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Alveolar macrophages stimulate lung fibroblast proliferation in vitro

Senft, James Alan, Ph.D.
The Ohio State University, 1988
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ALVEOLAR MACROPHAGES STIMULATE LUNG FIBROBLAST PROLIFERATION IN VITRO

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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The Ohio State University 1988

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ALVEOLAR MACROPHAGES STIMULATE LUNG FIBROBLAST PROLIFERATION IN VITRO

INTRODUCTION

The chronic interstitial lung diseases are a heterogenous group of inflammatory disorders of the lower respiratory tract in which the normal alveolar walls are progressively thickened by a fibrotic process characterized by a distorted accumulation of fibroblasts and a collagenous extracellular matrix secreted by these cells (34, 36, 40, 145). Approximately 160 of these disorders have been identified, each of which is broadly classified as to whether the etiology is known or unknown. Of the more than 120 agents known to cause interstitial lung disease, each usually falls within one of a few general categories including: infectious organisms, drugs (e.g., antineoplastics), poisons (e.g., paraquat), radiation, or inhalation of inorganic dusts (e.g., silica, asbestos), noxious fumes, gases (e.g., oxygen, nitrogen dioxide), or organic antigens (often associated with occupational hazards, e.g., Farmer's lung, Mushroom worker's lung, Wood dust worker's lung) (40). In two-thirds of all cases, however, no cause can be identified (36). This latter group, commonly referred to as interstitial lung diseases of unknown cause, has a prevalence of 5 to 10 cases per 100,000 population and is responsible for more than 10,000 hospital admissions in the United States each year. It includes the two most widely studied chronic interstitial lung diseases, idiopathic pulmonary fibrosis (IPF) and sarcoidosis (36, 40). Because the interstitial disorders are associated with a restrictive ventilatory defect and reduced lung volumes that lead to
insufficient oxygen delivery to vital organs, many of these disorders are often fatal within three to six years after the onset of symptoms (34).

Fibrosis of the alveolar wall is such a common characteristic of all the chronic interstitial disorders that they are often referred to as the "fibrotic lung diseases" (40). The fibrotic changes in these diseases classically have been conceptualized as fibrous thickenings within the pulmonary interstitium (40, 106, 151), i.e., those portions of the alveolar walls bounded by the alveolar epithelial and endothelial basement membranes that contain, under normal conditions, a connective tissue matrix composed of collagen and elastin fibers, proteoglycans, other glycoproteins, and the mesenchymal cells that produce this matrix (128, 157). More recently, however, Basset et al. (7) have reported that 'intraluminal' fibrosis of alveoli, alveolar ducts, and terminal or respiratory bronchioles is also an important event in the pathogenesis of interstitial lung disease. The intraalveolar fibrosis results in obliteration of alveoli and coalescence of alveolar walls. In these cases, the fibrosis is thought to represent the migration or extrusion of interstitial contents through defects in the epithelial cell lining and its basement membrane. However, by altering both the architecture and mechanical properties of the lower respiratory tract, both interstitial and intraluminal fibrosis may cause alveolar-capillary dysfunction, such that ultimately there is ineffective transfer of oxygen from atmosphere to blood (7, 34, 36, 40). Since fibrosis in the interstitial lung diseases is generally an irreversible process, determining the mechanisms that underlie the fibrotic state is necessary both to understand the pathogenesis of these disorders and to develop appropriate therapeutic strategies.

Independent of the specific etiology, it is generally accepted that interstitial lung disease is a chronic inflammatory disorder. Because the inflammation involves primarily the alveolar walls and lumina, it is usually referred to as an 'alveolitis', although it is recognized that small pulmonary blood vessels and bronchioles also may be involved (52). The importance of the alveolitis in the pathogenesis of the disease was
not appreciated until it was realized that the alveolitis preceded most of the
derangements in the alveolar walls that characterize these disorders (34, 35, 36, 40, 70,
81, 88). For example, biopsies from early stages of idiopathic pulmonary fibrosis (IPF)
are characterized by intense inflammation with only mild derangements of the
alveolar wall; in contrast, the morphology of late IPF usually shows less inflammation
but marked derangements of the alveolar structures (34, 88). In addition, Keogh et al.
(89) have shown that IPF patients with prolonged high-intensity alveolitis usually had
deterioration in lung function, while those with prolonged low-intensity alveolitis
showed little functional deterioration. Furthermore, bronchoalveolar studies by
Bitterman et al. (11) demonstrated that children of patients with familial pulmonary
fibrosis (a disease similar to IPF except that it has a genetic basis) often had mild
alveolitis, even though these children were asymptomatic and had normal chest
roentgenograms and pulmonary function tests. Hence, the current consensus is that the
alveolitis develops very early in the disease process, and is then responsible for the
subsequent injury and fibrosis of the alveolar walls (34, 40, 88). Therefore, in order to
understand the pathogenesis of the interstitial lung disorders, it is important to
understand the character and function of the effector cells comprising the alveolitis.

Four basic types of alveolitis are present in the interstitial disorders:
macrophage dominant, lymphocyte dominant, neutrophil dominant, and eosinophil
dominant (34, 35, 36, 37, 70, 88, 142). Most commonly, however, two basic mixtures of
effector cells are found, e.g., a macrophage-neutrophil alveolitis is typical for
idiopathic pulmonary fibrosis, while a macrophage-T lymphocyte alveolitis is
common of sarcoidosis (81).

The consequences of the alveolitis depend on three features: the numbers of
effector cells present, the types of effector cells, and the state of activation of each type
of cell (34, 40). Although all interstitial disease is generally characterized by a marked
increase in the number of effector cells (35, 36, 70, 88, 136), an alveolitis composed of a
twofold increase in the number of macrophages will likely have fewer consequences
than that with a tenfold increase in the same cell type (40). Likewise, an alveolitis dominated by neutrophils (a cell with a potent array of inflammatory mediators) will have more of an impact than an alveolitis dominated by eosinophils (a much less potent inflammatory cell). However, aside from the number and type of inflammatory cells is the fact that the consequences of their presence depend on whether the cells have been activated (34, 40). For example, although alveolar macrophages are capable of releasing a variety of mediators that can damage the alveolar structures, they do so only when activated by exogenous stimuli (34).

Activation is an important phenomenon since different inflammatory cells have different arrays of mediators (40, see review 144). In turn, the specific mediator released may determine the nature of the injury to the alveolar wall. For example, in idiopathic pulmonary fibrosis, macrophages are increased in number by three to four times (34), and may be activated by immune complexes (77) to release neutrophil chemotactic factor (34, 78, 130). Neutrophils then are attracted to the lower respiratory tract where they are activated by neutrophil chemotactic factor (154), thereby stimulating the release of highly reactive oxygen radicals (e.g., superoxide anion, hydrogen peroxide, hydroxyl radical) that are cytotoxic to lung parenchymal cells (55, 111, 112, 155). Neutrophils also release proteases, including collagenase (56, 57) and elastase (54, 56, 95) which are capable of damaging alveolar-wall connective tissue matrix components. In addition, the action of neutrophil proteases can cleave fibronectin, thereby releasing some parenchymal cells, including fibroblasts, from their substrate (114). Neutrophil proteases also can inhibit protein and DNA synthesis in Type II pneumocytes, thereby interfering with the normal proliferative response of the alveolar epithelium to injury (4). Further aggravating the injury caused by neutrophils is the fact that activated alveolar macrophages also produce collagenase (76) as well as release oxidants capable of causing cytotoxic damage to lung parenchymal cells (59, 111). The net effect of the macrophage/neutrophil mediator-induced damage is the disruption of the normal histological relationships between the
parenchymal cells and extracellular matrix, thus impairing normal respiratory function and setting the stage for remodeling of the alveolar wall with abnormal collagen deposition. (The precise molecular mechanisms by which phagocytic cell-derived inflammatory mediators initiate lung injury and disease have been reviewed elsewhere (51).)

In contrast to the inflammatory scheme described for IPF, alternative mediators function in other interstitial diseases. For example, in sarcoidosis there also may be an increase in the number of alveolar macrophages, but rather than releasing neutrophil chemotactic factor, the cells release Interleukin-1 which is chemotactic for lung T lymphocytes (79). Interleukin-1 also initiates T cell activation and mediates an increase in T cell numbers. Release of Interleukin-2 by activated T lymphocytes can contribute to further expansion of lymphocyte numbers. In addition, T lymphocytes release monocyte chemotactic factor and macrophage migration inhibition factor (40). The consequence of this alveolitis, therefore, is granuloma formation in addition to fibrosis. Thus, not only is the damage to the alveolar walls in the interstitial disorders mediated by the alveolitis, but the specific derangement is determined by the specific types of effector cells (and mediators) comprising the alveolitis.

From studies of human interstitial disease and of experimental pulmonary fibrosis induced by agents such as bleomycin (30, 118, 147), asbestos (23, 75, 137), silica (25, 73), paraquat (124, 126), radiation (66, 143), and hyperoxia (31, 90), it has been determined that fibrosis is initiated after injury occurs to the existing cells and connective tissue matrix of the alveolar wall. Some of this injury may be induced directly by the causative agent (40), but the majority of investigators believe that most of the injury is due to the effector processes and mediators of the cells comprising the alveolitis, e.g., proteases and cytotoxic highly reactive oxygen metabolites secreted by neutrophils (55, 111). Typically, the type I epithelial cells are injured, and in many cases, replaced by proliferating type II cells and cuboidal epithelial cells that have
migrated down from terminal bronchioles (86). Endothelial cells are also particularly sensitive to injury and easily destroyed (113), resulting in a loss of pulmonary capillaries. Interstitial edema ensues, and the basement membranes become thickened. As the disease progresses, the alveolar interstitium expands and is characterized by an increased cellularity and an accumulation of mostly type I collagen (107, 140, 152). With further progression of the disease, over half of the lung tissue becomes interstitial intercapillary tissue (compared to ~16% in normal lung) with concomitant twofold reductions in airspaces, capillary spaces, and alveolar capillary tissues (53). In more severe fibrosis, dense bundles, composed almost exclusively of type I collagen, develop into a well organized matrix within the interstitium (152). Indeed, the histological identification of type I collagen by conventional connective tissue stains (e.g., Masson trichrome) is commonly used to diagnose interstitial fibrosis (69).

The accumulation of type I collagen in the alveolar wall may result from a shift in the proportion of parenchymal cells in the alveolar structures (34). In this regard, the most significant change is the marked increase in the numbers of fibroblasts within the alveolar wall. Qualitative studies in humans (22, 29, 35, 136), as well as morphometric studies in animals (32, 72, 153) indicate a several-fold increase in fibroblast number in these disorders. Since fibroblasts are the main producers of type I collagen in the lower respiratory tract (80 to 90% of the collagen produced by fibroblasts is type I) (21, 68, 69, 128), an increase in the proportion of fibroblasts likely leads to an increase in the relative amount of type I collagen in the interstitial matrix (8, 68, 140), a process that contributes to the loss of functional alveolar-capillary units. Thus, to understand the underlying mechanisms of fibrosis, it is necessary not only to define the role of the effector cells present in the alveolitis (as described above), but also to determine the mechanisms mediating the accumulation of fibroblasts within the alveolar wall, i.e., processes and/or signals that stimulate fibroblast proliferation. One link between the chronic inflammation and the expansion of fibroblast numbers in these disorders may be the alveolar macrophage.
The importance of the monocyte/macrophage in fibroblast infiltration and proliferation was first studied in wound repair by Leibovich and Ross (98). They studied skin wounds in guinea pigs made monocytopenic by systemic administration of hydrocortisone. Following local injection of antimacrophage serum around the wound site, there was a marked delay in fibroblast infiltration, a retarded rate of fibroblast proliferation, and poor healing of the wound in general. In contrast, injection of antineutrophil serum had no effect on fibroplasia, suggesting to them that macrophages played a role in fibroblast proliferation. Next, Leibovich and Ross examined culture medium (free of platelet-derived mitogens) conditioned by peritoneal macrophages and demonstrated that cultured macrophages secreted a factor that stimulated proliferation of cultured guinea pig wound fibroblasts (99). Subsequent investigations by others have described and confirmed the release of a similar factor (or factors) from cultured mouse (5, 110, 122), rat (132), and human (141) peritoneal macrophages, rabbit macrophages in wound chambers (65), mouse macrophage cell lines (48, 159), human monocytes (3, 41, 43, 85), and human monocytes stimulated to become macrophages (3, 60, 85, 104, 135). Moreover, human (10, 13, 116, 141), guinea pig (105), and rat (9, 66, 93, 100, 101) alveolar macrophages also have been shown to elaborate a similar macrophage-derived growth factor(s) (MDGF) following stimulation with a variety of agents, including silica (66, 106), asbestos (9, 100, 101), bleomycin (93), zymosan (9, 10, 13), lipopolysaccharide (13), and immune complexes (13). The factor secreted by macrophages is capable of stimulating proliferation of dermal (3, 85, 135), foreskin (41, 60, 141), and lung (9, 66, 93, 104, 106, 122) fibroblasts, fibroblast cell lines such as 3T3 (48, 60, 110, 141, 159), WI-38 (100, 101, 135), and HFL-1 cells (10, 13), smooth muscle cells (60, 65, 110), arterial endothelial cells (5, 110), osteoblast-like cells (132), and chondrocytes (132). Thus, it has been shown that factors derived from wound, peritoneal, and alveolar macrophages, monocytes, and monocyte-derived macrophages stimulate proliferation in a variety of cell types.
Potential problems arise when attempting to determine the physiological relevance of the previous studies to the proliferation of fibroblasts that occurs during the pathogenesis of pulmonary fibrosis. First, in many of these earlier investigations, an absence of tissue specificity existed between macrophages and the target cells, and often at least one of the cell types used was of non-lung origin (3, 60, 85, 99, 104, 122, 135, 141). Second, earlier studies often used macrophages and fibroblasts (or other target cells) that were xenogeneic, i.e., there was a lack of species specificity between the two cell types (5, 65, 104, 110, 122). For example, Leslie et al. (104) tested conditioned medium from human monocyte-derived macrophages on target rabbit lung fibroblasts, and Phan et al. (122) tested murine peritoneal macrophage supernates on rat lung fibroblasts. Third, several studies have tested the proliferative effect of MDGF on target cells (i.e., fibroblasts, smooth muscle cells, endothelial cells, etc...) that were actually cell lines (10, 13, 16, 43, 48, 60, 65, 100, 101, 116, 135, 141, 159). For example, Bitterman et al. (10, 13) assayed supernates of human alveolar macrophages on HFL-1 cells, while Estes et al. (48) tested conditioned medium of P388D1 cells (a macrophage-like line) on BALB/c 3T3 fibroblasts. The effect MDGF has on cell lines, however, may not represent its true effect on normal cells. Thus, several of these previous studies may not be relevant models to study the role of macrophages and MDGF in the pathogenesis of pulmonary fibrosis because of the use of: a) xenogeneic and tissue mismatched macrophages and fibroblasts, b) macrophages and/or fibroblasts from non-lung sources, and c) cell lines in assays to determine the proliferative effect of MDGF.

The interaction between macrophages and fibroblasts is particularly complex because it appears macrophages have the capacity to secrete a variety of factors that both stimulate and inhibit fibroblast proliferation. Human alveolar macrophages, for example, release both fibronectin and alveolar macrophage derived growth factor (AMDG), two mediators thought to be responsible for stimulating the accumulation of fibroblasts within the alveolar wall in some interstitial lung diseases (10, 13, 26, 34, 129). Alveolar macrophage fibronectin, a 440,000 dalton glycoprotein, is thought to
recruit fibroblasts to sites of inflammation via its chemotactic properties (58, 129), mediate the attachment of the newly arrived fibroblasts to the extracellular matrix (91), and stimulate the fibroblasts to enter the G1 phase of the cell cycle (12). AMDGF, a 16,000- to 18,000-dalton protein distinct from other described growth factors (including growth hormone, insulin, fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and interleukin-1 (IL-1)), stimulates fibronectin-primed fibroblasts to progress through G1, synthesize DNA and proliferate (12, 13). In addition, alveolar macrophages, and macrophages of other types, may stimulate mesenchymal cell proliferation by releasing additional factors such as PDGF or PDGF-like molecules (113, 116), FGF (5), and IL-1 (16, 137).

In contrast to their stimulatory role, macrophages also appear to be capable of suppressing fibroblast growth. Elias et al. (47) showed that supernatants from normal human alveolar macrophages inhibited proliferation of lung fibroblasts in their log-phase of growth, and that the inhibitory capacity of the supernatant was directly related to its capacity to stimulate fibroblast prostaglandin production. Other investigators (66, 84) have confirmed the inhibitory effect of alveolar macrophage supernatants on fibroblast proliferation. However, the mechanisms by which macrophages regulate fibroblast proliferation become even more complex when evaluating studies in which specific fibrogenic agents were used. For example, Gritter et al. (66) demonstrated that the phase of the cell cycle of the target fibroblasts was critical, since supernates from silica-treated alveolar macrophages stimulated slowly dividing fibroblasts to proliferate, but inhibited rapidly dividing cells. Lugano et al. (106) found that the duration of exposure to the fibrogenic agent also was important, since supernates from alveolar macrophages exposed to silica for a short time (2 days) in vivo inhibited proliferation of cultured fibroblasts, whereas long term exposure of macrophages to silica (42 and 180 days) stimulated fibroblast proliferation.
Thus, it is evident that some of the mechanisms controlling fibroblast proliferation in the lung may be a complex interaction between multiple stimulatory and inhibitory factors released by alveolar macrophages. More work in this area is clearly needed. Furthermore, it must be noted that many of these previous investigations determined the effects of macrophage stimulatory and inhibitory factors in assays using cell lines. For example, the activity of AMDGF (10, 13) and alveolar macrophage-derived fibronectin (12, 129), PDGF (113, 116, 141), IL-1 (16, 48), and inhibitory factors (47, 84) were determined using HFL-1 cells (10, 12, 13, 16, 113, 116, 129), 3T3 cells (48, 141), and other fibroblast cell lines (47, 84), cells which may be physiologically different from normal fibroblasts. Finally, it should be noted that although studies have shown microscopically that macrophages and fibroblasts are physically close to each other during wound repair (98) and in pulmonary fibrosis (7, 19), no studies have investigated the effect this direct physical interaction may have on proliferation.

Therefore, the major objective of my study was to develop an in vitro model, using normal cells of the same species, to investigate several aspects of the effect of alveolar macrophages on lung fibroblast proliferation. In the first phase of the investigations, the cells used in the in vitro model were characterized morphologically, histochemically and immunocytochemically, and their normal growth kinetics determined (Results - Part 1). In the second phase of the study, the model was validated by demonstrating the proliferative effect of macrophage factors on fibroblasts, effects similar to those reported in some other systems (Results - Part 2). In the third and final phase of the study, and unlike any study reported previously, use of the model was extended using co-cultures to test the effect of the direct physical interaction between alveolar macrophages and lung fibroblasts on proliferation (Results - Part 3).
Ancillary to the main objective, the influence of lymphocytes and neutrophils on fibroblast proliferation was also examined. More specifically, the aims of this study were:

1) to characterize and determine the purity of both primary cultures and subcultures of rat lung fibroblasts by morphological, histochemical, and immunocytochemical procedures;

2) to determine the cellular composition of rat bronchoalveolar lavage cells, and to characterize the macrophages found therein by their morphology, as well as by histochemical and phagocytic tests;

3) to determine the normal growth kinetics of both asynchronous and synchronous populations of lung fibroblasts, so that these cells could be used as a bioassay to detect macrophage-derived fibroblast proliferation factors;

4) to determine whether alveolar macrophages could be activated by various agents (including opsonized zymosan, lipopolysaccharide, immune complexes, and surface adherence) to release a factor(s) into their culture medium that had the capacity to stimulate quiescent lung fibroblasts to a) re-enter the cell cycle and synthesize DNA and b) replicate;

5) to determine the effect of alveolar macrophage supernates on the log-phase growth of lung fibroblasts;

6) to determine at what point in the fibroblast cell cycle the macrophage-derived factor exerts its effect, according to the competence/progression theory proposed by Stiles et al. (151).
7) to determine if macrophages could stimulate proliferation when co-cultivated with fibroblasts, and if so, to determine which cell types proliferated and if macrophage activation by exogenous agents was required;

8) to determine a) the effect of cell contact between alveolar macrophages and lung fibroblasts on fibroblast proliferation, and b) whether cell contact or macrophage supernatants were more effective in stimulating fibroblast proliferation;

9) to determine whether pharmacologic agents could inhibit proliferation in macrophage/fibroblast co-cultures; and

10) to determine the capacity of lymphocytes and neutrophils, alone or in combination with macrophages, to stimulate proliferation when co-cultivated with lung fibroblasts.

For the sake of clarity, the macrophage factor described in the present study has been termed Alveolar Macrophag-derived Fibroblast Proliferation Factor (AMFPF). It may represent one or more factors, and may be the same as or different from the macrophage-derived growth factor(s) (MDGF) described by other investigators.
MATERIALS AND METHODS

ANIMALS

A colony of Sprague-Dawley rats was maintained in The Ohio State University College of Medicine Vivarium, an AAA-LAC accredited facility. All animals were kept in a climate controlled environment and had access to food and water ad libitum. SD rats used for bronchoalveolar lavage or cell culture preparation were obtained from random breedings. The conduct of all studies adhered to the NIH "Guide for the Care and Use of Laboratory Animals".

CULTURE MEDIUM

For initial cell plating, Dulbecco's modified Eagle's medium (DMEM; KC Biological, Lenexa, Ka) was supplemented with l-glutamine (2mM), gentamicin (.05mg/ml; Gibco, Grand Island, NY), NaHCO3 (184mg/100ml), and 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). In assays of alveolar macrophage-derived fibroblast proliferation factor(s) (AMFPF), macrophage and fibroblast cultures were grown in complete DMEM as described above, except either 0.4%, 0.8%, or 1% FBS (noncycling medium) was used instead of 10% FBS. Serum-free DMEM supplemented with 0.05% bovine serum albumin (BSA) was used in a few experiments.
LUNG FIBROBLAST CELL CULTURES:

Primary Fibroblast Monolayers

Pulmonary fibroblast primary cell cultures were established using a modification of the method by Bradley et al. (21). Two to three day old Sprague-Dawley rats were sacrificed by decapitation, and the intact lungs removed aseptically and trimmed of bronchi and large blood vessels. Lungs were rinsed and finely minced in Ca\(^{2+}/Mg^{2+}\) free Dulbecco's phosphate buffered saline (CMF-DPBS) supplemented with gentamicin (1 ul/ml). Pieces were further rinsed with 0.25% trypsin in CMF-DPBS and transferred to a trypsinizing flask. Tissue fragments were incubated at 37°C in 10 ml fresh 0.25% trypsin solution containing 2 mg DNase (Aldrich Chemical Co.), and agitated using a magnetic stirrer. After 20-30 min, fragments were allowed to settle and the supernatant removed and diluted with an equal volume of culture medium plus 10% FBS. Residual tissue fragments were trypsinized twice more and each supernatant fraction diluted and saved. The pooled cell suspension was filtered through sterile fine gauge Nitex nylon mesh and spun at 200 x g. The cell pellet was resuspended in DMEM plus 10% FBS and the cells counted. Primary cultures were seeded with 2 x 10^6 cells/35 mm culture dish and incubated at 37°C in humidified air gassed with 5% CO\(_2\). Sixteen hours after plating, cultures were washed 3 times with DPBS plus 0.1% BSA to remove nonadherent and nonviable cells, and fed with 2 mls fresh DMEM plus 10% FBS. The medium was changed every 3 days.
Fibroblast Subcultures

Primary cultures were subcultured when confluent, approximately 5-7 days after initial cell plating. Fibroblast monolayers were washed three times with CMF-DPBS, rinsed once with 0.25% trypsin, and then incubated in 1 ml of 0.25% trypsin for 20 min at 37°C. DNase (10 µg/ml) and DPBS (100 µl/ml) were then added to the culture vessels with agitation in order to disperse clumped cells into a single cell suspension. Resulting cell suspensions were pooled and diluted with an equal volume of DMEM + 10% FBS (to inactivate the trypsin) and spun at 200 x g. After resuspension in fresh medium, the cells were counted and secondary cultures established by seeding 1.25 x 10^6 cells/25 cm² Falcon plastic tissue culture flask (1:3 split ratio). Secondary cultures were subcultured every 5-7 days. By the third passage, fibroblast cultures were substantially pure and characterized by the criteria described below. Third to fourth passage cells were used in all assays of macrophage-derived factors.

