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STUDIES OF THE ROLE OF GROWTH FACTOR SECRETION BY LUNG MACROPHAGES IN A RABBIT MODEL OF PULMONARY FIBROSIS

A THESIS SUBMITTED TO THE UNIVERSITY OF LONDON FOR THE DEGREE OF DOCTOR OF MEDICINE

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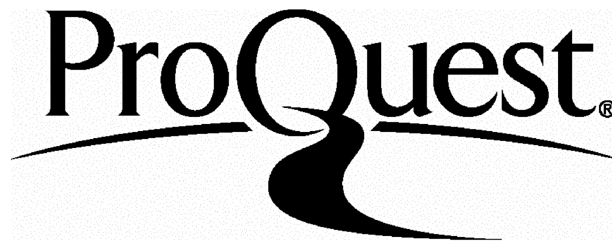

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ABSTRACT

Deposition of collagen in the alveolar structures of the lung is a central feature of all forms of pulmonary fibrosis. This is preceded by or associated with an influx of inflammatory cells, and in turn there is an increase in fibroblast numbers. This thesis examines the role of growth factor secretion by lung macrophages in the control of fibroblast replication. A rabbit model of pulmonary fibrosis, induced by intratracheal bleomycin, was used. Inflammatory cells were lavaged from the lungs of saline and bleomycin treated animals, cultured for 24 hours and the level of growth factor secretion measured on two fibroblast cell lines. This showed that the administration of bleomycin lead to a rapid and marked influx of inflammatory cells, of which macrophages remained the predominant cell. These cells secreted growth factors, although the secretion rate per cell was not higher in the bleomycin treated animals. Nonetheless the increased numbers of inflammatory cells in the alveoli resulted in an increased alveolar burden of growth factor which may account for the increase in lung collagen seen in this animal model.

As part of this work three subsidiary problems were addressed. The effect of culture of alveolar macrophages on their state of activation was quantitated by the measurement of protein synthesis rates, *in vivo* and *in vitro*. The rate was fivefold higher *in vitro*. A colorimetric method for estimating fibroblast numbers in 96 well plate cultures was developed and validated. Using this method the ability of alveolar macrophages from normal rabbits to secrete growth factor was confirmed and some of the physicochemical properties of the growth factors were determined.

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ABBREVIATIONS

A515	Absorbance measured at 515 nm
A650	Absorbance measured at 650 nm
AM	Alveolar macrophage
DMEM	Dulbecco's modification of Eagle's medium
GF	Growth factor
IF	Inhibitory factor
NCS	Newborn calf serum
NSE	Nonspecific esterase
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PGE_2	Prostaglandin E_2
SD	Standard deviation
SEM	Standard error of the mean
SR	Specific radioactivity
TCA	Trichloroacetic acid
TNF	Tumour necrosis factor

Statement of Personal Contribution to the Work

All of the work described in this thesis was carried out by myself. The animals used were kept in the animal house of the Cardiothoracic Unit and cared for by the Curator and staff of the animal house.

Chapter 1

INTRODUCTION

The last two decades have seen an explosion of interest in cell biology fuelled by improvements in cell culture techniques. These developments have illuminated many medical problems. In particular the description of growth factors has provided a tool for the study of local control of growth, remodelling of tissues and repair mechanisms in damaged tissue. It is to be hoped that this knowledge may finally help in the treatment of many human diseases which have remained resistant to our present day therapeutic armamentarium.

One group of conditions greatly in need of such advances are the diffuse fibrotic disorders of the lung. These are a heterogeneous group of diseases characterised by diffuse pulmonary fibrosis which dominates the final clinical picture. In most of these disorders therapy is either unavailable, or uncertain of benefit and associated with major side effects. The need for understanding of the pathogenetic mechanisms in this situation is pressing.

1.1 HUMAN DISEASE

1.1.1 Diffuse Pulmonary Fibrosis

The number of diseases in this category exceeds 100 (Crystal et al, 1981). Although not as common as disorders such as asthma, chronic bronchitis or bronchial carcinoma, many of these fibrotic diseases are not rare. They would be

part of every chest physician's weekly clinical load. They are all responsible for much morbidity in the form of breathlessness which can be incapacitating, and in the later stages damage to the pulmonary circulation causing pulmonary hypertension and right ventricular failure. Many of these diseases have a significant mortality rate. Cryptogenic fibrosing alveolitis, for example, has a five year survival from diagnosis of 50% (Turner-Warwick et al, 1980), no better than many malignancies. In none of them is a completely satisfactory form of therapy available, and in some therapeutic decisions can be very difficult. These problems are best illustrated by an outline sketch of three of these diseases.

1.1.2 Cryptogenic Fibrosing Alveolitis

This disease can start at any time in adult life. Examination of lung biopsies shows a pattern of alveolar wall damage and thickening together with the formation of increasing amounts of fibrous tissue. Biochemical analysis has confirmed the presence of increased amounts of collagen (Selman et al, 1986), in keeping with the histological picture. In the end stages, the fine alveolar structure of the lung is replaced by bands of thick fibrous tissue separating enlarged airspaces. Varying degrees of cellularity of the lung biopsies are seen, with some suggestion that the more cellular patterns are associated with potential for improvement spontaneously or with treatment (Crystal et al, 1976; Winterbauer et al, 1978).

The major symptoms are cough and breathlessness. The course of the disease is very variable ranging from mild symptoms with no change over many years of observation, to inexorable progression to death within months of diagnosis. Spontaneous remissions and relapses are seen.

Treatment is unsatisfactory (Johnson et al, 1986). High doses of corticosteroids benefit an uncertain proportion of patients, but in many make no perceptible difference.

cytotoxic drugs such as cyclophosphamide or azathioprine are used. For none of these is there convincing evidence from controlled trials of their benefit, although the general consensus is that they do benefit some 20% of patients, at a cost of major side effects in some (Johnson et al, 1989). This marked variability in tempo, rate of progression, and response to therapy of the disease makes decisions on therapy for an individual patient very difficult. This is compounded by the fact that response, if it occurs, is often slow so that assessment of the success of therapy is difficult. So the clinician has desperate need of (1) a marker of disease activity which would point to the need for therapy, (2) a marker of response to treatment and (3) new forms of therapy which are more effective and less toxic. The hope must be that research into the pathogenesis of the disease might satisfy some of these needs.

1.1.3 Sarcoidosis

Sarcoidosis is a multisystem disease of unknown aetiology characterised by the presence in affected tissues of organised collections (granulomata) of chronic inflammatory cells. It can involve almost any system in the body although the brunt is most often borne by the lungs. Histological examination of involved tissue generally shows non-caseating granulomata consisting of collections of lymphocytes, modified macrophages (epithelioid cells) and other chronic inflammatory cells but without the central caseating necrosis of the granulomata of tuberculosis. This abnormal pattern may resolve with little or no permanent mark in the tissues, but it may be replaced by bands of hyaline amorphous tissue which becomes increasingly fibrotic with time.

For many patients pulmonary shadowing may be a chance finding on a chest X-ray or discovered on investigation of minor respiratory or other symptoms. In some 50% of cases this X-ray shadowing will clear without treatment leaving a normal X-ray and an asymptomatic patient. In others,

treatment with corticosteroids is necessary to achieve the same result. In perhaps 25%, inspite of therapy, the radiographic shadows evolve into those characteristic of bands of pulmonary fibrosis. Often the process will arrest after a time leaving some disability, but in a small proportion of cases it is progressive, leading on to life threatening respiratory insufficiency. The same problems in assessment of likelihood of progression, response to therapy and individual variations in tempo are seen with sarcoidosis as with cryptogenic fibrosing alveolitis, accepting that the overall prognosis is better. Here, where most patients will not require therapy, the particular problem is to select at an early stage those who will, before permanent lung damage has occurred.

1.1.4 Mineral Dust Pneumoconiosis

This is a group of conditions characterised by pulmonary fibrosis in response to the inhalation of mineral dusts such as coal, silica, or asbestos. They vary as to the radiographic pattern of the fibrosis and in the degree of respiratory disability which results. Although the dose of inhaled dust does correlate with severity, there are marked individual variations in response. With a well defined and controllable aetiological agent, reduction in dust exposure offers the best hope of reducing future morbidity and mortality. In patients with established pulmonary fibrosis there is no therapy which can halt the progression of the disease. Fortunately many patients see only minor progression.

The pneumoconioses have become less prevalent because of the adoption of dust control measures in the workplace. Nonetheless they remain important as models for other forms of pulmonary fibrosis because in their case the aetiological agents are known.

1.2 THE MACROPHAGE, GROWTH FACTORS AND PULMONARY FIBROGENESIS

Tissue destruction, derangement of tissue architecture and fibrosis are all features of the pathology of the pulmonary fibrotic diseases. An increase in macrophage numbers is a consistent finding in all of these conditions. Several historical threads have come together to identify the macrophage as having a central role in one of these pathological processes - fibrosis itself.

1.2.1 Lessons from Silicosis

This example of human pulmonary fibrosis has been well documented clinically and pathologically for a number of decades. The aetiological agent is readily available for experimental study and so it is not surprising that the early work in pathogenesis of pulmonary fibrosis focused on this condition.

Two themes have run through this literature:- the direct cytotoxicity of silica and the role of the macrophage in the development of fibrosis (reviewed by deShazo, 1982). It would be expected that lung macrophages would ingest particulate inhaled silica. The pathologists confirmed that this process could be seen in lung tissue sections. Experiments *in vitro* confirmed that macrophages did ingest silica particles and that this process lead to the final lysis of the cells with the release of lysosomal enzymes (Davies and Allison, 1976) and free radicals (Gabor et al, 1980), which could cause lung damage. A more direct role for the lung macrophage in fibrogenesis was suggested by Hepplestone and Style's seminal paper (1967) where they reported that macrophages exposed to silica *in vitro* released substances which could stimulate fibroblasts to produce more collagen. This observation lead to contributions from many workers (well reviewed by Reiser and Last (1979)). Although

this early work is confused by the variations in experimental conditions used by the different investigators, it did establish that viable macrophages exposed to silica could secrete factors which modified fibroblast replication and collagen production. A study by Bateman et al (1980) of the effects of fluid phase factors from mixtures of macrophages and mineral dust contained in small diffusion chambers provided evidence for the importance of continuing viability of the macrophages in the development of fibrosis. The chambers were implanted into the peritoneal cavities of mice and the development of fibrosis observed. Asbestos, and silica in low concentration permitted viable macrophages to remain inside the chamber for two weeks, and the development of fibrosis was noted. Silica in higher concentration rapidly killed the macrophages and little fibrosis around the chambers resulted. This evidence demonstrated the importance in fibrogenesis of active secretion by macrophages of mediators, rather than the passive release of substances following macrophage cytotoxicity. This hypothesis is attractive as it is of general application to fibrotic disorders not linked to cytotoxic agents.

It seemed that the macrophage might have a central role in the pathogenesis of all forms of diffuse pulmonary fibrosis. Other developments in cell biology in the last decade provided insights and tools for the study of macrophage-fibroblast interaction.

1.2.2 Alveolitis

To the clinician studying the chest radiograph of patients with end-stage disease, or present at a post mortem examination of such a patient, the fibrosis is obvious and extensive. For years the morphologists have been pointing to the presence of accumulations of inflammatory cells - macrophages, lymphocytes, neutrophils etc - in the alveolar structures of patients with pulmonary fibrosis. This "alveolitis" is a feature common to all forms of pulmonary

fibrosis although its nature may differ between different conditions (Crystal, 1982).

There is now good evidence, provided by lung biopsy techniques, that this alveolitis is the earliest manifestation of these conditions, and may resolve completely (Lacronique et al, 1981; Carrington et al, 1978). These inflammatory cells have the potential to damage tissue by the release of free radical species, and hydrolytic enzymes; to secrete factors which activate nearby cells to contribute to the inflammatory process; and also to secrete chemotactic factors which further increase the local numbers of inflammatory cells (Shock and Laurent, 1990). Thus if the alveolitis persists then increasing disorganisation and fibrosis of the lungs can follow. This model of the pathogenesis of pulmonary fibrosis has its parallels in other organs e.g., the importance of the inflammatory infiltrate seen in early cirrhosis of the liver to the subsequent tissue destruction, derangement and fibrosis seen in this condition. Thus it is hoped that study of these inflammatory cells may illuminate pulmonary fibrogenesis, and treatment directed at the alveolitis may forestall the development of the changes apparent in end stage disease.

1.2.3 Growth Factors

It has long been known that diploid cells require serum to replicate in culture. In 1971, Samuel Balk made a critical observation about this effect of serum. He had designed a culture medium which was more "physiological" in that serum (derived from clotted whole blood) was replaced by plasma where the platelets had been removed by centrifugation. In this new medium the cells remained healthy but divided much less readily. Balk suspected that a "wound hormone" was released into serum during clot formation.

These observations were extended in 1974 by a number of researchers (Ross et al, 1974; Kohler and Lipton, 1974; Scher et al, 1974), who confirmed that platelets contain a

polypeptide which stimulates the growth of many cells in culture. This is known as platelet derived growth factor (PDGF). It is released when platelets are activated, for example during coagulation of whole blood. Plasma also contains growth promoting activity, different but complementary in its action to PDGF. Both forms of growth factor need to be present to sustain replication. Mesenchymal cells in tissues are bathed in extracellular fluid (effectively a platelet free ultrafiltrate of plasma) which is not sufficient to cause growth. After tissue damage with platelet activation and degranulation, the stimulus to replication is complete. Thus PDGF provides control over local variations in growth.

The partnership between PDGF and platelet poor plasma in controlling the cell replication cycle has been analyzed further (Scher et al, 1979). The two stimuli can be applied sequentially but if so, the cells must be exposed to PDGF before plasma to allow replication to proceed. PDGF induces cells to become "competent" to respond to the subsequent stimulus of plasma, so that they "progress" from G_0 into the G_1 and S phases of the cell cycle. Thus PDGF is known as a "competence" growth factor and plasma contains a "progression" factor or factors.

Since this pioneering work a large number of growth factors have been identified and the list now exceeds thirty (Deuel, 1987). The field is made particularly complex because the detailed chemical structure of the majority is not yet known. Some have, on stringent analysis, been shown to be identical, so that as analysis of others is achieved, the list may diminish and the picture become clearer. In general each has been found to have either competence or progression factor activity but not both. The target tissues of these growth factors vary as to their sensitivity to each GF, no doubt due to variable expression of the specific receptors for each GF.

The discovery of this class of compounds and their coordinate mechanism of action provides an explanation of the

control of tissue growth locally, of differential growth of different cell types in a single tissue and of the uncontrolled growth of tissue in malignant disease (Deuel, 1987; Druker et al, 1989).

1.2.4 Macrophages and the Fibroblast

In the cellular changes of wound healing, monocytes move from the capillaries into the granulation tissue of the wound where they evolve into tissue macrophages, and this is followed one to three days later by the appearance and multiplication of fibroblasts. Collagen is laid down by these fibroblasts to form scar tissue. Liebovich and Ross (1975) studied the effects of monocyte depletion on this progression of changes in healing skin wounds in guinea pigs. They showed that fibroblast proliferation and collagen deposition were much reduced in the monocyte-depleted animals. Their work suggested that tissue macrophages had a major role in controlling fibroblast proliferation and possibily collagen secretion.

The increasing literature on serum and tissue growth factors provided an impetus and new techniques to examine the effects of monocytes and macrophages products on fibroblast replication. The picture that has emerged is complex and still evolving rapidly (Nathan, 1987). This complexity which is partly due to the range of species and tissues studied and partly due to the problems in characterising biochemically the multiple activities secreted by macrophages, also reflects the genuine intricacies of the networks of cell-cell interaction being discovered.

Points of agreement are that

(a) Macrophages have the capability to produce a wide range of mediators (cytokines) which can modulate the function or replication of other cells in the local environment (Nathan, 1987).

(b) The macrophage cytokines which have major effect on fibroblast growth have proved to be polypeptides with M.Wts.

in excess of 12,000 (Kelley, 1990).

(c) These polypeptide cytokines may have growth promoting activity, or growth inhibiting activity or in the case of interleukin-1, exhibit both activities depending upon the experimental conditions (Schmidt et al 1982; Rainer et al, 1989)

(d) Most of these cytokines can be secreted by many cell types and are not unique to macrophages (Kelley, 1990).

Thus the macrophage has been found to have a central role in wound healing and the formation of collagen in scars, is present in increased numbers in the alveolitis which is the forerunner of all forms of pulmonary fibrosis, and is capable of secreting polypeptide cytokines which modulate fibroblast replication. Fortunately this key cell can be obtained from the lung for study both post mortem and in life by the technique of bronchoalveolar lavage.

1.2.5 Bronchoalveolar Lavage

Standard clinical techniques such as respiratory function tests, measurements of gas exchange and radiology shed little light on the pathogenesis of pulmonary disease. Biopsy samples allow histological study and biochemical analysis but the risks involved in lung biopsy precludes its use as a purely research tool. Samples will only be available if the management of the patient indicates the need for biopsy. In practice many patients with pulmonary fibrosis never undergo lung biopsy and it would be rare for it to be performed more than once in any patient. Sequential samples are not available therefore.

The central role of an alveolitis in the development of pulmonary fibrosis does offer a way out of this impasse. Much may be learned from samples of inflammatory cells obtained from the lung without sampling the parenchymal cells. The technique of bronchoalveolar lavage, developed by Reynolds and Newball (1974), achieves this.

Inflammatory cells are to be found on both sides of the alveolar wall i.e., in the interstitium and in the alveolar fluid. The cells free in the alveolar fluid can be sampled by the technique of bronchoalveolar lavage, whereby in life a pulmonary segment, or post mortem the whole lung, can be filled with physiological saline which is then aspirated. In humans the procedure is carried out using a fibreoptic bronchoscope wedged in a bronchus and is well tolerated with a low morbidity rate. It can be repeated and therefore permits sequential sampling.

Bronchoalveolar lavage has been accepted as both a clinical and a research tool. In the former setting, the interpretation of results finally rests on the pragmatic question of whether they answer the questions posed by the clinician i.e., questions of diagnosis or treatment. In a research setting the value of bronchoalveolar lavage depends on the answer to the question "do lavage cells reflect events in the lung interstitium"? A rapid inspection of a sample of lavage cells would show that the structural cells of the interstitium - epithelial, endothelial, fibroblast etc - are not represented. The cells seen are all "inflammatory" in type. Within this group some differences between the alveolar (lavage) and interstitial cell populations are apparent. The epithelioid cells of granulomata are not seen in lavage samples (Danel et al, 1983). Plasma cells and lymphocytes predominate in the interstitium in cryptogenic fibrosing alveolitis but eosinophils and neutrophils more commonly accompany macrophages in lavage samples. However Hunninghake and colleagues (1981) showed almost identical percentage counts in lavage and lung extract samples in a number of disease states. It is probable that the distinction between interstitium and alveolar space becomes less clearcut in many disease states (see Section 6.4.3 for a fuller discussion of this). Thus, in disease, bronchoalveolar lavage is likely to accurately reflect events in the interstitium.

Bronchoalveolar lavage has established a central role for

itself in research into the pathogenesis of pulmonary fibrosis both in human disease and in animal models, and a more limited role in the diagnosis of interstitial lung disease in humans.

1.2.6 Macrophage Activation

The macrophage is sensitive to both specific and non specific stimuli which can activate some or many of its functions (Nathan, 1987). The investigator about to embark on a study of cells *in vitro* must be concerned that the techniques involved in isolating and culturing the cell may of themselves stimulate or activate the cell, so that measurements of basal level activity are falsely high. No comparative study of a macrophage function measured both in vitro and *in vivo* has been published, primarily because of the problems of quantitating this *in vivo*. The difficulty in addressing this question has been to identify an index of activation which can be measured and compared for cells *in vivo* and *in vitro*. Many secretion products of macrophages are not unique to that cell e.g., arachidonic acid metabolites, oxygen metabolites, complement components and probably also growth factors. Therefore it would not be possible to confidently assign a secretion rate of these products measured *in vivo* to alveolar macrophages alone. Even for those which are unique to the cell, calculation of secretion *in vivo* is prevented by an unknown clearance rate. Recently Laurent (1982) developed and validated a method of measuring *in vivo* the protein synthesis rate of tissues such as muscle, liver and heart. It seemed that this could be applied to alveolar macrophages *in vivo* and thus provide a parameter of alveolar macrophage activation which could be compared *in vivo* and *in vitro*.

1.3 ANIMAL MODELS OF PULMONARY FIBROSIS

1.3.1 The Need for Animal Models of Pulmonary Fibrosis

It is a characteristic of most forms of human diffuse pulmonary fibrosis that in the established case a large open lung biopsy will reveal areas of early alveolitis and other areas of heavy inflammatory infiltrate, and areas of mild and marked fibrosis (Winterbauer et al, 1978). Thus all the different "stages" in pulmonary fibrosis will be represented. For the clinical investigator, this makes analysis of the component steps of the pathogenesis of the disease very difficult. The analysis would be easier if the moment of exposure to the aetiological agent was known so that a temporal sequence of events could be charted. In the adult respiratory distress syndrome (ARDS) the lung responds to an insult by developing widespread acute inflammatory changes which then either resolve or progress to fibrosis. In many cases the insult is known, e.g., inhalation of toxic gas, ingestion of a toxic chemical such as paraquat or a cytotoxic drug, or widespread sepsis. Unfortunately ARDS has a high mortality and these patients are a group in which research investigation is constrained by ethical considerations and by the presence of other pathological processes such as sepsis. It has been possible to set up models of pulmonary fibrosis in experimental animals often using the same agents seen to cause ARDS in humans. Examples include paraquat (Schoenberger et al, 1984), radiotherapy (Adamson and Bowden, 1983), oxygen (Rinaldo et al, 1982) and cytotoxic drugs such as bleomycin (see next section). In these models the potential exists for correlating the histological, biochemical, inflammatory and physiological events, and attempting "therapeutic" manipulation of these. Armed with these insights it may be possible to return to our patients, and develop diagnostic and therapeutic approaches of real benefit.

1.3.2 Bleomycin and the Lung

Bleomycin is an agent active against several solid tumours. Its use is limited by the dose-related development of pulmonary fibrosis (Blum et al, 1973). The histology of the affected lung shows epithelial and endothelial cell damage, interstitial oedema and fibrosis with derangement of lung architecture i.e., similar to that of cryptogenic fibrosing alveolitis.

The mechanism of action of bleomycin is dependent on the presence of reduced iron i.e., Fe^{2+} (Sausville et al, 1978; Burger et al, 1981). Disruption of cellular DNA with the production of toxic derivatives of nucleoside bases and free radical damage to cell membranes have been shown to be important (Umezewa, 1974; Grollman, 1988).

Bleomycin also causes pulmonary fibrosis in a range of experimental animals, including mice (Adamson and Bowden, 1977), hamsters (Snider et al, 1977), rats (Thrall et al, 1979), baboons (McCullough et al, 1978) and rabbits (Laurent et al, 1981). In early experiments the drug was given parenterally but Snider et al (1977) induced pulmonary fibrosis in hamsters by endotracheal instillation. This technique permits the use of lower doses of bleomycin with less toxicity to other organs. This has proved the most popular animal model of pulmonary fibrosis for study. The cellular changes which follow bleomycin have been well described (Snider et al, 1977; Thrall et al, 1979; Chandler et al, 1983). Chandler and co-workers (1983) charted these changes in detail using morphometric techniques in hamsters. They studied animals 4, 7, 21, 28, 35 and 42 days after instillation. They showed an early rise in neutrophil numbers peaking at 7 days and falling to low levels by 21 days. Monocytes and macrophages also showed an early rise but this was sustained throughout the study period with macrophages being relatively more common at 21 and 28 days. Fibroblasts on the other hand showed a steady rise in numbers from normal (control) level at 4 days to a peak at 28 days

and only a minor fall thereafter. These data fit in with the more qualitative impressions gained in other animal models. Thus the pattern seen is that observed in the wound healing experiments referred to earlier. The same deductions may well hold, i.e., macrophages play a central role in increasing fibroblast numbers in bleomycin induced pulmonary fibrosis.

1.3.3 The Rabbit Bleomycin Model of Pulmonary Fibrosis

Laurent and co-workers (1981, 1983) have described the histological and biochemical changes seen in the lungs of rabbits following the intratracheal instillation of bleomycin at a dose of 10mg/kg body weight. Their observations of the pathological changes agree with reports from other animal models. They commented that at four weeks there was still only a little excess extracellular material staining for reticulin, and for collagen with Masson's trichrome method. At eight weeks "young fibrous tissue" was obvious and a stronger trichrome reaction for collagen was seen. The slow onset of histologically apparent fibrosis contrasts with the biochemical evidence. In a first set of experiments (Laurent et al, 1981), examining animals at two, four and eight weeks after bleomycin, the lung content of collagen, elastin, total protein and DNA were all maximal by two weeks. Thereafter the total protein content fell markedly although the other components decreased only a little. This conflict between the views of the histologist and the biochemist can be explained by consideration of the nature of collagen and of their respective techniques for detecting collagen.

Collagen exists in at least eleven different forms or types, which differ in the detailed amino acid sequences of the component polypeptide chains. Types I and III collagens are the major fibrous interstitial proteins. They consist of three polypeptide chains (the α chains) covalently linked (Laurent, 1986). This fibrillar protein is then assembled into collagen fibres, rather like strands of hemp in a rope,

and linked by covalent bonds to other matrix components such as basement membrane collagens and proteoglycans. It is a feature of all collagens that the amino acids glycine, proline and hydroxyproline are a high proportion of the total amino acid composition of this protein. Hydroxyproline is found in a few other proteins (elastin, anticholinesterase and the C1q component of complement) but collagens are by far the largest source of hydroxyproline in all tissues. Therefore the biochemist uses the tissue content of protein bound hydroxyproline as a measure of the amount of collagen present. This measure of collagen pays no regard to what form the collagen is in. It is probably the complex chemical environment of fully matured type I collagen which is detected by Masson's trichrome, and this maturation may lag behind the initial collagen synthesis. Therefore in looking to unravel the cellular control of collagen production we must turn to the biochemical data to guide us in choosing time points to study.

This work confirmed that in the rabbit bleomycin model the deposition of excess collagen is an early and marked feature of the development of pulmonary fibrosis. Subsequent work provided more detail of the early time course of these changes and of the relative importance of increased synthesis and decreased degradation. This will be described in the introduction to chapter 5.

1.3.4 Questions Raised by the Biochemical Data

This work with the rabbit model has provided the most detailed description published of the biochemical events leading to net collagen deposition in an experimental animal model of pulmonary fibrosis. The predominant collagens in lung, as in most tissues, are type I and type III. It is tissue fibroblasts that are the major source of these collagen types. Therefore we must address ourselves to the control of fibroblast numbers and function if we are to trace backwards the chain of events which leads to collagen

deposition in this animal model.

An increase in tissue collagen might either be due to an increase in fibroblast numbers or a change in collagen metabolism (rise in synthesis, or fall in degradation) with constant fibroblast numbers, or both mechanisms might operate. The histological picture points to the first as an important mechanism. Unfortunately we do not have quantitative histological data in the rabbit bleomycin model. In the hamster bleomycin model the increase in fibroblast numbers - a peak increase of 273% over control values - was more than sufficient to account for the increase in lung collagen observed (Chandler et al, 1983; Chandler and Giri, 1982). This is a rather naive calculation but it does support the view that an increase in fibroblast numbers is the main mechanism of increased collagen deposition.

Locally an increase in fibroblast numbers might be achieved either by replication or migration of cells into the area. Rather surprisingly fibroblasts are capable of movement, within tissues, rather than being a fixed structural cell. This process is certainly important in the control of the local distribution of fibroblasts (Rennard et al, 1981) but will not be relevant to their overall numbers. The latter can only increase as a result of replication. These arguments suggest that to understand what leads to the deposition of collagen in damaged lung we need to look at the stimuli to the increase in fibroblast replication seen.

1.4 AIMS OF THIS THESIS

The central objective of this work was to examine the role of growth factor secretion by inflammatory cells obtained by bronchoalveolar lavage in the pathogenesis of lung fibrosis. This built upon the detailed picture described by Laurent and coworkers (1981, 1983) of the biochemical events occurring in bleomycin induced pulmonary fibrosis in rabbits. The hypothesis tested was that the increase in collagen synthesis rates seen might be mirrored by, and perhaps due to, a preceeding increase in secretion of growth factors by inflammatory cells in the pulmonary alveoli of bleomycin treated rabbits.

The likely source of such growth factors was the lung macrophage, and their secretion was to be studied *in vitro*. Accordingly, three subsidiary problems were identified and also investigated:-

1. Culture of alveolar macrophages *in vitro* might activate these cells, complicating interpretation of secretion rates of growth factor. This was to be assessed by measuring the macrophage protein synthesis rate *in vivo* and *in vitro*, allowing a comparison of their states of activation.

2. There was a need for a rapid assay of fibroblast numbers in microwell culture, as part of the measurement of levels of growth factors secreted by macrophages. A novel assay based on a colorimetric method was developed and validated.

3. The ability of rabbit alveolar macrophages to produce growth factor had not previously been described. This was confirmed and some of the physicochemical properties of the growth factors were delineated.

CHAPTER 2

MACROPHAGE ACTIVATION IN VIVO AND IN VITRO ASSESSED BY MEASUREMENT OF PROTEIN SYNTHESIS RATE

2.1 INTRODUCTION

2.1.1 Macrophage Activation

Recently Laurent (1982) developed and validated a method of measuring *in vivo* the protein synthesis rate of tissues such as muscle, liver and heart. It seemed that this could be applied to the small mass of tissue of alveolar macrophages *in vivo* and thus achieve for the first time the measurement of protein synthesis rate of a population of macrophages *in vivo*.

It is technically simpler to estimate protein synthesis of a cell population in culture. The quantitation of protein synthesis rate provided a measure of alveolar macrophage activation which could be compared *in vivo* and *in vitro*.

2.1.2 Principles of Measurement of Protein Synthesis Rate

Measurement of the rate of protein synthesis of intracellular protein requires a technique which labels the newly synthesised protein. The use of a radioisotopically labelled amino acid achieves this and has become the basis of the standard biochemical methods in this field. After exposure to radiolabelled amino acid of known specific radioactivity (SR), the tissue is sampled and protein isolated from free amino acids and other contaminants by standard techniques. The proteins are hydrolysed and the SR of the relevant amino acid is measured. The protein

synthesis rate is calculated as follows:-

Fractional protein synthesis rate (%/day)

$$= \frac{\text{SR of amino acid in protein}}{\text{SR of amino acid in precursor pool}} \times \frac{100}{\text{time (days)}}$$

This formula requires that the SR of the amino acid pool is constant - the mathematics becomes more complicated if this does not hold. A constant precursor SR is readily achieved *in vitro* when the extracellular pool of amino acid (i.e., in the culture medium) is large in relation to the metabolic capacity of the tissue (e.g., cells in culture). *In vivo* the same effect is achieved by the use of a large dose of injected proline (Laurent, 1982).

2.1.3 Choice of Experimental Conditions *in Vitro*

The present study is the first to examine macrophage protein synthesis *in vivo*. However other workers have looked at protein synthesis *in vitro* for AMs from guinea pigs (Airhart et al, 1981) and from New Zealand White rabbits (Hammer and Rannels, 1981). It seemed wise to use New Zealand White rabbits in this study of protein synthesis as they were to be used in the subsequent bleomycin work. The culture conditions described by Hammer and Rannels (1981) were chosen to allow direct comparison with their work.

2.1.4 Choice of Assay

The measurement of specific radioactivity requires knowledge of both the radioactivity and the molar amount of amino acid present. For the *in vivo* experiments these were provided by a chemical method (Peterkofsky and Prockop, 1962) involving Chloramine-T oxidation of the proline. This method has been modified and validated in this laboratory (Laurent et al, 1982).

A limitation of the chemical method was that the amounts

of proline to be obtained from the protein in AMs from a rabbit was near it's lower limit of sensitivity. The effect of different culture conditions on the protein synthesis rate was to be studied and this required using smaller number of cells in each individual culture. A different biochemical technique for measuring proline (and phenylalanine - see later) specific radioactivity was therefore employed. This was the dansyl chloride double isotope method which is much more sensitive than chemical assays for molar amounts of proline but needs higher specific radioactivities to ensure adequate radioactive counts from the smaller amounts of proline. This could easily be achieved *in vitro* (but would have been prohibitively profligate of radioisotope *in vivo*).

2.2 MATERIALS AND METHODS

2.2.1 MATERIALS

Laboratory stock chemicals were all of ANALAR reagent grade.

Special chemicals were obtained from Sigma UK, unless commented upon in the text. Where alternative Sigma preparations are available, the catalogue number is quoted. Radiochemicals were obtained from Amersham International.

PREPARATION OF ALVEOLAR LAVAGE CELLS

2.2.2 Animals

New Zealand White male rabbits were obtained from HOP Laboratories, Cork Farm, Chilham, Kent, at a body weight of 1.7-2.0kg. These were specific pathogen free animals, and the animals were screened regularly by animal house staff for the development of ear, nose or respiratory illness. Any

animal showing signs of disease was removed from the colony. They were fed *ad libitum*.

Animals were weighed regularly and often showed an initial fall after arrival related to the stress of transport. They were not used until they were gaining weight. They had usually been in the rabbit colony one to three weeks and were between 1.7 and 2.3kg in weight at the time of experiment.

2.2.3 Lung Lavage

Animals were killed with sodium pentabarbitone, 100 mg/kg body weight (Euthatal, from May and Baker, Dagenham, England) injected into an ear vein. The rabbit was then placed on its back and the fur of the neck, thorax and abdomen soaked in 95% ethanol. The skin of the neck was opened by a vertical incision and the trachea exposed by blunt dissection. A silk stay suture was placed behind the trachea. A tracheotomy was formed with sterile scissors and the trachea intubated with a sterile 2.5mm polyvinylchloride tube. The silk suture was tied to secure the tubing which was connected to a sterile 50ml polypropylene syringe via two 3-way taps connected in series. Tubing led from the two side ports, so created, one to a 500ml bag of 0.9% saline for injection (Travenol, U.K.) and the other to the collecting vessels held in crushed ice. In the protein synthesis experiments sterile glass bottles whose interior surface had been treated with silicone (Repelcote, BDH Chemicals) were used.

Saline (35ml) was drawn into the syringe and then slowly injected into the lungs of the rabbit. The chest wall of the animal was gently massaged, the lavage fluid aspirated back into the syringe, and then transferred into the collecting vessel. This process was repeated to a total of three times.

2.2.4 Lavage Cell Preparation

Cells were separated from lavage fluid by centrifugation (200g, 10min). They were then washed by resuspension in

phosphate buffered saline (PBS) and recentrifugation. Some lavage returns were slightly contaminated with erythrocytes. If so the cells were resuspended in PBS, layered onto Ficoll/sodium metrizoate (d = 1.077, "Lymphoprep", Nyegaard Ltd., Norway) and centrifuged at 400g for 10 minutes. The nucleated cells were collected from the interface with a silicone treated pasteur pipette, and washed twice in PBS. An aliquot of the cell suspension was taken for cell differential count. The lavage macrophages were then either placed in incubation vessels as detailed below, or mixed with 5% trichloroacetic acid as appropriate.

Throughout this preparation stage the cells were maintained at 0-4°C. All manipulations were carried out in a laminar air flow hood to maintain sterility.

2.2.5 Cell Viability

The percentage of viable cells was determined by Trypan Blue dye exclusion (Phillips, 1973). Equal volumes of the cell suspension and 1% Trypan Blue in 0.15M NaCl were mixed and allowed to stand at room temperature for 10 minutes, then loaded into a haemocytometer for examination under a microscope. Two hundred cells were counted. The proportion of viable cells, i.e., those whose cytoplasm did not stain blue, was expressed as a percentage of the total.

2.2.6 Cell Counts

Cell counts were performed by mixing 100μl aliquots of cell suspension and stock 0.1% Crystal Violet in 0.15M saline. A drop of the mixture was transferred into a haemocytometer, and sufficient squares examined to count at least 200 cells. From this the cell concentration was calculated.

2.2.7 Cell Differential Count

The Shandon Cytocentrifuge system was used. 100μl aliquots of a 10^6 cells/ml cell suspension were pipetted into the cytocentrifuge cups, and the system operated at 300 rpm for 10 minutes. The slides were then air dried before staining. May-Grunewald-Giemsa staining was carried out by the haematology department of the Brompton Hospital, Fulham Rd, London. In addition slides were examined using the non specific esterase method, which stained the cytoplasm of macrophages (see below). Differential counts by microscopy were performed by counting all the cells wholely within a high power field before moving on to another part of the slide until at least 400 cells had been examined.

