

CHARACTERIZATION OF THE HUMAN PULMONARY FIBROBLAST
DERIVED FROM THE NORMAL LUNG AND
FROM THE LUNG OF PATIENTS WITH
IDIOPATHIC PULMONARY FIBROSIS

by

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ABSTRACT

Fibrosis of the lung is a disease of chronic nature characterized by a profound derangement in the lung architecture caused by an alteration in the composition, distribution and organization of the lung extracellular matrix proteins. Two of the main histologic features of this disease are the increase in the number of fibroblasts (extracellular matrix-producing cells) in the lung parenchyma and the presence of a continuing inflammatory reaction. It is generally thought that the interactions between fibroblasts and inflammatory cells, particularly the monocyte/macrophage, represent a central component in the pathogenesis of this disease.

The proliferative behaviour of human lung fibroblasts in vitro was examined. Fibroblasts derived from the lung of patients with idiopathic pulmonary fibrosis proliferated faster compared to fibroblasts from control lung tissue. An examination of clonally-derived fibroblast lines showed a substantial degree of fibroblast heterogeneity which followed a normal distribution, and also the existence of a significantly greater number of fast-growing clones in the panels of clones derived from primary fibroblast lines established from fibrotic tissue. Heterogeneity with respect

to the expression of collagen genes was also documented.

The effect of an acute challenge of peripheral blood monocyte and/or alveolar macrophage supernatants on fibroblast proliferation was examined. Supernatants from peripheral blood monocytes and alveolar macrophages stimulated with lipopolysaccharide elicited a dose-dependent inhibition of fibroblast proliferation, likely induced by interleukin 1. This effect could be prevented by pre-treating the fibroblasts with indomethacin and reconstituted by adding exogenous prostaglandin E₂, thus indicating the involvement of the prostaglandin E₂ pathway of the fibroblast. Exogenous prostaglandin E₂ directly caused an inhibition of fibroblast proliferation, and fibroblasts derived from fibrotic tissues were shown to be hyporesponsive to this mediator. Supernatants from unstimulated alveolar macrophages obtained from rats which had been given intratracheal bleomycin caused a similar effect on the proliferation of rat lung fibroblasts.

In an attempt to reproduce the situation of chronic inflammation which exists in vivo, fibroblasts were chronically exposed (4-5 weeks) to peripheral blood monocyte supernatants and their response to acute challenges with this supernatant as well as to prostaglandin E₂ examined. Primary lines of fibroblasts chronically exposed to peripheral blood monocyte supernatants became hyporesponsive to both these supernatants and prostaglandin E₂, and released greater amounts of prostaglandin E₂, upon rechallenge compared to

unexposed fibroblasts. An examination of clonally-derived lines showed marked heterogeneity in the responsiveness of individual clones to peripheral blood monocyte supernatants and prostaglandin E_2 as well as a change in the clonal distribution after chronic exposure to peripheral blood monocyte supernatants. Chronic exposure of sensitive clones to peripheral blood monocyte supernatants did not alter their level of responsiveness.

These studies: a) provide evidence of fibroblast heterogeneity; b) demonstrate that fibroblasts derived from fibrotic tissues express distinct phenotypic characteristics compared to control fibroblasts in vitro; c) demonstrate that the prostaglandin E_2 pathway of the fibroblast is centrally involved in the regulation of fibroblast proliferation induced by interleukin 1; d) demonstrate that chronic exposure of human lung fibroblasts to inflammatory cell mediators in vitro results in a change in the responsiveness of these cells to these mediators, most likely by a mechanism of clonal selection. Together, these data would suggest that one mechanism by which fibroblasts accumulate in the lung of patients with pulmonary fibrosis could include the loss of the ability of these cells to self-regulate their growth via the prostaglandin E_2 pathway.

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List of Abbreviations

AM	alveolar macrophage
AMGDF	alveolar macrophage growth-derived factor
ARDS	adult respiratory distress syndrome
BAL	bronchoalveolar lavage
CF	control fibroblasts
Ci	curie
CM, FCM	conditioned medium, fibroblast-CM
cpm	counts per minute
DNA, cDNA	deoxyribonucleic acid, complementary DNA
ECM	extracellular matrix
FCS	fetal calf serum
FF	fibrotic fibroblasts
Fig.	figure
GAG	glycosaminoglicans
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony- stimulating factor
HLF	human lung fibroblast
HSF	hepatocyte stimulating factor
IFN γ	interferon gamma
IGF-1	insulin-like growth factor 1
IL-1	interleukin 1
IL-6	interleukin 6

IL-8	interleukin 8
IPF	idiopathic pulmonary fibrosis
kd	kilodalton
L	liter
LAF	lymphocyte activating factor
LPS	lipopolyssacharide
M	molar
m	mean
M-CSF	macrophage colony-stimulating factor
MEM	minimum essential medium
μ Ci	microcurie
μ g	micrograms
mg	milligram
ml	milliliter
PBM	peripheral blood monocyte
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PGE ₂	prostaglandin E ₂
RGM	regular growth medium
RNA, mRNA	ribonucleic acid, messenger RNA
SD	standard deviation
SE	standard error of the mean
TGF β	transforming growth factor β
TNF	tumor necrosis factor
TSC	tissue structural cells

INTRODUCTION

Prevalence and etiology of pulmonary fibrosis

Human pulmonary fibrosis is a disease with an estimated prevalence of three to five cases per 100,000 population (1). The disease may occur in all age groups but there is an increased prevalence in the fourth to sixth decades. There is also a slight prevalence in males (2). Fibrosis of the lung may occur as a result of exposure to a great deal of agents including infectious agents, inorganic dusts such as asbestos, silica and aluminium, inorganic chemicals such as mustard gas, nitrogen oxide and sulfur dioxide, therapeutic agents such as oxygen and radiation therapy, and drugs such as bleomycin, busulfan and nitrofurantoin. In general, the disease is restricted to the lung but the presence of pulmonary fibrosis in neoplastic diseases such as bronchoalveolar carcinoma and lymphangitis carcinomatosa as well as the association between pulmonary fibrosis with connective tissue diseases such as rheumatoid arthritis, dermatomyositis and scleroderma have been well documented. All in all, more than 100 agents with the potential to cause lung fibrosis have been identified (3). However, an etiologic agent cannot be found, after careful

investigation, in approximately 2/3 of the diagnosed cases, and these are thus termed idiopathic pulmonary fibrosis (IPF). The equivalent term in the British literature is "fibrosing alveolitis" which accurately describes the two essential histologic features of this disease (4).

Clinical course of pulmonary fibrosis

Pulmonary fibrosis is a rather devastating disease with a five-year survival of approximately 50% (5,6). The pulmonary functional deterioration which characterizes this disease is relatively slow in most cases (7). However, a number of patients have been described with rapid deterioration leading to death between 1 to 6 months from the onset of symptoms. This is referred to as the "Hamman-Rich Syndrome" (8). This rapidly fatal course can occur not only in IPF but also in instances with a recognized etiology. For example, whereas it is well known that diamond polishers may develop slowly progressive fibrosis of the lung (9), the occurrence of rather rapid and lethal disease has been recently reported (10). A number of experimental models resembling human pulmonary fibrosis have been developed. The course of the disease in these models is also heterogeneous. For example, the intratracheal instillation of bleomycin into rats causes marked fibrosis of the lung which peaks at around the second week after exposure (11), but the lungs of animals

which received bleomycin show relatively little fibrosis one year later (12). In contrast, the administration of paraquat causes a rapidly intense and sustained lung fibrotic response (13).

Histology of pulmonary fibrosis

The histologic abnormalities of pulmonary fibrosis consist of varying degrees of tissue injury, inflammatory cell accumulation and fibrosis. There is a rather general agreement that early in the disease the preponderant changes comprise a patchy alveolitis with mild to moderate thickening of the alveolar walls. As the process continues, the cellular population in the interstitium and alveoli expand and the structural abnormalities in the parenchyma become more apparent (14-17). In the final stages, a so-called honeycombing pattern characterized by a marked restructuring of distal air spaces, obliteration of small airways and formation of macroscopic cysts separated by areas of dense interstitial fibrosis, is frequently observed (18). In addition to the interstitial changes, there is evidence in human pulmonary fibrosis of alterations in both the endothelium and the epithelium (19-20). Detailed studies in experimental models of pulmonary fibrosis have shown that these changes always precede the changes in the lung parenchyma.

Treatment of pulmonary fibrosis

Based on the assumption that fibrosis is an inflammation-driven disease, corticosteroids have represented until recently the mainstay of the treatment of patients with pulmonary fibrosis. However, the patients response to these powerful antiinflammatory agents is rather poor. A few studies suggest that those patients with a more intense inflammatory, rather than fibrotic, component in the lung biopsy tend to respond better to this treatment. More recently, therapeutic regimes including corticosteroids together with cytotoxic drugs such as cyclophosphamide have been tried with apparently better results. In general, it is thought that approximately 30-50% of patients with IPF show some objective response to therapy (21-24). However, there are no sufficiently large properly controlled studies examining the effect of these drugs on important outcomes such as mortality. Therefore, there appears to be a large component of the disease which cannot be altered with any drug strategy currently available.

REVIEW OF THE LITERATURE

The extracellular matrix in pulmonary fibrosis

Lung fibrosis has been frequently thought of as fibrous thickening of the pulmonary interstitium resulting from increased collagen deposition. However, the structural abnormalities which characterize this disease are in fact of a more complex nature. Studies in animal models of pulmonary fibrosis have shown that injury to either the epithelium or the endothelium precedes the interstitial changes (25,26). The presence of areas with a defective epithelial lining, to the point of denudation, has been well documented in human interstitial lung disease (27,28). Fibroblasts can migrate through these areas and develop various patterns of intraluminal organization which, in some instances, incorporate distal bronchioles. In addition, these areas may fold and eventually collapse to permanent apposition of their walls. Further evidence of basement membrane disruptions has been documented in the distribution of laminin and type IV collagen in specimens from patients with pulmonary fibrosis (29). Kuhn et al, in an elegant immunohistochemical study of human pulmonary fibrosis, have clearly shown that the epithelium is abnormal, with bronchiolar and squamous

metaplasia in some areas and hyperplastic alveolar epithelium in others (20). They also showed that most areas of scarring exhibit an abnormal vascular pattern consistent with either ingrowth of granulation tissue or with residual alveolar capillaries.

Alterations in a number of extracellular matrix proteins other than collagen have been reported in pulmonary fibrosis. For example, Starcher et al have shown increases in elastin in the lungs of hamsters exposed to bleomycin (30), and qualitative and quantitative changes in glycosaminoglicans (GAG) have been reported in fibrotic lungs (31). Furthermore, alterations in the distribution of cellular fibronectin, both in foci at the surface of fibrotic septa and discretely localized in subepithelial regions, have been clearly documented (20). Thus, it seems more accurate to visualize fibrosis of the lung as characterized by a profound derangement of the lung architecture resulting from alterations in the deposition, distribution and/or organization of ECM proteins. The abnormalities in these various ECM proteins highlight the key role of tissue structural cells such as fibroblasts, epithelial and endothelial cells in the development of the fibrotic event.

Collagen metabolism in fibrosis

Amongst the various lung ECM proteins which

deposition, distribution or organization are altered in pulmonary fibrosis, collagen is the best studied protein to date. Thus, to examine the metabolism of collagen with some detail may provide important insights into the pathogenesis of pulmonary fibrosis.

Collagen is the most abundant constituent of the interstitial connective tissue matrix which comprises 60-65% of the total non-cellular mass (32). The fundamental unit of collagen is the tropocollagen molecule, which by its ability to copolymerize with other tropocollagen molecules, is capable of forming the cytoskeleton of the interstitium. Tropocollagen is composed of three alpha chains in a right-handed helical arrangement. The different types of collagen are distinguished by a combination of the five known alpha chains. There are 13 types of collagen, the product of 23 genes, described to date. Most of the collagen genes are developmentally regulated and a given tissue, or cell type, expresses only a subset of the collagen genes. The most abundant collagen in the lung is type I, which contains two α -1 chains and one α -2 chain (33,34). This type of collagen comprises 60-70% of total interstitial collagen in normal individuals. Type III collagen, which usually accounts for 30-40% of interstitial collagen, influences the way type I collagen forms fibrils by mechanisms not well understood (32,35).

The major abnormality in IPF appears to be an

increase in the ratio of type I:type III collagen; a predominance of type III collagen in the early stages of the disease (36) and of type I collagen in late or advanced disease has been reported (16). Collagen metabolism is a complex and dynamic process involving a number of intracellular and extracellular feedback mechanisms (37). In fact, degradation of collagen presumably occurs continually in the normal lung as the concentration of collagen remains unchanged in spite of continued synthesis (38). In this regard, an interesting paradox is that fibroblasts secrete, in addition to collagen, collagenase and stromelysin which are enzymes responsible for collagen breakdown, as well as TIMP, an inhibitor of collagenase (39). Thus, the actual amount of collagen of any type being laid down is in effect the balance between the production of these various proteins and enzymes or, in other words, between synthesis and breakdown.

Fibroblasts normally account for 35-40% of cells in the pulmonary interstitium (40), and thus abnormalities in fibroblast biology are likely to be a central component in pulmonary fibrosis. The observation that the number of fibroblasts is increased in the lungs of patients with pulmonary fibrosis supports this notion as it represents an increase in the number of collagen (and other extracellular matrix proteins)-producing cells (41). In addition to increased proliferation, fibroblast activation could be

another mechanism contributing to collagen alterations in the lung. By using antibodies to the propeptides of collagen types, thus identifying cells which are actively synthesizing collagen, Khun et al showed the presence of foci of active collagen synthesis in the majority of biopsies from IPF patients (20). These foci were identified as the sites of invasion by fibroblasts actively synthesizing extracellular matrix proteins. This is in contrast to normal lungs where most fibroblasts are essentially in a quiescent state. Interestingly, a substantial proportion of these collagen synthesizing fibroblasts resided in subepithelial areas, thus raising the issue of potential interactions between fibroblasts and epithelial cells. A similar observation has been made in scleroderma, a fibrotic condition of the skin, where the proportion of cells expressing collagen genes has been shown to be increased by means of in situ hybridization compared to normal skin (42). Furthermore, Selman et al showed decreased collagenolytic activity in lung tissue samples of patients with pulmonary fibrosis (43), and Perez-Tamayo reported a similar observation in experimentally-induced fibrosis of the liver (44). Therefore, there may be several, perhaps independent mechanisms, involved in the dysregulation of collagen metabolism in pulmonary fibrosis: a) an increased number of collagen-producing cells, b) an increased proportion of fibroblasts actively synthesizing collagen, c) an increased collagen output per cell, and d)

decreased collagen breakdown.

Inflammation and fibrosis

The sustained presence of an inflammatory process is a major observation in the lungs of patients with pulmonary fibrosis. Whereas the association of an inflammatory reaction to the fibrotic event has been long recognized, the notion that inflammation and fibrosis might be causally related is a more recent one. This has come about for several reasons. Firstly, the detailed examination of experimental models of fibrosis has clearly established that the development of fibrosis is always preceded by an inflammatory reaction. Secondly, the administration of fibrogenic stimuli, such as silica and hyperbaric oxygen, to species such as fowl and amphibia, in which inflammatory cells do not accumulate in the lung, does not result in pulmonary fibrosis (45,46). The third reason, and perhaps the most important one, stems from the burgeoning amount of knowledge documented in the last few years with regards to the effector potential of inflammatory cells, specifically, the ability of molecules released by these cells to modulate the function of tissue structural cells, particularly fibroblasts.

The AM as a central effector cell in lung fibrosis

The articulation of a physiological response to an injurious agent is not a simple isolated event. Rather, it involves a complex set of direct interactions between cells of various types. This dialogue between cells is mostly conducted by messenger signals which are hormone-like peptides, also referred to as cytokines. Amongst the various inflammatory cells which are thought to orchestrate the inflammatory response which occurs during the course of lung fibrosis, the AM is considered to play a central role. Indeed, this cell has, in addition to major scavenging and antigen presenting abilities (47) a considerable effector armamentarium.

Alveolar macrophages can release mediators with a wide range of pharmacologic effects such as prostaglandins and leukotrienes, which modulate the behaviour of numerous lung cells (48,49). AM's also secrete a number of enzymes including elastase and collagenase which are important to tissue integrity (50). Furthermore, the alveolar macrophage has been recognized during the last few years as a major source of cytokines. Interleukin-1 (IL-1) was the first cytokine to be assigned an "interleukin" nomenclature to indicate the messenger role of this molecule "between-leukocytes", most significantly its ability to stimulate lymphocytes. It is now well known that this cytokine mediates

numerous biological activities including the modulation of various functions of fibroblasts (51). Transforming growth factor β (TGF- β) is a 25-kD homo- and heterodimer initially identified by its ability to cause phenotypic transformation of rat fibroblasts and now recognized to have many other regulatory functions. For example, it has been shown that TGF- β is chemotactic for fibroblasts (52), and also that this molecule stimulates expression and biosynthesis of both collagen and fibronectin in dermal fibroblasts (53). TGF- β also inhibits collagenase gene expression and also enhances the secretion of tissue inhibitor of metalloproteinases (54). Tumor necrosis factor α and β (TNF α , β) are two cytokines originally recognized because of their cytotoxic and antitumor properties. There is now evidence that TNF stimulates collagenase and PGE₂ synthesis by human synovial and dermal fibroblasts (55) and Elias et al recently reported that this molecule increases GAG production by human lung fibroblasts (56).

In addition to these cytokines with significant effects on fibroblasts, monocyte/macrophages can produce other molecules with potentially important pro-inflammatory effects. For example, recent investigations have distinguished a novel polypeptide that possesses potent and selective activities, particularly chemotaxis and activation, for neutrophils (57) as well as for lymphocytes (58). This cytokine has received a variety of names but the term

interleukin-8 (IL-8) is beginning to be accepted by most. IL-8, which has a molecular weight of approximately 10kD was first identified in monocytes (57) but Strieter et al recently showed that human alveolar macrophages also release this activity (59). Interleukin-6 (IL-6) is a 25-30 kD protein produced by monocytes and macrophages which has been shown to be identical to the molecules previously known as hybridoma growth factor (HGF), interferon β 2 (IFN^C β 2) and hepatocyte-stimulating factor (HSF) (60). One of the main functions of this molecule is to stimulate hepatocytes to release the acute-phase response proteins (61), thus mediating some of the systemic effects which occur during the course of inflammatory processes. In addition, IL-6 has been recently shown to induce maturation of platelets (62) and, alone or in combination with other cytokines, to stimulate differentiation of hemopoietic cells (63) as well as proliferation and activation of lymphocytes (64) and neutrophils (65).

Furthermore, alveolar macrophages secrete a series of molecules better known as growth factors which include platelet-derived growth factor (PDGF), fibronectin and alveolar macrophage-derived growth factor (AMGDF). PDGF is a 39-33,000 dalton dimeric glycoprotein composed of an A chain and B chain linked by disulfide bonds which was first isolated from the alpha granules of platelets (66). The biologically active B chain of PDGF is coded for by a 30kb

gene located on chromosome 22 designated the C-sis proto-oncogene which is an analogue of the retroviral V-sis oncogene originally isolated from a simian sarcoma virus (67). Although this gene appears to be important in the malignant transformation of fibroblasts, there is evidence that the 4.2kd mRNA transcript of the C-sis gene is found in normal AM and monocytes (68). PDGF has been shown to be chemotactic for fibroblasts (69) and also to act as a growth factor for these cells (70). In addition, PDGF has been shown to be chemotactic for smooth muscle cells which also accumulate in the interstitium during the course of IPF (71). Fibronectin is a 440,000 dalton glycoprotein with specific domains that bind to the fibroblast surface (72). Fibronectin is also chemotactic for fibroblasts (73) and stimulates proliferation of resting fibroblasts at doses in the nanomolar range in a dose-dependent fashion (74). However, the optimal proliferative effect of fibronectin on fibroblasts is seen in combination with a progression factor such as alveolar macrophage-derived growth factor (AMDGF). AMDGF was described as an 18,000 dalton protein (75), recently being characterized as similar to insulin-like growth factor (IGF-1) (76), released by AM upon stimulation which induces replication of fibroblasts primed with fibronectin.

It is then readily apparent that the alveolar macrophage may secrete a great many cytokines and growth

factors in vitro which are capable of interacting with, thereby activating, fibroblasts. However, a central issue is whether this effector function is exercised in vivo and, more importantly, what its relationship is to disease processes. One approach to address this issue involves examining the effector ability of cells obtained from subjects with the disease, or from animals exposed to the appropriate agent, and compare these activities to those of cells obtained from normal subjects or control animals. An important point of interest is that cells obtained from normal hosts release spontaneously, that is without further stimulation in vitro, very small amounts of cytokines. In contrast, alveolar macrophages isolated from patients with fibrotic lung diseases spontaneously release increased amounts of AMDGF (75) and PDGF (77) without further stimulation in vitro. Similarly, Rom et al have recently demonstrated that AM derived from patients with fibrotic disease caused by exposure to occupational agents including asbestos, silica and coal spontaneously synthesize both IGF-1 and fibronectin (78). Driscoll et al have recently shown in a model of silica-induced pulmonary fibrosis that AM release increased amounts of TNF and fibronectin in vitro without further stimulation (79). In a rat model of bleomycin-induced pulmonary fibrosis, Khalil et al have shown that total lung TGF β was elevated within 2h of intratracheal bleomycin administration into rats and peaked 7d later at levels 30-

fold higher than controls, and that this peak preceded the maximum collagen and noncollagen protein synthesis in the lung (80). Furthermore, they demonstrated, in an excellent immunohistochemical study, intense TGF β staining of macrophages dispersed in the interstitium and in organized clusters at day 7 after bleomycin. Finally, Piguet et al recently showed, also in this experimental model, that there is an increase in the lung TNF α mRNA level from days 5 to 15 as shown by Northern gel analysis of whole lung RNA (81).

To summarize, alveolar macrophages from patients with a variety of interstitial/fibrotic lung disorders, or from animals undergoing experimental lung fibrosis, release increased amounts of a number of cytokines including AMGDF/IGF-1, fibronectin, TNF α and TGF β all of which can, alone or in concert, stimulate fibroblasts to display activities consistent with the development of fibrosis.

Other inflammatory effector cells in lung fibrosis

Although earlier studies of BAL did not find an increase in lymphocytes (82), several subsequent studies have shown an elevation of these cells in IPF (83). In fact, recent studies indicate that a lymphocyte alveolitis may be an early event in the disease. There are several mechanisms by which lymphocytes could contribute to the fibrotic event. Perhaps the most attractive one would involve B cell

stimulation. In this regard, there is evidence that the number of antibody-producing cells in the BAL of patients with IPF is markedly increased (84). The presence of immunoglobulin and immune complexes could mediate lung injury by activation of neutrophils to produce toxic oxygen species and fixation of complement after attachment to the epithelial or endothelial cell surface (85). In addition, lymphocytes could contribute to the inflammatory process by virtue of their ability to release cytokines such as GM-CSF (granulocyte-macrophage colony-stimulating factor), IL-3 (interleukin-3) and IL-5 (interleukin-5). GM-CSF mediates a broad spectrum of activities in mature inflammatory cells including their survival, activation and proliferation (86). IL-3 and IL-5 are mostly involved in growth, differentiation and activation of mast cells, eosinophils and basophils (87-90). Studies in experimental models of fibrosis have produced conflicting results. Thrall et al showed that the administration of anti-lymphocyte globulin partially suppresses fibrosis in rats which received bleomycin (91), and they found similar results in T-cell depletion by adult thymectomy (92). However, Schrier et al (93) and Szapiel et al (94) found that collagen synthesis was either increased or unchanged in nude animals compared to euthymic animals after bleomycin. Furthermore, fibrosis is a rare outcome in a human lymphocyte-driven disease (95) such as sarcoidosis (96).

Lung mast cell hyperplasia has been documented in a

variety of human fibrotic conditions (97,98) as well as in several experimental models of fibrosis including exposure to asbestos (99), bleomycin (100) and irradiation (101). However, the role of these cells in the pathogenesis of lung fibrosis remains far from being elucidated. A number of interesting interactions between mast cells and other lung cells have been recently described. For example, Liechtenstein has shown that the AM is capable of releasing a molecule, as yet not characterized, which stimulates histamine release from human lung mast cells and peripheral blood basophils (102). On the other hand, we have shown that histamine stimulates proliferation of human lung fibroblasts (103). The interaction between mast cells and fibroblasts appears to be bidirectional because fibroblasts can phagocytose mast cell granules and in the process actively secrete b-hexosaminidase and collagenase (104). Furthermore, there is now evidence that rat mast cell lines can express the gene for a number of cytokines including TNF, IL-6, IL-3 and GM-CSF (105). Whether human mast cells can release these cytokines is an important issue which remains to be clarified. Two independent groups have recently studied an experimental model of bleomycin-induced pulmonary fibrosis using mutant mast cell deficient mice (106,107) and observed that the lung hydroxyproline content of these mice, an indication of collagen deposition, was significantly lower than control littermates. A decrease in the total cell number

as well as in the percentage of neutrophils in the BAL in the mast deficient mice was also reported. However, it is difficult without further studies to conclude whether the differences in collagen content seen are specifically related to the mast cell deficiency or to other associated cellular abnormalities present in these mutant mice.

