populations, compared with the sample from the uninoculated (control) segments.

The control segments (second lateral segmental bronchus) from both horses had low neutrophil percentages (4.3% and 5.1%), which are consistent with normal bronchoalveolar lavage neutrophil percentages (Mair \textit{et al} 1987; McKane \textit{et al} 1994). The samples from the 4 inoculated segments contained neutrophil counts of 12.2% and 13.1% for the 0.01% acetic acid inoculation segments, and 15.4% and 16.9% for the 0.05% acetic acid inoculations. Based on the results of these lavages it was decided to use 20 ml, 0.01% acetic acid solutions to induce localised pulmonary inflammation in the horses used in the main part of this study, because this concentration produced inflammatory lesions of similar intensity to that seen in chapter 5, 24 hours after intrapulmonary blood inoculation (10.5 ± 2.8% neutrophils). Neither the 0.01% nor 0.05% acetic acid inoculations resulted in pulmonary haemorrhage in these non-exercised pilot study horses.

\textsuperscript{9} Colonoscope LB3R-CF, Olympus, New Hyde Park, N.Y. 11042, U.S.A.
\textsuperscript{10} Polyethylene tubing, Cat. No. 112102, Critchley Electrical Products, Auburn, N.S.W. 2144, Australia
\textsuperscript{11} Mepivicaine, Nature Vet, Agnes Banks, N.S.W. 2753, Australia.
\textsuperscript{12} Xylazil-100, Troy Laboratories, Smithfield, N.S.W. 2164, Australia.
\textsuperscript{13} Cytospin 2, Shandon Southern Products, Cheshire, England.
\textsuperscript{14} Diff Quik, Lab-Aids, Narrabeen, N.S.W. 2101, Australia.
Figure 1: Schematic diagram of the equine bronchial tree. (L1.1 = cranial branch of first lateral segmental bronchus of caudal lobe; L1.2 = caudal branch of first lateral segmental bronchus of caudal lobe; L2 = second lateral segmental bronchus of caudal lobe.)

Main Study Protocol
Seven Standardbred horses ranging from 5 to 8 years of age were all rested from exercise for a period of 6 months. Each horse had the second lateral segmental bronchus of the caudal lobe of one lung inoculated with 20 ml of 0.01% acetic acid, as described above. The inoculations were tolerated well by the horses with no clinically evident adverse effects observed.

Twenty four hours later the horses were each subjected to treadmill\textsuperscript{15} exercise at 10 m.s\textsuperscript{-1} on a 10\% (6\textdegree) slope until they could no longer keep pace with the treadmill speed. After the cessation of exercise the horses were allowed a recovery period of approximately 1.5 hours and then subjected to bronchoscopy and lavage of the inoculated segment and the second lateral bronchus of the caudal lobe of the opposite lung (control). At the same time as the lavage samples were obtained a venous blood sample was obtained and stored in a chilled tube containing lithium heparin to facilitate harvesting of plasma for plasma urea nitrogen determination.

Sample Handling
Total leucocyte counts were determined from each sample using a standard haemocytometer\textsuperscript{16} and differential cell counts determined from cytocentrifuge smears prepared in the same way as described for the pilot study. A 10 ml aliquot of lavage fluid was centrifuged at 600g to remove cells and mucus, before analysis to determine the lavage urea nitrogen concentration, and the plasma/lavage urea nitrogen ratio determined to provide the dilution factor of epithelial lining fluid (ELF) with the 5\% glucose lavage solution.

Statistical Analysis
The means ± sem for the differential cell counts and the absolute cell counts per µl of ELF are

\textsuperscript{15} Beltalong, Euroa, Victoria 3666, Australia.
\textsuperscript{16} Levy Double Neubauer counting chamber, Becton Dickinson, Parsippany, N. J. 07054, U.S.A.
presented in tables 1 and 2 respectively. The data for each cell count was compared between inoculated and control segments using 2 sample t-tests, conducted at the 95% confidence level. An analysis of the relative risk of developing EIPH following provocation of pulmonary inflammation is presented in table 3, although it should be noted that the number of horses and samples is too small to confidently accept the results of this table. The statistical package used to conduct t-test comparisons was Minitab 8.21\textsuperscript{17}.