Noncycling Fibroblast Cultures

Noncycling (quiescent) lung fibroblasts were grown by a modification of Bitterman's (13) method for HFL-1 cells, and were used in proliferation assays to test the effects of macrophage-derived stimulating factors. Second or third passage fibroblasts were subcultured into either: a) Corning 96-well tissue culture plates at 2 x 10^4 cells/well; b) Miles Lab-Tek 8 chamber slides at 4.5 x 10^4 cells/chamber; or c) 12mm Millicell HA tissue culture inserts (Millipore, Bedford, MA) at 7.03 x 10^4 cells/insert. Cells were maintained in DMEM + 10% FBS for 24 hrs, during which time attachment took place. For cell-cycle arrest, cells were rinsed twice with DPBS + 0.1% BSA, fed with noncycling medium (DMEM plus 0.4%, 0.8%, or 1% FBS) and then
returned to the incubator. After 4 days, fibroblasts became quiescent, or noncycling.
(In this study, the terms quiescence and noncycling were used synonymously, and
defined a population of cells in which less than 10% of the cells synthesized DNA as
determined by tritiated thymidine autoradiography.)

Characterization of Fibroblast Cultures:

Cultures of lung fibroblasts were tested by a variety of morphological methods to
characterize the cell types growing in the cultures. Live monolayers were examined
routinely by phase contrast microscopy for spindle-shaped cells in parallel arrays that
are characteristic of cultured lung fibroblasts (21). Fixed monolayers were examined
periodically by electron microscopy to confirm the presence of fibroblast
ultrastructure. In addition, histochemical, phagocytic, and immunocytochemical tests
were performed as described below. For immunocytochemical tests, third passage cells
were plated on glass coverslips at a density of $4.5 \times 10^5$ cells/35 mm dish and grown
1 day in DMEM + 10% FBS before staining.

Enzyme Histochemistry and Phagocytic Tests

Standard histochemical tests for acid phosphatase and nonspecific esterase (6)
were used to screen for macrophage contamination of fibroblast monolayers.
Phagocytic tests were also performed using 2 μm latex beads (Sigma Chem. Co.) added to
the monolayers for 1 hr at 37°C. Since fibroblasts do not actively ingest 2 μm beads in
so short a time, cells that contained latex beads and were acid phosphatase or esterase
positive were considered macrophages. Any cultures containing significant numbers of
macrophages were not used in assays of AMFPF.
Vimentin Immunocytochemistry

Cell cultures were immunostained for vimentin intermediate filaments to determine the proportion of mesenchymally-derived cells present. After rinsing with PBS, cells grown on coverslips were fixed for 15 min with 1.5% paraformaldehyde in PBS, rinsed again, and permeabilized for 10 min with 0.1% saponin in PBS plus 0.1% BSA. Cells were then treated with 5% normal sheep serum followed by incubation with rabbit anti-chicken vimentin (1:50; a gift from Bruce Granger, Cal Tech, Pasadena, CA) for 30 min. Cells were rinsed in PBS + 0.1% PBS and stained with sheep anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC, 1:50). Finally, cells were rinsed in DPBS + 0.1% BSA, counterstained with 4 µg/ml propidium iodide (Sigma) (9), and mounted in PBS:glycerol (1:1) or in Fluoromount (Southern Biotechnology Associates, Inc., Birmingham, AL).

Thy 1.1 Immunocytochemistry

Rat embryo and brain fibroblasts (125, 150) and adult mouse lung fibroblasts (150) express a surface antigen that reacts with a rat monoclonal antibody (OX-7) against murine Thy 1.1 antigen. Therefore, OX-7 antibody was used to identify lung fibroblasts in cultures as a means of monitoring the purity of the monolayers. Cells were fixed with 1.5% paraformaldehyde for 15 min, rinsed, treated with 5% normal sheep serum, and then incubated 30 min with mouse monoclonal anti-Thy 1.1 (1:10; Sera Lab, clone OX-7). After rinsing, cells were incubated an additional 30 min with the secondary antibody, FITC-conjugated sheep anti-mouse IgG (1:50), rinsed, and mounted in Fluoromount.
NBD Phallacidin Fluorescence

Nitrobenzoxadiazole (NBD) phallacidin (Molecular Probes, Inc., Junction City, OR) was used to stain F-actin in fibroblast cultures. Cells on coverslips were fixed with 1.5% paraformaldehyde, rinsed in PBS, permeabilized with 0.3% Triton for 15 min, and incubated in NBD phallacidin (10 units/ml) for 30 min. After rinsing, cells were mounted in Fluoromount.

Fibronectin Immunohistochemistry

To detect extracellular fibronectin within the fibroblast monolayer, cells were fixed in 1.5% paraformaldehyde for 15 min, rinsed in PBS, treated with 5% normal sheep serum for 15 min, and then incubated in rabbit anti-human fibronectin (1:100; Cappel Labs., Malvern, PA). After 30 min, cells were rinsed and incubated in FITC-conjugated sheep anti-rabbit IgG (1:100), and subsequently mounted in PBS:glycerol.

MACROPHAGE CELL CULTURE TECHNIQUES:

Bronchoalveolar Lavage

Pulmonary alveolar macrophages (PAM) were obtained by bronchoalveolar lavage (BAL). Four to five week or 10 to 12 week old SD rats were anesthetized intraperitoneally with either 2,2,2-tribromoethanol (238 mg/kg) or Nembutal (167 mg/kg; Abbott Laboratories, Chicago, IL) and then exsanguinated via the abdominal aorta. The chest cavity was opened and a canula was inserted into the trachea and
secured with a nylon ligature. Using a 10 ml plastic syringe, the lungs were lavaged with a total volume of 100 mls of warm (37°C) CMF-DPBS in 10 ml aliquots, 90% of which was recovered. The BAL cells were spun at 200 x g for 10 min at 4°C, washed in PBS, and finally resuspended in DMEM. Yields of 6-7 x 10^6 cells/animal were usually more than 90% viable by trypan blue exclusion, and were often >90% PAM, depending on the particular cell preparation.

Alveolar Macrophage Cultures

Alveolar macrophages obtained by bronchoalveolar lavage were suspended in DMEM + 10% FBS or in noncycling medium at 1 x 10^6 cells/ml and plated on glass coverslips in 35 mm culture dishes. The cells were incubated for 2 hrs at 37°C to allow the macrophages to adhere, and then washed with DPBS + 0.1% BSA to remove nonviable and nonadherent cells. These cultures were used either a) to characterize alveolar macrophages (as described below), or b) as one means of stimulating alveolar macrophages to release fibroblast proliferation factor(s) (also described below).

Characterization of Macrophages

Cytocentrifuge smears of 2.5 x 10^5 lavage cells were prepared and stained with Diff-Quick (American Scientific Products, McGraw Park, IL); differential cell counts were performed on 500 cells/slide. Additional samples of lavage cells were also examined by electron microscopy.

Macrophage cultures (free of non-adherent cells) were examined by phase microscopy and tested histochemically for nonspecific esterase and acid phosphatase
activity (6), as well as for phagocytic ability. Twenty-five microliters of a monodisperse suspension of 2 μm latex beads were added to macrophage cultures for 1 hr at 37°C. The cultures were washed with DPBS + 0.1% BSA, fixed, and stained with Diff-Quick or processed for enzyme histochemistry (esterase and acid phosphatase). The percentage of phagocytic cells was determined and correlated with cellular staining characteristics and enzyme activity.

Macrophage Enrichment Using Percoll

BAL cells that contained less than 85% macrophages and greater than 15% lymphocytes and/or neutrophils were fractionated using discontinuous gradients of iso-osmotic Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). This procedure produced enriched populations of alveolar macrophages that were usually 90-95+% pure. Stock iso-osmotic Percoll (1.129 g/ml) was diluted with 0.15 M NaCl to densities of 1.03, 1.065, 1.07, 1.09, and 1.12 g/ml (28). Gradients were built by starting with the heaviest layer and carefully adding successive lighter layers on top. The cell suspension in DMEM (1 ml volume) was carefully layered on top of the gradient, and then spun at 400 x g for 20 min. Macrophages banded at the 1.065/1.07 interface, lymphocytes at the 1.07/1.09 interface, and neutrophils at the 1.09/1.12 interface. Each cell population was washed 3 times before use in an experiment.

ALVEOLAR MACROPHAGE-LUNG FIBROBLAST CO-CULTURES

Third passage lung fibroblasts were grown in either 96 well tissue culture plates (2 x 10^4 cells/well), 8 chamber Lab Tek slides (4.5 x 10^4 cells/chamber), or in Millicell HA tissue culture inserts (Millipore) in noncycling medium until cells were quiescent.
After 4 days of culture, noncycling medium was removed and either fresh BAL-derived alveolar macrophages, or macrophages stimulated by IgG immune complexes were added directly to the fibroblast monolayer in fresh noncycling medium. Macrophages were added at either 1, 2, 4, or 8 x 10^4 cells/microtiter well or at 1 or 2 x 10^5 cells/Lab Tek chamber. Proliferation in co-cultures was assessed at several time points by a) incorporation of [^3]H-TdR (24hr pulse) into acid precipitable counts, and b) [^3]H-TdR autoradiography (as described below). Autoradiograms of co-cultures provided the advantage of being able to determine separate labeling indexes for macrophages and fibroblasts on any given day of culture.

Drug Pretreatment of Macrophages Used in Co-cultures

Macrophages were treated with protein synthesis inhibitors to test whether such inhibition affected fibroblast proliferation in co-cultures. BAL-derived alveolar macrophages were suspended in noncycling medium (10^6/ml) and incubated at 37°C with either puromycin (30 μg/ml; Sigma) or cycloheximide (10 μg/ml; Gibco) in 5 ml polypropylene tubes for 1 hr prior to co-cultivation. Cells were then washed 3 times in DMEM, seeded at 4 x 10^4 macrophages/well onto noncycling fibroblasts, and proliferation measured after 48 hrs of co-cultivation by [^3]H-TdR uptake.

Similarly, macrophage cytoskeletal elements were disrupted to test this effect on fibroblast proliferation in co-cultures. BAL-derived macrophages in noncycling medium (10^6/ml) were pretreated with Cytochalasin B (30 μg/ml; Sigma) for 1 hr at 37°C in polypropylene tubes. Macrophages were then washed 3 times in DMEM, seeded at 4 x 10^4/well onto noncycling fibroblasts, and co-cultivated for 48 hrs. Proliferation was assessed by [^3]H-TdR incorporation.
Paraformaldehyde (PFA) Fixation of Macrophages

Fixed alveolar macrophages were co-cultivated with fibroblasts to test whether a physical stimulus alone (i.e., the fixed cells) was sufficient to induce DNA synthesis in fibroblasts. Macrophages were fixed 5 min in fresh 1% PFA (diluted in 0.85% saline), then rinsed an additional 5 min in excess glycine buffer to stop the action of the PFA. Cells were spun, washed extensively in DMEM (minimum of 3 times), recounted, and plated at 4 or 8 x 10^4 macrophages/co-culture. In most cases, DNA synthesis was assessed after 48 hrs of co-cultivation.

OTHER CELL CULTURES USED:

Alveolar macrophage-derived fibroblast proliferation factor(s) (AMFPF) was tested on the following "non-lung" cell types.

Dermal Fibroblasts

Primary cell cultures of dermal fibroblasts were established from the dermис of 2-3 day old SD rats. Animals were sacrificed by decapitation, and the skin from the abdominal and thoracic regions removed and rinsed in cold CMF-DPBS. To obtain dermal cells, skins from 3 to 4 animals were stretched dermal side down onto sterile Whatman #1 filter paper in a 100 mm petri dish. Ten mls of 0.25% trypsin was then added to the bottom of the dish, causing the skins to float and exposing the dermal-epidermal junction to enzymatic digestion during a 6-8 hr incubation at 37°C, or an 18-24 hr incubation at 4°C. After rinsing, the dermis was easily stripped from the epidermis, and gently scraped to remove basal cells adherent to the basal lamina. The dermis was finely minced, transferred to a trypsinizing flask, and incubated 1.5 hrs at
37°C on a magnetic stirrer in 10 mls fresh 0.25% trypsin containing 2 mg DNAse. The resulting cells were washed, resuspended in DMEM + 10% FBS, and seeded at 2 x 10^6 cells/35 mm culture dish. Dermal fibroblasts were subcultured 3 times before use in an experiment.

Meningial Fibroblasts and Cerebral Astrocytes

Meningial fibroblast and cerebral astrocyte cell cultures established from SD rats were the generous gift of Dr. Douglas Kniss, and were used in experiments after the third subculture. The astrocytes were prepared using a method by Lasher (97). In some experiments, fibroblast growth factor (FGF, 5 ng/ml; a gift from Dr. Denis Gospodarowicz, Univ. of California - San Francisco, Cancer Research Institute) was used as an internal control, since it stimulates proliferation by astrocytes (personal communication from D. Kniss).

3T6 Cells

This fibroblast-like cell line was kindly provided by Dr. Robert DePhilip. FGF (5 ng/ml) and epidermal growth factor (EGF, 10 ng/ml; a gift from Dr. Stanley Cohen, Vanderbilt University) were used as controls in some experiments.

Thymic Epithelial Cells

Murine thymic epithelial cells were kindly provided by Dr. Kenneth Jones, and were used in experiments on the third passage.
Smooth Muscle Cells

Guinea pig aortic smooth muscle cells were kindly provided by Dr. Hang Fang Xang and used in experiments after 4 passages.

BC3H1 Cells

This myoblast-like cell line (passage 22) was kindly provided by Dr. Arthur Strauch.

ISOLATION OF PERIPHERAL BLOOD LYMPHOCYTES

Peripheral blood was collected from the aorta of anesthetized 8 to 10 wk old SD rats in a heparinized (10 units/ml) syringe. The blood was spun at 900 x g for 20 min, and the buffy coat collected and washed in osmotically adjusted saline (ACK buffer) so as to lyse the red cells. The leukocyte suspension was then placed on a discontinuous Percoll gradient and spun at 400 x g for 20 min. Peripheral blood lymphocytes (95-98+% enriched) were collected from the 1.07/1.09 density interface and washed three times in DPBS before use in experiments.

ELECTRON MICROSCOPY

Lung Fibroblasts

Third passage fibroblasts were grown 3 to 5 days in Corning 35 mm plastic culture dishes, rinsed in DPBS, and fixed in 0.05 M phosphate buffer containing
1.6% glutaraldehyde and 0.05 M sucrose for 30 min at room temperature. Cultures were rinsed in phosphate buffer, post-fixed in 1% osmium tetroxide (OsO₄) for 30 min, thoroughly rinsed again, dehydrated in ethanol, cleared in hydroxypropyl methacrylate and embedded in Epon 812. Once the Epon polymerized (60°C, 2 days), tissue culture dishes were peeled away and the hardened resin examined using phase contrast microscopy to find areas of cells to be sectioned. Selected areas were marked by an "ink objective", punched out, mounted on a resin block, and the sections cut on an ultramicrotome (Sorvall Porter-Blum, MT2-B). Sections were stained with uranyl acetate and lead citrate and examined with a Philips 300 transmission electron microscope.

Alveolar Macrophages

BAL macrophages were prepared as a cell suspension for electron microscopy using a modification of a method by Ackerman and Wolken (1). Cell suspensions were rinsed in 0.1 M phosphate buffer (pH 7.4 with 4% polyvinylpyrolodone), fixed with 2.5% glutaraldehyde in a 1.5 ml microfuge tube (Bio Rad), rinsed to remove fixative, and transferred to 400 μl microfuge tubes. After spinning 4 min at 750 x g, the cell pellet was sliced in half, post-fixed in OsO₄, dehydrated in acetone, cleared in propylene oxide, and embedded in Spurs resin. Sections were stained with uranyl acetate and lead citrate and examined on a Philips 300 electron microscope.
STIMULATION OF ALVEOLAR MACROPHAGES TO RELEASE FIBROBLAST PROLIFERATION FACTORS (AMFPF)

Alveolar macrophages were stimulated to release AMFPF by four different methods: a) surface attachment to plastic culture dishes or to glass coverslips; b) *Escherichia coli* lipopolysaccharide (LPS; 20 µg/ml; strain 0111:B4; Sigma Chemical Co., St. Louis, MO); c) zymosan A (Sigma) that was opsonized by incubating boiled, washed, and sonicated particles in 3-4 mls of fresh rat serum (37°C, 1 hr), followed by 5 washes in DMEM before use; and d) IgG immune complexes, prepared by incubating a 20% solution of sheep erythrocytes with an equal volume of anti-sheep red blood cell (SRBC) IgG (1:100 titer; Gibco) for 30 min at 37°C, and washing in DPBS to remove unbound serum. Immune complexes were added at a final concentration of 1% (vol/vol) to the macrophage incubation mixture; opsonized zymosan was used at 150 µg/ml.

To determine the ability of various agents to stimulate alveolar macrophages to elaborate AMFPF, 10^6 macrophages/ml were: a) incubated (37°C) in noncycling medium in suspension culture with zymosan, LPS, or IgG immune complexes as described above; or b) allowed to attach to a tissue culture dish and incubated with and without the above stimuli. After macrophages were incubated 24 hr, supernates were harvested, spun at 500 x g, filtered through 0.22 µm filters, and either assayed for fibroblast proliferative activity (described below), or stored at -70°C for later use.

To clarify the role of the small amount of serum (0.4% or 0.8%) present in noncycling medium used for macrophage incubations with various stimulating agents, a few experiments as described above were carried out in serum-free DMEM supplemented with 0.05% BSA. After 24 hrs of incubation, the macrophage-conditioned medium was processed as previously described.
Macrophages used in co-culture experiments were stimulated to produce fibroblast growth promoting factor(s) by incubating 10^6 macrophages/ml with IgG immune complexes in suspension culture (5 ml polypropylene tubes) at 37°C. Immune complexes were added at a final concentration of 1% (vol/vol) to the macrophage incubation mixture. After 1 to 3 hrs incubation in noncycling medium, macrophages were spun at 500 x g, resuspended in ACK buffer to lyse non-injested erythrocytes, washed 3 times in DPBS + 0.1% BSA, recounted, and seeded at the appropriate concentration.

ASSAY OF FIBROBLAST PROLIFERATION FACTOR(S):

Three techniques were used to determine whether alveolar macrophage supernatants, or macrophages themselves, could stimulate quiescent lung fibroblasts to proliferate. These techniques tested for both an increase in cell number and synthesis of DNA. For each method used, cells grown in noncycling medium served as a baseline control, while cells grown in 10% FBS served as a maximum control.

Cell Counts

To determine whether there was an increase in cell number over control values, fibroblasts treated with macrophage supernatants were detached from culture dishes by incubation with 0.25% trypsin for 20 min at 37°C; at the end of the incubation, trypsin was inactivated in an equal volume of DPBS + 0.1% BSA. The cells were suspended to a final volume of 8 ml in Isoton, and then counted in a Coulter Counter (Coulter Electronics Inc., Hialeah, FL) with the lower threshold set at 14.
DNA Synthesis Assay

Cellular proliferative activity was assessed by the ability of cells to enter the S phase of the cell cycle and incorporate tritiated thymidine ([³H]Tdr) into DNA. To determine the effect of macrophage-conditioned medium on fibroblast DNA synthesis, macrophage supernates were diluted with noncycling medium and placed on noncycling fibroblasts (2 x 10⁴/well) in 96 well plates. In co-culture experiments, macrophages were added directly to the fibroblasts. At specific time points after the addition of macrophages or macrophage supernate, cells in triplicate cultures were radiolabeled with 1 µCi/well (5 µCi/ml) of methyl-[³H]TdR (Sp. Act. 5 Ci/mmol, Amersham, Arlington Hts., IL) for 24 hrs prior to cell harvest. After the 24 hr pulse, labeled medium was removed and the cultures were incubated (37°C) with 0.25% trypsin for 20 min. Cells were collected on Whatman glass fiber filter paper with a Brandell Cell Harvester (model M24V), treated with trichloroacetic acid, and rinsed with 0.85% saline followed by methanol. Filter discs were air dried, transferred to glass minivials, and treated for 10 min with Scintigest tissue solubilizer (Fisher Scientific Co., Fairlawn, NJ). Four milliliters of acidified Scintiverse E scintillation cocktail (Fisher) was added to the vials, and incorporation of [³H]Tdr into acid precipitable counts was measured using a Beckman LS7800 liquid scintillation spectrophotometer with an average counting efficiency of 62%. Results were expressed as mean counts per minute (cpm) ± standard deviation (sd) for triplicate cultures. Data also were expressed as a) % of maximum control (cpm of fibroblasts incubated with macrophages or macrophage supernatant + cpm of fibroblasts incubated in 10% FBS), and b) the stimulation index (cpm of fibroblasts incubated with macrophages or macrophage supernatant + cpm of fibroblasts incubated in low serum medium).
[\textsuperscript{3}H]-Thymidine Autoradiography

A second method used to assess DNA synthesis was [\textsuperscript{3}H]Tdr autoradiography. Noncycling fibroblasts grown on 12 mm glass coverslips (1.24 \times 10^5 cells/16 mm well) or 8 chamber Lab Tek slides (4.5 \times 10^5 cells/chamber) were either a) incubated with macrophage supernatants or b) co-cultured with alveolar macrophages. Cultures were radiolabeled with [\textsuperscript{3}H]Tdr (2 \mu Cl/ml; Sp. Act. 5 Cl/mmol) for 18 to 24 hrs prior to termination of the experiment. The medium was then removed and cells were fixed in acid alcohol (acetic acid:methanol; 1:3), air dried, and processed for autoradiography (149). Coverslips or Lab Tek slides were dipped in Kodak NTB-2 autoradiography emulsion and stored in the dark at 4°C for 6 to 7 days. Autoradiograms were developed with Kodak D-19, fixed with Kodak rapid fixer, washed in tap water, stained with Glemsa (1:10) and dehydrated in acetone.

The labeling index of autoradiograms was quantified by counting at least 500 cells per culture using a light microscope and 40x objective.

COMPLEMENTATION ASSAY OF MACROPHAGE SUPERNATANTS

Stiles et al. (151) have described a complementation assay that allows classification of a growth factor as either a "competence" factor (e.g., fibroblast growth factor, platelet derived growth factor, fibronectin) or a "progression" factor (e.g., insulin, somatomedin C, epidermal growth factor) according to its point of action within the cell cycle. They have proposed that nonreplicating fibroblasts require the presence of both a competence factor and progression factor to initiate DNA synthesis and divide. Therefore, to evaluate the factor(s) in alveolar macrophage supernatants, a complementation test was conducted in which alveolar macrophage supernatants were
paired with either a characterized competence factor or a characterized progression factor, and the effect on DNA synthesis assessed. Serum-free conditions were used in some of these assays, so that the effect of the growth factors could be directly evaluated without interaction with undetermined serum constituents.

Third passage lung fibroblasts were cultured at $2 \times 10^4$ cells/microtiter well in noncycling medium (0.4% or 0.8% FBS) for 4 days. On the 5th day, fibroblast cultures were washed two times with DPBS + 0.05% BSA. Serum-free macrophage supernatants diluted in serum-free medium (DMEM + 0.05% BSA) were then added to the fibroblast cultures with either a competence factor (fibroblast growth factor, 5 or 10 ng/ml. Collaborative Research; or fibronectin, 10 µg/ml, Collaborative Research) or with a progression factor (insulin, 10 µg/ml, Sigma Chemical Co.). As negative controls, each factor or macrophage supernatant was used alone to determine its effect on fibroblast DNA synthesis. As a positive control, insulin was added to the fibroblast cultures with either fibroblast growth factor or fibronectin. In some experiments, the assay also was performed under low serum conditions (0.4% or 0.8% FBS). After adding growth factors and macrophage supernatant, fibroblasts were incubated at 37°C, pulsed after one day of culture with $[^3]$H]TdR, and harvested 24 hrs after radiolabelling. DNA synthesis was measured by the amount of $[^3]$H]TdR incorporated into acid precipitable counts.

**DATA ANALYSIS**

All data from experiments using scintillation spectroscopy or direct cell counts (Coulter Counter) are reported as either a) mean ± sd from at least triplicate samples, b) % of maximum control, or c) the stimulation index. In most cases, labeling indices are reported as the mean ± sd. However, the labeling indices of co-cultivated macrophages
and fibroblasts (Figure 17, Part 3 of Results) represent mean values ± sem from 3 separate experiments.