2.2.8 Non Specific Esterase Method

This method depends on the presence in the cytoplasm of cells of the monocyte/macrophage series, of a non specific esterase which converts a substrate α-napthyl butyrate into a coloured product which is deposited in the cells. Non specific esterase positive cells show an orange or rust brown cytoplasmic staining. Lymphocytes and neutrophils show at most a few granules of reaction product. Methyl Green is used as a nuclear counter stain.

The following solutions were prepared.

1. Formol and acetone buffer.

20mg of Na_2HPO_4 and 100mg of KH_2PO_4 were dissolved in 30ml of distilled water, to which was added 45ml acetone and 25ml formalin. This could be stored for up to three weeks at -40°C.

2. Non specific esterase stain.

A 4% pararosanaline/2M HCl solution was made by dissolving 2g pararosanaline hydrochloride by warming in 50ml 2M HCl.

This solution was mixed with an equal (50ml) volume of freshly prepared 4% sodium nitrite solution. The pH of the mixture was adjusted to 6.5 with 2M NaOH and filtered. The filtrate is referred to as hexazotised pararosanaline. Phosphate buffer was made by mixing, in approximately 5:1 proportions, 0.067M Na_2HPO_4 and 0.15M KH_2PO_4, with final pH adjustment to 7.6. 1,424ml of this phosphate buffer, 96ml of hexazotised pararosanaline and 800ml of a 2% solution of α-napthyl butyrate in ethylene glycol monomethyl ether, were mixed. The pH was adjusted to 6.1 and the solution left for fifteen minutes for the precipitate to form. After filtration a yellow solution is obtained which was aliquotted in 100ml amounts and stored at -70°C. This remains active for six to eight weeks. Once thawed an aliquot was discarded at the end of the day.

3. 1% Methyl Green in distilled water.

Slides were fixed using formol-acetone for thirty minutes at 4°C, before washing three times in distilled water and air drying. They were immersed in non specific esterase stain for one hour at 37°C, and again washed three times in water. Finally the counter stain was applied by immersion of the slides for four minutes in 1% Methyl Green, and washing three times in water. They were air dried and mounted. The percentage of non specific esterase positive cells was determined by a count of 400 cells per slide by light microscopy.

IN VIVO EXPERIMENTS

2.2.9 Injection of Animals

Animals in groups of four to six, were injected with L-[5-^{3}H]proline mixed with unlabelled L-proline (7mmol/kg body weight) dissolved in four ml sterile water at intervals of 30, 60, 90 and 120 minutes before they were killed. The

solution was injected into an ear vein over one minute, and the animals returned to their cages. The dose of [^{3}H]proline used (0.6-2.4mCi/kg) was highest for the 30 minute animals and lowest for the animals killed at 120 minutes ensuring that an adequate amount of radioactivity was incorporated into macrophage protein at all time points. The time of incorporation was taken from the start of the proline injection until the instillation of the first bolus of lavage saline. It was this measure which was used in calculation of synthesis rate, although in practice it was never more than six minutes adrift of the nominal time.

2.2.10 Separation of Samples for Assay of Specific Radioactivity

Macrophage Protein.

At the end of the washing steps detailed in section 2.2.4 the pellet of lavage cells was mixed with 5% (w/v) trichloroacetic acid (TCA). The resulting precipitate was washed three times in 5% TCA by centrifugation (600g, 5min) and then successively in acetone/12M HCl (400:1,v/v), ethanol/diethyl ether (2:1) and diethyl ether alone. The washed precipitate was dried and hydrolysed in one ml of 6M HCl (110°C, 16 hours) in sealed glass tubes.

Lavage fluid.

The lavage fluid was freeze dried and then redissolved in three ml of water before mixing with three ml 10% TCA. The TCA soluble fraction (containing free proline) was obtained by centrifugation (600g, 10 min), discarding the precipitated and sedimented protein.

Plasma.

Similarly the TCA soluble fraction of plasma was obtained by mixing one ml volumes of plasma and 10% TCA, and centrifuging down and discarding the precipitated protein.

2.2.11 Chloramine-T Assay of Proline Specific Radioactivity

Oxidation of proline by Chloramine-T gives a product, Δ^1-pyrroline, which is soluble in toluene (Peterkofsky and Prockop, 1962). Other amino acids and proline metabolites are not converted to a toluene extractable product. Therefore if the original sample contained radiolabelled proline, all the radioactive counts in a sample of the toluene extract are derived from [^{3}H]proline. By measuring chemically (using the ninhydrin assay) the quantity of Δ^1-pyrroline in another aliquot of the toluene extract the specific radioactivity can be calculated (with a simple correction for the volume of each aliquot).

2.2.12 Chloramine-T Reaction

The samples obtained were then assayed for proline specific radioactivity as follows:

(a) Samples were placed into labelled 20ml glass tubes fitted with screw tops.
(b) The pH was titrated with 1M and 0.1M potassium hydroxide to 8.3-8.9 using phenolphthalein as pH indicator.
(c) The volume of solution in the tubes was equalised with further water.
(d) Two ml of 1M potassium borate/boric acid buffer pH 8.7 was added.
(e) The tubes were vortexed, and then two ml of a 0.25M chloramine-T solution in 2-methoxyethanol was added to each tube, revortexed and left for 20 minutes at room temperature.
(f) The oxidation was stopped by adding six ml of 3.6M sodium thiosulphate solution.
(g) The mixture was saturated with KCl by adding approximately two grams of crystalline KCl to each tube (this improves the efficiency of extraction of the reaction product into toluene).
(h) 12ml of toluene was added to each tube. The tubes were

capped tightly and then put on a horizontal shaker for 10 minutes.

(i) The toluene containing the proline oxidation product Δ^1-pyrroline was aspirated and stored for subsequent assay.

2.2.13 Ninhydrin Reaction

Both proline and Δ^1-pyrroline react stoichiometrically with ninhydrin to produce an identical coloured reaction product, which could be measured photometrically. Aqueous solutions of proline were used to produce a standard curve of absorbance of the reaction product against molar concentration. Aliquots of the toluene extract were reacted similarly, the absorbance measured, and the concentration of Δ^1-pyrroline calculated.

(a) Standards of L-proline, 0.02-0.1μmol in 0.02μmol steps, were set up in one ml of aqueous solution, together with a tube containing one ml of water to act as a blank. The tubes used were screw-topped tissue culture glass tubes of 12ml volume.

(b) Aliquots of the toluene extract from the chloramine-T oxidation were pipetted into similar tubes. 0.2ml of the plasma samples were used while one ml of the lavage and macrophage protein samples were necessary because of the lower proline concentration.

(c) A solution of ninhydrin 2.5g in 60ml glacial acetic acid, 16ml of 15M phosphoric acid and 24ml H_2O was prepared. Three ml of this ninhydrin solution + three ml of glacial acetic acid were added to each reaction tube.

(d) The tubes were placed in a boiling water bath with gentle shaking for 60 minutes.

(e) The chromophore was extracted by shaking manually with additional toluene to a final toluene volume of three ml, and the tubes were allowed to cool, while the two liquid phases separated under gravity.

(f) Two ml of the toluene extract of the chromophore was

transferred to an additional tube. Occasionally clouding of the toluene solution was noted. This was attributed to further cooling of the toluene causing the small amounts of water which dissolved in the toluene layer at 100°C to separate from the organic phase. This was initially remedied by warming of the samples before placing them in the photometer. It was subsequently found that adding 50μl of ethanol to each tube kept the water in solution, and this modification was used thereafter.

(g) The absorbance (A515) of each toluene extract from the ninhydrin reaction tubes was measured on a Perkin-Elmer spectrophotometer at 515nm. The standard sample containing no proline constituted the blank needed to zero the spectrophotometer.

(h) A515 was plotted against proline content of standards on graph paper and the best straight line through these points and zero drawn by eye. The slope of this line is the conversion factor, C, from absorbance units measured to the quantity of proline present in sample.

2.2.14 Radioactive Counts in Toluene Extract

Aliquots, generally eight ml, of the toluene extract of the chloramine-T oxidation product were placed in glass scintillation vials, to which were added four ml of a 1.2% solution of 2,5-diphenyloxazole in toluene. The radioactivity for each vial was then measured in a Packard liquid scintillation counter (model 3380).

2.2.15 Calculation of Specific Radioactivity

Specific radioactivity was calculated from the following formula:-

$$\text{Specific radioactivity of proline} = \frac{R}{V_R} \times \frac{V_N}{A515 \times C}$$

where R = radioactivity in a counting vial
V_R = volume of toluene extract counted
V_N = volume of toluene extract in ninhydrin assay
A515 = measure absorbance in ninhydrin assay
C = conversion factor from (h) above

2.2.16 Calculation of the Protein Synthesis Rate

The information required for this calculation (see formula in Section 2.1.2) was now available. The precursor pool specific radioactivity was taken to be that of the free proline in the lavage fluid. The validity of this assumption will be discussed later.

MEASUREMENT OF PROTEIN SYNTHESIS IN VITRO

2.2.17 Cells

These were obtained as described previously using sterile equipment throughout. Alveolar macrophages (AMs) from three rabbits were pooled and a cell count performed and cytocentrifuge slide preparations obtained. Cell viability was assessed by Trypan Blue exclusion and was 97% prior to incubation, and 96% of a sample of cells from the control flasks at the end of the culture period.

2.2.18 Culture Conditions

AMs were incubated in five ml glass bijou bottles which had been cleaned, treated with silicone (Repelcote, BDH Chemicals) to inhibit adherence of the cells and then sterilized in an autoclave. The bottles were fitted with loose fitting caps to allow gas exchange, and maintained at 37°C in a water bath in an atmosphere of air/5% CO_2. Each culture contained 1.6×10^6 cells in 700μl of Krebs-Henselheit bicarbonate buffer with the following additions:-

glucose 10mM; benzylpenicillin 100u/ml; normal rabbit plasma concentrations of 18 amino acids (Block and Hubbard, 1962); phenylalanine 345μM (this is five times the physiological concentration) with L-[4-^{3}H]phenylalanine to a specific radioactivity of 100Ci/mol; L-[5-^{3}H]proline to a specific radioactivity of 100Ci/mol and various proline concentrations (detailed below).

In order to explore the effect of varying concentrations of proline and protein in the culture medium on the measured protein synthesis rate, cultures were set up containing 100μM, 1500μM and 4000μM proline, and in addition, cultures containing 1500μM proline with 6g/l and 20g/l bovine serum albumen (Fraction V, Sigma, catalogue no. A3350). Each set of cultures was set up in quadruplicate flasks.

Prior to starting the incubation, the culture bottles were placed in the water bath with the appropriate culture medium in each. The AMs were centrifuged, resuspended in buffer and aliquotted into the flasks to start the incubation. The flasks were shaken at 80 strokes/min to keep them in suspension.

After 126 minutes the flasks were removed and plunged into crushed ice to stop cellular metabolism. The cell suspension was transferred to centrifuge tubes in crushed ice, the original culture bottles rinsed with one ml ice cold phosphate buffered saline (PBS) into the centrifuge tubes, and a further ten ml of cold PBS added. Thereafter the cell processing was as described for *in vivo* experiments apart from a reduction in scale of equipment appropriate to the smaller numbers of cells involved in each sample.

2.2.19 Measurement of Specific Radioactivity of Proline and Phenylalanine by the Dansyl Chloride Double Isotope Method

The method of Airhart et al (1981) was followed. [^{14}C]dansyl chloride of known specific radioactivity is reacted with the sample containing [^{3}H]amino acids. The resulting dansyl amino acids can readily be separated by two

dimensional thin layer chromatography on polyamide coated plastic sheets. The position of these nanomolar quantities of dansylated amino acid can be identified by examining the sheets under ultra-violet light, when the dansyl residues fluoresce. By cutting out the appropriate "spot" on the polyamide sheets, a pure dansyl amino acid is obtained. The dansyl ligand and the amino acid must be present in equimolar amounts (because amino acid and dansyl chloride react stoichiometrically). Therefore the ratio of the ^{14}C and the ^{3}H counts measured for the "spot" will be in the ratio of the specific radioactivities of the [^{14}C]dansyl chloride and the [^{3}H]amino acid. As the former is known the latter can be calculated.

The elegance of the method lies in its technical simplicity, its great sensitivity to small amounts of amino acid (providing the specific radioactivity is sufficiently high) and its ability to measure more than one [^{3}H]amino acid in a mixture (potentially all 20 amino acids could be quantitated in a single assay). It does however require careful assay design to ensure adequate amounts of radioactivity are present in the dansyl amino acid spot and that the number of ^{14}C counts does not greatly exceed the ^{3}H counts, which make the ^{3}H count inaccurate (because of overlap of the energy spectra of their β emissions).

2.2.20 Reagents and Materials for Dansyl Chloride Assay

(a) A solution of unlabelled dansyl chloride in acetone (Sigma UK) was diluted as necessary with acetone. Final dansyl chloride concentrations were in the range 2-6 $\mu g/\mu l$. This solution could be kept at 4°C in a tightly stoppered glass container wrapped in aluminium foil to exclude light. Dansyl chloride will rapidly hydrolyse to the hydroxide when exposed to water and therefore care was needed to minimise this. The stock bottle was allowed to reach room temperature before opening to prevent condensation of water inside the flask.

(b) [^{14}C]dansyl chloride dissolved in benzene, specific radioactivity 37.8Ci/mol was obtained from Amersham International (catalogue no. 52). To make up [^{14}C]dansyl chloride solution of the appropriate specific radioactivity and concentration, an aliquot of the stock [^{14}C]dansyl chloride was transferred to a clean tube, the benzene evaporated in a stream of dry nitrogen before adding the appropriate volume of cold dansyl chloride. For this experiment two sets of [^{14}C]dansyl chloride were required to match the different [^{3}H]amino acid specific radioactivities to be measured.

(c) [^{3}H]amino acid standards were made up from [^{3}H]phenylalanine and stock non radiolabelled ("cold") phenylalanine solutions of precisely known molarity. In principle, either [^{3}H]dansyl chloride or [^{3}H]amino acid solutions could be used as calibration standards for the rest of the assay. In practice however the uncertain purity (due to hydrolysis) of cold dansyl chloride, and the problems of pipetting microlitre amounts of [^{14}C]dansyl chloride in organic solvents, prevented the preparation of [^{14}C]dansyl standards of precisely known SR.

(d) 10 x 50mm soda glass tubes were used for the dansylation reaction. The tubes were soaked in a 5% solution of Decon to eliminate traces of grease and contaminating amino acids, and repeatedly rinsed in distilled water. Thereafter the tubes were handled with forceps (the method is sensitive enough to detect the amino acids present in the fingerprints of a careless handler). The tubes were siliconised (Repelcote, BDH Chemicals) and again rinsed carefully before drying and storage in a dust free container. Silicone treatment of the tube ensured that the one to ten microlitre amounts of reagents pipetted into the tops of the tubes did not wet the glass, but remained as a globule which with gentle tapping of the tube would roll down the inside of the tube to mix completely with the other reagents at the bottom of the tube.

2.2.21 The Dansyl Chloride Assay

(a) Prior to dansylation all samples and standards were brought into aqueous solution pH 9.8. For samples of culture medium, amino acid mixtures, standards and plasma (after TCA precipitation as described previously), 10μl aliquots were placed in the reaction tubes and the pH adjusted by adding 1μl aliquots of 1M sodium bicarbonate buffer pH 9.8. The pH was checked by spotting the sample on to pH paper. The protein hydrolysate had a lower concentration of amino acid, dissolved in 6M HCl. To remove the acid and concentrate the specimen, 50μl aliquots of the hydrolysate were placed in reaction tubes, evaporated to dryness and redissolved in 10μl 0.1M sodium bicarbonate buffer pH 9.8.

(b) To each reaction tube (samples and standards) was added 10μl [^{14}C]dansyl chloride solution in acetone. The tubes were tapped to ensure mixing and covered in Parafilm to minimise evaporation. They were kept at 37°C for 30 minutes to allow the dansylation reaction to take place.

(c) Each reaction mixture was spotted onto separate 7.5cm square polyamide TLC plate (BDH Chemicals). To do this, reaction mixture was picked up in a 10μl Drummond microcapillary tube (Sigma UK, No P1924) which was then lightly touched onto a pencil mark two cm in from a corner of the plate. Some of the reaction mixture would transfer onto the plate spreading out from the pencil mark. After a minute or so, when the spot was nearly dry, the process was repeated, building up the sample without allowing it to spread beyond a radius of 2mm from the pencil mark.

(d) The plates were placed on edge in sealed chromatograph tanks containing a five mm depth of the first solvent (2% formic acid/98% H_2O) in a fume cupboard. When the solvent front had reached the top of the TLC sheets, they were removed and allowed to dry completely in a fume cupboard before chromatography in the second solvent (90% benzene/10% glacial acetic acid) in the second dimension at right-angles

to the first. At this stage the plates were removed when the solvent front was one cm short of the top edge. The plates were again dried.

(e) The "spots" of dansyl amino acid on the chromatography plate were identified under ultra-violet light and ringed with a soft pencil. The position of dansyl proline and dansyl phenylalanine spots were identifiable from previously published maps of all the dansyl amino acids, and from a plate run with larger amounts of proline and phenylalanine than other amino acids to make the former stand out. The separation of the dansyl proline and phenylalanine from the other spots was consistently good. If a dansyl proline or phenylalanine spot was not apparent on plates run from reagent blanks, the predicted positions of the spots were pencilled.

(f) The relevant spots were cut out of the plates using scissors, and placed in glass scintilation vials. To these were added 0.5ml "NCS" tissue solubiliser (Amersham International), and after 60 minutes 18μl of glacial acetic acid and 10ml 0.4% 2,5-diphenyloxazole in toluene. The vials were capped and the ^{3}H and ^{14}C radioactivity measured in a liquid scintillation counter equipped with double isotope counting and external standardisation facilities (Packard, Model 3380).

2.2.22 Calculation of Specific Radioactivity of Amino Acid

The reagent blank counts were used as the background to be subtracted from the sample and standard counts. For ^{3}H (proline and phenylalanine) these normally between 52 and 100dpm. For ^{14}C the reagent blanks were 18-30 dpm. For each spot two counts were available - the net ^{3}H count, derived from amino acid, and the net ^{14}C count derived from the dansyl ligand. As explained in Section 2.2.19 the observed ratio of ^{3}H:^{14}C counts was equal to the ratio of SRs of amino acid and dansylchloride. This ratio was calculated for each spot. Knowing the SR of the amino acid standard, the SR of the

[^{14}C]dansyl chloride solutions used could be calculated, and then in turn those of [^{3}H]amino acid in the samples.

2.2.23 Calculation of Protein Synthesis Rate

This was calculated from the formula detailed above (section 2.1.2). The SR of the amino acid in the culture medium was measured and used as the SR of the precursor pool. The SR of the amino acid in protein had been measured and calculated as described above.

2.2.24 Analysis of Results

Comparisons between the means of two groups of observations were performed using Student's unpaired t test. One way analysis of variance was used to assess whether the differences noted between several groups of observations were significant or might have arisen due to sampling error. Computation was performed on an electronic calculator using techniques from Armitage and Berry (1985).

2.3 RESULTS

2.3.1 Comparison of the Chemical and Dansyl Chloride Assays of [^{3}H]Proline Specific Radioactivity

The chloramine-T chemical assay had been validated in this laboratory by comparison with amino acid analysis using an LKB automatic analyser (Laurent et al, 1981). However the dansyl chloride method had not previously been validated in this laboratory. Plasma from three rabbits which had been injected with [^{3}H]proline of differing SR as described earlier were subject to assay by the chloramine-T method and later by the dansyl chloride method (one sample in

triplicate).

By the chemical method the three samples were found to have SRs of 306, 434 and 908 x 10^3 dpm/μmol, while by the dansyl chloride assay corresponding values were 301, 446 and 980 x 10^3 dpm/μmol respectively. The last figure is a mean of triplicate estimations viz 972, 979 and 988 x 10^3 dpm/μmol. There was good agreement between the two assays and the reproducibility of the dansyl chloride triplicate results was striking. This reproducibility is also shown in the results of dansyl chloride assays described later.

EXPERIMENTS IN VIVO

2.3.2 Total Cell Yields for Animals Injected *in Vivo* with [^{3}H]Proline

The total cell yield for animals injected *in vivo* ranged between 8.8 x 10^6 and 35.2 x 10^6 nucleated cells. The mean yield +/-SD was 17.2 +/-7.51 x 10^6 (16 animals). The cell yield from the control animals lavaged in the same experiments but not injected, was 14.9 +/-9.9 x 10^6 (mean +/-SD, 4 animals).

2.3.3 Differential Cell Counts for Animals Used in the Experiments *in Vivo*

Macrophages were much the most abundant cell. For the animals injected *in vivo*, staining by May-Grunewald-Giemsa indicated that macrophages constituted a mean of 99.16% of the total (range 96-100%), with lymphocytes 0.34% (range 0-1.5%), polymorphs 0.41% (range 0-2.5%), basophils 0.03% (0.5% of the count in a single animal), and epithelial cells 0.06% (0.5% of the count in two animals). Nonspecific esterase (NSE) staining supported this finding, with NSE positive cells being a mean of 99.4% (range 98-100%) of the total.

For the four animals used as uninjected controls the

picture was similar:- by May-Grunewald-Giemsa staining macrophages were a mean of 98.9% of the total, and 99.1% of the cells were NSE positive.

2.3.4 Measurement of Alveolar Macrophage Protein Synthesis Rate *in Vivo*

Table 2.1 displays the calculated synthesis rates of AM protein for each animal, together with the data used in this calculation.

It can be seen that the measured time of incorporation generally differs from the nominal time (30, 60, 90 or 120 min). These deviations were not large and were due to varying times needed to complete the process of injection of barbiturate, dissection of the neck and intubation of the trachea.

The mean +/-SEM of the synthesis rate for each group of animals were at 30 min: 6.9 +/-1.35 %/day; 60 min: 7.7 +/-1.59 %/day; 90 min: 10.9 +/-2.40 %/day; and 120 min: 9.3 +/-1.08 %/day.

2.3.5 Passage of Proline from Plasma into Alveolar Fluid

The validity of the experimental method used to measure AM protein synthesis rate *in vivo* depends upon rapid passage of proline from plasma into alveolar fluid. Alveolar fluid was sampled by the process of bronchoalveolar lavage. Table 2.2 details data on plasma and lavage fluid relevant to this issue. It can be seen that the mean quantity of proline recovered from the lavage fluid of the 30 min animals was 1.8 μmol. This contrasts with the situation in the control animals which were not injected with proline. In all of the control animals the amount of proline in the lavage fluid was below the limit of detection of the ninhydrin assay, which corresponds to a total content of less than 40 nmol. Thus, within 30 min of injection, the alveolar fluid proline concentration must have risen by more than 45 fold.

TABLE 2.1 **Measurement of Alveolar Macrophage Protein Synthesis *in Vivo***

Animal	Observed Time	Specific Radioactivity			Synthesis Rate
		Plasma	Lavage Fluid	AM protein	
Units	min	x 10^3 dpm/μmol		dpm/μmol	%/day
30 min					
R48	33	938	569	516	2.9
R 2	35	882	854	1,503	8.1
R 7	36	716	594	1,468	8.6
R 9	29.5	716	653	1,136	8.1
60 min					
R49	63	427	374	892	5.3
R 1	61	356	298	1,325	9.1
R 4	62	412	478	930	5.6
R10	57	334	266	863	6.4
90 min					
R51	91	306	214	1,277	7.9
R57	93	293	276	2,122	12.9
R 3	89	236	205	690	4.9
R 8	90	223	209	917	15.5
120 min					
R53	120	193	180	1,443	9.0
R55	120	192	208	1,174	7.4
R56	119.5	225	235	1,697	10.6
R 6	122	194	157	1,599	9.9

The measured time of incorporation generally differs from the nominal time. These deviations were due to varying times needed to complete the process of injection of barbiturate, dissection of the neck and intubation of the trachea.

The injected [^{3}H]proline was of highest SR in the 30 min animals and lowest in the 120 min animals to compensate for the varying duration of incorporation of the tracer into the AM protein, and therefore to achieve similar amounts of radioactivity in the protein.

Note that the units of SR for plasma and lavage fluid samples are "x 10^3 dpm/μmol", e.g. the SR of plasma from R48 was 938 x 10^3 dpm/μmol. This is the convention used throughout this thesis.

Although not used in the calculation of protein synthesis rate, the SR of proline in plasma was measured for each animal and is included in Table 2.1. This permits comparison of SRs of proline in plasma and lavage fluid which should be similar if there was rapid exchange of proline between plasma and alveolar fluid.

This relationship is best expressed as a ratio of the SRs of lavage fluid and plasma, detailed in Table 2.2. One-way analysis of variance indicates that there is no significant difference between the ratios observed for each group (F ratio 0.625, P>>0.05). This indicates rapid establishment of equilibrium.

2.3.6 Time Course of Incorporation of [^{3}H]Proline into Alveolar Macrophage Protein

To allow assessment of the time course of incorporation [^{3}H]proline into alveolar macrophage protein, correction needs to be made for different SRs of the injected proline solutions. These were higher for animals at the early time points to compensate for the shorter time of incorporation of radiolabelled amino acid into AM protein.

Figure 2.1 shows the time course of [^{3}H]proline incorporation into AM protein. There is some scatter of the points but the data fit best with a linear model of incorporation corresponding to the line drawn in Figure 2.1 by least squares regression analysis. The intercept on the time axis is 13min.

TABLE 2.2 Relationship between Specific Radioactivity of Proline in Plasma and Lavage Fluid, and the Amount of Proline Obtained in the Lavage Fluid

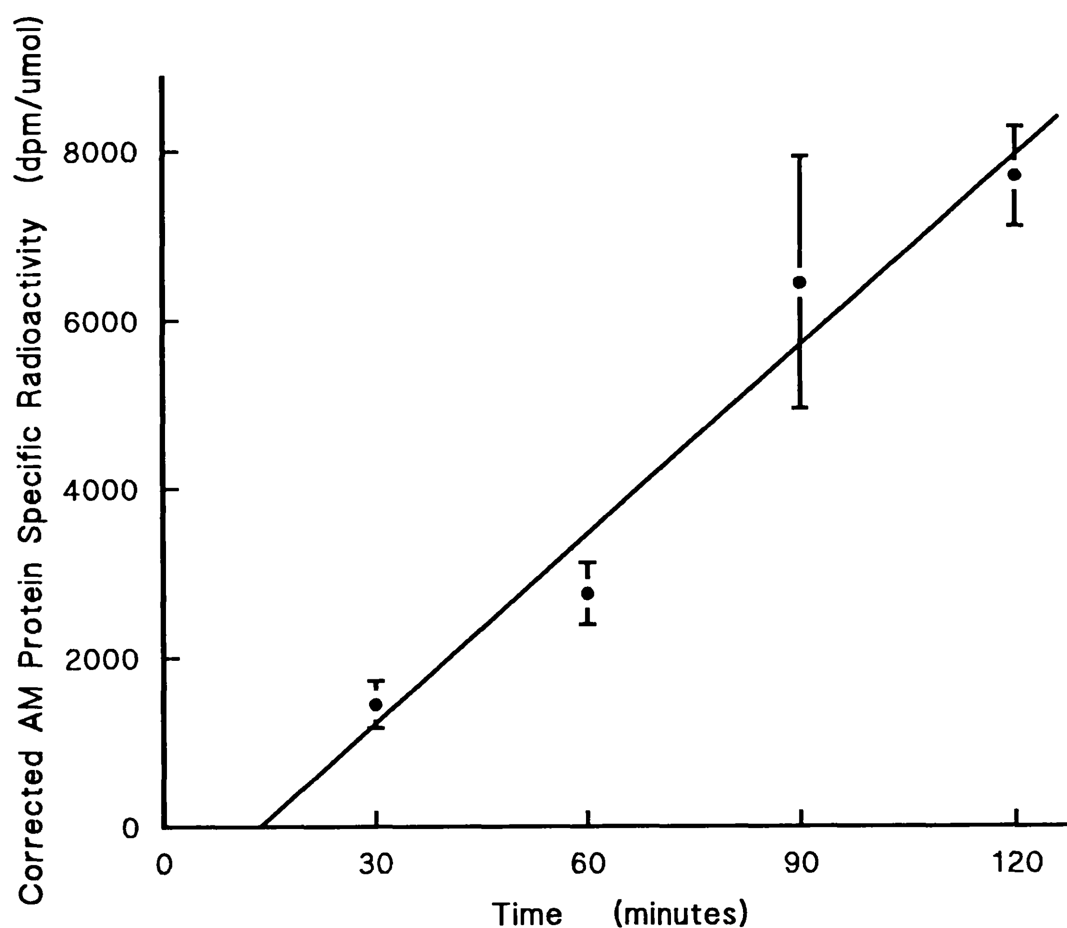
	Ratio of SR of Proline LF/Plasma (+/- SEM)	Yield of Proline in Lavage Fluid (μmol)
30 minutes		
R48	0.61	0.58
R2	0.97	3.56
R7	0.83	1.22
R9	0.91	1.84
Mean	0.84 +/-0.08	1.80
60 minutes		
R49	0.88	0.68
R4	1.16	9.89
R1	0.84	0.48
R10	0.80	0.28
Mean	0.92 +/-0.08	2.84
90 minutes		
R51	0.70	0.36
R57	0.94	0.28
R3	0.87	0.18
R8	0.94	0.36
Mean	0.86 +/-0.06	0.30
120 minutes		
R53	0.93	0.58
R55	1.08	1.18
R56	1.04	1.16
R6	0.81	0.24
Mean	0.97 +/-0.06	0.79

The ratios detailed in column 2 are derived from the data in columns 3 and 4 of Table 2.1. Values for each animal and the average ratio for each group of animals are tabulated.

The values of the yield of proline in lavage fluid assumes 50% extraction of Δ^1-pyrroline into the toluene phase during the chloramine-T assay. This figure derives from experience with the assay in this laboratory.

See Section 2.3.5 for discussion of this data.

Figure 2.1 **Time Course of Incorporation *in Vivo* of [^{3}H]Proline into Alveolar Macrophage Protein.**



The specific radioactivy of macrophage-protein proline has been normalised to an infused proline specific radioactivity of 10^{6} dpm/μmol. Error bars represent +/-SEM for groups of four animals, and the line was drawn by least-squares linear regression analysis.

EXPERIMENTS IN VITRO

2.3.7 Yield and Differential Cell Count of Lavage Cells

Lavage cells from three rabbits were pooled for the measurements *in vitro*. The mean cell yield was 12.7 x 10^6 cells. May-Grunewald-Giemsa staining indicated that 99.5% of these were macrophages, and this was supported by the finding that 99.75% of 400 cells were positive by non specific esterase staining. Viability was 97% before culture *in vitro* and 96% afterwards as judged by Trypan Blue exclusion.

2.3.8 Incorporation of [^{3}H]Proline and [^{3}H]Phenylalanine into AM Protein *in Vitro*

Table 2.3 details the results of the measured SR of [^{3}H]proline and [^{3}H]phenylalanine incorporated into AM cell protein for each individual culture of AMs.

It can be seen that incorporation of [^{3}H]proline into AM protein rises when the proline concentration in the medium is raised from 100μM to 1,500μM, but does not change again when the latter is increased to 4,000μM.

In contrast [^{3}H]phenylalanine incorporation into protein is not significantly affected by changing the proline concentration over this range (one way analysis of variance of the SR of phenylalanine in protein at 100μM, 1,500μM and 4,000μM; $P>0.05$). The trend is slightly downwards however, so that high proline concentrations may have a small effect on protein synthesis as measured by [^{3}H]phenylalanine incorporation.

The inclusion of protein, in the form of bovine serum albumin at a concentration of 6g/l, did not affect incorporation of [^{3}H]proline into protein, but at a higher concentration of 20g/l, bovine serum albumin did cause a significant fall in [^{3}H]proline incorporation ($0.01<P<0.02$, Student's t test comparing mean incorporation at 0 and 20 g/l albumin at 1500μM proline).

TABLE 2.3 **Alveolar Macrophage Protein Synthesis *in Vitro***

[proline] in medium	[albumin] in medium	SR of amino acid in AM protein	
		[^{3}H]proline	[^{3}H]phenylalanine
μM	g/l	dpm/nmol	dpm/nmol
100	0	3,758	9,479
		3,573	8,571
		3,575	8,961
		3,557	8,978
	Means +/-SEM	3,616 +/- 48	8,997 +/-186
1,500	0	8,018	8,873
		8,102	8,813
		8,626	8,547
		8,119	8,953
	Means +/-SEM	8,216 +/-138	8,797 +/- 88
4,000	0	8,523	8,656
		8,116	8,035
		8,358	8,464
		8,447	8,622
	Means +/-SEM	8,361 +/- 89	8,444 +/-143
1,500	6	8,261	9,133
		8,506	9,306
		8,701	9,071
		8,437	9,054
	Means +/-SEM	8,476 +/- 91	9,141 +/- 58
1,500	20	8,523	8,656
		8,116	8,035
		8,358	8,464
		8,447	8,622
	Means +/-SEM	7,594 +/-105	8,859 +/-166

These results are discussed in Section 2.3.8.

No similar effect on [^{3}H]phenylalanine incorporation was seen when album n was included in the medium, even at the higher concentration.

2.3.9 AM Protein Synthesis Rate *in Vitro*

The SR of the [^{3}H]proline and [^{3}H]phenylalanine in the culture medium of the flasks were measured in duplicate. For proline the values were 198,100 and 190,700 dpm/nmol (mean 193,900), and for phenylalanine 226,500 and 231,800 dpm/nmol (mean 229,400). From this, and the time of incorporation (126 min), the protein synthesis rate can be calculated, as described in Section 2.1.2.

For AMs cultured in a proline concentration of 4mM, and in the absence of albumen in the medium, the protein synthesis rate measured using [^{3}H]proline as the marker amino acid, was 48.9 +/-0.5 %/day (SEM, n=4). A similar calculation can be performed using [^{3}H]phenylalanine - the corresponding rate was 41.9 +/-0.9 %/day.

2.4 DISCUSSION

A fivefold higher rate of protein synthesis was seen for alveolar macrophages *in vitro* than *in vivo*. This suggests that AMs may be activated by the steps involved in their isolation for study *in vitro*. However the validity of this conclusion depends upon the accuracy of the determined rates and thus on the assumptions implicit in their calculation. The experimental basis for these assumptions will be discussed fully, as this work has been the first to measure *in vivo* the protein synthesis rate of alveolar macrophages (indeed of any leucocyte).

2.4.1 Assumptions Implicit in the Calculation of Protein Synthesis Rate *in Vivo*

The assumptions are:-

(1) that the specific radioactivity (SR) of proline in plasma is constant during the period of observation.

(2) that equilibration between the plasma and alveolar fluid, and hence into the AM is rapid, achieving constant SR of proline in these pools.

(3) that high proline concentrations do not affect protein synthesis.

(4) that it is reasonable to equate the proline SR in alveolar fluid with that in the precursor pool for AM protein synthesis, i.e. prolyl-RNA in the AM.

2.4.2 Constancy of Specific Radioactivity of [^{3}H]proline in Plasma

The use of a large "flooding" dose of injected proline ensures that assumption (1) holds. This method has been validated in previous studies of protein synthesis in various tissues in rabbits, including lungs (Laurent, 1982; Laurent and McAnulty, 1983). The method relies on the achievement of high proline concentrations in plasma and in tissues for the duration of the experiment, by the injection of large molar amount of radiolabelled proline. The diluting effect of endogeneously produced (and therefore unlabelled) proline is therefore minimal, until the proline concentration in plasma and tissue has fallen to near the normal plasma concentration, more than 180 minutes after injection.

2.4.3 Rapid Equilibration of Proline between Plasma, Alveolar Fluid and Alveolar Macrophage

Three lines of evidence show that assumption (2) is valid. The results describes in Section 2.3.5 demonstrated that within 30 minutes of injection of the radiolabelled proline,

the amount of proline in the lavage fluid had risen by more than 45 fold compared with uninjected controls. By the same token, the ratio of lavage fluid and plasma proline specific radioactivities had equilibrated within 30 minutes. It was not possible to measure the true proline concentration in alveolar fluid because the lavage saline diluted an unknown volume of alveolar fluid in each animal. Secondly, comparison of the specific radioactivities of proline in plasma and lavage fluid at the different time points shows that the labelled proline must have passed rapidly into alveolar fluid (Section 2.3.5). Finally the linear incorporation with time of [^{3}H]proline into AM protein (see Figure 2.1) indicated a constant SR of the precursor pool in the AM.

2.4.4 The Effect of High Concentrations of Proline on Protein Synthesis

This issue (assumption (3) above) was addressed by the experiments performed *in vitro*. The inclusion in the medium of [^{3}H]phenylalanine at constant concentration and SR, provided a marker of AM protein synthesis rate independent of proline. There was a trend to lower [^{3}H]phenylalanine SR in AM protein when the medium proline concentration was raised from 100μM to 4,000μM but the magnitude of the change was small (a 6% fall) and did not attain statistical significance. Thus proline concentrations up to 4,000μM do not have a significant effect on protein synthesis rates of alveolar macrophages.

