# Role of Fibronectin as a Growth Factor for Fibroblasts

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ABSTRACT    Fibroblast replication is regulated by exogenous signals provided by growth factors, mediators that interact with the target cell surface and signal the cell to proliferate. A useful model of growth regulation, the "dual control model," suggests that growth factors can be grouped either as competence factors or as progression factors, and that optimal replication of fibroblasts requires the presence of both types of growth factors. Although most growth factors are soluble mediators, recent studies have demonstrated that, for some cell types, the extracellular matrix can replace the requirement for a competence factor. Since fibronectin is an important constituent of the extracellular matrix that interacts with specific domains on the fibroblast surface, we examined the ability of fibronectin to act as a competence factor to promote the growth of human diploid fibroblasts. To accomplish this, fibronectins purified from two sources, human plasma and human alveolar macrophages, were tested for their ability to (a) stimulate fibroblast replication in serum-free medium containing characterized progression factors (insulin or alveolar macrophage-derived growth factor); (b) provide a growth-promoting signal early in $G_1$. Fibronectin stimulated fibroblast replication in a dose-dependent manner in the presence of a fixed dose of a progression factor. Conversely, fibronectin conferred on previously unresponsive fibroblasts the ability to replicate in a dose-dependent manner when cultured with increasing amounts of a progression factor. Moreover, fibronectin signaled growth-arrested fibroblasts to traverse $G_1$ ~4 h closer to S phase. No differences were observed in the ability of plasma or macrophage fibronectins to provide a competence signal for fibroblast replication. Since fibronectin is a major component of the extracellular matrix, these observations suggest that it may provide at least one of the signals by which the matrix conveys the "competence" that permits fibroblasts to replicate in the presence of an appropriate progression signal.

Fibroblast replication requires exogenous growth factors, mediators acting with temporal specificity in the $G_1$ phase of the cell cycle to promote DNA synthesis and cell replication (1–5). Analysis of this temporal specificity has led to the hypothesis that there are two major categories of growth factors, competence factors, and progression factors (5, 6). Competence factors, such as fibroblast growth factor or platelet-derived growth factor, act early in $G_1$, rendering the cell responsive, for a variable number of hours, to progression factors (7). Progression factors, such as insulin, insulin-like growth factors, and alveolar macrophage-derived growth factor, act later in $G_1$, signaling the cell to continue through the cell cycle and replicate (7, 8). Both categories of growth factors are thought to provide their signals to the target cell by interaction with specific cell surface receptors. Moreover, as

a general principle, cells require a signal from both competence factors and progression factors to manifest an optimal replication rate.

Normal fibroblast replication requires, in addition to soluble factors, that the cells attach to an extracellular matrix (9). Interestingly, studies by Gospodarowicz et al. (10–12) have suggested that, in addition to providing attachment sites for cells, the extracellular matrix can, at least for some cells, act to replace the requirement for growth factors such as fibroblast growth factor or platelet-derived growth factor. Since both fibroblast growth factor and platelet-derived growth factor are competence factors, these observations suggest that the overall requirements for fibroblast growth may be met by a progression factor together with the extracellular matrix serving both as a competence factor and as a site for attachment.

In this context, we have hypothesized that fibronectin, a glycoprotein component of the extracellular matrix with specific domains that bind to the fibroblast surface and to specific regions of various components of the extracellular matrix (13-15), can act as a competence factor. This report demonstrates that fibronectin is a competence factor by two criteria: (a) the ability of fibronectin to promote fibroblast replication in a serum-free complementation test with known progression factors; and (b) the action of fibronectin early in the temporal sequence of $G_1$.

## MATERIALS AND METHODS
### Preparation of Growth Factors

Plasma Fibronectin. Fibronectin was purified from human plasma by gelatin sepharose affinity chromatography as described by Engvall and Ruoslathti (16), followed by gel filtration. Briefly, 100 ml of peripheral blood were collected by venipuncture and anticoagulated with EDTA. Cells were separated from plasma by centrifugation, and the plasma was applied to a gelatin-sepharose column. Fibronectin was eluted from the column with 6 M urea followed by further purification of a 5-ml aliquot of the peak concentration (~2 mg/ml) on a Sephadex G-150 column. The fibronectin content of each fraction was quantified by an enzyme-linked immunosorbent assay as previously described (17). To reduce the probability that the fibronectin was contaminated by trace amounts of other growth factors found in plasma, some fibronectin preparations were purified by gelatin-sepharose followed by heparin-agarose chromatography. The gelatin-sepharose column was eluted with 4 M urea, and the eluate was applied directly to a heparin-agarose column followed by elution with 0.5 M NaCl in 60 mM Tris-HCl, pH 7.4, 5 mM EDTA, as described by Hayashi and Yamada (18).

All fibronectin samples were adjusted to a fibronectin concentration of ~1 mg/ml with 8 M urea, and stored in aliquots in liquid nitrogen vapor. As needed, an aliquot of fibronectin was thawed, dialyzed 1:500 against Dulbecco's modified Eagle medium (DME[1]; Biofluids, Rockville, MD.) for two changes at 22°C. For each sample, fibronectin was quantified by an enzyme-linked immunosorbent assay following dialysis.

Alveolar Macrophage Fibronectin: To purify alveolar macrophage fibronectin, ~50 × 10⁶ alveolar macrophages, obtained by bronchoalveolar lavage of patients with fibrotic lung disorders, were cultured for 24 h at 37°C in an atmosphere of 95% air-5% $CO_2$ in RPMI 1640 medium (Biofluids) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml) (19). Resultant supernatants were dialyzed against 50 mM NaCl, 20 mM Tris HCl, pH 7.4, 2 M urea, at 22°, and then chromatographed on DEAE cellulose (Whatman Laboratory Products, Inc., Clifton, NJ) in the same buffer with a NaCl gradient from 50 to 300 mM. To identify the fractions containing fibronectin, an aliquot of each fraction was dialyzed (1:500) against 150 mM NaCl, 20 mM Tris HCl, pH 7.4, and fibronectin content was determined by an enzyme-linked immunosorbent assay. Fibronectin-containing fractions were stored in liquid nitrogen vapor. Prior to use in growth experiments, the DEAE-purified macrophage fibronectin was dialyzed 1:500 against DME and assayed for fibronectin content by an enzyme-linked immunosorbent assay.