Statistical evaluation of data was performed by analysis of variance (ANOVA) or Student's 2-sample t test (two tailed). Probability values of <0.05 were considered significant. All statistical tests were conducted using the Statworks program by Cricket Software.
RESULTS
PART I

Characteristics of Lung Fibroblast Cell Cultures

Primary Fibroblast Monolayers

Pulmonary fibroblast primary cell cultures consisted of a heterogenous population of cells. Phase contrast microscopy of lung cells obtained from 2-day old SD rats and grown 2 days in vitro revealed cells of several types: a) round clusters of polygonal, epithelial-like cells arranged in cobblestone patterns, b) many stellate-shaped cells with elongated cytoplasmic processes that extended in various directions, c) spindle-shaped, fibroblast-like cells, d) small, spherical, refractile cells that did not spread out on the culture dish, and e) a number of macrophage-like cells with ruffled borders (Plate I). These latter cells were confirmed to be macrophages by enzyme histochemical tests performed in parallel with phagocytic tests (Plate I). Almost 11% of cells in 2-day primary cultures stained intensely for nonspecific esterase, 2.5% were acid phosphatase positive, and 13% had ingested 2 μm latex beads after a 1 hr incubation (Table 1).

After 5 to 7 days in vitro, the primary cultures became confluent, and nonadherent, spherical, refractile cells began to accumulate above the surface of the monolayer (Plate I). The numbers of these cells increased over the next few days. Since it was possible that these nonadherent cells contributed to the heterogenous nature of
primary cultures, an attempt was made to determine the nature of these cells. Primary fibroblast cultures were grown 1 week, and then rinsed repeatedly to collect the nonadherent, refractile cells. Nonadherent cells were subcultured an additional 7 days, during which time the cells attached. The morphology of these cells appeared similar to that of BAL-derived pulmonary macrophages grown in culture. Moreover, it was determined that these cells indeed were probably of the monocyte/macrophage lineage, since over 99% stained for nonspecific esterase and >92% were acid phosphatase positive and had ingested latex beads (Table 2). Thus, it was possible that the cells on the surface of primary monolayers were immature macrophages/monocytes unable to attach, or possibly they were macrophages that were simply crowded off the surface of the culture dish. Whatever mechanism involved, the nonadherent cells contributed to the cellular heterogeneity in primary fibroblast cultures.

Fibroblast Subcultures

Lung fibroblasts cultured in DMEM + 10% FBS grew vigorously through at least 4 subculturings. Soon after subculturing, fibroblasts appeared polygonal or stellate-shaped, with elongated cytoplasmic processes. As the cells achieved confluence, they became spindle-shaped and assumed the characteristic parallel arrangement of fibroblasts in tissue culture; almost all cells in 2nd and 3rd passage cultures attained this morphology. Cells grown more than 6-7 days in subculture appeared to produce an extracellular matrix and formed multiple layers 2 to 3 cells deep (Plate I).

Since the goal of initial studies was to examine the effect of a macrophage-derived factor on fibroblast proliferation, it was extremely important to determine that the target fibroblast monolayer was relatively macrophage-free. Following the first and second subculturings, it appeared that many macrophages were left behind in the primary and secondary culture vessels. In addition, histochemical and phagocytic tests
indicated that as fibroblasts were subcultured, the proportion of contaminating macrophages decreased. While both primary and secondary subcultures had approximately 10% contaminating macrophages, third passage fibroblasts contained less than 1-2% macrophages: $1.2 \pm 1.4\%$ of the cells stained for esterase, $0.4 \pm 0.3\%$ were acid phosphatase positive, and only $0.5 \pm 0.2\%$ ingested latex beads (Table 1). Third passage cultures, therefore, were considered relatively macrophage-free.

Immunocytochemistry

The subculturing procedure was not only an effective means of removing macrophages from cell cultures, but also a method that enriched for fibroblasts, as determined morphologically by phase contrast microscopy. However, it was recognized that while the monolayer was relatively macrophage-free and appeared highly enriched in fibroblasts, it was possible that other cell types still might be present. Therefore, immunocytochemical tests were employed to assess the purity of tertiary cultures. Third passage cells were grown 1 day and then incubated with the specific antibodies listed in Table 3. In summary, greater than 98% of cells in tertiary cultures expressed vimentin, an intermediate filament characteristic of mesenchymal cells (Plate II). In addition, nearly all cells had an actin-rich cytoskeleton as demonstrated by the phallacidin technique, and after an additional 3 days in culture, the monolayer contained abundant extracellular fibronectin (Plate II). Furthermore, greater than 98% of the cells stained positive with the rat anti-Thy 1.1 monoclonal antibody (OX-7 clone; Plate II). Collectively, therefore, these characteristics indicated that 3rd passage lung cells were predominantly (>98%) fibroblasts (Table 3).
Fibroblast Ultrastructure

As an additional means of confirming the purity of 3rd passage cultures, cells were examined by transmission electron microscopy to determine if they had ultrastructural features characteristic of fibroblasts. Confluent fibroblasts were often arranged side by side in parallel arrays. Nuclei were centrally located, elongated, euchromatic, and contained 1 to 3 prominent nucleoli (Plate III). The chromatin was evenly distributed and the contours of the nuclear membrane were smooth.

One of the most prominent features of the cultured fibroblasts was an abundance of well developed granular endoplasmic reticulum (RER) (Plate III). The many elongated cisternae of the RER were dilated and contained finely dispersed flocculent material of low electron density. Free ribosomes, often occurring as polysomes, were present between adjacent cisternae as well as throughout the rest of the cytoplasm. Among other cytoplasmic features were a well-developed Golgi (with associated vesicles present in some cells), lysosomes, occasional coated pits, and a few residual bodies. Long and slender rod-like mitochondria containing transversely oriented cristae were plentiful (Plate III).

Many bundles of actin-like microfilaments were another striking feature of fibroblasts in vitro. These bundles usually ran parallel to the surface of the cell just beneath the plasmalemma. In addition, dense bodies were occasionally present within the microfilament bundles (Plate III). Single microfilaments, as well as microtubules, were also seen throughout the cytoplasm. Therefore, morphologic observations in conjunction with immunocytochemical tests confirmed that the monolayer had been highly enriched (>98%) for fibroblasts after 3 subculturings (Table 3).
Characterization of Alveolar Macrophages

Morphology

Cytocentrifuge smears of BAL-derived alveolar macrophages suggested that the macrophage population was heterogeneous with respect to cellular size and degree of vacuolization. Macrophages were round and contained eccentric oval nuclei. Some cells, however, were small and non-vacuolated, while other larger cells contained from few to many vacuoles, often giving the cytoplasm a "foamy" appearance (Plate IV). Differences in size and vacuolization were also observed in cultivated alveolar macrophages (Plate IV), and may have reflected differences in maturity, phenotype, or state of activation of the cells. In addition, cultured macrophages varied in their attachment to the substrate; some cells flattened and spread completely, while others remained spherical and refractile (Plate IV). Macrophages that did flatten assumed either a round or elongated shape, possibly indicating sessile or mobile states, respectively.

Transmission electron microscopy of BAL-derived macrophages showed that they had a euchromatic, indented and frequently eccentric single nucleus, with a prominent nucleolus. Many finger-like projections, which formed irregular ruffled borders, extended from the surface of the cells (Plate V). Also, invaginations, a few coated pits, and coated vesicles along the cell surface were found. Vacuoles in the ectoplasm were frequent, but varied in size (Plate V).

The most striking ultrastructural feature of BAL-derived macrophages was the abundance of primary and secondary lysosomes (Plate V). These membrane-bound vesicles varied greatly in size and shape. Primary lysosomes were found in several stages of development, as indicated by varying degrees of electron density. Phagolysosomes contained myelin and often other unidentifiable material.
Within the cytoplasm, the organelles varied in number and disposition from one cell to the next, giving the macrophage population an apparent heterogeneity. Mitochondria often were numerous, rodlike, and contained several cristae. The Golgi was often compact. The endoplasmic reticulum varied in extent, being granular in some cells but agranular in others. In addition, the number of free ribosomes and polysomes varied from one cell to another. Therefore, the BAL cells used in my experiments had most of the ultrastructural characteristics ascribed to alveolar macrophages.

Histochemical and Phagocytic Tests for Cultivated Alveolar Macrophages

Table 4 shows the results of 3 experiments in which bronchoalveolar lavage cells were cultured for 3, 7, or 14 days, at which time histochemical and phagocytic tests were performed. It was found that 100% of the BAL-derived adherent cells had morphological features of macrophages, greater than 98% stained for acid phosphatase and nonspecific esterase, and over 95% of cells ingested latex beads. Thus, these cells had histochemical and phagocytic characteristics of macrophages (Plate IV).

Analysis of Growth Kinetics by Stimulation of DNA Synthesis: Effect of Serum Concentration on Asynchronous Fibroblasts

Initial experiments analyzed the growth kinetics of fibroblasts in response to varying concentrations of fetal bovine serum (FBS). Cells used in these experiments were assumed to be asynchronous; that is, the cell population was randomly distributed at different points throughout the cell cycle, including cycling portions (G1, S, G2).
and M) as well as noncycling or quiescent portions (Go). Second passage fibroblasts were grown to confluence in 10% FBS, subcultured at low density (10^4 cells/6.4 mm microtiter well), and then grown in DMEM plus either 0.4, 1.0, 5, or 10% FBS. Cell proliferation was assessed daily by [3H]TdR incorporation and liquid scintillation spectroscopy. In 2 separate experiments, there was an FBS concentration-dependent increase in fibroblast DNA synthesis over a 7 day period. Figure 1 illustrates the results of an experiment in which [3H]TdR incorporation was measured on days 2 and 3 following subculture. On day 2, both 5% and 10% FBS caused similar high levels of DNA synthesis in fibroblasts. However, on day 3 (and up through day 7), 10% FBS stimulated the highest levels of DNA synthesis, 0.4% FBS stimulated the lowest levels, and 1 and 5% FBS caused intermediate levels of DNA synthesis (p < 0.001 between treatments, ANOVA). Therefore, as assessed by [3H]TdR incorporation, proliferation by asynchronous fibroblasts was dependent on the concentration of FBS.

**Analysis of DNA Synthesis Stimulated by 10% FBS: Effect of Varying Cell Number**

Preliminary experiments were performed to determine the appropriate concentration of [3H]TdR required to measure DNA synthesis in proliferating fibroblasts. Inadequate concentrations of [3H]TdR might be exhausted by rapidly proliferating fibroblasts, and the scintillation counts resulting in these experiments would be lower than the actual potential of proliferating fibroblasts. Third passage fibroblasts were seeded at either 2, 4, 6, 8, 10, 12, or 15 x 10^3 cells/well in 10% FBS. Daily, for 7 days, DNA synthesis was assessed following an 18 hr label with 1 μCi/well of [3H]TdR. Results of 2 separate experiments showed that there was a linear relationship between the number of cells seeded per well and the amount of
incorporated $[^3\text{H}]\text{TdR}$. On day 3, for example, the amount of radioactivity measured in counts per minute (cpm) for $2 \times 10^3$ fibroblasts/well was $1.198 \pm 165$, but $16,050 \pm 1081$ for $15 \times 10^3$ cells. Thus, a concentration of $1 \mu\text{Ci/well}$ of $[^3\text{H}]\text{TdR}$ was adequate to measure cell number-dependent increases in DNA synthesis. In addition, $[^3\text{H}]\text{TdR}$ incorporation increased for each cell concentration over a 3 to 4 day period, indicating that 10% FBS stimulated DNA synthesis in cultures seeded at both low ($2 \times 10^3$) and higher ($14 \times 10^3$) cell concentrations.

Analysis of Growth Kinetics in Synchronized Fibroblast Cultures

Low Serum Concentration-Induced Quiescence

The previous experiments examined cell proliferation (assessed by DNA synthesis) in fibroblast cultures that were asynchronous. A potential problem using asynchronous cells as targets in growth factor assays is that it is difficult to detect the true effect of the factor on proliferation because some of the cells already are cycling at the start of the assay. In addition, when testing the effect of specific polypeptide growth factors on proliferation, cycling cells in an asynchronous population have the capability of masking the true proliferative response of the noncycling cells. Hence, to test adequately whether a specific growth factor can cause cellular proliferation requires that as many cells as possible be in a quiescent state ($G_0/G_1$). Cellular quiescence was achieved in previous studies by growing cells in low serum medium (13). The noncycling population of cells obtained is defined as one in which less than 10% of the cells synthesize DNA as determined by autoradiography (13). In the present study an attempt was made to induce quiescence in lung fibroblasts, so that most cells had an
equal opportunity to respond to specific proliferation-inducing stimuli. Furthermore, the use of noncycling, synchronous cells may increase the sensitivity of the assay and permit a more accurate measure of both a) the number and proportion of cells responding to a specific growth factor, and b) the total amount of DNA synthesis stimulated by a growth factor.

To test the above hypothesis, the first series of experiments examined whether low concentrations of FBS could be used to arrest DNA synthesis in lung fibroblasts, and thereby create a synchronous population of cells. Third passage fibroblasts (2 x 10^4/well) were grown 1 day in 10% FBS to allow attachment, then washed and subsequently cultured in low serum medium containing either 0.1, 0.2, 0.4, or 1.0% FBS; thymidine incorporation was monitored over the next 4 days. Fibroblast [3H]TdR incorporation declined over a 3 day period for each of the low serum conditions, and in most cases, leveled off after 4 days of culture (Figure 2). The decrease in [3H]TdR incorporation was also dependent on serum concentration. Thus, DNA synthesis decreased over a 4 day period in asynchronous cells cultured in low concentrations of FBS.

To reinforce the findings made by scintillation spectroscopy, additional experiments using autoradiography were performed. Fibroblasts were subcultured into either Lab Tek chamber slides (4.5 x 10^4/chamber) or onto 12 mm coverslips (1.24 x 10^5 cells/16 mm well), and grown in medium supplemented with low serum, varying from 0.2 to 1.0% FBS. Tritiated thymidine was added on the third day of culture; cells were processed for autoradiography 24 hrs later (day 4), and the percentage of labeled nuclei determined. After the 4 day culture period, the mean labeling indices calculated from at least 2 separate experiments were as follows:

a) 0.2% FBS = 0.38 ± .02%  
b) 0.4% FBS = 4.79 ± 3.0%  
c) 0.8% FBS = 4.38 ± 1.8%  
d) 1.0% FBS = 6.58 ± 5.0%
Thus, these data indicated that culturing fibroblasts 4 days in low serum resulted in a quiescent population of cells (less than 10% of the cells synthesized DNA); therefore, the cultures were considered synchronized.

In additional experiments, quiescent cells grown 4 days in 0.8% FBS were fed with fresh medium, cultured an additional 2 days, and then processed for autoradiography. The mean labeling index from 3 experiments was $2.13 \pm 1.3\%$. These data indicated that feeding quiescent cells with fresh low serum medium did not stimulate an increase in the proportion of cycling cells. This was an important finding because 0.8% FBS (and 0.4% FBS) was used as the baseline control value for proliferation assays described in Results, Parts 2 and 3 of this report.

Correlation of Low Serum Conditions with Cell Viability and DNA Synthesis

After demonstrating that fibroblast proliferation could be arrested, the next experiments determined whether the low serum culture conditions compromised cell viability. Fibroblasts were grown in either 0.2, 0.4, 0.8, or 1.0% FBS, radiolabeled with $[^3H]TdR$ after 3 days of culture, and processed for autoradiography 24 hrs later, on day 4. The labeling index was determined for each serum treatment and correlated with cell viability. Viability was assessed in autoradiograms by counting the proportion of pyknotic nuclei present in each culture condition.

It was found that serum concentration affected both the a) proportion of cells that synthesized DNA, and b) the survival of fibroblasts in vitro. After 4 days of culture (Figure 3a), serum concentration was inversely related to the proportion of pyknotic nuclei, but directly related to the proportion of labeled nuclei. Thus, at FBS concentrations of 0.2% and 0.4%, the labeling index was low, but the number of dead cells was high; in contrast, with 0.8% and 1% FBS the labeling index increased, but the number of dead cells decreased. For example, using 0.2% FBS resulted in a labeling
index of only 0.37%, but greater than 16% of the cells were dead; whereas, 0.8% FBS resulted in a labeling index of 4.4% with greater than 97% cell viability. These data indicated, therefore, that cell viability was compromised when 0.2% FBS was used to induce quiescence, but that viability increased linearly by using 0.4, 0.8, and 1.0% FBS, respectively. Furthermore, the use of higher serum concentrations (0.8 and 1.0%) produced a cell population that was 90-95% quiescent.

In a parallel experiment, cells grown in low serum were re-fed after the initial 4 day culture period with fresh medium containing the appropriate low serum concentration, and then cultured an additional 4 days and processed for [³H]TdR autoradiography (day 8). Results of this experiment were in agreement with the previous findings, i.e., cell viability was compromised using 0.2% FBS, but was improved using either 0.4, 0.8, or 1.0% FBS (Figure 3b). The results also indicated that culturing cells 8 days in low serum did not increase the proportion of dead cells, nor did it decrease the proportion of cells synthesizing DNA when compared to cells grown only 4 days.

In a third experiment, viability was assessed by staining with fluorescein diacetate and propidium iodide according to the method of Jones and Senft (10). Although precise quantification was difficult in confluent cultures, it was determined by morphological observations that far more dead cells were stained in cultures supplemented with 0.2% FBS than in cultures with 1% FBS, thus confirming the viability data obtained by counting the proportion of pyknotic nuclei (as described above).

Stimulation of Quiescent Cells with 10% FBS

Culturing cells in minimal concentrations of FBS proved to be an effective means of achieving quiescence in fibroblasts. The purpose of the next series of
experiments was to determine whether quiescent cells could be restimulated to 
proliferate, or if they had been rendered incapable of re-entering the cell cycle. The 
experiments performed measured proliferation by autoradiography and scintillation 
counting. After the initial plating, cells used for autoradiography were grown in either 
0.4% or 1.0% FBS for 4 days to achieve quiescence. After this time, 10% FBS was added 
back to one-half of the cultures, while control cultures received either fresh 0.4% or 
1.0% FBS. Cultures were radiolabeled daily with $[^3]$H]TdR for up to 4 days and processed 
for autoradiography.

Treatment with 10% FBS induced quiescent cells to enter S phase of the cell 
cycle within 24 hrs, and this recycling was possible for cells initially arrested in both 
0.4% and 1.0% FBS (Figure 4a). In contrast, low serum-treated controls did not increase 
the proportion of cells entering S phase during this same time period. After 2 days, up 
to 60% of the serum-stimulated cells incorporated $[^3]$H]TdR and entered S phase, while 
control cells still remained at quiescent levels; labeling indexes were 6.2% and 12.2% 
for 0.4% FBS and 1.0% FBS controls, respectively. By day 4, serum-stimulated cells 
continued to enter S phase at a level 4.8 to 7.7 times greater than control cells. In 
addition, there was little difference between arresting cells in either of the low FBS 
concentrations (0.4% and 1.0%) with regard to the maximum number of cells that 
entered S phase after serum stimulation. This result was confirmed in another 
experiment in which cells were arrested initially in 0.2% and 0.4% FBS. Therefore, 
these data demonstrated that quiescent lung fibroblasts arrested in G0/G1 of the cell 
cycle could be stimulated by 10% FBS to enter S phase.

Scintillation counting was used in the next series of experiments to examine 
further the ability of 10% FBS to stimulate quiescent cells to re-enter the cell cycle. 
Culture medium containing 10% FBS was added to fibroblasts that had been 
synchronized with either 0.2% or 0.4% FBS; control cultures received fresh low serum 
medium. Cultures were radiolabeled daily with $[^3]$H]TdR (24 hr pulse) for 4 days and
processed for scintillation spectroscopy. Cells treated with 10% FBS began to
synthesize DNA within 24 hrs after stimulation, confirming results observed with
autoradiography (Figure 4b). DNA synthesis peaked by 48 hrs, and attained levels that
were at least 20 times greater than that of low serum-treated controls. In contrast, DNA
synthesis in control cultures did not increase throughout the entire culture period, and
was never greater than 5% of the maximum levels achieved with 10% FBS. These data
indicated that cellular quiescence could be measured by the relative inability of cells to
incorporate \[^{3}H\]TdT into DNA. Furthermore, the data supported the previous
autoradiography findings, i.e., that serum could stimulate quiescent cells to recycle and
synthesize DNA.
Table 1. Serial Passage of Lung Fibroblasts Reduces the Numbers of Macrophages Found in the Cultures.

<table>
<thead>
<tr>
<th></th>
<th>% Positive Cells in Fibroblast Cultures&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1&lt;sup&gt;°&lt;/sup&gt;</td>
</tr>
<tr>
<td>Esterase</td>
<td>10.9 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acid Phosphatase</td>
<td>2.5 ± 2.2</td>
</tr>
<tr>
<td>Latex Beads (2 µm)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.0 ± 6.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 400 cells counted/cover slip in each of 3 experiments.

<sup>b</sup> results from 2 separate experiments.

<sup>c</sup> only cells that ingested ≥ 5 beads were counted as positive.
Table 2. *Nonadherent Cells from Confluent Primary Cultures of Lung Fibroblasts Exhibit Characteristics of Macrophages/monocytes*.a

<table>
<thead>
<tr>
<th>% POSITIVE CELLSb</th>
<th>Esterase</th>
<th>Acid Phosphatase</th>
<th>Latex Beads (2μm)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt;99</td>
<td>92±7</td>
<td>93±4</td>
</tr>
</tbody>
</table>

a nonadherent cells were isolated from 1 week primary cultures of fibroblasts, grown an additional 7 days, at which time histochemical and phagocytic tests were performed.

b minimum of 400 cells counted/culture from 1 experiment.

c cells ingesting ≥ 5 beads were determined positive.
Table 3. *Characterization of Lung Fibroblasts (3rd Passage) Used in Proliferation Assays.*

>98% FIBROBLASTS

- Morphology consistent with fibroblasts (phase and electron microscopy)
- Presence of Thy 1.1 (OX-7) antigen
- Presence of vimentin (intermediate filament)
- Actin-rich cytoskeleton
- Fibronectin positive monolayer

1-2% MACROPHAGES

- Esterase (1.2% ± 1.4)
- Acid Phosphatase (0.4% ± 0.3)
- Latex Bead Uptake (0.5% ± 0.2)
Table 4. Characteristics of Alveolar Macrophage Cultures.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Differential</th>
<th>Esterase</th>
<th>Acid Phosphatase</th>
<th>Latex Beads (2μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>97</td>
<td>97.3</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
<td>98.9</td>
<td>99</td>
<td>92.3</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>100</td>
<td>99.6</td>
<td>97.3</td>
</tr>
<tr>
<td>x±SD</td>
<td>100</td>
<td>99.6±0.6</td>
<td>98.5±1.4</td>
<td>95.6±2.9</td>
</tr>
</tbody>
</table>

*a* minimum of 300 cells counted/culture/test.