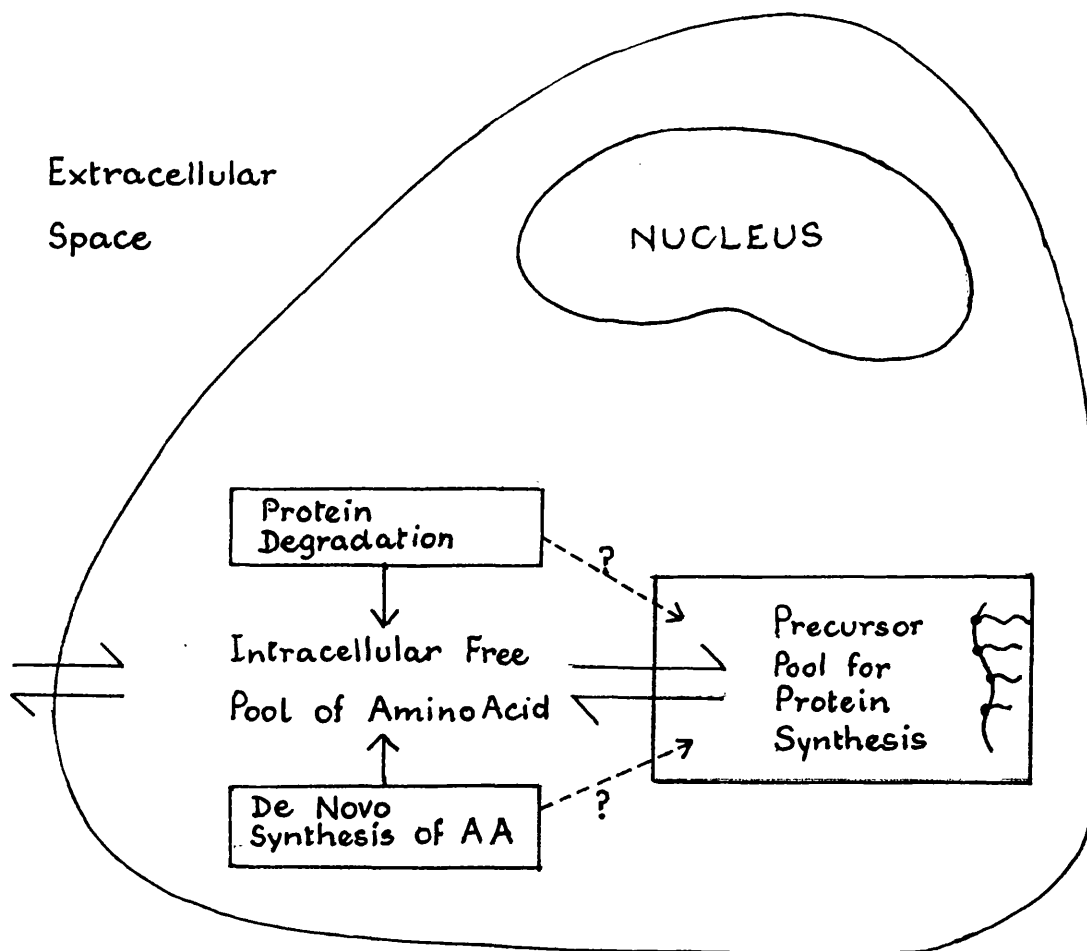
This result is in accord with the observations of Hildebran et al (1981) who found that changes in proline concentration upto 5,000μM did not affect total protein synthesis rates of human lung fibroblasts in culture, and with those of Baich et al (1980) who studied collagen synthesis rates in chick and mouse fibroblasts and human epithelial cells.

2.4.5 Amino Acid Precursor Pools *in Vivo* and *in Vitro*

In this work the calculation of the protein synthesis rate both *in vivo* and *in vitro* assumed that the SR of proline in the precursor pool for protein synthesis was equal to the SR of extracellular proline (assumption (4) above). The true precursor pool for protein synthesis is aminoacyl-tRNA. In an experiment to measure protein synthesis rate using radiolabelled proline, the pool of prolyl-tRNA receives contributions from unlabelled amino acid derived from within the cell, both by degradation of polypeptides and by endogeneous synthesis, as well as from the extracellular radiolabelled proline (see Figure 2.2). As extracellular labelled proline concentration is increased above physiological, so the extracellular amino acid contributes an increasing proportion of the precursor pool, and therefore the SR of prolyl-tRNA rises. At high extracellular proline concentrations, prolyl-tRNA SR plateaus, so that further increases in the former does not alter the latter. This effect was demonstrated in the results in Table 2.3. Although prolyl-tRNA SR does reach a plateau at high extracellular concentrations *in vitro*, it still may be less than the SR of the extracellular proline, i.e. there is an intracellular source of unlabelled proline for the tRNA pool, which is not swamped by flooding doses of extracellular proline. The effect of this will be that protein synthesis rates will be underestimated if extracellular or intracellular free proline SR is used in the calculation. Ideally the SR of prolyl-tRNA should be known.

Unfortunately, direct measurements of the prolyl-tRNA SR of AMs *in vivo* is precluded both by the rapid turnover of the tRNA pool (a few seconds) when compared with the time required to isolate and process the AMs, and by the difficulty in achieving in the intact animal a sufficiently high SR of radiolabelled proline to permit measurement of the SR of prolyl-tRNA in the picomolar amounts of tRNA which would be isolated from alveolar macrophages.

Figure 2.2 **Schematic Diagram of Fluxes of Amino Acid within the Cell Cytoplasm**



Degradation of protein and *de novo* synthesis are sources of amino acid for the intracellular pool in addition to amino acid from outside the cell. These intracellular sources may provide amino acid direct to the precursor pool of protein synthesis as well as via the general intracellular pool.

This appears to be a greater problem *in vitro* than *in vivo*. Hildebran et al (1981) studying lung fibroblasts *in vitro*, and Airhart et al (1981) working with guinea pig AMs *in vitro*, showed that aminoacyl-tRNA SR was lower than extracellular amino acid SR. On the other hand, studies carried out *in vivo* on heart (Everett et al, 1981), on perfused lung (Watkins and Rannels, 1980) and perfused liver (Khairallah and Mortimore, 1976) have indicated that when flooding doses of amino acid are used, the SRs of extracellular amino acid and aminoacyl-tRNA became equal. Using the present protocol of [^{3}H]proline administration, Laurent (1982) showed that the SR of skin procollagen (which has a half-life of less than 30 min) equalled that of the tissue free pool. This implies that the prolyl-tRNA of skin fibroblasts under these conditions also achieved a SR equal to that of the tissue free pool.

The evidence from the literature, therefore, suggests that prolyl-tRNA and tissue free pool proline SRs are equal *in vivo*, so that calculations of protein synthesis rate based on the tissue free pool value will be accurate. On the other hand a similar calculation from data obtained *in vitro* will lead to an underestimate of the protein synthesis rate, as the prolyl-tRNA SR is lower than the tissue free pool SR. This indicates that the much higher rate of AM protein synthesis we have seen *in vitro* than *in vivo* is not attributable to this technical problem - on the contrary the true discrepancy may be even larger than the fivefold difference seen.

CHAPTER 3

THE DEVELOPMENT OF THE METHYLENE BLUE ASSAY AND ITS APPLICATION TO THE BIOLOGICAL ASSAY OF GROWTH FACTORS

3.1 INTRODUCTION

3.1.1 Fibroblast Proliferation Assays *in Vitro*

The detection of growth factors depends on the demonstration of their effect on the growth of a suitable target cell. In this work the target cell was the fibroblast, which, for reasons of economies of scale and handling, were grown in 96 well microculture plates. The surface area of the bottom of a microwell is 0.28 cm^2, so that the number of cells present in a confluent fibroblast monolayer is only 5-10 x 10^4. This presents problems in the quantitation of such small numbers of cells. The most commonly used techniques to measure the growth of fibroblasts in microwell culture have employed [^{3}H]thymidine, and make no attempt to quantitate the number of cells present. Rather, they detect those cells which have replicated (and therefore synthesized DNA) since the addition of the [^{3}H]thymidine to the microculture. These techniques are either laborious (those which use autoradiography to identify individual newly replicated cells), or insensitive to low rates of replication (those which measure total uptake of radioisotope into the culture).

It would be preferable to have an assay which does measure cell number or some parameter proportional to it. This would allow the plotting of growth curves of cultures and avoid the problems of interpreting [^{3}H]thymidine uptake. Direct cell

counts of microwell cultures would be impossibly laborious and biochemical assays of the amount of protein or DNA in the cell layer would also be too time-consuming to be practical. The use of a cell stain offers the possibility of an *in situ* assay using a microwell plate photometer.

3.1.2 The Use of Methylene Blue in Assays of Cell Number

Stains such as Giemsa and Crystal Violet have long been used to visualise cells in monolayers in tissue culture. Attempts have been made to extend this to a semiquantitative measure of cell number (Fisch and Gifford, 1983; Hibbs et al,1977; Mirabelli et al, 1985). In none of these assays has there been a close and proportional relationship between absorbance and cell number. Lagneau et al (1977) used Methylene Blue to stain fibroblasts in microwell culture, which could then be eluted from the cell layer by lowering the pH and quantitated accurately in a photometer. The first description of this method was in the French literature (Lagneau et al, 1977) but no validation of the accuracy or reproducibility of the assay was given. It was subsequently briefly described in a paper in the English literature (Martin et al, 1978). Currie (1981) described a modification of this assay where the dye was left *in situ* after elution with a small volume of solvent, and the optical density measured using a microwell plate photometer. The validation of this assay was not published.

This assay potentially provided a rapid and convenient method of quantifying the numbers of adherent cells in microwell cultures. However, when the accuracy of the method was checked (see section 3.3.1) it was found that the relationship between absorbance and cell number was not linear. A modification of the assay to overcome this problem and details of accuracy, reproducibility and robustness of the assay are described in this chapter.

3.1.3 The Assay of Growth Factor Activity

Growth factors (GF) are at present detected and assayed by their influence on the growth of a target cell line growing in tissue culture. Even for the GFs which have been well characterised (e.g., platelet derived growth factor, epidermal growth factor) assays based on physicochemical or immunological techniques are only just becoming available.

The major disadvantage of biological assay is the effect of varying assay conditions upon the final result obtained. Parameters which needed to be fixed in this work included the target cell line used, the system for detecting increased replication of the target cell, the culture conditions and duration of exposure of target cell culture to the growth factors. The cells used in most of the experiments described in this thesis were Rat1 cells, and the reasons for this decision will be returned to in chapter 5. The choice of the Methylene Blue assay of measuring cell numbers in microwell plates has already been alluded to. The reasons for the choice of culture conditions will be presented in this chapter.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

1. Tissue Culture Medium.

Dulbeccos' modification of Eagle's medium (DMEM) was used throughout for the incubation of lavage cells and culture of the various fibroblast lines. This medium was provided by the central supplies department of the Chester Beatty Laboratories of the Institute of Cancer Research (Fulham Rd, London SW3). It was used within fourteen days of preparation, before any significant deterioration of the

components e.g., L-glutamine, of the medium would have occurred. The medium contained 100u/ml of benzylpenicillin and 100μg/ml of streptomycin. Cultures were incubated at 37°C in an atmosphere of 90% air/10% CO_2. Buffering was provided by the presence of 44mM sodium bicarbonate in equilibrium with the 10% CO_2 atmosphere. Calcium and magnesium free DMEM was used for the initial preparation of lavage cells to reduce cell to cell, and cell to vessel wall adherence.

2. Serum.

Newborn calf serum (NCS) was added to the culture medium as appropriate. The serum was obtained from Gibco Bio-cult, and was heat inactivated at 56°C for thirty minutes, aliquotted in 20 and 50 ml amounts and stored at -20°C. Care was taken to ensure that serum from a single batch was used throughout a set of experiments.

3. Other tissue culture materials.

Sterile liquid reagents such as trypsin mixtures and phosphate buffered saline were all supplied by the Chester Beatty Laboratories of the ICR.

4. Miscellaneous.

Chemicals were all of Analytical Reagent grade. Sterile disposable plastic equipment was obtained from Sterilin UK, unless otherwise indicated.

3.2.2 Cell Lines

1. Rat1.

This cell line was obtained from Dr. Margaret Knowles of the Marie Curie Research Institute, Oxted, Surrey. Rat1 cells are a fibroblast line derived from rat foetal lung, using a 3T3-like protocol (Freeman et al, 1973). The cells replicate rapidly, are robust in culture and are sensitive to exogeneous growth factors (Kaplan and Ozanne, 1983). They

were used in the majority of assays of growth factor activity.

2. IMR90.

This was obtained from the National Institute of Biological Standards, London. Their stock was derived from a sample supplied by the American Type Culture Collection (ATCC), Number CCL 186. IMR90 cells are a well differentiated human foetal lung fibroblast line.

3. Chang Cell line.

This was provided by Dr. Christopher Marshall of the Chester Beatty Laboratories of the Cancer Research Institute. It is a human hepatic epithelial cell line (ATCC Number CCL 13).

All cell lines were checked at six monthly intervals for the presence of *Mycoplasma* contamination. The tests were kindly carried out by Dr. Paul Taylor of the Brompton Hospital virology department using direct culture and staining with bisbenzamide (Hoechst 33258). No evidence of contamination by *Mycoplasma* was found.

3.2.3 Maintenance of Cell Cultures

When a cell line was brought into the laboratory, a culture in a 100mm petri dish was set up, passaged at confluence using standard techniques and again allowed to grow to confluence before samples of the daughter cultures were frozen down in DMEM containing 20% NCS and 10% dimethyl sulphoxide for storage in liquid nitrogen. Cultures were maintained by regular passaging at weekly intervals and the medium changed at intervals of two or three days. Cultures were discarded after eight passages, and another ampoule thawed out to provide a further set of cultures. Cells were used for an experiment within forty-eight hours of the culture reaching confluence.

3.2.4 Preparation of Fibroblasts for Experiments

The medium was aspirated from a confluent culture of fibroblasts and the monolayer washed gently with two changes of phosphate buffered saline (PBS). 1.5ml of a 0.02% EDTA/0.05% trypsin mixture was added to each petri dish, and the dishes incubated at 37°C for three to ten minutes. When the monolayer showed signs of disruption apparent to the naked eye, ten ml of PBS/10%NCS was added to each plate and repipetted until all the fibroblasts had been washed free from the bottom of the dish. The cell suspensions from the plates were combined, centrifuged at 100g for five minutes washed once with PBS alone and resuspended in a further ten ml of PBS. A 100μl aliquot of this suspension was placed in a haemocytometer and a cell count (200 cells) performed. The suspension was again centrifuged and the cells resuspended in a volume of appropriate culture medium to obtain the desired cell concentration.

3.2.5 Preparation of Cell Cultures in Microwell Plates

Fibroblast cultures for experiments were usually set up in 96 well plates (Microwell F96, Nunc, UK). These plates consist of an 8 x 12 array of 6mm diameter flat bottomed wells together with a lid to prevent airborne contamination. Preliminary experiments showed that cells grown in the outer perimeter of wells tended to display an uneven distribution in the bottom of the well and over a period of time grew at a different rate than cells in the inner 6 x 10 matrix of wells. In part this was due to greater evaporation from the outer wells (readily apparent if a microwell plate was left in the incubator for more than one week). Uniform growth was seen in the inner wells. For this reason the outer perimeter of wells was not used in experiments but filled with culture medium alone. This allowed the first column of eight wells to be used for blanking the microwell photometer in the Methylene Blue assay. In practice variations between wells

was sufficiently small for each sample to be tested in triplicate or quadruplicate only i.e., three, four or occasionally six wells in a column. The unused wells of the inner 6 x 10 matrix were filled with cells and control medium, providing an internal check on the uniformity of cell growth and the assay system.

In general the different culture conditions for an experiment were created by mixing aliquots of cell suspension and the different samples in each microwell. Test samples of medium were pipetted out first and then aliquots of cell suspension were dispensed from an Eppendorf Multipette. This consists of a handle containing the operating system and capable of adjustment to dispense repetitively and accurately aliquots of liquid from a sterile fine-tipped closed syringe which could be loaded with medium or cell suspension. Care was taken to ensure frequent agitation of the cell mixture to ensure a uniform suspension throughout the dispensing process.

Each microwell plate was covered with a lid and placed in the incubator for the appropriate period (routinely 72 hours).

3.2.6 Methylene Blue Assay

1. The culture medium was removed from the microwells by gentle vacuum aspiration. To prevent damage to the monolayer the medium was aspirated through a glass Pasteur pipette whose tip was bent through 180°, and then drawn out to a short fine capillary. The tip could then by run down the side of the microwell with the plate tilted slightly towards the operator. An alternative method was to gently immerse the microwell plate, held at 60° to the horizontal, and then invert it over a damp sponge cloth. The former method was used in most experiments.

In this and subsequent steps the outer rim of microwells (which had contained medium alone) were treated in an identical manner to the central wells.

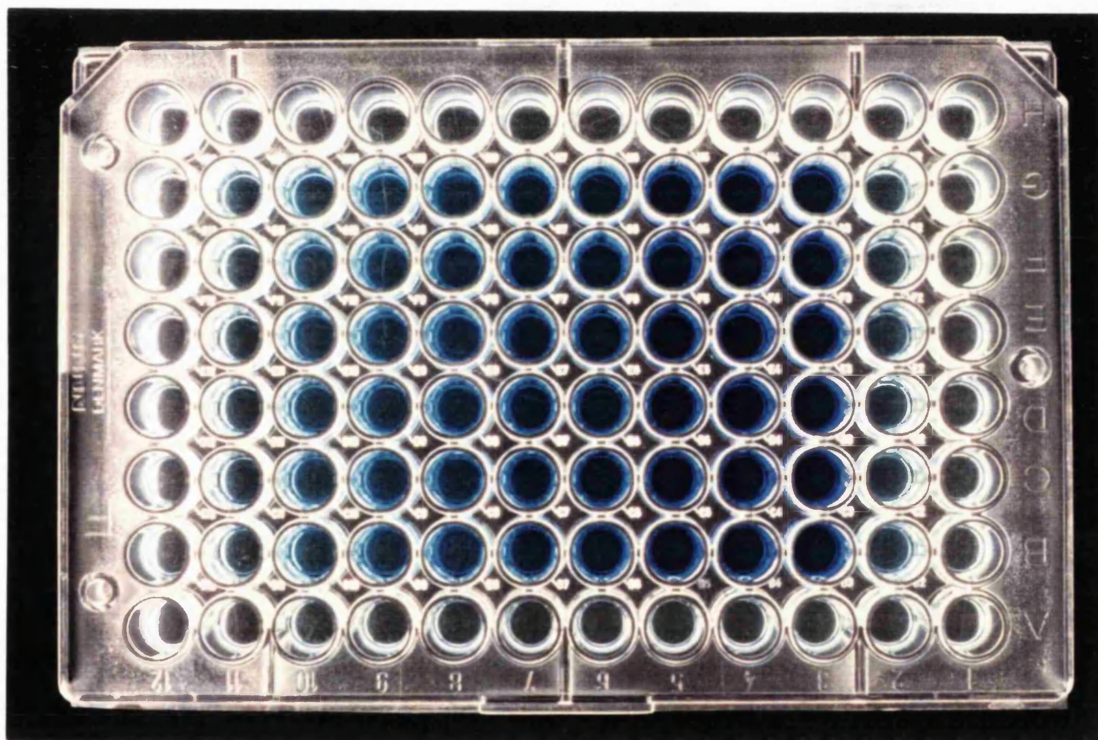
2. The monolayer was fixed with 100µl of 10% formol-saline (10% formalin/ 90% 0.15M saline) added to each well. All reagent pipetting was performed with a Titertek 12 channel 50-200µl adjustable pipette (Flow Laboratories Ltd., UK).
3. After 30 minutes the fixative was removed by shaking each plate held obliquely downwards over a sink, using a flick of the wrist. 100µl per well of 1% Methylene Blue in 0.01M borate buffer pH 8.5 was added to each well. The Methylene Blue solution was prepared, filtered and kept in a stoppered stock bottle for up to four weeks.
4. After 30 minutes the dye was removed by another flick of the wrist. The free dye remaining was rinsed off by dipping the plates into 0.01M borate buffer in four 1,000ml beakers, shaking off the buffer between each immersion. These rinsing steps were done in a uniform manner with no delay between each step, to minimise plate to plate variation in results.
5. The dye was eluted from the monolayer by adding 100µl of a 1:1 (v/v) mixture of ethanol and 0.1M HCl to each well. The plates were tapped gently to produce a homogeneous solution.
6. The bottom of the plates were dried with tissue and then the absorbance at 650nm (A650) for each well measured by a microplate photometer (a Titertek Multiskan MC fitted with a 650nm interference filter from Flow Laboratories). The machine produces a printout of the absorbance in all 96 wells in one minute. The photometer was blanked on the first column of "control" wells, which had contained medium alone.

A photograph of a microwell plate of fibroblast cultures which has been through the Methylene Blue assay is shown in Figure 3.1.

3.2.7 The Growth Factor Assay of Macrophage Supernatants

The general principle was that the test supernatants were mixed with a suspension of fibroblasts in 96 well plates and the cell density achieved after 72 hours of culture was

Figure 3.1 Photograph of a 96-well Plate Undergoing the Methylene Blue Assay



compared with that achieved by similar fibroblast cultures in the same microwell plate set up using control medium in place of the macrophage conditioned medium.

In more detail, a typical assay was performed as follows:-

1. The outer perimeter of wells of a 96 well microculture plate was filled with medium alone. 50μl aliquots of test supernatants or control media were pipetted into appropriate microwells in the central 10 x 6 matrix. Usually four or six microwells in a column of the plate would provide the replicate cultures for each sample. To allow accurate comparisons between plates control samples were always included in each plate. The plates were then placed in the incubator, while the fibroblast suspension was being prepared.

2. A suspension of fibroblasts were prepared as described in section 3.2.4. The concentration of fibroblasts was chosen so that a 50μl aliquot contained the number of fibroblasts intended for each microwell culture. For Rat1 fibroblasts the cell concentration was usually 1.2 x 10^5/ml giving 6 x 10^3 fibroblasts/microwell.

3. The culture was initiated by pipetting from an Eppendorf Multipette, 50μl aliquots of cell suspension into each of the test microwells. Each plate (containing upto 60 test microwells) could be filled in two minutes. The plates were then returned to the tissue culture incubator.

4. At the appropriate intervals, the microplates were removed from the incubator, the medium aspirated gently and 100μl of 10% formol-saline added to each well. The plates were wrapped in aluminium foil and stored for later assay by the Methylene Blue method.

5. The growth promoting activity of the sample was usually expressed as the growth factor ratio (GFR). To obtain this the cultures were harvested after 72h incubation, and assayed by the Methylene Blue method. The GFR was the ratio of the final cell densities (expressed as A650) achieved by the test sample and control cultures:-

$$GFR = \frac{\text{Mean A650 of replicate cultures with test sample}}{\text{Mean A650 of replicate cultures with controls}}$$

Clearly if the test supernatant contained growth promoting activity the GFR would be more than one, and if it was inhibitory the GFR would be less than one. However in both test and control fibroblast cultures, replication of the fibroblasts had occurred during the 72 hour period of incubation under the influence of the serum present in the culture medium.

In most experiments the culture medium for both alveolar macrophages and subsequently the fibroblast cultures, contained 2% NCS. This could be varied e.g., for macrophage supernatants containing no serum, the fibroblast cell suspension would be DMEM/4%NCS to give a final serum concentration of 2%.

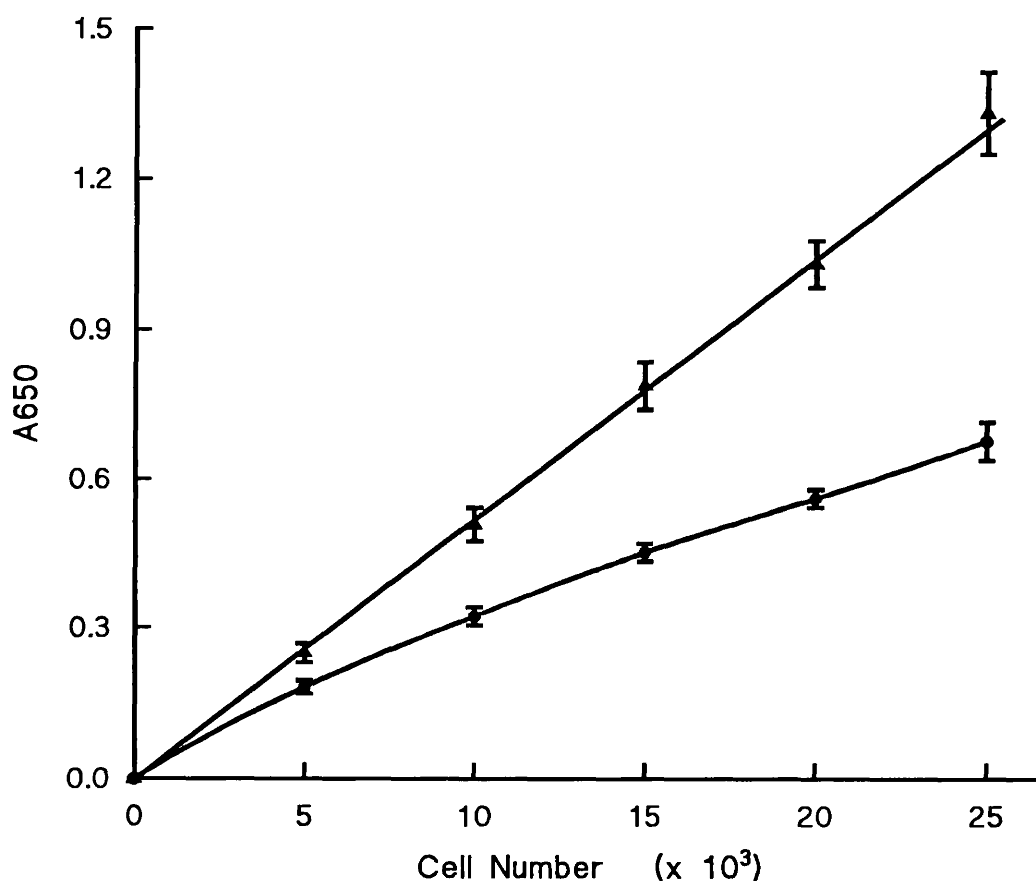
3.3 RESULTS

THE METHYLENE BLUE ASSAY

3.3.1 Choice of Solvent

Figure 3.2 shows that, when 0.1M HCl was used as the elution solvent in the Methylene Blue assay, the measured absorbance (A650) failed to rise proportionately with cell number. Trial of a number of solvents showed that the addition of ethanol to 0.1M HCl (1:1, v/v) eliminated this problem so that A650 was proportional to cell number (Figure 3.2).

Figure 3.2 **Effect of a Change in Elution Solvent on the Methylene Blue Assay**



Rat1 cells were plated out in DMEM/10%NCS at a range of cell densities, and harvested after 12 hours. This allowed cell adhesion and spreading to occur but cell replication would not have commenced. Thus the number of cells present at harvesting was very close to the number plated.

The Methylene Blue assay was initially performed using 100μl per well of 0.1M HCl as the elution solvent. The resulting A650 is plotted against the number of cells plated out in each well (-•-, error bars are standard deviations of 6 replicates).

Subsequently 100μl of ethanol was added to each well and the absorbance again measured after thorough agitation to mix the solvents. The upper plot (-▲-), shows the restoration of linearity between A650 and cell number.

3.3.2 Methylene Blue and Beer Lambert's Law

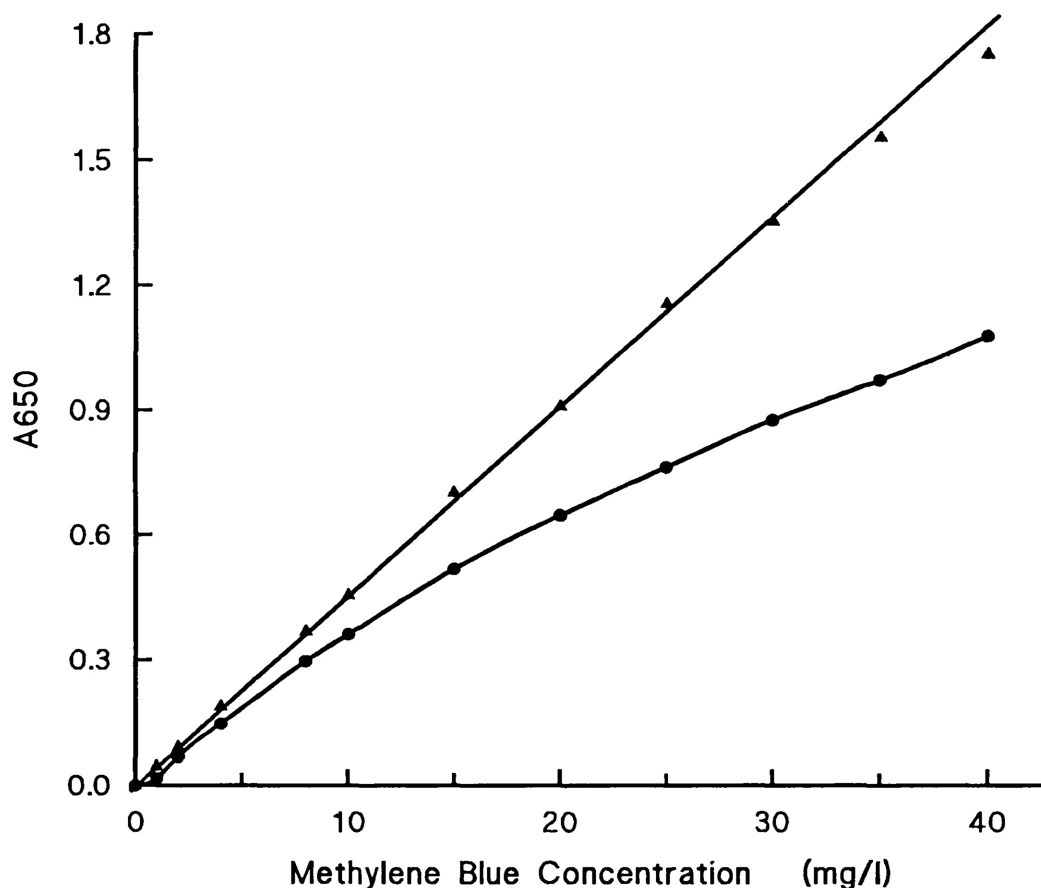
Figure 3.3 demonstrates that the nonlinearity observed when 0.1M HCl was used as the elution solvent was due to a failure of Methylene Blue to observe Beer Lambert's law in aqueous solution. Linearity was almost completely restored when Methylene Blue was dissolved in 0.1M HCl/C_2H_5OH. The mechanism underlying these problems was revealed by examining the absorption spectrum of Methylene Blue at different concentrations of the dye (Figure 3.4). This showed that at low concentrations, Methylene Blue had a major absorption peak at 660nm, with a minor peak at 605nm. The major peak is due to monomeric dye, and the secondary peak is due to absorption by dimeric Methylene Blue (Horobin, 1982). When the concentration of dye was increased fourfold, dimer formation was favoured, and the secondary peak became more prominent. Changing the solvent to 0.1M HCl/C_2H_5OH almost abolished the secondary peak at 605nm, with a corresponding increase in absorption at 660nm. This demonstrated that dimer formation was inhibited by the new solvent 0.1M HCl/C_2H_5OH, and explained why Beer Lambert's law was then observed.

3.3.3 Accuracy

Figure 3.5 displays the results of experiments to confirm that A650 was proportional to cell number in the assay for three different cell lines. The linearity was impressive. These cell lines had widely differing characteristics viz IMR90 - well differentiated human fibroblasts; Chang - human hepatic epithelial cells; Rat1 - rat lung fibroblasts which are less differentiated than the IMR90 cells. The absolute value of A650/cell (i.e., the slope of the lines) differed between cell lines reflecting their different contents of nucleic acid and proteins.

To confirm that this relationship held after growth of the cultures in various concentrations of serum, the results of

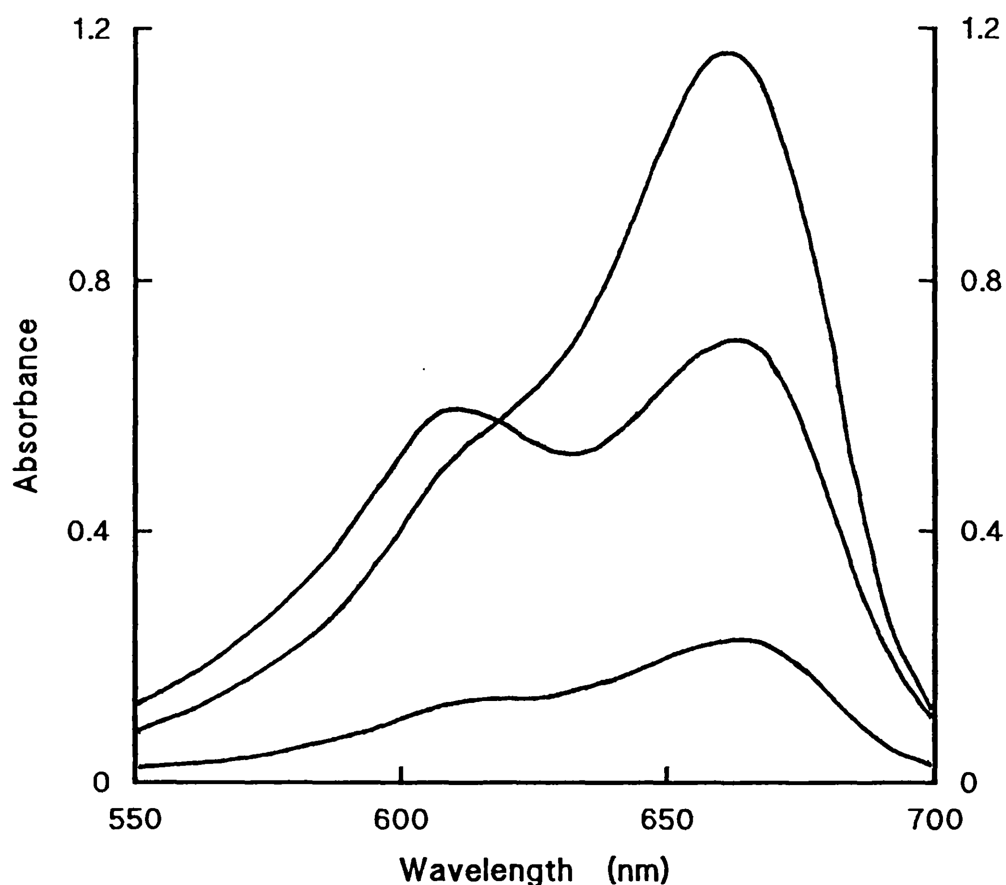
Figure 3.3 **Beer-Lambert's Law and Methylene Blue**



100μl aliquots of Methylene Blue dissolved in either 0.1M HCl or 0.1M HCl/C_2H_5OH at concentrations ranging from 2 to 40mg/l were pipetted into wells of a 96 well tissue culture plate. It can be seen that the concentrations of Methylene Blue chosen for this experiment gave values of A650 comparable to those achieved in the Methylene Blue cell number assay performed on microplates.

Absorbance at 650nm was measured using the microplate photometer and is plotted against Methylene Blue concentration.