Critical Appraisal of current concepts on the pathogenesis of pulmonary fibrosis

A number of leading researchers in the field of pulmonary fibrosis have proposed a hypothesis (Figure 1) which centers around the notion that interactions amongst alveolar macrophages, neutrophils and fibroblasts play a critical role in the cascade of events leading to fibrosis (108,109). Here, the alveolar macrophage is viewed as the central effector cell, the neutrophil as the cell chiefly responsible for causing tissue injury and the fibroblast as the major target cell undertaking a reparative role, a dysregulation of which results in fibrosis. Whilst there is a substantial amount of evidence in support of this hypothesis, it is important to examine to what extent this evidence is specific to the development of fibrosis as opposed to descriptive of a physiological reparative response.

In regard to the alveolar macrophage, it has been

shown that AM's from patients with sarcoidosis spontaneously release increased amounts of AMGDF in vitro (110). Furthermore, AM's from patients with sarcoidosis (111) and, as recently reported, with the Adult Respiratory Distress Syndrome (ARDS) (112), release increased amounts of IL-1. ARDS is an acute condition characterized by a very intense inflammatory reaction in the lung, primarily neutrophilic, with a despairing mortality rate. In contrast, sarcoidosis is a chronic disease, of a benign course in many instances, characterized by a lymphocyte-driven granulomatous reaction in the lung with marginal neutrophil accumulation. If activated AM's are the prime mediators of the neutrophil accumulation in the lung, it is difficult to explain the differences in neutrophil accumulation in diseases such as IPF, sarcoidosis and ARDS where the AM is activated. It is equally difficult to explain the distinct occurrence of pulmonary fibrosis in these three conditions. Indeed, fibrosis rarely occurs in sarcoidosis and is, on the basis of the data available, negligible in ARDS survivors one year after the acute episode (113-116).

Neutrophils have often been considered as the main "culprit" cell causing tissue injury leading to fibrosis. This has been based upon consideration of the ability of these cells to release products such as enzymes and oxygen species capable of damaging the tissue (117). To investigate

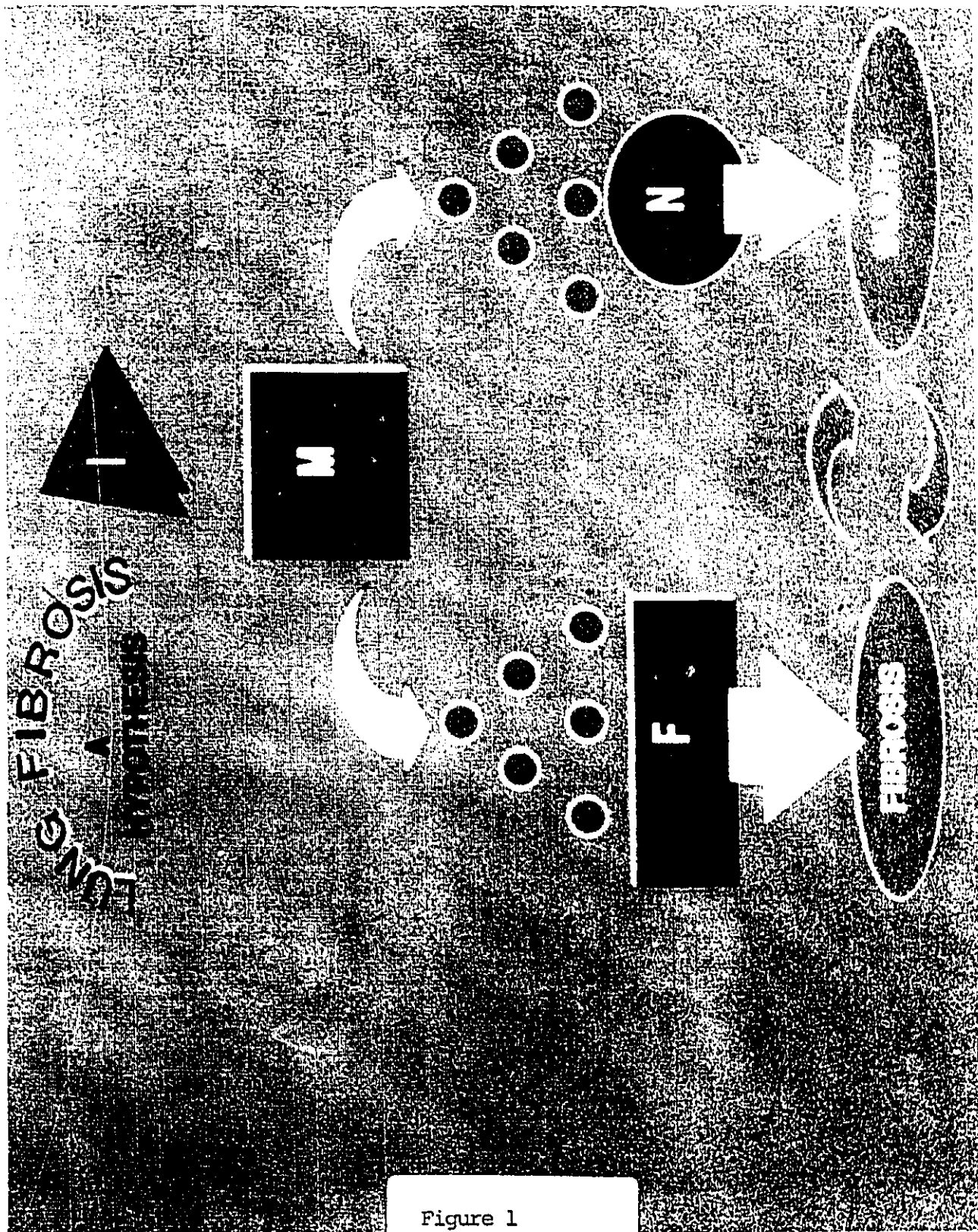


Figure 1

the role of neutrophils in fibrosis, Thrall et al studied the effects of bleomycin in rats depleted of neutrophils by an anti-neutrophil antiserum, and found an increased content of total lung collagen in these animals (118). Similarly, Phan et al examined the effect of bleomycin in beige mice, a mutant strain which has a functional defect in neutrophil function, and they also found that the rate of collagen synthesis and of the amount of lung collagen content was greater in these animals compared to normal mice (119). With regards to the mechanism by which neutrophils might disrupt the tissue, it has been proposed that the secretion of collagenase plays an important role in disrupting the ECM and, the finding of increased amounts of collagenase in the BAL fluid of patients with IPF could support this notion (120). However, the significance and implications of this observation are unclear. First, cells other than the neutrophil, such as the alveolar macrophage and the fibroblast, can secrete collagenase. Second, collagenase preferentially degrades type I and type II collagen which is difficult to reconcile with the alterations in type III collagen which occur in pulmonary fibrosis (39,121).

Finally, in the hypothesis under consideration, the fibroblast is considered merely a target cell, i.e. the expression of a particular function by the fibroblast is dependent on the signalling by inflammatory cell-derived cytokines. While this is certainly a conceivable possibility,

there is no definitive proof that this is the mechanism which determines the development of pulmonary fibrosis. In addition, there is substantial evidence now that fibroblasts, as well as other tissue structural cells (TSC), are not only targets but also effector cells themselves capable of releasing a number of powerful cytokines (Appendix). Given that TSC represent by far the most abundant cell compartment in the lung, it is clear that the hypothesis omits a potentially central player in the cellular interactions which occur in the lung particularly during the course of chronic inflammation.

Consequently, there are two fundamental issues which remain without adequate explanation at this point in time. The first one refers to the relationship between the initial event (inflammation) and the outcome (fibrosis). Effectively, each one of the inflammatory cells and each one of the cytokines which we have reviewed have a physiologic function. In the context of pulmonary fibrosis, their activities could represent the effort of the tissue response to heal itself. Thus, their presence in fibrosis might be coincidental but not causal. The second issue refers to why there is a sustained inflammatory response in the lung in chronic human fibrosis; specifically, what mediates the accumulation of activated inflammatory cells in the lung. Whereas a continued exposure to the offending agent could be invoked as the reason in instances such as occupational lung diseases, this

explanation would be difficult to support in idiopathic pulmonary fibrosis.

Hypothesis

The pulmonary interstitial fibroblast is the main cell responsible for the alteration of the metabolism of collagen and other extracellular matrix (ECM) proteins which characterizes pulmonary fibrosis. This disorder is also characterized, during its evolution, by the presence in the lung of an increased number of inflammatory effector cells that release mediators which modulate fibroblast function. The increased deposition of ECM proteins seen in pulmonary fibrosis may result from chronic stimulation of fibroblasts by lung effector cell-derived cytokines. Alternatively, similar signals may act on an existing heterogeneous fibroblast population to mediate the emergence, whether by selection or induction, of subpopulations of cells resulting in the predominance of the fibrotic phenotype in the lung.

Purpose of this study

The central objective of this project is to examine the role of the interstitial lung fibroblast in the pathogenesis of pulmonary fibrosis. This examination is focussed along two main lines. First, in the light of the

evidence showing that there are increased numbers of fibroblasts in the lung interstitium of patients with pulmonary fibrosis, to study whether fibroblasts isolated from the lungs of patients with this disease exhibit distinct intrinsic proliferative characteristics in vitro. In addition, to study whether there is human lung fibroblast heterogeneity and whether the profile of this heterogeneity is different within cells derived from diseased tissues. Second, to investigate the interactions between lung fibroblasts and the main inflammatory effector cell in the lung, the macrophage/monocyte. This investigation will include short term in vitro systems as well as long term in vitro systems in an attempt to recreate the chronicity which characterizes pulmonary fibrosis. The purpose of these studies is to examine whether certain phenotypic characteristics of the lung fibroblast may change, on a permanent basis, upon chronic exposure to inflammatory cell-derived mediators. These studies should provide insight into fundamental aspects of the pathogenesis of pulmonary fibrosis which may ultimately lead to the design of new therapeutic strategies.

MATERIALS AND METHODS

Patient characterization

Control fibroblast (CF) lines were derived from histologically normal areas of surgical specimens from patients undergoing resective surgery for cancer or hiatus hernia repair using a transthoracic approach. In addition, a series of fibroblast lines, referred to as "FF", were generated from specimens of grossly abnormal tissue from patients with clinically suspected active idiopathic pulmonary fibrosis undergoing open lung biopsy for diagnostic purposes. It should be pointed out that all our IPF patients had undergone the diagnostic procedure between 3 and 12 months since the beginning of symptoms and, thus, their disease was at a relatively early stage. None had been on immunosuppressive or corticosteroid therapy. Table 1 shows that the first three patients had a moderately severe restrictive defect. Patient 4, CIR, had a severe ventilatory impairment. All 4 biopsy specimens showed histologic evidence of fibroblast accumulation, increased collagen deposition and intimal fibroelastosis. However, none showed features suggestive of end-stage pulmonary fibrosis. Also, two fibroblast lines were established from tissues obtained from

Dr J. Maurer in Toronto of patients with very advanced pulmonary fibrosis who underwent a lung transplant. In addition, four lung fibroblast lines from neonates who had died from non-respiratory causes were established from tissue obtained within hours of death.

Isolation and culture of human lung fibroblasts (HLF)

Fibroblast cultures were established from lung tissue as previously described by Goldstein and Littlefield for forearm skin fibroblasts (Figure 2) (122). Briefly, lung specimens were chopped in pieces less than 1 mm³ and washed once with PBS and twice with RGM (Dulbecco's modified Eagle medium; Grand Island Biological Co., Grand Island, NY) containing 10% FCS; penicillin, 100 U/ml; streptomycin 100 µg/ml; and amphotericin B, 2.5 µg/ml. Three pieces were then plated in a 60-mm polystyrene dish (Corning Glassworks, Corning, NY) and overlaid with a coverslip adhered to the dish with sterile vaseline. Five ml of RGM were added, and the tissue was incubated at 37°C and 5% CO₂. The medium was changed twice weekly. When the bottom of the dish was coated with a monolayer of fibroblast-like cells at near confluency, usually 5 to 6 wk later, the explant tissue was removed, and the cells were trypsinized for 10 min, resuspended in RGM, and replated in 100-mm tissue culture dishes with 10 ml of RGM. Subsequently, the cells were split 1:2 at confluency and

TABLE 1
PATIENT DATA

Code	Age (yr)	Sex	Smoking History (pack-yr)	Diagnosis	FEV ₁ /VC*	Ga	BAL†‡
Control VEN	42	M	35	Poorly differentiated squamous cell carcinoma.	N/A	ND	ND
BRY	64	M	100	Poorly differentiated squamous cell carcinoma.	N/A	ND	ND
MCB	52	F	None	Hiatus hernia.	N/A	ND	ND
McL	30	M	18	Hiatus hernia.	N/A	ND	ND
Fibrotic SOM	59	M	None	Usual interstitial pneumonia. Consistent with asbestosis.	2.32/3.94 (3.5/4.45)	+	PMN 3% Ly 1% E 9%
JAC	22	F	None	Usual interstitial pneumonia. MCTD.	1.95/2.23 (3.56/4.08)	+	ND
RAC	50	M	35	Usual interstitial pneumonia.	2.75/2.97 (3.9/4.9)	+	PMN 13% Ly 11%
CIR	11	M	None	Desquamative interstitial pneumonia. §	0.45/0.75 (2.41/2.86)	+	ND

Definition of abbreviations: Ga = gallium scan; BAL = bronchoalveolar lavage; PMN = polymorphonuclear cells; Ly = lymphocytes; E = eosinophils; MCTD = mixed connective tissue disease; N/A = not applicable; ND = not done.

* Spirometric figures in brackets represent predicted values.

† Differential counts.

§ Developed after bleomycin treatment.

passaged, usually weekly. Aliquots of cells at each passage were frozen and stored in liquid nitrogen. In the experiments described below, cells at a passage earlier than the tenth were used.

Establishment of clonally-derived fibroblast lines

A limiting dilution method was used to prepare fibroblast clones (Figure 3). Initial attempts using 10% FCS did not yield any clones. Thus, 10^6 cells were suspended in 10 ml of RGM containing 20% FCS. Serial dilutions were performed to a final concentration of 50 cells in 10 ml of medium. One hundred microliters of this cell suspension were delivered to each well of a 96-microtiter plate. These cultures were maintained at 37°C and 5% CO₂ with a weekly medium change and examined visually at frequent intervals. Any wells having visual evidence of more than one cell at this initial plating were discarded. Once cells reached confluency in the microtiter wells, they were trypsinized as before and transferred to T-24 plates (Nunc, InterMed NUNC, Denmark). Similarly, when cells reached confluency in T-24 wells, they were trypsinized and transferred to 25-cm² Falcon tissue culture flasks (Becton Dickinson Labware, Oxnard, CA). Once the cells became confluent in these flasks, they were immediately harvested and assayed. Cells not used in the assay were stored in liquid nitrogen. Although the

limiting dilution technique we used here is not itself a guarantee that we actually generated monoclonal cells, the implication of seeding the equivalent of half a cell per well is that the probability of a given colony evolving from a single cell is very high.

Immunohistochemistry and electronmicroscopy of fibroblast monolayers

Confluent monolayers of fibroblasts were stained with anti-keratin (1:50 AE1/AE3, Hybritech, San Diego, CA), or with anti-vimentin (1/50 V9, Dako, Santa Barbara, CA) monoclonal antibodies using the Zymed streptavidin-biotin immunoperoxidase method (Zymed Histostain-SP Kit, Zymed Laboratories Inc., San Francisco, CA). Confluent monolayers of epithelial cells were also stained with these antibodies to control for specificity. Negative controls were included throughout by substituting the diluent (5% swine serum in tris buffered saline) for the primary antibodies.

For transmission electron microscopy, cultured fibroblasts grown on tissue culture dishes were rinsed with PBS (pH 7.4) and fixed in 2% cold glutaraldehyde in 0.1M cacodylate buffer (7.4) for 1h. After overnight rinsing in cacodylate buffer, they were then postfixed in 1% osmium tetroxide for 30 min and dehydrated with a series of increasing concentrations of ethanol. Monolayers were then

cut into small pieces and embedded in Spurr's resin. Ultrathin sections were doubly stained with uranyl acetate and lead nitrate and examined with a JEM 1000EX electronmicroscope.

Generation of fibroblast supernatants

To generate fibroblast supernatants, 8×10^6 HLF from various sources were seeded in 150 cm² Corning tissue culture flasks in 15 ml of RGM containing 10% FCS and incubated overnight at 37°C in 5% CO₂. This medium was removed the following day, and the cells washed three times with serum-free MEM. Fifteen ml of this serum-free medium were then added and the cells incubated for further 24 h, at which time the supernatant was harvested and stored at -20°C until used.

Generation of AM/PBM supernatants

Bronchoalveolar lavage (BAL) of normal volunteers was performed as previously described by Weinberger, Kelma and Elson (126). After centrifugation of the BAL fluid, the cell pellet was resuspended in RPMI containing 10% FCS and 2×10^6 cells in 1 ml were plated in each well of a T-24 NUNC plate. After 2 h of incubation at 37°C and 5% CO₂ to ensure adherence of the alveolar macrophages, the nonadherent cells population was carefully washed off. One ml of fresh medium

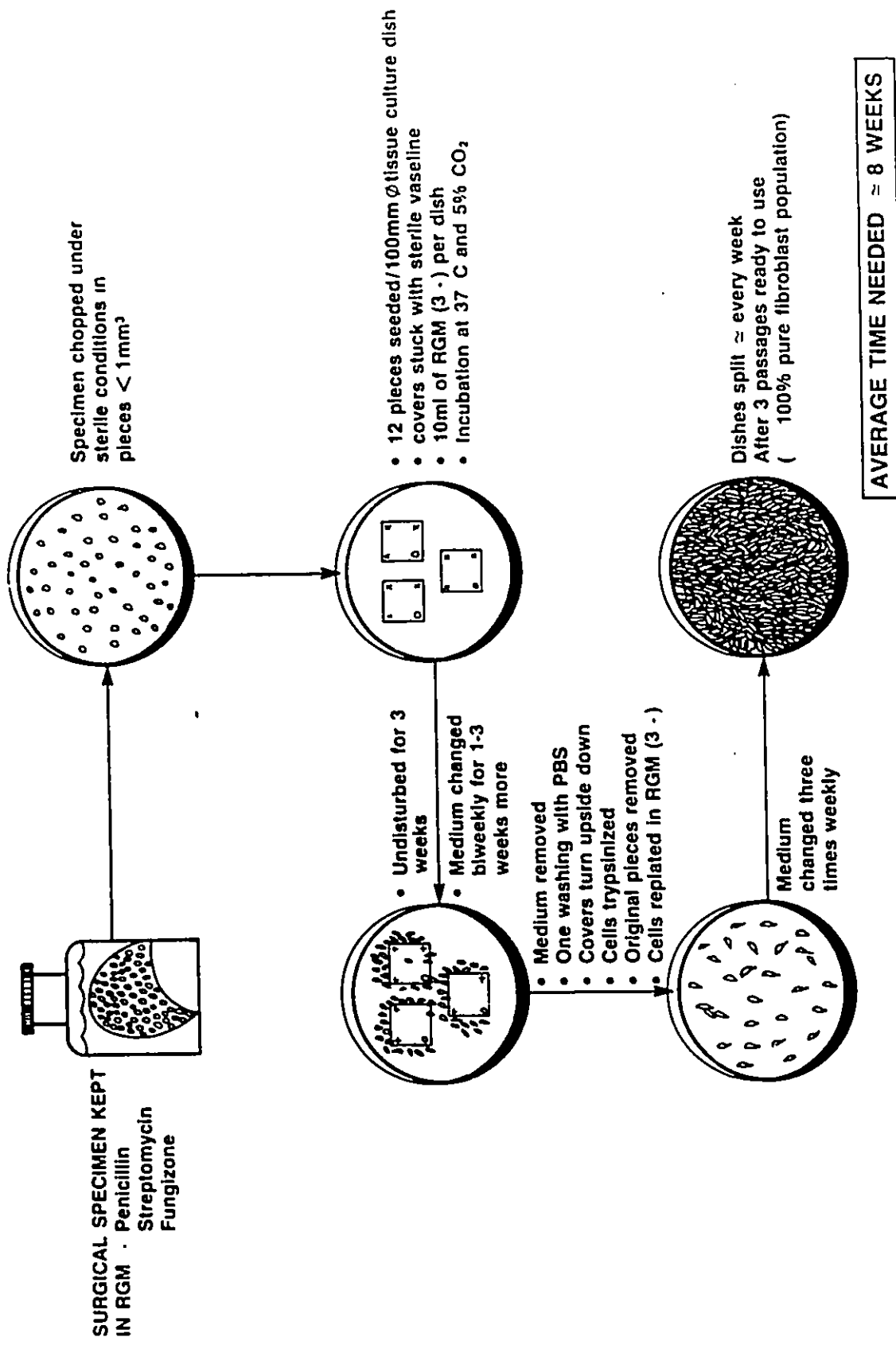


Figure 2

was then added and the adherent cells (95-99% alveolar macrophages) were incubated for a further 24 h with (stimulated) or without (unstimulated) lipopolysaccharide, 10 $\mu\text{g/ml}$ (LPS TCA precipitated; Sigma, St. Louis, Missouri). This level of LPS is not toxic to the cells and results in activation of macrophage/monocytes and the maximum generation of cytokines from the alveolar macrophage (127). The supernatant was collected, filtered, and extensively dialyzed against PBS using dialysis tubing with a molecular weight cut-off of 7-8,000 daltons. After dialysis, the samples were sterilized by micropore filtration (0.22 μ), aliquoted, and frozen at -20°C before being used. No samples thawed more than twice were ever used.

Peripheral blood monocyte supernatant was generated from normal volunteers in an identical manner after buffy-coat preparation and Ficoll-Hypaque centrifugation to prepare a mononuclear cell suspension. The cell suspension was further purified by adherence in RPMI 10% FCS at 37° in 5% CO_2 for 2 h at a cell density of 2×10^6 cells/ml in T-24 NUNC plates. After removal of the nonadherent cells by washing with PBS, the adherent cells were further incubated with 1 ml of medium with or without LPS for 24 h. The supernatant was removed and treated as for AM supernatant.

Fibroblast proliferation assays

Spontaneous fibroblast proliferation in vitro

Fibroblasts from confluent dishes were trypsinized and resuspended to 10^5 cells/ml of RGM. One hundred microliters of such suspension were delivered to each flat-bottom well of a 96-well microtiter plate (3.3×10^4 cells/cm²). The cells were incubated from 12 to 96 h. In all cases, 1 μ Ci/well of ³H-thymidine (20 Ci/mmol; NEN Corp., Boston, MA) was added during the last 12 h of the incubation period. The medium was then removed, the cells were washed with PBS, trypsinized and lysed, and cellular contents collected on a cell harvester (MA Bioproducts, Walkersville, MD). ³H-thymidine incorporation was detected by beta counting, and the resultant cpm expressed as the mean and standard deviation of 12 replicate wells.

Assessment of cell proliferation using cell counting was also performed. Unless otherwise indicated, 3.5×10^5 cells were seeded in 35-mm-diameter tissue culture dishes (3.6×10^4 cells/cm²; Corning) in 2 ml of RGM containing 10% FCS. As indicated in the RESULTS section, in some specific experiments several concentrations of FCS were used. The cells were incubated for various time intervals at 37°C and 5% CO₂. At the end of the incubation period, the medium was washed off, and the cells trypsinized and counted with a Coulter counter. Two dishes were prepared per data point, and

three counts were always performed per dish. All comparative examinations between FF and CF were carried out using the same batch of FCS. Cell viability was assessed by trypan blue exclusion and it was 95% or greater in all experiments.

Effect of fibroblast supernatants on fibroblast growth

To study the effect of fibroblast supernatants on fibroblast proliferation, 2×10^5 fibroblasts were seeded in 4 ml of RGM containing 10% FCS and incubated overnight to allow optimal adherence. The cells from three dishes were harvested and counted the following day. This cell count, which accounted for potential differences in cell adherence between experiments, represented the "background" count. The medium of the remainder dishes was removed and the cells washed once with serum-free medium. Four ml of medium containing 0.4% FCS were added and the cells incubated for further 24 h. At this time, the medium was removed again and fresh medium with or without fibroblast supernatant, at various concentrations, was added. The final concentration of FCS was equalized in all instances to 0.1%. The cells were then incubated for 4 days at which time they were trypsinized and counted with a hemocytometer. Three dishes were always prepared for each condition tested. The results plotted represent percent difference in relation to the "background" cell count.

Acute fibroblast challenge with AM/PBM supernatants

To study the response of HLF to either AM or PBM supernatants as well as to exogenous PGE₂ (Pharmacia, Piscataway, NJ), 10⁴ fibroblasts, removed by trypsinization from confluent cultures, were seeded in flat-bottomed microtiter wells of a 96-well microtiter plate in 100 μ l of RGM. The cells were incubated for 24 h at 37° in 5% CO₂. The medium was then removed and several concentrations of the test samples were added. It was ensured that regardless of the dilution used, the final concentration of FCS was 10% in all cases. The cells were incubated for an additional 24 h before being trypsinized and harvested. Tritiated thymidine, 1 μ Ci/well (20 Ci/mmol), was added during the last 12 h of the incubation period.