*b* cells that ingested ≥ 5 beads were determined positive.
Figure 1. **Effect of FBS Concentration on DNA Synthesis in Cultures of Asynchronous Fibroblasts.** Incorporation of $[^3]HdTdR$ by third passage lung fibroblasts grown in either 0.4, 1, 5, or 10% FBS was serum concentration-dependent on days 2 and 3 following subculture; (p < 0.001, within-group comparison for each day; ANOVA).
EFFECT OF FBS CONCENTRATION ON DNA SYNTHESIS IN CULTURES OF ASYNCHRONOUS FIBROBLASTS

FIGURE 1
Figure 2. **DNA Synthesis Decreases in Fibroblasts Grown in Low Concentrations of Serum.** Third passage fibroblasts were initially plated in DMEM plus 10% FBS for 1 day to allow attachment. The medium was then changed to either 0.1, 0.2, 0.4, or 1% FBS. [³H]TdR incorporation by fibroblasts declined over the next 3 days and reached minimal levels by day 4, when most cells were quiescent and in Go/G1.
DNA Synthesis Decreases in Fibroblasts Grown in Low Concentrations of Serum

Figure 2
Figure 3. Correlation of Cell Viability and DNA Synthesis in Fibroblasts Grown in Low Serum Concentrations for 4 and 8 Days. a) Third passage lung fibroblasts were grown in DMEM containing either 0.2, 0.4, 0.8, or 1.0% FBS, radiolabeled with [3H]TdR after 3 days, and processed for autoradiography on day 4. b) Conditions similar to those in (a) except that cells were fed with fresh medium on day 4, radiolabeled on day 7, and processed for autoradiography on day 8. Viability was assessed by counting pyknotic nuclei. Cell viability was compromised with low serum concentrations, but use of higher serum concentrations (0.8 and 1.0% FBS) produced a cell population that was at least 97% viable and still 90 to 95% quiescent.
Correlation of Cell Viability and DNA Synthesis in Fibroblasts Grown in Low Serum Concentrations for 4 and 8 Days

Figure 3
Figure 4. **10% FBS Stimulates Quiescent Fibroblasts to Undergo DNA Synthesis.** Cells were grown 4 days in noncycling medium (0.2, 0.4, or 1% FBS) until quiescent, and then fed on day 0 with either fresh noncycling medium or DMEM + 10% FBS. DNA synthesis was assessed daily for 4 days by (A) [\(^3\)H]TdR autoradiography and (B) incorporation of [\(^3\)H]TdR into acid precipitable counts (24 hr pulse). 10% FBS stimulated G\(_0\)/G\(_1\) arrested cells to (A) enter S phase of the cell cycle and (B) synthesize DNA within 24 hrs; low serum-treated control fibroblasts did not re-cycle.
10% FBS Stimulates Quiescent Fibroblasts to Undergo DNA Synthesis

Figure 4
Plate I. Characterization of Lung Fibroblast Cultures by Phase Contrast Microscopy and Histochemical Tests.

a. Phase contrast photomicrograph of a primary culture of lung cells grown 2 days. Note the nest of epithelial cells (arrowheads), spindle-shaped fibroblast-like cells (closed arrow), and macrophage-like cell with ruffled border (open arrow). Stellate-shaped cells and a few spherical birefringent cells are also present. (magnification 252x).

b. Photomicrograph of a primary culture of lung cells grown 2 days, then incubated 1 hr with 2 μm latex beads, washed, and stained for nonspecific esterase. Several cells have phagocytosed the beads and stain intensely for esterase (open arrows). (magnification 600x).

c. Primary culture of lung fibroblasts grown 7 days. Many spherical, refractile cells accumulated on the surface of the confluent monolayer. (phase contrast microscopy; magnification 112x).

d. Phase contrast photomicrograph of third passage lung fibroblasts grown 7 days. Cells in the middle of the field are in parallel arrays, whereas fibroblasts at the periphery are at least 2 cells deep. Note the absence of birefringent cells. (magnification 112x).
Plate II. **Immunocytochemical Characterization of Third Passage Lung Fibroblasts.**

Third passage lung fibroblasts were stained immunocytochemically for (a) vimentin intermediate filaments, (c) cell surface Thy 1.1 antigen, and (d) extracellular fibronectin. The phalacidin technique was used to stain fibroblasts for actin (b). Cell nuclei in (a) and (b) were counterstained with propidium iodide. (magnification 648x).
PLATE II

a

b

c

d
Plate III. **Ultrastructure of Third Passage Lung Fibroblasts.**

a. Electron micrograph of cells in a third passage culture. Cells are arranged in parallel arrays and contain elongated nuclei with prominent nucleoli (n), numerous lysosomes (arrows), rodlike mitochondria, and bundles of microfilaments arranged parallel to the cell surface (open arrow). (magnification 9230x).

b. Higher magnification of microfilament bundles beneath the plasmalemma. Note the dense bodies amongst the microfilaments (arrows). (magnification 33,862x).

c. Granular endoplasmic reticulum (arrow) is abundant within the cytoplasm. Cisternae are dilated and contain finely dispersed flocculent material. (magnification 27,300x).
Plate IV. Characterization of Alveolar Macrophages by Light and Phase Contrast Microscopy and by Histochemical and Phagocytic Tests.

a. Cytocentrifuge smear of BAL-derived cells. Cells have typical monocyte/macrophage morphology, and are heterogeneous with respect to size and degree of vacuolization. (magnification 297x).

b. Phase contrast photomicrograph of adherent BAL-derived cells cultured 1 day. Macrophages either flattened and spread on the substrate (arrows), or remained spherical and birefringent. Note heterogeneity in size, shape, and vacuolization amongst the flattened cells. (magnification 252x).

c. Photomicrograph of cultured BAL-derived cells that were incubated 1 hr with latex beads, washed, and stained for nonspecific esterase. (magnification 600x).
Plate V. **Ultrastructure of Alveolar Macrophages.**

a. Transmission electron micrograph of a BAL-derived macrophage. The nucleus (n) is eccentric, indented, and euchromatic. The cytoplasm contains phagocytic vacuoles (v) and numerous lysosomes (open arrow). (magnification 14,087x).

b. Higher magnification of an alveolar macrophage. Many finger-like projections extend from the surface of the cell. Within the cytoplasm are vacuoles (solid arrow), coated vesicles (arrowheads), and several lysosomes (open arrows) in various stages of maturity. (magnification 16,931x).
RESULTS

PART 2

Overview of the In Vitro Model to Study Alveolar Macrophage-Derived Fibroblast Proliferation Factor(s) (AMFPF)

To test the hypothesis that alveolar macrophages produce growth regulatory molecules for fibroblasts, a model was developed in which macrophage products could be tested for their ability to stimulate fibroblast proliferation. The characteristics and normal growth kinetics of the cells used in this model have been described in Part 1 of the Results.

The model itself can be described schematically as "two-armed" (Figure 5). In one arm, BAL macrophages are characterized morphologically and histochemically, then stimulated in vitro in noncycling medium and the supernatants collected after 24 hrs. In the other arm, primary lung fibroblast cultures are established from 2 day old rats and subcultured weekly, during which time histochemical tests are performed on monolayers to test for contaminating macrophages, and both immunocytochemical and morphological techniques are used to determine fibroblast purity. Both arms of the model are joined in the proliferation assay.

In the proliferation assay (Figure 6), third passage fibroblasts were grown 4 days in noncycling medium (DMEM + 0.4% or 0.8% FBS) until quiescent. At the start of the assay (day 0), the noncycling medium was aspirated and replaced with alveolar macrophage supernatant to test for AMFPF activity. A baseline (background)
control as well as a maximum control (10% FBS) were added also at this time.

Fibroblast proliferation was monitored daily for up to 4 days by $[^3\text{H}]$TdR incorporation. For example, to measure proliferation on day 2, cells were radiolabeled with $[^3\text{H}]$TdR on day 1, and 24 hrs later were either harvested and processed for scintillation spectroscopy, or fixed and processed for autoradiography (Figure 6). In some experiments, proliferation was assessed on day 3 by enumerating cells with a Coulter Counter.

Lung Fibroblast Proliferation Induced by Alveolar Macrophage Supernatants

Initial experiments were performed to determine if macrophages activated by surface adherence elaborated a fibroblast proliferation factor. Alveolar macrophages were plated on glass coverslips at $1 \times 10^6$ cells/ml and cultured in noncycling medium for 24 hrs, after which time the supernatants were collected and added to quiescent lung fibroblasts. Figure 7 shows that incorporation of $[^3\text{H}]$TdR increased linearly over a 3-day period in fibroblasts cultured with supernatant from adherence-stimulated alveolar macrophages. By day 3 of the assay, DNA synthesis in supernatant-treated fibroblasts was 50% of maximum control levels (10% FBS), and was 3.3 times greater than the low serum baseline control ($p < 0.001$). In additional pilot studies ($n = 5$), supernatants from adherence-stimulated macrophages consistently induced fibroblast proliferation, in some experiments as high as 66.2% of maximum control levels, and always at least 1.7 times greater than baseline values ($p < 0.005$; Table 5).
Stimulation Methods Used for Alveolar Macrophage Release of AMFPF

To determine the ability of various agents to induce alveolar macrophages to release AMFPF, 10^6 macrophages/ml were stimulated in noncycling medium in suspension culture or while attached to culture dishes with either: a) opsonized zymosan, b) lipopolysaccharide (LPS), or c) IgG immune complexes. In several experiments, parallel cultures were set up in which macrophages were stimulated by surface adherence alone. In all cases, macrophage supernatants were harvested after 24 hrs and assayed for AMFPF activity.

Upon stimulation by each of the methods cited above, alveolar macrophages released AMFPF into their culture medium within 24 hrs. This was evidenced by a significant increase (p < 0.01) in [3H]TdR incorporation by fibroblasts that were incubated for 48 or 72 hrs in the presence of alveolar macrophage supernates. Table 6 shows that fibroblast proliferation, expressed as mean percent of maximum control (10% FBS), ranged from 45% for immune complex-stimulated macrophage supernates to 66% for LPS-stimulated macrophage supernates. Supernates from opsonized zymosan and LPS-stimulated macrophages appeared to induce the greatest amount of proliferation, with mean values that were 63% and 66% of maximum control values, respectively; in a few individual experiments, proliferation was even as high as 75 to 80% of the maximum control (Figure 8).

When fibroblast proliferation was expressed as percent maximum control, the amount of variation in group mean values made it difficult to determine which stimulatory agent/method was optimum (Table 6). Variation appeared to be reduced, however, when fibroblast proliferation was calculated as a stimulation index (SI = [cpm of fibroblasts incubated with supernate + cpm of fibroblasts incubated in low serum control medium]/cpm of fibroblasts incubated in low serum control medium]). Since the SI measured proliferation relative to baseline controls, and actually determined the degree to which cells were stimulated by AMFPF to re-enter
the cell cycle, it was felt the SI might be a more meaningful measure for determining the amount of DNA synthesis induced by each type of supernate. Using the SI, therefore, it appeared that supernates from IgG immune-complex activated macrophages induced the most proliferation, with mean values that were $9.4 \pm 0.4$ times greater than low serum baseline controls (Table 6). Supernates from surface adherence, opsonized zymosan, or LPS activated macrophages produced much lower fibroblast SIs, ranging from $2.6 \pm 1.1$ to $2.9 \pm 0.9$. It was important to note, however, that in each of the 22 experiments conducted (Table 6), supernates from macrophages stimulated by any of the 4 methods induced significant fibroblast proliferation ($p < 0.01$), even in cases in which the SI was as low as 1.7.

Figure 8 demonstrates that AMFPF activity was dilutable, and often caused significant DNA synthesis at dilutions that ranged from 1:2 up to 1:16 ($p < 0.05$). However, the greatest activity usually was found at dilutions of 1:2, 1:4, or 1:8, while undiluted or more dilute supernates had less of a proliferative effect.

In general, supernates from either adherent or suspension cultures of macrophages stimulated significant proliferation by fibroblasts. Figure 8 shows that although the amount of $[^3]$H]TdR incorporation was somewhat less when fibroblasts were incubated with supernates of LPS-stimulated macrophages from suspension culture compared to parallel adherent cultures (64.6% of max. vs. 79.3% of max., respectively), significant DNA synthesis resulted from both methods at four supernate dilutions. Results were similar when supernates from suspension and adherent cultures of opsonized zymosan or IgG immune complex-stimulated macrophages were compared. It is possible, therefore, that stimulating macrophages already attached to a surface with an additional exogenous agent, such as LPS, results in release of a higher concentration of AMFPF than by macrophages stimulated by surface adherence alone, or by macrophages in suspension culture stimulated by only 1 agent.
Macrophage Release of AMFPF in Serum-free Conditions

In most experiments, macrophages were stimulated to release AMFPF in medium containing low serum concentrations. To determine whether the small amount of FBS (0.4, 0.8, or 1.0%) present in noncycling medium played a role in stimulating macrophages to release AMFPF, a few experiments were carried out in which macrophages were stimulated under serum-free conditions (DMEM + 0.05% BSA). Serum-free supernatants from surface adherence and IgG immune complex-stimulated macrophages were collected after 24 hrs and tested for fibroblast proliferative activity in a serum-free assay. In each of four experiments, there was a significant increase in fibroblast $[^{3}H]TdR$ incorporation, as evidenced by a mean stimulation index of $7.2 \pm 1.2$ (range = 5.5 to 8.4; $n = 4$). In other words, fibroblasts incubated with serum-free macrophage supernatants incorporated greater than 7 times as much $[^{3}H]TdR$ as did serum-free baseline controls. The paradigm used in these experiments made it difficult, though, to determine whether the same amount of AMFPF was released from macrophages cultured in serum-free DMEM compared to those cultured in noncycling medium containing 0.4% or 0.8% FBS. However, the results did suggest that serum was not required for release of AMFPF, and that lung fibroblast proliferation was possible in a serum-free assay system.

Macrophage Supernatant-induced DNA Synthesis by Fibroblasts: Effect of Initially Synchronizing Cells in Two Different Concentrations of Low-Serum Medium

Experiments were performed to determine whether the concentration of FBS used initially to synchronize fibroblasts could subsequently affect the amount of DNA synthesis in fibroblasts stimulated by macrophage supernates. Supernates for these
experiments were obtained from macrophages stimulated with IgG immune complexes and grown in 0.4% or 1% FBS. The target fibroblasts were synchronized in G0/G1 of the cell cycle also using either 0.4% or 1% FBS. After four days in this noncycling medium, fibroblasts were incubated with the appropriate low-serum macrophage supernate (1:4 dilution); control cultures received fresh noncycling medium or 10% FBS. Cells were radiolabeled each of the next three days with \[^{3}H\]TdR (24 hr pulse), and processed for scintillation spectroscopy.

By 48 hrs of incubation, supernate-stimulated DNA synthesis was significantly higher (two-fold) in fibroblasts initially arrested in 1% FBS than in cells arrested in 0.4% FBS (p < 0.01) (Figure 9). The difference in \[^{3}H\]TdR incorporation between the 0.4% and 1% FBS supernate-treated cultures was almost 7 x 10^4 cpm, whereas the difference between the 0.4% and 1% FBS baseline controls was much less (874 cpm) and not significant (p > 0.05). In addition, fibroblasts incubated with supernates in 1% FBS had an SI as high as 9, while the SI was only 5.9 for supernate-treated cells in 0.4% FBS. These results demonstrated, therefore, that macrophage supernates stimulated more DNA synthesis by fibroblasts arrested in higher concentrations (1% FBS) of low-serum medium, than in cells arrested in lower concentrations (0.4% FBS). The findings suggest that DNA synthesis was greater in the 1% FBS supernate-treated cultures because not only were the cells in a "healthier" state and better able to respond to AMFPF than cells in 0.4% FBS, but also, perhaps, that there were greater numbers of cells actually present in the 1% FBS cultures. This hypothesis is supported by data from Part 1 of the Results which demonstrated that viability in cultures arrested in 0.4% FBS was only 92%, but increased to 98.4% when cells were arrested in 1% FBS.
Validation of the Proliferation Assay: Correlation of Scintillation Spectroscopy with Autoradiography and Cell Counts

Sets of cultures incubated with macrophage supernate or control medium were processed in parallel for either scintillation spectroscopy or autoradiography in order to test whether both methods were equally capable of measuring DNA synthesis in fibroblasts. Noncycling fibroblasts were incubated with fresh 0.4% FBS noncycling medium, 10% FBS, or macrophage supernate (1:3 dilution; IgG immune complex stimulation). DNA synthesis was assessed daily by $[^3]$H]TdR incorporation over a 4 day period. Figure 10 shows that the graphs obtained from scintillation counting and autoradiography data mirrored each other for all treatments, i.e., low and high serum controls and macrophage supernate. In addition, standardizing the data using stimulation indices demonstrated further that results obtained from each method correlated. For example, on day 2 of the assay, the stimulation indices from scintillation counting and autoradiography for macrophage supernate-treated cells were 9.5 and 8.9, respectively, and for 10% FBS-treated control cultures were 22 and 28.6, respectively. Therefore, these data demonstrated that autoradiography and scintillation counting each provided a similar evaluation of DNA synthesis as assessed by $[^3]$H]TdR incorporation, and that the indirect method of estimating DNA synthesis by scintillation counts was confirmed and validated by the more direct method of counting labeled cells by autoradiography.

To be certain that the DNA synthesis in fibroblasts observed using autoradiography and scintillation counting was directed toward replication and not repair synthesis, a second series of experiments was performed in an effort to correlate increased $[^3]$H]TdR incorporation with an increase in actual cell numbers. Briefly, parallel cultures of noncycling lung fibroblasts were incubated with several different lots of macrophage supernates or control media (0.8% FBS or 10% FBS). One half of the
cultures were radiolabeled with \[^3\text{H}]\text{TdR} on day 1 of the assay, harvested 24 hrs later (day 2), and processed for scintillation spectroscopy. The other half of the cultures were trypsinized from dishes after 3 days of culture, and the cells enumerated with a Coulter Counter.

Table 7 shows that proliferation by fibroblasts cultured in macrophage supernates was significantly higher compared to fibroblasts cultured in low-serum medium. At 48 hrs, there was a marked increase in DNA synthesis in supernate-treated cultures (SI range = 10 to 22; p < 0.001). Moreover, by 72 hrs incubation in supernates, there was a significant increase in the number of fibroblasts, ranging from 149% to 176% above controls grown in noncycling medium (p < 0.01). For example, in one experiment the cell number in supernate-treated cultures (Table 7, Supernate D) increased to 28,384 compared to a count of 16,102 cells in the control. Thus, these data demonstrated that in cultures incubated with macrophage supernates, an increase in \[^3\text{H}]\text{TdR} incorporation (compared to control) on day 2 of the assay correlated with an increase in cell number on day 3, i.e., macrophage supernates stimulated the cells to undergo cytokinesis and divide. Therefore, increased fibroblast DNA synthesis (as measured by scintillation counting or autoradiography) did indeed reflect the cell's ability to replicate.

Although fibroblasts stimulated with 10% FBS underwent DNA synthesis in these experiments, actual cell numbers in 10% serum increased by only 14% and 55% above controls (Table 8). These rather low increases in cell number most likely were due to a technical artifact that arose during the counting procedure, rather than a true indication of the actual cell number. Cells grown in 10% FBS tended to clump together during the trypsinization procedure. As a result, the Coulter Counter probably counted clumps of cells instead of single cells, which then led to inaccurate and rather low counts. This problem was aggravated when the clumps of cells clogged the orifice of the counter, resulting in counts even more inaccurate.
Effect of Macrophage Supernatants on Fibroblasts Proliferating in 10% FBS

Previous experiments in this study demonstrated that macrophage supernatants stimulated quiescent fibroblasts to undergo DNA synthesis. Other studies with human cells (47) have shown, however, that although macrophage supernatants stimulated DNA synthesis by quiescent fibroblasts, they inhibited proliferation by fibroblasts that were in log phase growth (in 10% FBS). Therefore, an effort was made to determine whether a similar phenomenon occurred in my culture system. Supernates were prepared by stimulating alveolar macrophages with IgG immune complexes in 10% FBS. Fibroblasts were synchronized over a 4 day period in 1% FBS, after which time the medium was changed to either fresh noncycling medium or 10% FBS. Twenty-four hours later, 1/3 of the medium was removed from the cultures treated with 10% FBS, and replaced with an equal volume of undiluted macrophage supernatant. Parallel 10% FBS-treated control cultures did not receive supernate. Proliferation was monitored daily over a 4 day period by \([^3H]TdT\) incorporation.

Log phase incorporation of \([^3H]TdT\) began on day 1 and peaked on day 2 of the assay for fibroblasts grown in either macrophage supernates or 10% FBS control medium (Figure 11). Twenty-four hours after the addition of the supernate, stimulation indexes were nearly identical for both supernate-treated and 10% FBS-treated control cultures (15 and 14.7, respectively). By day 3 of the assay, \([^3H]TdT\) incorporation was sustained at high levels in supernate treated cultures, but declined in 10% FBS control cultures to a level that was only 58% of that in the supernate-treated cultures. However, incorporation of \([^3H]TdT\) in supernate-treated cultures was not significantly different from 10% FBS control cultures on days 2 thru 4 of the assay (p > 0.05). Thus, these data and the results of a second experiment indicated that supernates from stimulated rat alveolar macrophages do not inhibit log phase DNA synthesis in fibroblasts stimulated with 10% FBS.
**Effect of Macrophage Supernatants on DNA Synthesis in Cell Types Other than Lung Fibroblasts**

The ability of macrophage supernatants to stimulate DNA synthesis in cell types other than rat lung fibroblasts was examined. All cell types tested were synchronized over a four day period in either 0.4% FBS or 0.8% FBS, after which time cells were incubated 48 hrs with either macrophage supernate, fresh low serum control medium, or 10% FBS. After the first day of incubation, cells were radiolabeled with $[^3]$H]TdR, cultured an additional 24 hrs, and processed for scintillation spectroscopy (day 2). Table 8 shows that macrophage supernates stimulated significant DNA synthesis in rat derived cells, including cerebral astrocytes and dermal and meningial fibroblasts; stimulation indices ranged from 1.8 to 1.9 ($p < 0.05$). DNA synthesis was inhibited slightly (compared to controls) in the 3T6 fibroblast cell line, and in mouse thymic epithelial cells. Guinea pig smooth muscle cells, and the BC3H1 myoblast line had rather low stimulation indices of 1.1 and 1.3, respectively, and were not significant ($p > 0.05$). These data, along with results of the previous experiments, demonstrated that the proliferative activity of AMFPF on rat fibroblasts was not organ specific, since fibroblasts from lung, dermal, and meningial sources were all stimulated to synthesize DNA. In addition, the factor was not cell-type specific in the rat, since both fibroblasts and astrocytes were stimulated to enter S phase. Although supernates did not stimulate DNA synthesis in the fibroblast cell line (3T6 cells) or in xenogeneic epithelial and smooth muscle cells, more experiments would have been necessary to determine the extent of AMFPF's activity, i.e., its cell type and species specificity.
Complementation Test

A serum-free complementation test was performed to allow classification of AMFPF as either a competence or progression factor. This evaluation of fibroblast DNA synthesis (and determination of AMFPF's point of action in G₀/G₁ of the fibroblast cell cycle) required the pairing of alveolar macrophage supernates with either a characterized competence factor (fibroblast growth factor [FGF] or fibronectin) or a characterized progression factor (insulin).

AMFPF appeared to function as a competence factor for the fibroblasts. Neither FGF alone, fibronectin alone, or insulin alone increased DNA synthesis of the cells compared with control cultures (p > 0.05) (Figure 12). Incubation of fibroblasts with macrophage supernate alone significantly increased DNA synthesis compared to controls (p < 0.05). However, the addition of FGF or fibronectin (competence factors) to the supernate was no more effective in stimulating [³H]TdR incorporation than was macrophage supernate alone, i.e., AMFPF did not act as a progression factor. Increasing the concentration of FGF or fibronectin did not change this finding (data not shown). In marked contrast, macrophage supernate cultured with insulin (progression factor) caused almost a two-fold increase in fibroblast [³H]TdR incorporation compared to cells cultured in supernate alone (p < 0.01, compared with control cultures).

Fibroblast SIs increased from 3.6 for cells incubated in supernate alone to 7.0 for cells cultured in supernate plus insulin. Thus, in this serum-free complementation test, the macrophage-derived proliferation factor (AMFPF) acted as a competence factor since DNA synthesis increased markedly when a characterized progression factor was added to the supernate, but not when a characterized competence factor was added.

Additional complementation tests performed in low-serum medium (0.4% and 0.8% FBS) confirmed that AMFPF probably acted as a competence factor. Table 9 shows that a similar trend was found in most assays, i.e., FGF or insulin alone cultured
with fibroblasts did not stimulate DNA synthesis, whereas macrophage supernates caused a significant increase in \[^{3}\text{H}]\text{TdR} \text{incorporation (p < 0.01), and supernates cultured with insulin induced a significant (p < 0.05) two-fold increase in the SI compared to cultures incubated with macrophage supernates alone. In three of four experiments, the effect on DNA synthesis obtained when insulin was added to the macrophage supernate appeared more synergistic than additive, further suggesting that proliferation was due to the presence of both a competence factor (AMFPF) and a progression factor (insulin). (It should be noted, however, that since the complementation assay is based on the theory that quiescent fibroblasts require the presence of both a competence and progression factor to re-enter the cell cycle and initiate DNA synthesis, it was possible that macrophages actually elaborated both types of factors because significant \[^{3}\text{H}]\text{TdR} \text{incorporation occurred when supernates alone were incubated with the fibroblasts.)}

As a positive control in each complementation test, fibroblasts were cultured with FGF plus insulin (a characterized competence and progression factor, respectively). In each experiment, though, this control failed to stimulate DNA synthesis. Since it had been found, however, that insulin augmented DNA synthesis when cultured with supernate, only the activity of the FGF was in question. Therefore, in an effort to clarify its proliferative role on lung fibroblasts, FGF was obtained from a variety of sources (Dr. Denis Gospodarowicz, BRL (2 lots), and Collaborative Research Inc.) and tested individually or together with insulin to determine if it could stimulate DNA synthesis. In addition, because reconstituted FGF loses activity rapidly after 2 to 3 months of freezer storage, fresh lots of FGF were used in some assays to negate the effect of any storage time-related breakdown. It was found that FGF from any source, either alone or together with insulin, did not stimulate DNA synthesis in lung fibroblasts. Moreover, additional experiments confirmed that the FGF had not lost its activity, since it stimulated DNA synthesis in both rat cerebral astrocytes and 3T6 cells to levels
that were twice that of baseline controls. These findings suggested, therefore, that FGF neither stimulates DNA synthesis nor functions as a competence factor for rat lung fibroblasts derived from primary cultures.