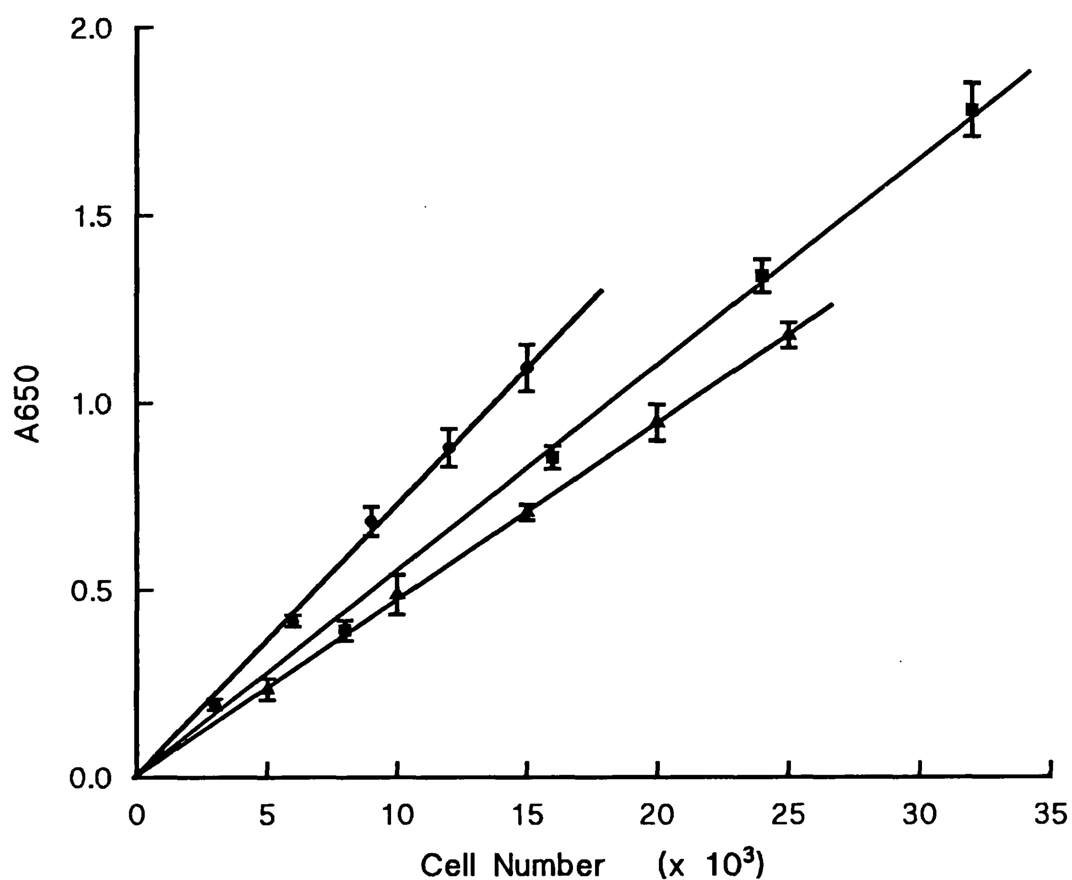
Figure 3.4 Absorption Spectra of Methylene Blue in 0.1M HCl and in 0.1M HCl/Ethanol



The absorption spectrum of three solutions of Methylene Blue, measured in a Gilford spectrophotometer, are displayed.

The concentrations of Methylene Blue studied were:-
A 7.8mg/l in 0.1M HCl
B 31.2mg/l in 0.1M HCl
C 31.2mg/l in 0.1M HCl/C_2H_5OH (1:1, $^v/_v$)
These concentrations span those achieved in the wells of a 96 well microculture plate in the Methylene Blue assay (compare Figures 3.3 and 3.5).

Figure 3.5 Linear Relationship between Cell Number and A650 in the Methylene Blue Assay for three Cell Lines



A650 is plotted against cell number plated out for three cell lines:- Rat1 (-▲-); Chang cells (-■-); and IMR90 (-●-). Error bars are for standard deviations for 12 replicate microwells (6 for the Chang cells) at each cell density.

The cells were plated out in DMEM/10%NCS, incubated for 12 hours and then harvested. The standard Methylene Blue assay was performed, using 100μl/well of 0.1M HCl/C_2H_5OH to elute the bound dye.

assay by the Methylene Blue method and by a direct cell counting method were compared. The results are displayed in Figure 3.6 and confirm that the Methylene Blue assay remained accurate for cells which have been replicating rapidly in culture. It can be seen that haemocytometer counts were subject to much greater variability than the results from the Methylene Blue assay. At the higher densities in this experiment, the monolayers were confluent.

3.3.4 Reproducibility

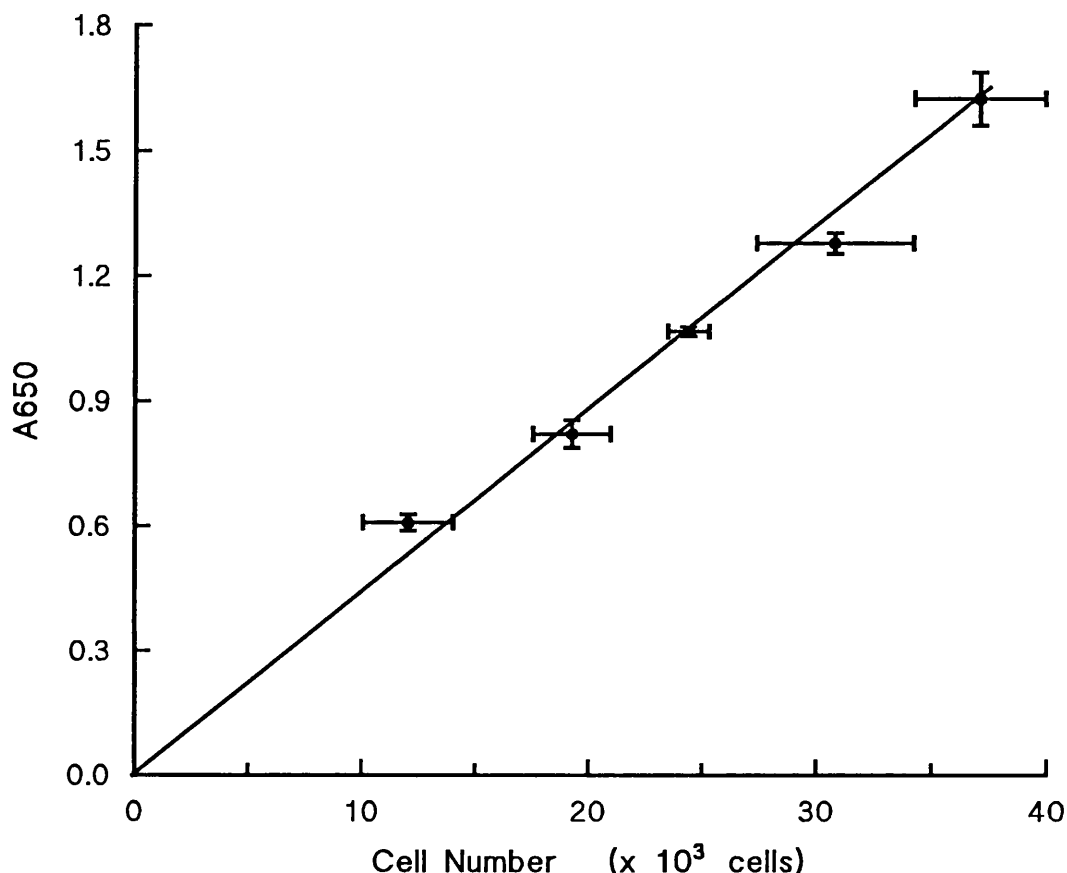
The results of an experiment to measure the reproducibility of the Methylene Blue assay are summarised in Table 3.1. Sixteen microplates, each containing 36 identical cultures of IMR90 cells, were assayed in groups of four at intervals over 10 days. The mean A650 for each of the 16 plates are listed in the table.

The coefficient of variation of A650 within a plate was 4.4%. One way analysis of variance of the mean A650s for the 16 plates revealed that the coefficient of variation between plates in a single run was 2.03%, and there was no additional variation attributable to the assay of plates on different days. There was no trend in A650 with duration of storage (which ranged from one day for assay run#1 to ten days for run#4).

3.3.5 Effect of Presence of Cell Monolayer on A650

The previous experiment also provided information on whether the presence of the monolayer of fixed cells in the microwell might contribute to the final A650 measured by causing scattering of the vertical light beam of the microplate photometer. An additional plate of IMR90 cells was put through the assay with the omission of the Methylene Blue staining step. The absorbance of the fixed, unstained monolayers was measured on the microplate photometer. The mean A650 was 0.034, representing 5.5% of the A650 obtained

Figure 3.6 Plot of Absorbance (A650) against Haemocytometer Count for Microwell Cultures



Rat1 cells were plated out at a density of 6 x 10^3/well in the central 10 x 4 matrix of a 96 well tissue culture plate. The medium employed was DMEM with 1.5%, 3%, 4.5%, 6% and 9% NCS for groups of 8 replicate wells. After incubation for 72 hours the medium was aspirated. 100μl of 10% formol-saline was added to alternate wells, and 50μl 0.05% Crystal Violet in 0.1M citric acid to the remainder. Vigorous pipetting of the wells containing Crystal Violet lysed the cytoplasm of the fibroblasts and produced a suspension of stained nuclei which was aspirated and counted in a haemocytometer. From this the original number of nuclei in each well was calculated. When this process was complete, the plate was put through the Methylene Blue assay to measure the A650 of the microcultures fixed in formol-saline.

Corresponding values of mean A650 and mean haemocytometer count (error bars represent standard deviations, n=4) are plotted for each group of cultures. The first point on the graph is derived from the microcultures grown in 1.5% NCS and the last, from those in 9% NCS.

TABLE 3.1 **Reproducibility of Methylene Blue Assay**

Run	#1	#2	#3	#4
Mean A650 (+/- SD) for each plate Units are 10^{-3} AU	635 (26)	626 (25)	623 (29)	617 (25)
	639 (24)	634 (30)	614 (33)	627 (46)
	614 (24)	653 (29)	597 (22)	621 (23)
	614 (18)	605 (26)	622 (27)	624 (28)
Average of run	626	630	614	622

10^4 IMR90 cells per well were pipetted into each of a 6 x 6 matrix of wells in each of 16 microculture plates (i.e. a total of 576 wells). These were all harvested after incubation for 16 hours to allow attachment, and then stored before assay in groups of four at intervals over the following ten days.

The Table shows the mean A650 (+/- SD, n=36) for each plate, and grand mean for the four plates assayed on each day. A more detailed analysis of the component part of the variance observed is described in the text.

Note the units of 10^{-3} AU. For example, the mean absorbance of the first plate of run#1 was 0.635 and the SD was 0.026. This is the convention used in all tabular results of A650 in this thesis.

by the Methylene Blue assay, for the other plates. Thus the presence of the monolayer during photometric measurement in the Methylene Blue assay made only a minor contribution to the observed A650, and therefore variations in distribution of the cells in the microwells would not have a perceptible effect.

3.3.6 How Robust is the Assay?

In any assay some of the steps require particular attention to achieve reproducible results, while other steps are "robust" and their exact timing or execution do not have a significant effect.

Once the monolayer was fixed in formol saline the monolayer did not appear vulnerable to cell detachment. This was not the case beforehand however. The method of removal of culture medium described by Currie (1981), i.e. by dipping the plates in PBS and inverting them over a sponge cloth, did cause cell loss with some lines (R9ab cells and IMR90 cells were examples). Monolayers at low cell density were particularly vulnerable. It was for this reason that the aspiration method described in the previous chapter was developed and could be used with the most delicate monolayers. An attraction of the Methylene Blue assay was that, following staining, it allowed the cell layer to be examined under an inverted microscope to look for damage and other changes in cell morphology, before the dye was eluted. It seemed very probable that a monolayer of cells would be rapidly fixed by formalin, and the effect of the duration of fixation was not examined. Other fixatives (glutaraldehyde, methyl alcohol) were tried and were equally effective but did result in different final measured absorbance (data not shown).

The duration of Methylene Blue staining beyond 15 minutes was not critical. When groups of 12 wells containing equal numbers of IMR90 cells were stained with Methylene Blue for 15, 30, 45, and 60 minutes, the mean A650 (+/-SD) obtained

were 0.526 (0.034); 0.519 (0.031); 0.540 (0.034); and 0.536 (0.034) respectively. One way analysis of variance confirmed that there was no significant difference between these means ($F=1.38$, $P>>0.05$). Staining times shorter than 15 minutes were not studied. In subsequent work a staining time of 30 minutes was used, which was convenient and within the period where staining time was not critical.

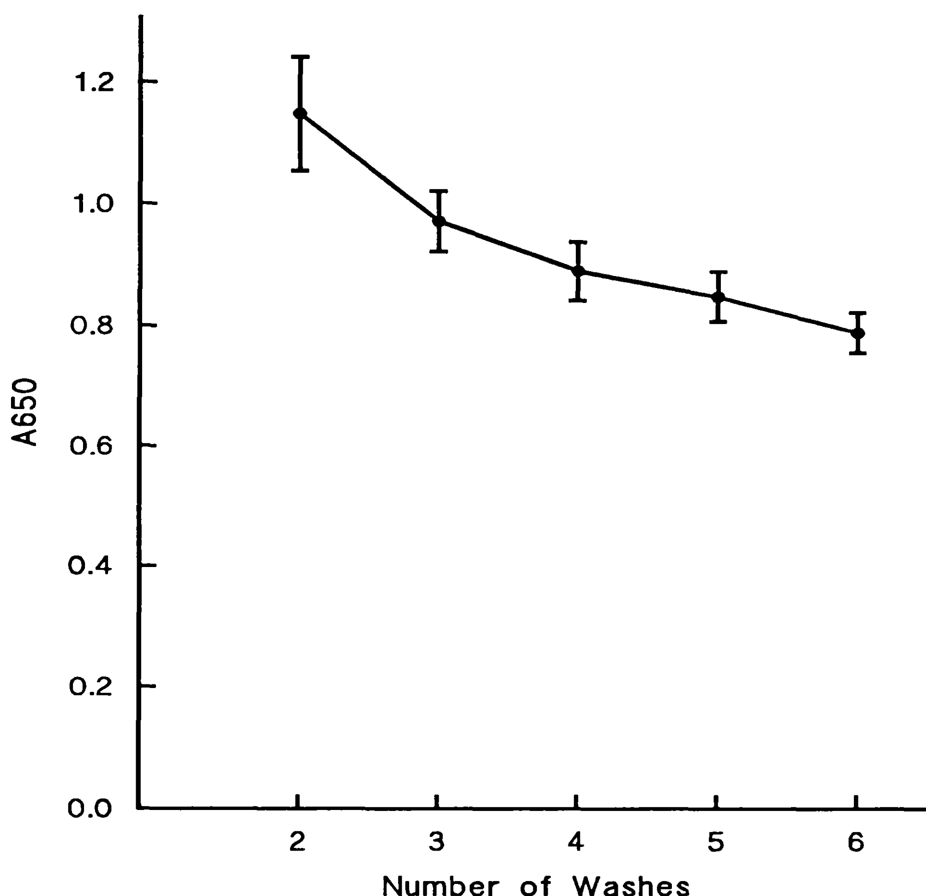
In contrast the number of washing steps had a marked effect on the observed A650 (Figure 3.7). Each wash caused a reduction in A650 with no sign of a plateau. The largest fall in A650 occurred between washes 2 and 3, but continued at approximately 6% per wash thereafter. In subsequent assays four washing steps were used. This decline also suggested that the duration of each washing step would have a significant effect. Care was taken to ensure that the washes were done in a rapid but uniform manner.

THE GROWTH FACTOR ASSAY

3.3.7 Choice of Serum Concentration and Duration of Culture

As a preliminary step in the selection of culture conditions for the assay of growth promoting activity, the effect of different concentrations of newborn calf serum on the growth of Rat1 cells was examined. Figure 3.8 shows the resulting growth curves. It can be seen that 2% NCS causes moderate stimulation of Rat1 cells, with the potential for detection of both inhibition and further increases in growth rate. It can also be seen that the Rat1 fibroblasts in this concentration of serum are in log phase growth for the 72 hours after initiation of the cultures. This was a convenient duration for the growth factor activity assay.

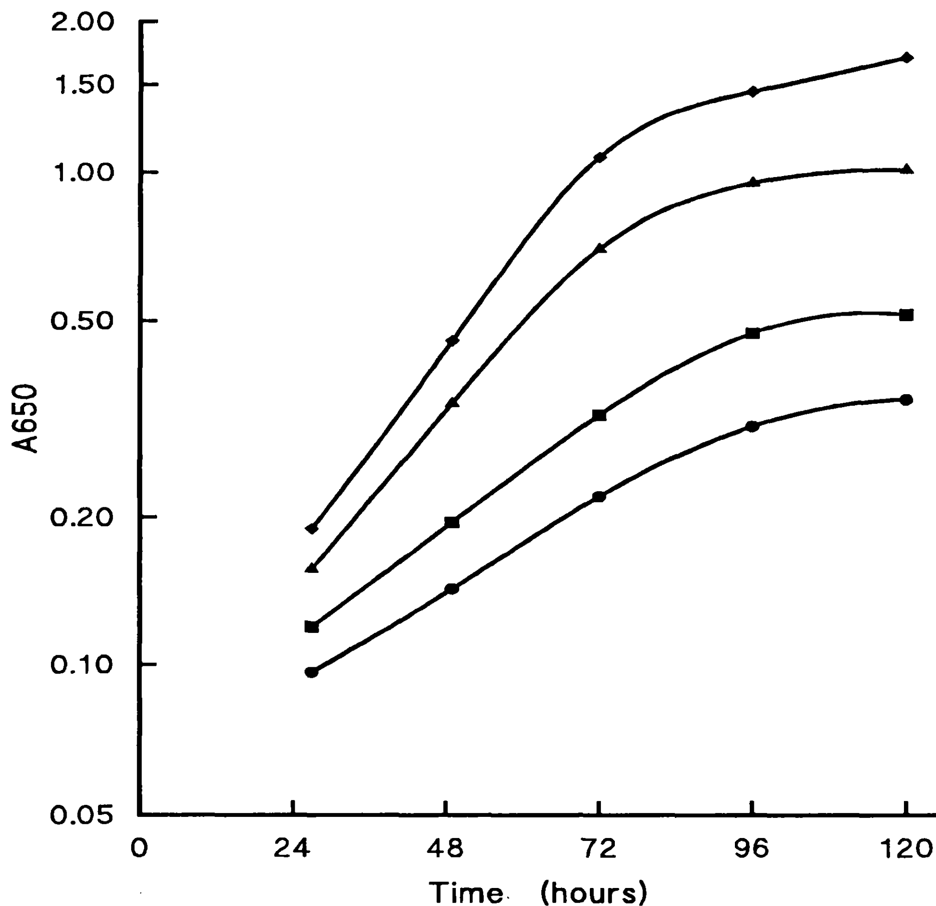
Figure 3.7. The Effect of the Number of Washes in the Methylene Blue Assay on the Absorbance Obtained



2 x 10^4 Rat1 cells were plated into the central 10 x 6 matrix of a 96 well microculture plate. After 14 hours incubation the plate was harvested, and assayed by the Methylene Blue method with the exception the the plate was immersed to a lesser extent in successive beakers of washing buffer. By this method columns 10 and 11 were washed twice, columns 8 and 9 three times, and so on.

The mean A650 (error bars = SD, n=12) obtained for each set of microwells is plotted against the number of washes they received.

Figure 3.8 **The Effect of Serum Concentration on Growth of Rat1 Cells in Culture**



Rat1 cells were plated out at a density of 4 x 10^3 cells/well in 1% (-•-), 2% (-▪-), 5% (-▲-) and 10% (-♦-) newborn calf serum in five 96 well plates. At daily intervals, a plate was removed from the incubator, the medium aspirated from the microwells, to be replaced by formol-saline before storage. After five days, all the plates were assayed by the Methylene Blue method.

The points plotted are of mean A650 (n=4). For clarity the error bars are not shown, but the mean coefficient of variation of A650 was low at 2.9%. Note that A650 is plotted on a logarithmic scale.

3.3.8 The Growth Factor Assay

The estimation of growth promoting activity as GFR (see methods section), is likely to vary between different assay runs, because of minor variations in the culture conditions of the assay (e.g., serum concentrations, plating density, trypsin damage to the fibroblasts when brought into suspension). To assess this interassay variations of GFR, 10 samples of rabbit lavage cell conditioned medium were assayed for GFR on three different occasions. The results are displayed in Table 3.2. The numbers in brackets are the ranking of each sample in the group for that assay run. Two way analysis of variance shows that the observed differences in assay grand means are statistically significant ($P<0.005$), although not excessive. More importantly the ranking order of the samples is very similar in each assay. The problem of interassay variations in GFR measurement could have been reduced if the purified growth factor being quantified had been available to provide a set of standards for each assay run. In the absence of such standards, the problem was circumvented by measuring the GFR of all the samples from one experiment in a single assay run.

These results also show that GF levels do not decrease markedly with storage. The interval between first and last assays was four months, and the samples had been stored at least two weeks at the time of first assay.

TABLE 3.2 Reproducibility of the Growth Factor Assay

Assay	#1	#2	#3
R80	1.00 (1)	1.10 (1)	0.98 (1)
R85	1.14 (2)	1.23 (4)	1.09 (2)
R66	1.16 (3)	1.18 (3)	1.13 (4)
R76	1.19 (4)	1.14 (2)	1.12 (3)
R71	1.25 (5)	1.35 (6)	1.19 (5)
R73	1.32 (6)	1.25 (5)	1.23 (6)
R67	1.35 (7=)	1.42 (7)	1.29 (7)
R74	1.35 (7=)	1.44 (8)	1.33 (8)
R75	1.44 (9)	1.53 (9)	1.44 (9)
R83	1.53 (10)	1.57 (10)	1.53 (10)
Mean GFR of assay run	1.273	1.321	1.233

Ten samples of conditioned media were assayed on three separate occasions over an eight week period. The results are tabulated above. The figures in brackets are the ranking in order of increasing GFR of the specimens in that assay run. This ranking is very consistent between the three runs. However two way analysis of variance shows that there is a significant difference ($F = 15.4$, $P<0.005$) between the runs.

3.4 Discussion

3.4.1 Assessment of Methylene Blue Assay

The assay has proved to be rapid, convenient and technically simple. Fifteen 96 well plates representing upto 1,320 individual microwell cultures can be processed through the assay in under two hours. The equipment used is relatively inexpensive. The largest capital item is a microplate photometer, already available in laboratories where ELISA assays are performed. These machines may be interfaced to computers for data capture and statistical analysis. A particularly convenient feature is that, after the cell monolayer has been fixed with formol saline, microplates can be stored almost indefinitely until a batch of plates has been assembled for assay. The first application (Lagneau et al, 1977) of Methylene Blue staining of cell monolayer to allow quantitation of cell numbers, involved dilution of the aqueous solution of the dye which was transferred to a photometer cuvette. Currie (1981) and subsequently Finlay et al (1984) described the use of a microplate photometer to allow in situ measurement of optical density of the aqueous solution of Methylene Blue in the microwell. Currie did not provide any evidence of the accuracy of the method. Finlay and coworkers (1984) showed a moderately good correlation between absorbance of the Methylene Blue solutions and cell number, but commented that at high cell densities, there was "a slight decrease in the amount of stain bound per cell". The present work shows that this observation is, in fact, due to the failure of Methylene Blue to follow Beer Lambert's law at the high concentrations achieved in the microwell (see Section 3.3.2). The introduction of a 1:1 mixture of 0.1M HCl and ethanol as the elution solvent remedied this problem. With this modification the assay is remarkably accurate throughout the range of cell densities achieved in adherent cultures in 96

well plates (see Figures 3.2, 3.5 and 3.6).

It is also noteworthy that the impressive reproducibility of the assay described in this work (coefficients of variation of A650 typically 2-5%) is better than that indicated in the paper by Finlay et al (1984). The reasons for this are not clear, although the importance of the washing steps in the final A650 measured (see Section 3.3.6) pointed to the need for this stage to be performed quickly and consistently.

Methylene Blue is a basic dye which is positively charged at pH 8.5. It binds electrostatically to negatively charged groups within the cell, predominantly the phosphate groups of nucleic acids (both DNA and RNA) but also to some negatively charged groups in proteins (Horobin, 1982). As the dye binds to RNA and protein, as well as DNA, the amount of the dye binding per cell will vary with the amount and composition of the cell cytoplasm. This explains the variation in dye bound per cell for different cell types from the same species, which would have the same content of DNA. It may also account in part for the lower coefficient of variation for the Methylene Blue assay seen with cloned lines compared with the less homogeneous cell strains (compare the results for Rat1 and IMR90 cells in Figure 3.5).

These principles suggest one potential problem with the assay - it would be possible that a stimulus to cells in culture might radically increase the ratio of cytoplasm to nucleus, and that this would lead to an increase in A650 without a change in cell numbers. In the optimal conditions generally used in the study of cell growth and the effects of growth factors this is unlikely to be a problem. The experiment illustrated in Figure 3.6 showed that for cultures growing at widely different rates in response to different serum concentrations, A650 did remain proportional to cell number.

3.4.2 Limitations of the Methylene Blue Assay

These are few and minor. The importance of the washing steps has already been alluded to. This probably explains the between plate variation in absorbance which was found (see Section 3.3.4). Nonetheless this between plate variation was small (approximately half of the already low within plate variation) and can be allowed for in experimental design, if need be, by the use of controls within each plate. It was reassuring to see that there was no additional variance created by assaying similar plates on different days. The assay is not applicable to microwell cultures of nonadherent cells such as lymphocytes. Care is needed to avoid disruption of weakly adherent monolayers, but this was not a problem with the cell lines used in this work.

3.4.3 Alternative Colorimetric Assays

Other cell stains have been used as the basis for colorimetric assays of cell number or viability. Much of this work has been in the field of cytotoxicity. For example Fisch and Gifford (1983) stained monolayers in 96 well plates with Crystal Violet, and quantitated the number of cell free plaques caused by activated macrophages by measuring the transmitted light of a microplate photometer beam. This assay did not attempt to measure cell number as such and would not discriminate between confluent monolayers of different densities.

Giemsa has also been used by Hibbs et al (1977) in similar fashion to the method of Fisch and Gifford (1983) described above. This technique was improved by Mirabelli et al (1985) who eluted the bound Giemsa with 0.1M HCl pipetted into the microwells. Their limited data showed that a linear relationship between absorbance (at 600 nm) and cell number only existed for lower cell densities, above which absorbance failed to rise with cell density.

The conversion by living cells of tetrazolinium into a coloured product, formazan, is the basis of an assay described by Mossman (1983) and subsequently refined by Carmichael et al (1987) and by Alley et al (1988). The assay is limited by the instability of the coloured product (Alley et al, 1988) so that it is not possible to build up "batches" of plates for assay; by interference from the presence of serum and some exogeneous chemicals (Alley et al, 1988); and by activation of the cells causing increased levels of enzyme expression and therefore of rates of conversion of the substrate to coloured product (Carmichael et al, 1987). In the latter study, the relationship between cell number and absorbance was examined in detail and found not to be linear.

None of the alternative colorimetric assays combine the accuracy, reproducibility and convenience seen with the Methylene Blue assay.

3.4.4 Cell Proliferation Assays Based on [^{3}H]Thymidine

The main alternative for rapidly assessing the growth rate of microwell cultures is the use of [^{3}H]thymidine labelling as a measure of DNA synthesis. This can be used in one of two ways:- (a) identification by autoradiography of the proportion of cells which have undergone mitosis during the period of [^{3}H]thymidine labelling; (b) quantitation of the amount of [^{3}H]thymidine taken up and bound (i.e., in DNA) by the cell culture. The former of these is accurate but requires manual counting of labelled and unlabelled nuclei, and is therefore extremely laborious. The latter method is the more widely used but has many disadvantages. Because of its popularity as an assay of cell growth of microwell cultures it is worth discussing in more detail the problems of this assay.

Firstly the use of radioisotopes in the laboratory requires the provision of special handling facilities, and expensive capital equipment such as scintillation counters.

Secondly it is necessary to achieve a minimum level of [^{3}H]thymidine incorporation for precise quantitation, above the natural background levels. Fibroblast microwell cultures contain typically 5-20 x 10^{3} cells, i.e. 10 fold less than is seen with lymphocyte microwell cultures. To compensate for this the duration of [^{3}H]thymidine exposure or the specific radioactivity of the thymidine must be increased. This risks radiation damage to the cells and a consequent falling off in culture growth (Wiezsaccker et al, 1981). Another consequence of the need to achieve a minimum level of [^{3}H] thymidine incorporation is the use of a high serum level in the culture medium to maintain a steady baseline of cell replication. This constrains the experiment, making it difficult to design experiments using more slowly growing cultures in low concentrations of serum or artificial media systems devoid of growth factors.

Thirdly, the [^{3}H]thymidine uptake measured in a culture is a function not only of the percentage of cells entering S-phase but also of the total number of cells present. This, in turn, depends on the previous growth of the culture. Thus, the value obtained derives from the previous history of the culture as well as the present stimulus to replication.

Fourthly, [^{3}H]thymidine uptake of proliferating cells depends for its validity on its being a measure of DNA synthesis. Production of cold thymidine by cells in culture (Opitz et al, 1975), secretion of enzymes which degrade the [^{3}H]thymidine in the medium (Palu, 1980), or changes in the rate of endogenous synthesis of thymidine by the cell (Rannels et al, 1982) will all affect the specific radioactivity of [^{3}H]thymidine in the precursor pool for cellular DNA synthesis. In these circumstances changes in [^{3}H]thymidine uptake will not accurately reflect changes in DNA synthesis. Many workers, therefore, feel obliged to validate results produced by [^{3}H]thymidine uptake by repeating key experiments using a cell counting method.

Finally, [^{3}H]thymidine uptake studies, even with the use of a cell harvester, are substantially slower than the Methylene Blue assay.

3.4.5 Conclusion

The Methylene Blue assay for numbers of adherent cells in microwell cultures seems to be a significant advance over existing assays. It is technically simple and uses only one piece of relatively expensive equipment i.e., a microplate photometer often available in laboratories which perform enzyme linked immunosorbent assays (ELISA). It avoids the use of radioisotopes. It is accurate and reproducible with the caveat that plate to plate variation is small but significant in an assay run. This can be circumvented by the use of internal controls if necessary. It is of wide application and allows the study of cultures of low cell density and/or low proliferation rates. These qualities of the assay made it well suited for the work described in subsequent chapters.

CHAPTER 4

SECRETION OF GROWTH FACTOR BY ALVEOLAR MACROPHAGES FROM NORMAL RABBITS

4.1 INTRODUCTION

The evidence that macrophages can secrete growth factors which stimulate fibroblast replication has been presented in Chapter 1. Although this has been shown for macrophages from many species, including man, it had not been shown for rabbit macrophages whether derived from peritoneum, lung or elsewhere. Before addressing the question of the relevance of this mechanism to the pulmonary fibrosis seen in the bleomycin rabbit model, it was necessary to show that alveolar macrophages (AMs) from normal rabbits could secrete growth factors (GFs). This required the demonstration that alveolar macrophages secreted a substance or substances which stimulated fibroblast growth in a nutritionally adequate medium and this growth promoting activity had the expected physicochemical characteristics of a GF. This work is described in this chapter.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The New Zealand White rabbits from HOP laboratories were handled as described previously. In these experiments the weights of the animals at the time of sacrifice ranged from

1.7 to 3.4 kg. They had all recovered from the stress of transportation before use.

4.2.2 Lung Lavage

Animals were killed by intravenous injection of sodium pentobarbitone (100mg/kg body weight, from May and Baker, Dagenham, UK). After soaking of the ventral fur of the animal in 95% ethanol, the thorax and abdomen of the animal was opened. The inferior vena cava and the abdominal aorta were transected to exsanguinate the animal. This effectively eliminated contamination of the lavage cells with erythrocytes (this technique was not appropriate in the experiments described in chapter 2 because of the presence of large quantities of radioactive [^{3}H]proline). Bronchoalveolar lavage was performed as described in Section 2.2.3 with the exception that each lavage return was collected in a separate 50ml sterile polypropylene centrifuge tube (Falcon, Becton Dickinson, UK). Each tube contained 10ml of Ca^{++} and Mg^{++} free DMEM (Dulbecco's Modification of Eagle's Medium) as a source of glucose and other metabolites. These were fitted with screw caps which prevented contamination of the lavage returns while further animals were being processed.

4.2.3 Lavage Cell Preparation

All subsequent cell manipulations were performed in a laminar air flow hood to maintain sterility. The cells were kept either in tubes plunged into crushed ice or at 4°C in, for example, the centrifuges, before initiation of the cultures.

The cells were separated from lavage fluid by centrifugation (200g, 10min, 4°C) and the cell pellets from the three tubes derived from a single animal, combined and resuspended in Ca^{++} and Mg^{++} free DMEM. The suspension was recentrifuged and the supernatant discarded. The cells were

resuspended in 10ml of Ca^{++} and Mg^{++} free DMEM and 100μl aliquots removed for cell count by haemocytometer, measurement of cell viability using Trypan Blue and preparation of cytocentrifuge smears as described in Sections 2.2.5 to 2.2.7. Viability was always greater than 95% and subsequent cell differential counts using May-Grunewald-Giemsa staining confirmed that macrophages constituted at least 98% of the lavage cells in all experiments. NSE staining was not routinely performed as, in the experiments described in chapter 2, the two different stains showed complete concordance in results.

4.2.4 Alveolar Macrophage Culture

The cells were centrifuged once more and then resuspended in an appropriate volume of culture medium to achieve the desired cell concentration.

In general terms, if the alveolar macrophages were to be co-cultured with other agents such as zymosan, this was achieved by adding an equal volume of cell suspension to the suspension of particles to be phagocytosed already pipetted into the culture tube. The basic culture medium was always DMEM equilibrated with humidified 10% CO_2/air in a CO_2 controlled incubator at 37°C. The AMs were incubated in small aliquots in 15ml polypropylene culture tubes (Falcon, Becton Dickinson, UK) with loose fitting caps to allow gas exchange. In parallel to the cell cultures, control tubes containing medium and the other reagents were set up and incubated for the same times.

4.2.5 Preparation of Additional Reagents

Zymosan

This was obtained from Sigma UK (catalogue no. Z4250). Before use it was boiled in distilled water for 60 minutes, and then washed twice in PBS by centrifugation and resuspension. In many experiments the boiled zymosan was

opsonised in rabbit serum before use. To achieve this the zymosan was suspended by vigorous vortexing in a mixture of 50% rabbit serum and 50% PBS, and kept at 37°C for 60 minutes. To ensure that all traces of free serum had been removed the zymosan then underwent six washes in fresh PBS, a further wash in DMEM, before resuspension in the final culture medium. This opsonisation was always performed on the day of the experiment. The concentration of zymosan in a suspension was determined by haemocytometer count at high magnification. For most experiments a concentration of 5 x 10^7 particles/ml was used, giving a particle : cell ratio of 50:1.

Sepharose 4b

This is an ion exchange resin (obtained from Pharmacia UK). It is in the form of very fine beads which can be ingested by phagocytic cells. It was prepared for use by washing twice by centrifugation with PBS, soaking in DMEM for two hours and then washing once more in DMEM.

Indomethacin

This was supplied by Sigma UK. It is poorly soluble in water and therefore the initial stock solution was made up in ethanol at a concentration of five mg/ml. 100μl of this stock solution was added to three ml of DMEM. This in turn was added to the cell culture in a ratio of 1:166 to give a final concentration of indomethacin of one μg/ml.

4.2.6 Harvesting and Assay of Macrophage Supernatants

After the appropriate intervals the cultures were taken out of the incubator and the tubes were plunged into crushed ice. The conditioned media were centrifuged (300g, 5 min, 4°C) to sediment larger particles of cellular debris. They were then aspirated into plastic hypodermic syringes (Gilette, UK) and then passed through 0.22μ Millipore filters into storage containers. The filters sterilised the

supernatants and excluded any cell debris or other particulate matter. For small volumes, two ml screw-top polypropylene vials (liquid nitrogen vials, Sterilin, UK) were used, and for larger amounts various sizes of clear polystyrene sterile pots (Sterilin, UK). These samples were stored at -30°C for periods up to one month, and at -70°C for periods longer than this. Assay of growth factors in the macrophage supernatants was performed as described in section 3.2.7, using Rat1 cells as the fibroblast line for detection of growth factor.

4.2.7 Dialysis of Samples

Samples to be dialysed were sealed in Visking No.1 tubing (M.Wt. cut-off 14,000; supplied by Medicell International, London and prepared as directed by the manufacturer). Dialysis was against a total of three changes of 500 ml volumes of DMEM at 4°C over a period of 24 hours, with constant agitation of the dialysis fluid by a magnetic stirrer.

4.2.8 Effect of Trypsin on Growth Factor Activity

10^7 alveolar macrophages from the pooled lavage returns from three normal rabbits were incubated in ten ml of serum free DMEM in a petri dish for one hour. The medium was then aspirated, the adherent cells (i.e., macrophages) washed with one change of DMEM, before a further ten ml of DMEM containing opsonised zymosan (5×10^7 particles/ml) was added. The cultures were incubated for 24 hours before the supernatant was harvested and filter sterilised. A 1.8ml aliquot of this was mixed with 0.2ml of a solution of trypsin (Sigma UK, catalogue no. T8253), 1mg/ml, in DMEM, achieving a final trypsin concentration of 100μg/ml. This was incubated for 30 min at 37°C. At the end of this incubation, the trypsin was inactivated by adding 0.5ml of a 4mg/ml solution of soyabean trypsin inhibitor (Sigma UK, catalogue

number T9003) in DMEM. 1.8ml samples of conditioned medium and also DMEM alone were mixed with both the trypsin and trypsin inhibitor solutions as detailed above prior to the 30 min incubation, to act as a controls. All these samples were then put into the fibroblast growth factor assay, together with a sample of DMEM which had been incubated at 37°C for 30 min without addition of reagents.

