ALVEOLAR-CAPILLARY PERMEABILITY CHARACTERISTICS IN CLINICAL AND EXPERIMENTAL ACUTE LUNG INJURY

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ABSTRACT

This thesis reports the examination of microvascular and alveolar epithelial permeability indices in clinical and experimentally-induced acute lung injury. It was prompted initially by the requirement for a direct and specific method of diagnosing and monitoring lung injury in patients with the adult respiratory distress syndrome (ARDS). An additional objective was the use of these techniques as sensitive end-points of lung injury, in studies which examined important aspects of the syndrome's pathogenesis and modulation. Previous experience with alternative methods of directly or indirectly assessing alveolar-capillary integrity, is, as discussed in the initial chapters, largely unsatisfactory.

Two nuclear medicine techniques were modified and developed - the pulmonary transvascular flux of ^{113m}In-transferrin and the pulmonary clearance of aerosolised ^{99m}TcDTPA (diethylene triamine pentacetate) - as indices of microvascular and epithelial integrity respectively. The first study used both techniques to investigate patients with established ARDS. All patients had increased ^{99m}TcDTPA clearance, but the finding was non-specific as the increased rate of solute clearance was similar to that found in smoking control subjects. Increased transvascular protein flux appeared however, to be specific to the ARDS patients. A close correlation was demonstrated between the two permeability indices in the ARDS patients, implying proportionate functional injury to both components of the alveolar-capillary unit. Studies which examined aspects of lung injury pathogenesis, focussed on both cellular and humoral factors. The role of the neutrophil was addressed in a series

of clinical and experimental investigations. Pulmonary clearance of ^{99m}TcDTPA was assessed in nonsmoking neutropaenic patients developing ARDS after bone marrow transplantation. Increased clearance was a uniform finding and histological studies confirmed both severe diffuse alveolar damage and an absence of sequestered intrapulmonary neutrophils. This study demonstrated for the first time that, at least in a specific sub-group of ARDS patients, a neutrophil-independent mechanism could be invoked. Experimentally, lung injury was induced in dogs by cardiopulmonary bypass, a model which was felt to have more widespread pathophysiological and clinical relevance than a direct toxic injury. This procedure was associated with increased transvascular protein flux and morphological endothelial cell injury. The microvascular permeability index correlated well with both intrapulmonary neutrophil sequestration and lung tissue peroxidation. This suggested a possible role for the neutrophil and its oxidant products in this model of lung injury. In contrast, endotoxin infusion in dogs produced severe persistent neutropaenia, implying intrapulmonary sequestration, but no change in transvascular transferrin flux. Of the humoral mediators examined, only platelet activating factor appears likely to play a role in the clinical syndrome. Infusion in dogs produced an increase in extravascular lung water, increased transferrin transvascular flux, and morphological confirmation of endothelial injury. Haemodynamic changes were pronounced but transient; the permeability increase however, was delayed, becoming significant an hour after infusion. Histamine produced a transient increase in ^{99m}TcDTPA clearance in humans and this appeared to be H_2-receptor mediated. Bradykinin did not alter pulmonary clearance of the solute.

Further studies assessed the effect of positive end-expiratory pressure (PEEP) on either or both permeability index, before and after lung injury. PEEP increased DTPA clearance markedly in normal nonsmokers but had no effect in smokers. After lung injury induced by canine cardiopulmonary bypass, PEEP had no effect on DTPA clearance. Prior to bypass, it produced a marked increase in clearance. Transvascular protein flux was unaffected by PEEP either before, or after bypass. The influence of beta adrenergic agonists on established lung injury was studied in an acid-aspiration model in the rat. Salbutamol produced significant accentuation of both increased DTPA clearance and extravascular lung water in acid-treated rats, pointing to a pulmonary haemodynamically-mediated effect.

This research programme has particularly, pointed to the specificity and sensitivity of transvascular transferrin flux as an index of microvascular permeability. Additionally, important insights have been gained into central issues of lung injury pathogenesis and modulation. The pathophysiological and clinical implications of these findings are outlined and discussed.

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DECLARATION

I, Stanley Braude, hereby declare that the work upon which this thesis is based is original and my own work (except where acknowledgements indicate otherwise). Neither the whole work, nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

PLAN OF THESIS

This thesis is presented in four sections. Section 1 starts by reviewing, from a historical perspective, the physiological principles and experimental observations pertaining to acute lung injury. The development of the clinical concept of a syndrome of ''acute respiratory distress'' in adults is traced and the aims of the thesis described. The requirement for a marker of alveolar-capillary integrity is discussed, and previous experience reviewed. In Chapter 3, the methodology of the permeability techniques used in these studies is discussed in detail, and the initial validating study where they are examined in patients with established adult respiratory distress syndrome, is outlined in Chapter 4.

Section 2 (Chapters 5 to 10) describes the use of these techniques in clinical and experimental studies which assessed pathogenetic aspects of acute lung injury. The role of the neutrophil is examined in a variety of studies including experimental cardiopulmonary bypass and endotoxin infusion. Neutropaenic patients developing acute respiratory dysfunction after bone marrow transplant were studied as they constituted a natural clinical experiment to examine the possibility of a neutrophil-independent mechanism. The latter chapters of this section are devoted to an assessment of putative humoral mediators of lung injury. The following section (Chapters 11 to 13) assesses the possible modulating effect of selected interventions, including positive end-expiratory pressure and beta adrenergic agonists, on the permeability indices. These are studied in humans and in animal experiments, both in the context of

pulmonary injury and in normal lungs.

Although each study is discussed in the relevant chapter, the final section (Chapter 14) summarises the most important findings and discusses them in a broader, overall perspective. Figures and tables, where appropriate, are presented at the end of each chapter.

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CHAPTER 1

HISTORICAL REVIEW and INTRODUCTION

1. HISTORICAL REVIEW AND INTRODUCTION

Acute diffuse injury to the alveolar-capillary gas exchanging units of the lung results in a constellation of physical signs that, over the last 20 years, have come to be recognised as the adult respiratory distress syndrome (ARDS). This syndrome represents a final common pathway for many insults that injure the lung directly or indirectly. It is characterised by pulmonary oedema with normal left heart filling pressures in association with specific abnormalities of gas exchange and lung mechanics. Although clinical recognition is relatively recent, the basic physiological concepts governing lung water and solute balance (whose derangement is the key initial step in the syndrome's pathophysiology) were described at the end of the 19th century. In this historical review the evolution of our understanding of the physiological principles governing transcapillary fluid and solute balance and the early experimental work pertaining to acute lung injury will be outlined. In addition the increasing clinical awareness of ARDS-like syndromes over the last 40 years will be reviewed and the requirement for a specific marker of alveolar-capillary integrity discussed. From this perspective, the aims of this thesis are outlined.

1.1 - Physiological principles

Starling, working at the physiological laboratories of Guy's Hospital, described the principles governing transcapillary fluid balance in 1896 (1), although these were not expressed in equation form until 1948 (2). At the time of Starling's original experiments

the secretion/filtration controversy of lymph formation had not yet been resolved. Heidenhain, a German worker, dominated physiological thinking at the time with his secretion theory of lymph formation (3). Starling, in collaboration with Bayliss, initially repeated Heidenhain's experiments and showed like him that ligation of the inferior vena cava dropped arterial blood pressure and increased lymph production (4). Unlike Heidenhain who interpreted this as being incompatible with filtration, Starling showed that inferior vena caval ligation actually increased capillary pressure and that the increased flow of lymph was readily accounted for by filtration. It was however, in his seminal paper in 1896 (1) that he expanded on the original idea of filtration and investigated further the forces governing fluid filtration. His hypothesis is summarised in one of the concluding paragraphs of the paper:

''....whereas capillary pressure determines transudation the osmotic pressure of the proteids of the serum determines absorption.....the osmotic attraction of the serum for the extravascular fluid will be proportional to the force expended in the production of this latter, so that, at any given time there must be a balance between the hydrostatic pressure of the blood in the capillaries and the osmotic attraction of the blood for the surrounding fluids.''

In equation form this states that net fluid flow is the product of the barrier conductance and the driving pressure. In symbols the relationship is expressed as:

$$Q = K_f (Pmv - Ppmv) - r (IImv - IIpmv)$$

where Q is the nett transvascular filtration rate

K_f is the hydraulic conductance or porosity of the microvascular barrier

P is the hydrostatic pressure in the microvascular lumen (mv) and perimicrovascular interstitial fluid (pmv)

r is a reflection coefficient for solutes which describes the effective transvascular oncotic pressure difference. This is dependent on how truly the membrane can act as a semi-permeable membrane and tends to decrease with decreasing molecular size of solute.

II is the oncotic pressure in the plasma (mv) and permicrovascular compartments (pmv).

The corollary of this equation is that oedema in any tissue is due either to increasing driving pressure or altered integrity of the microvascular membrane. Support for the applicability of Starling's original hypothesis to human tissue was provided initially by Landis and Gibbon (5). They showed a linear relationship in the forearm between fluid filtration and increasing venous pressure. It was not however until 1959 that the Starling equation was applied to lung fluid balance studies by Guyton and Lindsey (6). In a classic study, they demonstrated that the critical left atrial pressure at which pulmonary oedema develops decreases with reduction of the plasma protein concentration. In additional support of Starling's relationship they showed that once the critical left atrial pressure had been exceeded the rate of fluid accumulation was unaffected by plasma protein concentration. Thus, although animals with lower plasma oncotic pressures had significant lung water accumulation at a lower critical left atrial pressure, once that pressure had been

exceeded the rate of water accumulation was similar to animals with normal plasma oncotic pressure who had reached their own critical left atrial pressure. This finding was of great importance as it confirmed in the lung the validity of the Starling concept of a balance between capillary and oncotic pressures. The actual rate of water accumulation once the critical pulmonary microvascular pressure had been exceeded was dependent only on K_f, the hydraulic conductance of the barrier to water.

As Staub (7) and Michel (8) have pointed out, the Starling equation refers essentially to fluid flux; solute flux is the sum of simple diffusive and convective flux. The equation describing solute flux is derived from the work of Kedem and Katchalsky (9) and is expressed as follows:

$$Q_s = PS_s\ (Cmv - Cpmv) + 1 - r(C^mQ)$$

where Q_s is the solute flux

PS_s is the permeability:surface area product for diffusion of solute

C is the solute concentration in the microvascular lumen (mv) or perimicrovascular (pmv) space

r is the reflection coefficient

Q is the fluid flux and C^m is the mean concentration of solute across the microvascular membrane

In acute lung injury the pulmonary microvasculature becomes very leaky to solute and when these leaks are large enough to permit proteins to cross the wall, the reflection coefficient falls towards

zero and the osmotic pressure gradient disappears. The hydraulic conductivity, K, increases and (from Starling's equation) the fluid flux is now crucially dependent on the pulmonary microvascular pressure gradient. From consideration of the solute flux equation it is apparent that in this situation marked increases would be seen because of changes in both diffusive and convective flux. This is because acute lung injury results in increased capillary permeability; this alone would increase diffusive flux although it is negated by loss of solute concentration gradient. As the reflection coefficient falls to zero, convective solute flux becomes more important. Thus in clinical or experimental lung injury, alterations in all three inherent properties of the pulmonary microvascular membrane (hydraulic conductivity, reflection coefficient and the permeability:surface area product) are seen. Theoretical models which represent fluid and protein flux do however allow separation of fluid and protein flow (10). A model based on pores of different sizes would allow increased flow of water and electrolytes through very small pores while still remaining totally impermeable to protein. Alternatively, purely increased protein permeability is feasible if a pathway such as endothelial vesicles, which is independent of water flow is hypothesised. Practically however, permeabilty oedema is always associated with increases in water and solute flow (7).

Little is known about the relative importance physiologically of alveolar epithelial and microvascular endothelial integrity in preventing alveolar flooding in pulmonary oedema. Staub maintains that, on the basis of his group's experimental work, that injury to the microvascular endothelial component of the barrier is crucially

significant in permeability oedema (7). Of particular interest however, is the realisation recently that the lung interstitium is not ''dry'' physiologically and that there is constant transfer of fluid from the microvasculature into the lymphatics. The lymphatics have emerged as a key defence mechanism against extravascular lung water accumulation, as they are able to deal with a several fold increase in lymph flow (8).

1.2 - Experimental and pathological observations

Drinker, a Harvard professor of physiology in the interwar years was a pioneer in the investigation of pulmonary oedema pathogenesis. He was a remarkably innovative worker whose contributions included the use of right thoracic duct lymph flow and protein concentration as indices of lung fluid and protein flux (11). Although it is now recognised that lymph flow from the right thoracic duct is significantly influenced by non-pulmonary sources (12), this work did lead to the development of more selective methods of lymph collection which, particularly in the sheep (13), are now established investigative tools. In addition he developed what was probably the first experimental model of acute lung injury, using a thiourea derivative to produce fulminant pulmonary oedema (11). He did however, perpetuate the myth that hypoxia per se was responsible for, and accentuated increased capillary permeability. This contention was based on an experiment in which a dog breathed 10% oxygen and 90% nitrogen for over an hour (14). Towards the end of an hour the cardiac output dropped dramatically, lung lymph flow increased while its protein concentration dropped. In this case increased lymph flow was due not to increased permeability but increased pulmonary microvascular pressures and surface area probably due to a failing left heart. Subsequent studies by another group using isolated perfused dog lungs showed no increase in lung weight even with 0% oxygen ventilation (15). A further study by this latter group used albumin as the tracer to examine alveolar-capillary permeability (16). The results here confirmed their previous study and have established conclusively that alveolar hypoxia per se has no effect

on microvascular or alveolar epithelial permeability. Although true increases in capillary permeability and hypoxemia did co-exist in some of Drinker's experiments they were almost certainly epiphenomena rather than cause and effect relationships.

Moon, an American pathologist, provided an early pointer to the existence of a syndrome of acute lung injury occurring without primary insult to the lungs. In 1941 he published data showing a high incidence of pulmonary oedema in servicemen dying from non-thoracic trauma, burns, acute pancreatitis and infections (17). Interestingly, in the light of current thinking of acute lung injury pathophysiology, some of the patients he described showed histologically (in addition to intra-alveolar haemorrhage and oedema) pulmonary leucocytic infiltrates. At about the same time Eaton published his experimental work carried out in dogs, confirming that acute blood loss with resultant hypovolemia produced increased pulmonary lymph flow with evidence morphologically of pulmonary oedema and endothelial cell damage (18). Like Drinker and others at the time, he considered that pulmonary endothelial cell injury with consequent oedema formation was the direct result of hypoxemia. Eaton did however, make the highly original observation that the oedema fluid in the dogs with blood loss-induced lung injury was relatively protein-rich. This was a key observation as it implied that this type of pulmonary oedema was associated with increased pulmonary capillary permeability. Systematic clinical confirmation of this chance experimental finding only came nearly 30 years later (19).

1.3 - Recognition of clinical acute lung injury

During the second world war surgeons of the United States armed forces provided one of the first clinical descriptions of an ARDS-like syndrome (20). Although their description of ''wet lung in war casualties'' dealt largely with blood in the bronchial tree from intra-pulmonary haemorrhage, they did describe a case of true pulmonary oedema developing days after the initial trauma. They also stated that, pathologically, pulmonary oedema was the most significant finding at autopsy in patients who survived the initial shock but died later of their injuries. In the immediate post war years a similar syndrome was recognised largely by the description of the lung pathological features at autopsy. The term ''congestive atelectasis'' was used to describe the gross and diffuse lung changes of intra-alveolar haemorrhage and oedema noted in patients who developed respiratory failure after a variety of precipitating events. Although this was thought initially to be a complication of intravenous fluids (21), Berry et al pointed out in 1963 that these changes could develop with normal central venous pressures (22).

The first comprehensive clinical description of a syndrome of acute lung injury was by Ashbaugh and his co-workers in 1967 (23). Impressed by the resemblance of the clinical and pathological features to infant respiratory distress, they termed the syndrome ''acute respiratory distress in adults''. Although this was purely a clinical contribution they did speculate accurately about possible pathophysiological mechanisms:

''In view of the similar response of the lung to a variety of stimuli a common mechanism of injury may be postulated. The loss of lung compliance, refractory cyanosis and microscopic atelectasis point to alveolar instability as a likely source of trouble''.

Further evidence of a syndrome of acute lung injury developing after major trauma came from the pathological and clinical findings in American combat casualties during the Vietnam war. An extremely high incidence of pulmonary oedema and intra-alveolar haemorrhage was noted in soldiers who died within a few days of sustaining major trauma (24). Furthermore, similar pathological changes were noted in the vast majority of soldiers dying after head injury, including those who died within minutes of trauma (25).

Clinical and experimental observations over the last 15 years have supported the original concept of a common clinical and pathological syndrome of acute lung injury developing after numerous, diverse precipitating events. Pulmonary oedema has been recognised as a basic abnormality in this syndrome (26); the advent of the Swan-Ganz flow directed catheter into clinical medicine (27) allowed the further accurate separation of this syndrome, with its normal left heart filling pressures, from hydrostatic pulmonary oedema. As previously mentioned, altered capillary permeability with protein-rich oedema fluid allows further differentiation from hydrostatic oedema. Adult respiratory distress syndrome represents however not a single disease, but a syndrome of acute diffuse lung injury defined only by the presence of specific physiological and

radiological abnormalities. Indeed the very coining of the term by Petty and Ashbaugh in 1971 (28) aroused intense nosological debate (29,30,). Murray argued that not only was the pathophysiology entirely different from infant respiratory distress but that '' lumping together'' diverse entities would obscure their probably different pathophysiology and reponses to therapy (30). Despite these misgivings the term has become established and is now virtually synonomous with permeability pulmonary oedema, although it is defined to include only the most severe cases of permeability type oedema.

Usual criteria include abnormalities of gas exchange, reduced lung compliance, with diffuse radiological airspace infiltrates and normal left heart filling pressures as gauged by the pulmonary capillary wedge pressure. Exact values neccesary in order that these variables fulfill syndrome criteria are not uniform; in clinical or experimental studies reported in this thesis, specific values accepted for syndrome fulfillment will be outlined where appropriate. The pathological correlate of these clinical findings is diffuse alveolar-capillary injury with intra-alveolar oedema and haemorrhage (31). The development of the pathological features is usually divided into exudative, intermediate and proliferative phases. The exudative phase is predominant in the first few days after lung injury. Particular features include proteinaceous exudate in the interstitium and terminal airways, intra-alveolar haemorrhage and inflammatory infiltration of the alveolar walls. This merges into the intermediate phase where hyaline membranes are prominent and obvious oedema and exudate diminishes. During the proliferative phase, which is usually only seen after a week, hyperplasia of type 2 pneumocytes

as a healing response to type 1 cell injury is seen. Later fibroblastic proliferation with laying down of collagen is noted. It appears that despite marked proliferative changes virtually full resolution can take place (31). The dichotomy of functional and structural injury should however be emphasised; experimental work suggests that increases in vascular permeability and pulmonary oedema can be demonstrated by physiological measurements where there is no obvious structural abnormality (32),(33). It is possible, as Staub has emphasised (7), that early or mild cases of pulmonary oedema may not necessarily be associated with electron microscopic morphological changes.

1.4 - Requirement for a specific marker of alveolar-capillary injury

The above clinical and histological changes are passive or reactive changes to alveolar-capillary injury. As such they reflect only the sequalae of injury and probably represent an end-stage of the pathogenic process. Early in the development of lung injury, conventional measurements of gas exchange or lung mechanics are insensitive and do not quantitate the specific abnormalities that produce increased transcapillary water and solute flux and result in oedema formation (34). Because of this numerous groups world-wide have intensively investigated possible markers of tissue injury in the syndrome, in the hope that at-risk patients can be identified and appropiate therapy instituted as early as possible in the clinical course. This research effort has been spurred not only by the imprecision of current diagnostic modalities, but also by the knowledge that mortality in ARDS (50-70% in most series) has not changed over the years since the syndrome was first reported (23).

An additional motivation for developing such techniques is that clinical or experimental studies of lung injury pathogenesis or modulation, require specific and sensitive end-points of injury. These should preferably be based on dynamic information and for clinical studies the techniques should be as non-invasive as possible. Since altered permeability of the pulmonary endothelium and alveolar epithelium appear to be cardinal features of the syndrome the particular approach employed in this thesis was based on the development of nuclear medicine techniques to investigate the barrier

characteristics of the alveolar-capillary unit. Details of these techniques as well as the motivation for using them will be described fully in the following chapters. Initially, previous experience with alternative methods of alveolar-capillary functional assessment will be reviewed, with particular reference to their specificity and sensitivity as early markers of lung injury.

1.5 - Aims of the study

1) To develop and assess two nuclear medicine techniques to provide indices of permeability of both lung microvascular endothelium and alveolar epithelium.

To use these techniques to address the following specific issues in lung injury diagnosis, pathogenesis and modulation:

2) the assessment and comparison of both techniques in patients with established severe acute lung injury manifesting clinically as the adult respiratory distress syndrome.

3) the use of either or both techniques as sensitive end-points of lung damage in order to investigate:

a) key aspects of lung injury pathogenesis including the role of the neutrophil and that of putative humoral factors.
b) the modulation of lung permeability characteristics by selected mechanical or pharmacological interventions.

4) To develop an animal model of acute lung injury with clinical and pathophysiological relevance, to facilitate aspects of the study described above.

CHAPTER 2

THE SEARCH FOR A MARKER OF ALVEOLAR-CAPILLARY INTEGRITY IN LUNG INJURY: A CRITICAL REVIEW

2. THE SEARCH FOR A MARKER OF ALVEOLAR-CAPILLARY INTEGRITY IN LUNG INJURY: A CRITICAL REVIEW

The assessment of tissue dysfunction in disease requires an understanding of both normal cell biology and the pathophysiology of the particular disease state. This is exemplified by consideration of alveolar-capillary injury assessment in ARDS, since all markers studied have been based either on an alteration of normal endothelial and epithelial function, or on factors involved in the pathophysiology of injury. This review will therefore include a discussion of relevant cell biology as well as a brief consideration of current concepts of acute lung injury pathophysiology. Particular aspects of the latter will be discussed in more detail, where appropriate in succeeding chapters.

2.1 - Aspects of normal pulmonary endothelial and alveolar cell biology

2.1.1 - Pulmonary endothelial cell

Understanding of the cell biology of endothelium in general, and pulmonary endothelium in particular, has been transformed over the last 20 years (35). The original concept of a metabolically inert, semi-permeable barrier has been altered dramatically by the realisation that the endothelium has specific metabolic and synthetic functions as well as being important to haemostasis. In the lung the importance of the endothelium is magnified, since not only does it

have an important barrier function to leakage of water and macromolecules, but its venous effluent reaches the systemic arterial blood.

a) Handling of biologically active substances by the lung

Both serotonin and noradrenaline are taken up intracellularly and are enzymatically degraded by a process that is energy dependent. The extent of this removal is over 95% of serotonin and 30-40% of noradrenaline. Prostaglandins of the E and F series are also inactivated (36), but prostacyclin is unaffected by passage through the pulmonary circulation (37). Likewise the leukotrienes (LTC_4 and LTD_4) are inactivated but the mechanism for this is uncertain (38).

Angiotensin converting enzyme is situated on the luminal surface of predominantly pulmonary endothelium. It catalyses both the inactivation of bradykinin and the formation of angiotensin II from angiotensin I. The caveolae or pinocytic vesicles on the endothelial cell surface have enzymes including ATPase, ADPase and 5' nucleotide (39) which degrade adenosine mono, di and triphosphate in passage through the lung. Numerous important biological compounds, including adrenaline and angiotensin II are unaffected by passage through the lungs.

b) Pulmonary generation of biologically active compounds

The most important role here is with regard to prostaglandin synthesis; they are formed from arachidonic acid, which is released from membrane phospholipids by the action of phospholipase A_2. Some of the cycloxygenase and lipoxygenase products of arachadonic acid have potentially important roles in acute lung injury (40), and only these are discussed here. Prostacyclin and thromboxane A_2 have contrasting effects on platelet aggregation; prostacyclin is not only a potent inhibitor of platelet and neutrophil aggregation, but also is a potent pulmonary vasodilator. In contrast thromboxane increases pulmonary artery pressure (41) and promotes the interaction of neutrophils with the vessel wall (42). The leukotrienes particularly LTC_4 and LTD_4 (slow releasing substance of anaphylaxis) are chemoattractant for neutrophils and increase microvascular permeability (43). In a preliminary study exogenous LTD_4 caused significant lung injury with increase in extravascular lung water (44).

c) other important synthetic functions

Pulmonary and other endothelium produces one of the components of the Factor 8 complex, the Factor 8 related antigen protein which contains von Willebrand's factor (45). This component of Factor 8 is responsible for platelet adhesion to exposed collagen on damaged endothelium. Factor 8 procoagulant protein, the other component of the complex is not synthesised by endothelial cells (46) and is also distinct from von Willebrand factor; it (Factor 8:c) is a co-factor for Factor 10 in blood clotting.

Fibronectin is a large molecular weight (440,000) opsonically active glycoprotein. In tissue where it is insoluble, it exists in the extracellular matrix of cells as well as between endothelial cells and in the subendothelial space (47,48). It augments adhesion of fibroblasts and endothelial cells to a collagen matrix. Plasma fibronectin mediates clearance of blood-borne non-bacterial particulate matter by the reticuloendothelial system. It can be incorporated into the insoluble tissue pool of fibronectin (49) and is found in increased concentrations in areas of tissue injury and repair (50).

d) Role in local haemostatic balance

Adenosine diphosphate (ADP) is a potent platelet aggregator which is, as mentioned, degraded by ADPase in the caveolae to adenosine, which inhibits platelet aggregation (51). Thus, in addition to releasing adenosine and prostacyclin, inhibitors of platelet aggregation, the lung degrades ADP as well as serotonin (a platelet aggregator) and avoids intravascular thrombosis, maintaining microvascular integrity.

2.1.2 - Aspects of alveolar epithelial cell function

Only type II pneumocyte function will be discussed, since it is the cell that both produces surfactant (52) and is involved in repair after diffuse alveolar injury (53). Surfactant is composed largely of

a highly surface active phospholipid which allows it to meet its physiological function of reducing surface tension and preventing alveolar collapse during expiration. Corticosteroids, adrenergic and cholinergic agents as well as a number of other physiological and pharmacological factors modulate surfactant secretion (54).

Type I pneumocytes, which comprise over 90% of the alveolar surface, are particularly susceptible to injury. Necrosis is followed by Type II proliferation and subsequent differentation to Type I cells (55). In addition Type II cells are postulated to defend the lung against oxidant injury by increasing antioxidant enzyme activity (56).

2.2 - Current concepts of acute lung injury pathophysiology.

Although multiple humoral and cellular factors undoubtedly play a role in lung injury, evidence suggests that the neutrophil is a pivotal cell in initiating the inflammatory response (57). Neutrophils migrate and sequester in the lungs in response to numerous chemotaxins; the most frequently cited is activated complement (58), although experimental studies suggest a role for prostaglandins (59,60), alveolar macrophage products (61), fibrinogen fragments (62) and immune complex mediation (63). The sequestered, activated neutrophil injures the pulmonary endothelium by three principal mechanisms. These are discussed comprehensively in a recent authoritative review (57), and as they are not directly relevant to this thesis will only be summarised here:

(i) The release of oxygen free radicals which are generated by the burst of oxidative metabolism that occurs in neutrophils following membrane stimulation. These include superoxide anion, hydrogen peroxide and the hydroxyl radical which is formed by the action of a granular enzyme myeloperoxidase on hydrogen peroxide. These radicals directly injure endothelial cells and indirectly promote injury by inactivating alpha Protease inhibitor (PI).

(ii) Release of proteolytic enzymes, including elastase, collagenase, myeloperoxidase and others from neutrophil cytoplasmic granules. In addition to degrading lung tissue components, they lyse many other proteins including fibronectin and αPI. They also activate complement leading to further neutrophil activation and sequestration

in the lung.

(iii) Production and release of a range of arachidonate derived products. The possible role of prostacyclin, thromboxane and the leukotrienes have been alluded to previously.

A further possible mediator of neutrophil induced lung injury is the release of platelet activating factor (PAF). This is a potent inflammatory mediator released by a variety of activated inflammatory cells including human granulocytes, platelets, monocytes and alveolar macrophages (64). Once released it aggregates platelets and neutrophils with subsequent release by the neutrophil of proteolytic enzymes, oxygen free radicals and arachidonate derived products. It increases systemic microvascular permeability (65) and preliminary studies suggest it increases pulmonary microvasular permeability (66). The potential of this mediator to effect changes in pulmonary microvascular permeability is addressed in detail in Chapter 10 of this thesis.

Disseminated intravasular coagulopathy is an occasional associated feature in patients with ARDS (67). Radiolabelled platelet kinetic studies in this group of patients demonstrate that platelet consumption and organ sequestration is a prominent finding (68). The radiological correlate of these findings are multiple pulmonary artery filling defects which occur in nearly half of ARDS patients (69). This suggests that platelet-fibrin thrombi may be playing a role in lung injury: possible mechanisms include

activation of complement by thrombin with subsequent pulmonary leucostasis and activation, physical entrapment by the fibrin network of platelets (with release of serotonin, thromboxanes and lysosomal enzymes) and leucocytes in the pulmonary microvessels or a direct permeability enhancing effect of fibrin-derived peptides (70). Additionally, the activation of the Hageman factor by tissue injury and activated complement not only activates factors 11 and 7, but may also proteolytically convert prekallikrein to kallikrein with subsequent release of bradykinin, thrombin and plasmin (71) and potential for further capillary permeability increase.

2.3 - Markers of alveolar-capillary injury.

2.3.1. - Indices of tissue function.

a) Handling of biologically active substances.

Although animal studies with experimentally induced pulmonary injury showed an initial transient rise in serum ACE (72-74), this has not been found in clinical studies. Fourrier and his associates showed that the level of serum ACE fell in the first few days of ARDS evolution reaching a nadir at 72-96 hours; thereafter levels rose gradually to reach baseline levels by the 7th day (75). In this study, the level of serum ACE had no prognostic value, and was not related to any particular precipitating cause. There was however, a tendency for patients with the lowest ACE levels to have disseminated intravascular coagulopathy suggesting that a low ACE may be reflecting loss of pulmonary microvascular perfusion. A more recent study confirmed a tendency to lower serum ACE in ARDS (76). The finding was, however, non-specific in that critically ill patients without ARDS also had low values and the level in an individual patients neither reflected disease severity nor outcome.

This study (76) also examined serum concentrations of another peptidase, neutral endopeptidase, in patients with ARDS. This enzyme, unlike ACE, is not associated with the endothelial luminal surface being found predominantly within alveolar epithelial cells in terminal airways. Concentrations of this enzyme, unlike ACE, were

vastly elevated in ARDS patients, probably reflecting loss of alveolar capillary-integrity and release into the blood stream. Patients with cardiogenic pulmonary oedema and chronic obstructive lung disease also showed some rise in enzyme activation, although not to the extent of ARDS patients.

In experimentally induced lung injury serotonin uptake appears to be a sensitive index of endothelial injury. With rats exposed to hyperoxia, depression in uptake appeared to antedate morphological and physiological alterations (77). This sensitivity was borne out in a later study with alpha naphthylthiourea (ANTU) with uptake depression antedating gravimetric lung water and morphological changes (78). Similar uptake depression has also been noted with a microembolisation model of lung injury (79). Additionally, serotonin is a potent platelet aggregator and may increase vascular permeability; this depression of uptake may actually play a physiological role in injury pathogenesis and progression. Morel and colleagues examined these considerations in patients with ARDS (80). They found that not only was serotonin uptake depressed compared to normal and at risk controls, but uptake depression appeared to correlate with disease severity.

b) Synthetic function : factor 8 complex.

Grant and his co-workers were the first to point out that an altered factor 8 complex was a feature of pulmonary endothelial injury (81). They showed that in patients with acute respiratory insufficiency the

factor 8 related antigen was raised disproportionately to rises seen in factor 8 procoagulant activity, while the Von Willebrand factor was normal. These observations were later confirmed and extended by another group who demonstrated selective rises in factor 8 related antigen in acute lung injury patients (82). This was in contrast to critically ill patients without lung injury, where levels of the three components of the factor 8 complex were proportionately elevated. Additionally, an abnormal electrophoretic pattern of factor 8 related antigen was noted in lung injury patients, which returned to normal in patients who recovered. More recently, a preliminary study also indicated that factor 8 related antigen may be a predictor of progression to acute lung injury in septic patients with minimal or no lung injury when first identified (83).

2.3.2. - Markers implicated in pathogenesis or formed as a result of lung injury

a) Complement.

Hammerschmidt and his co-workers in Minnesota measured plasma C5a concentrations in patients either with - or at risk of developing - ARDS (84). They found that not only were C5a assays positive in the vast majority of ARDS patients but that the assay predicted accurately which at risk patients, followed prospectively, developed ARDS. This potentially exciting finding suggesting that evidence of complement activation may be a useful predictor of ARDS development, has not been borne out in follow-up studies by other groups. Indeed the very basis of the C5a assay used by the Minnesota group - the ability of plasma to aggregate polymorphonuclear leucocytes - may not have been accurate. Weinberg and colleagues in San Francisco measured C5a des arg and C3a des arg directly by radioimmunoassay in patients with sepsis and found that values correlated poorly with plasma neutrophil aggregating activity (85). In addition, they found that neither immunological nor biological markers of complement activation correlated with the initial severity nor predicted the development of the associated acute injury. A similar study recently reported by a Belgian group suggested that although C5a was significantly elevated in early ARDS, it could not be regarded as a specific marker since similar elevations were noted in septic patients who did not develop lung injury (86).

b) Proteolytic enzymes.

Patients with ARDS showed increased concentration of neutrophil-derived elastase in bronchoalveolar lavage (BAL) fluid. In an initial study, this appeared to be associated with reduced protease inhibitor functional activity and unassociated with any physiological index of lung injury (87). The basic finding of markedly elevated neutrophil elastase concentrations in ARDS lavage fluid has been confirmed by subsequent studies (88,89) although the most recent study (89) did demonstrate an association between BAL elastase and alveolar-arterial PaO_2 gradient. Similarly BAL fluid in ARDS patients shows collagenolytic activity directed against both types I and III collagen (90). The data in this study however, neither predicted nor was related to any gas exchange or haemodynamic index of lung injury severity.

c) Markers of oxidant injury.

Although animal studies suggest a role for free oxygen radicals in acute lung injury (91,92), few studies have examined this aspect clinically. A recent demonstration of increased levels of hydrogen peroxide in expired breath condensate in ARDS patients not only supports the notion that oxidants are involved in the syndrome's pathogenesis but suggests future use as a possible marker of oxidant-mediated lung injury (93). Since oral commensals influence peroxide production, this technique is probably limited to intubated patients.

d) Prostanoid measurements.

In patients with septic shock, measurements of plasma thromboxane B_2 were very significantly elevated in non-survivors compared to survivors (94). The implication of this finding with regard to development of an early predictor of lung injury in septic patients is uncertain. Patients who died did however, demonstrate a more severe gas exchange deficit, suggesting possible thromboxane involvement in respiratory distress development. A further study confirmed elevation of TXB_2 level in three groups of at risk patients who developed ARDS compared to those patients who did not (86). Sequential measurement after development of ARDS showed that TXB_2 was relatively slow to rise, reaching maximal levels only by the 3rd to 4th day, thus ruling out its use as an early marker of lung injury. Measurement of 6 keto prostaglandin $F_{1\alpha}$ (the stable metabolite of prostacyclin) showed no predictive ability for either ARDS development or mortality (86).

e) Miscellaneous markers.

The fibrinogen degradation product, D-antigen, was elevated in ARDS patients compared to disease matched controls who did not develop the syndrome (95). Concentrations of the antigen tended to parallel the clinical course but this study did not provide evidence to support an early diagnostic or prognostic role for this marker.

Recent work has pointed to significant elevation of immunoreactive trypsin (IRT) in septic patients with ARDS of non-pancreatic origin (96). Follow-up studies by a Swiss group have confirmed these findings with peak levels of IRT and serum lipase in ARDS patients comparable to those seen in patients with primary acute pancreatitis (97). The rise in IRT in ARDS was associated with a six-fold increase in trypsin inhibitory capacity (TIC) and a low TIC/IRT ratio appeared to be a poor prognostic sign. Levels of IRT do not, however, hold any promise of being a reliable early marker of ARDS evolution as peak values were only seen at 5-6 days.

Significant plasma prekallikrein activation into plasma kallikrein was demonstrated in ARDS patients compared to critically ill patients without ARDS (98). This study was based on a small number of patients and blood samples were drawn at varying intervals after fulfilment of ARDS criteria. Clearly further studies are required to clarify the possible clinical usefulness of this marker.

2.3.3 - Assessment of barrier function of pulmonary microvascular endothelium and alveolar epithelium in acute lung injury

As previously discussed in Chapter 1, acute lung injury with consequent loss of pulmonary microvascular and alveolar epithelial integrity results in increased transvascular water and solute flux. In this section, previous experience with assessment of both of these latter variables will be reviewed.

2.3.3.1 - Measurement of extravascular lung water.

Gravimetric lung water.

Use of this technique as an index of extravascular lung water (EVLW) is obviously confined to experimental work. Assessment of lung weight change during an experimental procedure cannot be performed in an intact animal and is confined to studies with isolated perfused lungs. In intact animals, assessment of lung weight can only be made at one point in time (post mortem) and is usually expressed as the ratio of lung weight to body weight. This particular method of lung water assessment is insensitive, with over 50% increase required for clear evidence of pulmonary oedema (7). The ratio of wet to dry weight, although also only measurable in whole lungs at one point in time, is more sensitive and more widely used. The sensitivity of this method is enhanced particularly by the classic correction for residual blood first introduced by Pearce et al. (99). This

correction is performed either by measurement of haemoglobin concentration in lung tissue or by tagging red blood cells isotopically. In much of the current literature this technique is the "gold standard" against which other parameters of lung water accumulation are compared.

Chest radiography.

Pistolesi and Guintini studied patients with cardiogenic pulmonary oedema, comparing EVLW using a multiple indicator dilution technique, with radiographic grading of oedema (100). Although there were occasional non-specific radiological abnormalities with no or minimal increase in EVLW, a linear relationship between these two variables was noted when EVLW was increased by more than 50%. This important observation was extended by Snashall and colleagues in 1979; they compared lung wet weight to dry weight ratios and radiographic grade in dog lung after experimentally induced hydrostatic and permeability oedema (101). No clear difference in radiographic patterns between the two types of oedema emerged from the study. The most striking feature was that increases in EVLW of greater than only 30% were necessary before oedema was unequivocally recognised by the radiologist. Thus the radiograph appears to be a reasonably sensitive index of lung EVLW accumulation with the particular advantages of being non-invasive, inexpensive and allowing assessment of regional differences.

Indicator dilution.

This technique, once virtually abandoned in clinical practice, has increased in popularity over the last 7 years, largely because of the introduction of an easier and more reproducible method which, with an on-line dedicated mini-computer, allows repeated measurements. The indicator dilution measurement is based on the different volumes of distribution available to an intravascular, non-diffusible (usually isotopically labelled red cells or albumin), and a diffusible indicator (usually tritiated water). Both indicators are injected into a central vein and the extravascular lung water calculated from the difference in transit times between the intravascular and diffusible indicators. The transit times for both indicators are calculated by regular automated sampling of blood from a major artery (femoral artery clinically) where its concentration at each moment in time is calculated relative to the concentration of the injected indicator. Both concentration curves are then plotted semi-logarithmically versus time. The concentration curve of the non-diffusible indicator will obviously rise and also fall quicker than that of the diffusible indicator but the recovery of both indicators from the arterial catheter (expressed as area under the curves) should be equal if both indicators have the same flow characteristics (102). The transit time itself is calculated by fitting a straight line to the descending limb of the curve and extrapolating it to the modal value (103). The EVLW is then calculated as -

$$\text{EVLW} = \text{Intravascular water flow } (t_{diff} - t_{non\text{-}diff})$$

where intravascular water flow = cardiac output x fractional water content of blood.

t_{diff} = transit time of diffusible indicator

$t_{non\text{-}diff}$ = transit time of non-diffusible indicator

This technique, with the indicators as described above, is tedious to perform, requires large samples of blood to be drawn and probably underestimates true extravascular lung water calculated gravimetrically by as much as 50% (7). Because of this, the technique fell into disrepute; there has however, been a marked resurgence of interest with the introduction of an elegant modification which, aided by the introduction of microprocessor technology, has overcome many of the previous theoretical and practical problems. It is based on the use of a thermal and dye marker as diffusible and non-diffusible indicators respectively. The particular indicators used are indocyanine green dye and iced dextrose solution which are prepared and injected in the same syringe. Blood is withdrawn in a sterile manner from the femoral artery, and dye concentration and thermal signal interpreted by a densitometer and a thermistor in the femoral artery catheter. A mini-computer accepts signals from both and computes cardiac output and mean transit time for each indicator.

In contradistinction to the previous method, this technique is suitable for bedside use and all blood withdrawn is injected back into the patient at the end of the measurement. Since its original description by Lewis and colleagues (104), EVLW as measured by this

technique has been demonstrated to correlate well with gravimetric EVLW in animal (105) and clinical studies (106). Clinical studies with this technique suggest that patients with permeability pulmonary oedema have greater lung water content than patients with hydrostatic oedema, although there is significant overlap between the two groups (107). In this study (107), there was a positive but poor correlation between EVLW and pulmonary capillary wedge pressures. Brigham and colleagues however, showed no correlation between EVLW (using a multiple tracer rather than thermal-dye technique) and either pulmonary microvascular pressure or gas exchange deficit (108). Despite the greater clinical use of EVLW measurement in recent years, substantial theoretical and practical problems remain:

(i) the invasive nature of the procedure, which requires either a central venous or pulmonary artery catheter in addition to a femoral artery catheter, restricts its use to intensive care patients only.

(ii) information derived from an individual measurement is non-dynamic and even if serial measurements were used, would give only an approximate guide to clinical progress.

(iii) available data suggests that the extent of EVLW increase in ARDS is not predictive of outcome (108).

(iv) the technique is highly perfusion dependent and this poses difficulties in critically ill ARDS patients. This is because associated inhomogeneities in perfusion may theoretically result in underestimation of lung water, and additionally any small error in

transit time calculation would be magnified should cardiac output be high.

(v) theoretically in early or mild lung injury transvascular water and solute flux may be increased, but observed EVLW will not reflect this until lymphatic clearance is overcome. This theoretical consideration appears to have practical basis since mild experimental lung injury, induced with a low dose thiourea, results in increase in an index of microvascular permeability, but no increase in EVLW (109).

(vi) measurement of EVLW in an individual patient does not appear to discriminate totally between cardiogenic and permeability pulmonary oedema (107).

Other methods of assessing lung water.

Soluble gas uptake.

This is based on the Cander-Foster principle (110) that the concentration in lung gas (relative to their inspired concentration) of two inspired gases of different solubility depends on their solubility coefficient and tissue volume. Since the solubility coefficients of the insoluble (usually helium) and soluble (acetylene) are known, the tissue volume can be calculated. This technique does not however, differentiate between vascular and

extravascular compartments while tissue and water in non-ventilated areas of the lung cannot be detected.

Measurement of extravascular lung density.

Positron lung scanning provides tomographic images of the distribution of both vascular and extravascular lung density (111). Although non-invasive and non-traumatic, this technique because of the very sophisticated technology required, can only be used as a research tool in major centres.

Transthoracic electrical impedance.

The principle here is to measure the impedance of the lung and thoracic wall to alternating current. Severinghaus and co-workers used specially modified electrodes to measure lung impedance independently of chest wall and mediastinal structures (112). The technique, however, appears poorly sensitive with an increase in lung water of over 50% required before a significant change in impedance was noted.

2.3.3.2 - Assesment of pulmonary microvascular and alveolar epithelial permeability characteristics.

Pulmonary microvascular permeability techniques

As discussed in Chapter 1, the advent of the Swan Ganz flow-directed catheter revolutionised the perception of ARDS pathogenesis. The original description by Ashbaugh and colleagues (23) is essentially a clinical description of the syndrome although a prescient allusion to "alveolar instability" as a possible contributory factor was made. Separate descriptions in the early 1970s of cases presenting with pulmonary oedema and normal pulmonary capillary wedge pressures (113-115) resulted in the awareness that -

(i) acute respiratory distress and non-hydrostatic pulmonary oedema were part and parcel of the same clinical problem.

(ii) since wedge pressures were normal, the likelihood was that pulmonary oedema resulted from an increase in microvascular permeability. This view was reinforced by the demonstration that oedema fluid protein concentration was generally over 0.7 of plasma protein concentration (19,113,114).

Systematic clinical measurements of lung vascular permeability would represent a crucial advance in lung injury diagnosis and management, since it would allow direct assessment of an index of pulmonary capillary damage. The vast majority of vascular permeability

techniques measure protein permeability. This is logical since macromolecules both provide a meaningful index of capillary injury and also have important physiological implications. Clinical and experimental experience of methods of permeability measurement will be reviewed by reference to dynamic and non-dynamic techniques. The latter, like indicator dilution lung water estimations, provide an index of content, while dynamic techniques measure flow. Staub has emphasised the insensitivity of content measurements in early pulmonary oedema (7); the theoretical basis for this is (as discussed earlier in the chapter) that fluid and solute flux may be increased in early or mild lung injury but content will not increased until the major inherent lung oedema defence mechanism, lymphatic clearance, is overcome.

Non-dynamic techniques.

The studies described here are all based on the finding of either increased protein concentration in pulmonary oedema fluid or increased clearance of an exogenous isotopically labelled protein into oedema or lavage fluid. Sibbald and his group in Ontario, Canada, have concentrated on clearance studies using ^{131}I-labelled albumin. They reported initially in 1979 that sepsis-associated pulmonary oedema patients with normal wedge pressures demonstrated 10 times the mean albumin clearance of patients with left ventricular failure (116). Subsequently they extended these observations to compare clearance into lavage fluid of labelled albumin with a small molecular weight chelate

(^{111}-InDTPA) in patients with cardiogenic and non-cardiogenic pulmonary oedema. The clearance of both molecular weight solutes was increased in the non-cardiogenic group, with the extent of clearance, particularly of labelled DTPA, showing a significant positive correlation with the net driving pressure (wedge pressure minus colloid oncotic pressure) (117). A further study from this group showed positive correlation in ARDS between albumin clearance and indices of a hyperdynamic haemodynamic state (118).

Potential criticism of the accuracy of these studies relates to the actual method of data acquisition. Since it is a clearance measurement, the calculation is based on -

$$\frac{\text{Tracer concentrations in BAS}}{\text{Tracer concentrations in plasma}} \times \text{BAS volume}$$

where BAS = brochoalveolar secretions

Secretions are aspirated by passing a catheter blindly "deep into the right and left bronchial trees". Since the total volume aspirated, which inherently must be subject to many extraneous variables, is crucial to the clearance calculation, there is potential for subjectivity and inaccuracies. Additionally this clearance method may be measuring airway rather than microvascular tracer clearance.