\textsuperscript{17} Minitab 8.21, Minitab Inc, State College, PA 16801-3008, U.S.A.
2.3 Results

Table 1: Differential cell count of bronchoalveolar lavages obtained from 0.01% acetic acid inoculated and control segmental bronchi following strenuous exercise.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Acetic acid</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophage</td>
<td>59.6 ± 1.7 *</td>
<td>67.4 ± 1.4</td>
<td>0.004</td>
</tr>
<tr>
<td>Erythrophage</td>
<td>1.3 ± 0.4 *</td>
<td>0.0 ± 0.0</td>
<td>0.025</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>27.6 ± 1.0</td>
<td>27.3 ± 1.1</td>
<td>0.85</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>12.3 ± 1.1 *</td>
<td>4.4 ± 0.3</td>
<td>0.0002</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>52.3 ± 15.0 *</td>
<td>0.29 ± 0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Mast Cell</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.5</td>
</tr>
</tbody>
</table>

a Percentage of leucocyte population  
b Percentage of macrophage population containing phagocytosed erythrocytes  
c Percentage of all cells present  
* Mean is different to control mean

Table 2: Absolute cell count of bronchoalveolar lavages obtained from 0.01% acetic acid inoculated and control segmental bronchi following strenuous exercise.

<table>
<thead>
<tr>
<th>Cells, µl⁻¹ ELF</th>
<th>Acetic acid</th>
<th>Control</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Leucocyte</td>
<td>5038 ± 987</td>
<td>4724 ± 1054</td>
<td>0.72</td>
</tr>
<tr>
<td>Macrophage</td>
<td>3046 ± 659</td>
<td>3182 ± 721</td>
<td>1.0</td>
</tr>
<tr>
<td>Erythrophage</td>
<td>33.8 ± 37.3 *</td>
<td>0.0 ± 0.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>1358 ± 239</td>
<td>1280 ± 273</td>
<td>0.69</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>609 ± 113 *</td>
<td>218 ± 59</td>
<td>0.01</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>15135 ± 7865 *</td>
<td>3.9 ± 3.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>19.7 ± 7.5</td>
<td>33.5 ± 10.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Mast Cell</td>
<td>16.2 ± 5.8</td>
<td>24.9 ± 7.4</td>
<td>0.37</td>
</tr>
</tbody>
</table>

* Mean is different to control mean

Table 3: Relative risk of developing EIPH 24 hours following inoculation of 0.01% acetic acid compared with control treatment.

<table>
<thead>
<tr>
<th>EIPH Positive</th>
<th>EIPH Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Acetic acid</td>
<td>5 (71.4%)</td>
</tr>
<tr>
<td>Control (no inoculation)</td>
<td>1 (14.3%)</td>
</tr>
</tbody>
</table>

Relative risk = 71.4 / 14.3 = 5

When a McNemar’s test, corrected for correlated proportions was conducted on the figures it showed that this risk value was not significant at the 95% confidence level but was significant at the 90% confidence level. Alternatively if the sample from the control site which was suspected of being contamination rather than a true episode of EIPH is discounted the test yields a significant result with a p-value of 0.03.

Bronchoalveolar lavage samples from 5 of the 7 acetic acid inoculated segments were observed to have a red tint when aspirated. None of the lavages from the control segments
displayed this gross indication of haemorrhage. Of the 5 inoculated bronchial segments which haemorrhaged, the minimum percentage of erythrocytes observed was 39%, however in the 1 control lavage which contained erythrocytes there was only 2.9%, suggesting that this apparent haemorrhage may in fact be induced by the lavage procedure, as occasionally can occur. Both the percentage and absolute cell counts of erythrocytes differed significantly between the acetic acid treated and control segments following exercise. There was insufficient time for the degradation of erythrocytes and production of haemosiderin.

The percentage of neutrophils in the control samples (4.4 ± 0.3%) is consistent with normal values for BAL samples, and was significantly different to the elevated neutrophil percentage (12.3 ± 1.1%) in samples from inoculated segments. This apparent inflammatory response, 24 hours following inoculation with 0.01% acetic acid, was equivalent in intensity to the response observed in the pilot study horses and in samples collected in the previous study (10.5 ± 2.8% neutrophils) 1 day after inoculation of intrapulmonary blood. There was an associated reduction in the percentage of macrophages in the acetic acid inoculated segments, which appeared to be reciprocal to the increased neutrophil percentage. The absolute numbers of macrophages per µl of ELF were not different between the inoculated and control segments, although there was a significant difference in the absolute neutrophil concentration. Percentages and numbers of lymphocytes, eosinophils and mast cells did not differ significantly between treatments.

