Bronchoalveolar Lavage and Alveolar Macrophage Function in Acute Lung Injury

By

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Dedication:

...to my parents and my family

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SUMMARY

Acute lung injury was studied in community-acquired pneumonia (CAP), smoke inhalation injury in fire victims and radiation injury following radiotherapy for bronchial carcinoma. *In vitro* methods for processing broncho-alveolar lavage (BAL) specimens and for studying alveolar macrophage (AM) function were developed, assessed and used in comparing these patient groups with control subjects (CS).

The AM was the predominant cell in BAL samples from radiogically clear areas (RCA) in CAP patients and in CS. The proportion of AM subgroups in BAL fluid from RCA as determined by macrophage markers was similar to CS apart from NA1/34 (T6) positive (Langerhans) cells which was lower in specimens from RCA. Furthermore BAL fluid from CS and RCA in CAP were similar in their levels of albumin, complement components and products of complement activation.

Both unstimulated and stimulated migration of AM from RCA in CAP towards zymosan activated (ZAS), casein and f-met-leu-phe was impaired. The impaired migration was associated with reduced generation of the respiratory burst by AM from RCA. This impairment of AM function could not be attributed to an inhibiting factor in the BAL fluid from these areas. Examination of peripheral blood cells did not reveal similar functional impairment thus making it unlikely to be due to a systemic effect of

pneumonia.

Neutrophils were the predominant cells in BAL fluid obtained from areas of consolidation (AOC) in CAP patients. AOC also contained significantly higher proportions of $UCHM_1$ positive cells (monocytes) and RFD7 positive cells. In addition BAL fluid from these lung areas showed significantly high chemotatic activity to neutrophils, along with very high levels of albumin, components of complement and products of complement activation. Due to technical difficulties limited information was obtained on function of AM from AOC. This suggested depression of generation of the respiratory burst.

The cell composition in BAL fluid from patients with smoke inhalation injury alone or with burns showed significantly higher proportions of neutrophils than CS. This increase in neutrophils was not seen in patients with burns only. There was a significant increase in the proportion of RFD9 positive cells in patients with smoke inhalation only and $UCHM_1$ positive cells in those with smoke and burns injury.

AM from patients with smoke inhalation injury showed increased migration compared to those from CS. This was most marked in AM obtained from patients with combined smoke inhalation and burns injury. They showed significantly higher AM unstimulated and stimulated migration towards casein, ZAS and f-met-leu- phe. Again patients with burns only did not show such increase in AM

migration. Furthermore, BAL fluid from patients with combined injury showed significantly higher functional chemotatic activity than those from CS; associated with significantly higher levels of albumin, complement components and products of complement activation. BAL fluid from patients with burns only did not show any chemotatic activity.

BAL samples from patients with bronchial carcinoma before radiotherapy showed similar proportions of cells to CS. However, BAL from tumour areas following radiotherapy contained significantly lower proportions of macrophages and significantly higher proportion of neutrophils than CS. There were no significant differences in the proportions of macrophage subgroups between CS and patients with bronchial carcinoma before or after radiotherapy. There was functional impairment of AM from patients with bronchial carcinoma and this was more marked in samples obtained from tumour areas. Some improvement in AM function appeared to occur following radiotherapy.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction:

Acute lung injury is a pathological process of the lung usually associated with inflammatory changes and occurring in various disease states. It can result from inhalation of many toxic substances such as ammonia (Sobonya, 1977), nitrogen dioxide (Lowry, 1956), fumes of a smoke bomb (Milliken, 1963) or smoke inhalation in fire victims (DiVincenti, 1971). Pneumonias caused by various microbiological agents such as viruses (Martin, 1959), bacteria (Tilghman, 1937; Austrian, 1964), fungi (Young, 1974) or protozoa (Burke, 1973), are another form of acute lung injury. It may also result from radiation (Smith, 1963a&b), drug therapy (Rosenow, 1972) or oxygen therapy (Clark, 1971).

These are but a few examples of lung injury and different though they may be aetiologically, they often manifest themselves with similar clinical features. The pathophysiological mechanisms of lung injury are poorly understood and most of the data available are based on animal studies. The use of animal models undoubtedly contributes a great deal to our understanding of various mechanisms in disease states but technical limitations in imitating natural disease states in animal models, and species differences make interpretation of data difficult.

The use of well circumscribed acute pathological

conditions such as pneumonia, smoke inhalation in fire victims, and radiotherapy as models of acute lung injury avoids such problems. It was proposed therefore to apply bronchoalveolar lavage, a well established diagnostic and research tool, to the study of acute lung injury represented by these conditions.

1.2 Lung defences

Lung defences can be broadly divided into four main groups:

I Mechanical barriers and airway reflexes.
II Mucociliary transport.
III Humoral mucosal factors.
IV Cellular defences.

However this review will focus on humoral mucosal factors and cellular defences only.

1.2.1 Humoral mucosal factors:

The humoral factors supplement the physical clearance of foreign particulates by the mechanical barriers. The humoral factors consist of local non-specific soluble products and immunological factors.

1.2.1.1 Non-specific soluble products:

Most of these products are secreted by alveolar macrophages and their protective role principally lies in their antimicrobial activity. However some products serve to neutralize enzymes while others serve to cleave complement precursors.

1.2.1.1.1 Lysozyme:

Although a product of macrophages and polymorphonuclear cells, its main function is in extracellular rather than intracellular digestion. It has a molecular weight of 14,000 daltons and is also found in other body fluids like tears and serum. It is known that it acts on the peptidoglycan of bacterial cell walls specifically hydrolysing the 1-4 glycosidic linkages between n-acetylmuramic acid and n-acetyl glucosamine (Unanue, 1976). Its production is increased in activated macrophages (Heise, 1966), but it is not modulated by a phagocytic stimulus like other enzymes.

1.2.1.1.2 Lactoferrin:

This is thought to be produced by glandular mucosal cells (Tourville, 1969) and polymorphonuclear leucocytes. It has a potent bacteriostatic activity.

1.2.1.1.3 Alpha-1-Anti-trypsin:

This is found in alveolar macrophages and in bronchial secretions (Cohen, 1973). It is capable of inhibiting bacterial enzymes but its better known role is in protection against proteases and lysosomal enzymes released by leucocyte granules. Deficiency of this enzyme is associated with emphysema and this appears to be accelerated by smoking.

1.2.1.1.4 Complement:

The role of complement in opsonisation and chemotaxis is well established. Further discussion of this aspect of host defence will be made under appropriate sections.

1.2.1.2 Immunological factors:

Early work on lung secretions concentrated on surfactant but it became clear that many other substances unrelated to surfactant are also found. It is now widely accepted that a local immune secretory system exists in the respiratory tract. Bull & McKee (1929) demonstrated that rabbits could be immunised against pneumococci by intranasal inoculation of these organisms without demonstrable serum antibodies. It has also been shown that intravenous inoculation with killed pneumococci results in production of large amounts of antibodies in the lungs (Askonas, 1958).

1.2.1.2.1 IgA:

Studies of bronchial secretions have shown that IgA is the major immunoglobulin component. Unlike serum IgA, which is a 7-S monomer, the secretory IgA is an 11-S dimer linked by a joining (J) chain to the secretory component (SC) (South, 1968). The dimeric IgA with the J-chain is produced by local plasma cells and is exported into the interstitial spaces where it comes in contact with the epithelial cells. There is also evidence that some dimeric IgA, is selectively transported to the lung. The secretory component is a product of the epithelial cells and appears to be important in transportation of the IgA-J component before it is exported to the mucosal surface. Secretory IgA (sIgA) is resistant to most forms of enzymatic degeneration. sIgA may play a role in lung defence by neutralizing toxins and viruses, agglutinating bacteria and impairing bacterial adherence to the epithelial cells and therefore possibly preventing colonisation. IgA-deficient patients are known to have an increased incidence of respiratory tract infections or associated allergic disorders such as eczema, allergic rhinitis or asthma (Schwartz, 1971). It should be mentioned that these atopic individuals do not necessarily have an increased incidence of IgA deficiency (Platt-Mills, 1976). Allergen-specific sIgA has been demonstrated in nasal secretions from atopic individuals (Platt-Mills, 1976). There is therefore evidence that sIgA plays a role in defence against microbial agents and possibly also in allergic disorders.

1.2.1.2.2 IgG:

IgG in pulmonary secretions was thought to originate solely from the serum by transudation, but several workers including Platt-Mills (1979) have reported that local synthesis also takes place. However the contribution of locally produced IgG in the normal lung appears to be smaller than that transuding from the serum. IgG in the respiratory tract agglutinates bacteria, and promotes phagocytosis by alveolar macrophages. IgG immune complexes are not found in the healthy lung but have been identified in respiratory disorders.

The quantity of IgG in relation to IgA in the respiratory tract appears to vary according to the area of the respiratory tract examined. Kaltreider (1976) estimated levels of immunoglobulins in stimulated saliva, tracheal washings and bronchopulmonary lavage supernatants in dogs and found higher levels of IgA in the upper respiratory tract compared to the lower respiratory tract and the converse with regard to IgG. Underlining the predominance of IgA even in the lower respiratory tract, is the finding that there were more IgA than IgG secreting lymphocytes per million lymphocytes in the lavage fluid of normal volunteers (Laurence, 1978). It is interesting that in the same study, both IgA and IgG secreting lymphocytes per million lymphocytes in the lavage fluid were more numerous than in the blood although the IgG secreting cells predominated in the blood.

1.2.1.2.3 IgM:

This large immunoglobulin is found in very small amounts in the normal lung and because of its size it is thought that it is entirely locally produced. Lawrence (1978) found IgM secreting cells in four out of seven lavage specimens although all corresponding blood samples demonstrated these cells. Recent work which has taken into account its rate of secretion into alveolar space relative to albumin, and also relative to the other body spaces such as the gut suggests that local synthesis of IgM is small (Delacroix, 1985). In disease states IgM in the lung has been found to be high and some workers have reported that this immunoglobulin allows differentiation between hypersensitivity pneumonitis and other interstitial lung diseases (Weinberger, 1978) although this has not been confirmed by Delacroix 1985.

1.2.1.2.4 IgE:

In the lower respiratory tract, IgE transudes from the serum since the levels in the two compartments are similar. However, high levels of IgE in the upper respiratory tract indicate that local synthesis is important. The role of IgE in the lung is not clearly understood but the finding of IgE receptors on a subpopulation of lymphocytes (Yodoi, 1979) and alveolar macrophages (Joseph, 1980) may indicate an important function. IgE secreting cells have been identified in lavage

fluid from healthy individuals (Lawrence, 1978).

1.2.1.2.5 IgD:

This immunoglobulin has not been detected in the lavage fluid (Huninghake, 1979) and has generally received little attention.

1.2.1.2.6 $Alpha_2$ macroglobulin:

Very low levels of this protein have been found in the normal lung but up to a 120- fold rise has been found in disease states (Delacroix, 1985). It appears to offer protection to lung tissue against metabolites secreted by various cells including neutrophils and macrophages.

1.2.2 Cellular defences:

Particles which escape entrapment by mechanical barriers or clearance by the mucociliary transport system and airway secretions, reach the alveoli and are dealt with by the cellular defences in conjunction with the humoral factors. In health, the backbone of the cellular defence is the alveolar macrophage (AM) which plays a pivotal role in its interaction with the lymphocytes. Furthermore the alveolar macrophage's ability to recruit other cells such as neutrophils in immune or inflammatory reactions is crucial to host defence. Each of the cell types (macrophages, lymphocytes, neutrophils and

eosinophils) will be briefly reviewed.

1.2.2.1 The alveolar macrophage

The AM is found mainly at the alveolus but is also found in the alveolar interstitial spaces.

1.2.2.1.1 Ontogeny:

The AM is part of the mononuclear macrophage system and originates from the bone marrow. Convincing evidence regarding its origin has come from bone marrow transplantation studies. Macrophages obtained from recipients of marrow from donors of the opposite sex have the sex chromosomes of the donor. Identification of the Y-limb in macrophages from female recipients of male donor marrow has been particularly convincing (Thomas, 1976; Winston, 1982). Alveolar macrophages have been shown to be capable of *in vitro* proliferation. Bowden *et al* (1972) and others showed that the alveolar interstitial cell has the propensity to multiply and support the macrophage population locally. Macrophages from a male recipient of a female marrow donor have been shown to be of male origin up to four and a half years after successful transplantation underlining the ability of the alveolar macrophage to replicate locally within the lung. Following tracheal instillation of carbon particles in monocyte-depleted mice, AM increased four-fold in two weeks (Bowden, 1982). Thus although the ultimate origin of the AM is the bone

marrow, local replication also plays a part in maintaining their population.

1.2.2.1.2 Life span and fate of AM:

Meuret (1973) estimated that the peripheral blood monocyte in man has a half life of about eight and a half hours in the circulation before it disappears into the tissues. Kinetic studies in mice have shown that about 70% of monocytes leaving the circulation end up as Kuppfer cells, 15% as alveolar macrophages and 8% as peritoneal macrophages (Van Furth, 1983). In mice the turnover of AM was estimated to be about six days. In human bone marrow transplants, Thomas et al (1976) estimated that the life span of the AM is about eighty-one days. It is thought that once macrophages reach their destination organ they do not re-enter the circulation although firm evidence for this is lacking. AM disappear from the lung via the airspaces or the lymphatics. Their exit via the lymphatics is considered to be important in disease states (Corry, 1984).

1.2.2.1.3 Maintenance of macrophage populations:

Using a mathematical approach, it has been estimated that in a normal steady state in mice about 75% of the alveolar macrophages are from the monocyte influx and 25% from replication of the local immature mononuclear cells originating from the bone marrow. Investigating the mechanism

of increased production of monocytes in inflammatory reactions, Van Waardle and colleagues (1977a) injected particulate substances into the mouse peritoneum to induce monocytosis. They described a Factor Increasing Monocytopoiesis (FIM) produced by macrophages at the site of inflammation, transported via the circulation to the bone marrow where it exerts its stimulatory action on the bone marrow. It is cell-line specific, not related to complement products, and has no chemotactic or colony-stimulating activity (van Waardle, 1977(b)). It has a short half life of about 20 minutes. Decreased monocyte production has been attributed to a Monocyte Production Inhibitor (Van Waardle, 1978). The regulation of the macrophage population in a steady state is not clear. AM obtained from patients with chronic lung disorders in vitro have been shown to have an increased propensity for in vitro multiplication indicating a possible mechanism for the expansion of macrophage populations in these conditions (Bitterman, 1984). Increased proportions of broncho-alveolar cells bearing monocyte markers have been documented in disease states (Hance, 1985).

1.2.2.1.4 Morphology of the AM:

Alveolar macrophages are heterogeneous in size ranging from 15-50 um in diameter. They have a large cyto-plasmic:nuclear ratio of about 3:1, single eccentric nucleus but are occasionally multinucleated. They have prominent nucleoli. Fusion of alveolar macrophages gives rise to larger

multinucleated cells and this is thought to be influenced by cytokines such as interferon gamma (Nagasawa, 1987). Higher proportions of the larger multinucleated macrophages are found in smokers (Martin, 1973). The surface of AM is usually ruffled due to the presence of lamellipodia and filopodia. The cytoplasm of the AM is intensely granular because of the large numbers of the membrane-bound primary lysosomal enzymes and also heterogeneous secondary lysosomes and inclusion bodies. These inclusions consist of fused primary lysosomes and digestive vacuoles, such as the unique "smokers' inclusions" seen in macrophages obtained from cigarette smokers. The AM stains strongly with nonspecific alpha naphthyl esterase, periodic-acid Schiff reagents and it is peroxidase-negative. In wet preparations these cells show intense autofluorescence which can also be detected by a fluorescence-activated flow cytometer; (Hance, 1985).

1.2.2.1.5 Surface receptors:

AM can express more than thirty different receptors (Du Bois, 1985) but comprehensive discussion of these is beyond the scope of this thesis and only a few will be mentioned here. Receptors for the Fc fragment of IgG are amongst the most important and a single macrophage may have up to 1×10^6 of these and this may double with cell activation. The AM was thought to lack receptors for IgA (Huninghake, 1979; McDermott, 1982), but a recent study reported the presence of Fc receptors for IgA on AM (Gauldie, 1983). AM will sometimes

interact indirectly with an immunoglobulin through some of its receptors. For example C3b receptors which are abundant on the AM will allow interaction with an IgM-antigen immune complex. Other receptors expressed on AM include complement components C5a and C3d, glucocorticoids, beta-adrenergic agonists, lysosomal glycosidases, and lactoferrin. The mechanisms by which the regulation of different receptors are expressed on the surface of the cell to facilitate a particular function are not understood. It is clear that during maturation from monocyte to macrophage, a cell can lose receptors for some antigens or biochemical fragments while it acquires the ability to express other types of receptors (Zwaldo, 1985; Hance, 1985).

1.2.2.1.6 Metabolism of AM:

Most of the data on metabolism of macrophages have come from animal work. Macrophages differ in their metabolism from other phagocytes like polymorphonuclear leucocytes. These latter cells for example depend on glycogen as an endogenous supply of energy for their metabolism, can handle only one phagocytic load and are unable to synthesize new granules or lysosomal enzymes. Macrophages may process repetitively and are capable of synthesizing new lysosomal enzymes (Mason, 1977). Macrophages are larger than peripheral blood monocytes, contain more lysosomes and mitochondria, but have very little peroxidase so that they cannot iodinate bacteria. The resident AM shows a high level of glucose consumption but

this does not appear to be affected by phagocytosis. AM metabolise glucose by glycolysis, the pentose phosphate pathway and Krebs cycle. The rate of phagocytosis is diminished by inhibitors of glycolysis and of the Krebs cycle. AM can produce lactate in the presence of glucose under anaerobic conditions. AM are able to digest various ingested sugars because they contain enzymes such as hexosidases and hexosaminidases, components of complement, immunoglobulins, interferon, endogenous pyrogen and colony stimulating factor. They can also digest proteins which have been ingested by exposing them to enzymes intracellularly. The complexity of the intracellular organelles in the AM enables it to digest ingested proteins selectively so that it can leave highly antigenic molecules undigested before transporting them to the cell surface for delivery to other cells such as T-lymphocytes.

1.2.2.1.7 Functions of AM

AM are capable of movement towards and phagocytosis and killing of microorganisms, secretion of various factors and interaction with other cells to modulate the immune and inflammatory reactions of the lung.

(i) Chemotaxis:

Locomotion is an important cellular characteristic and it is now considered as an integral component of cell function. This is because a number of studies with polymorphonuclear

leucocytes have established the co-existence of defective leucocyte locomotion with either a demonstrable impairment of intracellular killing, or with recurrent infections. Ward (1969) described a patient with recurrent infections associated with impaired leucocyte locomotion. The neutrophils from this patient were also defective in their bactericidal activity. The "lazy leucocyte syndrome" attributed to recurrent infections in two children with neutropenia and depressed neutrophil locomotion but with normal myeloid precursors and mature neutrophils in the bone marrow was described by Miller in 1971. Since then the literature covering various aspects of neutrophil chemotaxis has proliferated. AM locomotion is less well studied but there is evidence that AM can migrate from the bronchoalveolar space to the hilar lymph nodes. Corry (1984) reported that a proportion of live chromium or indium labelled AM intratracheally instilled into syngeneic guinea pigs migrated to the hilar lymph nodes. The importance of the capacity of macrophages to migrate to hilar nodes in antigen presentation and possible dissemination of infection to distant sites of the body is evident. In vivo migration of alveolar macrophages to sites of particle deposition has also been demonstrated in other studies. Warheit and colleagues (1984) have shown that following deposition of aerosolised asbestos fibres, there is accumulation of AM at the alveolar duct bifurcations where these cells are normally rarely found. In these experiments complement- deficient rats failed to achieve the same degree of macrophage accumulation compared to

normal rats. Analysis of lavage fluid showed that the chemotactic factor responsible for attracting alveolar macrophages to these sites was C5a. In vitro studies have also shown that C5a is chemotactic for alveolar macrophages (Fine, 1981; Richards, 1984). Winston (1982) reported depressed AM chemotaxis in a group of bone marrow transplant patients. Furthermore macrophages from these patients showed defective phagocytosis and killing of Candida albicans. Bone marrow transplant recipients often succumb to opportunistic infections (Deeg, 1985). Impaired migration of alveolar macrophages obtained from patients with malignant lung tumours has been reported by Le Marie, 1984. Demarest (1979) reported impaired chemotaxis of alveolar macrophages from a small group of smoke inhalation patients and concluded that this might be a predisposing factor to the increased incidence of pulmonary infections in fire victims. This study is of particular interest and will be discussed later.

There is little doubt therefore that AM chemotaxis is important in vivo, but this aspect of macrophage function is less well studied and the mechanisms regulating it are less well defined.

(ii) Phagocytosis:

The AM is responsible for clearing airborne particulates from the lower respiratory tract as well as locally produced debris or sequestrae. Asbestos-laden macrophages obtained from patients exposed to asbestos, and macrophages containing

proteinaceous material obtained from patients with alveolar proteinosis (Golde, 1977) are two examples of this interaction *in vivo*.

Ingestion of microorganisms by AM depends upon recognition of IgG- or C3b-opsonised microorganism by respective surface receptors on the macrophages, followed by attachment to the cell membrane (Reynolds, 1975). There is evidence that surfactant can also act as an opsonin for microorganisms (O'Neill, 1984). Until recently it was thought that IgA was not capable of mediating phagocytosis, but Richards *et al* (1985) reported IgA-mediated phagocytosis by AM but not by peritoneal macrophages. Attachment to the macrophage of unopsonised particles is also possible and may be due to charge interactions (Huninghake, 1979). Human plasma fibronectin has also been shown to augment opsonin-independent phagocytosis (Czop, 1982). Whatever the mechanism of attachment to the macrophage, a series of events appears to follow. It is believed that the attachment of the particle traps and immobilizes proteins in the vicinity, allows them to form a cluster and increases permeability of the membrane to sodium ions. This is followed by depolarization which allows influx of calcium ions into the macrophage. This triggers contraction of microfilaments situated beneath the cell membrane in the peripheral cytoplasm and allows the plasma membrane to move forward over the attached particle, completing phagocytosis (Alison, 1977).

Phagocytosis by AM depends on energy from the Krebs cycle, the pentose phosphate pathway and glycolysis. This is

the only phagocytic cell which uses the Krebs cycle for a major part of its energy production (Mason, 1977). Cellular events associated with phagocytosis including increased oxygen consumption and activity of the pentose phosphate pathway can be triggered by soluble factors alone (without phagocytosis taking place) like endotoxin, digitonin and detergents.

Once phagocytosis is achieved, the particle encased by the cytoplasmic membrane fuses with the lysosome to produce the phagolysosome. This primary phagolysosome finally detaches to become a secondary phagolysosome. Phagocytic vacuoles in AM have been found to lack enzymes such as myeloperoxidase, but have been found to be rich in lysosomal hydrolases, catalase and lipid hydroperoxidase (Gee, 1977). It is in these phagocytic vacuoles that intracellular killing or degradation of particles takes place.

(iii) Killing:

Killing of microorganisms can be intracellular or extracellular. Intracellular killing follows phagocytosis. Phagocytic activity or stimulation of the membrane by soluble substrates is accompanied by intracellular biochemical changes including increased oxygen consumption and activation of the hexose monophosphate shunt pathway. This "respiratory burst" is associated with generation of reactive oxygen species such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals (Adams, 1984). The generation of superoxide is thought to be due to oxidation of NADP to NADPH by an oxidase

enzyme called NADPH oxidase. The metabolic process leads to a change in the cell pH which is bactericidal. In addition there is release of extracellular enzymes which are also bactericidal. The mechanisms by which the release of these enzymes is regulated in other phagocytes such as polymorphonuclear leucocytes depend on the levels of cyclic AMP and cyclic GMP, but in the AM this is less clear. Lysozyme does not depend on phagocytic stimuli for its release. In addition to the above changes, the fusion of the phagolysosomes to form the phagocytic vacuole exposes particulate matter to lysosomal enzymes in the microbicidal environment of the phagocytic vacuoles. The release of lysosomal enzymes to the extracellular compartment may lead to tissue damage and this will be dealt with later in greater detail.

1.2.2.1.8 Secretory functions of the AM

The macrophage can secrete more than fifty products (Du Bois, 1985) and a few of these have been referred to earlier. These include products with potent antimicrobial activity, products which are capable of protecting the host from proteases released by other cells or even by the macrophage itself, and others which mediate macrophage interaction with other cells in modulating immune and inflammatory reactions. Equally important is the destructive effect of many of the products secreted by the macrophages and the role of these in the pathogenesis of interstitial lung diseases has become the

subject of extensive research.

(i) Lysozyme

This is a low molecular weight protein produced by the macrophage continuously, independent of the phagocytic stimulus. BCG-activated macrophages (Heise, 1967) secrete large amounts of this protein and this is not influenced by a high concentration of serum as occurs with lysosomal enzymes. The macrophage has the capacity to secrete lysozyme and increase the extracellular concentration four-fold while keeping the intracellular concentration unchanged (Gordon, 1974). Its bactericidal activity depends on its ability to hydrolyse the peptidoglycan of the bacterial cell wall.

(ii) Lysosomal enzymes:

These enzymes unlike lysozyme are released by the activated macrophage following a phagocytic stimulus during the stage of interiorization of the particle as the lysosome fuses with endocytic vacuole (reviewed by Unanue, 1976). Macrophage interaction with antigen-antibody complexes leading to perturbation of the cell membrane can cause release of lysosomal enzymes. The lymphokine interferon gamma can also activate the macrophage to release these enzymes. Release of lysosomal enzymes is energy dependent and can be modulated by cyclic GMP. It is a selective process so that following a phagocytic stimulus there is a release of acid hydrolases

without any lactic dehydrogenase which appears in the extracellular compartment following cell death.

(iii) Neutral proteases:

These enzymes including collagenase, elastase, plasminogen activator and cytolytic proteinase are poorly secreted by the resident alveolar macrophage; but are readily produced by the exudate macrophage. Their production is a two-stage process heralded by a priming signal which can be from a lymphokine or endotoxin followed by a second signal triggering the secretion. Secretion of these enzymes can be triggered in a variety of ways depending on the protease, and the secretion can be shut off by binding with alpha$_2$ macroglobulin-trypsin complex (Adams and Hamilton, 1984).

(iv) Alveolar macrophage-derived neutrophil chemotactic factor:

Huninghake and colleagues (1978) reported that pig alveolar macrophages secreted a chemotactic factor for neutrophils following ingestion of heat-killed S. aureus. This factor was less than 5000 daltons in molecular weight and was not a peptide or a complement fragment. Further work revealed that this factor did not only stimulate neutrophil migration but also stimulated the cells to release lysozyme and lactoferrin (Huninghake, 1980). This underlines the potential of the AM in both lung defence and also in lung injury. In

humans this factor was found to be released by the AM from smokers but not from non-smokers (Huninghake, 1983).

(v) Arachidonic acid metabolites:

Macrophages are a major source of these products which have important functional roles in vivo. The production of these metabolites depends on the presence of arachidonic acid, calcium, and phospholipase A_2. The levels of cyclooxygenase and lipoxygenase control the metabolism of arachidonic acid. Prostaglandin E_2 (PGE_2) has an important role in tumour cytolysis. PGF_2, PGE_2, leukotrine C_4 (LTC_4), LTB_4 and thromboxane B_2 (TxB_2) are potent chemotactic agents. LTD_4 is an extremely potent bronchoconstrictor having 1000-fold greater potency than histamine. Alveolar macrophages do not normally release prostaglandins or leukotrienes spontaneously and often in vitro experiments require a phagocytic stimulus or calcium ionophore A23187. LTB_4 is more chemotactic to neutrophils than monocytes or alveolar macrophages. This underlies the potential mechanism for recruitment by AM of neutrophils to the lungs. There appears to be some controversy regarding the synthesis of LTD_4 by the AM. Martin (1984) failed to find any LTD_4 release by AM whereas this was reported by Damon and colleagues (1983). In animal models immunologically activated macrophages produce less arachidonic acid metabolites than human alveolar macrophages from patients with sarcoidosis, cryptogenic fibrosing alveolitis, and AM from smokers produce significantly less

arachidonic acid metabolites than non-smokers' macrophages (Bachwich, 1986).

(vi) Interferon:

Interferon (IFN) was discovered in 1957 by Isaacs and Lindenmann following the observation that viral replication in a cell previously infected with another virus was inhibited (virus interference). Three major types of interferons are now recognised. These are INF, alpha, beta and gamma respectively produced by leucocytes, fibroblasts and lymphocytes, though not exclusively. These various cells need induction by an RNA-virus, double stranded RNA or a variety of immune stimuli. IFNs are proteins with a molecular weight between 10,000 to 50,000 daltons consisting of 166 aminoacids for INF alpha and beta and 142 aminoacids for INF gamma (reviewed by Burke, 1985). Their anti-viral activity is not due to direct neutralization but they protect the cell by producing a series of changes in cellular metabolism which interfere with nucleic acid and protein synthesis and also with the assembly of the virus particles. IFN gamma has been found to be identical to Macrophage Activating Factor and has a myriad of effects on macrophages and lymphocytes. Apart from its antiviral activity it can induce expression of DR- determinants, enhance presentation of antigens, activate mononuclear phagocytes for tumour cell lysis and intracellular parasite killing, stimulate T-lymphocytes in expression of interleukin-2 receptors, enhance natural killer cell activity and induce

expression of DR-determinants on B-cells.

The human alveolar macrophage, like other mononuclear phagocytes has been shown to be capable of producing interferon. Nugent (1985) reported that human alveolar macrophages from healthy volunteers released IFN alpha and IFN gamma following induction by influenza-A virus and the mitogen (concavalin A) respectively. Alveolar macrophages and lung T-lymphocytes from patients with active sarcoidosis were shown to release IFN gamma spontaneously (Robinson, 1985), but peripheral blood cells from these patients failed to do likewise. Alveolar macrophages from these patients showed an increased propensity to kill tumour cells compared to control macrophages, and this was further augmented by purified IFN gamma. Murray (1985) showed increased generation of H_2O_2 *in vitro* by alveolar macrophages from patients with acquired-immunodeficiency syndrome (AIDS) following stimulation with IFN gamma. These macrophages also showed increased inhibition of replication of *Toxoplasma gondii* and *Chlamydia psitaci*. This report underlined the normal antimicrobial activities of macrophages from patients with AIDS and their normal responsiveness to T-cell products.

Interferons have been used widely in a variety of clinical conditions often with an ameliorating rather than a curative effect.

1.2.2.1.9 Interaction of AM with lymphocytes:

This arm of lung defence is one of the most important and

forms the basis of delayed immunity. Macrophages are capable of ingesting foreign material (antigen), digesting (processing) it, and delivering (presenting) it to lymphocytes which in turn release a variety of lymphokines which can affect the macrophage. After the initial controversy on the role of alveolar macrophages in antigen presentation and interaction with lymphocytes, Huninghake in a recent editorial (1987), pointed out that this controversy was due to differences in techniques in various studies, and that the AM is capable of all these functions. There are three requirements for a cell to be capable of antigen presentation: i) express Class II (Ia antigens) glycoproteins on its surface, ii) process antigens and iii) synthesize and release interleukin-1 (IL-1) (Unanue, 1984a). Hocking (1981) reported that human alveolar macrophages can express Ia-like antigens and since then various workers have confirmed this (Clerici, 1984; Campbell, 1986). The extent of expression of Class II MHC antigens on cells is a dynamic process, so that expression of Ia antigens on young macrophages in culture and in vivo rapidly decays (Unanue, 1984b). The macrophages have a reciprocating interaction with the lymphocyte in the chain of events in immunoregulation. For example, following the activation of T-helper cells by Ia positive macrophages a lymphokine is produced by T cells which in turn induces expression of Ia antigens on the surface of the macrophage. Using a sophisticated technique of scanning and integrating microdensitometry in conjunction with antihuman HLA-DR antibody, Campbell and colleagues (1986) found increased

expression of antigen on macrophages obtained from patients with sarcoidosis, a condition with a preponderance of helper T-cells in the lungs.

1.2.2.1.10 Alveolar macrophages from smokers and non-smokers:

Cigarette smoking contributes to many diseases especially in the lung. The AM of smokers and non-smokers show some differences and a few of these will be described. Macrophage yields of lavages from smokers are three to five times higher than from non-smokers. There is an increase in the large-sized and multinucleated macrophages (Martin, 1973). Their surface morphology is often plate-like in appearance and has a ridge-like membrane compared to the ruffled surface of the AM from non-smokers (Ando, 1984). They often contain smokers' inclusions and they autofluoresce more commonly. Although macrophages obtained from smokers are usually in a more active state, they show functional impairment in many respects. Harris (1984) reported abnormal phagolysosome fusion in the AM from rats exposed to cigarette smoke although the phagocytic rates between these and control macrophages were similar. In vitro migration of alveolar macrophages from smokers is increased (Richards, 1984). These macrophages also contain more hydrolase enzymes (Martin, 1973). Both neutrophil elastase (serine enzyme) and macrophage elastase (metalloenzyme) are increased in the lavage fluid of cigarette smokers (Janoff, 1983). The macrophage elastase cannot be

inhibited by alpha-1 antitrypsin unlike neutrophil elastase. $Alpha_2$ macroglobulin is thought to be the main inhibitor of macrophage elastase (Du Bois, 1985) but a recent study reported the secretion of a separate tissue inhibitor of metalloproteinase (Albin, 1987). AM from smokers were found to contain a two-fold increase in cathepsin-B (like) activity compared to non-smokers and the lavage content of this enzyme from smokers was ten times that of non-smokers (Chang, 1986). Lavages from smokers contain not only increased numbers of macrophages but also of neutrophils. The presence of excessive neutrophils in the lungs in smokers and the resultant imbalance between neutrophil elastase and $alpha_1$ proteinase inhibitor form the basis of the widely published hypothesis of the pathogenesis of emphysema. This theory appears to be more convincing in alpha-1 anti-trypsin deficiency. In this condition (Pi ZZ phenotype) the enzyme is reduced in the alveolar macrophage.

1.2.2.2 The lymphocyte:

1.2.2.2.1 The lymphatic system of the lung

Interaction of lymphocytes with macrophages forms the backbone of long term immune surveillance. The lymphoid system of the lung consists of the lymphatic system and bronchus associated lymphoid tissue (BALT). The lymphatic system comprises of a complex drainage system and the bronchial lymph nodes. Detailed description of this system is

beyond the scope of this work and only a brief mention will be made (reviewed in detail by Leak, 1977). It is an unidirectional drainage system whose major vessels are the thoracic duct and the right lymphatic duct which empty at the junction of the jugular and subclavian veins. The lymphatic drainage serves to return to the circulation the connective tissue fluids which have escaped from the pulmonary capillaries.

1.2.2.2.2 Bronchus-associated lymphoid tissue (BALT):

Most of the information arises from work on the rabbit and after some initial controversy, it is now accepted that BALT exists in man, at least in children (reviewed by McDermott, 1982). It is absent immediately after birth but appears at the age of one week and development continues throughout infancy and early childhood. BALT resembles lymphoid tissue of the gut (GALT), and occurs along the entire length of the bronchial mucosa. Structurally two main features can be recognised namely the lymphoepithelium and the lymphoid follicles. The lymphoepithelium consists of lymphocytes and flattened non-ciliated epithelial cells with microvilli. Goblet cells are notably absent but macrophages are present and this may be important functionally. Beneath the lymphoepithelium lie the lymphoid follicles, which exhibit discretely organised areas, domed, follicular and parafollicular. BALT cells consist of 50% B cells and 18% T cells, but discrete B or T cell areas have not been

identified. The lymphoid tissue is concentrated at the bronchial bifurcations where antigens are maximally deposited. BALT is poorly developed in germ-free animals and in antigen-free transplanted foetal lungs.

Histological observations suggest that blood-borne lymphocytes enter these structures. Discontinuities in the basal lamina of the lymphoepithelium are occupied by lymphocytes. Cytokinetic studies have shown that labelled follicular cells migrate into the lymphoepithelium. Thus cells may enter from the blood, proliferate locally following antigenic stimulation and migrate either into the bronchial lumen via the lymphoepithelium or to the other sites in the body via the lymphatics.

Functionally BALT is less well understood. However it is known that in the rabbit, lymphoepithelium is capable of absorbing antigens by pinocytosis. Carbon particles instilled into the respiratory tract were not found in the lymphoepithelium or in follicles but accumulated in the alveolar macrophages. Although lymphoepithelium can absorb antigens and can transfer these to the follicular areas which are abundant in lymphocytes, their ability to process antigen is unknown. It is speculated that M cells (which have derived their name because of numerous microfolds) in the lymphoepithelium are functionally similar to macrophages in that respect.

Migration of lymphocytes to and from the lungs is less well studied. Small lymphocytes can reach the lungs via the high epithelial venules found in the lymphoid tissue but their

return to the circulation is only presumed to be through the bronchial lymph nodes to the lymphatics and thence to the blood stream. The mechanisms regulating the preponderance of one subgroup of lymphocytes in a disease state are not clear. There is a tendency for cells taken from one anatomical site (e.g. the intestine) to return to the same area although a significant proportion may accumulate at distant sites (McDermott, 1979). This propensity is in keeping with the predominance of pathology in one organ in some diseases e.g. lung more than gut in sarcoid and overt disease in gut in Crohn's colitis with subclinical alveolitis in the lung (Wallert, 1985). In the steady state, lymphocytes in lavage fluid correlate well with blood lymphocytes but this correlation is not maintained in some disease states. The mechanisms for this are not well understood. In active sarcoidosis there is a disparity between blood and lung lymphocytes (Reynolds, 1987). The observation that alveolar macrophages from patients with this condition release IL-1 (Huninghake, 1984) and that interleukin-1 is chemotactic to helper T-cells) (Huninghake, 1987) provides a possible explanation for this difference between lung and blood lymphocytes.

In brief the lymphocyte plays an essential role in lung defence; can enter the lung from the circulation; can replicate locally with antigenic stimulation and its concentration in the lung is disturbed in disease. But our understanding of the various facets of its regulation both in health and disease is far from complete.

1.2.2.3 Neutrophils:

1.2.2.3.1 Origin and Function

Under normal circumstances the main phagocyte of the lung is the alveolar macrophage but in inflammation neutrophils gain access to the lung and may even outnumber the macrophages. Research into the effector mechanisms of neutrophils and potential injurious effects has been extensive and the literature on the subject is formidable. No attempt is made here to cover even the tip of this iceberg and only a short summary is given.

Neutrophils originate from the bone marrow and at the promyelocyte stage clearly recognizable granules are seen. During the early stage of development these primary granules stain only with azure dyes (azurophilic) and later become neutrophilic and stain with either acidic or basic dyes. At this stage new and more profuse secondary granules appear and outnumber the initial (primary) granules by more than two to one. The primary granules are peroxidase positive and the secondary granules are peroxidase negative. Primary granules contain acid hydrolases, neutral proteases (including collagenase and elastase) cationic proteins, myeloperoxidase, lysozyme and acid mucopolysaccharide. Secondary granules contain lysozyme, lactoferrin, cobalophilin (vitamin B-12 binding protein), collagenase and acid proteins reviewed by Wright (1982). Because lactoferrin and cobalophilin (regarded as specific cytochemical markers for neutrophils) are contained in secondary granules, these are also known as

specific granules.

It is now well established that both phagocytic and various humoral stimuli can cause degranulation of neutrophils, which can be intracellular as well as extracellular. Secondary granules are released extracellularly more readily than primary granules. Granule extrusion is energy dependent, requires extracellular calcium and can be influenced by the guanyl and adenylcyclase systems.

Phagocytic and humoral stimuli also induce an oxidative metabolic burst with consequent production of metabolites; hydrogen peroxide, superoxide anion, hydroxyl radical and singlet oxygen. This oxidative apparatus is located at the plasma membrane and the respiratory burst is not necessary for the process of degranulation. Neutrophils from Chronic Granulomatous Disease patients are not capable of this metabolic burst but are still able to undergo degranulation albeit at a reduced rate. Neutrophil activation leads to production of other metabolites such as arachidonates, prostaglandins, thromboxanes, hydroxyeicosotetranoic acid (HETE) and platelet-activating factor. HETE stimulates granule exocytosis and it has been suggested that it is an endogenous neutrophil ionophore.

Secretory products of neutrophils provide antimicrobial activity; amplify, facilitate and regulate the inflammatory process and influence the functions of other exudate cells. Lysozyme participates in the control of bacterial colonization. Lactoferrin when not fully saturated with iron is bacteriostatic but this activity is lost when fully

saturated. Cobalophilin has also been shown to have antibacterial activity as have products of oxygen metabolites. Chemotactic factors stimulate granule extrusion, promote directed migration and initiation of the oxidative burst. Exocytosis of granules also increases neutrophil adhesiveness to the endothelium. HETE promotes neutrophil adhesion. Neutrophils are capable of loosening intercellular and surface attachments of endothelial cells and fibroblasts in culture. All these events lead to accumulation of neutrophils and other cells at the site of inflammation and through the effects of primary and secondary granules, digestion of collagen and fibronectin can take place.

1.2.2.3.2 Neutrophils and the lung:

In an infection, migration of neutrophils into the lungs can be stimulated by activation of complement by bacterial products or as a result of cytokines released by macrophages and lymphocytes. The alveolar macrophage-derived chemotactic factor for neutrophils has been referred to previously. The arrival of large numbers of neutrophils into the lung during an infective episode serves to provide potent scavengers against microbes and is free of long term effects except in rare conditions. However perpetuation of increased neutrophils within the lung, even when not in such large numbers, appears to be related to the development of chronic lung disorders.

Broncho-alveolar lavage fluid from patients with

cryptogenic fibrosing alveolitis contains increased numbers of neutrophils and eosinophils with or without increased lymphocytes. Frequently patients with increased lymphocytes show improvement with treatment, but in those who fail to improve, neutrophil and eosinophil counts tend to remain elevated. (Turner Warwick, 1987). Neutrophils have been implicated in the pathogenesis of emphysema. Elastase purified from pig neutrophil granules was shown to be capable of producing emphysema in the same species (Sloan, 1981). The relationship of neutrophils in smokers and the subsequent development of emphysema has been referred to earlier. This is most severe in the rare $alpha_1$-antitrypsin deficiency especially the Pi-ZZ phenotype. The mechanism involved here is the lack of $alpha_1$ protease inhibitor which normally serves to inhibit the neutrophil protease. Cigarette smoke not only activates the macrophages to attract the neutrophils into the lungs but also stimulates the release of proteases by neutrophils. In a recent study, Anderson (1987) reported that neutrophils from cigarette smokers released more extracellular and intracellular oxidants when activated with the chemotactic agent f-met-leu-phe, a synthetic tripeptide. There were more receptors for this agent on neutrophils from smokers than from non-smokers. Ayars in 1984 reported direct toxic effects on pneumocytes of metabolites released by neutrophils. In an attempt to try to identify the exact injurious agent, Kuroda (1987) and colleagues found that the hydroxyl radical was more damaging than either hydrogen peroxide or the superoxide radical. Neutrophils as a potential source of lung injury

have been implicated not only in chronic lung diseases but also in acute situations. For example neutrophils from the pulmonary artery in patients with adult respiratory distress syndrome (ARDS) showed higher chemiluminescence and chemotactic index compared to normal controls (Zimmerman, 1983).

Thus the neutrophil is extremely important in lung defence in acute infection, but it has also been associated with chronic lung damage and shows a similar potential in acute lung injury in ARDS.

1.2.2.4 Eosinophils:

1.2.2.4.1 Structure

The eosinophil contains prominent cytoplasmic granules and has an unilobular(especially in tissues) or multilobular nucleus. The granules are characteristically eosinophilic (hence the name) and under the electron microscope they show a dense core with electron-lucent matrix. Eosinophils are rich in arginine, histamine and peroxidase, but lack lysozyme and phagocytin. The granules when disrupted show a major electrophoretic protein band with a molecular weight of 11000 daltons and which behaves like a basic molecule. This protein has been called major basic protein (MBP). It has antigenic and a weak antibacterial activity but a potent antiparasitic action. Eosinophils but not neutrophils can bind irreversibly to parasites such as the schistozomule in the presence of

antibody and calcium ionophore A23187. In experiments on guinea pig trachea MBP caused extensive damage including exfoliation of epithelial cells, and impairment of ciliary beating with loss of the tubular structure of axonemes. There was also bronchial mucosal oedema and separation of collagen fibrils. These features especially excessive shedding and desquamation of epithelium are similar to those in bronchial asthma (reviewed by Ackerman, 1982). Patients with bronchial asthma have high serum and sputum levels of MBP, which , fall after therapy. It is believed that this finding in asthma and other diseases is due to degranulation of eosinophils with consequent release of granule constituents. The eosinophil peroxidase (EPO) differs from the neutrophil myeloperoxidase (MPO) both biochemically and functionally. EPO is less efficient in bacterial killing than MPO. In vitro studies have shown that in the presence of H_2O_2 and an halide, EPO induces mast cell degranulation, and is toxic to the schistosomule of S. mansoni. Another eosinophil constituent are the eosinophil cationic proteins (ECP), thought to originate from the granule matrix. ECP have no bactericidal activity but have both coagulant and fibrinolytic activities. Their blood levels correlate poorly with disease activity. Charcot-Leyden crystal (CLC) proteins are lysophospholipases biochemically and originate from the eosinophil plasma membrane. Their serum levels are raised in patients with eosinophilia. Eosinophil derived neurotoxin (EDN) originates from the granule matrix and is a potent toxin to myelinated neurones. When injected intrathecally, it causes predictable

neurological manifestations (Gordon phenomenon) characterised by stiffness in the limbs and ataxia associated with histological abnormalities in the cerebellum, pons and the spinal cord.

1.2.2.4.2 Functions of eosinophils:

Many in vitro studies have shown that the eosinophil can phagocytose a variety of substances, but in vivo phagocytosis is rarely observed (reviewed by Bass, 1982). Eosinophil phagocytic and bactericidal capacities are significantly less efficient than those of neutrophils. The antiparasitic activities of eosinophils have been referred to earlier. Evidence from in vitro studies point to the effects of eosinophils in detoxification of mediators of anaphylaxis. For example, the eosinophil can deactivate histamine, slow-reacting-substance of anaphylaxis (leukotriene C) and platelet activating factor by histaminase, arylsulfatase-B and phospholipase-D respectively. The potential for the harmful effects of the eosinophil is underlined by the pathogenetic actions of its various constituents as mentioned above.

1.2.2.4.3 Eosinophils and the lung:

Analysis of effector cells using bronchoalveolar lavage and mechanical lung disruption has shown that eosinophils are only rarely found in the normal human lung. Accumulation of the eosinophils in the upper airways can occur following mast

cell degranulation with release of eosinophil chemotactic factor of anaphylaxis or IgE induced release of LTB_4 which is also chemotactic to eosinophils. Mechanisms for accumulation of eosinophils in the lower respiratory tract in pulmonary fibrosis are not clear. In the normal lung, eosinophils constitute less than 1% of bronchoalveolar cells but in patients with interstitial lung diseases up to 20% of lavages have been found to have more than 5% eosinophils (Davis, 1984). Increased proportions of eosinophils amongst bronchoalveolar cells from patients with pulmonary fibrosis has been found to indicate a bad prognosis (Haslam, 1980; Peterson, 1987). These data suggest deleterious effects of eosinophils in these patients but there have been only a few studies investigating this possibility. Haslam (1981) reported increased histamine levels in lung lavage fluid from patients with cryptogenic fibrosing alveolitis. In a more direct approach, Davis and colleagues evaluated the effects of guinea pig and human eosinophils on lung connective tissue. They found that eosinophil granules contained a collagenase which specifically cleaved human collagen types I and III (Davis, 1984). More importantly eosinophils purified from bronchoalveolar cells from patients with lung disease demonstrated spontaneous cytotoxicity to lung cells. It should be mentioned however that chronic eosinophilic pneumonia and histiocytosis-X are two exceptions in which lung eosinophilia does not carry a bad prognosis. In another adverse report on eosinophils, Godard (1982) found that viability of alveolar macrophages from asthmatic subjects

inversely correlated with the percentage of eosinophils. Furthermore phagocytosis by AM was significantly less in asthmatics.

The eosinophil then appears to be less useful in the lung than the neutrophil in that its phagocytic and bactericidal capacities are less efficient. Apart from the ability of the eosinophil to deactivate mediators of allergy, there is ample evidence both from its biological effects and from circumstantial clinical observation to indicate that the eosinophil is potentially more harmful than useful in the lower respiratory tract.

The role of the alveolar macrophage in acute lung injury forms the theme of this thesis and before describing the investigations carried out I shall review the clinical conditions studied - community acquired pneumonia (CAP), smoke inhalation injury and radiation injury following radiotherapy.

1.3 Community-Acquired Pneumonia (CAP)

1.3.1 Historical Background:

"Peripneumony", "Peripneumonia" and finally pneumonia are the terms of this common inflammation of the "substance" of the lung. As far back as the 1800's an extensive description of this disease was written by Laennec in his book - "A Treatise on the Diseases of the Chest and on Mediate Auscultation" translated by Forbes in 1827. The description

of macroscopic pathology and the various stages of lobar pneumonia given in this treatise have largely remained unchanged.

1.3.2 Incidence:

It is extremely difficult to obtain reliable data on the incidence of pneumonia in the community, since patients are often treated at home and the disease is not notifiable unless it is fatal. Furthermore in death certificates pneumonia is often a convenient label especially in the elderly. In this population it is regarded as the most common infectious cause of death and the fourth most common causes of death (reviewed by Varghese and Berk, 1983). The incidence of pneumonia increases during influenza epidemics. A survey conducted in West Germany in 1983, revealed an incidence of 1970/100,000 respiratory disease, 50% of which was infective, with pneumonia specifically 9/100,000. In 1984 bronchitis and pneumonia was the primary cause of death in 16,308 cases (population 61 million) (Ringlemann, 1986). In the majority of cases pneumonia is fortunately a single lifetime episode but recurrent episodes occur in patients with underlying pulmonary and extrapulmonary disease.

1.3.3 Classification of Pneumonia:

Pneumonia is commonly classified anatomically, aetiologically or whether it is acquired at home (community-

acquired) or in hospital (nosocomial). Anatomically it can be lobar when it is confined to one lobe, or bronchopneumonia when incomplete and usually non-adjacent areas of more than one lobe are involved. Aetiological classification (pneumococcal, staphylococcal etc.), though more logical is limited by the frequent inability to identify the causative agent.

1.3.4 Aetiology:

Pneumonia in the community can be caused by viruses, gram-positive or gram-negative bacteria, fungi or protozoa. Some of the more common types of CAP will be briefly outlined below.

1.3.4.1 Pneumococcal Pneumonia:

The causative agent is Streptococcus pneumoniae, a gram-positive diplococcus which has 83 serological types. This organism accounts for 50-80% of hospitalised cases of pneumonia, with serotype 3 being the most virulent. It is most common between the ages of 30-50 with males more commonly affected than females. It affects previously healthy individuals, but those with cirrhosis of the liver, diabetes mellitus, renal failure, leukemia, multiple myeloma or sickle cell disease have an increased risk. Previous viral infection appears to predispose to it, and it tends to be fulminating in alcoholics and the elderly (Fraser & Peter Pare, 1978).

1.3.4.2 Staphylococcal pneumonia:

During influenza epidemics, Staphyloccal aureus is one of the principal secondary bacterial agents. Sporadic cases commonly occur among drug abusers and occasionally as a complication of post-operative staphylococcal septicemia.

1.3.4.3 H. Influenzae pneumonia:

H. influenzae and S. pneumoniae are the most common organisms isolated from patients with chronic bronchitis. H. influenzae exists in capsulated or non-capsulated forms and the pathogenicity of the latter in the respiratory tract is not clear and has been largely regarded as a colonizing microorganism. Serotype b of the capsulated form accounts for the vast majority of pneumonia due to this species. In one study H. influenzae accounted for 12% of all cases of CAP (Carbon, 1986). Special conditions and growth factors in the media are needed for the isolation of H. influenzae.

1.3.4.4 Mycoplasma pneumonia:

Mycoplasma pneumoniae is the second commonest cause of CAP. It is responsible for up to 20% of cases of CAP in the population at large and up to 50% in closed communities such as military bases, although radiological changes develop in 3-10% of infected cases (reviewed by Moskal, 1987).

1.3.4.5 Klebsiella pneumonia:

Types 1, 3, 4 and 5 of this non-motile, encapsulated gram-negative rod, account for < 1% of all pneumonias. Victims are often vagrants and alcoholics. It commonly affects the upper lobes or apical segments of lower lobes and often cavitates.

1.3.4.6 Legionellosis:

The causative agent Legionella pneumophila has been identified as a gram-negative bacterium belonging to several serological types (I to IV). This disease has tended to occur in outbreaks in institutions such as hospitals, but sporadic cases also occur especially among those who have recently returned from abroad. The agent relishes in warm and humid conditions and has been isolated in air-conditioning systems during outbreaks. Mortality can be as high as 20%. Pontiac fever is a milder form of legionellosis which manifests with constitutional symptoms without associated pneumonia or mortality. Other members of this species (Legionella-like organisms (LLO)) such as L. micdadei (Pittsburg pneumonia agent - PPA) and its genetically related organisms Tatlock and Heba have been implicated in nosocomial pneumonia (Muder, 1983).

1.3.5 Lung defences in pneumonia:

Lung defence mechanisms are normally capable of protecting the host from serious lower respiratory infections but when these are compromised such as in tracheostomy, development of pneumonia is likely. In 1956 Leper reported the development of pneumonia in 100% of patients with anterior poliomyelitis, who had undergone tracheostomy and received intermittent positive pressure ventilation.

The spread of bacterial agents such as the pneumococcus into the lungs sufficient to cause significant infection depends on the interplay between the initial dose of organisms, excessive mucus in the air passages, the presence of interlobar septa which provide a mechanical barrier against spread, virulence of the organisms and the response of the host to infection, including hypersensitivity and other abnormal immunological responses (reviewed by Spencer, 1985).

Onoforio and colleagues (1983) investigated infecting doses and demonstrated that precise inocula of S. aureus may be delivered to the lung without causing lung injury and a progressive increase in the size of the inoculum was inversely related to clearance capability by the host defences.

Epidemiological data suggest that cellular defences in the host are compromised in viral infections which accounts for the high mortality during viral epidemics. Jakab (1974) has shown that in mice previously exposed to aerosolized Sendai virus, clearance of S. aureus from non-consolidated

lungs was impaired. Further work revealed that this might be due to a phagolysosome fusion defect in the alveolar macrophage exposed to the virus infection (Jakab, 1980). Later antilymphocyte serum was found to be capable of reducing the virus-induced AM phagocytic defect (Jakab, 1982). More recently virus-induced immune complexes were thought to be at least partly responsible for this suppressed AM activity (Astry, 1984). Using a purely *in vitro* approach, Nugent (1979) failed to demonstrate impairment of ingestion of *S. aureus* by alveolar macrophage monolayers previously exposed to a virus infection.

The AM rids the lower respiratory tract of particulates and microorganisms through the opsonic and non-opsonic dependent mechanisms referred to earlier. Rehm and colleagues have demonstrated that the early phase of pneumococcal killing in decomplemented rats was normal (1982). There is now evidence which suggests that even non-phagocytic cells such as lung lymphocytes exert an IgA-dependent natural antibacterial activity in the lung (Sestini, 1988). However a large enough dose of a pathogen in the lung eventually attracts polymorphonuclear leucocytes into the lung, either by the secretion of the AM-derived neutrophil chemotactic factor or activation of complement products by bacterial factors (Heidbrink, 1982). Vial *et al* (1984) found that following pulmonary inoculation of *S. pneumoniae* there is rapid production of chemotaxins with consequent recruitment of neutrophils within four hours. Furthermore, this study has demonstrated that the numbers of neutrophils

were proportional to the size of the dose of pneumococci instilled. The requirements for the clearance of microorganisms from the lower respiratory tract are not the same for all pathogens. Heidbrink (1982) has shown that complement acted chiefly as a chemotaxin in clearance of pneumococci but as an opsonin in clearance of pseudomonas.

The increased incidence of pneumonia in some groups may be related to relatively compromised host factors. In alcoholics not only the mechanical barriers may be compromised because of altered level of consciousness and increased risk of aspiration, but nutritional deficiency often prevalent in these individuals may impair the cellular defences. Animal data suggest that alveolar macrophage phagocytic activity can be adversely affected by nutritional deficiencies (Moriguchi, 1983; 1984; Shennib, 1984). Children with protein-calorie malnutrition have been shown to have reduced immune reactivity (Edelman, 1973). CAP is responsible for high morbidity and mortality in the elderly population and there is ample evidence that aging affects both cellular and humoral immunity. In experimental pneumonia, Eposito and Pennington (1983) showed that the host response to pulmonary bacterial challenge was different in scenescent and young mice (1983). Howells (1975) compared the antibody response to influenza vaccine in elderly nursing home patients with a group of young adult controls and found lower antibody levels in the elderly. T-cell function is also impaired in the elderly (Roberts-Thompson, 1974; Canguly, 1987).

The AM and other lung defences in the normal state

are capable of protecting the host from development of pneumonia but when these are compromised, recruitment of additional phagocytes mainly neutrophils is needed and the final outcome depends on the interplay between host and pathogen factors.

1.3.6 Pathology of Pneumonia:

The classical pathological changes of pneumonia are seen in pneumococcal pneumonia. Four stages are recognised in pneumococcal pneumonia i) Spreading of inflammatory oedema this is usually a clinically imperceptible stage which results from the tissue reaction to the causative agent and the fluid is usually rich in the organism. This stage is seen only in those patients who die rapidly within a few hours of the illness and is followed rapidly by exudation into the alveolar space of red blood cells and neutrophils associated with more oedema fluid and fibrin. The alveoli are congested leading to compression of the alveolar capillaries. Macroscopically the lungs look red and this is called red hepatization. Increase in fibrin and polymorphonuclear leucocytes with a slight reduction in red blood cells leads to further engorgement of the alveoli and compression of the bronchial capillaries with almost complete shunting of the blood supply to the unaffected areas of the lung. The lung looks bloodless and grey - grey hepatization. The pulmonary arteriole may become thrombosed possibly due to a direct microbial toxic effect on the endothelium. There then follows the stage of resolution which

is heralded by the arrival of macrophages which progressively increase as they replace the neutrophils, which they phagocytose together with their contents. The neutrophils engulf the pneumococci but do not destroy them and the presence of the macrophage reaction is necessary for the resolution of the exudate (Spencer, 1985).