Sprung and co-workers compared total protein, albumin and globulin concentrations in oedema fluid and plasma in patients with

cardiac and non-cardiac pulmonary oedema (119). Oedema fluid to plasma ratio for all three measurements was significantly greater in the non-cardiogenic group, but there was significant overlap between the groups. The authors concluded that the differentiation of cardiogenic from non-cardiogenic oedema was less important, than actually determining the degree of permeability. This concept of a spectrum of permeability change in pulmonary oedema is not only intellectually attractive, but it also avoids classifying cases on the basis of an arbitrarily chosen value of pulmonary capillary wedge pressure. Use of lung lavage studies to assess microvascular permeability is however problematical, in that changes in protein concentrations may reflect only faster or slower water clearance. Additionally, the data reflect not only microvascular permeability but also that of the alveolar, and to a varying extent, the airway epithelium.

Dynamic techniques.

Study of lung lymph.

This is an excellent kinetic method for examination of lung fluid and protein balance which, because of its inherently invasive nature, is confined to experimental animals. As mentioned in Chapter 1, Drinker at Harvard was the first to popularise the technique but his cannulation of the right lymph duct was only suitable for acute

studies and was significantly contaminated by non-pulmonary sources. Staub and associates in San Francisco have subsequently modified the method so that it is now suitable for chronic studies with pure lung lymph obtainable from awake sheep for up to 4 weeks (120). The preparation involves detailed knowlege of sheep lymphatic anatomy and requires cannulation of an efferent of a large mediastinal lymph node which receives the majority of lung lymph. The procedure entails three separate thoracotomies at 3 to 5 day intervals with careful ligation of all systemic lymph contributions. Two major indices are routinely studied:

(i) Lymph flow - increases in flow per se, could reflect either hydrostatic or permeability causation.

(ii) Lymph to plasma protein (L:P) ratio is used to calculated transvascular protein clearance (flow x L:P protein ratio). Thus hydrostatic oedema is associated with increased flow but reduction in L:P protein ratio. In permeability oedema, although flow increases, the L:P ratio does not fall and, therefore, protein clearance increases.

The preparation is eminently suitable for chronic studies with repeated interventions, and hitherto has been virtually the standard animal investigative tool for lung fluid and protein balance studies. It has been pointed out that the preparation should be used with caution acutely, since baseline L:P ratios in this situation are higher than in chronic studies (121). This implies that the permeability of the microvasculature is greater in acutely prepared

animals, possibly because of the more immediate surgical trauma to the lungs.

Scintigraphic techniques.

a) Pulmonary microvascular permeability

Gorin and co-workers were the first to describe a scintigraphic technique for kinetic measurement of transvascular protein flux in man (122). They used a two compartment model in which the flux of a labelled protein from the intravascular pool (monitored with blood samples) into the lung (monitored with a scintillation probe detector) was examined. Flux into the lung of labelled protein was calculated relative to an intravascular indicator, labelled red cells. They had previously shown that transvascular protein flux with this method correlated closely with protein clearance into lymph in the sheep model (123). Although the mathematics for these flux measurements were relatively complex and no studies were performed in clinical lung injury, Gorin's work did point to the validity and suitability of external radioflux techniques in clinical investigation. At approximately the same time, Prichard and colleagues published their data showing that a similar radioflux technique could be used successfully for vascular permeability assessment in the dog (124).

Despite these innovative early studies, little work has subsequently been done to adapt these techniques for routine bedside

vascular permeability measurements. Computerised gamma scintigraphy was used to study in ARDS patients the flux of a single intravenously injected tracer (^{99m}Tc human serum albumin) from the intravascular pool into the lung, using the heart as the region of interest for the former (125). This was a small study with normal values derived from only 5 control studies and lack of an intravascular marker meant that any change in pulmonary blood volume was uncorrected for. These factors probably accounted for the wide variation in results in the ARDS patients studied - only 6 out of 11 patients showing a significant protein leak.

A more recent study in ARDS patients used a similar approach to that described above, although a diffusible and intravascular radiotracer was used, thus correcting for any pulmonary blood volume change during the course of the study (126). All patients with ARDS demonstrated abnormal pulmonary transvascular protein leak, although as in the above study the normal range was derived from very few control studies. The major problem with this study however, was that only one scintillation detector was used. This detector was moved from lung to heart so that each region of interest was consecutively monitored. This meant that, not only were counts from the two regions not assessed simultaneously, but that potentially a new region of lung interest was being studied with each counting period. The authors recognised this methodological problem and marked the skin in all their studies. My unpublished experience however, is that after any movement of a portable scintillation detector, it is virtually impossible to monitor counts again from the identical region of interest as previously. All results in the above study

(126) were based on only 15 to 20 data points and together with the above reservations would tend to invalidate the study's results and conclusions.

Multiple indicator dilution technique.

This technique is used almost exclusively by Brigham's group at Vanderbilt University in Nashville, USA (127). Two intravascular indicators, labelled red cells and labelled albumin, together with two diffusible indicators, triated water and ^{14}Curea, are injected into a central vein. Indicator curves are constructed as discussed in the lung water section, although the intravascular curve here is a composite of both intravascular tracer curves. The permeability:surface area product for urea is derived by complex mathematical manipulation of all four indicator curves. Further disadvantages of this technique are the multiple radiotracers required, the numerous blood samples and the invasive procedures necessary. An advantage of this technique over the others discussed, is that it alone provides an index of both vascular permeability and exchanging vessel surface area.

b) Alveolar epithelial permeability technique.

Clinical and experimental interest in solute exchange across pulmonary epithelium has grown considerably over the last 10 years. The rationale for the study of alveolar epithelial permeability

characteristics arises particularly from the awareness that it, rather than vascular endothelium, is the rate limiting step to solute exchange across the alveolar capillary barrier. Taylor and Gaar showed that the reflection coefficient for a solute depended on its molecular radius and tended to decrease as the molecular radius decreased (128). For any given molecular radius the alveolar epithelium had a reflection coefficient vastly greater than the endothelium. Staub, reviewing the published data, suggested that over the range of molecular weight solutes that hitherto had been studied, the permeability of the endothelium was at least ten times that of the epithelium (7). Thus on theoretical grounds, the permeability characteristics of the alveolar epithelium should reflect the permeability of the whole alveolar-capillary barrier, especially if the transport of a solute from the alveolar to vascular space (thus including the resistance of the endothelial layer) was studied.

Based on the above considerations, a technique has been introduced over the last 10 years which enables measurement of an index of alveoar permeability to be made routinely in humans. The key initial work was performed virtually simultaneously in three centres: Los Angeles (UCLA), San Francisco (Cardiovascular Research Institute) and London (Northwick Park Clinical Research Centre). It involves measurement of the lung to blood clearance of an aerosolised low molecular weight, inert and neutral hydrophilic solute, diethylene triamine pentacetate (DTPA). This chelate is usually labelled with ^{99m}Tc and is aerosolised with particle size modified to ensure predominant deposition on peripheral non-ciliated epithelium. Since the technique employed in this thesis is a

modification of this method, the detailed methodology will be outlined in the next chapter. Previous clinical and research experience with this technique will be discussed in Chapter 4.

CHAPTER 3

PULMONARY MICROVASCULAR AND ALVEOLAR EPITHELIAL PERMEABILITY INDICES: METHODOLOGY.

3. PULMONARY MICROVASCULAR AND ALVEOLAR EPITHELIAL PERMABILITY INDICES: METHODOLOGY

3.1. - Pulmonary microvascular permeability index.

This was determined by assessing the dynamic accumulation in the extravascular tissues of the lung of an isotopically labelled, intravenously injected protein, transferrin. The technique represents a modification of that initially described by Gorin and colleagues (122) and more recently by Dauber and his co-workers (109).

Like these studies, the method described here is based on external counting of two intravenously introduced isotopes and a simple two compartment model of the lung. The rationale is discussed in some detail in section 3.1.2 but will be briefly summarised here: The flux of intravascular labelled transferrin into the extravascular compartment is dependent on the concentration gradient and the permeability characteristics of the pulmonary microvasculature. Scintillation detectors over the lung and heart detect activity due to ^{113m}In-transferrin. Since the lung probe is ''seeing'' both intra and extravascular protein, dividing its counts by the blood pool counts (from the heart probe) allows correction for the intravascular component of the lung probe counts. Subsequent increases in the quotient:

$$\frac{\text{lung}}{\text{heart}}\ {}^{113m}\text{In-transferrin}$$

over time must therefore represent either increases in lung extravascular protein or lung intravascular blood volume. Changes due only to alterations in pulmonary blood volume are corrected for by simultaneously monitoring ^{99m}Tc labelled red cells in an identical fashion. An increase in the corrected ratio

$$\frac{\text{lung/heart labelled protein}}{\text{lung/heart red cells}}$$

over time can therefore be due only to an increase in lung extravascular protein accumulation. This method has advantages over conventional lung lavage estimations of pulmonary vascular permeability since it is non-invasive, provides dynamic information and is independent of alveolar epithelial permeability. Additionally, it provides a true index of macromolecular transvascular flux, while the lung lavage protein concentration may reflect only changes in lung water clearance, as discussed.

3.1.1. - Radiopharmaceuticals.

Red cell labelling.

Subjects were pretreated with 0.03 ml/Kg of Stannous agent (4.0 mg stannous fluoride, 6.8 mg sodium medronate, Amersham International,

Amersham, UK) intravenously which had been reconstituted with 6 ml of normal saline. When erythrocytes are preloaded with stannous ion, the ^{99m}Tc when subsequently injected is reduced, and becomes tightly bound to the beta chains of globin (129). When red cells are not preloaded with stannous ion, pertechnetate can diffuse freely into and out of the erythrocyte. Twenty minutes after injection of Stannous agent, a 10 ml sample of heparinised blood was drawn from a central venous line in patients, and from a 21 gauge forearm butterfly needle in control subjects. Using sterile solutions and containers, the heparinised blood was centrifuged and the supernatant plasma discarded. After washing the packed red cells in saline, they were incubated in vitro at room temperature with approximately 1 millicurie (mCi) of ^{99m}Tc for five minutes. The labelled cells were then again washed in saline prior to the suspension being made up to 10 ml with normal saline. These labelled cells were then injected intravenously and used as the blood pool marker. Repeated estimation showed red cells binding efficiency using this method to be consistently greater than 95%.

Labelling of transferrin with ^{113m}Indium.

Together with the autologous labelled red cells approximately 1 mCi of ^{113m}In (half-life 100 minutes) was injected intravenously. The ^{113m}In was obtained from a commercially available generator (Amersham International, Amersham, UK) in which the Indium is formed by isomeric transition from the long half-life (118 days) parent radionuclide ^{113m}Tin (Sn). The formed

^{113m}In is then eluted as the hydrochloride by the injection into the generator of 6 ml of 0.04 molar hydrochloric acid.

In vivo Indium binds to the beta globulin transferrin (molecular weight 76,000) (130). The specificity of this binding to plasma transferrin was verified in the following experiment:

Thirty minutes after injection of ^{113m}In, 10 ml of heparinised blood was drawn and the plasma separated. This plasma was then ultrafiltrated (Micropartition system, Amicon, Gloucester, UK) with the intention of separating protein bound from free ^{113m}In. This is performed by convective filtration of free radiotracer through a hydrophilic ultrafiltration membrane with the driving force for filtration provided by centrifugation at 2,000 g. Plasma components (including ^{113m}In bound transferrin) larger than the membrane pores are retained above the membrane while ultrafiltrate containing free radiotracer collects below in the filtrate cup. This system normally results in retention of more than 99.9% of serum protein. By counting radioactivity in the filtrate cup as a percentage of the total radioactivity in the plasma subjected to ultrafiltration, an index of ^{113m}In binding to macromolecular protein was derived. Five separate experiments showed binding of the tracer to protein was never less than 97%. An additional experiment was performed to confirm that the ^{113m}In was bound exclusively to transferrin rather than any other plasma protein. Plasma was electrophoresed on acrylamide gel; subsequently the gel was sliced and the components counted in a gamma scintillation well counter. This confirmed that ^{113m}In was bound exclusively to a protein whose electrophoretic

mobility was consistent with the known molecular weight of transferrin.

3.1.2. - Special instrumentation.

Fifteen minutes after the injection was allowed for isotopic equilibration. Scintillation detectors were then placed over the lung (right upper zone) and heart (as a measure of blood pool activity). The lung detector was placed in the same position in all human studies irrespective of the relative distribution of radiological abnormalities in the ARDS patients studied. The centre of the probe was placed anteriorly, just lateral to the mid-clavicular line at the level of the third interspace. The position of the heart detector was optimised by placing it where counts from ^{99m}Tc labelled red blood cells were maximal. Each detector comprised a 5.1 cm x 5.1 cm sodium iodide crystal (NE 5545, Nuclear Enterprises, Edinburgh, UK) fitted with a flat field collimator. Included in the scintillation detector is a photomultiplier tube whose basic function is to convert the light energy into electrical energy. The magnitude of the photomultiplier tube output is proportional to the energy of the incident gamma ray photon with a mono-energetic beam incident on a sodium iodide crystal producing a main photopeak and a broader scatter band (the Compton scatter) (131). The signal output from the probes was processed by the spectrometer (NE 4697, Nuclear Enterprises, Edinburgh, UK) which was specially modified to include two pulse height analysers and two ratemeters. The pulse height analyser looks at the frequency distribution of the pulse amplitude

and selects the part of the photo-electric spectrum to be counted, while the ratemeter counts the pulses fed to it by the pulse height analyser. Having a second pulse height analyser and ratemeter enables two radioisotopes of different energy peaks (^{99m}Tc = 0.14 MeV; ^{113m}In = 0.392 MeV) from each scintillation detector to be assessed simultaneously.

The output of the ratemeter was interfaced with a dedicated mini-computer (Dragon 64, Compusense Ltd, London, UK) which facilitated display and recording of data. The computer was programmed (see Appendix 2) to define the time period over which scintillations were to be counted. Counting was continuous except for 1 second breaks between counting periods. Thus for each counting period, counts were obtained for activity due to both ^{99m}Tc and ^{113m}In from both the lung and heart detectors. In all studies counts were recorded for at least 30 minutes. Initially the background in the room was subtracted and a correction factor for the Compton scatter effect of the higher energy ^{113m}In on ^{99m}Tc was derived, and applied to subsequent ^{99m}Tc counts. For each counting period the computer calculated (see Appendix 2) a lung to heart ratio for ^{113m}In transferrin and to correct for any dynamic changes in pulmonary blood volume that value (for transferrin) was divided by the lung heart ratio for labelled red cells. The rate of change of this corrected lung heart labelled transferrin ratio was used as the dynamic index of pulmonary transvascular protein leak.

To reiterate, the rationale for the above is as follows: at the time of injection of both radioisotopes, all radioactivity is

intravascular and, therefore, the radioactivity the lung probe is "seeing" at that point is an indicator only of the blood volume in the field of the probe. Additionally it is influenced by physical factors which may affect the relationship of true counts to external counts, i.e. chest wall geometry, tissue penetrance of isotope, the contribution of the chest wall and the counting efficiency of the detector system. To the extent that all red cells remain intravascular and the physical factors remain constant, subsequent changes in red cell counts could be due to changes in activity of the blood or changes in blood volume under the lung probe. Since the intravascular activity is being constantly monitored by the heart probe, changes in the external lung probe ^{99m}Tc red cell signal relative to the heart probe are due to changes in blood volume. Similarly for labelled protein, changes in the lung to heart ratio could be due either to changes in blood volume or extravascular movement of protein. Since changes in blood volume are known from the respective red cell ratio, any changes over time in the corrected ratio reflect transvascular protein flux.

$$\text{Corrected ratio} = \frac{^{113m}In \text{ lung counts}}{^{113m}In \text{ heart counts}} \Big/ \frac{^{99m}Tc \text{ lung counts}}{^{99m}Tc \text{ heart counts}}$$

The rate of change of this corrected ratio was quantitated by plotting the individual points versus time and drawing a computer-fitted linear regression line. The slope of this line, derived from the linear regression equation, was used as an index of lung transvascular protein leak. Extrapolating the line back to time

0, allow calculation of the Y intercept of the regression line. The rate of increase in the corrected lung to heart ratio of ^{113m}In-transferrin (the slope) was divided by this intercept to correct for the physical factors involved in external measurement of gamma counts. This correction was introduced by Dauber and colleagues (109) and is an elegant method of dealing with the only remaining uncorrected variable. Since at time 0 there is no extravascular labelled transferrin, the ratio at this time point reflects only how the physical factors have altered the intravascular volume within the probe field. Dividing the slope by the intercept in each experiment, corrects for physical factors differing between experiments. This quotient was used as the index of vascular permeability and expressed a protein flux units ($x10^{-3}$/min). A representative study is shown in Figure 1.

This method differs from that described by Dauber (109) and earlier Gorin (122) who both used blood sampling instead of probe counts over the heart as their measure of intravascular isotope activity. In three studies, the heart probe was compared with blood samples taken every five minutes to assess the comparability of monitoring isotope activity with both methods. A 2 ml blood sample was drawn on each occasion, the haematocrit determined and the sample weighed. The ^{99m}Tc and ^{113m}In activity of each sample was then measured in a well counter and corrected for haematocrit and weight. The corrected lung to blood pool ratio versus time was then computed using both methods. The results obtained indicated that the heart probe was a reliable indicator of the intravascular pool and consequently this method was continued in all studies. Its advantage

is that it does not required repeated blood sampling and analysis and in addition gives a continuous assessment of intravascular activity. Since ratios are being determined, there is no requirement to correct values for radioactive decay as each isotope is decaying at an equivalent rate in the fields of both detectors.

3.2 - Alveolar epithelial permeability index: pulmonary clearance of 99mTc-DTPA.

An index of alveolar epithelial permeability was derived from the lung to blood clearance of the stable hydrophilic chelate, diethylene triamine pentacetate (DTPA). Its molecular size and radius are approximately 500 daltons and 0.57 nm respectively (132). It was labelled with the gamma emitting isotope ^{99m}Tc, as discussed in chapter 2. Since DTPA diffuses slowly across intact alveolar epithelium, and this accounts for well over 90% of the resistance to its paracellular transport, accelerated clearance of DTPA into the blood suggests that the permeability of the epithelium is increased (132). Factors other than the integrity of the alveolar epithelium may theoretically influence DTPA clearance : pulmonary blood flow, lymphatic drainage or mucociliary clearance could conceivably affect tracer clearance. The molecular weight of DTPA and it's hydrophilic character does however, suggest that clearance is largely diffusion, rather than perfusion dependent (133). This has been borne out in an experimental study by Rizk and co-workers who showed that clearance of DTPA was not affected by changes in pulmonary blood flow, unless the pulmonary artery to the relevant lobe was obstructed completely (134). Lymphatic drainage does not appear to play a significant role because DTPA diffuses readily through lymphatic walls (135). Mucociliary clearance could be important, but this process is too slow to account for overall DTPA clearance (136), and certainly could not affect alveolar clearance.

Clearance of DTPA was measured largely as previously described (137), but because of modifications, the method will be outlined in detail here. A 4 ml solution of ^{99m}TcDTPA (1 mCi per ml) in normal saline was nebulised (airflow 8 litres/minute) using a commercially available Acorn device, shielded in a lead pot. Since the technique's aim was to measure alveolar rather than airway permeability, it was crucial to ensure that the vast majority of particles were appropriately deposited. The Acorn nebuliser alone produces particles with a mass median aerodynamic diameter (MMAD) of 3.8 micrometers (138) and at this MMAD there will be significant airway deposition (139), particularly in patients with chronic airflow obstruction (140). To ensure maximal alveolar region and minimal tracheobronchial deposition, particles should have aerodynamic diameters in the range 0.5 to 2.0 microns (141). A modification to the Acorn nebuliser has been developed by Royston and colleagues which ensures aqueous particles with an MMAD of 0.44 microns (141). Using this particular system, only 6% of all particles are above 2 microns in aerodynamic diameter. With this particle size, the DTPA aerosol will be deposited in the alveoli and distal conducting airways - large airway labelling is not observed (137). Because the surface area of the respiratory tract increases immensely distal to the terminal bronchioles, alveolar and respiratory bronchiolar deposition will predominate, even if a small amount of tracer solute is deposited on small conducting airways.

The separator, consisting of a tube with 2 cm inner diameter partially filled with 3 mm ball bearings attached to the outlet of the nebuliser, is now commercially available (CIS Ltd, London, UK)

and was utilised in this study. The output from the nebuliser and separator is passed into a 2 litre reservoir bag with a one way valve distal to the bag. The bag and one way valve allows subjects to breathe at their own rate and tidal volume. In subjects breathing spontaneously the nose was occluded and each subject inhaled the aerosol for 2-3 minutes until 20,000 to 30,000 counts per minute were recorded over the chest. In intubated patients being ventilated, the radioactivity was introduced by manual ventilation using a non-rebreathing system.

Two scintillation detectors identical to those described earlier were placed over the right anterior chest (same position as in protein flux study, in patients or volunteers having both studies) and right thigh. Once sufficient counts had been achieved, the breathing circuit and aerosol generator were removed from the vicinity of the detectors. With the subject resting supine, counts were monitored for 40 second periods of each minute. With time, the counts over the lung fell as the radiotracer particles were cleared into the blood. Accumulation in blood and tissue was verified by gradually increasing counts from the field detected by the thigh detector. All data was plotted on a semilogarithmic scale versus time (see Figure 2), once corrected for background activity in the room. When the chest counts had fallen to approximately 50% of their initial value, a bolus of ^{99m}TcDTPA (approx. 100 uCi) was injected intravenously and counted continued for a further 10 to 15 minutes. The purpose of this intravenous injection was to correct for ^{99m}TcDTPA accumulating intravascularly and in the chest wall in the field of the lung probe. This produces a curvilinear

disappearance curve to the plotted chest probe counts (see Figure 2). The intravenously introduced isotope is distributed in the fields seen by both probes producing a step-wise increase in counts in the two regions. Once both curves have equilibrated and stablised, they are extrapolated backwards to the moment of injection and a correction factor (increase in lung counts/increase in thigh counts) derived. All thigh counts are then multiplied by the correction factor and subtracted from the chest count to produce a lung clearance curve corrected for blood and tissue background (see Figure 2). Thus:

Corrected chest count = chest count - (correction factor X thigh count)

The rationale of the correction factor manoeuvre is that it attempts to relate accumulation of isotope in blood and tissue of the thigh to similar accumulation (due to recirculation) in the field of the chest probe. The product of the correction factor itself (based on relative accumulation of intravenous isotope in the counting fields) and the thigh count is then an estimation of the blood and tissue radioactivity in the field of the chest probe. The corrected clearance curve thus loses its curvilinearity and is normally mono-exponential.

What are the relative advantages and disadvantages of using scintillation detectors, rather than a gamma camera, to assess DTPA clearance. The most important disadvantage is the lack of regional information; although clearance is increased apically compared to the bases (143), assessment of a region of interest in the upper lobe (as

performed in this thesis) does appear to give reliable data, since increased clearance is reflected in all zones (143). Jefferies and associates compared a portable scintillation detector with a gamma camera (144). They showed that results with both methods correlated closely, thus establishing the probe as a valid method of assessing alveolar epithelial permeability, providing regional information is not specifically required.

Pulmonary ^{99m}TcDTPA clearance is expressed either as the half time of clearance, i.e. the time taken for the corrected initial lung counts to halve, or as the rate constant (per cent per minute) of corrected disappearance. This rate constant K is derived from the half time by the use of the following formula:

$$K = \frac{\log_e 2}{\text{half-time of clearance}}$$

The relationship between these two parameters of DTPA clearance is described in figure 3. The values themselves are derived from a linear regression line fitted to the semilogarithmic plot of the corrected curve. In this thesis, all DTPA data has been calculated with a specially written computer programme (see Appendix 1) used on an Apple II mini-computer (Apple Computer Inc, California, USA). In the studies to be described in this thesis, either the half-time or rate constant of DTPA clearance has been used.

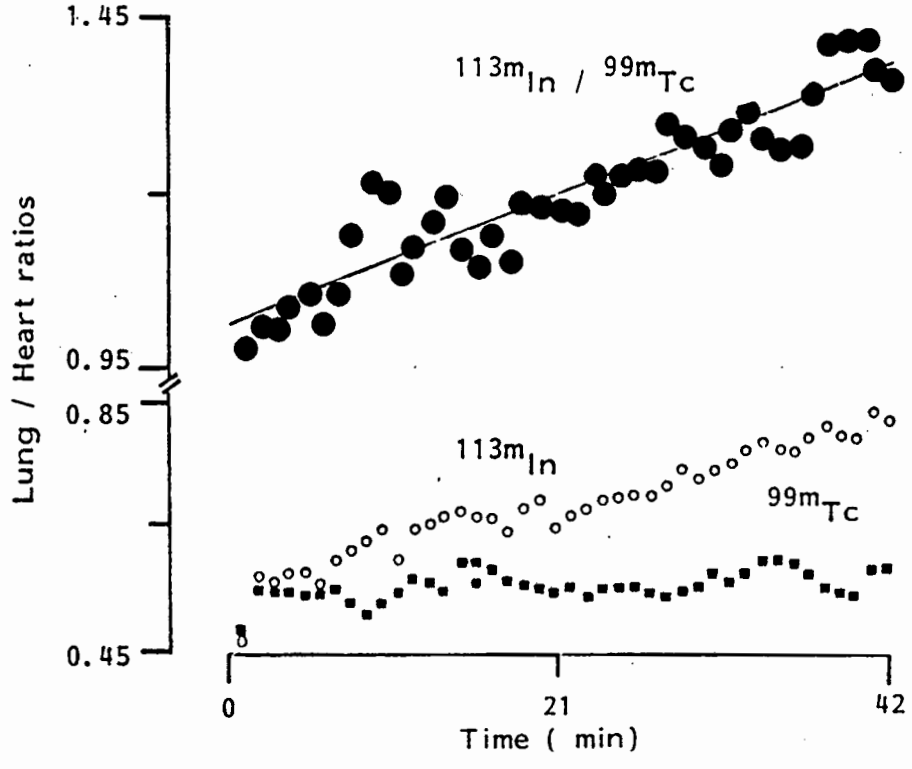

Figure 1

A representative protein flux study in a patient with severe ARDS. The lung: heart ratio for ^{99m}Tc labelled red cells is essentially unchanged over the period of study. In contrast, a similar ratio for ^{113m}In-transferrin gradually rises. The uppermost slope represents the corrected data; the linear regression equation is y = 1.01 + 0.0096 x 1 (r=0.94). Therefore the calculated protein flux index, allowing for correction for physical factors (see text) is 9.5 ($\times 10^{-3}$).

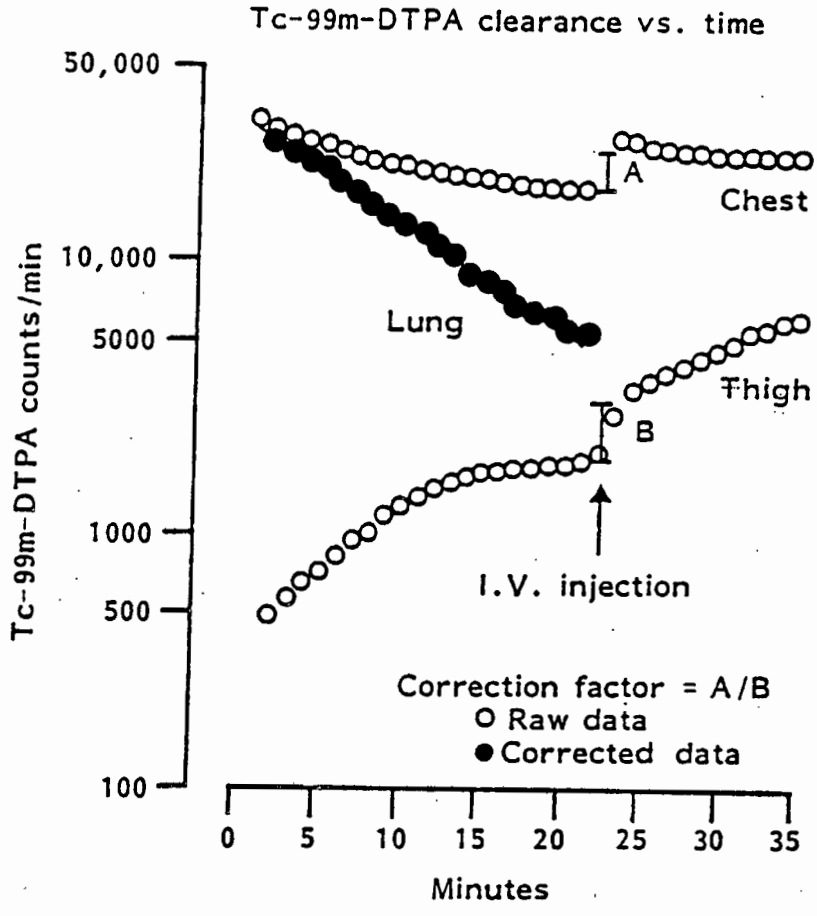

Figure 2

A representative DTPA clearance study showing the curvilinear appearance to the uncorrected data (open circles). The use of the intravenous injection of ^{99m}TcDTPA to yield a correction factor for blood and tissue background is illustrated (see text). The half time or rate constant of disappearance of the corrected (filled circles) data is used as an index of alveolar epithelial permeability.

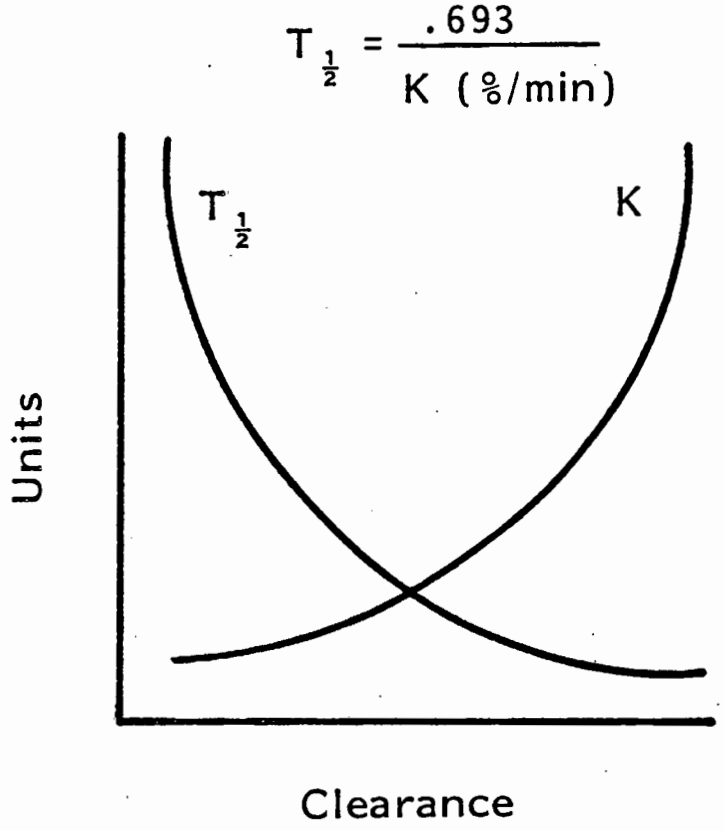

Figure 3

A diagram to illustrate the relationship between the 2 methods of expressing DTPA clearance, both of which are used in this thesis. As the half-time falls clearance and therefore, by implication, epithelial permeability increases; the contrary is obviously true for the rate constant, K. The figure 0.693 is used as it is the $\log_e 2$.

CHAPTER 4

CLINICAL VALIDATION: ASSESSMENT OF LUNG PERMEABILITY INDICES IN PATIENTS WITH ESTABLISHED ADULT RESPIRATORY DISTRESS SYNDROME.

4. CLINICAL VALIDATION: ASSESSMENT OF LUNG PERMEABILITY INDICES IN PATIENTS WITH ESTABLISHED ADULT RESPIRATORY DISTRESS SYNDROME.

4.1 - Introduction.

This initial study sought to examine and compare the specificity and sensitivity of the indices of pulmonary microvascular and epithelial permeability which were described in the previous chapter. Of the two techniques, pulmonary clearance of inhaled, aerosolised ^{99m}TcDTPA (an index of epithelial permeability) has received by far the more attention both clinically and experimentally, in recent years. Although increased clearance of this tracer solute has been documented in a number of clinical situations, few studies have addressed the specificity of the technique in the assessment of patients with acute lung injury.

The sensitivity of the pulmonary ^{99m}TcDTPA technique has been appreciated virtually from the outset, with the observation that otherwise normal smokers demonstrate markedly increased rates of solute clearance. This observation was made initially by Jones and co-workers in London (145) and later confirmed by Mason and associates in Los Angeles (146). Both groups demonstrated 3 to 4 fold increased rates of solute clearance from the lungs of smokers compared to non-smokers, with rapid return towards the normal range 14 days after cessation of smoking (146,147). This finding was felt to represent subclinical respiratory or membranous bronchiolitis, especially since these airways are known to be susceptible to inflammation in smokers (148,149). It was not attributed to alveolar or major airway injury as

respectively, the alveoli do not show inflammatory change in young smokers (148) and both studies excluded airway deposition of tracer solute by scintigraphic studies (145,146).

Further studies have shown increased pulmonary DTPA clearance in patients with systemic sclerosis and pulmonary involvement (150), chronic interstitial lung disease, including cryptogenic fibrosing alveolitis, sarcoidosis and various occupational lung diseases (151), and more recently in neonatal hyaline membrane disease (152). Only one study hitherto however, has assessed the technique clinically in patients with cardiogenic and permeability pulmonary oedema (143). These workers showed that in all but 3 of 14 patients with non-cardiogenic pulmonary oedema (NCPO) studied, clearance rates of DTPA was increased above the 99% confidence limits derived from normal non-smokers. Patients with cardiogenic pulmonary oedema did not show significantly increased clearance rates compared to normals. The data in this study is unfortunately difficult to interpret as 10 of the 14 patients with NCPO had a recent smoking history. In these patients an interval of 3 days to 3 weeks was documented between cessation of smoking and the study. Although the increased clearance in smokers is reversible after cessation, as discussed above, complete normalisation, although usually rapid, can occasionally take up to 3 months (146). Thus the increased clearance demonstrated in the NCPO group was undoubtedly contributed to by the patient's known smoking history. There is additional difficulty in differentiating the clearance in the 2 groups as only one of the patients with cardiogenic pulmonary oedema had smoked in the six months prior to study.

An experimental study has however, pointed to the technique's specificity with the demonstration that dogs with elevated left atrial pressure had clearance rate constants for ^{99m}TcDPTA similar to control dogs. Those animals who had lung injury induced with oleic or hydrochloric acid had clearances significantly greater than both the above mentioned groups (153). Notwithstanding this experimental data, clinically no study has assessed in a valid fashion the specificity of this technique in permeability pulmonary oedema.

Even if pulmonary clearance of the tracer solute is truly increased in acute lung injury, it is imperative to compare the extent of this accelerated clearance with that found in otherwise normal smokers. This comparison, which has not previously been made, is crucial to the assessment of whether increased clearance in a known smoker with lung injury reveals anything about the acute pulmonary disease process. This study compared, therefore, the rate of clearance of inhaled aerosolised ^{99m}TcDTPA in patients with established ARDS with that found in otherwise normal smokers.

Previous clinical experience (125,126) with pulmonary microvascular permeability assessment was discussed in Chapter 2. As was mentioned, the data was both sparse and open to methodological criticism. The technique employed in this thesis overcomes the methodological shortcomings with continuous assessment of both lung and intravascular labelled protein and red cell isotopic activity. Additionally, previous studies did not define the potential role of smoking in modulating pulmonary microvascular permeability. This is potentially important, since a lung lavage study has shown increased

IgG : IgA ratios in smokers compared to non-smokers (154). This finding implies possible transvascular leak of IgG in smokers, suggesting increased microvascular permeability. The relevance of this finding is however, uncertain as the contribution of local airway IgG synthesis and the amounts of airway versus alveolar spaces lavaged are both unkown. Despite these reservations, in order to clearly establish the specificity of the technique further, the extravascular accumulation of labelled transferrin was assessed in both smokers and non-smokers, together with ARDS patients. This study represented the first attempt clinically to define the relationship, if any, in injured lungs between techniques which assessed the barrier function of both components of the alveolar-capillary barrier. This was of particular interest, since morphologists have noted in ARDS disproportionate destruction of the alveolar squamous epithelium compared to the relatively intact capillary endothelium (155).

4.2 - Methods

Subjects.

The diagnosis of ARDS was based on the fulfillment of the following criteria:

1) Sudden onset of acute respiratory failure requiring mechanical ventilation.
2) Diffuse airspace infiltrates on chest radiograph.
3) Evidence of severe gas exchange deficit with the ratio of arterial to alveolar partial pressure of oxygen less than 0.25. The alveolar PO_2 was derived from the simplified form of the alveolar gas equation:

$$PAO_2 = PIO_2 - \frac{PACO_2}{R}$$

PIO_2 is the partial pressure of inspired oxygen. This was derived from the known fraction of inspired oxygen and the barometric air pressure by use of the following formula:

PIO_2 = the fraction of inspired oxygen x (barometric pressure -47)

$PACO_2$, the partial pressure of alveolar CO_2, was assumed to be equal to the arterial PCO_2.
The value of 0.8 was used for R, the respiratory exchange ratio.

The arterial to alveolar tension ratio is more stable an index of gas exchange function than the alveolar minus arterial oxygen difference (A:a gradient) with changing values of inspired oxygen concentration (156). Thus it was felt more valid to use this former index when attempting to set a single standard for patients being ventilated with different fractions of inspired oxygen.

4) Total thoracic compliance less than 35 ml/cmH_2O. This measures the static compliance of the total respiratory system by dividing the delivered tidal volume by the inspiratory plateau pressure. The latter is obtained by temporary occlusion of expiratory flow at the height of inspiration. This provides an index of elastic recoil pressure (157).

5) Pulmonary capillary wedge pressure less than 16 mmHg or central venous pressure (from the mid axillary line) of less than 6 cmH_2O.

Clinical details of the 14 non-smoking ARDS patients studied consecutively are summarised in Table 1. Aetiological predisposing factors were as follows:

Bone marrow transplantation (n = 6)

Cardiopulmonary bypass (n = 2; patient 7 had associcated Staphylococcal septicaemia)

Gram-negative septicaemia with renal failure (n = 1)

Blood transfusion of more than 100 units in association with 3 laparotomies (n = 1).

Post-operative aspiration (n = 1)

Acute fibrosing alveolitis (desquamative interstitial pneumonia or

Hamman-Rich) (n = 1).

Acute pancreatitis (n = 1)

Fulminant hepatic failure with clinical features of raised intracranial pressure (n = 1)

All patients were studied as soon as possible after syndrome criteria were fulfilled. In all cases studies were performed within 24 hours of initiation of assisted positive pressure ventilation. Ten smoking and 10 non-smoking volunteers (respective age ranges 23-58 and 22-64 years) were studied as control groups. All were healthy and none had a recent history of upper respiratory tract infection or medication use. The study was approved by the hospital Ethical Committee and subjects or their next of kin gave informed consent.

Radionuclide techniques.

The pulmonary clearance of ^{99m}TcDTPA and the pulmonary transvascular flux of ^{113m}In-transferrin were performed as outlined in Chapter 3. The salient features, including any particularly modifications introduced will be briefly reiterated. All patients and volunteers were studied in the supine position. All ARDS patients were being mechanically ventilated at the time of study and in 4 patients greater than 6 cmH_2O positive end-expiratory pressure was being applied (maximum 10 cm). Studies were performed consecutively; the DTPA clearance was measured initially and the transferrin flux study started within 30 minutes of its completion. This order of study was chosen as ^{99m}Tc which was used to label DTPA has a lower energy than ^{113m}In.

Pulmonary DTPA clearance.

The DTPA aerosol was generated with a nebuliser equipped with a specially developed particle separator as previously described (142). In subjects breathing spontaneously, the nose was occluded and the aerosol inhaled for 2-3 minutes until approximately 30,000 counts/minute were recorded in the field of the chest probe detector. In all of the ARDS patients radioactivity was introduced by manual ventilation using a non-rebreathing system. Radioactivity was countered with 2 scintillation detectors placed over the right anterior chest and right thigh. An intravenous dose of ^{99m}TcDTPA was used for correction of blood and tissue background as previously described. The decrease in corrected lung counts was plotted semilogarithmically versus time. A regression line was fitted by computer, using the specially written computer programme previously described (for full details see appendix). From this the clearance rate constant K (per cent decline in corrected lung activity per minute) was derived.

Transvascular protein flux.

For this study, scintillation detectors were placed over the lung (right upper zone) and heart (to reflect blood pool activity). The position of the lung probe was uniform in all studies irrespective of the relative distribution of radiological abnormalities. The probe was centred anteriorly just lateral to the midclavicular line in the 3rd right intercostal space. The heart detector was placed in

accordance with its known surface anatomy and the position then optimised by fine movements which maximised counts due to labelled red blood cells. Autologous red cells were labelled in vitro with 1mCi ^{99m}Tc as discussed in Chapter 3. Counts due to labelled red cells were in all cases at least 10 times those due to residual ^{99m}Tc counts from the ^{99m}TcDTPA study. Accordingly ^{99m}Tc counts from the latter source were ignored in the study. After injection of 1mCi of ^{113m}In, the effect of Compton scatter on ^{99m}Tc counts was noted and a correction factor derived which was applied to subsequent ^{99m}Tc counts. After 15 minutes to allow for isotope equilibration, counts due to both ^{99m}Tc and ^{113m}In were monitored continuously from both scintillation detectors for at least 30 minutes.

The ^{113m}In-protein lung:heart ratio for each minute was computed as was a similar ratio for ^{99m}Tc labelled red cells. This latter ratio allowed for any dynamic changes in pulmonary blood volume over the course of the study. The rate of change of the corrected lung:heart ^{113m}In-transferrin ratio quantitated by plotting the individual points versus time and computing the linear regression equation. The linear regression equation-derived slope was divided by the Y intercept at time 0 to correct for differences in physical factors between studies, as discussed in Chapter 3. This quotient was used as the index of vascular permeability and expressed as protein flux units ($\times 10^{-3}$/minute). Since data acquisition was continuous, the linear regression equation was derived from at least 100 data points. This greater density of data allowed statistically reliable slopes to be derived in shorter periods of study than

reported by Dauber and colleagues (109).

Statistics.

For the DTPA clearance study, the 3 groups were analysed by one way analysis of variance and unpaired t tests on logarithmically transformed data. Transferrin flux was compared using the Mann-Whitney U test because of the larger variances. A p value less than 0.05 was deemed significant. All results are expressed as mean ± standard error of the mean.

4.3- Results

The population of ARDS patients studied consisted of 9 men and 5 women with an overall mean age of 40 ± 5.4 years. The disease processes precipitating alveolar-capillary injury (Table 1) reflect the special interests at the Hammersmith Hospital which has an active bone marrow transplantation programme. One patient (no 14) was studied at Kings College Hospital (London) - the equipment and study protocol was identical to that of the other patients. Values for both permeability indices in the 14 ARDS patients are outlined in Table 2, along with relevant physiological and haemodynamic information. The physiological characteristics of the patients studied were determined within an hour of study and reflect in all cases severe ARDS.

Individual data for pulmonary clearance of ^{99m}TcDTPA in the three study groups are represented in Fig.4. The linear correlation coefficient of the corrected DTPA clearance data plotted against time, from which the rate constant of clearance is derived, ranged between -0.96 and -1.0 in the 34 DTPA clearance studies performed. The rate constant K for DTPA clearance in normal non-smokers was 1.12 ± 0.1. This differed significantly from both smokers (4.40 ± 0.8) and ARDS patients (5.71 ± 1.0), $p < 0.001$. All 14 ARDS patients had rate constants for DTPA clearance that were greater than the 95% confidence limits (upper limit 1.7) derived from 10 normal non-smokers. Although the mean rate constant of clearance in the ARDS group was higher than that found in smokers, this did not reach statistical significance ($p = 0.31$).

Transvascular protein flux data in the 3 groups is shown in Fig.5. Unlike the DTPA data, no significant difference existed between the smokers (0.36 ± 0.1 protein flux units) and non-smokers (0.31 ± 0.1) and therefore their data was combined to form 1 control group (0.33 ± 0.1, n = 20). Results in this control group differed significantly from that of the 14 ARDS patients (3.70 ± 1.0, $p < 0.005$). Within the ARDS group, there was significant Spearman's rank correlation between the index of protein flux and rate constant for DTPA clearance in each individual patient ($r_s = 0.77$, $p < 0.01$) (Fig. 6). Unlike the data for DTPA clearance, 3 of the ARDS group had values for the protein flux study that were within the 95% confidence limits established in the combined control group. Failure to detect increased protein flux did not imply a more favourably prognosis as none of these 3 patients survived.

As outlined in Chapter 3 and in the methods section of this Chapter, the value for transvascular protein flux upon which these statistics are based, is derived from the slope of the corrected ^{113m}In-transferrin lung:heart ratio (plotted versus time) and the intercept of this line. Both of these values are in turn derived from the computed linear regression equation. The linear correlation coefficients for the plots of the corrected lung:heart ratios versus time in those patients with positive results outside the control value 99% confidence limits ranged from 0.50 to 0.94 (mean r = 0.74). These regression lines were in all cases statistically significant.

Only 3 out of 14 patients studied (patients 7, 8 and 13) recovered from respiratory failure, and 1 of these died during the

same admission from an unrelated cause (patient 7). Autopsies were performed in 7 cases. Six showed diffuse alveolar damage but in 1 case (patient 10) the features were consistent with desquamative interstitial pneumonia.

4.4 - Discussion

The key features arising from the study are as follows:

1) Pulmonary clearance of the aerosolised solute ^{99m}TcDTPA is increased similarly in otherwise healthy smokers and critically ill ventilated patients with ARDS.

2) Increased transvascular protein flux was demonstrated in the ARDS group but this was not, unlike increased DTPA clearance, an invariable finding. Three patients with classical ARDS by conventional criteria had values for transvascular protein flux within the 95% confidence limits established in the combined control group.

3) Close rank correlation was noted in the ARDS patients between the indices of lung injury used to assess lung microvascular and epithelial integrity.