Alveolar Macrophage-derived Growth Factor (AMDGF): AMDGF was purified from the supernatants of alveolar macrophages obtained by bronchoalveolar lavage of normal individuals as previously described (8). The macrophages were activated by surface adherence and then cultured (24 h, 37°C) in DME supplemented with pencillin/streptomycin. The AMDGF was partially purified from the macrophage supernatants by ion exchange chromatography followed by gel filtration. Briefly, 600 ml of macrophage supernatants, containing 2.1 mg of macrophage secretory protein (determined by a colorimetric assay [Bio-Rad Laboratories, Richmond CA] using BSA [Sigma Chemical Co., St. Louis, MO] as a standard), was chromatographed on DEAE cellulose and eluted at 4°C with a NaCl gradient (150-300 mM) buffered with 20 mM Tris-HCl, pH 7.0. The peak of bioactivity (at ~270 mM) was detected by its ability to promote the replication of human lung fibroblasts (8). This peak was lyophilized, resuspended in PBS, then chromatographed on Sephadex G-50 in PBS, pH 7.4, at 4°C. The peak of activity eluted at 18,000 daltons, and was stored in liquid nitrogen vapor at a concentration of 1.2 μg/ml. Serial dilutions

of the pooled peak demonstrate the half-maximal growth response at a dilution of $10^{-4}$ with the maximum response at a dilution of $10^{-2}$. This represented an approximately 300- to 400-fold purification.

Other Growth Factors: Pituitary fibroblast growth factor (FGF) was obtained from Collaborative Research Inc. (Waltham, MA) and insulin from Sigma Chemical Co. Platelet-derived growth factor (PDGF) was partially purified as described by Vogel et al. (20).

## Evaluation of Plasma Fibronectin as a Growth Factor

All experiments were performed on HFL-1 fibroblasts (American Type Culture Collection CCL 153), previously frozen in liquid nitrogen vapor at the tenth to twelfth population doubling. Cells were cultivated on 100-mm tissue culture dishes (Falcon Labware, Cockeysville, MD) in DME supplemented with 10% calf serum (Colorado Serum, Denver, CO) as previously described (21), and used for growth studies between the fifteenth and twentieth population doubling.

To evaluate the effect of plasma fibronectin on fibroblast replication in a defined medium, the complementation test of Stiles et al. (7) was adapted for serum-free conditions. To accomplish this, fibroblasts were cultured on 35-mm dishes (Falcon Labware, 5 × 10⁴ cells/dish) in DME plus 0.4% serum for 3 d. Cells were then rinsed three times with serum-free DME followed by cultivation for 1 d in "complementation test medium" (DME + 5 μg/ml transferrin (Sigma Chemical Co. plus 0.1% BSA [Sigma catalogue 7888]). Under these conditions, fibroblasts did not replicate without subsequent addition of growth factors. For convenience, fibroblasts cultured for 3 d in serum-free DME + 0.4% serum followed by culture for 1 d in "complementation test medium" will be referred to as nonreplicating fibroblasts.

The basic principle of the complementation test is that nonreplicating fibroblasts require both a competence factor and a progression factor for an optimal replication rate. Thus, to evaluate the role of plasma fibronectin as a growth factor, fibronectin was added to cultures of nonreplicating fibroblasts in combination with either a characterized competence factor (FGF) or a characterized progression factor (insulin or AMDGF), and fibroblast growth was assessed. To accomplish this, complementation test medium containing each growth factor individually or plasma fibronectin in combination with either FGF, insulin, or AMDGF (at the concentrations indicated in the text) was placed on nonreplicating fibroblasts, and the cultures were continued at 37°C. Cell counts were determined daily for 4 d with an electronic particle counter (Coulter Electronics, Hialeah, FL). Cell counts in complementation test medium alone served as negative controls. As a positive control, in each experiment FGF was added to nonreplicating fibroblasts with either insulin or AMDGF to verify a synergistic increase in cell number.

Since Clemmons (22) has demonstrated that confluent fetal fibroblasts can release their own competence factor(s), the nonreplicating HFL-1 cells at low density used for the bioassay were evaluated for production of competence factor(s) that might obscure the effect of exogenously added growth factors. To accomplish this, fibroblasts were seeded at low density in DMEM plus 0.4% serum for 3 d and rinsed as described above, then cultured in complementation test medium for 1, 2, 3, or 4 d, and the "conditioned medium" was collected each day. Culture of nonreplicating fibroblasts (37°C, 3-5 d) with conditioned medium (undiluted, 1:2, 1:4, 1:8) alone, or supplemented with insulin (10 μg/ml) or AMDGF (10 ng/ml), caused no increase in cell number above control. Thus, under the conditions used, exogenously added factors were necessary to provide competence.

One potential problem with attributing growth-promoting properties to fibronectin was the possibility that the preparation was contaminated with small amounts of PDGF. Since PDGF is heat stable (23), a fibronectin preparation (1 μg/ml) with or without supplemental PDGF (100 ng/ml), was boiled (100°C, 10 min) and then evaluated in a complementation test as described above.

To determine the effect of plasma fibronectin on the replication of fibroblasts that had been growth arrested by density inhibition rather than serum deprivation, confluent monolayers of fibroblasts were cultured with growth factors and the effect on DNA synthesis was assessed. To accomplish this, fibroblasts were seeded (5 × 10⁴/well in 0.25 ml DME plus 10% calf serum) onto 8-well glass slides (Lab-Tek, Miles Laboratories, Naperville IL) and cultured in DME plus 10% calf serum with the medium changed every other day until the cells were confluent and no mitotic cells were observed by phase contrast microscopy. The confluent cells were cultured one additional day without a medium change, rinsed three times with serum-free DME, and then cultured (30 h, 37°C) in complementation test medium containing 0.1 μCi/ml [³H]thymidine (2 Ci/mMol, Amersham Corp., Arlington Heights, IL) plus each growth factor individually, or both a competence factor (PDGF, FGF, or fibronectin at the

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[1] *Abbreviations used in this paper:* AMDGF, alveolar macrophage-derived growth factor; DME, Dulbecco's modified Eagle medium; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor.

concentrations indicated in the text) and a progression factor (AMDGF, 10 ng/ml or insulin, 10 µg/ml). After this interval, cells were fixed in methanol:acetic acid (3:1) and processed for autoradiography was previously described (8). DNA synthesis in complementation medium alone served as a control. Each condition was performed in quadruplicate with >200 nuclei counted per well.