In bronchopneumonia the focus of the inflammatory process is around the respiratory bronchioles and the surrounding alveoli, unlike lobar pneumonia where it is in the alveolar spaces. Staphylococcal and Klebsiella pneumonia are prone to cavitation whereas this rarely occurs in pneumococcal pneumonia.

1.3.7 Bronchoalveolar Lavage in CAP:

Bronchoalveolar lavage has been used widely as a research tool in experimental pneumonia and is employed increasingly as a diagnostic tool in immunocompromised patients. However there is a notable deficiency of human data in the literature from bronchoalveolar lavage in CAP, especially in terms of the cellular aspects. Lafitte (1983), performed serial lavages on patients with pneumonia and found that during the initial period there was polymorphonuclear leucocytosis followed about ten days later by increased lymphocytes of up to 70%. The lymphocytosis was delayed in alcoholics. This study also found positive pneumococcal antigen in the concentrated lavage fluid both in bacteraemic and non-bacteraemic patients. Alveolar macrophage function was not

investigated in this study.

1.4 Smoke Inhalation:

1.4.1 Historical Background:

The lethal effects of inhalation injury were recognised as early as the first Century A.D. when it was reported by Pliny that the Romans executed their prisoners by placing them over smoke of green wood fires (Dressler, 1976). In 1840 Long published postmortem findings of burns and described hepatization of the lungs and associated pleural effusion (Long, 1840). In the more recent past the contribution to mortality from smoke inhalation was highlighted by the Coconut Grove Night Club fire. Aub (1943), in his clinical description of the victims of that disaster, described how some survived by covering their mouths with wet cloths underlining the importance of smoke inhalation in those who died. Phillips (1962) pinpointed respiratory damage as the principal killer in the Massachussetts experience of fatal burns over a period of eighteen years. The effects of experimentally induced thermal injuries to the lung were documented by Moritz and colleagues (1945).

1.4.2 Incidence:

The precise incidence of inhalation injury and its contribution to mortality is difficult to measure because of

the difficulty in separating the contribution from inhalation and cutaneous thermal injuries towards lung complications and mortality amongst fire victims. In Massachussetts General Hospital between 1939 to 1958, there were 106 deaths among 1140 cases of burns and 42% died of respiratory complications with or without respiratory sepsis (Phillips, 1962). Divincenti (1971), reported the incidence of 2.9% in inhalation injury among a total of 2,297 patients treated for burns but those with respiratory tract injury sustained a mortality of 76.3% compared to the overall mortality of 9.5%. In 1985 there were 978 deaths from fires in the United Kingdom, 155 in Scotland and 81 in the Strathclyde Region. While the overall mortality from fires has remained largely unchanged between 1979 to 1985, it is striking that among those who die from smoke or gas, mortaility has slowly increased (U.K. Fire Statistics, 1978-1985). Since the majority of fires occur in habitated dwellings, there is no sexual proponderance but the effect of age is important. In 1985, children below the age of five years had twice the risk of dying from fire compared to those aged between 15-59 years. In the same year 48% who died were aged over 60 years and 29% were aged above 75 years. In the latter group the risk of dying from fire was seven fold that of those aged between 15-59 years.

1.4.3 Physiopathology:

Death from inhalation injury can be early, delayed or

late. This chronological sequence is roughly in keeping with different operative mechanisms. Early death from a few minutes to several hours can be attributed to various noxious products of combustion. Carbon monoxide poisoning with resultant hypoxemia plays a major role during the early phase. Carbon monoxide (CO) forms a stable compound carboxyhaemoglobin (COHb) due to its high affinity for haemoglobin. Because it does not dissociate easily, COHb levels can rapidly build up and critically impair oxygen delivery to the tissues. Blood gas analysis can be misleading as typically PaO_2 is normal and it may be assumed that SaO_2 is also normal, but the measured SaO_2 is low. A fatal hypoxemic episode with levels of COHb greater than 60% is frequent (Cahalane, 1984). The carotid body is less sensitive to oxygen concentration and therefore there is often no compensatory tachypnea. Pyrolysis reduces the oxygen content of air leading not only to reduced inhaled oxygen but also reducing the dissociation of COHb which depends on oxygen concentration. Hyperbaric oxygen rapidly dissociates CO from haemoglobin.

In this acute phase, another problem is cyanide poisoning. Partial combustion of many household items such as furniture, nylon, asphalt, wool, silk and polyurethane-containing materials, produce cyanide. This cell poison inhibits cellular oxidation. Pyrolysis products of these synthetic materials are also toxic to the central nervous system and coupled with hypoxemia and the frequently associated alcoholism often leads to collapse in the enclosed environment increasing the incidence of a fatal outcome.

Underlining this sequence of events in early deaths for example is the finding that among the U.K. fatalities in 1985, 63% of fatal casualties from fires in dwellings were found in the room of origin of the fire compared to 20% found elsewhere on the same floor.

Those patients who survive the "biochemical effects" of fire in the form of hypoxemia, carbon monoxide poisoning and cyanide poisoning still face the threat of structural damage to the respiratory tract. Structural damage can be due to thermal or chemical injury. Direct thermal injury of the lower respiratory tract is rare unless one inhales steam. This is because of excellent cooling capacity of the upper respiratory tract (URT) and very low specific heat (heat carrying capacity) of dry air which is 1/4000 that of steam. Moritz (1945) demonstrated that pumping hot dry air at 500^{o}C transorally, its temperature dropped to around 270^{o}C when it reached the larynx and 50^{o}C when it reached the trachea. This explains why the upper respiratory tract bears the brunt of thermal injury. The larynx undergoes reflex closure when it is exposed to high heat or chemical irritants.

Presence of facio-oral burns or singeing of nasal hairs may indicate upper respiratory thermal damage. Hoarseness of voice needs to be taken seriously and may indicate the presence of laryngeal edema. In a recent study the severity of oedema of laryngeal structures characterised by nasopharyngoscopy correlated with increased surface burns, burns of face and neck and with rapidity of fluid therapy (Haponik, 1987). Damage to the URT beyond the larynx is also

caused by hot smoke debris which has a higher specific heat than dry air. Tracheitis and denudation of the respiratory mucosa with loss of cilia occurs within the first 24 hours. This is accompanied by excessive mucus production and increases the hazards of infection in the lower respiratory tract. Clark (1985) found that severe changes (Grade 3) of endobronchial architecture diagnosed bronchoscopically were associated with 100% mortality.

Chemical injury to the lower respiratory tract (LRT) or the URT may result from inhalation of toxic gases, depending on the solubility of these gases. The more soluble gases dissolve in the URT while the less soluble ones dissolve in the LRT (Carpo, 1981).

Damage to the LRT accounts for the fatalities during the delayed phase of 1-5 days post injury and the late phase at about fourteen days. In severe cases, within 24 hours exudation of fluid into lungs with radiological changes may take place and this occasionally may progress within the next few days to the full picture of the Adult Respiratory Distress Syndrome (ARDS). Patients with pure smoke inhalation injury usually do not develop severe pulmonary complications but those with both burns and inhalation injuries often succumb to these. These are brought about by a number of factors including shallow breathing from pain due to burns or skeletal injuries, narcotics, treatment factors such as fluid overload, hyperoxia, sepsis from ventilation, wounds or aspiration. Other extrapulmonary factors include coagulation problems and cardiac causes.

Late deaths are usually a result of sepsis of the LRT and/or pulmonary embolism (Aucher, 1973). For those who survive, various long-term sequelae have been reported. These include bronchiectasis, tracheal stenosis (Donnellen, 1965), endobronchial polyposis (Adams, 1979). Chronic airways obstruction has been reported following inhalation of overheated cooking oil fumes (Simpson, 1985) and amongst fire fighters (Loke, 1980).

1.4.4 Cellular and humoral changes in fire victims:

From the above discussion it is clear that pulmonary complications among fire victims are a result of an interplay of complex patho-physiological processes. Most of the data that are currently available have addressed the changes that occur in burns. Among the most important of these, is complement activation, and consumption. Dhenin (1978) found a transient fall in complement due to leakage at the sites of burn wounds followed by increased complement levels. Heidenman (1979) reported aggregation of platelets with entrapment in the lungs associated with initial leucopenia followed by leucocytosis and activation of the complement system in dogs exposed to thermal injury. Neutropenia which follows intravascular complement activation was thought to be due to increased neutrophil adherence with consequent margination (O'Flaherty, 1977). The neutrophils become entrapped within the pulmonary vasculature (Ward, 1981). Furthermore neutrophils have been found to be activated

following thermal injury although interestingly they show reduced chemotactic response towards C5a (Moore, 1986), but only a transient depression of this response towards f-met-leu-phe. This indicates incomplete paralysis of neutrophil migration apparatus. In a number of studies impaired chemotaxis of neutrophils associated with increased lysosomal enzyme release has been found in patients with ARDS (Fowler, 1984). In a study of potential ARDS patients an early reduced neutrophil migratory response towards C5a but not f-met-leu-phe was seen in all those patients who developed ARDS (Solomkin, 1985).

Reduced neutrophil accumulation at sites of burns wounds is explained by this impaired chemotaxis. The accumulation of neutrophils in the lungs can be explained by increased aggregation of neutrophils, but it is conceivable that other factors which might attract these neutrophils (largely unresponsive to C5a) may also take part. It is interesting that Fowler and colleagues (1984) failed to reproduce these changes in donor neutrophils exposed to serum from patients with ARDS, thus excluding a serum factor for depressed chemotaxis. Bronchoalveolar lavage fluid from patients with ARDS has been shown to have chemotactic activity to neutrophils, and preliminary data indicate that the chemotactic activity could not be attributed to C5a, C3, fibrinogen or C5a desarg (Parsons, 1985). Whichever mechanisms operate in the accumulation of neutrophils in the lungs in ARDS, their injurious effects are thought to be responsible for the ensuing lung damage. Till and colleagues

(1982) reported damage to endothelial cell lining, destruction of endothelial cells and plugging of pulmonary capillaries by neutrophils following intravascular activation of complement. The injurious effects of neutrophils on pneumocytes has been referred to earlier (Ayars, 1984). In the study by Solomkin (1985) neutrophils of patients with ARDS were found to have a reduced cellular content of lysozyme and beta-glucuronidase indicating previous degranulation. Cochrane (1983) reported oxidative and proteolytic cleavage of alpha-1-proteinase inhibitor by BAL fluid from patients with ARDS. Elastolytic activity of BAL fluid from similar patients was shown by other workers (Lee, 1981). There is thus ample evidence implicating the neutrophil in the acute lung injury of ARDS.

ARDS has been overshadowed by the predominance of neutrophil-derived injury. This is clearly important but the macrophage which may be equally important has received little attention. In thermal injury and smoke inhalation for example the evidence in the literature appears largely to underline depression of the alveolar macrophage function. Dressler (1974) reported increased bacterial phagocytosis and killing which became depressed when the animals became septic. Fick and colleagues (1984) reported impairment of phagocytic activity of alveolar macrophages from rabbits exposed to woodsmoke. In his study the lavage was performed shortly after the exposure and there were no polymorphonuclear leucocytes (PMN) in the lavage fluid. Interestingly the viability of macrophages showed positive correlation with longer exposure and CoHb levels. In another recent report by

Loose and colleagues (1984) alveolar macrophages from burned rats showed depressed phagocytic activity and chemotaxis but increased microbicidal activity. Demarest et al (1979) reported impairment of AM chemotaxis in a group of patients with smoke inhalation. It is notable that the bronchoalveolar cell population in this study consisted of a high proportion of neutrophils which could have interfered with migration of macrophages in the membrane assay deployed. However in a study involving a larger group of fire victims, in which serial lavages were performed in the course of 24 hours after admission demonstrated that there was a depression of respiratory burst of the AM obtained early after injury. Macrophages obtained later from a different site of the lung demonstrated increased initiation of the respiratory burst and phagocytic activity compared to those from control subjects. (Gemmell 1987). The recent finding of synergistic effects of leucocytic proteases and macrophages in oxygen radical responses (reviewed by Ward, 1986) underlines the importance of examining different aspects of AM function in this complex issue of lung injury in smoke inhalation with or without associated burns injury.

Another aspect of lung injury which has been investigated following smoke inhalation is surfactant activity. Neimann (1980) using a surface area curve as an indicator of the surfactant activity in dogs exposed to kerosine-ignited wood smoke found that there is immediate reduction in surfactant activity with consequent dense non-segmental atelactasis. However direct estimation of

surfactant in patients with inhalation injury was found to be normal (Head, 1980).

1.4.5 Diagnosis of Smoke Inhalation:

Diagnosis of smoke inhalation depends largely on clinical history and physical examination and arterial CoHb estimation. Until recently accurate quantification of severity of smoke inhalation was difficult but the work of Clark (1985) has demonstrated that the use of a clinical scoring system coupled with CoHb measurement allows fairly accurate estimation of the severity of inhalation injury. The following criteria each given a score of one were used. A score of more than two indicated significant smoke inhalation: i) A history of being trapped in a house or industrial fire in an enclosed space. ii) Production of carbonaceous sputum. iii) Perioral facial burns - affecting the nose, lips, mouth or throat. iv) Altered level of consciousness at any time after the incident including confusion. v) Symptoms of respiratory distress. vi) Signs of respiratory distress including stertorous or laboured breathing or auscultatory abnormalities. vii) Hoarseness or loss of voice.

In this study a clinical score of seven was associated with 100% mortality. Measurement of COHb and use of a normogram enabled one to extrapolate COHb back to the time of injury.

The chest X-rays are not helpful in the diagnosis of smoke inhalation injury as they are commonly normal. However

they are helpful in the follow-up of patients. Nasopharyngoscopy and bronchoscopy have been used to characterise URT and LRT damage. These may give an early indication for intubation although initial normal airways do not preclude later need for this procedure. Early intubation is advised for high risk patients as intubation later may be difficult (Achauer, 1973). Detection of high risk patients can be made with the use of Clark's mortality probability equation (Clark, 1986). Ventilation perfusion scanning using xenon has been reported to detect early small airways abnormality (Cahalane, 1984). However this is not routinely used in clinical practice for management of smoke inhalation injuries. Spirometry and analysis of flow volume loops may yield the same results.

1.5 Radiotherapy

1.5.1 Historical background

Radiotherapy for malignant conditions started during the first and second decades of this century and in 1921 the first paper was presented on the effects of radiation on the lung. The effects of radiation on skin and lung were compared and since then several reports have appeared describing the pathological changes, respiratory function impairment, effect of dosage and rate of delivery and the use of steroids.

1.5.2 Radiation Injury:

1.5.2.1 Incidence

Radiation injury is probably much commoner than the reported incidence of radiation pneumonitis, since it is likely that injury can occur without development of radiological changes. However radiation pneumonitis based on radiological changes is not infrequent but symptomatic pneumonitis is much less common. Using megavoltage for breast cancer, reports of radiation pneumonitis have varied between 24.5% to 70%, with a mean occurrence of about 41% (reviewed by Gross 1977a). In one series radiological changes following radiotherapy for lung cancer were observed in 13% of patients at three months, 33% at six months, and 66% at twelve months and 100% at thirty months. In this series 4.6% developed symptoms. In another series only 6% radiation pneumonitis was reported while in yet another, 15% of patients were reported to have developed radiation pneumonitis of whom a third had a fatal outcome. Radiation pneumonitis can also develop following mediastinal radiation with rates of between 6.4% to 65% in different series. Fatalities attributed to pneumonitis have been reported between 0.25% to 5.8% in these series. It should be mentioned that the above rates pertain to the higher dosage schedules employed in the past and efforts still continue to define dosage and fractionation of radiation with improvement in shielding techniques in order to

reduce the incidence of radiation pneumonitis. In a recent prospective study acute radiation damage as detected by computerised tomography scans occurred in thirty six of fifty four patients (Mah, 1988).

1.5.2.2 Biologic aspects of radiation damage:

Absorption of X-rays by tissues leads to ionisation of chemicals with production of free radicals. In the presence of oxygen molecules these radicals produce organic peroxides which aggravate the damage, (Gross 1981). Tissue damage can be either on genetic (DNA) or on non-genetic material (proteins and carbohydrates). Mitosis is the crucial stage at which genetic material is affected by radiation, where chromosomal aberration may result in anaphase arrest. Genetic material damage is usually apparent much later than non-genetic material damage, and the speed with which this appears is a function of rate of mitosis. Damage to non-genetic material is usually more widespread and acute. Functional impairment can occur fairly acutely due to increased permeability of membranes with the consequent exudation of tissue fluids, followed later by less acute manifestations and repair of membranes. Data from cytokinetic studies in small mammals indicate that radiation-induced chromosomal damage is most marked on bronchial epithelial cells, capillary endothelial cells and type II pneumocytes. Type I pneumocytes are not affected genetically because they do not enter into mitosis but suffer non-genetic damage. The capillary endothelium has

been viewed as the common target determining radiation tolerance in many organs. In the lung type II pneumocytes have also been considered important in this regard because of their secretory function. Chronologically radiation injury is usually considered under three somewhat overlapping phases which can be dissociated; the early phase occurring up to two months following radiation, the intermediate phase from two to nine months and the late phase after nine months.

1.5.2.3 Pathological Changes:

Typically early pathological changes are characterised by exudation of fluid into the alveoli and interstitial oedema. This may resolve leaving no radiological abnormality or may progress to a chronic phase. Warren and Spencer in 1940 described hyaline membrane formation as a reliable diagnostic finding of radiation pneumonitis (reviewed by Rubin and Casarett, 1968). Jennings and Arden in 1962 attempted to correlate pathological changes with the radiation dose and time interval and found fibrin (hyaline) membranes in 41% of all irradiated lungs usually and most prominently at 6 months to 2 years after irradiation with a dosage greater than 2000R. Other features include vascular lesions with engorged and thrombosed capillaries and arterioles, oedema, intimal proliferation and medial changes together with subintimal accumulation of lipid-laden macrophages. Atypia, hyperplasia and epithilial desquamation are also common findings. Inflammatory cells are notably absent. Necrosis of bronchial

mucosa and bronchiectasis also occur. Pulmonary function tests in patients with radiation pneumonitis show a restrictive defect with a drop in the transfer factor and lung volumes. Deterioration in lung function in patients who have undergone bone marrow transplantation is multifactorial, but radiation injury is one of the contributing factors. In a recent study (Sutedja, 1988) a large total body irradiation dose was found to be one of the major factors associated with early deterioration of lung function.

1.5.2.4 Factors that may influence development of radiation pneumonitis:

The incidence and severity of radiation injury depends on the volume of the lung irradiated, the total dose of radiation, the rate and quality of radiation and other modifying factors. The larger the lung volume irradiated the greater is the likeliehood of producing significant radiation injury even when using the same dosage delivered in the same manner.

Rubin and Casarret (1968) regard lung volume as the most important single factor determining radiation tolerance. Fractionation of dose allows repair of sublethal damage between fractions and therefore the rate of delivery of radiation is more important than the total dose. Errors in technique and dosimetry can influence the radiation absorbed and the incidence of radiation damage. Mah and colleagues (1988) found that a 5% increase in lung dosage higher than the

uncorrected prescribed dosage produced a 12% increase in acute radiation injury and the average lung dose can be up to 17% higher depending on lung geometry, technique and densitometric considerations. Other factors that may influence the development of radiation injury include repeat radiotherapy, concomitant or previous chemotherapeutic agents and use of steroids. Radiation injury can occur following direct lung radiation, or radiation to contralateral lung only, mediastinal irradiation, total body irradiation or irradiation of the upper half of the body. In one study Prato and colleagues (1977) found that mediastinal irradiation was most important in development of radiation pneumonitis.

1.5.2.5 Bronchoalveolar lavage in radiation injury:

Studies on this well recognised complication of radiotherapy have been largely histopathological and broncho-alveolar lavage as an approach has been rarely used. Among the few studies is that by Cordier et al. (1984) who have reported cellular data from six patients with early radiation pneumonitis. Lavage from these patients showed increased numbers of lymphocytes and in one patient these lymphocytes were activated. The lavage fluid also showed collagenolytic activity for type I human collagen. There were also increased serum proteins including high molecular weight species. These workers concluded that the radiation injury seen in their patients was a lymphocytic alveolitis possibly perpetuated by activated lymphocytes and collagenolytic

activity in alveolar structures contributed to possible fibrosis. In another study, Tsao and Ward (1985) reported increased numbers of macrophages in rats lavaged between two and six months following irradiation to the hemithorax. Fifty to sixty per cent of these macrophages were larger than control macrophages and were foamy in appearance and contained many lipid vacuoles. These macrophages also showed reduced plasminogen activator activity with some degree of recovery at six months. Alveolar macrophages have been shown to be affected by radiation. Sabloniere and colleagues (1983) reported an initial reduction in phagocytic activity of rat alveolar macrophages at 14 days after whole-body irradiation, followed by an increase to 75% above control macrophages at 21 days and returning to normal again at 35 days. The number of macrophages was also increased in the irradiated mice. Ultrastructural examination of the alveolar macrophage cytoskeleton following in vitro irradiation showed that at a dose of 120 Gy reproducible changes in the cytoskeleton take place but this change undergoes repair within hours (Ladyman, 1984). Patients undergoing allogeneic bone marrow transplantation are normally conditioned with total body irradiation and often succumb to interstitial pneumonitis. Alveolar macrophage function in a group of bone marrow transplant patients was reported as defective. (Winston, 1982).

Thus radiation injury to the lung is a common complication of radiotherapy and can be fatal. Its diagnosis is crude as it depends on X-ray changes and probably

underestimates the true incidence. Studies in its pathogenesis have been largely histopathological with few data from broncho-alveolar lavage. It is likely that in future with more sophisticated radiation techniques the incidence will be reduced.

1.6 Bronchoalveolar Lavage (BAL)

1.6.1 Historical Background:

Following the optimal development of the rigid bronchoscope at the beginning of this century, bronchial washing was used as a therapeutic procedure (Reynolds, 1987). Later various catheters were designed to be used through the rigid bronchoscope. In 1961 the technique of obtaining pure macrophage populations from a rabbit by bronchoalveolar lavage was described (Myrvick, 1961). The concept of flexible fibre optic bronchoscopy was developed by Ikeda in Japan in the early 60's but it was not until 1967 that the first fibre-optic bronchoscope was produced by the Machida and Olympus companies. In the West this equipment became available a few years later and its widespread use started in the early 70's.

1.6.2 Safety and Complications of BAL:

Compared to many invasive techniques BAL is very safe with an incidence of less than 5% minor complications with no major ones (Strumpf, 1981). Transient pyrexia can occur in a

small proportion (2.5%) of patients (Strumpf, 1981) but in a study where four separate lobes were lavaged pyrexia occurred in 50% of cases (Pingleton, 1983). This was unusually high and could not be explained. Transient pyrexia is thought to be due to pyrogen release and not due to infection (Reynolds, 1987). Bronchospasm especially in asthmatics may occur but nebulized bronchodilators are usually adequate to deal with this complication. Special guidelines for bronchoscopy and BAL have been laid for asthmatics (Bernstein, 1985). Bleeding can occur but this is usually bronchoscopy related and not lavage related. Finally it is not unusual to have non-infective radiological infiltrates a few hours after the procedure but these disappear spontaneously.

1.6.3 Distribution and yield of the lavage fluid:

Kelly et al (1987) using digital subtraction radiography demonstrated that following instillation of the first aliquot of fluid, it remained near the tip of the bronchoscope and an even distribution is obtained with further aliquots. The retrieval from the first aliquot is usually small but this increases subsequently. In general 50-60% of the total instillate is recovered from the normal lung, but this is affected by diseases such as emphysema where the yield can drop to as low as 10-40% (Reynolds, 1987). The right middle lobe and the lingula have been found to give greater fluid yields than the lower lobes, though the content of lavage

fluid from these different sites was similar (Pingleton, 1983). The temperature of the instillate was not found to affect the yield in this study but in a recent state of art review by Reynolds (1987) it is stated that warming the fluid increases the recovery slightly. Local anaesthetic was not found to affect either the volume or the content of the fluid recovered (Davidson, 1986).

1.6.4 Content of the BAL Fluid:

The lavage fluid consists of cellular and acellular or soluble components. These may be affected by procedure variables, smoking and disease states.

Alveolar macrophages constitute by far the highest proportion of cells in BAL fluid from normal individuals with a range of 85-93% (Huninghake, 1979; Reynolds, 1987); lymphocytes 7-12% and polymorphonuclear leucocytes (PMN) 1-2%. Eosinophils and basophils constitute less than 1%. T-cells make up to 80% of all the lymphocytes with a ratio of T_4:T_8 of 1.5 - 1.6:1 similar to that in the peripheral blood. A good BAL fluid lavage should contain less than 5% epithelial cells. A high epithilial cell count indicates bronchial rather than alveolar origin (Robinson, 1988). Red blood cells normally constitute less than 5%, but bronchoscopic or suction trauma can increase the red cell population.

BAL fluid contains proteins, carbohydrates, lipids, electrolytes and enzymes. The contents of soluble components in the BAL fluid is much more variable than the cellular

contents. These components are much more sensitive to the effect of dilution and variability of the volumes of the instillate. In an attempt to standardise these constituents, a ratio of the particular constituent to the albumin content is used. Another source of variability of the soluble components in BAL fluid is the small quantities of these constituents. This means they are more likely to be affected by methodological considerations. The concentration of many of the soluble contents in the BAL fluid is influenced by diffusion from the circulation. Small proteins of 150,000 daltons are fairly diffusible but those of larger molecular weight are progressively less diffusible so that alpha-2 macroglobulins and betalipoproteins are not found in the normal lung (Huninghake, 1979). A few examples of these constituents and their levels in smokers and non-smokers are given below.

1.6.5 Effects of aliquots:

The first aliquot retrieved differs from subsequent aliquots both in volume and content. Cell yields in sequential aliquots do not follow dilution models and often the second aliquot especially in smokers show maximal cell numbers which drop only a little in subsequent aliquots (Davis, 1982). Dohn (1982) found increased numbers of PMN and lymphocytes in the first aliquots of control patients but an increasing proportion of these cells in the later two aliquots from patients with interstitial lung diseases (ILD). However

Robinson et al (1988) did not confirm these findings. In their study, they found reducing proportions of PMN in subsequent aliquots from ILD patients. Similar findings of high content of PMN in the initial aliquot was reported by Martin (1985) from patients with or without airflow obstruction.

Merrill and colleagues (1982) assessed the effect of sequential aliquots on proteins and found that there was a progressive reduction of total proteins, albumin, immunoglobulins and free secretory component in subsequent aliquots. However the ratios of different proteins remained constant in all the aliquots. The effect of volume of instillate in this study revealed that when a smaller aliquot was used the content of the fluid was more attributable to airways origin rather than alveolar origin. The first aliquot then contains larger numbers of PMN and a higher concentration of proteins.

1.6.6 The Effect of Smoking:

Cigarette smoking increases the proportion of neutrophils but reduces the lymphocytes. It also affects the soluble components. Some examples of the changes in content of the BAL fluid in smokers are given overleaf, (table 1.1).

1.6.7 BAL-Versus Histology:

Lung biopsy tissue provides the most direct assessment of

Table 1.1: BAL in smokers and non-smokers

Constituent	Non-Smokers	Smokers	References
Volume of fluid		Reduced	Finley* 1972 Bell 1981 Villager 1981 Huninghake 1983
Cell Yields /ml BAL fluid	2.6 (± 0.6)x 10/ml BAL fluid	6.3(± 1.1)x 10 /ml BAL fluid	Yeager* 1974
Total cells	15.8 (± 2.8)x 10 (Total cells)	78(± 7.0) x 10	Merrill 1981*
% Macrophages	88 92 (± 4)	93.8 96 (± 3)	Merrill 1981* Costabel 1986*
% Neutrophils	1 (± 1) 1.5 (± 0.3) 0.2 (± 0.1)	1 (+) 3 (± 0.5) 4.4 (± 1)	Costabel Merrill 1981 Huninghake 1983*
% Lymphocytes	7.0 (± 3.0) 7.2 (± 1.0) 8.7 (± 1.2)	3 (± 2) 2.9 (± 1.0) 3.1 (± 0.3)	Costabel 1986* Bell 1981* Merrill 1981
T_4:T_8	1.9 (± 0.8)	0.9 (± 0.4)	Costabel 1986*
Immunoglobulins			
IgG /Albumin (g/ g)	0.082 (± 0.01)	0.196 (± 0.032)*	
IgG " "	0.043 (± 0.006)	0.045 (± 0.015)	
IgG	0.003 (± 0.002)	0.084 (± 0.003)	Merrill
IgG " "	0.009 (± 0.006)	0.0162 (± 0.116)	1985
IgGA " "	0.319 (± 0.04)	0.222 (± 0.04)	
IgGE " "	0.017 (± 0.004)	0.08 (± 0.06)	
Fibronectin ug/mg albumin	7.3 (± 2.9)	11.3 (± 3.9)	Villager 1981*
Carcino-embryonic Antigen ng/mg protein	0.04	0.08	Merrill 1981*

* indicates significant difference between smokers and non-smokers.

inflammation but is anatomically selective and invasive. The availability of a procedure less invasive which can be performed at multiple sites repeatedly, such as BAL is opportune provided it can reliably give comparable information to that which has hitherto been regarded as the gold standard. Like many tests in clinical use, BAL has limitations.

Biopsies from patients with cryptogenic fibrosing alveolitis (CFA), were found to have more airspace cells, mainly PMN and eosinophils, whereas the interstitium showed more lymphocytes (Davis, 1978). Comparison of airspace cells in open lung biopsies showed a close linear relationship between the percentages of lymphocytes in the BAL (Davis, 1976). Thus the lung lavage showed good correlation with free luminal cells but did not get over the discrepancy between lung interstitium cells and free alveolar cells. Haslam et al (1984) using semi-quantitative analysis of cells in histological specimens, quantification of cells in lung extracts and analysis of BAL from patients with ILD, found that the eosinophils and PMN correlated well in the latter two techniques but lymphocytes did not. There was no correlation between BAL or lung extraction with histological semi-quantitative analysis. Huninghake comparing lung extract cells and lavage cells in individual patients showed excellent correlation of cell proportions between these two techniques. In a recent study by Watters et al (1986) lymphocyte content of lavage fluid was said to show significant correlation with histopathological evidence of potentially reversible alveolar septal inflammation. Lymphocyte content showed negative

correlation with pathologic honeycombing. Thus the main message in CFA is the lack of correlation between predominantly lymphocytic cells in the interstitium with predominant neutrophils and eosinophils in the lavage.

In sarcoidosis light microscopy of biopsy specimens normally shows very few cells of any type in the alveolar airspaces while lavage fluid shows predominance of lymphocytes. Using immunocytochemical techniques on cryostat preparations from transbronchial biopsies (TBB) and on lavage cells from five patients with sarcoidosis, Campbell (1985) reported that subsets of lymphocytes in BAL accurately reflected type and proportion of subsets present in TBB. The value of T_4:T_8 cells in BAL from each individual patient was in accord with the TBB specimen. Macrophage phenotypes however only showed qualitative but not quantitative correlation. In another study Semenzato et al (1985) used similar histochemical technique and monoclonal markers on lung biopsies and BAL specimens from 33 patients with sarcoidosis and hypersensitivity pneumonitis and showed that the relative proportions of inflammatory and immunocompetent cells were well correlated. However BAL tended to overestimate percentages of lymphocytes especially in high intensity alveolitis and particularly in hypersensitivity pneumonitis. Thus despite the light microscopy findings in sarcoidosis referred to earlier, BAL analysis appears to give some correlation with cells in biopsy specimens.

1.6.8 Uses of BAL:

1.6.8.1 Therapeutic:

BAL had been used as a therapeutic measure in the past in conditions such as bronchiectasis, asthma or cystic fibrosis. Therapeutic bronchoscopic suction of bronchial secretions especially in the intensive care setting is more common. However proper therapeutic lavage of the alveolar compartment is now rare and is indicated in the rare condition of alveolar proteinosis, and microlithiasis. In the former condition bilateral whole lung lavage using large volumes of fluid is repeated at intervals (du Bois, 1983).

1.6.8.2 Diagnostic:

Several reports have now shown that BAL can be used successfully in the diagnosis of pneumonia of the immuno-compromised patients. It was recently recommended in making the diagnosis of pneumonia in a hospital outbreak of Legionaires disease (Winter et al., 1987). The role of BAL in the diagnosis and staging of ILD is controversial, especially in CFA. The BAL changes in CFA are variable and cannot be used to make a firm diagnosis in the individual patient. In sarcoidosis some authorities appear more confident with BAL. In a recent editorial Springmeyer (1987), stated that in their experience a T-lymphocyte predominant lavage with a T_4:T_8 ratio of 4:1 or greater was nearly 95% specific for the

diagnosis of sarcoidosis. Hypersensitivity pneumonitis, however has a much larger lymphocyte predominance usually, with the T_4:T_8 ratio of less than 1:1. Use of monoclonal markers to detect B-lymphocytes has been reported to be diagnostic of lymphoma (Davis, 1987). Pulmonary alveolar proteinosis can be diagnosed by the characteristic lamellar bodies in the alveolar macrophage (du Bois, 1983). Langerhans cells which are OK T6 (or the equivalent NA1/34) positive cells with the Langerhans granules in a proportion of 5% or more of BAL cells may confirm the diagnosis of histiocytosis X (Chollet, 1984). Demonstration of siderin granules in alveolar macrophages indicates pulmonary haemorrhage.

Finally the impact of BAL cell analysis on clinicians' diagnostic evaluation of ILD patients was recently assessed by Stoller et al (1987). In this study clinicians were asked in a questionnaire about the likeliest diagnosis for the patient and the confidence of each diagnosis and any proximate plans, when they submitted lavage specimens and when they obtained the results. The study revealed that BAL results influenced the clinicians' diagnostic thinking in 59% of cases; and in 52% of cases this change was appropriate compared to 9% inappropriate. In addition clinically impressive change occurred in 9% of cases including an unexpected case of AIDS among the total of 78 patients in the study.

1.6.8.3 Patient Follow-Up:

While controversy exists regarding the diagnostic

capability of BAL in ILD some use regarding prediction of prognosis has been reported. Turner Warwick et al (1987) reported that CFA patients with predominantly lymphocytic lavage, were more likely to respond to corticosteroids while those with predominant neutrophil and eosinophil counts often responded to cyclophosphamide. With regards to sarcoidosis, another study by Turner Warwick (1984) in which BAL, angiotensin converting enzyme (ACE) and gallium scan were performed but not used in decision making, revealed that there was a clear improvement of many parameters in many patients in the absence of high initial lymphocyte count of the lavage fluid. In addition in some individuals some of the parameters including chest x-rays, physiology, gallium scan and ACE returned to normal while lymphocyte counts remained high.

Huninghake (1986) has reported that in their experience lymphocyte counts might remain high following treatment with corticosteroids and for this reason it is their policy to continue corticosteroids until BAL lymphocyte counts normalize. This policy ensured that only few patients relapsed.

1.6.9 Processing of the Lavage Fluid:

BAL fluid is collected into the container in individual aliquots by direct suction through the bronchoscope with a syringe on the suction pump; or pooled together in one larger container. Some workers use siliconised containers to minimise cell loss. Some recommend collecting samples on ice

(Huninghake, 1979) while others are not so specific (Reynolds, 1987). An attempt should be made to examine the fluid as soon as possible. Buffering of the lavage fluid is known to improve the viability of cells (Haslam - personal communication) and it is conceivable that delay in examination of cells can adversely affect the viability.

Once the BAL fluid is in the laboratory, excess mucus is removed by a pipette and an original total cell yield is made before further processing of the fluid. Some workers use Coulter counters for this purpose (Haslam, 1986) but most use one of the many counting chambers and a white cell dilution fluid. Sometimes it is necessary to sieve the BAL fluid on a layer of surgical gauze to remove mucus and debris. As this treatment can affect the cell counts (Kelly, 1987) substantially, it is important that the original count should have been made before this step as mentioned above. Cytospin preparations are made from the original fluid for differential counts. The use of cytospin preparations has been found to underestimate lymphocyte counts (Saltini, 1984), due to the less adherent property of these cells compared to macrophages. However most authorities regard cytospin preparations adequate (Huninghake, 1979; Haslam, 1986; Reynolds, 1987). The most commonly used stain for differential counts is May-Grunwald-Giemsa stain.

CHAPTER 2

METHODS

2.1 Bronchoalveolar Lavage (BAL)

2.1.1 The technique:

The procedure is performed under local anaesthetic coupled with some premedication. The patient who has been previously fasted for about eight hours receives intramuscular premedication in the ward about half an hour earlier or intravenous (i.v.) premedication in the bronchoscopy suite. Our regimen consists of i.v. atropine immediately prior to bronchoscopy followed by i.v. diazepam with or without fentanyl depending on the age and the general condition of the patient. Local anaesthesia is achieved by 10% lignocaine nasal (Plate 2.1(a)) and pharyngeal spray. The bronchoscope is introduced nasally and anaesthesia of the larynx and major airways is achieved by spraying aliquots of 4% lignocaine under vision through the bronchoscope channel. Supplemental oxygen is given through nasal prongs if needed. Usually a quick survey of the bronchial tree is done before the bronchoscope is wedged into the subsegment chosen for lavage. At this point it is our practice to change the collecting trap to minimise the content of lignocaine. Buffered normal saline (pH 7.2) is then instilled into the chosen subsegment (Plate 2.1(b)) and fluid is sucked back into the collecting pot.

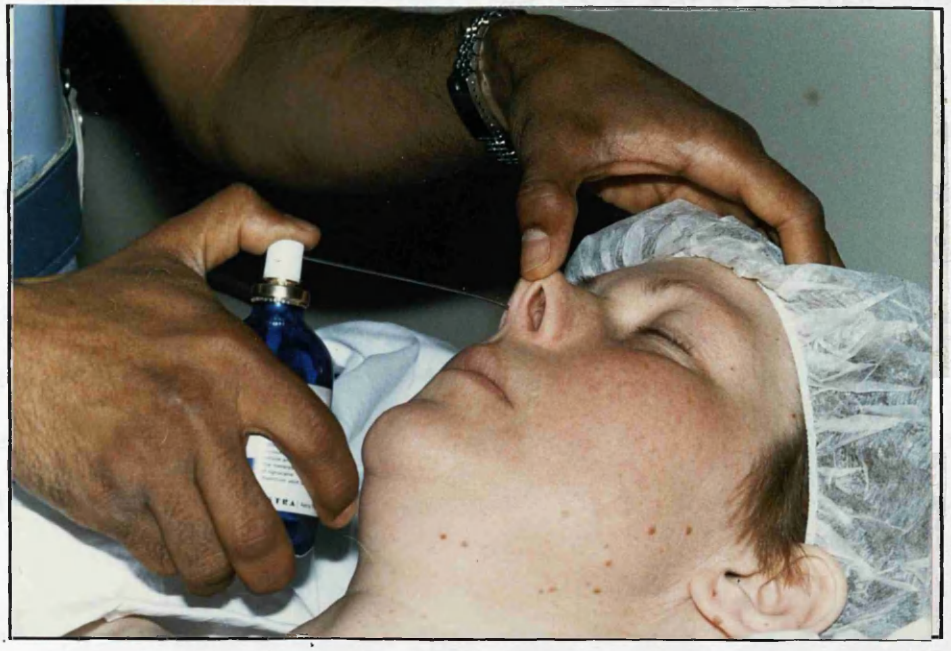


Plate 2.1(a): Local anaesthesia of the nose with 10% lignocaine spray.

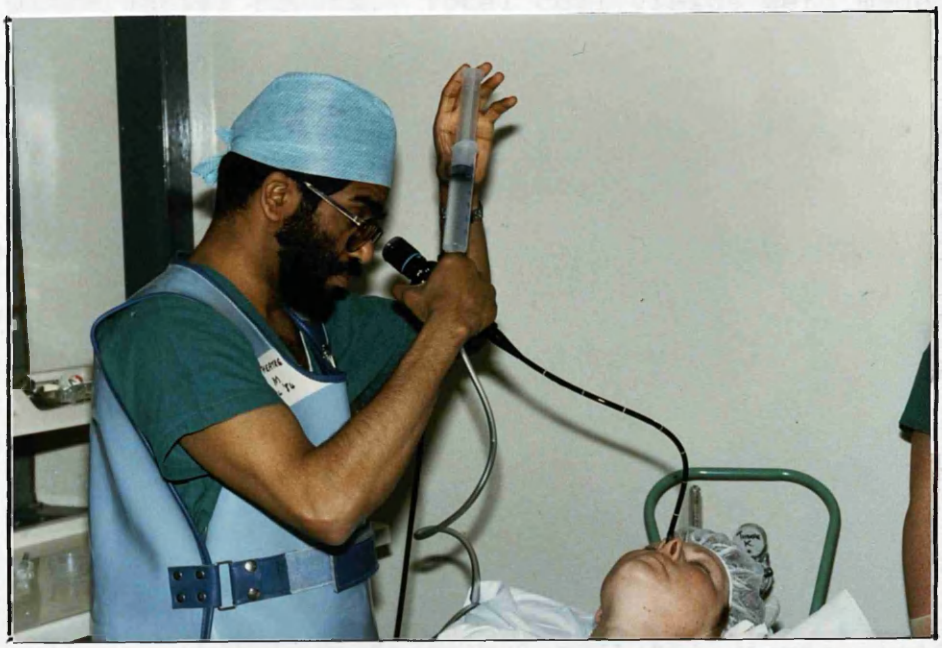


Plate 2.1(b): Instillation of buffered normal saline.

Aliquots of 50 mls are instilled to a total of 150-200 mls for unilateral lavages and 300-400 mls for bilateral lavages, depending on the volume of the fluid recovered. These volumes are recorded in each case. Most centres use 300 mls but up to 400 mls have been used (Pingleton, 1983). Ear oximetry and cardiac rhythm monitoring is recommended in sick patients by some authorities (Reynolds, 1987) but this practice is not widespread in this country and we have not found it necessary. Facilities for resuscitation have always been at hand.

2.1.2 Processing of BAL fluid

2.1.2.1 Pooling and cleaning:

The fluid was pooled, excess mucus aspirated and an aliquot of 5 mls set aside for original (total) cell* yield and differential counts. Total cell yields were made by using a Fuchs-Rosenthal counting chamber with or without white cell dilution fluid. In some cases especially at the beginning of the project it was found necessary to clear BALF of mucus by passing through 2 layers of gauze. The effect of this was assessed (see Tables 2(a) and (b)).

2.1.2.2 Preparation of cystospins:

Cystospins were made using a cytocentrifuge (Shandon).

*Throughout the test "original cells" indicate cell counts done on the unprocessed BAL fluid other than clearing of mucus by aspiration and excluding sieving.

The concentration of cells was adjusted to 1 x 10^5 cells/ml for Leishman's staining and 5 x 10^4/ml for esterase staining. 0.2 mls was placed in the cytospin bucket which was centrifuged at 900 r.p.m. for 4 minutes. Labelled double frost microscope slides (25 mm x 76 mm) were used to collect the cells. These were fixed appropriately according to the stain used.

2.1.2.3 Wright-Giemsa stain:

Cytospins (2 x 10^4 cells) were heat fixed, before being covered with 2.5 mls of Leishman's stain for 30 seconds. The stain was then diluted by an equal volume of tap water. After 10 minutes excess stain was rinsed off and the slides air dried. Differential counts were made under oil immersion from a total of 500 cells (Plate 2.2a & b).

2.1.2.4 Non-specific esterase:

Cells were fixed in citrate-acetone-methanol mixture for 30 seconds. The esterase stain was prepared from a commercially obtained kit (see Appendix). In a Coplin jar protected from light, slides were incubated at 37^{o}C for 10 minutes, gently washed in running water for 3 minutes before counterstaining with Mayer's haematoxylin for 15 minutes. Differential counts were made from a total of 500 cells. Esterase positive cells were recognised by their dark brown to black granules (see Plate 2.3(a) & (b)). Data comparing

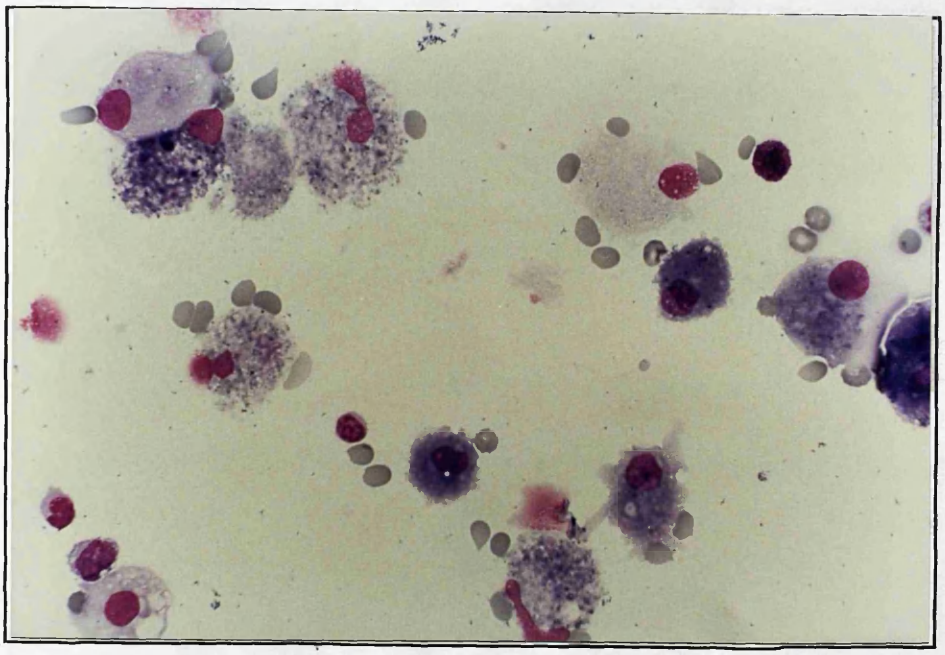


Plate 2.2(a): Leishman's stain of BAL cells from a non-smoking control.

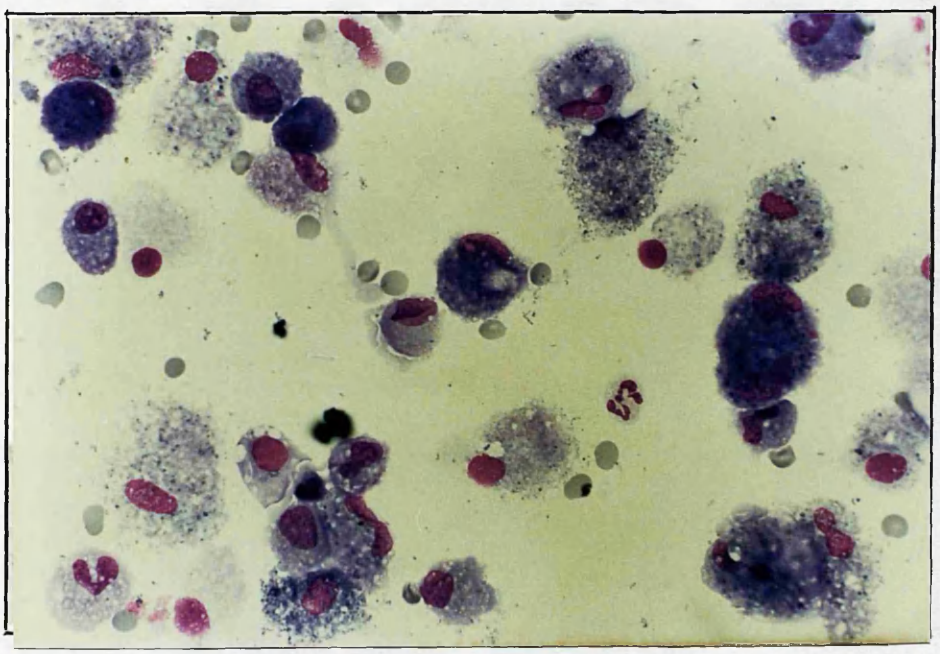


Plate 2.2(b): Leishman's stain of BAL cells from a smoking control.

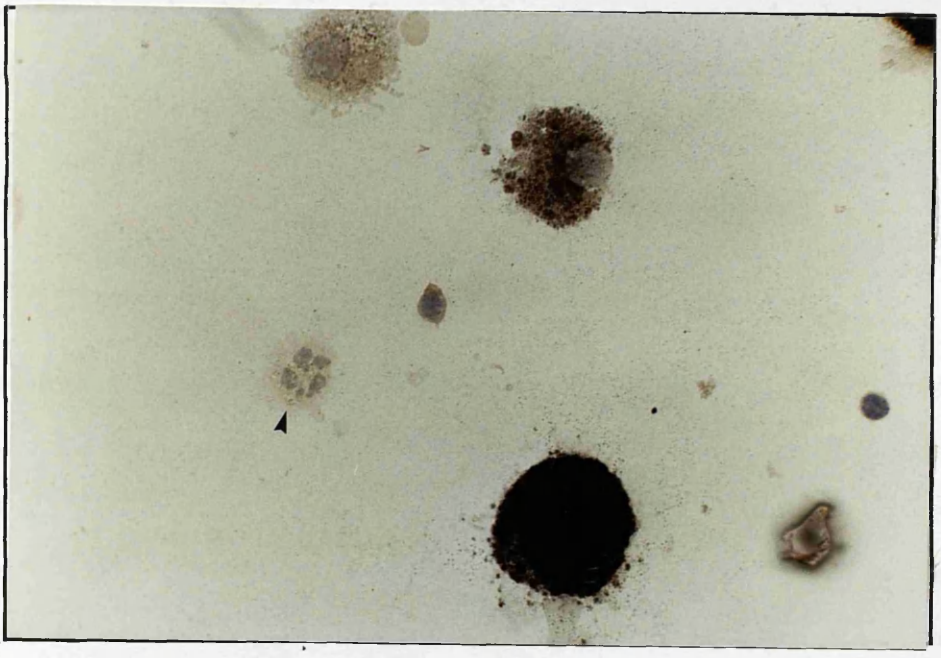


Plate 2.3(a): Showing varying degrees of esterase positive AM and an esterase negative cell (PMN) (arrow)

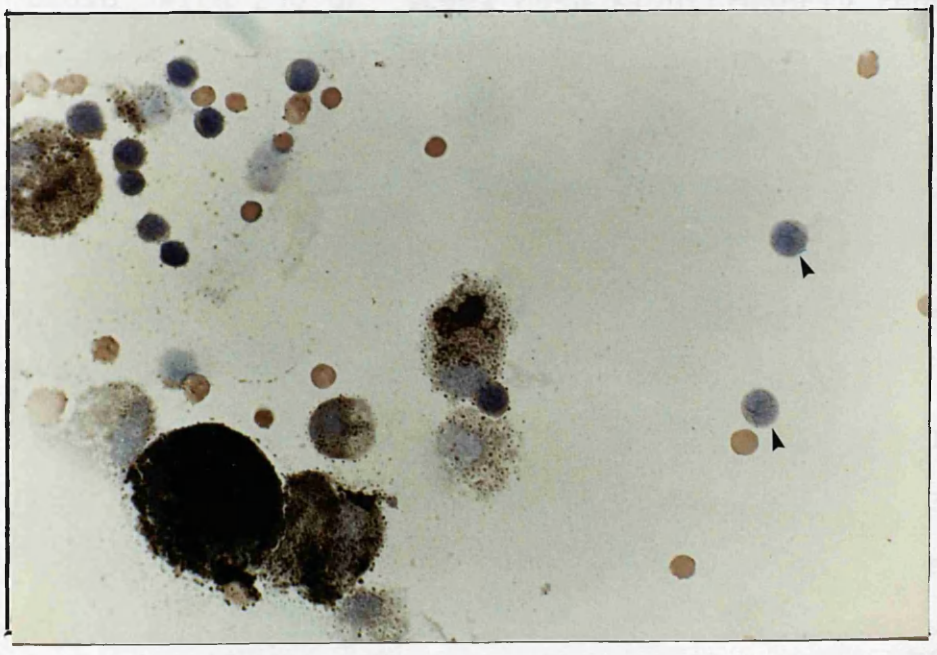


Plate 2.3(b): Strongly esterase positive AM and esterase negative cells (lymphocyte) (arrow).

esterase and Leishman's stain is presented later in this chapter.

2.1.2.5 Assessment of the effect of sieving:

BAL fluid was obtained from patients undergoing routine diagnostic bronchoscopy mainly for bronchogenic carcinoma. Fluid was pooled, an aliquot removed for original counts, before being divided in two equal parts. One part was passed through 2 layers of surgical gauze and the other through 4 layers. Total and differential cell counts were made and compared to the original counts.

There was no significant change in differential counts of BAL cells following sieving through either 2 or 4 layers of surgical gauze. These results are partially in agreement with those by Kelly et al (1986) who found that filtration of BAL fluid with 200 um^2 steel mesh significantly reduced the total cells. However the differential cell counts were not affected in this study. This could be due to the physical differences between surgical gauze and stainless steel mesh.

2.1.2.6 Enrichment of macrophages using adherence technique:

The adherence technique described by Ackerman (1978) was assessed using samples with high neutrophil content. Following the initial processing of the BAL fluid outlined above, cells were centrifuged at 200 g for 5 minutes in 20 ml universal containers. The cells were washed twice and resuspended in

Table 2.1(a): Effect of sieving on total cell yields.

n = 8	Unprocessed Fluid	2 layers	4 layers
Cell yields x 10^5 /ml BAL fluid	3.40	2.75*	2.56*
Cell loss	NA	19.1%	24.7%
Viability (%)	79.4	78.5	79.5

NA = Not applicable.

* There was significant cell loss following passage of BAL fluid through surgical gauze 2 layers (P = 0.025) and 4 layers (P = 0.036). Viability however was not affected by sieving.

Table 2.1(b): Effect of sieving on cell composition.

	Mean (%) differential cell counts				
n = 8	Macro-phages	Neutro-phils	Eosino-phils	Lympho-cytes	Epithel-ial cells
Unprocessed fluid	89.05	5.06	0.14	2.56	4.08
2 layers	90.2	4.63	0.20	2.97	2.35
4 layers	89.75	5.06	0.28	3.15	2.30

medium 199/20% foetal calf serum (FCS). An aliquot was taken for total and differential counts. 10 mls of a known number

of cells were placed in previously used tissue culture flasks and incubated at 37°C in an atmosphere containing 5% CO_2 in air for 45 minutes to allow macrophages to attach. Non-adherent cells including lymphocytes and neutrophils were decanted and rinsed with 3 changes of PBS (pH 7.4) prewarmed to 37°C. Macrophages were detached by addition of 3.5 mls each of 10 mM EDTA/PBS and medium 199/20% FCS and incubation at 37°C (5% CO_2) for 15 minutes. The flasks were removed and shaken vigorously and macrophages were decanted into the test tubes. Further vigorous rinsing of the flasks with 5 mls 199/20% FCS was done to remove the remaining macrophages. Cells were centrifuged for 5 minutes at 200g, and resuspended in M199. Cell loss and purity and viability of macrophage was assessed.

Table 2.2: Total cell yield viability and differential counts following macrophage enrichment using adherence technique.

n = 3	Macrophages x 10^6	% via-bility	% differential cell counts of BAL fluid			
			Macro-phages	Neutro-phils	Eosino-phils	Lympho-cytes
Pre	22.1	84	74.7	23.4	0.6	1.3
Post	5.17	53	96.0	4	0	0

Excellent macrophage enrichment was obtained using adherence technique but this resulted in an unacceptable high cell loss (macrophages) both in numbers (> 76%) and viability.

2.1.2.7 Enrichment of Macrophage using density gradient:

Method:

7 mls of BAL fluid was placed in conical tubes and 3 mls of lymphocyte separation medium was injected beneath it through a size 20 gauge lumbar puncture needle. This was centrifuged for 25 minutes at 160 x g. The cell-free supernatant was aspirated to within 1 cm of the interface layer of cells (IFC) and placed in universal containers for further processing. The IFC were aspirated, pooled, centrifuged at 200xg, washed and resuspended once in medium 199 (M199). Total and differential cell counts were made, and viability was assessed by trypan-blue exclusion test. The concentration of cells was adjusted as needed.

2.1.2.8 Trypan-blue dye exclusion test:

8 ul of cells were mixed with an equal volume of 0.1% trypan blue and covered by a coverslip (24 mm x 24 mm) coverslip before examination under the microscope. A total of 200 cells were counted and dead cells were recognised by the blue staining of their nuclei.

2.1.2.9 Assessment of macrophage-enrichment using density gradient:

The effect of cell loss, viability, and purity of the macrophage population was assessed using the procedure outlined above, (tables 2.3a&b).

Table 2.3(a): Macrophage enrichment in samples with low neutrophil content using Ficoll-hypaque density gradient centrifugation.

n = 8	Total macrophages x 10^6 (Mean)	% viability	% Differential cell count of BAL fluid			
			Macrophages	Neutrophils	Eosinophils	Lymphocytes
Pre	44.20	76.50	92.42	2.52	0.52	4.50
Post	20.04	71.62	96.25	1.31	0.17	2.57
P value	0.018	NS	0.028	0.018	NS	0.043

Ficoll hypaque density centrifugation also resulted in substantial and significant loss of about 54% macrophages but viability of the cells was not significantly affected. There was also significant macrophage enrichment and neutrophil reduction in these samples. It is interesting that the effect of Ficoll-hypaque (lymphocyte separation medium) centrifugation resulted in a reduction rather than enrichment of lymphocytes in BAL fluid, unlike in blood samples.

Centrifugation of BAL fluid with a high neutrophil content on Ficoll-hypaque resulted in significant loss of

Table 2.3(b): Macrophage enrichment in samples with a high neutrophil content using Ficoll-hypaque density gradient centrifugation.

n = 5	Total cells x 10^6	% Viability	% Differential cell counts of BAL fluid			
			Macrophages	Neutrophils	Eosinophils	Lymphocytes
Pre	138.36	90.4	26.82	62.64	0.32	10.24
Post	66.43	87.4	36.04	55.34	0.12	8.66
P value	0.043	0.195	0.068	0.225	0.593	0.345

cells. There was also a trend towards some macrophage enrichment but this was not significant. The reduction in neutrophil proportion was not significant although it was apparent on examining the pelleted cells that these contained even higher neutrophil proportions (data not shown).

Comparison of Leishman's staining and esterase staining on 20 random samples from controls, smoke inhalation and CAP patients was performed to demonstrate correlation between these procedures.

