

# Bleomycin-induced Pulmonary Fibrosis in Hamsters

## AN ALVEOLAR MACROPHAGE PRODUCT INCREASES FIBROBLAST PROSTAGLANDIN E<sub>2</sub> AND CYCLIC ADENOSINE MONOPHOSPHATE AND SUPPRESSES FIBROBLAST PROLIFERATION AND COLLAGEN PRODUCTION

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**ABSTRACT** Bleomycin-induced pulmonary fibrosis in hamsters is associated with collagen accumulation that results from increased lung collagen synthesis rates. However, 1-2 wk after intratracheal instillation of bleomycin, lung collagen synthesis rates decline toward control values. To evaluate the potential role of the bronchoalveolar macrophage in regulating lung collagen production, we studied the effects of macrophages from normal and bleomycin-treated hamsters upon fibroblasts in vitro. We observed: (a) Medium from macrophage cultures decreased fibroblast [<sup>3</sup>H]thymidine incorporation and nondialyzable [<sup>3</sup>H]hydroxyproline production in a dose-dependent manner. Fibroblast cell counts were decreased in exposed cultures, and fibroblast viability was unchanged. Procollagen prolyl hydroxylation and prolyl-transfer RNA-specific activity were not altered by macrophage medium; this indicates that [<sup>3</sup>H]hydroxyproline reflects collagen production rate under the experimental conditions. (b) The suppressive effect of macrophage medium was selective for collagen since collagen production decreased more than noncollagen protein production. (c) Medium from bleomycin-treated hamster macrophages suppressed fibroblast proliferation and collagen production to a greater degree than medium from normal hamster macrophages. (d) Fibroblast suppression by macrophage medium was associated with increased fibroblast endogenous prostaglandin E<sub>2</sub> production and intracel-

lular cyclic AMP (cAMP). (e) Incubation of fibroblasts with indomethacin before exposure completely inhibited prostaglandin E<sub>2</sub> production and increases in cAMP, and eliminated suppression of fibroblast proliferation and collagen production. The macrophage-derived suppressive factor has an apparent molecular weight of 20,000-30,000 and is heat stable. It does not appear to be species restricted since both hamster and human lung fibroblasts are similarly suppressed. It is at least in part preformed in macrophages obtained by lavage, but its production can also be stimulated in vitro. We concluded that alveolar macrophages release a product that stimulates endogenous fibroblast prostaglandin E<sub>2</sub> production and cAMP formation with resultant suppression of fibroblast proliferation and collagen production. Enhanced release of suppressive factor by macrophages during a time when lung collagen production is declining in bleomycin-induced pulmonary fibrosis suggests that macrophages may limit collagen accumulation in pulmonary fibrosis.

### INTRODUCTION

Diffuse interstitial pulmonary fibrosis is a clinically heterogeneous condition caused by a variety of injuries to the lung. Inflammatory cells are invariably present, and a characteristic feature of the disorder is histologically increased and disorganized interstitial collagen (1-3). Because biochemical correlates have been difficult to obtain in patients, studies of experimentally induced pulmonary fibrosis in animals have been useful in delineating the pathogenesis of abnormal collagen deposition in the disorder.

In bleomycin-induced pulmonary fibrosis in hamsters, collagen content increases (4, 5) as the result of greatly increased collagen synthesis rates that occur promptly

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after administration of the drug (6). However, collagen synthesis rates decline 1–2 wk later and are subsequently restored to normal even though histological fibrosis persists and collagen content remains elevated. We recently described a factor released from normal lung explant cultures that suppresses lung fibroblast proliferation and collagen synthesis by stimulating endogenous fibroblast prostaglandin (PG)<sup>1</sup>E<sub>2</sub> production and intracellular cyclic AMP (cAMP) formation (7). In the first week after bleomycin administration, fibroblast-suppressive activity is decreased, but at 2 wk increases to greater than normal levels. At this time, lung collagen synthesis rate is declining and a mononuclear cell infiltrate is present along with increased numbers of bronchoalveolar lavage macrophages (4–6, 8). These observations suggest that mononuclear phagocytes might play a role in the regulation of lung fibroblast proliferation and/or collagen synthesis. In this report, we present studies of the effects of products from cultured bronchoalveolar macrophages (BAM) on lung fibroblasts. The results suggest that bronchoalveolar macrophages may participate in limiting lung collagen accumulation in pulmonary fibrosis.

## METHODS

**Bleomycin treatment.** 10-wk-old male Syrian hamsters received a single intratracheal dose of 1 U of bleomycin (Blenoxane; Bristol Laboratories, Syracuse, NY) in 0.1 ml of sterile saline (0.9 g/dl). Age-matched, uninjected hamsters served as controls. Bleomycin-treated animals were studied at 1–3 wk after instillation of the drug.