113m In-transferrin flux

What is the explanation for the failure to detect increased protein flux in patients with classical ARDS? If this is a false negative finding, this reflects a lack of sensitivity of the technique. This is, however, contrary to the experimental findings of Dauber and associates (109) and to experimental work to be presented later in this thesis. The former study (109) showed, using a graded dose of thiourea, that a low dose caused increased protein flux without concomitant increase in extravascular lung water. This,

together with the similar finding to be reported here of increased protein flux in association with minimal morphological injury in one study and minimal increases in lung water in another, underlines the experimental sensitivity of this method of microvascular permeability assessment. It would be reasonable to assume that this applies also in the clinical situation. If, therefore, this is not a false negative finding, what alternative explanations exist for the normal protein flux in 3 of the ARDS patients.

Since blood flow to the more injured areas is reduced (158), normal flux data in these patients may represent more active derecruitment of these regions compared to the other patients. Brigham and associates have however, shown that a low permeability:surface area product for urea, implying active derecruitment of injured areas, was associated with improved oxygenation and a more favourable prognosis (108). The 3 patients in this study all had severely impaired gas exchange and all died during the acute illness. This tends to negate derecruitment as a possible explanation for these findings. Alternatively and probably most likely, is the transient nature of transvascular protein leak in acute lung injury. Although this is not an aspect which has received much clinical or experimental attention, morphological observations suggest that pulmonary oedema formation (and presumably increased vascular permeability) usually reach a maximum within 24 hours of acute lung injury (31). Thereafter the oedema fluid is gradually resorbed and hyaline membranes and interstitial inflammation become more prominent histologically. Although all 3 patients were studied within 24 hours of fulfilment of ARDS criteria, increased protein flux may have been

too transient in these cases for detection with this technique.

Notwithstanding the normal findings in 3 patients, the group as a whole differed significantly from the control subjects and this finding at present appears specific for the syndrome. Complete validation of this would require more systematic study in a group of patients with cardiogenic pulmonary oedema. Although Dauber's study showed experimentally that the technique was unequivocally specific with normal findings in animals with increased left atrial pressure (109), I have subsequent to this initial clinical study assessed 4 patients with cardiogenic pulmonary oedema. All patients had:

1) Radiological evidence of pulmonary oedema.
2) A known predisposing cause (in 2 patients myocardial infarction, and 2 patients acute renal failure with iatrogenic volume overload).
3) Pulmonary capillary wedge pressures > 16 mmHg.

In all 4 cases, pulmonary transvascular protein flux was within the 95% confidence limits established in the combined smoking and non-smoking control group. This finding supports the experimental data and underlines the specificity of the technique.

Inhaled DTPA clearance

In contrast to the data for protein flux, clearance of inhaled ^{99m}TcDTPA was increased in every ARDS patient studied, but this finding was not specific to the syndrome. This study shows conclusively that as far as the extent of increased clearance is concerned, this technique cannot distinguish between ARDS patients and

otherwise normal smokers. This is an important finding, and makes data from the technique impossible to interpret in patients with acute lung injury who have a recent smoking history. A possible criticism relates to the relative paucity of subjects studied for this comparison. This reduces the power of the statistic and makes a statistical type 2 error (false acceptance of the null hypothesis that no difference exists between the 2 groups) more possible. It is however, unlikely in this case that a type 2 error occurred as other larger studies in smokers have demonstrated similar means and variances for the DTPA clearance rate constant to that in the study (159,146).

How can similar tracer solute clearances in the 2 groups be reconciled with their widely disparate clinical situations. The healthy smoking subjects must have nearly normal lung morphology in contrast to the severe diffuse lung injury of the ARDS patients. Similarly increased solute clearance in both groups may support previous suggestions that the site of increased solute flux in smokers is in respiratory bronchioles rather than in alveoli (160). As mentioned, the alveoli do not show inflammatory change in smokers (148). Implicit in these inferences from the DTPA clearance data is the actual deposition of aerosolised tracer solute on peripheral non-ciliated epithelium including respiratory bronchioles and alveoli. Undoubtedly, there must have been some deposition of aerosol on more proximal airways; the relative surface areas of airspaces and airways, together with the DTPA particle size, must however, imply a contribution from the alveoli which is orders of magnitude greater than airways. It does therefore, seem reasonable to regard this

technique as an index of alveolar epithelial permeability.

The DTPA clearance results show that in non-smoking patients a single study showing increased solute clearance implies an active inflammatory process while serial studies have been shown to provide useful information on recovery of alveolar-capillary barrier integrity (161). The use of PEEP in many of the ARDS patients introducing a possible source of error for the clearance data since PEEP increases the clearance of DTPA in normal non-smokers as will be outlined later in this thesis. However, as is discussed in Chapter 12, in the presence of acute lung injury clearance is unaffected by PEEP. This experimental data is consistent with that reported clinically by Mason and co-workers (143). They showed that ARDS patients with PEEP had similar DTPA clearance to ARDS patients not receiving PEEP. It, therefore, seems unlikely that in this study PEEP affected DTPA clearance in the ARDS patients.

Correlation between indices of microvascular and epithelial permeability.

The statistically significant rank correlation in the ARDS patients between DTPA clearance and transvascular protein flux was a surprising finding. These techniques not only employ solutes of very different molecular weights (transferrin approximately 76,000 daltons and DTPA approximately 500 daltons) but also assess discretely different components of the alveolar-capillary units. The microvascular permeability technique, being based purely on a 2 compartment model of the lung (intra- and extravascular), is

independent of epithelial permeability. Clearance of DTPA, although indirectly assessing both epithelium and endothelium since the solute is cleared into blood, has the epithelium as its rate limiting step. This is because the alveolar epithelium and pulmonary capillary endothelium have very different reflection coefficients for a substance of known molecular radius (128). This is particularly marked for small molecules like DTPA (molecular radius 0.6 nm) where the epithelium is virtually ten times less permeable than the endothelium. Thus most of the resistance to diffusion across the barrier of a hydrophilic solute like DTPA is in the epithelium rather than the endothelium. These relationships apply to normal rather than to injured lung. Nonetheless, the strong relationship found between the two indices of barrier integrity, despite the heterogeneity of predisposing conditions to lung injury, suggest that both epithelium and endothelium were damaged proportionately. Thus despite the relatively more severe epithelial rather than endothelial injury morphologically, the functional evidence would imply a positive correlative relationship between damage to each component of the barrier. This is in accord with a recent study of patients with acute alveolitis, where a significant correlation was shown between DTPA clearance and lung lavage albumin concentration (as an index of microvascular permeability) (162).

In summary, although pulmonary ^{99m}TcDTPA clearance is a sensitive technique in ARDS, a single finding of increased clearance in a patient who smokes is non-specific. The use of this technique, therefore, in smokers with clinical acute lung injury cannot be advocated. Transvascular protein flux is not affected by smoking and

appears at present specific for the syndrome. Although this was primarily a validating study and therefore assessed patients with established ARDS, the applicability for these techniques in future should include early or mild injury when lung water and the chest radiograph may be normal. The use of these techniques will hopefully focus attention on this early phase of acute lung injury when the potential benefit of appropriate therapy is greatest. In addition, in established disease and used appropriately, serial studies should aid management by providing more dynamic information about recovery of alveolar-capillary integrity.

TABLE 1: Clinical details of ARDS patients

No.	Age	Sex	Precipitating event	Background clinical information
1.	27	M	Post BMT	Associated GVHD; no pulmonary pathogen
2.	53	F	Massive transfusion	Laparotomies x 4 for uncontrolled GI bleeding
3.	22	F	Post BMT	Cytomegalovirus on open lung biopsy
4.	72	F	Post cardiopulmonary bypass	For closure of post infarct VSD
5.	26	F	Post BMT	Herpes simplex on sputum cytology
6.	27	M	Post BMT	No pulmonary pathogen found
7.	48	M	Post cardiopulmonary bypass	For replacement endocarditic mitral valve
8.	19	M	Postoperative aspiration	Major hepatic surgery
9.	28	M	Post BMT	No pulmonary pathogen found
10.	74	M	Acute fibrosing alveolitis	Known seropositive RA; DIP on autopsy biopsy
11.	70	M	Sepsis	Acute renal failure
12.	23	M	Post BMT	No pulmonary pathogen found
13.	47	M	Acute pancreatitis	Associated renal failure
14.	26	F	Fulminant hepatic failure	Associated raised intracranial pressure

Abbreviations used:

BMT = bone marrow transplant; GVHD = Graft-versus-host disease; GI = gastrointestinal
RA = Rheumatoid arthritis; DIP = Desquamative interstitial pneumonia

TABLE 2: Physiological and permeability data in ARDS patients

	Physiological status at time of study			Permeability indices	
No.	Arterial/alveolar pO_2 ratio	Total thoracic compliance (ml/cm H_2O)	PCWP (mmHg)	Rate constant (DTPA)	Flux units (protein)
1.	0.14	14	10	6.9	9.5
2.	0.18	20	CVP 3cm	1.9	-0.9
3.	0.15	17	12	3.8	3.2
4.	0.23	22	15	2.6	1.6
5.	0.19	24	11	3.7	0.2
6.	0.18	17	CVP 4cm	3.3	-0.3
7.	0.15	20	4	4.6	4.1
8.	0.18	23	10	5.8	7.6
9.	0.15	14	CVP 3cm	7.9	1.6
10.	0.10	18	CVP 2cm	4.9	2.1
11.	0.09	29	10	3.9	1.6
12.	0.10	20	CVP 6cm	13.3	8.5
13.	0.18	22	14	3.4	2.8
14.	0.24	26	7	14.0	10.2

Abbreviation:

CVP: Cental venous pressure (cm water) taken from the mid-axillary line

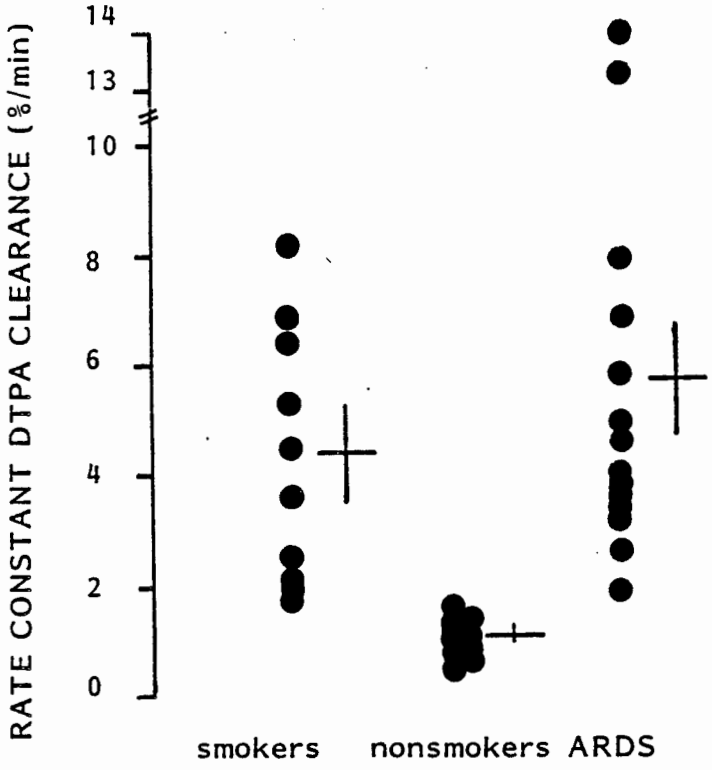

Figure 4

Clearance of DTPA in individual subjects and patients, expressed as the rate constant K (%/min). The means and standard errors are given by the crossed symbol alongside. Nonsmokers versus both smokers and ARDS patients: $p < 0.001$.

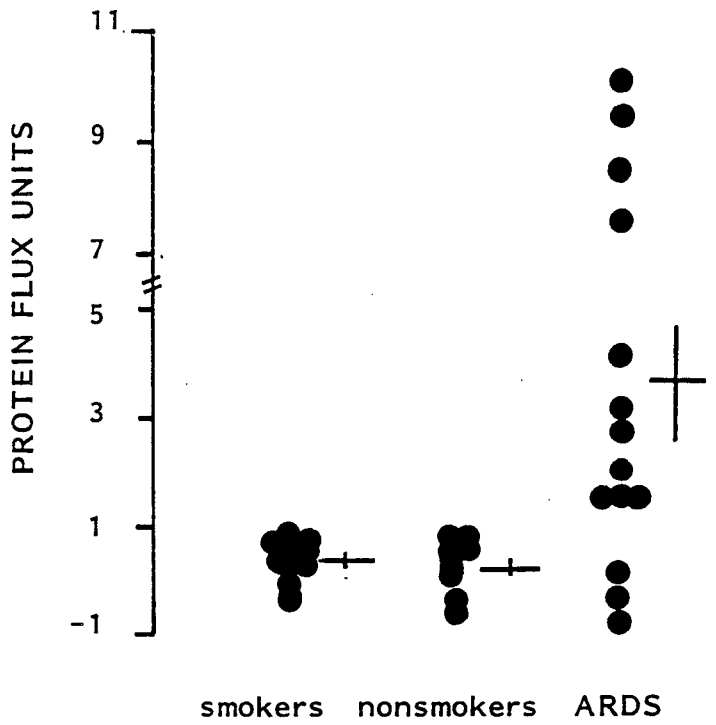

Figure 5

Transvascular transferrin flux expressed as protein flux units (see text) in the 3 study groups. Smokers and nonsmokers do not differ, but this combined group differ significantly from the ARDS group ($p < 0.005$).

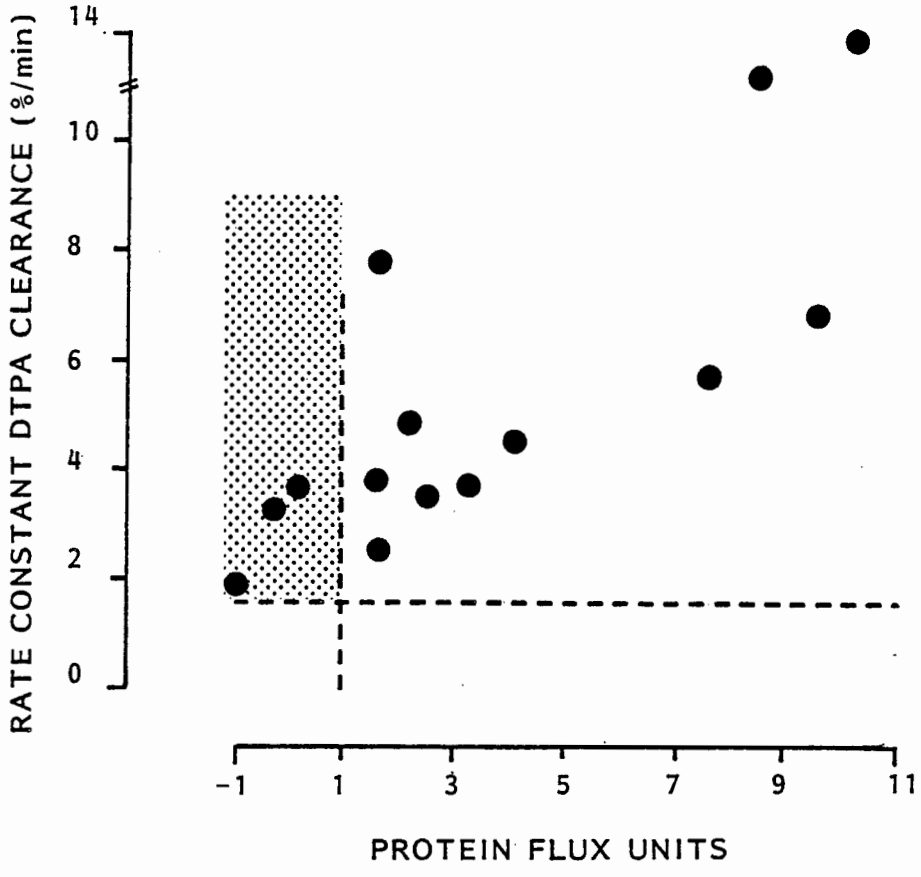

Figure 6

Protein flux units plotted against the rate constant K for DTPA clearance in each individual ARDS patient. Spearman's rank correlation, $r_s = 0.77$ ($p < 0.01$). Vertical and horizontal lines dotted lines are the 95% confidence intervals in controls (see text). The diagonal lined area represents significantly increased DTPA clearance but normal protein flux.

SECTION 2

USE OF LUNG PERMEABILITY TECHNIQUES TO EXAMINE ASPECTS OF ACUTE LUNG INJURY PATHOGENESIS

CHAPTER 5

THE NEUTROPHIL and ACUTE LUNG INJURY - A CRITICAL REVIEW.

5. THE NEUTROPHIL AND ACUTE LUNG INJURY - A CRITICAL REVIEW

In recent years, much attention has been focussed on the neutrophil as a key mediator of alveolar-capillary injury in ARDS. This contention is based principally on extensive experimental work where the neutrophil and its toxic products have been shown to have the potential to cause lung tissue injury. Despite this, confirmatory evidence from clinical pathophysiological studies has been unconvincing. The issue remains controversial and presently constitutes one of the major challenges for workers in this field. Theoretical mechanisms of lung injury mediation by neutrophils were discussed in Chapter 2; therefore, this chapter will deal only with a critical review of data implicating the cell in acute lung injury. The evidence can be summarised as follows:

1) Neutrophils sequestrate in the pulmonary microvasculature and migrate into the alveolar spaces in animal models and patients with ARDS.

The role of complement as a major chemotactic factor for pulmonary neutrophil sequestration was discussed in Chapter 2. The importance of lung neutrophil sequestration to ARDS-related research developed largely from the observations of a group of workers at the University of Minnesota, USA. They were studying the phenomenon of haemodialysis-associated leucopaenia, an observation initially reported in 1968 (163). The Minnesota group showed conclusively that pulmonary vascular leucostasis in animals could be produced by infusion of plasma that had been incubated with a cellophane-membrane

dialyser (58). Additionally, their studies suggested that the dialyser cellophane activated complement and it was this plasma factor which was responsible for the profound pulmonary vascular leucostasis. Although this finding at the time appeared to have little clinical importance, their proposal (164) that an exaggerated version of these events - complement activation, intrapulmonary neutrophil aggregation and neutrophil-mediated pulmonary dysfunction - could eventually produce ARDS, aroused much attention and fundamentally altered the thrust of research at the time.

Over the last 6 years, two major research groups in the United States have addressed themselves to the relationship between complement activation, neutrophil intrapulmonary sequestration and lung injury. Ward and associates have consistently shown that complement activation by cobra venom factor produces lung injury with neutrophil accumulation in rats and mice (165-167). Shaw and his co-workers initially concurrred, showing that intra-tracheal installation of C5a produced intra-alveolar neutrophil accumulation with endothelial injury and oedema formation (168). This injury appeared neutrophil-dependent. Later studies from this latter group showed that neutrophil migration into the airspaces and microvascular injury only occurred when, in addition to complement activation, there were additional stresses including surgical manipulation, hypoxia or prostaglandin E_2 infusion (169). The contrasting results may reflect species difference; nontheless the controversy of whether complement activation per se can produce neutrophil activation and lung injury continues. The clinical finding of the Minnesota group that C5a concentrations in plasma predicted the subsequent onset of

ARDS appeared to give the hypothesis further scientific credibility (84). Although as outlined in Chapter 2, subsequent more sensitive measurements have shown no predictive value for intravascular complement activation in ARDS development, the work of the Minnesota group did focus attention on the aggregated, activated intravascular pulmonary leucocyte as a possible effector cell for lung injury.

Histological evidence to support the hypothesis that pulmonary vascular leucostasis is an important feature in ARDS is limited. This is possibly because of the difficulty of accessibility to tissue early in the clinical course - this is usually limited to autopsy assessment. Existing electron micrographic studies performed early in the course of the syndrome do however, reveal increased numbers of neutrophils in lung capillaries, especially with sepsis-related ARDS (170). Experimental models of lung injury, especially endotoxin infusion, support the clinical evidence with marked neutrophil sequestration in the pulmonary microvasculature (171-173). Neutrophil sequestration has also been demonstrated with other experimental models of lung injury including haemorrhagic shock (174), pneumococcal bacteraemia (175) and inhalation injury (176).

The accessibility of the airspaces for sampling of inflammatory cell populations has led to the popularising of broncho-alveolar lavage as a major tool in acute lung injury research. A recent clinical study indicated that neutrophils constituted the vast majority of the lavage cell population in ARDS compared to mechanically ventilated and normal volunteer subjects (177). This study also indicated that neutrophil recovery in lavage fluid

correlated with gas exchange abnormality and lung vascular permeability. Like previous workers (87,88), they demonstrated neutrophil secretory products including collagenase and myeloperoxidase in ARDS lavage fluid. The major criticism of this work (177) relates to the lack of controls with at-risk factors for ARDS but without clinical features of the syndrome. Another group of workers indeed showed that neutrophils accumulate in the airspaces in patients predisposed to, but without clinical features of ARDS (178). A further recent study examined pulmonary neutrophil kinetics by assessing scintigraphically neutrophil-endothelial interaction in ARDS. Additionally they studied control groups which included patients with sepsis but without respiratory dysfunction and patients with cardiogenic pulmonary oedema (179). They demonstrated significantly greater pulmonary localisation of neutrophils in ARDS than in any of these control groups. This elegant scintigraphic study tended to confirm the work of Thommasen and associates (180) who showed that a fall in total white cell count was an early feature in ARDS pathogenesis. Although it was inferred in the latter study that this transient leucopaenia reflected pulmonary sequestration, these workers produced no supportive data for this assumption.

2) Neutrophils in the pulmonary and/or systemic circulation of ARDS patients exhibit abnormal functional activity.

One of the first studies to address the issue of the functional activity of neutrophils from ARDS patients in vitro was published by Zimmermann and co-workers (181). They demonstrated enhanced chemotactic activity in neutrophils from patients with established

ARDS as compared to critically ill patients without ARDS. They also studied the phenomenon of chemiluminescence; this accompanies the increased oxygen consumption resulting from stimulation of the neutrophil plasma membrane. Increased resting and stimulated chemiluminescence responses were observed from neutrophils of ARDS patients, suggesting that the cell was primed and likely to generate enhanced quantities of oxygen-free radicals in response to membrane stimulation. Fowler and associates however, showed depressed neutrophil chemotactic activity in both ARDS and at risk patients (182). They did show enhanced neutrophil secretory activity as judged by basal lysozymal enzyme release. Thus, although both studies implied enhanced functional activity of neutrophils from ARDS patients, the two studies produced conflicting results with regard to chemotactic activity. This is possibly because Zimmermann's group examined blood from the pulmonary artery while the study of Fowler et al looked at peripheral blood neutrophils. Presumably in the latter situation, those neutrophils with enhanced chemotactic activity were not studied since they were preferentially already sequestrated in the pulmonary microvasculature. Both groups however, showed that chemotaxis of neutrophils from healthy control subjects was not enhanced by incubation with ARDS plasma, tending to negate an important chemotactic role for complement components.

A further study from Zimmermann's group examined granulocyte adherence in ARDS (183). They found interestingly that although plasma from ARDS patients enhanced in vitro adherence of granulocytes from healthy subjects, the ARDS granulocytes themselves demonstrated reduced adherence. This suggested that ARDS granulocytes were

desensitized to a circulating adherence-promoting mediator. The apparent paradox of in vitro hypoadherence and in vivo scintigraphic white cell accumulation in the lung, implied that the determinants of pulmonary neutrophil accumulation were either inflammatory mediators or changes in endothelial cell characteristics.

3) Neutrophil depletion or scavenging of neutrophil-generated oxygen-free radicals attenuates experimentally induced lung injury.

Neutrophil depletion attenuates lung injury induced by hyperoxia (184), endotoxin (185) and microembolisation (186,187). Likewise, scavenging of neutrophil-derived oxygen-free radicals ameliorates injury in a number of experimental models (188-190).

A retrospective clinical series has examined the contrary hypothesis; that increase in leucocyte numbers during remission of chemotherapy-induced leucopaenia accentuates pre-existing respiratory insufficiency (191). Although uncontrolled, the data appeared to confirm the hypothesis, with worsening oxygenation and increased microvascular permeability.

Discussion of evidence presented.

Despite the demonstration that neutrophils sequestrate in the pulmonary microvasculature and are recovered in abnormal numbers in the lavagable airspaces of ARDS patients, this by no means proves that the cell is causing the associated injury. It is well recognised experimentally that neutrophils accumulate in the lungs in response to

oleic acid administration (192). Most studies suggest however, that neutropaenia does not protect against injury induced by this agent (193). This experimental finding may mirror the clinical situation in that the presence of the neutrophil reflects an inflammatory response to the tissue injury, rather than the cell being responsible for it. The neutrophil may be performing any of a number of functions inherent in the inflammatory response, including phagocytosing cellular debris or being involved in the process of tissue repair. Alternatively, its presence in the lung may be a non-specific epiphenomenon associated with the precipitating event rather than the lung injury itself. The finding of increased neutrophil activation in ARDS may also reflect a response to tissue injury rather than a causative relationship, since neutrophil activation has been demonstrated in a variety of inflammatory disorders (194).

Evidence has recently been published suggesting that in vitro findings of neutrophil-generated oxygen radical injury to endothelial cells may be modulated in vivo. The respiratory burst of activated neutrophils is inhibited by the presence of an endothelial monolayer (195), while the erythrocyte can act as a potent scavenger of superoxide anion (196). These protective factors which operate only in vivo would tend to influence or even negate many of the conclusions about neutrophil-mediated cytotoxicity derived from in vitro studies. Thus despite the intense interest that this cell has aroused in lung injury research, its precise role has yet to be completely defined.

CHAPTER 6

EVIDENCE FOR A NEUTROPHIL-INDEPENDENT MECHANISM IN A SPECIFIC GROUP OF ADULT RESPIRATORY DISTRESS SYNDROME PATIENTS.

6. EVIDENCE FOR A NEUTROPHIL-INDEPENDENT MECHANISM IN A SPECIFIC GROUP OF ADULT RESPIRATORY DISTRESS SYNDROME PATIENTS

6.1 - Introduction

The previous chapter reviewed critically the extensive evidence suggesting that neutrophils have a fundamental role in mediating acute lung injury in ARDS. Central to the question of whether or not the cell represents a common effector mechanism for lung injury development in ARDS, would be the demonstration that the classical syndrome can develop in certain situations by a neutrophil-independent mechanism. This clinical phenomenon would, if shown, contrast with the experimental observations previously reviewed, where neutrophil depletion protected against increased pulmonary vascular permeability in several animal models of acute lung injury. Patients undergoing bone marrow transplantation (BMT) are rendered grossly neutropaenic routinely as part of the conditioning for grafting. After marrow transplantation, they remain neutropaenic until engraftment takes place, which, if successful, is usually within four weeks (197). Despite initial successful engraftment, subsequent neutropaenia may develop in association with acute or chronic graft-versus-host disease (GVHD) which directly impairs marrow granulocyte development (198). Careful study of respiratory complications developing in patients after BMT represents a natural clinical experiment similar to assessing the protective effect of experimentally-induced neutropaenia.

Pulmonary complications of BMT cause significant morbidity and mortality and occur in 40-60% of transplanted patients (199). The common syndrome within 100 days of BMT is interstitial pneumonitis which is often related to infection (199). Cytomegalovirus (CMV) is the most common organism associated with pneumonitis; it is extremely virulent with up to 90% mortality (200). The herpes virus, including simplex and zoster, is the other most important cause of pneumonitis (200). Bacterial pneumonitis, including gram-positive and negative, organisms occurs particularly in the context of neutropaenia, or with extensive chronic GVHD (201). Fungal pneumonitis is usually associated with systemic infection while pneumocystis is now much less of a problem as chemoprophylaxis is routine. In a significant percentage of patients, pneumonitis develops without a demonstrable infectious cause. Late chronic respiratory complications, including restrictive (202) and obstructive ventilatory defects (203), are not relevant to this discussion.

A fulminant ARDS-type presentation has been described, particularly when donors and recipients have been mismatched for HLA antigens (204). In this chapter, 5 patients are described in whom acute respiratory failure developed after BMT for chronic myeloid leukaemia. All patients received fully matched sibling grafts and all were neutropaenic to varying degrees at the onset of respiratory symptoms. During their clinical course, all patients fulfilled the characteristic radiological, physiological and haemodynamic features of ARDS. An index of pulmonary epithelial permeability, clearance of ^{99m}TcDTPA, was examined in order to confirm alveolar-capillary barrier dysfunction, as all patients were non-smokers. Since

peripheral blood neutropaenia may be simply a reflection of intrapulmonary neutrophil sequestration, evidence for this feature was sought histologically. In addition, the general morphological lung features were assessed. Studies confirmed that:

a) patients had classical ARDS with histological evidence in all cases of diffuse alveolar damage

b) peripheral blood neutropaenia was not associated with intrapulmonary neutrophil sequestration

This implies a neutrophil-independent mechanism and in the light of these findings the relationship between the neutrophil and ARDS pathogenesis is further discussed.

6.2 - Methods

Patients

Five patients, whose clinical features are summarised in Table 3, were studied. The diagnosis of chronic myeloid leukaemia had been made a median of 14 months before transplantation (range 9-42 months), at which time only patient 2 was in the accelerated phase of the disease. All patients had received intermittent busulphan therapy initially and only 2 patients (no 4 and no 5) had pre-transplant evidence of a mild reduction in gas transfer coefficient (KCO) of less than 10%. Pre-transplant conditioning consisted of daunorubicin 60 mg/m^2 on day -6 and cyclophosphamide 60 mg/kg on day -5 and day -4. Total body irradiation was given as 5 fractionated doses of 200 cGy at a dose rate of 15 cGy per minute, using a linear accelerator (days -3 to -1). Additional splenic irradiation was given at a dose of 500 cGy on two consecutive days.

All patients received marrow from HLA identical, mixed lymphocyte culture non-reactive siblings with no other evidence of histoincompatibility. Prophylaxis against GVHD consisted of T cell depletion with CAMPATH 1 (205) in patients 1, 2 and 3 and a monoclonal anti-T antibodies RFT8 and MBG (206) in patient 4. Patient 4 received cyclosporin in addition. Patient 5 also received cyclosporin, in this case as the sole therapy. Definite GVHD developed in 4 patients (see Table xx) and was graded by the criteria of Thomas and associates (207). It was treated with methylprednisolone except in the 2 patients in whom GVHD developed concomitantly with the acute

respiratory illness.

Studies

Alveolar-capillary barrier function was studied by examining clearance of aerosolised ^{99m}TcDTPA as previously described. Pulmonary transvascular flux of ^{113m}In-transferrin was not assessed as these patients presented prior to proper validation of this technique. All patients were studied within 24 hours of fulfilling the identical criteria for ARDS that were outlined in Chapter 4.

All patients studied were non-smokers. Since all patients were ventilated at the time of study, the aerosol was introduced by manual ventilation using the ventilation circuit described in Chapter 3. Radioactivity was again counted with 2 scintillation detectors placed over the right anterior upper chest and right thigh, providing a correction factor for tissue background. The placement of the probe detectors was made irrespective of the relative distribution of radiological abnormalities, although by definition all patients had diffuse infiltrates. Corrected lung counts were plotted semilogarithmically versus time and the computer fitted regression line was used to calculate the half-time clearance (T1/2) and rate constant of DTPA clearance (K). Eight aged matched non-smoking adults were studied as control subjects.

Histology

Post mortem lung histology was available in all cases. The tissue was processed conventionally and examined under light microscopy.

Statistical analysis.

Where applicable results are expressed as mean ± standard deviation. The differences between groups were assessed with Student's unpaired t test with p values less than 0.05 deemed as significant.

6.3 - Results

DTPA clearance

Half-time clearance of the tracer solute was significantly less in the post BMT patients than in the control group, implying disordered alveolar-capillary barrier function (K values 4.3 ± 1.5 and 1.1 ± 0.2 respectively, $p < 0.001$) (Table 4). Pulmonary clearance of DTPA was increased in each individual post BMT ARDS patient studied and all were outside the 99% confidence interval established in the non-smoking control group. The correlation coefficients of the corrected DTPA disappearance curves, upon which calculation of the rate constant is based, was greater than -0.96 in all cases.

Clinical outcome

All patients died within 10 days of the start of artificial ventilation support. One patient had no evidence of pulmonary infection. All the other patients had ante or post mortem diagnoses of CMV or herpes simplex pneumonitis (see Table 4).

Histology

Autopsies were performed on all patients. All showed diffuse exudative alveolar damage with hyaline membrane formation and intra-alveolar haemorrhage. There was no evidence of increased numbers of intracapillary neutrophils and the oedematous interstitium showed only a mild inflammatory infiltrate. CMV pneumonitis was

diagnosed histologically in 2 patients (see Table 4).

Neutrophil counts

The counts detailed in Table 4 refer to those obtained at the time of onset of respiratory symptoms. Subsequent counts during the clinical course were in all cases lower.

6.4 - Discussion

Notwithstanding marked neutropaenia at the start of respiratory symptoms, all these patients went on to develop fatal acute respiratory insufficiency. Patients demonstrated accepted physiological and haemodynamic features consistent with the diagnosis of ARDS. This was further supported by the autopsy lung histological findings which showed diffuse alveolar damage with rare or absent neutrophils. Pulmonary clearance of ^{99m}TcDTPA was vastly increased in all cases. This occurrence of ARDS in neutropaenic patients is of particular interest as it implies that the syndrome can in certain clinical situations develop independently of neutrophils. Although all patients had neutropaenia of varying severity at the onset of respiratory symptoms, this in itself might just be a reflection of the often precipitious fall in total white cells at the onset of ARDS (180). The failure, however, to demonstrate histologically any evidence of intrapulmonary neutrophil aggregation and the minimal cellular infiltration observed, would appear to negate this possibility.

Many possible precipitating factors, including total body irradiation, cytotoxic chemotherapy, infection and GVHD were present in these patients. Previous studies have suggested that the total dose of irradiation delivered to the lungs (208,209) and the dose rate (210) are both important in determining the incidence of interstitial pneumonitis after BMT. It does appear however, that the dose required to produce pathological and radiographic changes is appreciably higher than that administered to these patients as part of their

pre-transplant conditioning (211). Cyclophosphamide-related interstitial pneumonitis is well described (212) and thoracic irradiation appears to enhance the pulmonary toxicity of cyclophosphamide (213). Although lung injury has been demonstrated histologically (214) in experimental studies , clinical ARDS has only been reported on one occasion in association with cyclophosphamide (215). Thus neither radiation nor the cytotoxic chemotherapy appears likely to have played a role in mediating the pulmonary damage in these patients. GVHD itself produces defined abnormalities in humoral and cellular immunity (216). Its occurrence in 4 of the 5 patients described, in all of whom fatal respiratory failure subsequently developed, is in keeping with previous studies showing that the incidence and severity of pneumonia increases in association with the incidence and severity of acute GVHD in other organs (217).

The most common clinical syndrome after viral infection is interstitial pneumonitis and in 4 of these patients a viral pulmonary pathogen was demonstrated. Interstitial pneumonitis probably represents a less severe clinical and pathological expression of the same syndrome as post BMT ARDS. Furthermore, some of the cases described in the literature as interstitial pneumonitis probably had ARDS by standard criteria. Other workers have pointed to the association of high levels of cyclosporin and subsequent development of ARDS after BMT, particularly in view of a putative effect of cyclosporin on capillary permeability (218). This factor almost certainly had no bearing on this syndrome in this study. Only 2 patients had cyclosporin as part of GVHD prophylaxis and in both patients serum levels were consistently at or below the lower end of

the therapeutic range.

The finding that the clearance of DTPA was significantly higher in the study group than in normal controls implies an impairment in alveolar-capillary barrier function. As all patients studied were non-smokers, this does seem a valid finding reflecting abnormal alveolar epithelial integrity. Is the finding specific to ARDS? As discussed in Chapter 4 and as will be outlined later in the thesis, increased clearance has been noted in a variety of clinical situations including chronic interstitial lung disease and ventilation at high lung volumes. Although none of these factors apply to these patients, the specificity of the data to "full-blown" ARDS is uncertain. Little data is available on the clearance of DTPA in the context of a localised pneumonic process. However, as an adjunct to this study, 2 further patients with CMV pneumonitis were studied; these patients did not fulfil gas exchange or radiographic criteria for ARDS. The patients who were both non-smokers presented after renal and bone marrow transplants respectively. Clearance rate constants for inhaled DTPA over the area of maximum radiographic change were 3.85 and 3.15 respectively, both well outside the 99% confidence limits established in normal non-smokers. These patients have by definition an inflammatory process in their peripheral airspaces and thus the result is hardly surprising. Abnormal DTPA clearance in a non-smoker, breathing at normal lung volumes (see Chapter 11), is thus indicative only of an inflammatory process. ARDS is defined by physiological criteria which may not necessarily reflect the intensity of inflammation or injury - highly abnormal gas exchange is more likely to be a reflection of gross ventilation:perfusion mismatch rather than

"alveolar-capillary block". Thus as these two preliminary studies of a pneumonic area suggest, clearance in ARDS and in pneumonia may not be dissimilar. Therefore, although this finding appears to reflect alveolar epithelial injury, the increased clearance suggests an inflammatory alveolitis rather than ARDS per se.

What bearing does this finding of a neutrophil-independent mechanism have on our overall perception of ARDS pathogenesis? In Chapter 5, the clinical and experimental data suggesting an important effector role in lung injury for the neutrophil was reviewed. Although as discussed, there are numerous experimental models in which neutrophil depletion appears to attenuate injury, other investigators using different models have not shown similar protection with neutrophil depletion (219-221). This may be because of animal species differences or different mechanisms of injury in the different experimental models used. In addition, even where attenuation of injury was shown with neutrophil depletion, this was always only partial. This experimental situation may well mirror that pertaining clinically, in that pathogenetic pathways of injury depend on the particular precipitating event and are not common to all cases of ARDS. This heterogeneity of ARDS pathogenesis is in contrast to the concept that the neutrophil is a central component of the final common effector pathway in acute lung injury.

The assumption that arises from this clinical study as well as from some of the experimental studies to be presented later in this thesis is that the neutrophil is of importance in <u>some</u> models or with <u>particular</u> precipitating causes of lung injury. In view however, of

this neutrophil-independent mechanism, clearly other humoral and cellular factors are also playing an important role. The possible role of humoral factors in lung injury pathogenesis is addressed in Chapters 9 and 10. It has been shown experimentally that activated macrophages are capable of releasing toxic substances that can damage the alveolar-capillary barrier (222-224). Since alveolar macrophages appeared histologically to be present in normal numbers in these patients, it is thus possible that this cell had a role in mediating the lung tissue injury.

In conclusion, this study demonstrates that classical ARDS can in certain circumstances develop independently of neutrophils. This points to a more complex relationship between the neutrophil and lung injury development and suggests an important role for other humoral and cellular factors.

TABLE 3: Clinical details in bone marrow transplant patients

No.	Age	Sex	Onset of respiratory symptoms days after transplant	Acute GVHD (grade *)	Duration of clinical course (from onset mechanical ventilation)
1.	27	M	17	III	9 days
2.	24	F	73	-	10 days
3.	26	F	148	II	4 days
4.	26	M	12	II	5 days
5.	22	M	46	II	6 days

Abbreviation:

GVHD = graft-versus-host disease; graded (*) according to Thomas et al (reference 207)

TABLE 4: Relevant pathological and physiological data in bone marrow transplant patients

No.	Neutrophil count ($\times 10^9/l$)	DTPA clearance (rate constant)	Pulmonary pathogen
1.	0.2	6.4	None isolated
2.	1.2	3.6	CMV - Lung lavage and postmortem histology
3.	1.2	3.0	Herpes simplex- tracheal aspirate
4.	0.1	5.2	CMV - Lung lavage and postmortem histology
5.	1.4	3.2	CMV - lung tissue culture post mortem

Mean = 4.3 ± 1.5 (standard deviation)

(Controls = 1.1 ± 0.2, $p < 0.01$)

Abbreviation:

CMV = Cytomegalovirus

CHAPTER 7

LUNG MICROVASCULAR INJURY AFTER CANINE CARDIOPULMONARY BYPASS: PATHOPHYSIOLOGICAL IMPLICATIONS

7. LUNG MICROVASCULAR INJURY AFTER CANINE CARDIOPULMONARY BYPASS: PATHOPHYSIOLOGICAL IMPLICATIONS

7.1 - Introduction

Cardiopulmonary bypass (CPB) is associated with a small but significant incidence (2%) of severe alveolar-capillary injury presenting as the adult respiratory distress syndrome (post perfusion lung syndrome) (225). Since approximately 117000 operations for coronary artery bypass surgery were performed in the U.S. in 1980 (226), post perfusion lung syndrome appears to represent an important clinical problem. Prospective ultrastructural studies suggest however, that minor degrees of cellular injury after CPB are virtually invariable, particularly with prolonged duration of bypass (227). In support of these morphological findings, physiological studies demonstrate increased pulmonary clearance of aerosolised ^{99m}TcDTPA after CPB, implying impairment of alveolar-capillary barrier integrity (161).

The pathophysiology of this injury is unknown, although evidence suggests a central role for complement activation and associated neutrophil sequestration (228). There is histological evidence for neutrophil sequestration in the pulmonary microvasculature after CPB and this phenomenon appears to correlate with evidence of cellular injury (229). As has been discussed, neutrophil accumulation in the lung microvasculature does not necessarily in itself, imply that these cells are responsible for the associated tissue damage. Neutrophils release at least three groups of products which could result in lung

tissue injury: proteolytic enzymes, oxygen free radicals and arachidonate derived products. Oxygen radicals cause tissue injury by amongst other mechanisms, peroxidizing cell membrane lipid (230). Products of this peroxidation, including principally malondialdehyde, can be quantitated using the thiobarbituric acid (TBA) reaction (231). Increased TBA reactivity, implying an oxygen free radical mechanism, has been demonstrated in experimental endotoxin-induced acute lung injury (232) and after experimental CPB (233). It has also been demonstrated after human CPB, where the time sequence of rise in peroxidation products closely mirrored lung neutrophil sequestration (234).

This model of lung injury was used as, not only did it have clinical relevance, but in addition it involved pathogenetic mechanisms that were of more general importance in lung injury research. These factors represented advantages over chemical means of inducing lung injury (eg. alloxan, alpha-naphthylthiourea, oleic acid) which act by a direct toxic effect on lung microvascular endothelium. The objective of this study was to determine whether accumulation of neutrophils in the lung was associated with egress from the lung of plasma peroxidation products (measured by TBA reactivity) and the development of increased pulmonary microvascular permeability. Egress from the lung of peroxidation products (measured by the left atrial : central venous gradient for TBA reactivity) was used in this study as the index of lung tissue peroxidation. These associations, if shown, would imply that the sequestered neutrophil was playing a role in mediating lung injury by release of oxygen free radicals. Such a relationship could be postulated on the basis of

recent experimental work using a different model of lung injury (167). In that model neutrophil depletion significantly reduced plasma peroxidation products and a biochemical index of lung injury. In the present study these relationships were investigated in a canine model of CPB using the lung transvascular flux of ^{113m}In-transferrin as an index of pulmonary vascular permeability.

7.2 - Methods

Animals and Anaesthesia

Fourteen mongrel dogs (mean weight 27 Kg, range 18.5-37 Kg) were studied on two separate occasions. The first, seven days prior to the main study day, was used for control measurements of lung transvascular protein flux as described below. The animals were anaesthetized with intravenous thiopentone (10 mg/Kg) and intubated and ventilated using a Starling pump (rate 14/minute, tidal volume 10 ml/Kg) with an inspired gas mixture of nitrous oxide in oxygen (1:1 by volume). This was supplemented by halothane (0.5-1%).

Operative procedure and conduct of bypass

Seven days after the control measurement the dogs were again anaesthetized and ventilated as described previously, with the addition of meperidine (2 mg/Kg) to provide analgesia. Eight dogs then underwent thoracotomy and a period of cardiopulmonary bypass, while in the remaining six control animals the effects of thoracotomy alone, with no diversion of pulmonary blood flow, hypothermia or anticoagulation, were investigated. In all dogs carotid arterial and internal jugular vein catheters were inserted to provide continuous monitoring of heart rate, systemic arterial blood pressure and central venous pressure. A right thoracotomy was performed and another catheter introduced into the left atrium for blood sampling and pressure recording. Following the administration of bovine lung heparin (300 units/Kg), the eight dogs allocated to the bypass group

underwent bypass according to the following schedule: cannulae were inserted directly into the ascending aorta and through the right atrium into the superior vena cava. Cardiopulmonary bypass was initiated with bubble oxygenators (BOS-5, Bentley Labs, Irvine, California) and non-pulsatile perfusion using Sarns roller pumps (Sarns, Ann Arbor, Michigan) at a flow of 100 ml/Kg/min. Hartmann's solution (1,000-1,300 ml) was used to prime the oxygenator. When full flow was achieved, hypothermia (28-30^{o}C) was induced, the aorta was cross-clamped and cardiac arrest produced by infusion of 30 ml/Kg of 4^{o}C St. Thomas' cardioplegic solution. This contained magnesium chloride 3.25 gm, potassium chloride 1.93 gm and procaine hydrochloride 272.8 gm in 1 litre of Hartmann's solution. Mechanical ventilation of the lungs was then discontinued. After 45 minutes of total bypass, the aortic cross-clamp was removed and rewarming commenced. Restoration of sinus rhythm always required 10 J DC counter shock to the heart.

Forty five minutes following cross-clamp removal and at an oesophageal temperature of 37^{o}C, pulmonary ventilation was recommenced and the animals weaned from bypass. The aortic and caval catheters were removed and residual heparinisation reversed with protamine sulphate (1 mg/300 units heparin) administered via the left atrial line. During the bypass procedure, blood gas, electrolyte and acid base status were kept within normal limits with sodium bicarbonate and potassium supplementation if required. After haemostasis, the ribs were apposed and the thoracotomy closed. The animals continued to be ventilated for a further four hours until all studies were completed. The six remaining thoracotomy control dogs

were ventilated for 90 minutes open chested to simulate temporally the bypass procedure. As in the bypass animals, the chest wound was then closed and ventilation continued until all studies were completed.

Studies

White blood cell and neutrophil counts.

Blood was diluted 1:20 with 2% acetic acid and methylene blue and counts performed in duplicate in Neubauer chambers. Differential cell counts were performed on 200 consecutive cells on Wright's stained blood films. The effect of hemodilution was corrected by simultaneous measurement of the packed cell volume.

TBA reactive products.

Separated plasma was assayed in duplicate using a modification of the thiobarbituric acid reaction (231). Briefly, thiobarbituric acid, freshly prepared as a 1% solution in 0.05 molar sodium hydroxide, was buffered with 4 molar potassium hydrogen phthalate in water and the pH adjusted to 3.5 with hydrochloric acid. Fifty microlitres of plasma and 2.4 ml of this buffered TBA reagent were heated to 95^{o}C for 1 hour. After cooling, the resultant thiobarbituric acid complex was extracted into 3 ml of Butan-1-ol (British Drug House, Poole, England) by whirly mixing for 10 seconds. Following centrifugation, the upper layer was transferred to polystyrene tubes containing anhydrous sodium sulphate to dry the Butan-1-ol. With excitation at 535 nM, the

fluorescent emission at 560 nM was then measured using a fluorimeter. Results were compared against a standard curve. The effect of hemodilution on plasma TBA concentration was corrected by simultaneous measurement of the total protein concentration.