To examine the kinetics of the effect of PBM supernatants on fibroblast proliferation, 10⁴ fibroblasts per well were seeded and incubated for 24 h. The medium was then removed and monocyte supernatant was added to the cells for various periods of time ranging from 30 min to 24 h (Figure 4). After each exposure, the medium was removed and the cells washed with fresh medium. Fresh RGM was then added and the incubation continued to a total of 24 h. In all cases, the fibroblasts were incubated for a total of 48 h before being trypsinized and harvested, with thymidine being added for the last 12 h. To confirm that thymidine incorporation reflected

fibroblast proliferation, 3.5×10^5 cells were seeded in 35-mm dishes (the same density as in the thymidine assay), the supernatants added as described above and the cultures terminated by trypsinization. Cells were then counted using trypan blue exclusion and a hemocytometer.

Determination of cytokines

IL-1. Lymphocyte-Activating Factor (LAF) assay. Assay of LAF activity was carried out essentially as described by Simon and Willoughby (128). Thymocytes from C3H/HeJ mice (1.5×10^6 cells) in 100 μ l of medium (RPMI with 10% FCS) were cultured for 30 min at 37° and 5% CO₂ in the presence of 1 μ g of PHA-P (Difco Laboratories, St Louis, MO). One hundred μ l of medium, or AM/PBM culture supernatant, or dilutions of such were added, and the thymocytes cultured for a further 48 h. Thymocyte proliferation was detected after cell harvest and counting as the uptake of ³H-thymidine (20 μ Ci/ml) added for the last 16 h of culture. Results are expressed as mean cpm \pm SD of 3-5 wells.

IL-6. Hepatocyte-Stimulating Factor (HSF) assay.- HSF was assayed as previously described (129). Briefly, hepatocytes were isolated from the livers of Sprague-Dawley rats weighing 200 to 250 g by digestion upon perfusion with collagenase. Single cell suspensions of hepatocytes were adjusted to 2×10^6 cells/ml in Williams's E medium (GIBCO)

supplemented with 5% FCS, 1 μ g dexamethasone (Organon, Toronto, Ontario), 1 μ g/ml insulin (Sigma Chemical Co., St Louis, MO), 5 μ g/ml heparin (Organon), 10 mM Hepes, 0.075% bicarbonate, and 1% mixture of penicillin, streptomycin and gentamicin. The cells were pipetted (200,000 cells/well) into T-24 well NUNC plates precoated with collagen (Flow Laboratories, Hamden, CT). After a 2-h incubation at 37°C in 5% CO₂, the nonadherent cells were washed off and 200 μ l of fresh medium were added followed by 50 μ l of test material. The cultures were then incubated at 37°C in 5% CO₂ for 72 h with daily changes of medium and addition of test material. At 72 h of culture, the supernatants were harvested and tested for levels of acute-phase reactants by rocket immunoelectrophoresis. HSF activity was defined as the ratio of the amounts of α_2 -macroglobulin to albumin synthesized by hepatocyte cultures. This ratio has been shown to maximize sensitivity in detection of this hepatocyte response in vitro. This assay has a coefficient of variation of 4% within assays and 10% between assays.

Experimental model of pulmonary fibrosis

Adult male Lewis inbred rats weighing 250 g were purchased from Trudeau laboratories (Syracuse, NY). For the duration of the experiment, the animals were fed with commercial rat food and water ad libitum. The instillation of

intratracheal bleomycin, 0.5 to 0.6 U/100 g body weight (Blenoxane; Bristol Laboratories, Syracuse, NY) in 0.3 ml of sterile PBS was performed via tracheostomy with the animals under anesthesia, ketamine 10 mg/100 g body weight (Ketalar; Parke Davis, Scarborough, Ontario). Beginning at 1 h after bleomycin and at several time intervals for as long as 28 days, the animals, three per data point, were anesthetized with sodium pentobarbital (Somnotol. MTC Pharmaceuticals, Mississauga, Ontario), were subjected to BAL as described below, and were then killed. In addition, another group of control animals kept in the same quarters under the same living conditions underwent tracheostomy using the same anesthetic protocol and received 0.3 ml of PBS without bleomycin, intratracheally. Bronchoalveolar lavage was performed 6 h after the instillation of PBS.

Lung lavage of each animal was performed with the slow infusion of 50 ml (in 5-ml aliquots) of sterile PBS through a syringe and Teflon^R tubing. The fluid was aspirated after each aliquot infusion, placed in centrifuge tubes on crushed ice, and then centrifuged at 300 g for 5 min. The cell pellet of three animals were pooled and suspended in RPMI 1640 with 10% FCS. Cells were then resuspended in RPMI containing 10% FCS to a concentration of 2×10^6 cells/ml. One ml per well of this suspension was placed in T-24 NUNC plates and incubated for 2 h at 37°C in 5% CO₂. The medium was then removed and the cells washed 4 or 5 times with RPMI to

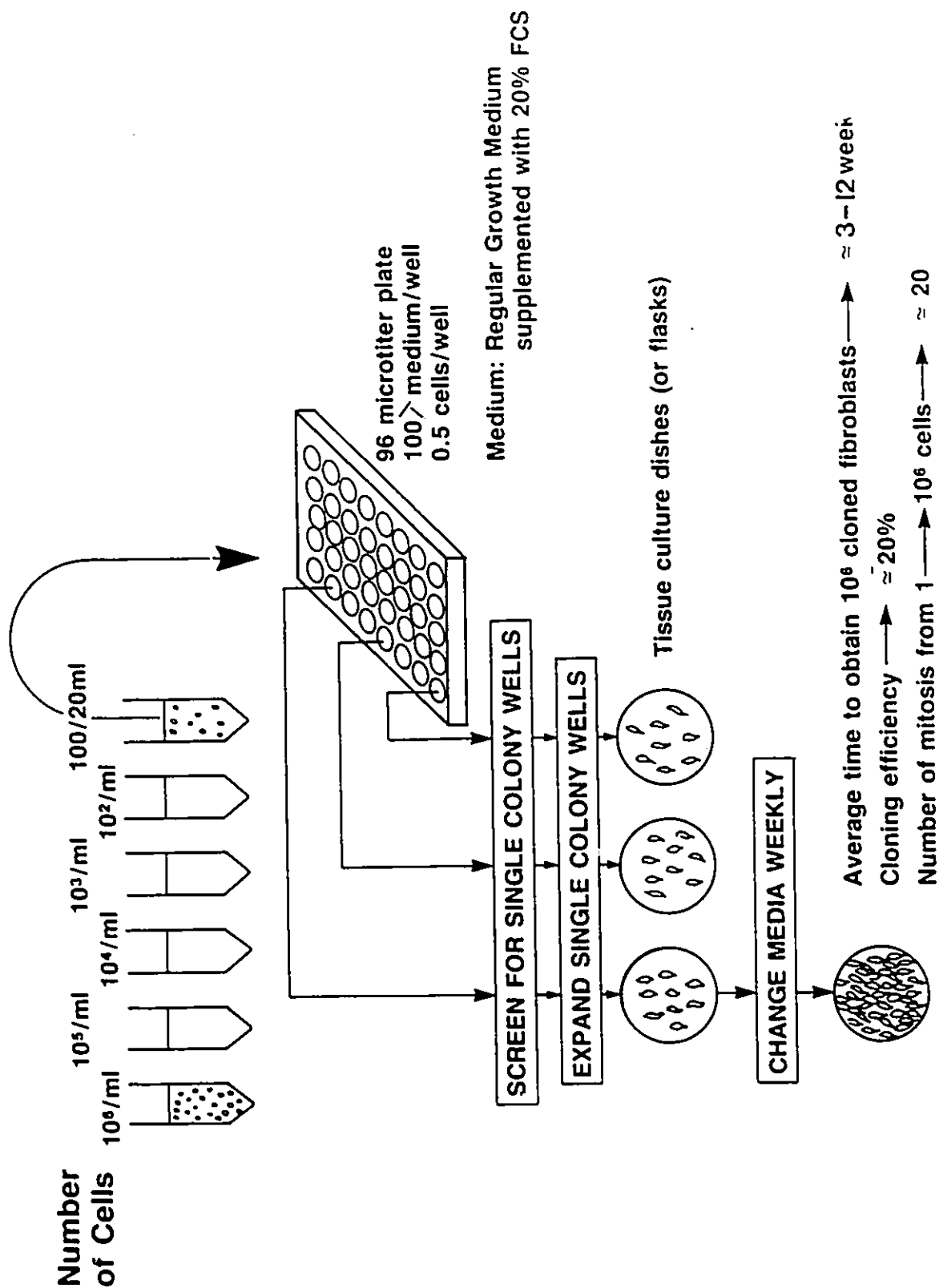


Figure 3

EXPERIMENTAL DESIGN

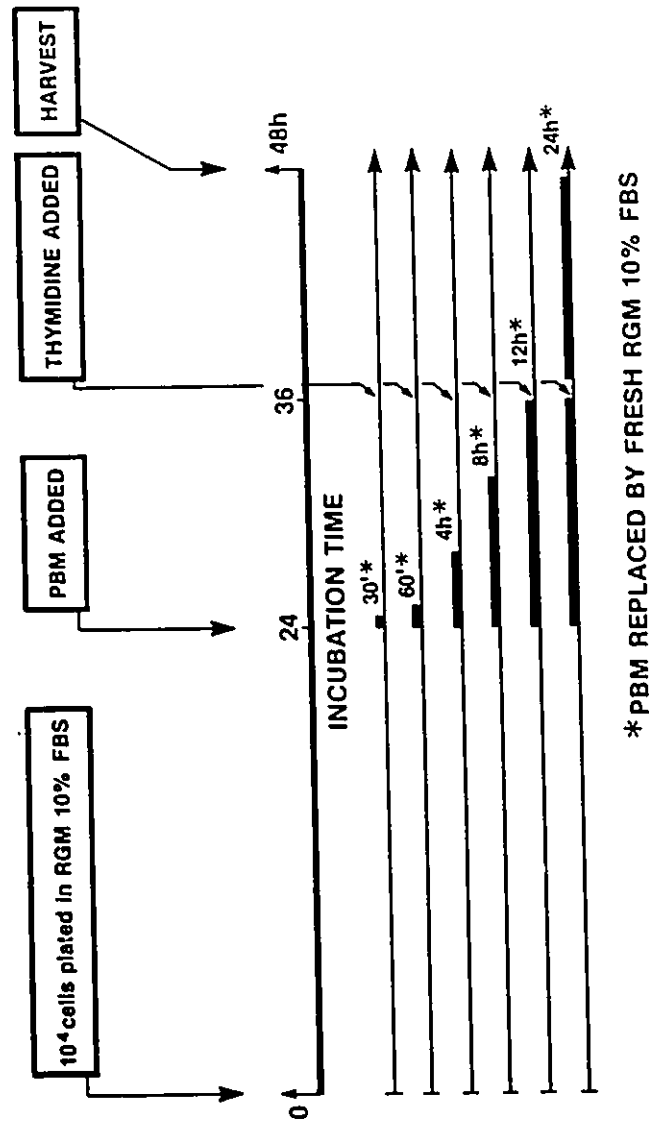


Figure 4

remove all non-adherent cells. One ml of fresh RPMI containing 10% FCS was added per well, with or without 10 ug of LPS. After 24 h, the AM supernatants were collected, sterilized by millipore filtration and stored at -20°C . In all instances, the supernatants were dialyzed for 24 h against PBS using dialysis tubing with a molecular weight cut-off of 7-8,000 daltons.

Experimental protocol for the chronic exposure of HLF to PBM supernatants

Between 4 and 5 x 10⁶ human lung fibroblasts were seeded in 25 cm² Falcon tissue culture flasks with 5 ml of RGM containing 10% FCS. Several dishes were prepared for each cell line tested. All dishes were fed three times a week; half with fresh RGM (heretofore referred to as "unexposed" cells), and half with RGM containing PBM supernatant at a dilution of 1/20. All media used contained a final FCS concentration of 10%. This dilution of PBM supernatant had induced a half maximal effect (inhibition of fibroblast proliferation) in the acute challenge assay. This dose was increased to 1/10 approximately 2 weeks later since the start of the experiment. Between 4 and 5 weeks from the beginning of the experiment, the cells were harvested after being cultured for a few days in RGM medium without PBM supernatant, and their response to acute challenge to both

PBM supernatants and PGE_2 was examined as detailed above. Throughout this period, the cultures were split 1:2 when confluent. The same experimental protocol was subsequently applied to individual clonally-derived cell lines. In addition, primary fibroblast lines which had been chronically exposed to PBM supernatants were cloned, and the response of individual clones to acute challenges of both PBM supernatants and PGE_2 examined.

Statistical analysis

Statistical analysis were made using a two-tailed unpaired Student's t-test in Minitab software. p values less than 0.05 were considered not significant. Correlation coefficients were obtained using the same software package.

RESULTS

Characteristics of polyclonal primary fibroblast lines

Cell monolayers from the control and fibrotic tissues were stained with hematoxylin-eosin. All cells in the culture showed the typical stellate-to-spindle-shape morphology of fibroblasts (Figure 5). Differences in cell size could be noted, but these were unrelated to whether fibroblasts were derived from control or fibrotic specimens. In addition, immunohistochemistry with vimentin and keratin of cell monolayers was performed. As shown in Figure 6, whereas virtually all cells were positive for vimentin (d), there were no cells positive for keratin (c). For comparison, a monolayer of human airways epithelial cells is depicted, showing that the vast majority of cells are positive for keratin (a) and there are no cells positive for vimentin (b). Cell monolayers were also examined by electron microscopy (Figure 7). All these cells had the recognizable ultrastructural features of fibroblasts. Multinucleoli were frequently seen. Differences in width and length as well as in the presence of microfilaments and rough endoplasmic reticulum were common amongst the 8 fibroblast lines examined

but, again, these were not related to whether cells were control or fibrotic tissues. There were no abnormal karyotypes noted in the FF cell lines, and no cells tested positive for reverse transcriptase indicating the lack of retrovirus infection.

Proliferative characteristics of primary fibroblasts lines

Representative results of experiments using thymidine incorporation and involving 4 CF and 4 FF cell lines are shown in Figure 8. The data represent 4 separate experiments, each of which included 1 CF and 1 FF lines. The pattern of thymidine incorporation over time in both CF and FF lines was similar, with peak values observed at 60 h. Sometimes, individual cell lines achieved peak thymidine incorporation at 48 h. On average, FF lines incorporated 3 times as much thymidine as did CF lines ($p \leq 0.005$).

The in vitro fibroblast proliferation of 4 fibroblast lines as measured by cell counting is shown in Figure 9. The cell density used in these experiments and that used in the thymidine incorporation assay were equivalent, approximately 3.5×10^4 cells/cm². In this and other cell counting experiments, the pattern of cell proliferation was very similar. Cell numbers increased during the first 72 h and tended to plateau during the last 24 of the incubation period. The increase in cell number in the FF lines was

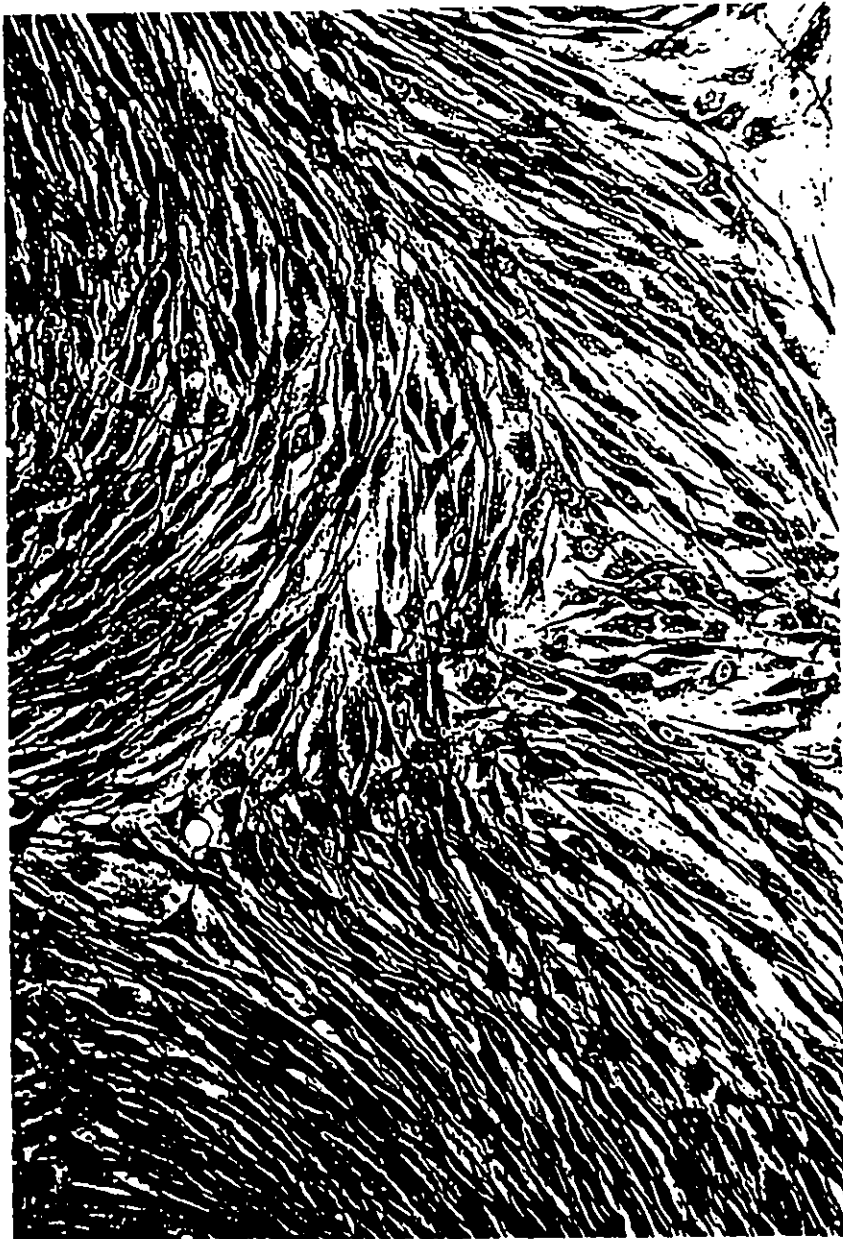


Figure 5. Light microscopy of a human lung fibroblast monolayer stained with the Diff-Quick modification of the Giemsa stain. (Magnification, x40)

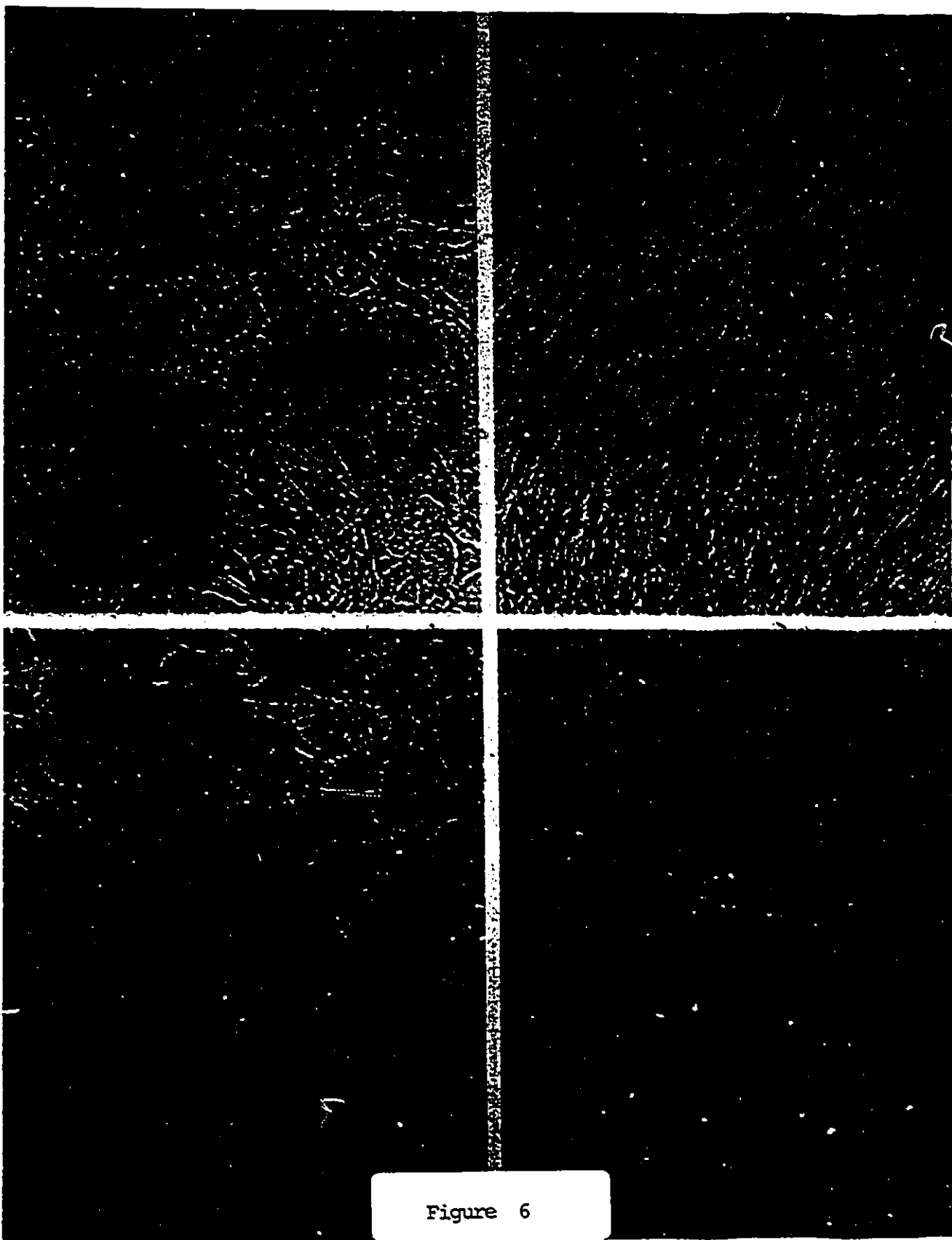


Figure 6

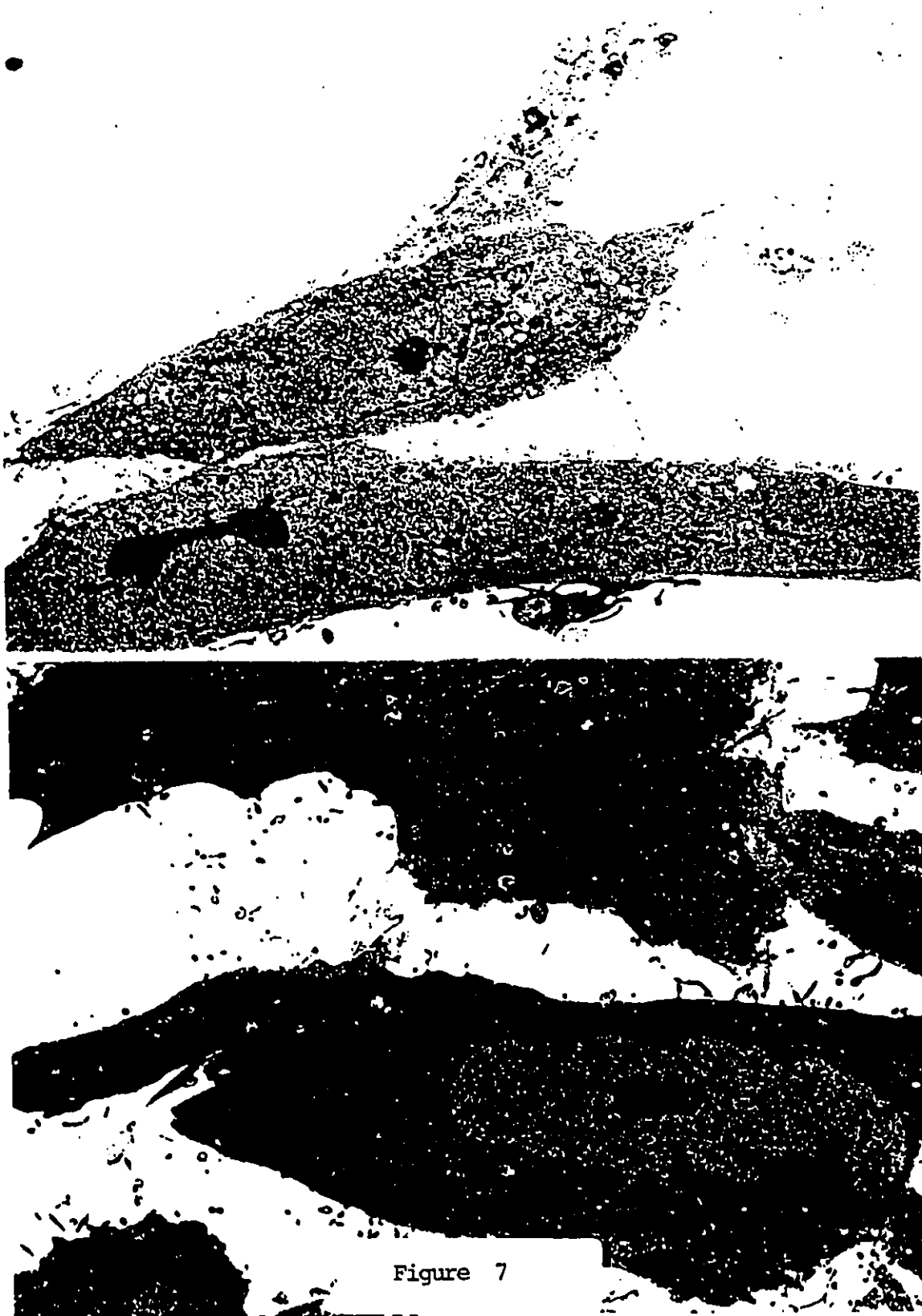


Figure 7

consistently higher than the CF lines at 72 h and 96 h. ($p \leq 0.05$).