4.2.9 Analysis of Results

The standard deviation of each GFR is derived from that of the A650 of the test sample as follows:-

$$\text{SD of GFR} = \frac{\text{SD of A650 of test sample}}{\text{A650 of control}}$$

Comparison of two measures of GFR was made by Students two-tailed t test. Multiple comparisons were based on one way analysis of variance.

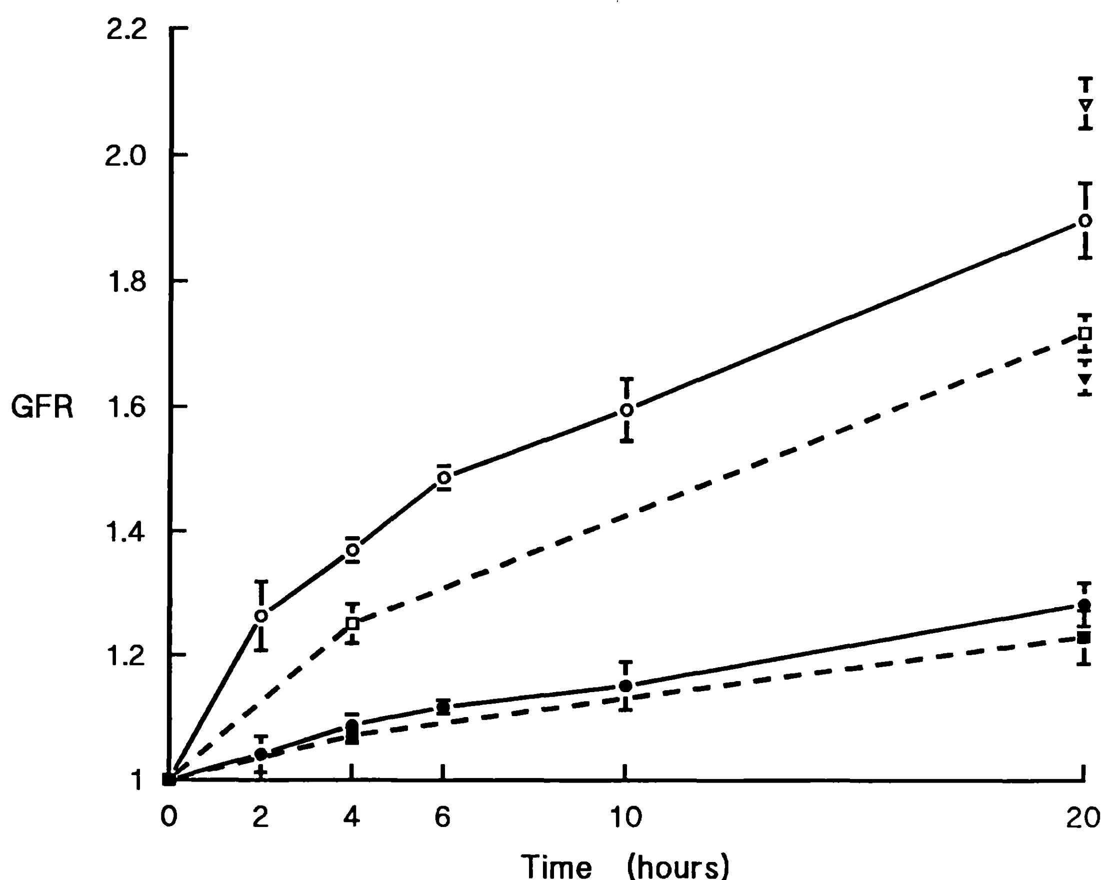
4.3 RESULTS

4.3.1 Time Course of Secretion of Growth Factor Activity and Effect of a Phagocytic Stimulus

Figure 4.1 shows the time course of GF secretion by alveolar macrophages from two normal rabbits, in response to stimulation by phagocytosis of Sepharose 4b and zymosan. It can be seen that the secretion of GF activity continued for 20 hours and the secretion of GF by Sepharose 4b stimulated and control macrophages followed a similar time course.

Opsonised zymosan was a more potent stimulus to secretion of GF than Sepharose 4b. It can also be seen that there was a basal level of GF secretion from unstimulated AMs.

Figure 4.1 **Production of Growth Factor by Alveolar Macrophages in Response to Phagocytic Stimuli**



AMs were obtained from two rabbits, R34 and R37 and were set up in suspension culture in DMEM (without serum) at a concentration of 6 x 10^5cells/ml together with opsonised zymosan (3 x 10^7 particles/ml), sepharose 4b (3 x 10^7 particles/ml) or alone (control tubes). Separate cultures were used for each time point which were stopped by cooling to 0°C at the time indicated. Cell free controls contained medium alone, medium and sepharose 4b, medium + zymosan were also incubated for 20 hours.

Growth promoting activity was measured as described in the methods. There was no significant difference between the cell free controls (by one way analysis of variance) so that the A650 of medium alone (which was included on each 96 well plate) was used in the calculation of GFR.

Solid data points are from rabbit R37 and empty data points from R34. Symbols:- squares = controls; circles = sepharose 4b; triangles = zymosan stimulated cells.

4.3.2 The Effect of Macrophage Concentration on Growth Factor Secretion

The influence of cell concentration on the secretion of growth factor by AMs is illustrated in Figure 4.2. GFR rose with cell concentration although above 10^6/ml, increasing AM density yields only minor increases in GFR.

To confirm that, at high cell concentrations, there was a true reduction in GF production per alveolar macrophage, the effect of dilution of the AM supernatants on the measured GFR was examined. Threefold dilution of the samples from the highest concentration of AMs (3 x 10^6 cells/ml) produced a lower GFR than the samples from the cultures with an equivalent cell concentration of 10^6 cells/ml. For R59, the GFR +/-SEM obtained from the culture containing 10^6 AMs/ml was 1.639 +/-0.036, and from the culture containing 3 x 10^6 AMs/ml, diluted threefold was 1.519 +/-0.016 (Student's t test, P=0.04). The corresponding figures for R60 were 1.684 +/-0.032, and 1.437 +/-0.023 (P=0.004). This is also illustrated in Figure 4.2.

4.3.3 Effect of Dialysis on Growth Factor Activity

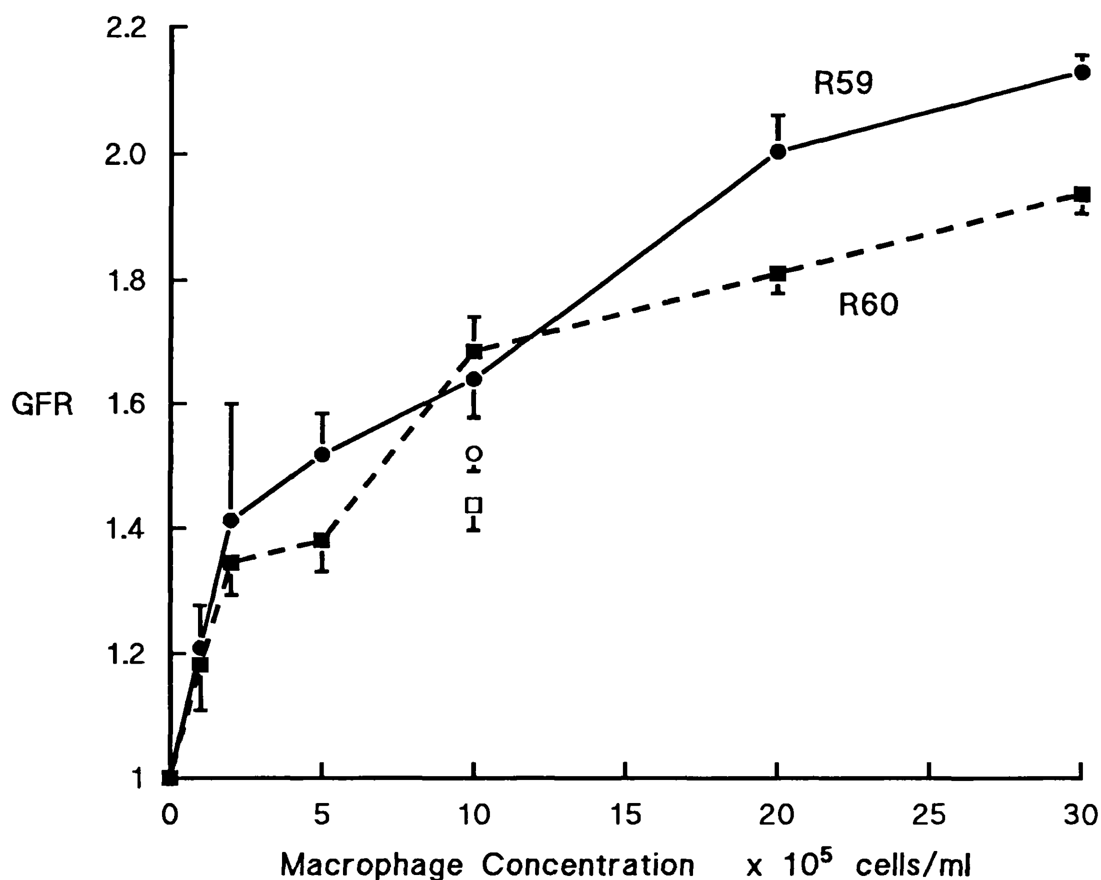
To exclude the possibility that the GF activity was due to low molecular weight molecules, the effect of dialysis on the GF activity of samples of medium conditioned by zymosan stimulated AMs was studied. The results are shown in Table 4.1.

The GFR of conditioned medium was unaffected by dialysis (columns 4 and 5 in Table 4.1), nor did dialysis reduce the nutritional qualities of control medium (columns 1 and 2).

4.3.4 Effect of Cycloheximide on Secretion of Growth Factor Activity

Table 4.1 also shows the effect on growth factor production of inclusion of cycloheximide (an inhibitor of

Fig 4.2 Effect of the Concentration of Macrophages on Growth Factor Secretion



Alveolar macrophages from two rabbits (R59 and R60) were set up in one ml aliquots in suspension culture at the cell concentrations indicated in DMEM/2%NCS together with opsonised zymosan at a concentration of 5 x 10^7 particles/ml. A control containing medium + zymosan but no cells was also set up.

After 24 hours incubation the supernatants from these cultures were obtained, filter sterilised and then tested on the Methylene Blue growth factor assay. GFR was calculated as described in the methods using the A650 of the zymosan medium controls measured on the same plate. GFR (mean +/-SD, n=3) is plotted against concentration of AMs.

The open symbols indicate the effect of threefold dilution of samples of conditioned medium from the cultures at an AM concentration of 30 x 10^5 cells/ml. These diluted samples had a significantly (by Student's test) lower GFR than the samples from cultures with an equivalent cell concentration of 10 x 10^5 cells/ml (Section 4.3.2).

TABLE 4.1 The Effect of Cycloheximide on Secretion of GF by Alveolar Macrophages, and of Dialysis of Conditioned Medium on Measured Levels of GF

	1	2	3	4	5	6
	Control Media			AM Conditioned Media		
Cycloheximide	No	No	Yes	No	No	Yes
Dialysis	No	Yes	Yes	No	Yes	Yes
A650 (10^{-3} AU)	542	528	533	821	813	594
SD (10^{-3} AU)	12.2	5.8	16.3	24.5	13.2	18.9
GFR	-	-	-	1.54	1.52	1.11
SD of GFR	-	-	-	0.046	0.025	0.035

Comparison by Student's t test: columns 1 vs 2: $P=0.09$; columns 1 vs 3: $P=0.41$; columns 4 vs 5: $P=0.61$; columns 4 vs 6: $P \ll 0.001$

Alveolar macrophages obtained from three normal rabbits were pooled, and then cultured in DMEM/2%NCS at a concentration of 10^6 AMs/ml together with opsonised zymosan (5×10^7 particles/ml) in suspension culture. One culture contained cycloheximide 100 mg/l in the medium. Controls of medium + zymosan, with and without cycloheximide were also set up. After 16 hours the conditioned and control media were separated by centrifugation. Aliquots from each of these samples were dialysed against fresh DMEM at 4°C as described in the methods.

Samples of each of these dialysed specimens, together with undialysed samples of control and AM conditioned media (without cycloheximide) were tested on the Methylene Blue GF Assay. Final A650 (4 replicate cultures) are displayed above. As the controls did not differ significantly, their mean A650 (0.534) was used to calculate the GFR of the conditioned medium shown above.

Note that the units of A650 are "10^{-3} AU", e.g., the A650 in column 1 is 0.542, and the SD is 0.0122. This convention makes the tabulated figures for A650 less unwieldy.

protein synthesis) in the culture medium of AMs stimulated by opsonised zymosan. The cycloheximide was dialysed out of the samples of AM conditioned and control media before they were tested in the fibroblast growth factor assay. Cycloheximide reduced GF secretion to a low level (compare columns 5 and 6 in Table 4.1). That this reduction in observed GFR of the samples from cycloheximide treated cultures was not due to the failure of dialysis to completely remove the cycloheximide was shown by the results for the control detailed in column 3 of the Table. This sample of unconditioned medium had contained cycloheximide, but was dialysed and then achieved a similar final density of fibroblasts in the GF assay as the other control media.

4.3.5 Effect of Cell Lysis by Freeze-Thawing

If GF was stored, preformed, in the cytoplasm of the AM, then lysis of a suspension of AMs should release this GF into the medium. Table 4.2 shows the GFR of the supernatant of a suspension of AMs lysed by freeze-thawing and compares this with the GFRs achieved by suspensions of AMs incubated for 24 hours with and without opsonised zymosan. It can be seen that lysis does release some growth factor activity (GFR 1.09) but this is much less than that secreted by viable cells either unstimulated (GFR 1.25) or stimulated by zymosan (1.58).

4.3.6 Effect of Trypsin on Growth Factor Activity

Table 4.3 shows that incubation with trypsin (100 μg/ml) of serum free medium from a 24 hour culture of zymosan stimulated AMs, destroyed the growth factor activity of the medium. The presence of trypsin neutralised by soyabean trypsin inhibitor had no effect on either the medium controls or the conditioned medium.

TABLE 4.2 **Release of Growth Factor from Alveolar Macrophages by Cell Lysis**

	1	2	3	4	5	6
	Control Media			AM Conditioned Media		
Zymosan	No	No	Yes	No	No	Yes
Freeze-Thaw	Yes	No	No	Yes	No	No
A650 (10^{-3} AU)	513	504	493	548	630	798
SD (10^{-3} AU)	10.6	9.8	10.7	14.2	15.4	11.6
GFR	-	-	-	1.09	1.25	1.58
SD of GFR	-	-	-	0.028	0.031	0.023

Comparison by Student's t test: columns 1–2 P=0.26; columns 2–3 P=0.17

Alveolar macrophages obtained from three normal rabbits were pooled, and aliquots suspended at a concentration of 10^6 AMs/ml in DMEM/2%NCS. One aliquot was immediately subject to five freeze-thaw cycles between -20°C and room temperature to lyse the cells (confirmed by microscopy). Two further aliquots were incubated at 37°C for 24 hours, with opsonised zymosan in one culture (10^7 particles/ml). The media were separated by centrifugation and filtration before measurement of GF levels by the Methylene Blue assay. Medium controls were treated similarly. The results are tabulated above as mean final A650 (4 replicate cultures). Neither freeze-thawing nor the presence of zymosan affected the control media (columns 1, 2 and 3), and so a pooled A650 (0.503) for the medium controls was used for the calculation of GFR.

The differences observed in GFR between columns 4,5 and 6, and each from unity are all highly significant by Student's t test ($P<<0.01$ in all cases).

TABLE 4.3 **Effect of Trypsin on Growth Factor**

	1	2	3	4	5	6
	Control Media			AM Conditioned Media		
Trypsin (T) Inhibitor (I)	-	T	T+I	-	T	T+I
A650 (10^{-3} AU)	503	507	492	811	503	796
SD (10^{-3} AU)	27.4	16.1	22.9	8.9	12.2	13.8
GFR	-	-	-	1.62	1.00	1.59
SD of GFR	-	-	-	0.018	0.024	0.028

Comparison by Student's t test: columns 1–2 P=0.80; columns 1–3 P=0.57; columns 4–6 P=0.12

AM conditioned serum free medium and control media were incubated alone or with trypsin or soyabean trypsin inhibitor as described in the methods (Section 4.2.8). The resulting samples were assayed by the Methylene Blue assay, and the results tabulated above.

The presence of trypsin alone, or trypsin + inhibitor in the control samples did not affect the final A650 obtained for these samples. Therefore the mean A650 (0.501) of the three controls was used to calculate the GFR of the conditioned media.

4.3.7 Temperature Stability of Growth Factor Activity

The effect of elevated temperature on the GF activity in AM conditioned medium was studied. Aliquots of serum-free conditioned medium, obtained as described in Section 4.2.7, were used. The GFR +/-SD (n=6) of an untreated sample was 1.47 +/-0.015. After heating to 56°C for one hour the GFR was 1.45 +/-0.017. The GFR of a sample heated to 80°C for one hour was 1.03 +/-0.023, and of a sample exposed to 100°C for three minutes was 0.98 +/-0.012. On the other hand GF activity was unaffected by freeze-thawing (five cycles) of the conditioned medium (GFR = 1.48 +/-0.018).

4.3.8 Effect of Indomethacin in Macrophage and Fibroblast Cultures

It has been shown that rabbit AMs can secrete products which stimulate fibroblasts to replicate. To examine the possible role of prostaglandin production by AMs in this process, the effect of the inclusion of indomethacin in the culture medium of the AMs and of the target fibroblasts was studied and the results are shown in Table 4.4.

It can be seen that supernatants from zymosan stimulated AMs had the expected stimulatory effect on fibroblast growth (compare columns 3 and 6 with the medium control, column 1). The presence of indomethacin in the culture medium of the AMs did not affect the final fibroblast density achieved (compare columns 3 and 5, and 6 and 8). The indomethacin in the supernatants from the AM cultures was transferred to the fibroblast cultures and might have had a direct effect on fibroblast growth. To study this, samples of control medium and AM conditioned medium which did not contain indomethacin were added to fibroblast cultures to which indomethacin had been added. This had no effect on the fibroblast density achieved by the cultures stimulated to more rapid growth by AM conditioned medium (columns 4 and 7), but the unstimulated fibroblasts did reach a higher final density than those not exposed to indomethacin at any stage (cf. columns 2 and 1).

TABLE 4.4 The Effect of Indomethacin on Growth Factor Production by Alveolar Macrophages.

	1	2	3	4	5	6	7	8
Indomethacin	Control		R34			R37		
in AM medium	No	No	No	No	Yes	No	No	Yes
in Fibroblast	No	Yes	No	Yes	No	No	Yes	No
A650 (10^{-3} AU)	318	371	662	664	678	523	531	529
SD (10^{-3} AU)	7.6	10.0	12.6	3.2	15.2	8.6	10.6	15.0
GFR	-	-	2.08	-	-	1.65		

Comparison by ¦ $P<0.001$ ¦ ¦ P=0.9 ¦ ¦ P=0.29 ¦
Student's t test ¦ P=0.17 ¦ ¦ P=0.52 ¦

Alveolar macrophages from two rabbits, R34 and R37, were obtained and cultured for 24 hours at a concentration of 10^6/ml with opsonised zymosan, 10^7/ml, with and without indomethacin 1 μg/ml. Parallel samples of medium alone, with and without indomethacin, were also incubated for 24 hours. At the end of the 24 hours, the culture media were harvested and tested in the growth factor assay. Those samples which did not contain indomethacin, were tested on two sets of fibroblast cultures, one to which indomethacin had been added (columns 2, 4 and 7 above), and one with no indomethacin (columns 1, 3 and 6). The indomethacin containing AM supernatants were tested on fibroblast cultures to which no indomethacin had been added.

Student's t test shows that the difference in A650 between columns 2 and 1 is significant but between 4 and 3, 5 and 3, 7 and 6, and 8 and 6 are not.

4.4 DISCUSSION

4.4.1 Evidence for Secretion of Growth Factor by Alveolar Macrophages

It has been demonstrated that rabbit alveolar macrophages can secrete, into both serum containing and serum free media, over a period of at least 20 hours in culture, a substance or substances which promotes the replication of fibroblasts. This growth promoting activity is not dialysable and therefore has a M.Wt. of more than 14,000. Its secretion is almost completely inhibited by cycloheximide and it is destroyed by trypsin, both of which indicate that it must be largely or wholly polypeptide. Its secretion is unaffected by the presence of indomethacin. This observation and the fact that it is not dialysable confirm that this is not an arachidonic acid metabolite, e.g. a leukotriene or prostaglandin. It therefore has the properties of a polypeptide growth factor (Hyashi and Sato, 1976). Secretion of growth factors by cells of the monocyte/macrophage line has been well documented, but there are reports to suggest that lymphocytes may also secrete GFs (Wahl and Gately, 1983; Neilson et al, 1980). AMs always constituted at least 98% of the cells obtained by lavage from these normal rabbits. In one experiment, (see Section 4.3.6), the lavage cells were allowed to adhere to tissue culture plates, and the medium then aspirated which would remove nonadherent cells such as lymphocytes. Supernatants from these cultures yielded the expected levels of GF activity. From these observations it is clear that the alveolar macrophages were the source of the growth factor detected.

4.4.2 Effect of Indomethacin in Macrophage and Fibroblast Cultures

The arachidonic acid metabolite, prostaglandin E_2 (PGE_2), has been shown to inhibit fibroblast growth in culture (Ko et al, 1977; Clark et al, 1985). The alveolar macrophage can produce PGE_2 and the ingestion of zymosan is a potent stimulus of this, increasing PGE_2 production more than fourfold higher than basal levels (Hsueh, 1980). Fibroblasts themselves secrete PGE_2 into their culture medium. Thus there are two possible mechanisms mediated by PGE_2 by which alveolar macrophages might inhibit fibroblast growth:-(a) direct secretion of PGE_2 by the alveolar macrophage, and (b) production of a macrophage factor which stimulates PGE_2 secretion by the target fibroblast. The latter process has been shown to operate for both hamster (Clark et al, 1983) and human (Elias et al, 1985) alveolar macrophages, and seems to be quantitatively important.

These issues were addressed in the experiment described in Section 4.3.8. The concentration of indomethacin in the AM culture medium was $1\mu g/ml$, sufficient to effectively abolish PGE_2 production by macrophages as shown by Hsueh (1979), and by Clark and co-workers (1983).

The data showed that stimulated rabbit alveolar macrophages do not secrete sufficient amounts of prostanoid to affect Rat1 fibroblast growth (in agreement with the lack of effect of dialysis of conditioned medium), and that there is no evidence of secretion by AMs of an inhibitory factor acting by increasing prostaglandin production by the target fibroblasts. In fact the opposite effect was noted, i.e., that the presence of indomethacin in the medium of fibroblasts growing under the influence of 2% serum lead to a higher final density, but that indomethacin had no effect when the fibroblasts were growing more rapidly under the influence of AM growth factor. This is compatible with the following conclusions:-

(1) That Rat1 fibroblasts growing in 2% serum secrete a

prostanoid (perhaps PGE_2) which is inhibitory for fibroblast growth and (2) that the GF present in the macrophage supernatants either blocks the effect of Rat1 cell autologous prostanoids or acts by inhibiting the autologous production of this prostanoid by Rat1 cells.

4.4.3 Comparison with Other Macrophage Growth Factors

The finding that rabbit AMs secrete a polypeptide growth factor for fibroblasts is in accord with reports of studies of a wide range of species e.g. rat (Kovacs and Kelley, 1985), sheep (Lemaire et al, 1983), monkey (Schoenberger et al, 1984) and man (Dayer et al, 1985; Rom et al, 1988). Similar, if not identical, GFs are secreted by peritoneal macrophages (Martin et al, 1981), by peripheral blood monocytes (Glenn and Ross, 1981) and by macrophage-like cell lines (Turck et al, 1988).

The present work found that polypeptide GF accounted for all of the mitogenic activity of AM conditioned medium. Thus trypsin completely destroyed the mitogenic activity of macrophage conditioned medium, in agreement with the experience of Glenn and Ross (1981) and Rutherford et al (1982) with human monocytes, and of Martin et al (1981) with mouse peritoneal macrophages. By the same token there is general agreement that mitogenic activity is not lost by dialysis of the macrophage conditioned medium.

There is less agreement about the ability of lysis of macrophages by freeze thawing to release GF into the medium. Liebovich and Ross (1976) found that a lysed suspension of guinea pig peritoneal macrophages released approximately 50% of the mitogenic activity of a similar aliquot of macrophages cultured for six hours. On the other hand Glenn and Ross (1981) and Rutherford et al (1982), working with human monocytes found that lysis did not release GF from the cells. The data in Section 4.3.5 shows that lysis by freeze-thawing of rabbit AMs yielded 16% of the mitogenic activity produced by an equal concentration of AMs stimulated by zymosan over

24 hours (36% of the mitogenic activity of unstimulated AMs cultured for 24 hours). Thus the amount of preformed GF present within AMs is very low as compared with their synthetic potential in 24 hours. This accords with the effect of inclusion of cycloheximide, a protein synthesis inhibitor, in the culture medium of AMs (Section 4.3.4) which reduced the mitogenic activity secreted by zymosan stimulated AMs to 21% of cycloheximide free cultures. In this work the M Wt of the GF was assessed indirectly by showing that it was nondialysable using tubing with a M Wt cut-off of 14,000. More detailed study by molecular weight sieve chromatography would be of interest, and has been performed by a number of investigators. The M Wt of macrophage derived GF has given the first clues of the complexity of this field. The wide scatter of results obtained suggested that more than one GF was being studied.

Rabbit AM derived GF was stable at 56°C but inactivated by heating to 80°C, and 100°C (Section 4.3.5). This pattern is keeping with all of the reported work on macrophage derived GFs (Lemaire et al, 1983; Martin et al 1981; Liebovich and Ross, 1976; Kovacs and Kelley 1986). On the other hand, platelet derived growth factor (PDGF), obtained from platelets, is unaffected by heating to 100°C for a few minutes (Scher et al, 1979). This difference in heat stability seemed to indicate that GFs produced by alveolar and other macrophages were distinct from PDGF. However with the development of antisera directed against PDGF and gene probes able to detect the expression of the PDGF gene, it has been shown that alveolar macrophages can synthesize and secrete a PDGF like substance (Shimokado et al, 1985; Mornex J-F et al, 1986). Shimokado and coworkers found that AMs produced two different forms of PDGF, with M Wts of 14,000 and 38,000, as compared with a M Wt of 31,000 of PDGF from platelets. Thus PDGF exists as a "family" of polypeptides, varying with the source of the GF.

4.4.4 Conclusion

The work described in this chapter has shown that rabbit alveolar macrophages secrete a growth factor (or factors) which can stimulate the replication of fibroblasts. The final stage of this work detailed in the next chapter, examines the role of growth factor production by inflammatory cells in the pathogenesis of the pulmonary fibrosis seen after the instillation of bleomycin.

CHAPTER 5

GROWTH FACTOR SECRETION BY LAVAGE CELLS FROM BLEOMYCIN TREATED RABBITS

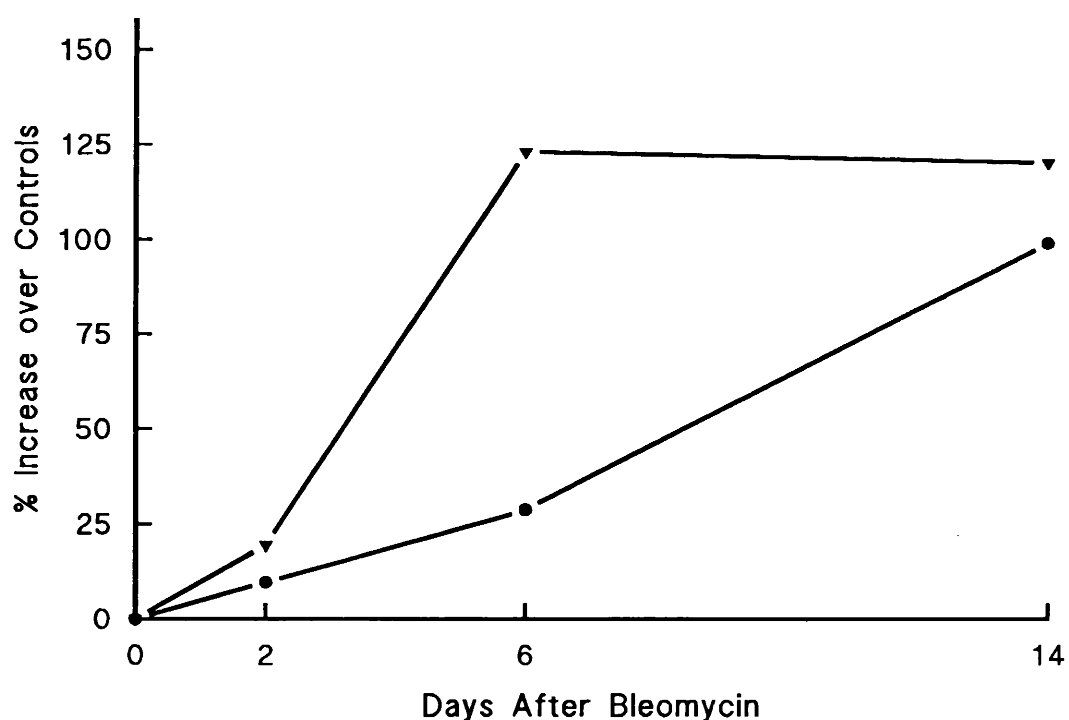
5.1 INTRODUCTION

The intratracheal instillation of bleomycin in animals provides a model of pulmonary fibrosis. The histological events and the changes in lung content of collagen and other biochemical components of the lung in the ensuing eight weeks have been described by Laurent et al (1981) and summarised in chapter 1 (Section 1.3.3). This work was extended and provides a guide to when changes in the modulation of fibroblast replication and function might be expected. Laurent and McAnulty (1983) examined the biochemical events of the first fourteen days in more detail. They found that total protein rose early but the increase seen in collagen content was most marked at fourteen days. The early rise in total protein need not depend on local synthesis of the new protein, but is probably explained by the leaking of protein rich exudate and inflammatory cells from the circulation through damaged capillaries. Collagen on the other hand is not a circulating protein and therefore its appearance must follow an increase in local synthesis or decrease in degradation.

The latter question is addressed by their data on synthesis and degradation rates of collagen measured *in vivo*. There was an early fall in degradation rate at two days but synthesis rate rose to a maximum at six days with little change at fourteen days. The major changes in synthesis and degradation rates occurred before those in content, as might be expected. Their findings for the content and synthesis rate of collagen in the lungs are illustrated in Figure 5.1.

Figure 5.1 **Changes in Lung Collagen Content and Synthesis Rates during Bleomycin-induced Pulmonary Fibrosis in Rabbits**

drawn from Laurent and McAnulty (1983)



Changes in lung collagen content (-•-) and collagen synthesis rates (-▾-) following instillation of bleomycin sulphate 10mg/kg into the lungs of New Zealand White rabbits. The figure is drawn from data taken from Tables 1 and 2 of the paper by Laurent and McAnulty (1983).

Collagen synthesis rates might rise either by an increase in fibroblast numbers or by a higher collagen secretion rate per cell or a combination of these mechanisms. These influences must occur around or just before the time of maximum collagen synthesis. The evidence for an increase in fibroblast numbers in bleomycin models of lung fibrosis is outlined in chapter 1. One source of the stimulation of fibroblast replication is likely to be inflammatory cells recruited to the lung. In this chapter is described the results of experiments to quantify the release of growth factors by inflammatory cells lavaged from the lungs of rabbits treated with bleomycin.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Design

The secretion of growth factors by lavage cells was measured in rabbits two, six and fourteen days following the intratracheal instillation of bleomycin. Five animals were studied at each time point. To exclude the possibility that intratracheal injection itself, rather than the bleomycin might account for any observed change, groups of four rabbits who had been given intratracheal saline only, were examined at each time point. These were designated saline controls. In addition four uninjected animals constituted a group of normal controls.

Levels of growth factor in medium conditioned by the lavage cells were assayed as described previously (see section 3.2.7) on two cell lines - Rat1 cells and R9ab cells. Additionally, samples of conditioned medium were dialysed and assayed on Rat1 cells with a parallel set of undialysed samples for comparison.

5.2.2 Tracheal Instillation of Bleomycin or Saline

New Zealand White rabbits were obtained and handled as described earlier (section 2.2.2).

The animals were given an intramuscular injection of 0.6ml/kg body weight of Hypnorm (fentanyl 0.2 mg/ml, fluanisone 10 mg/ml from Crown Chemical Co Ltd, Lamberhurst, Kent). When anaesthetised they were placed on their back, the fur of the anterior neck clipped, and a vertical incision through the skin was made. The trachea was exposed by blunt dissection. A 25g intravenous cannula (Venflon, from Viggo-Spectramed) was passed distally into the trachea, and the inner metal stylet removed. To the cannula was attached a syringe containing 5ml of sterile 0.9% saline, with bleomycin sulphate (Lundbeck Ltd, Luton, Beds, UK) 10mg/kg body weight, if appropriate. Air was aspirated to check correct placement and the syringe contents injected over one minute. During this time the head of the rabbit was supported to ensure all of the injected solution entered the lungs.

Following injection the cannula was removed, and the skin closed using surgical clips. The effect of the opiate was reversed by intravenous injection of 100μg naloxone and the animals were returned to their cages. In early experiments two animals died within five minutes of intratracheal injection. The use of naloxone reversed the respiratory depression caused by the anaesthetic agent and the instillation of saline into the animal's lungs. No "anaesthetic" deaths occurred in subsequent experiments. Animals were weighed at two to three day intervals after instillation of bleomycin.

5.2.3 Lung Lavage and Preparation of Lavage Cells

The instillation of bleomycin (or saline alone) was organised so that animals from different time points were sacrificed in groups of four to six. The details of the bronchoalveolar lavage and preparation of the lavage cells

were as described in Sections 4.2.2 and 4.2.3, with one exception. Some lavage returns contained erythrocytes. These were removed by 30 sec exposure of the cell pellet to five ml lysis buffer. 20ml DMEM was then added and the cells washed in DMEM by centrifugation (200g, 10min, 4°C). Aliquots of the cell suspension were taken for the measurement of total cell count, viability by Trypan Blue exclusion and cell differential using May-Grunewald-Giemsa stain, as described previously (section 2.2.3 to 2.2.6).

5.2.4 Culture of Lavage Cells

Once the haemocytometer and viability counts had been completed, the lavage cells were again sedimented by centrifugation and resuspended in the final culture medium (DMEM/2%NCS) at a concentration of 10^6 viable cells/ml. Cultures were then set up in polypropylene tubes in small volume aliquots (maximum five ml) and incubated for 24 hours. The conditioned medium was harvested, filter sterilised and stored at -30°C.

5.2.5 Measurement of Growth Factor Activity

Growth factor activity was measured as described previously (see sections 3.2.6 and 3.2.7). Samples from all the rabbits were assayed together. In the first experiment one set of samples of conditioned medium was assayed on Rat1 cells (initial cell concentration 6 x 10^3/well), and a parallel set on R9ab cells (3 x 10^3/well). In a second experiment aliquots of the supernatants were dialysed against three changes of DMEM at 4°C (as described in section 4.2.7). There was insufficient supernatant derived from two control rabbits, R73 and R83, to allow dialysis. The dialysed supernatants were then assayed on Rat1 cells, together with a further set of undialysed samples.

The R9ab cells were obtained from the American Type Culture Collection, number CCL327. They are a fibroblast

line derived from the lung of a New Zealand White rabbit. As R9ab cells grew rapidly in 2% NCS, a lower starting density was used than for Rat1 cells.

5.2.6 Analysis of Results

As will be described later, one way analysis of variance suggested that the differences between the control groups in cell counts or mean GFR, were not significant. The results for the control groups (saline and normal) were therefore pooled to produce a grand mean and standard error of the mean. Results for each time point of bleomycin treated animals were treated separately. Comparisons between these and the combined control group were made with Student's t test.

5.3 RESULTS

5.3.1 Mortality of Study Group of Rabbits

Two animals died following bleomycin instillation, after six and thirteen days. In both cases the animals had continued to lose weight after injection. It was probable that they died from pulmonary insufficiency. Two further animals were used to fulfill the experimental protocol. All the other bleomycin treated animals showed an initial fall in weight after injection but had started to regain weight by seven days.

LAVAGE CELL POPULATIONS

5.3.2 Total Cell Yield and Cell Viability

The results for individual animals are laid out in

Appendix 1 and summarised in Table 5.1 as means (+/- SEM). In the controls, lavage cell yields were highest for the uninjected animals and declined in the days after injection. However one way analysis of variance indicated that the differences seen between the mean cell yields from the different control groups could have arisen by sampling variation. Examination of the detailed results (Appendix 1) reveals that one saline control, R87, produced an usually large number of lavage cells, at 58.5 x 10^6. The animal appeared healthy at the time of sacrifice, and the lavage cell differential was unremarkable with 99.1% macrophages. The significance of this was not clear.