**Conditioned medium from BAM cultures.** BAM were obtained by bronchoalveolar lavage of anesthetized hamsters with an 18-gauge intratracheal catheter by using 10 ml of 0.9 g/dl NaCl containing 3 mM Na<sub>2</sub>EDTA. The lavage fluid was centrifuged at 250 g for 15 min at 25°C, and the cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) and recentrifuged. Cells were suspended in DMEM at 5 × 10<sup>6</sup> cells/ml. The percentage of macrophages was estimated by examination of cytocentrifuge preparations stained with hematoxylin-eosin. 5 × 10<sup>5</sup> BAM were plated in 1.0 ml of DMEM in 1.7-cm diameter multiple well culture plates (Multiwell Tissue Culture Plate, Falcon Labware, Oxnard, CA). After incubation for 1 h at 37°C in 5% CO<sub>2</sub>:95% air atmosphere, cell layers were rinsed once with DMEM to remove nonadherent cells, and adherent cells were cultured in basal medium (DMEM supplemented with 10% fetal calf serum, 200 U/ml penicillin, 200 μg/ml streptomycin, and 2 mM glutamine). In one experiment, the serum concentration of basal medium was varied. Lavage cells were also cultured in suspension in polypropylene tubes. Conditioned medium (CM) was removed after 24 h and stored at –20°C. Adherent cells were >95% viable as indicated by trypan blue exclusion, and >95% demonstrated specific esterase positivity. Before

addition to fibroblast cultures, CM was dialyzed (12,000 mol wt cutoff, Spectrum Medical Industries, Los Angeles, CA) against three changes of DMEM (100× sample volume) for 36 h at 4°C and filter-sterilized (0.2 μm pore size).

To determine if protein synthesis was required for appearance of activity in CM, adherent BAM were cultured in basal medium containing cycloheximide (100 μg/ml). As a control, cycloheximide (100 μg/ml) was added to BAM-CM before dialysis against fresh medium. Parallel BAM cultures were incubated with [<sup>3</sup>H]proline and the CM was dialyzed exhaustively against tap water. Nondialyzable radioactivity was measured as an indication of newly synthesized protein. In other BAM cultures, Sepharose 4B (10% wt/vol) was added to the incubation medium to increase release of suppressive activity (7). BAM lysate was obtained from a BAM sample by freezing (–70°C) and thawing (25°C) the cells three times.

**Fibroblast cultures.** Human fetal lung fibroblasts (IMR-90, Institute for Medical Research, Camden, NJ) were plated at 3 × 10<sup>4</sup> cells/1.7-cm diameter well in 1 ml of basal medium and incubated in a 5% CO<sub>2</sub>:95% air atmosphere at 37°C. Cells were in the tenth to fifteenth passage (1:4 split ratio).

Hamster lung fibroblasts were cultivated as previously described from 10-wk-old male Syrian hamsters (7). Hamster lung fibroblasts obtained by this method required 2–3 wk to reach visual confluence, while IMR-90 fibroblasts plated at the same density were confluent in less than 1 wk. Because of their slow growth, hamster lung fibroblasts were plated at high density and used in the first to third passage. Unless otherwise specified, we used IMR-90 cells, which are referred to as fibroblasts.

When fibroblasts were nearly confluent (usually 5 d after passage), varying amounts of CM from cultured BAM were added with fresh basal medium to a total volume of 500 μl/well. In one experiment, serum concentration in basal medium was reduced 2 d before addition of CM. Fibroblasts were incubated in the presence of CM, and proliferation and collagen synthesis assayed as described below.

**Fibroblast proliferation assays.** After incubation of the fibroblasts with CM, 1 μCi tritiated thymidine ([<sup>3</sup>H]dThd) (25 Ci/mol; Amersham Corp., Arlington Heights, IL) in 100 μl of basal medium was added to each well, and the incubation was continued for an additional 4 h. Incorporation of [<sup>3</sup>H]dThd was measured as previously described (7). In some experiments, cell layer protein (9) or DNA (10) was measured as an index of cell number. Cell counts were performed with a hemocytometer. Cell viability was determined by trypan blue exclusion.