Figure 10 shows the proliferative characteristics as assessed by thymidine incorporation of two primary fibroblast lines from control tissues, two from patients with IPF and four from neonatal lung tissues. The proliferative rate of the control and IPF lines is similar to previous experiments. Neonatal fibroblasts exhibited in comparison the highest rates of thymidine incorporation. We also examined the proliferative characteristics of two primary fibroblast lines derived from the lungs of patients with very advanced pulmonary fibrosis and found no differences compared to control lung fibroblasts (data not shown).

It is recognized that the proliferation of fibroblasts in vitro depends on free surface area and the presence of appropriate growth factors and nutrients in the culture medium. The data in Figure 11 show the proliferative response of 2 CF and 2 FF cell lines to several concentrations of FCS. In this experiment, 3.5×10^5 cells/35-mm dish were seeded in 2ml of RGM containing 10% FCS for 24 h to allow for maximal adherence. The cells from two dishes were then released with trypsin and counted. The medium of the remaining dishes was changed to medium containing various concentrations of FCS. The cells were incubated for a further 96 h period and counted as before. The results, which represent the percentage change in cell

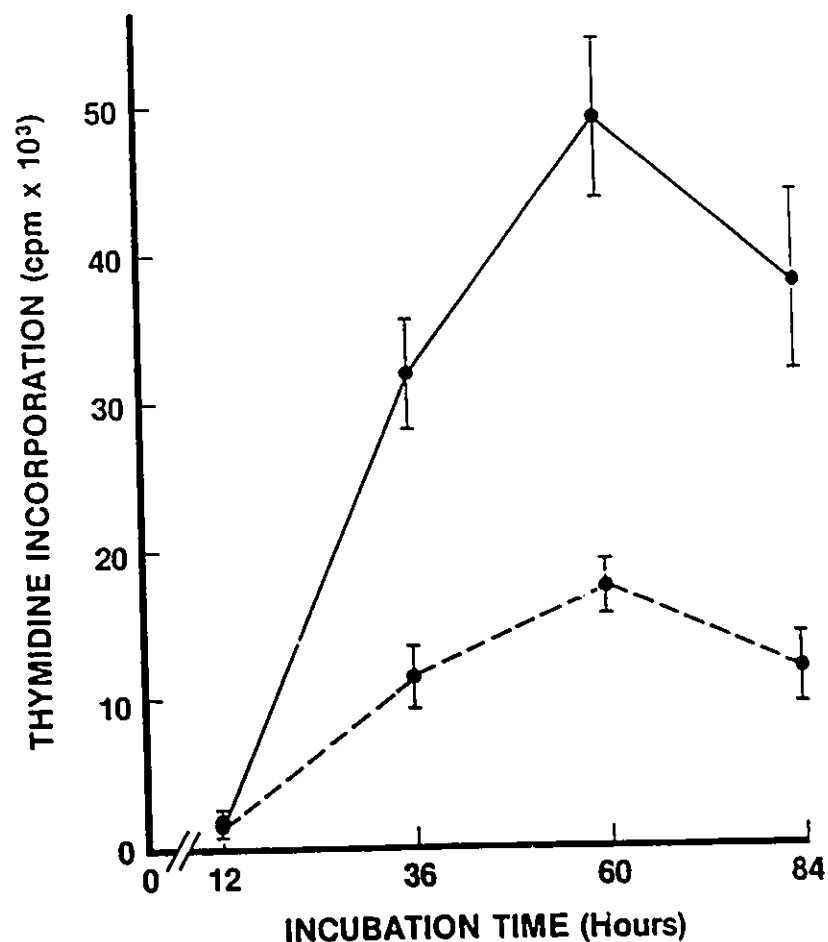


Figure 8. In vitro thymidine incorporation of human adult lung fibroblasts. Results expressed as mean \pm SEM of 4 control (C, dashed line) and 4 pulmonary fibrosis (F, solid line) fibroblast cell lines. The value of each data point for each cell line was the mean of 12 replicate wells. Differences between C and F were statistically significant ($p < 0.05$) at 36, 60 and 84h.

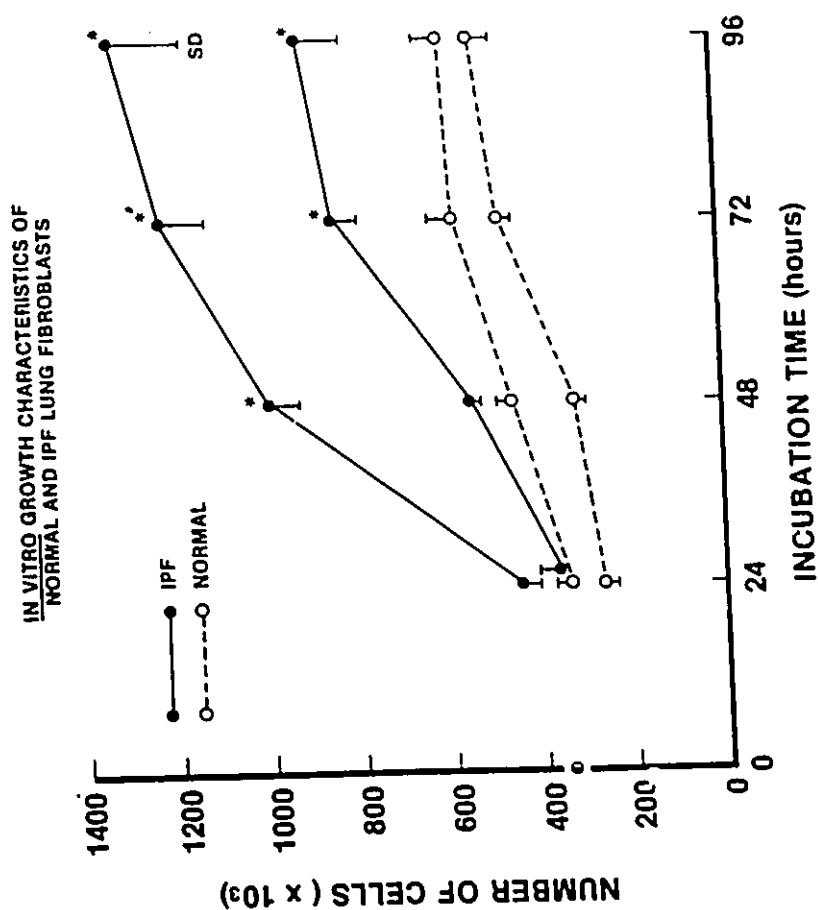


Figure 9. In vitro proliferation as assessed by cell counting, of 4 fibroblast lines: 2 control, McL and VEN (open circles), and 2 pulmonary fibrosis, SOM and CIR (closed circles). Results are expressed as mean \pm SEM of 6 counts (3 counts per dish). Statistical comparisons as in Figure 8.

TIME-COURSE OF THYMIDINE INCORPORATION BY
NEONATAL AND ADULT FIBROBLAST LINES

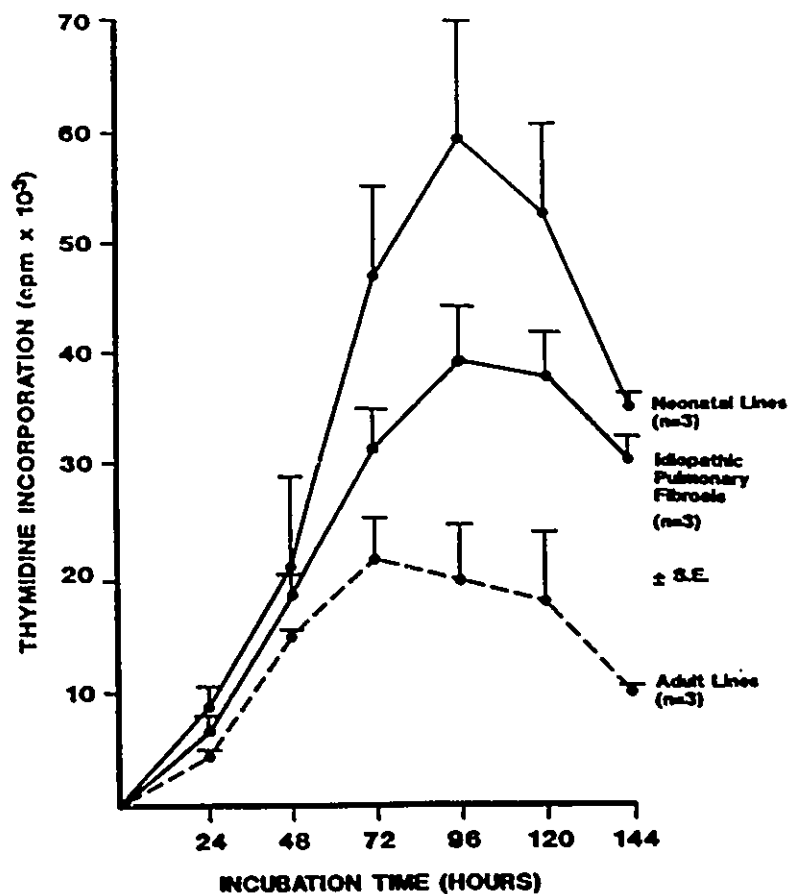


Figure 10. In vitro thymidine incorporation of human lung fibroblasts. Results expressed as mean \pm SD of 3 CF (control adult), 3 FF (idiopathic pulmonary fibrosis) and 3 neonatal fibroblast primary lines. The value of each data point for each cell line was the mean of 6 replicate wells. The differences between CF, FF and neonatal fibroblasts were statistically different at 72, 96 and 120 h.

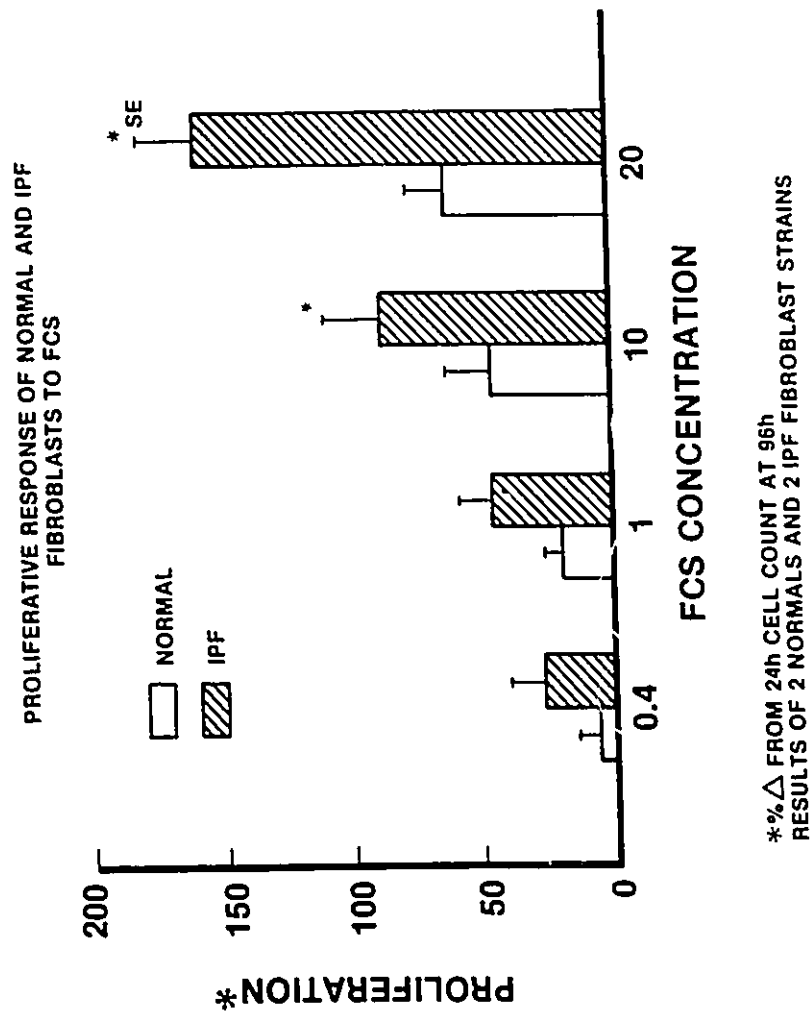


Figure 11. Proliferative response of 2 control, McL and McB (open bars), and 2 pulmonary fibrosis, JAC and SOM (shaded bars), primary lung fibroblast lines upon exposure to different concentrations of FCS for 96 h. Results represent the mean percentage change \pm SEM from cell number after 24 h of adherence. The value for each cell line was the mean of 3 counts. Statistical comparisons as in Figure 8.

number compared to the 24 h count, showed that FF exhibited a higher increase in cell number compared to CF at every FCS concentration, although the differences were statistically significant only at 10% and 20% FCS.

Profile distribution of clonally-derived HLF lines

A summary of the cloning efficiency for the 4 fibroblast lines that were cloned is shown in Table 2. Initial attempts to clone cells in 10% FCS medium were unsuccessful. Only when we used 20% FCS were there significant numbers of clones that developed. From three primary lines, VEN, BRY and JAC, almost two thirds of the clones expanded to T-24 wells did not reach full confluency in T-flasks. From the fourth primary line, SOM, 25.5% of the expanded clones were not recovered. These losses were presumably due to handling and to natural senescence. The final cloning efficiency was similar for 2 CF and FF panels of clones, 5, 5.5 and 5%, respectively. The cloning efficiency for SOM, a FF cell line, was almost double, i.e. 10%.

The in vitro proliferative characteristics as assessed by cell counting of 44 CF and 56 FF-derived clones are shown in Figure 12. These numbers are slightly lower than those showed in Table 2 because, when assayed, 6 CF and 7 FF clones, though viable, did not proliferate at all. These clones were excluded from the initial comparisons because

they were considered to be terminally differentiated clones, thus not contributing to the pool of proliferating clones. The index of cell proliferation in the Y axis represents doublings in cell number over 96 h of incubation. The results depicted demonstrate a marked heterogeneity in terms of proliferative behaviour of both CF and FF-derived clones. The average proliferation index of FF clones, 1.80 ± 0.7 , was significantly higher compared to that of CF clones, 1.46 ± 0.65 ($p \leq 0.05$). This difference remained significant when data were re-calculated including the 6 CF and 7 FF clones initially excluded. Eleven clones of the proliferating pool, 1 CF and 10 FF, had an index of cell proliferation that lay outside of 2 standard deviations of the mean index of proliferation of control clones. The mean index of proliferation of these 11 clones was 2.9 ± 0.2 . The percentage of these so-called fast-growing clones was similar in each individual panel of FF clones, 13% for JAC and 17,5% for SOM. Figure 13 illustrates the proliferative characteristics of 31 clones derived from lung neonatal tissues compared to CF and FF clones, and shows that the average growth potential of these clones is significantly greater than FF clones and also that the proportion of fast-growing clones is the greatest amongst the three panels of clones tested.

The reproducibility of the determination of proliferation was assessed by examining 33 clones, 22 FF and

11 CF, on 2 repeated occasions. We deliberately chose clones with a wide range of proliferation indices and studied them several passages apart and some had been frozen in liquid nitrogen for several weeks. The mean index of proliferation was 1.95 ± 0.84 when first tested and 1.91 ± 0.81 when assayed on a second occasion. The results for each individual clone of the 33 clones included in these experiments are plotted in Figure 14. The coefficient of correlation between these repeat experiments was 0.70.

Effect of fibroblast supernatants on the proliferation of fibroblasts

Fibroblast-derived supernatants did not induce any detectable effect on fibroblast proliferation when these cells were cultured in the presence of 10% FCS. The most likely explanation is that fibroblasts are already maximally stimulated under these experimental conditions. Therefore, a system of quiescent fibroblasts and minimal serum supplementation was established. Figure 15 shows the effect of supernatant from neonatal lung fibroblasts on the proliferation of both C and F fibroblasts. The data in the Y axis represent percent change over the negative control, i.e. the cell counting obtained at day 4 in cells cultured with medium without fibroblast supernatant. The data show that supernatant from neonatal lung fibroblasts significantly

TABLE 2
RELATIVE CLONING EFFICIENCY OF 4 FIBROBLAST LINES

Line Designation	Wells Seeded* (n)	Clones Expanded ∞ (n)	Clones Recovered η (n)
VEN§	576	100 (17%)	32 (5.5%)
BRY§	384	43 (11%)	18 (5%)
SOM•	384	51 (13%)	38 (10%)
JAC•	480	81 (17%)	25 (5%)

* Seeded at the equivalent of half a cell/well.

∞ Clones successfully transferred to wells of T-24 plates.

η Number of clones assayed after successful transfer to flasks.

§ Control lines.

• Pulmonary fibrosis lines.

**IN VITRO PROLIFERATIVE CHARACTERISTICS
OF HUMAN ADULT LUNG FIBROBLAST
DERIVED CLONES**

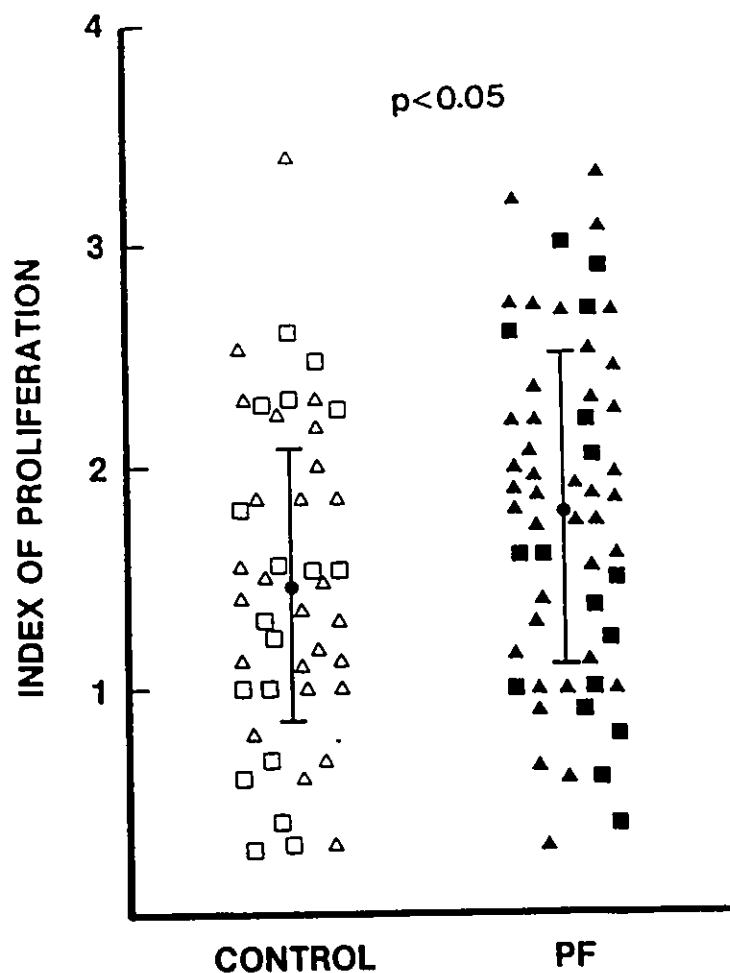


Figure 12. In vitro proliferative characteristics of 44 control (open squares, open triangles) and 56 pulmonary fibrosis (PF)-derived (closed squares, closed triangles) clones. Index of cell proliferation in the vertical axis represents number of doublings over 96 h of incubation. Statistical comparisons were made using an unpaired t test; $p \geq 0.05$ was considered not significant (solid line, SD).

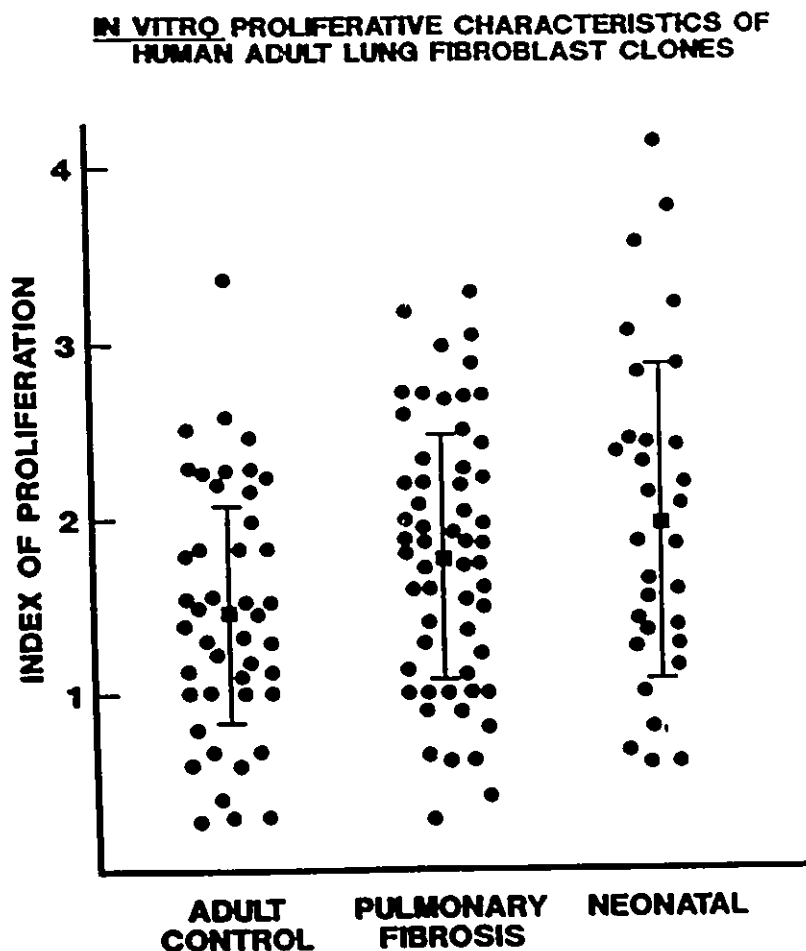
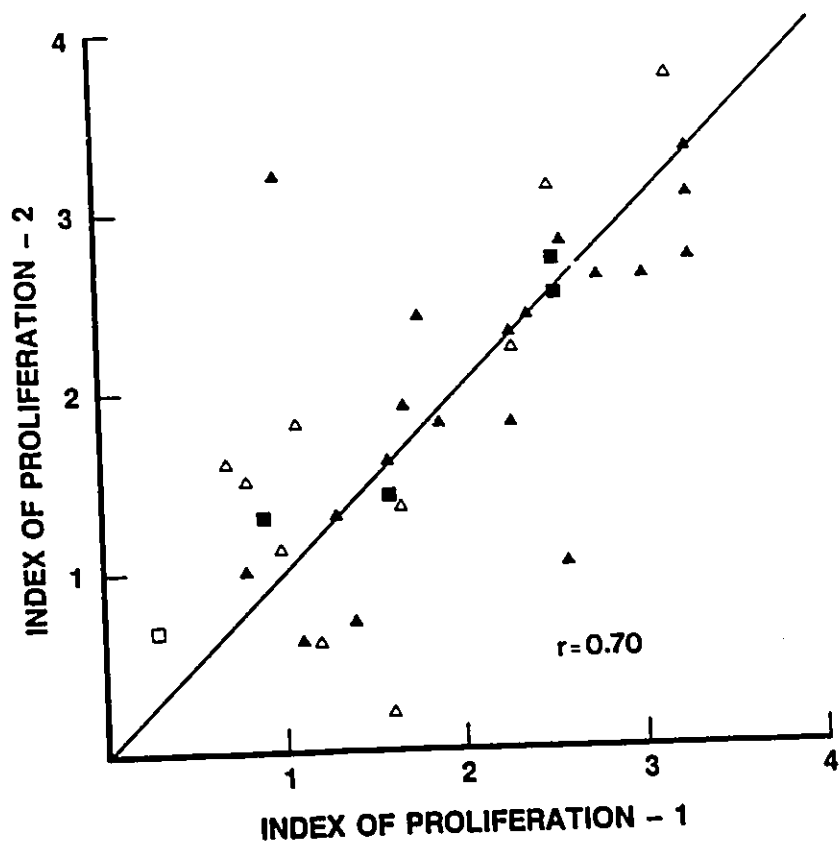


Figure 13. In vitro proliferative characteristics (thymidine incorporation) of 33 clones derived from a primary line of neonatal lung fibroblasts. Results for adult control (AC) and pulmonary fibrosis (PF) are the same as in figure 12. Index of cell proliferation in the vertical axis represents number of doublings over 96 h of incubation. Differences between neonatal and AC clones were statistically significant (unpaired t test).