Lavage returns from bleomycin treated animals contained more cells than from the control animals, reaching a peak at six days. The difference between saline control and bleomycin treated animal was not statistically significant at two days ($P=0.18$), but was so at the two later time points ($P<0.005$ for both comparisons).

Table 5.1 also displays the percent viability of the lavage cells, which was unaffected in the saline injected animals, but fell markedly for the cells lavaged from bleomycin treated animals to a nadir of 56.3% (+/-8.4) at six days.

The total yield and percentage viability can be combined to give a figure for the yield of viable cells harvested from each animal. This is detailed in the last column of Appendix 1 and is summarised in Table 5.1. The low mean viability of the lavage cells from six day bleomycin treated animals depresses the yield of viable cells, so that the highest number of viable cells was obtained from the fourteen day animals.

5.3.3 Differential Cell Counts

Macrophages accounted for more than 99% of the lavage cells from the control animals, with a single exception, R110 (a six day saline control), where the differential count was

TABLE 5.1 Total Cell Yields and Viability of Bronchoalveolar Lavage Cells

Days after injection	0	2	6	14
Controls				
Total cell yield $x10^6$ cells	29.1 (3.95)	27.3 (10.6)	13.9 (4.6)	10.3 (2.1)
Viability %	95.9 (1.8)	98.4 (0.7)	95.6 (1.2)	97.1 (0.9)
Viable cells $x10^6$ cells	28.0 (4.0)	26.9 (10.5)	13.1 (4.1)	10.0 (2.0)
Bleomycin treated animals				
Total cell yield $x10^6$ cells		41.8 (6.6)	92.5 (14.8)	88.9 (14.0)
Viability %		85.3 (3.7)	56.3 (8.4)	79.9 (6.5)
Viable cells $x10^6$		36.2 (6.4)	47.5 (7.4)	71.7 (14.8)

The Table summarises the yields of cells obtained at bronchoalveolar lavage from each group of rabbits as means (+/-SEM) of total cells, percentage viability as judged by Trypan Blue exclusion, and the yields of viable cells (derived from the first two measurements).

Treated animals received 10mg/kg body weight (1mg bleomycin = 1 International Unit).

95.6% macrophages, 3.4% lymphocytes and 1% neutrophils. The differential counts for the bleomycin animals are summarised in Table 5.2. In addition to the appearance in greater numbers of cell types other than macrophages, many degenerate cells were seen. These were characterised by their pyknotic, denser and less distinct nuclei. These were readily distinguishable from cells damaged by the cytocentrifuge preparation technique. In the latter the nuclear outline was sharp and well defined but the cell membrane was ruptured. The appearance of the "degenerate" cells strongly suggested that cell death had occurred some time before sacrifice of the animal. Almost without exception these degenerate cells could be recognised as neutrophils. The percent differential counts can be combined with total cell yield for each animal to calculate the numbers of each cell type obtained. The mean results for each group of animals are displayed in graphical form in Figure 5.2. Within each segment of the histogram is inscribed the mean yield for that cell type.

It can be seen from Figure 5.2 that in the acute phase of bleomycin injury, two days after instillation of the drug, there has been a massive efflux of neutrophils into the alveolar space. At later intervals after instillation the number of neutrophils falls markedly. Alveolar macrophages are recruited to the alveolar space with a slower time course, with yields rising up to fourteen days post bleomycin instillation. Other cell types are remarkable by their scarcity. Lymphocyte yields are highest at fourteen days but still only average 1.44×10^6 i.e., 2% of the total viable cell population. Eosinophils and basophils were seen in such small numbers as to not allow any general conclusion. It is not possible to combine measurement of cell viability with cell differential counting in a single technique. Most differential stains are applied to fixed preparations of cells on a slide, and therefore do not provide data for the viability of each separate cell type present. The techniques used to identify a particular type in unfixed viable preparations do not reliably distinguish between dead cells

TABLE 5.2 Differential Cell counts of the Lavage Cells from the Bleomycin Treated Rabbits

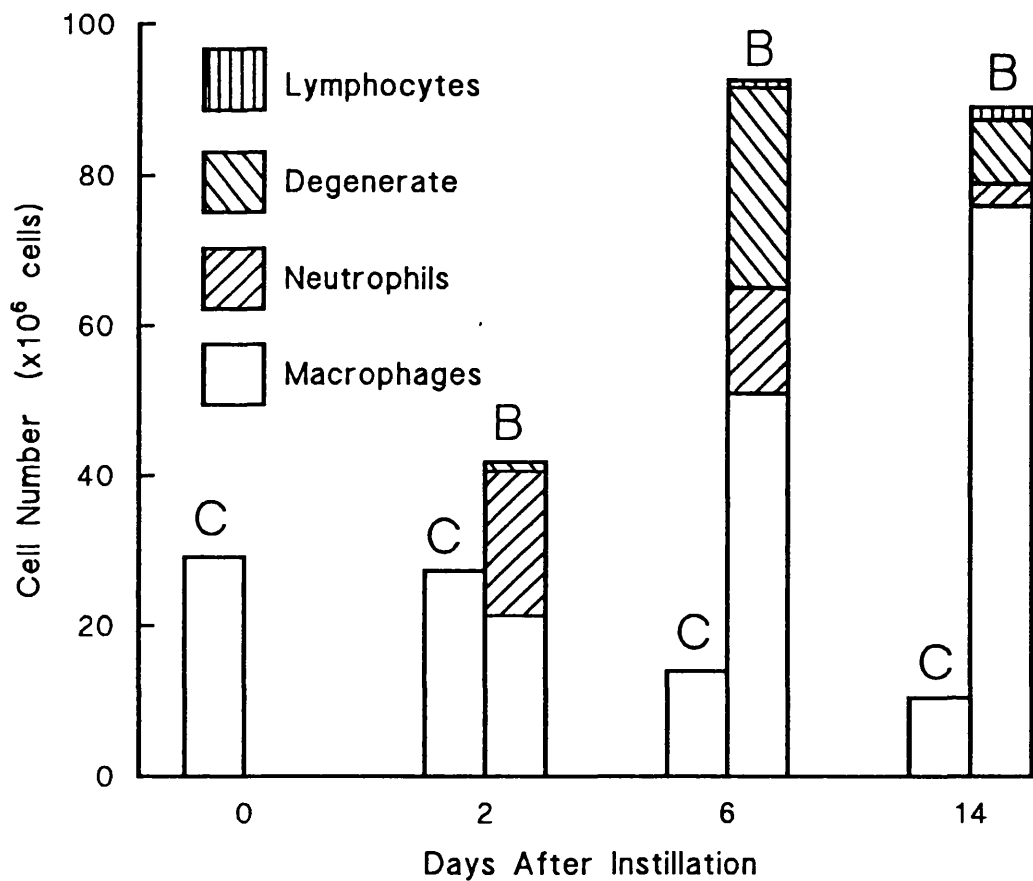
	Mφ	Nφ	Lφ	Deg	Other
	%	%	%	%	%
2 Day Bleomycin Animals					
	49.1 (4.8)	47.0 (5.1)	0.2 (0.1)	3.5 (1.0)	0.2
6 Day Bleomycin Animals					
	59.4 (7.6)	15.8 (2.0)	1.3 (0.6)	23.4 (8.2)	0
14 Day Bleomycin Animals					
	85.5 (4.4)	3.3 (0.8)	1.7 (1.2)	9.4 (4.4)	0.1

This Table summarises the differential counts of the bronchoalveolar cells lavaged from the bleomycin treated animals. The results are means (+/-SEM) for each group.

As the lavage cells from the control animals were an almost pure population of macrophages (see Section 5.3.3), the corresponding results for the controls are not tabulated.

Mφ = macrophage; Nφ = neutrophil; Lφ= lymphocyte;
Deg = degenerate cells; Other = basophils or eosinophils.

Figure 5.2 **Mean Cell Yields from Bronchoalveolar Lavage of Rabbits after Instillation of Saline or Bleomycin**



The mean total and differential cell yields for each group of animals are displayed above. The majority of degenerate cells were probably neutrophils (see section 5.3.3).

C = controls, B = bleomycin treated animals.

of that cell type and other cell types viable or not. However in the samples studied in these experiments, two cell types were predominant i.e., alveolar macrophages and neutrophils. As the macrophages were much larger than the neutrophils, and this was apparent in the preparations stained with Trypan Blue, it is possible to make some comment on the relative viabilities of the macrophages and neutrophils seen. Nonviable cells constituted a much greater proportion of the small diameter cells, i.e., neutrophils, than of the larger cells i.e., mostly macrophages.

The morphology of the alveolar macrophages from the bleomycin animals differed from that from control animals. In the case of the latter, alveolar macrophages had a very uniform size and appearance. Alveolar macrophages from bleomycin animals ranged in diameter up to three fold that of the "normal" alveolar macrophages. The cytoplasm of many of these cells contained many large vacuoles.

SECRETION OF GROWTH FACTORS BY LAVAGE CELLS

5.3.4 Growth Factor Assay Results

The results of assay of the lavage cell supernatants on Rat1 and R9ab cells and after dialysis are summarised in Table 5.3 and detailed in Appendix 2.

The average standard deviations of A650 seen in these assays for the replicate cultures was 0.0166 for Rat1 cell assays, and 0.0222 for R9ab assays which correspond to coefficients of variation of 2.1% and 3.6% respectively.

5.3.5 Correlation of the Observed GFR Obtained for Undialysed Samples in Two Separate runs on Rat1 Cells

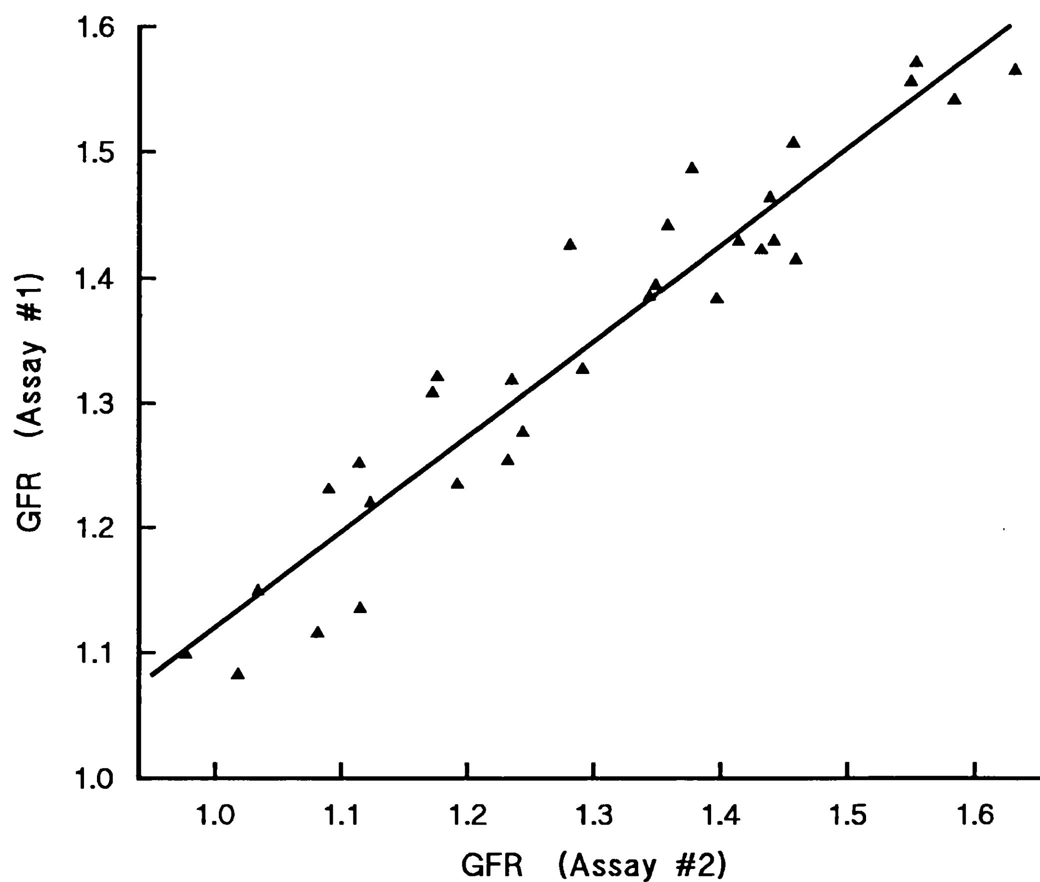
The correlation between values of GFR of these samples of conditioned medium in the two separate runs on Rat1 cells was good (Figure 5.3). Overall the second assay produced slightly lower GFRs than the first run. The mean difference

TABLE 5.3 **Growth Factor Secretion by Lavage Cells from Control and Bleomycin Treated Rabbits**

	Assay 1		Assay2		Mean of (1)&(3)
	(1) Rat1	(2) R9ab	(3) Rat1	(4) Rat1 dialysed	
Normal Controls					
	1.44 (0.021)	1.31 (0.039)	1.38 (0.022)	1.35 (0.021)	1.41 (0.017)
Saline Controls					
2 days	1.37 (0.075)	1.22 (0.066)	1.38 (0.100)	1.36 (0.035)	1.38 (0.088)
6 days	1.41 (0.104)	1.15 (0.033)	1.39 (0.116)	1.24 (0.116)	1.40 (0.110)
14 days	1.35 (0.011)	1.21 (0.041)	1.31 (0.119)	1.36 (0.133)	1.33 (0.099)
Bleomycin Treated Animals					
2 days	1.33 (0.029)	1.23 (0.033)	1.23 (0.054)	1.27 (0.029)	1.28 (0.041)
6 days	1.31 (0.074)	1.18 (0.030)	1.24 (0.092)	1.27 (0.085)	1.27 (0.078)
14 days	1.26 (0.060)	1.21 (0.031)	1.19 (0.065)	1.25 (0.099)	1.23 (0.068)

This Table summarises the data detailed in Appendix 2, of the results of the growth factor assays performed on media conditioned by cultures of lavage cells from control and bleomycin treated animals. The samples were assayed on two occasions on Rat1 cells (columns 1 and 3), and the mean of these two assays is shown in column 5. The samples were also tested on R9ab cells (column 2), and after dialysis on Rat1 cells (column 4). The results are means (+/-SEM) for each group.

Figure 5.3 **Correlation between the Two Assays for Growth Factor on Rat1 Cells**



The results from the two assays of the samples of lavage cell conditioned medium on Rat1 cells is displayed above as a scatter plot. The line is the best fit by least squares linear regression analysis.

of corresponding pairs of GFR was 0.050, the standard deviation of the differences was 0.060, and the correlation coefficient (linear regression) between the two sets of data was 0.954.

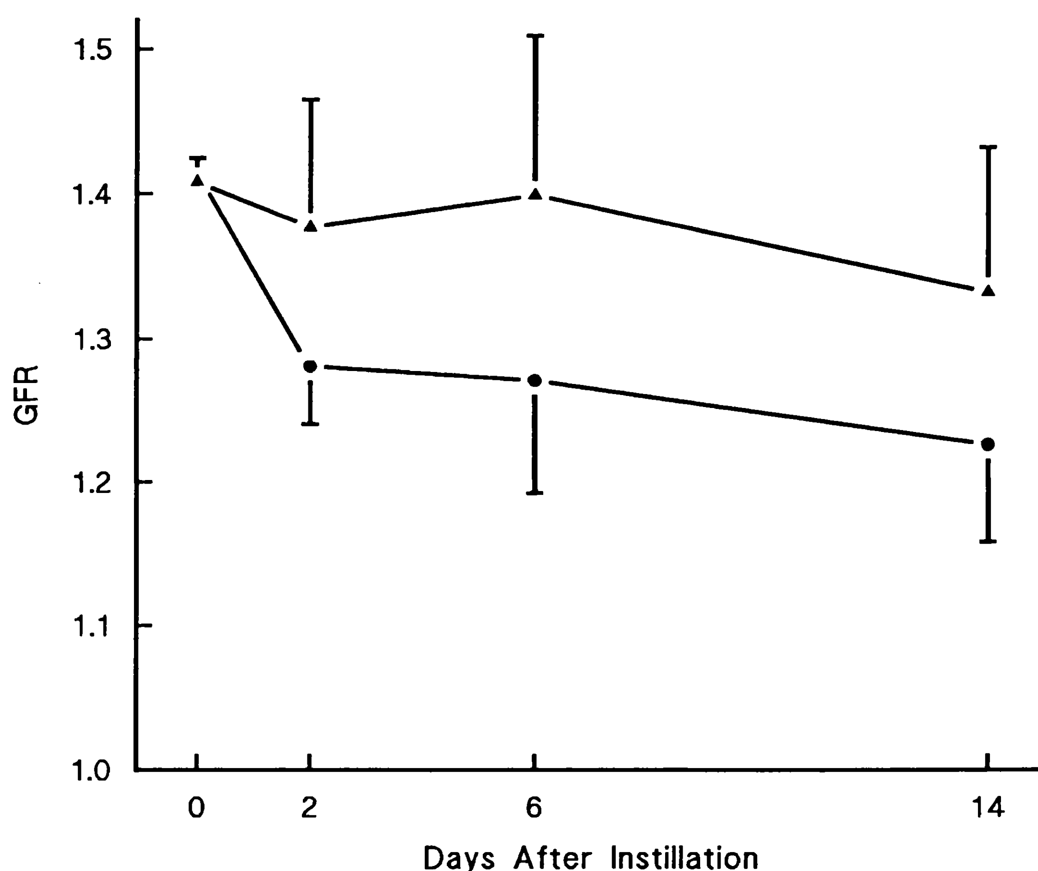
Conditioned medium from lavage cells from uninjected animals, saline control and bleomycin treated animals stimulated additional growth of Rat1 cells when compared to medium controls. There was one sample which seemed to be an exception to this. In the second assay run, conditioned medium from a six day bleomycin animal, R80, gave a GFR of 0.977 i.e., a 2.3% lower final density (A650) of the target fibroblasts than seen with medium control. However the confidence limits of the GFR obtained extended above unity, (0.936 to 1.018). It is probable that this minor apparent inhibition arose by chance variation in the replicate culture. In support of this the GFR of this sample assayed in the first run was 1.098.

5.3.6 Growth Factor Secretion Assayed on Rat1 Cells

Table 5.3 details the mean GFR +/-SEM for each group of animals tested in each assay run. Corresponding results have been calculated from the mean of each pair of estimations of GFR of the undialysed samples on Rat1 cells. The subsequent description of the results is based on these combined data. Figure 5.4 displays the latter results graphically. The mean GFR for all four groups of control rabbits are similar and one way ANOVA confirms that there is no significant difference between them. The injection of saline does not stimulate alveolar cells (largely macrophages) to produce more (or less) growth factor activity.

Each of the mean GFRs for the bleomycin treated groups is lower than its corresponding control group. However these differences do not attain statistical significance (Student's t test). The same conclusion holds when comparisons are made with the pooled results of all the control animals (normal + saline).

Figure 5.4 **Growth Factor Secretion by Lavage Cells from Bleomycin Treated and Control Animals Assayed on Rat1 Cells**



This figure illustrates the effect of instillation of saline or bleomycin on GF secretation by lavage cells, assayed on Rat1 cells. Each point is the mean of 4 (controls) or 5 (bleomycin treated) animals, and error bars are standard errors of the mean. These results are derived from the mean of two assays (See Table 5.3, and Section 5.3.6).

The differences observed between control and bleomycin treated animals are not statistically significant (Student's unpaired t test, two tailed, $P>0.05$ in all cases).

Data from control animals are plotted as ▲, and from bleomycin treated animals as •.

5.3.7 Growth Factor Secretion Assayed on R9ab Cells

Appendix 2 and Table 5.3 show the detailed results and the group means, respectively. The latter are displayed graphically in Figure 5.5.

The pattern of results of testing on these rabbit lung fibroblasts (R9ab cells) is similar to that for samples tested on Rat1 cells. One way analysis of variance showed no significant difference between the normal and saline injected control groups. The overall mean GFR for all four control groups tested on R9ab cells was 1.22 +/-0.026. The GFR secretion of lavage cells from bleomycin treated animals was very similar to their corresponding saline injected control groups. At each time point the mean GFR observed in the bleomycin animals did not differ significantly from that of the corresponding saline controls, nor from the overall mean GFR of all 16 control rabbits. The lack of demonstration of increased growth factor activity measured on Rat1 cells in bleomycin animals is not due to species difference.

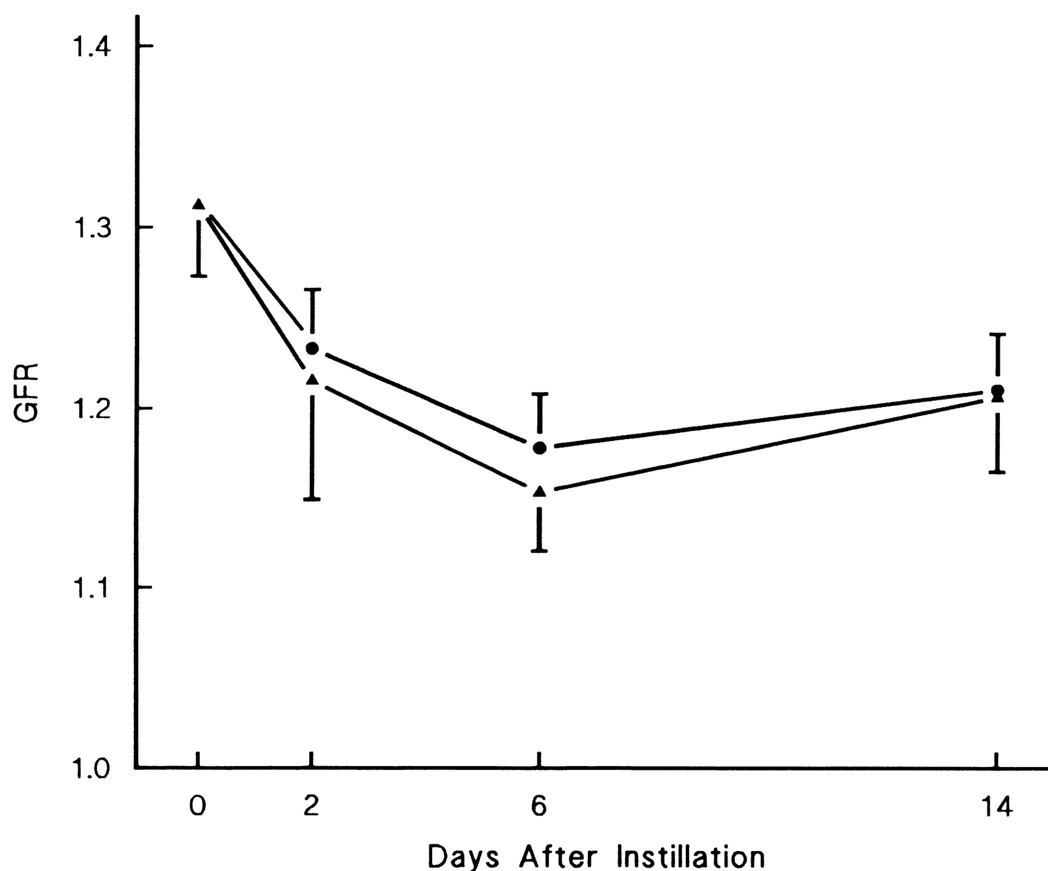
5.3.8 Correlation Between GFR Obtained with Rat1 and R9ab Cells

The values of GFR obtained on R9ab cells are plotted against the corresponding values estimated using Rat1 cells (mean of two assays) in Figure 5.6. There was a positive correlation between them, but this was weak ($r = 0.465$). Overall the GFRs observed were lower than the absolute values seen with Rat1 fibroblasts. No sample inhibited replication of the rabbit fibroblasts, however.

5.3.9 Effect of Dialysis of the Conditioned Medium

The results obtained are detailed in Appendix 1 and summarised in Table 5.3. The correlation is illustrated in Figure 5.7 as a scatter plot. There was a slight trend for higher GFRs in dialysed specimens from bleomycin animals

Figure 5.5 Growth Factor Secretion by Lavage Cells from Bleomycin Treated and Control Animals Assayed on R9ab Cells.

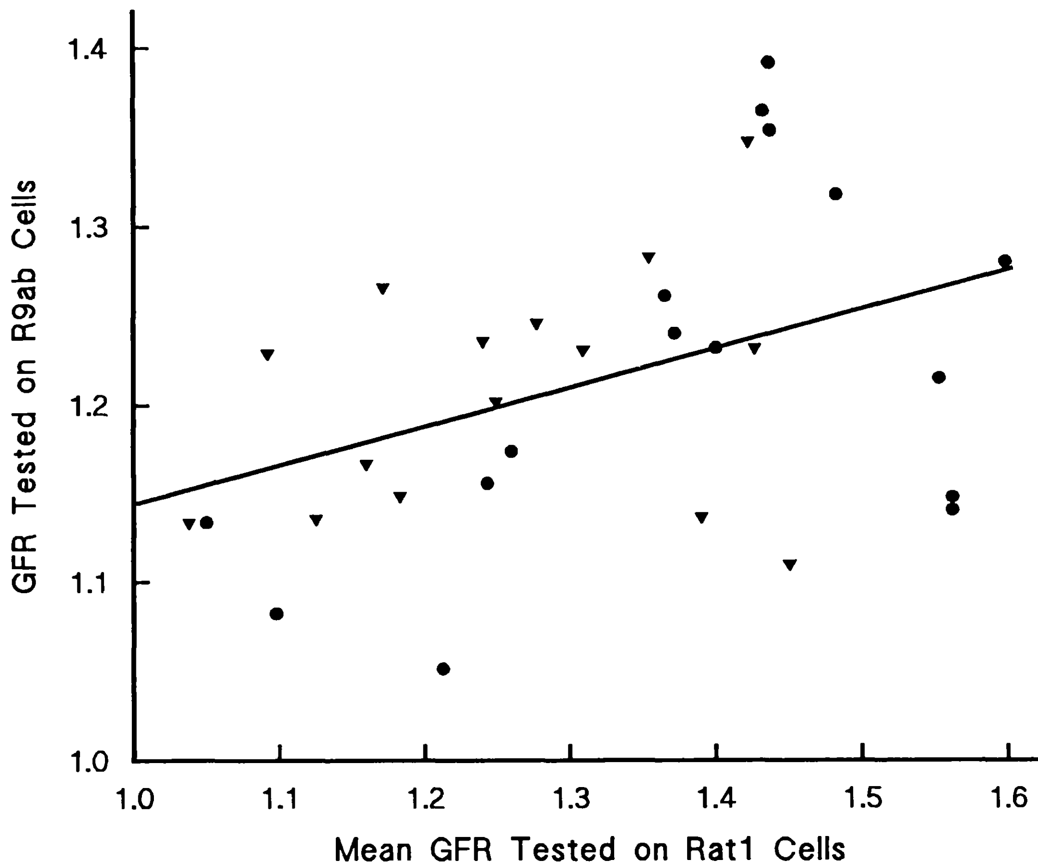


This illustrates the effect of instillation of saline or bleomycin on GF secretion by lavage cells, as assayed on R9ab cells. The layout of the figure is identical to Figure 5.4.

GF secretion by lavage cells from control and bleomycin treated animals do not differ significantly at each time point (by Student's unpaired t test, $P>0.05$).

Data from control animals are plotted as ▲, and from bleomycin treated animals as •.

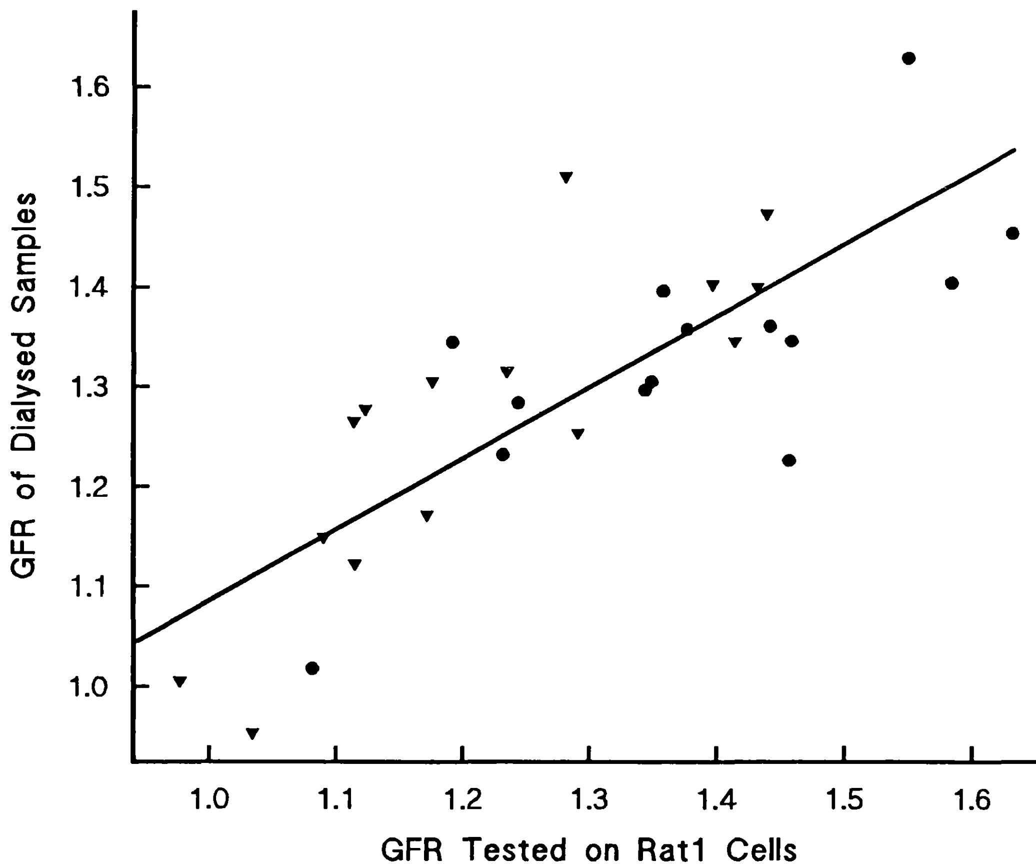
Figure 5.6 **Correlation between Assays for Growth Factor Secretion on Rat1 Cells and R9ab Cells**



This is a scatter plot of the results of the GF assay for the samples of conditioned medium assayed on R9ab cells, and Rat1 cells (mean of two assays). The correlation is weak. The line is the best fit by least squares linear regression analysis.

Data from control animals are plotted as •, and from bleomycin treated animals as ▼.

Figure 5.7 **Correlation between Assays for Growth Factor Secretion on Dialysed and Undialysed Specimens, using Rat1 Fibroblasts**



This is a scatter plot of the results of the GF assay performed on samples of conditioned medium which had been dialysed (as described in Section 4.2.7), and on undialysed samples. Both sets of samples were assayed simultaneously on Rat1 cells. The line is the best fit by least squares linear regression anaylsis.

Data from control animals are plotted as •, and from bleomycin treated animals as ▾.

compared with undialysed. However this trend was not completely consistent and not of great magnitude so that the effect on the mean GFR of each group of rabbits was small. The low molecular weight mediators are not having a significant effect on the measured GFR, even in conditioned medium from bleomycin treated animals.

Statistical comparison of control and bleomycin treated animals at each time point supports this conclusion. The unavailability of results for dialysed specimens from two saline control animals (at six and fourteen days) complicates interpretation, but accepting this limitation, no significant difference is seen at any time point.

5.4 DISCUSSION

5.4.1 Lavage Cells

Intratracheal instillation of saline alone did not produce any discernible change in the cell types and cell viability seen at lavage. Macrophages continued to account for more than 96% of cells. There was a trend to lower overall cell yields in the saline controls in the days after injection, but this probably arose by chance, as indicated by analysis of variance. Closer examination of the detailed results supports this suggestion. The apparent falling trend in cell yield in the days after instillation of saline is in large part due to the high cell yield of the normal controls (mean 29.1×10^6). This was higher than was obtained from normal rabbits in other experiments - for example compare the mean yield of 17.6×10^6 of the 16 animals used in the measurement of AM protein synthesis *in vivo* (Section 2.3.3). It was also higher than found by Shen et al (1988) who obtained an average cell yield of 16.1×10^6 from lavage of New Zealand White rabbits. The mean cell yield of the two day saline

controls was inflated by the presence of an outlier with an exceptional cell yield of 58.5 x 10^6 in the two day saline control group. Exclusion of this outlier reduces the mean cell yield of this group of animals to 16.9 x 10^6. Accepting that the instillation of saline does not affect cell yields, the figures for all control animals were pooled for comparison with the yields of the bleomycin treated animals.

The tracheal instillation of bleomycin lead to a rapid and marked rise in alveolar cell numbers, and an evolving pattern of influx of other cell types in keeping with an acute inflammatory insult. The initial response was the appearance of neutrophils which were most numerous at two days. As would be expected in any acute inflammatory cellular exudate the neutrophils, which have a short lifespan, were mostly viable at two days but by six days many were degenerate. Thereafter the proportion and absolute numbers of neutrophils fell and they were replaced by increasing numbers of macrophages, and a much smaller proportion of lymphocytes. These results are in accord with the findings of Kaelin et al (1983), who performed bronchoalveolar lavage on rats following intratracheal instillation of bleomycin. One day after bleomycin, neutrophils were 52.2% of the lavage cells obtained (as compared with 1.9% in controls), falling to 41.6% at four days, and 11% at eight days. Although there is not a direct link between the populations of lavage, i.e., alveolar cells and the inflammatory cells in the interstitium, it is reasonable to assume that changes in the former do broadly reflect changes in the latter. This is supported by the work of Chandler et al (1983) who examined by morphometric techniques the interstitial cell populations after bleomycin administration in hamsters. They found an early rise in neutrophil numbers, maximal at seven days, and a slower rise in macrophages, peaking at twenty eight days. Intratracheal bleomycin causes considerable tissue damage (Laurent et al, 1981), leading to increased phagocytic activity by macrophages. This would account for the dramatic cytoplasmic changes seen in the alveolar macrophages with the

appearance of large and heavily vacuolated cells, which have been described by other authors (Suwabe et al, 1989).

5.4.2 Growth Factor Studies

These experiments found no increase in growth factor secretion per cell from inflammatory cells lavaged from the lungs of bleomycin treated rabbits. The experiments also provided information to rule out a number of explanations of this observation.

Assay of the samples on two separate occasions on Rat1 cells yielded similar results. The good correlation seen was in accord with the experiments described in chapter 3. Thus the assay findings were reproducible.

It is possible that a number of modulators of fibroblast replication were being secreted by the lavage cells. The conflict between their inhibitory and stimulatory effects might mask any increase in growth factor secretion. To completely address this question would require the development of techniques to identify and separate these putative mediators, so that they each could be quantitated individually. This was not attempted in this work. Although a number of AM products may have an inhibitory effect on fibroblast growth, two classes of mediators seem likely candidates. One is a medium molecular weight polypeptide secreted by hamster (Clark et al, 1983) and human (Dayer et al, 1985; Elias et al, 1985) macrophages which causes fibroblasts to increase their autologous PGE_2 secretion and hence downregulate fibroblast replication. Earlier experiments (Section 4.3.8, discussed in Section 4.4.2) found no evidence of secretion of this factor when rabbit AMs were stimulated by zymosan.

Another class of inhibitory mediators secreted by macrophages and neutrophils which might have confounded the measurement of growth factors in this system is the arachidonic acid metabolites, particularly PGE_2 itself. It was for this reason that the samples were dialysed and

reassayed on Rat1 cells. However dialysis did not lead to a qualitative change in GFR, so that secretion of arachidonic acid metabolites in sufficient amounts to affect fibroblast replication can be excluded.

The correlation between the GFR of dialysed and undialysed samples (Figure 5.7) was not as close as that between the two assay runs on the same undialysed samples. It is noteworthy that there is no significant trend in the change in GFR resulting from dialysis. It is possible that there is more than one low molecular weight factor present in these conditioned media, perhaps with some having growth promoting and some with growth inhibiting activity. Thus dialysis would have an effect on the GFR, with the direction of change depending upon the relative proportions of these factors. However such factors cannot have been present in large amount, as dialysis did not qualitatively change the observed GFR.

A more mundane and likely explanation for the scatter seen in Figure 5.7 is that the handling of these small samples in the process of dialysis might affect the GFR. An example of this is that if a sample contained in dialysis tubing is left in an air stream, water will pass through the tubing and evaporate, concentrating the sample. These were small samples and they were manipulated in a laminar flow hood for a time, so such an effect may have occurred. Another possibility is adsorption of the growth factor to the dialysis tubing. These effects may have contributed to the unpredictable but minor changes in GFR seen with dialysis.