Physical Characterization of AMFPF

To determine the heat stability of AMFPF, serum-free macrophage supernates were heated to either 56°, 80° or 100°C. It was found that the activity of AMFPF was stable at 56° and 80°C (15 min), and in two experiments, supernates retained at least 94% activity at 100°C (10 min). In a third experiment, 46% of the activity was lost at 100°C.

Freeze-thawing fresh supernatants (5 times) resulted in minimal loss of activity (87% of activity retained; n = 3). However, AMFPF’s activity decreased markedly when supernatants were stored longer than 6 months at -20° or -70°C.

Although data suggested that the proliferative activity in supernatants was trypsin sensitive, results were inconclusive because cell viability appeared to be reduced after addition of the trypsin-treated sample (n = 2).
Table 5. **Effect of Supernatants from Adherence-Stimulated Alveolar Macrophages on Lung Fibroblast DNA Synthesis.**

<table>
<thead>
<tr>
<th>Exp'1 #</th>
<th>Supernatant (%)</th>
<th>0.4% FBS (%)</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50.0%</td>
<td>15.1%</td>
<td>3.3*</td>
</tr>
<tr>
<td>2</td>
<td>66.2</td>
<td>25.6</td>
<td>2.6*</td>
</tr>
<tr>
<td>3</td>
<td>61.3</td>
<td>36.0</td>
<td>1.7*</td>
</tr>
<tr>
<td>4</td>
<td>64.3</td>
<td>37.7</td>
<td>1.7*</td>
</tr>
<tr>
<td>5</td>
<td>49.3</td>
<td>16.5</td>
<td>3.0*</td>
</tr>
</tbody>
</table>

*a DNA synthesis assessed by [3H]TdT incorporation on day 3 of the assay.

b % Maximum Control = (cpm of fibroblasts incubated with supernatant or 0.4% FBS + cpm of fibroblasts grown in 10% FBS) x 100.

c Stimulation Index = (cpm of supernatant-treated cultures + cpm of low serum control cultures).

* p < 0.005
Table 6. Summary of Fibroblast Proliferation Induced by Alveolar Macrophage Supernatants: Effect of Macrophage Stimulation Method

<table>
<thead>
<tr>
<th>MØ Stimulation Agent(^b)</th>
<th># of Exp's</th>
<th>Stat. Signif.(^c)</th>
<th>% Maximum Control(^d)</th>
<th>Fibroblast S(_{l}^{de})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x</td>
<td>(range)</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Surface Adherence</td>
<td>11</td>
<td>11/11</td>
<td>52.4 ± 13.6 (22-69.5)</td>
<td>2.6 ± 1.1 (1.5-5.5)</td>
</tr>
<tr>
<td>Opsonized Zymosan</td>
<td>4</td>
<td>4/4</td>
<td>62.9 ± 11.3 (49-74.4)</td>
<td>2.7 ± 0.8 (1.9-3.7)</td>
</tr>
<tr>
<td>LPS</td>
<td>3</td>
<td>3/3</td>
<td>65.7 ± 13.2 (53-79.4)</td>
<td>2.7 ± 0.3 (2.4-3)</td>
</tr>
<tr>
<td>IgG Immune Complexes</td>
<td>4</td>
<td>4/4</td>
<td>44.6 ± 11.4 (33.2-59.7)</td>
<td>9.4 ± 0.4 (9-9.9)</td>
</tr>
</tbody>
</table>

\(^a\) proliferation measured by incorporation of \(^{3}\text{H}\)TdR into acid precipitable counts on either day 2 or 3 of the assay.

\(^b\) supernatants from stimulated macrophages were collected after 24 hrs incubation.

\(^c\) p < 0.01

\(^d\) % Maximum Control = (cpm of fibroblasts incubated with macrophage supernate + cpm of fibroblasts incubated in 10% FBS) x 100.

\(^e\) (SI) = Stimulation Index = (cpm of fibroblasts incubated with macrophage supernate + cpm of fibroblasts incubated in low serum medium).
Table 7. Alveolar Macrophage Supernates Stimulate both DNA Synthesis and Cell Division by Fibroblasts.

<table>
<thead>
<tr>
<th>Exp't#</th>
<th>Treatment</th>
<th>Day 2</th>
<th></th>
<th>Day 3</th>
<th></th>
<th>% Increase in Cell# above control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm</td>
<td>SI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cell #</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>268 ± 48</td>
<td>-</td>
<td>16,920 ± 1920</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supernate A</td>
<td>2685 ± 338*</td>
<td>10</td>
<td>25,744 ± 2448*</td>
<td></td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Supernate B</td>
<td>3954 ± 390*</td>
<td>14.8</td>
<td>28,296 ± 2048*</td>
<td></td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Supernate C</td>
<td>3363 ± 412*</td>
<td>12.6</td>
<td>25,209 ± 7072&lt;sup&gt;ns&lt;/sup&gt;</td>
<td></td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>10% FBS</td>
<td>4585 ± 897*</td>
<td>17</td>
<td>19,300 ± 752*</td>
<td></td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>Control&lt;sup&gt;b&lt;/sup&gt;</td>
<td>260 ± 23</td>
<td>-</td>
<td>16,102 ± 2528</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supernate D</td>
<td>5907 ± 882*</td>
<td>22.7</td>
<td>28,384 ± 2192*</td>
<td></td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>10% FBS</td>
<td>17,908 ± 3652*</td>
<td>68.8</td>
<td>25,061 ± 3008*</td>
<td></td>
<td>55</td>
</tr>
</tbody>
</table>

<sup>a</sup> (SI) = Stimulation Index = (cpm of fibroblasts incubated in macrophage supernate + cpm of fibroblasts in control medium).

<sup>b</sup> 0.8% FBS.

<sup>c</sup> not significant (p > 0.05).

<sup>*</sup> (p < 0.01).
Table 8. Effect of Macrophage Supernatants on DNA Synthesis in Cell Types Other than Lung Fibroblasts.  

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Macrophage Supernate</th>
<th>0.8% FBS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SI(^b)</td>
<td>% of Max.(^c)</td>
</tr>
<tr>
<td>rat dermal fibroblasts</td>
<td>1.9*</td>
<td>36.4</td>
</tr>
<tr>
<td>rat meningial fibroblasts</td>
<td>1.9*</td>
<td>35.6</td>
</tr>
<tr>
<td>rat cerebral astrocytes</td>
<td>1.8*</td>
<td>39</td>
</tr>
<tr>
<td>3T6 cells</td>
<td>0.9</td>
<td>25.5</td>
</tr>
<tr>
<td>mouse thymic epithelial cells</td>
<td>0.8</td>
<td>24.6</td>
</tr>
<tr>
<td>guinea pig aortic smooth muscle cells</td>
<td>1.1</td>
<td>19.5</td>
</tr>
<tr>
<td>BC3H1 cells (myoblast-like)</td>
<td>1.3</td>
<td>46.6</td>
</tr>
</tbody>
</table>

\(^a\) DNA synthesis assessed by \(^{3}\)HTdR incorporation (24 hr pulse into acid precipitable counts.

\(^b\) SI = Stimulation Index = (cpm of cells incubated in macrophage supernate + cpm of cells incubated in low serum control).

\(^c\) % Maximum = (cpm of cells incubated in macrophage supernate + cpm of cells incubated in 10% FBS) x 100.

* \(p < 0.05\).
Table 9. *Complementation Test Stimulation Indexes*\(^a\)

<table>
<thead>
<tr>
<th>Exp'</th>
<th>[Serum Conc.]</th>
<th>FGF(^b)</th>
<th>Insulin</th>
<th>MØ Supernate</th>
<th>FGF + MØ Supernate</th>
<th>Insulin + MØ Supernate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(serum-free)</td>
<td>0.7</td>
<td>1.3</td>
<td>3.6*</td>
<td>2.8*</td>
<td>6.7*</td>
</tr>
<tr>
<td>2</td>
<td>(0.4% FBS)</td>
<td>0.8</td>
<td>1.2</td>
<td>6.7*</td>
<td>8.6*</td>
<td>13.9*†</td>
</tr>
<tr>
<td>3</td>
<td>(0.4% FBS)</td>
<td>N.D.</td>
<td>1.2</td>
<td>3.5*</td>
<td>N.D.</td>
<td>7*†</td>
</tr>
<tr>
<td>4</td>
<td>(0.8% FBS)</td>
<td>0.8</td>
<td>1.3</td>
<td>1.4*</td>
<td>1.4*</td>
<td>1.9*†</td>
</tr>
</tbody>
</table>

\(^a\) Stimulation Index = (cpm of fibroblasts incubated with growth factors or macrophage supernate + cpm of fibroblasts incubated in noncycling control medium).

\(^b\) Fibroblast Growth Factor.

\(^*\) (p < 0.01); compared to control fibroblasts grown in noncycling medium.

\(^†\) (p < 0.05); compared to fibroblasts grown in macrophage supernate alone.

N.D. = not done
Figure 5. **Overview of the In Vitro Model.** The purity of alveolar macrophage and lung fibroblast cultures is determined in each arm of the model by morphological, histochemical, and immunocytochemical techniques as described in Materials and Methods. Both arms of the model are joined in the proliferation assay when macrophage supernatants are added to quiescent fibroblasts.
OVERVIEW OF MODEL

ALVEOLAR MACROPHAGES

BRONCHOALVEOLAR LAVAGE OF 10-12 WK RATS

DIFFERENTIAL COUNTS

CELLS CULTURED IN DMEM + 0.4% FBS

HISTOCHEMICAL TESTS

SUPERNATANTS COLLECTED AFTER 24 HRS

LUNG FIBROBLASTS

LUNGS OF 2 DAY RATS MINCED AND TRYPsinIZED

PRIMARY CULTURES ESTABLISHED IN DMEM+10%FBS

CELLS SUBCULTURED WEEKLY; 3-4 X

3RD-4TH PASSAGE FIBROBLASTS PLATED IN 0.4% FBS

4 DAYS

PROLiferATION ASSay

Figure 5
Figure 6. **Overview of Fibroblast Proliferation Assay.** Third passage lung fibroblasts are grown 4 days in noncycling medium until quiescent. At the start of the AMFPF assay (day 0), noncycling medium is aspirated and replaced with alveolar macrophage supernatant; 0.4% or 0.8% FBS is added to baseline controls and 10% FBS is added to maximum controls. Proliferation is assessed by $[^3H]TdR$ incorporation on day 2, or by cell counts on day 3 of the assay.
OVERVIEW OF FIBROBLAST PROLIFERATION ASSAY

ALVEOLAR MACROPHAGES OR SUPERNATANT ADDED
(CONTROLS: 0.4% OR 0.8% AND 10% FBS)

3RD PASSAGE FIBROBLASTS
0.4% OR 0.8% FBS (4 DAYS)

1) HARVEST CELLS
2) MEASURE CPM
3) AUTORADIOGRAPHY
{4) CELL COUNTS}

PULSE WITH [³H]-TdR
(24 HRS)

Figure 6
Figure 7. **Lung Fibroblast DNA Synthesis: Time Study.** As measured by $[^3H]TdR$ incorporation, DNA synthesis increased linearly over a 3-day period in fibroblasts incubated with conditioned medium from adherence-stimulated alveolar macrophages ($p < 0.005$; compared to noncycling control fibroblasts).
LUNG FIBROBLAST DNA SYNTHESIS: TIME STUDY

Figure 7
Figure 8. Effect of Supernate Dilution on Fibroblast DNA Synthesis. Supernates of macrophages stimulated by LPS, while in either adherent or suspension culture, were diluted and added to noncycling fibroblasts. DNA synthesis was measured after 48 hrs incubation by $[^3H]$TdR incorporation. AMFPF stimulated significant DNA synthesis by fibroblasts at several dilutions ($p < 0.05$; compared to noncycling controls).
Effect of Supernate Dilution on Fibroblast DNA Synthesis

**SUPERNATES:**
- Adherent MØ + LPS
- Suspension MØ + LPS

**FIGURE 8**
Figure 9. **Macrophage Supernates Stimulate More DNA Synthesis in Fibroblasts Synchronized in 1% FBS than those in 0.4% FBS.** Fibroblasts were synchronized in Go/G1 of the cell cycle by growing the cells 4 days in either 0.4% or 1% FBS. Supernates from IgG immune complex-stimulated macrophages (1:4 dilution), or control medium, was then added to the cultures, and DNA synthesis was assessed after 48 hrs by the incorporation of $[^3H]$TdR (24 hr pulse). Supernate-stimulated $[^3H]$TdR incorporation was significantly greater in cells initially arrested in 1% FBS than in cells arrested in 0.4% FBS ($p < 0.01$); there was no significant difference between 0.4% and 1% FBS noncycling controls ($p > 0.05$).
Macrophage Supernates Stimulate More DNA Synthesis in Fibroblasts Synchronized in 1% FBS than those in 0.4% FBS

Figure 9
Figure 10. Correlation of DNA Synthesis by Scintillation Counting and Autoradiography in Macrophage Supernate-treated Fibroblast Cultures. Quiescent fibroblasts were incubated with supernates from IgG immune complex-stimulated alveolar macrophages (1:3 dilution), 10% FBS, or fresh noncycling medium. DNA synthesis was assessed daily over a 4 day period by incorporation of \(^{3}H\)TdR into acid precipitable counts (open symbols) or by \(^{3}H\)TdR autoradiography (closed symbols).
Correlation of DNA Synthesis by Scintillation Counting & Autoradiography in MØ Supernate-treated Fibroblast Cultures

Figure 10
Figure 11. **Macrophage Supernatant Does Not Inhibit DNA Synthesis in Fibroblasts Stimulated with 10% FBS.** Noncycling lung fibroblasts were incubated with 10% FBS or fresh low serum medium on day 0. Twenty-four hrs later (day 1), of the medium was removed from the 10% FBS cultures and replaced with an equal volume of undiluted supernate from IgG immune complex-stimulated macrophages; control 10% FBS cultures did not receive supernate. DNA synthesis was monitored by [³H]TdR incorporation over a 4 day period. There was no significant difference (p > 0.05) in DNA synthesis between supernate-treated or 10% FBS-treated control cultures on days 2 thru 4 of the assay.
Macrophage Supernatant Does Not Inhibit DNA Synthesis in Fibroblasts Stimulated with 10% FBS

Figure 11
Figure 12. **Serum-free Complementation Assay of Macrophage Supernatant.** Alveolar macrophage supernatant was added to noncycling lung fibroblasts with either a characterized competence factor (FGF or fibronectin) or a characterized progression factor (insulin); noncycling medium (DMEM + 0.5% BSA) and each factor added alone served as controls. DNA synthesis was assessed by [3H]TdR incorporation after 48 hrs incubation (24 hr pulse). Macrophage supernate + insulin stimulated almost a two-fold increase in DNA synthesis compared to cells cultured in supernate alone (p < 0.01; compared to noncycling control).
TREATMENT COMPLEMENTATION ASSAY (SERUM FREE)

- DMEM + 0.5% BSA
- FGF (10 ng/ml)
- FIBRONECTIN (10 ug/ml)
- INSULIN (10 ug/ml)
- 1/4 ALV MØ SUPER
- FGF + MØ SUPER
- FIBRONECTIN + MØ SUPER
- INSULIN + MØ SUPER

(CPM x 10^3)

FIGURE 12

* p < 0.05
RESULTS

PART 3

Overview of Co-culture Model

Studies described in this section utilized the same 2-armed macrophage-fibroblast model described in Parts 1 and 2 of the Results, except with the following variations (Figure 13). First, bronchoalveolar lavages were performed on 10 to 12 wk old rats (instead of 4 to 5 wk rats) because it was found that the number of macrophages obtained was 2 to 4 times greater than that from younger animals. Secondly, unlike experiments in Part 2 that used macrophage supernates, most experiments described in this section used macrophages themselves co-cultivated directly with fibroblasts. Finally, proliferation was assessed only by $[^3\text{H}]$TdR incorporation (scintillation counting and autoradiography) and not by cell counts.

DNA Synthesis in Co-cultures: Effect of Macrophage Concentration

Findings in Part 2 demonstrated that supernates from stimulated alveolar macrophages caused significant proliferation in lung fibroblasts. The following series of experiments was designed to explore further the role of macrophages in enhancing
fibroblast proliferation, and to approximate more closely the possible early cellular pathophysiological events leading to fibrosis in vivo. More specifically, experiments tested a) whether macrophages themselves stimulated proliferation when co-cultivated directly with fibroblasts, and b) if proliferation ensued, was the degree of proliferation related to the number of macrophages added. To conduct these experiments, IgG immune complex-stimulated alveolar macrophages were added in noncycling medium at 1, 2, 4, or 8 x 10^4 cells microliter/well to quiescent fibroblast monolayers; DNA synthesis was measured daily for 4 days by the incorporation of [3H]Tdr (24 hr pulse) into acid precipitable counts.

Results of several experiments demonstrated that co-cultivating stimulated macrophages with noncycling fibroblasts caused significant DNA synthesis, and that the amount of DNA synthesized (i.e., the amount of [3H]Tdr incorporated) was directly proportional to the number of macrophages present in the co-culture. This macrophage concentration-dependent response on cells entering S phase was usually most evident by 48 hrs of co-cultivation. For example, Figure 1 shows a typical experiment in which, on day 2 of the assay, [3H]Tdr incorporation increased significantly from a baseline of 1298 ± 245 cpm in control fibroblast cultures to 5626 ± 1027 cpm (SI = 3.3) in co-cultures with 10^4 macrophages present; increasing the concentration to 2, 4, or 8 x 10^4 macrophages/co-culture increased [3H]Tdr incorporation even further to 9240 ± 716 cpm (SI=5), 13,792 ± 1232 cpm (SI = 10.6), and 16,452 ± 2562 cpm (SI = 12.7), respectively (p < 0.005, all comparisons to control). In this experiment, co-cultures with 8 x 10^4 macrophages/well synthesized DNA at levels as high as 86% of those attained with the 10% FBS maximum control; in a few other experiments, DNA synthesis in co-cultures even exceeded that in the 10% FBS controls. In marked contrast, macrophages cultured alone (negative control) in noncycling medium did not undergo DNA synthesis (62 ± 12 cpm; SI = 0.05); this finding was confirmed in several additional...
experiments using both scintillation counting and autoradiography. Thus, these data indicated that macrophages and fibroblasts did not enter S phase of the cell cycle when cultured separately in noncycling medium. However, co-cultivation of macrophages and fibroblasts stimulated DNA synthesis to levels that were directly proportional to the number of macrophages added. Additional experiments using both scintillation counting and autoradiography, and cells arrested in either of three low serum concentrations (0.4%, 0.8%, or 1% FBS), confirmed the macrophage concentration-dependent effect on DNA synthesis in co-cultures.

Effect of Fresh Lavage Macrophages vs Stimulated Macrophages on DNA Synthesis in Co-cultures

In the preceding experiments, macrophages "stimulated" by an exogenous agent were found to modulate DNA synthesis in co-cultures by a concentration dependent mechanism. To determine whether fresh lavage (possibly "nonstimulated") macrophages could also modulate DNA synthesis in co-cultures, fibroblasts were co-cultivated with either fresh lavage macrophages or with macrophages that had been stimulated by IgG immune complexes for 1 to 3 hrs prior to co-cultivation. Both stimulated and fresh lavage macrophages were added at low and high concentrations to noncycling fibroblasts and DNA synthesis was assessed after 48 hrs of co-cultivation by incorporation of [3H]TdR (24 hr pulse) into acid precipitable counts.

Figure 15 shows a representative experiment in which it was found that: a) fresh lavage macrophages stimulated significant DNA synthesis in co-cultures (p < 0.01), b) DNA synthesis was proportional to the number of fresh lavage macrophages added, and c) the effect on DNA synthesis in co-cultures was similar whether fresh lavage or stimulated macrophages were used. For example, stimulation indices for co-cultures
containing either $10^4$ stimulated or $10^4$ fresh lavage macrophages were 3.4 and 3.6, respectively. Increasing the concentration to $8 \times 10^4$ macrophages/co-culture also increased the stimulation indexes, but again there was little difference between levels of DNA synthesis in co-cultures with stimulated macrophages (SI = 8.9) and those with fresh lavage cells (SI = 8.6). As observed in previous experiments, co-cultivating fibroblasts with $8 \times 10^4$ macrophages/well induced DNA synthesis to near maximum levels obtained with 10% FBS.

Results of additional experiments comparing effects of co-cultivating 2, 4, or $8 \times 10^4$ stimulated vs. fresh lavage macrophages are presented in Table 10. In each experiment at a given cell concentration, and in all but one comparison, stimulated and fresh lavage macrophages induced DNA synthesis to levels that were not significantly different from each other ($p > 0.05$). Thus, these and the above data demonstrated that both stimulated and fresh lavage macrophages modulate DNA synthesis in co-cultures by a concentration-dependent mechanism and, furthermore, that BAL macrophages induced similar levels of DNA synthesis whether they had been stimulated or not by an exogenous agent prior to co-cultivation.

Effect of Macrophage and Fibroblast Co-cultivation on Cell Type-Specific DNA Synthesis

$[^3H]TdR$ autoradiography was used in an attempt to identify which type of cell synthesized DNA in co-cultures, i.e., macrophages, fibroblasts, or both. Noncycling lung fibroblasts grown on Lab Tek chamber slides were co-cultivated with $10^5$ alveolar macrophages/chamber for 48 or 72 hrs; cultures were radiolabeled with $[^3H]TdR$ 24 hrs
prior to the end of the incubation. Cells were fixed, processed for autoradiography, stained with Giemsa, and the labeling indices determined.

Morphologically, it was apparent that macrophages could be distinguished from fibroblasts in autoradiograms. Macrophages appeared as randomly distributed round cells positioned on the surface of the fibroblast monolayer, and were identified by their small relative size, eccentric nuclei, and densely stained basophilic cytoplasm that contained vacuoles and azurophilic granules (Plate VII). In contrast, fibroblasts were confluent, larger, and possessed a lightly stained, basophilic cytoplasm with pale violet nuclei that were approximately equal in diameter to most entire macrophages (Plates VI, VII). There did not appear to be a specific pattern in which the two cell types were arranged; macrophages appeared singly or in small groups (2 to 5 cells) and were situated over the cytoplasm or nuclei of fibroblasts. In addition, analysis of thick section autoradiograms from Epon-embeded co-cultures indicated that the round cells resting on the surface of the monolayer had morphological characteristics typical of monocytes/macrophages, and often made cellular contact with the underlying fibroblasts. In cases where no contact was made, fibroblasts and macrophages were usually within a few micrometers of each other. Occasionally, a few macrophages were insinuated between adjacent fibroblasts, and in a few instances, macrophages were actually found beneath the fibroblast monolayer.

The labeling indices of co-cultivated macrophages and fibroblasts are presented in Table 11. In each experiment, there was an inverse relationship between the proportion of labeled fibroblasts and labeled macrophages that was time-dependent. Fibroblast labeling indices were maximum on day 2, but decreased on day 3; whereas, macrophage labeling was low on day 2, but increased on day 3 of the assay. For example, in experiment #2 (Table 11, Plates VI and VII), 32% of fibroblasts entered S phase of the cell cycle after 48 hrs of co-cultivation (SI = 9.5), while less than 3% of macrophages cycled during this same time. In contrast, after 72 hrs of co-cultivation only 14% of fibroblasts synthesized DNA (SI = 2.6), while the proportion of
macrophages in S phase rose to higher than 24%. This represented more than a 55% decrease in the proportion of fibroblasts synthesizing DNA between days 2 and 3, and almost a 9-fold increase in the proportion of macrophages entering S phase during this same period.