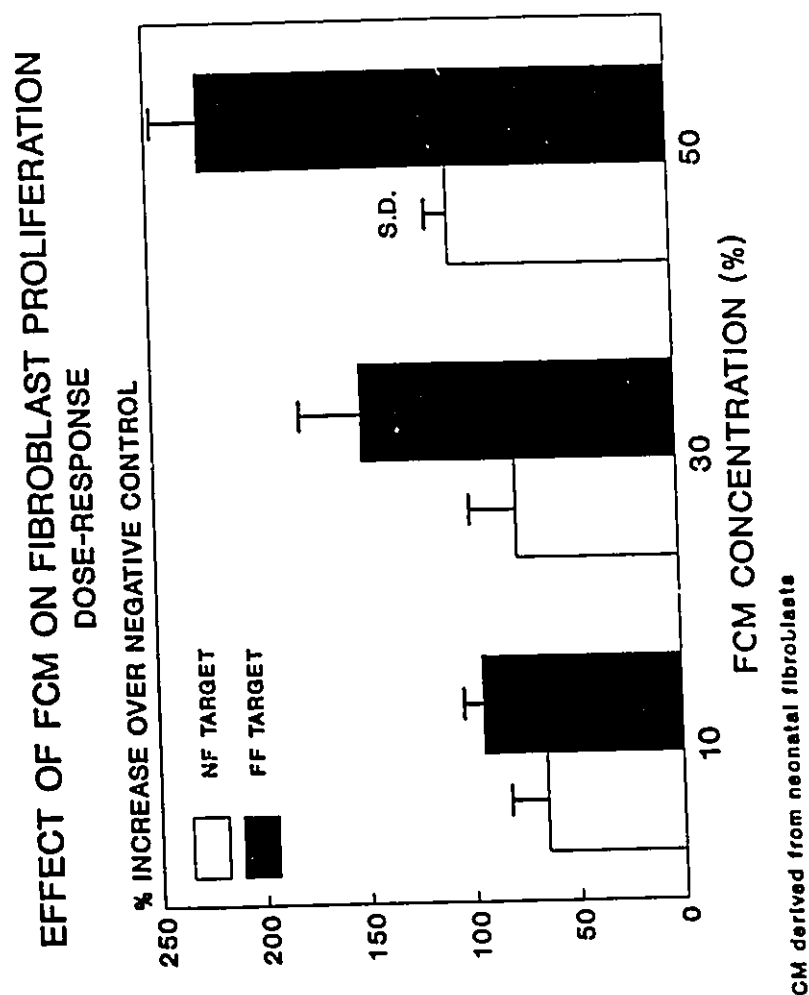


Figure 15. Proliferative response of NF and FF fibroblasts to conditioned medium (FCM) derived from human neonatal lung fibroblasts. Dose-response. The data in the vertical axis represents percent increase over the cell count obtained after 4 days of culturing the cells in medium containing minimal amounts of serum and before fibroblast supernatant was added. Results expressed as mean \pm SD of three experiments.

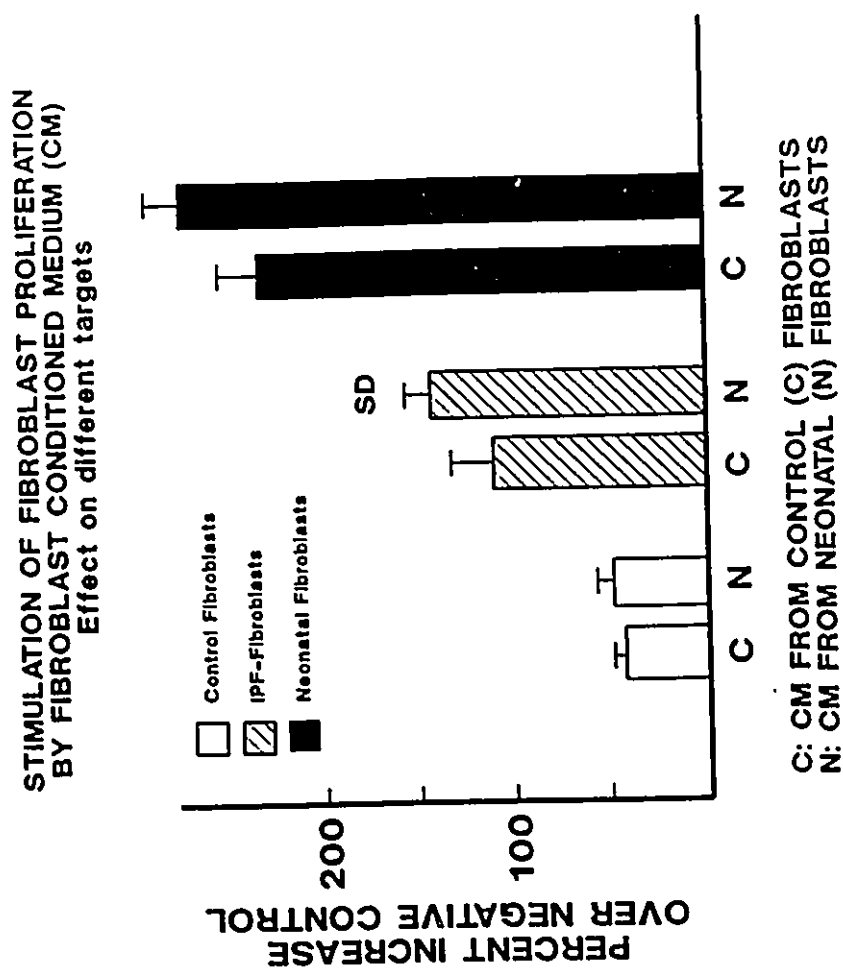


Figure 16. Proliferative response of CF, FF and neonatal fibroblasts to pooled conditioned media (FCM) obtained from both CF and neonatal fibroblasts. The data in vertical axis as in Figure 15. Results expressed as mean \pm SD of three experiments using different cell lines as targets.

stimulates fibroblast proliferation in a dose-dependent manner. They also show that the response of FF to this supernatant was significantly greater than that of CF. Figure 16 summarizes the data of a series of experiments in which the response of CF, FF and neonatal fibroblasts to supernatants from both control and neonatal fibroblasts was examined. Both supernatants stimulated fibroblast proliferation in a similar manner. However, FF and, particularly, neonatal fibroblasts showed a greater proliferative response to either supernatant compared to CF. Thus, the nature of the responding cell rather than the origin of the supernatant appears to be the significant variable in the system.

Effect of PBM/AM supernatants and exogenous PGE₂ on the proliferation of HLF

When fibroblasts were cultured under normal growth conditions, the rate of incorporation of ³H-thymidine over the first 60 h of culture was directly related to cell proliferation (Figure 17), and this time period was used to examine the effect of monocyte supernatants on fibroblast proliferation in subsequent experiments with ³H-thymidine uptake alone.

As shown in Figure 18, unstimulated AM supernatant or medium alone containing LPS did not significantly affect

fibroblast proliferation. In contrast, LPS-stimulated AM supernatant inhibited fibroblast proliferation in a dose-dependent manner, from up to 70% inhibition at 1/4 dilution to approximately 40% at 1/64 dilution. On further dilution, normal proliferation was observed. LPS-stimulated PBM supernatants caused similar inhibition, and some preparations had higher titers than AM supernatants (Figure 19). Because both AM and PBM supernatants had a similar effect, most of the subsequent experiments were carried out with PBM supernatants because of the easier access to these cells. At no time did we see any stimulation of fibroblast proliferation by AM or PBM supernatants in our assay. These experiments were performed on several normal fibroblast cell lines with similar results. The inhibitory effect of AM or PBM supernatants on fibroblast proliferation was not due to a cytotoxic effect as shown by trypan blue exclusion assessment of viability ($\geq 90\%$ at 96 h).

Mechanism of fibroblast growth inhibition by AM/PBM products

Figure 20 shows the effect of activated monocyte supernatant, indomethacin and exogenous PGE_2 alone and in combination on fibroblast proliferation. The inhibition caused by PBM supernatants could be duplicated by adding exogenous PGE_2 alone (500 ng/ml added twice at 12 h

intervals). Indomethacin at 1 $\mu\text{g/ml}$ caused no detectable modulation of fibroblast proliferation by itself, but could reverse the inhibition caused by PBM supernatants. However, indomethacin could not reverse the inhibition caused by the addition of exogenous PGE_2 , implicating the arachidonic acid pathway of the fibroblast as being involved in the modulation of that cell by cytokines. Dexamethasone (1 μM) in the culture system had a very similar effect (data not shown).

Kinetics of cytokine-mediated modulation of fibroblast proliferation

In order to examine the rate of induction of inhibition, we cultured fibroblasts with PBM supernatants for increasing periods. Significant inhibition was seen with as little as 30-min exposure to cytokines. When we compared normal and fibrotic cell lines by this method, the FF showed a greater inhibition at each time of exposure, but the maximum degree of inhibition over 24 h was indistinguishable between the two types of cell lines. Figure 21 depicts the typical result of one such experiment. When we added indomethacin 1h either at the start or variously throughout the culture with PBM supernatant, we were able to totally or partially abrogate the inhibition. Pretreatment of fibroblasts for 1h with indomethacin followed by PBM supernatants for 23 h resulted in completed abrogation of the

previously shown inhibition of fibroblast growth. Indomethacin had no protective effect if added after fibroblasts had been exposed for 12 h to PBM supernatants.

Effect of exogenous PGE_2 on fibroblast proliferation

The effect of exogenous PGE_2 addition on fibroblast thymidine incorporation is shown in Figure 22. Four cell lines, two CF and two FF, were tested three times. To allow comparisons, the results are expressed as the percentage change in thymidine incorporation compared to background (cells grown in RGM alone). The addition of exogenous PGE_2 to the fibroblast cultures induced a dose-dependent inhibition of fibroblast proliferation on both CF and FF cell lines. However, the proliferation of FF lines was significantly less depressed by PGE_2 at each dose tested. The value assigned to each cell type was its average response to PGE_2 on repeated testing.

Characterization of the active AM/PBM product

In order to characterize the AM/PBM product(s) active on lung fibroblasts, crude dialyzed supernatants from both activated AM and PBM were chromatographed in PBS on a G-100

Sephadex column (3.0 x 110 cm) as previously described, and the fractions were microfiltered and tested directly in both the fibroblast and LAF assays. Figure 23 shows that fractions 30 to 32 eluting at approximately 15 kD were active in both the fibroblast and LAF assays, and the activities appeared coincident. The inhibitory effect of these active fractions on fibroblasts could be abrogated by incubating the fibroblasts with indomethacin (data not shown). As a direct assessment of IL-1 being the active component in the supernatant, we tested the effect of highly purified human IL-1^C, kindly supplied by Dr J. Van Damme. Table 3 shows that pure IL-1^C causes a similar inhibition of proliferation, as does the purified factor from alveolar macrophage and peripheral blood monocyte. This inhibition could also be reversed by the addition of indomethacin.

Biological effects of rat AM supernatants

The effects of supernatants of unstimulated AM (spontaneous release) from bleomycin-treated animals, as well as from normal (untreated) animals stimulated in vitro with LPS (positive control), on thymocyte and fibroblast proliferation assays is shown in Figure 24. Supernatants from LPS-activated AM markedly stimulated thymocyte proliferation as much as 450% and inhibited fibroblast proliferation as much as 65% compared with background (cells incubated with

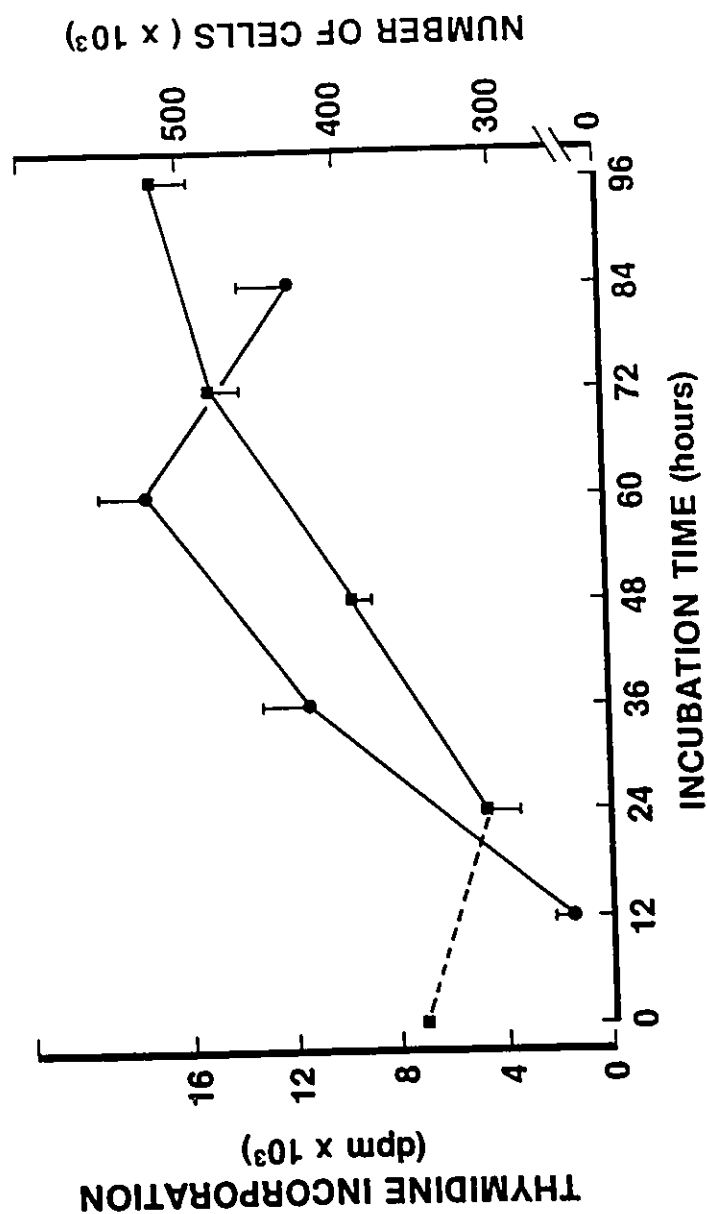


Figure 17. Growth characteristics of normal human adult lung fibroblast cell lines. Over each 24 hr interval, cell counts (\blacksquare - \blacksquare) by trypan blue exclusion and ^3H -thymidine uptake (\bullet - \bullet) were determined. Data for ^3H -thymidine is expressed at mean cpm \pm SD of six replicates.

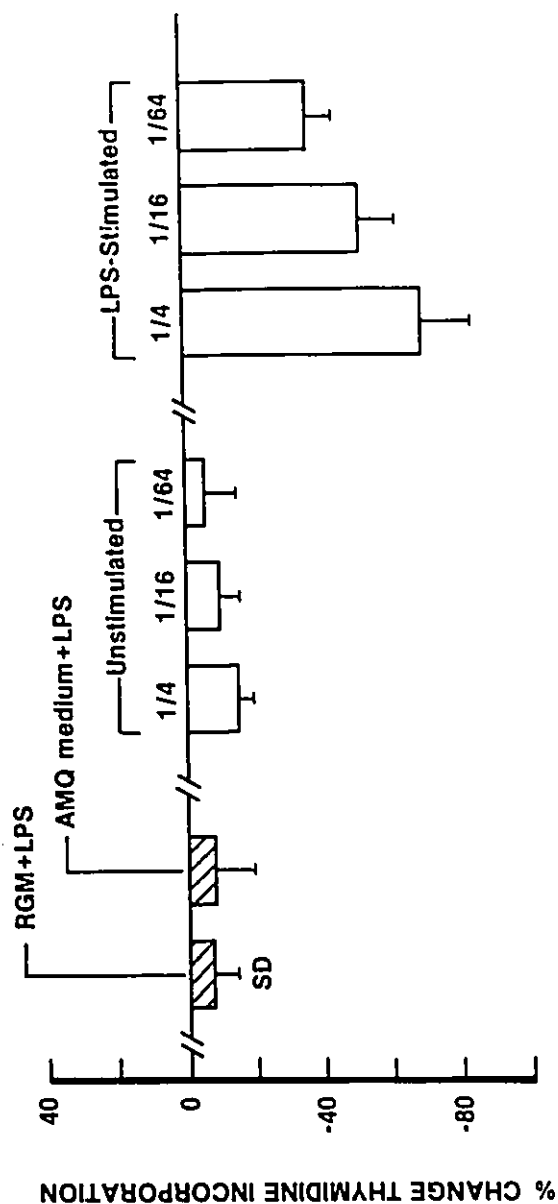


Figure 18. Effect of alveolar macrophage supernatants (AM) on in vitro fibroblast proliferation. Data are expressed as percentage change from a control culture of the same fibroblasts (incubation in RGM with 10% FBS alone). Each data point represents mean \pm SD of four replicates.

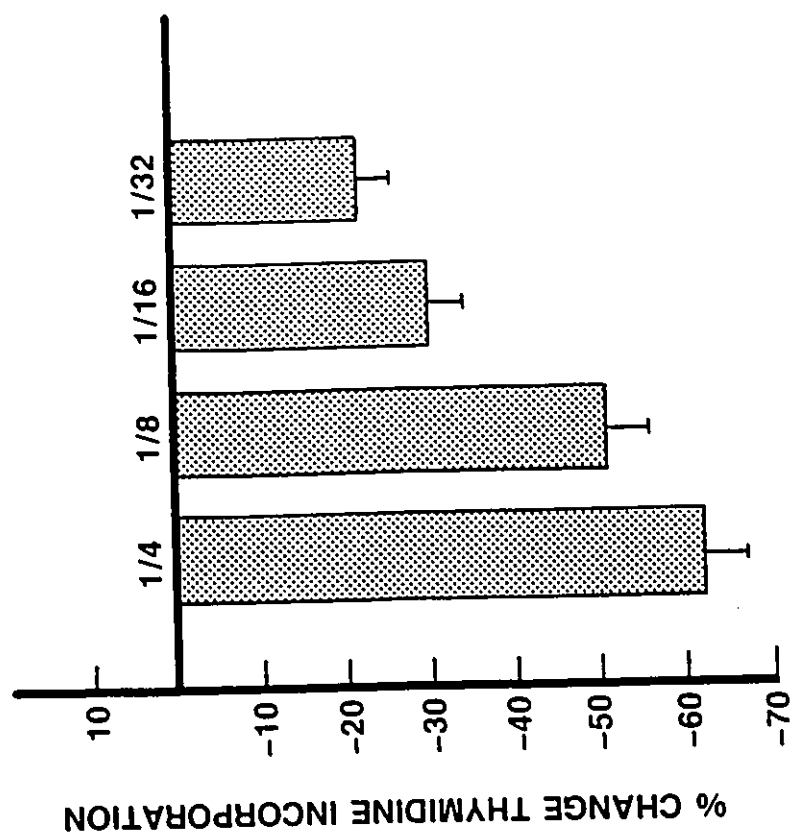


Figure 19. Effect of LPS-stimulated peripheral blood supernatants on in vitro fibroblast proliferation. Data expressed as in Figure 18. Each data point represents mean \pm SD of four replicates.

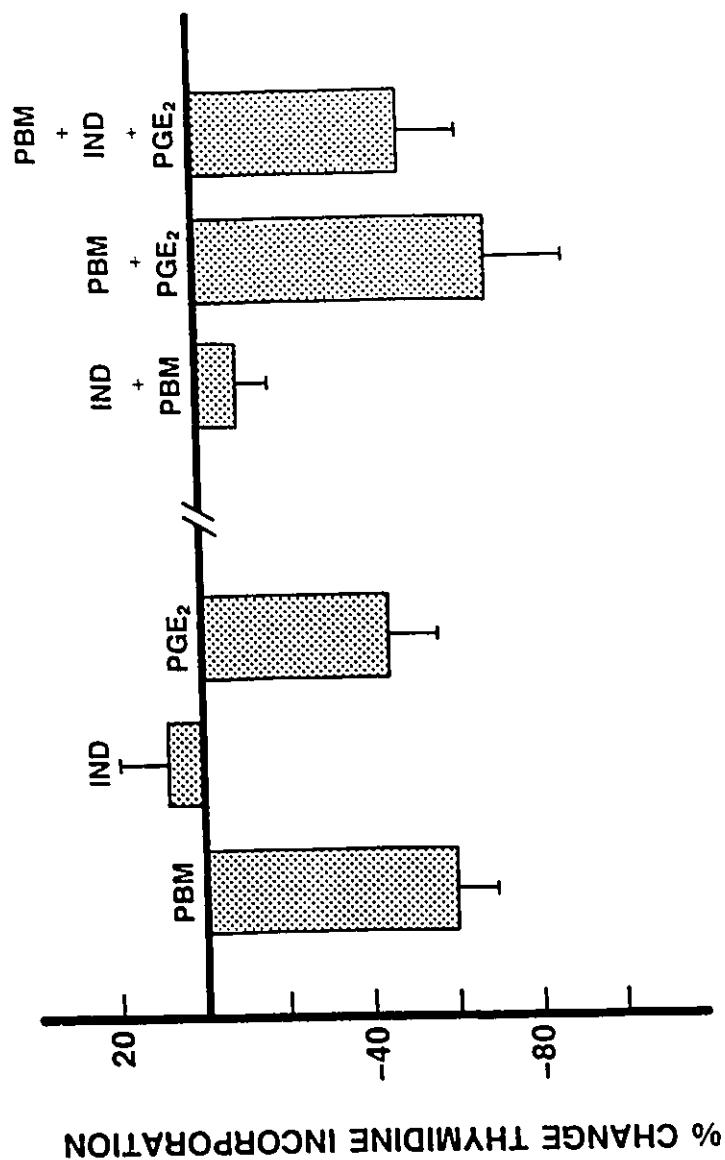


Figure 20. Effect of LPS-stimulated peripheral blood monocyte supernatants (PBM), indomethacin (1 μ g/ml), and prostaglandin E₂ (500 ng/ml added twice), alone and in combination on in vitro fibroblast proliferation. Data expressed as in Figure 18. Each data point represents mean \pm SD of six replicates.

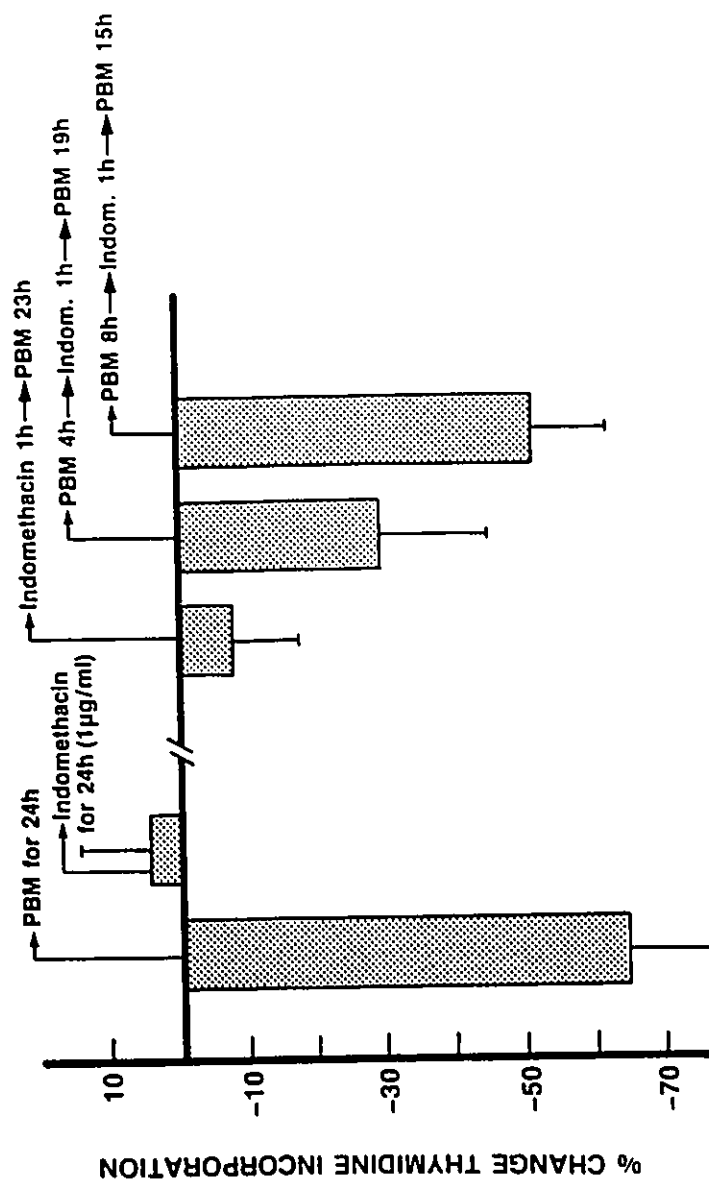


Figure 21. Effect of indomethacin ($1\mu\text{g/ml}$) for 1h added at various intervals during the incubation period on fibroblast proliferation. Data expressed as in Figure 18. Each data point represents mean \pm SD of eight replicates.