2.4 Discussion

The increase in neutrophil percentages and concentration strongly suggest that the 0.01% acetic acid solution inoculated, provokes a mild inflammatory reaction within the lower airways of the lung, similar to that caused by the presence of blood in the airways. The fact that this level of inflammation was sufficient to predispose the lung to EIPH has important implications for horse management. Neutrophil percentages of approximately 10% are not uncommon in horses undergoing training (McKane et al 1993; McKane and Rose 1995), and lavages from horses with clinically mild respiratory disease may have neutrophil percentages as high as 25-30% (Fogarty and Buckley 1991). If these clinically silent levels of pulmonary inflammatory disease have the same ability to predispose the equine lung to EIPH as the inflammation caused by the inoculation of 0.01% acetic acid then the role of inflammatory disease in the aetiology of EIPH has been greatly underestimated in recent years. Even if the situations are not directly comparable there is obvious reason for equine trainers and veterinarians to reconsider the practice of working a horse through a mild bout of respiratory disease.

The results of this study support the idea that the inflammatory response provoked by previous episodes of EIPH could play a role in the development of new and perhaps more severe episodes of haemorrhage. This would be particularly so if horses are strenuously exercised within 3 days of an episode of EIPH, as may happen during seasonal racing carnivals, contradicting an old trainers' anecdotal theory that if a horse 'bleeds' during track work on Thursday it will do well racing on Saturday. The ability of previous episodes of EIPH to increase the severity of future EIPH agrees with the epidemiological data which indicates an increase in prevalence and severity of EIPH with the increasing age of racehorses (Mason et al 1983; McKane et al 1993).

In this study the segments inoculated were located in the cranioventral regions of the caudal
lobe, which are generally considered not to be involved in EIPH. These segments were chosen partly because they were expected to be free from the effects of previous EIPH and because they are more easily accessible to bronchoscopic examination. This study proves that under circumstances of inflammatory disease the cranioventral pulmonary segments of the horse are subjected to sufficient forces to induce haemorrhage during strenuous exercise. It must therefore be concluded that racehorses do naturally haemorrhage into the cranioventral lung segments at certain times, although the prevalence of this may be low enough that the changes known to occur in association with EIPH (O'Callaghan et al 1987h) are infrequently seen and far more subtle than those lesions observed in the caudodorsal regions of the lung. The cause of the increased propensity for EIPH in the caudal lung, would appear to be generally more important in the development of EIPH than low grade pulmonary inflammatory disease.

The obvious validation to the conclusions of this study would be a study of the effect intrapulmonary blood inoculation has on the development of EIPH. This was not possible at this time because it is not possible to determine whether exercise 3 days after intrapulmonary blood inoculation resulted in fresh haemorrhage into the inoculated segment, or whether the inoculated blood is still present. This is because of the slow removal of extravascular erythrocytes from the alveoli and the lack of a method to exactly determine the volume of EIPH in the lung at any single moment. One technique to overcome this situation would be to add a label to the circulating blood which would be detectable on bronchoalveolar lavage only if haemorrhage had occurred into the region. Unfortunately a labelling technique which provides this characteristic and is not diluted too greatly by the blood volume of the horse has not been identified, despite extensive research and testing in our laboratory.

### 2.5 Implications

This research indicates that minor inflammation predisposes to EIPH, at least when inflammation is acute. The effects of chronic inflammation are unknown. However, minor acute inflammation has been described in horses exposed to urban pollutants, stable dusts and to infectious agents. A heightened awareness of the potential risks of non-specific inflammation precipitating EIPH or making it more severe should be of interest to the racing industry and would suggest that the use of anti-inflammatory drugs in the non-specific treatment of EIPH should be investigated.
3 Estimation of Intrapulmonary Haemorrhage in Horses With Experimental EIPH using $^{99}$Technetium-labelled erythrocytes.