2.1.2.10 Comparison of esterase and Leishman's stain:

In the earlier part of this project both the original and the interface layer of cells were stained with both Leishman's and esterase stains, but in the later part of the study the interface layer of cells were stained with Leishman's stain

only. Correlation of these two stains was assessed.

Table 2.4: Comparison of Leishman's stain with esterase stain.

	% Differential cell counts		
n = 20	Macrophages (Esterase positive)	Neutrophils & Eosin (Esterase negative polymorphonuclear cells)	Lymphocytes (Esterase negative mononuclear cells)
Leishman's Stain	62.58	28.06	9.28
Esterase Stain	64.47	25.89	9.64
r_s value	0.9594	0.9759	0.9477

r_s = Spearman rank correlation.

Excellent correlation was obtained between Leishman's and esterase staining. Although this was clear from very early on during the study, it was decided to continue using both stains simultaneously to ensure that proportions of cells were adequately assessed especially in samples with a high neutrophil content some of which may be immature with atypical forms. These results are also presented in Figure 2.1(a-c).

2.1.2.11 Concentration of lavage fluid:

Various methods including pressure filtration, lyophilization, oncotic methods or membrane filtration of the soluble constituents of BAL fluid have been used by different

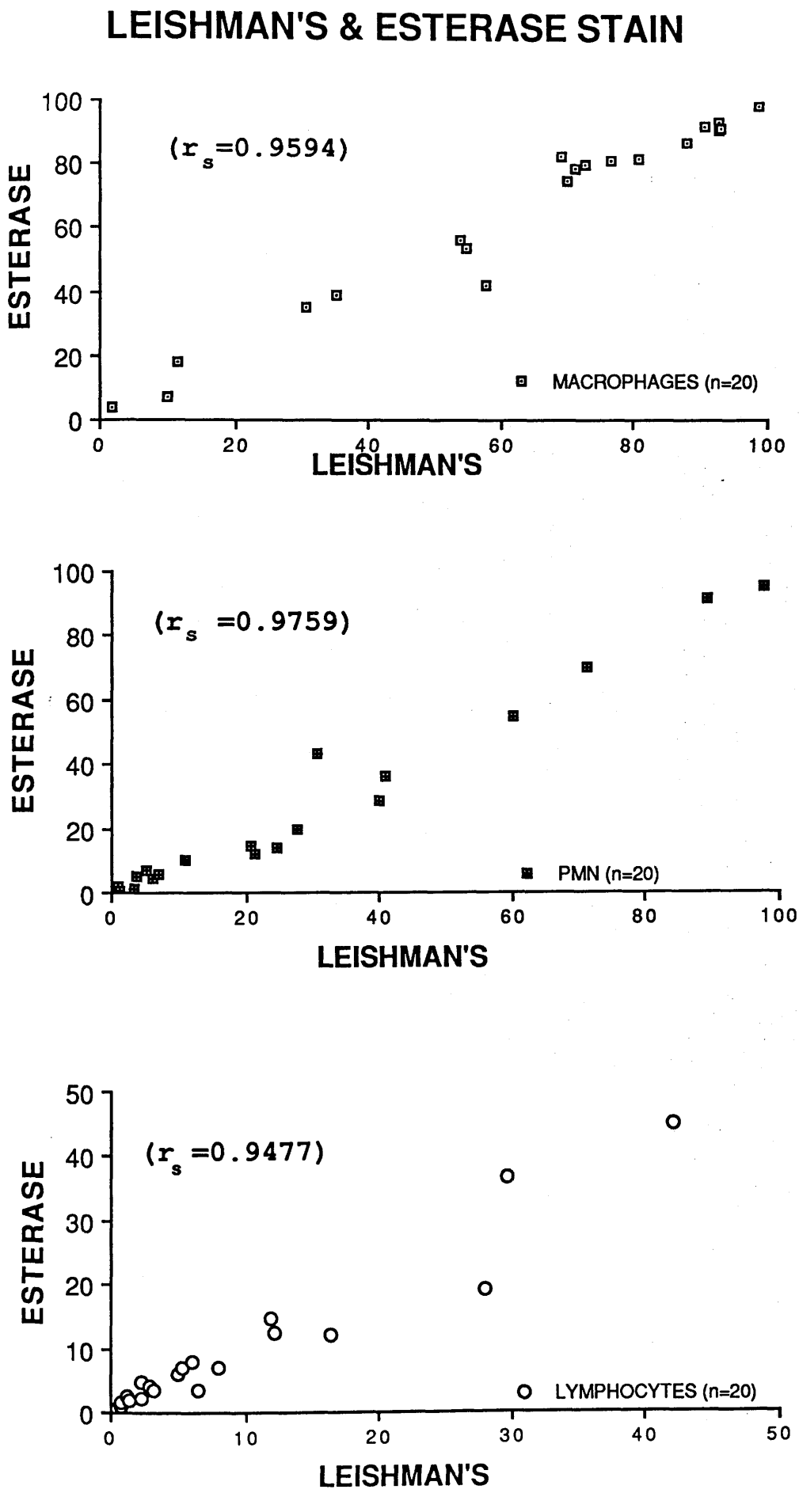


Fig. 2.1(a-c): Correlation of Leishman's and esterase stain of BAL cells.

workers (Huninghake, 1979). Membrane filtration was chosen in this work because it is by far the cheapest and does not need elaborate apparatus.

Method:

The cell free supernatant was centrifuged in sterile universal containers at 2000 g for 10 minutes to remove any remaining debris or bacteria. This was then passed through 0.22 u filters, placed in the membrane tubing* and covered with polyethylene glycol overnight until completely dry. Sterile distilled water was injected. into the tubing to reconstitute the fluid to 1/10 of its original volume. Thorough mixing was achieved by gentle massaging of the tubing. The concentrated fluid was aliquoted into five equal portions and stored at -20°C (1 portion) and -70°C (4 portions) until they were used or analysed.

2.2 Isolation of peripheral blood cells:

2.2.1 Neutrophils and peripheral blood mononuclear cells

This was done by the method described by Boyum (1968). 7 mls of heparinised blood was mixed with 3 mls of 5% dextran-saline and allowed to sediment for 30 minutes. 7 mls of leucocyte-rich plasma was placed in conical tubes and 3 mls of Lymphocyte-Separation Medium was injected beneath it using a lumbar puncture needle. This was centrifuged for 30 minutes at

* Chemotactic factors with molecular weight of less than 12000 daltons could have been lost during the concentration procedure.

289xg. The interface layer of cells was aspirated and centrifuged at 289xg for 5 minutes, washed once and resuspended in gel-Hanks. This was used as unpurified monocytes (peripheral blood mononuclear cells - PBMC) (Plate 2.4). The pellet cells were resuspended in 0.875% cold ammonium chloride for 15 minutes to lyse any contaminating red blood cells. These were centrifuged at 289xg for 5 minutes, resuspended in gel-Hanks and washed again once. An aliquot of cells was diluted in white cell dilution fluid and counted in Rosenthal-Fuchs counting chamber. These cells were more than 95% neutrophils (PMN) with more than 98% viability.

2.2.2 Isolation of monocytes using nycodenz monocytes (Boyum, 1983)

10 mls EDTA venous blood was mixed with 1 ml 6% dextran-saline and allowed to sediment at room temperature for 45 minutes. 7 ml of leucocyte-rich plasma was placed in conical tubes and 3 mls of monocyte nycodenz solution was injected beneath it with a lumbar puncture needle, before centrifugation at 600xg for 15 minutes. The leucocyte-free supernatant plasma was aspirated up to 3 mm above the interface layer of cells and was reconstituted (6 parts supernatant: 94 parts saline) for use as a "washing fluid". Monocyte-rich supernatant including the monocyte-nycodenz solution was aspirated up to 3 mm above the cell pellet, and mixed with an equal volume of washing fluid before centrifugation at 600xg for 10 minutes. Cells were resuspended

Plate 2.4: Ficoll-hypaque separation of PMN (pellet cells) and mononuclear cells (interface layer).

in washing fluid and the procedure repeated once before resuspending the cells in gel Hanks and adjusting the concentration as needed. Cytospin preparations were made and purity was checked using non-specific esterase stain. In our hands this method yielded 80% purity.

2.3 Phagocytosis and killing:

2.3.1 Introduction:

Phagocytosis involves recognition of the particle by the phagocyte, followed by attachment and ingestion. For optimal ingestion particles need to be opsonised and this is achieved by a specific antibody of IgG subclass with or without complement. The IgG antibodies are bound to particles at their $(Fab)_2$ sites leaving the Fc segment exposed thus allowing the phagocyte to recognise the particle through the Fc receptor on its surface. Weak IgA-mediated phagocytosis by alveolar macrophages has been shown (Richards, 1985). IgM has no opsonic activity but its ability to induce complement activation leads to deposition of C_3b on the particle which is then recognised by the phagocytes C_3b receptors. Receptor-ligand interaction and receptor-opsonin interaction leads to engulfment of the particle by the phagocyte membrane, fusion of the phagocyte pseudopodia, dislodging of the particle into the phagosome and fusion of the lysosome leading to formation of the phagolysosome. The phagocytic process evokes a number of oxygen and non oxygen-dependent mechanisms increased oxygen

consumption and production of superoxide anion, hydrogen peroxide and hydroxyl radical, leading to intracellular killing of micro-organisms.

Many *in vitro* methods have been employed in the assessment of phagocytosis and killing, and though they may be different in detail their general principles are similar. These are summarised overleaf.

2.3.3 Assessment of phagocytosis and killing:

This was done using prelabelled bacteria, labelling bacteria after phagocytosis or killing and pour plate method.

2.3.3.1 *Uptake method*:

Bacterial ingestion using the method described by Peterson *et al* (1977) was used. Experiments were performed using PMN, PBMC (1×10^7/ml) from healthy donors, and alveolar macrophages from patients with negative bronchoscopy, and normal chest x-rays. Bacteria were prepared as follows: A clinical isolate of *Staph aureus 502A* was grown overnight in 10 ml Mueller-Hinton (M-H) broth to which 100 mcl ^{3}H-Adenine was added. Bacteria were washed thrice in sterile saline and their concentration was adjusted to 1×10^7 colony forming units (c.f.u.)/ml. Using a spectrophotometer an optical density of 0.025 was found to correspond to a concentration of 1×10^7 cfu/ml. For opsonisation the bacteria were incubated with an equal volume of 10% pooled serum in an orbital shaker

2.3.2 In-vitro methods in phagocytosis and killing

Principle	Example	Comment
PHAGOCYTOSIS		
Determination of increase in number of intracellular particles	Light and/or electron microscopy	- labour intensive - Difficulty of distinguishing between extracellular and intracellular particles.
	Labelling particles with fluorescent or radioactive probe	- Some methods do not distinguish between intracellular ingestion and extracellular attachment. - Allows kinetic studies.
Determination of decrease in number of extracellular organism	a) employ direct microbiological methods b) use of radioactive probe	- Bactericidal factors to the extracellular organisms not accounted for. - often lack distinction between ingestion and attachment.
KILLING		
Direct measurement of: microbicidal activity	Measurement of decrease in total number of live microorganisms by radioactive probe or microbiological methods.	- Affected by rate of ingestion and extracellular microbicidal mechanisms.
Indirect measurement of microbicidal activity of phagocytes	Measurement of metabolic burst accompanying phagocytes.	- Non-specific activation accompanied by metabolic burst may not be accompanied by intracellular killing.

(150 r.p.m.) at 37°C. After 15 minutes the bacteria were centrifuged at 2020xg for 10 minutes and resuspended to their original volume in gel-Hanks. Phagocytosis mixtures were set up incubating 0.1 ml bacteria and 0.1 ml phagocytes for 15, 30 or 45 minutes in triplicate according to the protocol shown overleaf.

2.3.3.2 Killing assay:

Unlabelled bacteria was used. Phagocytes and bacteria were incubated in 0.1 ml volumes for 15, 30, or 45 minutes at 37°C, before lysing the cells by adding 3 mls distilled water to the mixture. For total bacterial counts cells were lysed at 0 minutes. An aliquot of 0.1 ml was taken from each tube and serial dilution with saline was made before plating 20 ul volumes on blood agar and counting c.f.u. % Killing was calculated according to the formula shown.

2.3.3.3 Phagocytosis and killing - labelling of bacteria after phagocytosis and killing

2.3.3.3.1 Principle of the method

The method used is based on the principle described by Lam and Mathison (1979) that uridine is not significantly incorporated by PMN while bacteria in the logarithmic growth phase will do so. The killing part of the assay was adopted from the method described by White and Walker (1981) who employed freezing and thawing to disrupt the phagocytes.

Assessment of Ingestion using ^{3}H-adenine Labelled Staph aureus 502A

Tube A	Tube B	Tube C
0.1 ml ^{3}H-Adenine labelled Bact. + 0.1 ml phagocytes	0.1 mls ^{3}H-Adenine labelled Bact. + 0.1 ml phagocytes	0.1 ml gel Hanks + 0.1 ml phagocytes
Incubate at 37°C for phagocytosis	Incubate at 37°C for phagocytosis	Incubate at 37°C
No washing or spinning	Centrifuge at 162xg and wash x 3	Centrifuge at 162xg and wash x 3
3 mls Scintillation Fluid	3 mls Scintillation fluid	3 mls Scintillation fluid
Total (A) bacteria cpm	Cell Associated (B) bacteria cpm	Background cpm (C)

$$\% \text{ Bacterial uptake} = \frac{(B) - (C)}{(A)} \times 100.$$

Killing: Counting live c.f.u.

Tube A	Tube B
0.1 ml cell + 0.1 ml Bact.	0.1 ml cells + 0.1 ml Bact.
At 0 min Add 3 ml distilled water	Incubate for phagocytosis and killing at 37°C for desired period.
	Add 3 mls distilled water
Total Bacteria (A)	Live Bacteria (B)

Serial Dilution + Pour plating

$$\% \text{ Killing} = \frac{\text{Total Bact.} - \text{Live Bact.}}{\text{Total Bact.}} \times 100$$

$$\text{i.e.} = \frac{A - B}{A} \times 100$$

2.3.3.3.2 Preparation of bacteria:

On the morning of the experiment, a fresh suspension of Staph aureus 502A was prepared in an M-H broth from a similar overnight culture. The bacteria was washed twice in saline and the concentration adjusted to 1 x 10^7 c.f.u. before opsonisation in 10% serum.

2.3.3.3.3 Phagocytosis:

Mixtures were set up in triplicate as shown in the protocol overleaf. Phagocytosis was terminated by placing the tubes on ice before adding ^{3}H-uridine. For assessment of killing, ^{3}H-uridine was added following the disruption of phagocytes by freezing and thawing, on methanol - CO_2 mixture, and the mixtures were incubated for one hour at 37^{o}C to allow labelling of bacteria. 1 ml of gel Hanks was added to each tube and an aliquot of 200 ul was passed through a filter (0.22 um pore size) and washed to remove excessive unbound label. The filter was placed in scintillation tubes and 3 mls of scintillation fluid was added before readings were made on a scintillation counter.

Comparison of each of the three assays to assess the ingestion or killing of Staph. aureus by donor neutrophils showed some discrepancy in the results between the different methods. More important was the fact that in some experiments, ingestion and killing (by ^{3}H-uridine labelling) failed to work completely. The reason for this failure could not be identified readily (Tables 2.5a-c).

Assessment of ingestion: post-phagocytosis labelling of bacteria

Tube A	Tube B	Tube C
0.1 ml Bact. + 0.1 ml Gel Hanks	0.1 ml Phagocytes + 0.1 ml Bact.	0.1 ml phagocytes +0.1 ml Gel Hanks
Incubate at 37°C for desired period	Incubate at 37°C for desired period	Incubate at 37°C for desired period
Place on ice	Place on ice to terminate phagocytosis	Place on ice
Add 20 ul ^{3}H-Uridine	Add 20 ul ^{3}H-Uridine	Add 20 ul ^{3}H-Uridine
Incubate at 37°C for 1 hr to label all the bacteria	Incubate at 37°C for 1 hr to label extracellular bact.	Incubate at 37°C for 1 hr to obtain background cpm.
Add 1 ml cold Gel-Hanks	Add 1 ml cold Gel-Hanks	Add 1 ml cold Gel-Hanks
Filter 200 ul aliquot and wash unbound label	Filter 200 ul aliquot and wash unbound label	Filter 200 ul aliquot and wash unbound label
Place dry filter into scint. tube and add 3 ml scint. fluid	Place dry filter into scintillation tube and add 3 ml scint. fluid	Place dry filter into scint. tube and add 3 ml scint. fluid
Total bact. cpm	Extracellular Bact. cpm	"Phagocytes" only" Background cpm
A	B	C

$$\% \text{ Ingestion} = \frac{A - B - C}{A - C} \times 100$$

Killing assay using freezing and thawing of phagocytes and labelling of bacteria.

Tube A	Tube B	Tube C
0.1 ml bact. + 0.1 ml gel Hanks	0.1 ml cells + 0.1 ml bact.	0.1 ml phagocytes + 0.1 ml gel Hanks
Incubate at 37°C for desired period	Incubate at 37°C for desired period	Incubate at 37°C for desired period
Freeze in dry ice & thaw in warm water x 2	Freeze in dry ice & thaw in warm water x 2	Freeze in dry ice & thaw in warm water
Add 20 ul ^{3}-H uridine	Add 20 ul ^{3}H-uridine	Add 20 ul ^{3}H-uridine
Incubate for 1 hr at 37 C to label all bacteria	Incubate for 1 hr at 37 C to label live bacteria	Incubate for 1 hr at 37 C to obtain background cpm
Filter on 0.22 u Sweenex filters or microtitre plates	Filter and wash to remove unbound label	Filter and wash to remove unbound label
Place filter in scint. tube and add 3 ml scint. fluid	Place filter in scint. tube & add 3 ml scintillation fluid	Place filter in scint. tube & add 3 mls scint. fluid
Total bact. cpm	Live Bact.	Phagocytes only Background cpm
Total cpm A	Live Bact. B	C

$$\% \text{ Killing} = \frac{A - B - C}{A - C} \times 100$$

Table 2.5(a): Ingestion and killing of staph. 502A by healthy donor neutrophils.

Experiment No.	Ingestion (%) ^{3}H-Adenine	Ingestion (%) ^{3}H-uridine	Killing (%) ^{3}H-uridine	Killing (%) colony counting
1	ND	66	77	89.4
2	85.7	0	0	90
3	2.2	83	89	100
4	60	56.3	4.29	ND
5	ND	40.1	51.8	68
6	0	0	41	31
7	ND	ND	9.6	28

ND = Not done.

Table 2.5(b): Ingestion and killing of Staph. 502A by peripheral blood mononuclear cells.

Experiment No.1	Ingestion (%) ^{3}H-Adenine	Ingestion (%) ^{3}H-Uridine	Killing (%) ^{3}H-Uridine	Killing (%) Colony counting
1	0	0	0	ND
2	ND	ND	16	33
3	ND	ND	49.4	35
4	91.9	0	0	ND
5	61.9	17.2	0	20.9
6	ND	0	0	26.7

ND = Not done.

Similar problems were encountered in these assays when mononuclear cells were used.

Table 2.5(c): Ingestion and killing of Staph. 502A aureus by alveolar macrophages.

Experiment No.	Ingestion (^{3}H-Adenine) (%)	Ingestion (^{3}H-Uridine) (%)	Killing (^{3}H-Uridine) (%)	Killing (Colony counting) (%)
1	ND	0	0	ND
2	ND	27	93	ND
3	29.3	77.3	77.7	31.5
4	36	0	ND	ND
5	57.8	79	14	ND
6	ND	9.24	0	26.9
7	ND	0	0	8.2
8	ND	12.7	23.7	ND
9	ND	7.6	0	38.6
10	ND	9.3	19.5	ND
11	0	21.4	25.5	0
12	28.40	58	11.2	16.5

ND = Not done.

These experiments represent a selection of the results of ingestion and killing assays using alveolar macrophages and it is clear that discrepancies are apparent. The lack of reproducibility especially in the freeze and thaw cycle

method was a major problem. Various manoeuvres were tried including change of bacteria-cell ratio, ensuring that fresh bacteria were used, and variation of incubation period. It was often noted that tubes which contained bacteria and cells had higher bacterial counts than those with bacteria only. Thus these live phagocytic cells were "enhancing" bacterial multiplication rather than killing them.

Because of these problems which could not be overcome these methods had to be abandoned.

2.4 Chemotaxis

2.4.1 Historical background:

Studies on inflammation began during the early nineteenth century by many workers including Addison 1843, Waaler, 1846, Cohnheim, 1867, Hess, 1888. Observations of adhesion of leucocytes to blood vessels and their subsequent accumulation into the extravascular compartment had been made during that early period but it was not until 1888 when definite reports of chemotaxis were made by Theodor Leber. Metchnikoff, working on phagocytosis, recognised the importance of Leber's observations, and with his co-worker Gabrietchevsky confirmed that dead or live bacteria were able to attract leucocytes. Metchnikoff (reviewed by Wilkinson, 1982) recognised that macrophages and microphages moved differently and realized the presence of chemotaxins. Commandon demonstrated the first successful *in vitro* experiment in 1917 using time-lapse

cinematography. His work provided the first insight into the sequence of morphological changes of white cell during locomotion and phagocytosis. A great deal of work was carried out by McCutcheon, using time lapse cinematography. His work defined the relative speed of cell locomotion including lymphocytes and the role of chemotaxins. In 1955 Rebuck and Crowley described the skin window technique. The introduction in 1962 of the membrane assay by Boyden marked a turning point in contemporary chemotaxis work. The under-agarose technique was reported by Nelson (1975).

2.4.2 Definitions:

Locomotion of a cell (or organism) is the process which involves physical transfer of the cell from one point to another. It is limited by the intrinsic capacity of the cell to do so, and can be influenced by many environmental factors. Locomotor responses are generally described as "tactic" or "kinetic". Tactic responses are directional toward a gradient source and essentially involve physical orientation towards the source. Kinetic reactions relate to speed of migration in which cells move faster or slower. These are positive or negative kinetic responses respectively.

Random locomotion: describes that tendency of the cell to migrate from one point to another without a special orientation to a stimulus or gradient and any inherent orientation exhibited in that process is determined solely by chance. Random locomotion is often spuriously used to mean

unstimulated locomotion but these two terms are not synonymous. In systems where spatial orientation cannot be demonstrated such as membrane assays, the terms chemotaxis and random migration are best replaced by stimulated and unstimulated migration respectively. Another term conveniently defined here is "leading front". The foremost cells in a population of cells migrating towards a gradient form the leading front and the methods where the distance from the starting point to the front is measured bear this name.

2.4.3 Behaviour of cells in migration:

The physiology of locomotion of cells is a complex subject and is beyond the scope of this thesis. For a detailed description the reader is referred to the excellent reviews of Wilkinson (1981, 1982) and Zigmond (1981). A short summary on cell behaviour will be given here.

Both neutrophils and monocytes undergo morphological changes during locomotion. Neutrophils exposed to a gradient polarize within a minute or two with their leading rounded end (lamellipodia) facing the gradient source. The accuracy of this orientation is governed by the potency of the gradient and occurs before translocation takes place. This orientation and the changes that take place when the gradient changes has led to the belief that neutrophils can sense different concentrations of the gradient along its body. It has been proposed that detection of a gradient by the cells is achieved by sensing differences in the number of receptors occupied on

their surfaces over some distance along the gradient (Zigmond, 1981). At certain concentrations of some chemotoxins the rate of locomotion correlates with the number of receptors occupied. Receptors for several different chemotaxins have been described for PMN (Wilkinson, 1982) and macrophages (Wilkinson, 1985). An important property determining cell locomotion is adhesion. Too rigid adhesion to the substratum renders the cell immotile and reduction of adhesion can increase locomotion to an optimum beyond which, further reduction leads to impaired locomotion. Physiological adhesion such as on protein-coated surfaces shows temperature and divalent cation dependency unlike adhesion to uncoated glass or plastic. The magnitude and duration of adhesion in PMN appear to depend on receptor occupancy. A suitable substratum is essential for locomotion. For example PMN on an uncoated filter or glass surface in the presence of a gradient do not show locomotion but when exposed to the same gradient on albumin coated surfaces they show vigorous migration.

2.4.4 Methods Used in Chemotaxis:

An exhaustive review of these methods is deemed unnecessary and beyond the scope of this work. A brief discussion of the principles involved in the main methods in use will be made here: Assays for chemotaxis fall into two main categories. The first include those which examine the behaviour of individual cells over a period of time. These employ visual assays. The second are those which examine the

response of populations of cells and the majority of assays fall under this category.

2.4.4.1 Visual Assays:

These assays were developed before membrane assays but nevertheless they are becoming more popular again (Wilkinson, 1985). The principle involves filming of a cell as it moves towards a gradient and its course can be plotted. Both chemotaxis and chemokinesis are best studied by these assays but membrane assays are superior in obtaining dose response data of pharmacological agents (reviewed by Wilkinson, 1982).

2.4.4.2 Membrane Assays:

These consist of porous membranes separating two chambers, one containing the cells and the other usually below it, containing the chemoattractant. The pore sizes of these membranes are such that they prevent the cells dropping through by gravity but allow them to actively squeeze themselves through the membrane matrix towards the attractant. For PMN 3 micron pore size membranes are used, monocytes 5 microns, macrophages 5 or 8 microns and lymphocytes usually 8 microns. The membranes are either thick or thin.

2.4.4.2.1 Thick membranes:

The thickness of these membranes are of the order of 150

microns and measurement of the distance travelled by the leading front of cells is usually determined. This is done by focusing through the mounted membrane and the distance is determined by a micrometer attached to the focusing apparatus of the microscope. Some workers use the leading front as well as determining the total number of cells that have migrated to a given distance through the membrane. The thick membranes allow development of a gradient across their thickness but do not allow morphological appreciation of the cells and it is imperative that only pure populations of cells be studied.

2.4.4.2.2 Thin membranes:

These are of the order of 10-12 microns thick and measurement of the numbers of cells that have migrated to the undersurface of the membrane is made. This system allows appreciation of morphology and in a mixed population a reliable differential count can be made. However some authorities believe that the thickness of these membranes is not sufficient to allow the development of a gradient (Wilkinson, 1985). Another criticism against thin membranes is the possibility that the faster cells may drop off once they reach the undersurface making cell counts unreliable. To get over this problem some workers have deployed membranes without a wetting agent (polyvinyl-pyrrolidone-free membranes) and have found that cells do not drop off (Harvath, 1980). Others have employed a double membrane system where a cell-impermeable membrane (pore size 0.20 - 0.45 microns) is

placed below these membranes and have counted cells on both membranes. Apart from direct microscopic counting of cells various ingenious methods including image analysis (Falk, 1978), enzyme estimation (Mroweitz, 1986) or radiolabelling of cells (Gallin, 1978) have been used.

2.4.4.2.3 Chambers used in membrane assays:

Since the introduction of Boyden chambers in 1962, several chambers have been described and a brief description of the one we have used will be given here. This 48-well microchemotaxis chamber was produced by Neuroprobe and described by Falk et al (1978). It consists of two acrylic plates (see Plate 2.5). The lower plate consists of 25 mcl wells for chemotaxins, on which the membrane (25 mm x 80 mm) is laid followed by the gasket before screwing the top plate in position. Once this is in place it forms the upper wells which are 50 mcl in volume. This chamber has a number of advantages including economy in cells and chemotaxins, ability to test many chemotaxins or different samples of cells simultaneously and reducing the variability in filters which is often a problem in Boyden chambers. Because many samples can be examined simultaneously, this enables better standardization of the microenvironment.

2.4.4.3 Checkerboard assays:

This is not a separate method but rather a protocol often

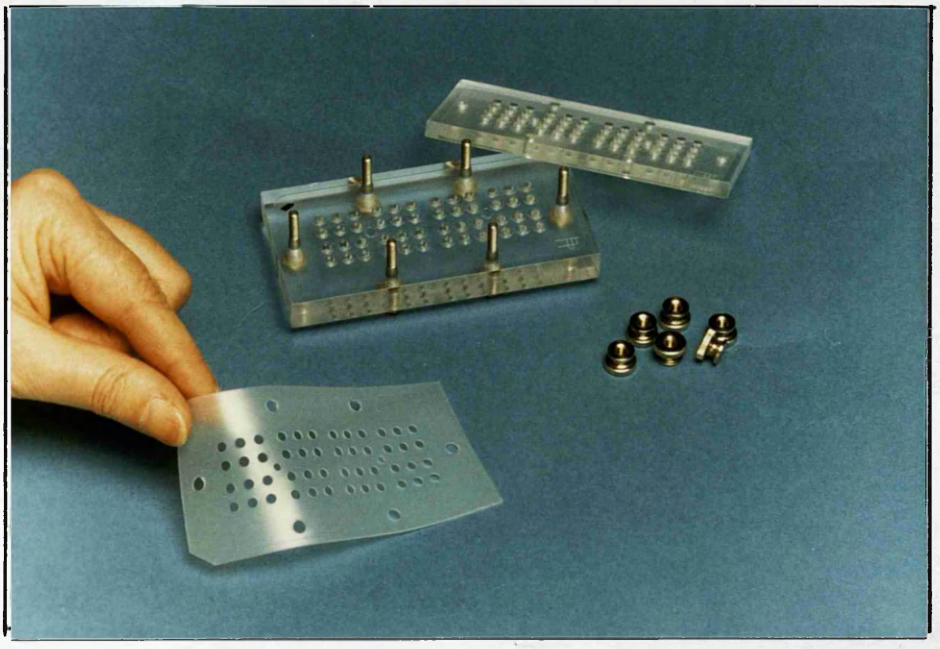


Plate 2.5: 48-well microchemotaxis chamber.

used in the membrane assays in an attempt to differentiate chemotactic from chemokinetic responses. The protocol involves setting up of different gradients across a number of chambers in two directions so that the chemotaxin concerned is both below and above the membranes. Thus a positive gradient (higher concentration below) a negative gradient (higher concentration above) or zero gradient (equal concentrations) can be achieved. In this way a chemokinetic or chemotactic response can be implied, since a kinetic response can be observed even in the absence of a gradient.

2.4.4.4 The under-agarose assay:

This system consists of a series of three wells punched out in an agarose gel. Cells are placed in the middle well, and a chemotaxin or control medium is placed in each of the other two wells. The cells crawl under the agarose towards the chemotaxins and the distance (A) migrated by the leading front can be measured. Subtraction of the distance (B) migrated by the cells towards the control medium gives rise to the chemotaxis differential (A-B). In the original description of this method, albumin or serum were incorporated into the agarose and Chenowith (1979) modified this by substituting gelatin for serum. This assay also offers cell and chemotaxin economy and works well for PMN. Despite the authors' claim that this assay works for monocytes many workers have had no success with it in monocyte chemotaxis (Wilkinson, 1985). Very few studies have used this method successfully in AM

migration. Some workers have succeeded in studying chemotactic responses by filming the under surface (Wilkinson, 1982) but on the whole this method is used mainly in studying populations of cells. Attempts at setting up checkerboard assays have been met with limited success.

2.4.4.5 The skin window technique:

This in vivo method involves scraping an area of skin on the forearm with a scalpel or abrasing it with a high speed drill until the dermal papillae are evident. The chemotaxin under test is applied and the cells migrating to the area are collected in a coverslip or a collecting chamber and counted. The main pitfall of this method is that it does not distinguish between cell accumulation and cell migration. The former can be due to vascular injury or change in permeability.

2.4.5 Problems in Monocyte and Macrophage Chemotaxis

Locomotion of mononuclear cells is less well studied and their responses less well understood. This is mainly because of the technical problems involved in their study. Among these is the difficulty of obtaining pure cell populations in sufficient quantities. Many macrophage enrichment methods involve an adhesion procedure following which non-adherent cells are washed off. The problem then arises of removing adherent cells for locomotion studies without damaging them.

Again because these cells are adherent most of the studies have had to depend on membrane assays which are not adequate in elucidating cell responses. Finally it is becoming increasingly clear that macrophages are a heterogenous population even those from a single site such as the lung. Whether a subpopulation is concerned principally with locomotion is not yet known.

2.4.6 Methodological details of assays used

2.4.6.1 Under-agarose assay:

Before assessment of AM migration using this assay PMN (2.5×10^7/ml) and PBMC from healthy donors were used. Cells were prepared as previously described. Agarose-gelatin (final concentrations 1% - 0.25% respectively) in minimal essential medium (MEM) was prepared as detailed in Appendix I. Unstimulated migration as well as migration towards zymosan activated serum (ZAS), FMLP 10^{-5}M and E.coli supernatant (all prepared as detailed in the Appendix) was assessed. 10 ul volumes of cells or chemotaxins were placed in the inner wells or outer wells respectively and were incubated at 37°C in an atmosphere containing humidified 5% CO_2 for 4 hours (PMN) or 18 hours (PBMC). Cells were fixed by covering with 5 mls methanol for 30 minutes followed by 10% buffered formalin for another 30 minutes. The agarose gel was removed and the plates were stained with Leishman's stain before examining under an inverted microscope. The linear distances

migrated by the leading edge of cells towards the chemotaxin (stimulated migration - A) and towards the medium (unstimulated migration - B) were measured by a micrometer (units) attached to the microscope eyepiece. Chemotactic differential (CD) was obtained by subtracting B from A.

2.4.6.2 AM chemotaxis using under-agarose method

BAL cells were prepared as previously described and were suspended in medium 199 containing antibiotics and concentration was adjusted to 5 x 10^6/ml and 2.5 x 10^6/ml. The remaining part of the procedure was as described above apart from incubation period which was 18 hours initially and later 24 hours. No migration was achieved and it was clear that only the few contaminant PMN had actually migrated. The failure of AM migration was persistent with all above chemotaxins despite preparation of fresh agarose plates and rechecking the pH media etc. Six experiments were performed before the procedure was finally abandoned.

2.4.6.3 Microchemotaxis chamber:

The 48-well microchemotaxis chamber was used instead of the standard Boyden chambers because of cell economy. Macrophages were enriched by density gradient as described earlier and resuspended in medium 199 containing antibiotics and concentration was adjusted to 1 x 10^6 cells/ml (minimum viable cells 8 x 10^5/ml). This concentration was found

optimal (See Table 2.6(a). Unstimulated migration (UM) and stimulated migration towards ZAS, casein and FMLP was assessed (See Tables 2.6(b,c & d) and Figures 2.3 a,b,c). 25 ul volumes of medium 199 (for U.M.) or a chemotaxin (for SM) were placed in the lower wells. A 10 u thick 8 u pore size polycarbonate membrane (25 mm x 80 mm) was carefully laid on the lower wells, followed by the gasket and the top plate was screwed in position. The upper wells were covered with a microscope slide and the chamber was incubated at 37^{o}C in an atmosphere containing humidified 5% CO_2 in air, for 10 minutes to allow temperature stabilization. The upper wells were filled with 50 ul volumes of cells and the chamber was incubated in the same environment for 4 hours before removing the membrane, carefully wiping the top surface cells (avoiding the disturbance of the undersurface cells which have migrated through the membrane). The membrane was air-dried overnight before fixing the cells for 2 minutes in methanol for Leishman's stain or for 1 minute in citrate-acetone-methanol mixture for esterase stain. Leishman's staining was done by immersing the membrane in undiluted stain for 3 minutes followed by 50% diluted stain for 5 minutes before rinsing the excess stain in water. Esterase stain (20 minutes incubation) was performed according to previously described method. The membrane was mounted (spirit based for Leishman's and aqueous based for esterase) on a double width microscope slide (50 mm x 76 mm) before examination under the microscope. Cells in 5 fields (magnification x 400) from each well were counted and a mean of 3 wells was used for UM or SM towards each chemotaxin.

For PMN chemotaxis cells were suspended in gel Hanks and 3u pore size membrane was used with an incubation period of 30 minutes. For monocytes 5 u pore size membranes with 45 minutes incubation.

Experiments were performed to determine optimum concentration of cells and chemotaxins. Tables 2.6(a-d) show the results of experiments using different concentrations of cells and chemotaxins.

Table 2.6(a): Macrophage migration, with variable cell concentration (mean of 3 wells x 400 n = 4).

Concentration of cells x 10^5		5	7.5	10	25	50
Unstimulated Migration	Mean	15.6	21	24.4	27.5	39.6
	(SD)	(6)	(9.8)	(13.7)	(9.1)	(14.0)
Casein 10 mgs/ml	Mean	20.3	30.6	32	59	71
	(SD)	(2.1)	(7)	(11.3)	(36.3)	(52.4)
ZAS	Mean	49.6	54	55.6	85.6	92.3
	(SD)	(27.2)	(27.5)	(35)	(55.9)	(60.5)

Increases in the cell concentration showed that there was a higher but disproportionate increase in cells migrating through to the undersurface of the membrane. For ease of counting and "cell economy" it was decided to use a concentration of 1 x 10^6 cells/ml for the study. These results are also shown in Figure 2.2.

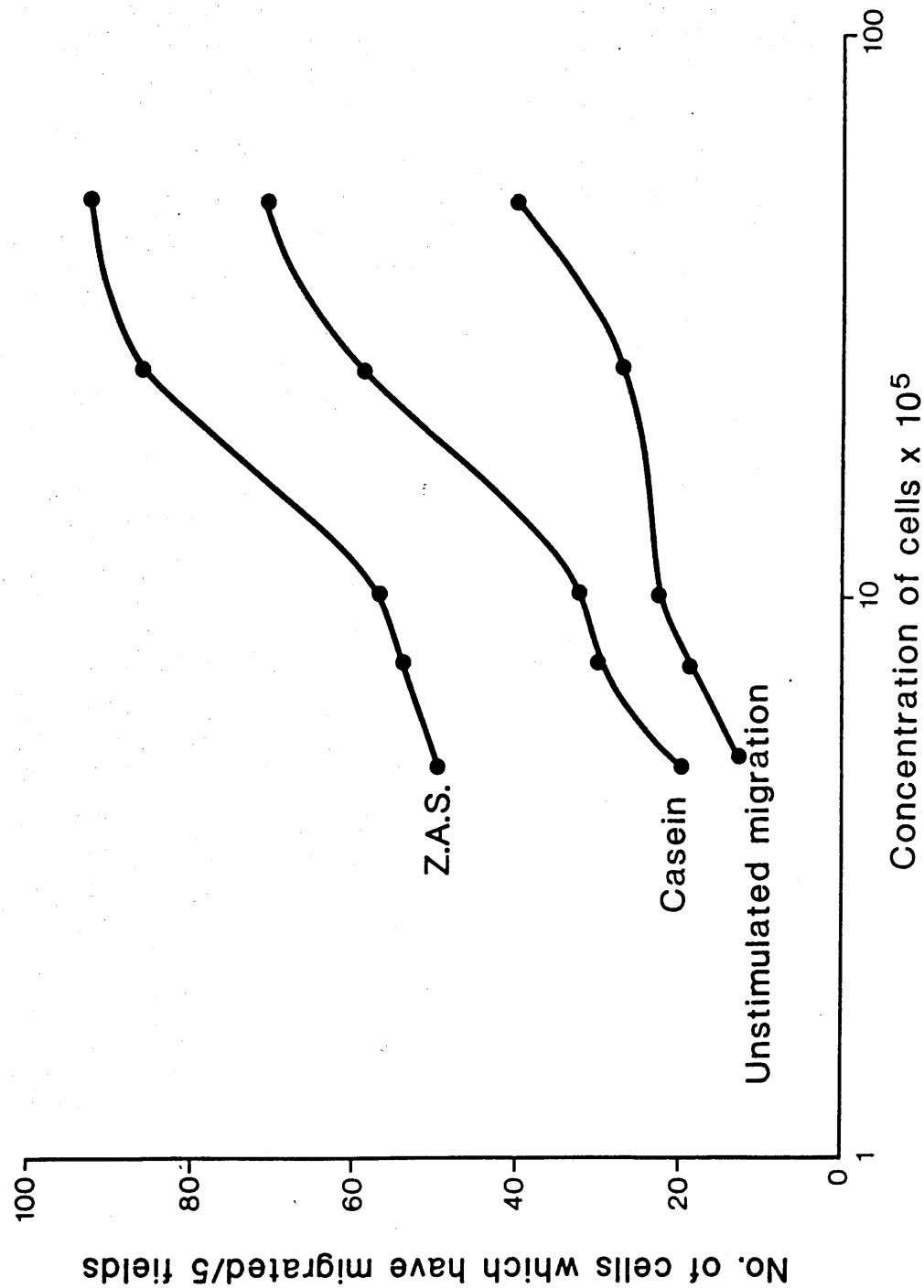


Fig. 2.2: Effect of varying BAL cell concentration on AM migration.

Table 2.6(b): Migration of AM towards different concentrations of casein.

	Concentration casein mg/ml				
n = 4	1.5	3	6	10	20
Mean (SD) No. of cells/ 5 fields	48 (7.0)	55.8 (17.4)	45.8 (16.0)	62 (34.9)	46.8 (10.7)

There was no definite dose response to different concentrations of casein. However the concentration of 10 mgs/ml gave the maximum response and this concentration was used for the study.

Table 2.6(c): Migration of AM towards various concentrations of ZAS.

	ZAS Dilution Factor					
n = 3	1 (Neat)	2	4	8	16	32
Mean (SD) cells/5 fields (mean of 3 wells)	49.6 (1.5)	56 (18.5)	74.8 (40.3)	74.3 (20.7)	61.6 (21.7)	47.6 (15.0)

AM migration towards ZAS showed a dose-response which peaked at a four-fold dilution. However it was decided to use undiluted ZAS for the study because of ease of cell counting.

Table 2.6(d): Migration of AM towards various concentrations of FMLP.

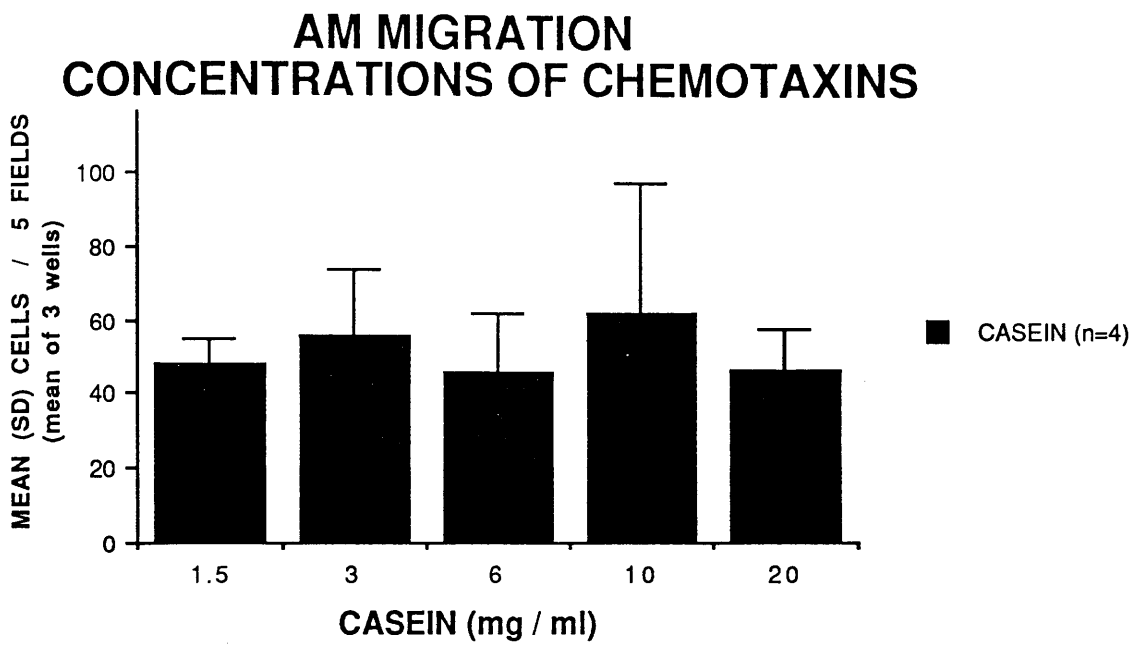
	Molar Concentration of FMLP						
n = 3	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}
Mean (SD) Cells/5 fields (mean of 3 wells)	27.0 (9.6)	34.6 (17.2)	31.3 (9.5)	33.3 (2.5)	35 (6.2)	41.6 (14.2)	25.6 (5.7)

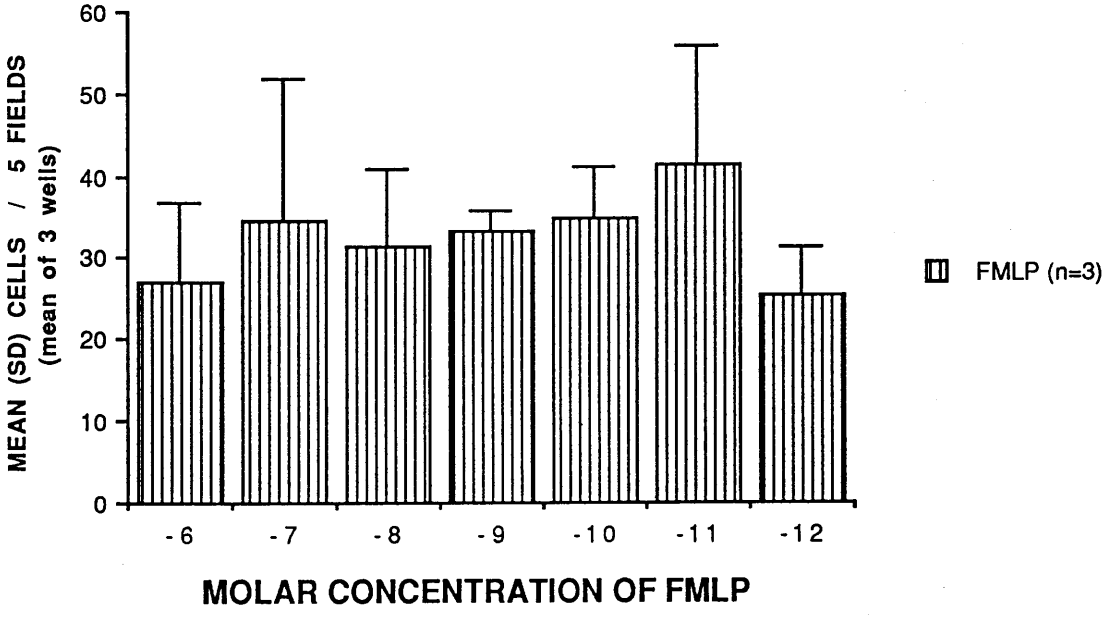
No definite dose-response curve was obtained but there was a major peak at a concentration of 10^{-11}M and a minor one at 10^{-7}. A concentration of 10^{-7} was used in the study.

The results of AM migration towards varying concentrations of chemotaxins are presented in Figure 2.3(a-c) Intrasubject variability of the migration data was assessed using cells from 3 control patients. Measurement of unstimulated migration and migration towards ZAS was done using six wells in each case. Table 2.7 shows the number of cells in each well and the means, standard deviation and coefficient of variation for each set of six wells.

Some variability is seen between different wells but on the whole, measurement of macrophage migration using microchemotaxis chamber is fairly well reproducible.

Comparison of esterase and Leishman's staining of cells which have migrated through the membranes was done in 5 control subjects and 4 patients with smoke inhalation injury.





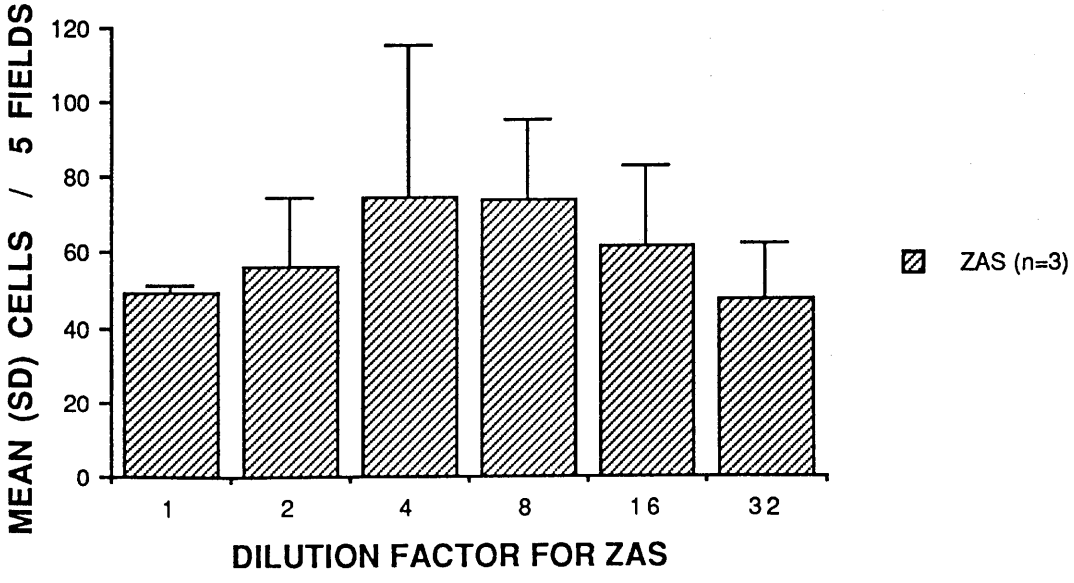


Fig. 2.3(a-c): Effect of varying concentrations of chemotaxins on AM migration.

These data are conveniently presented here (Tables 2.8(a) and (b)) (Plates 2.6a&b).

Table 2.7: Variability between wells in cells which have migrated through the membrane.

	ZAS			Unstimulated Migration		
Exp. No./ Well No.	1	2	3	1	2	3
1	46	30	47	35	21	33
2	48	52	42	40	24	37
3	50	40	38	35	23	36
4	54	48	35	27	39	27
5	38	61	49	37	38	30
6	64	38	36	51	40	26
Mean	50	44.8	41.2	37.5	30.8	31.5
S.D.	8.67	11	5.8	7.9	9	4.6
Coefficient of variation	0.17	0.24	0.17	0.21	0.29	0.14

Table 2.8(a): Comparison of Leishman's and esterase staining of chemotaxis membranes (control subjects, n=5)

	Mean (SD) cells /5 fields			
	Unstimulated	Casein	ZAS	FMLP
Leishman's stain	31.4(10.4)	31.6(16.0)	50.2(16.6)	39.8(17.1)
Esterase stain	31.0(12.3)	32.0(18.5)	52.6(17.2)	33.8(17.3)
r_s value	0.80	0.90	0.9211	0.80

r_s = Spearman rank correlation.

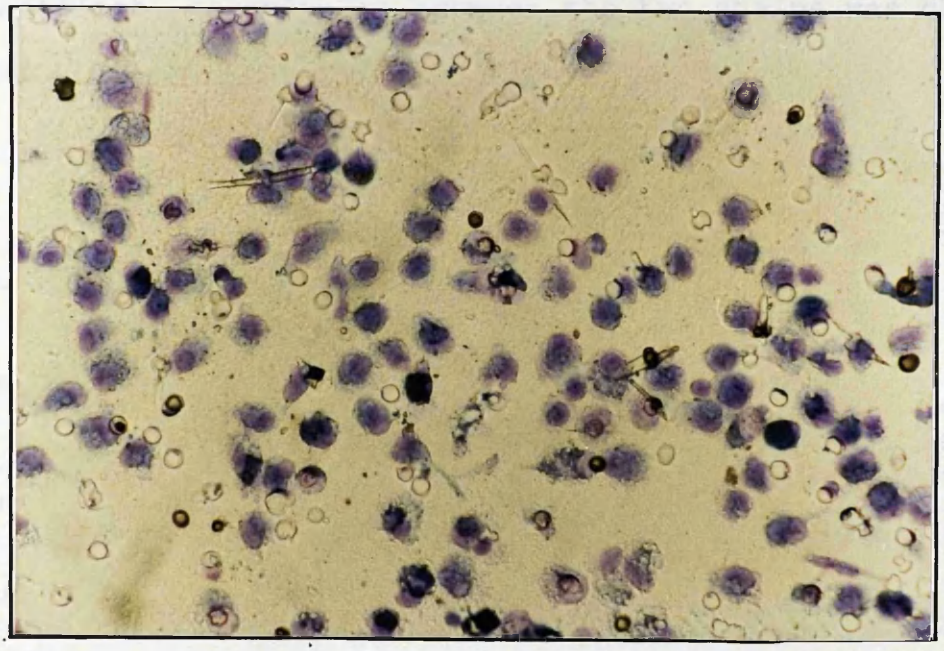


Plate 2.6(a): Leishman's stain of BAL cells from a patient with smoke inhalation and burns injury.

Chemotaxis membrane

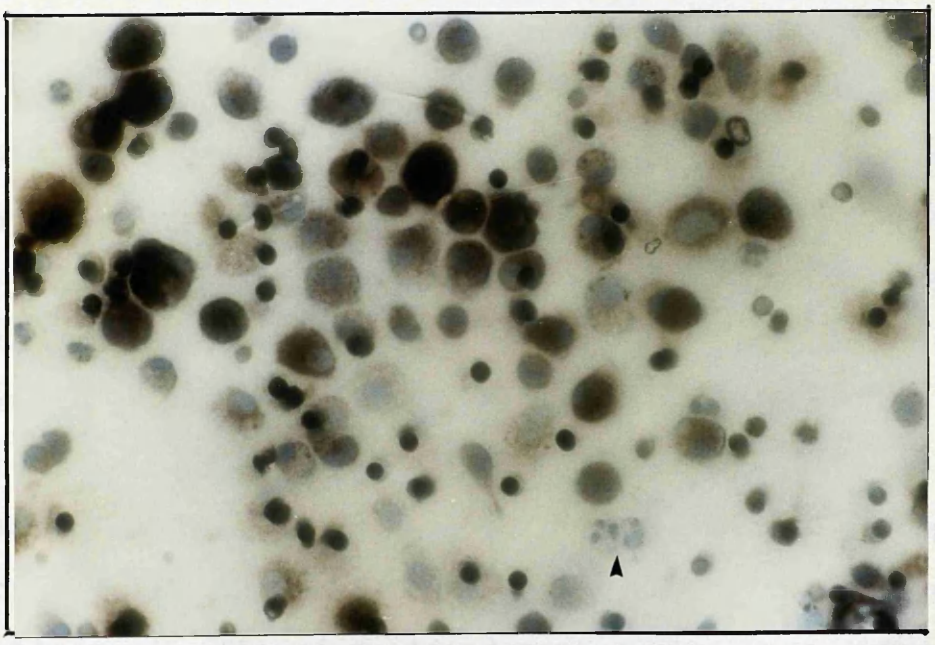


Plate 2.6(b): Esterase stain of BAL cells from a patient with smoke inhalation injury. A few PMN can be seen (arrow).

Chemotaxis membrane

Excellent correlation between the two stains was obtained in samples from 5 control subjects.

Table 2.8(b): Comparison of Leishman's and esterase staining of chemotaxis membranes (smoke inhalation patients (n = 4)).

	Mean (SD) cells / 5 fields			
	Unstimulated	Casein	ZAS	FMLP
Leishman's stain	67.3(29.3)	91.8(34.4)	246.0(148.7)	66.0(47.4)
Esterase stain	63.0(35.4)	103.8(45.1)	274.5(136.6)	68.0(42.2)
r_s	0.971	0.907	0.992	0.996

r_s = Spearman rank correlation.

A good correlation was obtained between the two stains although there was a tendency to higher numbers of positive cells in the wells with high migration counts (such as ZAS) than the corresponding number of macrophages in the Leishman's stain. This could be due to the presence of monocytes which are more easily recognized with esterase stain than with the Leishman's. Alternatively this could be due to an artefact resulting from non-specific staining of the esterase negative cells by the intense esterase stain originating from the numerous surrounding macrophages.

The effect of neutrophils on migration of macrophages was studied on cells from two control patients. Increasing proportions (5-60%) of purified neutrophils were mixed with purified macrophages. Migration of these cells towards ZAS

was measured (Table 2.9).

Table 2.9: Effect of neutrophils on migration of AM through polycarbonate filters: (Mean cells/5 fields).

	Macrophage content (%) of cell mixtures							
	99	95	90	80	70	60	50	40
Patient 1	50	41	42	37	23	18	14	12
Patient 2	55	49	40	38	39	24	21	10

Increasing proportions of neutrophils in the cell mixtures resulted in progressive reduction of macrophages which migrated through the membranes. This could be due to the fact that neutrophils migrate faster and they physically compete with the macrophages through the pores, or it could be due to the injurious effect of neutrophils on macrophage migration. Whatever the underlying cause may be, it is clear that substantial neutrophil contamination has an effect of underestimating the true macrophage migration.

2.5 Chemiluminescence

2.5.1 The principle of chemiluminescence (CL):

Light emission by phagocytes was first noted by Allen (1972) in a PMN-bacterial phagocytosis system. Later he

described luminol-dependent CL in peritoneal and alveolar macrophages. The precise mechanism by which photon emission demonstrable as CL is not clear. Allen proposed that PMN non luminol-dependent CL (unamplified CL) was due to relaxation to ground state of electronically excitable carbonyl groups released during the oxidative respiratory burst. Later Cheson et al. (1976) suggested that this might be due to reaction between oxidative metabolism of leucocytes and bacterial products. Whatever the exact mechanism involved there is a strong evidence identifying the importance of the myeloperoxidase-halide-hydrogen peroxide system. The evidence lies firstly in the observation that CL of normal PMN can be inhibited by catalase (H_2O_2 scavenger), superoxide dismutase (superoxide scavenger) and sodium azide (myeloperoxidase inhibitor). Secondly, neutrophils deficient of myeloperoxidase have been found to be deficient in CL activity (Allen, 1975). CL has been shown to be quantitatively proportional to bacterial and neutrophil populations in phagocytic systems and also to the concentration of luminol in luminol-dependent CL (Quie, 1982). Non phagocytic membrane stimulation by soluble stimulants such as phorbol myristate acetate (PMA) and FMLP or adherence of phagocytes to vial surfaces, has been shown to produce CL similar to phagocytic stimulation. The pattern of PMN-CL produced by soluble stimulants was recently defined. Luminol-dependent CL was found to have bimodal peaks; an early one during the first two minutes and a second one at 10 minutes. Using cell-free and cell-containing oxidation systems with or without

inhibition of CL it was possible to attribute these peaks to extracellular (first peak) or intracellular (second peak) CL activity (Brieheim, 1984). The diffusion of luminol to the intracellular compartment was thought necessary for the production of the second peak. The finding in some later work (Dahlgren, 1985) that lucigenin, a substrate with a large molecular weight, thought incapable of diffusing into the cell is associated with only one (first) peak is in keeping with this concept.