5.4.3 Choice of Target Cell Lines

The species difference between the experimental animals and the fibroblast used in the GF assay might explain the failure to detect a change in secretion of growth factors. The choice of cell line is partly philosophical and partly pragmatic. One philosophical issue was whether it was important that the target cell line was a "published" one and

therefore available to other investigators. The advantages of this seemed significant and the cell lines used (Rat1, R9ab) reflected this. A second philosophical question was whether the target cell culture system is simply a biological assay for GF or whether it is a model of events *in vivo*. The largest part of these experiments adhered to the first principle. This allowed the pragmatic choice of a target cell line, Rat1, which was robust in cell culture, strongly adherent to the plastic culture vessels, and very sensitive to growth factor stimulation. Preliminary experiments had shown Rat1 cells to be more sensitive than a variety of other human and rabbit fibroblasts to both serum GFs and rabbit AM secreted products.

The samples of conditioned medium were also assayed on R9ab cells. This is closer to being a model of events *in vivo* in that these cells are fibroblasts obtained from New Zealand White rabbit lung. Moreover they are a published line available from the American Tissue Culture Collection, and therefore accessible to any research worker. Again no significant difference in growth factor secretion was seen between control animals and bleomycin treated animals. This supports the findings of the assays on Rat1 cells and confirms that species difference between experimental animal and fibroblast employed in the GF assay did not account for tha absence of significant response in GF secretion by lavage cells following bleomycin administration. A difference in the absolute values of GFR estimated on the two fibroblast lines was to be expected, but the correlation between the two measures was not strong. The reasons for this were not investigated.

5.4.4 Culture Conditions of Lavage Cells

The lavage cells were cultured in medium containing 2% NCS and without prior separation of the different cell types. In consequence there was a reduction in the absolute

concentration of AMs in the cultures from bleomycin treated animals as compared with controls. In the latter cultures other cell types (lymphocytes, neutrophils) amounted to less than 4% of the total, so that the concentration of AMs was very close to the total cell concentration of 10^6/ml. In the cultures of lavage cells from bleomycin animals, AMs were diluted by other cell types, so that their concentration was reduced, to a mean of 4.91 x 10^5/ml for the two day animals, and higher concentrations for animals lavaged later. Assuming that AMs were the source of the GFs detected in the assay, then this dilution of AMs by other cell types in the lavage cell cultures might explain the trend to lower GFR noted in the samples from bleomycin animals. A precise correction for this dilutional effect is not possible - the relationship between AM concentration and GFR is not proportional as was demonstrated in Section 4.3.2. However, as GF levels from cells from the bleomycin animals, tested on Rat1 cells, were lower than controls it is unlikely that the use of equal concentrations of AMs would have altered the final conclusions.

The decision to culture the lavage cells without prior separation of cell types stemmed from the desire to mirror the environment *in vivo* where the different inflammatory cell types are present in the inflamed lung. Although the AM is the most likely source of GF secretion, lymphocytes have been shown to secrete GF (Wahl and Gately, 1983; Neilson et al, 1980). Lymphocytes were only a small part of the lavage cell population from bleomycin treated animals, even at fourteen days post instillation (when they were 1.7% of the lavage cells), and are unlikely to have made a significant contribution to GF secretion.

The lavage cells were cultured in medium containing 2% NCS. Workers in this field have used a variety of different culture conditions. Bitterman et al (1982) used fibroblasts quiescent in 0.4% serum, Kovacs and Kelley (1986) a similar system with quiescent fibroblasts in 1% serum, while Martin et al (1981) used a fibroblast culture medium containing

plasma derived serum to induce quiescence. All of these systems permit only the detection of stimulation of growth. Others (Elias et al, 1985; Clark et al, 1982) have used fibroblasts actively growing in 10% serum. This choice favours the detection of inhibitory effects of the test supernatants (the findings in both of those papers). The complexities of the situation are illustrated in work by Clark et al (1983) who describe an inhibitory effect on fibroblast growth in 10% serum by medium conditioned by AMs from bleomycin treated hamsters, but a stimulation of fibroblast growth in 0.4% serum by the same conditioned medium.

The choice of 2% NCS for the culture medium ensured that the fibroblasts were in log phase but submaximal growth for 72 hours, so that the potential existed for the detection of either a predominant stimulatory or inhibitory effect of the lavage cell supernatants.

5.4.5 Basal Secretion of Growth Factors by Lavage Cells from Control Animals

All the control supernatants showed growth promoting activity. Reported basal levels of secretion of growth or inhibitory factors have varied greatly. Direct comparisons are difficult because of the enormous variety of species, culture media and techniques of quantitation of proliferation used. Examples include a 160% increase in [^{3}H]thymidine incorporation by fibroblasts in response to medium conditioned by unstimulated AMs from sheep (Lemaire et al, 1983); a 114% increase seen with conditioned medium from human AMs (Dayer et al, 1985); while zero basal secretion of growth factor by unstimulated human AMs was found by other authors (Bitterman et al, 1983). Reports of inhibitory factors have described significant basal levels of secretion by AMs. Elias et al (1985) found 44% inhibition of fibroblast [^{3}H]thymidine incorporation by supernatants from control human macrophages, and Clark et al (1983) found 41.9%

inhibition by samples from hamster AMs.

Thus the finding in the present work of high levels of mediator secretion by unstimulated AMs from control animals is in accord with the experience of many but not all of the workers in the field. It is possible that this is an artefact of the experimental procedures, and that alveolar macrophages *in vivo* are truely quiescent. The results of experiments described in chapter 2 on AM protein synthesis *in vivo* and *in vitro* support this view. Evidence to the contrary is provided by the demonstration that bronchoalveolar lavage fluid from normal animals also shows similar activity to that secreted by AMs in culture systems *in vitro*. Clark et al (1982) found that lavage fluid from control animals inhibited [^{3}H]thymidine uptake of fibroblasts by 35%, (and lavage fluid from bleomycin treated animals caused a 53% inhibition). This suggests that the alveolar macrophages were actively secreting this inhibitory factor *in vivo*. Until other measures of macrophage activation *in vitro* and *in vivo* are available for study, or until techniques are developed to quantitate the secretion rate of polypeptide mediators *in vivo* this question will remain unresolved.

5.4.6 The Role of Growth Factor Secretion by Lung Inflammatory Cells in Bleomycin-Induced Lung Fibrosis

The total lung burden of macrophage growth factors will be the product of the number of macrophages present and the rate of secretion per cell of growth factor. The present work has shown that the total number of inflammatory cells obtained by bronchoalveolar lavage rose rapidly after bleomycin instillation. In particular the increase in macrophage numbers was marked by six days and was maximal at fourteen days, when the numbers of AMs obtained by lavage was approximately sixfold higher than from the corresponding saline controls. Thus, with an unchanged but significant secretion rate of growth factor per inflammatory cell, the total lung burden of growth factor will mirror the changes in

inflammatory cell numbers, i.e., increase substantially after bleomycin instillation. Therefore the increase in number of lung fibroblasts seen after bleomycin instillation is probably attributable to these growth factors. Laurent and McAnulty (1983) found that the collagen synthesis rate in the lungs of rabbits treated with bleomycin is highest at six days, and had fallen a little by fourteen days. One might expect that the stimulus to fibroblast replication would precede the peak in collagen synthesis, yet alveolar macrophages numbers and therefore total growth factor "burden" were greater at fourteen days. This discrepancy may not be as great as it first appears. In the absence of multiple time points for the data between six and fourteen days post bleomycin (both for cell yields, and for lung collagen metabolism) it is not possible to be confident of the precise time of peak lavage cell yield and lung collagen synthesis - both might lie close together in this interval.

CHAPTER 6

CONCLUSIONS, FUTURE DIRECTIONS AND AN OVERVIEW OF THE PATHOGENESIS OF PULMONARY FIBROSIS

This work has focused on the alveolar macrophage, starting with a comparison of its state of activation, as measured by protein synthesis rate *in vivo* and *in vitro;* then turning to the characterisation of one of its secretory products - growth factor for fibroblasts; and finally studying the role of growth factors secreted by alveolar macrophages and possibly also by other inflammatory cells in a rabbit model of pulmonary fibrosis. As part of this process the Methylene Blue assay of adherent cells in microwell culture was developed, and applied to the assay of growth factors.

6.1 CONCLUSIONS

6.1.1 Alveolar Macrophage Protein Synthesis Rate and Activation

The macrophage is responsive to a wide range of stimuli, soluble and particulate; and of mineral, microbiological or mammalian origin. In response to these stimuli it may replicate, migrate, attach to appropriate surfaces, phagocytose particles, or release some of its approximately 100 different secretory products (Hocking and Golde, 1979; Nathan, 1987). Not all of these functions are enhanced at one time, so that "activation" of the macrophage will have different meanings, in different contexts. Nonetheless the rate of synthesis of intracellular proteins of the macrophage

provides a general measure of activation, and its quantitation in the alveolar macrophage permitted a comparison of the state of activation a single cell population *in vitro* and *in vivo*. This work has shown the alveolar macrophage in suspension culture to have a fivefold higher rate of protein synthesis than the same cells *in vivo*. By this parameter at least, the alveolar macrophage has been activated by the process of isolation and suspension culture. The steps involved in the isolation of these cells were atraumatic by the standards of most protocols for the establishment of cell lines. No enzymatic or mechanical disaggregation of tissue was necessary. They were subject only to three washing steps by low speed centrifugation before suspension culture. The cells were obtained as an almost pure population by lavage of the alveolar spaces of the lung. Thus it is difficult to envisage techniques of isolation which would be less likely to stimulate the cell.

The isolated alveolar macrophage is an active cell as judged by the synthesis rate of intracellular protein. This synthetic activity will involve many proteins, and these have not been characterised in these studies. It would be difficult to achieve a sufficiently high specific radioactivity of individual proteins *in vivo* to determine which particular proteins are produced at a greater rate with activation of the cell *in vitro*. An alternative parameter which could be studied is the level of transcription of individual, well characterised genes *in vivo* and *in vitro*. The present work did not look at synthesis rates for secreted proteins such as growth factors. It is conceivable that synthesis of intracellular proteins is selectively activated, and it cannot be assumed that growth factor secretion by alveolar macrophages is increased by harvesting and culture of these cells. Nonetheless these findings indicate that caution is needed in extrapolating results obtained by experiment on alveolar macrophages *in vitro,* to the situation *in vivo*.

6.1.2 The Methylene Blue Assay and Growth Factor Secretion by Alveolar Macrophages

The Methylene Blue assay proved rapid, accurate and reproducible. Its utility has been demonstrated by the experiments described in Chapters 4 and 5. In particular the figures detailed in Appendix 2 represent the results of assay of two sets of approximately 400 individual fibroblast cultures in microwell plates. Each set was assayed in a single day. Although immunoassays of well documented cytokines are now available, there will still be a need for biological assays of the functional activity of samples containing known of unknown cytokines (see Section 6.2.2). The Methylene Blue assay is ideally suited for the assay of growth factors and will find wide application. The work described in Chapter 4 demonstrated that rabbit alveolar macrophages are able to secrete factors which increase the replication of fibroblasts. This activity has the properties of a cytokine growth factor i.e., it is nondialysable (M.Wt. 14,000), heat and trypsin sensitive, and its secretion is active and largely inhibited by cycloheximide.

6.1.3 Growth Factor Secretion by Inflammatory Cells Lavaged from the Lung of Bleomycin Treated Rabbits

In Chapter 5 it was shown that after instillation of bleomycin into the lungs of New Zealand White rabbits, there is a rapid influx of inflammatory cells into the alveoli, predominantly macrophages at six and fourteen days. These cells continue to secrete growth factors. The combination of these two observations implies that the burden of growth factor within the alveolar spaces rises rapidly at six and even higher at fourteen days after bleomycin instillation, and is likely to be an important pathogenetic mechanism in the pulmonary fibrosis which follows.

6.2 THE EXPERIMENTAL STUDY OF CYTOKINES AND THEIR EFFECTS

6.2.1 Complexities of Cytokine Networks

The work described in Chapter 5 involved testing crude supernatants from cultures of a mixture of cell types lavaged from an inflamed lung. This approach is relevant to the situation *in vivo,* but further experimental examination of this complex model of pulmonary fibrosis should attempt to define the nature of the mediators present in this supernatant, their actions and to confirm that macrophages rather than other inflammatory cells are their source. The design of these experiments must take into account evidence which has accumulated in the last decade of the complexity of the network of cytokines and their effects. Growth factors are examples of a class of compounds called cytokines. These are extracellular signalling proteins which alter the behaviour of other nearby cells, via a receptor binding mechanism. Although this thesis has been in large part concerned with the growth promoting or inhibiting actions of cytokines, many other types of activity have been demonstrated such as the stimulation of secretion of cell products or the activation of cell functions (chemotaxis, cytotoxicity, phagocytosis, expression of cell receptors). Of the approximately 100 secretory products of macrophages which have been identified (Nathan, 1987) some 20 would be described as cytokines. The dividing lines between different classes of secretory products are not clear cut so that other products may have cytokine-like actions e.g., macrophages can secrete fibronectin, a cell adhesion protein, which has also been shown to stimulate fibroblast growth (Rennard et al, 1981).

The range of products of a single cell type is echoed, fortunately to not quite the same extent, by the number of inflammatory cell types involved in pulmonary fibrosis. In the present work, macrophages and neutrophils were increased

in the lavage returns from bleomycin treated rabbit lungs, and at later time points lymphocytes, and low numbers of eosinophils and mast cells were observed. All of these cells can secrete cytokines (Shock and Laurent, 1990), although the macrophage is preeminent. Most cytokines are not unique to a single cell type, and many are secreted by many different cells, both inflammatory and structural. Thus, PDGF is synthesized by megakaryocytes, endothelial and epithelial cells (Sariban et al, 1988), activated monocytes (Martinet et al, 1986), macrophages (Martinet et al, 1987; Bauman MD et al, 1987) and by mesenchymal cells.

A complex situation is made more so by the observation that individual cytokines have many distinct actions and these overlap with other cytokines. Nathan (1987) lists approximately 65 actions of the two cytokines interleukin-1 and tumour necrosis factor (TNF), most of which are common to both. Thus the culture medium of even a pure population of a single inflammatory cell, such as the macrophage, will contain multiple cytokines, of which several may have a direct effect on the particular activity of the target cell being assessed. These other cytokines may also have indirect effects of equal importance, either by modulating (generally inhibiting) the rate of secretion of the index cytokine or by altering the sensitivity of the target cell to the index cytokines. An example of the former effect is the demonstration that macrophages may secrete PGE_2 and the presence of this prostaglandin in the macrophage culture medium inhibits secretion of PDGF by the cell. An important corollary is that the rate of secretion of the index cytokine might fall off with time because of a rising concentration of other cytokines in the medium. An example of modulation of target cell sensitivity was described by Bitterman et al (1986) who showed that Interleukin-1 was not itself active as a "primary" growth promoting signal for fibroblasts, but that its presence in the fibroblast culture medium augmented the effect of alveolar macrophage derived growth factor. The target cell in a biological assay may itself secrete

cytokines which alter its own behaviour in the assay. This "autocrine" behaviour is illustrated by the secretion by fibroblasts of PGE_2, an inhibitor of fibroblast growth (discussed in more detail in Section 4.4.2). This provides a mechanism of action of an inhibitory polypeptide secreted by alveolar macrophages (Clarke et al, 1985). By the same token, switching off of autocrine production by fibroblasts of PGE_2 may be a mechanism of action of cytokine growth factors. Experimental evidence for this was described in Section 4.3.8 and discussed in Section 4.4.2. The precise experimental conditions used in a biological assay of a putative cytokine may have a profound effect on the result achieved. An example of this is the marked influence of the concentration of serum used in the assay of growth factors on fibroblasts (see Section 5.4.4).

Equally important is the choice of target cell used in the assay. An enormous number of cell lines are now available from both national organisations such as the American Type Culture Collection and from commercial suppliers of tissue culture reagents. These cells are derived from a range of different species, organs and at different stages of development of the donor animal. It is to be expected that these fibroblast cell lines will differ both in the degree of expression of cytokine receptors and in the level of autocrine secretion of growth factors by the cells. For example, autocrine secretion of growth factors may be a feature of some cell lines but not others. Clemmons et al (1981) showed that human fibroblasts but not BALB/c 3T3 mouse fibroblasts produced somatomedin (a progression growth factor) in response to PDGF. Thus some lines may be highly dependent on exogeneous growth factors for replication and others may be largely self sufficient (as in transformed cells).

6.2.2 Pitfalls of Physicochemical and Immunological Assays of Cytokines

The complexity of cytokine actions and interactions described above make it difficult to design a specific and reproducible biological assay for a cytokine, and therefore make very attractive the alternative approach of a direct assay of the concentration of a cytokine in a sample of medium.

Separation of the polypeptides in a sample of medium by physical means such as molecular sieve chromatography, gel electrophoresis etc, has been an essential stage in the isolation and purification of cytokines. However these procedures are too laborious to be used routinely for assay purposes. The availability of polyclonal antibodies to individual cytokines has permitted the development of radioimmunoassays and ELISAs. Unfortunately the detection of a cytokine in a sample by immunoassay does not necessarily equate with the presence of a functional form of the polypeptide - it may require proteolytic cleavage for activation, as has been shown for transforming growth factor B (Lyons et al, 1988), or be bound to another protein rendering it inactive e.g. the binding of PDGF to α_2-macroglobulin (Shimokado et al, 1985). Thus tests of biological activity of a cytokine will always be essential to their study, as well as providing the first indication of the presence of a new cytokine and providing a rapid screening test for established mediators.

6.2.3 Study *in Vitro* or *in Vivo*??

The study of the secretion of cytokines by cells maintained *in vitro,* and of the functional activity of these cytokines on pure populations of target cells has lead to an explosion of knowledge of these complex control systems. It is important that it is not assumed that cells isolated from an experimental animal are always in the same functional

state as existed *in vivo*. The work described in Chapter 2 of this thesis provides evidence of activation of alveolar macrophages by the process of their isolation. The other side of the coin may be equally important. It is conceivable that cells activated *in vivo* by some pathological process, may not remain in a stable activated state after prolonged culture *in vitro*. This possible functional decay should be looked for in experimental systems *in vitro,* and, where possible, confirmatory evidence of activation sought *in vivo*. The presence of cytokines in appropriate tissue fluids will support the finding of active secretion of cytokines by isolated cells *in vitro*. With the development of gene probes, it is now possible to show transcription of the genes for certain cytokines in tissue from experimental animals.

6.3 FUTURE STUDIES IN THE ROLE OF CYTOKINES IN THE PATHOGENESIS OF PULMONARY FIBROSIS

Decisions as to future directions of study of the bleomycin model of pulmonary fibrosis must take into account the growing appreciation of the complexity of cytokine networks referred to in the preceding sections.

6.3.1 Characterisation of Secreted Cytokines

To define further the importance of the secretion of cytokines by lung inflammatory cells it would be necessary to

1. Achieve separation of the different cell types obtained at lavage of bleomycin treated animals.
2. Confirm that the growth promoting activity derives from alveolar macrophages.
3. Separate and characterise the different cytokines produced which have mitogenic activity. As a first step this might be on a physicochemical basis - molecular sieve

chromatography to determine molecular weight, and ion exchange chromatography or gel electrophoresis for further purification and characterisation. The use of absorbing antibodies might allow assignment of the growth factor activity to one of the cytokine "families".

6.3.2 Quantitation of the Rate of Cytokine Secretion

The measurement of secretion rates of the cytokines in a way which allows comparison with the findings of other workers would require careful examination of the effect of differing protocols of culture of these inflammatory cells. Questions to be addressed include:-

1. Is the secretion rate of these cytokines steady and if so over what period of time?
2. If there is a decline in secretion rate with time, is this due to the build up of other cytokines in the culture medium? Can this be overcome by a programme of frequent changes of culture medium? What is the precise nature of these negative feedback loops on cytokine secretion?
3. Is it possible to develop a standard serum free medium for the culture of these inflammatory cells? This would simplify subsequent bioassay of the conditioned medium.
4. Is the basal secretion of cytokine by alveolar macrophages, observed in this work, a result of the isolation procedure used? The examination of lavage fluid for the presence of these cytokines and if available, the use of gene probes for the detection of synthesis of the cytokine m-RNA in tissues would allow this question to be answered.
5. If the isolation procedures are the cause of the cell activation, can they be modified to minimise that effect?

6.3.3 The Growth Factor Assay

The Methylene Blue assay is a powerful tool in the study of growth promoting effects of cytokines. Bioassay will continue to be a vital part of the study of growth factors

and is complementary to, and not supplanted by, quantitation of cytokines by immunoassay. However bioassay needs to be refined, so that the assay answers more specific questions, and standardised, so that the results of different laboratories can be compared. These aims could be achieved by:-

1. Examination of a range of widely available fibroblast lines, to choose those which have high sensitivity to individual cytokines. It may prove possible to screen samples for growth promoting activity against a battery of fibroblasts, and the resulting pattern of results may indicate the presence of individual cytokines.
2. The development of standardised assay protocols, in terms of growth rate and serum concentration, for bioassays of growth promoting cytokines on fibroblasts in culture.

6.4 THE PLACE OF MACROPHAGE - FIBROBLAST INTERACTION IN THE PATHOGENESIS OF PULMONARY FIBROSIS

6.4.1 "Primary Fibrogenesis"

It is clear that macrophages have the potential to control fibroblast replication and function. There is also good evidence that macrophages do have a central role in the conditions characterised by fibrosis. Macrophages react dramatically to soluble and particulate stimuli, and such stimuli have been demonstrated in many forms of pulmonary fibrosis. In cryptogenic fibrosing alveolitis, to take an example, immune complexes have been demonstrated in alveolar walls (Dreisen et al, 1978), and it is known that they are a potent stimulus to lung macrophages to secrete growth factors (Bitterman et al, 1983). Thus, a chain of events can be envisaged which might be termed "primary" fibrogenesis, where immune complexes are deposited in alveolar walls, bind to

macrophages, and stimulate growth factor secretion causing the fibrosis which is the most obvious manifestation of this condition.

This is an attractive model and brings with it the hope that pharmacological manipulation of this chain of events could decrease the fibrosis and therefore the deranged function of the lung. Unfortunately examination of the histological evidence points to a more complex picture and the need to examine macrophage/fibroblast interactions in the context of derangements of tissue architecture.

6.4.2 Alveolar Epithelial Damage in Pulmonary Fibrosis

The list of agents which can cause pulmonary fibrosis is long and varied and all share the ability to damage lung epithelium. This is the dominant process in the early stages of acute respiratory distress syndrome (ARDS). Parallels exist with disease where pulmonary fibrosis develops over a long time scale. In all of these conditions foci of epithelial damage in the alveolar walls are seen. In cryptogenic fibrosing alveolitis this process can be so pronounced as to give rise to an alternative histological description, viz. desquamative interstitial pneumonia. Bleomycin illustrates the point well. Given as a single dose to animals, an ARDS like picture results, with damage to the alveolar epithelium occurring within days, and the subsequent development of fibrosis. Given therapeutically to humans with solid tumours, pulmonary fibrosis can develop insidiously over months or years. Given with another pulmonary toxin such as high concentration oxygen, an ARDS-like picture can result. It seems that alveolar epithelial damage is a common denominator in most forms of pulmonary fibrosis.

Not all illnesses involving damage to alveolar epithelium go on to pulmonary fibrosis. For example, the majority of survivors of ARDS are left with no or minor pulmonary impairment (Ghio et al, 1989). It is likely that the

persistence of epithelial disruption beyond a certain period may cause fibrosis. Bleomycin is particularly potent in this regard, both because of its slow clearance from the lung after injection (Hay et al, 1987) and because its mechanism of action of damage to DNA, will slow attempts at repair of the epithelium. Witschi and coworkers (1980) showed that sequential exposure of mice to 70% O_2 for the six days after pulmonary injury induced by butylated hydroxy toluene, produced fibrosis, while each insult given separately or a week apart (allowing repair after the first injury) did not.

6.4.3 Intraalveolar Events in Pulmonary Fibrosis

The pattern and distribution of the collagen deposition in human pulmonary fibrosis reinforces the importance of the loss of integrity of alveolar epithelium in these conditions (Basset et al, 1986). Patterns that can be recognised at alveolar level include the formation of buds of intraluminal organising connective tissue, obliterative changes where loose connective tissue masses obliterate the lumen of alveoli, mural incorporation of previously intraluminal connective tissue masses (superficially resembling interstitial thickening of the alveolar wall) and atelectasis to form compacted masses of fibrotic tissue derived from several alveoli. These patterns are seen commonly in most forms of human pulmonary fibrosis, although they are not typical of a few - sarcoidosis, lymphangioleiomyomatosis and the pneumoconioses (Basset et al, 1986; Kuhn et al, 1989). These processes occur in the bleomycin model of lung fibrosis. Disruption of alveolar epithelium allows the movement of interstitial cells including fibroblasts and macrophages into the alveolar lumen, and alveolar collapse, obliteration and intramural incorporation of intraalveolar connective tissue all follow (Kawamoto and Fukada, 1990; Lazenby et al, 1990).

This evidence indicates that study of the macrophage - fibroblast axis in isolation will not provide a complete

picture of the events leading to irreversible pulmonary fibrosis. The influence of damage to the alveolar structures, and restoration of their integrity, on macrophage -fibroblast interactions must also be explored.

6.5 THE FUTURE

These are exciting times for research into pulmonary fibrosis. The importance of the subject, the use of bronchoalveolar lavage in patients to obtain samples of inflammatory cells from the damaged tissues, the availability of a wealth of animal models of human disease and the techniques of cell and molecular biology have combined to produce an explosion of knowledge in this field. The answers obtained may illuminate inflammatory conditions in other organ systems, as well as pulmonary disorders, and will provide, in time, therapies for these challenging diseases.

APPENDIX 1 Lavage Cell Yields (see Section 5.3.2)

	Total Cell Yield	Viability	Viable Cell Yield
	(x 10^6 cells)	%	(x 10^6 cells)
Normal Controls			
R118	23.7	97.8	23.2
R120	31.6	90.5	28.6
R123	39.7	98.5	39.1
R124	21.9	96.7	21.2
2 Day Saline Controls			
R 86	11.8	98.7	11.7
R 87	58.5	99.1	58.0
R119	19.7	96.4	19.0
R107	19.2	99.5	19.1
6 Day Saline Controls			
R 83	7.4	94.0	6.9
R 89	9.0	97.0	8.8
R 91	11.7	98.6	11.5
R110	27.5	93.5	25.1
14 Day Saline Controls			
R 73	12.0	99.6	12.0
R 72	6.2	96.0	5.9
R 77	15.4	95.5	14.7
R 79	7.5	97.1	7.3
2 Day Bleomycin Animals			
R 90	50.9	89.0	45.3
R108	56.8	86.2	48.9
R112	49.5	92.2	45.6
R105	24.7	88.1	21.8
R122	27.2	70.9	19.3
6 Day Bleomycin Animals			
R 80	65.8	79.0	52.0
R 85	58.0	71.4	41.4
R 88	141.4	35.8	50.6
R115	102.4	40.9	41.9
R116	95.0	54.3	51.6
14 Day Bleomycin Animals			
R 76	47.0	92.3	43.4
R 78	85.3	75.9	64.8
R 81	75.8	60.2	45.6
R 82	106.8	75.1	80.2
R100	129.4	96.1	124.4

APPENDIX 2 Growth Factor Secretion by Lavage Cells

	Assay 1		Assay 2	
	Rat1	R9ab	Rat1	Rat1 dialysed
Normal Controls				
R118	1.486	1.365	1.377	1.358
R120	1.441	1.232	1.358	1.396
R123	1.385	1.261	1.344	1.297
R124	1.429	1.392	1.442	1.361
2 Day Saline Controls				
R 86	1.276	1.174	1.244	1.284
R 87	1.234	1.051	1.192	1.345
R119	1.414	1.354	1.459	1.346
R107	1.564	1.280	1.632	1.453
6 Day Saline Controls				
R 83	1.570	1.141	1.554	-
R 89	1.394	1.240	1.349	1.305
R 91	1.540	1.148	1.584	1.404
R110	1.115	1.082	1.081	1.018
14 Day Saline Controls				
R 73	1.082	1.134	1.018	-
R 72	1.253	1.156	1.232	1.232
R 77	1.506	1.318	1.457	1.226
R 79	1.555	1.215	1.550	1.628
2 Day Bleomycin Animals				
R 90	1.308	1.236	1.172	1.172
R108	1.321	1.202	1.176	1.306
R112	1.251	1.149	1.114	1.266
R105	1.327	1.231	1.291	1.254
R122	1.429	1.348	1.414	1.346
6 Day Bleomycin Animals				
R 80	1.098	1.134	0.977	1.006
R 85	1.230	1.167	1.090	1.149
R 88	1.318	1.246	1.235	1.316
R115	1.422	1.232	1.432	1.400
R116	1.463	1.110	1.439	1.473
14 Day Bleomycin Animals				
R 76	1.135	1.136	1.115	1.122
R 78	1.383	1.137	1.397	1.403
R 81	1.426	1.283	1.281	1.511
R 82	1.219	1.266	1.123	1.278
R100	1.190	1.229	1.034	0.954

These data are discussed in Section 5.3.4 et seq.

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A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors

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Summary

There is currently much interest in the role of mediators that regulate cell proliferation. Methods to assay proliferative effects of such mediators usually involve cell counting techniques, which are tedious to perform, or methods based on uptake of radiolabelled thymidine, which may be prone to errors caused by precursor pool artefacts. We describe here an assay for estimating the number of adherent cells present in a microculture and its application to the study of growth factors. The assay depends on the binding of Methylene Blue to the fixed monolayer at pH 8·5 and, after washing the monolayer, release of dye by lowering pH. The use of an elution solvent containing acidified ethanol ensures a linear correlation between absorbance of the dye and cell number, and enables the assay to be carried out in 96-well plates measuring absorbance with an automated vertical light-path microplate photometer. The assay is rapid, highly reproducible and easy to perform, making it ideal for screening large numbers of samples. It was shown to be applicable to a number of foetal and adult cell lines derived from man and experimental animals. It was also demonstrated to be useful for assaying purified growth factors and detecting growth promoting activity in cell and tissue extracts.

Key words: fibroblast proliferation, growth factors.

Introduction

At present there is much research that is directed towards elucidation of the role and mechanism of action of growth factors. Measurement of these factors depends on *in vitro* bioassays of their effect on the rate of cell division in culture. Such cultures are often set up in 96-well microplates, for ease of handling and economy of scale. Existing assays of cell replication rates in microwell culture generally depend on the incorporation of [^{3}H]thymidine into dividing cells, and then either estimating the total [^{3}H]thymidine incorporation or, by autoradiography and microscopy, counting the percentage of labelled cells. The former requires several assumptions about precursor pool radioactivity, which may not be valid, and the latter, although accurate, is laborious.

A rapid assay for counting adherent cells in microwell cultures that avoids the use of radioisotopes would have considerable application to the study of cell proliferation. We have modified the Methylene Blue assay of cytotoxicity (Lagneau *et al.* 1977) for use in microwell plates. This enables batches of plates, representing hundreds of individual cultures, to be read by an automated microplate photometer. This study describes the assay in detail and demonstrates its use for the study of growth factors on a range of cell lines.

Materials and methods

Cell culture

Cell lines used in these experiments were: Rat 1, a fibroblast line from foetal rat lung; IMR90, a cloned human foetal lung cell line; HFL, a fibroblast line from human foetal lung; Chang cells, a cloned liver epithelial cell line, and Rabbit SIRC, a fibroblast line from rabbit cornea. Cells were maintained in Dulbecco's modification of Eagle's medium containing penicillin and streptomycin, with 5% newborn calf serum (DMEM–5% NCS). Serum was supplied by Gibco (Bio-Cult Ltd, Paisley). Cell cultures were replenished after 10 passages from stocks frozen in liquid nitrogen. No evidence of mycoplasmic contamination in these cells was found, either using a DNA probe for mycoplasmic ribosomal RNA (Gen-Probe Mycoplasma T.C.II), or by staining with bisbenzamide (Hoechst 33258).

To set up microwell cultures, cells were brought into suspension with 0·02% EDTA/0·02% trypsin, washed with

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calcium- and magnesium-free phosphate-buffered saline and resuspended in medium that, unless otherwise indicated, was DMEM–5% NCS. The cells were counted in a haemocytometer, and the cell suspension was diluted with DMEM–5% NCS to give an appropriate cell concentration. Samples (100 μl) of cell suspension were introduced into 96-well tissue culture plates using a multichannel pipette with sterile tips. In general, only the central 10×6 matrix of wells was used, the outer wells (where edge effects on prolonged culture present a problem) were filled with 100 μl DMEM–5% NCS alone.

Methylene Blue assay

Fixation of cells. The culture medium in each well was removed by holding the plate at 60° to the horizontal, dipping it in a beaker of 0·15 M-saline, then gently inverting it over absorbent paper to remove excess fluid. For less strongly adherent cell lines this method can cause damage to the monolayer (easily apparent when stained with Methylene Blue) in which case gentle vacuum aspiration using a Pasteur pipette with a fine angled tip is an alternative. The cell layer was then fixed by adding 100 μl of 10% formol saline to each well for at least 30 min. Other fixatives (e.g. 100% methanol) may be used but the choice of fixative does affect the final absorbance value. At this stage the plates can be stored, by wrapping them in aluminium foil to prevent evaporation of the fixative, and later assayed as a batch.

Cell staining. The fixative was shaken off each plate by a flick of the wrist and 100 μl of filtered 1% (w/v) Methylene Blue in 0·01 M-borate buffer (pH 8·5) was added to each well. After 30 min, excess dye was removed by another flick of the wrist. The remaining dye was then washed off by serially dipping the plate into each of four tanks of 0·01 M-borate buffer (pH 8·5), shaking the buffer off between each immersion. This must be done in a uniform manner to minimize between-plate variation. After the last rinse and shake, the cell layer, still stained with Methylene Blue, can be examined microscopically if desired. To elute the dye, 100 μl of 1:1 (v/v) ethanol and 0·1 M-HCl were added to each well. The plates were then gently shaken and the absorbance at 650 nm (A_{650}) measured for each well by a microplate photometer (Titertek Multiskan MC, Flow Laboratories Ltd, Irvine, Scotland). The photometer was blanked on the first column of control wells containing elution solvent alone.

To demonstrate the wider application of the assay, the effects of platelet-derived growth factor and PGE_2 on fibroblast replication were investigated. The assay was also used to identify growth factor activity in cell-conditioned media and tissue extracts. Details of these experiments are given in legends to the appropriate figures.

Statistical analysis

Mean values ± standard deviation were calculated for cell numbers determined by haemocytometer count and optical density (A_{650}). In some instances the standard deviation for replicate samples was very small and fell within the symbol shown in the figures. Correlations between optical density and cell number, were assessed using Kendall's correlation coefficient (t).

Results

Absorption spectra of Methylene Blue in aqueous and organic solvents

Methylene Blue in aqueous solution at low pH does not obey Beer-Lambert's Law (Fig. 1) because of the formation of dimers, particularly at high concentrations (Fig. 2). We therefore used an ethanol/0·1 M-HCl mixture to elute the dye from the cell monolayer. This suppressed the formation of dimers giving a single absorption peak at 650 nm (Fig. 2) and Beer-Lambert's Law was obeyed (Fig. 1).

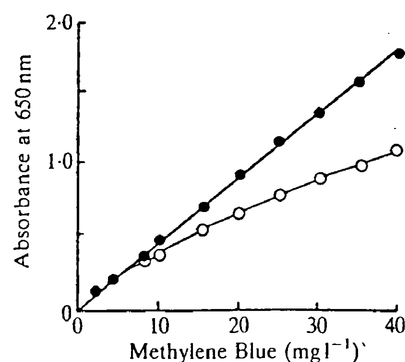

Fig. 1. The A_{650} of 100 μl of solutions of Methylene Blue in 0·1 M-HCl (○) and in 1:1 (v/v) 0·1 M-HCl/ethanol (●) measured in the Multiskan MC photometer. The usual range of Methylene Blue concentration obtained in the cell counting assay is 0–32 mg l^{-1} and the maximum absorbance measurable on the Multiskan is 2·0.

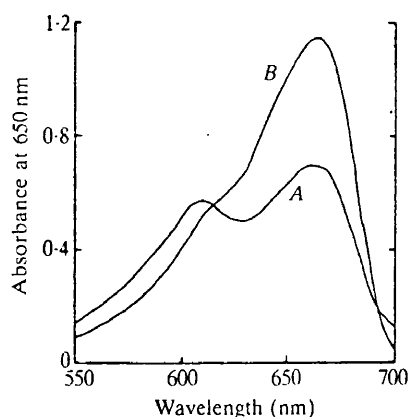

Fig. 2. Absorption spectra of Methylene Blue at a concentration of 32 mg l^{-1}, in curve *A* in 0·1 M-HCl, and in curve *B*, 1:1 (v/v) in 0·1 M-HCl/ethanol, measured in a Gilford spectrophotometer.