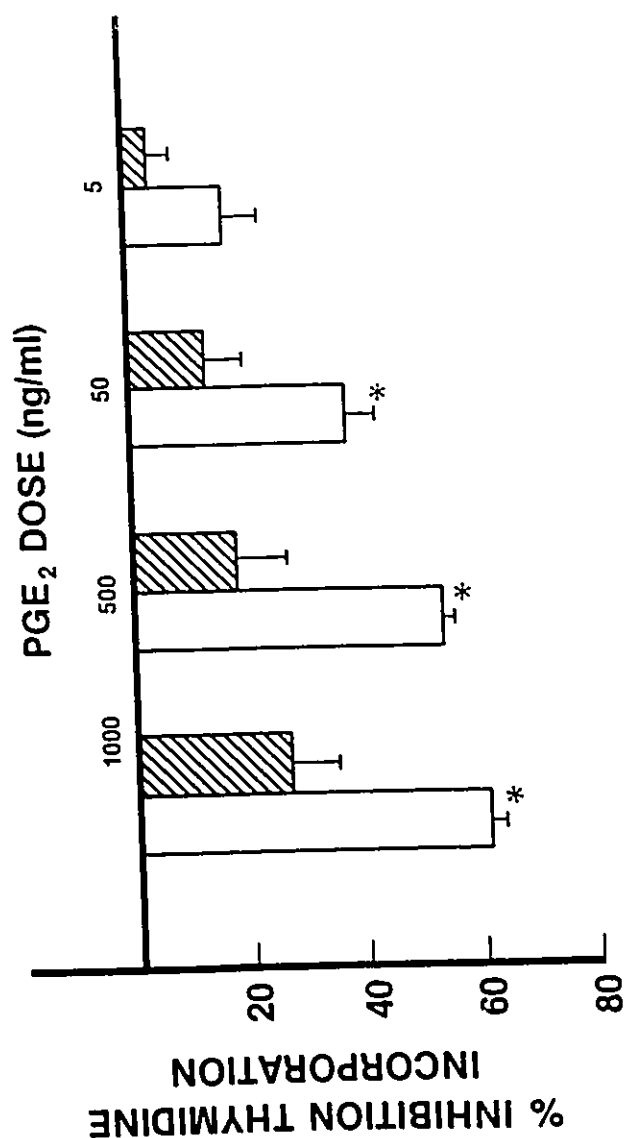


Figure 22. Effect of exogenous PGE₂ on in vitro proliferation of lung fibroblasts. Results are expressed as mean \pm SD of two normal (empty bars) and two fibrotic (dashed bars) cell lines. The value assigned to each cell type was the mean response (% change compared to control) to any given concentration of PGE₂ on repeated testing. Control value was obtained by incubating fibroblasts in RGM with 10% FBS alone.

* $p < 0.05$, normal vs fibrotic.

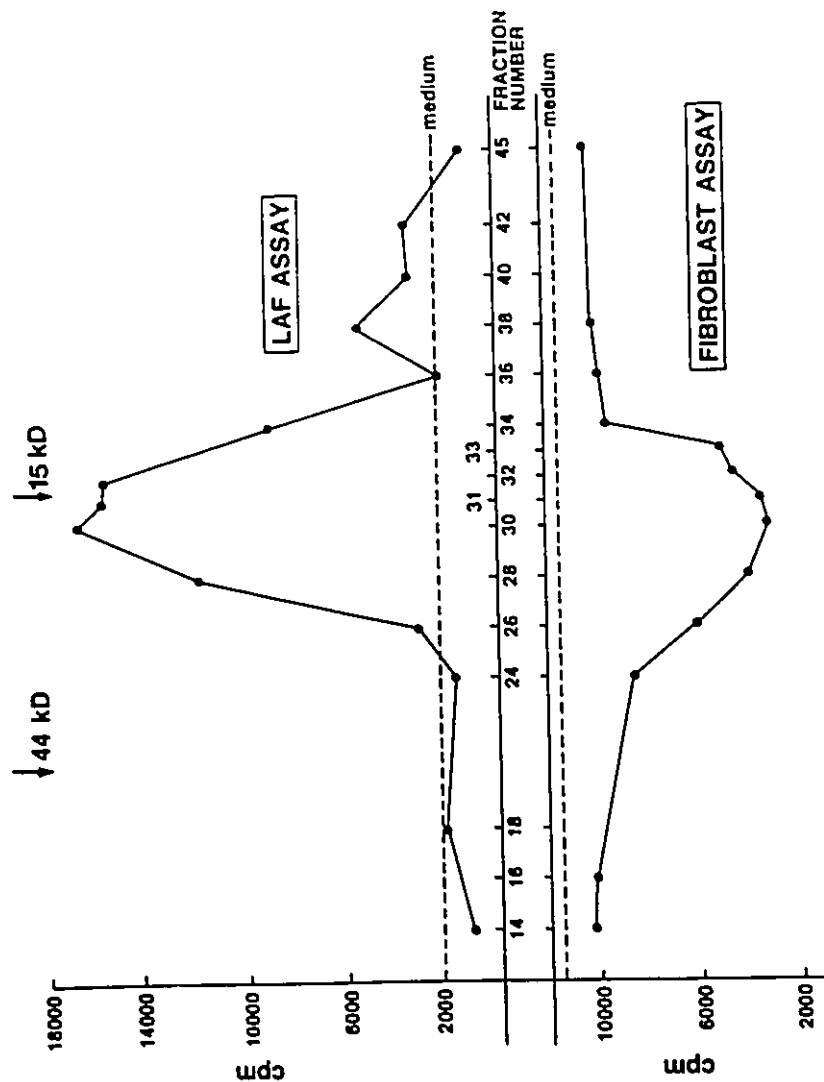


Figure 23. Effect of fractionated (Sephadex G-100 column, PBS) LPS-stimulated alveolar macrophage supernatant on the fibroblast and lymphocyte activating factor (LAF) assays. Each data point represents mean of three replicates. SD was less than 15% in all cases in the fibroblast assay and $\leq 20\%$ in the LAF assay. The molecular weight markers used to calibrate the column were ovalbumin, 44 kD and cytochrome C, 13.5 kD.

TABLE 3

EFFECT OF PURIFIED HUMAN IL-1 β ON
NORMAL LUNG FIBROBLAST PROLIFERATION

Units IL-1 β ^a	Inhibition (%) ^b
12.5	53.5 \pm 17.6
25	74.1 \pm 8.9
50	82.6 \pm 2.9
100	82.2 \pm 7.6

^a) IL-1 β activity is expressed as anti-viral units [34] added to fibroblast cultures in 10% FBS.

^b) Inhibition is calculated as percent inhibition of ³H-thymidine incorporation compared to fibroblasts cultured in 10% FBS alone
(n = 6).

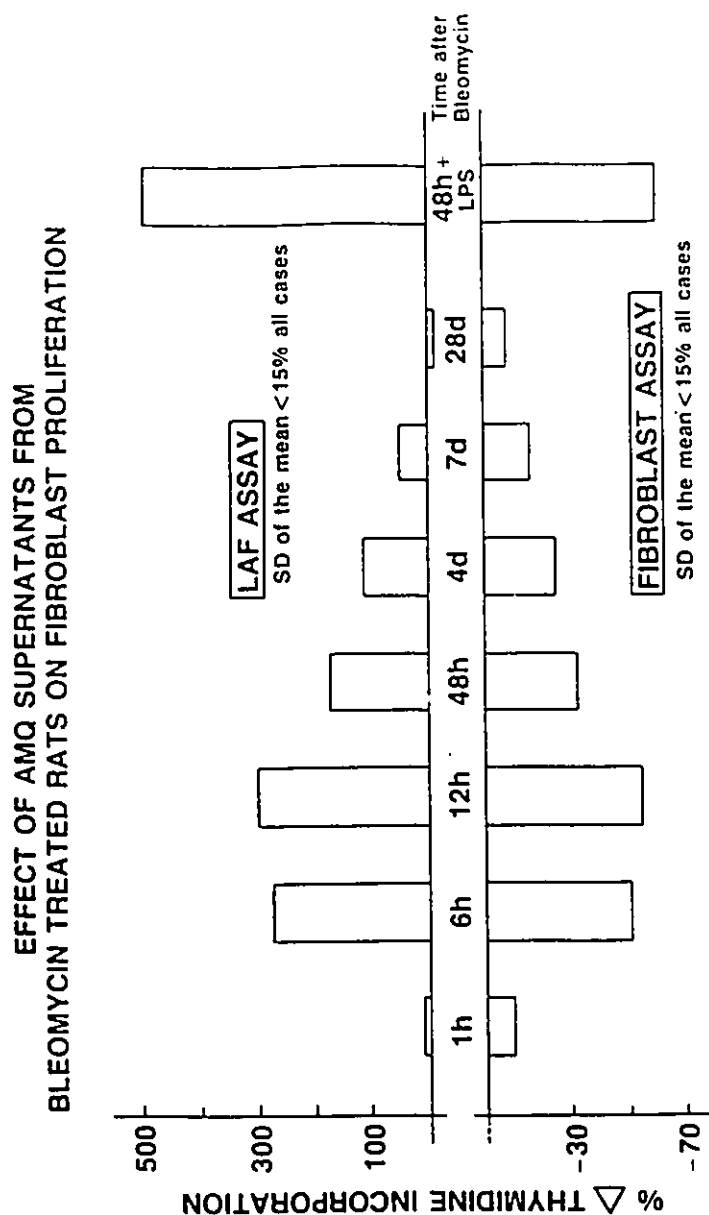


Figure 24. Effect of pooled AM supernatants from 3 animals harvested at several intervals after the intratracheal administration of bleomycin (open bars) with no stimulation in vitro. Bar on the far right of the graph indicates results of in vitro LPS-stimulated macrophages from control rats. Effect on thymocyte (LAF) and fibroblast proliferation. Results are expressed as percent change from control and represent the mean of 6 replicates in the fibroblast assay and 4 in the LAF assay. Control value was obtained by incubating cells in medium with 10% FCS alone (SD < 15% in the fibroblast assay and $\leq 20\%$ in the LAF assay).

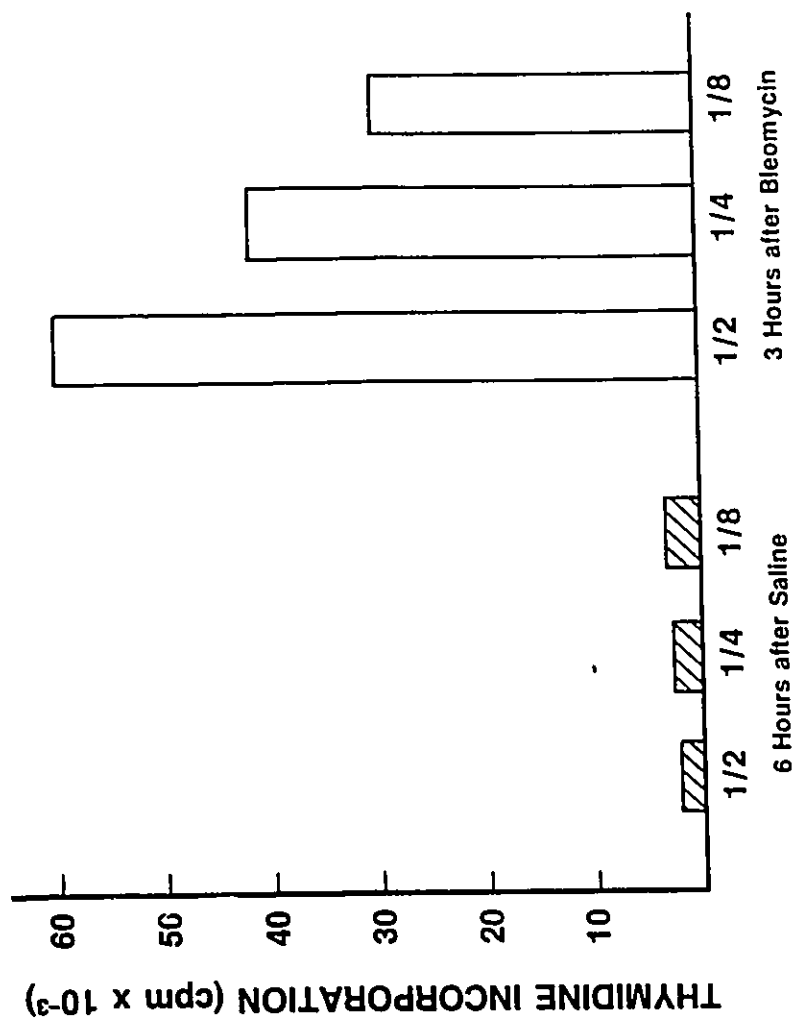


Figure 25. Effect of unstimulated AM supernatants harvested 3 and 6 h after the intratracheal administration of either bleomycin (open bars) or saline (hatched bars), respectively, on the thymocyte proliferation assay. The fractions on the horizontal axis represent dilutions of the supernatant.

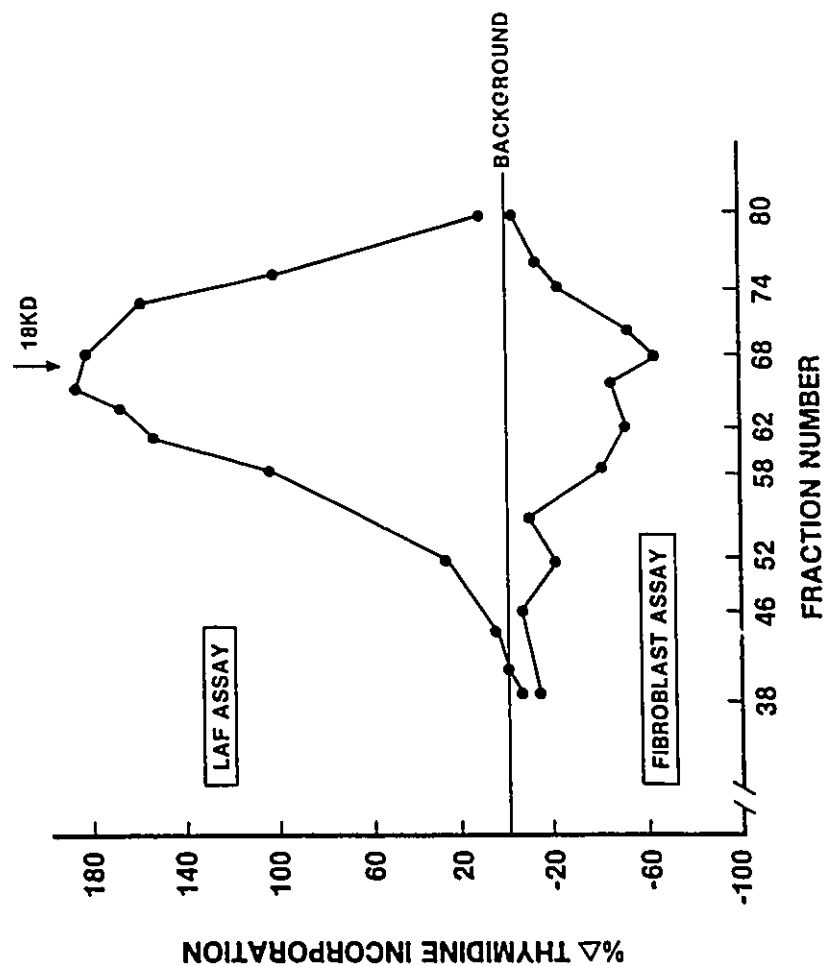


Figure 26. Effect of fractionated (Sephadex G-100 column unstimulated AM supernatants obtained 6 h after bleomycin on fibroblast and thymocyte proliferation. Each data point represents mean of 3 replicates (SD < 15% in the fibroblast assay and ≤ 20% in the LAF assays. Results are expressed as percent change compared with background (cells incubated with RGM in 10% FBS alone).

medium alone). Supernatants from AM recovered from bleomycin-treated animals obtained at 6, 12 and 48 h after bleomycin without further stimulation with LPS in vitro, elicited marked effects of a similar nature on both fibroblast and thymocyte proliferation. In this particular experiment, the maximal effect was seen at 12 h, but in others the maximal effect occurred at 6 h after bleomycin. Supernatants from AM obtained 4 days after bleomycin still consistently showed some effect on both LAF and fibroblast assays, but of substantially less magnitude than shown previously, whereas effects seen with 7-, 21-, or 28-day AM supernatants were not different from control. The inhibitory effect of AM supernatants on fibroblast growth was not due to a cytotoxic effect as shown by trypan blue exclusion assessment of viability ($\geq 95\%$ viable) at the end of the assay. To confirm that in vivo AM activation was induced by the administration of bleomycin and was not related to the surgical or anesthetic protocol, AM supernatants from sham-saline rats were obtained at intervals at which maximal activation was seen in bleomycin-treated rats and tested in the LAF assay. Supernatants from AM obtained as early as 3 h after the instillation of bleomycin induced a marked dose-dependent stimulation of thymocytes. In contrast, supernatants from AM harvested for as long as 6 h after the administration of saline showed negligible LAF activity (Figure 25).

To characterize the AM product active on both

fibroblasts and thymocytes, crude dialyzed supernatants from AM obtained at 6 h after bleomycin were chromatographed on a G100 Sephadex column (110 x 2.5 cm) and the fractions collected were simultaneously tested in the fibroblast and LAF assays. Figure 26 shows that fractions 64 to 78 eluting with an apparent molecular weight of 15 to 18 kD induced marked inhibition of fibroblast proliferation and stimulation of thymocyte proliferation.

Chronic exposure of primary and clonally-derived fibroblast lines to PBM supernatants

The response of four primary fibroblast lines to acute challenge with PBM supernatant is shown in Figure 27. As expected, this supernatant induced a dose-dependent inhibition of proliferation in the unexposed cell lines. In contrast, this inhibition was substantially abrogated in the cells which had been chronically exposed to PBM supernatant. In fact, PBM supernatant at a 1/4 dilution induced an average inhibition of 17% in these cells compared to 57% in the same cells which had been cultured for the same length of time in RGM without PBM supernatants. Figure 28 shows the proliferative response of these cells to an acute challenge with PGE_2 . Whereas PGE_2 induced a marked dose-dependent inhibition of proliferation of the unexposed cell lines, the effect on the cells chronically exposed to PBM supernatants

was rather minimal, $54 \pm 7\%$ versus $13 \pm 6\%$ with a concentration of PGE_2 of 10^{-6} M.

Figure 29 shows the response of individual clonally-derived fibroblast lines to an acute challenge with a single dilution of peripheral blood monocyte supernatant. The data clearly illustrate that there is a great degree of heterogeneity in the response of clones derived from unexposed cell lines to this acute challenge. Figure 30 shows the distribution of PBM responsiveness in two panels of clones derived from the same cell line. Panel A represents clones established from fibroblasts never before exposed to PBM supernatants; and panel B is comprised of clones established after primary cultures of fibroblasts had been chronically exposed to PBM. The data illustrate a skewing of phenotype distribution in that there are far fewer clones which can be substantially inhibited by an acute challenge with this supernatant in the panel of clones established after the cell line had been chronically exposed to PBM (15% versus 46%). Conversely, a greater proportion of clones obtained from the cell line chronically exposed to PBM supernatant were resistant to significant inhibition when acutely challenged with PBM supernatant compared to clones derived from a fibroblast culture never exposed before to PBM supernatants (40% versus 21%). Similar results in terms of both heterogeneity and responsiveness were found when the acute challenge was carried out with PGE_2 . In order to

determine whether the degree of responsiveness to PBM supernatants is a characteristic which the cells can acquire, individual clonally-derived fibroblast lines which upon acute challenge to PBM were shown to be responsive, were chronically exposed to PBM supernatants and then their response to an acute challenge with PBM supernatant examined. Table 4 shows the result of such an experiment in four individual clones, and illustrates that the clones continue being responsive.

Finally, the growth response of fibroblast clones to PBM supernatants was assessed in a series of clones (n=22) derived from a primary line of normal (N) fibroblasts as well as in clones established from a primary line of idiopathic pulmonary fibrosis (IPF) fibroblasts (n=27). These were further divided between clones exhibiting a normal pattern of in vitro thymidine incorporation (IPF-I, n=19) and those showing a fast proliferative rate (IPF-II, n=8). As shown in Figure 31, these fast growing clones (IPF-II) were significantly less inhibited by PBM supernatants (average of 9%) compared to either normal fibroblasts-derived clones (N) or fibrotic fibroblasts-derived clones with normal proliferative rates (IPF-I) (average of 40 and 31% respectively).

Human lung fibroblasts release small amounts of PGE_2 under normal culture conditions but release substantial amounts upon challenge with PBM supernatants or with

individual cytokines such as IL-1 and TNF. Table 5 shows that fibroblasts which had been chronically exposed to PBM supernatants release, upon an acute challenge with this supernatant, much greater amounts of PGE_2 compared to unexposed cells.

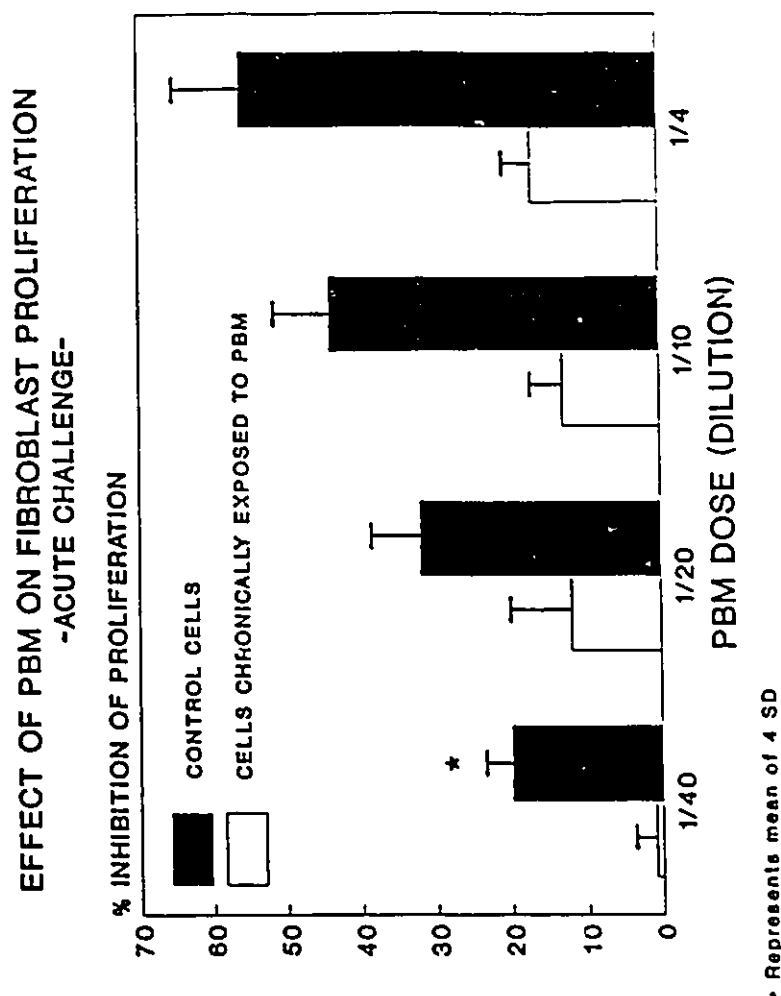


Figure 27. Effect of LPS-stimulated peripheral blood monocyte (PBM) supernatants on in vitro fibroblast proliferation (thymidine incorporation). Open bars represent control (unexposed) fibroblasts. Solid bars represent these same fibroblasts tested after chronic exposure to PBM supernatants (see text for details). Results expressed as mean \pm SD of 4 primary cell lines. Differences between control and chronically exposed fibroblasts are statistically significant at all PBM dilutions.

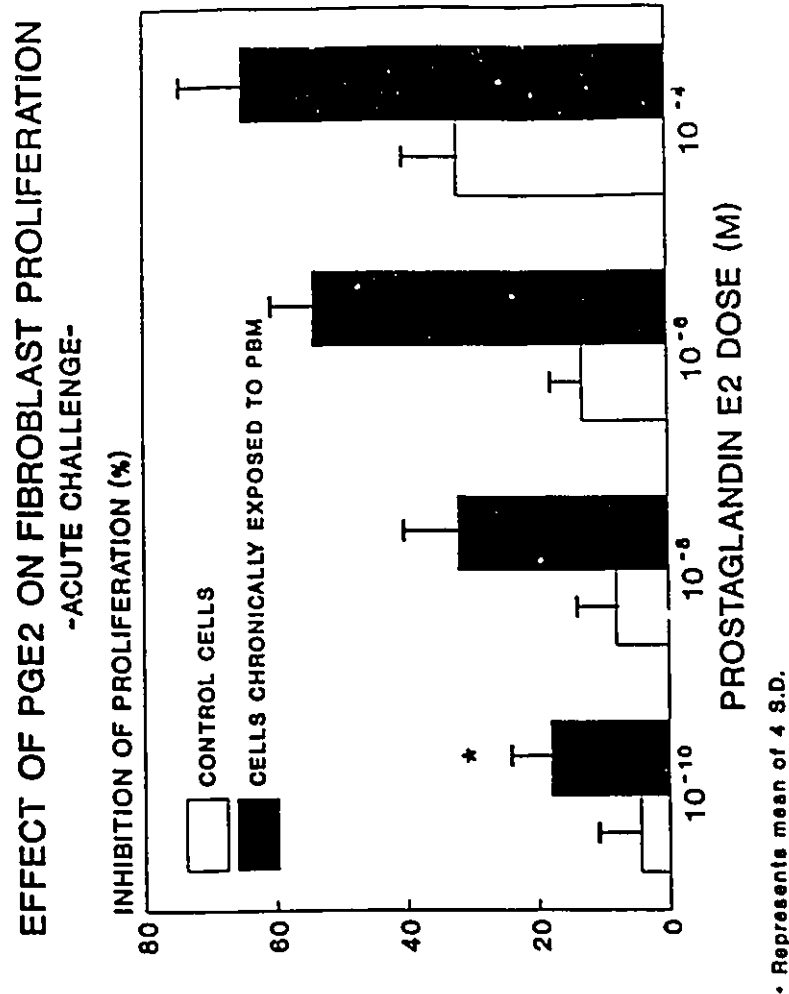


Figure 28. Effect of exogenous PGE₂ on in vitro fibroblast proliferation (thymidine incorporation). Open and solid bars as in figure 27. Results expressed as in Figure 27. Differences between control and chronically exposed fibroblasts are statistically significant at all PGE₂ concentrations.