2.5.2 CL of Alveolar Macrophages:

In a study of metabolic activity of peritoneal and alveolar macrophages, Allen (1976) failed to elicit any chemiluminescent response from these phagocytes either in a resting state or following phagocytosis. However addition of luminol elicited a strong CL which was inhibited by addition of superoxide dismutase (superoxide scavenger) and sodium benzoate (hydroxyl radical scavenger). Production of CL in this system was proposed to be due to relaxation to ground state of electronically excited aminophthalate anion molecule produced during the phagocytic process. Further work by Williams et al. (1981a) revealed that luminol-dependent CL activity of bronchoalveolar cells correlated well with their PMN content and that human alveolar macrophages produced little or no luminol-dependent CL. This was attributed to low peroxidase activity of the AM. These workers also found that alveolar macrophages produce lucigenin-dependent CL of the

order of magnitude produced by PMN (Williams, 1981b). Because this activity was found to be more than 95% suppressed by superoxide dismutase, superoxide radical is implicated in its production.

2.5.3 CL-Method:

BAL cells, PMN and monocytes were prepared as described above and the concentrations adjusted to 2.5 x 10^6/ml, 1 x 10^7/ml and 2.5 x 10^6/ml respectively. For preliminary experiments, the cell concentration was adjusted as required. An overnight growth of Staph aureus 502A in MH broth was washed, its concentration adjusted to 2.5 x 10^8cfu/ml and opsonised in 10% serum. Luminol 10^{-5}M and lucigenin 10^{-4}M previously found to be optimal for PMN and AM respectively were used. A Packard luminometer with a computerised protocol was operated at a stable temperature of 37°C, and was programmed to make 10 second counts at five minute intervals for a total of 70 minutes. 0.1 ml phagocytes were added to 4 luminometer tubes placed in luminometer chambers before adding bacteria 0.05 mls (phagocytes + bacteria in 2 tubes), followed by the addition of luminol or lucigenin 0.05 mls. In 2 tubes with phagocytes only (spontaneous CL) gel Hanks was added instead of bacteria. A printout is obtained at the end of the experiment, and peak counts were used for analysis. Experiments were performed to determine optimum cell concentration and reproducibility of chemiluminescence data.

2.5.4 Effect of varying concentration of cells in chemiluminescence of AM.

The intensity of the chemiluminescent response of a phagocytic cell exposed to a particulate stimulus such as Staphylococcus aureus depends to some extent on the particulate:cell (bacteria:cell) ratio. To determine the optimum bacteria:cell ratio, varying concentrations of cells were used against a constant bacterial concentration with an optical density of 0.45 (approximately 2.5 x 10^8 cfu/ml).

Table 2.10: Effect of varying bacteria:AM ratio on the chemiluminescent response.

n = 4		Mean (SD) counts/sec/10^3 cells	
Cellsx10^5/ 0.1 ml	Bact:cell	Lucigenin	Luminol
10	12.5:1	2.73 (1.86)	0.51 (0.57)
5	25:1	13.70 (10.96)	2.1 (3)
2.5	50:1	28.64 (24.4)	4.34 (5.70)
1	125:1	26.60 (11.55)	N.D.

ND = Not done.

For both luminol and lucigenin there was an increase in CL response with the higher bacteria:cell ratios. On that basis a concentration of 2.5 x 10^5 AM/0.1 ml was used for the study.

The reproducibility of chemiluminescence was tested using

mononuclear cells with luminol as a substrate (n = 3) and using alveolar macrophages with luminol (n = 5) and lucigenin (n = 4) as substrates. Table 2.11(a&b) shows the

Table 2.11(a): Variability in luminol-dependent chemiluminescence of mononuclear cells and AM.

	Counts/second/10^3 cells							
	Mononuclear cells + Luminol			AM + Luminol				
Exp. No.	1	2	3	1	2	3	4	5
Range From	195	129.2	26.5	3.84	0.029	11.92	2.52	1.21
to	223	242	50	4.03	0.035	12.8	2.76	1.36
Replicates	5	10	9	5	3	2	2	3
Mean	203	187.7	39.7	3.95	0.031	12.39	2.6	1.29
S.D.	10.6	39.5	6.99	0.09	0.003	0.66	0.14	0.07
Coeff. Var.	0.05	0.21	0.17	0.02	0.10	0.05	0.05	0.05

Table 2.11(b): Variability of Lucigenin dependent CL of AM. (All experiments were done in duplicates)

Exp. No.	Counts/second/10^3 cells 1	2	3	4	5
Range	120-150	4.30-4.58	8.68-9.16	30-30	160-170
Mean	135	4.44	8.92	30	165
S.D.	21.2	0.19	0.33	0	7.07
Coefficient Variation	0.16	0.04	0.04	0	0.04

range, mean, standard deviation of counts/second/x 10^3 cells and coefficient of variation in each experiment.

These results show that while there is an enormous intersubject variability, there is little intrasubject variability and the test is highly reproducible.

Finally the effect of neutrophils on AM lucigenin dependent chemiluminescence was studied in 3 samples. Purified preparations of AM were mixed with increasing proportions of neutrophils before measuring Staphylococcus aureus stimulated lucigenin-dependent chemiluminescence (Table 2.12). Neutrophil lucigenin-dependent chemiluminescence was of the same order as that of AM (data not shown).

Table 2.12: Effect of neutrophils on lucigenin-dependent chemiluminescence of AM.

		Counts/second/10^3 cells							
		% Neutrophils added							
Sample No.	*M(%)	0	5	10	20	30	40	50	60
1	84.4	110	90	110	170	120	ND	ND	ND
2	98	140	150	150	150	210	140	100	170
3	92	100	100	100	100	90	100	ND	ND
Mean		116.6	113.3	120	140	143.3	120	100	170
S.D.		20.8	32.1	26.4	41.6	58.5	28.2	-	-

ND = Not done.

*M(%) = Percentage of macrophages: before addition of PMN.

These results suggest that mixing of neutrophils with AM does not have an adverse effect on lucigenin-dependent chemiluminescence of AM.

2.6 Monoclonal markers

2.6.1 Introduction and historical background:

Labelling of antibody with fluorescent dye was first achieved by Albert Coons and his colleagues in 1941. Since then various labels including fluorescein dyes such as fluorescein or rhodamine isothiocyanate; enzymes such as peroxidase (Nakane & Pierce, 1966), alkaline phosphatase (Mason & Summons, 1976) have been widely used. The technique of indirect linking of a label to the antibody, exploitation of strong attraction between avidin and biotin and linking of enzymes to electron dense materials such as colloidal gold or radioactive materials have greatly increased the sensitivity of these methods. Early monoclonal antibody work was hampered by limitation in production techniques and lack of specificity. The major breakthrough in this field came with the publication by Kohler and Milstein (1975) of their method of production of monoclonal antibodies of predetermined specificity by culture of spleen cells "immortalized" by fusion with myeloma cells.

2.6.2 Use of Monoclonal Markers in BAL Cells:

T-cell markers have been widely used and substantial data in various disease states are now available. The relevance of these data to the clinical situation has been discussed

earlier and will not be repeated. Monocyte/macrophage lineage cell markers have been used less extensively than T-cell markers and their role in clinical practice is still uncertain. Campbell et al (1986) have applied these to BAL cells from patients with interstitial lung diseases and found quantitative differences in subpopulations of alveolar macrophages, as identified by different markers, between patients and control groups. A similar approach has been employed by other workers (Hance et al). It was therefore regarded reasonable to explore this approach in the investigation of acute lung injury.

2.6.3 Monoclonal Markers - Methodology:

Two methods were assessed involving either labelling of cells in suspension or after fixation on glass slides.

2.6.3.1 Labelling of Cells in Suspension:

BAL cells were prepared from an original aliquot which was cleared of mucus (sieved if necessary), washed in PBS twice before adjusting the concentration to 5×10^6 cells/ml. A 5 ul aliquot of primary antibody was added to 200 ul of BAL cells (1×10^6) and was left standing on ice for 30 minutes (gently mixed every 10 minutes). Cells were washed by centrifuging at 200g at $4^{o}C$ for 5 minutes and resuspending the cells in PBS twice before finally discarding the cell free supernatant leaving behind the cell pellet suspended in about

100 ul PBS. 100 ul of appropriate secondary antibody (goat antimouse-FITC) was added to the cells and placed on ice for 30 minutes before washing in PBS twice and resuspending the cells in 1 ml of PBS. Cells were examined as wet preparations, by flow cytometry (FACS analyser) or cytospins.

2.6.3.1.1 Wet preparation:

15 ul aliquot of cells suspended in PBS placed on a microscope slide was covered with a cover slip (24 mm x 24 mm) and was examined under a fluorescent microscope. All cells in a field were counted using a phase contrast and positive cells from the same field were recognised by their lemon green fluorescent ring when examined under fluorescence. This method was technically acceptable apart from nonspecific staining of dead cells which had a characteristic appearance. However this method had to be abandoned because it was very labour intensive since each preparation needed at least 20 minutes of microscopy.

2.6.3.1.2 Cytospins:

These were fixed in formalin and stained in 0.1% methylene green for 45 seconds, and mounted with PBS/glycerol and coverslip edges sealed with a moist varnish. The nuclei appear red when examined under a filter of the fluorescent microscope, and positive cells were recognised by the fluorescent ring. Examination of cytospins was much less

labour intensive compared to the wet preparations but "smudging" and widespread nonspecific background fluorescent staining was a major problem that rendered the technique unsuitable.

2.6.3.1.3 Use of FACS analyser:

At Glasgow Royal Infirmary, this equipment has been set up at the Leukaemia Research Institute for diagnosis and follow-up of hematological malignancies. With the help of the senior technician in charge, counting of labelled BAL cells was assessed. The following problems were encountered and this method was abandoned:

i) Alveolar macrophages often showed spontaneous autofluorescence of high intensity which precluded identification of positive cells from pseudo-positive cells. Indeed some of unlabelled specimens showed between 40-45% positive cells! This problem as mentioned earlier has been experienced by other workers (Hance, 1985).

ii) "Gating" of small cells such as lymphocytes could not be achieved adequately because of their small numbers. Superimposition of sizes of small macrophages, monocytes (MO_1 or MO_2 positive cells) and large lymphocytes was another problem.

iii) Because the very nature of this machine involves flow of

cells in "single file" through the very small orifice (maximum size 100 u) BAL fluid preparations often resulted in blockage of the channel, necessitating elaborte cleaning procedures.

2.6.3.2 Labelling of cells on cytospins:

Both immunoperoxidase (IPX) and immunoalkaline phosphatase (IAP) methods were assessed but the former was soon abandoned because of non-specific peroxidase activity of macrophages from specimens of heavy smokers. A resume of these methods is given overleaf.

2.6.3.2.1 IAP staining:

Cytospins were prepared from the original aliquot of cells on microscope slides covered with a thin layer of polylysine to enhance sticking of the cells, before fixing in chloroform:acetone (1:1) mixture for 5 minutes. These were air dried for at least one hour, circled with a diamond cutter and stored at -20^{o}C in slide carriers wrapped in cling film until they were stained. Preliminary experiments were performed on cryostat tonsillar sections to determine appropriate dilutions of primary antibody (see Appendix I). Labelled slides were placed on a flat (spirit levelled) surface in a humidified chamber and cytospins were covered with 75 ul of 1/5 normal rabbit serum for 15 minutes. After this excess serum was discarded and the cells were uniformly covered by a 50-75 ul

LABELLING CELLS ON CYTOSPIN PREPARATIONS

IMMUNOPEROXIDASE		IMMUNOALKALINE PHOSPATASE	
75 ul normal sheep serum) 1/5 in tris-buffer)	15 min.	75 ul Normal Rabbit Serum) 1/5 in tris-buffer)	15 min
Primary Ab. 50-75 ul)	2 hrs	Primary Ab 50-75 ul)	2 hrs
Wash tris-saline	2 minutes	Wash tris-saline	2 mins
	Double Staining Technique		
Sheep-Antimouse HRP conjugate)	45 mins	Rabbit Anti-mouse AP-conjugate)	1 hr
Wash tris-saline	2 minutes	Wash tris-saline	2 mins
DAB	10 minutes	Solution A AB-Substrate)	15 mins
Tap water	1 minute	Tap water	2 minute
Haematoxylin	30-60 sec.		
Tap water	10 sec.	Haematoxylin	30-60 sec.
STWS	10 sec.	Tap water	10 sec.
Tap water		STWS	10 sec.
Water		Tap water	
Microscope check		Microscope check	
	If excess stain		Mount or repeat stain if needed
Acid Alcohol			
Industrial Alcohol	Acid Alcohol		
Xylene X2			
Mount Harleco Synthetic resin.			

aliquot of the primary antibody for 2 hours. Control cytospins were covered with PBS instead of primary antibody. Cells were gently washed in Tris-saline for 3 minutes, excess Tris saline wiped off, and were covered with 50 ul 1/20 alkaline phosphate conjugated for one hour, before they were washed again in Tris saline for 3 minutes. Freshly prepared solution A was applied for 15 minutes on the cytospins which were then washed in distilled water and counterstained in Gill's haematoxylin for 30-60 seconds. To render nuclei blue, slides were immersed in Scots tap water substitute for 1 minute and placed in warm tap water before mounting. Avoiding any drying of the cells, the cytospins were mounted in glycerol. Cells were examined at 400x and positive cells were recognised by the red staining of the plasma membrane. (Plate 2.7(a)&(b).

2.6.3.2.2 Immunoperoxidase and double staining technique:

IPX is similar to IAP with minor variations as shown schematically (page 139). This method was assessed first but was soon abandoned because of nonspecific peroxidase activity of alveolar macrophages especially from heavy smokers. IAP was found suitable and was adopted as the method of choice in this work. However in samples obtained from areas of consolidation in CAP patients the monocyte marker $UCHM_1$ appeared to be taken up by neutrophils and this was not observed among controls or other patient groups. To obviate this problem a double staining technique was employed.

Specimens were labelled initially with $UCHM_1$ using IPX up to the stage following the application of the chromogen diaminobenzidine (DAB) and then a second antibody HLADR was applied on the same specimen using IAP technique. Cells which were both HLADR and $UCHM_1$ positive showed a mixed colour of brown and red and were regarded as being of true monocyte lineage, thus excluding the granulocytes which took up $UCHM_1$ alone. (Plate 2.8). All the other marophage markers were also tested using the double staining technique but were not found to be taken by neutrophils. Technical help with the double staining method given by Mr J. Richmond, Immunocytochemistry Department, Glasgow Royal Infirmary, is gratefully acknowledged.

2.6.3.3 Problems encountered with IAP and IPX techniques:

i) Wiping off or denudation of cells (?during washing) from the slides was the most common problem. When this happened a fresh specimen was stained. This underlined the need to have sufficient cytospins stored.

ii) Unexplained failure of staining: This problem occurred more commonly with RFD_7 and on a couple of occasions it was attributed to a poor batch of the antibody.

iii) Pale staining of nuclei: This was a problem with T_4 marker which for some reason appeared to interfere with haematoxylin staining of the nuclei. No other marker did

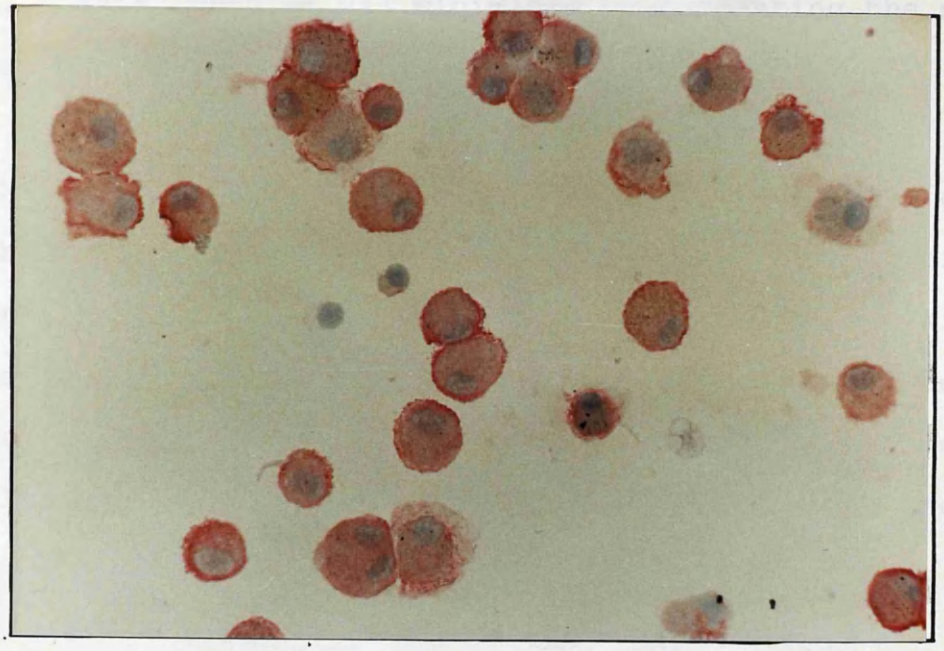


Plate 2.7(a): IAP staining of AM with RFDR marker (HLA-DR) showing that virtually all are positive.

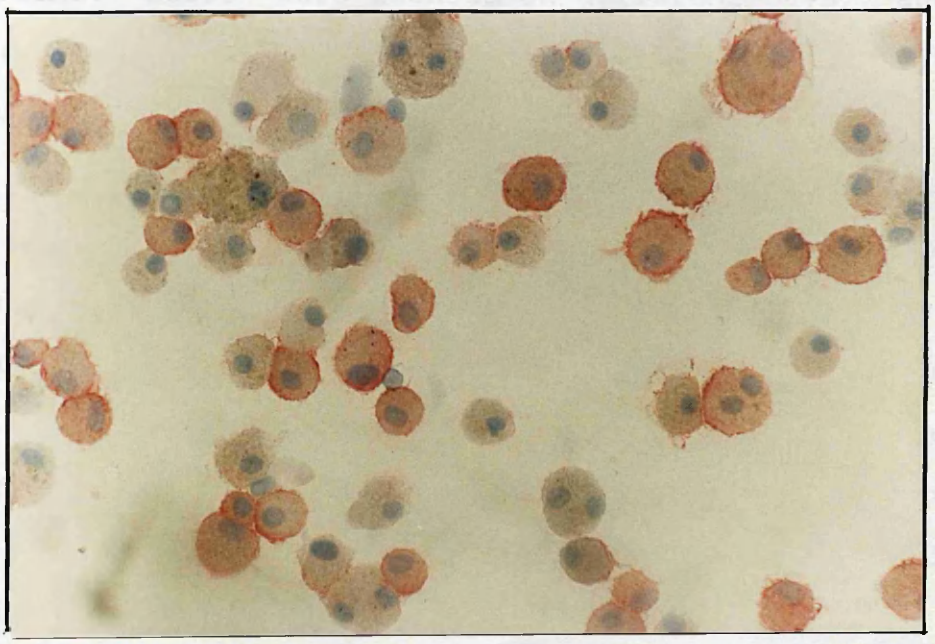


Plate 2.7(b): IAP staining of AM with RFD_9 marker.

this. This problem was minimised by increasing the staining period time to 2 minutes.

iv) Non-specific staining of macrophages: T_4 marker appeared to have this special problem and this has been reported by other workers (Wood et al, 1983). This problem was minimised by diluting the primary antibody in 20% normal human serum instead of tris-buffer, but this procedure unfortunately made problem number (iii) above worse. Morphology of the cells was also helpful.

2.6.3.4 Reproducibility of immunoalkaline phosphatase technique

This was tested by staining cytospin preparations from 3 patients with T_4, T_8, RFD_1 and RFD_9 markers on separate occasions. Cells were counted on different days, and Table 2.13(a) & (b) show the results of each batch of staining. However, the presence of very low numbers of T-cells in the BAL fluid of these patients (cigarette smokers) means that the coefficient of variation is exaggerated. Presentation of the raw data is more helpful (Table 2.13(a)). Plate 2.9 shows two T_8 positive cells from a non-smoking control subject.

These experiments show that while the staining technique is fairly well reproducible when the raw data is examined, the fact that there are such small numbers of cells in each specimen, renders the coefficient of variation between experiments very high. This effect is not seen with staining

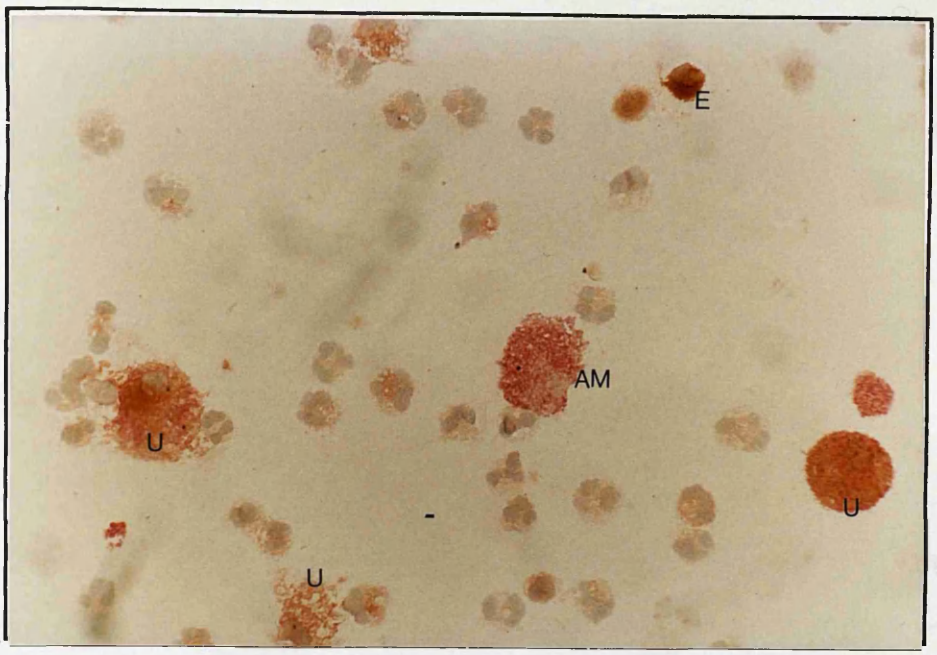


Plate 2.8(a): Shows i) non-specific staining of neutrophils with $UCHM_1$
ii) one AM stained only with $RFDR_1$ marker (AM)
iii) AM stained both by RFDR and $UCHM_1$ (U)
iv) Intense non-specific IPX activity of an eosinophil (E).

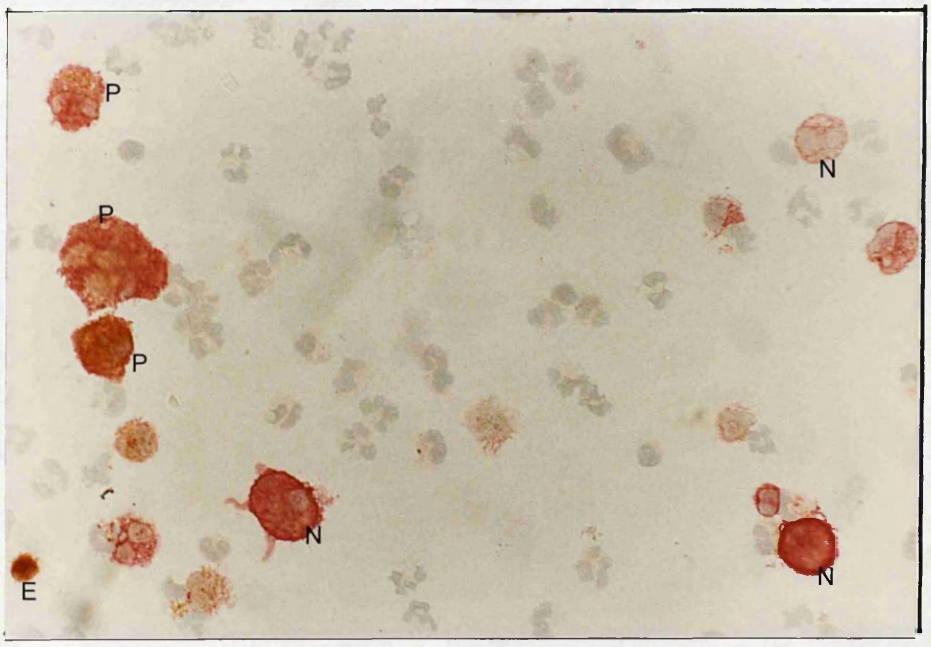


Plate 2.8(b): Double staining with $RFDR_1/RFD_7$. Note
i) $RFDR_1$(HLADR) + AM but RFD_7 - (N)
ii) $RFDR_1$ + /RFD_7 + AM (P)
iii) Neutrophils take up little or no stain unlike Plate 8(a) above.
iv) Intense non-specific peroxidase stain of an Eosinophil (E).

for macrophage markers in BAL where high proportions of cells are positive. Table 2.13(b) presents the results of 4 batches of staining with RFD_1 and RFD_9 markers.

Table 2.13(a): Variability in proportions of T-cells stained by immunoalkaline phosphatase technique.

	No. of positive cells/500 cells (%)					
	Patient 1		Patient 2		Patient 3	
Batch No.	T_4	T_8	T4	T8	T4	T_8
1	1 (0.2)	1 (0.2)	1 (0.2)	5 (1)	0 (0)	0 (0)
2	2 (0.4)	0 (0)	4 (0.8)	9 (1.8)	0 (0)	0 (0)
3	1 (0.2)	0 (0)	2 (0.4)	7 (1.4)	2 (0.4)	1 (0.2)
4	3 (0.6)	1 (0.2)	2 (0.4)	4 (0.8)	0 (0)	0 (0)
Mean	1.75 (0.35)	0.5 (0.1)	2.25 (0.45)	6.25 (1.25)	0.5 (0.1)	0.25 (0.05)
S.D.	0.95 (0.19)	0.57 (1.12)	1.25 (0.25)	2.2 (0.44)	1 (0.2)	0.5 (0.1)
Coeff. Variat.	0.54	1.5	0.56	0.35	2	2

Table 2.13(b): Variation of staining of macrophage markers in BAL on different occasions.

	% positive cells					
	Patient 1		Patient 2		Patient 3	
Batch No.1	D_1	D_9	D_1	D_9	D_1	D_9
1	78.8	68.8	88.0	70.4	85.4	56.8
2	94.4	67.8	97.5	74.8	90.0	56.8
3	85.0	59.0	94.0	59.6	90.0	70.0
4	ND	82.8	ND	67.0	98.4	70.8
Mean	86.1	69.4	93.2	67.9	91.0	63.6
S.D.	7.8	9.48	4.8	6.4	5.4	7.9
Coeff. Variat.	0.09	0.14	0.05	0.09	0.06	0.12

ND = Not done.

These results show that immunoalkaline phosphatase staining with these macrophage markers is fairly reproducible when the same preparation is stained on more than one occasion.

2.7 Measurement of albumin and complement levels in concentrated BAL supernatants:

Measurement of albumin was kindly made by Dr D.S. O'Reilly and his team (D. McLean, J. Cooper and R. Martin) using double label radioimmunoassay (RIA). Complement levels

were measured by Professor Whaley's assistant, Dr. Holmes, using an ELISA assay for all complement fragments except C5a and C3a where a RIA was employed. I am deeply grateful to all of them.

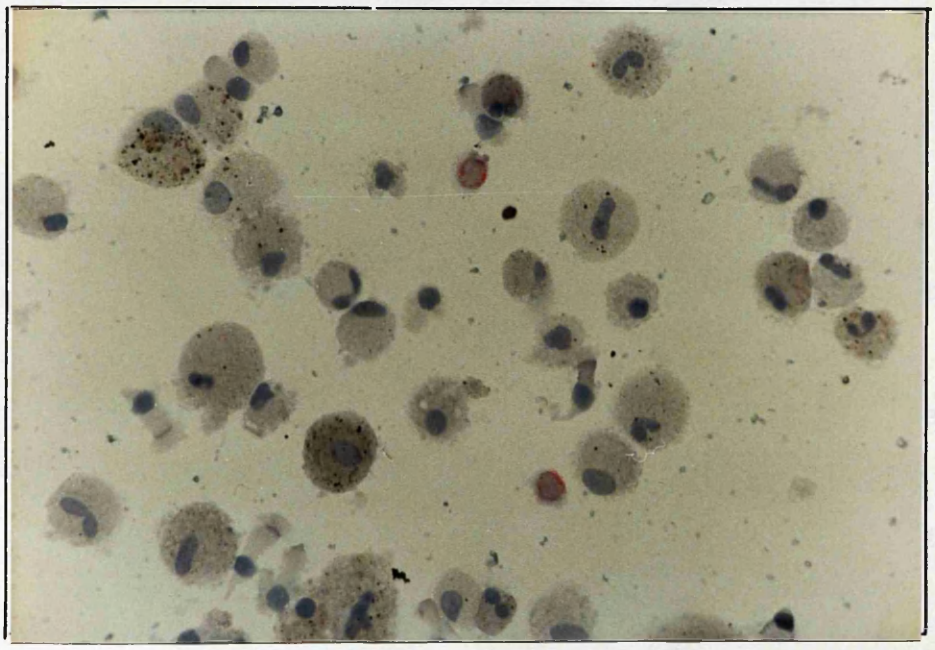


Plate 2.9: T_8 positive cells from a non-smoking control.
(Immunoalkaline phosphatase)

CHAPTER 3

COMMUNITY-ACQUIRED PNEUMONIA (CAP)

3.1 Introduction:

Acute lung injury sustained in pneumonia represents microbiologically triggered injury and is one of the commonest in clinical practice. The reason for choosing this group of patients is evident.

3.2 Aims of the Study:

1. to catalogue cell types and proportions of BAL cells and compare with control subjects.
2. to identify BAL cell profiles according to aetiological agents.
3. to assess complement activation in BAL fluid from these patients.
4. to assess AM function in these patients.

3.3 Definition of Study Groups

3.3.1 Control Subjects (CS):

Because of the lack of normal healthy volunteers it was decided to use patients undergoing bronchoscopy for the following indications as control subjects.

i) Patients with haemoptysis, normal chest X-rays and negative bronchoscopies and other relevant investigations. This group formed the vast majority of control subjects.

ii) Doubtful hilar shadow on chest X-rays with negative bronchoscopy and other relevant investigations.

All patients gave informed written consent.

A total of 36 CS were recruited in the study. Two patients' data were excluded from final analysis: They had elevated calcium and abnormal liver function tests and were presumed to have underlying malignancy. Further details of the 34 control subjects is given in Table 3.1.

3.3.2 Community Acquired Pneumonia:

Patients were recruited from the acute general medical admission ward. The following criteria were applied for inclusion into the study or the final analysis of the results:

i) Clinical and radiological evidence of pneumonia with or without microbiological identification of the aetiological agent.

ii) No known underlying medical condition predisposing to pneumonia including bronchial carcinoma, diabetes, multiple myeloma. None of these patients was on steroid or cytotoxic

therapy.

iii) Age below 70 years.

iv) Tolerability of bronchoscopy: Safety of the procedure was assessed on clinical grounds and arterial blood gases, often by a consultant chest physician. In the few doubtful cases "unbiased" independent advice was sought from another consultant.

v) Informed consent.

vi) Normal bronchoscopic findings other than those attributable to pneumonia.

vii) Complete resolution of chest X-rays to normal at follow-up.

Most of the patients were bronchoscoped within 24 hours of admission and a few within 48 hours. All X-rays were within 24 hours of bronchoscopy. Clinical management of these patients was done by the physician in charge and the decision to resume antibiotic therapy was not influenced by bronchoscopy request. Most of the patients had received one or two doses of antibiotics (usually intravenous ampicillin with or without erythromycin) by the time they were bronchoscoped.

There were no serious complications attributed to

bronchoscopy. One patient died of type 3 pneumococcal pneumonia. 27 patients were recruited in the study but four patients were excluded in the final analysis because of other diagnoses (2 cases pulmonary tuberculosis, 1 case pulmonary embolism and 1 case portal vein thrombosis, deep venous thrombosis and cardiomyopathy).

Of the remaining 23 patients, 13 patients had bilateral lavages, 5 had lavages performed on radiologically clear areas (RCA) and another 5 on areas of consolidation (AOC) only. Further details of these patients is given in Table 3.1.

Table 3.1: Age and sex distribution and smoking history of patients groups.

	Age Range (Mean)	Sex Distribution		Smoking History	
		M	F	N-smokers	Smokers
Control Subjects (n = 34)	30-63 (48.9)	22	11	8	26
CAP Patients (n = 23)	19-65 (47.8)	16	7	2	21

Both in the pneumonia group and control subjects there is male preponderance, and similar to many chest disorders, the influence of smoking is apparent even in these small groups.

The following data was obtained in this study group.

1. Cell yields and differential counts.
2. Monoclonal markers (macrophage and T-cells).
3. AM migration (microchemotaxis chamber).
4. Chemiluminescence.
5. Effect of BAL supernatants on neutrophils from healthy donor.
6. Function of peripheral blood cells from another group of patients with community acquired pneumonia.
7. Albumin and complement levels of the BAL supernatants.

3.4 Results.

3.4.1 BAL fluid volumes, original cell yields and differential counts.

The proportion of fluid volumes recovered between smoking and non-smoking controls and between CAP and CS were similar. However the proportion of fluid recovered from AOC when compared to RCA in CAP patients was significantly lower ($P < 0.02$). This could be due to mucosal oedema in these areas. (Table 3.2(a)).

The cell yield was significantly lower in non-smoking controls when compared to smoking controls or CAP-RCA ($P < 0.05$). Cell yields from RCA in CAP and smoking controls were similar. There was more than a four-fold rise in cell yields from AOC in CAP patients when compared to control subjects or

Table 3.2a: BAL-fluid volumes and cell yields.

		BAL Fluid Volumes (mls)		Proportion of recovered fluid	Cellsx10^5 /ml BAL Fluid
		Instilled	Recovered		
Control Subjects Non-smokers	Range	150-300	25-125	0.13 - 0.52	0.5 - 3
	Mean	(205)	(83.75)	(0.40)	(1.3)++
Control Subjects Smokers	Range	120-280	35-160	0.20 - 0.70	0.6 -12.5
	Mean	(202.6)	(94.0)	(0.4)	(3.67)
CAP - RCA	Range	60-240	20-110	0.4 - 0.73	0.75 - 16.5
	Mean	(153.5)	(75.5)	(0.51)	(4.57)
CAP - AOC	Range	50-240	25-135	* 0.25 - 0.60	**2.0 - 27.5
	Mean	(147.3)	(54.3)	(0.37)	(20.2)

++ when compared to smoking controls P = 0.033.
* when compared to RCA significantly lower. P < 0.02.
** when compared to smoking controls (P < 0.02).
** when compared to RCA (P < 0.002).

RCA (Figure 3.1). Table 3.2(b) shows further breakdown of data on cell yields in these patients and smoking controls.

These results show that there was a highly significant increase in total cells from areas of consolidation compared to control subjects and CAP (RCA). This was mainly due to increases in neutrophils and to a lesser extent the lymphocytes. The increase in neutrophils and lymphocytes in RCA is a reflection of those few patients who had developed pneumonic changes in radiologically clear areas. This is demonstrated further in Table 3.3 which shows proportions of cells in control groups and CAP patients.

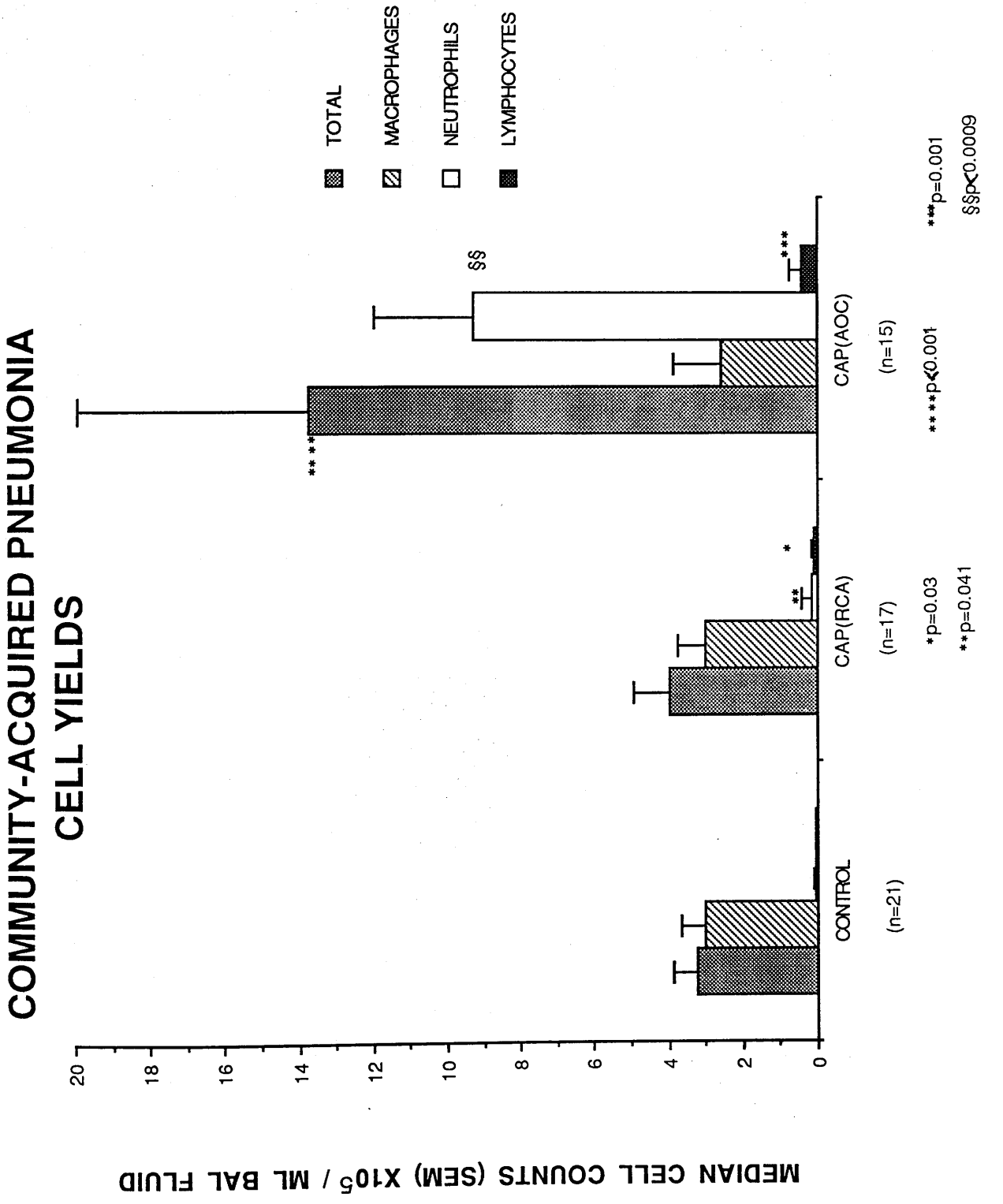


Fig. 3.1: Cell yields in community-acquired pneumonia (CAP).

Table 3.2(b): Absolute cell yields.

Patients' groups	Mean / Median	Total	Macrophages	Neutrophils	Lymphocytes
		Cells x 10^5/ml BAL fluid			
Control subjects n = 21	Mean (SD)	3.67(2.97)	3.50(2.97)	0.07(0.05)	0.07(0.07)
	Median (SEM)	3.2 (0.65)	2.998(0.65)	0.008(0.01)	0.045(0.01)
CAP RCA n = 17	Mean (SD)	4.57(3.98)	3.75(3.01)	0.67(1.13)	0.18(0.21)
	Median (SEM)	4.02(0.96)	3.02(0.73)	0.17(0.27)	0.08(0.05)
	P value	NS	NS	P = 0.041	P = 0.03
CAP AOC n = 15	Mean (SD)	20.23(24.2)	4.13(4.9)	10.2(10.26)	0.93(1.18)
	Median (SEM)	13.75(6.23)	2.60(1.26)	9.32(2.65)	0.44(0.30)
	P value	*P < 0.002	NS	**P < 0.002	***P = 0.001

All P values in the table refer to comparison with control subjects.

* when compared to RCA P < 0.002.

** when compared to RCA P < 0.002

*** when compared to RCA P < 0.05.

Table 3.3: Original differential cell counts.

		Original differential cell counts			
		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Control Subjects (Non-smokers) n = 8	Range	71.2-93.6	0 - 5.4	0 - 2.8	6.4 - 28
	Mean (SD)	85.5 (9)	1.8 (1.95)	0.02 (0.07)	12.4 (8.5)
	Median	86.8	1	0	8.5
Control Subjects (Smokers) n = 26	Range	78.8-99.6	0.2-8.3	0 - 2.4	0 - 12.2
	Mean (SD)	93.8 (4.75)	2.8 (2.23)	0.36(0.57)	2.79(2.91)
	Median	94.95	2.4	0.1	2
CAP-All patients (including 2 non-smokers) RCA n = 18	Range	50 - 99	0 - 48	0 - 1	0.8 - 16
	Mean (SD)	84.2 (13.8)	11.7 (13.1)	0.21(0.32)	3.8(3.5)
	Median	88.6	7.8	0.1	2.85
	P value	NS	NS(P=0.052)	NS	NS
CAP-(Smokers) excluding 5 patients with > 10% Neutrophils RCA n = 11	Range	75.5-98.4	0 - 8.6	0 - 0.9	0.8 - 16
	Mean (SD)	92.5(6.7)	3.94(3.5)	0.2(0.31)	3.5(4.3)
	Median	94.5	2.6	0	2.0
	P value	NS	NS	NS	NS
CAP All patients. AOC n = 18	Range	0.8 - 69.5	3 - 99.2	0 - 1	0 - 60
	Mean (SD)	27.2(22.8)	59.8(34.7)	0.14(0.25)	12.8(18.8)
	Median	24.3*	72.4**	0	3.0
	P value	P < 0.001	P < 0.001	NS	NS

P values in the table relate to comparison with C.S.

* when compared to RCA significantly lower P < 0.002.
** significantly higher than RCA P < 0.002.

The above table shows the cell composition of unprocessed BAL fluid from control subjects and CAP patients. The two control groups show differences in their lymphocyte and neutrophil contents. Cells from radiologically clear (RCA) show some neutrophil contamination although in the

majority of patients this contamination is small. The cell composition from areas of consolidation (AOC) show a predominance of neutrophils with a reduction in the proportion of macrophages. Few patients only show an increased lymphocyte population. Further examination of these data according to aetiological agent of pneumonia is helpful (Table 3.4).

Table 3.4: BAL cell composition according to the nature of the aetiological agent.

		(%) Differential counts of original cells. Means and ranges according to aetiological agent			
Aetiological agent		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Streptococcus Pneumomoniae	RCA n=4	84.8 (62.2-94.5)	12.7 (2.3-34.3)	0.2 (0 - 0.7)	2.3 (1.4-3.5)
	AOC n=4	9 (1-18)	89 (83.6-95.6)	0.05 (0 - 0.2)	1.9 (0.2 - 3)
Legionella Pneumophila	RCA n=3	97.6 (96.5-98.4)	1 (0 - 2.6)	0.1 (0 - 0.3)	1.4 (1 - 2.6)
	AOC n=2	47.3 (37 - 57.6)	12.7 (3 - 22.4)	0 0	40 (20-60)
Mycoplasma sp.	RCA n=2	85.2 (71.5-99)	12.2 (0 - 24.3)	0.1 (0 - 0.2)	2.5 (0.8-4.2)
	AOC n=2	50 (30.6-69.5)	26.5 (12.5-39.6)	0.7 (0.4 - 1)	23.3 (17-29.6)
Undetermined aetiology	RCA n=9	79.2 (50 - 94)	14.8 (1.2 - 48)	0.23 (0 - 1)	5.6 (2 - 16)
	AOC n=11	26 (2 - 56.3)	63.8 (9.2 - 95.6)	0.09 (0.03)	9.97 (0.4 - 58)

The above table shows cell content according to aetiological agent. Patients with Legionnaire's disease show least contamination of their macrophage population from RCA. In addition these patients appear to have a lymphocyte preponderance in areas of consolidation unlike patients with pneumococcal pneumonia. The cell composition from the two patients with mycoplasma infection show a mixture of neutrophils and lymphocytes from AOC and with some degree of contamination in RCA. Cells from patients with pneumococcal pneumonia contained the largest proportion of neutrophils.

3.4.2 Monoclononoal markers

3.4.2.1 Macrophage markers

(Table 3.5): There was considerable variation between subjects in all groups in most of the markers. However BAL cells from CS and from CAP-RCA showed a remarkable similarity in their profile as determined by the panel of macrophage markers used. One exception to this was the proportion of Langerhans cells NA1/34 (T_6 positive cells) which was 1.57% ($\pm$ 0.91) in control subjects compared to 0.65 ($\pm$ 0.63) in CAP-RCA, which was significantly lower ($P < 0.02$).

BAL cells from AOC in CAP patients showed significant differences in some markers when compared to those from CS or from RCA with $UCHM_1$ positive cells constituting only 0.97% ($\pm$ 1.38) in CS, 1.28% ($\pm$ 0.84) in RCA and 49.2% ($\pm$ 31.7) in AOC ($P < 0.002$). Some of this difference was due to neutrophils

Table 3.5: Presence of macrophage markers on BAL cells from CAP and control subjects.

	(%)	RFD_1	RFD_7	RFD_9	$RFDR_1$	T_6 (NA1/34)	$UCHM_1$	$UCHM_1/DR$
Control Subjects (Smokers) n = 11	Range	34.9-98.8	9-73.0	28-86.9	92-100	0.4-3	0.2-4.8	
	Mean (SD)	81.1(16.3)	43.0(22.9)	60.0(19.3)	98.1(2.73)	1.57(0.91)	0.99(1.34)	-
	Median	86.1	37.6	62.1	99.1	1.8	0.60	
CAP - RCA n = 11	Range	25.2-89	18-74.7	29.8-91.2	91-100	0.2-1.6	0 - 2.4	
	Mean (SD)	76.3(20.3)	40.9(16.5)	63.6(19.1)	98.2(2.5)	0.65(0.63)	1.28(0.84)	-
	Median	86	38	67.5	99.2	0.4	1.2	
	P value	NS	NS	NS	NS	P < 0.02++	NS	
CAP - AOC n = 9	Range	54-100	27.5-79	34-73.8	93-100	0.2-2.0	6 - 80.5	8 - 58
	Mean (SD)	79.1(16.3)	56.4(18.9)	50.9(16.7)	98.9(2.32)	0.8(0.21)	49.2(31.7)	29.24(21.6)
	Median	84	55	48.7	100	0.8	57.5	24
	P value	NS	NS**	NS	NS	P < 0.05	*P < 0.002	*P < 0.002

* When compared with $UCHM_1$ in CS and CAP-RCA.

** NS. When compared to CS but significantly higher when compared with RCA (P = 0.02).

++ When compared with CS.

taking up the $UCHM_1$ marker. However the presence of higher proportions of (monocytes) $UCHM_1$ positive cells was confirmed by higher proportions of $UCHM_1$ DR positive cells 29.24% (± 21.6) ($P < 0.002$). It is interesting that the few patients with increased neutrophil content of BAL cells from RCA did not show any increase in the proportion of $UCHM_1$ positive cells. This can be illustrated in two patient's profiles (Table 3.6).

Table 3.6:

Patient	Differential count of cells from RCA				
	Macrophage	PMN	Eosinophil	Lymphocyte	$UCHM_1$ +ve
A.N.	72.8	21	0.2	6	1.2
M.Y.	50	48	0	2	2

There was also a significant increase in RFD_7 positive cells from AOC compared to those from CS ($P = 0.02$). The proportions of $UCHM_1$ positive cells is presented in Figure 3.2.

3.4.2.2 T cell markers:

These were prepared as previously described (Table 3.7). The absolute proportion of T-cells among the smoking controls were low as previously documented (Costabel, 1986). The T_4/T_8 ratio of 1.37 was slightly higher than the

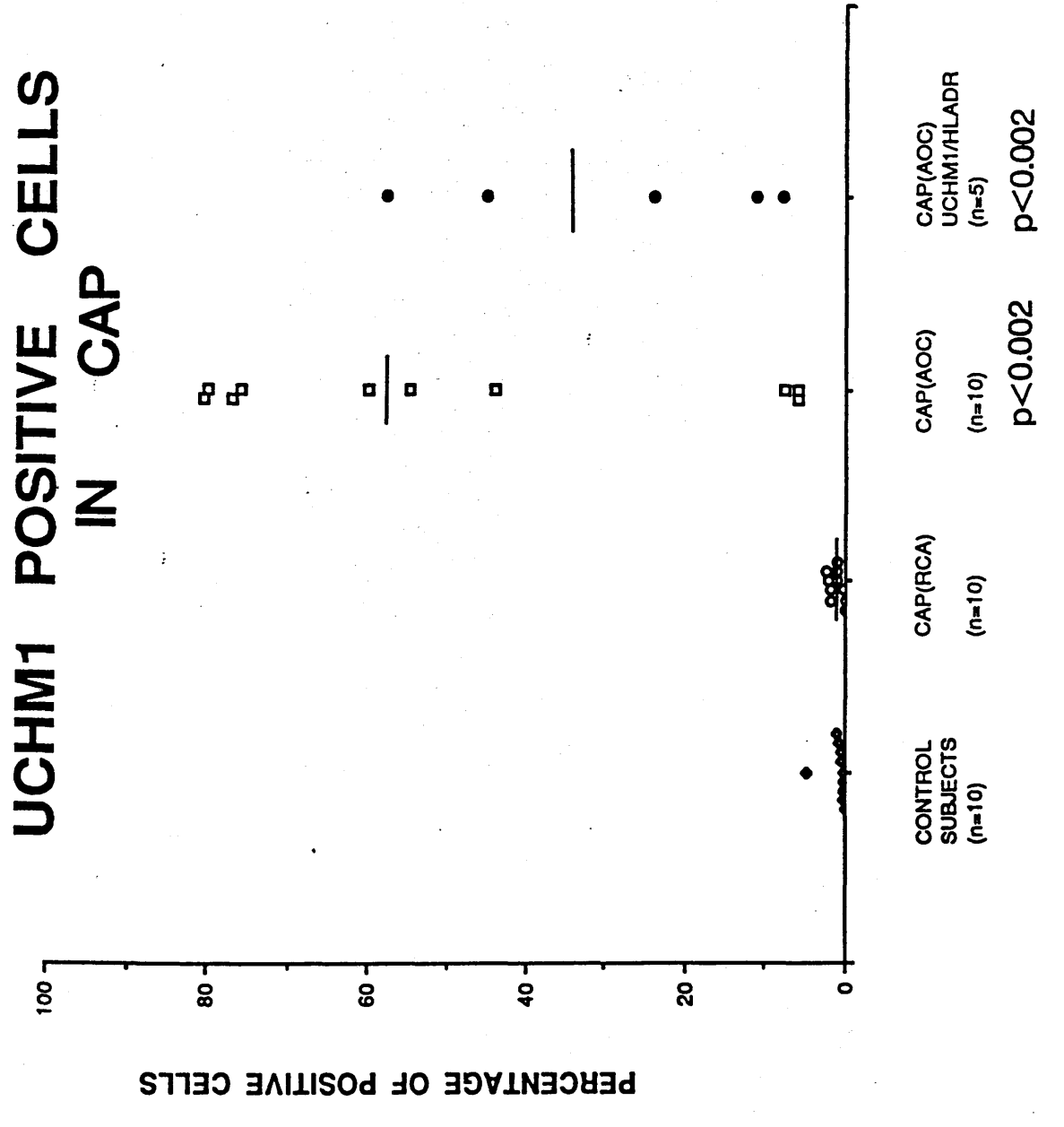


Fig. 3.2: $UCHM_1$ positive cells in CAP.

Table 3.7: Proportions of T-cell subsets in CAP and CS BAL samples.

Mean % Counts (SD)	T_3	T_4	T_8	T_4/T_8
Control Subjects (Smokers) n = 13	2.6 (3.35)	1.35(1.19)	1.88(2.60)	1.37(1.16)
CAP RCA (Smokers) n = 11	5.51 (6.5)	2.39(2.59)	3.31(5.55)	1.49(0.85)
CAP - AOC (Smokers) n = 9	*19.6 (22.2)	13.2(15.6)**	7.4 (5.1)	2.0 (1.87)

* Compared to CS. $P < 0.02$.
** When compared to RCA. $P < 0.05$.

previously reported ratio of 0.9 for smokers. The T-cell profile was similar between CS and CAP-RCA; and although the T_4/T_8 ratio was slightly higher (1.49) there was no significant difference between them. Proportions of T_3 positive (Pan T-cells) and T_8 positive cells were also higher from AOC compared to those from CS. The T_4/T_8 ratio of cells from AOC was not significantly different to those from CS or from RCA. Scrutiny of these data according to the nature of the aetiological agent does not show any pattern of T_4/T_8 ratio amongst any patient group, although there was a total increase of T-cells in patients with Legionnaires' Disease (Plate 3.1(a) and (b)) and mycoplasma pneumonia. This is a reflection of an increase in the total proportion of lymphocytes from AOC in these patients. However these subgroups are too small from which to draw any clear

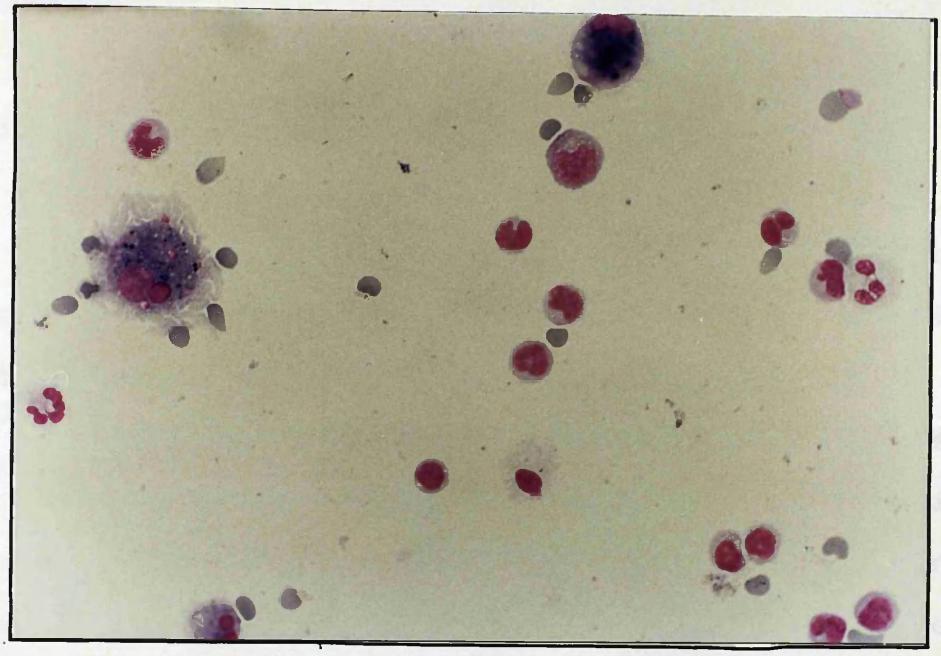


Plate 3.1(a): BAL lymphocytosis (60%) from AOC in one of the patients with Legionnaire's Disease.

Leishman's stain

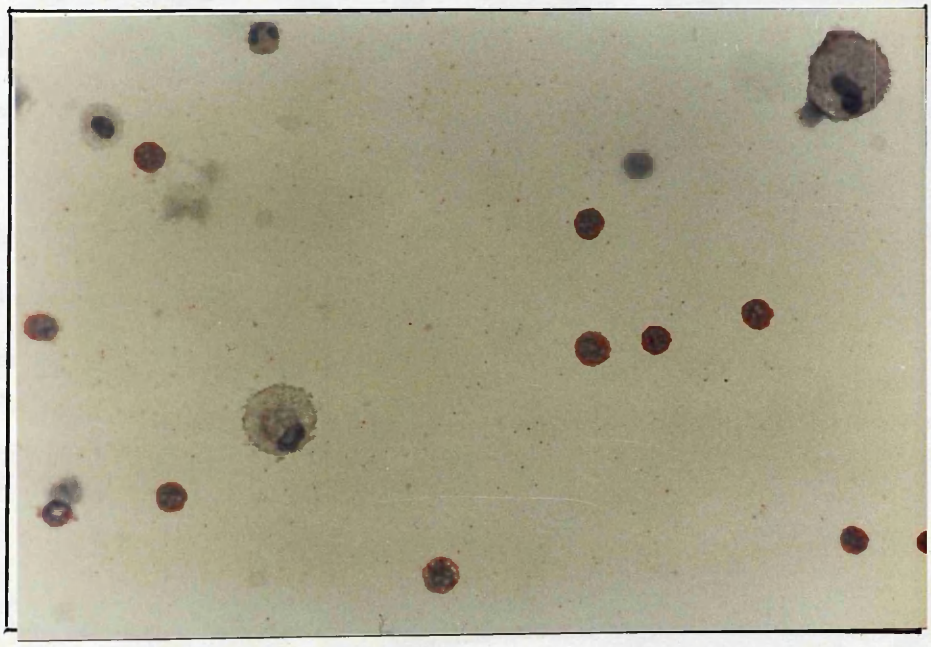


Plate 3.1(b): T_3 positive cells from same patient.

IAP stain

conclusions.

3.4.3 Purification of alveolar macrophages from BAL

Ficoll hypaque centrifugation gave excellent macrophage enrichment in BAL samples from CS and from RCA in CAP patients (Table 3.8).

The final macrophage and neutrophil content of samples from RCA as a whole group showed no statistically significant differences with CS although the neutrophil content was slightly higher in the CAP group. This was due to incomplete clearing in samples (5) which contained more than 10% neutrophils originally. When these samples are excluded from analysis, none of the remaining samples (RCA) contained more than 3% neutrophils. Purification of macrophages from areas of consolidation was less satisfactory. Specimens from these areas generally contained more mucus and debris and this rendered density gradient centrifugation less effective in removing neutrophils. (Plate 3.2(a)). Migration of AM from such specimens is impaired by the neutrophil contamination. (Plate 3.2(b)). The mean viability (%) (SD) for CS, CAP (RCA) and AOC was 67.0 (15.0), 73.6 (12.8) and 82.3 (11.4) respectively.

3.4.4 AM Migration

Using microchemotaxis technique as described in chapter two, migration of AM from RCA in CAP patients was compared to

Table 3.8: Cell composition of BAL fluid after density gradient centrifugation.