Measurement of lung transvascular protein flux.

This technique, as described in previous chapters, examines dynamically the accumulation of ^{113m}In-transferrin in extravascular tissues of the lung. The technique used was identical to that already outlined and only the essential features, relevant to this particular study are discussed. Autologous red cells labelled with ^{99m}Tc (1mCi, approximately = 37mBq) were injected along with 0.5 mCi of ^{113m}In. Acrylamide gel electrophoresis of dog plasma after ^{113m}In injection and subsequent counting of sliced gel in a scintillation well counter confirmed binding to a protein with electrophoretic mobility consistent with transferrin (molecular weight 76,000).

After 15 minutes to allow for equilibration, scintillation detectors comprising 5.1 cm x 5.1 cm collimated sodium iodide crystals were placed over the heart and right lung. The lung probe was placed over the midlung field and, since the dogs were studied open-chested, the heart probe was placed by direct visualisation. The latter probe's position was further optimised by ensuring that counts from it due to ^{99m}Tc red cells, as a reflection of the intravasular pool, were maximised. This method of assessing intravascular isotope

activity offers advantages over regular blood sampling as it allows continuous monitoring without blood loss; both features may be important in haemodynamically labile subjects. Additionally, continuous monitoring results in greater density of data points, reducing the time required for valid, accurate studies. A correction factor for influence of ^{113m}In on ^{99m}Tc counts (Compton scatter) was derived initially and applied to subsequent ^{99m}Tc counts. In all studies counts were recorded serially for at least 30 minutes. The lung to heart ratio for ^{113m}In-transferrin for each minute was divided by the corresponding ^{99m}Tc ratio to correct pulmonary blood volume changes. The rate of change of this corrected ^{113m}In-transferrin lung:heart ratio was quantitated by plotting the individual points versus time and fitting a computer-derived linear regression line. The regression equation derived slope was divided by the Y intercept at time 0 to correct for differences in physical factors between studies. This quotient was used as an index of vascular permeability and expressed as protein flux units ($x10^{-3}$/min). This method of data acquisition and analysis was identical to that employed in the human study reported in Chapter 4.

Alveolar to arterial oxygen tension gradient

The alveolar partial pressure of oxygen was calculated from the alveolar gas equation. Arterial oxygen and carbon dioxide tensions were determined on an automated analyser and the inspired oxygen concentration (50%) was verified with a paramagnetic oxygen analyser.

Protocol

Pre-anaesthesia blood was taken from a leg vein for measurement of neutrophil counts and TBA reactivity. After anaesthesia blood was taken from the central venous and left atrial lines at the following times:

(1) just after anaesthetic (arterial blood from carotid arterial line)
(2) 10 minutes into the bypass procedure
(3) just prior to removal of aortic cross-clamp
(4) 10 minutes after cross-clamp removal
(5) 30 minutes after cross-clamp removal
(6) 15 minutes after protamine administration

In the control group, blood was taken at matched times. The samples were collected anaerobically into heparinzed, cooled syringes which were immediately capped and stored on ice. Separation of plasma was undertaken within 20 minutes of sampling.

Transvascular protein flux was measured 3 hours after closing of the chest wound in both bypass and thoracotomy groups. The alveolar to arterial partial pressure of oxygen gradient was determined prior to surgery and 3 hours after closure of the thoracotomy.

Histology

Lung biopsies from the right middle lobe were taken prior to bypass and just before the protein flux study. They were inflated to 10cm H_2O and the tissue divided for light and electron microscopy. For light microscopy the tissue was fixed in 10% neutral buffered formalin prior to being stained and examined. Tissue for electron microscopy was fixed in phosphate buffered 2% glutaraldehyde and post-fixed in 1% osmium tetroxide.

Statistical analysis

When serial data were compared with one baseline sample, paired t tests were used with the critical p value corrected by the Bonferroni method (235). A p value of less than 0.01 was taken as significant for these data; otherwise a p value of less than 0.05 was taken as significant. Because of large variances, Wilcoxon signed rank test was used to compare lung protein flux on the control and study days. Correlation coefficients between variables were compared with Spearman's rank correlation coefficient. All results are expressed as mean ± standard error of the mean (SEM).

7.3 - Results

Induction of anaesthesia caused no significant change in either neutrophil counts or concentration of TBA reactive products. Because of this, the first blood sample, just after induction of anaesthesia, was regarded as the baseline value for both neutrophil counts and TBA reactivity measurements.

Neutrophil counts

Left atrial and central venous neutrophil counts before, during, and after the bypass procedure are shown in Fig.7. Just prior to removal of the aortic cross clamp both left atrial (LA) and central venous (CV) neutrophil counts had fallen significantly in a parallel fashion from initial values of 5.3 ± 0.7 ($\times 10^9$ cells / l) and 5.3 ± 0.7 to 3.0 ± 0.5 and 2.9 ± 0.5 respectively ($p < 0.001$). This trend persisted after removal of the aortic cross clamp in the left atrial samples particularly, where it was still present 30 minutes later (3.7 ± 1.1, $p< 0.01$). Significant gradients of neutrophil counts across the lung were demonstrated only in the samples taken 10 minutes (0.7 ± 0.2) and 30 minutes (1.2 ± 0.5) after cross clamp removal ($p< 0.01$ and $p< 0.001$ respectively, versus baseline gradients).

Neutrophil counts in the control thoracotomy dogs showed similar patterns in both CV and LA lines with a gradual rise during the course of thoracotomy from baseline values of 6.3 ± 1.6 (CV) and 6.6 ± 1.9 (LA) to respective values 90 minutes later of 8.3 ±

2.2 and 8.8 ± 2.6. This rise was not however statistically significant, and at no stage was a significant transpulmonary (CV-LA) gradient present.

Trends in total white cell counts were mirrored closely by the neutrophil counts, and no significant changes were shown in other polymorphonuclear or in mononuclear cells.

TBA reactive products

There was a progressive rise in TBA reactivity in both CV and LA samples over the course of CPB (Fig.8). Baseline levels of 6.4 ± 0.5 nmol/l (CV) and 6.5 ± 0.5 nmol/l (LA) rose 30 minutes after cross clamp removal to 8.6 ± 1.0 nmol/l and 9.4 ± 1.0 nmol/l respectively ($p < 0.01$). Corresponding values after protamine administration (9.4 ± 1.6 nmol/l and 9.8 ± 1.0 nmol/l) were likewise significantly different from baseline values. A significant transpulmonary gradient was shown only in the sample taken 30 minutes after cross clamp removal (0.8 ± 0.3 nmol/l, $p < 0.01$).

The concentration of TBA reactive products tended to rise in the control thoracotomy dogs with baseline CV and LA levels of 5.9 ± 0.6 nmol/l and 6.0 ± 0.7 nmol/l rising at the end of the procedure to 7.7 ± 1.0 nmol/l and 7.5 ± 1.0 nmol/l respectively. This rise was not however, statistically significant. As with the neutrophil counts in the control thoracotomy dogs, LA and CV

concentrations were similar throughout and at no time did a significant transpulmonary gradient exist.

Lung transvascular protein flux

Individual results of lung ^{113m}In-transferrin flux studies are represented in Fig.9. In the CPB dogs the mean baseline flux was -0.2 ± 0.3 units and after bypass there was a significant increase to 3.3 ± 1.0 units ($p < 0.01$). Five out of eight dogs showed protein flux outside the normal range (defined by the 95% confidence limits in all 14 dogs on their baseline study day, 0.0 ± 0.2). The control thoracotomy dogs had protein flux results similar to baseline studies of the CPB group (0.2 ± 0.3) but no significant increase was seen after thoracotomy (0.2 ± 0.2). No individual animal in this control group had a value for protein flux outside the normal range.

Alveolar to arterial oxygen tension gradient

The gradient rose from 154 ± 12 mmHg before bypass to 296 ± 6 mmHg three hours after bypass ($p < 0.001$). The control animals did not show a significant increase in gradient with respective values of 140 ± 17 mmHg and 152 ± 11 mmHg.

Correlation between variables

Significant neutrophil and TBA reactive product concentration transpulmonary gradients were demonstrated only in the bypass

animals after removal of the aortic cross clamp, when coronary and pulmonary perfusion were restored after a period of asystole. Fig.10 shows the transvascular protein flux results after bypass for individual animals, plotted against the difference in LA-CV TBA concentration gradients 30 minutes after and just prior to cross clamp removal. This transpulmonary gradient was used as an index of net lung lipid peroxidation when pulmonary perfusion recommenced. There was a significant Spearman's rank correlation coefficient between these variables ($r_s = 0.81$, $p < 0.05$). Similarly Fig.11 shows individual values for net pulmonary neutrophil sequestration after cross clamp removal (gradient 30 minutes after - gradient just prior to clamp removal) plotted against the gradient difference for TBA reactivity concentrations over this same time period. Spearman's rank correlation for these two variables was again significant ($r_s = 0.76$, $p < 0.05$). In individual animals this index of neutrophil sequestration also correlated with transvascular protein flux after bypass ($r_s = 0.66$, $p < 0.05$).

No relationship existed between each animal's alveolar:arterial oxygen tension gradient and value for lung protein flux.

Histology

Examination of biopsied lung tissue under light microscopy showed accumulation of granulocytes in alveolar capillaries after bypass only. No intra-alveolar granulocytes were seen. In addition, no perivascular or intra-alveolar edema and no interstitial

inflammatory cell infiltrate were noted. Electron microscopy did however show signs of cellular injury after bypass with endothelial swelling, disruption of the basement membrane, and neutrophils tightly apposed to endothelial cells. In addition mild interstitial edema was noted. These features are demonstrated in Figs. 12 to 15.

7.4 - Discussion

This study demonstrates that CPB in dogs is associated with significantly increased lung transvascular protein flux, neutrophil sequestration and egress from the lung of products of lipid peroxidation. Significant correlations were demonstrated between peroxidation product egress from the lung and both increased vascular permeability and pulmonary neutrophil sequestration.

The finding of abnormal protein flux in five out of the eight bypass dogs, but in none of the six dogs subjected to thoracotomy alone, suggests that this feature was not a non-specific response to anaesthesia and major thoracic surgery, but was a feature related to CPB or it's specific associated perturbations (extra-corporeal circulation and pulmonary blood flow diversion, hypothermia and protamine administration). Since none of the bypass dogs showed morphological evidence on light microscopy of parenchymal damage after bypass, this increased protein flux probably reflects a mild injury. This was confirmed by electron microscopy which also revealed interstitial edema. Presumably this finding reflects a less severe microvascular injury where lung lymphatic clearance is still able to deal with increased fluid and solute flux. The animals in this study thus avoided frank intra-alveolar edema as in the post-perfusion lung syndrome. Nontheless these data represent one end of the spectrum of lung injury after CPB; this may have been either a transient abnormality or alternatively there may have been, in time, progression to frank pulmonary edema. The bypass dogs showed a significant increase in

alveolar:arterial oxygen tension gradient after the procedure; the lack of relationship between this variable and lung protein flux, together with the absence of intra-alveolar edema histologically, would suggest that the main determinant of the profound hypoxemia after bypass was ventilation:perfusion inequality.

Neutrophil counts in the bypass group showed a different pattern to those dogs who underwent thoracotomy alone. The initial parallel fall in both CV and LA neutrophil counts early in the bypass procedure, without a transpulmonary gradient, implies sequestration of cells outside the lungs, probably in the splanchnic or mesenteric vascular beds. This fall cannot be ascribed to haemodilution as this was specifically corrected for in all samples. After release of the aortic cross-clamp and reversal of complete cardiac asystole, a significant neutrophil gradient across the lungs was noted, implying intrapulmonary sequestration. Failure to establish a transpulmonary gradient for neutrophils or TBA reactivity in those dogs not undergoing CPB, suggests that bypass itself is necessary for significant cell sequestration and peroxidation product egress from the lung. The temporal relationship between aortic cross clamp removal and lung neutrophil sequestration and peroxidation product egress is similar to that reported after human CPB (234). In contrast to the findings in that study however, is the observation that dogs show progessive falls in neutrophil counts in the pre-cross clamp removal phase of bypass. Similar falls in neutrophil counts have however, been reported in another human bypass study, although transpulmonary counts were not obtained and neutropaenia was only a transient

phenomenon (236). An additional contrast to the previously reported human CPB study (234), is the timing in dogs of lung neutrophil sequestration and oxidant production. In humans both features were present five minutes after cross clamp release. In this study although significant neutrophil sequestration was noted within 10 minutes, a transpulmonary gradient for TBA reactivity was shown only in the sample obtained 30 minutes after cross clamp removal.

Notwithstanding these differences between human and dog studies, this study supports the concept that resumption of pulmonary vascular perfusion, after the period of total pulmonary blood diversion, is crucial in the mediation of lung neutrophil trapping, tissue peroxidation and increased pulmonary vascular permeability. Complement activation and pulmonary leucostasis have been demonstrated in extracorporeal systems (237) including CPB (228,236). Polymorphonuclear leucocytes when exposed to activated complement, aggregate (238) and release oxygen radicals which in vitro damage endothelial cells (190). After cross clamp release, rewarming and reversal of asystole, complement activated neutrophils perfuse the pulmonary vascular bed for the first time. This could account for neutrophils sequestrating in the lungs at this particular phase of the bypass procedure. Alternatively, reperfusion of the relatively ischaemic coronary and pulmonary vasculature could result in a burst of oxygen free radical production (239) and pulmonary endothelial cell injury.

Since protamine administration has been associated with transient neutropaenia (240) and increase in pulmonary vascular permeability both clinically (241) and experimentally (242), it's possible role in this study should be addressed. As the effect of CPB and its associated perturbations were examined, only the bypass group received protamine. No exacerbation of neutropaenia was noted in the blood sample taken after protamine (point 6) and the role of the drug in influencing this variable can thus be disregarded. The role of protamine in mediating the alteration in pulmonary microvascular integrity remains speculative however. Frater and his associates showed that clinically, protamine administration via the left atrial line minimised the associated haemodynamic effects of systemic arterial hypotension and decrease in systemic vascular resistance (243). Accordingly in this study protamine was given via the left atrial line and no significant change in haemodynamic variables resulted. Clinical descriptions of protamine-associated non-cardiogenic pulmonary edema featured marked peripheral vascular collapse within an hour of administration (241); our finding of less severe pulmonary vascular injury at three hours without significant haemodynamic alterations, would tend to negate, although not totally rule out, a role for the drug in the pulmonary vascular permeability changes observed.

Does measurement of plasma TBA reactivity reflect lung tissue peroxidation. Considerable theoretical and practical difficulties exist in attempting to demonstrate tissue lipid peroxidation using the thiobarbituric acid technique. Peroxidised cell membrane lipids are rapidly lost from the cell into the plasma as has been

demonstrated in vitro (244). These workers showed that tissue lipid peroxidation induced hydrolytic enzyme activity which could profoundly alter analysis of tissue for TBA reactivity. These considerations were probably responsible for the inability of Ward and colleagues (167) to demonstrate lung tissue peroxidation using this technique. Because of the rapid loss of TBA reactive products into the plasma, it was reasoned that measurement of plasma reactivity across the lung would better reflect lung tissue peroxidation. Accordingly, the left atrial :central venous gradient was used as an index of egress from the lung of tissue oxidants and this was interpreted as an indirect measurement of lung lipid peroxidation.

The strong rank correlation demonstrated between lung neutrophil accumulation and peroxidation egress from the lung, as well as the temporal relationship between these two phenomena, suggests that the neutrophil was in part responsible for production of the tissue oxidants. Similarly, the significant association between peroxidation egress, as measured by the difference in transpulmonary gradients before and after cross clamp removal, and lung protein flux suggests that oxidants were, at least in part, responsible for this increase in pulmonary vascular permeability. It is unlikely however, that oxidant release was the sole mechanism responsible for this finding, although other potential toxic substances were not specifically investigated. Neutrophil proteolytic enzymes have been implicated in the pathogenesis of the adult respiratory distress syndrome (88). This may involve directly, digestion of lung elastin, or indirectly, amplification

of the inflammatory process (245). Activated neutrophils may also release arachidonate derived products with potential deleterious effects on vascular and airway smooth muscle tone and vascular permeability. The probable interrelationship of all these potential mechanisms of tissue injury is underlined by the observation that arachidonic acid metabolism in macrophages or neutrophils is associated with oxygen radical production (246).

In conclusion, this study suggests that neutrophils sequestered in the lung play a role in the development of pulmonary micovascular injury, which occasionally presents clinically as the'post-perfusion lung syndrome'. Although these data imply that release of tissue oxidants is one of the mechanisms that may be responsible for these findings, firm conclusions cannot be drawn until the findings are verified in a study where appropriate protective interventions are introduced. Nonetheless, the data support the concept outlined in Chapter 6 that the neutrophil is important in particular models of lung injury.

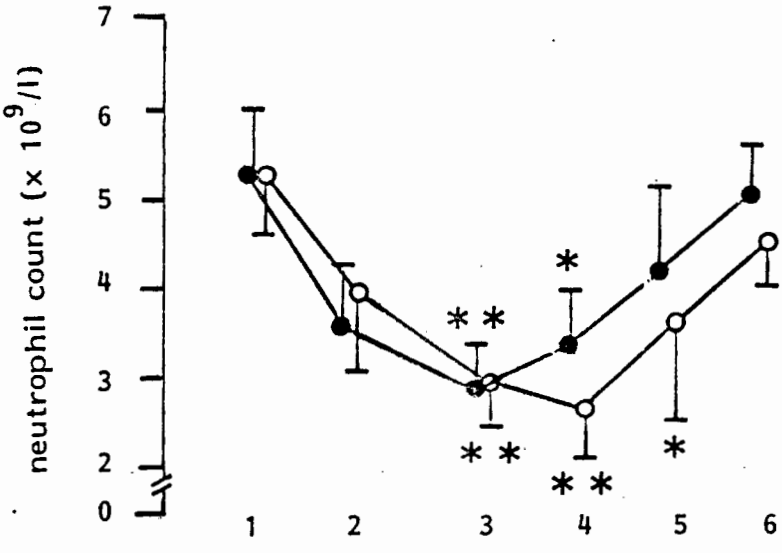

Figure 7

Neutrophil counts (x 10^9/l, mean ± SEM) in central venous (filled circles) and left atrial (open circles) samples for the dogs that underwent cardiopulmonary bypass. Numbers on the x axis refer to standard times of sampling, as described on Page 146. * $p < 0.01$; ** $p < 0.001$; versus respective central venous or left atrial values at baseline (time 1).

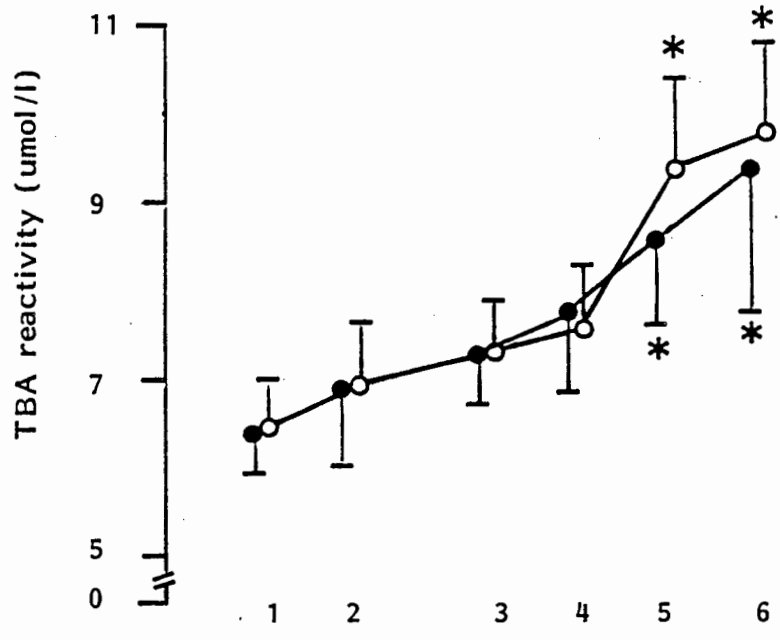

Figure 8

Concentration of thiobarbituric acid (TBA) reactive substances (mean ± SEM) in central venous (filled circles) and left atrial samples for bypass dogs (n=8). Numbers on the x axis refer to the standard times of sampling outlined on Page 146. * $p < 0.01$ versus respective central venous or left atrial value at baseline (time 1).

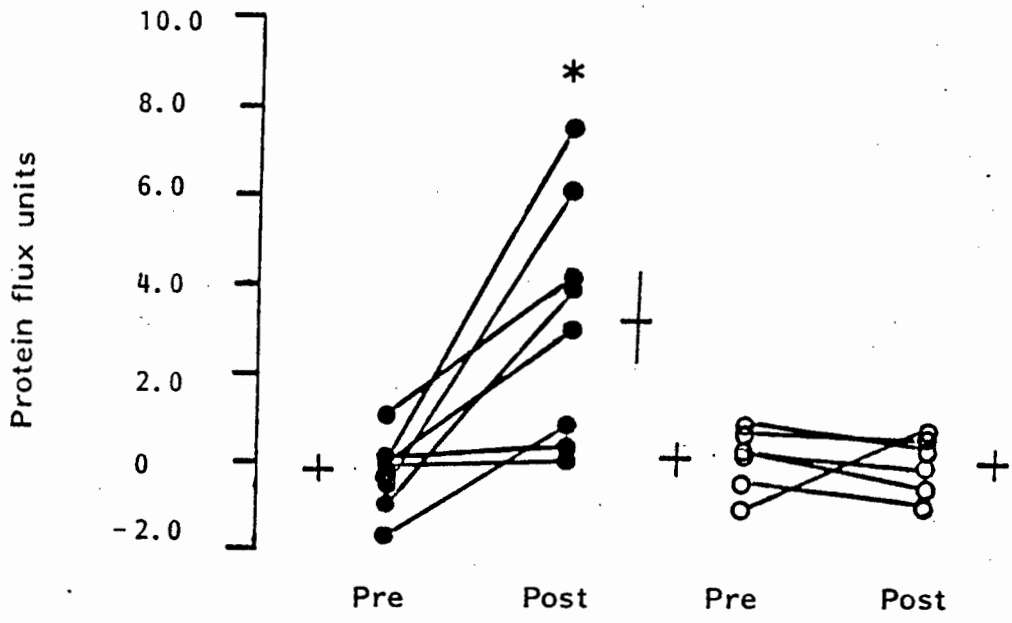

Figure 9

The rates of transvascular flux of ^{113m}In-transferrin expressed as protein flux units are shown in individual bypass dogs (n=8, filled circles) and control thoracotomy animals (n=6, open circles). Both groups have values shown for the baseline study one week before (left) as well as the study 3 hours after thoracotomy closure (right). * $p < 0.01$ versus baseline study 1 week prior to bypass.

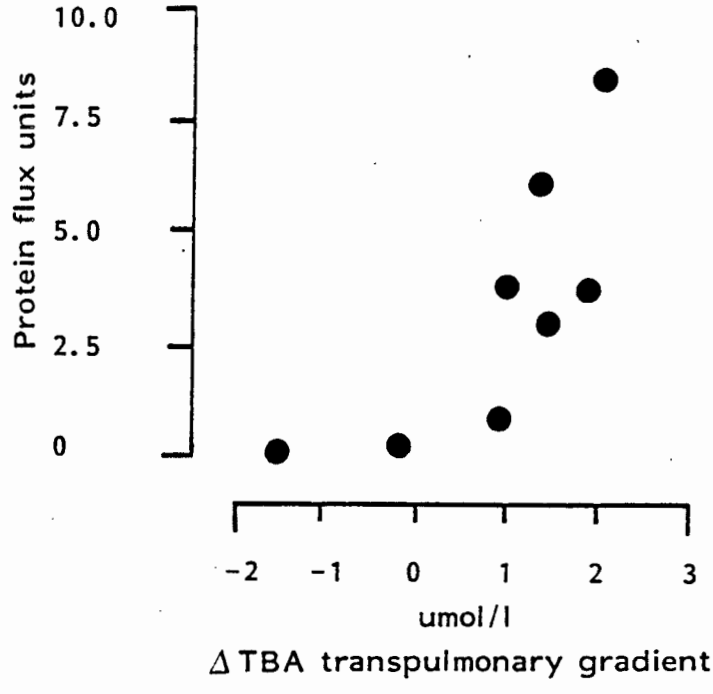

Figure 10

Protein flux units for each individual dog after bypass (n=8) plotted against the difference in transpulmonary gradient (LA-CV) 30 minutes after and just prior to removal of the aortic cross-clamp. Spearman's rank correlation coefficient, $r_s = 0.81$ ($p < 0.05$).

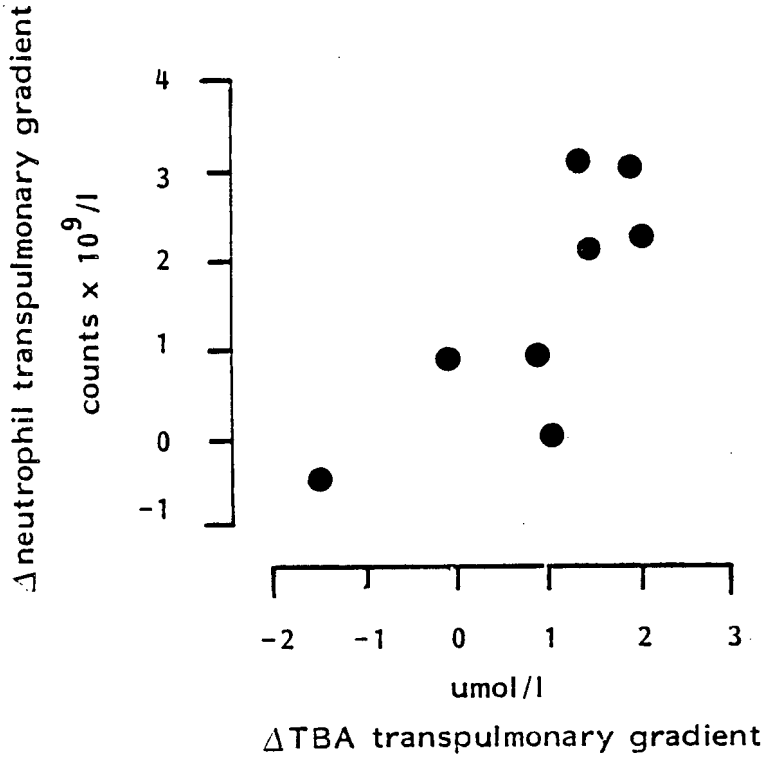

Figure 11

The difference in transpulmonary neutrophil gradient (CV-LA) for each individual bypass dog (n=8) 30 minutes after and just prior to removal of the aortic cross-clamp, plotted against the transpulmonary gradient over the same time period for TBA reactivity. $r_s = 0.76$, $p < 0.05$.

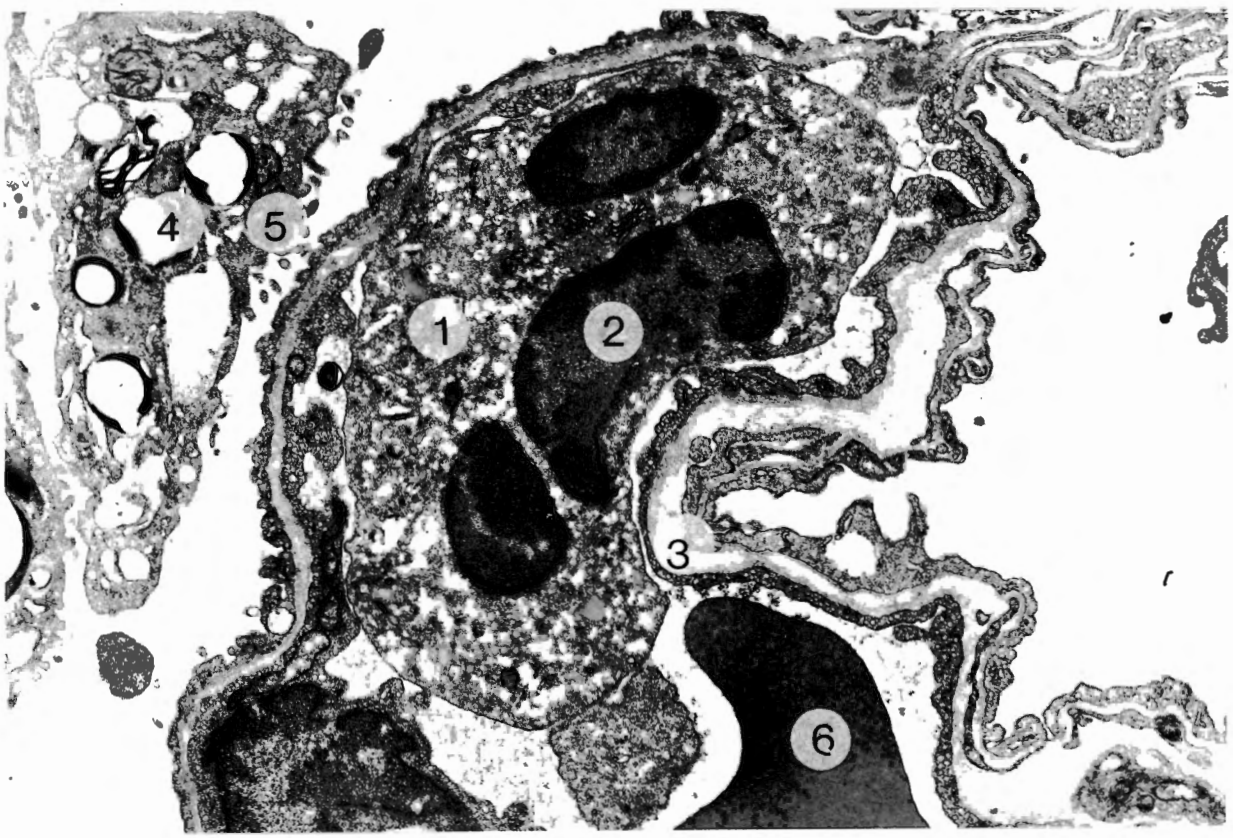

Figure 12

Electron micrograph (x 20,000) showing the relationship between the neutrophil (1) and the endothelial cell lining (3) before institution of cardiopulmonary bypass. Important is the clear space visible between the neutrophil and the endothelial lining, demonstrating that the cell is not apposed to the endothelial surface. Of note also is the multi-lobed neutrophil nucleus (2), and alongside the neutrophil is a red cell (6), both lying within the capillary lumen. Also visible, apparently protruding into the alveolar space, is a Type 2 pneumocyte (4) with its lamellar cytoplasmic bodies and microvilli (5).

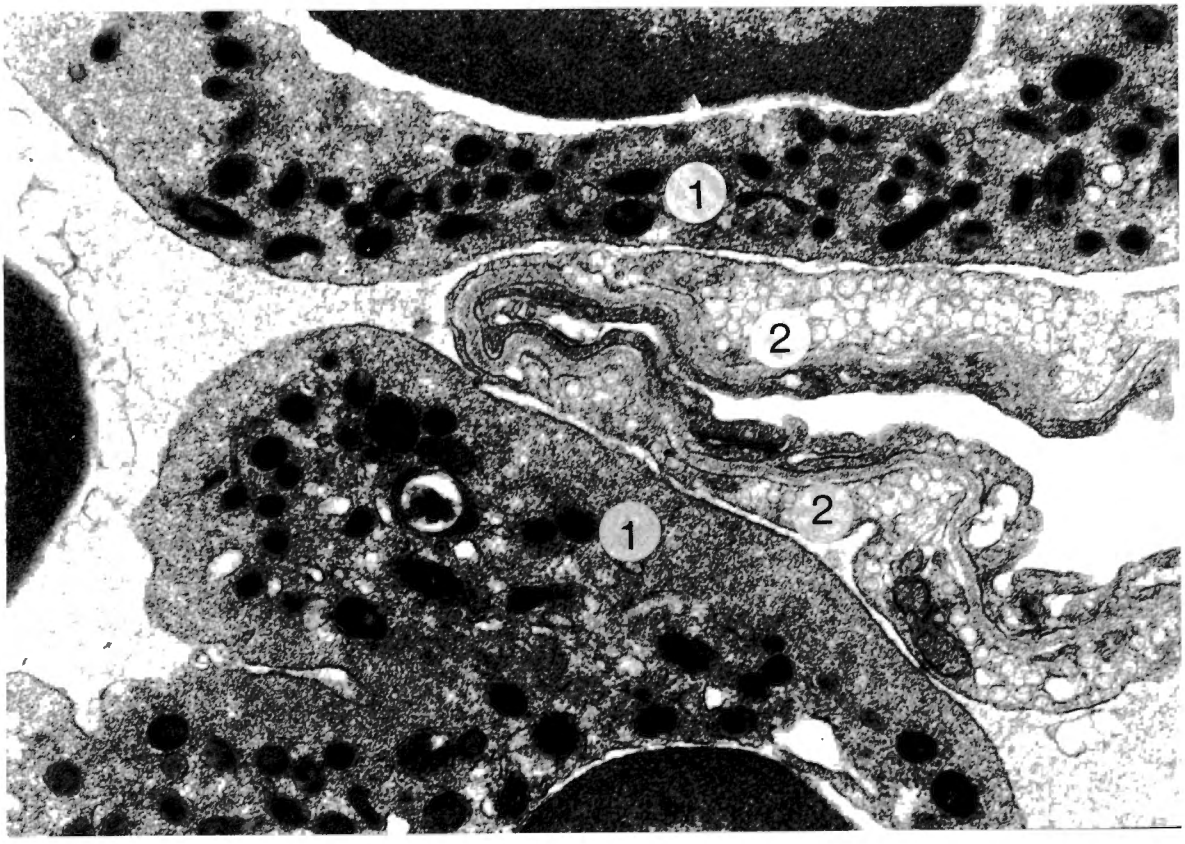

Figure 13

Further detail on a higher power electron micrograph (x 50,000) taken again before institution of cardiopulmonary bypass. The demarcation between the neutrophil (1) and the endothelial cell lining (2) is clearly visible. Of interest are the neutrophilic granules (electron-dense bodies in the neutrophil cytoplasm) and the endothelial caveolae (pinocytic vesicles) adjacent to the symbol (2).

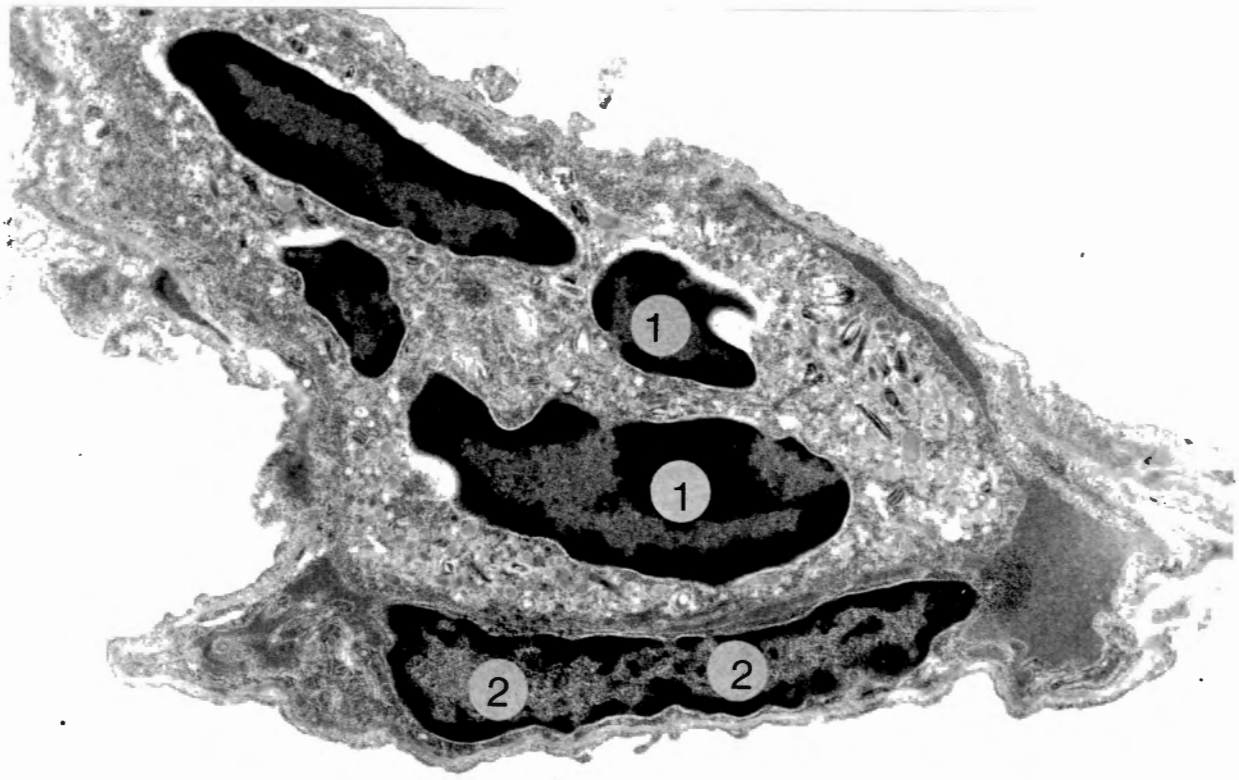

Figure 14

Electron micrograph (x 20,000) from a lung biopsy taken just after coming off bypass. This shows the neutrophil with its multilobed nucleus (1) tightly apposed to the endothelial lining. This latter feature can be appreciated by the position of the elongated endothelial cell nucleus (2).

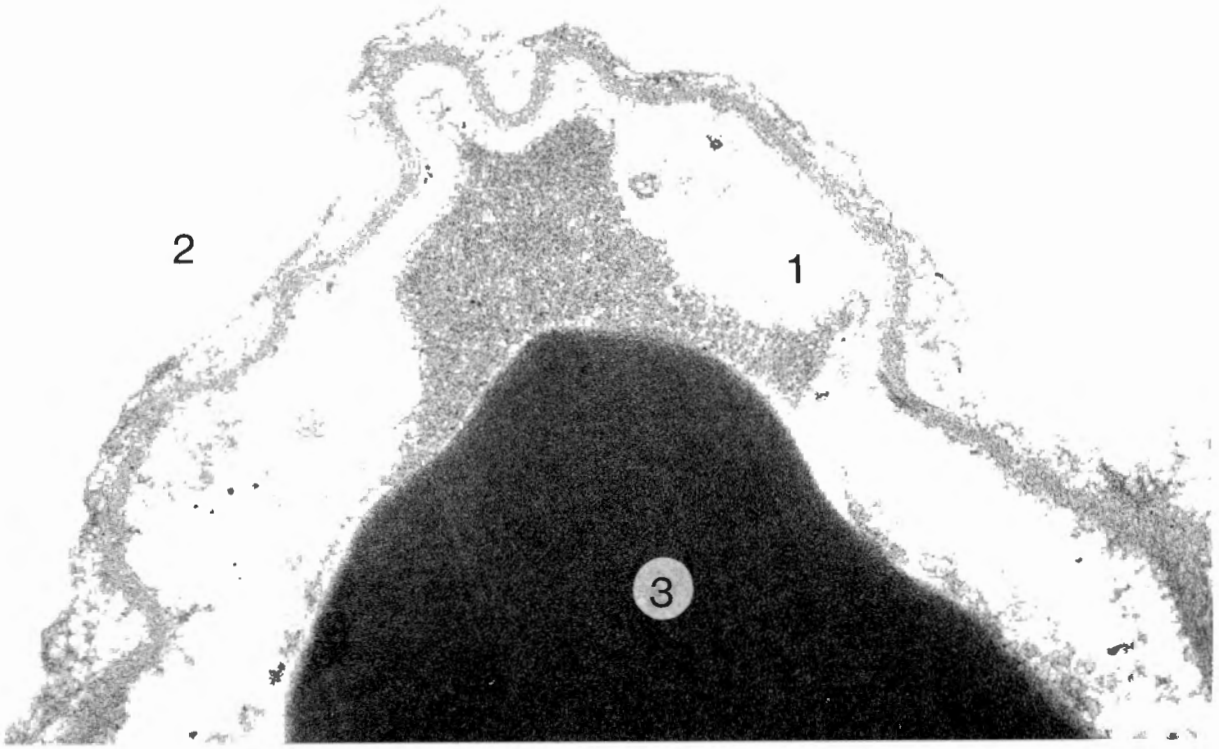

Figure 15

High power electron micrograph (x 50,000) from a lung biopsy 2 hours after coming off cardiopulmonary bypass. The important feature is the gross endothelial swelling (1); orientation is facilitated by the red cell lying within the capillary lumen (3) and the alveolar space (2).

CHAPTER 8

ENDOTOXIN INFUSION IN DOGS: DICHOTOMY BETWEEN LUNG NEUTROPHIL SEQUESTRATION AND LUNG INJURY DEVELOPMENT.

8. ENDOTOXIN INFUSION IN DOGS: DICHOTOMY BETWEEN LUNG NEUTROPHIL SEQUESTRATION AND LUNG INJURY DEVELOPMENT

8.1 - Introduction

Gram-negative sepsis represents one of the most common precipitating events for lung injury development. Because of this many investigators have used endotoxaemia as a convenient, clinically relevant model, to study the pathophysiology of the associated lung injury. This model has been particularly extensively studied in sheep where the pulmonary functional abnormalities mimic many of the features of human ARDS (32). A 2-phase response is classically described with rise in pulmonary artery pressure and alterations in lung mechanics occurring within the first hour. From 2 hours onward, increase in lung vascular permeability is noted in association with lung endothelial cell injury. The relative sensitivity of the sheep to endotoxin is not, however, common to all animal species. The rat is able to tolerate enormous doses of endotoxin without obvious evidence of lung injury (247), while the dog similarly requires large doses to produce significant haemodynamic effects (248). Even with large doses of endotoxin in the dog, the cardiovascular effects outweigh the relatively mild lung injury; this is in contrast to the sheep where severe lung injury can occur in the absence of shock (249).

The original motivation for this study was to attempt to define canine endotoxin-induced lung injury more accurately by the

dynamic assessment of pulmonary transvascular flux of ^{113m}In-transferrin. The intention was to develop a model of lung injury that would result in loss of endothelial cell integrity similar to that induced by cardiopulmonary bypass, which was outlined in the last chapter. The particular reason for choosing endotoxin was that it was known to involve similar pathogenetic pathways to bypass. Endotoxin infusion produces complement activation and the subsequent intrapulmonary neutrophil sequestration and activation is thought to be a putative mechanism for the effect of endotoxin on the lungs.

If endotoxin did produce consistent lung injury in dogs, it would represent a simple, relatively non-invasive model for further investigation. Despite however, producing persistent and significant neutropaenia suggestive of intrapulmonary sequestration, the dogs studied showed no evidence of acute lung injury. The relevance of this finding to current concepts of lung injury pathophysiology is further addressed in section 8.4.

8.2 - Methods

Six mongrel dogs (mean weight 23 Kg, range 18-27 Kg) were studied. The animals were anaesthetized with intravenous thiopentone (10 mg/Kg) and intubated and ventilated using a Starling pump (rate 14/minute, tidal volume 10 ml/Kg) with an inspired gas mixture of nitrous oxide in oxygen (1:1 by volume). This was supplemented by halothane 0.5-1.0%.

The initial preparation included catheterisation of the carotid artery to produce continuous monitoring of systemic arterial blood pressure. The internal jugular vein was catheterised with a Swan Ganz flow directed catheter so that the catheter tip was in the pulmonary artery and a satisfactory trace verifying wedging of the catheter obtained. Cardiac output was determined by thermal dilution using a cardiac output computer. After preparation, each dog was allowed to stablilise for 20 minutes before baseline transferrin transvascular flux was determined. In addition, baseline haemodynamic and arterial blood gas data was acquired and blood taken for white cell and differential counts from the pulmonary artery and carotid artery lines.

Experimental protocol.

After 30 minutes baseline measurement of transvascular transferrin flux, 4 dogs received an infusion of E. Coli endotoxin (serotype 055:B5, Sigma Ltd, Dorset, UK) 2 mg/Kg given at a dose

rate of 2 mg/minute. After endotoxin infusion, cardiovascular measurements and arterial blood gas estimations were performed every half hour until completion of the study. The 2 remaining dogs were studied in a similar fashion but did not receive endotoxin and acted as time course controls. All animals were studied for two and a half hours after endotoxin infusion. During this time period, ventilation settings were held constant.

Studies.

Transvascular ^{113m}In-transferrin flux.

This was performed in a similar fashion to that used in the bypass study described in Chapter 7. In this study, however, all animals were studied closed-chested. Accordingly the heart scintillation probe was placed initially to be just over the observed cardiac impulse. As before, this position was optimised by movements aimed to maximise counts resulting from ^{99m}Tc-labelled red cells. The lung probe was placed over the right mid lung field in all studies. Once the initial probe positions had been determined, they were not moved until all studies had been completed. Baseline data pre-endotoxin was collected for 30min and after endotoxin infusion counts due to both isotopes were monitored continuously for two and a half hours. The data was analysed in 30 minute time periods and as previously the data for each time period was expressed as protein flux units (x 10^{-3}/minute).

Assessment of oxygenation defect.

This was assessed by calculating the arterial to alveolar partial pressure of oxygen ratio before endotoxin and at half hour intervals thereafter. The alveolar partial pressure of oxygen was calculated from the alveolar gas equation as described in Chapter 4. Arterial oxygen and carbon dioxide tensions were determined on an automated analyser and the inspired oxygen concentration (50%) verified with a paramagnetic oxygen analyser.

White blood cell and neutrophil counts.

White cell counts were performed in duplicate in Neubauer chambers and differential counts assessed using Wright's stained blood films. Blood samples were taken before endotoxin and at 5, 30 and 150 minutes after endotoxin.

Haemodynamics.

Heart rate, systemic and pulmonary arterial blood pressures were monitored continuously. Cardiac output estimations were performed in triplicate before endotoxin and at half hourly intervals thereafter. The mean of these 3 estimations was used as the value for that time period. Pulmonary vascular resistance was calculated by the use of the following formula:-

$$\frac{\text{Mean pulmonary artery pressure - pulmonary capillary wedge pressure}}{\text{cardiac output}}$$

and was expressed in arbitrary units.

Statistics

All data is expressed as mean ± standard error of the mean. When serial data was compared with one baseline sample, paired t tests were used with the critical p value corrected by the Bonferroni method (235). Otherwise the p value of < 0.05 was taken as significant.