TABLE 4
PGE₂ RELEASE BY HLF CELL LINES
(ng/ml)

Control			After Chronic Cytokine Exposure	
Cell Line	Spontaneous	Upon Cytokine Challenge	Spontaneous	Upon Cytokine Challenge
1	1.1	43	31	501
2	2.5	290	10	852
3	6.3	19	21	625
4	0.5	156	2.5	454

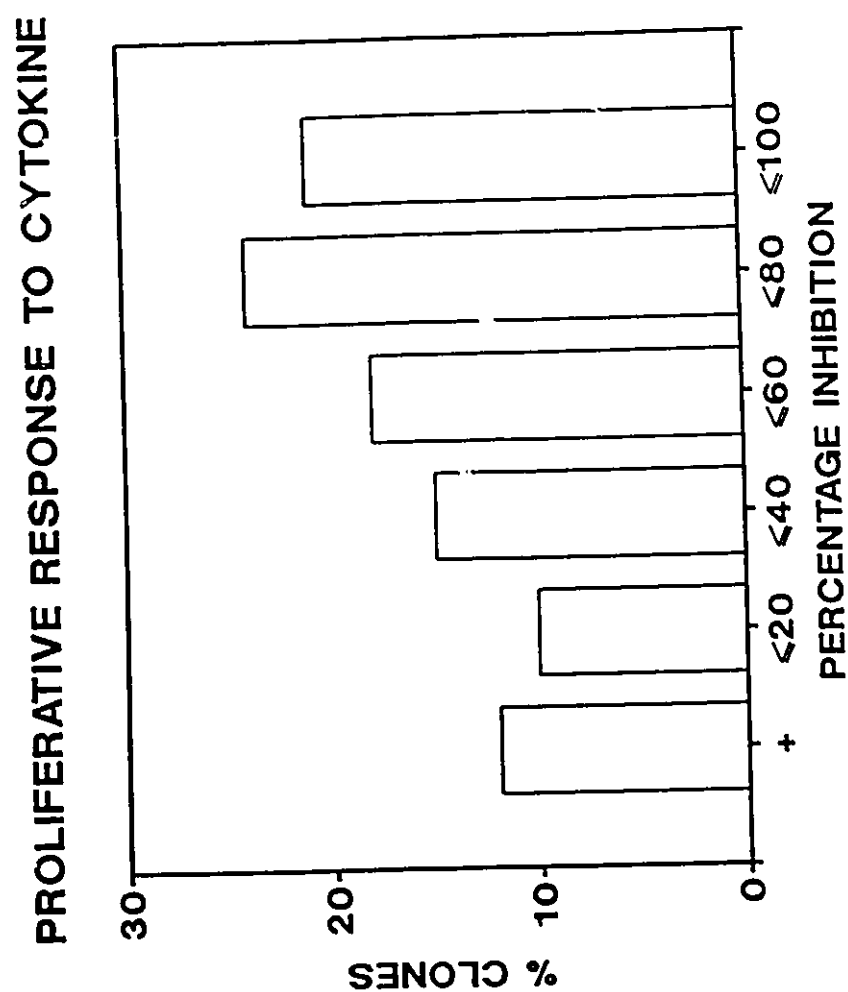


Figure 29. Proliferative response (thymidine incorporation) of human lung fibroblast clones to an acute challenge with peripheral blood monocyte supernatants.

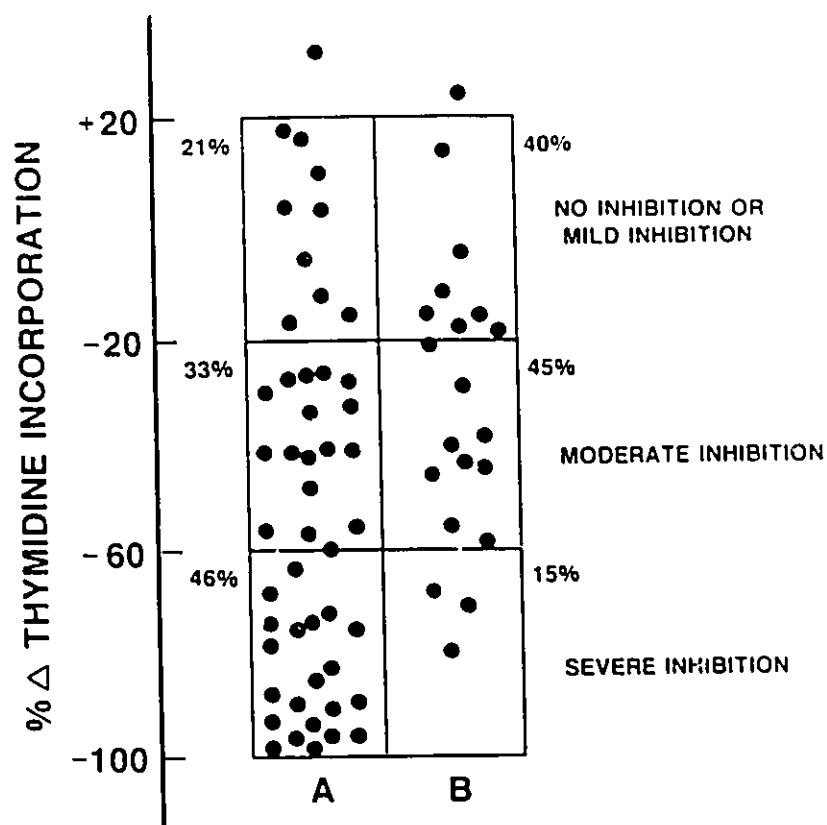


Figure 30. Proliferative response of human lung fibroblast clones to an acute challenge with PBM supernatant. Clones in Panel A were established from a control (unexposed) primary line. Clones in Panel B were established after this primary line had been chronically exposed to PBM supernatants. The data in the vertical axis represent percent inhibition (or stimulation) of each individual clone to this supernatant.

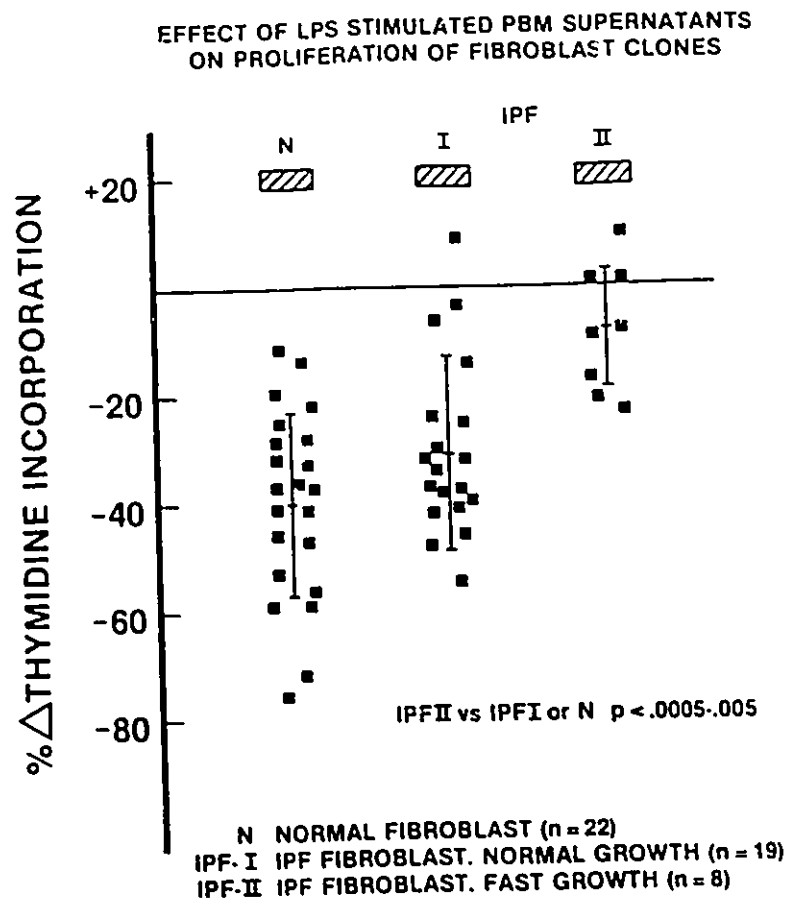


Figure 31. Proliferative response of human lung fibroblast clones established from primary lines derived from normal lung tissue (N) and from tissue of patients with idiopathic pulmonary fibrosis (IPF) to PBM supernatants. The selection of IPF clones with "normal" (IPF-I) and "fast" growth (IPF-II) was made on the basis of prior testing as in Figure 12. (Vertical lines represent SD).

RESPONSE OF FIBROBLAST CLONES TO ACUTE CYTOKINE CHALLENGE

CLONE	CONTROL CONDITIONS			CHRONIC CYTOKINE CONDITIONS		
	% Inhibition Of Growth	PGE ₂ Release		% Inhibition Of Growth	PGE ₂ Release	
		Unchallenged	Challenged		Unchallenged	Challenged
1	96	1.2	>200	85	6.7	> 200
2	71	3.8	128	81	4.1	129
3	61	12.4	106	78	17	109
4	89	5.9	>200	60	10	> 200
5	89	3.1	>200	87	6.7	> 200
6	80	1.3	>200	75	5.7	> 200

TABLE 5

DISCUSSION

Growth behaviour of fibroblasts in vitro

Ever since the first studies by Hayflick (130), there is full agreement that the replicative life-span of mass cultures of fibroblasts is limited. The genotype of the donor influences the life-span of fibroblasts. For example, skin fibroblasts derived from patients with Werner's syndrome have a striking decrease in growth potential (131) and, on the other hand, cultures of fibroblasts which have been transformed by an oncogene virus such as SV-40 become immortal (132). The life-span of fibroblasts is inversely related to the age of the donor (133). Whereas fetal lung strains achieve 50 to 60 population doublings, fibroblast cultures from adult skin fibroblasts average 35 population doublings. However, it is important to understand that the number of population doublings is not equivalent to the number of divisions a cell is capable of undertaking before death. In other words, that the behaviour of a mass culture is not, as it was initially thought, a reflection of this behaviour in all cells in the culture. In this regard, the study of the proliferative potential of clonal skin fibroblast lines has clearly demonstrated that a mass culture is in fact comprised of a very heterogeneous population of

cells with different population doublings (133). It could be argued that in such a situation there would be a continual selection for those cells with the shortest generation time. Thus, slow growing cells would be progressively eliminated leaving the fast growing ones to prevail in the culture. However, the number of cells capable of dividing exponentially decreases with the age of the culture. For example, Smith and Hayflick showed that 50% of the cells of a culture of human fetal lung fibroblasts at passage 9 were able to divide but only 5% of the cells were able to divide at least once 50 passages later (134). Based on extensive studies with clonally-derived skin fibroblast lines and mathematical models, Kirkwood and Holliday (135) and Jones and Smith (136) have proposed a hypothesis to explain the limited life-span of fibroblast cultures. The central component of the hypothesis is that there is a limited number of "uncommitted" cells (stem-like cells), with an unlimited growth potential, in a culture. Every time an uncommitted cell divides there is an increasing probability of a cell to become "committed", i.e. with a defined (limited) growth potential. Commitment is irreversible, so that division of a committed cell would always result in two committed cells. The hypothesis would predict that, over time, there would be in the culture an accumulation of committed cells diluting the pool of uncommitted cells to a point where there would be a high probability of culture to become stationary and,

eventually extinct.

Proliferative characteristics of fibroblasts from normal and fibrotic tissues

To study in vitro fibroblast proliferation, a thymidine incorporation assay was established for the initial series of experiments, because it has been shown that the amount of thymidine incorporated reflects cells proliferation (137). In some instances, cell counting was performed to substantiate the results. The data shown in Figure 8 demonstrate that both CF and FF lines have a similar pattern of in vitro incorporation of thymidine. However, FF fibroblasts consistently showed increased rates of thymidine incorporation throughout the entire incubation period and, on average, reached peak values 3 times higher compared to control fibroblasts. It is extremely unlikely that the age of the donors had an influence in these results because the average donor's ages were very similar, 47 years for CF and 35.5 years for FF. Figure 9 confirms that FF proliferated faster than those from control tissues. However, FF fibroblasts showed both a serum dependence of growth (Figure 11) as well as, ultimately, a saturation density (Figure 9) indicating that these cells did not exhibit an "uncontrolled" proliferative behaviour or, in other words, did not exhibit a "transformed" phenotype.

The observation that fibroblasts derived from fibrotic lung tissue proliferate in vitro faster than do control fibroblasts is similar to the observation by Oohira and colleagues showing that skin fibroblasts from patients with mucopolipidosis III have a deficiency of density-dependent regulation of growth (138). However, our observation differs from those reported in fibroblasts from skin fibrotic disorders such as scleroderma and keloid tissue (139,140), suggesting that the mechanisms contributing to the increased collagen deposition in various fibrotic disease may well be different. Indeed, the interpretation of our findings should be essentially restricted to relatively early and active human pulmonary fibrosis of unknown cause.

The observed faster proliferation of FF compared to CF could be the result of polyclonal fibroblast activation or, alternatively, the result of the presence in the fibrotic cell lines of certain fibroblast subpopulations with distinct growth characteristics. To examine this question, the primary fibroblast lines were cloned. An examination of Table 2 indicates that the cloning procedure itself selected cells with certain characteristics. However, the cloning efficiency for both CF and FF lines was very similar, suggesting that is unlikely that this process of technical selection differentially affected CF and FF lines. Figure 12 demonstrates that both CF and FF-derived clones are considerably heterogeneous with respect to in vitro

proliferative behaviour. Because the development of adequate cell numbers for assays requires a single cell to go through about 20 doublings, this indicates the stability of the phenotypes seen. The mean proliferation of FF clones was significantly higher than that of CF clones. In addition, a number of clones, 15%, showed a distinctly fast growth rate (twice as fast as the mean control population), and this percentage was similar, 13% and 17%, in 2 separate panels of FF clones. This small number of fast growing clones could in fact have important relevance in the development of fibrosis. Given the appropriate conditions for proliferation in vivo, such as those that might be present in chronic tissue injury, it would take a relatively short period of time for that particular group of clones to reach a mass that would alter the overall proliferative behaviour of the fibrotic tissue.

The concept of functional heterogeneity among morphologically similar cell populations of human lung fibroblasts is consistent with previous data on dermal fibroblasts. Heterogeneity in terms of proliferation and synthesis of extracellular matrix proteins by dermal fibroblasts has been reported by Botstein and colleagues (141) and Hassell and Stanek (142). Bordin et al have also documented similar observations and related the population of cells that grow and produce high levels of proteins to the presence on these cells of high affinity binding sites to the first component of complement (C1q) (143). However, since

there clearly is substantial evidence of site heterogeneity (144-146), it is apparent that extrapolation of data from one tissue source to another is not appropriate and that characterization of the target cell relevant to each pathologic condition is thus necessary.

Effect of fibroblast supernatants on the proliferation of fibroblasts

Human lung fibroblasts release factors which stimulate the proliferation of fibroblasts in vitro (Figures 15 and 16). The nature of the factor responsible for this autocrine growth stimulatory effect has not been characterized as yet. However, there are reasons suggesting that this factor could be PDGF. First, there is evidence that tissue structural cells including lung fibroblasts are capable of secreting, and responding to, PDGF (147,148). Second, there is clear evidence that the PDGF gene is developmentally regulated. For example, Seifert et al showed that cultured rat aortic smooth muscle cells isolated from pups, but not those derived from adult animals, spontaneously produce a PDGF-like molecule (149). Majesty et al also showed that although the gene encoding for PDGF-A chain is, both in newborn and adult rats, expressed at similarly low levels in the intact aorta, when these cells were isolated and placed in culture, expression of mRNA for PDGF-A markedly increased

only in newborn-derived cells (150). Third, Libby et al have shown that in atherosclerosis, a disease characterized by the presence of a chronic inflammatory reaction and smooth muscle cell hyperplasia, smooth muscle cells derived from atheromatous plaques spontaneously express the gene, and release the gene product for the PDGF-A chain (151). Although enhanced production of a growth factor may be one of the components underlying this autocrine mechanism of growth regulation, there may be others. Our data examining the effect of fibroblast supernatants on the proliferation of fibroblasts indicate that the main detected difference between CF and FF was not in the ability of a particular supernatant to stimulate proliferation but rather in the extent of the target cell to respond to any given supernatant. This observation is consistent with the data examining the response of CF and FF to various concentrations of FCS (Figure 11), thus suggesting that changes either in the receptors for these growth factors or in intracellular signal transduction pathways may also contribute to this distinct proliferative behaviour between CF and FF fibroblasts. In fact, Antoniades and Selman have recently examined the distribution of PDGF in lung biopsies of patients with idiopathic pulmonary fibrosis by means of in situ hybridization and immunohistochemistry, and have shown that the greatest proportion of cells synthesizing this growth factor are epithelial cells followed by macrophages,

and that only a few fibroblasts synthesize PDGF. They also show that very few cells express this activity in control lung tissues (152).

In addition to PDGF, it is quite probable that in vivo tissue structural cell-derived growth factors, particularly from epithelial cells, are involved in paracrine pathways in the accumulation of fibroblasts. Kuhn et al demonstrated that in human pulmonary fibrosis there is a greater proportion of fibroblasts actively synthesizing collagen near the air-tissue interface, particularly in areas with an hyperplastic epithelium (20). Nasal polyposis is a condition which shares some basic analogies with pulmonary fibrosis including the presence of a chronic inflammatory infiltrate, abnormalities in the epithelial lining, from metaplasia in some areas to hyperplasia in others, and varying degrees of fibrosis in the polyp stroma. In this condition, Petrusson et al studied the tissue distribution of IGF-1, a molecule shown to stimulate the growth of many cell types including fibroblasts (153,154), and found an intense staining exclusively in the epithelium (155). Therefore, the implication would be that the increase in fibroblast numbers seen in the lungs of patients with pulmonary fibrosis, or the increased proliferative rate observed in FF in vitro, could be related to an enhanced responsiveness of these fibroblasts to stimulatory factors released by other cells in the lung, such as the epithelial cells, or present in the serum.

One can only speculate at this time with respect to the origin of the fast-growing cells found in fibroblast cultures derived from fibrotic tissue. Figure 10 shows that primary lines of neonatal fibroblasts showed the highest rates of proliferation, and Figure 13 illustrates that the proportion of fast growing clones is even greater within neonatal fibroblast cultures in relation to that of FF cultures. It is tempting to suggest that the increase reparative demand which occurs during the course of continuing tissue injury due to chronic inflammation, as it happens in pulmonary fibrosis, results in the emergence of an increase proportion of cells with more "stem-like" characteristics, such as the neonatal cells. It is of interest to note that fibroblasts obtained from the lungs of patients with very advanced fibrosis of the lung do not proliferate faster than control fibroblasts, which would be consistent with the concept of finite replicative life-span of fibroblasts discussed before.

The notion that the persistent expression of a fetal fibroblast phenotype might be related to disease development has been indicated by Schor et al (156). Indeed, fetal fibroblasts distinctly express a number of features including their ability to form colonies in semisolid medium (157), production of a growth factor with PDGF-like activity (158), differential proliferative response to TGF β (159) and synthesis of certain isoforms of matrix macromolecules (160).

These characteristics influence the migratory behaviour of these cells, the arrangement of these cells in culture, the orientation and packing density of collagen fibers in gels and the pattern of epithelial cell morphogenesis and differentiation. Interestingly, McNeal et al showed that the stroma of tissues with benign prostatic hyperplasia displayed a morphologic resemblance to fetal stroma (161). Similarly, lung fibrosis might be viewed as an aberrant remodelling process and, perhaps, the basis of this aberrancy is more related to a change in fibroblast subpopulations in the lung, specifically the emergence of cells with a neonatal/fetal phenotype, rather than to changes in specific functions of the adult cells themselves.

Acute effects of AM/PBM products on fibroblast proliferation

Evidence is presented that in vitro LPS-activated alveolar macrophages or peripheral blood monocytes release a non-dialyzable factor (Mr approx. 15kD) which inhibits in vitro fibroblast proliferation in a dose-dependent manner (Figures 18 and 19). The data also show that the activity present in AM/PBM supernatants is consistent with the presence of IL-1, since the two activities (fibroblast growth inhibition and lymphocyte activating factor) co-elute on molecular sieve chromatography (Figure 23), and highly

purified IL-1 β causes similar changes in lung fibroblast behaviour (Table 3). Figure 20 shows that indomethacin, which by itself did not have a significant effect on fibroblast proliferation, entirely reversed the inhibition induced by AM/PBM products. Initially, fibroblasts were exposed to indomethacin for 24 h, concurrently with PBM supernatants, but it was equally effective to simply pre-expose the target cell for 1 h to indomethacin before adding the PBM supernatants (data not shown). The fact that the addition of exogenous PGE₂ reconstituted the growth inhibition even when indomethacin was present suggests that the response of fibroblasts to PBM cytokines is mediated through the prostaglandin pathway of the fibroblast. Indeed, Elias and colleagues found that AM products stimulated PGE₂ release, and indomethacin, at a dose similar to that in our experiments, markedly prevented the release of PGE₂ (162). In addition, Mizel et al showed that a partially purified IL-1 preparation stimulated PGE₂ production by rheumatoid synovial cells (163), and recent reports using rIL-1 confirm that the fibroblast is a target cell for this cytokine and responds by activation of the arachidonic pathway and generation of PGE₂ (164). The results in Figure 21 with various exposures to PBM supernatant and indomethacin indicate that the cytokines rapidly activate the arachidonate pathway of the fibroblast and that although the inhibition can be halted at any stage, it can not be reversed.

Effect of rat AM products on rat lung fibroblast proliferation

The preceeding experiments involved the examination on the proliferation of fibroblasts of products released by AM or PBM stimulated with LPS in vitro. The following experiments were performed in order to demonstrate that the effects seen were not restricted to these defined experimental conditions. The intratracheal instillation of bleomycin into rodents elicits a substantial inflammatory lung reaction followed by the development of fibrosis. The cellular constituents in the BAL fluid, the histologic characteristics, the changes in lung function and a number of aspects related to collagen metabolism have been studied in this model (165-168), and it is widely accepted that the phenomena described in this system resemble those occurring in human pulmonary fibrosis.

The evidence shows that supernatants from AM obtained by lavage at an early stage after the intratracheal instillation of bleomycin, and cultured without further in vitro stimulation, contain cycokines that modulate in vitro fibroblast and thymocyte proliferation (Figures 24 and 25). LAF activity in the supernatants was detected as early as 3 h after the administration of bleomycin, with peak activities between 6 and 12 h. These effects could not be due to residual bleomycin since all supernatants had been thoroughly

dialyzed prior to testing. Supernatants from AM from animals that received saline, obtained at a time (6 h) when peak LAF activities were detected in animals which received bleomycin, did not contain any LAF activity indicating that AM activation had occurred in vivo and it was a result of bleomycin administration. The data also show that incorporation of ^3H -thymidine by rat lung fibroblasts was decreased by AM supernatants from animals which had received bleomycin. This inhibitory effect is likely due to IL-1 because both fibroblast and LAF activities exhibit a similar chronology. Also, when supernatants released by the AM recovered at 6 h after bleomycin were fractionated by size exclusion chromatography, both activities co-eluted in the 15 kD region (Figure 26) further suggesting that IL-1 was the molecule responsible for the inhibitory activity on fibroblasts. Therefore, the data in this experimental system is fully consistent with the data using human cell populations and in vitro stimulation of AM.

Evaluation of the effect of AM/PBM-derived IL-1 on the modulation of fibroblast proliferation

The evidence presented demonstrates that human AM or PBM activated by LPS in vitro or rat AM activated in vivo by the intratracheal instillation of bleomycin release IL-1 and that this molecule causes an inhibition of lung fibroblast

proliferation. Clearly, an increase in the number of fibroblasts within the lung parenchyma occurs in human as well as in experimentally-induced pulmonary fibrosis. Therefore, the implications of the findings reported to the development of fibrosis of the lung are not straightforward and require a careful analysis.

Leibovitch and Ross described a macrophage-derived factor that stimulates proliferation of guinea pig skin fibroblasts (169), and Bitterman et al showed that activated AM release a molecule termed AMGDF which stimulates proliferation of fibronectin-primed fibroblasts (170). In addition, IL-1 has also been shown to stimulate the proliferation of fibroblasts (171). On the other hand, Elias et al reported have demonstrated that LPS activated AM and PBM release a molecule, likely IL-1, which inhibit the proliferation of human lung fibroblasts (162). Interestingly, conflicting results have also been observed examining the effect of lymphocyte supernatants on fibroblast proliferation (172,173). In addition, similar discrepancies have been noted with supernatants of cells obtained from experimental animals. For example, Clark and coworkers showed that supernatants from hamster AM harvested between 1 and 3 weeks after exposure to bleomycin inhibited both proliferation and collagen synthesis of lung fibroblasts (174), whereas Bigby et al have shown that supernatants from rabbit AM obtained 3 and 8 weeks after bleomycin stimulated proliferation of lung

fibroblasts (175). A recent review by Freundlich et al have summarized the differences in outcomes in fibroblast proliferation assays (176).