Accuracy and reproducibility

In order to assess the accuracy of the assay, cultures were set up in replicate within a microwell plate for a range of cell concentrations. Fig. 3 shows that the A_{650} was directly proportional to the cell number for three cell types studied. In this experiment the standard deviations were in all cases less than 5% of the values shown and fell within the symbols for each cell line.

To assess further within- and between-plate variation, IMR90 cells in DMEM were pipetted into 36 wells of each of 16 plates at a seeding density of 10^4 cells per well. Cells were harvested after 16 h incubation, fixed with formol saline and stored before being assayed in groups of four at intervals over the next 10 days. The coefficient of

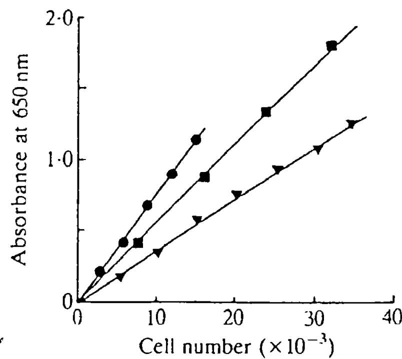

Fig. 3. Linear relationship between A_{650} and number of cells. The cultures were harvested after 12 h (i.e. before significant cell replication could have occurred). The results are expressed as means of six replicates: Rat 1 (▼); IMR 90 (●); Chang cells (■). Standard deviations fell within the symbols.

variation within a plate was 4·4%. One way analysis of variance of the mean A_{650} value for each plate revealed that the coefficient of variation between plates in a single run was 2·03% and there was no additional variation attributable to comparing plates assayed on different days. There was no trend in A_{650} to suggest an effect due to the varying lengths of storage before assay.

Duration of staining and washing steps

We also studied the effect of the duration of staining and washing steps on the final A_{650} value. The duration of staining was not critical. There was no significant difference between cultures of IMR90 cells stained for 15, 30, 45 and 60 min (data not shown). The final A_{650} value obtained did depend on the number of washing steps. For example, in one experiment, after 2, 3, 4, 5 and 6 washes, values for A_{650} were 1·13, 0·98, 0·86, 0·84 and 0·80, respectively. This implies that the number of washes should be standardized and performed in a consistent manner.

Effects of characterized mediators

Having established that the assay was suitable for estimating cell numbers, we investigated the problem of how to optimize cell culture conditions for assaying the effect of growth factors on cell proliferation. Fig. 4 demonstrates that cell growth rate is dependent upon seeding density (Fig. 4A) and concentration of serum in culture medium (Fig. 4B). On the basis of these data, in subsequent experiments studying the effect of growth factors we used a seeding density of 6×10^3 cells per well, cultured in DMEM+0·4% NCS for stimulatory factors and 2·0% NCS for inhibitory factors. An experiment demonstrating a dose–response effect for platelet-derived growth factor (PDGF) is shown in Fig. 5A. The inhibitory effect of PGE_2 (prostaglandin E_2) is shown in Fig. 5B.

Relationship between staining by Methylene Blue and cell number

Fig. 6 shows that a linear relationship existed between

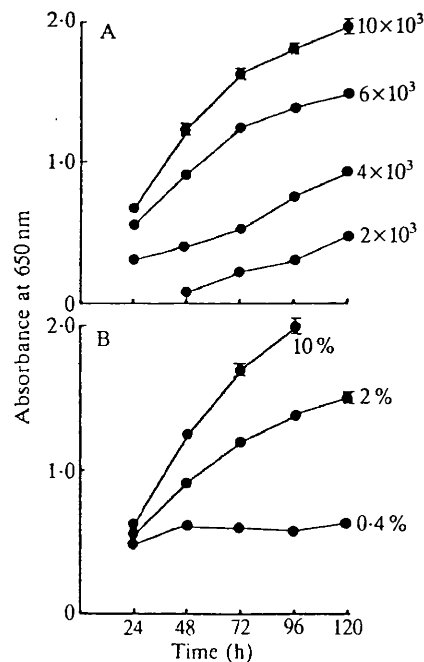

Fig. 4. Plot of A_{650} against time for HFL fibroblasts. A. Cells were seeded at varying cell densities (values shown indicate cells per well). Cells were all grown in microwell plates containing 100 μl of DMEM+2% NCS. B. Cells were grown in microwell plates containing 100 μl DMEM with varying concentrations of NCS (0·4%, 2% and 10%, as indicated). All cells were seeded at a density of 6×10^3 cells per well and each value represents the mean of 18 wells.

absorbance, determined in the assay, and cell number, determined by haemocytometer count of Crystal Violet-stained nuclei ($t = 0{\cdot}86$, $P < 0{\cdot}01$). This relationship held for cell proliferation in the presence of serum at different concentrations, and for cells treated with PDGF.

Effect of growth-promoting activity in cell-conditioned media and tissue extracts

Fig. 7 demonstrates the application of the Methylene Blue assay to assess the effect of cell-conditioned media on fibroblast proliferation. Dilution curves of macrophage-primed media from two different rabbits are compared. It can be deduced that sample A contains 16-fold more growth-promoting activity than sample B.

The assay was also appropriate for examination of mediators derived from whole tissues. In Fig. 8 we compare the effects of extracts from left and right ventricles of rabbits with pulmonary hypertension. The extract from the hypertrophied right ventricle contains more growth-promoting activity for any given dilution, and the data suggest four times more growth-promoting activity in right ventricles compared with the left.

Discussion

Colorimetric techniques are increasingly being used to assess cell number in assays of cell proliferation, in particular in studies of the effects of cytotoxic drugs on tumour cells (Mosman, 1983; Mirabelli *et al.* 1985; Alley

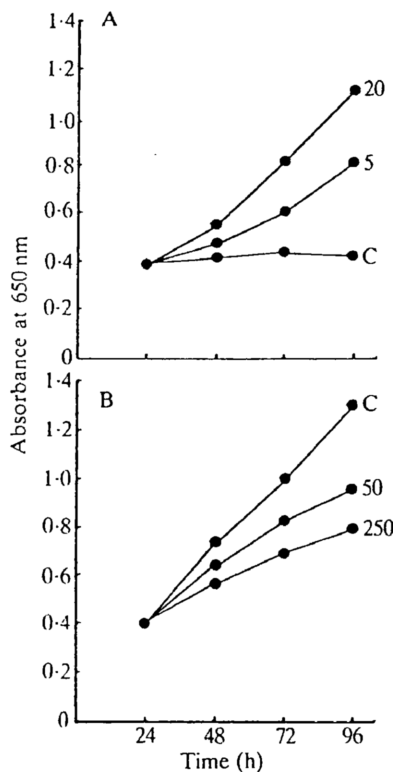

Fig. 5. Dose–response effect of: A, PDGF; B, PGE_2 on proliferation of HFL fibroblasts grown in microwell plates. In A cells were grown in DMEM + 0·4% NCS at a seeding density of 6×10^3 cells per well. Control cells and those grown in PDGF at $5\,ng\,ml^{-1}$ and $20\,ng\,ml^{-1}$ are indicated as C, 5 and 20, respectively. In B, cells were grown in DMEM + 2% NSC at a seeding density of 6×10^3 cells per well. Control cells and those grown in PGE_2 at $50\,ng\,ml^{-1}$ and $250\,ng\,ml^{-1}$ are indicated as C, 50 and 250, respectively.

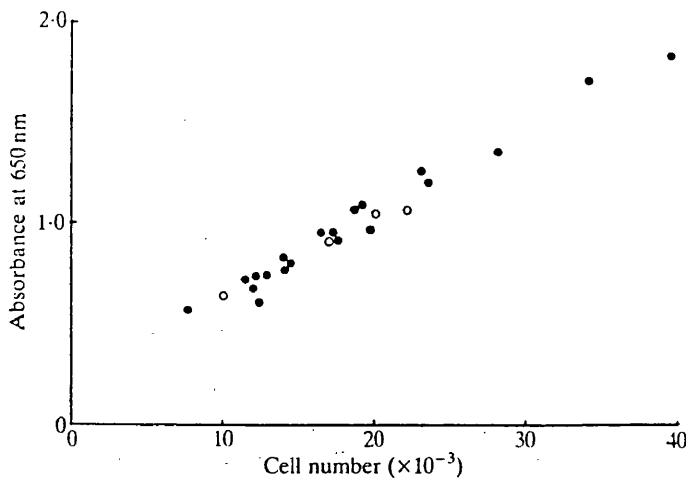

Fig. 6. Plot of absorbance against haemocytometer count of cell number. The data are derived from HFL cells seeded at 6×10^3 and 10×10^3 cells per well grown in DMEM in 0·4, 2, 5 and 10% NCS and harvested at time intervals from 24 to 96 h (●). Cells seeded at 6×10^3 cells per well grown in DMEM with 0·4% NCS containing $10\,ng\,ml^{-1}$ PDGF were harvested at similar time intervals (○). All values represent means of replicates in 15 different wells.

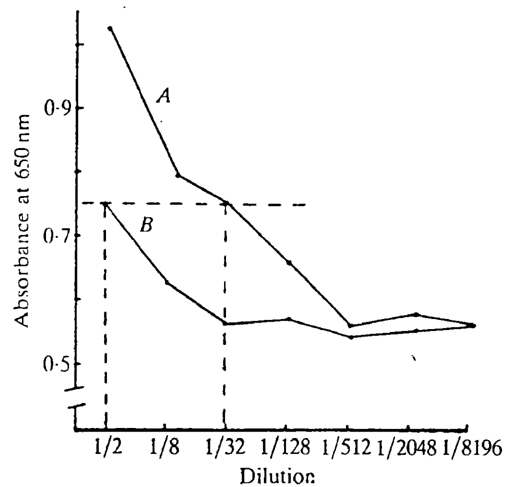

Fig. 7. Effect of macrophage-primed medium on Rat 1 fibroblast proliferation. Alveolar macrophages obtained by lung lavage from two rabbits, *A* and *B* (Oliver *et al.* 1984), were incubated at $5\times10^5\,cells\,ml^{-1}$ in DMEM for 24 h. The conditioned medium was then separated from the cells and serial dilutions made of each sample in fresh medium; 50 μl of each dilution was added to triplicate microwells in a 96-well plate. Control wells contained fresh medium alone. A suspension of 8×10^3 Rat 1 $cells\,ml^{-1}$ in 50 μl of DMEM–4% NCS was added to each well so that the final concentration of NCS was 2%. The cultures were incubated for 72 h before assaying cell number by the Methylene Blue method.

et al. 1988; Carmichael *et al.* 1987; Finlay *et al.* 1984). These techniques usually rely on the ability of live cells to induce the formation of a product that is detectable by colorimetry, but Methylene Blue staining of fixed cells has also been used (Finlay *et al.* 1984). In this paper we describe a modified assay using Methylene Blue, which is highly accurate over a range of cell concentrations. We also demonstrate the applicability of this assay to studies of mediators that can stimulate or inhibit cell replication.

Assessment of Methylene Blue assay

Methylene Blue is a basic dye that is positively charged at pH 8·5. It binds electrostatically to negatively charged groups within cells, predominately phosphate moieties of nucleic acids and some charged groups in proteins. This explains the variation in dye bound by cells from different species and also by different cell types within the same species (see Fig. 3).

Lowering the pH below 2 by adding HCl causes acidic groups to be protonated, liberating the Methylene Blue into the elution solvent. The demonstration that an elution solvent of ethanol/0·1 M-HCl (1:1) gives a single absorption peak for Methylene Blue at 650 nm is important because it means absorption at this wavelength is an accurate and reproducible reflection of the number of cells stained by the dye. This is not the case for Methylene Blue in aqueous solution at low pH without ethanol present (Lagneau *et al.* 1977; Horobin, 1982).

Limitations imposed by the automated microplate photometer for reading optical density restricts the range of cell numbers that can be estimated by this method to approximately 2×10^3 to 40×10^3 cells per well, depending

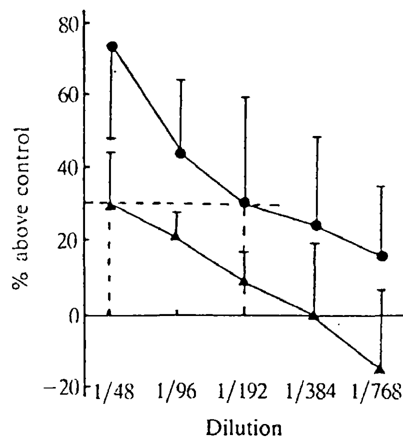

Fig. 8. Effect of rabbit heart extracts on fibroblast proliferation. Soluble extracts were obtained from right and left ventricles following the development of right ventricular hypertrophy in rabbits with bleomycin-induced lung disease (Turner *et al.* 1986). The ventricles were separated and homogenized in water (1:3, w/v), centrifuged at 15 000g, and the supernatant was recentrifuged at 30 000g, as described by Hammond *et al.* (1979). The final supernatant was applied in serial dilutions to 96-well plates containing 6×10^3 rabbit SIRC cells/well in DMEM with a final concentration of 2% NCS. The plates were incubated for 70 h and growth factor activity was estimated using the Methylene Blue assay. The stimulatory effects on fibroblast growth (% control) of serially diluted ventricular extracts are shown. For 30% stimulation a 1:192 dilution of extract from right ventricle (●) was required, compared with 1:48 dilution for left ventricle (▲). Values represent means ± S.E.M. for right ventricle of 12 animals and the left ventricle of five animals. There was no difference between the stimulation obtained from extracts of the left ventricle of bleomycin-treated animals and either ventricle of controls (data not shown).

on cell type. Whilst this limitation could cause constraints for some workers, the accuracy of the assay over a 20-fold range in cell numbers makes it applicable for most studies. However, this limitation does indicate the importance of performing preliminary experiments on cell numbers and culture conditions (such as demonstrated in Fig. 4) before applying the assay to studies of cell proliferation.

A potential source of error with this assay is that under certain culture conditions the mean cytoplasmic/nuclear ratio of cells may change, thus altering A_{650} without necessarily changing cell number. We found no evidence for this in our experiments. Cell counts gave a close correlation with A_{650} and this relationship held for a range of cell lines and culture conditions. Nonetheless, in critical experiments where variations in cell morphology could occur, workers may be advised to confirm results using another assay, preferably direct cell counting.

A potential problem of the assay is that the A_{650} of proliferating cultures could be underestimated because cells round up in metaphase and may be lost during the washing procedures. However, even in rapidly proliferating cultures, cells in metaphase usually represent less than 1% of the total. In practice, therefore, this does not lead to a significant error.

The only practical disadvantages in the assay are that it can be used only on adherent cell cultures (although some less strongly adherent cell lines may be used if vacuum aspiration of the medium is performed), and that between-plate variation occurs even when using the same cell line. This variation must be borne in mind when designing experiments, but is very small in most cases, and can be circumvented by the use of controls on each plate.

Comparison of Methylene Blue assays with methods based on uptake of radiolabelled thymidine

The main alternative for assessing the growth rate of cells in microwell cultures is the measurement of [^{3}H]-thymidine uptake. This has disadvantages because it is expensive, it involves the use of radioisotopes and it requires capital expenditure for equipment. There are also other problems, both conceptual and practical. First, [^{3}H]thymidine uptake in cell culture is a function not only of the percentage of cells entering S-phase, but also of the total number of cells present. This, in turn, depends on the previous growth of the culture. Therefore, uptake may be a reflection of the previous history of the cells together with the contemporary stimulus for replication. Second, the validity of [^{3}H]thymidine uptake as a measure of cell proliferation depends on it being incorporated into newly synthesized DNA. A variety of factors could potentially affect the rate of this metabolic process. For example, secretion of enzymes that degrade [^{3}H]thymidine in culture medium (Palu, 1980), or changes in the rate of endogenous synthesis of thymidine by the cell (Rannels *et al.* 1982; Opitz *et al.* 1975), may both affect the specific radioactivity of [^{3}H]thymidine in the precursor pool. In these circumstances changes in [^{3}H]thymidine uptake will not accurately reflect changes in DNA synthesis. Although such pool effects may be examined by measuring the specific radioactivity of free [^{3}H]thymidine (Rannels *et al.* 1982; Elliot & Johnson, 1983), many workers feel obliged to validate results of [^{3}H]thymidine uptake by repeating key experiments using a cell counting method. A third problem is that it may prove difficult to ensure that a microwell culture of fibroblasts (typically 5×10^4 to 10×10^4 cells) that are not proliferating maximally can incorporate sufficient [^{3}H]thymidine to achieve reasonable radioactive counts. Increasing the duration of exposure to [^{3}H]thymidine or the total amount of [^{3}H]thymidine in the plate could cause artefacts due to radiation damage to cells (Wiezsaccker *et al.* 1981). In practice this has meant that [^{3}H]thymidine uptake studies of fibroblast growth in 96-well plates have only been carried out in the presence of a near maximal growth stimulus (e.g. high percentages of serum). Finally, [^{3}H]thymidine uptake studies, even with the use of a cell harvester, can be time-consuming.

Use of Methylene Blue assay to assess growth-promoting activity

We have shown that the Methylene Blue assay can be used to determine stimulatory or inhibitory activity of

purified compounds (i.e. PDGF and PGE_2), as well as growth-promoting activity in two biological extracts: conditioned media from macrophages, and extracts of whole heart tissue. Despite the complexity of the biological fluids, which probably contain a host of inhibitors as well as promoters of cell growth, the dilution curves allowed accurate assessment of the relative activities of the fluids. This demonstrates how the Methylene Blue assay can be used to compare the growth-promoting activity of two or more biological samples. Our data was obtained from a single 96-well plate, which emphasized the convenience of this assay. Performing such experiments, which require analysis of five or more concentrations of fluid from multiple samples, would be enormously time-consuming using traditional cell counting methods.

In summary, the Methylene Blue assay we have described, harnesses the convenience and economy of cell culture in 96-well plates to a method that accurately quantifies the small number of cells present in such cultures. This permits the generation of growth curves and dilution analysis to quantify growth factors, and is ideal for the testing of large numbers of samples. The assay is also reproducible, rapid, inexpensive and easy to perform. It requires only a single item of special equipment: an automated vertical-path microplate photometer, widely used for ELISA studies. This permits automated data-capture in a microplate disk-storage system to be linked with programmed statistical analysis and printout. It is likely that the Methylene Blue assay will be of wide application in an area of research that is rapidly expanding.

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Comparison of protein-synthesis rate of alveolar macrophages *in vivo* and *in vitro*

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This paper describes and validates a novel method for measuring rates of protein synthesis of rabbit alveolar macrophages *in vivo*. A rate of 9.3%/day was obtained, compared with 48.9%/day measured *in vitro*. This study suggests that the procedures involved in the isolation of alveolar macrophages for study *in vitro* may themselves activate the cell.

Alveolar macrophages (AMs) can now be readily obtained from the lungs of both animals and humans by bronchoalveolar lavage (Hunninghake *et al.*, 1979). These cells are increasingly the subject of study in normal and in diseased animals and humans. Although the steps involved in the isolation of these cells are relatively simple, AMs are known to be 'activated' by surface stimuli, and it seems possible that the manipulations involved in lavage and washing the cells prior to study *in vitro* might be sufficient to activate the cells. There are many measures *in vitro* of macrophage activation, but most cannot be applied to AMs *in vivo*. The measurement of protein-synthesis rate of AMs is, as far as we are aware, the only measure of activation of AMs accessible both *in vitro* and *in vivo*.

In the present study we have developed methods of measuring the rate of protein synthesis of normal rabbit AMs *in vivo* by using L-[5-^{3}H]proline as a labelled precursor, and compared this with a rate measured *in vitro* by standard techniques. We obtain a rate *in vitro* that is 5-fold higher than *in vivo*. It is probable that AMs obtained by lavage and subsequent centrifugation are already partially activated.

Experimental

Alveolar macrophages

New Zealand White male rabbits (body wt. 1.7–2.3kg), supplied by HOP Laboratories, Cork Farm, Chilham, Kent, U.K., were killed with intravenous sodium pentobarbitone and intubated via a tracheotomy. The lungs were lavaged with three 30ml aliquots of 0.15M-NaCl at room temperature, and the returns collected in a silicone-treated glass bottle precooled in ice. Lavage cells were maintained at 4°C thereafter until either culture or addition of trichloroacetic acid. Blood was obtained by cardiac puncture and collected into heparinized tubes.

After lavage, cells were separated from lavage fluid by centrifugation (100*g*, 10min) and then washed three times by resuspension in phosphate-buffered saline and re-centrifugation. Some lavage returns were slightly contaminated by blood; if so, the cells were suspended in phosphate-buffered saline, layered on to Ficoll/sodium metrizoate (d = 1.077) (Lymphoprep; Nyegaard Ltd., Oslo, Norway) and centrifuged at 400*g* for 10min. The nucleated cells were collected from the interface with a silicone-treated Pasteur pipette and washed twice in phosphate-buffered saline. Macrophages, identified by May–Grunewald–Giemsa and non-specific esterase stains, represented at least 96% of nucleated cells in all experiments.

Protein synthesis in vivo

Animals, in groups of four, received intravenous injections of L-[5-^{3}H]proline (Amersham International) (dose: 0.6–2.4mCi/kg body wt.), mixed with unlabelled L-proline (7mmol/kg body wt.) at intervals of 30, 60 and 120min before they were killed. The time of incorporation was taken from the start of the injection of proline until the instillation of the first aliquot of lavage saline.

At the end of the washing steps detailed above, the cell pellet was mixed with 5% (w/v) trichloroacetic acid. The resulting precipitate was washed

Abbreviations used: AM(s), alveolar macrophage(s); phosphate-buffered saline, 137mM-NaCl/2.6mM-KCl/8.1mM-Na_2HPO_4/1.5mM-KH_2PO_4; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

three times in 5% (w/v) trichloroacetic acid by centrifugation, and then successively in acetone/12M-HCl (400:1, v/v), ethanol/diethyl ether (2:1, v/v) and diethyl ether, before drying and hydrolysis in 6M-HCl (110°C, 16h).

Free proline was extracted from both the lavage fluid, after removal of cells as described above, and the plasma. The lavage fluid was freeze-dried, and was redissolved in 3ml of water before mixing with 3ml of 10% (w/v) trichloroacetic acid. For both samples the acid-soluble fraction was obtained by centrifugation (600*g*, 10min).

For each animal the specific radioactivities of proline in AM protein hydrolysate, and free in lavage fluid and plasma, were measured by a chloramine-T oxidation method (Stegemann, 1958) with recently described modifications (Laurent *et al.*, 1982).

The protein-synthesis rate was calculated as described previously (McNurlan *et al.*, 1979; Laurent, 1982) from the equation:

$$\text{Fractional protein-synthesis rate (\%/day)} = \frac{\text{specific radioactivity of amino acid incorporated in protein} \times 100}{\text{specific radioactivity of amino acid in precursor pool} \times \text{time (days)}}$$

The precursor-pool specific radioactivity was taken to be that of the free proline in the cell-free lavage fluid.

Protein synthesis in vitro

AMs were obtained from three rabbits, as described above, using sterile equipment throughout. Cell viability was assessed by Trypan Blue exclusion, and was 97% prior to, and 86% at the end of, the culture period. The cells were pooled and incubated in suspension in silicone-treated 5ml glass bijou bottles with loose-fitting caps while being shaken at 80 strokes/min in a water bath at 37°C in CO_2/air (1:19). Each culture contained 1.6×10^6 cells in 700μl of Krebs–Henseleit (1932) bicarbonate buffer with the following additions: glucose, 10mM; benzylpenicillin, 100 units/ml; normal rabbit plasma concentrations of 18 amino acids (Block & Hubbard, 1962); phenylalanine, 345μM (this is five times the physiological concentration) with L-[4-^{3}H]phenylalanine (Amersham) to a specific radioactivity of 100Ci/mol; L-[5-^{3}H]proline to a specific radioactivity of 100Ci/mol and various proline concentrations (detailed below). In addition, some cultures contained bovine serum albumin (fraction V; Sigma). Each set of cultures was set up in quadruplicate flasks.

At the end of a 2h incorporation period, metabolic activity was stopped by rapid cooling to 0°C, the AMs separated by centrifugation (100*g*, 10min) and washed by centrifugation three times with phosphate-buffered saline, before addition of 5% (w/v) trichloroacetic acid. Washing of the resulting precipitate and acid hydrolysis was as described for experiments *in vitro*. The specific radioactivities of [^{3}H]proline and [^{3}H]phenylalanine in the protein hydrolysate and the culture medium was measured by using a [^{14}C]dansyl chloride double-isotope method exactly as described by Airhart *et al.* (1979). The higher sensitivity of this method as compared with chloramine-T-oxidation assay was required because of the small number of cells in each culture *in vitro*. The specific radioactivities of the [^{14}C]dansyl chloride allowed to react with the amino acid samples were 12200d.p.m./nmol (culture medium) and 1030d.p.m./mol (macrophage AM protein hydrolysate). The quantity of [^{14}C]dansyl amino acid derived from AM protein spotted on to the polyamide sheets contained approx. 4nmol of dansyl-proline and 1.5nmol of dansyl-phenylalanine. After chromatography the spots of these two dansyl amino acids were readily identified, well separated from the other dansyl amino acids (although, at this high loading, resolution of some dansyl amino acids was not complete). The spots were cut out with scissors, as were corresponding regions of the polyamide sheets from reagent blanks put through the assay. Each 'spot' was placed in a glass scintillation vial containing 0.5ml of NCS tissue solubilizer (Amersham International). After 1h, 17μl of 16M-acetic acid, followed by 10ml of FisoFluor scintillation cocktail (Fisons) were added to each vial, and radioactivity was measured in a Packard liquid-scintillation spectrometer, model 3380, with external standardization and automatic correction for spill-over from the ^{14}C channel to the ^{3}H channel. After subtraction of the radioactivity in the spots obtained from the reagent blank ([^{3}H]phenylalanine, 90d.p.m.; [^{3}H]proline, 100d.p.m.; and [^{14}C]dansyl chloride, 20d.p.m.), the ratio of ^{3}H to ^{14}C radioactivity was calculated for each spot, and from this and the known [^{14}C]dansyl chloride specific radioactivity, the corresponding [^{3}H]proline or [^{3}H]phenylalanine specific radioactivity could be obtained. The protein-synthesis rate was calculated, as described above, by assuming the specific radioactivity of the amino acids in the culture medium to be that of the precursor pool.

Results and discussion

Alveolar-macrophage protein-synthesis rates

Rates of macrophage protein synthesis were calculated both *in vivo* and *in vitro* on the basis of the incorporation of [^{3}H]proline over 2h. The rate obtained *in vivo* after injection of radioactivity with a large amount (7 mmol kg body wt.) of unlabelled proline was 9.3±1.5 (S.E.M., $n = 4$) %/day, compared with a rate of 48.9±0.5 ($n = 4$) %/day obtained *in vitro*, again in the presence of high levels of unlabelled proline (concn. in medium, 4mM). This fivefold difference between the rates *in vivo* and *in vitro* suggests that AMs may be activated by the steps involved in their isolation for studies *in vitro*. However, the validity of this conclusion is dependent on the accuracy of the determined rates and thus on various assumptions associated with their calculation. The experimental basis for the assumptions involved for the study *in vivo* will be discussed fully, as this approach has not been used previously.

Assumptions implicit in the calculation of protein-synthesis rate in vivo

The assumptions are (1) that the specific radioactivity of proline in plasma is constant during the period of observation, and that equilibration between the plasma and alveolar fluid, and hence into the AM, is rapid, (2) that high proline concentrations do not affect protein synthesis, and (3) that it is reasonable to equate alveolar fluid proline specific radioactivity with that of the precursor pool for AM protein synthesis, i.e. prolyl-tRNA in the AM.

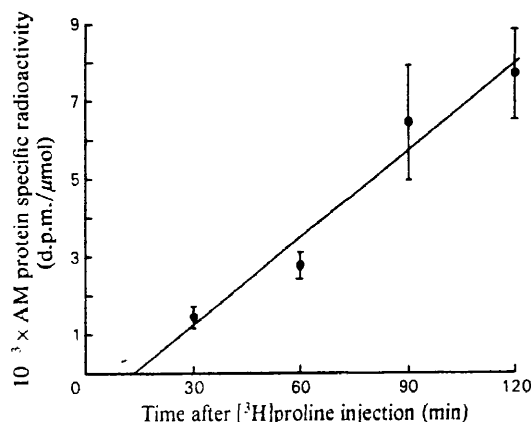

Fig. 1. *Time course of incorporation in vivo of [^{3}H]proline into alveolar-macrophage protein*

The specific radioactivity of macrophage-protein proline is normalized to an infused proline specific radioactivity of 10^6 d.p.m./μmol. Error bars represent ±S.E.M. for groups of four animals, and the line was drawn by least-squares regression analysis.

(1) The use of a large 'flooding' dose of injected proline ensures that assumption (1) holds. This method has been validated in previous studies of protein synthesis in various rabbit tissues, including lung (Laurent, 1982; Laurent & McAnulty, 1983). In the present study, the proline must in additio pass rapidly into alveolar fluid (which bathes the AM), achieving 'flooding' levels of proline to ensure a constant precursor-pool specific radioactivity in the AM.

To examine this question we calculated the ratio of proline specific radioactivity free in lavage fluid (which has sampled alveolar fluid) and in plasma, for the animals 30, 60, 90 and 120min after [^{3}H]proline injection. The means (±S.E.M.) of these ratios for each group of animals were respectively 0.84±0.08, 0.92±0.08, 0.86±0.06 and 0.97±0.06. There is no significant difference between these ratios (one-way analysis of variance, $P > 0.05$), confirming rapid passage of proline into the alveolar fluid. Fig. 1 shows that the incorporation of [^{3}H]proline into AM protein is linear with time, confirming that the proline precursor pool for protein synthesis is of constant specific radioactivity. The intercept of the least-squares regression line suggests that incorporation of [^{3}H]proline starts at 13 min. This apparent delay in incorporation may be due to a finite time for equilibration of proline between the various free pools. However, accepting the observed delay of 13min, this period is still short compared with the total incorporation time, and correction by this amount does not substantially alter the rates obtained (10.4%/day versus 9.5%/day). We were not able to measure alveolar fluid proline concentration directly because the 90ml of lavage saline diluted an unknown volume of alveolar fluid. This was also made difficult because in controls (which did not receive injected proline), the lavage returns contained amounts of proline which were at the

Table 1. *Effect of various extracellular proline and albumin concentrations on [^{3}H]proline and [^{3}H]phenylalanine incorporation into proteins of cultured macrophages*

The specific radioactivity of both amino acids was constant under all conditions used (see the Experimental section). Values are means ±S.E.M. for four determinations.

[Proline] (μM)	[Albumin] (g/litre)	Protein specific radioactivity (d.p.m./nmol) [^{3}H]Proline	[^{3}H]Phenylalanine
100	0	3616±48	8997±186
1500	0	8216±138	8797±88
4000	0	8361±89	8444±143
1500	6	8476±91	9141±58
1500	20	7594±105	8859±166

lower limits of the assay employed. However, the total proline content of the lavage fluid returns of controls was less than 40 nmol, compared with a mean of 1800 ± 640 (S.E.M., $n = 4$) nmol in the group of animals killed at 30 min after injection. This, together with results discussed above, is further evidence supporting our contention that passage of proline into alveolar fluid was rapid and sufficient to achieve high 'flooding' levels of the amino acid for the duration of the experiment.

(2) The second assumption, that high proline concentrations do not affect protein synthesis, has been discussed previously (Laurent, 1982). We have further examined this question in the present study by using techniques *in vitro*. To do this, [^{3}H]phenylalanine was included in the culture medium at constant concentration and specific radioactivity to provide a measure of protein synthesis independent of variations in intracellular proline pool concentrations and specific radioactivities. Table 1 shows that [^{3}H]phenylalanine incorporation was unaffected by proline concentrations over the range 100–4000 μM (one-way analysis of variance, $P > 0.05$). This range includes the proline concentration in plasma for normal rabbits (about 200 μM) and the concentration 2 h after injection of a flooding dose of proline (about 3000 μM; Laurent 1982).

(3) Our assumption that when flooding doses of proline are used the specific radioactivities of extracellular (i.e. alveolar fluid) proline and AM prolyl-tRNA *in vivo* are equal is supported by recent work. Studies carried out *in vivo* on heart (Everett *et al.*, 1981), and on perfused lung (Watkins & Rannels, 1980) and perfused liver (Khairallah & Mortimore, 1976) have indicated that, when high doses of amino acid are used, the specific radioactivities of extracellular amino acid and aminoacyl-tRNA become equal. In addition, one of us (Laurent, 1982) used the same protocol of [^{3}H]proline administration *in vivo* and demonstrated that the specific radioactivity of proline in skin procollagen (which has a half-life of less than 30 min) equalled that of the tissue free pool, a result implying that the prolyl-tRNA of skin fibroblasts under these conditions also achieved a specific radioactivity equal to that of the tissue free pool. Unfortunately, direct measurement of the prolyl-tRNA specific radioactivity of AMs *in vivo* is precluded by the rapid turnover of the rRNA pool when compared with the time required to isolate and process the AMs and also by the need to use prohibitively large amounts of [^{3}H]proline to achieve a measurable prolyl-tRNA specific radioactivity.

Notwithstanding the results discussed above, there is evidence obtained *in vitro* that suggests potential problems which may affect calculations *in vivo*. One is that exogenous protein can act as a source of amino acid for protein synthesis in AMs (Rannels *et al.*, 1982). In the alveolus, macrophages are bathed in a fluid containing protein, predominantly albumin, at an unknown concentration, although very probably much lower than that in plasma. This exogenous protein, because it does not contain labelled amino acid, might lower the precursor-pool specific radioactivity in the cell, leading to an erroneously low measurement of protein-synthesis rate *in vivo*. To assess this problem, cells were incubated in various concentrations of bovine serum albumin and [^{3}H]proline incorporation measured. The results are shown in Table 1. At an albumin concentration of 20 g/litre, there was a significant ($0.01 < P < 0.02$, Student's t test) but small fall in [^{3}H]proline incorporation, but this was not apparent with 6 g of albumin/litre. This effect is unlikely, therefore, to bias significantly a comparison of protein-synthesis rates *in vivo* and *in vitro*.

A potentially more profound methodological problem in the use of radiolabelled amino acids to measure protein-synthesis rates is illustrated by recent work *in vitro* on lung fibroblasts (Hildebran *et al.*, 1981) and guinea-pig AMs (Airhart *et al.*, 1981). At physiological levels, an increase in extracellular amino acid concentration at constant specific radioactivity will increase ^{3}H-labelled-amino acid incorporation (and therefore the apparent protein-synthesis rate) via an increase in aminoacyl-tRNA specific radioactivity. At high extracellular amino acid concentration, aminoacyl-tRNA specific radioactivity reaches a plateau, where it is unaffected by changes in the extracellular amino acid concentration. This effect is demonstrated in Table 1, where it can be seen that [^{3}H]proline incorporation rises when the proline concentration in the medium is raised from 100 mM to 1500 mM, but does not change again when the latter increased to 4000 mM. Our comparison is made at extracellular proline concentrations in excess of 1500 mM, i.e. in the plateau region.

Although aminoacyl-tRNA specific radioactivity does reach a plateau at high extracellular amino acid concentrations *in vitro*, it still may be less than the specific radioactivity of the extracellular amino acid (Hildebran *et al.*, 1981; Hammer & Rannels, (1981). This contrasts with the situation *in vivo* outlined above, where flooding doses of amino acid seem to be successful in equalizing the specific radioactivities of the two pools. Thus rates *in vivo* are likely to be unbiased, whereas rates *in vitro* may be underestimated because of this effect, which would amplify the difference reported in the present study.

The present study is the first to examine

macrophage protein metabolism *in vivo*. We obtained a protein fractional synthesis rate of 9.3%/day. This contrasts with the value of 35%/day for total lung protein measured in rabbits (Laurent, 1982), and suggests that AMs are metabolically relatively inert in the normal lung. When AMs were cultured in suspension *in vitro* we obtained a rate of 48.9%/day. This is very similar to the value of 50.7%/day obtained by Hammer & Rannels (1981) with New Zealand White rabbits and similar experimental conditions, except that phenylalanine was the radioactive precursor amino acid. When corrected for phenylalanine-tRNA specific radioactivity (which was 59.9% of that of culture-medium phenylalanine), their rate became 86%/day. The rate we have obtained *in vitro* should therefore be considered a minimum.

We have found at least a fivefold difference between the fractional synthesis rate obtained *in vitro* and that obtained *in vivo*. It therefore seems likely that, in the lungs of normal animals, the AM is in a quiescent state with a low synthetic rate *in vivo*, and that obtaining the cells by bronchoalveolar lavage and low-speed centrifugation is sufficient to activate them partially to a state with a substantially higher rate of protein synthesis. These results should be borne in mind when extrapolating observations made *in vitro* to the situation in the intact animal.

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