## Evaluation of Macrophage Fibronectin as a Growth Factor

Because of the difficulty of preparing sufficient amounts of macrophage fibronectin to perform the cell counting assay, the ability of macrophage fibronectin to act as a growth factor for fibroblasts was studied in a microassay using DNA synthesis rather than cell number as an index of fibroblast replication. The complementation test was performed in a way analogous to that described above except that the nonreplicating fibroblasts were cultured in microtiter wells (10⁴ fibroblasts/well, 96-well plates; Falcon Labware) in 0.2 ml of complementation test medium with or without exogenous growth factors. After a 14-h incubation at 37°C, each well was pulsed with [³H]thymidine (0.1 µCi/ml; 2 Ci/mMol, Amersham Corp.). After an additional 24-h incubation, acid-precipitable [³H]thymidine incorporation was determined by scintillation counting. [³H]Thymidine incorporation in the absence of added growth factors (1100 ± 300 dpm/10⁴ fibroblasts) was subtracted from the values in the presence of growth factors to correct for the [³H]thymidine incorporation unrelated to growth factor addition.

## Ability of Fibronectin to Signal Fibroblasts to Respond to Progression Factors

To evaluate the ability of fibronectin to signal fibroblasts to replicate in response to signals from progression factors, plasma fibronectin was added to fibroblasts suspended in complementation test medium at the time of seeding and then removed prior to addition of progression factors. To accomplish this, 35-mm tissue culture dishes were coated with collagen (prepared from lathyritic rat skins, the gift of H. K. Kleinman, National Institute of Dental Research) as previously described, and sterilized by exposure to ultraviolet irradiation. Fibroblasts were prepared by incubation with trypsin (Gibco Laboratories, Grand Island, NY 0.25%; 1 min, 22°C) to detach them from culture dishes, washed once in DME plus soybean trypsin inhibitor to inactivate the trypsin, washed three times in complementation test medium, and resuspended in that same medium in the presence or absence of plasma fibronectin (1.0 µg/ml). Fibroblasts with or without added fibronectin were then seeded at a density of 5 × 10⁴ cells/collagen-coated dish. After incubation overnight (14 h, 37°C), the fibroblasts were rinsed three times with DME to remove unbound fibronectin. Cell counts were then determined to confirm equal plating efficiency. The fibroblasts were then cultured for three d with either FGF, insulin, or AMDGF at the concentrations indicated. At the end of the 3-d culture, the number of cells was counted to determine the growth response.

## Cell Cycle Kinetics

Previous studies have indicated that competence factors act early in the G₁ phase of the cell cycle of nonreplicating BALB/C 3T3 cells (5). To determine where, in the temporal sequence of G₁, fibronectin provided its growth-promoting signal to lung fibroblasts, the kinetics of fibroblast DNA synthesis after pretreatment with fibronectin was determined. Nonreplicating fibroblasts (10⁴ cells/well, Falcon 96-well microtiter dishes, 0.2 ml of complementation test medium per well) were cultured for 12 h with (a) no further additions; (b) fibronectin (1.0 µg/ml); or (c) FGF (2 ng/ml). Following this 12-h incubation, calf serum was added to a final concentration of 10% (i.e., to provide the fibroblasts with a complete set of signals to replicate and thus to determine whether the pretreatment with fibronectin shifted the time of onset of DNA synthesis). [³H]Thymidine (0.5 µCi/ml, 2 Ci/mMol, Amersham Corp.) was added with the serum. At the time of serum and [³H]thymidine addition, and every 2 h for the subsequent 18 h, triplicate wells of fibroblasts were harvested for measurement of acid-precipitable [³H]thymidine incorporation. To establish the background for each time point in the assay, triplicate wells of fibroblasts were pretreated as described above and then cultured with [³H]thymidine but without serum. The background was then subtracted from the experimental time points. These background values ranged from 200 ± 75 dpm/10⁴ cells at 2 h to 900 ± 125 dpm/10⁴ cells at 18 h.

## Statistical Evaluation

Each condition for the cell counting experiments was performed in duplicate, with replicates varying by <7%. Each condition in the [³H]thymidine incor-

poration experiments was performed in triplicate. Experimental values were compared using a two-tailed Student's t-test. The data presented are the mean ± standard deviation of three experiments using fibroblasts with nearly identical tissue-culture histories.

## RESULTS

### Fibroblast Replication in Response to Plasma Fibronectin

Nonreplicating lung fibroblasts cultured in complementation test medium containing fibronectin and insulin were stimulated to replicate (Fig. 1). When fibroblasts were cultured for 3 d in DME plus 0.4% serum followed by 1 d in complementation test medium, they entered a nonreplicating state, maintaining constant cell numbers for an additional 5 d unless growth factors were added. In the example shown, cultivation of the nonreplicating fibroblasts for 5 d in complementation test medium maintained cell number at 15 × 10⁴ fibroblasts/dish. Each growth factor individually caused less than a 20% increase in cell number by the fifth day after growth factor addition (data not shown). The addition of fibronectin (0.2 µg/ml) and a competence factor (FGF, 1.0 ng/ml) to the nonreplicating fibroblasts resulted in only a slight stimulation of fibroblast replication, suggesting that fibronectin was not a progression factor. Within 2 d there were 17 × 10⁴ cells/dish, and by the fifth day after growth factor addition there were 18 × 10⁴ cells/dish, only a 20% increase in cell number. In contrast, the addition of fibronectin (0.2 µg/ml) and progression factor (insulin, 5 µg/ml) to nonreplicating fibroblasts markedly stimulated fibroblast replication, suggesting that fibronectin was a competence factor. In this regard, there were 32 × 10⁴ cells/dish by the fifth day after growth factor addition, representing an 110% increase in cell number.
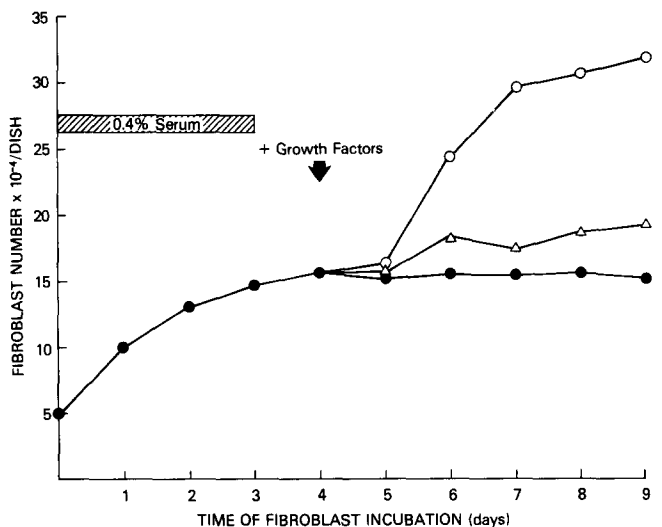