While differences in the species, the incubation period and the cell density may contribute to some of these differences, the single probably most important variable determining the outcome of the proliferation assay is the state of the cells. Stimulation of proliferation is seen in assays which use quiescent cells, generally achieved by serum starvation. Inhibition is seen when actively proliferating cells are used, that is with cells cultured in the presence of serum. The implications of this observation are not merely of a technical nature. First of all, the lung cell turnover, particularly that of the structural cell compartment is extremely low (177). Indeed, the vast majority of cells are in vivo in a quiescent state and, under these conditions, fibroblast signalling by AM-derived cytokines would result in stimulation of proliferation. However, when the fibroblast is actively proliferating, a situation virtually identical to that re-created in vitro by seeding these cells at a relatively low density and in the presence of serum, signalling by AM-derived cytokines, particularly IL-1, would result in inhibition. Since this effect is mediated by fibroblast released PGE₂, it is reasonably to argue that the ability of the fibroblast to respond (by inhibition) to this mediator forms the basis of a physiologic response in that it

would prevent uncontrolled fibroblast proliferation.

Chronic exposure of primary and clonally-derived fibroblast lines to PBM supernatants

An important consideration in the evaluation of in vitro experimental systems which examine the response of a target cell (the fibroblast) to a single exposure to AM-derived signals is that this is hardly a situation equivalent to what might be occurring in vivo during the course of pulmonary fibrosis, a disease of a chronic nature characterized by the continuing presence of inflammation. Under these chronic conditions, a number of inflammatory mediators could potentially promote the preferential expression of fibroblast subpopulations with distinct phenotypic characteristics including their responsiveness to AM/PBM-derived cytokines. The experiments involving the chronic exposure of fibroblasts to PBM supernatants were designed to explore this hypothesis.

Evidence is presented indicating that the responsiveness of fibroblasts to acute challenges with PBM supernatants, that is the ability of these supernatants to inhibit the growth of actively proliferating fibroblasts, changes after exposure of these fibroblasts to PBM supernatants for 4-5 weeks (Figure 27). There are two observations suggesting that this is likely not a lack of

responsiveness to cytokines contained in the PBM supernatant. First, cells chronically exposed to PBM supernatants release upon acute PBM challenge substantial amounts of PGE_2 , in fact even greater than unexposed cells (Table 5), which indicates that these cells do respond to cytokines present in the PBM supernatant. Second, cells chronically exposed to PBM supernatants become unresponsive to acute challenges with PGE_2 as well. The data is consistent with that of Korn, Torres and Downie showing that normal skin fibroblasts exposed to mononuclear cell supernatants for several weeks show a greater PGE_2 synthetic response upon re-exposure to those supernatants (178). The data is also consistent with the observation of Ko, Page and Narayanan showing that incubation of normal skin fibroblasts in PGE_2 -containing medium for two weeks results in the emergence of a PGE_2 -insensitive cell population (179). We are aware of only another clinical condition in which to some extent similar observations have been made. The fronto-ethmoidal mucocoele is a condition which causes obstruction of the fronto-nasal duct which is characterized at the histological level by chronic inflammation, the presence of connective tissue stroma and bone destruction. Lund et al examined fibroblasts derived from these mucocoeles and found that these cells synthesized upon stimulation with mononuclear cell supernatants significantly greater amounts of PGE_2 and collagenase in comparison to normal nasal fibroblasts (192).

The examination of individual clonally-derived fibroblasts lines to acute challenges with PBM supernatants provides dramatic evidence of fibroblast heterogeneity (Figure 29). Such examination in a panel of clones derived from a primary line which had been chronically exposed to PBM supernatant shows that the change in responsiveness of the primary line is due to a change in the clonal distribution, i.e. the presence of fewer clones capable of substantially responding (being inhibited) to acute PBM challenges (Figure 30). However, this examination did not allow us to conclude whether the change in clonal distribution was due to induction or to selection. The data in Table 4 demonstrates that clones sensitive to PBM supernatants upon acute challenge remain similarly sensitive after having being exposed for several weeks to PBM supernatants. Since "sensitivity" equates inhibition of proliferation, the implication would be that the relative proportion of these cells in the mass culture after several weeks is decreased. This process of clonal selection is subserving as the cells which are allowed to emerge also produce greater amount of PGE_2 upon recurrent exposure to PBM supernatants. We also examined the spontaneous in vitro proliferative characteristics of primary lines of fibroblasts chronically exposed to PBM supernatant and found no differences compared to unexposed fibroblasts. It is probable that the conditions of the experimental protocol, i.e. duration of the experiment

and repeated introduction of negative pressures, could have resulted in an excessive "aging" of the cultures and, thus affected the results. As a counterpoint to this, it is of particular significance the observation that fibroblasts derived from fibrotic tissue, i.e. a chronically inflamed tissue, are hyporesponsive to acute PGE_2 challenges compared to control fibroblasts (Figure 22), and also that clones derived from a primary FF line which exhibit fast proliferative characteristics in vitro are very little inhibited by PBM supernatants (Figure 31). Thus, the emergence in the tissue of fibroblast subpopulations with such characteristics would in fact result in the loss of a potentially prime mechanism of growth control. Ultimately, the increase in fibroblast numbers in the lung of patients with pulmonary fibrosis could be due as much as to the emergence of fibroblast subpopulations unable to downregulate themselves and to inhibit others as to the continuing presence of positive (stimulatory) signals.

CONCLUSIONS

Human pulmonary fibrosis is a condition characterized by a profound derangement of the lung architecture which is the result of alterations in the deposition and organization of a number of extracellular matrix proteins. This implicates the lung fibroblast as one of the key cells in the pathogenesis of this disease. In addition, fibrosis of the lung is associated with the continuing presence of an inflammatory process. This is defined by the accumulation in the lung of increased numbers of a variety of inflammatory cells which are, most or all, in a state of activation. Amongst the various cell types involved in this inflammatory process, the macrophage/monocyte is thought to play a central role. Therefore, to gain insight into the relationship between fibrosis and inflammation or, in other words, between the fibroblast and the macrophage/monocyte, appears critical to understanding the pathogenesis of pulmonary fibrosis. It is important to note that the structural derangement characteristic of pulmonary fibrosis occurs, in the majority of instances, over a protracted period of time and, also, that the administration of powerful anti-inflammatory agents to patients with this disease has a relatively poor therapeutic effect.

Human adult lung fibroblast are heterogeneous in regard to growth and proliferative response to proinflammatory cytokines synthesized by the macrophage/monocyte. In addition, human adult lung fibroblasts derived from patients with active lung fibrosis display a faster proliferative rate in vitro compared to fibroblasts derived from normal lungs. This proliferative behaviour appears to be the result of an increased proportion of fast-growing clones in the cultures of fibrotic fibroblasts (FF). Also, normal neonatal human lung fibroblasts exhibit the highest proliferative rate and fibroblasts derived from tissues with patients with very advanced fibrosis do not proliferate differently than normal fibroblasts. These findings suggest that the proliferative behaviour observed in FF could result from the emergence in the tissue of fibroblast subpopulations with neonatal characteristics.

Supernatants from human macrophage/monocytes stimulated in vitro with endotoxin, or from unstimulated rat macrophages obtained from animals which received bleomycin, inhibit the growth of proliferating lung fibroblasts in vitro. This effect is likely elicited by interleukin-1 and mediated by the prostaglandin pathway of the fibroblast because pretreatment of the fibroblasts with indomethacin prevents this inhibition, but is reconstituted by the addition of exogenous PGE₂. Also, fibrotic fibroblasts are significantly less growth inhibited by PGE₂ compared to

normal lung fibroblasts.

The chronic exposure (4-5 weeks) of human lung fibroblasts to macrophage/monocyte (PBM) supernatants alters the response of these cells to subsequent acute challenges with these supernatants. Indeed, fibroblasts become non-responsive (not growth-inhibited) but release substantially greater amounts of PGE_2 upon further acute challenges. This change in the behaviour of primary fibroblasts lines appears the result of a change in the distribution of clones in the culture as the proportion of clones which are sensitive, i.e. capable of being inhibited, by PBM supernatants decreases after chronic exposure of the primary lines. Furthermore, the change in clonal distribution appears related to selection rather than to induction, as the specific response of a particular clone to acute challenges with PBM supernatants can not be modified by chronic exposure to these supernatants. These results indicate that chronic exposure to proinflammatory mediators are capable of inducing a phenotypic change on the fibroblast affecting its growth regulatory mechanisms.

Despite evidence of fibroblast heterogeneity in collagen gene expression, we have not found evidence, nor is there indication in the literature, that the overall synthesis of collagen per cell is increased in FF. Whether the synthesis of collagenase, stromalysin or TIMP, i.e., of enzymes involved in collagen breakdown, is altered in these

fibroblasts is not known to date. Until such evidence is available, our preliminary conclusion would be that the increased deposition of collagen, and other ECM proteins, characteristic of pulmonary fibrosis, is primarily due to an increase in the number of fibroblasts in the lung parenchyma. Clearly, an examination of these issues, is an important future direction of these studies. In addition, to pursue the question of increased fibroblast responsiveness to specific growth factors directly in situ by asking whether there is an increase proportion of fibroblasts in fibrotic tissues expressing the PDGF or the IGF-1 receptors may provide further basic insights into the pathogenesis of the fibrotic diseases.

REFERENCE MATERIAL

APPENDIX I

Work and Concepts Undertaken as a Follow-up to the Thesis Studies

Chronic Inflammation and the Fibroblast

The central point developed during the previous studies is that, during the course of chronic inflammation, there is a selection of subpopulations of lung fibroblasts with phenotypic characteristics commensurate with the development of lung fibrosis. However, the issue which has not been addressed is what determines the chronicity of the inflammatory response or, in other words, what mediates the accumulation in the lung of activated inflammatory cells. It is reasonable to argue that there are instances of pulmonary fibrosis where continuing exposure to a given agent can be identified. This, for example, could be the case in pulmonary fibrosis due to the chronic inhalation of potentially fibrogenic agents such as silica and asbestos. However, in the majority of instances of pulmonary fibrosis either








chronic exposure to a identified agent or even the agent itself cannot be recognized. In this situation, it is reasonable to speculate that a single or short term exposure to an unknown agent could have triggered a tissue response which became self-perpetuating.

It has become apparent in recent years that tissue structural cells in general, and fibroblasts in particular, are effector cells themselves, i.e. cells with the ability to synthesize a number of cytokines the source of which was initially thought to be restricted to the traditional inflammatory cells. Indeed, fibroblasts are capable of releasing cytokines including the CSF's, IL-6 and IL-8. These cytokines are, on the one hand, capable of regulating the differentiation of hemopoietic progenitors into functionally mature inflammatory cells of various lineages (180-182). On the other hand, they can interact with these mature inflammatory cells and modulate their survival, proliferation and activation (86,183,184). An important point of interest is that macrophage-derived cytokines such as IL-1 and TNF markedly stimulate the effector function of fibroblasts (185,186). This effect is demonstrated in Figure 32 which shows the level of GM-CSF gene expression by fibroblasts. Of particular relevance is the data in Figure 33 which shows the level of expression of IL-6 in normal fibroblasts as well as in fibroblasts derived from fibrotic lung tissue. There are marginal differences under unstimulated conditions. However,

the level of IL-6 expression by fibrotic lung fibroblasts is, under .stimulated conditions (by PBM supernatants) substantially higher compared to control lung fibroblasts. This hyperactive response of fibrotic fibroblasts might represent yet another phenotypic characteristic of these cells.

The effector capacity of fibroblasts is not limited to their ability to release cytokines. It is increasingly clear that the extracellular matrix itself is not an inert structure but rather an inductive one. Indeed, the ability of the ECM to modulate the shape, organization and movement of the cells which sit on it has been well documented and the concept of the extracellular matrix as an "informational structure" has been elegantly reviewed in the context of embriogenesis (187-189). More recently, new evidence has emerged examining the effect of the ECM on the resident inflammatory cells. In this regard, Newman and Tucci have shown that culture of monocytes on a collagen gel induces, compared to plastic, a rapid activation of the phagocytic and bactericidal activity of these cells (190). With regards to the lung, Figure 34 illustrates a fibroblast-derived ECM, and Figure 35 shows the effect of this ECM and of fibroblast supernatant, alone or combined, on the level of expression of IL-1 by human peripheral blood monocytes. It is apparent that each of these conditions upregulates the expression of the IL-1 gene compared to cells cultured on plastic with RGM

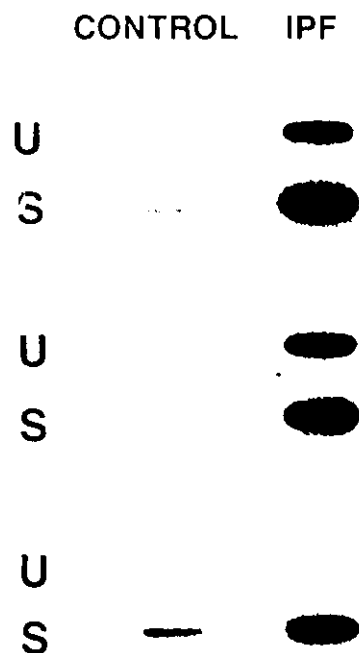
FIBROBLAST GM-CSF MODULATION BY IL-1 AND STEROIDS

	GM-CSF GENE EXPRESSION	GM-CSF CONTENT (pg/ml)
RPMI 10% FCS		51
Bud 10^{-8} M		0
Bud 10^{-6} M		0
IL-1 α		420
Bud 10^{-8} M + IL-1 α		62
Bud 10^{-6} M + IL-1 α		64
calf rRNA		—

* HUMAN NASAL FIBROBLASTS

Figure 32. Autoradiogram of human lung fibroblast RNA hybridized with a cDNA probe for human GM-CSF (left column). Data on the right column represent GM-CSF content in the supernatant measured by ELISA. The examination was carried out in unstimulated fibroblasts (cultured in RPMI 10% FCS) and in cultures of fibroblasts stimulated with IL-1 α . In addition, some fibroblast cultures were pretreated with steroids (budesonide, Bud). Calf rRNA was used as a negative control. (Additional methodological details in Ref. 180).

INTERLEUKIN-6 GENE EXPRESSION BY HUMAN LUNG FIBROBLASTS



U=Unstimulated

S=IL-1 Stimulated

IPF=Idiopathic Pulmonary Fibrosis

Figure 33. Autoradiogram of human lung fibroblast RNA hybridized with a cDNA probe for human IL-6. "Control" represent human adult normal lung fibroblasts. "IPF" represent fibroblast cultures established from lung tissue from patients with idiopathic pulmonary fibrosis. RNA was isolated from cultures of unstimulated (U) fibroblasts (cultured in RPMI 10% FCS) and from cultures of fibroblasts stimulated (S) with IL-1 α .

alone. It is also apparent that maximal upregulation occurs when monocytes are exposed to both conditions, a situation which may better reflect the in vivo microenvironment. Thus, the emerging concept is that, at any given time, the cells in the tissue articulate a message which is the synthesis of a number of signals presented in a unique context. The signals are the cytokines and growth factors secreted by the cells; the cells and the ECM's they produce define the physical context. It follows that the behaviour of any cell, mature or progenitor, entering a tissue is largely determined by both the signals and the context in which these are presented.

To summarize, a chronic inflammatory response can be defined as a situation where there is a persistent accumulation in the tissue of inflammatory cells which, most or all, are in a state of sustained activation. The TDR (Tissue Directed Response) concept which we have recently put forward (191), evolves from the simple notion that what occurs at a particular tissue site is regulated in, and to a large extent by, this tissue (Figure 36). The key aspects of this concept are capsulized in the words "in" and "by", signifying that the tissue is not a passive battleground but rather a powerful inductive context. One mechanism by which chronic inflammation, and eventually structural derangement such as fibrosis, might occur involves a change in the effector phenotype of the tissue whereby the tissue itself determines the nature and consequences of the

response. Fibroblasts, epithelial, endothelial and smooth muscle cells (TSC) are not merely targets but effector cells themselves. Indeed, cytokines and other products released by TSC can elicit a broad range of modulatory effects on inflammatory cells. Thus, an upregulation in the effector phenotype of TSC would result in a "hot microenvironment", that is a context rich in messages active on both hemopoietic progenitors and mature inflammatory cells. Since TSC can also respond to cytokines derived from inflammatory cells, subserving interactions between these two cell compartments could conceivably lead to a longlasting, or even self-perpetuating process. An important prediction of the "TDR Concept" is that once the phenotype of the tissue has changed, the specificity and the threshold of the exposure would become secondary issues, so that agents which were previously innocuous, can now elicit a pathogenic response.

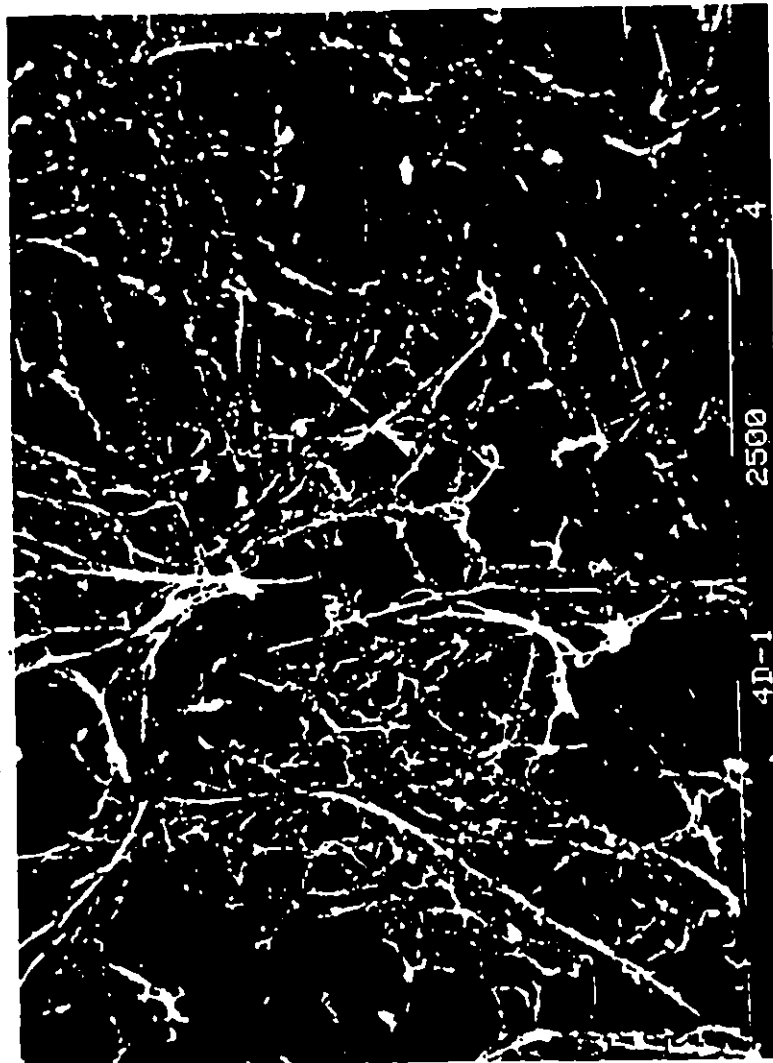
FIBROBLAST ECM

Figure 34. Fibroblast-derived extracellular matrix (ECM). Human lung fibroblasts were grown on a monolayer in RPMI medium with 10% FCS for 4 days. At this time, cells were removed by permeabilization of the monolayer by exposure to 0.5% Triton X-100 ammonium hydroxide. Firmly attached ECM was extensively washed 5 times with PBS.

MONOCYTE IL-1 β EXPRESSION EFFECT OF FIBROBLAST-CM AND EXTRACELLULAR MATRICES (ECM)

RPMI ON PLASTIC

50% FbCM ON PLASTIC

RPMI ON ECM

50% FbCM ON ECM

calf rRNA



2-DAY EXPOSURE

Figure 35. Autoradiogram of human peripheral blood monocytes mRNA hybridized with a cDNA probe for human IL-1 α . RNA was isolated from monocytes cultured either on a plastic surface or on top of a fibroblast-derived extracellular matrix (ECM). On both surfaces, monocytes were cultured in RPMI medium with 10% FCS with or without fibroblast-derived conditioned medium (FbCM) at a concentration of 50%. Calf rRNA was used as a negative control.

Figure 36. The TDR (Tissue Directed Response) concept. The hypothesis emphasizes the contribution of the cells that make up a particular tissue, fibroblasts (Fib), epithelial cells (EpC) and endothelial cells (EnC) in the regulation of the inflammatory response. As a result of activation by a single or repeated insults, these cells become activated and produce extracellular matrix (ECM) components as well as cytokines which then interact, thereby regulating, the inflammatory cells entering the tissue. This regulation involves differentiation, proliferation, activation and survival. Further activation of the tissue cells can be accomplished by the release of cytokines by the inflammatory cells. This bidirectional, and subserving, interaction between inflammatory and tissue cells may be central to the perpetuation (chronicity) of the inflammatory response.

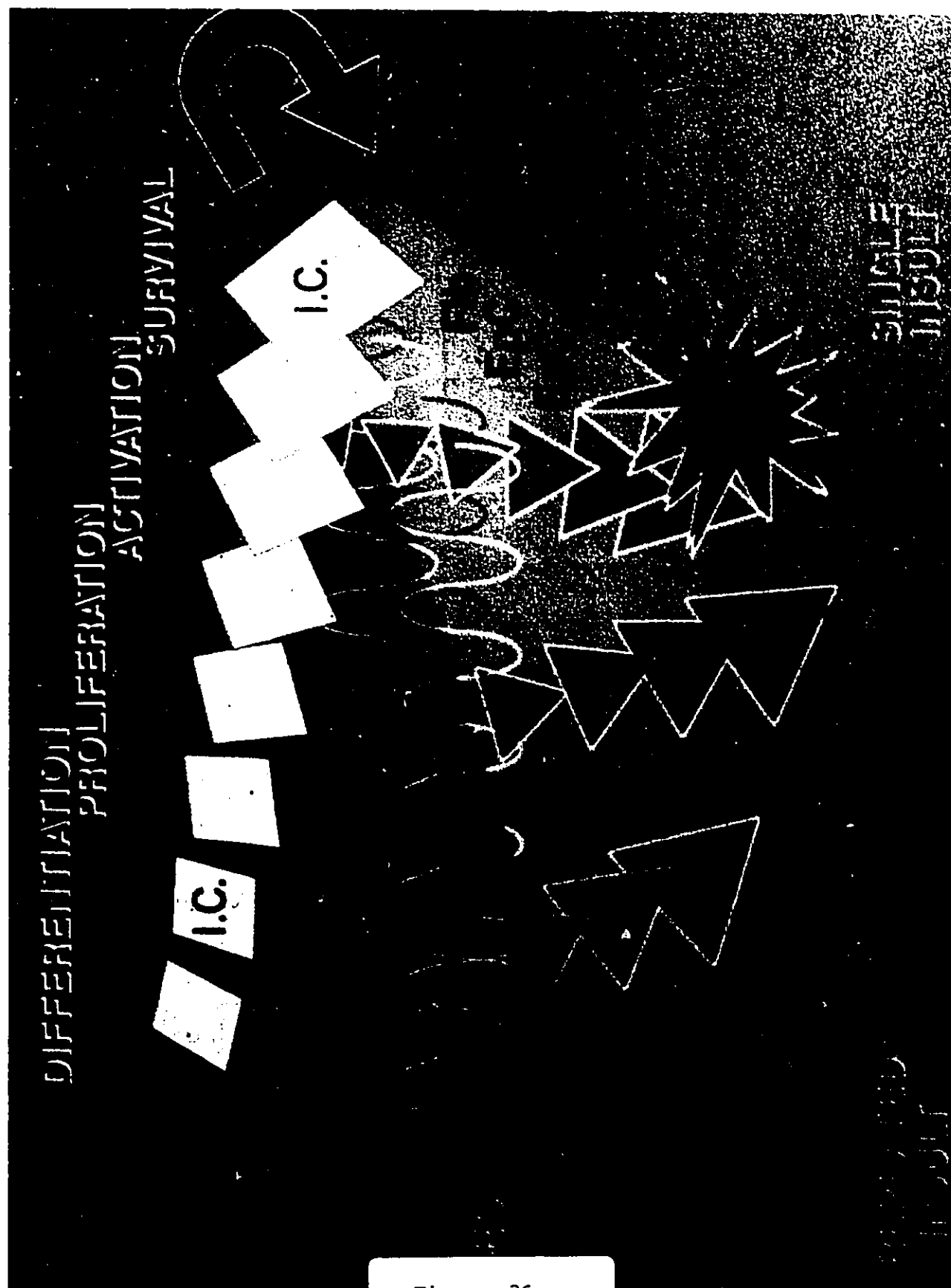


Figure 36

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