8.3 - Results

Neutrophil counts

Counts taken after endotoxin were expressed as a percentage of the baseline pre-endotoxin sample. Values 5, 30 and 150 minutes after endotoxin were 29.8 ± 3.2, 18.4 ± 5.8 and 10.8 ± 4.9% respectively of the baseline count ($p < 0.001$). This indicates a severe and persistent neutropaenia over the whole course of the study. Trends in total white cell counts were mirrored closely by the neutrophil counts, and no significant changes were shown in other polymorphonuclear or in other mononuclear cells. Although in all cases mean pulmonary artery counts were greater than mean systemic arterial counts, this did not at any of the time periods reach statistical significance.

Haemodynamics and oxygenation data.

This is summarised in Table 5. Persistent significant falls in mean arterial systemic blood pressure were noted ($p < 0.01$) which were maximal half an hour after endotoxin infusion (68.3 ± 5.8 mmHg). Cardiac output fell after endotoxin infusion from 3.1 ± 0.3 litres/minute pre-endotoxin to 2.3 ± 0.3 litres/minute half an hour after endotoxin infusion ($p < 0.01$). Although the other mean values for cardiac output were less than the mean baseline value, they did not reach statistical significance. Pulmonary vascular resistance increased from 2.7 ± 0.1 units pre-endotoxin to 4.0 ± 0.3 and 3.5 ± 0.2 units ($p < 0.01$) half an hour and one and a half

hours after endotoxin, respectively. The value at two and a half hours (2.9 ± 0.2 units) was no longer statistically significant.

Arterial oxygenation deficit expressed as the partial pressure of arterial oxygen to the partial pressure of alveolar oxygen ratio was within normal limits and did not differ from the baseline value significantly throughut the study.

Pulmonary transvascular protein flux.

No mean value for any of the 5 half hour time periods after endotoxin differed significantly from the baseline value (0.01 ± 0.1, 0.07 ± 0.1, 0.2 ± 0.1, 0.1 ± 0.2 and 0.4 ± 0.2 protein flux units). Indeed no individual animal had a single value after endotoxin that was outside the 99% confidence limits derived from the 14 dogs studied on the baseline day in the bypass project described in Chapter 7.

Control studies.

The 2 control dogs demonstrated no significant change in neutrophil counts, haemodynamics, oxygenation or transvascular protein flux over the temporally matched period of study.

8.4 - Discussion

These data demonstrate that infusion into dogs of 2mg/kg of E.coli endotoxin produces significant haemodynamic effects, severe persistent neutropaenia indicative of sequestration within the pulmonary vasculature, but no evidence of microvascular injury. These findings are of principal interest since they provide evidence for a more complex relationship between intrapulmonary neutrophil sequestration and lung injury development.

This study did not include histological assessment; therefore conclusions about two of the major experimental variables - lung injury and intrapulmonary neutrophil sequestration - are based on functional information. The data do show profound and persistent neutropaenia after endotoxin infusion but this per se does not necessarily indicate neutrophil sequestration within the lungs. Although the gradient of cells across the lungs was at no stage significant, this factor does <u>not</u> in itself mean that neutrophils didn't sequestrate within the lungs. If, as seems likely, neutrophils sequestered not only within the lungs but also within extra-pulmonary tissue including the mesenteric and splanchnic vascular beds, any gradient across the lungs would be lost. In support of this interpretation is previous histological assessment after endotoxin infusion in dogs clearly showing marked pulmonary leucostasis (250).

The finding of an absence of microvascular injury, based on no change in pulmonary transvascular protein flux, emphasises the

species-specific nature of the response to endotoxin. The lack of change in the microvascular permeability index does it seems, provide valid evidence for a lack of significant lung injury despite the absence of confirmatory morphological assessment. This contention is supported by:

i) the work reported in this thesis. In the cardiopulmonary bypass studies pulmonary transvascular protein flux was increased despite relatively subtle changes morphologically. This is similar to the findings reported in Chapter 10 in the study of platelet activating factor.

ii) the finding of Dauber et al, previously alluded to (109), showing that transvascular protein flux is increased before increase in extravasacular lung water occurs.

Both these aspects point to the extreme sensitivity of this microvascular permeability technique, and tend to rule out the possibility of a false negative finding. Further evidence of the minimal nature of the respiratory dysfunction associated with this model, is the lack of significant deterioration in arterial oxygenation over the course of this study. Although no previous study has examined a similar microvascular permeability index after canine endotoxin infusion, McCaffree and colleagues reported a lack of change in gravimetric lung water with this model (250). They also noted a discrepancy between the lack of lung injury and a significant haemodynamic response as in the present study. These workers did however, as mentioned, use extravascular lung water accumulation as their index of lung injury.

Workers at Vanderbilt University, Nashville U.S.A, have clearly shown that endotoxin produces significant intrapulmonary neutrophil sequestration in the sheep with evidence of lung injury (251). In contrast they have demonstrated that infusion of zymosan-activated plasma (ZAP) produces similar cellular sequestration but minimal lung injury (252). This ZAP-related phenomenon appears similar to the results of this study. The difference between the neutrophil-endothelial cell interactions in these two situations is crucial to future understanding of lung injury pathogenesis. Brigham and Meyrick have pointed to the difference not being explainable simply by a quantitative difference in the numbers of granulocytes sequestered (253). These data would support their contention, although based indirectly only on the profound and persistent neutropaenia observed. It is more likely that there is a qualitative difference, with actual injury only being produced in specific circumstances. Henson and colleagues have shown accentuation of lung injury resulting from intratracheal instillation of chemotactic stimuli, with hypoxaemia or infusions of vasodilator prostaglandins (169). A further possibility is that injury only occurs when either neutrophils produce a specific toxic product (possibly a protease or oxygen free radical) or in the presence of a specific humoral mediator (possibly platelet-activating factor or an arachidonate metabolite). At present these are merely speculations, but undoubtedly in future the dichotomy pointed to by this study will be the subject of much research.

In summary, endotoxin infusion in dogs produced no evidence of transferrin accumulation as a measure of lung microvascular injury, despite indirect evidence for intrapulmonary neutrophil sequestration. These features point to a more complex relationship between the latter finding and the development of acute lung injury.

TABLE 5: Haemodynamic and oxygenation data after canine endotoxin infusion

		Time (hours) after endotoxin		
	Baseline (Pre-endotoxin)	0.5	1.5	2.5
HR (/min)	117 ± 3.4	130 ± 4.8	132.8 ± 3.2	139.3 ± 3.2
MABP (mmHg)	108.8 ± 6.3	68.3 ± 5.8*	59.5 ± 4.3*	83.5 ± 4.9*
PVR (mmHg/l/min)	2.7 ± 0.1	4.0 ± 0.3*	3.5 ± 0.2	2.9 ± 0.2
Cardiac Output (l/min)	3.1 ± 0.3	2.3 ± 0.3*	2.8 ± 0.5	3.2 ± 0.4
Arterial/Alveolar pO_2 ratio	0.66 ± 0.03	0.61 ± 0.07	0.63 ± 0.04	0.59 ± 0.09

Abbreviations:

PVR = Pulmonary vascular resistance; MABP = Mean arterial blood pressure; HR = Heart rate.

Results expressed as mean ± standard error. * = $p < 0.01$, versus baseline sample

CHAPTER 9

HISTAMINE AND BRADYKININ: THEIR EFFECT ON AN INDEX OF ALVEOLAR EPITHELIAL PERMEABILITY IN HUMANS

9. HISTAMINE AND BRADYKININ: THEIR EFFECT ON AN INDEX OF ALVEOLAR EPITHELIAL PERMEABILITY IN HUMANS

9.1 - Histamine

9.1.1 - Introduction

The role of histamine as a mediator of altered alveolar-capillary permeability in acute lung injury is controversial. Its potential role as a mediator was initially suggested by the studies of Brigham and Owen in 1975 (254). They showed in the sheep that histamine infusion caused a 2-6 fold increase in lung transvascular protein clearance as assessed by the product of lung lymph flow and lung lymph:plasma protein ratio. This implied an increase in pulmonary microvascular permeability, suggesting a possible role for histamine in clinical lung injury. Later more detailed studies from Brigham's group implied that any permeability response was only transient and that the sustained response seen was explainable by an increase in exchanging vessel surface area rather than microvascular permeability (255).

These studies from Brigham's group were in contrast to other studies which did not show a permeability enhancing effect of histamine on the pulmonary microcirculation (256,257). The conflicting data may be a reflection of the different molecular size substances studied and also the different component of the alveolar-capillary unit assessed; Pietra and associates used a static model with a large molecular weight probe (256) while

Goetzman's and Visscher's study examined alveolar epithelial permeability to albumin (257). Propst and associates extended this latter study by examining the effect of histamine on alveolar epithelial permeability to different molecular weight substances, using the in vivo saline-filled dog lung model (258). They showed that histamine increased permeability to substances of molecular weight up to 10,000 daltons, but like the study of Goetzman, permeability to albumin was unaffected. The exact site however, of any permeability-increasing effect of histamine is uncertain. Pietra and co-workers injected colloidal carbon intravenously to delineate the site of vascular leakage after histamine infusion (256). They found the marker to be confined to the cellular junctions of the bronchial circulation. This was in contrast to the original Brigham study (254) which showed an increased effect of histamine on lung lymph protein clearance when it was infused into the pulmonary circulation rather than the left atrium. The role of histamine in effecting an increase in bronchial vascular permeability was further assessed by Hutchison and colleagues, again using the sheep lung lymph model (259). They showed that aerosol histamine increased lung lymph flow with a higher protein concentration than under baseline conditions. These findings were interpreted as being consistent with an increase in bronchial rather than pulmonary vascular permeability.

This study sought to examine further the effect of histamine on lung permeability characteristics by assessing the response of pulmonary ^{99m}TcDTPA clearance to inhalation of a histamine aerosol in humans. A preliminary study by other workers showed an

apparent increase in lung DTPA clearance in response to histamine inhalation (260) but the time course of this response and the histamine receptor subtype response for mediating this effect had not been studied. Assessment of cutaneous vascular permeability showed that both H_1 and H_2 antagonists reduced histamine-induced skin weals in man, indicating a possible role for both receptor subtypes in vascular permeability (261). The H_2 antagonist cimetidine however, had little or no effect on resting bronchial tone or on histamine-induced bronchospasm which was exclusively H_1 mediated (262-264). This study examined the effects of inhaled histamine on the lung epithelial permeability index and determined the histamine receptor responsible for this by prior H_1 or H_2 blockade in a double blind randomized cross-over fashion. In addition, in the same subjects the effect of receptor blockade on histamine-induced bronchoconstriction was assessed.

9.1.2 - Methods

Subjects

Seven healthy non-smoking men (age range 24-36 years) were studied. Although not clinically asthmatic, all were atopic and had previously demonstrated bronchial reactivity to histamine. None had a history of recent upper respiratory tract infection and none was on medication. The study was approved by the hospital Ethical Committee and all subjects gave informed consent.

Lung epithelial permeability

Pulmonary clearance of inhaled ^{99m}TcDTPA was measured using identical equipment and technique to that previously described. Throughout this study DTPA clearance was expressed as the half-time clearance ($T_{1/2}$) rather than the rate constant of clearance as in previous chapters. Each subject had the clearance study performed on six separate occasions; the total dose of radioactivity inhaled by each subject was calculated to be less than 1 mCi. The respone to histamine (inhaled concentration described under Experimental Protocol) was verified on two separate occasions. Subsequently the effect of saline on DTPA clearance was assessed before entry into the placebo controlled study of H_1 and H_2 receptor antagonists. In the 2 initial histamine permeability studies, it was apparent that after histamine inhalation the radioactivity clearance was biexponential with an initial period of relatively steep fall, which in all subjects was

complete by 7 minutes. Thereafter the slope approximated closely to the pre-histamine challenge clearance slope. Because of this biexponential slope, data from the first 5 minutes was analysed separately.

Bronchial reactivity.

This was measured by determining the concentration of inhaled histamine which produced a 40% fall in expiratory flow at 70% expired vital capacity from a partial flow volume manoeuvre. This was expressed as the provocation concentration (PC_{40}). The partial flow volume technique is a sensitive measurement of small airway calibre and avoids possible changes in bronchomotor tone produced by a full inflation (265). A rolling-seal dry spirometer (P.K. Morgan, Ltd., Gillingham, Kent) was interfaced to a Hewlett Packard 85B dedicated minicomputer (Collingwood Measurement, Leicester, UK). Histamine was inhaled from a Wright nebulizer driven by air at 8 litres/minute for 2 minutes at doubling concentrations every 5 minutes from 2 mg/ml until the required bronchoconstriction.

Experimental Protocol

In 2 of the 7 subjects, inhalation of a concentration of histamine one multiple above the PC_{40} for 2 minutes either produced an insigificant increase in DTPA clearance or a significant increase that was not reproducible. Five subjects had a significant reproducible permeability increase in response to

histamine and they were studied further in a double blind randomized manner at weekly intervals. Subjects were pre-treated orally with either an H_1 blocker (terfenadine 60 mg), H_2 blocker (ranitidine 150 mg) or matched placebo at the same time on each of the three study days. Baseline airway calibre was assessed just before ingestion of the matched capsule and 2 hours later. This was to exclude an effect of either ranitidine or terfenadine itself on airway calibre. Immediately thereafter (2 hours after ingestion of the matched capsule) subjects inhaled via the ventilator circuit previously described, a nebulized solution of ^{99m}TcDTPA. After satisfactory counts of ^{99m}Tc over the lung was achieved (20-30,000/minute) baseline assessment of ^{99m}DTPA clearance was performed. After 15 minutes data collection, subjects inhaled a histamine aerosol generated by a Wright nebulizer at a concentration 1 multiple above the PC_{40} for 2 minutes. While subjects maintained the exact position relative to the scintillation counters they held previously, lung and thigh radioactivity counts were recorded for a further 25 minutes. Thereafter, bronchial reactivity was again measured with a histamine challenge as described above. This was designed to test the effect of pre-existing H_1 or H_2 blockade or placebo on histamine bronchial responsiveness.

Stastistical Analysis

Results were expressed as mean ± standard error of the mean. Differences between the groups were analysed using analysis of variance and paired student's t tests.

9.1.3 - Results

Lung epithelial permeability response to histamine.

Mean baseline half-time of clearance was 72.4 ± 5.4 minutes which did not fall significantly after saline inhalation (66.8 ± 9.9 minutes). Histamine caused a pronounced increase in clearance in the first 5 minutes after inhalation. In the 2 preliminary measurements, the half-time for this initial period fell by 64.6 ± 2.8% compared to the pre-histamine half-time ($p < 0.01$). The mean fall in half-time for the remaining 20 minutes of the study was 22.7 ± 8.5% and this fall did not reach statistical significance. Neither placebo nor terfenadine pre-treatment had any effect on the initial 5 minute permeability increase to histamine with respective falls in half-time of 62.6 ± 3.9% and 52.6 ± 6.0% (see Fig.16). Rantidine pre-treatment however, conferred significant protection with the half-time falling only 27.6 ± 4.9% in the first 5 minutes ($p < 0.01$ compared to terfenadine and placebo pre-treatment). On the 3 study days, mean pre-histamine half-time did not differ significantly.

Airway response to histamine

Terfenadine and ranitidine produced a mean change on baseline expiratory flow of 6.6 ± 3.6% and -4.9 ± 1.5% respectively. Neither change was significant and neither differed from that produced by placebo. PC_{40} values were derived for each subject on each of the 3 study days. With placebo pre-treatment mean

PC_{40} was not significantly different from the value obtained when bronchial reactivity was defined before entry into the study. PC_{40} rose from 5.1 ± 0.8 mg/ml (geometric mean ± SEM) after placebo to 38.0 ± 1.9 mg/ml after terfenadine pre-treatment ($p < 0.001$) - see Fig.17. These values underestimate the degree of protection conferred by terfenadine on histamine-induced bronchoconstriction since 2 subjects had to discontinue the histamine challenge at 64 mg/ml because of unacceptable systemic side-effects before the required change in lung function. These subjects were arbitrarily assigned 64 mg/ml as their PC_{40} to facilitate statistical analysis. After rantidine pre-treatment the mean PC_{40} (5.5 ± 1.5 mg/ml) did not differ significantly from that after placebo pre-treatment.

9.1.4 - Discussion

This study demonstrates that histamine inhalation, at a dose that caused significant changes in airway calibre, produced profound but transient increase in ^{99m}TcDTPA clearance. Prior H_1 receptor blockade had no significant effect on this histamine-induced response. In contrast however, H_2 receptor blockade did result in marked protection of the initial DTPA clearance increase. Assessment of bronchial responsiveness to histamine revealed that terfenadine pre-treatment significantly increased PC_{40} while ranitidine had no effect. This confirms the sole role of the H_1 receptor in mediating histamine-induced bronchoconstriction.

The failure of H_1 receptor blockade and the ability of H_2 receptor blockade to ameliorate histamine-induced DTPA clearance increase was a surprising finding. This discussion will therefore, focus on possible reasons to explain the result as well as its likely significance. The lack of response to the H_1 blocker could be due to inadequate receptor blockade. This however, appears unlikely as terfenadine resulted in significant shift to the right of the dose-response curve of bronchial responsiveness to histamine. Terfenadine is a specific H_1 antagonist whose plasma concentration peaks 1-2 hours after oral ingestion (266). Thus the lack of effect of this drug on the alveolar epithelial permeability index appears to be a true finding, as receptor blockade was theoretically and practically adequate. The apparent H_2 receptor mediation of increased

pulmonary solute clearance may reflect a role for this receptor in modulating pulmonary or bronchial blood flow. In vitro studies on human pulmonary arteries show that the H_2 receptor mediates pulmonary dilatation (267). Therefore, this ranitidine-mediated effect may be due to an increase in microvascular surface area due to recruitment of alveolar capillaries. The transient nature of the increased DTPA clearance after histamine would tend to support an H_2 "vascular blush" with resultant capillary recruitment as a possible mechanism. Interestingly, a recent study in sheep showed that increased bronchial arterial blood flow after histamine is mediated predominantly by H_2 receptors (268). This could promote airway mucosal oedema and the resultant change in epithelial cell configuration increase epithelial permeability (269). The relative importance of bronchial and pulmonary blood flow in influencing ^{99m}TcDTPA clearance in humans is difficult to assess precisely. In animal studies, solute clearance appears to be dependent on pulmonary rather than bronchial blood flow (134); this is in keeping with the known predominant peripheral deposition of the ^{99m}TcDTPA aerosol.

In further support of these data is the finding that H_2 receptor-blocking drugs have apparent anti-permeability effects in other models of lung injury. This raises the speculative possibility that this effect is independent of its ability to competitively antagonize histamine at H_2 receptor sites. This notion is supported by 3 diverse experimental findings:

(1) Cimetidine, a selective H_2 antagonist, when infused

intravenously, is a pulmonary vasodilator (270). The mechanism for this is unknown.

(2) In mice with acute lung injury induced by cobra venom factor (a complement activator), cimetidine produced a 44% reduction in an index of lung vascular permeability (166). In contrast, an H_1 antagonist had no protective effect.

(3) In an acid aspiration model of lung injury in rats, ranitidine produced significant reduction in the increased pulmonary ^{99m}TcDTPA clearance and lung water accumulation which occurred in association with the lung damage (271).

Although it is conceivable that histamine release is a component of both of the above models of lung injury, it is unlikely that its antagonism alone would produce the significant protection observed. If the mechanism of these findings is related to histamine antagonism, the important physiological effect blocked is likely to be pulmonary vasodilatation; otherwise the drug must possess some other unknown protective effect unassociated with histamine blockade.

In summary, histamine produced only a short-lived increase in pulmonary solute clearance which appeared to be H_2 receptor mediated. This is probably a haemodynamic effect related to pulmonary or bronchial vasodilatation. These data do not appear to support the notion that histamine plays an important role in mediating acute lung injury. In vivo endogenous histamine release may however, result in much higher tissue concentrations. Caution is therefore required in extrapolating these data to clinical lung

injury.

9.2 - Bradykinin

9.2.1 - Introduction

Like histamine, bradykinin is unequivocally a potent mediator of vascular permeability in the systemic microcirculation (272,273). Its effect however, on the pulmonary circulation is less well defined. In sheep in the presence of hypoxia, bradykinin induced an increase in lung lymph flow and lung lymph protein clearance (274). The authors ascribed this increase in microvascular permeability to hypoxia-mediated depression of angiotensin converting enzyme, which normally degrades bradykinin. Subsequent studies have not confirmed these data; using the same sheep lung lymph model, bradykinin, with or without angiotensin converting enzyme inhibition, had no effect on microvascular permeability (275). More recently, other workers have examined the effect of bradykinin infusion, with or without simultaneous hypoxia, on sheep pulmonary clearance of ^{99m}TcDTPA (276). Bradykinin did not increase lung solute clearance either with or without coexistent alveolar hypoxia. Additionally, they showed no impairment in the ability of the lungs to clear bradykinin despite profound alveolar hypoxia.

This study assessed in humans the effect of bradykinin inhalation - at a dose that had previously caused significant bronchoconstriction - on pulmonary ^{99m}TcDTPA clearance. This represented the first attempt to extend previous animal studies of the bradykinin effect on pulmonary alveolar-capillary permeability

to humans.

9.2.2 - Methods

Four healthy non-smoking subjects (age range 22-36 years) were studied. All subjects were atopic and mildly asthmatic. None had a history of recent upper respiratory tract infection and none was on medication. The study was approved by the hospital Ethical Committee and all subjects gave informed consent. The subjects had previously taken part in another study which assessed bronchial responsiveness to bradykinin. In that study, the provocative concentration required to cause a 35% fall in specific airways conductance (PC_{35}) using a computerized body plethysmograph had been assessed. Bradykinin had been delivered via a compressed air nebuliser controlled by a breath-actuated dosimeter. Each dose consisted of 5 slow vital capacity breaths from the nebuliser. Full details of this protocol are described elsewhere (277). The required fall in airways conductance was reached in the subjects at bradykinin concentrations ranging from 0.03 to 16 mg/ml. For the assessment of ^{99m}TcDTPA clearance, each subject inhaled their PC_{35} dose in an identical manner to that described above.

Lung epithelial permeability.

This was performed as previously described in this thesis. After collection of baseline DTPA clearance data, subjects inhaled their own PC_{35} dose while reclining semi-supine. As with the histamine study, this inhalation was accomplished without moving from the position held during the DTPA clearance study. The dose of bradykinin was delivered by 5 slow vital capacity breaths from

the dosimeter-controlled nebuliser. Immediately thereafter clearance data of DTPA was collected for a further 20 minutes. The half-time of clearance for each of the 4 subjects was calculated both before and after bradykinin inhalation.

Statistics

Results are presented as mean ± standard error of the mean. The paired student's t test was used to compare half-times before and after bradykinin inhalations.

9.2.3 - Results

The linear correlation coefficients of the corrected ^{99m}TcDTPA clearance data plotted against time, from which the clearance half-time is derived, ranged between -0.95 and -1.0 in the 8 DTPA clearance slopes analysed. Half-time clearance before bradykinin was 66.4 ± 5.8 minutes and after bradykinin was 60.8 ± 7.9 minutes (non-significant difference). No individual subject had a significant fall in DTPA clearance half-time after bradykinin.

9.2.4 - Discussion

These data indicate that bradykinin inhalation does not increase pulmonary epithelial permeability to a small molecular weight solute in humans. This finding is compatible with that of O'Brodovich and colleagues who studied the effect of bradykinin infusion on the pulmonary clearance of ^{99m}TcDTPA in sheep (276). Clearance from the lung of this solute is, as previously discussed, largely diffusion-dependent with the airway epithelium rather than the microvascular endothelium, the rate-limiting step. Thus it is possible that bradykinin had a small effect on endothelial permeability which is not reflected in these data. This is however, unlikely in view of the equivocal, largely negative, previous experimental studies of bradykinin and pulmonary vascular permeability.

Thus notwithstanding the potent effects of bradykinin on the systemic microcirculation, this study supports the concept that this mediator is unlikely to play a role in the increased alveolar capillary permeability associated with clinical lung injury.

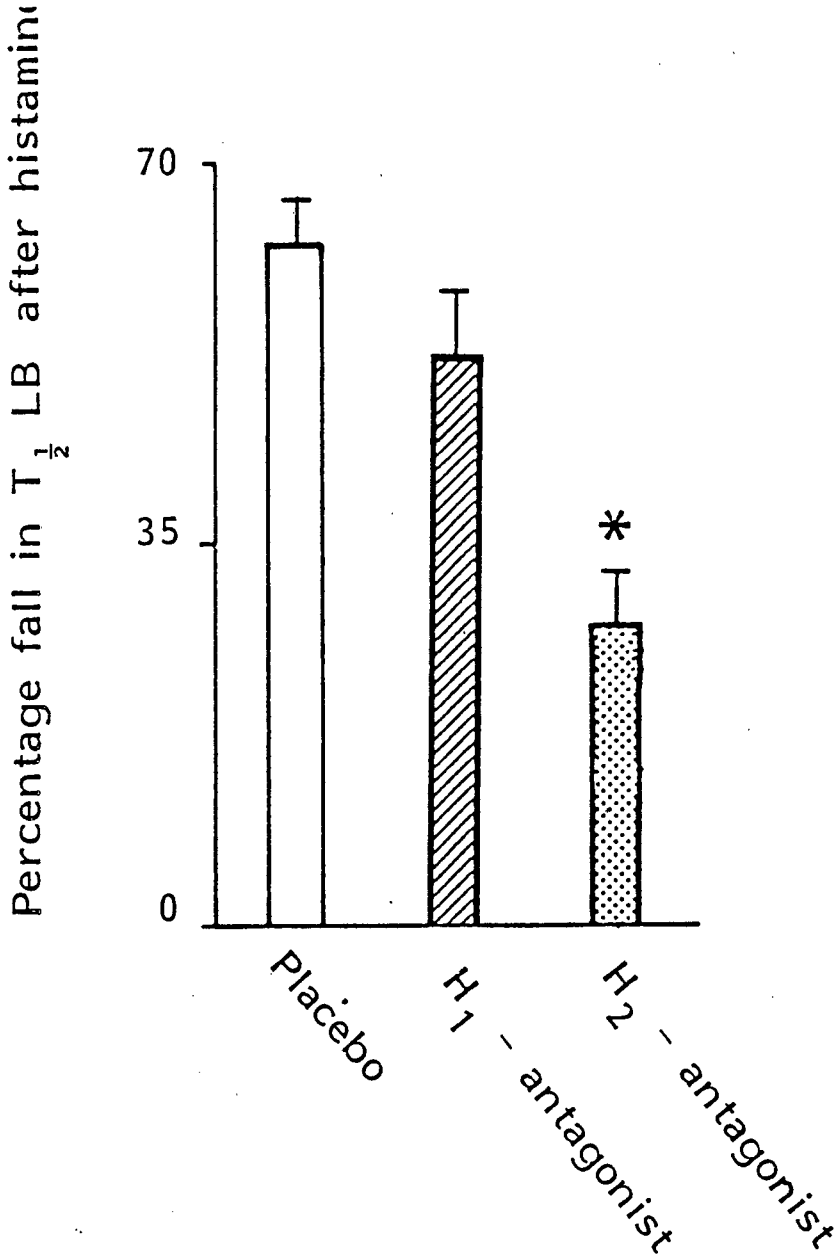

Figure 16

The effect of pretreatment with placebo, H_1-antagonist (terfenadine) or H_2-antagonist (ranitidine) on the pulmonary clearance of ^{99m}TcDTPA after inhaled histamine. The figure shows the mean ± SEM percentage fall in the half-time of clearance in the first five minutes after histamine inhalation (n=5). * $p < 0.01$ versus placebo or terfenadine pretreatment.

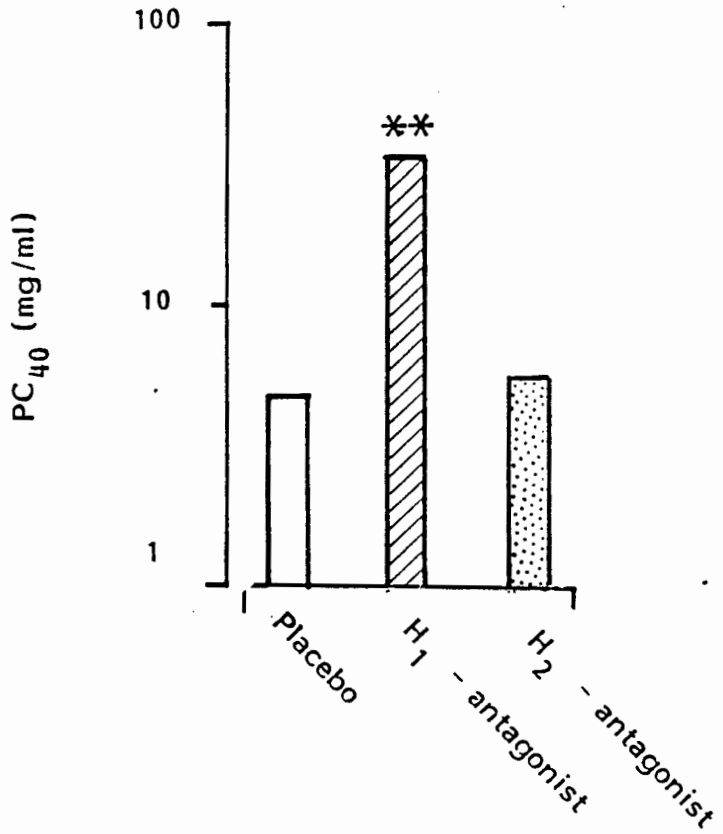

Figure 17

The effect of pretreatment with placebo, H_1-antagonist (terfenadine) or H_2-antagonist (ranitidine) on bronchial responsiveness to histamine. Values shown are the geometric mean PC_{40} for all subjects (n=5) on the 3 study days. ** $p < 0.001$ versus the other pretreatments.

CHAPTER 10

PLATELET ACTIVATING FACTOR INFUSION IN DOGS: ASSESSMENT OF LUNG TRANSVASCULAR PROTEIN FLUX AND RELATED CARDIORESPIRATORY VARIABLES.

10. PLATELET ACTIVATING FACTOR INFUSION IN DOGS: ASSESSMENT OF LUNG TRANSVASCULAR PROTEIN FLUX AND RELATED CARDIORESPIRATORY VARIABLES

10.1 - Introduction

Platelet activating factor (PAF) is one of the most potent inflammatory mediators to be recently identified. Henson reported originally the isolation of a soluble factor from antigen-sensitized basophils, that induced histamine release from platelets (278). Subsequently, it has been shown to be released by a variety of inflammatory cells including neutrophils, platelets, macrophages and monocytes (279,280). The mediator has been identified as a low molecular weight phospholipid, acetyl glyceryl ether phosphorylcholine (281), and is usually referred to as AGEPC in the American literature. It has a wide spectrum of biological activities including in vitro platelet and neutrophil aggregation and stimulation (281-283). Intravenous infusion of PAF into rabbits produces acute neutropaenia and thrombocytopaenia together with profound cardiopulmonary changes, including systemic hypotension, increased pulmonary artery pressures and increased airway resistance (284,285). These latter changes are typical of those which develop during IgE-induced systemic anaphylaxis in the rabbit and suggest that this phospholipid is an extremely important mediator of allergic and inflammatory responses.

An additional important proinflammatory property of PAF is its ability to increase vascular permeability. This was first alluded

to by McManus and co-workers in 1981 (286); they showed that PAF infusion into baboons caused a dramatic increase in haematocrit. They interpreted this increase as being due to loss of intravascular fluid due to altered vascular permeability, although the sites of extravascular water accumulation were not further investigated. In subsequent work from this group, they investigated a morphological correlate of increased vascular permeability in extrapulmonary tissue (287). In this study, PAF was potent in inducing Evans blue dye extravasation in skin and cremaster muscle. Concomitant with the vascular permeability changes, leucocyte emigration from venules was noted. These findings have been confirmed and extended by other groups focussing on the systemic microcirculation. Using the hamster cheek pouch, PAF was shown to induce macromolecular extravasation from venules as well as polymorphonuclear leucocyte adherence and emigration (288).

Few studies have assessed the effect of PAF on the permeability characteristics of the pulmonary microcirculation in vivo. Available evidence suggests that exogenous PAF can induce pulmonary oedema in an isolated perfused guinea pig lung system and this is associated with increased levels of metabolites of prostacyclin and thromboxane in the perfusate (289). This effect of PAF was platelet-independent in contrast to earlier work in isolated rabbit lungs where the PAF-induced pulmonary oedema was dependent on the presence of human platelets (290). Mojarad and co-workers did examine the effect of PAF in vivo using a dose that did not change pulmonary haemodynamics or platelet and neutrophil

counts (66). In this canine study, microvascular permeability was assessed by lung lymph protein clearance. Small but significant increases were noted in this index but these were unassociated with obvious morphological injury.

Prompted by the paucity of data available on the effect of PAF in vivo, this study aimed to assess this putative mediator further by examining in dogs its effect on a dynamic index of pulmonary microvascular integrity. The transvascular protein flux of ^{113m}In-transferrin was studied rather than the pulmonary clearance of ^{99m}TcDPTA as the former index alone could provide a dynamic, continuous assessment. In order to mirror the clinical situation, a dose of PAF was selected which, based on previous canine studies, was likely to elicit major but non-lethal haemodynamic effects. In this study, acute lung injury was assessed by:

(1) Examining continuously the pulmonary transvascular flux of ^{113m}In-transferrin to provide a dynamic index of pulmonary microvascular permeability.

(2) Measurement of gravimetric lung water before and after PAF infusion to provide an index of extravascular lung water accumulation.

(3) Histological (including electron microscopic) evaluation of lung biopsies to provide a morphological correlate for the above functional indices. In addition to assessment of alveolar

capillary injury, histological evidence for platelet and neutrophil sequestration within the pulmonary microvasculature was sought.

The results of the study demonstrate that PAF induces acute lung injury in vivo, supporting a role for the mediator clinically in ARDS.

10.2 - Methods

Animals and anaesthesia.

Ten greyhound dogs (mean weight 26 Kg, range 22 to 33 Kg) were studied. The animals were anaesthetized with intravenous thiopentone (10 mg/Kg) and intubated and ventilated using a Starling pump (rate 14/minute, tidal volume 12 ml/Kg) with an inspired gas mixture of nitrous oxide in oxygen (1:1 by volume). This was supplemented by 0.5-1.0% halothane.

Operative procedure and instrumentation.

After induction of anesthesia, femoral arterial and internal jugular vein catheters were inserted to provide continuous monitoring of heart rate, systemic arterial blood pressure and central venous pressure. A right thoracotomy was performed and another catheter introduced directly into the left atrium for blood sampling and pressure recording. A 7 French thermistor tipped, flow directed Swan Ganz catheter was inserted through the right atrium and right ventricle into the pulmonary artery, where its position was verified by the appropriate pressure tracing. The tip of the catheter was not wedged as left atrial pressure was monitored directly. Through this catheter cardiac output was determined by thermal dilution using a cardiac output computer.

Drug administration.

Known amounts of PAF aliquots, prepared from bovine heart lecithin by a modification of a previously described method (281), (Sigma Ltd, Dorset, UK) were stored at -20^{o}C having been dissolved in chloroform. On the day of study, the solvent was evaporated and the PAF redissolved in saline containing 2 mg bovine serum albumin/ml. Each dog received 1 ug/Kg PAF (usual volume of injectate 6-7 ml) as a bolus injection over 2 minutes directly into the pulmonary artery line.

Studies performed.

(1) Measurement of transvascular lung protein flux.

The technique used here was identical to that described in the canine bypass study which was outlined in Chapter 7. Briefly, autologous ^{99m}Tc labelled red cells were injected along with ^{113m}In which had been demonstrated to bind to transferrin in vivo (approximately 1 and 0.5 mCi of isotope activity respectively). Scintillation detectors (5.1 x 5.1 cm sodium iodide crystals) were placed over the heart and right mid lung field. The probe over the heart was placed by direct vision and its position optimized as previously by ensuring that counts due to ^{99m}Tc red cells, as a reflection of the intravascular pool, were maximized. The isotopes were injected 30 minutes after thoracotomy and instrumentation were completed. Subsequently, data was acquired continuously until completion of the study. Initial data was collected for 30 minutes prior to injection of platelet activating factor (n=6) or equivalent volumes of diluent (n=4) and

for three and a half hours after injection. All data was analysed in 30 minute time periods. Thus, a value of protein flux units (x 10^{-3}/minute) was derived from the pre-PAF or control injection periods as well as 7 values to represent the 210 minutes of study after injection. The method of data acquisition and analysis was identical to that already described in Chapters 4 and 7.

(2) Haemodynamic Studies

Heart rate, mean systemic arterial blood pressure, mean pulmonary artery and left atrial pressures were recorded and displayed continuously. Cardiac output measurements were performed before PAF or control administration and at 1, 5, 10, 15, 30 minutes afterwards and thereafter at half hourly intervals until 210 minutes when all studies were completed. On each occasion, cardiac output measurements were performed in triplicate and a mean value taken. Pulmonary vascular resistance was calculated as the -

$$\frac{\text{mean pulmonary arterial pressure - mean left atrial pressure}}{\text{cardiac output}}$$

and expressed in arbitrary units.

(3) White cell and platelet counts.

These were performed manually using Neubauer chambers. Differential cell counts were performed on 200 consecutive cells

using Wright's stained blood film. The effect of haemodilution was corrected for by simultaneous measurement of the packed cell volume. Blood samples were taken at each time point (pre-PAF or control) and thereafter at 5, 15, 30, 60 and 210 minutes, from both the left atrial and central venous lines.

(4) Alveolar to arterial oxygen tension gradient.

The alveolar partial pressure of oxygen was calculated from the alveolar gas equation described in Chapter 4. Arterial oxygen and carbon dioxide tensions were determined on an automated analyser and the inspired oxygen concentration was verified with a paramagnetic oxygen analyser. Arterial blood was sampled before and immediately after PAF or control injection and at half hourly intervals thereafter until all studies were completed.

(5) Peak inflation pressure.

This was monitored before PAF or control injection, immediately thereafter and at 5,15,30 minutes and then at 30 minute intervals until completion of the study. This was used as an indirect index of airway calibre.

(6) Gravimetric lung water.

Biopsies were taken from the right middle lobe before drug or control administration and at the end of the study. Gravimetric lung water was calculated by a modification of the method of Pearce

et al (99). Correction for residual blood in tissue was facilitated by counting known weights of blood and lung tissue for ^{99m}Tc labelled red cells which had already been introduced for the study of transvascular protein flux. An example of the calculation involved in deriving the baseline wet to dry weight ratio in one of the PAF studies is outlined here:

	wet weight (gm)	dry weight (gm)
Lung tissue	1.4297	0.3031
Blood	1.0782	0.2682

Calculation of correction for residual blood:

a) 1.0392 gm tissue homogenized in 9 ml saline
1 ml of homogenate yields 427 counts/minute (cpm)
Therefore total count in tissue = 427 x 9 = 3847 cpm

b) 1.0134 gm of blood gave 16,725 cpm

c) Weight of blood in tissue = (3847 ÷ 16,725) x 1.0134
= 0.233 gm/1.0392 gm tissue

d) True wet weight of tissue = 1.4297 - (0.233 x 1.4297 ÷ 1.0392)
= 1.4297 - 0.3206
= 1.109 gm

e) Dry weight of same tissue = 0.3031 - (0.3206 x 0.2682 ÷ 1.0782)
= 0.2242 gm

f) Corrected wet/dry weight ratio = 1.109 ÷ 0.2242 = 4.946

(7) Lung histology

Lung biopsies from the right middle lobe were taken before PAF or control administration and at the end of the study. The tissue was divided immediately for light and electron microscopic study. For light microscopy the tissue was fixed in 10% neutral buffered formalin prior to being stained and examined. Tissue for electron microscopy was fixed in phosphate buffered 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. The histologist was unaware whether a particular dog had been given PAF or control solution.

Summary of protocol.

After initial instrumentation and equilibration, baseline measurement of transvascular protein flux was performed. Thereafter blood samples were drawn and a lung biopsy performed for baseline histology and weight:dry weight ratio estimations. In 6 dogs, PAF was then administered over 2 minutes as described above, while in the remaining 4 dogs the diluent was given in an identical volume. Both groups were then studied in identical fashion with continuous transvascular protein flux, haemodynamic monitoring and intermittent blood sampling from the left atrial and pulmonary artery lines as described. At 210 minutes after PAF or control solution, a further lung biopsy was performed for histology and lung water estimation and the study completed. In all animals, only intravenous normal saline was infused during the study (approximately 10 ml/Kg/hr) irrespective of the haemodynamic state.

Statistics.

All results are expressed as mean ± standard error of the mean. All haemodynamic data is expressed as a percentage of the baseline value. When serial data were compared with one baseline sample, paired t tests were used with the critical p value (0.05) corrected by the Bonferroni method (235).

10.3 - Results

Pulmonary transvascular protein flux.

A value for protein flux units was derived for each 30 minute study period - 1 before PAF or control administration and 7 thereafter. The results in the PAF groups are represented graphically in Fig 18. Baseline pre-PAF protein flux was 0.4 ± 0.1 units and in the first half hour time period after PAF administration only 1 of the 6 dogs had a value (6.3) that was outside the 95% confidence limits derived from the baseline studies. As a group, protein flux was significantly increased from baseline values from the 4th period (60-90 minutes, 1.7 ± 0.5) through the remaining 2 hours of the study (2.5 ± 0.5, 2.5 ± 0.6, 2.3 ± 0.6, 2.4 ± 0.6 respectively, $p < 0.01$ for all these periods compared to baseline). Despite the PAF group as a whole showing increased transvascular protein flux from 60 minutes to the end of the study, this response was not uniform as 1 animal had a normal value for each post-PAF time period. None of the 4 control dogs who were subjected to the same protocol had a single value for any of the time periods that was outside the 95% confidence limits established from their baseline study (0.2 ± 0.2 units). Baseline values in the 2 groups did not differ significantly.

Gravimetric lung water.

This was examined in 4 of the 6 PAF dogs and all of the 4 control animals. In the PAF group, wet:dry weight ratios increased

from 4.60 ± 0.2 to 5.75 ± 0.3 ($p < 0.01$). This constitutes a 25% increase in an index of extravascular lung water accumulation. In the control group, no significant difference existed between the initial and final wet:dry weight ratio (4.73 ± 0.1 and 4.66 ± 0.1 respectively).

Lung histology.

Light microscopic appearances were in all cases within normal limits. Only the electron microscopy studies in the PAF group revealed significant abnormalities. Gross endothelial cell swelling was noted in all PAF animals (see Fig.19) and in some cases clear disruption of the endothelial cell layer was seen (Fig.20). There was marked accumulation of granulocytes in alveolar capillaries and many of these cells were tightly apposed to the endothelial cell (Fig.21). Some granulocytes appeared to be disintegrating (Fig.22). No structural abnormalities in or accumulation of platelets in the pulmonary microvasculature was noted.

Haemodynamic studies

Mean systemic arterial blood pressure (MABP) fell to 42.0 ± 3.5% of the baseline value 1 minute after completion of PAF administration ($p < 0.001$). A significant fall in MABP persisted until 15 minutes after PAF administration (Fig.23) at which point it was 67.2 ± 5.8% of the baseline value ($p < 0.01$). Thereafter MABP gradually recovered towards the normal range and at completion of the study was 94.0 ± 0.8% of its baseline value. Fall in MABP

was accompanied by an initial dramatic fall in cardiac output to 36.0 ± 4.9% of baseline value 1 minute after PAF administration. This fall persisted at 5, 10 and 15 minutes (47.1 ± 7.3, 57.6 ± 6.0 and 63.8 ± 4.4% of baseline value respectively, $p < 0.01$). Thereafter like MABP, cardiac output gradually returned towards baseline values and at completion of the study at 210 minutes after PAF was 81.6 ± 1.9 of the baseline value. Pulmonary vascular resistance (PVR) was, as outlined, derived from the mean pulmonary arterial and left atrial pressures and the cardiac output. Immediately after PAF administration (Fig.24) there was a marked increase in PVR to 273.8 ± 57.9% of the baseline pre-PAF value ($p < 0.01$). This significant PVR increase persisted at 5 and 10 minutes (188.2 ± 22.7 and 187.1 ± 16.5% of baseline value respectively, $p < 0.01$). Thereafter, although mean PVR remained above baseline value, the p value was above the significant threshold taking Bonferroni's correction for multiple comparisons into account. At study completion, the PVR was 132.0 ± 13.0% of baseline value ($p = 0.047$). Control animals showed no signficant variation in any haemodynamic parameter throughout the experiment.

Neutrophil and platelet counts.

These are displayed in Tables 6 and 7. Neutrophil counts (Table 7) before PAF administration in central venous and left atrial blood were 2.1 ± 0.5 and 2.1 ± 0.4 x $10^3/mm^3$ respectively. After PAF significant falls were noted ($p < 0.01$) which persisted to the 15 minute sample (Fig.25). Only in the 15 minute sample did a significant transpulmonary gradient (central

venous - left atrial) exist. A significant rebound neutrophilia was noted in the final sample at 210 min (5.3 ± 1.3 and 5.0 ± 1.0 respectively). Platelet counts (Table 7) followed a similar pattern with initial central venous and left atrial counts of 103.9 ± 19.6 and 98.4 ± 15.1 falling to 54.7 ± 3.5 and 46.2 ± 5.3 x $10^3/mm^3$ respectively 5 minutes after PAF ($p < 0.001$). Platelet counts remained significantly depressed until the 15 minutes (64.4 ± 4.0 and 70.5 ± 8.8 respectively, $p < 0.01$) but by 30 minutes were similar to baseline levels. At no stage was there a significant transpulmonary gradient for platelet counts. No significant alteration in platelet or neutrophil counts were noted in the control animals.

Peak inflation pressure.

This increased from 18.0 ± 2.0cm H_2O before PAF administration to 23.8 ± 2.3 cm H_2O immediately thereafter ($p < 0.01$). This rise was transient and by 5 minutes peak inflation pressure was similar to baseline levels (19.2 ± 1.7 cm H_2O) where it remained for the rest of the study. No significant change was noted in this variable in the control group over the course of the study.

Alveolar-arterial oxygen difference.

This gradient increased from 152.4 ± 15.2 mmHg at baseline to 307 ± 35.4 mmHg at the end of the study in the PAF group ($p <$

0.001). No significant increase occurred in the control group.

10.4 - Discussion

This study emphasises both the wide range of biological actions, and potential importance as a inflammatory mediator of PAF. A single infusion of 1ug/kg of PAF in dogs produced important haemodynamic effects with systemic hypotension, reduction in cardiac output and increased pulmonary vascular resistance. Most significantly from the viewpoint of this study, was the demonstration that in vivo, infusion of the mediator produced unequivocal evidence of acute lung injury. Functionally, increased microvascular permeability was noted with abnormal pulmonary transvascular protein flux; additional evidence for loss of microvascular integrity came from the 25% increase in extravascular lung water. These findings were extended by the morphological demonstration of endothelial cell injury on electron microscopy which also showed intrapulmonary neutrophil sequestration. Using an indirect index, PAF infusion resulted in only a transient airway calibre decrease, but widening of the alveolar-arterial oxygen gradient was noted. All these changes appeared specific to PAF, since these features were not noted in the control group who were treated in all other respects similarly.