Figure 1 Growth of nonreplicating fibroblasts in response to plasma fibronectin. Fibroblasts were cultured in DME + 0.4% serum until day 3, and then in complementation test medium until day 4. Growth factors, in fresh complementation test medium, were added to these nonreplicating fibroblasts on day 4 (arrow). Shown is the number of fibroblasts in the presence of fibronectin (0.2 µg/ml) + insulin (5.0 µg/ml, O); fibronectin (0.2 µg/ml) + FGF (1.0 ng/ml, △); or in complementation test medium without added growth factors (●).

## Dose Dependence of the Stimulation of Fibroblast Replication by Plasma Fibronectin

Nonreplicating fibroblasts cultured in complementation test medium manifested a dose-dependent increase in replication rate in response to fibronectin only when a progression factor (i.e., insulin or AMDGF) was also present (Fig. 2). The addition of fibronectin alone to the fibroblast cultures resulted in a modest increase in cell number. Furthermore, the addition of fibronectin and a fixed dose of a competence factor (FGF, 2.0 ng/ml) to the fibroblast cultures resulted in little further increase in cell number. In contrast, the addition of fibronectin along with a fixed dose of a progression factor (either insulin [10 μg/ml] or AMDGF, [10 ng/ml]) resulted in a marked increase in cell number up to ~170% above control with a fibronectin concentration of 2.0 μg/ml. This pattern of growth-promoting activity indicated that fibronectin acted as a competence factor.

In view of these findings, it was important to demonstrate that the dose-dependent competence signal provided by fibronectin was due to the fibronectin itself and not a contaminant of the fibronectin preparation. To exclude the possibility that part of the observed growth response was due to a contaminant of the fibronectin preparation, fibronectin was purified by two methods: gelatin-sepharose affinity chromatography followed by (a) gel filtration chromatography; or (b) heparin-agarose affinity chromatography. The results with both preparations were identical, suggesting that fibronectin and not a contaminant was responsible for the growth-promoting effect.

In addition, to rule out the possibility that PDGF (a heat-stable competence factor with high positive charge) might still be contaminating the preparation, the heat stability of the fibronectin-induced growth response was assessed. Boiling the fibronectin resulted in complete loss of activity in the assay described above. Supplementing the fibronectin with authentic PDGF (100 ng/ml) followed by boiling resulted in that amount of fibroblast growth observed with unboiled PDGF (data not shown). Therefore, contamination of our fibronectin with PDGF did not account for the growth-promoting effects observed.

Fibronectin not only provided a competence signal to fibroblasts that had been growth arrested at low density by serum deprivation, but also provided a dose-dependent competence signal to fibroblasts arrested in $G_1$ by culture to saturating density (Table I). When confluent fibroblasts were cultured with the characterized competence factors, PDGF or FGF, in the presence of a progression factor (AMDGF, 10 ng/ml or insulin, 10 μg/ml) there was a dose-dependent increase in DNA synthesis. The maximum response ranged from 26 to 33% labeled nuclei representing ~50 to 75% of the DNA synthesis produced by the addition of 10% calf serum. Similarly, the addition of fibronectin to the density-arrested fibroblasts in the presence of either AMDGF or insulin resulted in a significant though smaller increase in fibroblast DNA synthesis ($p < .01$, compared with media alone). The maximum DNA synthesis in response to fibronectin was 16% labeled nuclei representing about one-third of the response to 10% calf serum. In contrast, DNA synthesis in response to each factor individually was far less, ranging from 1 to 7%. In addition, only for PDGF and FGF individually was there a small dose-related increase in nuclear labeling.

## Fibroblast DNA Synthesis in Response to Macrophage Fibronectin

When nonreplicating fibroblasts were incubated in the presence of a progression factor there was an increase in the rate
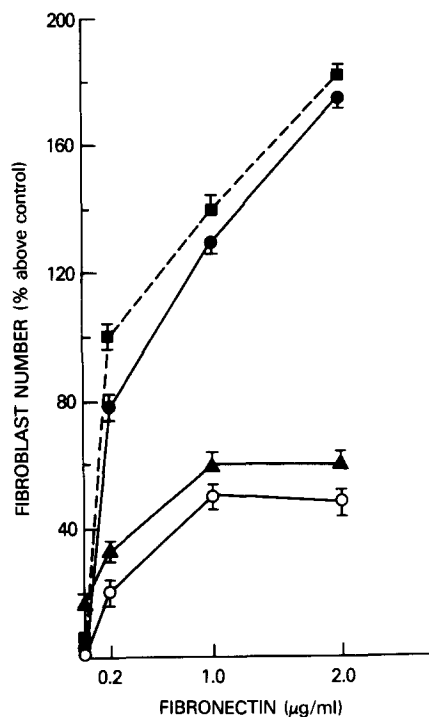


FIGURE 2  Dose dependence of the stimulation of fibroblast replication by plasma fibronectin. Nonreplicating fibroblasts were cultured with increasing amounts of fibronectin and either a progression factor or a competence factor. The resultant increase in fibroblast number is expressed as the percent increase in cell number above control. Shown is the effect of fibronectin on fibroblast replication alone (○); in the presence of the progression factor AMDGF (10 ng/ml, ■); in the presence of the progression factor insulin (10 μg/ml, ●); or in the presence of the competence factor FGF (2.0 ng/ml, ▲).