3.1 Introduction

Currently, there is no reliable method to identify the volume of intrapulmonary haemorrhage after an episode of EIPH, yet this information is probably the most important of all in order to devise individual, rational treatment for the disorder. This project seeks to test whether the volume of haemorrhage in the lungs can be detected accurately using $^{99}$Technetium labelled erythrocytes. For this method to work, labelled cells free within the lung must be discriminated from background arising from labelled cells present within the pulmonary vasculature. From previous physiologic studies we know that as little as 50 ml of inoculated blood is sufficient to impair performance in maximally exercising horses. Therefore, for this technique to be of clinical value, minimum volumes of intrapulmonary haemorrhage in the order of 50 ml should be detectable.

3.2 Methods

Part A: Determination of signal with no vascular background

Erythrocytes were obtained by jugular venipuncture into ACD solution as anticoagulant, washed and labelled according to identical protocols for $^{99}$Technetium labelling of human blood. Aliquots of labelled blood were injected into regions under endoscopic guidance and gamma imaging of the thorax performed. The intensity of any radiolabelled signal was compared with control samples placed either side of the chest in order to determine attenuation of radiation caused by tissue absorbence.

Part B

Providing a reliable signal can be identified under "no background" circumstances, studies were to proceed with a second phase, where similarly labelled blood was injected intravenously, horses were to be treadmill exercised at intensities expected to cause minor EIPH, and then thoracic scintigraphy performed to determine whether "hot spots" consistent with sites of haemorrhage developed. These sites of putative haemorrhage were to be confirmed by lavage of affected regions and haemorrhage confirmed both by determining the radioactivity of lavage samples and by routine cytology.

3.3 Results

A: Initial Feasibility Studies

Five ml of autologous blood from a healthy horse were labelled with 39.7 mCi Te99m using an Ultratag Kit. Labelled blood was inoculated into the right caudal lung field under endoscopic guidance. External gamma scintigraphy detected labelled erythrocytes in the caudal dorsal lung at the inoculation site, and no background activity was noted in the remaining lung fields. In order to evaluate a different labelling method, 200 mCi of Te99m-DTPA was administered intravenously and allowed to distribute within the circulation for 30 min. At this time, 25 ml of venous blood was collected and immediately inoculated into the right caudal lung field under endoscopic guidance as previously. External gamma
scintigraphy failed to detect labelled erythrocytes in the caudal dorsal lung. In addition, significant background activity was present in the remaining lung.

Two additional pilot studies were conducted. Using an Ultratag kit, 97 mCi Tc99m was used to label 5 ml of venous blood and this was injected intravenously. After 30 minutes to allow for redistribution and thorough mixing in the circulation, the horse was treadmill exercised (10 m/s) for 15 minutes, and post-exercise venous blood collected. External gamma scintigraphy failed to detect labelled erythrocytes in caudal dorsal lung, but lavage of this area obtained after imaging contained radioactivity. The ratio of activity BAL / Post-Exercise blood = 0.000615. This protocol was repeated using another horse with 121 mCi Tc99m Ultratag labelled blood, and in this case, external gamma scintigraphy detected slightly increased activity in the caudal dorsal lung.

Lavage was collected as before after imaging and the ratio of activity BAL / Post-Exercise blood = 0.000430

**B: Treadmill Studies**

Normal thoroughbred horses, (n=8) were studied using the experimental protocol developed in feasibility trials. Each horse’s individual VO2 max had been previously determined and all horses exercised at treadmill speeds to give the same exercise intensity. Using Ultratag kits, 5ml of labelled autologous venous blood was injected intravenously, horses stood quietly for 15 min to allow even distribution of labelled erythrocytes within the circulation, and the animals then exercised at 90% VO2 for 10 min. Immediately post exercise, horses were scanned, again at 15 and 30 min post exercise, and then the caudal lung fields lavaged as in the pilot protocol.

BAL/Post-Exercise blood ratios ranged from 0.00011655 to 0.007964. The analysis of the scintigraphic images showed definitive increased activity in the caudal dorsal lung field (suggesting EIPH and the accumulation of labelled erythrocytes) but changes were subtle and not detectable on initial subjective evaluation. Substantial background activity was present throughout the lung fields interfering with evaluation in the caudal dorsal lungs in particular. Computer determined activity levels within regions of interest centred over the caudal dorsal lungs appear to show a marginal increase activity relative to background lung when absorption using external calibration standards are taken into account, but computer modelling to date has not allowed accurate estimation of the volume of haemorrhage.