Analysis of the mean labeling index (calculated by taking a mean of labeling indices from all 3 experiments) revealed a similar trend as described above. The proportion of fibroblasts that synthesized DNA decreased from a maximum of 38.6 ± 1.9% on day 2 to 12.9 ± 3.6% on day 3 (55% reduction), while the proportion of macrophages in S phase increased from 4.6 ± 1.7% to 16.5 ± 4.5% during this same period (Table 11, Figure 16). Thus, initial DNA synthesis occurred predominantly in fibroblasts, but by 72 hrs of co-cultivation, fibroblast DNA synthesis decreased while the proportion of macrophages in S phase increased to levels that were similar but slightly higher than that of fibroblasts. Therefore, these results indicated that, when co-cultivated under low serum conditions, fibroblasts as well as macrophages were stimulated to synthesize DNA in a time-dependent manner.

**Cell Contact Alone Between Macrophages and Fibroblasts Does Not Stimulate DNA Synthesis in Co-cultures**

The effect of co-cultivating fibroblasts with paraformaldehyde (PFA) fixed macrophages was examined in an attempt to clarify whether cell contact alone between these two cell types was a sufficient stimulus for fibroblasts to initiate DNA synthesis. Forty thousand nonfixed or PFA-fixed alveolar macrophages were added to quiescent fibroblasts maintained in 0.8% FBS; parallel control cultures received fresh noncycling medium or 10% FBS. DNA synthesis was measured after 48 hrs of co-cultivation by the incorporation of [³H]TdR (24 hr pulse) into acid precipitable counts. At the end of the incubation, and before being processed for liquid scintillation
spectroscopy, the cultures were examined by phase contrast microscopy to verify that fixed as well as viable macrophages still rested on the surface of the fibroblast monolayers.

In each of three experiments, PFA-fixed macrophages failed to stimulate DNA synthesis in co-cultures, while nonfixed (live) macrophages induced significant synthesis of DNA. Figure 17 shows a typical experiment in which $[^3H]TdT$ incorporation was almost 25 times higher in co-cultures with live macrophages than in those with fixed macrophages (SI for live cells = 43; SI for fixed cells = 1.7). Increasing the concentration of fixed macrophages to $8 \times 10^4$/well increased the SI by only 1 point (data not shown). In addition, it was important to note that co-cultures with PFA-fixed macrophages incorporated at least baseline levels of $[^3H]TdT$, thereby indicating that the fibroblast monolayers had not been poisoned, and that all of the PFA had been removed from the macrophage preparations by extensive washing prior to co-cultivation (0.8% FBS control = 219 ± 32 cpm; $40 \times 10^3$ fixed macrophages/co-culture = 380 ± 104 cpm; $40 \times 10^3$ live macrophages/co-culture = 9,407 ± 397 cpm). Thus, these data indicated that physical contact alone between fibroblasts and macrophages was not a sufficient stimulus to induce DNA synthesis under co-culture conditions.

**Cell Contact Between Viable Macrophages and Fibroblasts Enhances DNA Synthesis in Co-cultures**

Experiments reported thus far have shown that DNA synthesis in lung fibroblasts was stimulated consistently by either incubating fibroblasts with macrophage supernates, or by co-cultivating fibroblasts directly with viable macrophages. Under co-culture conditions, macrophages and fibroblasts were often in contact. Hence, the next series of experiments was performed to determine whether the
proximity of macrophages to fibroblasts in co-cultures influenced the degree of fibroblast proliferation. More specifically, I investigated whether fibroblast proliferation was stimulated to the same extent under conditions in which fibroblasts were in direct contact with macrophages, compared to conditions in which there was no physical contact between the two cell types. To conduct these experiments, alveolar macrophages were co-cultivated either directly with fibroblasts, or separated 1mm from the fibroblasts by a permeable molecular membrane (0.22 um pore size; Figure 18). Use of this particular experimental design also permitted me to determine whether macrophage supernates or macrophages themselves were more effective in stimulating fibroblast proliferation. (Since it had been found previously that macrophages elaborated AMFPF into culture medium for up to 72 hrs after stimulation (data not shown), it was believed that the factor would be continuously generated in the co-culture conditions just described.)

Fibroblasts were seeded at 7.03 x 10^4 cells/culture into 16 mm wells or onto the membrane of 12 mm Millicell HA tissue culture inserts (placed within 16 mm wells), and grown four days in noncycling medium until quiescent. Two hundred eighty-one thousand fresh lavage macrophages were then a) added directly to noncycling fibroblasts grown in 16 mm wells, or b) plated in wells 1 mm beneath fibroblast monolayers grown on membranes of the culture inserts (each insert had legs approximately 1 mm high). In both cases, macrophages and fibroblasts were considered co-cultivated, since both cell types were grown within the same 16 mm well (Figure 18). Control fibroblasts received fresh 0.8% FBS noncycling medium or 10% FBS. DNA synthesis was assessed after 48 hrs incubation by the incorporation of [3H]TdR (24 hr pulse) into acid precipitable counts.

There were two important and consistent findings from these experiments. First, macrophages co-cultivated directly with fibroblasts stimulated significantly greater [3H]TdR incorporation compared to a) conditions in which there was no contact between the two cell types (cells separated by a membrane), or b) the 10% FBS
maximum control (p < 0.01; Figure 19). Second, even when the cells were separated by a
membrane, macrophages still stimulated fibroblasts to incorporate [3H]TdR at levels
similar to the 10% FBS maximum controls.

With regard to the first point, experiment #1 in Table 12 shows that [3H]TdR
incorporation was 165,763 ± 370 cpm for co-cultures with direct cell contact, but only
97,264 ± 8,424 cpm (fibroblasts) and 1,696 ± 8 cpm (macrophages) in co-cultures where
there was no cell contact between the two cell types. This difference in [3H]TdR
incorporation was equally apparent by comparing the mean stimulation indices for
each treatment; SI = 46.3 for co-cultivation with direct cell contact vs. 28.9 for
co-cultivation but no contact between the two cell types, or 25.7 for the 10% FBS
maximum controls (Table 12). In other words, macrophages co-cultivated directly with
fibroblasts stimulated an average of: a) 160% more thymidine incorporation compared
to conditions in which macrophages and fibroblasts were physically separated in
culture, and b) 180% more thymidine incorporation compared to the 10% FBS controls.

In regard to the second point above, co-cultures in which macrophages were
separated from fibroblasts by the membrane still incorporated [3H]TdR at levels a)
similar to the 10% FBS controls, and b) significantly greater than the low serum
controls (p < 0.005; Figure 19). For example, in experiment #1 (Table 12), [3H]TdR
incorporation was 97,991 ± 10,822 cpm for 10% FBS controls (SI=20.1), and
97,264 ± 8,424 cpm for fibroblasts separated from macrophages by the membrane
(SI=20); noncycling control fibroblasts were only 4,864 ± 2,421 cpm (SI=1).
Macrophages separated from fibroblasts by the membrane incorporated minimal
thymidine.

Thus, the above data taken collectively demonstrated that macrophages
stimulated significant DNA synthesis in co-cultures whether or not they were separated
from the fibroblasts. However, DNA synthesis was enhanced substantially when the
macrophages and fibroblasts were in direct contact. Therefore, the proximity of
macrophages to fibroblasts in co-cultures did indeed influence the degree of fibroblast
proliferation. Furthermore, since AMFPF would have had to diffuse through the culture medium (and therefore condition the culture medium itself) to reach the fibroblasts under conditions in which there was a physical separation between the two cell types, these data may have indicated that co-cultivating macrophages directly with fibroblasts was more effective in stimulating fibroblast DNA synthesis than was incubating fibroblasts with macrophage supernatants alone.

Treatment of Macrophages with Inhibitors of Protein Synthesis or Cytoskeletal Disrupting Agents Decreases Macrophage-Stimulated DNA Synthesis in Co-cultures

Macrophages were incubated with puromycin or cycloheximide for 1 hr prior to co-cultivation to determine whether macrophage-stimulated DNA synthesis in co-cultures depended upon the ability of the macrophages to undergo protein synthesis. To ensure that mostly fibroblast DNA synthesis was measured in these experiments, co-cultures were radiolabeled with [3H]TdR on day 1, and harvested after a total of 48 hrs (day 2) of co-cultivation. As shown in Table 13, co-cultures that contained either puromycin- or cycloheximide-treated macrophages incorporated significantly less [3H]TdR than did control co-cultures that contained untreated fresh macrophages (p < 0.01). In experiment #3 (Table 13) for example, [3H]TdR incorporation in control co-cultures was 7,397 ± 493 cpm, but decreased 58% to 3,128 ± 216 cpm in co-cultures containing puromycin-treated macrophages, and by 66% to 2,478 ± 77 cpm in co-cultures with cycloheximide-treated macrophages. Overall, pretreating macrophages with inhibitors of protein synthesis decreased [3H]TdR incorporation in co-cultures by as much as 39 to 66% (range). Thus, these data indicated that macrophages must undergo protein synthesis to stimulate maximal levels of DNA synthesis by fibroblasts in co-culture.
In this same series of experiments, macrophages also were incubated with cytochalasin B prior to co-cultivation to determine whether disruption of cytoskeletal elements affected the ability of macrophages to stimulate fibroblast DNA synthesis in co-culture. Table 13 shows that in each of three experiments, cytochalasin B treatment significantly inhibited macrophage-stimulated [3H]TdR incorporation in co-cultures (p < 0.005), in one case by as much as 62%. Increasing the concentration of cytochalasin B from 30 to 60 µg/ml decreased [3H]TdR incorporation in a dose-dependent manner (data not shown). These data indicated, therefore, that disruption of cytoskeletal proteins had a negative influence on the macrophage's ability to induce fibroblast proliferation in co-cultures.

**Effect of Co-cultivating Fibroblasts with Macrophages, Lymphocytes, or Neutrophils**

During the course of experiments over a three and one-half year period, it was determined that lavage cell preparations varied with respect to macrophage purity and yield. Differential counts showed that lavage preparations often contained approximately 85 to 95% macrophages, with the remainder of cells made up of lymphocytes and neutrophils; these relatively pure macrophage populations were used in most experiments. Periodically, though, BAL cell preparations from some rats consisted of greater than 30 to 40% lymphocytes and/or neutrophils, and less than 50 to 70% macrophages. The reason for the increased proportion of apparent inflammatory cells was not determined. However, it was demonstrated in several experiments that these mixed lavage cells (i.e., macrophages, lymphocytes, and neutrophils) together could stimulate marked DNA synthesis when co-cultivated with noncycling fibroblasts. In one experiment, for example, lavage cells, which contained 73% macrophages, 21% lymphocytes, and 5% neutrophils, were added at 40 x 10^3 macrophages/well to fibroblast monolayers; DNA synthesis was assessed daily for 3
days by $[^3]H$TdR incorporation. After 48 hrs of co-cultivation, $[^3]H$TdR incorporation was 93% of maximum control values (i.e., fibroblasts grown in 10% FBS), and 15.9 times higher than baseline control values (i.e., noncycling fibroblasts). In a similar co-culture experiment in which the differential was 45% macrophages, 17% lymphocytes, and 38% neutrophils, $[^3]H$ TdR incorporation was stimulated to levels that were 48% of maximum controls, and over 7 times greater than baseline controls. Thus, these data indicated that, in co-cultures, fibroblast DNA synthesis could be stimulated by a mixed population of lavage cells that contained significant numbers of lymphocytes and neutrophils in addition to macrophages.

To be certain that macrophages were the primary cells responsible for stimulating DNA synthesis in co-cultures, additional experiments were performed in which mixed BAL cells were fractionated on a discontinuous gradient of iso-osmotic Percoll, so as to enrich the population for macrophages and remove contaminating lymphocytes and neutrophils. This procedure usually produced populations of macrophages that were 90 to 95% pure. Enriched macrophage preparations then were co-cultivated with noncycling fibroblasts, and DNA synthesis assessed. As shown in Figure 20, a cell population, which was 96% pure in macrophages after Percoll enrichment (only 4% lymphocytes), stimulated a significant dose-dependent increase in $[^3]H$TdR incorporation in co-cultures ($p < 0.001$). Also, using purified cells, it was once again demonstrated that there was little difference in the amount of $[^3]H$TdR incorporation stimulated by fresh lavage macrophages compared to that stimulated by IgG immune complex-activated macrophages ($p > 0.05$) (Figure 20). Percoll enriched macrophages produced similar results in other co-culture experiments. In addition, macrophages occasionally were obtained directly from animals in >98% purity. In these cases, it was found that highly pure macrophages stimulated thymidine incorporation in co-cultures to levels that were similar and sometimes even higher than in the 10% FBS maximum controls (Figure 19). Collectively, therefore, these data
reconfirmed that macrophages were the cells primarily responsible for stimulating DNA synthesis in co-cultures, although other cell types might play a minor role.

In the next experiments, lymphocytes or neutrophils were co-cultivated with fibroblasts in order to determine whether either of these individual cell types was capable of stimulating DNA synthesis in co-cultures. Forty or eighty thousand peripheral blood lymphocytes (98% enriched) or lavage-derived neutrophils (99% enriched) were co-cultivated with noncycling fibroblasts for 48 hrs; noncycling fibroblasts alone were the baseline control, while co-cultivated BAL macrophages (90% enriched) served as the positive control. DNA synthesis was assessed during the final 24 hrs of incubation by the incorporation of [3H]TdR into acid precipitable counts. As shown in Figure 21, neutrophils were unable to stimulate [3H]TdR incorporation in co-cultures, while lymphocytes stimulated only a slight but statistically significant increase in [3H]TdR incorporation (p < 0.05). Macrophages, however, stimulated by far the greatest increase in [3H]TdR incorporation by fibroblasts (p < 0.001). For example, [3H]TdR incorporation was 215 ± 44 cpm for noncycling fibroblasts (SI = 1), and 361 ± 39 cpm (SI = 1.7) or 756 ± 289 cpm (SI = 3.5) for 4 x 10^4 co-cultivated neutrophils or lymphocytes, respectively. Conversely, [3H]TdR incorporation in co-cultures containing macrophages ranged from 8,200 ± 979 cpm (SI = 38; 4 x 10^4 macrophages/well) to 12,806 ± 857 cpm (SI = 59.6; 8 x 10^4 macrophages/well). In a second experiment (Figure 22), lymphocytes (>98% enriched) stimulated [3H]TdR incorporation to levels that were only 1.5 to 1.8 times higher than in control fibroblasts. And in a third experiment using autoradiography (Figure 23), lymphocytes, but not neutrophils, again stimulated a slight but significant increase in the proportion of labeled fibroblast nuclei (p < 0.01). Macrophages, however, stimulated the greatest increase in proportion of labeled fibroblast nuclei, with labeling indices similar to that
of the 10% FBS maximum control. More specifically, fibroblast labeling indices were:

1 ± 0.8 (0.8% FBS controls); 3 ± 1 (co-cultivated neutrophils); 6.6 ± 3.4 (co-cultivated lymphocytes); 25.6 ± 7.1 (co-cultivated macrophages); and 26.8 ± 5.5 (10% FBS maximum control). Lymphocytes or neutrophils themselves were never labeled. Thus, these data indicated that in co-cultures: a) neutrophils did not stimulate DNA synthesis, b) lymphocytes were capable of stimulating a slight increase in $[^3\text{H}]$TdR incorporation, and c) macrophages, by far, stimulated the greatest amount of DNA synthesis.

Since neutrophils had no effect on stimulating DNA synthesis in co-cultures, and lymphocytes had only a slight effect, a final series of investigations was performed to determine the extent to which DNA synthesis could be stimulated by co-cultivating fibroblasts with a combination of either neutrophils plus macrophages or lymphocytes plus macrophages. These particular cell combinations were chosen in order to test the hypothesis that neutrophils or lymphocytes (or their products) could contribute to the stimulation of fibroblast DNA synthesis only under conditions in which macrophages themselves, or the correct combination of macrophage-derived competence or progression factors, were present. In each of two experiments, lymphocytes caused a concentration-dependent increase in $[^3\text{H}]$TdR incorporation when co-cultivated with macrophages and fibroblasts, and in each case, the increase appeared to be more than just the additive effect of the lymphocytes, i.e., an increase greater than that obtained when lymphocytes were co-cultivated alone with fibroblasts. In the first experiment (Figure 22), for example, $[^3\text{H}]$TdR incorporation in noncycling control fibroblast cultures was 8.7% of maximum levels; the addition of 4 or 8 x $10^4$ lymphocytes/well increased $[^3\text{H}]$TdR incorporation to only 12.8% or 15.4% of maximum levels, respectively. In contrast, co-cultivation of fibroblasts with 4 x $10^4$ macrophages/well significantly increased $[^3\text{H}]$TdR incorporation to 41.6% of maximum (p < 0.001).
The addition of 4 or 8 x 10^4 lymphocytes/well to these macrophage/fibroblast co-cultures, however, further increased [^3H]TdR incorporation to 52% or 62% of maximum levels, respectively (p < 0.05). Hence, co-cultivating fibroblasts with 4 or 8 x 10^4 lymphocytes alone increased [^3H]TdR incorporation only slightly (by 4.1 to 6.7%), whereas the addition of the same number of lymphocytes to macrophage/fibroblast co-cultures increased [^3H]TdR incorporation by 10% or 20%, respectively. A similar trend was demonstrated in a second experiment. Thus, in the presence of macrophages, lymphocytes stimulated [^3H]TdR incorporation a) by a concentration-dependent mechanism, and b) to a greater extent than when they were co-cultivated alone with fibroblasts.

The findings based on scintillation counting data were confirmed in a third experiment using autoradiography. Figure 23 shows that the labeling index increased significantly (p < 0.01) from 25.6 ± 7.2 for macrophage/fibroblast co-cultures, to 42.2 ± 9.7 for macrophage/fibroblast co-cultures that contained lymphocytes (48 hrs incubation). Thus, the labeling index was approximately 17% higher in co-cultures to which lymphocytes had been added. In contrast, the addition of neutrophils to macrophage/fibroblast co-cultures did not increase the labeling index above that of co-cultures containing macrophages and fibroblasts alone (Figure 23). Therefore, the addition of lymphocytes to co-cultures containing macrophages once again stimulated an effect on DNA synthesis that was additive at the very least, while neutrophils stimulated no effect.

The final experiment in this series was designed to test the hypothesis that any effect lymphocytes might have on [^3H]TdR incorporation (when co-cultivated with macrophages/fibroblasts) could be negated by removing the lymphocytes from the BAL cell population. In this experiment, a comparison was made between the following two
cell populations in their ability to stimulate $[^3\text{H}]\text{TdR}$ incorporation in fibroblasts: a) fresh BAL cells that contained 83% macrophages, 15% lymphocytes, and 2% neutrophils, and b) the same cell population after Percoll enrichment, which resulted in >95% macrophages, and <5% lymphocytes. Figure 24 shows that when lymphocytes were removed from the lavage cell preparation, there indeed was a decrease in $[^3\text{H}]\text{TdR}$ incorporation in co-cultures. For example, with $8 \times 10^4$ macrophages/well, $[^3\text{H}]\text{TdR}$ incorporation decreased significantly ($p < 0.05$) from $32,776 \pm 2,455 \text{ cpm} \ (SI = 13.9)$ for co-cultures containing 15% lymphocytes, to $26,437 \pm 1,799 \text{ cpm} \ (SI = 11.2)$ for co-cultures with Percoll enriched cells containing less than 5% lymphocytes (a difference of 6,339 cpm). In contrast, there was a difference of only 3,367 cpm between noncycling control fibroblasts and fibroblasts co-cultivated with $4 \times 10^4$ lymphocytes alone.

Therefore, the above data indicated that: a) macrophages were responsible for stimulating most of the DNA synthesis in co-cultures, b) when co-cultivated, a combination of macrophages and lymphocytes could stimulate DNA synthesis in fibroblasts to levels above those stimulated by macrophages alone, c) in the presence of macrophages, lymphocytes had a concentration-related effect on DNA synthesis in co-cultures, and d) the enhanced DNA synthesis in co-cultures containing lymphocytes plus macrophages might be more than just the additive effect of co-cultivating each of these cell types alone with fibroblasts. Finally, the results suggested that neutrophils had no role in stimulating DNA synthesis in co-cultures.
Table 10. **Effect of Fresh Lavage Macrophages vs. Stimulated Macrophages on DNA Synthesis in Co-cultures: Analysis by Stimulation Index.**

<table>
<thead>
<tr>
<th>Exp't #</th>
<th>2 x 10^4 MØ/Co-culture</th>
<th>4 x 10^4 MØ/Co-culture</th>
<th>8 x 10^4 MØ/Co-culture</th>
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<tr>
<td></td>
<td>Stim.</td>
<td>Fresh</td>
<td>Stim.</td>
</tr>
<tr>
<td>1</td>
<td>ND</td>
<td>ND</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>8.8</td>
<td>9.7</td>
<td>7.7</td>
</tr>
<tr>
<td>3</td>
<td>4.2</td>
<td>6.1</td>
<td>6.4</td>
</tr>
</tbody>
</table>

**STIMULATION INDEX**<sup>a,b</sup>

<sup>a</sup>Stimulation Index = (cpm of macrophage/fibroblast co-culture + cpm of fibroblasts in noncycling control medium).

<sup>b</sup>DNA synthesis was measured after 48 hrs of co-cultivation by incorporation of [³H]TdR into acid precipitable counts.

* (p < 0.05); compared to stimulated macrophages co-cultured at same concentration.

ND = not done.
Table 11. Effect of Co-cultivation on DNA Synthesis in Fibroblasts and Macrophages.

% LABELLED NUCLEI$^a$

<table>
<thead>
<tr>
<th>Exp't #</th>
<th>10% FBS$^{b,c}$ Fibroblasts</th>
<th>Co-cultured Fibroblasts</th>
<th>Co-cultured Macrophages</th>
<th>10% FBS$^{b,d}$ Fibroblasts</th>
<th>Co-cultured Fibroblasts</th>
<th>Co-cultured Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.8</td>
<td>28</td>
<td>8</td>
<td>22.8</td>
<td>6.2</td>
<td>16.7</td>
</tr>
<tr>
<td>2</td>
<td>30.6</td>
<td>32.2</td>
<td>3</td>
<td>17</td>
<td>14.2</td>
<td>24.2</td>
</tr>
<tr>
<td>3</td>
<td>63.8</td>
<td>25.6</td>
<td>3</td>
<td>45.6</td>
<td>18.3</td>
<td>8.6</td>
</tr>
<tr>
<td>x ± sem$^e$</td>
<td>40.4 ± 11.8</td>
<td>28.6 ± 1.9</td>
<td>4.6 ± 1.7</td>
<td>28.5 ± 8.7</td>
<td>12.9 ± 3.6</td>
<td>16.5 ± 4.5</td>
</tr>
</tbody>
</table>

$^a$ minimum of 500 cells/slide were counted.

$^b$ 10% FBS = Maximum Control.

$^c$ day 2 noncycling control fibroblasts = 2.13 ± 0.75% (x ± sem); (range = 0.8 to 3.4).

$^d$ day 3 noncycling control fibroblasts = 4 ± 0.76% (x ± sem); (range = 2.8 to 5.4).

$^e$ mean ± standard error of the mean.
Table 12. **Cell Contact Between Viable Macrophages and Fibroblasts Enhances DNA Synthesis in Co-cultures.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cell Type</th>
<th>[(^{3})H]-Thymidine Incorporation (cpm)</th>
<th>% Max. Control</th>
<th>Stimulation Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8% FBS (Noncycling Control)</td>
<td>Fibroblasts</td>
<td>Exp't 1(^a) 4864</td>
<td>39.3 ± 1.3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Exp't 2(^a) 2319</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-cultivation with NO cell contact(^c)</td>
<td>Fibroblasts</td>
<td>92,264(^*)</td>
<td>112 ± 7</td>
<td>28.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110,368(^*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td>1696</td>
<td>1.3 ± 0.5</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>761</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Co-cultivation with DIRECT cell contact</td>
<td>Fibroblasts</td>
<td>165,763(^*)†</td>
<td>180 ± 0.6</td>
<td>46.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>166,982(^*)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Macrophages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% FBS (Maximum Control)</td>
<td>Fibroblasts</td>
<td>97,991(^*)</td>
<td>100</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>86,716(^*)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Each value represents the mean of triplicate cultures harvested on day 2 of the assay.

\(^b\) Stimulation index = (mean cpm of control or co-cultivated cells + mean cpm of fibroblasts grown in noncycling control medium).

\(^c\) Fibroblasts separated from macrophages by membrane of HA millicell tissue culture insert.

\(^*\) (p < 0.01); compared to noncycling control fibroblasts.