TABLE I
Evaluation of the Effect of Fibronectin on Proliferation of Density-arrested Fibroblasts

| Growth factor present | Nuclei Labeled* | | |
|---|---|---|---|
| | No added factors | +Insulin[‡] | +AMDGF[§] |
| | % | % | % |
| None | 1 ± 1 | 2 ± 1 | 1 ± 1 |
| PDGF (ng/ml) | | | |
| 1 | 2 ± 1 | 5 ± 3 | 6 ± 2 |
| 10 | 3 ± 2 | 16 ± 3 | 18 ± 3 |
| 100 | 7 ± 2 | 26 ± 4 | 21 ± 3 |
| FGF (ng/ml) | | | |
| 1 | 3 ± 2 | 12 ± 4 | 15 ± 2 |
| 10 | 5 ± 3 | 26 ± 3 | 27 ± 5 |
| 100 | 6 ± 2 | 31 ± 5 | 33 ± 7 |
| Fibronectin (μg/ml) | | | |
| 0.1 | 3 ± 3 | 3 ± 2 | 5 ± 3 |
| 1.0 | 4 ± 2 | 11 ± 1 | 10 ± 2 |
| 10 | 4 ± 3 | 16 ± 4 | 14 ± 5 |

* Data presented as percent fibroblasts incorporating [³H]thymidine into nuclei during 30 h, 37°C; mean ± SD of three experiments. In the presence of medium + 10% calf serum, the percent nuclei labeled = 45 ± 6%.
‡ Insulin = 10 μg/ml.
§ AMDGF = 10 ng/ml.

of DNA synthesis in response to increased amounts of macrophage fibronectin (Fig. 3). As observed with plasma fibronectin, there was a macrophage fibronectin dose-dependent increase in the fibroblast growth response in the presence of either insulin (10 µg/ml) or AMDGF (10 ng/ml). The result was similar to that observed when [³H]thymidine incorporation was used as the index of fibroblast replication in response to plasma fibronectin in the presence of AMDGF or insulin (data not shown). Comparison of the dose-response relationships between macrophage fibronectin and plasma fibronectin revealed that 50% of the maximum response occurred within a twofold concentration range of fibronectin (80 ± 15 ng/ml for macrophage fibronectin and 200 ± 23 ng/ml for plasma fibronectin), indicating that there was not a major difference in growth-promoting potency of the fibronectins from these two different sources. Thus, both plasma and macrophage fibronectin provided a dose-dependent competence signal for lung fibroblasts.

## Ability of Fibronectin to Signal Fibroblasts to Respond to Progression Factors

Nonreplicating fibroblasts seeded onto collagen-coated dishes in the presence of fibronectin manifested a rapid rate of replication after the addition of a progression factor (Fig. 4). Under the conditions used, fibroblasts seeded onto collagen-coated dishes in complementation test medium had similar plating efficiencies in the absence or presence of exogenous fibronectin (1.0 µg/ml). When fibroblasts were allowed to adhere in the absence of fibronectin, they did not replicate when a progression factor (insulin or AMDGF) was added. In marked contrast, when fibroblasts were allowed to adhere in the presence of fibronectin, there was a dose-related increase in replication rate after addition of insulin or AMDGF. For example, at an insulin concentration of 5 µg/ml, cell number increased 93% above control and at 50 µg/ml cell number increased 125% above control. Addition of AMDGF resulted in a similar stimulation of cell replication, with an 80% increase in cell number above control at a concentration of 0.1 ng/ml and a 140% increase at an AMDGF concentration of 10 ng/ml. Thus, fibronectin was able to provide a signal to fibroblasts that allowed them to replicate in response to a progression signal.

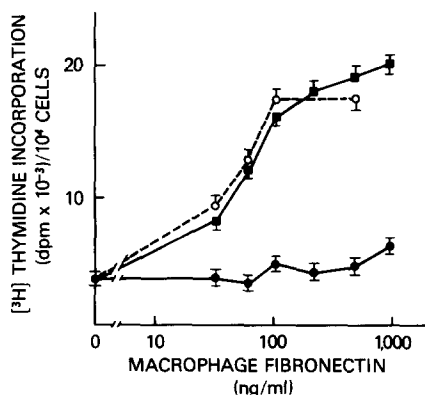Further confirmation that the growth-promoting effect of

FIGURE 3   Dose dependence of the stimulation of fibroblast DNA synthesis by macrophage fibronectin. Nonreplicating fibroblasts were cultured with increasing amounts of fibronectin and a progression factor. Shown is fibroblast DNA synthesis in response to fibronectin alone (●), or in the presence of the progression factors insulin (10 µg/ml, ■), or AMDGF (10 ng/ml, ○).
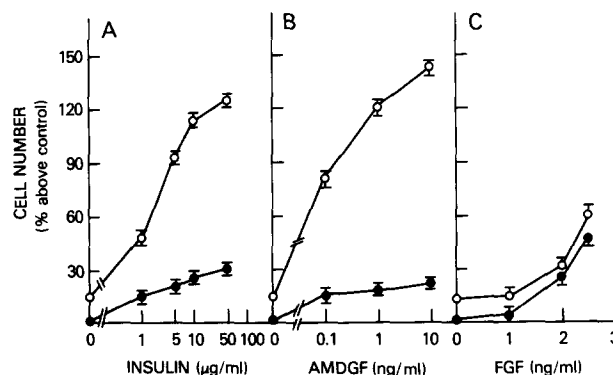
FIGURE 4   Ability of plasma fibronectin to signal fibroblasts to respond to progression factors. Fibroblasts in suspension culture were added to collagen-coated dishes in the presence or absence of fibronectin (1.0 µg/ml) and then cultured for 14 h. The plates were rinsed three times to remove unbound fibronectin, exposed to different concentrations of progression factors in fresh complementation test medium, and the cultures continued for an additional three days. Resultant effects on fibroblast growth are expressed as the percent increase in cell number above control. (A) Growth of fibroblasts in the presence of the progression factor insulin: O, cells exposed to fibronectin prior to insulin addition; ●, no fibronectin exposure prior to insulin addition. (B) Growth of fibroblasts in the presence of the progression factor alveolar macrophage-derived growth factor (AMDGF) with (O) or without (●) prior exposure to fibronectin. (C) Growth of fibroblasts in the presence of the competence factor fibroblast growth factor (FGF) with (O) or without (●) prior exposure to fibronectin.

fibronectin for fibroblasts was not due to a progression signal was provided by the absence of a dose-related increase in fibroblast replication in the presence of FGF, a known competence factor (Fig. 4C). Seeding fibroblasts in the presence of fibronectin did not enable FGF to substantially augment their rate of replication compared with fibroblasts seeded in the absence of fibronectin, suggesting that fibronectin was not providing a progression signal. As a positive control, fibroblasts exposed to FGF (1–2.5 ng/ml) in the presence of insulin (5 µg/ml) or AMDGF (10 ng/ml) demonstrated a significant increase in fibroblast replication, an effect that was not substantially augmented by the addition of fibronectin (1.0 µg/ml) to the cultures (data not shown).