**Control Studies:**

Four of the horses used in the above exercise protocol were used in a “control” group 4 weeks after the first treadmill studies. Experimental protocols were identical to the treadmill study except the horses were not exercised, and were restrained in stocks for the same time period required to complete the treadmill exercise.

BAL/Post-Exercise blood ratios ranged from 0.006865 to 0.131635.

This is a completely surprising result, as the control group’s BAL/Blood ratios were greater than the exercised group’s BAL/Post-Exercise ratio.

The computer analysis of the scintigraphic images from this group fails to show any definitive increased activity in any specific region of the lung field. Substantial background activity
was also present throughout the lung fields, interfering with evaluation in the caudal dorsal lungs. Computer determined activity levels within regions of interest centred over the caudal dorsal lungs are currently being compared to background lung regions but controls are invalid because of radioactivity present in the lavages.

3.4 Discussion

These studies were undertaken with substantial technical difficulties. For example, all equipment had to be decontaminated, and where this was not possible, items such as animals, endoscopes and treadmill had to be isolated for ~48 hours after each use. Provision had to be made to ensure all urine voided during treadmill exercise or immediately after injection of the label was collected, since this has a high level of radioactivity because technetium is largely excreted in urine. Lavage samples for cytology had to be stored refrigerated until radiation had decayed to a safe level before routine processing. The tri-State imaging facility has a highly trained staff and state of the art equipment that allowed high resolution scans to be attempted, but nonetheless computer image analysis has been laborious.

While some evidence exists to indicate that EIPH may produce a detectable change in scintigraphy scans of the thorax using radiolabelled autologous blood, changes are subtle, require extensive computer manipulation and currently is totally impractical as a diagnostic tool for evaluation of client animals. The differences between control and treadmill horses remain unclear, and the mechanism for post exercised enhanced background radiation is also not known.

3.5 Implications

Without a clear indication that this method, with minimal modification, could lead to accurate assessment of the extent of EIPH in vivo, further research was not considered appropriate under the current RIRDC grant. This problem remains an unsolved one for the industry, and semi quantitation of the severity of haemorrhage using lavage methods remains the only practical method. Similar studies to these described above have been conducted in Belgium by Votion and co-workers, and similar difficulties in interpreting results arose. With little prospect of high resolution scintigraphy available for horses within this country in the near future, our assessment is that research efforts should now be directed elsewhere.
4 General Discussion

In the studies described above several key areas concerned with the effects of the presence of intrapulmonary blood on the function and health of the equine lung have been examined. Practical experience in the diagnosis and evaluation of horses suffering episodes of EIPH had provided the authors with several preconceived ideas of how intrapulmonary blood altered alveolar cytology and also how erythrocytes were removed from the lung. The results of these studies correlate well with these clinical experiences and have helped to clarify the exact manner in which EIPH alters the pathophysiology of the equine lung. The responses of the equine lung to intrapulmonary blood are significant and the effects on performance and pulmonary health have great importance to the Thoroughbred racing industry, because of its high prevalence, no preventative therapy, and no cure.

The results of these studies indicate that not only does the blood haemorrhaged during EIPH evoke an acute inflammatory response but may also may predispose to new episodes of EIPH. However, it is the chronic inflammatory changes and possible pulmonary fibrosis that may have the greatest effect on the health of the lung and development of future episodes of EIPH. Further studies are required to determine the role of alveolar fibrosis in the development of subsequent episodes of EIPH and also if fibrosis does predispose to EIPH, research to mediate this response is also indicated.

Autologous intrapulmonary blood was seen to provoke a mild but persistent inflammatory response within the alveoli of the lung, which was characterised initially by an increase in neutrophil numbers. The intensity of this neutrophilic inflammatory response observed appeared consistent with an inflammatory lesion that is capable of predisposing the local area to EIPH. The duration of this acute phase of the inflammatory response after intrapulmonary blood inoculation was noted to be approximately 3 days. In some cases, horses competing in racing carnivals may be required to race a second time before this acute phase of the inflammatory response has resolved. The potential for the inflammatory response induced by the presence of intrapulmonary blood to predispose the lung to new episodes of EIPH has great implications for the management of racehorses, particularly older horses which may have a pre-existing level of chronic lung injury and an exaggerated tendency to haemorrhage. It would seem logical that these horses require rest periods of 2 - 3 weeks following the development of a significant volume of intrapulmonary blood, to allow resolution of the inflammatory process and removal of the blood from the parenchyma.