		Differential counts of interface layer of cells			
		Macrophages	Neutrophils	Eosinophils	Lympho-cytes
Control Subjects (Non-smokers) n = 7	Range	71.7-97.4	0.2-4.3	0 - 0.2	2-24
	Mean (SD)	89.4(8.3)	1.3(1.4)	0.02(0.07)	8.9(7.0)
	Median	92.4	0.8	0	7.2
Control Subjects (Smokers) n = 26	Range	87.0-99.6	0 - 5.0	0 - 1	0 - 8.0
	Mean (SD)	96.7(3.1)	1.61(2.07)	0.15(0.25)	1.6(1.99)
	Median	98.0	0	0	0.90
CAP - All patients. RCA n = 18	Range	65 - 99.6	0 - 35	0 - 0.6	0 - 5.3
	Mean (SD)	92.2(9.4)	6.0(9.4)	0.06(0.16)	1.76(1.69)
	Median	95.5	2.0	0	1.1
	P value*	0.160	0.052	0.147	0.675
CAP-RCA (Smokers) excluding 5 patients with > 10% PMN in original cell counts RCA n = 11	Range	92.6-99.6	0 - 3.0	0 - 0.4	0 - 4.8
	Mean	95.9(3.7)	2.5(3.32)	0.03(0.11)	1.5(1.48)
	Median(SD)	98.0	1.7	0	1.1
	P value	0.63	0.86	0.107	0.863
CAP - AOC patients. n = 18	Range	2 - 86.2	1 - 96.4	0 - 0.6	0 - 42.4
	Mean (SD)	38(25.88)	52.6(35)	0.06(0.18)	9.4(15.2)
	Median	30.0	70.0	0	2.0

* P values: when compared to smoking controls.

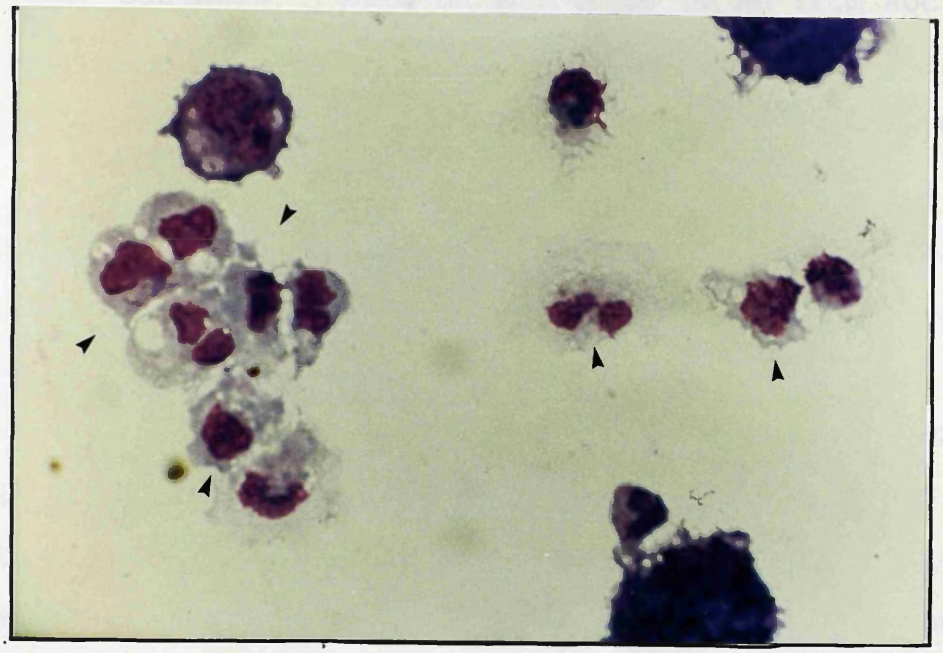


Plate 3.2(a): BAL cells from AOC in a CAP patient, after Ficoll-hypaque centrifugation. Typical immature PMN (arrows) which are bilobed or unilobed are commonly seen. Unlike AM or monocytes these are esterase negative.

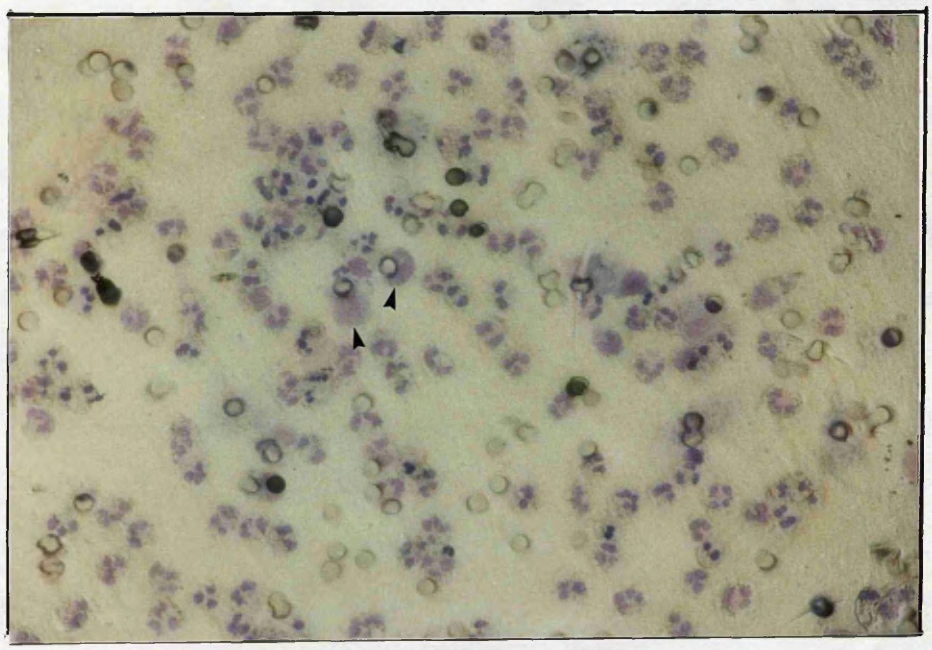


Plate 3.2(b): Chemotaxis membrane flooded with PMN allowing the occasional macrophage migrating through. (arrow).

control subjects. Data on migration of AM from AOC are not satisfactory as purification of macrophages from these areas was not achieved. Table 3.9 shows data from RCA and CS only.

Measurement of macrophage movement shows a marked impairment of unstimulated migration and stimulated migration of AM from CAP (RCA) towards casein, ZAS (C5a) and f-met-leu-phe.

In analysis 3 comparison of AM migration from BAL fluid samples with comparable cell profile to those of CS, shows that the impairment of AM migration from CAP in RCA is genuine and not merely a function of neutrophil contamination in these samples. These results are demonstrated in Figures 3.3(a) and (b).

3.4.5 Respiratory burst generation in alveolar macrophages

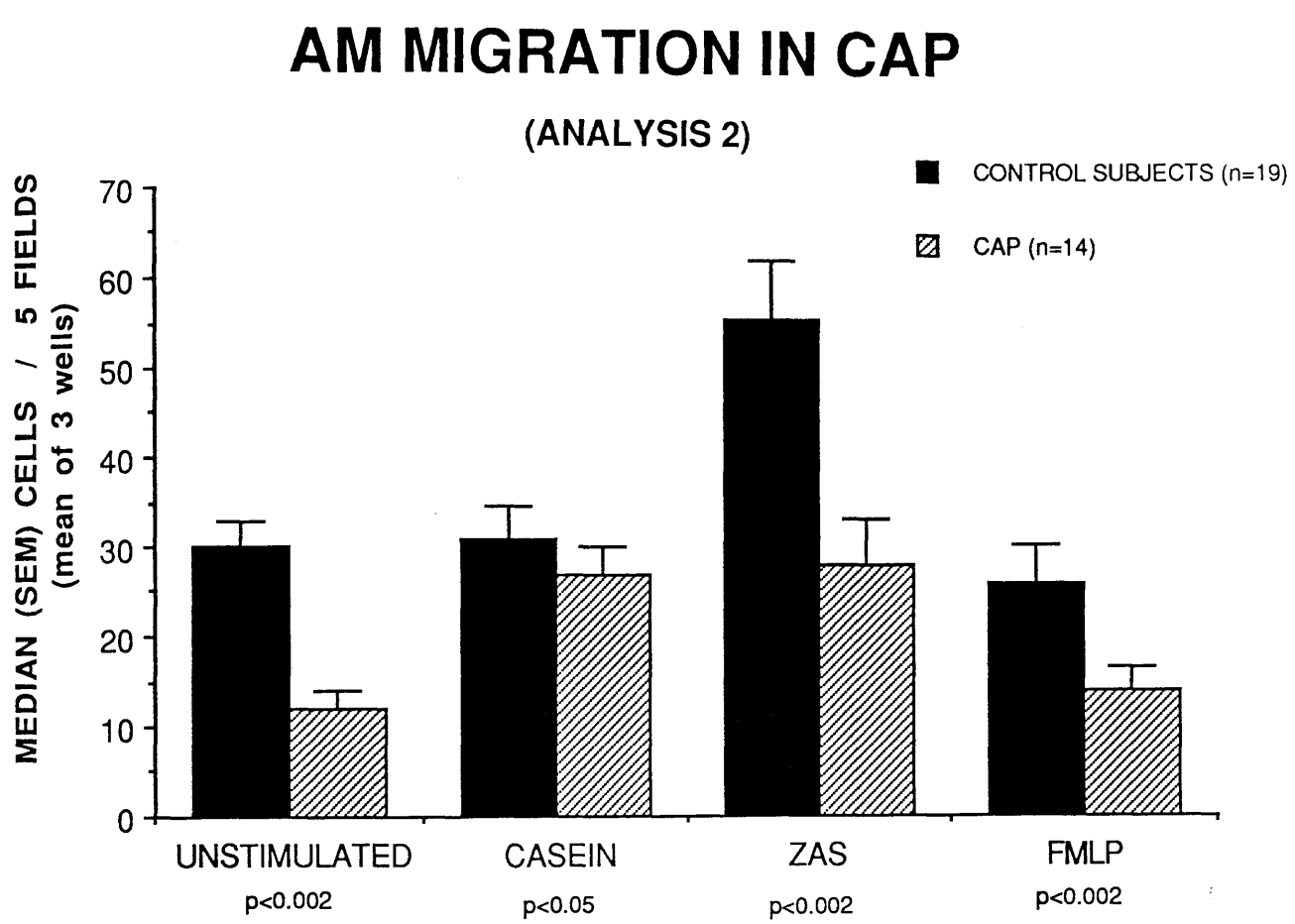
Assessment of generation of respiratory of burst by AM from these patients was compared to CS by measurement of chemiluminescence. Both luminol and lucigenin dependent chemiluminescence was measured for 70 minutes with readings taken every 5 minutes for 10 seconds. Peak counts per second (cps) generated by 10^3 cells are presented in Tables 3.10(a) and (b).

Lucigenin dependent chemiluminescence (Luc-D-CL) of cells from CAP-RCA tended to be lower as compared to CS. This trend was not significant. Spontaneous (cells + gel Hanks) Luc-D-CL of cells from AOC was low compared to those of CS and CAP-RCA. This was statistically significant when

Table 3.9: Chemotaxis (stimulated and unstimulated migration of purified alveolar macrophages.

		Unstimulated migration	Casein	ZAS(C5a)	FMLP
Control Subjects n = 19	Mean (± SD)	29.1(12.2)	37 (15)	61 (2)	33.4(17.6)
	Median(±SEM)	30(2.8)	31.0(3.5)	55.5(6.6)	26.0(4.3)
CAP n = 16	Mean(±SD)	12.5(6.8)	22.4(12.1)	30.3(18.2)	13.6(9.5)
	Median(±SEM)	12.0(1.7)	21.5(3.0)	26.0(4.5)	13.5(2.4)
Analysis 1	P value	P < 0.002	P < 0.02	P < 0.002	P < 0.002
CAP n = 14	Mean(±SD)	13.0(7.1)	24.4(11.6)	31.9(18.8)	15.0(9.3)
	Median(±SEM)	12.0(1.9)	27.0(3.1)	28.0(5.0)	14.0(2.6)
Analysis 2	P value	P < 0.002	P < 0.05	P < 0.002	P < 0.002
CAP n = 10	Mean(±SD)	14.0(7.6)	25.6(10.8)	39.8(16.1)	16.4(10.0)
	Median(±SEM)	13.0(2.4)	26.5(3.4)	37.5(5.1)	24.5(3.2)
Analysis 3	P value	P < 0.01	P < 0.05	P < 0.05	P < 0.01

All P values relate to comparison with control subjects.



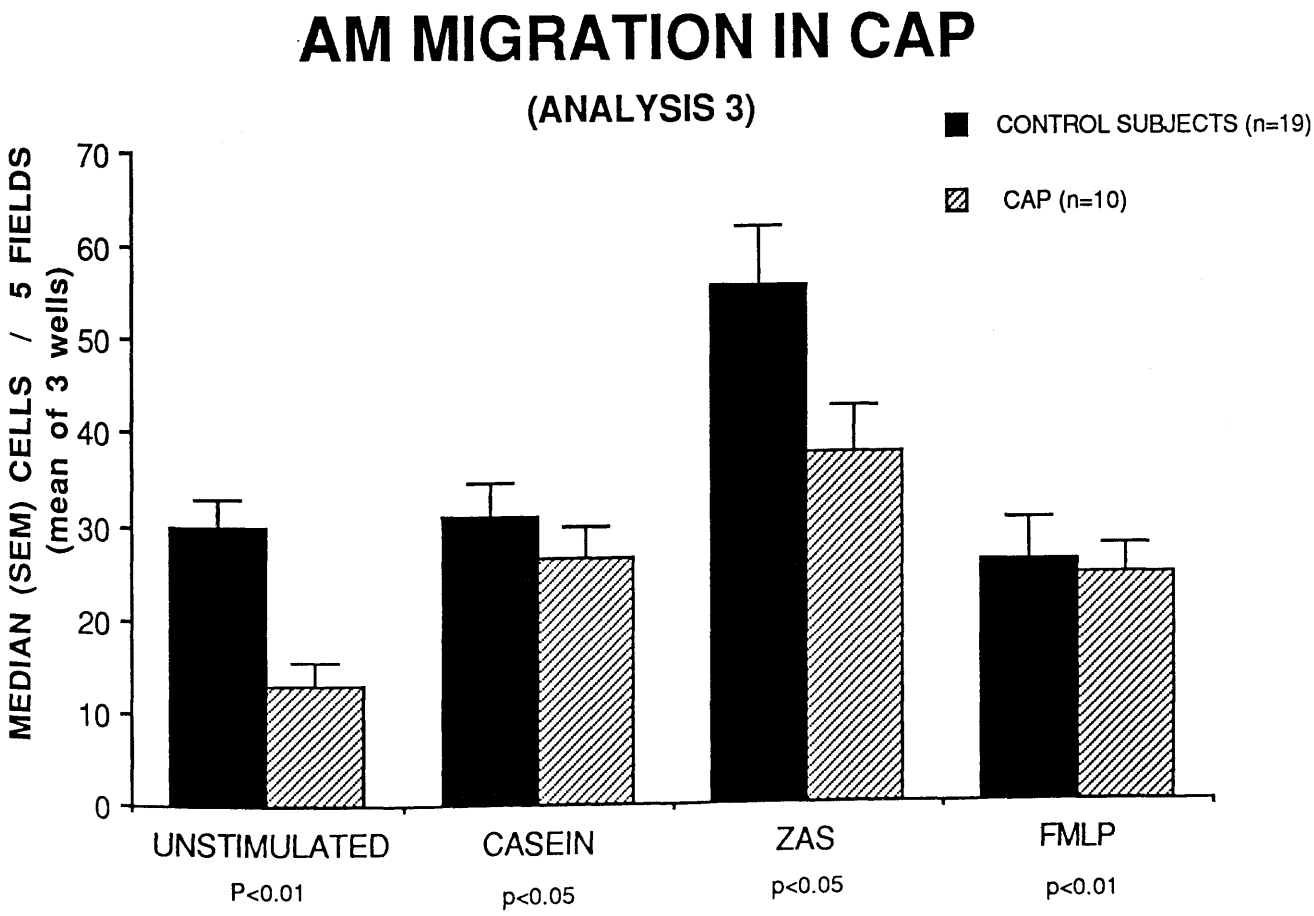


Fig. 3.3(a&b): AM migration in CAP.

Table 3.10(a): Lucigenin dependent chemiluminescence of AM from CS and CAP patients.

Peak/cps/10^3 cells		Lucigenin Dependent	
		Cells + Bact.	Cells + Gel Hanks
Control Subjects	Mean (SD)	812.4(852.5)	821.1(876.9)
	Median (SEM)	400.0(269.6)	415.0(277.3)
	n = 10		
CAP - RCA	Mean (SD)	453.2 (587)	548 (644)
	Median (SEM)	180 (195)	235 (228)
	n =	9	8
	P value	NS	NS
CAP-AOC	Mean (SD)	85.5 (81)	56.3 (70.2)
	Median (SEM)	50 (27)	20 (22.2)
	n =	9	10
	P value	NS	**P < 0.01

** when compared to CAP-RCA and CS.

Table 3.10(b): Luminol-dependent chemiluminescence of AM from CS and CAP patients.

Peak counts/ sec x 10^3 cells		Cells + Bacteria	Cells + gel Hanks
Control Subjects n = 17	Mean (SD)	21.5 (58.4)	14.4 (39.5)
	Median (SEM)	3.8 (14.1)	2.7 (9.5)
CAP - RCA n = 11	Mean (SD)	30.3 (38.4)	8.9 (10.5)
	Median (SEM)	10.0 (11.5)*	4.5 (3.7)
CAP - AOC n = 14	Mean (SD)	77.5 (101.3)	3.7 (18.2)
	Median (SEM)	25.0 (27.0)**	4.2 (5.2)

* P < 0.05
** P < 0.01
} when compared to CS

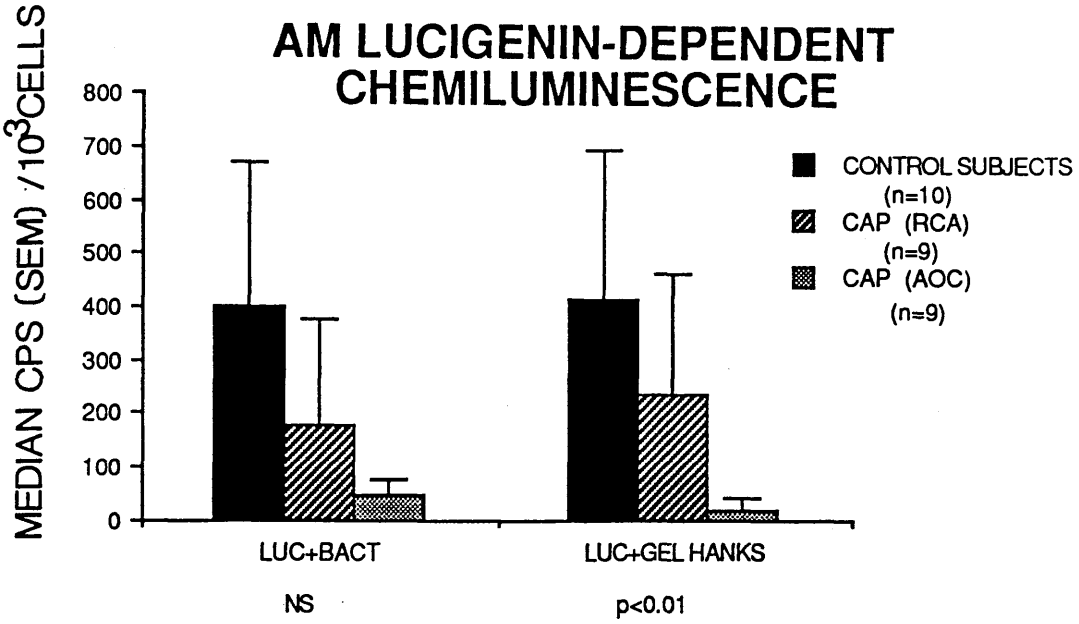
compared to CAP-RCA ($P < 0.01$). (Figure 3.4(a)).

Luminol dependent chemiluminescence (Lum-D-CL) of cells from CAP patients (both RCA and AOC) was significantly higher than CS and this is likely to be due to higher neutrophil content of cells from those areas. (Figure 3.4(b)).

3.4.6 Effect of BAL supernatants on migration of neutrophils from healthy donors

The presence of large numbers of neutrophils in areas of consolidation in CAP patients was thought likely to be due to various chemotactic products including AM neutrophil derived chemotactic factor and complement activation products. In order to test this, the effect of 10-fold concentrated BAL supernatants from CS, and from RCA and AOC in CAP patients were used as chemotaxins for healthy donor neutrophils. Neutrophils were obtained on two different days from two healthy donors and BAL concentrates were tested against each donor's cells. Normal saline was used as a negative control and ZAS (C5a) was used as a positive control.

Because of a wide variation between the two donors, neutrophil migration (donor 1 unstimulated migration (U.M.) = 151; donor 2 UM = 5), the chemotactic differential of each supernatant was used. (Chemotactic differential = Migration towards a chemotaxin (supernatant) - migration towards normal saline). A mean of two donors' chemotactic differentials against BAL supernates for each patient group was obtained, (Table 3.11 and Figure 3.5)



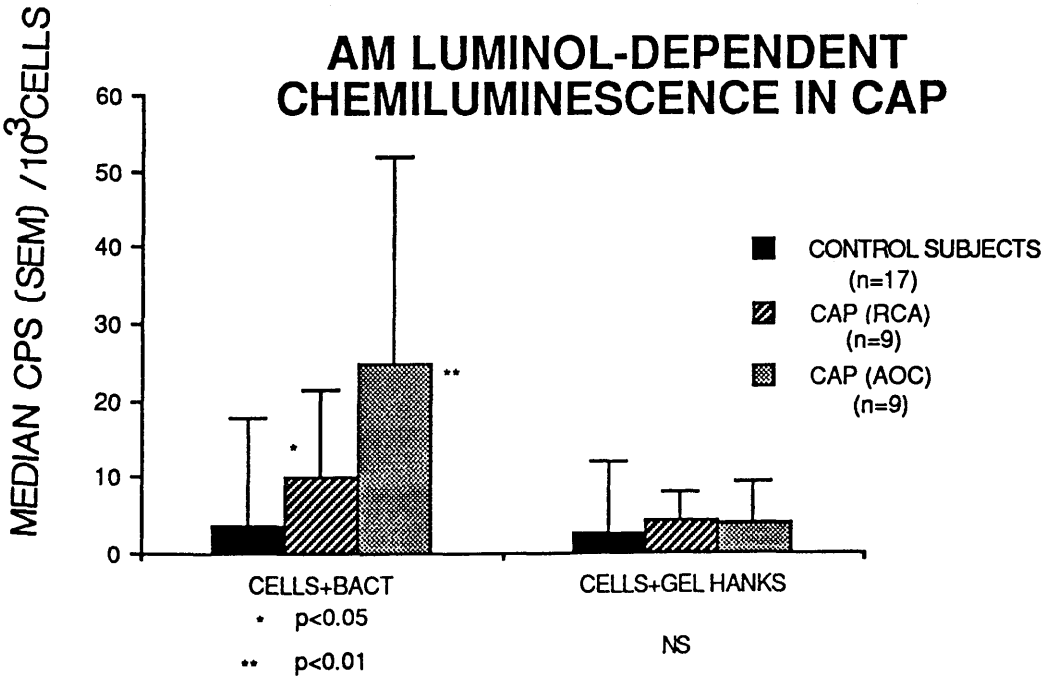


Fig. 3.4(a&b): AM lucigenin and luminol-dependent chemiluminescence in CAP

Table 3.11: Effect of BAL supernatants on healthy donor neutrophils.

Cells/5 fields (x 400)	Control Subjects (n = 9)	CAP-RCA (n = 6)	CAP-AOC (n = 7)
Mean (SD)	32.3 (95.2)	56.6 (95.7)	212.1 (125.7)
Median (SEM)	36.0 (31.7)	52.0 (39.0)	160.5 (47.5)
*P value		NS	P < 0.05

* compared to control subjects.

These results indicate that BAL supernatants from CAP were more active chemotactically towards donor neutrophils than supernatants from CS. This chemotactic activity was significantly greater in BAL supernatants from AOC ($P < 0.05$). Furthermore there was no indication to suggest the presence of any inhibitory activity of supernatants from the RCA which might have accounted for the observed impairment of migration of AM obtained from those areas.

In order to compare the chemotactic activity of supernatants from RCA and AOC in CAP, matched samples in patients with bilateral lavages were analysed. (Table 3.12).

In keeping with a much higher neutrophil content from areas of consolidation, the supernatant fluid from these areas showed significantly greater chemotactic activity towards healthy donor neutrophils.

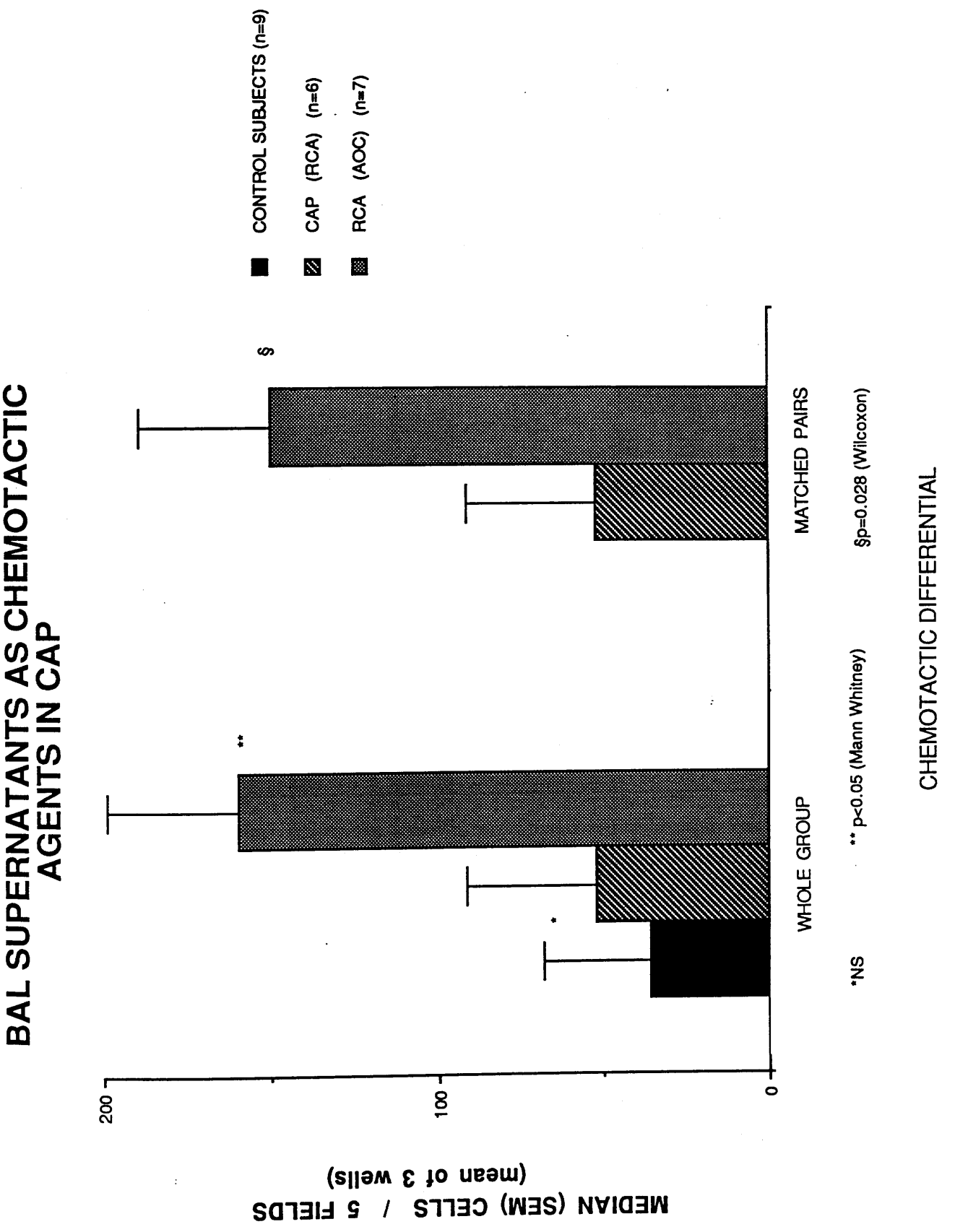


Fig. 3.5: BAL supernatants as chemotactic agents in CAP.

Table 3.12: Effect of BAL supernatants from CAP with bilateral lavage as a chemotactic stimulus for health donor neutrophils.

	Neutrophil Migration	
n = 6	CAP - RCA	CAP - AOC
Mean	56.6 (95.7)	185.4 (114.0)
Median	52.0 (39.0)	150.0 (46.5)
*P value		P = 0.028

* Wilcoxon

3.4.7 Peripheral Blood Cell Function in CAP

Having demonstrated that AM migration and chemiluminescence is impaired in this group of CAP patients, the question that arises is whether this impairment is part of a systemic effect of the pneumonic illness, or whether the impairment of AM function could have predisposed these patients to pneumonia. In an attempt to answer this question, data were obtained on peripheral blood neutrophil and monocyte migration from another group of CAP patients and compared to healthy donor controls, (table 3.12).

3.4.7.1 Monocytes and neutrophil migration

Monocytes were enriched using monocyte-nycodenz gradient (Boyum, 1983) with a purity of 80% esterase positive

Table 3.13: Patients from whom peripheral PMN and MN were obtained.

	Age Range (Mean)	Sex Distribution	
		M	F
Controls n = 10	24.56 (38.2)	6	4
CAP n = 11	28-73 (54.1)	8	3

cells, (Plate 3.3). Unstimulated migration and migration towards ZAS (C5a) and f-Met-Leu-Phe was assessed, Table 3.14(a). Migration of neutrophils was assessed in the same way, Table 3.14(b).

Table 3.14(a): Monocytes migration.

		Monocyte Migration		
		Unstimulated	ZAS (C5a)	FMLP
CS	Mean (SD)	33.4 (23.3)	130.7 (79.4)	38.3 (22.5)
	Median (SEM)	29.0 (7.7)	122.0 (26.4)	33.0 (9.2)
		n = 9	n = 9	n = 9
CAP	Mean (SD)	34.7 (20.8)	79.5 (36.5)	42.1 (16.4)
	Median (SEM)	25.0 (7.8)	93.0 (13.8)	48.0 (6.2)
		n = 7	n = 7	n = 7
		NS	NS	NS

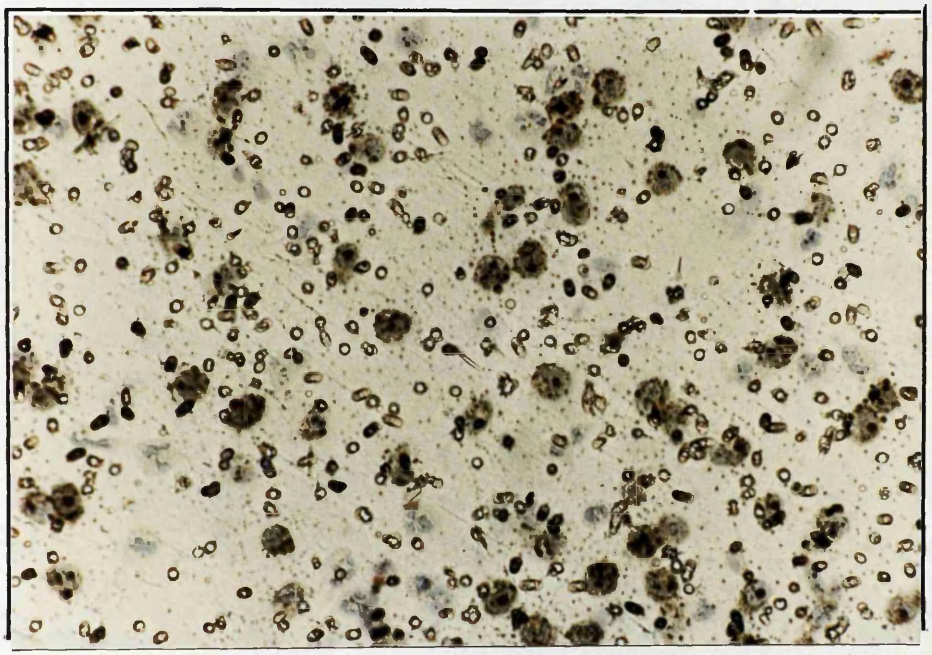


Plate 3.3: Monocyte (esterase positive cells) migration through polycarbonate membrane pore size 5μ.

Table 3.14(b): Neutrophil migration.

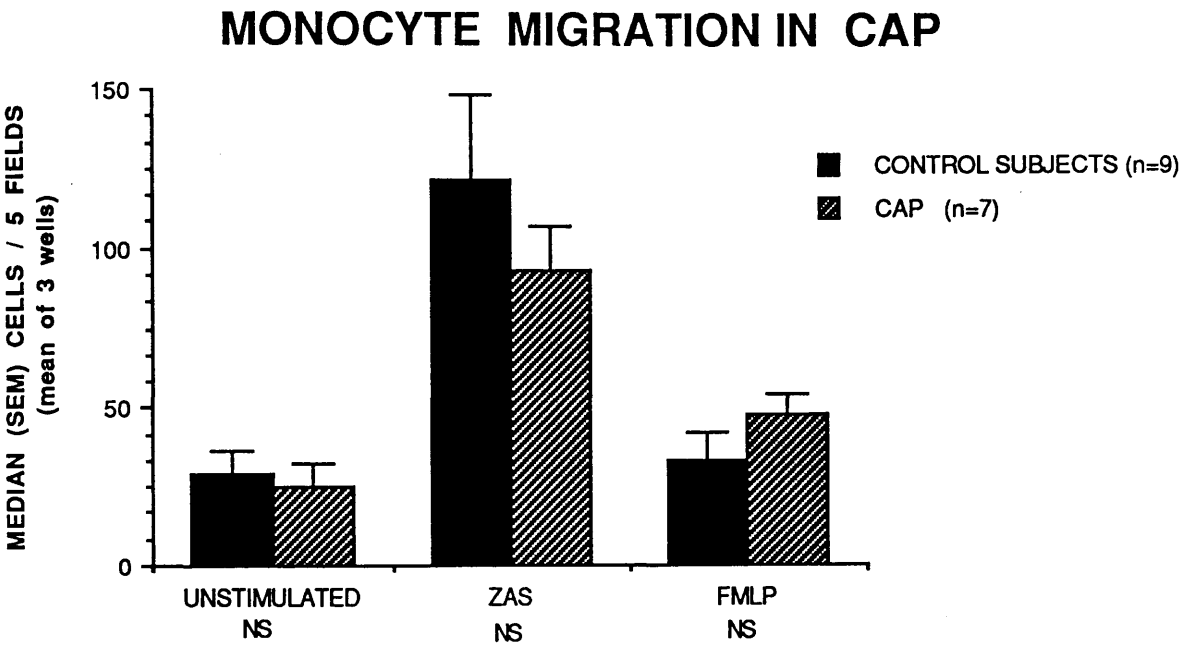
		Unstimulated	ZAS (C5a)	FMLP
CS	Mean (SD)	48.0 (52.9)	214.1 (79.3)	86.8 (32.2)
	Median (SEM)	31.5 (18.7)	192.0 (28.0)	80.6 (11.4)
		n = 8	n = 8	n = 8
CAP	Mean (SD)	62.3 (36.5)	* 113.0 (116.1)	122.8 (51.8)
	Median (SEM)	41.0 (11.0)	51.0 (35.0)	105.0 (16.4)
		n = 11	n = 11	n = 10

*P < 0.05.

Unstimulated migration and stimulated migration of monocytes towards ZAS and FMLP were similar between CS and CAP patients. Unstimulated neutrophil migration and migration towards FMLP from CAP patients showed an increased trend but this was not significant. In contrast however, migration of neutrophils towards ZAS was significantly impaired in CAP patients. (Figure 3.6(a) and (b)).

3.4.7.2 Measurement of chemiluminescence in peripheral blood monocytes and neutrophils.

Generation of respiratory burst in peripheral blood



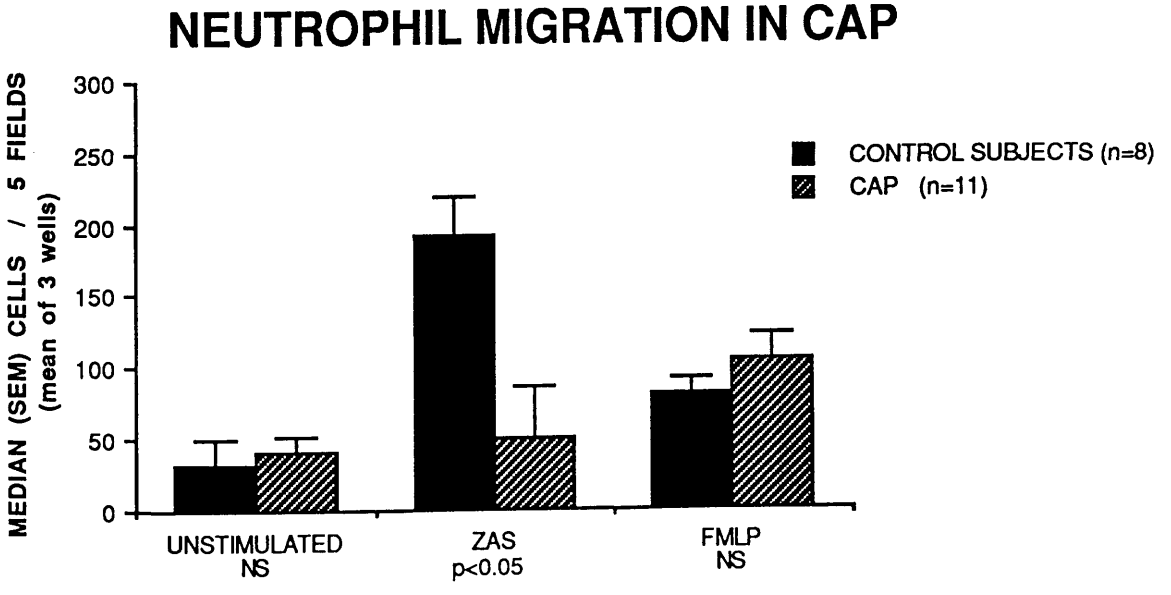


Fig. 3.6(a&b): Monocyte and neutrophil migration in CAP.

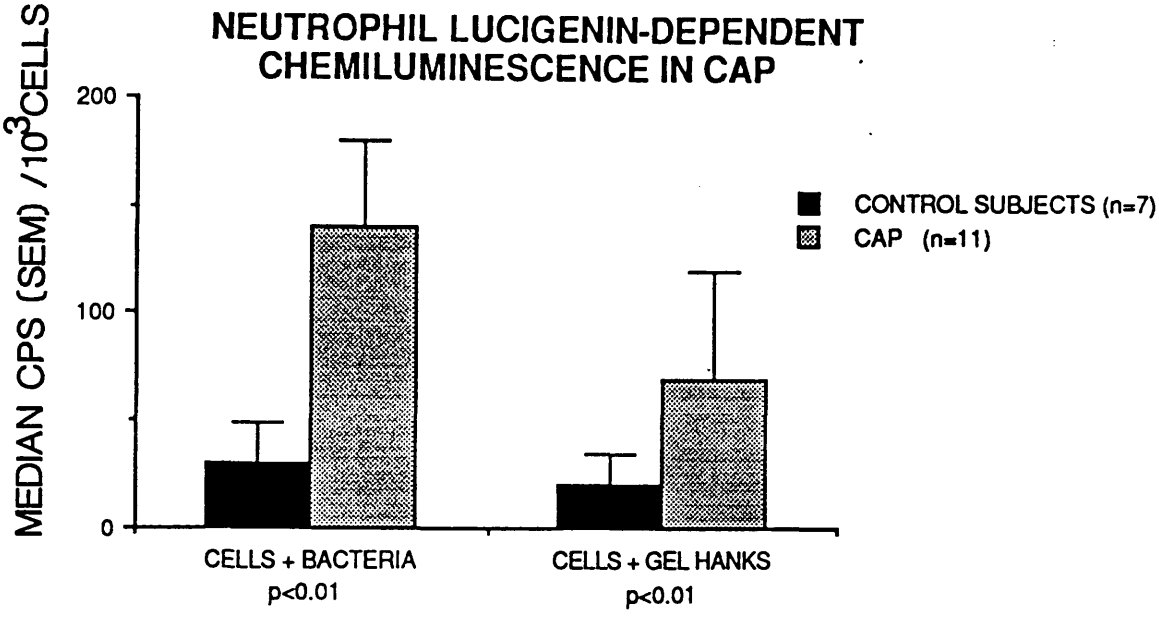
cells in CAP patients was assessed by measuring lucigenin and luminol dependent chemiluminescence and was compared to healthy donor cells. Cells were prepared as previously described. Both monocytes and neutrophils showed a trend of increased lucigenin and luminol dependent chemiluminescence in the CAP group compared to the CS. (Tables 3.15 a-d). This heightened activity was highly significant in the neutrophil lucigenin-dependent chemiluminescence ($P < 0.01$) (Table 3.15b) and (Figure 3.7(a)).

Lucigenin Dependent Chemiluminescence

Table 3.15(a): Monocytes Luc-D.CL.

	Peak cps/10^3 cells	Cells + Bacteria	Cells + gel Hanks
CS	Mean (SD)	112.8 (49.5)	85.0 (55.7)
	Median (SEM)	100.0 (18.7)	95.0 (22.7)
		n = 7	n = 6
CAP	Mean (SD)	304.5 (475.8)	445.0 (509.3)
	Median (SEM)	130.0 (143.0)	95.0 (207.9)
		n = 11	n = 6

No significant differences between the two groups. (Figure 3.7(b)).



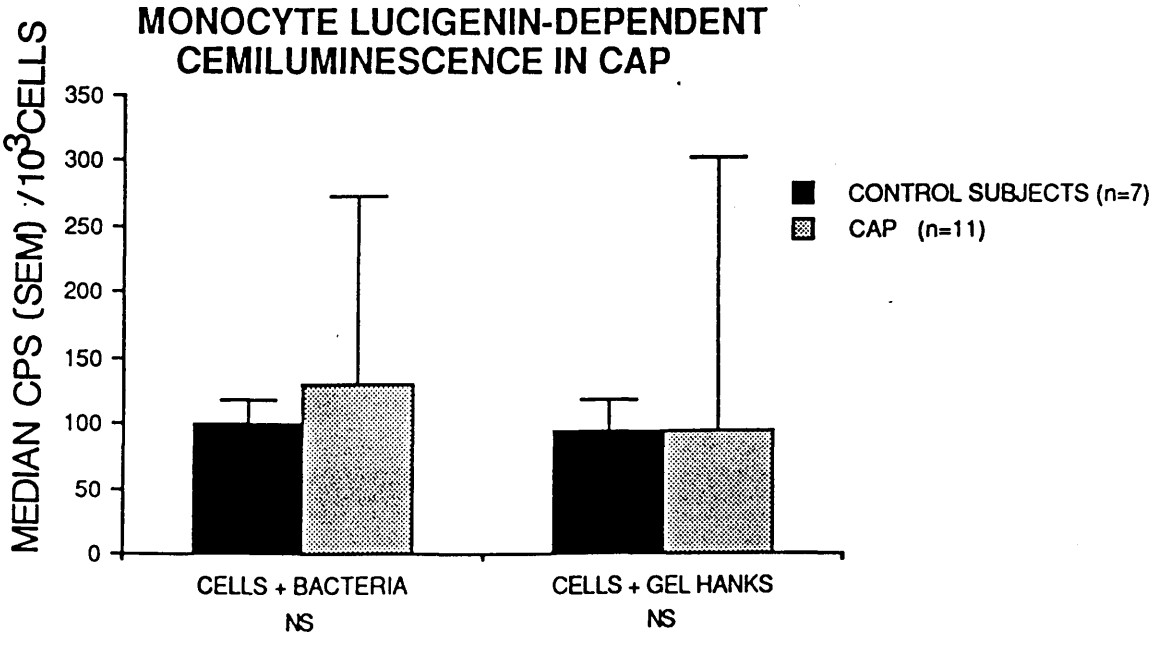


Fig. 3.7(a&b): Neutrophil and monocyte lucigenin-dependent chemiluminescence in CAP.

Table 3.15(b): Neutrophil Luc-D-CL

Peak cps/10^3 cells		Cells + Bacteria	Cells + gel Hanks
CS	Mean (SD)	44.2 (51.9)	32.0 (40.5)
	Median (SEM)	30.0 (19.6)	20.0 (15.3)
		n = 7	n = 7
CAP	Mean (SD)	180.9 (131.0)	155.4 (163.0)
	Median (SEM)	140.0 (39.5)	70.0 (49.2)
		n = 11*	n = 11*

* P < 0.01

Luminol Dependent Chemiluminescence

Table 3.15(c): Monocyte (Lum.-D-CL).

Peak cps/10^3 cells		Cells + Bacteria	Cells + gel Hanks
CS	Mean (SD)	205.7 (89.0)	4.6 (2.7)
	Median (SEM)	220.0 (33.6)	3.5 (1.1)
		n = 7	n = 6
CAP	Mean (SD)	633.3 (775.4)	40.0 (17.3)
	Median (SEM)	360.0 (316.5)	50.0 (10.0)
		n = 6	n = 3

P value: No significant differences between the two groups.

Table 3.15(d): Neutrophil Lum-Dep-CL.

	Peak cps/10^3 cells	Cells + Bacteria	Cells + gel Hanks
	Mean (SD)	265.7 (136.0)	1.8 (0.6)
CS	Median (SEM)	220.0 (51.4)	2.0 (0.2)
		n = 7	n = 7
	Mean (SD)	447.2 (412.0)	17.4 (22.5)
CAP	Median (SEM)	440.0 (124.2)	10.0 (6.8)
		n = 11	n = 11

P value: No significant differences between the two groups.

3.4.8 Measurement of albumin and in BAL supernatant.

Albumin measurement was done using a double-label radio immunoassay in the Biochemistry Department - Glasgow Royal Infirmary. The results are presented in Table 3.16a.

These results show that although albumin levels in BAL supernatant from RCA in CAP are slightly higher than those from CS they are not significantly different. Levels of albumin from areas of consolidation are significantly higher than those from CS ($P < 0.002$) and RCA ($P = 0.011$). This observation is in keeping with the degree of inflammation in AOC.

Table 3.16(a): Albumin levels in mg/L for BAL fluid from patients with CAP.

	Control Subjects n=16	CAP (RCA) n=12	CAP (AOC) n=11
Range	3.8 - 111.8	0 - 500.7	0.528-1345
Mean (SD)	37.12(31.27)	77.93(138.5)	542.31(549.73)
Median (SEM)	27.45(8.36)	30.55(39.9)	216.6(165.75)
*P value	NA	NS	P<0.002**

NA = Not applicable.

* = All P values relate to comparison with control subjects.

** = Significantly higher than RCA for matched samples P = 0.011 (Wilcoxon).

3.4.9 Measurement of complement components and products of complement activation.

Measurement of complement components was done using an ELISA assay. Baseline values for the assay are given in Appendix I. The results are presented here in mg/L and and since the lower limits for these components are in ng/ml (ug/L), where these were not detectable a level of 0 was assigned for the purpose of analysis Table 3.16b.

It is clear from these results that the levels of complement components in supernatants from AOC are significantly higher than CS and from RCA.

In order to assess the contribution of passive exudation of these components into the alveoli as a

Table 3.16(b): Complement levels in mg/L of unconcentrated BAL fluid in CAP patients.

		C1q	C1r	C1s	C4	C3	C1-Inh	Factor B	Factor H
C.S.	Mean	0.006	0.009	0(0)	0.013	0.753	0.068	0.138	0.087
	(SD)	(0.024)	(0.034)		(0.034)	(1.22)	(0.13)	(0.154)	(0.186)
n = 16	Median	0(0.006)	0(0.009)	0(0)	0(0.009)	0.40	0(0.033)	0.10(0.039)	0(0.046)
	(SEM)					(0.306)			
CAP	Mean	0.186	0.062	0.043	0.213	2.267	1.083	0.667	0.378
	(SD)	(0.457)	(0.073)	(0.074)	(0.359)	(2.80)	(2.09)	(1.06)	(0.52)
(RCA)	Median	0.004	0.08	0(0.221)	0.05	1.10	0.35	0.40	0.45
	(SEM)	(0.132)	(0.021)		(0.104)	(0.809)	(0.605)	(0.31)	(0.15)
n = 12	P value	NS	NS	NS	NS	NS	NS	NS	NS
CAP	Mean	0.65	0.364	0.54	5.058	8.09	8.68	6.54	1.56
	(SD)	(0.579)	(0.233)	(1.32)	(5.98)	(3.97)	(12.53)	(7.12)	(0.86)
(AOC)	Median	0.55	0.50	0.135	2.65	7.55	5.95	3.70	1.40
	(SEM)	(0.167)	(0.067)	(0.381)	(1.72)	(1.1)	(3.64)	(2.15)	(0.28)
n = 12	*P value	P<0.002	P<0.002	P<0.002	P<0.001	P<0.002	P<0.002	P<0.002	P<0.002
Comparing RCA with AOC (Wilcoxon)		P=0.012	P=0.008	P=0.017	P=0.008	P=0.005	P=0.008	P=0.008	P=0.018

*P values: relates comparison with control subjects. The u values are very much lower than the critical u value at a probability level of 0.002 but this was the lowest P value obtained from the reference tables.

Table 3.16(c): Complement levels in BAL from CAP patients in mg/gm albumin.

		C1q	C1r	C1s	C4	C3	C1-Inh	Factor B	Factor H
C.S.	Mean (SD)	0.056(0.22)	0.078(0.31)	0.039(0.16)	0.969(2.68)	44.31(115.1)	2.12(4.13)	4.66(6.77)	6.33(18.5)
n = 16	Median (SEM)	0(0.056)	0(0.078)	0(0.39)	0(0.67)	11.85(28.78)	0(1.03)	3.38(1.69)	0(4.87)
RCA	Mean	0.737	1.95	1.55	9.39	42.50	14.27	10.85	3.13
	(SD)	(1.20)	(3.86)	(3.81)	(11.76)	(41.78)	(19.43)	(10.27)	(4.69)
n = 12	Median (SEM)	0(0.345)	0.34(1.115)	0(1.10)	2.11(3.39)	38.82(12.06)	7.93(5.60)	9.33(1.48)	0(1.48)
	P value	NS	NS	NS	NS	NS	NS	NS	NS
AOC	Mean	1.22	0.92	3.52	8.70	25.47	9.02	19.07	4.24
n = 11	(SD)	(1.48)	(1.01)	(10.63)	(7.65)	(27.83)	(7.33)	(28.35)	(3.49)
	Median	0.60	0.52	0.14	7.55	11.78	8.61	8.92	3.28
	(SEM)	(0.45)	(0.30)	(3.20)	(2.31)	(8.39)	(8.96)	(8.96)	(1.24)
	P value	P<0.002	P<0.002	P<0.002	P<0.002	NS	P<0.002	NS	P<0.05
Comparing RCA with AOC (Wilcoxon)		NS	NS	NS	NS	NS	NS	NS	NS

result of the inflammatory process, these levels were related to albumin levels; Table 3.16c).

Relating the levels of complement components to albumin levels, shows the degree of difference between AOC and CS is less than when no correction is made for albumin. The majority of the components remain significantly higher than the CS. There are now no significant differences between AOC and RCA and, indeed in some circumstances some of the complement components from inflamed areas are lower than in RCA and even in CS. A case in point is C3 levels, which are lower (though not significantly so) than in RCA or CS. Whether this indicates consumption must be viewed together with the products of complement activation. These are shown in Table 3.17. Complement activation and cleavage of the components is a local phenomenon and there is no justification in correcting for albumin (Whaley - personal communication).

All but one product of complement activation from AOC in CAP patients are higher than in CS. C5a was higher than either the control subjects or RCA but was not significantly so when compared to the latter. C5b-9 showed undetectable levels in the majority of the specimens throughout the project and the reason for this is not clear. Hence no significant differences were found in this fragment between groups. Significantly higher levels of C1s-CInh complex ($P < 0.01$), C3-P ($P < 0.05$) and C3a ($P < 0.05$) are higher in AOC than in RCA from the

Table 3.17 : Products of complement activation in unconcentrated BAL in CAP.

		Units/litre			ug/L	
		C15-CInh	C3-P	$C5_{-9}$	C5a	C3a
Control subjects	Mean(SD)	138.5(499.0)	76.9(277.3)	0(0)	1.72(0.9)	10.71(4.97)
n = 14	Median(SEM)	0(138.5)	0(76.9)	0(0)	1.60(0.24)	9.8(1.33)
CAP(RCA)	Mean(SD)	772.7(1312.3)	345.5(867.6)	0(0)	2.96(1.64)	25.64(42.63)
n =	Median(SEM)	0(395.7)	0(261.6)	0(0)	3.10(0.49)	14.4(12.9)
	P value	NS	NS	NS	NS	NS
CAP(AOC)	Mean(SD)	5700(4073.5)	1657.1(1661.2)	714(160)	4.57(4.48)	126.1(105.2)
	Median(SEM)	6200(1539.6)	1600(627.9)	0(620.1)	3.10(1.24)	100(33.3)
	P value	P<0.002	P<0.01	NS	P<0.002	P<0.05
Comparing RCA and AOC		P<0.01	P<0.05	NS	NS	P<0·05

same patients. These results suggest that there is both classical and alternative pathway complement activation in AOC in CAP patients.

3.5 Discussion

The AM is the predominant cell in BAL fluid from the normal lung but is superseded by exudate cells in disease states such as pneumonia or ARDS. This study has investigated both "normal" and inflamed areas of the lung by assessing BAL cells both phenotypically and functionally. This was compared with BAL cells from as near normal a control group as could possibly be obtained. The proportion of BAL cells obtained from the control group and RCA in CAP were similar in the original yields and differential counts as those reported in other studies (Huninghake, 1979; Reynolds, 1987). Cells obtained from RCA of the lung from the CAP patients were very similar to those from CS in many patients, and exudate cells were only found in a few patients. Density gradient centrifugation on Ficoll-hypaque rendered final differential counts between the two groups similar with no statistical differences in the cell composition as a whole. Exclusion of those patients who had more than 10% neutrophils in their original count, yielded a subgroup whose final differential count was almost identical to that of the control subjects.

Use of a panel of macrophage markers which have been used in previously published work (Campbell, 1986; Poulter, 1986; Alegre, 1986), has enabled further comparison between the macrophage populations of these two groups. In five of the six macrophage markers tested there were no differences detected between CS BAL cell composition and CAP (RCA)

patients. The one exception is the NA1/34 (T_6 equivalent) marker which identifies Langerhans cells (Alegre, 1986; Chollet, 1982, 1984; Murphy, 1983, 1987; Kawanami, 1981). The proportion of NA1/34 positive cells from CAP patients was significantly lower than the control subjects.

This is a hitherto unreported finding and it is difficult to compare the results of this work with the literature for several reasons. Firstly, all the work using this monoclonal antibody on lung cells by various workers (Chollet, 1981, 1984; Murphy, 1981, 1983; Kawanami, 1981) employed an OK T_6 which may not be identical to NA1/34. Secondly these workers have used different techniques from that used in this study and particularly that used by Chollet et al. (1984). In that study they employed immunoperoxidase labelling of live cells which preserves cisternal peroxidase activity which is apparently helpful in identifying Langerhans cells. The third and perhaps the most important reason is that in all the studies quoted, the control groups have been patients with various disease states, and only 3 normal controls whose proportion of T_6 positive cells was not mentioned in these papers. Our experience with this monoclonal antibody has been similar to others, in that it appeared very highly specific and gave a strong reaction (see plate 3.4). Chollet (1984) quoted among the miscellaneous group one case of viral pneumonia with 1.2% T_6 positive cells but it is not clear whether these cells were from normal or inflamed areas of the lung and the range for our pneumonia (RCA) is 0.2 - 2%. These workers have compared various

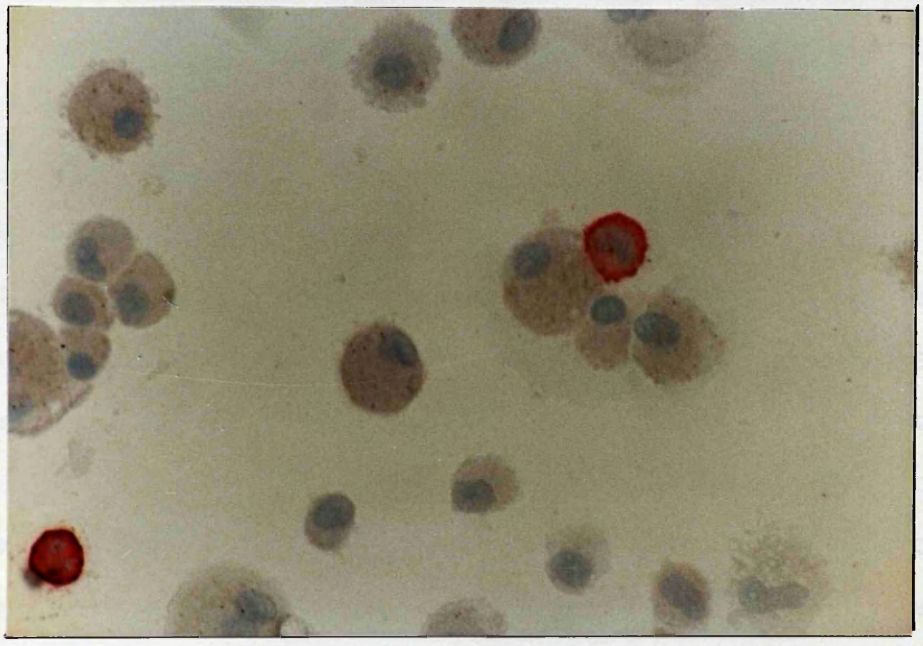


Plate 3.4: NA 1/34 (T_6) positive cells are typically strongly positive.

patient groups with histiocytosis-x patients who were found to have much higher proportions of positive cells in their BAL fluid. It is possible that the true "normal controls" have "intermediate values" comparable to those in this study (0.4 - 2.6%). If that were the case then the lower proportion of T_6 positive cells may be related in an as yet unidentified immunological mechanism perhaps in keeping with other functional impairment of AM found in this study. It is known for instance that antigen presentation form one of the other functions of Langerhans cells (Silberberg-Sinakin, 1980). However it is clear that these findings need to be confirmed by others before such an interesting speculation is considered more seriously.