\(^†\) (p < 0.01); compared to fibroblasts grown either on HA membrane insert or in 10% FBS.
Table 13. Treatment of Alveolar Macrophages with Protein Synthesis Inhibitors or Cytoskeletal Disrupting Agents Decreases Macrophage-stimulated DNA Synthesis in Co-cultures.  

<table>
<thead>
<tr>
<th>MACROPHAGE TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp't #</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
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<tr>
<td>2</td>
</tr>
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<td></td>
</tr>
<tr>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> DNA synthesis assessed by [3H]Tdr incorporation (24 hr pulse) after 48 hrs of co-cultivation.
<sup>b</sup> 40 x 10^3 mØ/well.
<sup>c</sup> (30 ng/ml).
<sup>d</sup> (10 ng/ml).
<sup>e</sup> cpm ± sd of triplicate cultures.
<sup>f</sup> Stimulation Index = (cpm of co-culture + cpm of noncycling control fibroblasts).
<sup>g</sup> Percent Inhibition compared to control co-cultures containing untreated macrophages.
<sup>*</sup> (p < 0.01); compared to control co-cultures.
ND = not done.
Figure 13. **Overview of Co-culture Model.** Macrophages and fibroblasts were characterized as described in Materials and Methods. For co-culture experiments, alveolar macrophages from 10 to 12 wk old rats were added directly to cultures of noncycling third passage lung fibroblasts. Proliferation was assessed either by incorporation of $[^3\text{H}]\text{TdR}$ into acid precipitable counts or by $[^3\text{H}]\text{TdR}$ autoradiography.
OVERVIEW OF CO-CULTURE MODEL

ALVEOLAR MACROPHAGES

BRONCHOALVEOLAR LAVAGE OF 10-12 WK RATS

DIFFERENTIAL COUNTS

CELLS CULTURED IN DMEM + 0.8% FBS; 24 HRS

LUNG FIBROBLASTS

LUNGS OF 2 DAY RATS MINCED AND TRYPsinIZED

PRIMARY CULTURES ESTABLISHED IN DMEM + 10% FBS

CELLS SUBCULTURED WEEKLY; 2-3 X

3RD PASSAGE FIBROBLASTS PLATED IN 0.4%, 0.8% OR 1% FBS

4 DAYS

PROLIFERATION ASSAYS:
SUPERNATANTS ONLY
CELL-CO-CULTURE

FIGURE 13
Figure 14. **DNA Synthesis Is Directly Proportional to Numbers of Macrophages in Co-culture.** Alveolar macrophages stimulated by IgG immune complexes were added in noncycling medium at 1, 2, 4, or $8 \times 10^4$ cells/well to quiescent fibroblast monolayers. DNA synthesis was measured daily by incorporation of $[^3H]TdT$ into acid precipitable counts. By 48 hrs of incubation, DNA synthesis in co-cultures containing each concentration of macrophages was significantly greater than noncycling control cultures ($p < 0.005$). DNA synthesis in co-cultures containing $8 \times 10^4$ macrophages was not significantly different from the 10% FBS maximum control ($p > 0.05$). Macrophages cultured alone in noncycling medium did not undergo DNA synthesis.
DNA Synthesis is Directly Proportional to Numbers of Macrophages in Co-culture

Figure 14

* p < 0.005
Figure 15. DNA synthesis is Similar in Co-cultures Containing Either Fresh Lavage Macrophages or Stimulated Macrophages. Noncycling fibroblasts were co-cultivated with 1 or 8 x 10^4 fresh lavage or IgG immune complex-stimulated macrophages, and DNA synthesis was measured after 48 hrs by incorporation of [^3H]TdT (24 hr pulse) into acid precipitable counts. DNA synthesis in co-cultures containing either concentration of macrophages was significantly greater than 0.8% FBS controls (p < 0.01). However, no significant difference in [^3H]TdT incorporation was detected between co-cultures containing fresh macrophages and those with immune complex-stimulated macrophages (p > 0.05).
DNA Synthesis is Similar in Co-cultures Containing Either Fresh Lavage MØs or Stimulated MØs

Figure 15

* \( p < 0.01 \)
Figure 16. **Effect of Co-cultivation on DNA Synthesis in Fibroblasts and Macrophages.**

Quiescent lung fibroblasts were co-cultivated with $10^5$ alveolar macrophages/chamber for 48 and 72 hrs; cultures were radiolabeled with $[^3H]TdT$ 24 hrs prior to the end of the incubation. Cells were processed for autoradiography as described in Materials and Methods. Fibroblast labeling indices (A and B) were significantly higher than noncycling controls on day 2 (\*; $p < 0.001$) but not on day 3, whereas macrophage labeling indices were higher than control on day 3 (\++; $p < 0.05$), but not on day 2. (Data represent mean values ± sem from 3 experiments.)
EFFECT OF CO-CULTIVATION ON DNA SYNTHESIS
IN FIBROBLASTS AND MACROPHAGES

Figure 16
Figure 17. **DNA Synthesis in Co-cultures is Stimulated by Viable and Not PFA-Fixed Macrophages.** Noncycling lung fibroblasts were co-cultivated 48 hrs with $4 \times 10^4$ fresh or paraformaldehyde (PFA)-fixed alveolar macrophages/microtiter well; DNA synthesis was measured by incorporation of $[^3H]Tdr$ into acid precipitable counts (24 hr pulse). (SI = 43 for co-cultures containing viable macrophages ($p < 0.001$), and 1.7 for co-cultures containing PFA-fixed macrophages.)
DNA Synthesis in Co-cultures is Stimulated by Viable and Not PFA-Fixed Macrophages

Figure 17

* p< 0.001
Figure 18. Co-cultivation Schemes. To determine the effect of cell contact on proliferation in co-cultures, alveolar macrophages were (A) co-cultivated directly with noncycling lung fibroblasts, or (B) separated 1 mm from fibroblasts by Millicell HA tissue culture membrane inserts.
CO-CULTIVATION SCHEME

A. Alveolar Macrophages Co-cultivated Directly With Lung Fibroblasts

B. Co-cultivated Macrophages and Fibroblasts Separated by a Molecular Permeable Membrane

Figure 18
Figure 19. **Cell Contact Between Viable Macrophages and Fibroblasts Enhances DNA Synthesis in Co-cultures.** Alveolar macrophages were either co-cultivated directly with lung fibroblasts or separated from fibroblast monolayers by a permeable molecular membrane as shown in Figure 18; the macrophage to fibroblast ratio was 4:1. DNA synthesis was assessed after 48 hrs of co-cultivation by incorporation of $[^3H]TdR$ into acid precipitable counts (24 hr pulse). Fibroblasts co-cultivated with macrophages, either directly or with membrane separation, incorporated significantly more $[^3H]TdR$ (*; p < 0.005) than did noncycling controls in 0.8% FBS. DNA synthesis was highest in direct co-cultures compared to membrane separated co-cultures or the 10% FBS controls (†; p < 0.01).
Cell Contact Between Viable Macrophages and Fibroblasts Enhances DNA Synthesis in Co-cultures

Figure 19

* p < 0.005
† p < 0.01
Figure 20. Percoll enriched alveolar macrophages: Fresh lavage vs. immune complex-stimulated macrophages. Quiescent lung fibroblasts were co-cultivated with 4 or 8 x 10^4 fresh lavage or IgG immune complex-stimulated macrophages that had been enriched by Percoll to 96% purity. DNA synthesis was assessed daily for 4 days by incorporation of [^3H]TdR into acid precipitable counts (24 hr pulse). After 48 hrs incubation, [^3H]TdR incorporation was macrophage concentration-dependent, and significantly greater than controls at both concentrations used (p < 0.001). There was no significant difference in DNA synthesis between co-cultures containing fresh lavage macrophages compared to those with immune complex-stimulated macrophages.
PERCOLL ENRICHED ALVEOLAR MACROPHAGES
Fresh Lavage vs. Immune Complex-Stimulated MØs

Figure 20

* p < 0.001
Figure 21. **Effect of Neutrophils, Lymphocytes, or Macrophages on DNA Synthesis in Co-cultures.** Forty or eighty thousand peripheral blood lymphocytes, lavage-derived neutrophils, or alveolar macrophages were co-cultivated with noncycling lung fibroblasts for 48 hrs. DNA synthesis was assessed during the final 24 hrs of co-cultivation by $[^3H]$TdR incorporation. Neutrophils did not stimulate DNA synthesis in co-cultures, whereas lymphocytes stimulated a small amount of DNA synthesis ($\dagger$: $p < 0.05$; SI = 1.7 to 3.5), and macrophages stimulated the greatest increase in DNA synthesis in co-cultures ($\star$: $p < 0.001$; SI = 38.4 to 59.6).
Effect of Neutrophils, Lymphocytes, or Macrophages on DNA Synthesis in Co-cultures

Figure 21

<table>
<thead>
<tr>
<th>BAL Differential</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mø 90%</td>
<td></td>
</tr>
<tr>
<td>Lymph 5%</td>
<td></td>
</tr>
<tr>
<td>PMN 5%</td>
<td></td>
</tr>
</tbody>
</table>

Percoll-enriched Peripheral Blood
Lymph >98%

* p < 0.001
† p < 0.05
Figure 22. **Lymphocytes Further Enhance DNA Synthesis in Macrophage/Fibroblast Co-cultures.** Noncycling lung fibroblasts were co-cultivated with either a) 40 or 80 x $10^3$ peripheral blood lymphocytes or b) 40 x $10^3$ alveolar macrophages alone or in combination with lymphocytes. DNA synthesis was measured after 48 hrs incubation by incorporation of $[^3H]TdT$ (24 hr pulse). Lymphocytes, alone, stimulated only a slight increase in fibroblast DNA synthesis (*; p < 0.05). Although macrophages, alone, stimulated significant fibroblast DNA synthesis (*; p < 0.001), the addition of lymphocytes to these macrophage/fibroblast co-cultures further increased DNA synthesis by 10 to 20% of maximum levels (†; p < 0.05).
Lymphocytes Further Enhance DNA Synthesis in Macrophage/Fibroblast Co-cultures

Figure 22

* p < 0.05 [to control]
† p < 0.05 [to 40 MØ]
Figure 23. The Effect of Alveolar Macrophages, Neutrophils, or Lymphocytes, Alone or in Combination, on Lung Fibroblast DNA Synthesis. One hundred thousand neutrophils, lymphocytes, or alveolar macrophages were co-cultivated alone or in combination with noncycling fibroblasts grown on Lab-Tek chamber slides. DNA synthesis was assessed after 48 hrs incubation by $[^3]$H]TdR autoradiography. Neutrophils alone were unable to stimulate DNA synthesis in co-cultures, but lymphocytes alone stimulated DNA synthesis slightly (*; p < 0.01). There was no significant difference between labeling indices of macrophage/fibroblast co-cultures and the 10% FBS maximum control (p > 0.05). DNA synthesis in macrophage/lymphocyte/fibroblast co-cultures was significantly higher than in either macrophage/fibroblast co-cultures or the 10% FBS maximum control (†; p < 0.01). Neutrophils had no effect on DNA synthesis when added to macrophage/fibroblast co-cultures.
The Effect of Alveolar MØs, PMNs, or Lymphocytes, Alone or in Combination, on Lung Fibroblast DNA Synthesis

Figure 23

* p < 0.01 [to control]
† p < 0.01 [to MØ alone]
Figure 24. **Lymphocyte Depletion from BAL Cells [by Percoll] Results in Decreased Fibroblast DNA Synthesis.** Quiescent lung fibroblasts were co-cultivated with a) 40 or 80 x 10^3 fresh BAL cells that contained 83% macrophages and 15% lymphocytes, and b) the same cell population after Percoll enrichment for macrophages (<5% lymphocytes).

Control cultures received 0.8% FBS, 10% FBS, or 40 x 10^3 lymphocytes co-cultivated alone with fibroblasts. DNA synthesis was measured after 48 hrs incubation by [3H]TdR incorporation (24 hr pulse). DNA synthesis decreased significantly (*; p < 0.05) when lymphocytes were removed from BAL cells co-cultivated with fibroblasts at a concentration of 80 x 10^3 macrophages/well.
Lymphocyte Depletion from BAL Cells (by Percoll) Results in Decreased Fibroblast DNA Synthesis

Figure 24
Plate VI. \[^{3}H\]Tdr Autoradiography of Alveolar Macrophage/Lung Fibroblast Co-cultures After 48 hrs Incubation.

a. 0.8% FBS noncycling control; only one labeled fibroblast nucleus (closed arrow) is present in the field. (magnification 120x).

b. Co-culture of alveolar macrophages and lung fibroblasts grown in 0.8% FBS. The field contains several labeled fibroblast nuclei; compare with (a) and (c). (magnification 120x).

c. 10% FBS maximum control; (no macrophages). (magnification 120x).
PLATE VI
Plate VII. \[^3\text{H}T\text{dR Autoradiography of Alveolar Macrophage/Lung Fibroblast}\]

Co-cultures After 48 and 72 hrs Incubation.

a. 48 hr co-culture of alveolar macrophages and lung fibroblasts grown in 0.8% FBS. Macrophages (open arrows) could be distinguished from fibroblasts by their smaller relative size, eccentric nuclei, and densely staining cytoplasm. Several labeled fibroblasts are present in the field, whereas macrophages usually were not labeled after 2 days incubation. (magnification 334x).

b. 10% FBS maximum control (48 hrs); no macrophages.

(magnification 334x).

c. 72 hr co-culture of alveolar macrophages and lung fibroblasts grown in 0.8% FBS. This field contains unlabeled macrophages (solid arrows) as well as a labeled macrophage (open arrow). Macrophages contained vacuoles and azurophilic granules. The labeled macrophage (open arrow) could be distinguished clearly from the labeled fibroblast (arrowhead).

(magnification 834x).
DISCUSSION

Alveolar macrophages and interstitial lung fibroblasts are increased in numbers during the pathogenesis of pulmonary fibrosis (29, 32, 34, 35, 72, 134, 135, 153). The interaction between these two cell types, however, is poorly understood. Therefore, an in vitro model was developed to investigate the influence of alveolar macrophages on lung fibroblast proliferation. The model may be important in determining some of the underlying mechanisms of pulmonary fibrosis, and may also contribute to an understanding of the pathogenesis of interstitial lung disease.

Most previous studies of macrophage-fibroblast interaction have used either cells isolated from different species, frequently from a non-pulmonary source, or fibroblasts from established cell lines, the functional state of which may not be typical of in vivo cells. The significance of the current model, therefore, is that it a) used "normal" alveolar macrophages and lung fibroblasts isolated from the same species (and animal colony), and b) tested fibroblasts after no more than three passages in culture.

This study has demonstrated that cultured rat alveolar macrophages can be activated to secrete a factor(s) (AMFPF) that stimulates the proliferation of quiescent lung fibroblasts and appears to function as a competence factor in a serum-free complementation assay. The factor, however, does not inhibit proliferation of fibroblasts in log-phase growth, nor is it fibroblast histospecific. Moreover, when alveolar macrophages and lung fibroblasts are co-cultivated, fibroblast proliferation is stimulated to levels similar to maximum controls (10% FBS), is enhanced by direct cell
contact with macrophages, and is not dependent on macrophage activation by exogenous stimuli. Furthermore, it was discovered that the interaction between co-cultivated macrophages and fibroblasts also stimulated macrophages to proliferate in a time-dependent manner. Finally, it was shown that lymphocytes, but not neutrophils, have the capacity to stimulate fibroblast proliferation to a small degree.

**Characteristics of Cells Used in the In Vitro Model.**

The mechanisms controlling the pathogenesis of human and experimental pulmonary fibrosis are numerous and highly complex. One way to approach the study of fibrosis is to circumvent the complexity of the in vivo process by reducing the variables involved. Tissue culture systems provide for such an approach, and in the present study, allow for the dissection of specific cellular and molecular interactions between alveolar macrophages and lung fibroblasts.

Since one aim of my study was to test the activity of a macrophage-derived factor on lung fibroblast proliferation, it was imperative, prior to the assay, to determine the purity of the target fibroblast monolayer, as well as to eliminate any contaminating macrophages. Primary cultures of fibroblasts were heterogenous and consisted of epithelial-like, fibroblast-like, and macrophage-like cells (Plate I). Although the hilar region (including bronchi and large blood vessels) was removed from the lungs prior to the tissue dissociation procedure, the cellular heterogeneity of primary cultures was not surprising since the peripheral lung parenchyma consists of several types of cells. By the third passage, however, macrophages were eliminated from the cultures (Table 1), and all cells had typical fibroblast morphology (by phase and electron microscopy) and grew in parallel arrays (Plates I, III). These findings are consistent with those of Bradley et al. (21), who noted that primary cultures of
human lung fibroblasts were also heterogenous, but by the fifth subcultivation, the cultures were entirely fibroblastic in appearance.

Unlike Bradley et al. (21) [or a few others who used early passage lung fibroblasts as targets for MDGF (9, 66, 93)], the present study used several criteria in addition to morphology to show that lung fibroblast subcultures were highly pure. Immunocytochemical techniques determined that >98% of cells in third passage cultures had the characteristics ascribed to fibroblasts (21, 158), i.e., the cells had cytoskeletons rich in actin and vimentin, and released fibronectin into the extracellular matrix (Table 3, Plate II). In addition, nearly all cells in third passage cultures had OX-7 immunoreactivity, and thus expressed the Thy 1.1 cell surface antigen (Table 3, Plate II). Although Thy 1.1 typically has been used as a marker for murine thymocytes and T lymphocytes, it has also been found on a few types of mouse fibroblasts, rat neurons, rat brain fibroblasts, and rat fibroblast cell lines (33, 125, 150). The present study, however, appears to be the first to demonstrate that Thy 1.1 is expressed on rat lung fibroblasts. Although it is possible that there was cros-reactivity with an antigen that was not necessarily Thy 1.1, use of the OX-7 antisera was effective in determining the purity of fibroblast cultures, and may be an important marker for other investigators using rat lung fibroblasts.

Thus, while there is no single unequivocal marker for fibroblasts, morphological observations and immunocytochemical tests demonstrated that third passage cultures were highly enriched with fibroblasts. Characterization of cells in the monolayer was necessary for accurate interpretation of data from subsequent proliferation experiments. It is possible, however, that some phenotypic variability existed in these apparently pure fibroblast cultures. For example, though most cells had ultrastructural features typical of fibroblasts, some cells actually had characteristics of myofibroblasts (Plate III), i.e., these cells contained "dense bodies" within large bundles of actin-like microfilaments that ran parallel to the plasma membrane (21, 158). Limited phenotypic variability was not surprising since the cells
had been subcultured only three times, and because it was possible that the in vitro conditions might select for certain cell types. Other studies also have shown heterogeneity within apparently homogenous fibroblast cultures (18, 46, 92). Importantly, though, Bradley et al. (21) have reported that differences between lung fibroblasts, myofibroblasts, and the so called "Interstitial cell" are only minor when compared to other interstitial mesenchymal cells such as pericytes and smooth muscle cells. Therefore, they have suggested that the three former cells be grouped in the same population and be classified as fibroblasts. Thus, it could be concluded that the cells in my culture system were in fact fibroblasts, and that the slight heterogeneity might reflect phenotypic variability amongst fibroblasts in vivo.

It was important that most cells (≤90-95%) obtained by bronchoalveolar lavage (BAL) were macrophages so that, in subsequent experiments, the appropriate cell was identified as having the capacity, or lack thereof, to regulate lung fibroblast proliferation. Macrophages recovered by BAL had morphological, enzyme histochemical, and phagocytic features typical of alveolar macrophages (Plates IV, V), and consistent with those characteristics described by others (19, 74, 158). The morphologic heterogeneity of macrophages in my study is typical of alveolar macrophages and is in agreement with the findings of Bowden (19). The heterogeneity likely indicates macrophage subpopulations with differences in age, function, state of activation, or quantity and quality of material that has been phagocytosed. Although Elias et al. (47) have shown that a subpopulation of smaller and denser human alveolar macrophages is responsible for release of certain fibroblast growth-regulatory factors, and have suggested that different alveolar macrophage subpopulations may have different effects on fibroblast function, the identification of a specific subpopulation of macrophages responsible for the release of AMFPF was out of the scope of the current study.
Analysis of Cell Cycle Kinetics in Synchronized Fibroblast Cell Cultures

To test adequately whether a specific growth factor can stimulate cellular proliferation requires that as many cells as possible be at the same temporal point of the cell cycle. Previous studies have shown that serum-deprivation in vitro causes 3T3 fibroblasts to cease proliferating, accumulate in the G1/G0 stage of the cell cycle, and thereby achieve quiescence (24). Upon restoration of serum or the addition of certain growth factors, the cells resume cycling and enter S phase in a synchronous wave after a well defined lag period (13, 24, 123).

In the present study, lung fibroblast DNA synthesis decreased in a time-dependent manner in response to serum depletion (Figure 2). After 4 days in low serum (0.8% FBS) medium, fewer than 5% of fibroblasts entered S phase of the cell cycle during a 24 hr period (Figure 3a). This finding is in general agreement with Bitterman et al. (13) who defined a population of "noncycling" lung fibroblasts as one in which less than 10% of the cells synthesized DNA. Moreover, the inability of cells to enter S phase was not due to decreased viability caused by serum-depleted medium, since >97% of cells grown in 0.8% or 1% FBS remained viable (Figure 3). Hence, because most serum-depleted cells in my study did not enter S phase, it was assumed that they had become arrested in G1/G0 and, therefore, were synchronized.

Upon re-stimulation with serum, G1/G0 arrested cells resumed active cycling in a synchronous manner (Figure 4), with entry into S phase occurring after a lag of approximately 18 to 20 hrs (data not shown). The rapid increase in DNA synthesis (Figure 4b) likely reflected a wave of cells entering S phase (Figure 4a). A much more gradual rise in DNA synthesis would have indicated an asynchronous (non-quiescent) population of fibroblasts. The finding that lung fibroblasts required about 18 to 20 hrs to traverse G1/G0 and enter S phase is in agreement with results of Brooks (24) who used 3T3 fibroblasts and Kovacs and Kelley (93) with rat lung fibroblasts.
It was evident, therefore, that normal lung fibroblasts could be manipulated in vitro with respect to cell cycle position. These investigations, which demonstrated that fibroblasts can be synchronized (arrested) in G1/G0 by serum-depletion and later stimulated by serum to re-cycle, were necessary to demonstrate that quiescent lung fibroblasts were responsive to mitogens. Thus, serum-depleted monolayers could be used as a bioassay in subsequent experiments that tested for growth promoting effects of alveolar macrophages and/or their products.

**Induction of Lung Fibroblast Proliferation by AMFPF.**

Alveolar macrophages, when stimulated by a variety of agents, released a factor(s) within the first 20 hrs of culture that induced noncycling lung fibroblasts to traverse G1/G0 of the cell cycle and proliferate (Figure 7, Tables 5, 6). AMFPF was a true mitogen in that it stimulated DNA synthesis (Figure 10) as well as cell replication (Table 7), was effective for at least 72 hrs in vitro (Figures 7, 10), and its activity in the medium was reduced by dilution (Figure 8). Though AMFPF was able to stimulate fibroblast proliferation even under serum free conditions, the factor may be classified as a competence factor, providing a growth-promoting signal to noncycling fibroblasts that is complementary to that provided by progression factors such as insulin (Figure 12).

Previously, alveolar macrophages from guinea pigs (106), sheep (102), monkeys (139), and humans (10, 13, 141), have been shown to release a factor(s) that stimulates fibroblast proliferation. Results of the present study using rat alveolar macrophages are in agreement with other recent reports of alveolar MDGF production by rats (9, 66, 93, 100, 101), and therefore support the concept that production of fibroblast-stimulating activity is not species-dependent, but rather a general phenomenon. Most
previous investigations, however, have measured the mitogenic activity of alveolar macrophage factors on fibroblast cell lines (10, 13, 100, 101, 102, 139, 141), but not on "normal" fibroblasts. Only a few recent investigations, using either bleomycin (93) or particulate-stimulated macrophages (9, 66), have demonstrated that alveolar macrophage factors also have the capacity to stimulate proliferation of early passage lung fibroblasts. The current study confirms and extends these previous findings that alveolar macrophage factors stimulate "normal" lung fibroblasts to proliferate.