The prolonged time for removal of blood from the alveoli also has additional effects because persisting blood in alveoli may cause local inhomogeneities in ventilation even though the extensive flooding of alveoli by acute phase oedema resolves rapidly. Data from these studies indicate that a greater awareness of the potential of one episode of EIPH or any other cause of pulmonary inflammation to predispose the lung to EIPH should be acknowledged. Central to this awareness is the need to rest horses that display even slight respiratory tract disease, and to provide sufficiently long periods of convalescence for horses after an episode of EIPH to allow resolution of the inflammatory process.

Many of the results of these studies have been previously suggested by authors speculating as to the possible causes and effects of EIPH, but very few had actually been proven to occur prior to this work. Now that these effects have been proven and their interrelationship demonstrated there is an obvious need for further work, directed at modifying the responses
of the lung to intrapulmonary blood and altering the rate of clearance of erythrocytes, to alleviate the adverse impact of EIPH. The key for this work appears to be a need to increase the removal rate of erythrocytes from the lung while minimising the adverse affects associated with prolonged macrophage activation. Perhaps the best way to reduce the impact of EIPH and preserve the athletic performance of older horses would involve limiting the development of pulmonary fibrosis after episodes of haemorrhage, commencing when the horses are young. Therapies designed to act on modifying the lung's response to EIPH will not provide a cure or a prevention, however they may retard the cumulative damage occurring throughout a racehorse's career and delay the onset of significant performance loss and thereby prevent premature retirement resulting from EIPH.

Bronchoalveolar lavage is a technique that may be used to qualitatively monitor the severity of EIPH within the lungs of racehorses (Fogarty and Buckley 1991; McKane et al 1993; Meyer et al 1998). Although an accurate volume of haemorrhage cannot be determined, alterations in differential cell percentages in samples from the caudodorsal lung regions help can be used to determine changes in the inflammatory response of the lung and also the relative number of erythrocytes present in the alveolar space. This technique is limited obviously because it does not provide information concerning the extent of lung parenchyma involved in the haemorrhage but it does provide an excellent indication of the health of the particular segment lavaged (Sweeney and Beech 1991). Rises in percentages of neutrophils, erythrocytes and haemosiderophages are all indicators that the lung damage is increasing and the horse may need to be rested to allow resolution of the damage, however further studies of racehorse pulmonary cytology are required to determine exactly what relative erythrocyte percentage indicates that a period of rest from racing is required. While exact determination of the extent of EIPH after exercise using scintigraphy was not possible, some relative estimates of blood volumes are likely given the quantitative cytology data generated in our studies using known volumes of inoculated blood.

It appears from the literature and current theories that Thoroughbred racehorses are destined to suffer EIPH for as long as they are involved in competition. The increase in severity with age appears to be associated with the development of progressive alveolar fibrosis, which is probably a side effect of the prolonged erythrophagocytic response. However further research is required to conclusively prove that alveolar fibrosis predisposes the lung to new episodes of EIPH, but for now it remains a strong probability and a discussion of potential research into ways of reducing the inflammatory response to intrapulmonary blood and reducing the rate of alveolar fibrosis is worthwhile. Delaying the increase in severity of EIPH would involve altering the inflammatory response to intrapulmonary blood so that less pulmonary fibrosis develops in association with each episode of EIPH. Therapy would need to begin in young horses at the commencement of their racing careers when the horse first encounters strenuous exercise and presumably EIPH. The following potential therapies are very speculative and will need to be studied extensively for efficacy and adverse responses, before use in horses could be recommended.

The first and most logical way to reduce the length of time blood remains in the lung is to increase its clearance via better ventilation and the mucociliary system. This may be attempted through conventional means such as the nebulisation of β-adrenergic agonists to provide bronchodilation and better ventilation of the collapsed regions, particularly if there is a local bronchoconstriction effect in response to intrapulmonary blood. Water vapour therapy, although it fails to prevent EIPH (Sweeney et al 1987 and 1988) may help moisten blood in the lower airways and enhance its transport out of the lung, and could be
administered concurrently with clenbuterol. The major benefit of water vapour therapy, if it proved to be effective is that it is not a drug and therefore not a prohibited substance under the rules of racing. Specific drugs have been tested in studies on human chronic obstructive bronchitis patients using erythrocytes as markers of the rate of clearance, and reported that oral tulobuterol and particularly inhaled fenoterol significantly accelerated the removal of erythrocytes via the mucociliary system (Matthys et al 1987).