Cells from areas of consolidation in the CAP group as a whole were predominantly neutrophils, although the same trend was evident when the results were analysed according to the aetiological agent. These results are in agreement with some and in conflict with others. Abrams et al. (1984) found a predominantly neutrophil-rich BAL profile in those pneumonia patients with confirmed aetiological diagnosis but none of these patients had Legionnaire's disease or mycoplasma pneumonia. Pierce and colleagues (1977), using an experimental model found neutrophils predominant amongst lavage cells following E.coli challenge but a weak neutrophil response after staphylococcal challenge. Data from experimental Legionnaire's disease in guinea pigs showed an early neutrophil response, with lymphocytes peaking at 7 days of infection (Davis, 1983). Our patients though

bronchoscoped early following admission, could be regarded as late in terms of evolution of the disease, which could explain this apparent lymphocytosis. However, this may be useful in clinical practice. This was demonstrated retrospectively in one of our patients with Legionnaire's disease whose serology and other microbiological tests were negative and whose lavage profile from AOC was 57.6% macrophages, 22.4% neutrophils and 20% lymphocytes. This patient seroconverted (titre of 1/1000 for legionella type I after three weeks). None of our patients with pneumococcal pneumonia showed lymphocytosis and all had very high neutrophil counts (83.6 -95.6%). Lafitte et al. (1983) serially lavaged patients with pneumonia up to 90 days and found neutrophils initially in pneumococcal pneumonia followed by lymphocytosis at about 10 days.

The results of macrophage markers were interesting. There were increased proportions of $UCHM_1$-positive cells (monocytes) from AOC compared to RCA or CS. This was slightly exaggerated by non-specific staining of neutrophils by this monocyte marker but the use of the double-staining technique (i.e. in conjunction with HLADR marker) confirmed increased proportions but established more accurate proportions of these cells. The finding that none of the specimens which contained substantial numbers of neutrophils in radiologically clear areas had any increase in the proportion of $UCHM_1$-positive cells, indicates that the influx of these cells is later compared to neutrophils. This is in keeping with the findings of Hudson et al. (1977) in an

experimental model. The other macrophage marker that showed a difference between AOC and RCA is the RFD_7 which has been shown to label mature macrophages (Poulter, 1986). This monoclonal antibody did not appear to label neutrophils and this was confirmed by double-staining technique. This suggests that some of the newly arrived mononuclear cells, have sufficiently "matured" and have acquired at least some of the characteristics of the resident macrophages. This marker has been shown not to cross react with $UCHM_1$-positive cells (Poulter, 1986). An increased proportion of RFD_7 positive cells has been shown in patients with cryptogenic fibrosing alveolitis (Campbell, 1986), a condition known to have increased turnover of the macrophage population (Bitterman, 1984). Such an increase of these cells in acute lung inflammation as seen in our patients underlies the versatility of the mononuclear phagocyte system.

The proportions of T-cells in our smoking and non-smoking controls are similar to those reported by other workers (Reynolds, 1987; Costabel, 1986). The T_4/T_8 ratio for our non-smoking controls is also similar to those reported earlier but for the smoking controls the ratio of 1.37 ($\pm$ 1.16) is slightly higher than (0.9 $\pm$ 0.4) reported by Costabel et al. (1986), but lower than the ratio for non-smokers which is in keeping with their findings. There was considerable intersubject variation in these counts and because the total numbers of lymphocytes on any one cytospin are small, missing one or two cells during the process of

counting could mean a wide margin of error. To reduce this often all the cells in the cytospin were counted rather than the standard 500 cells used in the study. The increase in the total numbers of T-cells in the community-acquired pneumonia patients is reflected by the increase in total numbers of lymphocytes in some patients but on the whole the ratio of T_4/T_8 was not significantly different compared to CS.

The AM is now widely accepted as the principal cell responsible for host defences in the lung. Some insight has been obtained as to the state of host defence in the CAP patients in "normal" and inflamed lung areas. Migration of AM obtained from RCA in CAP patients showed marked impairment compared to AM from CS. This depression was demonstrated towards the three chemotaxins tested as well as in unstimulated migration. There are no similar data available with which to compare our results, but impairment of AM function including migration and phagocytosis has been reported in a group of patients with pneumonia following allogeneic bone marrow transplantation (Winston, 1982). Lucigenin-dependent chemiluminescence of AM from RCA has shown a clear trend towards functional depression compared with CS although this did not achieve statistical significance, perhaps due to smaller numbers of patients involved. Such an impairment of AM function could be the result of a "systemic effect" of the pneumonic illness or to some predisposing factor to pneumonia.

This question was addressed in two ways. Firstly, the

supernatant fluids from RCA (and from AOC) were used as chemotaxins against healthy donor neutrophils although it might have been better to use AM or monocytes for this purpose. Given the limitation of such an approach, our data showed no suggestion of any inhibitory factor(s) in the supernatants from these lung areas. Indeed, these supernatants were slightly more chemotactic than those of C.S. Our complement data in the BAL supernatants confirm that there are significantly higher levels of complement components and products of complement activation from AOC than from RCA or CS. The second approach was the assessment of peripheral blood cell function. Both migration and chemiluminescence of monocytes from another group of CAP patients showed no differences when compared to healthy donor cells. Chemiluminescence of neutrophils from CAP patients was significantly enhanced compared to CS. The only exception to this overall lack of depression of function of peripheral blood cells in CAP patients compared to control subjects was the migration of neutrophils towards ZAS (C5a). Neither the unstimulated migration nor the migration towards F-met-leu-phe of cells from CS were "superior" to those from patients with CAP. Thus the data on peripheral blood cells in CAP patients do not suggest a systemic impairment of function that could also have accounted for the impairment of function of AM from RCA of the lung.

Thus bearing in mind the small numbers of peripheral blood cells examined it appears that a localised predisposing factor within the lung compartment is more likely to lead to

the development of pneumonia than some systemic change. One possible cause of depressed AM function sufficient to predispose to development of pneumonia is viral infection. Epidemiological data as well as experimental data suggest that viral infection can predispose to pneumonia. This is thought to be due to an AM phago lysosome fusion defect (Jakab, 1980) or immune complex deposition on the AM (Astry, 1984). In this study there was no comprehensive viral screen for all the patients. But when viral titres were obtained, nothing was found except in one patient with pneumococcal pneumonia an antibody titre towards para influenza virus of 1/64. Previous viral infection for some of our CAP patients cannot therefore be ruled out. The other possibility which might have accounted for impairment of AM function in these patients is chronic alcohol abuse. At least five of the sixteen patients who had AM migration studies admitted to having a "drink problem", although none showed obvious features of malnutrition. Patients with chronic alcoholism have an increased incidence of pneumonia (Bradsher, 1983 and Sullivan, 1972).

Migration data on BAL cells from AOC could not be interpreted adequately because of large numbers of neutrophils from these areas. However the use of lucigenin-dependent chemiluminescence allows comparison of AM from CS and from RCA. AM have been shown to produce lucigenin dependent chemiluminescence activity of the same order as neutrophils (Williams & Cole, 1981b). Thus lucigenin dependent chemiluminescence of BAL cells from AOC is

depressed compared to those from CS and those from CAP (RCA) ($P < 0.01$). These results seem to contrast with those from peripheral blood cells which show enhancement of lucigenin-dependent chemiluminescence of neutrophils in CAP compared to CS. However this is not entirely surprising since these lung cells (mainly neutrophils from AOC) could have degranulated by the time they were lavaged. Such degranulation is not unknown in acute lung injury (Yamada, 1982).

3.6 Conclusions:

1. BAL cells from "normal" areas of lung are comparable to those from CS, while those from AOC are predominantly but not exclusively neutrophils. An early lavage may give a useful cellular profile.
2. Lower than expected proportions of Langerhans cells were found in patients with CAP. This finding if confirmed could have important immunological implications regarding function of these cells in the lung.
3. Areas of consolidation contain significantly higher levels of complement components and products of complement activation.
4. Impairment of AM function in CAP patients may predispose to the development of pneumonia.

CHAPTER 4

SMOKE INHALATION INJURY

4.1 Introduction:

Lung injury due to smoke inhalation among fire victims has been identified as the single most important contributory factor to mortality in these patients. Fire victims may suffer from smoke inhalation only, smoke inhalation and burns or burns only. Patients who sustain both injuries carry by far the highest risk of mortality. This study endeavours to examine cellular and humoral changes in the lung, in the hope of filling some of the gaps in our understanding of the complex mechanisms involved. This study was planned and initiated by the Department of Anaesthesia at Glasgow Royal Infirmary in conjunction with the Department of Microbiology. I am grateful for being allowed to contribute to the study. Recruitment of patients was done for the most part by research registrars in the Department of Anaesthesia initially by Dr A. Pollok and later by Dr J. Kinsella. Without their cooperation this part of the study would have been impossible. I am deeply grateful to both of them. Patients were recruited directly from the Casualty Department in liaison with the Fire Officer who would often notify the research registrar of the fire incident even before the arrival of the patient(s) in the Casualty Department! Those patients who were initially admitted in other hospitals often

arrived here much later. Therefore, the time of bronchoscopy after the fire incident is variable (from 4-36 hours).

4.2 Diagnosis of smoke inhalation and definition of study group.

Diagnosis of smoke inhalation is not as easy as it may appear, and for this the system of clinical scoring introduced by Dr C.J. Clark, mentioned in Chapter 1, was followed. In brief, each one of the following points was allocated one score and a score of 2 or more was accepted as diagnostic of significant smoke inhalation:

1. A history of being entrapped in a fire in a closed space.
2. Production of carbonaceous sputum.
3. Perioral facial burns.
4. Altered level of consciousness at any time after the incident.
5. Symptoms of respiratory distress.
6. Signs of respiratory distress.
7. Hoarseness of voice.

Using these scores and the presence or absence of cutaneous burns, patients were categorised as follows: I. Smoke Inhalation only. II. Smoke Inhalation plus burns. III. Burns only. Measurement of carboxyhaemoglobin was made as soon as the patient was admitted and using a normogram it was possible to extrapolate the level of carboxyhaemoglobin at

the time of the incident. This gave a more accurate assessment of the intensity of smoke inhalation. Patients who were referred from other hospitals often lacked these data.

All patients included in this study gave informed consent. Often the procedure was performed because it was clinically indicated. BAL was performed as previously described between 4 and 36 hours and 13 patients were lavaged twice within 24 hours.

Control subjects were those described in the pneumonia study (Chapter 3). Table 4.1 gives further details of the patients in this study.

Table 4.1: Age, sex distribution and smoking history of study groups.

	Age Range (mean)	Sex Distribution		Smoking History	
		M	F	NS	S
Control Subjects n = 34	30-63 (48.9)	23	11	8	26
Smoke Inhalation Study Group n = 41	17-78 (46.2)	30	11	3	38

The above table underlines the importance of smoking in both the control group and to a greater extent the fire victims. It also shows that in both groups males

predominate. The following table shows a further breakdown of patients, subgroups and some clinical details.

Table 4.2: Clinical parameters of patients.

		Smoke Inhalation alone n=15	Smoke Inhalation + burns n=20	Burns alone n=6
% Burns	< 10	NA	5	
	11-29		9	1
	30-50		2	4
	> 50		4	1
% CoHb	10-20	1	5	
	21-30	4	5	
	31-40	4	3	NA
	41-50	1	4	
	> 51	4 (MD=1)	1 (MD=2)	
Symptom Score	3	3	5	
	4	4	4	
	5	3	4	NA
	6	2	2	
	7	-	2	
	MD	3	3	
No. of cases who died		1	8	1

NA = Not applicable. MD = Missing Data.

It is clear from Table 4.2 above that by far the highest mortality occurs in patients who sustain both inhalation and burn injuries.

The following data were obtained from these patients' bronchoalveolar lavages.

1. Cell yields and differential counts.

2. AM and T-cell subsets as determined by monoclonal markers.
3. AM migration (microchemotaxis chamber).
4. Chemiluminescence of AM.
5. Effect of BAL supernatants on migration of neutrophils from healthy donors.
6. Data of repeat lavages from some patients including functional assays.
7. Albumin and complement levels in BAL supernatants.

4.3 Results

4.3.1 BAL fluid volumes, original cell yields and differential counts

There was a slight variation in the volume of fluid instilled and recovered although these were generally comparable. (Table 4.3).

There was no difference between the proportion of fluid recovered between control subjects and different patients' subgroups. Cell yields were significantly lower in the non-smoking controls. There was a clear trend of increase in cell yields from patients with smoke inhalation and much more so in those with combined inhalation and burns injury. These however were not statistically significant. However there were significant differences between control subjects and patients with smoke inhalation in the yield of neutrophils as shown in Table 4.3(b).

Table 4.3a: BAL fluid volumes and cell yield.

		BAL Fluid Volumes (mls)			Cell
		Instilled	Recovered	Proportion of recovered fluid	Cells$x10^5$ /ml BAL fluid
Control Subjects (non-smokers)	Range	150-300	25-130	0.125-0.52	0.50-3.6
	Mean	205.0	83.75	0.40	1.61 *
Control Subjects (smokers)	Range	120-280	35-160	0.20-0.70	0.6-12.5
	Mean	202.6	94.0	0.46	3.67
Smoke inhalation only	Range	150-300	60-190	0.45-0.80	0.75-8.0
	Mean	220.9	119.1	0.56	4.44
Smoke inhalation with burns	Range	150-300	40-160	0.18-0.83	2.0-38
	Mean	236.7	116.7	0.50	6.06

* Significantly lower than cell yields of smoking controls.

P = 0.028.

Table 4.3(b): Yield of different cell types in smoke inhalation.

Patient Groups		Total	Macrophage	Neutrophils	Lymphocytes
		cells x 10^5/ml BAL fluid			
Controls n = 21	Mean(SD)	3.67(2.97)	3.50(2.97)	0.07(0.05)	0.07(0.07)
	Median(SEM)	3.2(0.65)	2.998(0.65)	0.008(0.01)	0.045(0.01)
Smoke Inhalation alone n = 12	Mean(SD)	4.44(2.1)	3.55(2.02)	0.80(1.03)	0.06(0.07)
	Med.(SEM)	3.93(0.60)	3.55(0.58)	0.39(0.29)	0.07(0.02)
	P value	NS	NS	P<0.001	NS
Smoke & Burns n = 17	Mean(SD)	6.05(8.56)	3.39(4.67)	2.55(4.34)	0.19(0.29)
	Med.(SEM)	3.30(2.07)	2.23(1.13)	0.80(1.05)	0.10(0.07)
	P value	NS	NS	P<0.001	NS
Repeat BAL (smoke with or without burns) n = 12	Mean	8.80(7.79)	3.98(2.98)	4.61(7.79)	0.08(0.1)
	Median	6.37(2.25)	3.05(0.86)	1.18(2.25)	0.04(0.02)
	P value	P = 0.01	NS	P<0.001	NS

These results show that there is a significant increase in neutrophils in patients with smoke inhalation with or without burn injury. Because of increase in the total cell yields the macrophage yields remain comparable to control subjects. (Fig. 4.1).

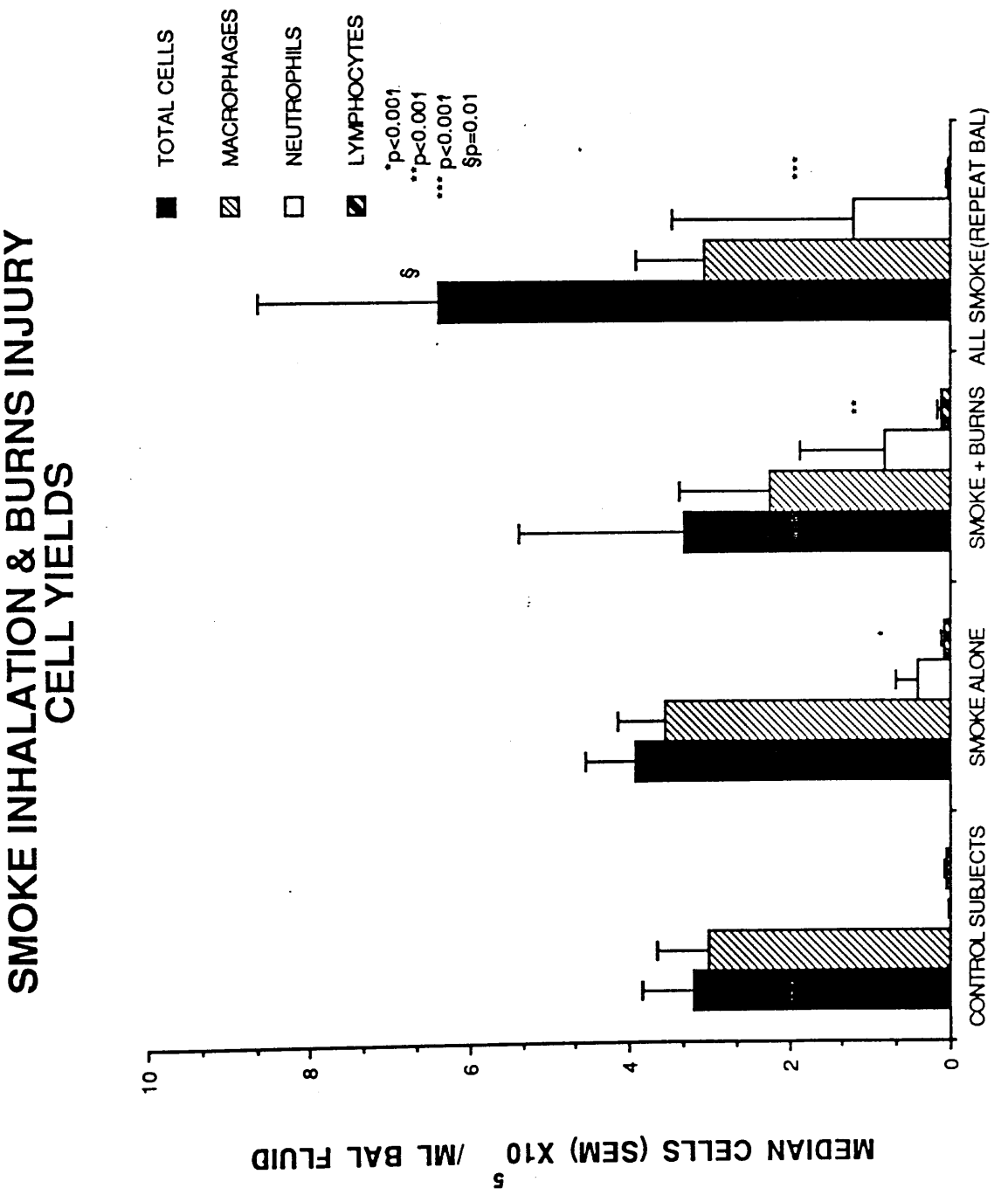


Fig. 4.1: Cell yields in smoke inhalation and burns injury.

The cell composition of BAL fluid from these patients, however, differs significantly from control subjects.

The cell composition of patients with smoke inhalation with or without burns showed a reduced proportion of macrophages and a reciprocal increase in neutrophil content, Table 4.4(a). This was highly significant in both subgroups but was much more pronounced in patients with combined inhalation and burn injuries. The increase in neutrophil content was greater with a longer delay of lavage following the inhalation injury and this is more apparent in repeat lavage data (Table 4.4(b)). It should be mentioned here that all repeat BAL were done on the opposite lung to the one previously lavaged, usually the right middle lobe or the lingula.

The cell yields from repeat BAL samples were significantly higher than the initial samples. Most of this increase is due to the influx of neutrophils, but it is clear that there is some increase in the absolute numbers of macrophages as well. The proportion of macrophages is however significantly lower in the repeat BAL samples than in the initial ones. This is associated with the significant reciprocal rise in the proportion of neutrophils ($P = 0.007$). The cell composition of BAL fluid from patients with burns only was remarkably similar to that from CS and did not show any increase in neutrophil population.

Table 4.4a: Original differential cell counts.

		Original differential cell counts (%)			
		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Control Subjects (Non-smokers) n = 8	Range	71.2 - 93.6	0 - 5.4	0 - 2.8	6.4 - 28
	Mean (SD)	85.5(9)	1.8 (1.95)	0.02 (0.07)	12.4 (8.5)
	Median	86.8	1	0	8.5
Control Subjects (Smokers) n = 26	Range	78.8 - 99.6	0.2 - 8.3	0 - 2.4	0 - 12.2
	Mean (SD)	93.8(4.75)	2.88(2.23)	0.36(0.57)	2.79(2.91)
	Median	94.95	2.4	0.1	2.0
Smoke Inhalation only n = 15	Range	30 - 97.8	2 - 69.8	0 - 1	0.2 - 27.6
	Mean (SD)	77.3(30)	18.7(22.6)	0.12(0.27)	3.76(7.15)
	Median	87.7*	10.4**	0	2.0
Smoke Inhalation + Burns n = 20	Range	7.4 - 95.0	1.8 - 91.8	0 - 2.2	0 - 10.4
	Mean (SD)	63.7(27.2)	33.4(27.2)	0.42(0.61)	2.46(2.3)
	Median	70.0***	27.85***	0.2	
Burns only n = 6	Range	76.3 - 98.4	0 - 19.5	0 - 0.2	0 - 8.2
	Mean (SD)	92.1(9.1)	4.7(8.2)	0.04(0.08)	3.1(3.1)
	Median	96.6	1.6	0	2.0

* Significantly lower than CS P = 0.0007 *** P<0.002 when compared to CS
** Significantly higher than CS P < 0.0009

Table 4.4b: Original cell yields and differential cell counts of initial and repeat BAL samples (n = 10).

		Time of Bronchoscopy (HRS)	Cell yield x 10^5 /ml	% Differential counts			
				Macro-phages	Neutro-phils	Eosino-phils	Lympho-cytes
Initial Lavage	Range	3 - 21	2.35-6.25	18.8-97.8	1.8-78.8	0 - 0.6	0 - 2.6
	Mean (SD)	9(5.2)	3.73(1.36)	75.3(27.1)	22.6(26.9)	0.16(0.24)	1.87(0.95)
Repeat Lavage	Range	27 - 43	2.70-31.5	5.6-91.5	8.2-94.0	0 - 3.0	0.4-6.0
	Mean (SD)	32.3(5.1)	12.69(10.2)	54.3(28.5)	43.0(29.3)	0.46(1.0)	2.12(1.94)
	P Value		0.005*	0.007*	0.007*	NS*	NS*
Control Subjects (Smokers) (mean = 21)	Range	-	0.60-12.5				
	Mean (SD)		3.67(2.97)**				

* Compared to the initial BAL.
** Compared to the 2nd BAL. Significantly lower P = 0.02.

4.3.2 Monoclonal markers

4.3.2.1 Macrophage markers

In an attempt to define the proportions of macrophage and T-cell subgroups monoclonal markers were used. Table 4.5a shows the result of macrophage markers. The proportions of subgroups of macrophages between different patient groups and control subjects is very similar except for RFD_9+ve cells in smoke inhalation only patients which were significantly higher than CS ($P < 0.02$). Surprisingly patients with combined injury also showed an increase, this did not achieve significance. There is a trend towards an increase in $UCHM_1$, positive cells in patients with smoke inhalation and significantly so in those with combined injury ($P < 0.02$). It is interesting that there is no increase in the proportion of RFD_7 positive macrophages which was observed in the AOC from CAP patients. The proportions of macrophages as defined by macrophage markers in the initial and repeat samples did not show any marked change, but this may be because of too few samples being studied (Table 4.5(b)).

Table 4.5(a): Percentage of macrophage subgroups in BAL of control inhalation patients as defined by Macrophage Markers.

	%	RFD_1	RFD_7	RFD_9	$RFDR_1$	T_6 (NAI/34)	$UCHM_1$
Control	Range	34.9-98.8	9-73.0	28.0-86.9	92-100	0.2-3.0	0-4.8
Subjects	Mean (SD)	81.1(16.3)	43.0(22.9)	60.0(19.3)	98.1(2.73)	1.57(0.91)	0.99(1.34)
n = 12	Median	86.1	37.6	62.1	99.1	1.40	0.60
Smoke	Range	78.0-98.0	16.0-76.0	70.8-88.8	98.0-100	1.0-2.8	0 - 10.8
Inhalation	Mean (SD)	87.7(6.9)	40.0(16.0)	77.7(5.1)	99.3(0.66)	2.02(0.63)	3.6(4.2)
only	Median	89.6	36.0	78.6	99.2		1.80
n=9	P Value	NS	NS	$P < 0.02$	NS	NS	NS
Smoke +	Range	75.0-98.0	10.2-76.0	37.9-93.8	97.2-100	0.4-3.2	0 - 14.7
Burns	Mean (SD)	88.7(6.99)	44.5(21.6)	67.85(17.7)	99.0(0.93)	1.97(0.77)	5.26(5.1)
n=14	Median	91.5	39.9	70.6	99.3		
	P Value	NS	NS	NS	NS	NS	P<0.02
Burns	Range	86-91.6	46.2-75.4	54.0-84.0	97.2-100	0.4-3.8	
only	Mean (SD)	88.8(2.8)	56.8(16.1)	73.6(17.0)	98.7(1.4)	1.66(1.85)	13.4(n=1)
	Median	89	49.0	83.0	99.0	0.8	
n=3	P Value	NS	NS	NS	NS	NS	NS

In Table 4.5(b) % Macrophage markers in initial and repeat BAL samples.

% (Means)	RFD_1	RFD_7	RFD_9	$RFDR_1$	$(NA1/34)T_6$	$UCHM_1$
Initial samples (n=6)	87.8	44.4	70.1	99.0	2.40	0.95 (n=5)
Repeat samples* (n=6)	88.1	49.3	71.0	98.8	2.0	2.6 (n=5)

*No significant differences were found between initial and repeat BAL samples in any of the macrophage markers.

4.3.2.2 T-cell markers:

These were done as previously described, Table 4.6a.

Table 4.6(a): (%) Mean T-cell subset in BAL of control subjects and smoke inhalation patients.

% Mean (SD)	T_3	T_4	T_8	T_4/T_8 Ratio
Control Subjects n = 13	2.6(3.35)	1.35(1.19)	1.88(2.60)	1.37(1.16)
Smoke only n = 7	1.80(2.09)	1.15(0.65)	0.65(0.41)	2.0(1.17)
Smoke + Burns n = 12	4.71(4.93)	1.9(1.73)	2.64(3.4)	1.10(0.8)
Burns only n = 2	3.48	2.30	2.61	1.16

There were no differences detected in the T-cell subsets and T_4/T_8 ratio between control subjects and patient groups. However in those patients where repeat BAL samples were examined, there was a clear trend emerging so that out of seven samples, six showed a relative reduction in T_4 positive cell proportions with a resultant reduction in T_4/T_8 ratio. The trend however was not statistically significant (P = 0.063).

Table 4.6(b): T-cell subsets in initial and repeat BAL cells from patients with smoke inhalation with or without burn injury (n=7).

% Means (SD)	T_3	T_4	T_8	T_4/T_8 ratio
Control Subjects (n = 13)	2.6(3.35)	1.35(1.19)	1.88(2.60)	1.37(1.16)**
Initial samples	3.2(2.1)	1.35(1.1)	1.42(0.93)	0.98(0.46)
Repeat samples	2.67(2.0)	0.74(1.1)	2.0(1.7)	0.33(0.39)*

*Compared to the initial lavage, all were not significantly different. T_4/T_8 ratio approached significance (P = 0.063).
**Compared to 2nd lavage, T_4/T_8 ratio is significantly higher (P < 0.01).

4.3.3 Differential cell counts following macrophage enrichment

Ficoll hypaque centrifugation allowed removal of substantial numbers of neutrophils so that the macrophage content of samples from patients with smoke inhalation alone was statistically not different to those from CS, (Table 4.7a). However neutrophil content in BAL samples from patients with smoke and burns injury although greatly reduced remained significantly higher than CS. Samples from patients with combined injury also achieved some degree of macrophage enrichment but neither the macrophage nor the neutrophil contents were comparable to those from CS.

Samples from patients with burns only compared extremely well with those from CS. The neutrophil content from this subgroup was low enough almost to be significantly different to those from patients with combined inhalation and burn injuries ($P = 0.05$). Purification of macrophages from repeat BAL samples was comparable to that of initial samples despite their increased neutrophil content.(see Table 4.7(b)).

The mean (SD) (%) viabilities of cells after ficoll hypaque centrifugation from CS patients with smoke inhalation only, smoke and burns only were 67(15); 85.5(6.8), 82.1(10.9) and 75.6(10.0) respectively.

Purification of alveolar macrophages from BAL:

Table 4.7(a): Cell composition of BAL samples after density gradient centrifugation.

		Differential Counts (%)			
		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Control Subjects (Non-Smokers) n=7	Range	71.7-97.4	0.2 - 4.3	0 - 0.2	2 - 24
	Mean (SD)	89.4(8.3)	1.3(1.4)	0.02(0.07)	8.9(7.0)
	Median	92.4	0.8	0	7.2
Control Subjects Smokers n=26	Range	87.0-99.6	0 - 9.0	0 - 1.0	0 - 8.0
	Mean (SD)	96.7(3.1)	1.51(2.07)	0.15(0.25)	1.6(1.99)
	Median	98.0	0.90	0	0.90
Smoke Inhalation only n=14 +	Range	52.0-99.6	0.2 - 48	0 - 0.18	0 - 27.6
	Mean (SD)	88.9(16.4)	8.3(13.4)	0.01(0.04)	2.9(6.9)
	Median	96.8	2.0*	0	0.6
Smoke Inhalation + Burns n=17 +	Range	14.0-100	0 - 85.6	0 - 1.0	0 - 12.0
	Mean (SD)	76.7(14.0)	21.2(28.5)	0.12(0.26)	1.8(2.7)
	Median	86.4**	12.0***	0	1.2
Burns only n=6	Range	91.2-98.3	0 - 3.4	0	0 - 8.2
	Mean (SD)	94.4(3.0)	1.86(1.23)	0	3.7(3.2)
	Median	94.1	1.9 ++	0	3.6

+ Data from non-smokers excluded and 1 sample from burns + smoke subgroup was unsuitable for further processing.
++ When compared to smoke and burns P = 0.05
* When compared to CS significantly higher neutrophil content. P = 0.014.
** Significantly lower compared to CS P = 0.001
*** Significantly higher than CS P < 0.0009

Table 4.7(b): Purification of AM from initial and repeat BAL samples of patients with smoke inhalation with or without smoke inhalation.

% Means (SD)	Macro-phages	Neutro-phils	Eosino-phils	Lympho-cytes
Initial Samples	77.4(33.6)	21.3(33.3)	0.07(0.27)	1.10(0.81)
Repeat Samples	75.2(25.4)	21.5(26.7)	0.16(0.24)	1.66(0.58)

4.3.4 A.M. Migration:

This was done on 10 samples from patients with pure smoke inhalation (including one non-smoker) and on 12 samples on those with mixed injuries (including 2 non-smokers). Data from 5 patients with pure burns were also obtained. The results were analysed in two ways: I. Comparing the whole group of smoke inhalation with or without burns to controls. II. Comparing the subgroups to controls. In both cases data from non-smokers are excluded.

Measurement of macrophage migration showed significantly higher unstimulated migration of AM from the patients with smoke inhalation (Table 4.8a and Figures 4.2a-c). There was also increased stimulated migration towards casein, ZAS and f-met-leu-phe although the latter did not achieve statistical significance. There was considerable intersubject variation

Table 4.8(a): AM migration of smoke inhalation with or without burns.

		Unstimulated	Casein	ZAS (C5a)	FMLP
Control Subjects	Mean (SD)	29.1 (12.2)	37.1 (15.4)	61.5 (28.3)	33.4 (17.6)
n = 19	Median (SEM)	30.0 (2.8)	31.0 (1.5)	55.5 (6.6)	26.0 (4.3)
Smoke Inhalation	Mean (SD)	53.6 (34.2)	93.3 (88.7)	196.3 (212.8)	70.1 (85.4)
with or without	Median (SEM)	47.0 (7.8)	73 (20.3)	107 (48.8)	45.0 (20.7)
burns	P value	P = 0.01	P < 0.01	P = 0.002	NS
n = 19					

NS = Not significant.

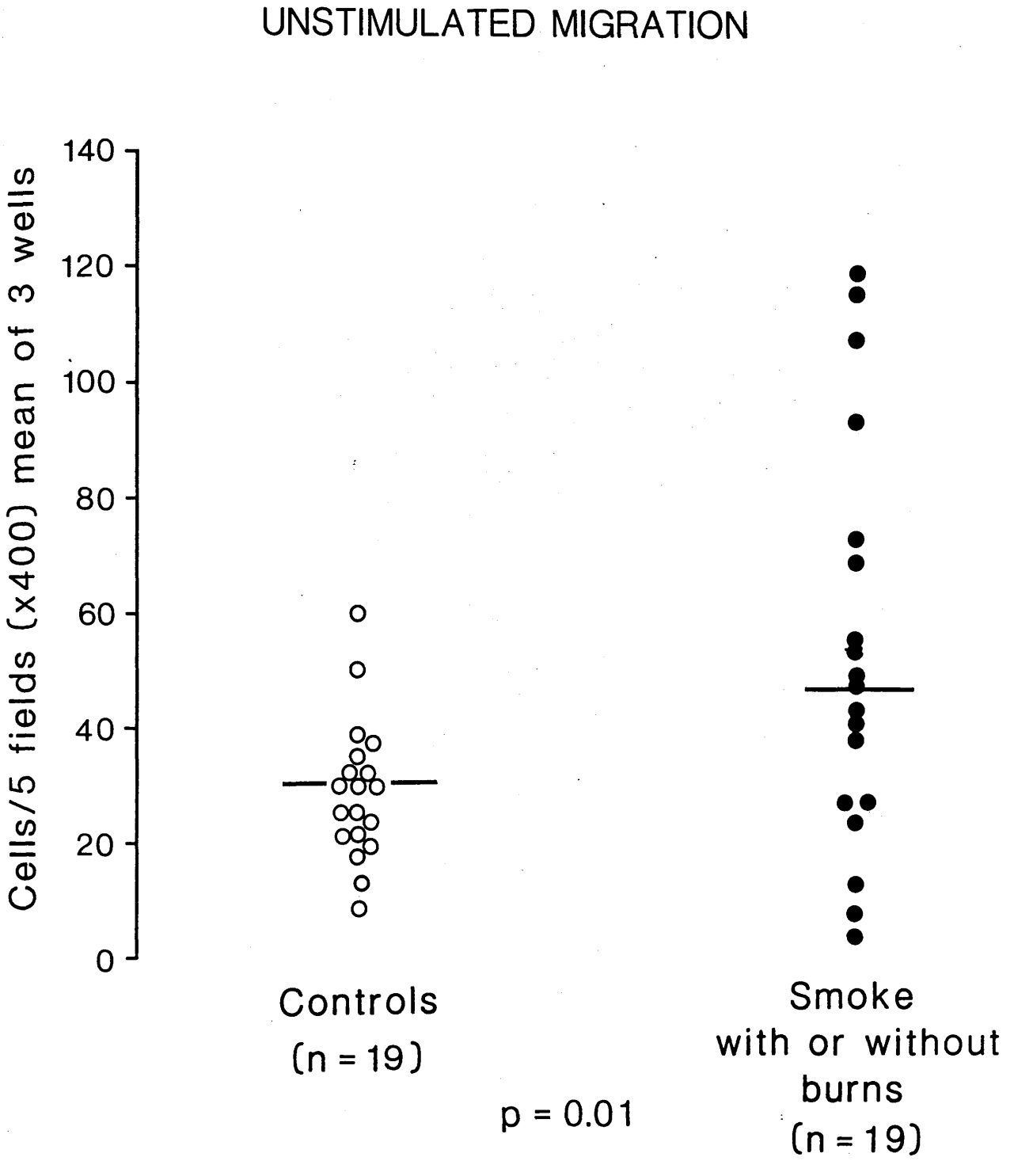


Fig. 4.2:(a): Unstimulated AM migration in smoke inhalation and burns injury.

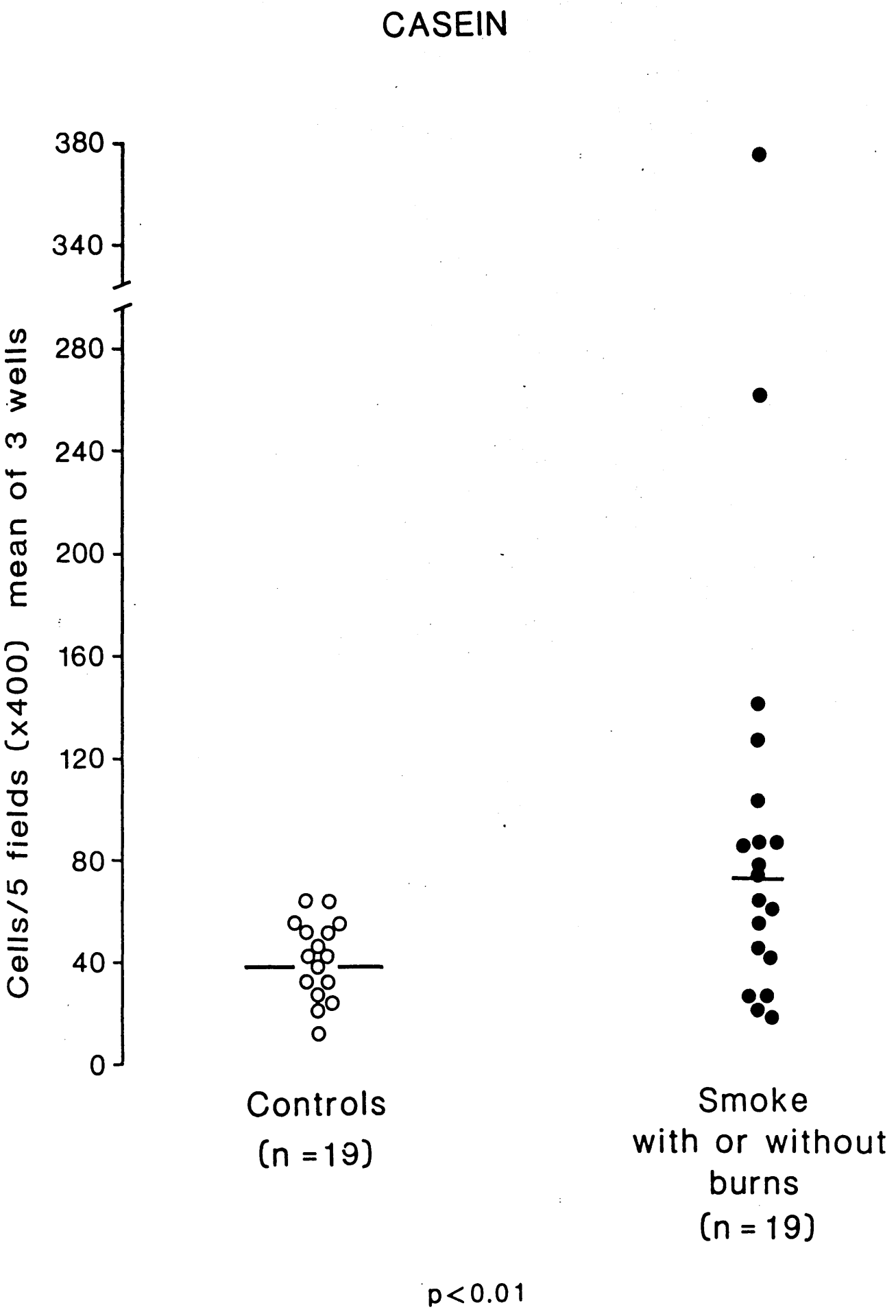


Fig. 4.2(b): Stimulated AM migration towards casein in smoke inhalation and burns injury.

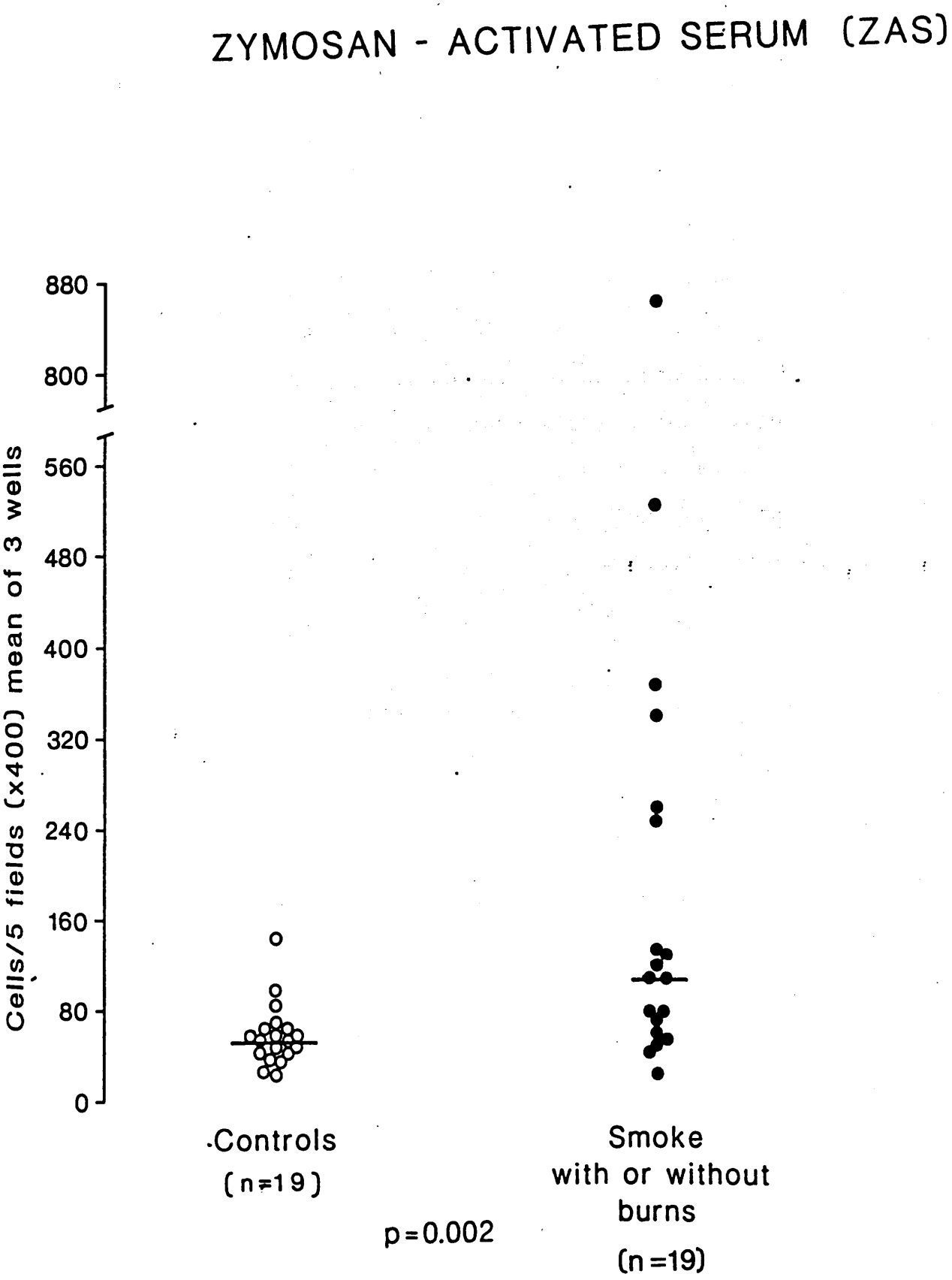


Fig. 4.2(c): Stimulated AM migration towards ZAS in smoke inhalation and burns injury.

as is apparent in the standard variations of these values. Further analysis of these data into subgroups gives valuable information. (Table 4.8(b) and Figure 4.3).

It is clear that AM from patients with inhalation injury alone show a trend of increased migration both stimulated and unstimulated but this is not significant. However, AM from patients with combined smoke inhalation and burn injury show significantly higher stimulated and unstimulated migration. Migration of AM from patients with burns alone is not different from those of CS. Measurement of migration of AM from repeat BAL samples was insufficient and data for 4 patients only is available (Table 4.8(c)) and no clear pattern emerged from these experiments.

4.3.5 Respiratory burst generation in alveolar macrophages:

Generation of respiratory burst in AM from patients with smoke inhalation was assessed using both lucigenin and luminol dependent chemiluminescence. Counts were measured for 10 seconds at five minute intervals for a period of 70 minutes. Results are presented as mean counts per 10^3 cells in tables 4.9a&b.

Measurement of AM lucigenin dependent chemiluminescence showed no difference between control subjects and patients with smoke inhalation either alone or with burns. (Fig. 4.4(a)).

Table 4.8(b): Migration of AM from smoke inhalation study subgroups.

		Unstimulated	Casein	ZAS (C5a)	FMLP
Control Subjects	Means (SD)	29.1 (12.2)	37.1 (15.4)	61.5 (28.3)	33.4 (17.6)
n = 19	Median (SEM)	30.0 (2.8)	31.0 (3.5)	55.5 (6.6)	26.0 (4.3)
Smoke Inhalation	Means (SD)	44.1 (29.1)	66.0 (43.7)	145.2 (123.9)	41.1 (40.7)
only	Median (SEM)	38.0 (9.7)	59.0 (14.5)	107.0 (41.3)	31.0 (15.3)
n = 9	P value	NS	NS	NS	NS
Smoke Inhalation	Mean (SD)	62.2 (37.6)	117.9 (112.48)	242.3 (268.3)	90.4 (103.8)
+ Burns	Median (SEM)	54.0 (11.8)	81.5 (35.5)	113.5 (84.8)	60.0 (32.8)
n = 10	P value	$P < 0.01$	$P < 0.005$	$P < 0.002$	$P < 0.05$
Burns only	Mean (SD)	24.0 (6.4)	67.2 (45.7)	116.6 (100.6)	30.6 (28.9)
n = 5	Median (SEM)	27.0 (2.9)	58.0 (24.0)	58.0 (42.0)	16.0 (12.0)
	P value	NS	NS	NS	NS

NS = Not significant

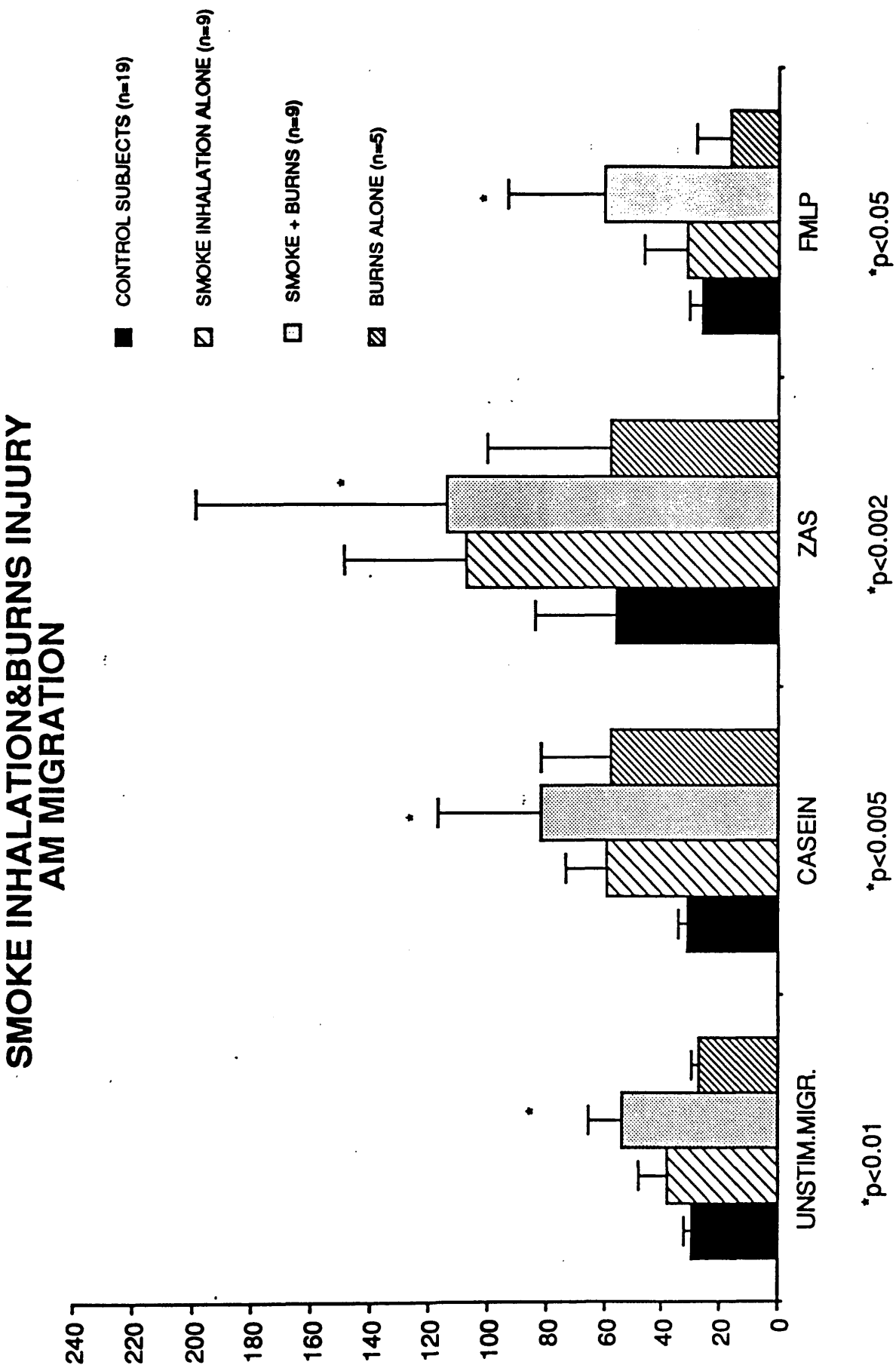


Fig. 4.3: AM migration in smoke inhalation and burns injury.

Table 4.8(c): Measurement of AM migration from initial and repeat BAL samples from patients with smoke inhalation with or without burn injury (n=4).

Mean (SD)	Unstimulated	Casein	ZAS	FMLP
Initial Samples	34.2(27.3)	49.0(31.2)	150.0(148.4)	27.2(26.9)
Repeat Samples	37.0(24.0)	49.0(41.5)	75.5(26.5)	37.7(7.8)

Table 4.9(a): Lucigenin dependent chemiluminescence of AM from smoke inhalation patients.

Peak counts per 10^3 cells		Cells + Bacteria	Cells + gel Hanks
Control Subjects	Mean (SD)	812.4(852.5)	821.1(876.9)
n = 10	Median	400.0(269.6)	415.0(277.3)
Smoke Inhalation	Mean	770.0(779.8)	835.0(718.2)
n = 10	Median	565.0(246.6)	545.0(227.1)
	P value	NS	NS
Smoke + Burns	Mean	671.7(812.4)	853.8(927.1)
n = 15	Median	350.0(209.7)	525 (247.7)
	P value	NS	NS

NS = Not significant.

Table 4.9(b): Luminol dependent chemiluminescence of AM from patients with smoke inhalation.

Peak counts/10^3 cells		Cells + Bacteria	Cells + gel Hanks
Control Subjects	Mean (SD)	21.5 (58.4)	14.4 (39.5)
n = 17	Median (SEM)	3.8 (14.1)	2.7 (9.5)
Smoke Inhalation only	Mean (SD)	72.2 (111.2)	4.7 (5.8)
n = 9	Median (SEM)	17.0 (37.0)	4.7 (1.7)
	P value	P < 0.01	NS
Smoke Inhalation + Burns	Mean (SD)	45.3 (42.8)	10.3 (6.9)
n = 13	Median (SEM)	43.6 (11.9)	10.2 (2.0)
	P value	P < 0.05	NS

NS = Not significant.

Measurement of luminol-dependent chemiluminescence of BAL cells from patients with smoke inhalation only or with burns was significantly higher than control subjects and this was in keeping with higher neutrophil content in samples from these patients. These results are illustrated further in Figure 4.4(b). To follow the capability of BAL cells in initiating respiratory burst, chemiluminescence was assessed in repeat lavage samples from patients with smoke inhalation with or without burns. These were not analysed into subgroups because of small numbers (Table 4.10(a) and 4.10(b)).

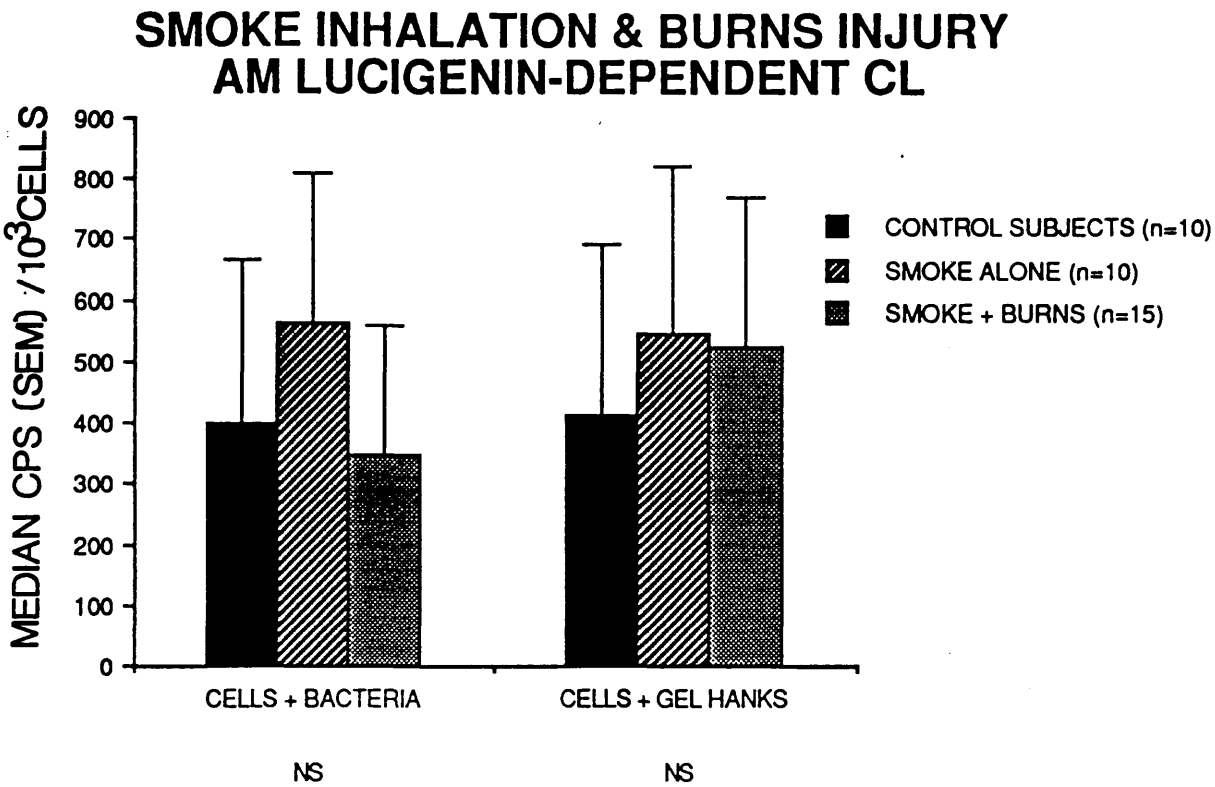


Fig. 4.4(a).

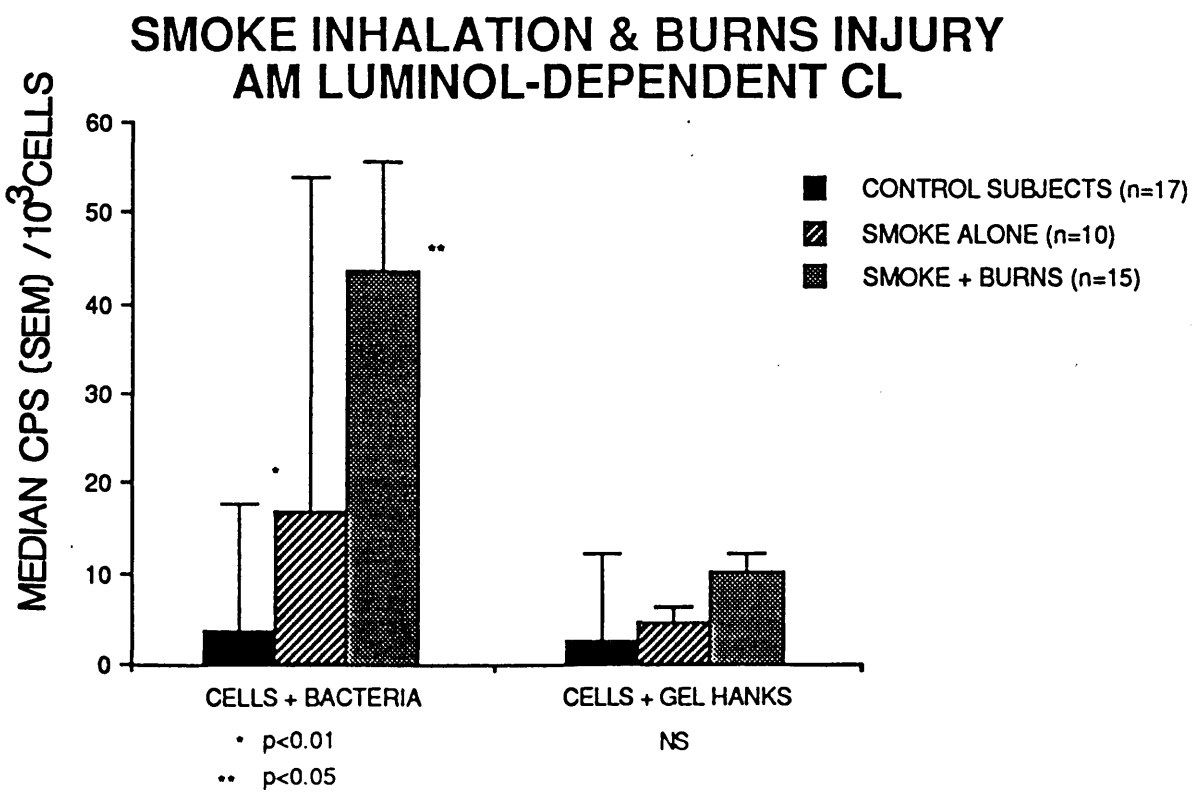


Fig. 4.4(a&b): AM lucigenin and luminol-dependent chemiluminescence in smoke inhalation and burns injury.