## Evaluation of the Position of Action of Fibronectin in the G₁ Phase of the Cell Cycle

Evaluation of the timing of DNA synthesis under various conditions demonstrated that fibronectin acted as a competence factor by providing a growth-promoting signal early in G₁ (Fig. 5). Under the conditions used in these studies, without the addition of growth factors, nonreplicating lung fibroblasts were arrested in the G₁ phase of the cell cycle, about 12–14 h before the onset of S phase. Nonreplicating fibroblasts cultured for 12 h with the competence factor FGF (2.0 ng/ml) traversed about one-third of the remaining interval of G₁, arresting 8–10 h from S phase. Within the framework of this temporal sequence of G₁, fibronectin (1.0 µg/ml) also signalled nonreplicating fibroblasts to traverse one-third of G₁, with DNA synthesis beginning 8–10 h after serum addition. In contrast, incubation of fibroblasts for 12 h with insulin or AMDGF prior to the addition of serum resulted in no traversal of G₁, i.e., there was still a 12–14-h lag for DNA synthesis, a value similar to that for cells in complementation test medium alone (data not shown).
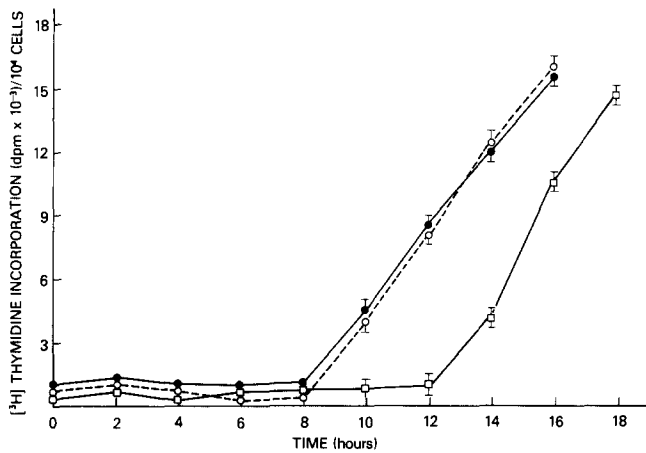
FIGURE 5 Effect of plasma fibronectin on the cell cycle kinetics of nonreplicating fibroblasts. Nonreplicating fibroblasts were cultured for 12 h with or without added growth factors followed by addition of serum and [³H]thymidine. Shown is [³H]thymidine incorporation into DNA by fibroblasts pretreated for 12 h with complementation test medium alone (□), fibronectin (●; 1.0 μg); or FGF (○; 2.0 ng/ml). Data are expressed as disintegrations per minute per 10⁴ cells as a function of time after addition of serum.

## DISCUSSION

Fibronectin, at concentrations in the nanomolar range, can, under the appropriate circumstances, stimulate nonreplicating human lung fibroblasts to replicate in a dose-dependent manner. In this context, fibronectin is able to complement the growth-promoting effects of the characterized progression factors, insulin and AMDGF, by providing a competence signal to nonreplicating fibroblasts. In providing this competence signal, fibronectin stimulates nonreplicating fibroblasts to traverse $G_1$ ~4 to 6 h. Thus, at least for diploid human fibroblasts, replication can be induced in serum-free conditions by a signal from the extracellular matrix component, fibronectin, plus an additional signal provided by a progression factor.

### Role of Extracellular Matrix in Growth Control

There are several lines of evidence that extracellular matrix elements can provide growth promoting signal(s) to parenchymal cells. For example, in addition to stimulating the replication of human lung fibroblasts as shown in the present study, fibronectin augments the replication rate of chick heart fibroblasts (24) and human dermal fibroblasts (25). In addition, type IV collagen, a basement membrane component intimately associated with the basal surface of epithelial cells, promotes the replication of rat mammary epithelial cells (26). Furthermore, bovine corneal endothelial extracellular matrix, a highly ordered collection of several matrix elements, promotes the replication of bovine vascular endothelial cells (10). Thus, extracellular matrix components have a role in attaching and orienting parenchymal cells within an organ, and in addition there is increasing evidence that at least some of these components are able to provide a signal for cellular replication.

### Separation of the Role of Fibronectin in Fibroblast Replication from its Role in Fibroblast Attachment and Spreading

Although attachment and spreading are important prerequisites for fibroblast replication, and fibronectin clearly

can mediate cell attachment and spreading (27, 28), three lines of evidence suggest that fibronectin provides a signal that stimulates cell replication beyond its ability to mediate attachment and spreading, i.e., fibronectin is able to provide a competence signal directing cell replication that is not provided by the process of attachment and spreading.

First, under the conditions employed in this study, normal human lung fibroblasts attached to collagen-coated dishes in equal numbers in the presence or absence of exogenous fibronectin. However, only fibroblasts exposed to exogenously added fibronectin were competent to replicate in response to progression factors. This finding suggests that attachment alone, perhaps mediated by endogenously produced fibronectin, by residual cell surface fibronectin, or by other cell surface components (29), is not sufficient to confer responsiveness to progression factors.

Second, consistent with our observations, Hall and Ganguly (25) have shown that, in serum-free medium, dermal fibroblasts attach to tissue culture plastic but do not replicate. However, the addition to plasma fibronectin to these attached cells promotes both spreading and replication in a dose-dependent manner. While this observation does not dissociate the effects of fibronectin on spreading and replication, it clearly provides further evidence that attachment is not sufficient to promote fibroblast replication.

Third, dissociation of the effect of fibronectin on cell spreading from the competence signal for replication is provided by our observation that fully spread lung fibroblasts cultured for several days but not exposed to fibronectin are unresponsive to progression factors. However, when exogenous fibronectin is added, the fibroblasts respond to the progression factors and replicate.