The finding of acute lung injury, supported by both functional and morphological evidence, developing after in vivo canine PAF infusion is novel, and supports the 2 previously published preliminary studies of PAF infusion in sheep (291,292). These studies however, only examined lung microvascular permeability, by assessing transvascular lung lymph protein clearance, and did not assess extravascular lung water or lung morphology. In this study

transvascular protein flux in the group as a whole only became abnormal 60 min after PAF infusion. This is in contrast to the above sheep studies (291,292), where lung lymph flow appeared to increase immediately after PAF infusion. While this may reflect genuine species differences, another interpretation is the increased sensitivity of the lymph protein clearance technique. This was not the case however, when Gorin and colleagues compared the two techniques and found a close correlation between them (123). If the temporal findings in this study are valid, it would resemble closely the sheep endotoxin model, where peak rises in pulmonary artery pressure are seen early and an increase in lung vasacular permeability occurs only from 2 hours onward (251). Interestingly, the similarity to the endotoxin model extends both to the haemodynamic findings and the apparent involvement of neutrophils in the production of lung injury.

Morphologically, intrapulmonary neutrophil sequestration was a prominent feature and this was associated with a profound early neutropaenia and the demonstration of a transpulmonary gradient. Although thrombocytopaenia was also noted in the first hour after PAF infusion, this was unassociated with a transpulmonary gradient and no increase in pulmonary capillary numbers was noted histologically. This would imply that the development of lung injury was independent of the platelet. This is consistent with Christman and associates who showed that platelet depletion did not attenuate lung injury, (292) and an in vitro study where PAF-induced pulmonary oedema in lungs perfused with platelet-free solution. As was discussed in earlier chapters, the presence of neutrophils in increased numbers in the pulmonary capillaries, even if as here they are tightly apposed to endothelial

cells, does not neccesarily imply a causative relationship with the observed lung injury. PAF may have promoted secondary effects such as the production of leukotrienes (293) or thromboxane A_2 (287), which could have initiated or accentuated pulmonary microvascular lung injury. Alternatively, PAF may have damaged the endothelium directly and the presence of the neutrophil only reflect an inflammatory response to injury. This is supported by work in the rat and rabbit systemic microcirculation showing that despite leucocytic infiltrates after PAF, neutrophil depletion did not alter the vascular permeability changes induced (287).

The haemodynamic results of this study are very similar to those reported by Kenzora and colleagues who assessed the effects in dogs of 0.4ug/kg PAF infusion (294). They found similarly, a marked drop in systemic arterial blood pressure and cardiac output gradually returning towards the normal range by 60 min. Unlike this study, the effect on pulmonary vascular resistance appeared more transient, lasting only a few minutes, although this may reflect the smaller dose used. Higher dose infusions (20-22ug/kg) produce profound circulatory collapse and occasional death in dogs (295). The particular interest of the haemodynamic findings relates particularly to whether PAF, since it appears to produce similar effects, is a mediator of endotoxic shock. A recent study addressed this notion, by assessing in rats the effect of a specific PAF antagonist on endotoxin-induced cardiovascular changes (296). The PAF antagonist reversed endotoxin-induced hypotension and improved survival, implying a central role for the mediator in endotoxaemia.

The haemodynamic data are of additional interest, since it is conceivable that the cardiovascular changes are playing a part in the genesis of the lung injury. Shock of any cause may predispose to ARDS (297) and the possibility exists that the lung injury in this study was initiated or accentuated by the sustained systemic arterial hypotension or indeed by the elevated pulmonary microvascular pressure. In an in vitro system, nitroglycerin infusion maintained baseline pulmonary artery pressure and prevented the development of PAF-induced pulmonary oedema (290). This suggested that the oedema was hydrostatic in nature. A similar mechanism undoubtedly played no part in the abnormalities observed in this study as left atrial pressure never rose above 9mm Hg and endothelial cell injury and vascular permeability increase were clearly demonstrated. It is still feasible however, that arterial hypotension played a role in the pathogenesis of lung damage, although this latter feature has been noted with PAF when there were no associated haemodynamic alterations (66). The extremely transient nature of the reduction in airway calibre, as inferred from the peak inflation pressure, is in keeping with data from human studies of aerosolised PAF (298,299). As in this study, the maximum response was seen within minutes of inhalation of the mediator.

This study has provided conclusive evidence that PAF can experimentally produce lung injury. This appears independent of platelets but the neutrophil or its toxic products may be playing a role in the response. The data implicate this unique phospholipid as an important mediator of acute lung injury. Further studies are required however, to define its complex mechanism of action.

TABLE 6: Platelet counts before and after platelet activating factor infusion

		CENTRAL VENOUS		LEFT ATRIAL
Pre PAF		103.9 ± 19.6		98.4 ± 15.1
Post	5 min	54.7 ± 3.5	*	46.2 ± 5.3
	15	64.4 ± 4.0	*	70.5 ± 8.8
	30	76.9 ± 10.3		73.9 ± 15.0
	60	96.1 ± 14.9		96.2 ± 9.4
END	210	94.6 ± 10.5		95.0 ± 6.0

* $p < 0.01$ versus baseline value

All counts are mean ± SEM (X $10^3/mm^3$).

TABLE 7: Neutrophil counts before and after platelet activating factor infusion

		CENTRAL VENOUS		LEFT ATRIAL
Pre PAF		2.1 ± 0.5		2.1 ± 0.4
Post	5min	1.1 ± 0.3	*	0.9 ± 0.3
	15	1.5 ± 0.3	* **	1.1 ± 0.4
	30	1.7 ± 0.4		1.4 ± 0.4
	60	2.6 ± 0.7		2.6 ± 0.7
END	210	5.3 ± 1.3	*	5.0 ± 1.0

* $p < 0.01$ versus baseline sample

** $p < 0.01$ versus baseline transpulmonary gradient

All counts are mean ± SEM (X $10^3/mm^3$)

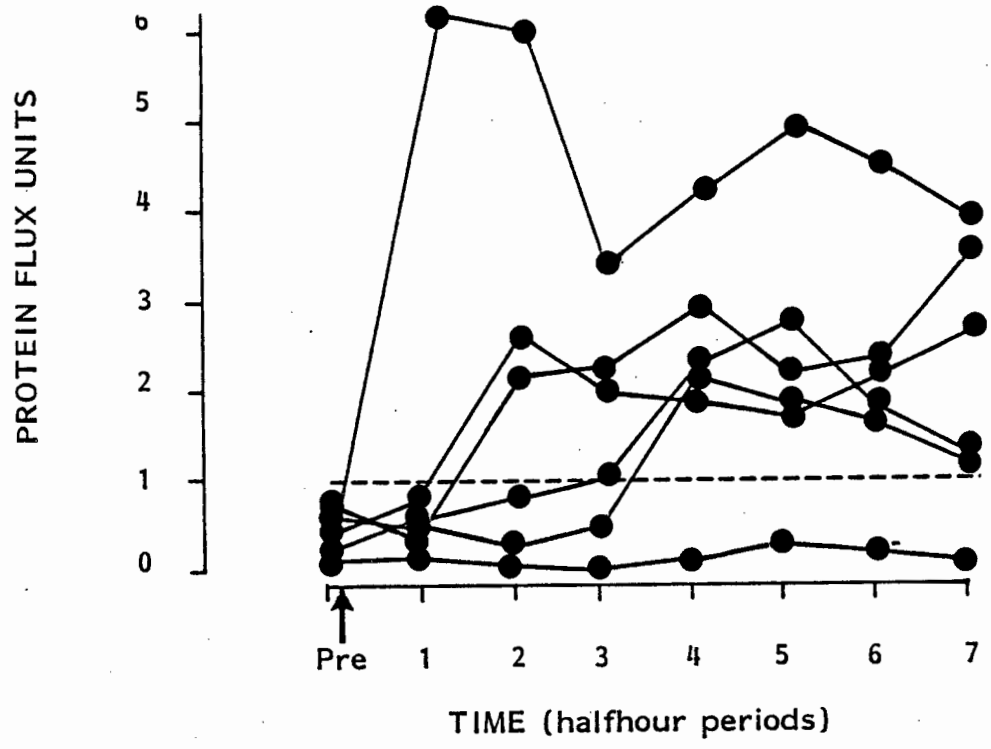

Figure 18

This demonstrates the individual canine transvascular protein flux at baseline (1st data point) and for consecutive half hour time periods after infusion of platelet activating factor. Mean transvascular protein flux became significantly increased only after 90 min (see text). One animal had an immediate increased value after infusion, while another stayed within the 95% confidence limits of the study (dotted horizontal line) throughout.

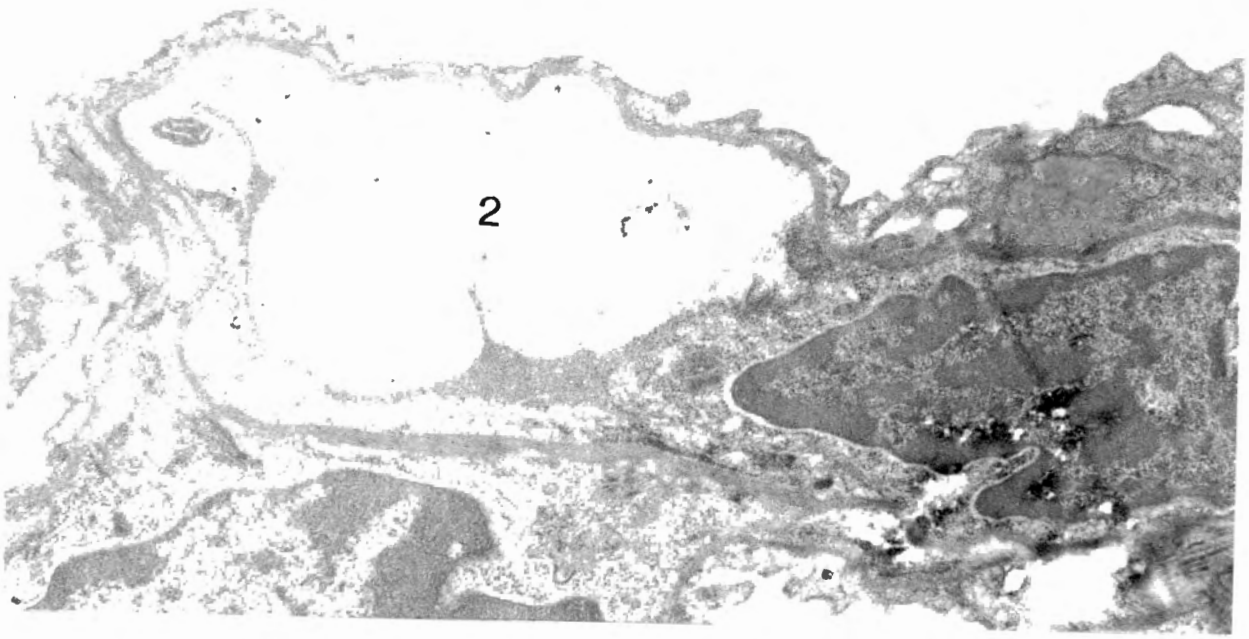

Figure 19

This electron micrograph (as with the others in this series) is from lung biopsies taken at the completion of the study (210 min after platelet activating factor infusion). This example (x 20,000) shows gross endothelial swelling (2)

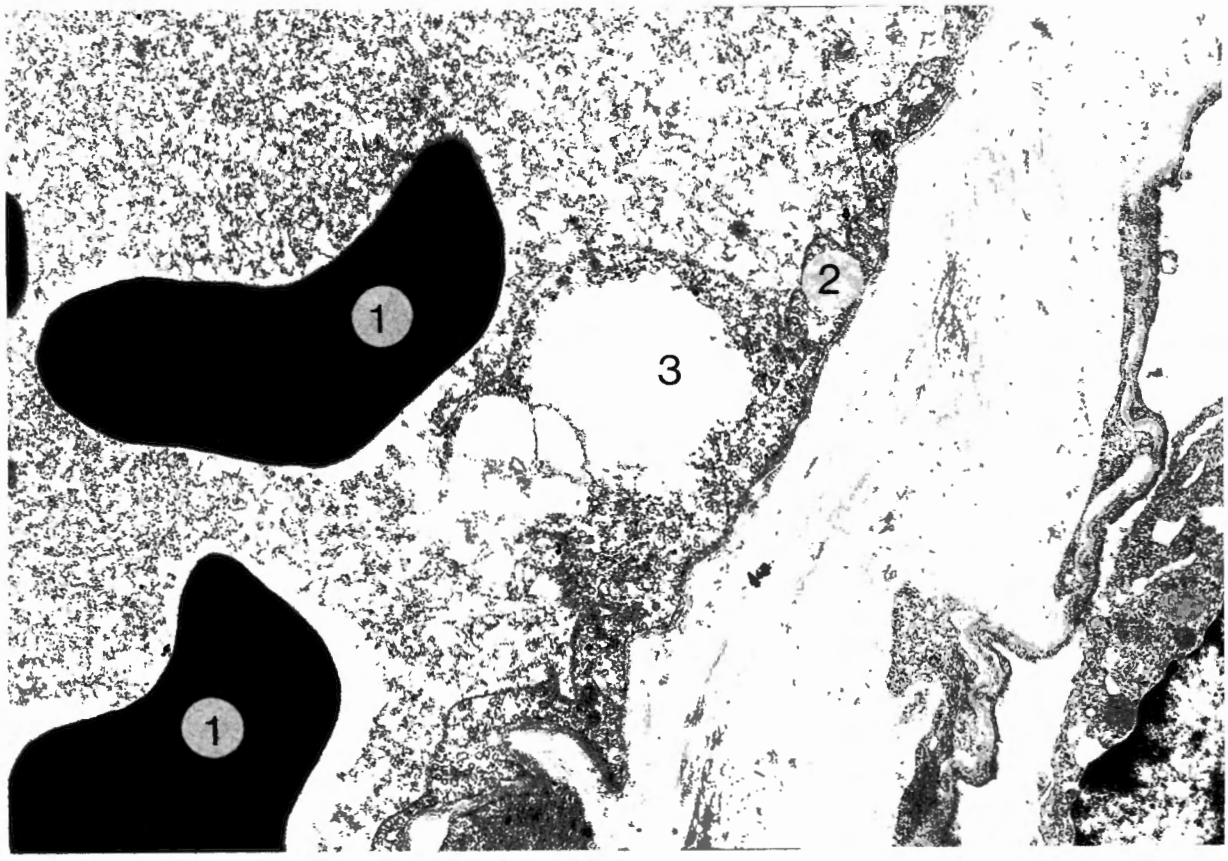

Figure 20

Electron micrograph (x 12,600) showing , on the left, a red cell (1) surrounded by plasma in the capillary lumen. On the right the endothelium is visible (2), which in parts is grossly swollen (3) and apparently disrupted.

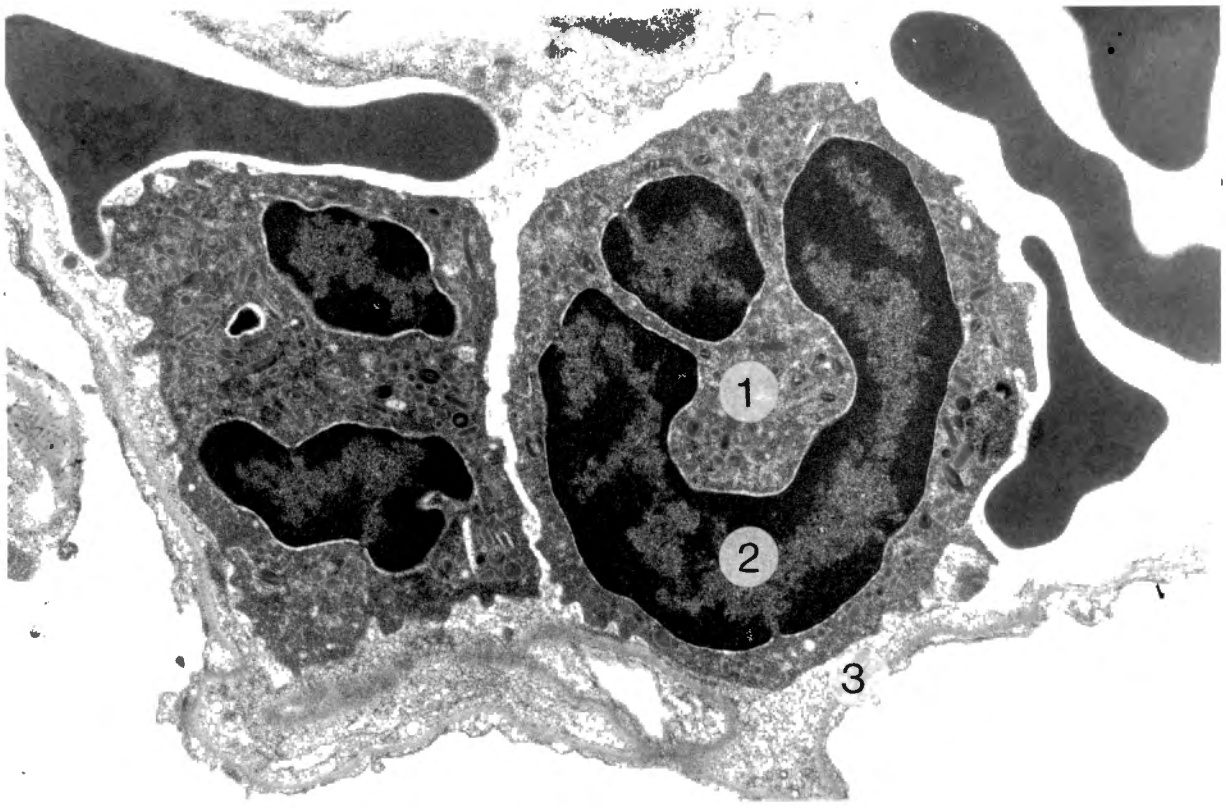

Figure 21

Electron micrograph (x 12,600) showing a neutrophil (1) with its multilobed nucleus (2), apposed to the endothelial lining (3).

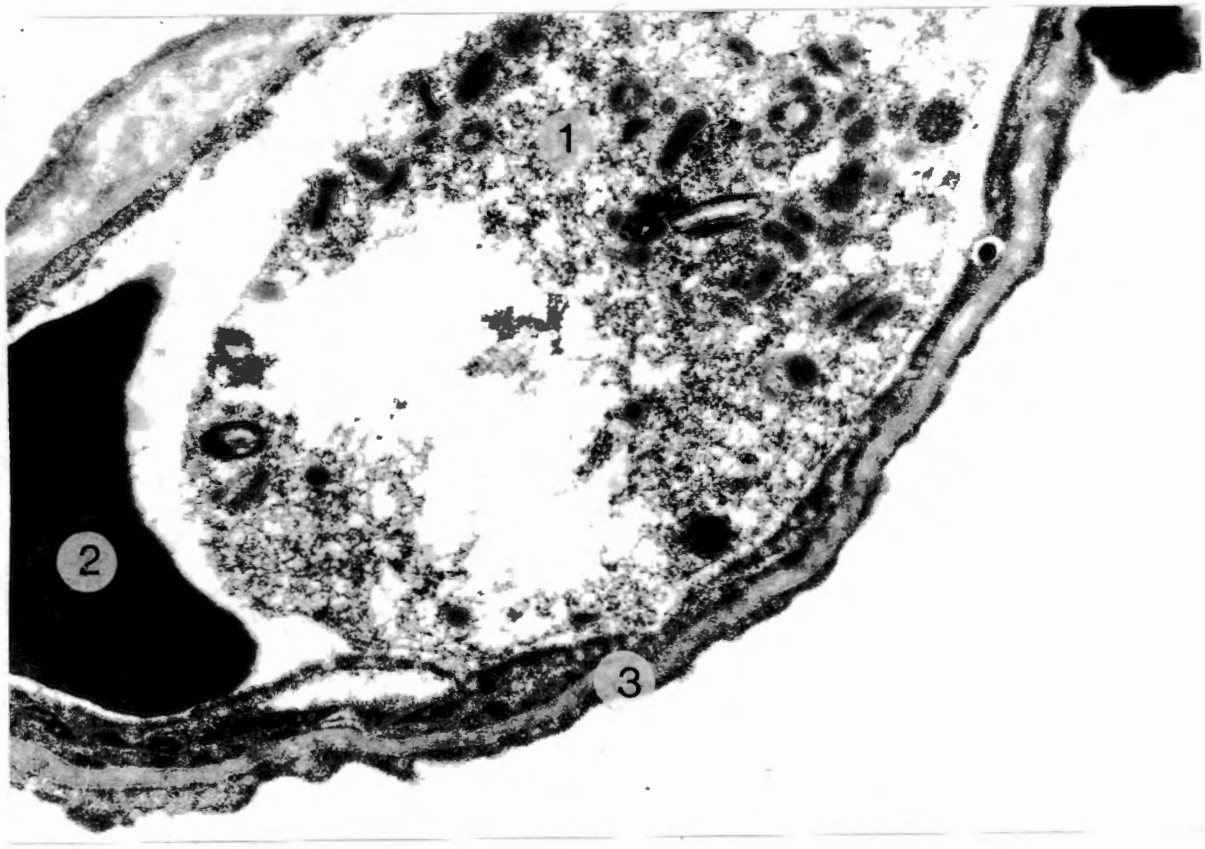

Figure 22

Electron micrograph (x 32,000) showing the neutrophil (1) and the red cell (2) within the capillary lumen. The neutrophil is apposed to the endothelial surface (3) and clearly disintegrating.

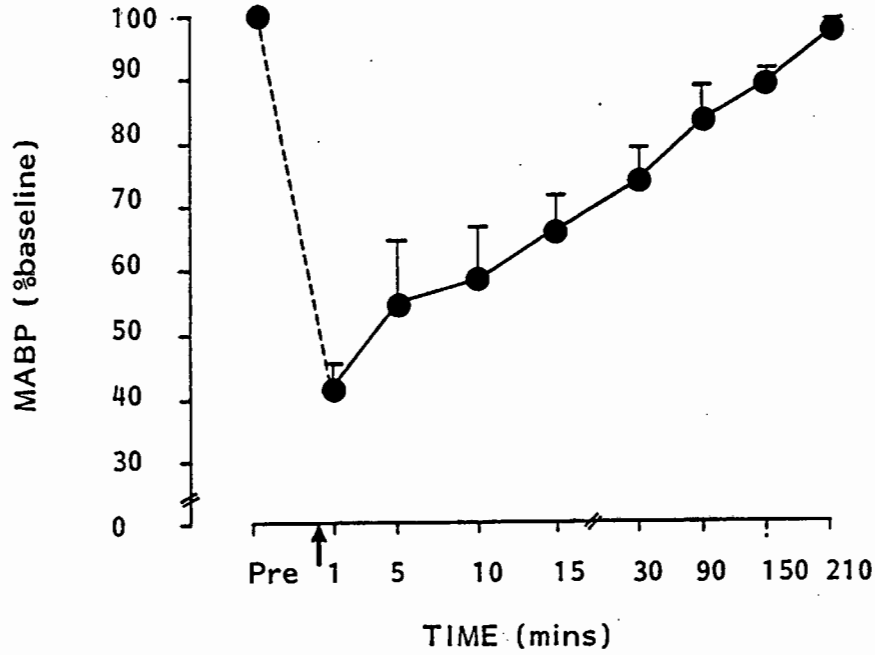

Figure 23

Mean arterial blood pressure (MABP) response to platelet activating factor infusion, expressed as the percentage of the baseline value. Significant falls were noted until 15 min after infusion, thereafter gradually returning towards baseline values.

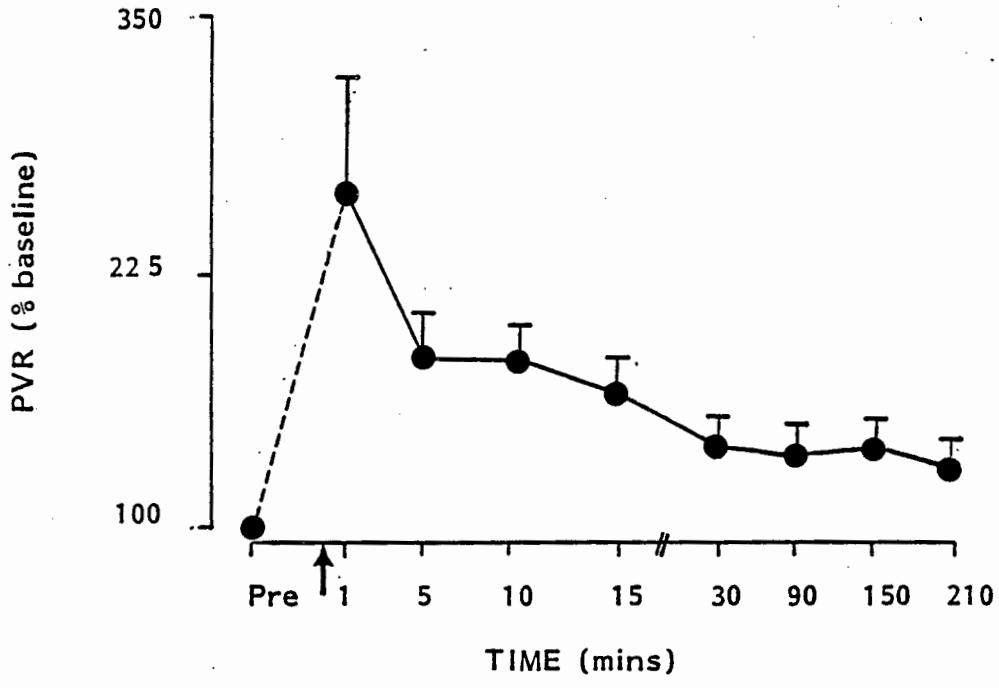

Figure 24

Pulmonary vascular resistance (PVR) response to platelet activating factor infusion, expressed as a percentage of the baseline value. Significant increases were transient, lasting until 10 min after infusion (187 ± 16.5% baseline value, $p < 0.01$.)

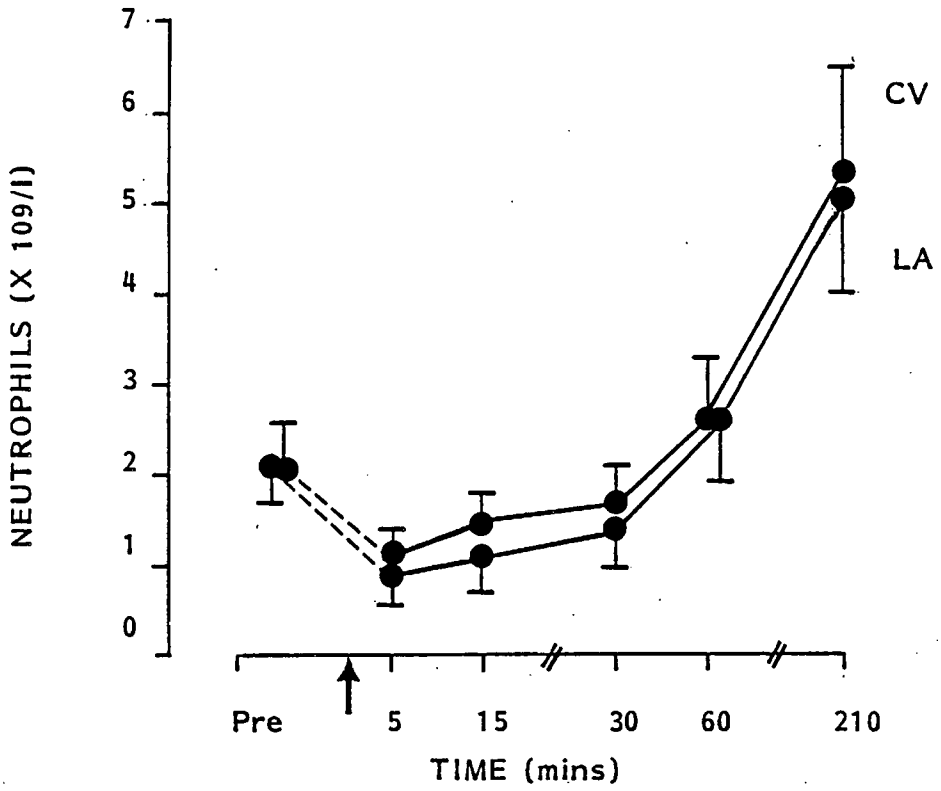

Figure 25

Graphic representation of neutrophil counts in central venous (CV) and left atrial (LA) blood after platelet activating factor infusion. Significant falls were noted until the 15 min sample ($p < 0.01$), and it was only in this sample that a significant transpulmonary gradient existed. By the end of the procedure a marked neutrophilia was noted in both CV and LA blood.

SECTION 3

MODULATION OF LUNG PERMEABILITY CHARACTERISITICS BY SELECTED MECHANICAL OR PHARMACOLOGICAL INTERVENTIONS

CHAPTER 11

THE EFFECT OF POSITIVE END-EXPIRATORY PRESSURE ON PULMONARY DTPA CLEARANCE IN NORMAL SMOKERS AND NONSMOKERS

11. THE EFFECT OF POSITIVE END-EXPIRATORY PRESSURE ON PULMONARY DTPA CLEARANCE IN NORMAL SMOKERS AND NONSMOKERS

11.1 - Introduction

The adult respiratory distress syndrome (ARDS) usually requires high inspired oxygen concentration during ventilatory support. In order that a lower inspired oxygen concentration can be used, positive end-expiratory pressure (PEEP) is often applied. Although its early application probably does not alter the pathophysiology of ARDS (300), its effect on the permeability characteristics of the alveolar-capillary unit in man is uncertain. If PEEP were to increase solute flux across the barrier, this could conceivably worsen alveolar flooding and therefore gas exchange.

As has been discussed, the clearance of inhaled ^{99m}TcDTPA from lung to blood has been used as an index of the permeability of the alveolar epithelium. As far as the effect of positive pressure breathing on this measurement is concerned, anaesthetised dogs show an increase in ^{99m}TcDTPA clearance during ventilation with 10 cm H_2O of PEEP (134). More recent studies in normal humans report either an increase in clearance (301) or no significant change (143) during positive pressure breathing. None of the previous studies however, measured the effect of PEEP on lung solute clearance in healthy smokers. The study described in Chapter 4 showed that chronic tobacco smoke inhalation increases ^{99m}TcDTPA clearance to the same extent as severe lung injury. Definition of the mechanism of increased solute clearance in healthy smokers is thus important and

should enhance the value of the technique in assessing lung disease. This study therefore compared the effects of 10 cm H_2O of PEEP on ^{99m}TcDTPA clearance in healthy smokers and nonsmokers.

11.2 - Methods

Subjects

Twelve subjects were studied, consisting of six nonsmokers (5 male, 1 female with a mean age of 32 years, range 24 to 40) and six healthy smokers (5 male, 1 female with a mean age of 31 years, range 27 to 35). Of the six smokers, four were moderate cigarette smokers (10-20/day), one smoked a pipe (50 g of tobacco/week), and one smoked 5-8 large cigars/day. None of the subjects had a recent history of upper respiratory tract infection or medication use. The study was approved by the hospital ethical committee, and all subjects gave informed consent.

Positive End-expiratory Pressure Apparatus

PEEP was provided by wide-bore tubing immersed in 10 cm of H_2O. A one-way valve allowed unrestricted inspiration. While on PEEP, each subject breathed through a mouthpiece with the nose occluded.

Measurement of Pulmonary DTPA Clearance

The methodology of study was identical to that outlined in other chapters. Briefly, with the nose occluded, each subject inhaled the aerosol through a mouthpiece during tidal breathing for three minutes. This produced a scintillation count rate of 40,000 per min.

After inhalation, radioactivity was counted for 20 min with two scintillation counters placed over the right anterior chest and the right thigh. On each study day, a small dose (100 uCi) of ^{99m}TcDTPA was given intravenously to allow correction for blood and tissue background within the chest, as previously described. From a computer-fitted regression line, the half-time clearance ($T_{1/2}$) of ^{99m}TcDTPA from lung to blood was calculated. All regression lines had an r value of 0.95 or greater.

Measurements of Lung Volume

Forced expiratory volume in one second (FEV_1), vital capacity (VC), and total lung capacity (TLC) were measured by body plethysmography. To determine the degree of hyperinflation induced by PEEP, volume measurements were recorded during relaxed and PEEP breathing in ten of the subjects (5 smokers and 5 nonsmokers). Ventilation was monitored with inductance plethysmography (Respitrace Ambulatory Monitoring Systems Inc., New York, USA) calibrated with a single posture technique as previously described (302). The net change in lung volume produced by 10 cm H_2O of PEEP was measured. For each subject, the mid-inspiratory level of breathing (functional residual capacity plus half the tidal volume) was determined at baseline and during PEEP.

Protocol

Each subject was studied in a semi-recumbent position on two separate days. On the first day, each subject inhaled the

^{99m}TcDTPA, and baseline clearance was measured for 10 min. The subject then breathed against PEEP, while clearance was measured for another 10 min period. An intravenous bolus of ^{99m}TcDTPA was then given to allow a correction factor to be derived while breathing against PEEP. On the second day, ^{99m}TcDTPA clearance was measured for 20 min without PEEP.and then corrected with a similar bolus of ^{99m}TcDTPA. The last 10 min of this period served as a second baseline.

Statistical Analysis

Results were analysed using paired Student t-tests within each group and unpaired Student t-tests between groups. A value of $P < 0.05$ was accepted as being statistically significant. Values are presented as means ± standard error of the mean.

11.3 - Results

Fig.26 shows the corrected values for ^{99m}Tc-DTPA clearance for both groups. The mean baseline $T_{1/2}$ value for nonsmokers was 48.6 ± 4.7 min (mean ± S.E.) and declined significantly on PEEP to 31.6 ± 4.7 ($p < 0.05$). All six subjects showed a fall in $T_{1/2}$ on PEEP, demonstrating increased clearance of ^{99m}TcDTPA from lung to blood. In contrast, the mean $T_{1/2}$ for the smokers was 27.7 ± 5.0 min at baseline and was unchanged by PEEP (30.4 ± 6.5 min). None of the smokers' individual values changed significantly.

To assess the possible influence of PEEP-induced changes in blood volume within the chest, the mean PEEP $T_{1/2}$ values, using day 1 correction factors, derived while on PEEP, were also compared with the baseline measurements obtained on the second day, when no PEEP was applied (Fig.27). For the nonsmokers, the mean $T_{1/2}$ on PEEP (31.6 ± 4.7 min) was significantly lower ($p < 0.05$) than either baseline value (48.6 ± 4.7 min on the same day, using that day's correction factor and 53.8 ± 6.3 min using the day 2 correction). In contrast, the mean $T_{1/2}$ for smokers on PEEP was no different from either baseline value (26.5 ± 5.1 min on the same day and 27.7 ± 5.0 min on the second day).

The mean FEV_1, VC, and TLC for the nonsmokers (3.61 ± 0.28, 4.64 ± 0.31, and 6.26 ± 0.30 liters respectively) were slightly higher but not statistically different from the same measurements in the smokers (3.29 ± 0.29, 4.00 ± 0.38, and 5.54 ± 0.71 liters respectively). No subject showed evidence of airway obstruction. While

breathing against PEEP, all subjects demonstrated an increase in tidal volume. The mean changes in lung volume produced by 10 cm H_2O PEEP were 925 ± 170 ml in the nonsmokers and 785 ± 203 ml in the smokers, which were not significantly different.

11.4 - Discussion

This study supports the conclusion of Marks and colleagues (301) that positive pressure breathing increases ^{99m}TcDTPA clearance in nonsmoking humans, and demonstrates in addition that PEEP has no effect on ^{99m}TcDTPA clearance in smokers.

In the nonsmokers, there are several possible mechanisms by which PEEP may accelerate the flux of solutes from alveoli to blood. Firstly, PEEP may increase ^{99m}TcDTPA clearance by slowly redistributing blood from the chest to the rest of the body during the course of the study. This would increase the apparent rate of decline of the radioactive count rate measured by the chest probe and accelerate the increase in count rate measured over the thigh, producing an artefactual increase in ^{99m}TcDTPA clearance from lung to blood. However, as demonstrated in Fig.27, the mean clearance $T_{1/2}$ for the nonsmokers during PEEP was lower than both the pre-PEEP baseline (Day 1) and the baseline value corrected for background blood in the chest while off PEEP (Day 2).

PEEP may also be increasing ^{99m}TcDTPA clearance through its effects on alveolar surface area. Although there are no studies on the effects of hyperinflation on human alveolar surface area, Gil et al. (303) showed in excised rabbit lungs that there was very little change in alveolar surface area with expansion to 80% of total lung capacity. The small increase which did occur was due mainly to unfolding and unpleating of the alveolar septa. Furthermore, during inhalation the ^{99m}TcDTPA presumably disperses throughout the alveolar surface

lining (301), so it is difficult to imagine how a small increase in surface area can significantly alter the transmembrane ^{99m}TcDTPA distribution or concentration gradient.

Another possible explanation in the nonsmokers is a true increase in permeability of the "alveolar-capillary barrier". There is both morphological (304,305) and physiological (306,307) evidence that the alveolar membrane has a low permeability to small solutes. Hyperinflation of atelectatic or isolated fluid-filled animal lung segments increases the flux of small molecular weight compounds (308). In the present study, alveolar wall stretching may have expanded slightly the intercellular junctions, allowing increased access of the ^{99m}TcDTPA to the lung interstitium.

The lack of effect of PEEP on ^{99m}TcDTPA clearance in smokers is difficult to explain. The protocol was identical to that followed by nonsmokers. There were no significant differences in lung expansion produced by PEEP, and the distribution of inhaled one micron particles has been shown to be similar in healthy smokers and nonsmokers (309). Because of the uncertainty regarding normal pathways for solute flux through the alveolar-capillary barrier (310), one can only speculate on the reason for this discrepancy between smokers and nonsmokers. The most likely explanation is that smokers may have relatively wide intercellular junctions that do not vary with lung volume. Simani and co-workers (311) showed in guinea pigs that cigarette exposure caused penetration of horseradish peroxidase into intercellular clefts of the alveolar and bronchiolar epithelium. Small hydrophilic molecules such as DTPA are thought to pass from alveoli into blood through such

junctions. Although a "pore radius" of about 1 nm has been calculated (306), a heterogenous population of small and large pores probably exists, as suggested by Theodore and colleagues (312) and Kim and Crandall (308). If the mechanism of PEEP's effects in normal lungs involves "opening up" of the small intercellular clefts, smokers may be immune because their junctions are already opened up by inflammation and therefore unable to respond to further changes induced by volume or pressure. The rate of solute clearance from lung to blood in smokers may be limited by the integrity of the alveolar-capillary barrier at some point distal to the alveolar epithelium. A final possible explanation for these data is that in smokers, the rate constants measured may partly reflect dissociation of the ^{99m}TcDTPA complex; this would yield free pertechnetate (TcO_4^-) which will be cleared from the lung at an increased rate. Support for this notion comes from recent preliminary observations (313).

In conclusion, these studies have confirmed that the pulmonary clearance of ^{99m}Tc-DTPA is increased by the application of positive end-expiratory pressure in healthy nonsmokers. This effect was not however, seen in otherwise healthy smokers. Further studies in patients with inflammatory lung disease and using differently labelled chelates may help elucidate the mechanism of these findings.

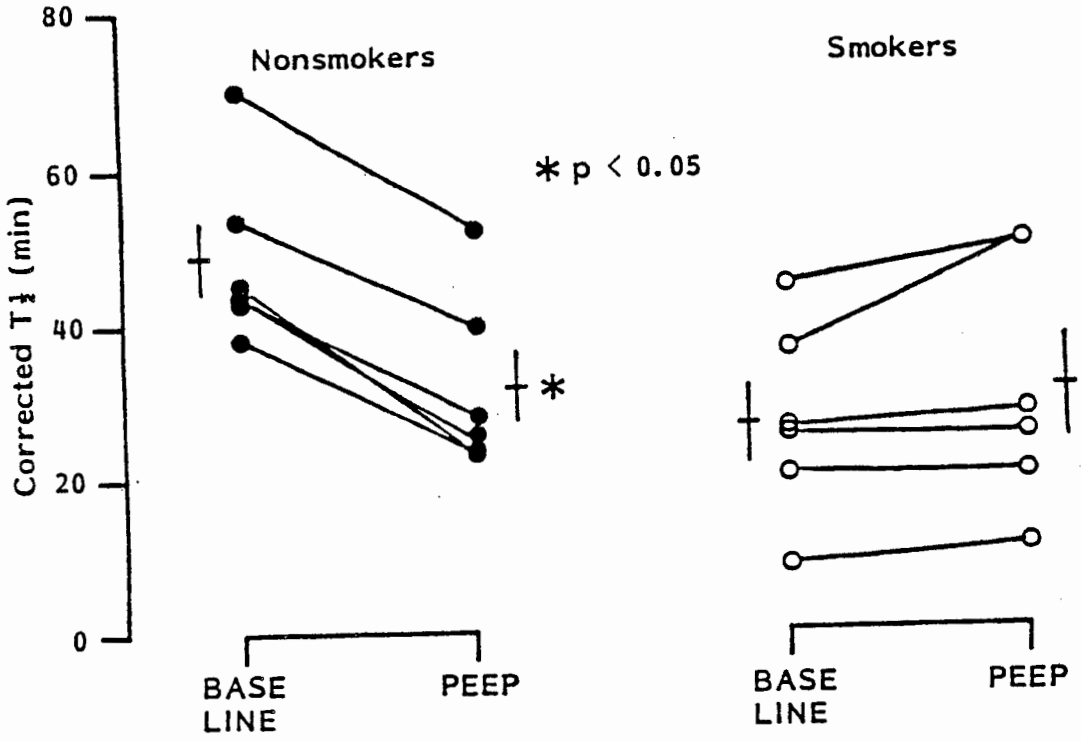

Figure 26

This shows the corrected half-times of ^{99m}TcDTPA clearance in smokers and nonsmokers, before and after breathing with positive end-expiratory pressure (PEEP). The nonsmoking group show a significant fall in half time with PEEP, a response not seen in the smoking group.

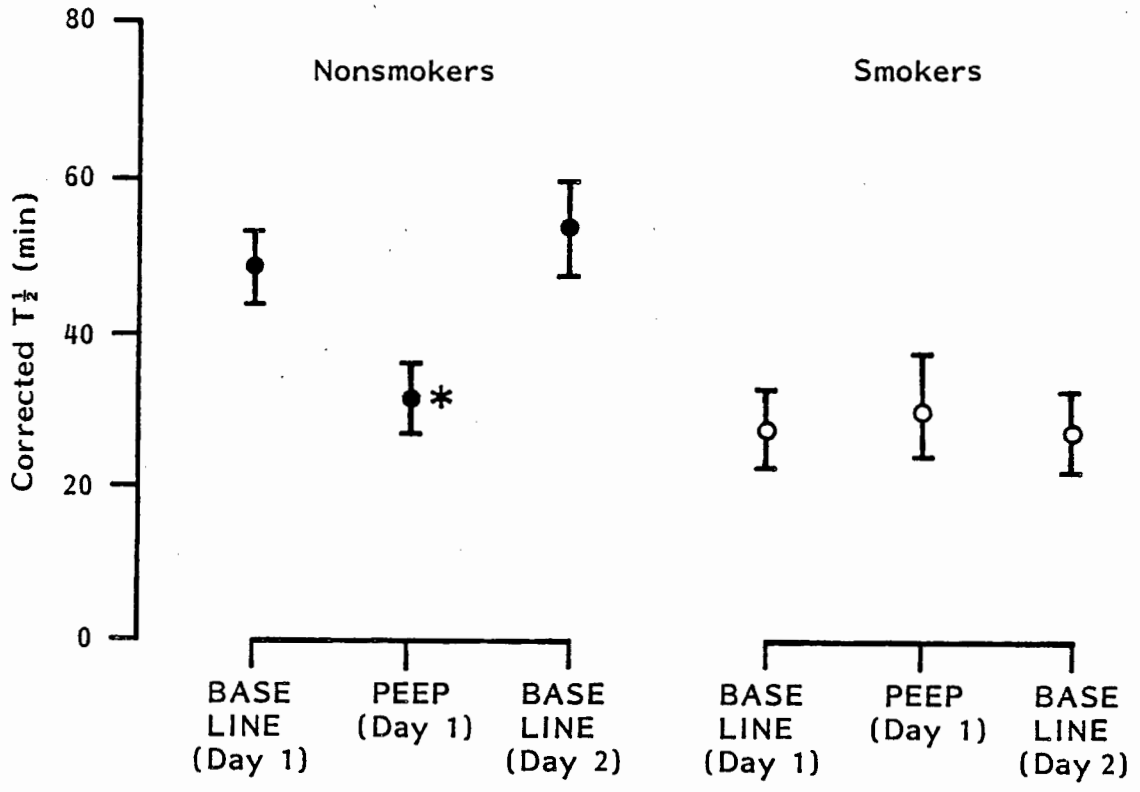

Figure 27

A comparison of baseline (off PEEP) half-time clearances of ^{99m}TcDTPA on Days 1 and 2. On day 1 the correction factor was derived while breathing with PEEP, unlike day 2, when it was calculated without positive end-expiratory pressure. This shows that in both groups clearances were similar on both their respective baseline days, negating a role for PEEP-induced changes in pulmonary blood volume in causing the fall in nonsmokers half-time.

CHAPTER 12

THE EFFECT OF POSITIVE END-EXPIRATORY PRESSURE ON CANINE LUNG PERMEABILITY CHARACTERISTICS AFTER ACUTE LUNG INJURY INDUCED BY CARDIOPULMONARY BYPASS

12. THE EFFECT OF POSITIVE END-EXPIRATORY PRESSURE ON CANINE LUNG PERMEABILITY CHARACTERISITICS AFTER ACUTE LUNG INJURY INDUCED BY CARDIOPULMONARY BYPASS

12.1 - Introduction

The use of positive end-expiratory pressure (PEEP) during the ventilation of patients with pulmonary oedema was first described in 1938 (314), and is now commonly used in treating patients with the adult respiratory distress syndrome (ARDS). Although the application of PEEP usually improves oxygenation, whether the use of PEEP can alter the course of the disease process remains controversial. In the late 1970's, two randomized controlled studies suggested that the early use of PEEP could reduce the incidence of ARDS in patients thought to be at risk (315,316). In a more recent study however (alluded to in Chapter 11), the prophylactic use of PEEP was not associated with differences in the incidence or mortality of ARDS (300).