Table 4.10(a): Lucigenin dependent chemiluminescence of repeat BAL samples from patients with smoke inhalation with or without burn injury (n = 9)

Peak counts/10^3 cells		Cells + Bacteria	Cells + Gel Hanks
Initial samples	Mean (SD)	753.3(881.8)	1065.2(1094)
	Median (SEM)	350.0(293.9)	490.0(364.8)
Repeat samples	Mean (SD)	196.6(133.7)	327.8(246.2)
	Median (SEM)	180.0(44.6)	370.0(82.0)
	*P value	0.038	0.066 (NS)

* Wilcoxon matched pairs signed Ranks Test.

Table 4.10(b): Luminol-dependent chemiluminescence of repeat BAL samples from patients with smoke inhalation with or without burn injury (n = 9).

Peak counts/10^3 cells		Cells + Bacteria	Cells + gel Hanks
Initial Samples	Mean (SD)	72.7 (109.2)	4.91 (3.6)
	Median (SEM)	30.0 (36.4)	4.50 (1.2)
Repeat Samples	Mean (SD)	60.6 (45.2)	7.75 (3.2)
	Median (SEM)	50.0 (15.0)	9.0 (1.0)
	¹ue	NS	NS

Stimulated lucigenin-dependent chemiluminescence showed significant reduction in subsequent BAL samples, but the reduction of spontaneous chemiluminescence of BAL cells from these samples was not significant (Table 4.10(a)). Luminol-dependent chemiluminescence did not show any change despite increased proportions of neutrophils in these samples.

4.3.6 Effect of BAL supernatants on neutrophil migration

To investigate a possible cause of neutrophil accumulation in the lungs, and also a measure of AM activation (which can result in the release of AM-derived neutrophil chemotactic factor), concentrated BAL supernatants were tested for their chemotactic activity to neutrophils from healthy donors. These were compared to chemotactic activity of supernatants from control subjects. These were done on two different days from 2 healthy donors and calculation of the chemotactic differential is taken as a measure of chemotactic activity. (See Table 4.11).

Table 4.11: Effect of 10-fold concentrated BAL supernatants from smoke inhalation patients on chemotaxis of healthy donor neutrophils.

Cells/5 fields (x 400)	Control Subjects n=9	Smoke only n=7	Smoke + burns n=5	Burns only n=3
Mean (SD)	32.3(95.2)	68.7(72.8)	158.4(114.8)	24.0(130.4)
Median (SEM)	36.0(31.7)	48.5(27.5)	182.0(51.3)	-19.0(75.3)
P value		NS	$P < 0.05$	NS

Measurements of chemotactic activity of concentrated BAL supernatants towards neutrophils from healthy donors showed that there was increased chemotactic activity of BAL supernatants from patients with smoke inhalation and this was significantly so in patients who had combined injury ($P < 0.05$). This is in keeping with the higher neutrophil content of lavages from this subgroup compared to control patients. BAL supernatants from patients with burn injury alone did not show any increased chemotactic activity above control patients. (Figure 4.5).

4.3.7 Albumin in BAL supernatants.

Data for BAL supernatants was obtained by using a double labelled RIA as previously acknowledged. The results are presented in Table 4.12.

Table 4.12: Albumin levels in mg/L of BAL fluid from patients with smoke inhalation injury.

	Control Subjects n = 16	Smoke Alone n = 7	Smoke + Burns n = 10
Range	0 - 500.7	19.2 - 200	0 - 555.6
Mean (SD)	77.93(138.5)	94.73(77.1)	161.74(183.9)
Median (SEM)	30.55(39.98)	60.0(29.1)	83.0(58.2)
P value	NS	NS	P<0.05

The results [illegible] a significant difference

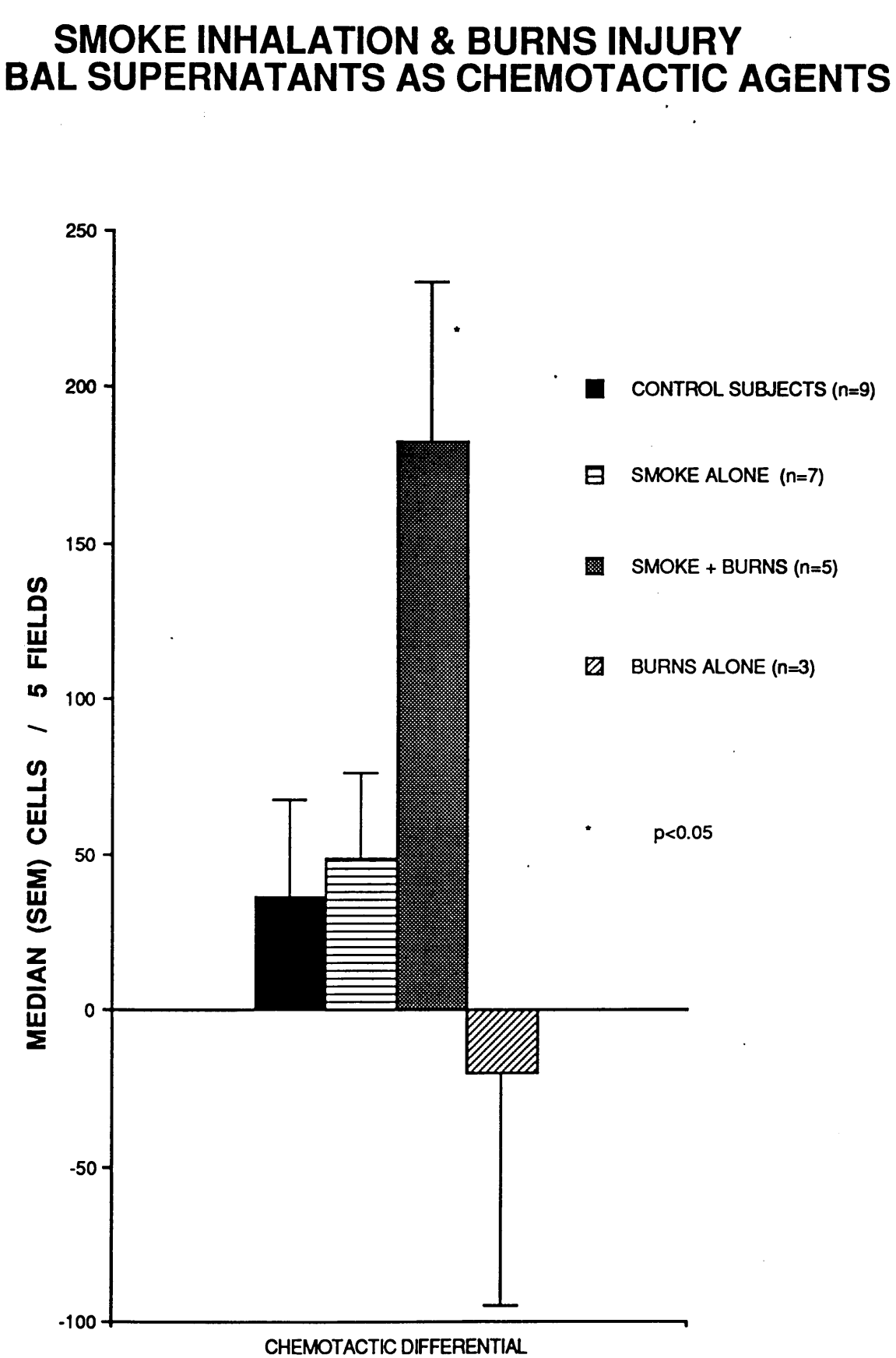


Fig. 4.5: BAL supernatants as chemotactic agents in smoke inhalation and burns injury.

in albumin levels between patients with combined inhalation and burns injury compared to either the CS or those with smoke inhalation only. Patients with combined injury also had higher levels of albumin in their BAL supernatants than those with smoke inhalation alone. However this was not statistically significant.

4.3.8 Estimation of complement components and products of complement activation in BAL supernatants.

Complement components were measured by an ELISA technique as previously mentioned, Tables 4.13a and b. These results show that there are significantly higher levels of complement on BAL supernatants from patients with combined injury. While there is a trend of increased levels in patients with smoke inhalation only, none of the products is significantly higher than the control subjects.

Comparison between patient subgroups shows that there is no significant difference in the levels of most of these products between the two except the C1s and the C3 which are significantly lower in the smoke inhalation only patients compared to the smoke inhalation with burns patients.

Relating the levels of complement components to albumin concentration in the BAL supernatants resulted in the reduction of the significance level of differences between smoke patients and control subjects.

Table 4.13(a): Complement levels in BAL fluid from patients with smoke inhalation injury in mg/L.

		C1q	C1r	C1s	C4	C3	C1-Inh	Factor B	Factor H
;ol	Mean (SD)	0.006(0.02)	0.009(0.03)	0(0)	0.013(0.03)	0.753(1.22)	0.068(0.13)	0.138(0.15)	0.087(0.19)
,ects 16	Median (SEM)	0(0.006)	0(0.009)	0(0)	0(0.009)	0.40(0.306)	0(0.033)	0.10(0.039)	0(0.046)
ıoke	Mean(SD)	0.028(0.04)	0.053(0.08)	0.016(0.05)	0.238(0.35)	1.513(1.44)	0.288(0.55)	0.438	0.3
ılone	Median(SEM)	0(0.01)	0(0.038)	0(0.02)	0(0.12)	1.10(0.51)	0(0.2)	0.40	0.1
n = 8 P value		NS	NS	NS	NS	*NS	NS	NS	NS
Smoke + burns	Mean(SD)	0.338(0.48)	0.173(0.16)	0.221(0.33)	3.255(5.34)	4.758(4.07)	1.875(2.44)	1.250 (1.78)	0.990 (0.94)
	Median(SEM)	0.07(0.14)	0.11(0.05)	0.07(0.01)	1.20(1.61)	3.50(1.18)	0.95(0.7)	0.60 (0.513)	0.70 (0.282)
n = 11 P value**		P<0.01	P<0.002	P<0.002	P=0.01	P<0.002	P<0.05	P<0.002	P<0.002

* Significantly lower than smoke and burns patients P = 0.05.

** All P values relate to comparison with control subjects.

Table 4.13(b): Complement levels in BAL fluid in mg/gm albumin in smoke inhalation injury.

		C1q	C1r	C1s	C4	C3	C1-Inh	Factor B	Factor H
ıtrol bjects nly ı= 16	Mean (SD)	0.056 (0.22)	0.078 (0.31)	0.039 (0.16)	0.969 (2.68)	44.3 (115.1)	2.12 (4.13)	4.66 (6.77)	6.33 (19.49)
	Median(SEM)	0(0.056)	0(0.078)	0(0.039)	0(0.67)	11.85(28.78)	0(1.03)	0(1.03)	0(4.87)
Smoke alone n=8	Mean (SD)	0.285 (0.463)	0.471 (0.704)	0.140 (0.396)	2.144 (3.117)	31.55 (19.629)	4.423 (6.557)	7.205 (8.128)	3.226 (2.64)
	Median(SEM)	0(0.164)	0(0.249)	0(1.139)	0(1.102)	23.42(6.94)	0(2.318)	5.385(2.874)	3.60(0.734)
	P value	NS	NS	NS	NS	NS	NS	NS	NS
Smoke + burns n=10	Mean (SD)	0.287 (0.388)	0.563 (0.411)	0.269 (0.250)	2.492 (2.492)	15.309 (9.830)	3.164 (3.89)	4.073 (1.905)	3.710 (3.94)
	Median(SEM)	0.14 (0.123)	0.71 (0.130)	0.27 (0.079)	2.83 (0.788)	14.065 (3.108)	2.185 (1.229)	4.18 (0.603)	2.245 (1.246)
	P value	NS	P<0.01	P<0.02	NS	NS	NS	NS	P<0.05

Levels of all components except C3 remained higher in smoke and burns patients than either the CS or those with single injury. Levels of C1r and C1s and Factor H were significantly higher in those with combined injury than CS.

Products of complement activation in smoke inhalation injury were also measured. These are presented in Table 4.14.

All these products were higher in patients with combined smoke inhalation and burns injury than CS or those with smoke inhalation alone. The level of C1s-CInh complex was significantly so ($P < 0.05$). The results for C3-P were particularly interesting in patients subgroups. In 10 lavage samples (8 initial and 2 repeat) from the smoke inhalation only group, none showed detectable levels of C3-P, while 5 out of 13 samples from those with smoke and burns injury showed detectable levels.

Table 4.14: Levels of complement activation products in BAL fluid in smoke inhalation injury.

	Units/L BAL fluid			ug/L BAL fluid	
	C1s-C-Inh	$C3_{-p}$	$C5b_{-9}$	C5a	C3a
ontrol subjects	138.5(499)	76.9(277.3)	0(0)	1.72(0.9)	10.71(4.97)
n = 14	0(138.5)	0(76.9)	0(0)	1.60(0.24)	9.8(1.33)
Smoke alone	300(669.7)	0(0)	237.5(477.9)	1.62(1.06)	19.37(10.5)
	0(236.8)	0(0)	0(168.9)	1.50(0.37)	23.2(3.5)
n = 8	NS	NS	NS	NS	NS
Smoke + burns	3327(4197.4)	560(653.5)	411.1(816.2)	2.53(2.21)	98.84(172.05)
	2500(1399.1)	250(206.6)	0(272.1)	1.75(0.70)	34.6(54.4)
n = 10	$P < 0.05$	NS	NS	NS	NS

4.4 Discussion:

Previous studies (Phillips, 1962; Achauer, 1973) have underlined the importance of lung injury in morbidity and mortality among fire victims. While there is an enormous amount of literature in burns injury, there is a conspicuous gap in the literature on smoke inhalation injury especially the changes in the lung. BAL offers a direct approach in studying some of the physiopathological changes that occur in the lung. Using this approach, Gemmell (1987) and others (Clark 1988(a&b); Demarest 1979) have presented data showing some of the cellular and functional changes that occur.

These changes include an influx of neutrophils into the lungs and an increase in total cell yields from BAL fluid in these patients. Data presented in this study confirm these findings. Patients with smoke inhalation and especially those with burns injury as well have a moderate increase in total cell yields but not significantly so during the early period. Patients who were lavaged a second time (from the opposite lung) showed significant increases in their total cell yields, compared both to their initial lavages ($p < 0.005$) and to the CS ($p = 0.002$). The increase is almost completely due to an influx of neutrophils into the lungs. This influx soon changes the cell profile in BAL fluid of these patients so that there is a reciprocal rise and fall in the proportions of neutrophils and macrophages respectively. Even in the first 24 hours the proportion of neutrophils becomes significantly higher in patients with

smoke inhalation alone ($p < 0.002$) and in those with combined injury ($p < 0.002$) than in CS (see Table 4.3(b) and (c)). The proportion of macrophages becomes significantly lower in smoke inhalation only ($p = 0.0007$) and smoke plus burns ($p < 0.002$), when compared with C.S. Patients with burns only do not show any increase in neutrophil population.

After the first 24 hours this situation progresses further so that the cell profiles in repeat BAL samples from the same patients with or without smoke inhalation show an even greater rise and fall in the proportions of neutrophils and macrophages respectively ($p = 0.007$), (Table 4.4b).

The influx of neutrophils in the lungs of these patients in our study is similar to that reported by others (Gemmell 1987; Demarest 1979; Clark 1988a&b) in smoke inhalation injury. This accumulation of neutrophils may be due (a) to the release of macrophage products such as AM-derived neutrophil chemotactic factor known to be released by activated macrophages (Huninghake, 1978, 1980) or (b) to products of complement activation or (c) LTB_4. Such factors have been shown to attract neutrophils into the lungs. Our data on the chemotactic effect on normal neutrophils of BAL supernatants from patients with smoke inhalation suggests the presence of similar factors (Table 4.11). Data on complement levels strongly support this view (Table 4.13a&b and 4.14).

Although the proportion of macrophages is lower in the BAL samples from [illegible]nts, with an increase in total cell yields, th[illegible] macrophages remain very

similar to those from control patients (Fig. 4.1).

Macrophage markers show a modest rise in $UCHM_1$-positive cells (monocytes) in patients with combined smoke and burn injury ($p < 0.02$) (Table 4.5(a)).

Data on macrophage markers also suggest an increase in the proportion of RFD_9-positive cells in the smoke inhalation only patients compared to CS ($p < 0.02$). Surprisingly in patients with combined injury the trend was not significant. The importance of this finding can only be conjectural because the function of the subgroups of macrophages defined by these markers is not yet known. RFD_9-positive cells are tingible macrophages with wide distribution in the body (Janossy, 1986).

It is interesting that these data did not show any significant change in RFD_7-positive cells which are supposed to be "mature" macrophages. This observation is different from that seen in BAL samples from areas of consolidation in the CAP patients in which there was an increase in RFD_7 -positive cells (Table 3.5). In addition CAP patients (AOC) showed a large increase in $UCHM_1$-positive cells.

It is not clear how soon these $UCHM_1$ positive cells acquire RFD_7 markers <u>in vivo</u> but in culture this takes five days (Linch, 1984) or 7 days (Poulter, 1986). Another interesting observation in the macrophage markers data is the similarity of proportions of NA1/34 (T_6) positive cells between these patient groups and CS (in contrast to CAP patients). This may be because these patients are similar in many ways to CS [illegible] been for the fire incident a few

hours earlier.

T-cell markers revealed no differences in the proportions of T-cell subsets between CS and patient groups in the initial lavage samples. In the repeat BAL samples, however, there was a trend towards a lower proportion of T_4-positive cells and an increase in the T_8-positive cells, which in themselves were not different from the initial lavage samples, but were sufficiently different to affect the T_4/T_8 ratio which was significantly lower than in the CS ($p < 0.01$) (Table 4.7).

Chemotaxis data on AM from patients with smoke inhalation showed increased unstimulated and stimulated migration towards the various chemotaxins. Patients with smoke inhalation alone showed only a trend but it was clear that patients with combined injury demonstrated highly significant differences in migration compared to control subjects.

This finding is in sharp contrast to that of Demarest (1979) who reported significantly lower AM chemotaxis from a group of seven patients with smoke inhalation compared to both smoking and non-smoking controls. There are some important differences between Demarest's study and ours. First the total number of patients whom they studied were few, three of whom had no burns, and another two had sustained only very mild burns. Second, the BAL profile from Demarest's patients consisted of a high proportion of neutrophils (35%) which in our experience physically interferes with t[illegible]n of macrophages through membrane

pores. BAL cell profiles in our patients who had chemotaxis studies, consisted of a mean of 85.8 (± 24.9)% macrophages and 11.2 (± 12.1)% neutrophils after purification with Ficoll -hypaque density gradient centrifugation. Thus these differences could account for the contrasting results between our studies and those of Demarest (1979).

The finding of increased migration of macrophages from patients with smoke inhalation may be due to the presence of a new population of macrophages as suggested by increased RFD_9-positive cells moving into the alveoli and capable of expressing various receptors for chemotactic agents such as C5a. Such a picture has been previously described, for example following inhalation of asbestos (Warheit, 1984). Alternatively this could be due to stimulation of the resident macrophages by a phagocytic load (smoke debris), plate 4.1(a&b), resulting in their activation and expression of receptors for chemotactic factors previously internalized.

Our data on lucigenin-dependent-chemiluminescence (luc-dep-cl) showed no difference between the patient groups and control subjects. Furthermore comparison of the initial luc-dep-cl with that of repeat samples from some patients shows a significant reduction in this activity ($p = 0.038$) in the repeated samples (Table 4.10a). Luminol-dep-cl from the initial and repeat BAL samples from smoke inhalation patients was significantly higher than CS. The latter is most likely to be related to the increased neutrophil proportions in these samples.

The apparent discrepancy between AM migration data and

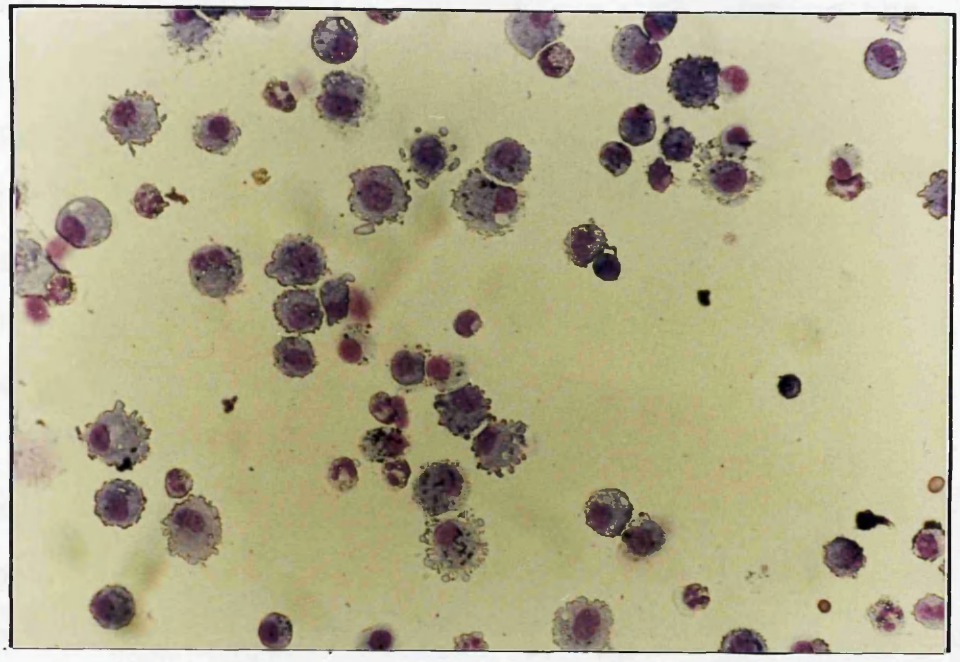


Plate 4.1(a): Leishman's Stain of BAL Cells From a Patient with Smoke Inhilation.

Note: Smoke Debris - Laden Macrophages.

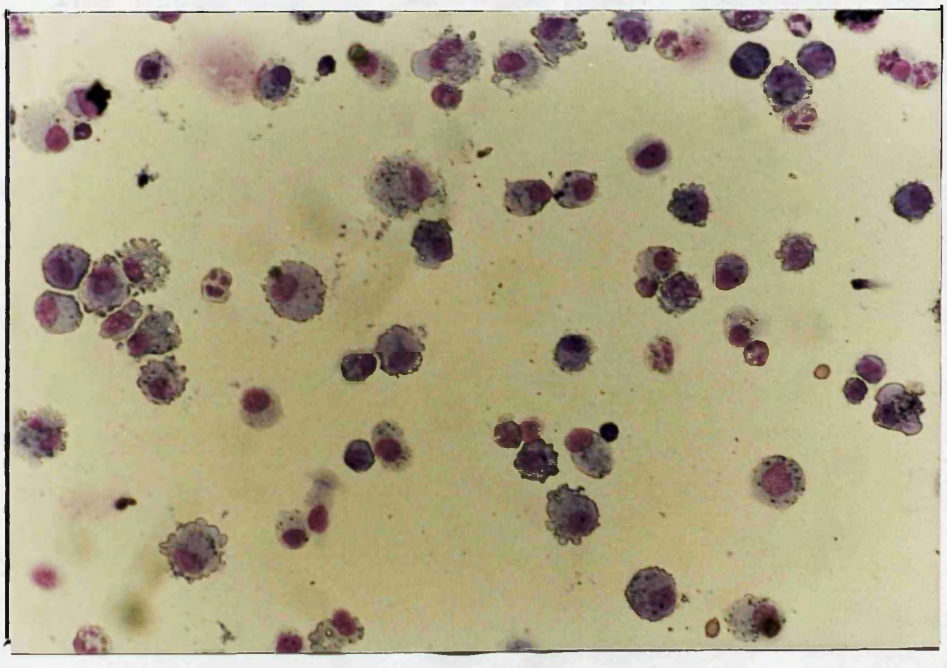


Plate 4.1(b): BAL Cells From Patient Above.

Note: Some Increase in PMN.

chemiluminescence activity is possibly due to the different time scale involved in these two functions. In vitro data suggest that initiation of the respiratory burst is a rapidly activated function, so that in most of our experiments, the peak counts are obtained within 5-15 minutes. This contrasts sharply with migration, which is of the order of 3-4 hours. The reduction of luc-dep-cl activity of AM from the repeat BAL samples, suggests that by the time these patients were lavaged this function of AM was "winding down". In a systematic study where patients were serially lavaged by Gemmell (1987) chemiluminescence of BAL cells was low at 0 hours (at admission to hospital), higher than normal at 4 hours and lower again at 24 hours. Our patients were not lavaged according to such a strict schedule, and there was a wide variation in the duration of time between smoke inhalation and lavage.

The picture that comes over then in these patients with smoke inhalation is that of accumulation of neutrophils and activation of AM. This phnomenon is accentuated in patients with burns and smoke inhalation and is qualitatively different from that of simple "depression" of systemic immune functions described in burns only. In addition there are increased levels of components of complement and complement activation products patients with combined injury.

4.5 CONCLUSIONS:

1. Accumulation of neutrophils in the lung of patients

with smoke inhalation.

2. Activation of AM in these patients.

3. Presence of products of complement activation in the lungs of patients with smoke inhalation.

4. Accentuation of all these findings in patients with smoke inhalation and burn injury.

CHAPTER 5

RADIATION INJURY

5.1 Introduction

Radiation pneumonitis is one of the most important complications limiting the dosage of radiotherapy. Radiation pneumonitis occurs within the first few months after irradiation but the cellular and biochemical events leading up to the development of the full clinical picture of the disease are poorly understood. The aim of this study was to catalogue cellular changes in the lung during the early period before the development of clinical disease. It was decided to lavage patients before and after radiotherapy. The initial lavage was performed during the diagnostic bronchoscopy on patients who were identified as being likely to receive radiotherapy; before histological diagnosis was available. Of the 20 patients so identified, 13 did receive radiotherapy but only 7 were available for repeat lavage following radiotherapy. Repeat lavages were done four weeks after the last dose of treatment in six patients and after three weeks in one patient. Three patients had received radiotherapy 12 weeks after a course of chemotherapy. In these patients their initial lavage was done four days before initiation of radiotherapy.

5.2 Definition of study group:

Six patients received palliative radiotherapy, and one patient received radical radiotherapy. The dosage of radiation varied from 2,500 cGy to 5, 00 cGy given over a period of 7-10 days. Further details of the patients with bronchial carcinoma are given in Table 5.1(a) and 5.1(b). All patients were smokers.

Table 5.1(a): Age and sex distribution of patients with bronchial carcinoma.

	Age range (mean)	Sex Distribution M	F
Control subjects (n = 26) (Smokers)	30 - 66 (48.0)	17	9
Bronchial carcinoma patients (n = 20)	36 - 75 (60.2)	14	6

The following data was obtained:

1. BAL cell yields and proportions.
2. Proportion of macrophage and T-cell subsets as defined by the monoclonal antibodies.
3. AM migration.
4. Measurement of chemiluminescence.
5. Albumin and complement levels in BAL supernatants.

Table 5.1(b): Details of patients who had radiotherapy.

No.	Age & Sex	Cell type & site of tumour	Chemo-therapy	Radiation & Dosage	Main area of radiat-iation
1	36 M	Squamous Right Upper lobe (RUL)	None	Radical 5500 cGy in 21 fractions over 5.5 weeks	Tumour area Right (R) upper chest & mediastinum
2	59 F	Adenocar-carcinoma Right lower lobe	None	3000 c Gy in 10 fractions over 2 weeks	R. chest & mediastinum
3	68 M	Squamous Left upper lobe (LUL)	None	3000 cGy in 10 fractions over 2 weeks	Left (L) chest and mediastinum
4	75 M	Undetermined LUL	None	2000 cGy in 5 fractions over 1 week	Left apex and mediastinum
5	62 M	Small Cell RUL	Vincristine Adriamycin, VP 16 and cyclophos-phamide	4000 cGy in 15 fractions over 3 weeks	R. chest and mediastinum
6	59 F	Small Cell RUL	As above	4000 cGy in 15 fractions over 3 weeks	R. chest and mediastinum
7	61 M	Small Cell Left lower lobe	As above	4000 cGy in 15 fractions over 3 weeks	L. chest and mediastinum

5.3 Results

5.3.1 BAL fluid volumes, original cell yields and counts.

BAL was performed as previously described. Lavages from "tumour area" were obtained by wedging the bronchoscope in subsegment nearest to the (often occluded) subsegment containing the tumour. In a few instances this was done in the nearest lobe to that containing the tumour. Lavage from the "opposite lung" was accordingly performed in the right middle lobe or the lingula. The retrieval of fluid from these patients was again on the whole not very different to control subjects. Table 5.2(a) shows the cell content and fluid volumes from these patients.

The proportion of fluid volume retrieved from both tumour areas of the lung and opposite lung before radiotherapy was similar to control subjects. Retrieval of fluid from tumour areas after radiotherapy was significantly lower than the opposite lung. This may be due to mucosal oedema. This finding was similar to that in areas of consolidation in the patients with CAP. The total cell yields were slightly higher in patients with bronchial carcinoma but these were not statistically different compared to control subjects. The cell yields are represented in Table 5.2(b) and Figure 5.1.

Table 5.2(a): BAL-fluid volumes and cell yields.

		BAL Fluid Volumes		Proportion of recovered fluid	Cell Yields x 10 /ml of BAL fluid
		Instilled	Recovered		
Control Subj. Smokers n=26	Range	120-180	35-160	0.20 - 0.70	0.60 - 12.50
	Mean	202.6	94.0	0.46	3.66
Tumour Area Before DXT** n = 14	Range	80-180	22-105	0.16 - 0.625	0.85 - 10.2
	Mean	145	63.0	0.43	(4.40)
Opposite lung Before DXT n = 20		100-270	30-160	0.16 - 0.70	1.30 - 16.0
		163.1	80.5	0.48	5.09
Tumour area After DXT n = 7		150-200	25-90	0.125 - 0.63	0.50 - 12.0
		171.4	59.2	0.37*	5.57
Opposite lung After DXT n = 7		120-150	50-110	0.33 - 0.73	2.35 - 10.0
		145	77.1	0.52	5.35

* Significantly lower than the opposite lung after DXT. P = 0.028.

** DXT = Deep x-ray therapy.

Table 5.2(b): Absolute cell yields in bronchial carcinoma.

Patient groups		Cells x 10^5/ml BAL Fluid Total	Macrophages	Neutrophils	Lymphocytes
Control Subjects n = 21	Mean (SD)	3.67(2.97)	3.50(2.97)	0.07(0.05)	0.07(0.07)
	Median(SEM)	3.2 (0.65)	2.99(0.65)	0.008(0.01)	0.045(0.01)
P Tumour Area n = 12	Mean (SD)	4.40(2.81)	3.86(3.12)	0.418(0.88)	0.07(0.1)
	Median(SEM)	3.68(0.81)	3.69(0.90)	0.05(0.2)	0.04(0.07)
	P value	NS	NS	NS	NS
D Opp. lung n = 17	Mean (SD)	5.09(4.47)	4.59(4.0)	0.33(0.69)	0.15(0.18)
	Median(SEM)	3.5(1.08)	3.2(0.97)	0.07(0.16)	0.07(0.05)
	P value	NS	NS	NS	NS
P Tumour area n = 7	Mean (SD)	5.57(4.63)	3.43(3.76)	1.99(3.63)	0.07(0.1)
	Median(SEM)	3.7 (1.75)	*1.84(1.42)	0.08(1.37)*	0.01(0.04)
	P value	NS	NS	NS(P=0.067)	NS
D Opp. lung n = 7	Mean (SD)	5.35(3.06)	5.21(3.06)	0.063(0.04)	0.02(0.03)
	Median(SEM)	4.55(1.16)	4.51(1.15)	0.07(0.01)	0.01(0.01)
	P value	NS	NS	NS	NS

P values in the table relate to comparison with control subjects.

*Significantly lower macrophage content than the opposite lung. P = 0.018 and higher neutrophil content approaching significant level (P = 0.063). (Wilcoxon).

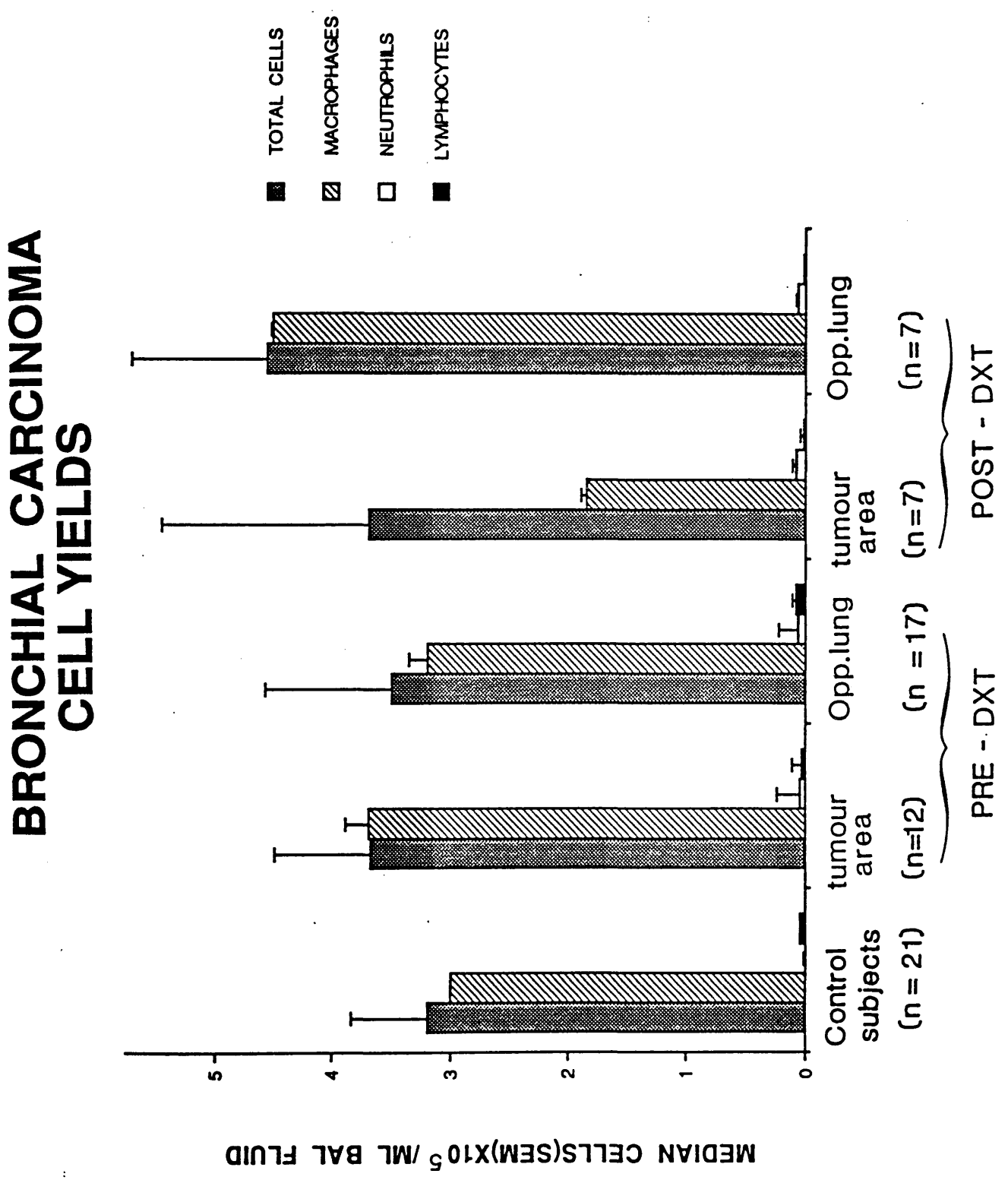


Fig. 5.1: Cells yields in bronchial carcinoma.

This shows that there was an increase in neutrophil content of BAL from areas of tumour after radiotherapy compared to the opposite lung. This was not statistically significant (P = 0.063). However the total macrophage yields were significantly lower from these areas compared to those from the opposite lung (P = 0.018), although these were not significantly different from control subjects. Table 5.3 shows proportions of cells in the original BAL fluid from these patients and control subjects.

Comparison of data before and after radiotherapy is limited by the disproportionately fewer patients lavaged following radiotherapy. However despite these constraints, it is clear that following radiotherapy there is a significant increase in proportions of neutrophils and reduction of macrophages from the tumour areas compared to control subjects. Interestingly, this is mirrored by a relative increase in the macrophage population from the opposite lung compared to proportions from the same area before radiotherapy.

5.3.2 Monoclonal markers

5.3.2.1 Macrophage markers (Table 5.4):

These did not show significant differences between control subjects and patients with bronchial carcinoma before radiotherapy. Comparison of proportions of macrophages in samples before and after radiotherapy was difficult because too few samples after radiotherapy were available. There was a clear trend emerging in the D_7 positive cells which were

reduced in tumour areas following DXT but this was not significant.

Table 5.3: Original differential cell counts in BAL from bronchial carcinoma patients.

		Cell Counts (%)			
		Macrophages	Neutrophils	Eosinophils	Lymphocytes
Control Subjects (Smokers) n = 26	Range	78.8-99.6	0.2-8.3	0 - 2.4	0 - 12.2
	Mean(SD)	93.8(4.75)	2.88(2.23)	0.36(0.57)	2.79(2.9)
	Median	94.95	2.4	0.1	2.0
Tumour Area (Before DXT) n = 14	Range	9.8-99.6	0 - 88.6	0 - 2.8	0 - 19.0
	Mean(SD)	81.1(30.8)	15.23(30.3)	0.59(0.85)	3.13(5.19)
	Median	92.5	1.8		0.8
Opposite Lung (Before DXT) n = 20	Range	61.6-98.4	0.3-23	0 - 2.8	0 - 28.0
	Mean(SD)	89.8(10.8)	5.3(7.2)	0.51(0.70)	4.34(6.31)
	Median	95.85	2.15	0.25	2.35
Tumour Area (After DXT) n = 7	Range	11-93.6	3 - 88.6	0 - 5.0	0 - 2.8
	Mean(SD)	68.57(37.5)	28.9(38.5)	1.22(1.81)	0.98(1.00)
	Median	+ 82.5*	+ 6.6**	0.8	0.45
Opposite Lung (After DXT) n = 7	Range	93.2-99.2	0.4-3.6	0 - 4.6	0 - 3.2
	Mean(SD)	97.1(2.35)	1.37(1.10)	0.82(1.69)	0.68(1.13)
	Median	98.0***	0.8	0	0.50

* Significantly lower than CS P = 0.004.

** Significantly higher than CS P = 0.021

*** Proportions of macrophages higher in the opposite lung after DXT compared to Pre-DXT values from same areas of the lung. P = 0.041. (Wilcoxon).

\+ Significantly higher neutrophil and lower macrophage proportions than the opposite lung. P = 0.018.

Table 5.4: Proportions of macrophage subsets as determined by macrophage markers in BAL patients with Br.Ca.

	%	RFD_1	RFD_7	RFD_9	$RFDR_1$	NA1/34 T6	$UCHM_1$
Control Subjects	Range	34.9 - 98.3	9.0 - 73.0	28.0 - 86.9	92.0 - 100.0	0.2 - 3.0	0 - 4.8
n = 12	Means(SD)	81.1(16.3)	43.0(22.9)	60.0(19.3)	98.1(2.73)	1.57(0.91)	0.99(1.34)
	Median	86.1	37.6	62.1	99.1	1.40	0.66
Tumour Area	Range	72.8 - 92.8	15.0 - 64.8	17.5 - 85.6	94.8 - 100.0	0.2 - 3.0	0 - 4.2
(Before DXT)	Mean (SD)	85.6(6.87)	36.2(18.0)	63.4(19.8)	98.9(1.59)	1.55(0.88)	1.74(1.42)
n = 12	Median	87.7	40.0	68.4	99.4	1.20	2.0
Opposite Lung	Range	40.5 - 99.2	11.6 - 80.8	32.4 - 81.6	81.8 - 100.0	0.1 - 2.8)	0 - 6.6
(Before DXT)	Mean (SD)	86.2(15.04)	34.2(18.7)	58.7(18.2)	97.9(4.6)	1.23(0.86)	1.51(1.75)
n = 15	Median	88.4	31.45	54.1	99.6	1.1	1.2
Tumour Area	Range	80.4 - 95.2	10.4 - 29.2	36.0 - 61.0	96.4 - 100.0	0.8 - 2.7	0 - 4.3
(After DXT)	Mean (SD)	88.4(6.0)	21.48(8.09)	52.0(10.2)	98.4(1.40)	1.66(0.9)	0.92(1.85)
n = 5	Median	90.8	25.0*	57.2	98.6	1.2	1.0
Opposite Lung	Range	85.2 - 94.0	15.0 - 42.8	36.4 - 73.4	96.0 - 99.2	0.4 - 4.6	0.2 - 6.6
(After DXT)	Mean (SD)	88.7(4.2)	24.8(12.3)	58.4(14.6)	97.6(1.42)	2.1(1.91)	2.04(2.64)
n = 5	Median	86.0	21.4	63.2	96.8	1.4	1.0

*For matched samples (n = 4) there was a clear trend of reduced proportions of D7 +ve cells in every specimen compared to pre-DXT values in tumour areas from some patients and this was not statistically significant. P = 0.068.

Interestingly there was no trend to increase in $UCHM_1$ positive cells.

5.3.2.2 T-cell markers:

These were assessed as previously described.

Table 5.5: T-cell subsets in patients with bronchial carcinoma.

Mean (%) (SD)	T_3	T_4	T_8	T_4/T_8
Control subjects n = 13	2.6(3.35)	1.35(1.19)	1.88(2.6)	1.37(1.16)
Tumour Area (Before DXT) n = 13	5.3(8.13)	2.75(4.54)	3.09(3.52)	1.46(1.33)
Opposite Lung (Before DXT) n = 16	4.78(5.26)	2.62(3.15)	2.97(3.71)	0.99(0.65)
Tumour Area (After DXT) n = 7	2.37(1.64)	1.04(0.73)	1.19(1.21)	1.52(1.25)
Opposite Lung (After DXT) n = 7	1.85(1.44)	0.48(0.29)	0.73(0.69)	1.64(1.64)

There were no differences detected in proportions of T-cell subsets or the T_4/T_8 ratio between control subjects and patients with bronchogenic carcinoma before or after radiotherapy. There were also no differences in these proportions in samples obtained from comparable areas in the same patients before and after DXT.

5.3.3 Purification of AM with Ficoll-hypaque gradient:

Excellent purification of macrophages was obtained in BAL samples from the tumour areas and from the opposite lung. Only two samples from the tumour areas before and after radiotherapy could not be adequately cleansed of neutrophils. (Table 5.6).

There were no significant differences in proportions of different cell types between patient groups and control subjects and subgroups of patients.

The mean (SD) (%) viabilities of BAL cells from CS and patients with bronchial carcinoma from tumour area and opposite lung before and after radiotherapy were 67(15), 74.6(12.1), 72.4(12.3), 73.4(15.5) and 68.4(12.3) respectively.

5.3.4 AM Migration:

Measurement of migration of AM from patients with bronchial carcinoma showed significant depression in both stimulated and unstimulated migration in patients with

Table 5.6: Differential cell counts following Ficoll-hypaque gradient centrifugation.

		Macro-phages	Neutro-phils	Eosino-phils	Lympho-cytes
Control Subjects n = 26	Range	87.6-99.6	0 - 9.0	0 - 1.0	0 - 8.0
	Mean (SD)	96.7(3.1)	1.51(2.07)	0.15(0.15)	1.6(1.99)
	Median	98.0	0.90	0	0.90
Tumour area before Dxt n = 13	Range	13.7-99.6	0 - 86.3	0 - 1.0	0 - 10.9
	Mean (SD)	88.5(23.3)	9.23(23.7)	0.16(0.32)	2.16(3.50)
	Median	97.0	1.20	0	0.80
Opposite lung before Dxt n = 19	Range	88.0-100	0 - 10.0	0 - 0.8	0 - 6.5
	Mean (SD)	96.9(3.7)	1.52(3.11)	0.11(0.22)	1.48(2.0)
	Median	98.8	0.4	0	0.60
Tumour area after Dxt n = 6	Range	14.0-98.6	0.4-85.2	0 - 1.2	0 - 0.8
	Mean (SD)	72.1(40.1)	27.0(40.19)	0.46(0.51)	0.3(0.35)
	Median	97.4	1.9	0.3	0.2
Opposite lung after Dxt n = 7	Range	92.4-100	0 - 6.0	0 - 1.6	0 - 1.6
	Mean (SD)	98.0(2.65)	1.31(2.19)	0.31(0.58)	0.37(0.57)
	Median	98.3	0.1	0	0.2

bronchial carcinoma before DXT compared to control subjects (Table 5.7). (Figure 5.2). None of the samples contained more than 10% neutrophil after Ficoll-hypaque centrifugation.

Migration of macrophages from tumour areas were much more depressed compared to control subjects than those from the opposite lung and this is reflected in significant differences of their stimulated migration to Casein and ZAS

Table 5.7: Migration of AM from patients with bronchial carcinoma.

Cells/5 fields (x400) (mean of 3 wells)		Unstimulated	Casein	ZAS (C5a)	FMLP
Control Subjects n = 19	Mean (SD)	29.1(12.2)	37.1(15.4)	61.5(28.3)	33.4(17.6)
	Median (SEM)	30.0(2.8)	31.0(3.5)	55.5(6.6)	26.6(4.3)
Tumour Area Before DXT n = 9	Mean (SD)	13.9(4.8)	18.7(5.6)	26.9(9.8)	15.1(5.6)
	Median(SEM)	15.0(1.6)	18.0(1.9)	23.0(3.2)	15.0(2.1)
	*P value	P < 0.002	P < 0.002 **	P < 0.002 **	P < 0.01
Opposite Lung Before DXT n = 14	Mean (SD)	18.6(9.1)	25.6(6.9)	38.3(19.2)	15.7(11.3)
	Median(SEM)	15.0(2.4)	24.5(1.8)	35.0(5.1)	12.5(3.5)
	*P value	P < 0.01	P < 0.05	P < 0.01	P < 0.002
Tumour Area After DXT n = 4	Mean (SD)	26.5(11.3)	34.5(17.3)	51.5(26.9)	20.0(2.6)
	Median(SEM)	23.5(5.7)	28.0(8.6)	46.0(13.4)	19.0(1.5)
	*P value	NS	NS	NS	NS
Opposite Lung After DXT n = 7	Mean (SD)	24.6(5.4)	38.4(20.6)	50.4(25.1)	23.2(5.6)
	Median(SEM)	23.0(2.0)	30.0(7.8)	44.0(9.5)	22.5(2.3)
	*P value	NS	NS	NS	NS***

* All P values indicate comparison with control subjects.

** Significantly lower than migration of AM from the opposite lung. $P < 0.05$.

*** Significantly higher than migration of AM from same areas before DXT. $P < 0.05$.

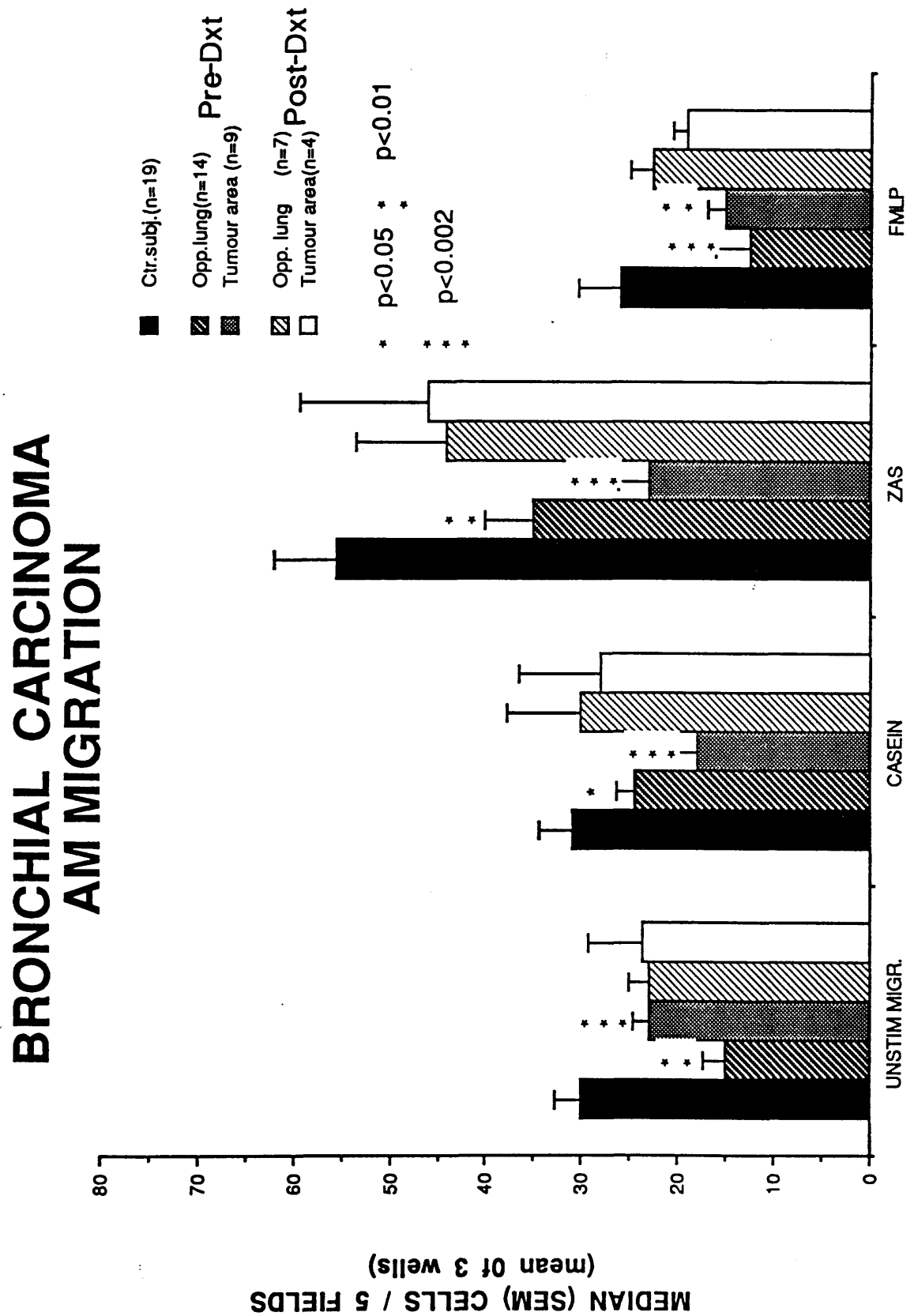


Fig. 5.2: AM migration in bronchial carcinoma.

($P < 0.05$). Migration of AM from these patients following radiotherapy showed consistent increase above their pre-treatment levels so that they were now comparable to control subjects. Unstimulated migration towards FMLP of AM from the opposite lung following DXT was significantly higher to pre-treatment levels ($P < 0.05$).

5.3.5 Measurement of the respiratory burst in AM from patients with bronchial carcinoma:

Assessment of generation of respiratory burst in AM from these patients was made by measurement of lucigenin and luminol dependent chemiluminescence (Tables 5.8 & 5.9).

Measurement of lucigenin-dependent chemiluminescence in patients and control subjects showed lower levels of chemiluminescence (CL) in AM from patients with bronchial carcinoma before radiotherapy. This was significantly lower in AM from the opposite lung ($P < 0.01$). Chemiluminescence of AM from the opposite lung post-treatment showed an increase above their pre-treatment levels ($P < 0.05$) (spontaneous CL) so that they were now almost comparable to control subjects. Chemiluminescence of AM from tumour areas remained low, Figures 5.3 and 5.4.

Measurements of luminol dependent chemiluminescence showed no differences between control subjects and patients with bronchial carcinoma before or after radiotherapy. There was a trend towards "recovery" of chemiluminescence activity in AM from the opposite lung but this was not significant.

Table 5.8: Lucigenin-dependent chemiluminescence.

Peak cps/10^3 cells		Cells + bacteria	Cells + gel Hanks
Control Subjects	Mean (SD)	812.3(852.5)	821.1(876.9)
n = 10	Median (SEM)	400.0(269.6)	415.0(777.3)
Tumour Area	Mean (SD)	378.9(522.5)	153.8(225.3)
Before DXT	Median (SEM)	160.0(174.1)	34.0(75.1)
n = 9			
Opposite Lung	Mean (SD)	*159.2(252.9)	138.75(205.8)
Before DXT	Median (SEM)	40.0(76.2)	30.0(62.0)
n = 11			
Tumour Area	Mean (SD)	147.5(74.1)	147.5(91.4)
After DXT	Median (SEM)	140.0(37.0)	130.0(45.7)
n = 4			
Opposite Lung	Mean (SD)	420.0(364.6)	522.0(307.2)
After DXT	Median (SEM)	260.0(163.1)	** 570.0(137.3)
n = 5			

* Significantly lower than control subjects. $P < 0.01$.

** Significantly higher than pre-treatment levels in AM from the same area. ($P < 0.05$). (Figures 5.3 and 5.4.

Table 5.9: Luminol-dependent chemiluminescence.

Peak cps/10^3 cells		Cells + Bacteria	Cells + gel Hanks
Control Subjects	Mean(SD)	21.5 (58.4)	14.4 (39.5)
n = 17	Median(SEM)	3.8 (14.1)	2.7 (9.5)
Tumour Area	Mean(SD)	20.06 (23.7)	38.8 (65.0)
Before DXT	Median(SEM)	20.0 (7.93)	10.0 (21.6)
n = 9			
Opposite Lung	Mean(SD)	14.09 (14.0)	16.8 (20.8)
Before DXT	Median(SEM)	10.0 (4.4)	10.0 (6.9)
n = 9			
Tumour Area	Mean(SD)	91.2 (165.8)	14.7 (13.3)
After DXT	Median(SEM)	9.75 (82.9)	8.8 (7.7)
n = 4			
Opposite Lung	Mean(SD)	100.1 (212.3)	9.1 (11.6)
After DXT	Median(SEM)	4.3 (94.9)	3.9 (5.2)
n = 5			

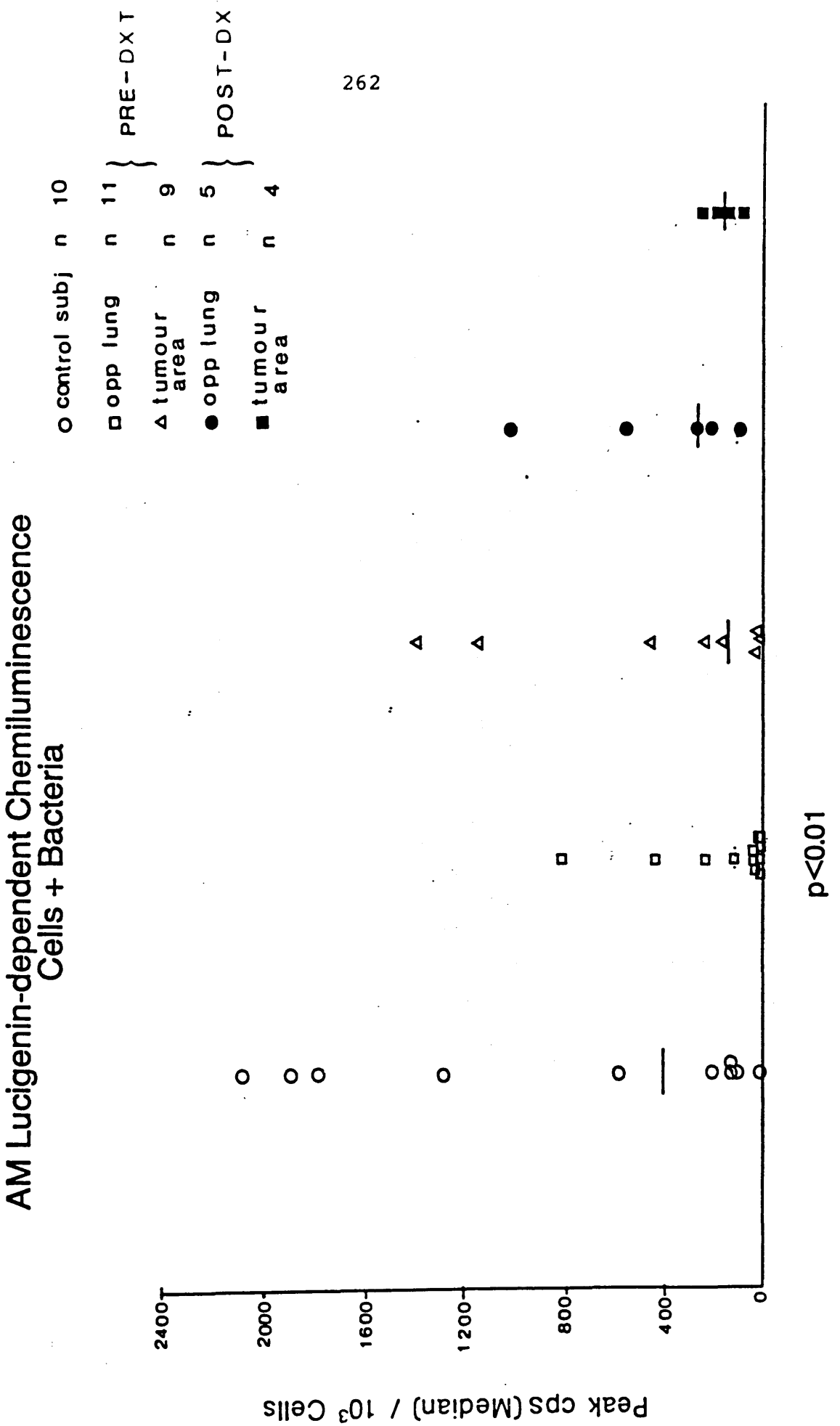


Fig. 5.3: AM lucigenin-dependent chemiluminescence (cells + bacteria) in bronchial carcinoma.