Also of interest is the role of surfactant replacement therapy following an episode of EIPH. Erythrocytes and other blood products bind to SP-A and inhibit surfactant activity (Holm and Notter 1987; Binfeld and Palm 1994; Bruni et al 1996), while elastases produced by neutrophils and macrophages cleave SP-A and also reduce surfactant activity in the alveolus (Pison et al 1989). Administration of exogenous surfactants may help to resolve the acute oedema response and also alleviate alveolar collapse and increase the local compliance. This would reduce the stress placed on surrounding parenchyma during subsequent strenuous exercise and also restore ventilation of the alveolus. Surfactants could be used in conjunction with the other agents mentioned above to attempt to restore the normal ventilatory mechanics of the lung and reduce tissue stress, and facilitate phagocytosis as they are known opsonins for alveolar macrophages.

The activation of macrophages to produce a significant rate of erythrophagocytosis took about 3 days to develop and was maximal at about 7 days. The therapies suggested above may help to accelerate erythrocyte removal and reduce the amount of erythrophagocytosis that occurs in response to intrapulmonary blood, although it is unlikely they will accelerate removal of erythrocytes to clear the blood from the lungs in less than 3 days. Although a reduction on the number of erythrocytes required to be removed via erythrophagocytosis would possibly reduce the amount and duration of macrophage activity and thus reduce the damage caused by the release of elastases and superoxide. Also a reduction in macrophage activity may reduce the resulting degree of fibrosis by limiting the amount of TGF-β released and therefore the degree of fibroblastic induction (Chegini 1997; Finkelstein et al 1997).

The process of macrophage release of TGF-β are central to the development of alveolar fibrosis (Zhang and Phan 1996). If the activity or duration of macrophage activity can be reduced then the amount of TGF-β released will be accordingly reduced as explained above, but there may be more direct methods to alter macrophage response. The duration of macrophage activity may be reduced by enhancing the rate of erythrophagocytosis via up regulation of macrophage activity using cytokines similar to those produced by lymphocytes. Specifically γ-IFN has been shown to activate macrophage phagocytic activity (Munk and Emoto 1995), which would accelerate the removal of erythrocytes from the alveolus and decrease the duration of macrophage activity. Testing would have to be conducted to see if this resulted in a greater overall release of TGF-β and if this translated into greater alveolar fibrosis.

Suppression of macrophage activity is more likely to be of benefit, providing there is sufficient mucociliary clearance and macrophage activity to prevent infection of the lung. Macrophage production and release can be potentially suppressed by a number of means, including α-IFN which has been shown to suppress production of TGF-β and reduce pulmonary fibrosis in mice (Dhanani et al 1994). Its application to the horse is uncertain but worthwhile investigating, as this is the central issue to the long term effects of EIPH on the pulmonary parenchyma. A more general method of suppressing macrophage activity could
be the administration of glucocorticoids via nebulisation and thereby reduce the activation of macrophages already present and possibly reduce the numbers of macrophages which are recruited into the lung (DeBowes and Anderson 1991). It may have other effects, including the alteration of surfactant levels and surfactant regulatory proteins, perhaps offering additional benefits.

The complex interactions and effects of many cytokine and growth factors on multiple target cells make the prediction of the best therapy or combinations of therapies almost impossible. Study of the effects of these potential therapies *in vitro* and *in vivo* are required to determine which if any could be successful in reducing the development of alveolar fibrosis in response to EIPH. Unfortunately even if an effective therapy is discovered it will take several years to prove its efficacy in racehorses because of the difficulty in obtaining data from enough horses to establish that the therapy has altered the epidemiological characteristics of EIPH. However there would be great benefits to the racing industry in reducing the impact of one of the largest causes of economic wastage, and extending the racing careers of many valuable racehorses.
5 References


6 Publications

Abstracts and proceedings


Articles


McKane, S.A. and Slocombe, R.F. Sequential changes in bronchoalveolar cytology after autologous blood inoculation. Equine Vet J. (ICEEP 5 supplement) accepted for publication.


McKane, S.A. Physiologic and pathologic effects of Exercise-Induced Pulmonary Haemorrhage on the equine lung.