Although many agents activated alveolar macrophages to release AMFPF, there appeared to be a hierarchy among the stimulatory agents with regard to the amount of AMFPF released. When evaluated by stimulation indices, supernates from IgG-immune complex-activated macrophages were far more effective in stimulating fibroblast proliferation than were supernates from either surface adherence, opsonized zymosan, or LPS-activated macrophages (Table 6). A similar hierarchy has been demonstrated by Bitterman et al. (13) using human alveolar macrophages, i.e., IgG-immune complexes stimulated the greatest release of growth factor, while particulates and heat-killed bacteria stimulated a moderate release, and surface adherence was the least stimulatory. In addition, Glenn and Ross (60) have shown that although endotoxin, concanavalin A, zymosan, and surface adherence all stimulate mononuclear phagocytes to release a growth factor for fibroblasts, endotoxin and concanavalin A are far more effective than zymosan or adherence. Moreover, other studies have shown that zymosan, latex beads, LPS, phorbol myristate acetate (PMA), and phytohemagglutinin (PHA) have a differential effect on MDGF release by human monocytes (43) and monocyte-derived macrophages (60, 104) and by murine peritoneal macrophages (110). Thus, the finding in the present study that various agents are differentially effective in promoting release of AMFPF is consistent with several previous reports concerning a hierarchy of MDGF release in response to a variety of activating agents.
There is increasing evidence which suggests that immune complexes are important in the pathogenesis of certain fibrotic disorders such as IPF. First, immunofluorescent and immunoperoxidase studies have demonstrated immunoglobulin deposits in the alveolar walls of patients with diffuse interstitial fibrosis (35, 50). Second, immune complexes have been detected in the lavage fluid of patients with high-intensity alveolitis (35, 77). Third, alveolar macrophages from IPF patients have their IgG Fc and C3b receptors occupied, consistent with the presence of immune complexes on their surfaces (77). And finally, alveolar macrophages from patients with IPF show intracytoplasmic fluorescence for IgG, suggesting that they have phagocytosed immune complexes (77). It is noteworthy that my results confirm the only previous report (13) that demonstrated the potent capacity of immune complexes to stimulate MDGF release by alveolar macrophages.

Variation in fibroblast proliferation was observed in AMFPF assays when different lots of supernate (each from macrophages stimulated by the same agent) were tested on the same fibroblast monolayer in a single experiment, and when the same lot of supernate was assayed on different fibroblast monolayers over the course of several experiments (Tables 5, 6). There are several possible explanations for the variation observed between lots of supernate. First, since crude supernates were used in these investigations, it is possible that the concentration of AMFPF varied among the lots. Second, certain lots of supernate may have contained fibroblast inhibitory factor(s) (as discussed below). Third, some of the BAL cell preparations used to make supernates may have consisted of certain macrophage subpopulations that preferentially influenced fibroblast function (47), while other BAL preparations may have been deficient in these subpopulations. Finally, differences in the fibroblast monolayer itself may have contributed to the variation. Such differences in the monolayer may also account for the variation seen when a single lot of supernate was assayed in different experiments. For example, because of the low-serum conditions used to make the monolayers quiescent, there may have been slight differences in the viability of
monolayers in different experiments, which might influence the potential proliferative response of a particular monolayer. In addition, because fibroblasts were derived from primary cultures, there might have been some slight phenotypic variation among the fibroblast populations in different experiments. Elias et al. (46) have shown that density-fractionated lung fibroblasts, derived from primary cultures, differ in their proliferative response to serum, and that the differential response is associated with phenotypic variability of the cells. Moreover, substrains of gingival fibroblasts differ in their ability to synthesize both DNA and collagen (18), while several clones of dermal fibroblasts obtained from primary cultures displayed tenfold differences in their response to mononuclear cell-derived mediators (92). Therefore, in the present study it is possible that cells of some monolayers were capable of a better proliferative response than cells of other monolayers. Finally, since three lots of $[^3]$H]TdR were used over a three year period, it is possible that the biodegradation of thymidine also contributed to some of the minor variations observed.

In the normal as well as the fibrotic lung, alveolar macrophage regulation of fibroblast proliferation may be the result of a delicate balance between both stimulatory and inhibitory factors. Because inhibitory factors are difficult to assay when using quiescent cells as targets, it is necessary to test for these factors using proliferating cells. It was concluded from proliferating cell assays that alveolar macrophage-conditioned medium neither inhibited nor decreased log phase DNA synthesis by fibroblasts stimulated to proliferate with 10% FBS (Figure 11), suggesting that rat alveolar macrophages failed to release fibroblast inhibitory factor(s). This result is in marked contrast to other reports (47, 84) that showed fibroblast proliferation is inhibited by supernatants from LPS-stimulated human alveolar macrophages, and that this inhibition is mediated by the capacity of the supernate to stimulate fibroblast prostaglandin synthesis. In addition, Gritter et al. (66) demonstrated that supernates from silica-treated rat alveolar macrophages inhibit DNA synthesis by fibroblasts grown in 10% FBS. It is unclear why my findings conflict
with these previous reports, although a number of reasons may be possible, including
the agents used to activate macrophages, the types of target cells, and the assay
conditions. For example, macrophages were activated by immune complexes in my
experiments, whereas the previous studies used silica (66) and LPS (47, 84). It is
difficult, therefore, to determine whether each of these agents stimulated release of the
same or different factor(s), and in equal concentrations. In addition, Elias et al. (47)
and Jordana et al. (84) tested their factor on lung fibroblast cell lines. Also, unlike the
present study which added macrophage supernate to synchronous cells, the previous
studies tested the macrophage factor on apparently asynchronous fibroblasts. Finally,
since it has been shown that cell density influences whether macrophage supernates
will inhibit or stimulate fibroblast proliferation (66), it is important to note that all
the above studies used target cells plated at different densities. Because of all the
variables involved in these previous studies, direct comparison of results is difficult.
Nonetheless, whether fibroblast proliferation is stimulated or inhibited by
monokines, the implications remain that mediators released by alveolar macrophages
can modulate the behavior of the lung fibroblast. The nature of these mediators
remains to be determined.

The relationship of AMFPF in the present study to other well-known growth
factors for fibroblasts is unknown. The possibility cannot be excluded that AMFPF is
identical or closely similar to either IL-1, PDGF, FGF, TGF (transforming growth
factor), EGF (epidermal growth factor), AMDGF, or other characterized growth factors.
Recent studies have shown that activated human alveolar macrophages release IL-1
(16), PDGF (113, 116), AMDGF (10, 13), and fibronectin (12). Only one recent study,
however, has attempted to characterize rat alveolar MDGF. Bauman et al. (9) have
shown that treatment of serum-free macrophage conditioned medium with a
monoclonal antibody to a synthetic peptide homologous to a part of the β chain of
PDGF inhibits MDGF activity in a concentration-dependent manner. This suggests
that rat alveolar MDGF is either homologous to PDGF or has some sequence homology
to a PDGF-like molecule. In addition, their rat macrophage factor was heat-stable (100°C, 10 min), which is consistent with the physical properties of PDGF, but not those of IL-1 (121), FGF (64), or AMDGF (13). The present results suggested that AMFPF also was heat-stable at 100°C, thus raising the possibility that AMFPF might be similar to PDGF, as Bauman (9) has proposed for MDGF. Interestingly, I found that purified PDGF added to quiescent fibroblasts did not stimulate DNA synthesis (data not shown). It is not clear why PDGF failed to stimulate DNA synthesis in my study. The discrepancy may suggest that only a PDGF-like molecule, but not purified PDGF itself, is capable of stimulating rat lung fibroblast proliferation. Alternatively, the findings may imply that macrophages release at least two factors that must work in concert to stimulate fibroblast proliferation, i.e., in Bauman's study (9) only one factor was present after treatment of conditioned medium with the anti-PDGF β chain antibody, and in my study, PDGF alone was incapable of stimulating DNA synthesis.

One useful system for classifying growth factors is based on Stiles' (151) dual control model of the cell cycle. According to this model, many growth factors can be divided into two categories, competence factors and progression factors. Competence factors act early in the G1 phase of the cell cycle rendering a cell competent for a variable number of hours to respond to progression factors, which act later by stimulating traverse of G1 and progression towards DNA synthesis. Cells not rendered competent do not respond to progression factors and cannot become committed to DNA synthesis (123). In the present study, pairing AMFPF with a characterized progression factor (insulin) in a serum-free complementation test consistently stimulated marked fibroblast DNA synthesis (Figure 12, Table 9). In this context, the AMFPF in my study appeared to act as a competence factor, in common with FGF, fibronectin, and PDGF. This finding agrees with investigations by Kovacs and Kelley (93) and Bauman et al. (9) who demonstrated that rat alveolar MDGF was a competence factor. In contrast,
Bitterman et al. (13) have shown that human AMDGF is a progression factor, a result which suggests species variation amongst MDGFs with respect to their point of action in the cell cycle.

The dual control model of the cell cycle is based on the premise that quiescent cells cannot be stimulated to traverse the cell cycle if only one growth factor or one general category of growth factors (i.e., competence or progression) is present in the culture medium (151). Consistent with this theory is my finding that neither FGF, fibronectin, or insulin stimulated fibroblast DNA synthesis above control levels (Figure 12). In contrast, and unlike results of any previous MDGF assays (9, 13, 93), alveolar macrophage supernatant cultured alone with fibroblasts stimulated DNA synthesis three and one-half fold higher than baseline controls, even under serum-free conditions (Figure 12, Table 9). Based on competence/progression theory, this result supports my hypothesis that the proliferative activity in rat alveolar macrophage supernates is actually derived from not just one factor, but rather from a combination of at least one competence factor and one progression factor. If this interpretation is correct, the reason for the apparent synergy between AMFPF and an additional progression factor (i.e., insulin) in stimulating DNA synthesis is unclear (Figure 12, Table 9), although one possible explanation may be that the proliferative effect depends on the relative concentrations of the macrophage-produced competence and progression factors and the added insulin. An alternative view is that AMFPF is indeed a single factor capable of stimulating fibroblast proliferation, and that since the dual control model has been determined from studies using 3T3 fibroblasts, a cell line (123, 151), the model may not be completely applicable to "normal" cells. In support of this view is my finding that the growth factor control used in the complementation test, FGF or fibronectin (competence factors) plus insulin (progression factor), consistently failed to stimulate DNA synthesis by "normal" lung fibroblasts (data not shown). A third possible explanation for AMFPF's capacity to stimulate fibroblast proliferation under serum free conditions is that AMFPF is a single competence factor that acts by
stimulating lung fibroblasts to produce their own insulin-like progression factors, similar to the mechanism of action proposed for AMDGF on human lung fibroblasts (14).

In summary, the present study demonstrates that rat alveolar macrophages release a factor(s) which stimulates "normal" quiescent lung fibroblasts to proliferate. These results confirm the findings of previous investigators, and support the concept that in the lung, one of the targets for mononuclear cell-derived growth factors can be regarded as a population of relatively slowly replicating fibroblasts that can be signaled rapidly to proliferate by monokines released from activated alveolar macrophages.

Co-cultivation of Alveolar Macrophages with Lung Fibroblasts.

Close physical association between macrophages and fibroblasts has been demonstrated microscopically in studies of wound repair (98) and human pulmonary fibrosis (7, 19). In addition, Davis et al. (39) determined that BAL fluid of patients with fibrotic lung disease usually contains fibroblasts as well as macrophages, further suggesting the close association of these cells during the pathogenesis of the disease. Moreover, macrophages contact connective tissue elements as they migrate from the interstitium into airspaces in a variety of chronic interstitial lung diseases (38). The effect on proliferation, however, of this direct physical interaction between alveolar macrophages and lung fibroblasts heretofore has not been investigated. Therefore, in the present study, I chose to co-culture macrophages and fibroblasts in order to examine several aspects of the direct interaction between these cells. The co-culture model may reflect more closely the interaction between macrophages and fibroblasts in vivo during the pathogenesis of pulmonary fibrosis, and may be more
physiologically relevant to the study of mechanisms by which macrophages regulate fibroblast proliferation than is testing the effects of macrophage supernatants alone.

Alveolar macrophages stimulated lung fibroblast DNA synthesis to near maximum control levels (10% FBS) when the cells were co-cultured at appropriate cell-to-cell ratios (Figures 14, 15, 16; Plate VI); in some cases this stimulation exceeded maximum control levels (Figure 19, Table 12). These findings indicated that the amount of lung fibroblast proliferation was regulated proportionally by the number of macrophages present (or by the concentration of their products), and that macrophages were as effective as serum mitogens in stimulating proliferation by fibroblasts when both cell types were in direct contact. In support of these findings, Diegelmann et al. (42) have shown that murine peritoneal inflammatory cells (70% macrophages) also stimulated DNA synthesis when co-cultivated with 3T3 fibroblasts, and that the amount of DNA synthesis was dependent on the number of peritoneal cells added. Other studies also support the concept that macrophages have the capacity to stimulate proliferation in co-cultures. Rabbit wound macrophages induce proliferation of bovine vascular smooth muscle and endothelial cells (65), and rat type II lung epithelial cell proliferation is proportional to the number of alveolar macrophages present in the co-cultures (103). Thus, these previous studies suggest that macrophages influence proliferation when co-cultivated with certain cell types. Only one previous investigation has co-cultivated alveolar macrophages with lung fibroblasts, however. Although Goldstein et al. (63) found that guinea pig alveolar macrophages stimulated increases in both protein and collagen synthesis by IMR-90 lung fibroblasts, the effect on proliferation was not determined. Hence, the present study appears to be the first to demonstrate that direct physical interaction between alveolar macrophages and lung fibroblasts stimulates fibroblast proliferation. This finding may be important in the context of pulmonary fibrosis, because during fibrosis, macrophages are increased in numbers (34, 40) and have access to both interstitial (38) and intra-alveolar fibroblasts (7).
The ability of macrophages to function as effector cells and release monokines is believed to be dependent on whether they are activated (2, 34, 120). In that regard, it is not entirely clear why fresh lavage macrophages (presumed to be nonactivated) and macrophages activated by exogenous agents via Fc receptors both stimulated fibroblast proliferation to the same extent in co-cultures (Figures 15, 20, Table 10). It is possible that macrophages were activated either by an agent(s) in vivo prior to lavage, or perhaps by the lavage and/or enrichment procedures themselves. In support of this interpretation, Kelley et al. (87) have suggested that some activation of monocytes may also occur during isolation and purification. Alternatively, macrophages attached to the fibroblast monolayer, and adherence itself has been shown to activate macrophages (87) and stimulate the release of alveolar MDGF (9, 13, 100; Tables 5, 6). Thus, it is possible that adherence alone activated fresh lavage macrophages to stimulate fibroblast DNA synthesis at levels similar to those of fibroblasts stimulated by macrophages activated by an exogenous agent prior to co-culture.

The magnitude of lung fibroblast proliferation in co-cultures is directly related to the proximity of the fibroblasts to alveolar macrophages. By using tissue culture inserts, it was determined that fibroblast DNA synthesis was enhanced markedly when macrophages and fibroblasts were in direct contact compared to when they were separated by a molecular permeable membrane (Figure 19, Table 12). Therefore, cell contact between macrophages and fibroblasts augments proliferation, and it appears that direct physical interaction between these two cell types is more effective in stimulating fibroblast DNA synthesis than is AMFPF alone, i.e., the mitogenic effect of alveolar macrophage conditioned medium. This finding is important in light of previous studies which have only assessed the proliferative capacity of alveolar macrophage supernates, and thus suggests the physiological relevance of the present co-culture model. Furthermore, these observations may be particularly significant in the context of pulmonary fibrosis, where macrophages and fibroblasts are found in close physical association (7, 19).
It is unclear why cell contact stimulated more fibroblast proliferation than when fibroblasts and macrophages were co-cultivated but physically separated. It may be that cell contact facilitated the direct transfer of AMFPF from macrophages to fibroblasts. It is also possible that contact between macrophages and fibroblasts stimulated the expression of greater numbers of receptors for AMFPF by fibroblasts. A third possibility may be that a concentration gradient was established with regard to AMFPF; the closer fibroblasts were to macrophages, the higher the concentration of AMFPF, and thus the greater the proliferative effect. Although the precise mechanism remains to be elucidated, it is presumed that the enhanced proliferation that ensues when macrophages and fibroblasts are in direct contact is the result of a biophysical interaction rather than the result of just a contact stimulus. Evidence for this was found when nonviable fixed macrophages failed to stimulate any fibroblast DNA synthesis in co-culture (Figure 17). It is important to note, however, that even when co-cultivated viable cells were separated by a membrane, macrophages were still able to stimulate fibroblast DNA synthesis to levels similar and even higher than the 10% FBS maximum control (Figure 19, Table 12). This indicates the potency of AMFPF as a mitogen, especially where it can be continually released by macrophages into the local milieu. These observations may also be particularly relevant to the pathogenesis of fibrosis because they suggest that activated alveolar macrophages have a profound effect on fibroblast proliferation when both cells are within the same microenvironment, but not necessarily in contact.

Although one concept of the tissue macrophage is that it is a terminally differentiated, nonrepli-cating cell, the current study presents evidence that direct interaction of alveolar macrophages and lung fibroblasts stimulates not only fibroblast proliferation, but DNA synthesis by macrophages as well (Plates VI, VII; Figure 16; Table 11). Study of the proliferation kinetics in macrophage/fibroblast co-cultures indicated that initial (48 hrs) DNA synthesis occurred predominantly in fibroblasts, but by 72 hrs of co-cultivation, fibroblast DNA synthesis decreased while
the proportion of macrophages in S phase increased by more than three-fold (Figure 16). Previous co-culture studies which reported that macrophages stimulated DNA synthesis in 3T3 cells (42), smooth muscle or endothelial cells (65), or lung type II epithelial cells (103), did not determine whether macrophages also were stimulated to proliferate. However, several other human and animal studies support the concept that tissue macrophages are capable of proliferating. For example, Golde et al. (61) have shown that a small percentage of both immature and mature human alveolar macrophages can synthesize DNA. In addition, Bitterman et al. (15) demonstrated that the proportion of alveolar macrophages that synthesized DNA increased 4- to 15-fold during chronic inflammation associated with interstitial lung disease, and that such macrophages also replicated in soft agar. These findings are in general agreement with animal studies that have also demonstrated DNA synthesis by alveolar macrophages in vitro (20, 105, 109), or during pulmonary injury in vivo (49). Moreover, bovine (115), murine (146), rat (105), and hamster (105) alveolar macrophages not only synthesize DNA, but also divide.

It is not clear how alveolar macrophages were stimulated to undergo DNA synthesis in co-culture. It was likely, however, that macrophage proliferation resulted from direct interaction of macrophages with fibroblasts because macrophages cultured alone did not synthesize DNA (Figures 14, 19; autoradiography results (data not shown)). Indirect evidence from other investigations supports this hypothesis. First, it has been shown that ingestion of effete cells or cellular debris causes murine peritoneal macrophages to proliferate in vitro (160). A similar phenomenon may have occurred in the present study because growing fibroblasts under low-serum conditions caused some cell death (Figure 3), and thus macrophage DNA synthesis may have been stimulated by ingested cellular debris. Second, lung fibroblasts (105, 119), certain fibroblast cell lines (105, 108, 109), and peripheral lung tissue cell lines (17, 146) produce factors that stimulate proliferation of peritoneal (108, 109, 148), bone marrow (119), and alveolar (17, 105, 146) macrophages. Hence, fibroblasts may have released similar proliferation
factors for macrophages in the present investigation. Third, activated human alveolar macrophages secrete colony stimulating factors (62), which have been suggested to be responsible for stimulating alveolar macrophage proliferation in vitro (148). Therefore, it is possible that there was autocrine stimulation of macrophage DNA synthesis by similar factors in the present study. However, because it has been shown that macrophage labeling indices may not necessarily correlate with the cell's mitotic activity (20), future studies must determine whether macrophages also divide in co-culture. Regardless of the mechanism, these findings suggest that direct interaction between macrophages and fibroblasts may be one possible means by which both cell populations expand during the pathogenesis of pulmonary fibrosis.

The therapeutic control of pulmonary fibrosis may lie in inhibiting the ability of macrophages to stimulate fibroblast proliferation. Although the classic therapy for interstitial lung disorders has been corticosteroids (34, 35, 36, 40), administration of these agents does not suppress the release of AMDGF by human alveolar macrophages (10, 96). In the present study, treatment of alveolar macrophages with cycloheximide or puromycin prior to co-cultivation resulted in inhibition of fibroblast DNA synthesis by as much as 66% (Table 13), indicating that protein synthesis by macrophages was necessary for sustained stimulation of fibroblast proliferation. In addition, this finding suggests that protein synthesis is required for continuous synthesis of AMFPF. Consistent with these findings are the facts that cycloheximide partially inhibits MDGF production and/or release by human monocytes (43), monocyte-derived macrophages (104), and alveolar macrophages (13), and that colchicine, a drug that modulates protein secretion by its effect on microtubules, inhibits AMDGF release from activated human alveolar macrophages (127). Although I did not use colchicine, I found that treatment of alveolar macrophages with cytochalasin B, another cytoskeletal disrupting agent, also inhibited the capacity of macrophages to stimulate fibroblast DNA synthesis in co-cultures (Table 13). Collectively, therefore, these findings demonstrated that use of pharmacological agents in vitro can inhibit the
proliferative response that ensues from direct interaction between alveolar macrophages and lung fibroblasts. Further studies, however, are required to investigate additional agents that may inhibit release of potential mediators of fibrosis.

Increased numbers of neutrophils and T lymphocytes in interstitial lung diseases, such as IPF, have been demonstrated in histological studies (35, 36, 142) and by cellular analysis of BAL fluid (35, 36, 71, 81, 131, 142). The contribution of these cells, however, to the pathogenesis of pulmonary fibrosis is unclear and controversial (142). For example, lymphocyte factors both stimulate (27, 156) and inhibit (44, 45, 100) DNA synthesis by fibroblasts. In the present study, the observation that peripheral blood lymphocytes directly stimulate a slight amount of DNA synthesis by fibroblasts in co-culture (Figures 21, 22, 23, 24) supports the contention that lymphocytes have a stimulatory role in fibroplasia. My findings are in general agreement with other investigators who have shown that peripheral blood T cells from both normal donors (27, 156) and IPF patients (27) also release a lymphokine that promotes proliferation of fibroblasts. Although the factor that induced DNA synthesis in lymphocyte/fibroblast co-cultures is unknown, it is possible that some monocytes, which are known to release MDGF (3, 41, 43, 85), contaminated the highly enriched lymphocyte populations, and thus were the cells responsible for stimulating fibroblast proliferation. Alternatively, it is known that lymphocytes produce I-interferon (80, 117, 133), a factor recently shown to stimulate lung fibroblast proliferation via its progression activity (80). Therefore, it is also possible that lymphocyte-derived I-interferon was responsible for stimulating DNA synthesis in lymphocyte/fibroblast co-cultures. In addition, the progression activity of a factor such as I-interferon in combination with the competence activity of AMFPF, may explain why DNA synthesis in co-cultures containing both lymphocytes and macrophages was greater than in co-cultures containing each of these cell types alone with fibroblasts, i.e., the effect was more than additive (Figures 22, 23). Cooperative interaction between lymphocytes and
Macrophages in stimulating fibroblast proliferation has also been suggested by Wahl (156) and Kovacs and Kelley (94). Finally, the inability of neutrophils to stimulate fibroblast proliferation in co-culture (Figures 21, 23) confirms and extends earlier findings that showed neutrophil culture supernatants did not enhance DNA synthesis by foreskin fibroblasts (41). Thus, it appears that macrophages and lymphocytes each can regulate fibroblast proliferation, whereas neutrophils cannot.

In conclusion, the in vitro model developed in this study has demonstrated that rat alveolar macrophages release a factor(s) that stimulates proliferation of "normal" lung fibroblasts, and that the proliferative effect is enhanced when fibroblasts are in direct contact with macrophages. In addition, the direct interaction of macrophages and fibroblasts also stimulates DNA synthesis by macrophages. It has also been determined that lymphocytes may cooperate with macrophages in stimulating lung fibroblast proliferation. Furthermore, use of certain pharmacologic agents can inhibit the macrophage's capacity to stimulate fibroblast proliferation. Collectively, these in vitro observations provide additional evidence to support the concept that alveolar macrophages may be important integrators of pulmonary fibrosis by stimulating fibroblast proliferation, which leads to increased elaboration of collagen. Moreover, the direct interaction between macrophages and fibroblasts in the inflamed lung may play an important role in the expansion of numbers of both of these cell types - a feature that characterizes many fibrotic lung diseases. Future studies need to determine the biochemical properties of AMFPF, and its relationship to other characterized growth factors. Finally, the mechanisms by which cell contact influences fibroblast proliferation and how lung fibroblasts stimulate macrophage DNA synthesis are important topics that need to be investigated.
LIST OF REFERENCES