As discussed in previous chapters, the initial pathophysiological consequence of diffuse lung injury is increased permeability of the capillary endothelium, alveolar epithelium, or both. Therefore, measurement of alveolar-capillary barrier integrity can provide a more direct assessment of the effects of therapy such as PEEP. In Chapter 11, the finding that positive airway pressure increased ^{99m}TcDTPA clearance was confirmed in nonsmoking humans. The effect of elevated airway pressure on microvascular protein flux has been studied in several animals. Ten cm H_2O of continuous positive airway pressure

had no effect on vascular protein and fluid flux in normal sheep lungs (317). However, peak airway pressures greater than 45 cm H_2O have been reported to produce increased microvascular fluid and protein flux in isolated dog lungs (318) and in rats (319,320). Data on the effects of raised airway pressure on microvascular protein flux in injured lungs are more limited. In two studies of oleic acid injury in dogs, PEEP had no effect on extravascular albumin accumulation (321) or the resolution of lung injury (322). In the rats reported by Webb and Tierney, however, the addition of 10 cm H_2O of PEEP to the high peak inspiratory pressures was associated with decreased edema and mortality (320).

PEEP thus appears to increase small solute flux from alveolus to blood in uninjured lung tissue, while its effect on damaged lung alveolar permeability is uncertain. If the application of PEEP also caused an increase in capillary endothelial permeability for large molecules, then the accumulation of solutes in the interstitium could adversely affect lung function and hasten the development of ARDS. In this study, the effects of PEEP on solute flux were determined before and after cardiopulmonary bypass (CPB) in dogs. As in all studies, the clearance of ^{99m}TcDTPA from lung to blood was used as an index of epithelial permeability, and the integrity of the pulmonary microvascular barrier was assessed by measuring the rate of accumulation in extravascular lung tissue of intravenous ^{113m}In-transferrin.

Lung injury was induced with CPB for reasons outlined in Chapter 7. Briefly, it was felt to be a model where the putative mechanism of

lung injury had more widespread relevance to the pathophysiology of ARDS than the more generally used chemical means of directly injuring the alveolar-capillary unit. In addition, the initial study reported in Chapter 7 did provide conclusive evidence that CPB induced an increase in microvascular permeability, and that this was associated with morphological features of lung injury. Clinically, CPB is associated with an early reversible post-operative increase in the clearance of ^{99m}TcDTPA (161). This study examined:

a) whether this was reproducible in canine CPB

b) if so, what the effect of PEEP was on this change

c) what the effect of PEEP was on the previously demonstrated CPB-induced microvascular permeability increase

12.2 - Methods

Animal Preparation

Fifteen mongrel and greyhound dogs were used. The anaesthetic protocol was virtually identical to that described in Chapter 7. Seven days prior to the day of surgery, each animal was anaesthetised with intravenous thiopentone (10 mg/kg), intubated, and ventilated using a Starling pump (rate of 14 per min, tidal volume 10 ml/kg) with an inspired gas mixture of 1% nitrous oxide in oxygen. Following determinations of solute flux, the animal was allowed to recover and returned to the kennel.

On the day of surgery, the animal was anaesthetized in the same way, except for the addition of a dose of meperidine (2 mg/kg) for analgesia. Indwelling lines were placed in the femoral artery and femoral vein for blood sampling and continuous pressure monitoring. A right thoracotomy was then performed, and a monitoring line was placed in the left atrium. Study animals (n = 9) were then given bovine lung heparin (300 IU/kg), and cannulae were inserted directly into the aorta and vena cava via the right atrium. Cardiopulmonary bypass was performed using the identical schedule to that outlined in Chapter 7. Fluids were administered to maintain central venous pressure between 5 and 10 mm Hg. Arterial oxygen and carbon dioxide tensions were measured with an automated analyzer. Following haemostasis, the ribs were apposed and the animals ventilated for a further four hour period.

Following thoracotomy in the six control dogs, a left atrial catheter was placed and positive pressure ventilation continued as a sham bypass procedure. In a similar fashion to the bypass animals, the chest wound was closed after 90 min and ventilation continued for four additional hours. This was also identical to the control protocol used in the initial study.

Positive End-Expiratory Pressure and Lung Volume Measurements

PEEP was provided by placing the exhaust tubing of the ventilator into a water cylinder to a depth of 10 cm. To assess the change in volume produced by PEEP, compliance curves were generated in five bypass and three control dogs. Expired tidal volumes were measured using an electronic Wright spirometer attached to the endotracheal tube. Each dog was placed on 5 cm H_2O of PEEP for several minutes until a stable tidal volume was recorded. PEEP was then withdrawn at end-inspiration, and the subsequent expired volume was recorded. This procedure was repeated at 10 and 15 cm H_2O of PEEP. The difference between the tidal volume and post-PEEP expired volume was plotted against the level of PEEP to obtain a compliance curve for each dog both before and after the operative procedure.

Measurement of Pulmonary DTPA Clearance

The clearance of inhaled ^{99m}TcDTPA (radioactive half-life 360 min) was measured as described previously. The aerosol was delivered by Ambu bagging through tubing equipped with one-way valves. After 2-3 min of nebulization (to achieve a lung count rate of 30,000/min),

radioactivity was counted for 20 min with scintillation counters placed over the right lateral chest and the right thigh. A small dose (50 uCi) of ^{99m}Tc-DTPA was given intravenously to allow correction for blood and tissue background within the chest. From a computer-fitted regression line of the corrected lung counts plotted logarithmically, the half-time clearance ($T_{1/2}$) of ^{99m}TcDTPA from lung to blood was calculated.

Measurement of Microvascular Protein Flux

An identical methodology to that used in the initial CPB study was employed. Data was also acquired and analysed in the same way.

Protocol

Each animal was studied on two days. On the control day, the dog was anaesthetized and ventilated as described. The clearance of inhaled ^{99m}TcDTPA was measured at baseline (10 min), during 10 cm H_2O of PEEP (10 min), and following injection of the 50 uCi of ^{99m}TcDTPA (10 min). A sample of blood was then taken for labelling with $^{99m}TcO_4^-$, and the labelled red cells were reinjected, along with ^{113m}Indium. After a 15 min equilibration period, ^{113m}In-transferrin microvascular flux was measured at baseline (20-30 min) and during PEEP (20-30 min). The animal was then allowed to recover.

On the following week, the animal was anaesthetized, ventilated, and instrumented as described previously. The pulmonary circulation

was bypassed, and the blood was oxygenated for 90 min. Two hours after restoration of the dog's circulation, measurements of inhaled ^{99m}TcDTPA clearance were made at baseline (10 min), during PEEP (10 min), and following the intravenous injection of ^{99m}TcDTPA (10 min). Transferrin flux was then measured at baseline (20-30 min) and during PEEP (20-30 min). The six control dogs were studied using a similar protocol but with no diversion of pulmonary blood flow.

Statistical Analysis

The results were analyzed using analysis of variance. Data are expressed as means ± standard error. A p value of < 0.05 was accepted as being significant.

12.3 - Results

Physiologic Status

The haemodynamic response to the operative procedure, in both gruops of animals, is summarised in Table 8. Central venous pressures were maintained between 5 and 10 mm Hg with fluid administration. In both groups of animals, left atrial pressures (referred to midsternal level) remained less than 5 mm Hg throughout the procedure. After closure of the chest, mean arterial pressures in both groups declined gradually during the subsequent four hours. Blood gas measurements during surgery, in both bypass and control animals, are shown in Table 9. In the bypass group, PO_2 levels were significantly lower after completion of the procedure, necessitating an increase in the inspired concentration of oxygen. The mean PO_2 in the thoracotomy group did not change as a result of the procedure.

DTPA Clearance

Measurements of ^{99m}TcDTPA clearance before and after CPB are shown in Fig.28. Prior to bypass, the mean $T_{1/2}$ for the group was 18.8 ± 1.9 min. When placed on PEEP, all dogs demonstrated an increase in ^{99m}TcDTPA clearance; the mean $T_{1/2}$ declined to 9.4 ± 2.0 min (a net decrease of 50%). The following week, two hours after CPB was completed, the mean $T_{1/2}$ was 8.1 ± 1.6 min at baseline. When placed on PEEP this time, only two dogs showed a decline in $T_{1/2}$, one showed an increase, while the remaining six

were essentially unchanged. For the group as a whole, the mean $T_{1/2}$ during PEEP (8.6 ± 2.9 min) was unchanged from the baseline value.

Values for the six control dogs are shown in Fig.29. Prior to thoracotomy, all dogs responded to PEEP with an increase in ^{99m}TcDTPA clearance. The mean $T_{1/2}$ fell from 14.9 ± 2.1 min during the baseline to 7.1 ± 1.3 min during PEEP (a net decrease of 52%). After thoracotomy, the baseline clearance was 6.4 ± 1.0 min. In contrast to the bypass group, five of the six control dogs showed an increase in ^{99m}TcDTPA clearance during PEEP, and the mean $T_{1/2}$ of 4.1 ± 0.9 min was slightly faster than the baseline clearance ($p < 0.05$).

Pulmonary transvascular transferrin flux

The overall effect of PEEP on ^{113m}In-transferrin flux in the animals was greater after the operative procedure compared to baseline studies ($p < 0.05$).

Fig.30 shows individual measurements for ^{113m}In-transferrin flux in the nine bypass dogs. On the control day, the mean protein flux units (slope/min X 10^{-3}/ intercept of line) was -0.36 ± 0.27 at baseline (i.e. not significantly different from zero) and 0.31 ± 0.34 during PEEP. Two hours after CPB, it was significantly increased to 3.25 ± 0.95 at baseline. Only five of the nine dogs however, showed a significantly positive slope index of transferrin flux, using as the normal range the mean value ± 2 standard deviations in

the 15 control measurements. During PEEP, the mean value was 1.71 ± 0.80, which was not significantly different from the baseline value ($p = 0.10$). Nonetheless, of the five dogs with a significantly positive value at baseline, four showed a decrease during PEEP. The one dog that failed to show a lower transferrin flux during PEEP, depicted in Fig.30 as a dotted line, died after only 6 min of PEEP.

Measurements of ^{113m}In-transferrin flux in the six control dogs are shown in Fig.31. Before thoracotomy, the mean values were 0.03 ± 0.30 at baseline and 0.57 ± 0.21 during PEEP. After thoracotomy, the mean values were unchanged at 0.30 ± 0.39 at baseline and 0.82 ± 0.32 during PEEP.

Lung Volume Measurements

For both the bypass and control animals, changes in lung volume produced by PEEP were higher after thoracotomy. In the experimental animals, 10 cm H_2O of PEEP increased lung volume by 556 ± 44 ml prior to surgery and 710 ± 77 ml after bypass. In the control animals, 10 cm H_2O of PEEP increased lung volume by 473 ± 135 ml at baseline and 697 ± 109 ml after thoracotomy.

12.4 - Discussion

In this model of early acute lung injury, the application of 10 cm H_2O of PEEP had contrasting effects on solute and protein flux through the alveolar epithelial and microvascular membranes. In the baseline state prior to thoracotomy, the effect of PEEP on canine ^{99m}TcDTPA clearance is consistent with a recent report in the literature; the changes in both the experimental group (50% increase) and control group (52% increase) are similar to the previous findings of a 49% increase in dogs (134). The mechanism by which hyperinflation increases ^{99m}TcDTPA clearance in normal lungs remains open to speculation, and may in part be due to the factors alluded to in Chapter 11: changes in the alveolar surface fluid lining (323), surface area (301,134), or intercellular pores (301,134).

In contrast to the normal lungs, the mean ^{99m}TcDTPA clearance after cardiopulmonary bypass did not change when hyperinflated. This was probably due to changes in the pathways by which ^{99m}TcDTPA usually passes from alveolus to blood, such that they no longer responded in the same way to hyperinflation, and thus were subject to a different rate-limiting step. Explaining the cause of this acquired lack of responsiveness is hampered by definitive knowledge about the normal pathways for solute flux through membranes in the lung (310). Nonetheless, the cardiopulmonary bypass procedure itself is directly implicated for two reasons: Firstly, the lack of responsiveness to PEEP after bypass theoretically could have been due a smaller change in lung inflation. However, the measured change in lung volume

produced by PEEP after thoracotomy (with or without CPB) was actually greater than before thoracotomy. This is likely to be due to an increase in chest wall compliance which offset any changes in lung tissue compliance caused by the operative procedure.

Additional evidence is provided by data from the control animals. Compared to their baseline measurements, all dogs after thoracotomy alone had an increased clearance of ^{99m}TcDTPA. In five of the six, the clearance accelerated slightly during PEEP, whereas the bypass animals showed no further change. The main conclusion to be drawn about ^{99m}TcDTPA clearance is that dogs do not show the same increase during PEEP after chest surgery that they show prior to surgery. Although most of this lack of responsiveness appears to be due to the thoracotomy alone, part of the change is due to the bypass procedure itself, which in some way renders them less responsive to PEEP.

Concerning the measurement of microvascular integrity, a similar percentage of dogs demonstrated a significantly positive value for transvascular protein flux as in the initial study. Four of the five dogs with a significant rate of extravascular ^{113m}In-transferrin accumulation demonstrated a slower rate during PEEP. The dog that failed to show this change died 6 minutes later from circulatory collapse. This merits comparison with previous reports of microvascular protein flux during positive pressure breathing. Woolverton and co-workers (317) showed that in chronically instrumented unanaesthetized sheep, 10 cm H_2O of positive airway pressure did not significantly change lymph protein concentration,

although the flow trended downwards. In two studies involving lung injury, 10 cm H_2O of PEEP was applied to dogs that had previously received oleic acid. Sugerman et al. (321) failed to show a change in ^{99m}Tc-albumin flux during 10 min of PEEP in pairs of dogs who received several doses of oleic acid. Luce and colleagues (322) applied PEEP for 21 hours to dogs that had received oleic acid 3 hrs previously and noted no difference in outcome. One must however, bear in mind that oleic acid produces a type of lung injury that probably bears little correlation with any type of human lung injury. As discussed in Chapter 7, cardiopulmonary bypass, although rarely causing ARDS, does usually produce complement activation, neutrophil retention, and gas exchange abnormalities, and thus can serve as a model of early, subtle lung injury. There are, of course, multiple events associated with CPB (e.g. anaesthesia, thoracotomy, hypothermia, protamine administration, and exclusion of the pulmonary circulation) that may be responsible for the changes observed. The possible influence of these associated perturbations on transvascular protein flux were discussed in Chapter 7. Normal transvascular protein flux in the control animals would suggest that the changes observed were not simply due to prolonged anaesthesia and thoracotomy.

The clinical significance of these data relates to patients with early lung injury that may progress to ARDS; based on these findings it appears that the application of PEEP is unlikely to affect solute flux from the alveolus to blood and interstitium. In this respect, they behave like the healthy human smokers whose results were discussed in Chapter 11. Likewise, PEEP is not likely to increase the flux of protein through the vascular endothelium, and in some cases

may actually slow the rate of protein accumulation in the interstitium.

TABLE 8: Haemodynamic response to operative procedure in bypass and control animals

Bypass animals

	Before Thoracotomy	During 90 min Bypass	0.5 Hr Post Bypass	2.0 Hr Post Bypass	4.0 Hr Post Bypass
CVP (mm Hg)	7.1 ± 0.5	4.2 ± 1.4	6.3 ± 0.4	5.4 ± 0.6	4.1 ± 1.2
LAP (mm Hg)	3.0 ± 0.3	0.7 ± 0.3	4.2 ± 0.3	3.0 ± 0.3	3.3 ± 0.3
MAP (mm Hg)	110 ± 3.8	68.0 ± 1.1	105 ± 4.2	98.0 ± 2.4	81.3 ± 1.0

Thoracotomy animals

	Before Thoracotomy	During 90 min Open Thoracotomy	0.5 Hr post Chest Closure	2.0 Hr post Chest Closure	4.0 Hr Post Chest Closure
CVP (mm Hg)	7.1 ± 0.5	6.9 ± 0.6	5.3 ± 0.7	5.4 ± 0.8	5.0 ± 0.8
LAP (mm Hg)	3.0 ± 0.4	3.1 ± 0.3	3.2 ± 0.3	2.7 ± 0.2	2.1 ± 0.3
MAP (mm Hg)	112 ± 4.6	115 ± 5.5	109 ± 4.0	103 ± 3.2	96.6 ± 2.5

Values are means ± S.E. of central venous pressure (CVP), left atrial pressure (LAP), and mean arterial pressure (MAP) for both groups of dogs on the day of surgery.

TABLE 9: Blood Gas Data in bypass in and control animals

Bypass animals

	Before Thoracotomy	During 90 min Bypass	0.5 Hr Post Bypass	4.0 Hr Post Bypass
FiO_2	0.5	0.95	0.5	1.0
pH	7.36 ± 0.01	7.47 ± 0.02	7.38 ± 0.01	7.32 ± 0.01
PO_2 (torr)	222 ± 8.7	305 ± 10	78 ± 4.2	85 ± 7.7
PCO_2 (torr)	36 ± 0.7	29 ± 1.1	35 ± 0.7	38 ± 0.9

Thoracotomy animals

	Before Thoracotomy	During 90 min Open Thoracotomy	0.5 Hr Post Chest Closure	4.0 Hr Post Chest Closure
FiO_2	0.5	0.5	0.5	0.5
pH	7.35 ± 0.01	7.36 ± 0.01	7.35 ± 0.01	7.33 ± 0.02
PO_2 (torr)	235 ± 15.2	213 ± 16.1	223 ± 8.6	198 ± 13.5
PCO_2 (torr)	36 ± 1.2	36 ± 1.6	37 ± 1.4	36 ± 1.6

Values are means ± S.E. of fraction of inspired oxygen (FiO_2), arterial pH, PO_2, and PCO_2 for both groups of animals on the day of surgery. Data are corrected for temperature.

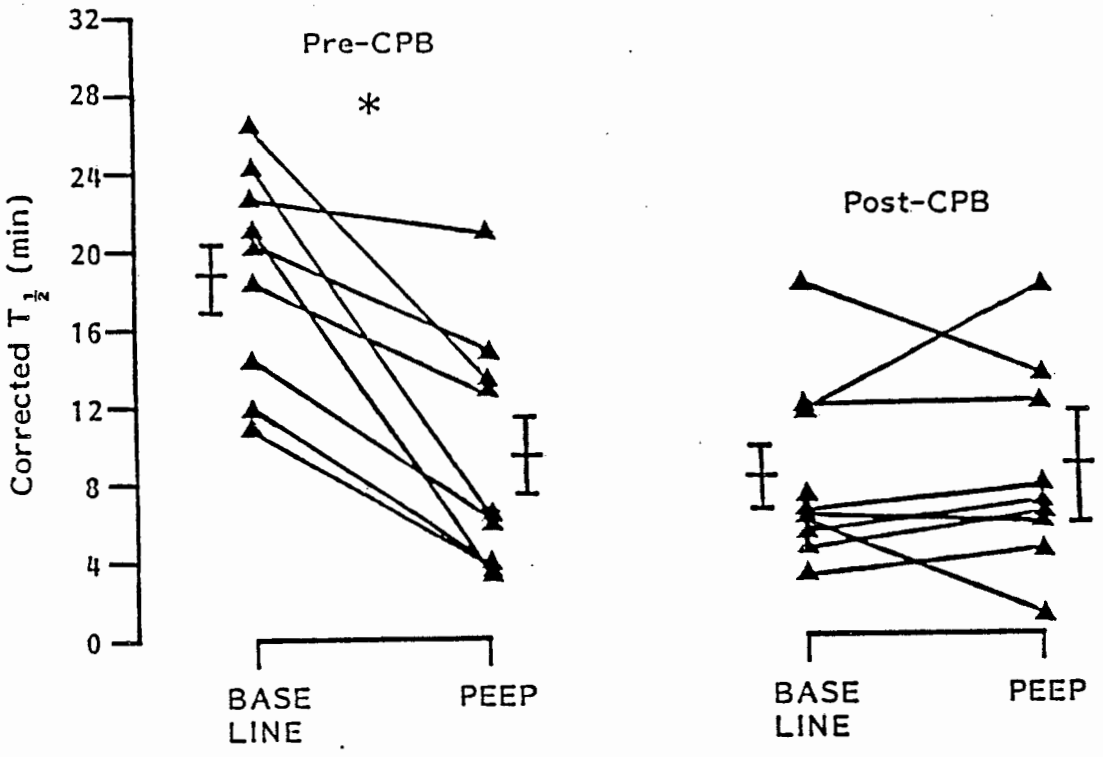

Figure 28

This shows in those animals undergoing cardiopulmonary bypass (CPB), the response in half-time clearances of ^{99m}TcDTPA to positive end-expiratory pressure before, and after the procedure (see text for protocol). As a group, a significant fall (*) in half-time clearance was noted only before CPB.

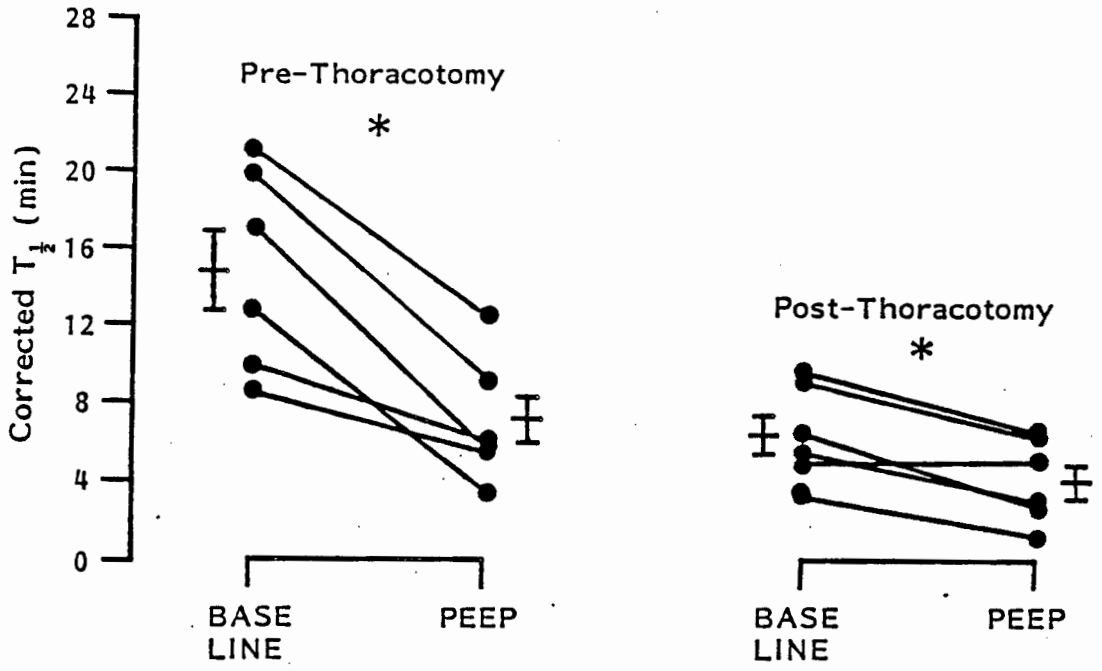

Figure 29

The change in half-time clearances of ^{99m}TcDTPA with positive end-expiratory pressure (PEEP) in the control animals (who underwent thoracotomy alone) before and after the procedure. In contrast to the animals who underwent cardiopulmonary bypass, this group retained responsiveness to PEEP after thoracotomy (*).

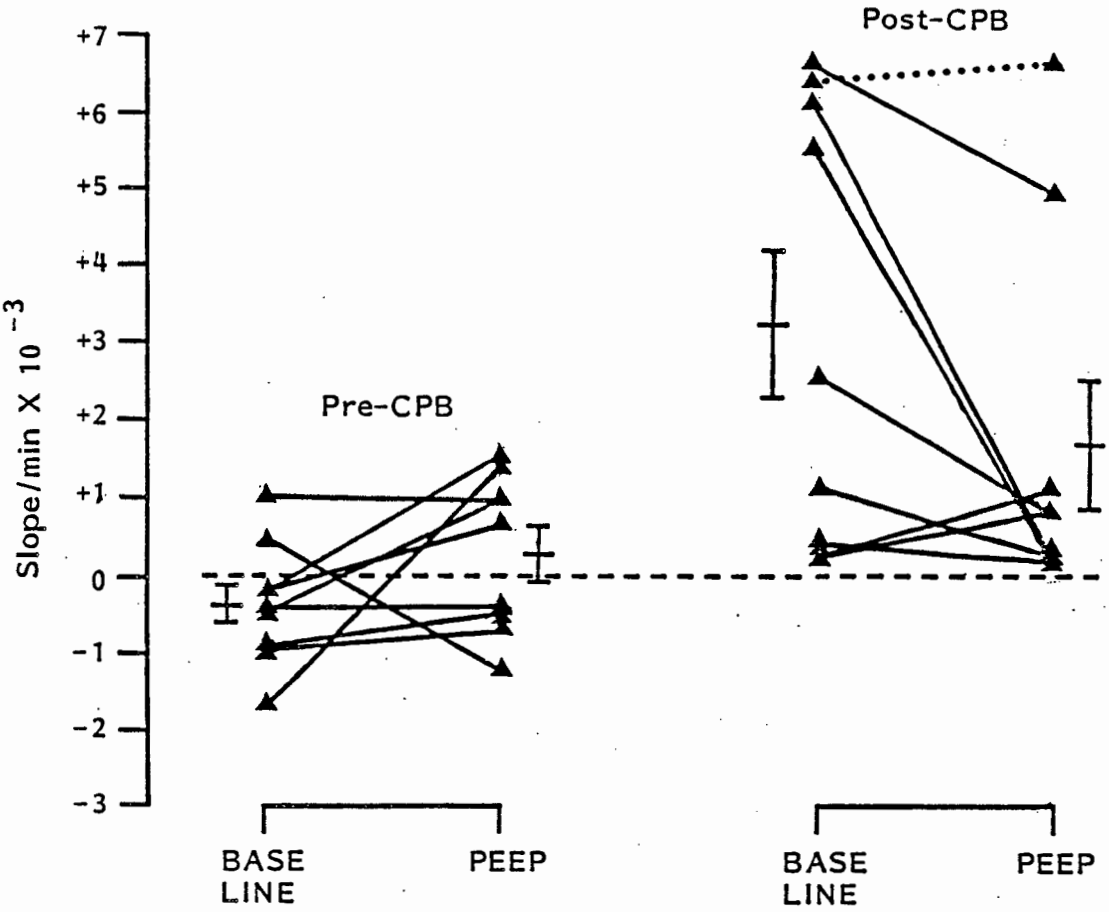

Figure 30

This shows ^{113m}In-transferrin transvascular flux in the cardiopulmonary bypass group, before and after the procedure. This was expressed as protein flux units (slope/intercept, not slope alone as in figure). No significant change is noted with PEEP before bypass, and although this is also true after bypass, 4 animals did show a downward trend. The dotted line refers to the data derived from a dog who died 6 min after PEEP institution.

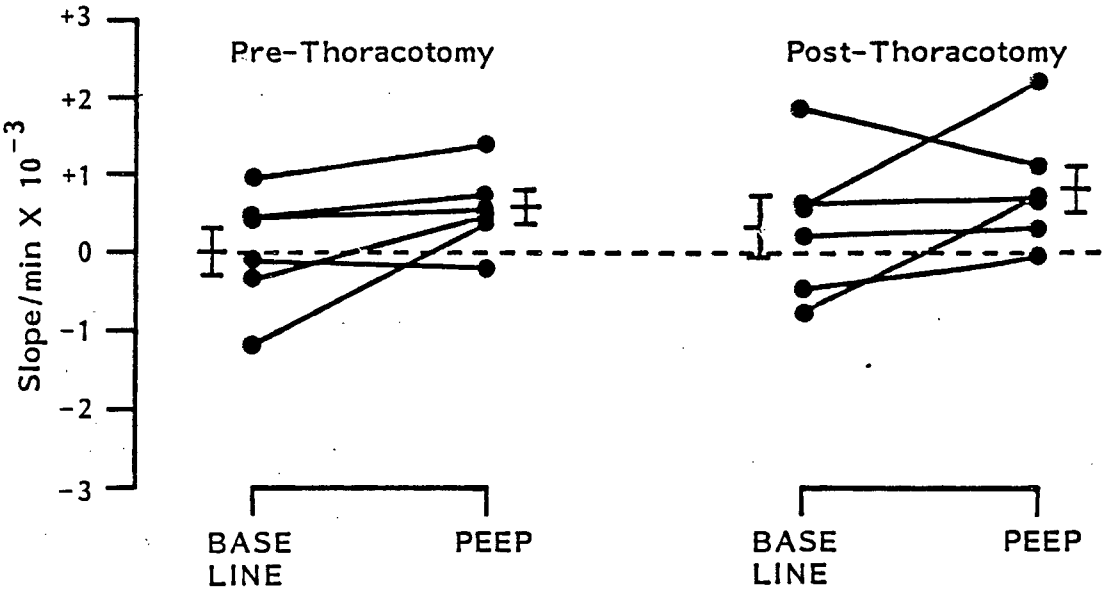

Figure 31

The pulmonary transvascular protein flux response, in the control (thoracotomy alone) animals to PEEP, before and after the procedure. PEEP produced no change in this variable, either before or after thoracotomy

CHAPTER 13

THE EFFECT OF INFUSED BETA-ADRENERGIC AGONIST ON ACID-INDUCED LUNG INJURY IN THE RAT

13. THE EFFECT OF INFUSED BETA-ADRENERGIC AGONIST ON ACID-INDUCED LUNG INJURY IN THE RAT

13.1 - Introduction

No clear consensus has emerged concerning the role of β-adrenergic agonists in modulating lung injury. Initial experimental work in guinea pigs suggested that terbutaline (a selective β_2-adrenergic agonist) pretreatment protected against histamine-induced pulmonary oedema (324). Subsequent studies using isolated perfused lung systems tended to confirm a protective role for β-adrenergic agonists in both acid (325,326) and endotoxin (327) mediated lung injury. In contradistinction however, pretreatment of monkeys with terbutaline prior to anaphylactic shock accentuated injury with over half the animals developing fulminant pulmonary oedema (328). Likewise clinically, the use of β_2-adrenergic agonists to treat premature labour is occasionally associated with the development of pulmonary oedema (329). In four out of five reported cases where pulmonary capillary wedge pressure has been measured (330-333), it was below 15mm Hg implying permeability rather than cardiogenic pulmonary oedema. The cause of this syndrome is unknown, but it appears to be an idiosyncratic rather than a dose related phenomenon.

To investigate adrenergic mechanisms in lung injury further, I have examined the effect in the rat of a β_2-adrenergic agonist, salbutamol, on lung injury induced by intratracheal acid instillation. Aspirated acid injures the lung immediately because of

a direct effect, although the resultant inflammatory response probably amplifies tissue damage (334). Alveolar-capillary permeability increases with resultant increase in transendothelial water filtration and solute flux. I sought to measure these latter parameters after experimental acid-induced injury by respectively determining

1) gravimetric lung water by the ratio of lung wet:dry weight ratio and

2) the clearance from the lung of ^{99m}Tc-DTPA as an index of alveolar epithelial permeability.

This latter technique is highly reproducible in rats (335) with clearance of the tracer solute increasing markedly after experimental acid-induced injury (D.Royston, submitted)

13.2 - Methods

Experimental technique

Studies were performed on female specific pathogen free Sprague-Dawley rats (weight 200-250 gm). Anaesthesia was induced with a 1:1 by volume mixture of nitrous oxide and oxygen together with halothane 3%. The tail vein was cannulated and atropine 1.5-2mg/kg given by slow intravenous injection. After tracheal intubation with a 16 gauge cannula (Abbot) the animals were mechanically ventilated using a small animal ventilator (Harvard apparatus, Kent, U.K.) with a tidal volume of 10ml/kg and rate of 80/min. The inspired gas mixture consisted of air with supplemental oxygen to achieve a concentration of approximately 40%; this was verified with an oxygen analyser (Servomex 570). Anaesthesia was maintained with an intraperitoneal injection of Diazepam 10mg/kg and intramuscular 'Hypnorm' 0.8ml/kg (Fentanyl 0.2 mg/ml, Fluanisone 10 mg/ml). The latter was repeated at hourly intervals throughout the study. After ten minutes mechanical ventilation hydrochloric acid (pH2, 1ml/kg), or saline in equivalent volumes in the control animals, was injected down a fine polyethylene catheter which protruded 3mm from the tip of the endotracheal cannula and this was flushed with a 1ml bolus of air. The animals were held vertical throughout this procedure. Immediately thereafter, infusions of salbutamol in saline (1ug kg^{-1} min^{-1}), flow rate 5ml kg^{-1} $hour^{-1}$) or equivalent volumes of normal saline in controls were begun. After three further hours of controlled ventilation the following studies were performed.

1) Pulmonary clearance of ^{99m}TcDTPA

A similar technique to that described by Minty et al (12) was employed. Briefly, with the animals held vertically 0.1 ml of a 5 mBq/ml (approximately 0.12 mCi) solution of ^{99m}TcDTPA was instilled through a polyethylene catheter of similar length to that used to introduce the hydrochloric acid. A lead shield was then placed around the animal with windows removed to expose the thorax and one of the rear legs. Scintillation detectors were placed above the windows and the clearance of ^{99m}TcDTPA from the lung (corrected for blood and tissue background in the field of the detector) determined. A computer fitted regression line enabled the half-time ($T_{1/2}$) lung to blood clearance to be calculated.

2) Gravimetric lung water (lung wet:dry weight ratio)

The animals were exsanguinated by percutaneous cardiac puncture. The heart and lungs were removed en bloc; The lungs were dissected free of attached tissue, weighed and immersed in liquid nitrogen for two hours. They were then dried to constant weight by vacuum desiccation for 24 hours and then reweighed.

3) Lung histology and verification of ^{99m}TcDTPA deposition

Two separate groups of rats (n=6) were studied to determine the pattern of DTPA deposition and to assess lung injury histologically (n=4, acid instillation; n=2, saline instillation). In studies examining DTPA deposition the DTPA solution was mixed with colloidal carbon particles. For conventional lung histology 10% neutral formaldehyde was instilled intratracheally with the lungs in situ to

a constant pressure of 15cm H_2O. The lungs of animals into which carbon particles had been instilled with the DTPA solution were inflated with 4 ml of air and then removed and fixed with 10% neutral formaldehyde.

Study design

Four experimental groups were studied:

1) Acid instillation and salbutamol infusion (n=14)
2) Acid instillation and saline infusion (n=14)
3) Saline instillation and salbutamol infusion (n=5)
4) Saline instillation and saline infusion (n=18)

Statistics

The four groups were compared by one way analysis of variance and unpaired t tests. Because of unequal variances data for DTPA clearance was logarithmically transformed. All results are expressed as mean ± standard error of the mean. P values of less than 0.05 were deemed significant.

13.3 - Results

Five animals (all Group 1) died during the three hour period of mechanical ventilation after acid instillation. Results in the remaining Group 1 animals (n=9), together with the other study groups are represented graphically in Table 10. Lung wet:dry weight ratios in the acid and saline infusion group were significantly greater (6.4 ± 0.3) than the group 4 controls (5.4 ± 0.2) ($p<0.01$). The Group 1 rats (acid+salbutamol infusion) however, showed a dramatic increase in lung weight ratios (10.0 ± 0.6) as compared to Group 2 (acid+saline infusion)($p<0.001$). Lung weight ratios were measured in three of the five rats who died and ranged from 8.5 to 11.1. These latter results were excluded from statistical analysis. In addition, lung dry weights in the salbutamol treated groups did not differ significantly from the other groups.

Pulmonary clearance of instilled ^{99m}TcDTPA was increased significantly in Group 2 rats ($T_{1/2}$ in minutes, 19.4 ± 2.6) as compared to Group 4 controls (98.1 ± 7.2)($p<0.0001$). As with the data for gravimetric lung water the group who had salbutamol infused after acid instillation had faster clearance of DTPA (11.5 ± 1.9) than the saline infused rats (Group2) (19.4 ± 2.6, $p<0.05$).

Group 3 rats (salbutamol infusion after saline instillation) had values for lung water (6.0 ± 0.3) and for DTPA clearance (126.2 ± 18.4) similar to Group 4 rats who were treated identically, but had saline rather than salbutamol infused.

Histology

Acid treated rats alone showed severe alveolar-capillary injury which was bilateral and represented in every lung lobe. Prominent features were marked alveolar and interstital oedema with neutrophil infiltration.

DTPA deposition pattern

In control animals there were virtually no carbon particles deposited in airways although this was noted to a minor degree in acid-treated animals. In the latter group however, the vast majority of carbon particles were seen in alveolated areas.

13.4 - Discussion

This study has demonstrated that after acid-induced injury there is significantly increased gravimetric lung water and pulmonary clearance of ^{99m}TcDTPA in anaesthetised, intubated and ventilated rats who had salbutamol rather than saline intravenous infusion. The histological findings together with the DTPA deposition data suggest strongly that the increased tracer solute clearance seen in the acid-treated animals was due largely to alveolar epithelial rather than airway injury.

There is little evidence that β_2-adrenergic agonists have a direct permeability enhancing effect. Experimental studies in skin and other extra-pulmonary tissue (336-338) show a protective role for these agents in mediator -induced permeability increase. The rationale for this could partly be explained by the Starling equation for transendothelial water filtration, which was outlined in the first Chapter of this thesis. It states that nett fluid filtration is the product of the driving pressure, the surface area and the hydraulic conductivity. Where β-adrenergic agonists have previously reduced water filtration in experimental lung injury in vivo, it has been as a function of reduction in pulmonary microvascular pressure (339). Our finding of markedly <u>increased</u> gravimetric lung water in salbutamol treated animals implies a predominant β_1 agonist inotropic effect with consequent increases in pulmonary artery pressure.

Salbutamol could possibly have impaired a physiological protective response. Both salbutamol (340) and other β_2-adrenergic agonists (341) inhibit hypoxic pulmonary vasoconstriction (HPV). This would increase perfused surface area and would tend to accentuate any β_1-agonist mediated increase in microvascular pressure, resulting in the vastly increased gravimetric lung water noted in the salbutamol treated animals. It is unlikely however that salbutamol influenced the gravimetric lung water data by increasing residual blood volume, since dry lung weights were similar in all study groups. This would suggest that dry blood weight did not contribute significantly to total dry weight in the salbutamol treated animals. Although an inspired oxygen concentration of 40% should in itself inhibit HPV, areas of poor ventilation with alveolar flooding will still remain hypoxic; thus in these areas the response will remain intact despite supplemental oxygen. Clinically, Brigham et al have shown that in the adult respiratory distress syndrome (ARDS), a persistent high permeability:surface area product for urea, implying continuing perfusion of already injured alveolar capillary units, correlated with the severity of the gas exchange deficit and was associated with a poor prognosis (108). It is interesting therefore to speculate whether inhibition of HPV by salbutamol, with subsequent impairment of pulmonary vascular autoregulation, exacerbated the pre-existing alveolar-capillary injury and increased pulmonary oedema and epithelial solute flux.

How can these data be reconciled with the studies (325-327), showing an apparent protective effect of β_2-adrenergic agonists in experimental acute lung injury? These studies all used an isolated

perfused lung preparation where the HPV response, although present (342), is undoubtedly more difficult to sustain (343). In this situation, after induced lung injury, there is presumably already progressive mismatching of ventilation and perfusion due to the lack of the HPV response and any further β_2-receptor mediated inhibition of HPV may be relatively unimportant. It may therefore be that the possible beneficial effects of a β_2-agonist (pulmonary vasodilatation and putative permeability protection) may actually confer a degree of protection in this in vitro preparation. In the whole animal however, HPV may represent a crucially important protective response and its inhibition correspondingly more detrimental.

In summary, salbutamol infusion mediated an exacerbation of acid-induced pulmonary injury with significant further increases in gravimetric lung water and ^{99m}TcDTPA clearance. Loss of protective HPV, but probably more importantly, β_1 agonist mediated increases in cardiac output and pulmonary artery pressures may be important factors accounting for these findings.

TABLE 10: Gravimetric lung water and 99mTc-DTPA clearance data in the 4 study groups

Group	n	W/D	DTPA clearance
1 (acid + salbutamol)	9	* 10.0 ± 0.6	** 11.5 ± 1.9
2 (acid + saline)	14	*** 6.4 ± 0.3	**** 19.4 ± 2.6
3 (saline + salbutamol)	5	6.0 ± 0.3	126.2 ± 18.4
4 (saline + saline)	18	5.4 ± 0.2	98.1 ± 7.2

Results are mean ± SEM. DTPA clearance is expressed as the corrected half time in minutes. W/D is the wet to dry lung weight ratio, an index of gravimetric lung water.

* $p < 0.001$; ** $p < 0.05$ vs Group 2.

*** $p < 0.01$; **** $p < 0.0001$ vs Group 4.

SECTION 4

CONCLUDING CHAPTER

CHAPTER 14

GENERAL DISCUSSION AND CONCLUSIONS

14. GENERAL DISCUSSION and CONCLUSIONS

The body of work upon which this thesis is based was prompted initially by the lack of a specific method of assessing alveolar-capillary barrier integrity in clinical acute lung injury. Two nuclear medicine techniques have been modified and developed to provide indices of the permeability characteristics of both microvascular endothelium and alveolar epithelium. Either or both of these techniques were utilised clinically and experimentally in order to fulfill the broad aims of the thesis as outlined in Chapter 1.

The initial study pointed to the non-specificity of ^{99m}TcDTPA pulmonary clearance in smokers and based on this, use of the method should be restricted clinically to nonsmokers. Attention should be focussed particularly on earlier diagnosis of lung injury and subsequent serial studies to monitor recovery or otherwise of lung epithelial integrity. This approach would identify patients at risk earlier and thereby maximise the efficacy of any pharmacological or other therapeutic intervention. The ^{113m}In-transferrin pulmonary transvascular flux method does appear - on the basis of these clinical studies and other experimental work, previously described - to be specific to microvascular injury. It is likely therefore, that a single study may be useful in the management of patients with pulmonary oedema of doubtful hydrostatic or permeability aetiology. As discussed, there is probably a spectrum of permeability alterations in pulmonary oedema and unless the clinical and haemodynamic status of the patient points unequivocally to a hydrostatic aetiology, an assessment of

microvascular permeability may be worthwhile. This approach would avoid dichotomising, based on an arbitrarily chosen value of the pulmonary capillary wedge pressure, assessed at one time point. This may provide misleading information as:

i) an elevated wedge pressure does not exclude a coexisting permeability alteration and

ii) a normal wedge pressure does not, per se, in the context of acute respiratory insufficiency with radiological infiltrates, imply a permeability derangement as the definition of ARDS would imply. A normal value may only reflect a reduction in a previously elevated value, usually in response to therapy.

Notwithstanding this, as with ^{99m}TcDTPA pulmonary clearance, future studies should be concerned particularly with patients at risk of developing lung injury, rather than those where the disease process is already well established.

Experimentally, canine cardiopulmonary bypass was used as a subtle model of lung injury to assess the role of the neutrophil. This model was used as it had more general pathophysiological and clinical relevance than the more widely recognised chemical methods of directly injuring the alveolar-capillary endothelium and/or epithelium. Invariable morphological lung injury was induced and in 2 separate studies the majority of animals demonstrated increased microvascular permeability. As a result of these studies, as well as further clinical studies in bone marrow transplant patients and

experimental work with endotoxin and platelet activating factor, the following conclusions about the role of the neutrophil in acute lung injury were reached:

1) The neutrophil is not a sine qua non for lung injury development as has been suggested. Classical ARDS was verified by permeability and subsequent histological studies in grossly neutropaenic patients after bone marrow transplantation.

2) Lung neutrophil sequestration can occur in dogs after endotoxin without lung damage ensuing. This suggests that the relationship between intrapulmonary neutrophils and the development of lung injury is complex with additional, hitherto undefined, humoral and cellular factors playing a role.

3) Studies after cardiopulmonary bypass show that in this model the neutrophil does appear to be playing a central role in the functional and morphological alterations observed. Similar conclusions apply to the studies with platelet activating factor, although in both cases definitive conclusions about the role of the neutrophil should await appropriate depletion experiments.

It seems therefore, that in certain specific models of lung injury the neutrophil is playing a key role, but in others injury can occur independently of the cell. The same is true of the clinical situation, where the neutrophil may be important with particular precipitating events. Additionally, the mere presence of intrapulmonary neutrophils, sequestrated within capillaries, does not

necessarily imply that alveolar-capillary injury will ensue. Thus the use of these permeability techniques has emphasised the heterogeneity of pathogenetic mechanisms and pointed to a more complex role for the neutrophil in acute lung injury.

Of the putative humoral mediators of lung injury, it is likely that only PAF has a significant role. The reservation should be expressed that histamine and bradykinin were studied after aerosolisation in humans, because of the limitation of administering a dose which provided limited but non-severe reduction in airway calibre. These studies were however, in line with previous experimental studies using higher doses which were described in Chapter 9. Thus the conclusion of a limited role, if any, of these mediators, is probably correct. Use of a dynamic technique, transvascular ^{113m}In-transferrin flux, to study pulmonary microvascular integrity emphasised the potential importance of PAF as an inflammatory mediator in the lung. The data demonstrated a temporal dissociation between the early haemodynamic changes and the later sustained permeability increase. This underlined its possible significance in endotoxaemia, where the time sequence of events is similar.

The studies reported in this thesis provide evidence of the non-specificity of ^{99m}TcDTPA pulmonary clearance. The changes seen in normal smokers have already been alluded to and the studies described in Chapters 11 and 12 have extended the technique's non-specificity. Although in normal nonsmokers solute clearance is clearly volume-dependent, this was not found in smokers or, more

importantly from the view point of clinical applicability, after experimental acute lung injury. The finding however, that thoracotomy alone increases DTPA clearance is difficult to explain; if this were true clinically it would severely circumscribe its potential usefulness. Taking all these factors into account it appears that in acute lung injury ^{99m}TcDTPA clearance has a limited role. There should be awareness of the various perturbations that may influence it and caution applied in the interpretation of data.

The study examining the role of beta adrenergic agonists in an acid-aspiration model (Chapter 13) used DTPA clearance and extravascular lung water as indices of lung damage. Although this was not a comprehensive examination of adrenergic modulation of water and solute balance, the study did focus attention on the potentially deleterious properties these agents may possess. This interesting and unexpected finding underlines the need for further careful studies of how pulmonary haemodynamic alterations may influence established acute lung injury.