### Mechanisms of Modulation of Cellular Replication by the Extracellular Matrix

For the process of fibroblast replication, the dual control model of Pledger and co-workers (6) suggests that most growth factors can be classified as competence factors or progression factors according to their position of action within the temporal sequence of $G_1$. Although this is still a working model that has not been completely validated, it emphasizes the importance of cell cycle specific signals in the control of cell replication and thus provides a useful framework in which to conceptualize growth signals. Competence factors, such as FGF, act early in $G_1$ and need be present for only a few hours to render a cell responsive to a subsequent progression signal. Progression factors, such as the insulin-like growth factors and AMDGF, provide a signal later in $G_1$ that, under the appropriate conditions, allows the cell to proceed to DNA synthesis. In this model, both a competence signal and a progression signal are necessary for significant cell replication (7, 30).

While the precise mechanisms of action of either competence or progression factors are not known, in principle, they may act directly by initiating the sequence of events leading to replication, or indirectly by stimulating the target cell to produce their own growth factors. With regard to progression factors, several defined signals are capable of stimulating human fibroblasts to produce insulin-like growth factors (31, 32), which are active at nanomolar concentrations as progression factors. Evaluation for an analogous inducible production of competence factors by fibroblasts has not been described. However, Clemmons (22) has demonstrated that

confluent monolayers of human fibroblasts release compe-
tence factor(s) into the medium constitutively. This raises the
theoretical consideration that nonreplicating fibroblasts (low
density or confluent) might be signalled to divide by a growth
factor that stimulates the target cell to produce its own com-
petence factor.

Within this framework, the present study demonstrates that
fibronectin acts as a competence factor by providing a signal
early in $G_1$, either directly or indirectly, permitting human
lung fibroblasts to proliferate in response to progression fac-
tors. Fibronectin provides this competence signal to fibro-
blasts that have been arrested in $G_1$ either by serum depriva-
tion or by growth to saturating densities. However, while
fibronectin is as potent as the well characterized competence
factors FGF and PDGF with regard to stimulating the repli-
cation of fibroblasts arrested by serum deprivation, it clearly
was not so potent as FGF or PDGF in stimulating DNA
synthesis by density-arrested fibroblasts. This difference in
potency, depending upon the method used to arrest fibroblasts
in $G_1$, distinguishes fibronectin from other competence factors
such as FGF or PDGF.

While the applicability of the dual control model has not
been directly assessed in cells other than fibroblasts, the
concept that extracellular matrix can provide a specific signal
for cellular replication is also in accord with the work of
Gospodarowicz and Ill (10). These workers demonstrated that
the requirement of bovine vascular endothelial cells for the
competence factor, FGF, was eliminated by culturing these
cells on an extracellular matrix produced by bovine corneal
endothelial cells. Interestingly, however, in contrast to the
situation with lung fibroblasts, fibronectin does not provide a
good competence signal to vascular endothelial cells when
compared with intact extracellular matrix. Together, these
observations are consistent with the concept that matrix com-
ponents provide a competence signal but that different cell
types may require different matrix components.

Although the signal provided by fibronectin in stimulating
lung fibroblast growth acts early in $G_1$, the growth-promoting
actions of fibronectin may occur at other times in the cell
cycle for other cells. In this context, Orly and Sato (33) have
demonstrated a marked increase in rat follicular cell replica-
tion in serum-free medium containing fibronectin, transfer-
rin, and insulin; analysis of the cell cycle specificity of the
growth-promoting signal provided by fibronectin demon-
strated that it was required for cytokinesis during M phase.
Thus, for various types of cells, fibronectin can serve an
important function in the control of replication, but these
functions may be cell specific.

## Various Roles for Fibronectin in Tissue Reconstruction and Fibrosis

The present study demonstrates that both plasma fibronec-
tin and macrophage fibronectin can provide a replication
signal to fibroblasts. Together with the known functions of
fibronectin in promoting fibroblast chemotaxis (34–36) and
attachment and spreading (27, 28), these observations suggest
a fundamental role for fibronectin in the reparative processes
following injury. For example, in wound healing where cap-
illary leak is significant and macrophages play an important
role (37), plasma fibronectin as well as macrophage fibronec-
tin may contribute in concert with other growth factors to the
increase in fibroblast numbers characteristic of this process.
Furthermore, in human fibrotic lung disease, alveolar mac-

rophages are releasing both fibronectin (34) and AMDGF
(38), factors that, together, can recruit fibroblasts (fibronec-
tin), attach fibroblasts (fibronectin), and signal replication of
fibroblasts (fibronectin and AMDGF). Consistent with this
concept, morphologic studies of the alveolar wall in these
diseases have demonstrated increased numbers of fibroblasts
(39). Thus fibronectin may by one of the stimuli that results
in expansion of the fibroblast population in various physio-
logical and pathological settings.