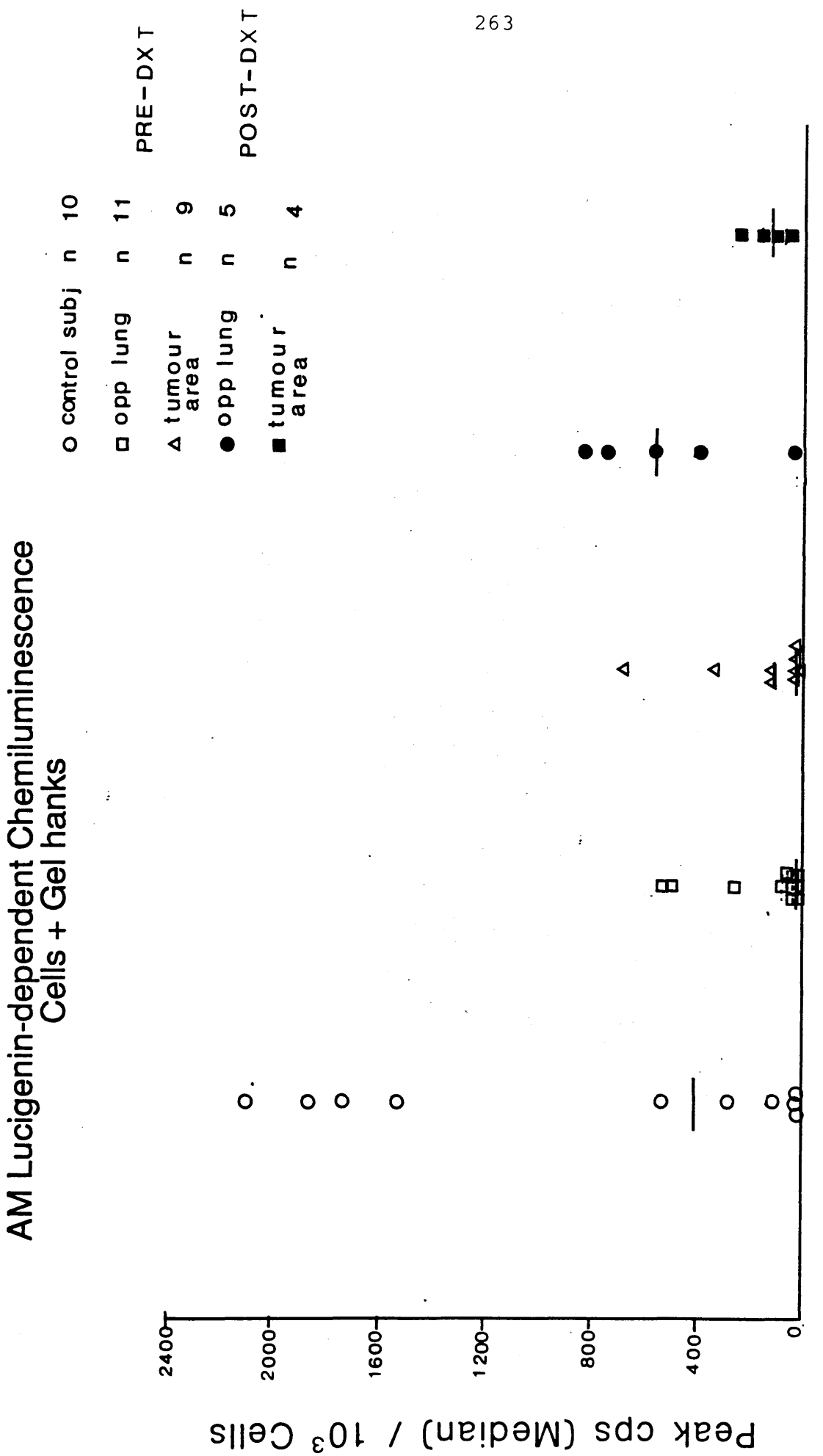


Fig. 5.4: AM lucigenin-dependent chemiluminescence (cells + gel hanks) in bronchial carcinoma.

5.3.6 Measurement of albumin in BAL supernatants.

These were done as previously described. Table 5.10a shows the results of albumin levels.

Table 5.10(a): Albumin levels in mg/L of BAL fluid from patients with bronchial carcinoma.

	Control Subjects n = 16	Pre-DXT		Post-DXT	
		Tumour Area n = 14	Opp.Lung n = 14	Tumour Area n = 6	Opp. Lung n=3
Range	3.8-111.8	1.5-156.5	2.9-117	9.8-214.9	0.6-64.0
Mean (SD)	37.12 (31.27)	45.07 (45.38)	35.06 (33.92)	91.67 (86.51)	86.51 (32.04)
Median (SEM)	27.45 (8.36)	30.40 (12.59)	23.95 (9.06)	68.15 (35.32)	24.2 (18.5)
**P value	*NA	NS	NS	NS	NS

* NA = Not applicable.

**P values: relate comparison with control subjects.

The albumin levels were similar between control subjects and patients with bronchial carcinoma before radiotherapy. Albumin levels from tumour areas after radiotherapy were threefold higher than CS but the difference between groups was not statistically significant. Only 3 samples from the opposite lung after dxt were available for examination.

5.3.7 Measurement of complement components in BAL supernatants.

Data on complement components are presented in Tables 5.10b and 5.10c. The majority of the components were similar in the bronchial carcinoma group to CS. C3 and factors B and H levels were significantly higher in tumour areas before radiotherapy. Factor B is significantly higher in the opposite lung than CS ($P = 0.05$).

Following radiotherapy tumour areas contained significantly higher levels of the same components (C3, B and H) than CS. Again factor B levels in the opposite lung is significantly higher than CS. Thus factor B has remained consistently significantly higher in the bronchial carcinoma patients both in the tumour areas and in the opposite lung before and after radiotherapy. Factor B takes part in the alternative pathway and plays an important role when complexed with C3b initially as a low activity convertase before being cleaved by activated Factor D. The significance of the observation of higher levels of Factor B in patients with bronchial carcinoma is not clear.

In bronchial carcinoma the significant differences seen in tumour areas after radiotherapy (C3, B & H) are now lost when albumin is corrected for. These however remain significant both in the tumour areas and in the opposite lung before radiotherapy but at low levels of

Table 5.10(b): Complement levels (mg/litre) in BAL fluid from patients with bronchial carcinoma.

		C1q	C1r	C1s	C4	C3	C1INH	Factor B	Factor H
Control Subjects n=16	Mean	0.006	0.01	0(0)	0.013	0.75	0.07	0.14	0.09
	(SD)	(0.02)	(0.03)	0(0)	(0.03)	(1.22)	(0.13)	(0.15)	(0.19)
	Median	0	0	0	0	0.40	0	0.1	0
	(SEM)	(0.006)	(0.01)	(0)	(0.01)	(0.31)	(0.03)	(0.04)	(0.05)
Tumour Area Pre-DXT n=14	Mean	0.07	0.23	0.06	1.79	3.21	0.07	1.43	0.73
	(SD)	(1.5)	(6.08)	(1.23)	(47.79)	(4.36)	(0.13)	(3.27)	(0.85)
	Median	0	0.04	0	0.5	2.05	0	0.45	0.20
	(SEM)	(0.004)	(0.16)	(0.03)	(1.33)	(1.17)	(0.03)	(0.88)	(0.23)
	*P value	NS	NS	NS	NS	P 0.002	NS	P<0.01	P<0.002
Opp. Lung Pre-DXT n=14	Mean	0.024	0.02	0.004	0.29	1.30	0.67	1.01	0.39
	(SD)	(0.04)	(0.04)	(0.02)	(0.56)	(1.42)	(2.03)	(2.48)	(0.53)
	Median	0	0	0	0	0.65	0	0.30	0.20
	(SEM)	(0.01)	(0.01)	(0.004)	(0.15)	(0.52)	(0.05)	(0.66)	(0.14)
	*P value	NS	NS	NS	NS	NS	NS	P=0.05	NS
Tumour Area Post-DXT n=6	Mean	0.01	0.04	0.02	0.30	1.25	0.28	0.62	0.28
	(SD)	(0.002)	(0.07)	(0.03)	(0.35)	(1.27)	(0.25)	(0.21)	(0.18)
	Median	0	0	0	0	0.65	0.30	0.60	0.20
	(SEM)	(0.01)	(0.03)	(0.01)	(0.14)	(0.52)	(0.10)	(0.09)	(0.08)
	*P value	NS	NS	NS	NS	P 0.05	NS	P<0.002	P<0.02
Opp. Lung Post-Dxt n=6	Mean	0.01	0.01	0	0.20	0.47	0.18	0.33	0.23
	(SD)	(0.02)	(0.03)	(0)	(0.33)	(0.44)	(0.15)	(0.20)	(0.22)
	Median	0	0	0	0	0.30	0.25	0.35	0.15
	(SEM)	(0.01)	(0.01)	(0)	(0.14)	(0.18)	(0.06)	(0.08)	(0.09)
	*P value	NS	NS	NS	NS	NS	NS	P=0.05	NS

*All P values relate comparison with controls. Comparison of tumour areas pre-and post-DXT (matched samples) revealed no differences. Comparison of opposite lung areas pre- and post-DXT also did not show any significant differences.

Table 5.10(c): Complement levels in BAL from bronchial carcinoma patients in mg/gm albumin.

		C_1q	C1r	C1s	C4	C3	C1-Inh	Factor B	Factor H
Control	Mean	0.06	0.08	0.04	0.97	44.31	2.12	4.66	6.33
Subjects	(SD)	(0.22)	(0.31)	(0.16)	(2.68)	(115)	(4.13)	(6.77)	(19.49)
n = 16	Median	0	0	0	0	11.85	0	3.38	0
	(SEM)	(0.06)	(0.08)	(0.04)	(0.07)	(28.78)	(1.03)	(1.69)	(4.87)
Tumour	Mean	0.66	1.06	0.47	12.59	74.60	7.71	41.72	17.31
Area	(SD)	(0.93)	(1.29)	(0.81)	(22.11)	(104.86)	(14.59)	(71.14)	(20.29)
Pre-DXT	Median	0	0.64	0	0	39.47	0	13.99	8.48
	(SEM)	(0.26)	(0.36)	(0.23)	(6.13)	(29.08)	(4.21)	(20.54)	(5.86)
n=13	*P value	NS	P<0.05	NS	NS	P<0.05	NS	P<0.002	P<0.01
Opp.Lung	Mean	0.13	0.04	0.03	5.71	40.70	12.26	17.78	10.77
Pre-DXT	(SD)	(0.38)	(0.12)	(0.11)	(15.99)	(24.54)	(27.52)	(26.22)	(11.48)
	Median	0	0	0	0	37.65	0	9.14	5.05
	(SEM)	(0.1)	(0.04)	(0.03)	(4.44)	(6.56)	(7.36)	(7.0)	(3.07)
n=14	*P value	NS	NS	NS	NS	P 0.05	NS	P 0.05	P 0.01
Tumour	Mean	1.27	0.73	18.35	4.0	75.24	9.14	29.88	35.13
Area	(SD)	(1.56)	(0.91)	(43.43)	(4.82)	(103.84)	(5.74)	(23.33)	(65.95)
Post-Dxt	Median	0.75	0.60	0.95	2.40	33.88	10.75	23.73	6.05
	(SEM)	(0.64)	(0.37)	(17.73)	(1.97)	(42.39)	(2.34)	(10.62)	(29.50)
n=6	*P value	NS	NS	NS	NS	NS	NS	NS	NS
**T/0 Pre-Dxt n=7		P = 0.075	P = 0.18	P = 0.043	NS	NS	NS	NS	NS

*P values relate to comparison with control subjects. ** Comparison of tumour area and opposite lung pre-DXT (matched pairs). Data for opposite lung post-DXT is available from two patients only, therefore is not included. (Wilcoxon).

significance. It should be noted that C3 levels in the opposite lung before radiotherapy has now become significantly higher than CS while this was not so before correction for albumin.

Interpretation of complement data in bronchial carcinoma group is particularly difficult because of the presence of inflammatory activity as evidenced by presence of neutrophils in some patients before radiotherapy. The contribution of radiotherapy (if any) to the differences, observed in some complement compounds is thus difficult to assess. It is unfortunate that data on complement activation products is not available in this patient group.

5.4 Discussion:

Data from bronchoalveolar lavage in radiation pneumonitis in man are scanty. One of the few studies is that by Cordier (1986) who reported cellular and humoral changes in BAL fluid from six patients with radiation pneumonitis. Among other findings in that study was an increase in the lymphocyte proportions in BAL from five out of the six patients studied. One patient had an increased neutrophil content. Our data do not show such an increase in lymphocytes but suggest an increase in the neutrophil counts. This difference between the two studies is likely to be due to the time interval between radiation and lavage. In the study by Cordier, patients were lavaged between 5-12 weeks after radiation except in one case when lavage was performed 1 week after the initiation of treatment. This patient developed hyper-acute radiation pneumonitis and showed an increase in neutrophil content. Irradiation of mice has shown that the number of macrophages are initially reduced reaching a minimum at around 2 weeks and this is followed by return to normal levels 6-10 weeks after irradiation (Gross, 1977b). The finding in our study of low normal (in comparison to pre-radiation) levels of macrophages from the tumour area and high normal levels from the opposite lung is in keeping with the experimental data. The lower numbers from the tumour area could reflect the higher dosage of radiation sustained by the tissue in and around the tumour in comparison to the opposite lung hence earlier "recovery" in the opposite lung (Fig.5.1). This is again reflected in

the fairly low levels of mature macrophages from the tumour areas as defined by the RFD_7 monoclonal marker. The absence of any increase in $UCHM_1$-positive cells (monocytes) in this study is interesting. Alveolar macrophages are regarded as radioresistant cells so that they are not completely eliminated by thoracic irradiation and regeneration partly depends on local multiplication of macrophages. Bowden (1969) showed that following irradiation of the hemithorax of rats there was no influx of monocytes into the alveoli of the irradiated mice, which would be consistent with our findings.

Our finding of depressed migration of AM from patients with bronchial carcinoma especially from areas of tumour is similar to that reported by Le Marie (1984). It is not clear whether the depression of AM chemotaxis in patients with bronchial carcinoma especially in macrophages obtained from the neighbourhood of the tumour is due to the presence of an "inhibiting factor" possibly released by the tumour or part of the poor general condition of these patients. The "recovery" of this function could thus be because of destruction of the tumour (albeit temporary) and reduction of the inhibiting factor or due to improvement in the general condition of the patient. The "recovery" of the capacity of AM from the opposite lung to generate the respiratory burst is in keeping with the chemotaxis data. AM obtained from patients with bronchial carcinoma are capable of cytotoxic activity against human lung tumour (Swinburne et al, 1982). This is achieved by non-specific binding (Adams, 1984) or through antigen-dependent cytolytic activity. This latter mechanism is

thought to be mediated through the release of H_2O_2 (Nathan, 1980). Whether the improvement in migration of AM and generation of respiratory burst following radiotherapy demonstrated in this study is translated into a more efficient anti-tumour capacity of these cells *in vivo* remains unclear.

5.5 Conclusion

In conclusion, data from this study suggest:

1. Increase in the proportions of neutrophil population in BAL fluid from patients who have undergone radiotherapy for bronchial carcinoma, before the development of overt clinical evidence of radiation pneumonitis.
2. Improvement of previously existing depressed function of AM from these patients following radiotherapy. Such a partial recovery occurs initially in the opposite lung.
3. Some components of complement are elevated in BAL supernatants from patients with bronchial carcinoma.

CHAPTER 6

GENERAL DISCUSSION

The purpose of this thesis was to examine cellular and humoral responses to acute lung injury. The types of acute lung injury studies were community acquired pneumonia, smoke inhalation injury in fire victims and radiation injury following radiotherapy for bronchial carcinoma.

This was done through examination of bronchoalveolar lavage cell composition, measurement of albumin, complement components, products of complement activation and functional chemotactic activity of the BAL supernatants. In addition AM function was assessed by measurement of unstimulated and stimulated migration towards three chemotaxins, and generation of the respiratory burst.

Data on patients with bronchial carcinoma showed preponderance of AM both before and after radiotherapy. There was however some increase in neutrophils after radiotherapy. There was a marked depression of migration of AM from these patients. This depression was greater in AM obtained from tumour areas than those from the opposite lung. Similar observations were made by Le Marie (1984). Generation of the respiratory burst was also impaired in AM from patients with bronchial carcinoma. There was a trend of improvement in AM

function following radiotherapy but the number of patients was too small to draw conclusions from.

The cell composition from RCA in CAP patients was similar to CS, but AM function appeared to be depressed. The latter did not appear to be due to a systemic effect of pneumonia since peripheral blood monocytes were not similarly depressed. This is a hitherto unreported observation in non-immunocompromised patients. The cause for such a depression of AM function in our patients is not clear.

Data on experimental pneumonia have documented functional impairment from animals previously subjected to viral infection (Jakab 1974; 1980 and 1982, Astry 1984) although this was not confirmed by Nugent (1979).

In clinical practice the evidence for predisposition to bacterial pneumonia by preceding viral infection has largely come from influenza epidemics (Schwarzmann 1971, Martin 1959). In non-epidemic situations, the association of viral infection and the development of bacterial pneumonia is more common in children (Nichol 1967) than in adults (Sullivan 1972; Mufson 1967; Fekety 1971; and Bath 1964). Alcoholism is another risk factor identified in pneumonia studies (Sullivan 1972; Mufson 1971). Thus the cause of apparent impairment of AM function in our patients may be multifactorial. The significance of this functional impairment in the development of disease in the infected areas (AOC) and the precise role AM play in limiting such infection is not clear.

The composition of cells from these areas was different from either the CS or RCA. There was extensive accumulation of neutrophils in AOC in most patients with CAP. Studies on migration of AM from AOC were hindered by difficulties in purification of AM. Chemiluminescence data however suggested that the respiratory burst in BAL cells from AOC in CAP was depressed. Assuming that the major part of chemiluminescence activity is from neutrophils which are the predominent cells this impairment of chemiluminescence contrasts with peripheral blood neutrophils from the CAP group examined later, which showed activation. Lung neutrophils appear to behave differently from peripheral blood neutrophils. This finding could be explained on the basis of prolonged stimulation of lung neutrophils by particulate (bacteria, debris) and soluble stimuli (C5a), to which the peripheral blood neutrophils have not been similarly subjected. Examination of neutrophils from lungs and peripheral blood in a model of lung injury has demonstrated marked degranulation occurring in the former, (Yamada, 1982). This was thought to be due to complement activation.

Supernatants from AOC showed significantly higher levels of complement components and products of complement activation than CS or RCA. There was also a suggestion of C3 consumption in these areas. The presence of significant levels of C5a and C3a is in keeping with the large numbers of neutrophils in AOC.

This observation is similar to that seen in patients with smoke inhalation injury but to a lesser degree.

Patients with smoke inhalation injury with or without burns showed accumulation of neutrophils within the first 24 hours, and this became more pronounced during the following 24 hours. Patients with burns alone did not show such a change. Macrophage function assessed by migration suggested some activation in patients with smoke inhalation only but in patients with smoke and burns such activation of AM was markedly increased. Again AM function from patients with burns alone did not show such activation.

BAL from patients with smoke inhalation only contained higher levels of complement components than CS but the differences were not statistically significant. However BAL supernatants from patients with both injuries showed significantly higher levels of C1r, C1s and Factor H. They also showed lower levels of C3 than CS along with higher levels of C3-P suggesting consumption of C3 with activation of the alternative pathway.

Although the difference in the levels of C3-P between patients with smoke inhalation alone (or CS) and those with combined injury was not significant, examination of raw data is very revealing. In smoke inhalation only out of 10 samples examined (from 8 patients) none showed detectable levels of C3-P. In smoke with burns patients, 5 samples (5 patients) out of 13 samples (9 patients) showed detectable levels. Four out

of five of these patients with combined injury died. Furthermore patients with both smoke inhalation and burns showed significant activation of the classical pathway as shown by the levels of C1s-CInh complex. Unfortunately data on complement from patients with burns alone are not available but it is reasonable to assume that BAL supernatants from these patients did not contain any significant levels of complement activation products since they did not show any chemotactic activity on functional assay. This is also in keeping with lack of neutrophil accumulation in the lungs of these patients during the study period.

In summary, patients with burns alone bear no resemblance at least during the early period to those with smoke inhalation injury in terms of AM function, neutrophil accumulation and probably complement profile. In contrast patients with smoke inhalation injury only and those with smoke inhalation and burns show some resemblance in the degree of neutrophil accumulation in the lungs and to a lesser extent macrophage activation. However it appears that complement activation occurs only in patients with smoke and burns.

Does this apparent activation of the alternative and classical pathway of complement play a key role in promoting further physiopathological changes in patients with combined smoke inhalation and burns injury with subsequent high mortality? Although patients with CAP (AOC) also show even greater complement activation, their

mortality is comparatively low. There are several possible causes for this. Firstly in most patients with pneumonia, the inflammation is a localized process and does not involve the whole bronchial tree as in smoke inhalation injury. Indeed patients with pneumonia sustain increasing mortality in proportion to the number of lobes involved (Austrian 1964). Secondly there is effective therapy in pneumonia not yet available in smoke inhalation and burns injury. Thirdly it is known that both serum and lung antiproteases are increased in pneumonia (Lonky 1980) although some workers have found decreased functional activity of such antiproteases (Abrams 1984).

Data on antiproteases are not available from patients with smoke inhalation and burns. It is not unreasonable however to speculate that loss of serum factors known to occur through burn wounds in these patients may include antiproteolytic enzymes. This would render the lungs of these patients vulnerable to the injurious effects of neutrophils. Such reduced antiproteolytic activity has been documented in some patients with ARDS, (Lee 1981; Cochrane 1983). Furthermore and perhaps more importantly the presence of products of complement activation such as C5a which is capable of neutrophil activation (Davis 1987) would lead to the release of proteolytic enzymes, resulting in further complement activation (McPhaden 1985). Thus a vicious cycle which results in lung damage is set in

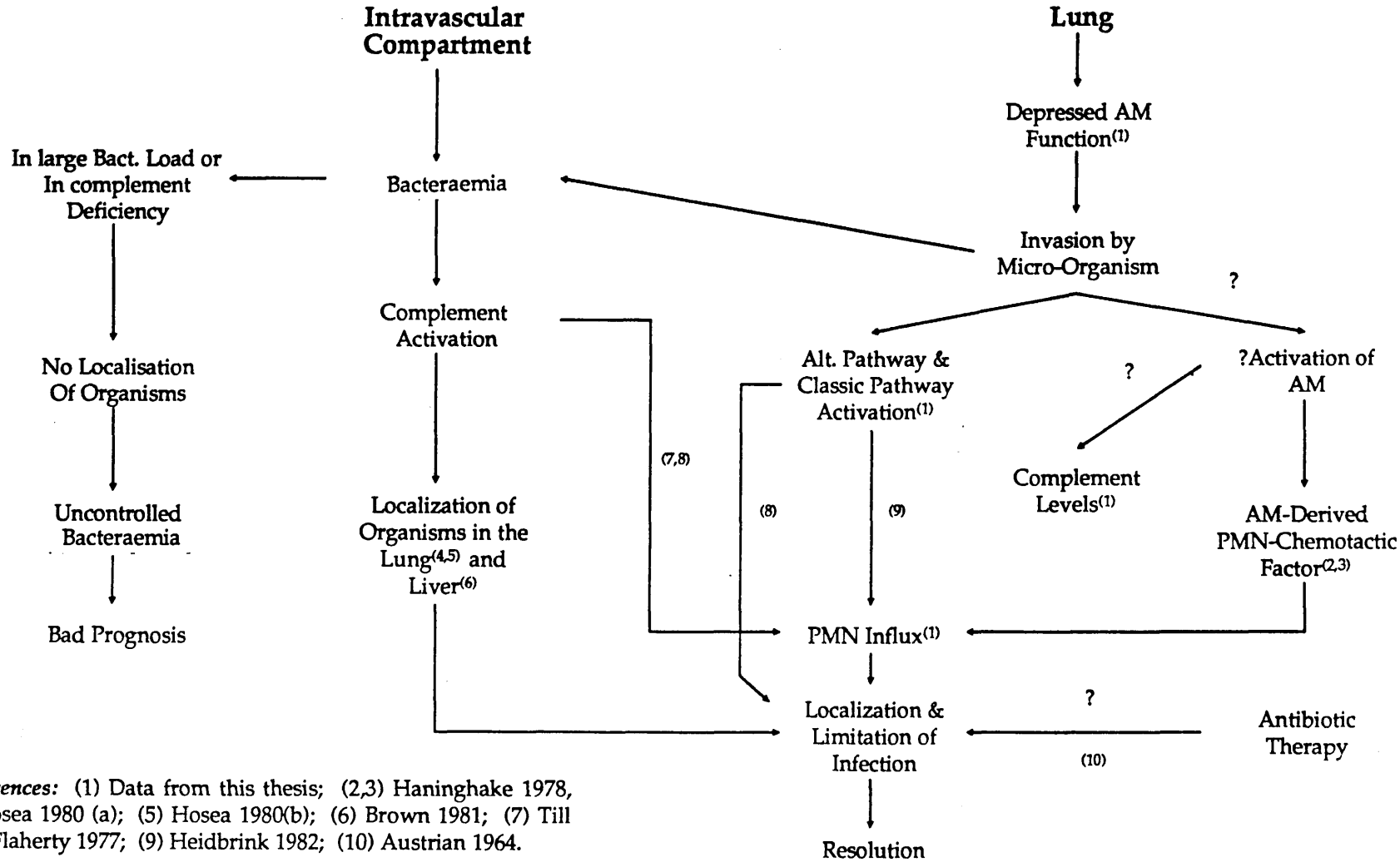
motion.

In smoke inhalation injury, AM activation is perhaps sufficient to recruit neutrophils. The presence of additional burns injury allows complement activation due to tissue damage with further neutrophil sequestration in the lungs. The consequent increase in permeability allows exudation of complement components into the lungs which are cleaved and activated by neutrophil proteases. In severe burns of sufficient magnitude, intravascular complement activation may be the starting point of the vicious cycle.

Thus in these studies pneumonia patients are seen to have intense but localised inflammation. Whereas patients with inhalation injury alone have mild but generalised inflammation, and those with combined smoke and burns injury have both intense and generalised inflammatory changes.

Based on the above discussion it is possible to put forward an hypothesis on lung injury in these conditions. This is outlined in flow charts (Figures 6.1,2 & 3) which also serve to highlight the areas about which we have no information and on which future studies may be undertaken.

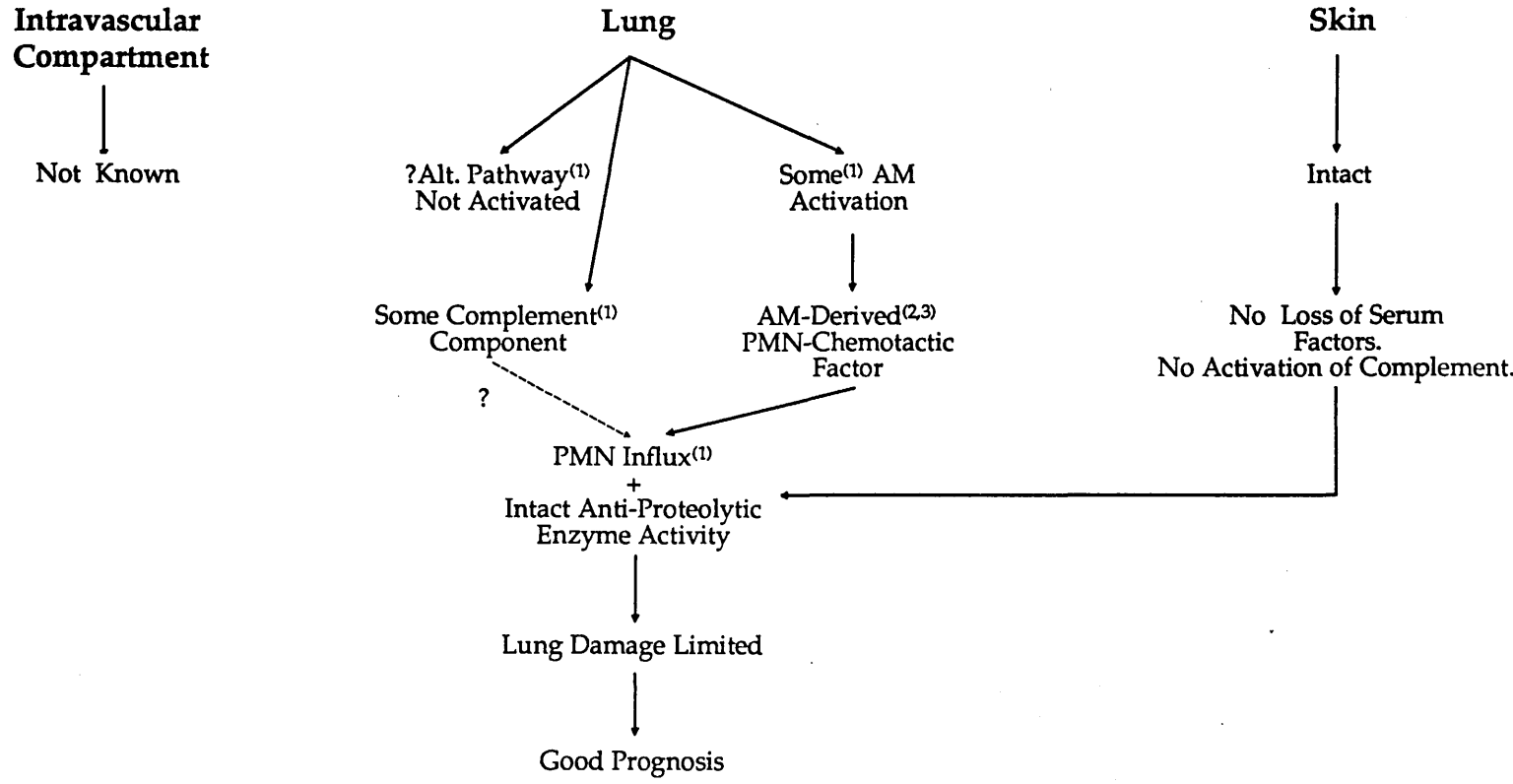
Finally an exciting area of research would be the investigation of potential use of antioxidants in ameliorating lung damage in patients with smoke inhalation and burns injury.



Key to References: (1) Data from this thesis; (2,3) Haninghake 1978, 1980; (4) Hosea 1980 (a); (5) Hosea 1980(b); (6) Brown 1981; (7) Till 1982; (8) O'Flaherty 1977; (9) Heidbrink 1982; (10) Austrian 1964.

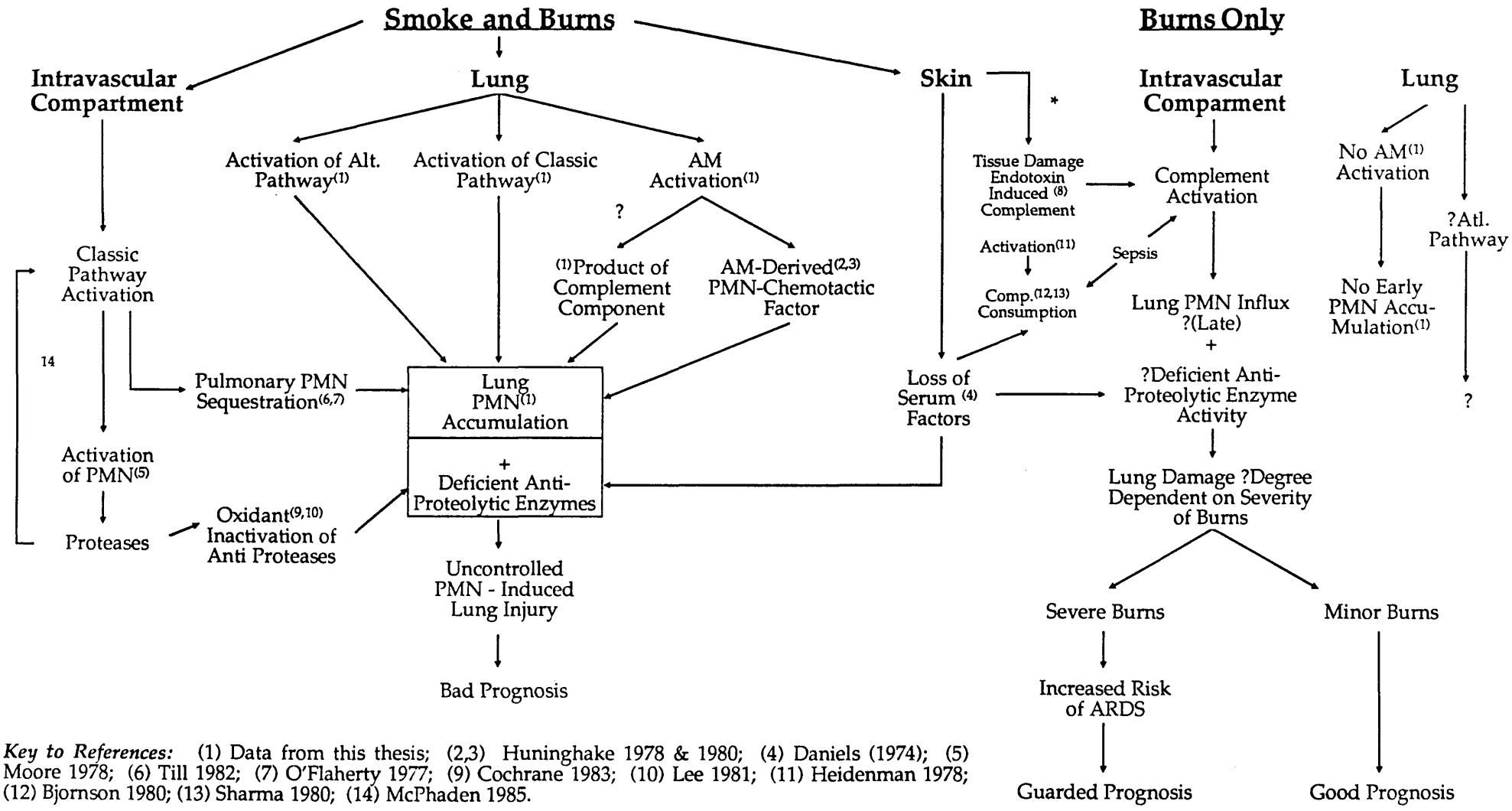
Figure 6.1: Hypothesis on mechanisms of acute lung injury in pneumonia.

Smoke Only



Key to References: (1) Data from this thesis;
(2,3) Huninghake 1978, 1980.

Figure 6.2: Hypothesis on mechanisms of lung injury in smoke inhalation only



Key to References: (1) Data from this thesis; (2,3) Huninghake 1978 & 1980; (4) Daniels (1974); (5) Moore 1978; (6) Till 1982; (7) O'Flaherty 1977; (9) Cochrane 1983; (10) Lee 1981; (11) Heidenman 1978; (12) Bjornson 1980; (13) Sharma 1980; (14) McPhaden 1985.

* All mechanisms under skin may operate in patients with burns alone or with smoke inhalation + burns

Figure 6.3: Hypothesis on mechanisms of lung injury in smoke inhalation & burns injury.

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APPENDIX I (TO CHAPTER 2 - MATERIALS)

PROCESSING OF BAL FLUID

Sterile Universal Containers 150 mls (STERILIN Ltd., Feltham, England)

Pasteur pipette (disposable plastic)

Surgical Gauze

Fuchs-Rosenthal counting chamber (Weber Scientific International Ltd., Sussex, England)

White-cell dilution fluid (Exogen, Clydebank, Scotland)

Leishman's Stain (Exogen, Clydebank, Scotland)

Non Specific-Esterase Staining

1. Kit No. 90-AI (Sigma Chemical Company Ltd., Fancy Road, Poole, Dorset, U.K. BH1 7NH) contains:

i) Citrate concentrate Solution Cat. No. 386-1 (Reagent A)

i) Trizmal Buffer concentrate pH 7.6 Cat No. 90-2C (Reagent B)

iii) Capsules each containing RR salt Cat. No. FBS-25 (Reagent C)

iv) Capsules containing 20 mgs alpha naphthyl acetate Cat. No. 90-6 (Reagent D)

v) Ethylene glycol monomethylether solution Cat. No. 90-1 (Reagent E)

vi) Mayer's Hematoxylin.

2. Acetone BP (Evans Medical Ltd., Langhurst, Horsham, England).

3. Methanol 99.85% w/w.

4. Distilled water.

Preparation of a fixative (citrate-acetone-methanol mixture).

1 ml Reagent A + 9 mls distilled water + 15 mls acetone. Discard 2.5 mls from this mixture and replace with 2.5 mls methanol.

Preparation of Esterase Stain:

5 mls Reagent B + 45 mls distilled H_20 prewarmed in a shaker at 37^oC + contents of 1 capsule of Reagent C. Immediately contents of Reagent D are dissolved in 2 mls of Reagent E and emptied into the mixture. Mix for 20 seconds and empty into coplin's jar (protected from light by covering with tin foil).

Enrichment of macrophages using Ackerman's method (1978)

1. Previously used tissue culture flasks obtained from Virology Department (Corning or Flow Laboratories).
2. x10 concentrated Medium 199 (Gibco).
3. Fetal Calf Serum (FCS) Gibco.
4. Phosphate Buffered Saline (PBS) Oxoid Ltd., (1 tab + 100

mls distilled water).

5. Distilled water.

6. EDTA (di Potassium) Mol. Wt. 404.29 (May & Baker, Dagenham, England).

Enrichment of Macrophages using Density Gradient:

1. Lumbar Puncture Needle gauge 20.

2. Lymphocyte-Separation Medium: (also referred to as ficoll-hypaque) specific gravity 1.077 (Flow Laboratories, U.K.).

3. x10 concentrated Medium 199 with Earle's Salt without $NaHCO_3$.

4. HEPES Buffer (1 Molar) pH 7.3.	5 mls) 1 ml)	Medium 199 (M199) prepared fresh each day
5. 7.5% Sodium Bicarbonate Solution.	1 ml)	
6. Distilled water.	43 ml)	

Concentration of BAL Fluid

1. Dialysis Tubing (Cellulose) width 1.7" cut off mol. wt. 12000 daltons (Sigma Chem.).

2. Polyethylene glycol (approx. mol. wt. 8000) (Sigma Chemical Co.).

3. Millipore filters 0.22 um (Gelman Sciences).

4. Sterile distilled water.

Isolation of Peripheral Blood Cells

(Ficoll-Hypaque method -(Boyum 1968)).

1. Heparin (Mucous) Injection BP (Sodium Heparin) 1000 units/ml (Leo Laboratories Ltd., U.K.).

2. 0.9% Sodium Chloride BP 100 mls) 5% Dextran -
3. Dextran 150 (M.wt. 100,000-200,000) (Fisons) 5 Gm.) Saline

4. Lymphocyte Separation Medium s.gravity 1.077 (Flow Laboratories Limited, Irvine, Scotland KA12 8NR).

5. Ammonium chloride (Sigma) 8.75 Gm)
6. Distilled water. 1 litre) 0.875% w/v

7. x10 concentrated Hanks Balanced Salt Solution (HBSS) (Flow Laboratories).

8. Gelatin (Type IV) (Sigma) (1% w/v in distilled H_2O sterilized in 10 ml vol.).

9. NaOH.

10. Conical tubes 110 x 17 mm. (Nunc, Kastrup, Denmark, DK 4000)

Gel Hanks = (x10 concentrated HBSS + 1% Geltain 10 mls + sterile distilled water 80 mls; adjust pH to 7.2 with few drops of NaOH).

Heparinized blood = 100 units heparin per 10 mls venous blood.

Isolation of monocytes using nydocenz (Boyum, 1983)

10 mls potassium EDTA tubes (Monoject KE/10)

Dextran T500 (Pharmacia, Uppsala, Sweden) 6 Gm)
Normal Saline (0.9% NaCl) 100 mls) 6% w/v dextran-saline

Nycodenz Monocytes (specific gravity 1.068, osmolality 337 (± 5 mosm) (Nyegaard Diagnostica, Post Box 4220, Torshov, N-0401, Oslo, Norway).

Gel Hanks.

Conical tubes (110 x 17 mm) (Nunc).

Preparation of Bacteria for phagocytosis and killing assays

1. Mueller Hinton Broth (Oxoid).
2. ^{3}H-Adenine (Specific activity 20 uCi/mmol, 0.74 TBq/mmol) (Radiochemical Centre, Amersham).
3. ^{5,6,3}H-Uridine (specific activity 38 Ci/mmol - 1.41 TBq/mmol). 1/10 Stock Solution in distilled water is prepared and stored at 4^{o}C.
4. 0.9% Sodium Chloride.
5. Cecil Spectrophotometer CE 272 (Cecil Instruments, Cambridge)
6. Pooled human serum.
7. Gel Hanks.
8. Blood agar plates.
9. Solid CO_2 and methanol.
10. Scintillation vials (Fisons Lab. Supplies, Belton Road West, Loughborough LE11 OTR).
11. Scintillation fluid (Hydro Luma) (May & Baker Ltd., Liverpool Road, Manchester M30).
12. Test tubes (70 x 11 mm) (Nunc).

Preparation of chemotaxins

1. Zymosan (Sigma).
2. N-Formyl-L-Methionyl-L-Leucyl-L-Phenylalamine (FMLP) (Sigma).
3. Mueller Hinton Broth (Oxoid Ltd., Basingstoke).

4. Casein Hammarsten (BDH).
5. Dimethyl Sulphoxide (DMSO) (Sigma).
6. Sodium Dihydrogen Orthophosphate ($NaH_2PO_4.2H_2O$ mol. wt. 156.01) (BDH).
7. Minimum Essential Medium (Flow Laboratories).

Zymosan Activated Serum:

125 mgs Zymosan + 5 mls human serum, incubated in a shaker at 37°C for 60 minutes. Centrifuge at 800xg for 10 minutes. Heat in a water bath at 56°C for 30 minutes. Store in aliquots of 0.4 ml at -70°C.

FMLP

43.76 mg FMLP + 10 mls DMSO = FMLP 10^{-2}M solution 10^3 fold dilution in MEM is done to give 10^{-5}M solution. Store at -70°C in aliquots of 0.1 ml.

Casein:

Final concentrations of 1.5 mgs/ml, 3 mgs/ml, 6 mgs/ml, 10 mgs/ml and 20 mgs/ml were prepared. Appropriate amounts of casein was dissolved in 10 mls MEM (pH 11 adjusted with NaOH drops) was incubated at 37°C for 60 minutes and the pH adjusted back to 7.2 by addition of a few drops of 1 molar $NaH_2PO_4.2H_20$ stored in 0.4 ml aliquots at -70°C.

Under-agarose chemotaxis Assay:

Agarose (B.D.H. Poole)

gelatin

x 10 Concentrated Minimum Essential Medium (MEM) (Flow Laboratories Ltd).

$NaHCO_3$ (Sigma)

NaOH

60 x 15 mm culture plates (Gibco, Paisley Ltd)

Rigid Template with 3 mm holes.

3 mm diameter cork borer.

Preparation of 2% agarose (w/v) and 0.5% gelatin w/v in MEM

Agarose 4 Gm + Gelatin 1 Gm + 200 ml distilled water, warmed to boiling, to dissolve gelatin, cooled to 48°C. (Solution A).

Preparation of x 2 MEM (Solution B)

x 10 concentrated MEM 40 mls)

$NaHCO_3$ 7.5% 4 mls) Adjust pH to 7.2 with few drops

Distilled water 156 mls) NaOH. Warm to about 50°C.

Solution A + Solution B warmed at 56°C in a water bath. On a flat surface pour 5 mls into each culture plate, refrigerate at 4°C.

5 pairs of wells are punched out using the cork borer through the solid template on the day of the experiment.

Microchemotaxis Assay

48-well microchemotaxis chamber with accessories (Neuroprobe Inc. 7621 Cabin Road, Cabin John, MD. 20818).
Polyvinylpyrrolidone-free polycarbonate membrane filters (25 mm x 80 mm) 8u, 5u or 3 u pore size, 10u thick. (Neuroprobe).
Medium 199 with antibiotics (Penicillin 10 i.u./ml, gentamycin 10 mcg/ml, amphotericin-B 1 mcg/ml).

Chemiluminescence:

Luminometer tubes (55 x 12 mm) (Sarstedt Ltd., 68 Boston Road, Beumont Leys, Leicester LE4 1AW).
Packard Picolite Luminometer - model 6500 (Packard Instruments C5. 2200 Warrenville Road, Downers Grove, IL. 60515).
Luminol (5-amino-2,3,dihydro-1,4 phthalazinedione) mol. wt. 177 (Sigma).
Lucigenin (dimethylbiacridinium nitrate) Mol. wt. 510.5 (Sigma)
x 10 Concentrated HBSS (Flow Laboratories)
HEPES Buffer 1 Molar (Gibco)
Dimethylsulfoxide (DMSO) (Sigma)
Sodium Bicarbonate 7.5% (Gibco)

Preparation of Luminol:

17.7 mg luminol was dissolved in 10 mls DMSO to obtain 10^{-2}M and serial dilutions were made in distilled water to 10^{-5}M.

Preparation of Lucigenin:

Buffered HBSS was prepared as follows:
x 10 HBSS 2 mls + HEPES 0.4 mls + $NaHCO_3$ 0.3 mls + Distilled H_2O 17.3 mls. Dissolve 51.05 mg lucigenin in 10 mls of buffered HBSS to obtain 10^{-2}M solution from which further dilution in buffered HBSS is made.

Monoclonal Markers

I. Labelling cells in suspension:

The following markers were used:

Primary antibodies used: Mouse antihuman antibodies of various Ig subclasses.

T-Cell Markers from Coulter Clone (Coulter Electronics Ltd., Northwell Drive, Luton, LU3 3RH, England).

T_3	Pan T-cell marker	IgG_1 subclass
T_4	Helper T-cell marker	IgG_1 "
T_8	Suppressor/Cytotoxic T-cell marker	IgG_1 "

Monocyte markers: (Coulter clone)

Mo_1 IgM subclass

Mo_2 IgM "

Macrophage Marker

Anti-Leu-M_5 (IgG_{2b}) (Beckton-Dickinson Immunocytochemistry Systems, P.O. Box 7375, Mountain View, California 94039).

Secondary Antibodies:

Goat-anti mouse - FITC conjugated antibodies (Coulter Clone).

Other Materials:

PBS (filtered through 0.22 u pore size filters)

Fluorescent microscope (Leitz Wetzlar - 48 Park Street, Luton).

Methylene green (M7766) (Sigma Chemical Co.)

FACS Analyser (Becton Dickinson)

II. Labelling cells on cytospins

(Immunoperoxidase (IPX)/Immunoalkaline phosphatase (IAP) techniques).

The above primary antibodies failed to work with these methods and the following were gratefully supplied by Scottish Antibody Production, Law Hospital, Carluke ML8 5ES) and were found to be satisfactory.

T-cell markers (T_3 1/10, T_4 1/5, T_8 1/20 dilutions)

Monocyte marker $UCHM_1$ 1/4 dilution

HLA-DR marker $RFDR_1$ 1/40 dilution

The following macrophage markers were kindly supplied by Dr L.W. Poulter, Department of Immunology, Royal Free Hospital, London

RFD_1	IgG_1)		1/10 dilution
RFD_7	IgG_1)	mouse antihuman)	1/10 "
RFD_9	IgG_1)		1/5 "
NA1/34	(T_6 equivalent)		1/5 "

Secondary antibody: for IPX - Horse Radish Peroxidase (Anti mouse IgG) (SAPU).

For IAP: Alkaline Phosphatase Conjugated Rabbit antimouse (RAM) D314 (Dakopatts, Glustrup, Denmark).

Preparation of secondary antibody

(1/20 alkaline phosphatase conjugate)

0.5 ml filtered normal human serum + 0.5 mls tris buffer + 50 ul RAM-AP (D314)

Normal Rabbit Serum (SAPU): Filtered and 1/5 dilution in Tris-Buffer.

Mountant: glycerol (Dako)

Microscope slides thoroughly cleaned in methylated spirit.

Poly-L-Lysine Hydrobromide 0.1% (BDH)

Slide carriers (Sterilin Ltd., Feltham, England).

Cling Film (H.D. Plastics Ltd., Letchworth, Herts.).

Coverslips (Chance Propper Ltd., Smethwick, Warley, England)

Gills Haematoxylin (Solution Gill No.2 - Sigma Diagnostics)

Scots Tap Water Substitute:

Sodium Bicarbonate (BDH) 7 g dissolved in distilled H_2O

Magnesium Sulphate (BDH) 40g " " "

Two solutions are mixed and made up to 2000 mls in distilled H_2O. A crystal of thymol is added to prevent mould growth.

Other Reagents (IPA)

Tris Buffer: 48.48 g Trizma HCl (Sigma)

11.12g Trizma Base (Sigma)

8 litres distilled water - adjust pH to 7.6

Tris Saline: 8.76g NaCl (BDH) + 1 litre distilled H_2O = saline

10 parts saline + 1 part Tris Buffer.

Veronal Acetate Buffer (pH 9.2)

5.886g Barbitone Sodium

3.886g Sodium Acetate Trihydrate (BDH)

990 mls distilled H_2O

10 mls N/10 HCl (BDH)

Store in a dark bottle at 4^oC

Solution A - AP substrate:

2.5 mg Naphthal-AS B_1 Phosphoric Acid (Sigma)

1 drop Dimethyl Formamide (Sigma)

2.5 mg Fast Red TR salt (Sigma)

5 mls Veronal Acetate Buffer pH 9.2

100 ul 1mM Levamisole (Sigma)

a) Suspend naphthol AS-B_1, Phosphoric acid in dimethyl formamide

b) Dissolve fast red TR salt in veronal acetate buffer.

c) Add b) to a).

d) Add Levamisole.

e) Mix well and filter through 0.22u pore size filter before use. Mixture tube used within one hour.

Immunoperoxidase

3,3 Diaminobenzidine tetra hydrochloride (DAB) (D5637 - Sigma)

Hydrogen Peroxide (H_2O_2) 30% w/v (10128 - BDH).

T

0.05% DAB = 50 mg DAB + 100 mls Tris-buffer. Mix and filter

DAB/H_2O_2 = 33 ul of 30% H_2O_2 is added to 100 mls of 0.05% DAB. Stir and use immediately. DAB is handled with gloves as it is a suspected carcinogen.

Industrial Alcohol + Methylated Spirit (Charles Tennent & Co., Whistleberry Road, Blantyre G72 OTQ).

Xylene: (May & Baker) 200 mls) Harleco Synthetic

Piccolyte Resin (AS 20944 - Kodak) 120 gm) Resin

Baseline levels of complement components and products of Complement Activation.

Complement Components: in ug/litre

C1q	5
C1r	6
C1s	8
C4	12
C3	2
C1-Inh	4
B	3
H	0.5

Products of Complement Activation

C1sCINH	1500	
C3-P	900	Units/Litre
C5b-9	4000	
C5a	10	ug/litre
C3a	40	ug/litre

Albumin	10	mg/litre

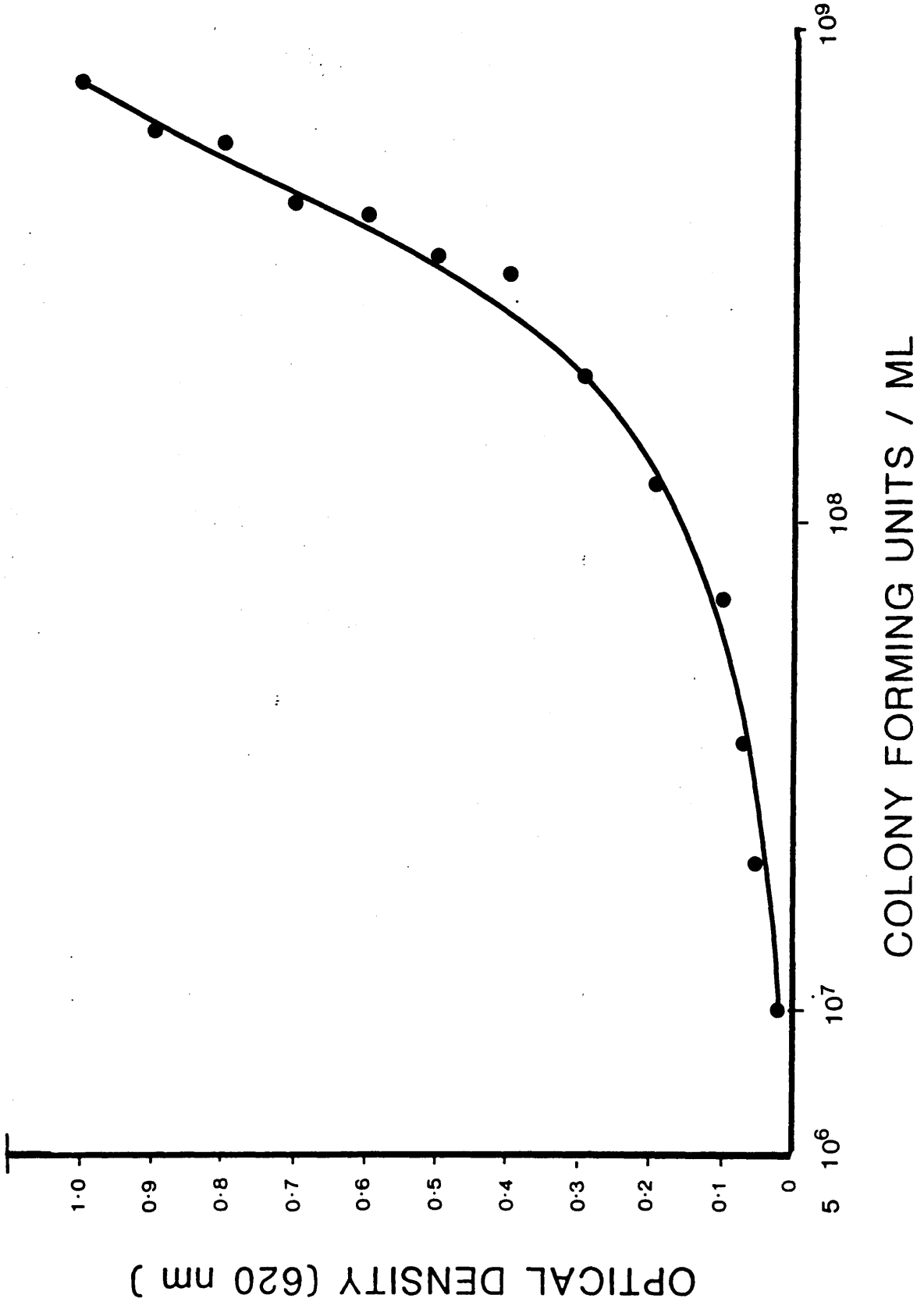

Fig. A. 1: Correlation between colony-forming units / ml of Staphylococcus 502A and optical density. (mean of 4 experiments)

APPENDIX 2

STATISTICAL ANALYSIS

Basic Definitions:

Means ($\bar{x}$): $= \dfrac{\text{Sum of all observations}}{\text{Total number of observations}} = \dfrac{1}{n}.\sum x$

where n = number of observations

Median = value of an observation which lies in the middle of all the observations if they were arranged in increasing or decreasing order. For an odd number of observations this is the:

$$\frac{n + 1}{2} \text{ th}$$

observation. For an even number this is the mean of the middle two observations.

Standard deviations (SD) a measure of a spread of observations about the mean. It is useful for data with normal distribution.-

$$= \sqrt{\frac{\sum x^2 - \frac{\sum x^2}{n}}{n - 1}}$$

where $\sum x$ = sum of all observations.

Standard Error of Mean (SEM): $\frac{\text{Standard Deviation}}{n - 1}$

Coefficient of Variation: $\frac{\text{Standard Deviation}}{\text{Means}}$

$$= \sqrt{\frac{\sum x^2 - \frac{\sum x^2}{n}}{n - 1}} \cdot \frac{1}{\bar{x}}$$

The Null hypothesis (H_o) = is the assumption that there is no difference between the two sets of data being compared.

Two tail test: Compares the two sets of data at both the lower end and the upper end of the distribution.

Probability (p): Denotes the number of times (%) an incident would occur by chance. It is conventional to accept that this was significant and not merely due to chance

if it occurred in 5% or less number of times. Thus when $p \leq 0.05$ this is significant and if it is ≤ 0.01 it is highly significant.

Statistical tests used in this thesis: All statistical analyses were made using a computer based Haessle statistical pack (prepared by Haessle university and Huddings Stockholm). The following tests were used.

1. Mann Whitney U test:

All comparisons between two sets of data in this thesis were made using this non- parametric test. All significance levels (p values) were for the two tail test. Mann Whitney u test does not assume criteria for the normal distribution and does not require that the standard deviations between the two groups be similar. It analyses ranks of scores between data. The scores of the two groups are rank ordered in a common pool and the sum of the ranks is obtained. Tied observations (i.e. observations with equal values) are assigned the mean of rank position that they would have occupied if there had been no ties.

$$u = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - R_1$$

R_1 = sum of ranks assigned to the groups with smaller number of observations (n_1).

R_2 = sum of ranks assigned to the group with larger number of observations (n_2).

It has been shown that the larger the sample size the sampling distribution of u value rapidly approaches normal distribution (Siegel 1956). Therefore when either of the groups is larger than 20, the significance level of observed u value is determined by computing the Z value. The P value associated with the occurrance of (or rejection) of H_o is determined by reference to the table for values as extreme as observed value of Z.

$$Z = \frac{u - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 n_2 (n_1 + n_2 + 1)}{12}}}$$

When ties occur between two or more values in both groups, the u value is affected and correction for u value needs to be applied. Thus Z will be computed as follows:

$$Z = \frac{u - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 n_2}{N(N-1)} \frac{N^3 - N}{12} - \sum T}}$$

$$N = n_1 + n_2 \qquad T = \frac{t^3 - t}{12}$$

t = number of observations tied for a given rank.

2. Wilcoxon matched pairs signed-rank test:

This test was used to compare matched pairs of observations from the same patients. Differences between pairs are ranked from smallest to largest and pairs with difference of 0 are dropped. Tied differences are assigned the average of the ranks had there been no ties. The ranks are then signed (+ or -). T is the formal test for the Wilcoxon matched pairs signed ranks test.

T = smaller sum of like signed ranks. For samples larger than 25, T value has to be transformed to a Z score.

$$Z = \frac{T - \frac{N(N + 1)}{4}}{\sqrt{\frac{N(N + 1)\ (2N + 1)}{24}}}$$

N = number of matched pairs minus the number of pairs whose differences = 0.

3. Spearman rank correlation coefficient (rs):

Was used to compare the degree of association between two groups:

$$rs = \frac{\sum x^2 + \sum y^2 + \sum d^2}{2\sqrt{\sum x^2 \sum y^2}}$$

x and y are the values for the two groups.

$$\text{or } rs = 1 - \frac{6 \sum_{i=1}^{N} di^2}{N^3 - N}$$

$$d = x - y$$

$$N = n_1 + n_2$$