In summary this research programme has provided important evidence for more routine use of these techniques - particularly pulmonary transvascular protein flux - in the management of critically ill patients with, or at risk of developing, adult respiratory distress syndrome. This latter permeability index appears at present the most specific and sensitive method of assessing clinically, pulmonary microvascular integrity. Based on studies presented here, and other work quoted in this thesis, the abnormalities in this index appear to antedate both significant

extravascular lung water accumulation and severe morphological lung injury. Experimentally, important insights have been gained into aspects of lung injury pathogenesis and modulation. This has led particularly, to an appreciation of the diversity of pathogenetic mechanisms which operate in this syndrome and the variable role played by the neutrophil in them. These suggest that a final common pathway of of lung injury does not exist. These clinical and experimental findings imply that examination of the permeability characteristics of the alveolar-capillary unit has a pivotal role to play in furthering understanding of pathophysiological mechanisms in - and aiding clinical management of patients with- the adult respiratory distress syndrome.

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PAPERS IN PREPARATION

Time course of change in cardiorespiratory variables after PAF infusion in dogs (1st author).

Pulmonary capillary injury after experimental canine cardiopulmopnary bypass: a morphological and physiological correlation (2nd author).

ABSTRACTS

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APPENDICES

APPENDIX 1:

Computer programme for analysis of pulmonary ^{99m}TcDTPA study

```
]B LOAD TEXT TABLE
]LIST

1  TEXT : HOME
5  LOMEM: 25000
10 D$ = "":G$ = "":Z = 70
12  HTAB (3): VTAB (8): PRINT "LUNG PERMEABILITY PROGRAMME": HTAB (3): PRINT
     "--------------------------": VTAB (16): PRINT  TAB( 3)"1 COUNT PER
     MINUTE": PRINT : PRINT  TAB( 3)Z;" MINUTES MAXIMUM"
15  PRINT D$;"BRUN HGR1.DIU#1.OBJ0"
16  PRINT D$;"BRUN HGR1.DIU#2.OBJ0"
20  PRINT D$;"BRUNTEXT TABLE,A23000"
30  SCALE= 1: ROT= 0
40  DIM A(Z + 1): DIM B(Z + 1): DIM LA(Z + 1): DIM LB(Z + 1): DIM U(Z + 1)

50 S1$ = "ENTER READING FOR CHEST - ":S2$ = "ENTER READING FOR LEG - "
60  ONERR  GOTO 9000
70  REM  DON'T ASK ME WHY THIS HAS TO BE  HERE
100 OS = 0:E = 0: FOR Q = Z TO 1 STEP  - 1: IF A(Q) = 0 THEN  NEXT :E = 1:
     GOTO 200
110 TL = 0:FX = B(Z + 1):TD = A(Z + 1):BC = A(0):BL = B(0)
200  TEXT : HOME : PRINT "PERMEABILITY PROGRAMME": VTAB (8): PRINT "1) ENT
     ER DATA": PRINT : PRINT "2) LOAD DATA FROM DISC": IF E = 1 THEN  GOTO
     250
210  IF C + K > 0 THEN  PRINT : PRINT "3) SAVE DATA TO DISC"
220  PRINT : PRINT "4) CORRECT DATA"
230  IF TL <  > TD THEN  PRINT : PRINT "5) CALCULATE CORRECTION FACTOR"
240  PRINT : PRINT "6) CALCULATE HALFTIMES"
250  PRINT : PRINT "7) DELETE RECORDS"
260  POKE 34, PEEK (37) + 2
300  HOME : POKE 37, PEEK (37) - 1: PRINT "ENTER NUMBER": HTAB (14): GET Q
     $:Q =  VAL (Q$)
310  ON Q GOTO 2000,7000
320  IF E = 1 THEN  GOTO 370
330  IF Q = 3 AND C + K > 0 THEN  GOTO 6000
340  IF Q = 4 THEN  GOTO 3000
350  IF Q = 5 AND TL <  > TD THEN  GOTO 4000
360  IF Q = 6 THEN  GOTO 5000
370  IF Q = 7 THEN  GOTO 8000
380  PRINT G$;G$;G$: GOTO 300
1000  PRINT G$:CV =  PEEK (37): POKE 37,CV - 1: PRINT S$: HTAB ( LEN (S$) +
     1): INPUT Q$:Q =  VAL (Q$): RETURN
1010  FOR N = 1 TO  LEN (S$):CH =  ASC ( MID$ (S$,N,1)): XDRAW CH: NEXT : RETURN

1100 X = ((T - 1) * C1) - 16: IF X > 235 THEN X = 235
1105  XDRAW 114 AT X,Y3 + 13:S$ = "=" +  STR$ (R1): GOSUB 1010: XDRAW 114 AT
     X,Y4 + 13:S$ = "=" +  STR$ (R2): GOSUB 1010: RETURN
1110 X = (T * C1) - 16: IF X > 235 THEN X = 235
1115  XDRAW 114 AT X,Y1 + 13:S$ = "=" +  STR$ (R1): GOSUB 1010: XDRAW 114 AT
     X,Y2 + 13:S$ = "=" +  STR$ (R2): GOSUB 1010
1130  RETURN
1200 S1 = 0:S2 = 0:S3 = 0:D1 = 0:D2 = 0:D3 = 0:D4 = 0:D5 = 0
1210  FOR N = N1 TO N2:S1 = S1 + LA(N):S2 = S2 + LB(N):S3 = S3 + N: NEXT :
     N = N2 - N1 + 1:M1 = S1 / N:M2 = S2 / N:M3 = S3 / N
1220  FOR N = N1 TO N2:D1 = D1 + ((LA(N) - M1) ^ 2):D2 = D2 + ((LB(N) - M2
     ) ^ 2):D3 = D3 + ((N - M3) ^ 2):D4 = D4 + ((LA(N) - M1) * (N - M3)):D
     5 = D5 + ((LB(N) - M2) * (N - M3)): NEXT
1230 R1 =  INT ((D4 * 100 /  SQR (D1 * D3)) + .5) / 100:R2 =  INT ((D5 * 1
     00 /  SQR (D2 * D3)) + .5) / 100:B1 = D4 / D3:B2 = D5 / D3: RETURN
1300 X2 = ( PDL (0) * 259 / 255) + 20:X = X2: GOSUB 1380:X3 = ( PDL (1) *
     259 / 255) + 20:X = X3: GOSUB 1380
1310 Q =  PEEK ( - 16384): POKE  - 16368,0: IF Q > 127 THEN  GOTO 1350
1320 X0 = ( PDL (0) * 259 / 255) + 20: IF X0 > X2 + 2 OR X0 < X2 - 2 THEN
     X = X2: GOSUB 1380:X = X0: GOSUB 1380:X2 = X0
1330 X1 = ( PDL (1) * 259 / 255) + 20: IF X1 > X3 + 2 OR X1 < X3 - 2 THEN
     X = X3: GOSUB 1380:X = X1: GOSUB 1380:X3 = X1
```

```
1340  GOTO 1310
1350 X = X2: GOSUB 1380:X = X3: GOSUB 1380:N1 = X0:N2 = X1: IF X0 > X1 THEN
     N1 = X1:N2 = X0
1360 N1 =  INT (((N1 - 20) / C1) + 1.5):N2 =  INT (((N2 - 20) / C1) + 1.5)
     : IF N2 - N1 > 0 THEN  RETURN
1370  GOTO 1300
1380  SCALE= 28: ROT= 48: XDRAW 95 AT X,139: ROT= 0: SCALE= 1: RETURN
1400  HGR : HPLOT 17,0 TO 20,0 TO 20,140 TO 279,140: IF Q < 31 THEN  FOR I
      = 0 TO Q: HPLOT 20 + (I * C1),141 TO 20 + (I * C1),143: NEXT : FOR I
      = 0 TO Q - 1 STEP 2:S$ =  STR$ (I):X = 18 + (I * C1):Y = 154: IF I >
     9 THEN X = 15 + (I * C1)
1410  IF Q > 30 THEN  FOR I = 0 TO Q STEP 2: HPLOT 20 + (I * C1),141 TO 20
      + (I * C1),143: NEXT : FOR I = 0 TO Q - 1 STEP 4:S$ =  STR$ (I):X =
     18 + (I * C1):Y = 154: IF I > 9 THEN X = 15 + (I * C1)
1420  DRAW 31 AT X,Y: GOSUB 1010: NEXT :T = 1
1430  FOR I = 0 TO 4:Y = 140 - ( LOG (10 ^ I) * 140 /  LOG (10 ^ 5)): HPLOT
     17,Y TO 19,Y: DRAW 31 AT 0,Y + 5:S$ = "10": GOSUB 1010: DRAW 31 AT 13
     ,Y - 4:S$ =  STR$ (I): GOSUB 1010: NEXT : RETURN
1500  REM  :THIS REQUIRES T1,T2 AND THE UTILITY ARRAY 'U' TO BE LOADED
1510 C1 = 259 / (T2 - T1)
1520 Y0 = U(T1):Y1 = Y0
1530  FOR T = T1 TO T2: IF U(T) < Y0 THEN Y0 = U(T)
1540  IF U(T) > Y1 THEN Y1 = U(T)
1550  NEXT
1560 C2 = 140 / (Y1 - Y0)
1570  HGR : HPLOT 17,0 TO 20,0 TO 20,140 TO 279,140: FOR I = 0 TO T2 - T1:
      HPLOT 20 + (I * C1),141 TO 20 + (I * C1),143: NEXT : FOR I = T1 TO T
     2 - 1 STEP 2:S$ =  STR$ (I):X = 18 + ((I - T1) * C1):Y = 154: IF I >
     9 THEN X = 15 + ((I - T1) * C1)
1580  DRAW 31 AT X,Y: GOSUB 1010: NEXT
1590 Y = 140 + (Y0 * C2): FOR I = T1 TO T2 - 1: HPLOT 20 + ((I - T1) * C1)
     ,Y - (U(I) * C2) TO 20 + ((I - T1 + 1) * C1),Y - (U(I + 1) * C2): NEXT

1600  RETURN
1700  TEXT : HOME : VTAB (8): HTAB (16): FLASH : PRINT " CAUTION ": INVERSE
     : PRINT : PRINT : PRINT " YOU ARE ABOUT TO ERASE UNSAVED DATA ": NORMAL
     : PRINT G$;G$;G$
1710  PRINT : PRINT : PRINT "ENTER '1' TO CONTINUE": PRINT : PRINT "PRESS
     ANY OTHER KEY FOR CONTENTS": VTAB (17): HTAB (34): GET Q$: IF Q$ = "1
     " THEN  RETURN
1720  POP : GOTO 100
1800  IF OS = 0 THEN OS = 1:S$ = "NOW PLOTTING OFF SCALE": DRAW 31 AT 126,
     135: GOSUB 1010
1810  RETURN
2000 N = 0:BC = 0:BL = 0: IF C + K > 0 THEN  GOSUB 1700
2002  FOR Q = 0 TO Z + 1:A(Q) = 0:B(Q) = 0: NEXT :C = 0
2005  TEXT : HOME : VTAB (8): PRINT "HELLO. BACKGROUND COUNTS FIRST:": POKE
     34,10:K = 1
2010 N = N + 1: HOME :S$ = S1$: GOSUB 1000: IF Q <  > 0 THEN BC = ((BC * (
     N - 1)) + Q) / N:A(0) = BC
2020 S$ = S2$: GOSUB 1000: IF Q <  > 0 THEN BL = ((BL * (N - 1)) + Q) / N:
     B(0) = BL: GOTO 2010
2030  TEXT : HOME : VTAB (8): PRINT "BEFORE YOU ENTER POST-NEBULISER READI
     NGS": PRINT "PLEASE ENTER THE MAXIMUM TIME THAT THE": PRINT : PRINT "
     EXPERIMENT IS LIKELY TO GO ON FOR, IN":S$ = "MINUTES - ": GOSUB 1000
2032  IF Q <  >  INT (Q) OR Q < 2 OR Q > Z THEN  PRINT G$,G$,G$: FOR Q = 0
      TO 1000: NEXT : GOTO 2030
2035 C1 = 259 / Q: GOSUB 1400
2040  POKE 34,20: HOME : PRINT "TIME - ";T;" MIN.": POKE 34,21:S$ = S1$: GOSUB
     1000: IF Q = 0 THEN  GOTO 2120
2050  HOME :A(T) = Q:LA(T) =  LOG (Q - BC):S$ = S2$: GOSUB 1000: IF Q = 0 THEN
      GOTO 2120
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2060 B(T) = 0:LB(T) =  LOG (Q - BL)
2065  IF T >  = (259 / C1) + .5 THEN  GOSUB 1800: GOTO 2090
2070 Y1 = 140 - ( LOG (A(T) - BC) * 140 /  LOG (100000)):Y2 = 140 - ( LOG
     (B(T) - BL) * 140 /  LOG (100000))
2072  IF Y1 < 1 THEN Y1 = 1
2074  IF Y2 < 1 THEN Y2 = 1
2076  IF T = 1 THEN Y3 = Y1:Y4 = Y2
2080  HPLOT 20 + ((T - 1) * C1),Y3 TO 20 + (T * C1),Y1: HPLOT 20 + ((T - 1
     ) * C1),Y4 TO 20 + (T * C1),Y2
2090  IF T > 10 THEN  GOSUB 1100
2100  IF T > 9 THEN N2 = T:N1 = T - 5: GOSUB 1200: GOSUB 1110
2110 Y3 = Y1:Y4 = Y2:T = T + 1: GOTO 2040
2120  TEXT : HOME : VTAB (8): PRINT "TIME = ";T: PRINT : IF T > 9 THEN  PRINT
     "REG. CO. FOR CHEST DATA = ";R1: PRINT : PRINT "REG. CO. FOR LEG DATA
      = ";R2
2130  VTAB (14): PRINT "1) GIVE I.V. DOSE": PRINT : PRINT "2) ADD MORE DAT
     A": PRINT : PRINT "3) CONTENTS": VTAB (18): HTAB (14): GET Q$
2150  IF Q$ = "1" THEN  HOME : VTAB (8): PRINT "GIVE DOSE NOW": VTAB (13):
      INPUT "PRESS <RETURN TO CONTINUE";Q$: POKE  - 16304,0: GOTO 2190
2160  IF Q$ = "2" THEN  POKE  - 16304,0: GOTO 2040
2170  IF Q$ = "3" THEN TD = T - 1:A(Z + 1) = TD: GOTO 100
2180  PRINT G$;G$;G$: GOTO 2120
2190 TD = T - 1:A(Z + 1) = TD: IF T > 9 THEN  GOSUB 1100
2200  POKE 34,20: HOME : PRINT "TIME - ";T;" MIN.": POKE 34,21:S$ = S1$: GOSUB
     1000: IF Q = 0 THEN  GOTO 2280
2210  HOME :A(T) = Q:LA(T) =  LOG (Q - BC):S$ = S2$: GOSUB 1000: IF Q = 0 THEN
      GOTO 2280
2220 B(T) = Q:LB(T) =  LOG (Q - BL)
2225  IF T >  = (259 / C1) + .5 THEN  GOSUB 1800: GOTO 2250
2230 Y1 = 140 - ( LOG (A(T) - BC) * 140 /  LOG (100000)):Y2 = 140 - ( LOG
     (B(T) - BL) * 140 /  LOG (100000))
2232  IF Y1 < 1 THEN Y1 = 1
2234  IF Y2 < 1 THEN Y2 = 1
2240  HPLOT 20 + ((T - 1) * C1),Y3 TO 20 + (T * C1),Y1: HPLOT 20 + ((T - 1
     ) * C1),Y4 TO 20 + (T * C1),Y2
2250  IF T > TD + 7 THEN  GOSUB 1100
2260  IF T > TD + 6 THEN N2 = T:N1 = T - 4: GOSUB 1200: GOSUB 1110
2270 Y3 = Y1:Y4 = Y2:T = T + 1: GOTO 2200
2280  TEXT : HOME : VTAB (8): PRINT "TIME = ";T: PRINT : IF T > TD + 6 THEN
      PRINT "REG. CO. FOR CHEST DATA = ";R1: PRINT : PRINT "REG. CO. FOR L
     EG DATA = ";R2
2290  PRINT : PRINT : PRINT "DO YOU WANT TO -": PRINT : PRINT "1 - ADD MOR
     E DATA": PRINT : PRINT "2 - SOMETHING ELSE": PRINT : PRINT : POKE 37,
      PEEK (37) - 1: PRINT "ENTER NUMBER": HTAB (15): GET Q$
2300  IF Q$ = "1" THEN  POKE  - 16304,0: GOTO 2200
2310  IF Q$ = "2" THEN  GOTO 100
2320  PRINT G$,G$,G$: GOTO 2280
3000  TEXT : HOME : PRINT "NO.   CHEST   LEG": POKE 34,2: HOME
3010  IF N > TL - 15 THEN N = TL - 15: IF N < 0 THEN N = 0
3020  FOR Q = N TO N + 15: IF TD <  > 0 AND Q = TD + 1 THEN  INVERSE : PRINT
      TAB( 20)"I.V. DOSE": NORMAL
3030  PRINT  TAB( 3 -  LEN ( STR$ (Q)))Q; TAB( 10 -  LEN ( STR$ (A(Q))));A
     (Q); TAB( 18 -  LEN ( STR$ (B(Q))));B(Q): NEXT
3100  POKE 34,20: HOME : PRINT "1) CORRECT": PRINT "2) CHANGE I.V. TIME": PRINT
     "3) ADD/DELETE": POKE 33,18: POKE 32,22: HOME
3102  IF N > 0 THEN  PRINT "4) EARLIER DATA"
3104  IF N < TL - 15 THEN  PRINT "5) LATER DATA"
3106  VTAB ( PEEK (37) + 1): PRINT "6) CONTENTS": POKE 32,0: POKE 33,40
3108  VTAB (23): HTAB (38): GET Q$:Q =  VAL (Q$)
3110  IF Q$ = "1" THEN  GOTO 3200
3120  IF Q$ = "2" THEN  GOTO 3400
3130  IF Q$ = "3" THEN  GOTO 3500
3140  IF Q$ = "4" AND N > 0 THEN N = N - 15: IF N < 0 THEN N = 0
3145  IF Q$ = "4" THEN  GOTO 3000
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3150  IF Q$ = "5" AND N < TL - 15 THEN N = N + 15: GOTO 3000
3160  IF Q$ = "6" THEN  GOTO 100
3170  PRINT G$;G$;G$: GOTO 3100
3200  HOME : INPUT "WHICH READING ? ";Q$:Q =  VAL (Q$): IF Q <  > INT (Q)
      OR Q < N OR Q > N + 15 OR Q > TL THEN  PRINT G$;G$;G$: GOTO 3100
3205 V = 3 - N + Q: IF Q > TD AND TD > N THEN V = 4 - N + Q
3210  HTAB (5): VTAB (V): CALL  - 868
3215  IF Q = 0 THEN  GOTO 3260
3220  INPUT Q$: IF Q$ <  > "" THEN A(Q) =  VAL (Q$):C = 1: IF  VAL (Q$) <
     A(0) THEN Q$ =  STR$ (A(0) + 1)
3225  IF Q$ <  > "" THEN LA(Q) =  LOG ( VAL (Q$) - A(0))
3230  VTAB (V): HTAB (12): INPUT Q$: IF Q$ <  > "" THEN B(Q) =  VAL (Q$):C
      = 1: IF  VAL (Q$) < B(0) THEN Q$ =  STR$ (B(0) + 1)
3235  IF Q$ <  > "" THEN LB(Q) =  LOG ( VAL (Q$) - B(0))
3240  GOTO 3000
3260  INPUT Q$: IF Q$ <  > "" THEN  GOSUB 3300
3270  VTAB (V): HTAB (12): INPUT Q$: IF Q$ <  > "" THEN  GOSUB 3350
3280  GOTO 3000
3300 BC =  VAL (Q$):A(0) = BC: FOR I = 1 TO Z:N = A(I): IF N < BC + 1 THEN
     N = BC + 1
3310 LA(I) =  LOG (N - BC): NEXT : RETURN
3350 BL =  VAL (Q$):B(0) = BL: FOR I = 1 TO Z:N = B(I): IF N < BL + 1 THEN
     N = BL + 1
3360 LB(I) =  LOG (N - BL): NEXT : RETURN
3400  HOME : PRINT "I.V. DOSE WAS GIVEN IMMEDIATELY": PRINT : INPUT "AFTER
      WHICH READING ? ";Q$:Q =  VAL (Q$): IF Q <  > INT (Q) OR Q < 0 OR Q
      > TL THEN  PRINT G$;G$;G$: GOTO 3100
3410 TD =  VAL (Q$):A(Z + 1) = TD:C = 1: GOTO 3000
3500  HOME : PRINT : PRINT "1) ADD    2) DELETE": PRINT : GET Q$
3510  IF Q$ = "2" THEN  GOTO 3600
3520  IF Q$ <  > "1" OR A(Z) <  > 0 THEN  GOTO 3100
3530  HOME : PRINT : PRINT "WHICH NUMBER DO YOU WANT THIS": PRINT : INPUT
     "TO BE ?  ";Q$:Q =  VAL (Q$)
3540  IF Q <  > INT (Q) OR Q < N OR Q > TL + 1 OR Q > N + 16 OR Q < 1 THEN
      PRINT G$,G$,G$: GOTO 3100
3550 TL = TL + 1: FOR I = TL TO Q + 1 STEP  - 1:A(I) = A(I - 1):B(I) = B(I
      - 1):LA(I) = LA(I - 1):LB(I) = LB(I - 1): NEXT : IF Q <  = TD THEN T
     D = TD + 1:A(Z + 1) = TD
3560  HOME : PRINT : INPUT "NEW READING  - CHEST  ";I$:I =  VAL (I$): IF I
     $ = "" THEN  GOTO 3100
3570  IF I <  > INT (I) OR I = 0 THEN  PRINT G$;G$;G$: GOTO 3560
3580 A(Q) = I:LA(Q) =  LOG (A(Q) - BC): PRINT : HTAB (14): INPUT "- LEG  "
     ;I$:I =  INT ( VAL (I$)):B(Q) = I:LB(Q) =  LOG (B(Q) - BL): GOTO 3000

3600  HOME : PRINT : INVERSE : INPUT "WHICH ENTRY DO YOU WANT DELETED ?  "
     ;Q$: NORMAL :Q =  VAL (Q$): IF Q <  > INT (Q) OR Q < 1 OR Q < N OR Q
      > TL OR Q > N + 15 THEN  PRINT G$;G$;G$: GOTO 3100
3610  FOR I = Q TO Z - 1:A(I) = A(I + 1):B(I) = B(I + 1):LA(I) = LA(I + 1)
     :LB(I) = LB(I + 1): NEXT :A(TL) = 0:B(TL) = 0:LA(TL) = 0:LB(TL) = 0:T
     L = TL - 1: IF Q <  = TD THEN TD = TD - 1:A(Z + 1) = TD
3620  GOTO 3000
4000  POKE 34,20: HOME : PRINT "INTERCEPTS FOR CORRECTION FACTOR": POKE 34
     ,21
4010  HOME : PRINT "BRACKET DATA FROM FIRST CHEST TRACE": PRINT "AND PRESS
      'RETURN'"
4020 T1 = 1:T2 = TD: FOR I = 0 TO Z:U(I) = LA(I): NEXT : GOSUB 1500: GOSUB
     1300
4030  GOSUB 1200:I1 = M1 + (D4 * (TD - M3) / D3): HOME : PRINT "CORR. CO.
     = ";R1;"  INTERCEPT "; INT ( EXP (I1)): INPUT "('1' TO RE-DO OR JUST
     'RETURN') ";Q$: IF Q$ <  > "" THEN  GOTO 4010
4040  HOME : PRINT "BRACKET DATA FROM FIRST LEG TRACE": PRINT "AND PRESS '
     RETURN'"
4050  FOR I = 0 TO Z:U(I) = LB(I): NEXT : GOSUB 1500: GOSUB 1300
4060  GOSUB 1200:I2 = M2 + (D5 * (TD - M3) / D3): HOME : PRINT "CORR. CO.
     = ";R2;"  INTERCEPT "; INT ( EXP (I2)): INPUT "('1' TO RE-DO OR JUST
     'RETURN') ";Q$: IF Q$ <  > "" THEN  GOTO 4040
4070  HOME : PRINT "BRACKET DATA FROM SECOND CHEST TRACE": PRINT "AND PRES
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    S 'RETURN'"
4080 T1 = TD + 1:T2 = TL: FOR I = 0 TO Z:U(I) = LA(I): NEXT : GOSUB 1500: GOSUB
    1300:N1 = N1 + TD:N2 = N2 + TD
4090  GOSUB 1200:I3 = M1 + (D4 * (TD - M3) / D3): HOME : PRINT "CORR. CO.
    = ";R1;"  INTERCEPT "; INT ( EXP (I3)): INPUT "('1' TO RE-DO OR JUST
    'RETURN') ";Q$: IF Q$ <  > "" THEN  GOTO 4070
4100  HOME : PRINT "BRACKET DATA FROM SECOND LEG TRACE": PRINT "AND PRESS
    'RETURN'"
4110  FOR I = 0 TO Z:U(I) = LB(I): NEXT : GOSUB 1500: GOSUB 1300:N1 = N1 +
    TD:N2 = N2 + TD
4120  GOSUB 1200:I4 = M2 + (D5 * (TD - M3) / D3): HOME : PRINT "CORR. CO.
    = ";R2;"  INTERCEPT "; INT ( EXP (I4)): INPUT "('1' TO RE-DO OR JUST
    'RETURN') ";Q$: IF Q$ <  > "" THEN  GOTO 4100
4130 FX = ( EXP (I3) -  EXP (I1)) / ( EXP (I4) -  EXP (I2)):FX =  INT ((10
    0 * FX) + .5) / 100: POKE 34,20: HOME : PRINT "CORRECTION FACTOR = ";
    FX
4140  PRINT : INPUT "PRESS 'RETURN' TO CONTINUE  ";Q$:B(Z + 1) = FX:C = 1:
     GOTO 100
5000 FX = B(Z + 1): IF FX <  > 0 THEN  GOTO 5005
5001  TEXT : HOME : VTAB (8): PRINT "NO CORRECTION FACTOR YET": POKE 34,11

5002  HOME : VTAB (11): PRINT "PLEASE ENTER VALUE AND PRESS 'RETURN": PRINT
    : PRINT "(JUST PRESS 'RETURN' FOR CONTENTS)": PRINT : PRINT : INPUT "
    CORRECTION FACTOR : ";Q$: IF Q$ = "" THEN  GOTO 100
5004 C = 1:FX =  VAL (Q$):B(Z + 1) = FX
5005  TEXT : HOME : VTAB (8): PRINT "CORRECTION FACTOR : ";B(Z + 1): PRINT
    : PRINT : PRINT "1) CHANGE VALUE": PRINT : PRINT "2) CONTENTS": PRINT
    : PRINT "3) CONTINUE": POKE 37,13: PRINT : HTAB (15): GET Q$: IF Q$ =
    "2" THEN  GOTO 100
5006  IF Q$ = "3" THEN  GOTO 5009
5007  IF Q$ = "1" THEN  GOTO 5002
5008  PRINT G$;G$;G$: GOTO 5005
5009 T1 = 1:T2 = TD - 1: FOR I = 1 TO TD:U(I) = (A(I) - BC) - ((B(I) - BL)
     * FX): IF U(I) < 1 THEN U(I) = 1
5010 U(I) =  LOG (U(I)): NEXT
5015  GOSUB 1500: HOME : VTAB (22): PRINT "BRACKET USEFUL DATA": GOSUB 130
    0:TA = N1:TB = N2
5020 T1 = TA:T2 = TB: GOSUB 1500: HOME : VTAB (22): PRINT "BRACKET DATA FO
    R HALFTIME CALCULATIONS": GOSUB 1300:N1 = N1 + TA - 1:N2 = N2 + TA -
    1
5030 S1 = 0:S3 = 0:D1 = 0:D3 = 0:D4 = 0: FOR N = N1 TO N2:S1 = S1 + U(N):S
    3 = S3 + N: NEXT :N = N2 - N1 + 1:M1 = S1 / N:M3 = S3 / N
5040  FOR N = N1 TO N2:D1 = D1 + ((U(N) - M1) ^ 2):D3 = D3 + ((N - M3) ^ 2
    ):D4 = D4 + ((U(N) - M1) * (N - M3)): NEXT
5050 N = N2 - N1:R1 =  INT ((D4 * 100 /  SQR (D1 * D3)) + .5) / 100:HF =  -
    ( LOG (2) * D3 / D4)
5051 A = M1 - (D4 * M3 / D3):I1 = A + (D4 * TA / D3):I1 = Y - (I1 * C2): IF
    I1 < 0 THEN I1 = 0
5052  IF I1 > 140 THEN I1 = 140
5053  HPLOT 20,I1
5055  FOR N = TA + 1 TO TB:I1 = A + (D4 * N / D3):I1 = Y - (I1 * C2): IF I
    1 < 0 THEN I1 = 0
5056  IF I1 > 140 THEN I1 = 140
5057  HPLOT  TO 20 + ((N - TA) * C1),I1: NEXT
5060  HOME : VTAB (22): PRINT "HALFTIME = "; INT ((HF * 10) + .5) / 10; SPC(
    6);"CORR. CO. = ";R1: PRINT : POKE 37,22: PRINT "1) REPEAT  2) STRIP
     3) CONTENTS": HTAB (35): GET Q$
5080  IF Q$ = "1" THEN  HOME : HGR : GOTO 5020
5090  IF Q$ = "2" THEN  GOTO 5110
5095  IF Q$ = "3" THEN  GOTO 100
5100  PRINT G$;G$;G$: GOTO 5060
5110 A = M1 - (D4 * M3 / D3): FOR N = TA TO TB:I1 = A + (D4 * N / D3):U(N)
     =  EXP (U(N)) -  EXP (I1): IF U(N) < 1 THEN U(N) = 1
5120  NEXT : FOR N = TA TO TB:U(N) =  LOG (U(N)): NEXT : GOTO 5020
6000  TEXT : HOME : PRINT
6005  PRINT D$;"CATALOG"
6010  PRINT : INPUT "ENTER F
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6020  IF Q$ = "" THEN  GOTO 100
6100  TEXT : HOME : VTAB (12): HTAB (20 - ((10 +  LEN (Q$)) / 2)): INVERSE
    : PRINT " SAVING "Q$" ": NORMAL
6110 AD =  PEEK (107) +  PEEK (108) * 256
6120  PRINT D$;"BSAVE ";Q$;",A";AD;",L"((((Z + 2) * 5) + 7) * 4)
6130 C = 0:K = 0: GOTO 100
7000  IF C + K > 0 THEN  GOSUB 1700
7010  TEXT : HOME : PRINT
7020  PRINT D$;"CATALOG"
7030  PRINT : INPUT "ENTER FILE NAME  ";Q$
7040  IF Q$ = "" THEN  GOTO 100
7102  TEXT : HOME : HTAB (20 - ((10 +  LEN (Q$)) / 2)): VTAB (12): INVERSE
    : PRINT " LOADING "Q$" ": NORMAL
7105 AD =  PEEK (107) +  PEEK (108) * 256
7110  PRINT D$;"BLOAD";Q$;",A";AD
7120  IF  PEEK (43616) +  PEEK (43617) * 256 = ((((Z + 2) * 5) + 7) * 4) THEN
     GOTO 7140
7130  PRINT G$,G$,G$: TEXT : HOME : VTAB (8): PRINT "THIS DATA FILE IS OF
    THE WRONG FORMAT": PRINT : PRINT "FOR THIS DISCETTE, AND HAS DAMAGED"
    : PRINT : PRINT "THE PROGRAMME": PRINT : PRINT : PRINT "PLEASE START
    AGAIN.": END
7140 C = 0:K = 0: GOTO 100
8000  GOSUB 1700
8010  HOME : PRINT
8020  PRINT D$;"CATALOG"
8030  PRINT : INPUT "ENTER NAME OF FILE  ";Q$
8040  IF Q$ = "" THEN  GOTO 100
8050  HOME : VTAB (12): HTAB (20 - ((11 +  LEN (Q$)) / 2)): FLASH : PRINT
    " DELETING ";Q$;" ": NORMAL : PRINT
8060  PRINT D$;"DELETE ";Q$
8070  GOTO 8010
9000 ERR =  PEEK (222): TEXT : HOME : VTAB (8): PRINT G$,G$,G$
9010  IF ERR = 6 THEN  PRINT "NO SUCH FILE AS "Q$: GOSUB 9100: GOTO 100
9020  IF ERR = 8 THEN  PRINT "I CAN'T READ THE DISCETTE.": PRINT : PRINT "
    PLEASE CHECK IT'S IN PROPERLY": GOSUB 9100: GOTO 100
9030  IF ERR = 11 THEN  PRINT "THAT IDIOT'S MADE ANOTHER SYNTAX": PRINT : PRINT
    "ERROR. YOU'D BETTER ASK JOHN TO": PRINT : PRINT "LOOK AT IT.": GOSUB
    9100: GOTO 100
9040  PRINT "THERE SEEMS TO BE AN ERROR SOMEWHERE.": PRINT : PRINT "PLEASE
     CHECK THE DATA FOR ME": GOSUB 9100:E = 0
9050  POKE 768,104: POKE 769,168: POKE 770,104: POKE 771,166: POKE 772,223
    : POKE 773,154: POKE 774,72: POKE 775,152: POKE 776,72: POKE 777,96
9060  CALL 768: GOTO 100
9100  FOR I = 0 TO 2000: NEXT : RETURN
```

APPENDIX 2:

Computer programme for acquisition and analysis of data for transferrin flux study.

In this study data is being acquired from two scintillation detectors simultaneously, from 2 different isotopes (^{99m}Tc and ^{113m}In). The programme was written specifically to deal with this methodology.

```
10 '**************************
20 '* COUNT305 (C) 19/02/85  *
30 '*  D HEMSLEY R.P.M.S.    *
40 '* INSTRUMENTATION DEPT   *
50 '*  JOB NUMBER 84/6       *
60 '**************************
70 CLEAR 200,&H5FFF : CLS
80 PRINT"LOADING MACHINE CODE" : PRINT"PLEASE WAIT"
90 LOAD"COUNT2CM.BIN"                    CLOAD"COUNT2CM"
100 EXEC &H7000 ' CSTART
110 PMODE 4,1
120 DEF FNP(N)=256*PEEK(N)+PEEK(N+1)
130 CLS
140 PRINT@0,"----------MENU----------"
150 PRINT"(S)TART DATA COLLECTION"
160 PRINT"(H)ALT DATA COLLECTION"
170 PRINT"DISPLAY (A)LL CHANNELS"
180 PRINT "DISPLAY CHANNELS"
190 PRINT "(1),(2),(3),OR (4)'
200 PRINT "DISPLAY (I)NDIUM"
210 PRINT "DISPLAY (T)ECHNETIUM"
220 PRINT "DISPLAY (R)ATIO I/T"
230 PRINT "(P)RINTOUT"
240 PRINT "CASSETTE (W)RITE (L)OAD"
250 PRINT"E(X)IT TO BASIC"
260 PRINT
270 NPOINT=(FNP(&H6000)-&H6006)/10
280 PRINT"NUMBER OF DATA POINTS=";NPOINT
290 IN$=INKEY$
300 IF IN$="" THEN GOTO 290
310 IF IN$="I" THEN GOSUB 2270
320 IF IN$="T" THEN GOSUB 2560
330 IF IN$="R" THEN GOSUB 2850
340 IF IN$="X" THEN GOSUB 2030
350 IF IN$="H" THEN POKE &H7FEC,0
360 IF IN$="L" THEN GOSUB 1290
370 IF IN$="W" THEN GOSUB 1450
380 IF IN$="P" THEN GOSUB 1600
390 IF IN$<>"S" THEN GOTO 480
400 INPUT"COUNTING PERIOD IN SEC ";PER$
410 PER=VAL(PER$)
420 IF PER=0  THEN GOTO 460
430 IF PER>255 THEN PRINT"NOT MORE THAN 255 SECS PLEASE" : GOTO 400
440 IF PER<1 THEN PRINT"NOT LESS THAN 1SEC PLEASE" :GOTO 400
450 POKE &H6004,PER ' &PERIOD
460 EXEC &H7003 ' WSTART
470 GOTO 130
480 IF IN$="A" THEN GOTO 510
490 CHAN=VAL(IN$)
500 IF CHAN >4 OR CHAN <1 THEN GOTO 140
510 AEND=FNP(&H6000) ' 6000=@DATPNT
520 NPOINT=(AEND-&H6006)/10
530 IF NPOINT=0 THEN NPOINT=1
540 XSTEP=256/NPOINT
550 MAX=FNP(&H6002)
560 IF MAX=0 THEN MAX=1
570 YSCALE=191/MAX
580 GOTO730
```

```
590 '============================
600 '= DRAW AXIS S/R            =
610 '============================
620 PCLS : SCREEN 1,0
630 FOR Y=191-STP TO 0 STEP -STP
640 PSET(0,Y) : PSET(1,Y)
650 NEXT Y
660 FOR X=10 TO 256 STEP 10
670 PSET(X,191) : PSET(X,190)
680 NEXT X
690 RETURN
700 '
710 '============================
720 '= DISPLAY CHANNELS 1 TO 4=
730 '============================
740 STP=191*10000/MAX
750 IF MAX<=10000 THEN STP=191*1000/MAX
760 IF MAX<=1000 THEN STP=191*100/MAX
770 IF MAX<=100 THEN STP=191*10/100 : YSCALE=191/100
780 GOSUB 610
790 IF IN$="A" THEN GOTO 820
800 GOSUB 920
810 GOTO 850
820 FOR CHAN=1 TO 4
830 GOSUB 910
840 NEXT CHAN
850 IN$=INKEY$
860 IF IN$="" THEN 850
870 GOTO 300
880 '
890 '============================
900 '=  DISPLAY SUBROUTINE    =
910 '============================
920 ADDR=&H6006
930 X=0 : Y=191-YSCALE*FNP(ADDR+CHAN*2)
940 LINE(X,Y)-(X,Y),PSET
950 FOR ADDR=&H6010 TO AEND-1 STEP 10
960 X=X+XSTEP
970 Y=191-YSCALE*FNP(ADDR+CHAN*2)
980 IF Y <0 THEN Y=0
990 LINE-(X,Y),PSET
1000 NEXT ADDR
1010 RETURN
1020 '
1030 '============================
1040 '= STOP COLLECTION S/R    =
1050 '============================
1060 CLS
1070 PRINT "DO YOU WISH TO STOP"
1080 PRINT "DATA COLLETION Y/N/";CHR$(127)
1090 IN$=INKEY$
1100 IF IN$="" THEN 1090
1110 IF IN$="Y" THEN POKE&H7FEC,0
1120 RETURN
1130 '
```

```
1140 '============================
1150 '= WARNING SUBROUTINE      =
1160 '============================
1170 PRINT "          **********"
1180 PRINT "          * WARNING *"
1190 PRINT "          **********"
1200 PRINT "THIS WILL STOP THE CLOCK"
1210 PRINT "AND DATA COLLECTION"
1220 PRINT "DO YOU REALY MEAN IT Y/N"
1230 IN$=INKEY$
1240 IF IN$="" THEN 1230
1250 RETURN
1260 '
1270 '============================
1280 '= LOAD FROM CASSETTE     =
1290 '============================
1300 CLS
1310 PRINT "LOAD FROM CASSETTE"
1320 PRINT "------------------"
1330 PRINT
1340 GOSUB 1160
1350 IF IN$<>"Y" THEN RETURN
1360 PRINT
1370 INPUT "ENTER FILE NAME ";NAME$
1380 PRINT "SEARCHING FOR ";NAME$
1390 CLOADM NAME$
1400 IN$=""
1410 RETURN
1420 '
1430 '============================
1440 '= WRITE TO CASSETTE      =
1450 '============================
1460 CLS
1470 PRINT "WRITE TO CASSETTE"
1480 PRINT "-----------------"
1490 PRINT
1500 GOSUB 1160
1510 IF IN$<>"Y" THEN RETURN
1520 PRINT
1530 INPUT "ENTER FILE NAME ";NAME$
1540 CSAVEM NAME$,&H6000,FNP(&H6000),&H7000
1550 IN$=""
1560 RETURN
1570 '
1580 '============================
1590 '= PRINTOUT               =
1600 '============================
1610 GOSUB 1050
1620 IF IN$=CHR$(8) THEN RETURN
1630 CLS
1640 PRINT "(T)ABULAR OR (G)RAPH. PRINTOUT"
1650 PRINT "------------------------------"
1660 PRINT
1670 LINE INPUT "NAME ";NAME$
1680 LINE INPUT "DATE ";DATE$
1690 MAX=FNP(&H6002)
1700 PER=PEEK(&H6004)
1710 PRINT#-2
1720 PRINT#-2,"NAME:- ";NAME$;"    ";DATE$
1730 PRINT#-2,"MAXIMUM COUNT=";MAX;"    COUNTING PERIOD=";
1740 PRINT#-2
```

```
1750 LNO=5
1760 PRINT#-2,"TIME SEC : CHAN1 : CHAN2 : CHAN3 : CHAN4 :";
1770 PRINT#-2," INDIUM : TECN.   : RATIO  :"
1780 PRINT#-2,"-----------------------------------------";
1790 PRINT#-2,"----------------------------"
1800 FOR ADDR=&H6006 TO (FNP(&H6000)-10) STEP 10
1810 PRINT#-2,": ";
1820 FOR CHAN=0 TO 4
1830 PRINT#-2, USING" ##### :";FNP(ADDR+CHAN*2);
1840 NEXT CHAN
1850 C1=FNP(ADDR+2)
1860 C2=FNP(ADDR+4)
1870 C3=FNP(ADDR+6)
1880 C4=FNP(ADDR+8)
1890 IF C1=0 OR C2=0 OR C3=0 OR C4=0 THEN 1930
1900 PRINT#-2,USING" #.#### :";C2/C4,
1910 PRINT#-2,USING" #.#### :";C1/C3;
1920 PRINT#-2,USING" #.#### :";(C2/C4)/(C1/C3),
1930 PRINT#-2
1940 LNO=LNO+1
1950 IF LNO=57 THEN LNO=0 : PRINT#-2,CHR$(&H0C)
1960 NEXT ADDR
1970 PRINT#-2,CHR$(&H0C)
1980 IN$=""
1990 RETURN
2000 '
2010 '=========================
2020 '= EXIT TO BASIC         =
2030 '=========================
2040 GOSUB 1050
2050 IF IN$=CHR$(8) THEN RETURN
2060 CLS
2070 PRINT "EXIT TO BASIC"
2080 PRINT "-------------"
2090 PRINT
2100 PRINT "TO RESTART WITH NEW DATA"
2110 PRINT "TYPE RUN100"
2120 PRINT
2130 PRINT "TO RESTART WITH OLD DATA"
2140 PRINT "TYPE RUN110"
2150 PRINT
2160 PRINT "DO YOU WISH TO STOP"
2170 PRINT "THE CLOCK Y/N "
2180 IN$=INKEY$
2190 IF IN$="" THEN 2130
2200 CLS
2210 IF IN$<>"Y" THEN END
2220 EXEC &H7009
2230 END
2240
2250 '=========================
2260 '= DISPLAY INDIUM        =
2270 '=========================
2280 STP=19
2290 GOSUB 610
2300 ADDR=&H6006
2310 C2=FNP(ADDR+4)
2320 C4=FNP(ADDR+8)
2330 IF C4=0 THEN C4=1
2340 X=0 : Y=191-191/1*C2/C4
2350 IF Y<0 THEN Y=0
```

```
2360 LINE(X,Y)-(X,Y),PSET
2370 AEND=FNP(&H6000)
2380 NPOINT=(AEND-&H6006)/10
2390 IF NPOINT=0 THEN NPOINT=1
2400 XSTEP=256/NPOINT
2410 FOR ADDR=&H6010 TO AEND-1 STEP 10
2420 X=X+XSTEP
2430 C2=FNP(ADDR+4)
2440 C4=FNP(ADDR+0)
2450 IF C4=0 THEN C4=1
2460 Y=191-191*C2/C4
2470 IF Y<0 THEN Y=0
2480 LINE-(X,Y),PSET
2490 NEXT ADDR
2500 IN$=INKEY$
2510 IF IN$="" THEN 2500
2520 RETURN
2530 '
2540 '=========================
2550 '= DISPLAY TECHNETIUM    =
2560 '=========================
2570 STP=19
2580 GOSUB 610
2590 ADDR=&H6006
2600 C1=FNP(ADDR+2)
2610 C3=FNP(ADDR+6)
2620 IF C3=0 THEN C3=1
2630 X=0 : Y=191-191/1*C1/C3
2640 IF Y<0 THEN Y=0
2650 LINE(X,Y)-(X,Y),PSET
2660 AEND=FNP(&H6000)
2670 NPOINT=(AEND-&H6006)/10
2680 IF NPOINT=0 THEN NPOINT=1
2690 XSTEP=256/NPOINT
2700 FOR ADDR=&H6010 TO AEND-1 STEP 10
2710 X=X+XSTEP
2720 C1=FNP(ADDR+2)
2730 C3=FNP(ADDR+6)
2740 IF C3=0 THEN C3=1
2750 Y=191-191*C1/C3
2760 IF Y<0 THEN Y=0
2770 LINE-(X,Y),PSET
2780 NEXT ADDR
2790 IN$=INKEY$
2800 IF IN$="" THEN 2790
2810 RETURN
2820 '
2830 '=========================
2840 '= DISPLAY IND./TECH.    =
2850 '=========================
```

```
2860 STF=63
2870 GOSUB 610
2880 ADDR=&H6006
2890 C1=FNP(ADDR+2)
2900 IF C1=0 THEN C1=1
2910 C2=FNP(ADDR+4)
2920 IF C2=0 THEN C2=1
2930 C3=FNP(ADDR+6)
2940 IF C3=0 THEN C3=1
2950 C4=FNP(ADDR+8)
2960 IF C4=0 THEN C4=1
2970 X=0 : Y=191-191/3*(C2/C4)/(C1/C3)
2980 IF Y<0 THEN Y=0
2990 LINE(X,Y)-(X,Y),PSET
3000 AEND=FNP(&H6000)
3010 NPOINT=(AEND-&H6006)/10
3020 IF NPOINT=0 THEN NPOINT=1
3030 XSTEP=256/NPOINT
3040 FOR ADDR=&H6010 TO AEND-1 STEP 10
3050 X=X+XSTEP
3060 C1=FNP(ADDR+2)
3070 IF C1=0 THEN C1=1
3080 C2=FNP(ADDR+4)
3090 IF C2=0 THEN C2=1
3100 C3=FNP(ADDR+6)
3110 IF C3=0 THEN C3=1
3120 C4=FNP(ADDR+8)
3130 IF C4=0 THEN C4=1
3140 Y=191-191/3*(C2/C4)/(C1/C3)
3150 IF Y<0 THEN Y=0
3160 LINE-(X,Y),PSET
3170 NEXT ADDR
3180 IN$=INKEY$
3190 IF IN$="" THEN 3180
3200 RETURN
3210 CSAVE"COUNT305
3220 CSAVEM"COUNT2CM",&H7000,&H717F,&H7000
```