## REFERENCES

1. Todaro, G., Y. Matsuya, S. Bloom, A. Robbins, and H. Green. 1967. Stimulation of RNA synthesis and cell division in resting cells by a factor present in serum. *In* Growth Regulatory Substances for Animal Cells in Culture. V. Defendi and M. Stoker, editors. Wistar Institute Press, Philadelphia. 103–116.
2. Ross, R., and G. H. Sato. 1979. Cell culture and endocrinology: an overview. *In* Hormones and Cell Culture. B. G. H. Sato and R. Ross, editors. Cold Spring Harbor Laboratory. 965–971.
3. Gospodarowicz, D. 1975. Purification of a fibroblast growth factor from bovine pituitary. *J. Biol. Chem.* 250:2515–2520.
4. VanWyk, J. J., and R. L. Hintz. 1979. Peptide growth factors. *In* Endocrinology. L. J. DeGroot, editor. 3:1767–1775.
5. Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher. 1978. An ordered sequence of events is required before BALB/C-3T3 cells become committed to DNA synthesis. *Proc. Natl. Acad. Sci. USA.* 75:2839–2843.
6. Pledger, W. J., C. D. Stiles, H. N. Antoniades, and C. D. Scher. 1977. Induction of DNA synthesis in BALB/C-3T3 cells by serum components: reevaluation of the com-mitment process. *Proc. Natl. Acad. Sci. USA* 74:4481–4485.
7. Stiles, C. D., G. T. Capone, C. D. Scher, H. N. Antoniades, J. J. VanWyk, and W. J. Pledger. 1979. Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proc. Natl. Acad. Sci. USA.* 76:1279–1283.
8. Bitterman, P. B., S. I. Rennard, G. W. Hunninghake, and R. G. Crystal. 1982. Human alveolar macrophage growth factor for fibroblasts: regulation and partial characteriza-tion. *J. Clin. Invest.* 70:806–822.
9. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell. Res.* 25:585–621.
10. Gospodarowicz, D., and C. Ill. 1980. Extracellular matrix and control of proliferation of vascular endothelial cells. *J. Clin. Invest.* 65:1351–1364.
11. Gospodarowicz, D., D. Delgado, and I. Vlodavsky. 1980. Permissive effect of the extracellular matrix on cell proliferation in vitro. *Proc. Natl. Acad. Sci. USA.* 77:4094–4098.
12. Gospodarowicz, D., and C. R. Ill. 1980. Do plasma and serum have different abilities to promote cell growth? *Proc. Natl. Acad. Sci. USA.* 77:2726–2730.
13. Kleinman, H. K., G. R. Martin, and P. H. Fishman. 1979. Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. *Proc. Natl. Acad. Sci. USA.* 76:3367–3371.
14. Kleinman, H. K., E. B. McGoodwin, and R. J. Klebe. 1976. Localization of the cell attachment region in types I and II collagen. *Biochem. Biophys. Res. Commun.* 72:426–432.
15. Yamada, K. M., D. W. Kennedy, K. Kimata, and R. M. Pratt. 1980. Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteo-lytic fragments. *J. Biol. Chem.* 255:6055–6063.
16. Engvall, E., and E. Ruoslahti. 1977. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer* 20:1–5.
17. Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P. Gehron-Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Bio-chem.* 104:205–214.
18. Hayashi, M., and K. M. Yamada. 1981. Differences in domain structures between plasma and cellular fibronectins. *J. Biol. Chem.* 256:11292–11300.
19. Hunninghake, G. W., J. E. Gadek, O. Kawanami, V. J. Ferrans, and R. G. Crystal. 1979. Inflammatory and immune processes in the human lung in health and disease: evaluation by bronchoalveolar lavage. *Am. J. Pathol.* 97:149–206.
20. Vogel, A., E. Raines, B. Kariya, M. J., Rivest, and R. Ross. 1978. Coordinate control of 3T3 cell proliferation by platelet-derived growth factor and plasma components. *Proc. Natl. Acad. Sci. USA.* 75:2810–2814.
21. Bradley, K. H., O. Kawanami, V. J. Ferrans, and R. G. Crystal. 1980. The fibroblast of human lung alveolar structures: a differentiated cell with a major role in lung structure and function. *In* Methods of Cell Biology. C. C. Harris, B. R. Trump, G. D. Stoner, editors. 21A:37–64.
22. Clemmons, D. R. 1983. Age dependent production of a competence factor by human fibroblasts. *J. Cell. Physiol.* 114:61–67.
23. Ross, R., and A. Vogel. 1978. The platelet-derived growth factor. *Cell.* 14:203–210.
24. Couchman, J. R., D. A. Rees, M. R. Green, and C. G. Smith. 1982. Fibronectin has a dual role in locomotion and anchorage of primary chick fibroblasts and can promote entry into the division cycle. *J. Cell. Biol.* 93:402–410.
25. Hall, W. M., and P. Ganguly. 1981. The relationship of serum fibronectin and cell shape to thrombin-induced inhibition of DNA synthesis in human fibroblasts. *J. Cell. Physiol.* 109:271–280.
26. Wicha, M. S., L. A. Liotta, S. Gaibisa, and W. R. Kidwell. 1979. Basement membrane collagen requirements for attachment and growth of mammary epithelium. *Exp. Cell Res.* 124:181–190.
27. Klebe, R. J. 1975. Isolation of a collagen-dependent cell attachment factor. *Nature (Lond.).* 250:248–251.
28. Grinnell, F., D. G. Harp, and D. Minter. 1977. Cell adhesion and spreading factor. *Exp. Cell Res.* 110:175–190.
29. Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J Cell Biol* 88:473–485.
30. Van Wyk, J. J., L. E. Underwood, A. J. D'Ercole, D. R. Clemmons, W. J. Pledger, W.

R. Wharton, and E. B. Leof. 1981. Role of somatomedin in cellular proliferation. *In* The Biology of Normal Human Growth. M. Ritzen, K. Hall, A. Zitterberg, A. Aperia, A. Larsson, and R. Zettertrom, editors. Raven Press, New York. 223:239.

31. Clemmons, D. R., L. E. Underwood, and J. J. Van Wyk. 1981. Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. *J. Clin. Invest.* 67:10–19.

32. Bitterman, P. B., S. I. Rennard, S. P. Nissley, S. Adelberg, and R. G. Crystal. 1982. Insulin-like growth factor production by fibroblasts: stimulation by alveolar macrophage derived growth factor. *Clin. Res.* 30:385*a*. (Abstr.)

33. Orly, J., and G. Sato. 1979. Fibronectin mediates cytokinesis and growth of rat follicular cells in serum-free medium. Cell. 17:295–305.

34. Rennard, S. I., G. W. Hunninghake, P. B. Bitterman, and R. G. Crystal. 1981. Production of fibronectin by the human alveolar macrophage: mechanism for the recruitment of fibroblasts to sites of tissue injury in interstitial lung diseases. *Proc. Natl.* *Acad. Sci. USA.* 78:7147–7151.

35. Gauss-Muller, V., H. K. Kleinman, G. R. Martin, and E. Shiffman. 1980. Role of attachment and attractants in fibroblast chemotaxis. *J. Lab. Clin. Med.* 96:1071–1080.

36. Postlethwaite, A. E., J. Keski-Oja, G. Balian, and A. H. Kang. 1981. Induction of fibroblast chemotaxis by fibronectin. Localization of the chemotactic region to a 140,000-molecular weight non-gelatin binding fragment. *J. Exp. Med.* 153:494–499.

37. Leibovich, S. J., and R. Ross. 1975. The role of the macrophage in wound repair. A study with hydrocortisone and antimacrophage serum. *Am. J. Pathol.* 78:71–91.

38. Bitterman, P. B., S. Adelberg, and R. G. Crystal. 1983. Mechanisms of pulmonary fibrosis: spontaneous release of the alveolar macrophage-derived growth factor in the interstitial lung disorders. *J. Clinical Invest.* 72:1801–1813.

39. Bitterman, P. B., S. I. Rennard, and R. G. Crystal. 1981. Environmental lung disease and the interstitium. *In* S. M. Brooks, editor. Clinics in Chest Medicine. Saunders, Philadelphia. 393–412.