**Fibroblast collagen production assay.** After incubation of the fibroblasts with CM, 10 μCi [<sup>3</sup>H]proline (130 Ci/mol; Amersham Corp.) in 100 μl of basal medium, containing ascorbic acid and β-aminopropionitrile to yield final concentrations of 50 and 20 μg/ml, respectively, was added to each well, and the incubation continued for an additional 6 h. The medium was removed and combined with a 500 μl Hanks' balanced salt solution wash of the cell layer. The cell layer was scraped into 1 ml of 0.5 M acetic acid. After the addition of protease inhibitors (10 mM N-ethylmaleimide, 20 mM disodium EDTA, and 0.3 mM phenylmethylsulfonyl fluoride in final concentration), the medium and cell layer were dialyzed separately for 24 h against running tap water, hydrolyzed in 6 N HCl at 110°C for 18 h, evaporated, and dissolved in 0.2 M sodium citrate buffer, pH 2.2. [<sup>3</sup>H]hydroxyproline and [<sup>3</sup>H]proline were separated chromatographically as previously described (11). Since [<sup>3</sup>H]hydroxyproline in medium was proportional to total [<sup>3</sup>H]hydroxyproline, measurements were made on medium alone unless otherwise indicated. In some experiments, me-

<sup>1</sup> Abbreviations used in this paper: BAM, bronchoalveolar macrophages; CM, conditioned medium; DMEM, Dulbecco's modified Eagle's medium; [<sup>3</sup>H]dThd, tritiated thymidine; PG, prostaglandin.

dium  $^3\text{H}$ -proteins were digested with bacterial collagenase (type VI, Sigma Chemical Co., St. Louis, MO), which was purified as described by Peterkovsky and Diegelmann (12).

Specific radioactivity of proline in fibroblast intracellular free amino acid pool and amino acyl-transfer RNA (tRNA) pool were measured by a method described by Airhart et al. (13) and modified by Hildebran et al. (14). In these experiments, confluent fibroblasts were cultured in P-60 culture dishes (Falcon Labware) with 2 ml of CM (30% concentration) or basal medium containing 200  $\mu\text{Ci}$  [ $^3\text{H}$ ]proline, ascorbic acid (50  $\mu\text{g}/\text{ml}$ ),  $\beta$ -aminopropionitrile (20  $\text{mg}/\text{ml}$ ), and proline (0.2 mM) for 18 h. The following is a brief description of the analysis. Medium was removed and analyzed for [ $^3\text{H}$ ]hydroxyproline and [ $^3\text{H}$ ]proline as described above. The rinsed cell layer was solubilized with cacodylate-buffered sodium dodecyl sulfate. An aliquot of the solubilized cell layer was precipitated with trichloroacetic acid; the supernatant containing the intracellular free amino acid pool was analyzed as described below to determine intracellular free proline-specific radioactivity. The remainder of the cell layer to be analyzed for prolyl-tRNA-specific radioactivity was deproteinized by addition of freshly distilled phenol. Nucleic acids were precipitated from the aqueous phase by addition of ice-cold ethanol, and amino acids bound to tRNA were released by alkaline hydrolysis after which nucleic acids were reprecipitated by addition of HCl.

Specific radioactivity of proline in the intracellular free amino acid pool and in the aminoacyl-tRNA pool was determined after reaction with [ $^{14}\text{C}$ ]dansyl chloride and separation of dansyl derivatives by two-dimensional ascending thin-layer chromatography. Dansyl proline (cold) was added to the sample before chromatography to clearly identify the position of proline on the chromatography plate. That spot was cut from the plate and  $^3\text{H}$  and  $^{14}\text{C}$  counted in a liquid scintillation counter equipped with automatic quench compensation (Beckman LS8000, Beckman Instruments, Inc., Fullerton, CA). Specific radioactivity of proline was calculated by the formula:

proline specific activity

$$= \frac{^3\text{H}(\text{dpm})}{^{14}\text{C}(\text{dpm})} \cdot \text{dansyl chloride specific activity} \cdot K \quad (1)$$

where  $K$  equals moles of dansyl group bound per moles of proline, which equals 1. Because dansyl chloride specific activity was not known with certainty, the values are presented as arbitrary numbers for comparison of control and CM-exposed cultures.

Intracellular free proline concentration was measured in some experiments by a photochemical method described by Troll and Lindsley (15).

Other calculations included:

Procollagen prolyl hydroxylation (%)

$$= \frac{([\text{H}] \text{hydroxyproline released by collagenase})}{\text{total radioactivity released by collagenase}} \times 100. \quad (2)$$

Noncollagen protein production

$$\begin{aligned} & (\text{noncollagen proline incorporation per cell}) \\ &= (\text{total } ^3\text{H-protein}/\mu\text{g DNA}) \\ & - \frac{([\text{H}] \text{hydroxyproline production}/\mu\text{g DNA})}{\text{procollagen prolyl hydroxylation}} \\ & \div (\text{prolyl-tRNA specific radioactivity}). \quad (3) \end{aligned}$$

Collagen production (hydroxyproline production per cell)

$$= \frac{([\text{H}] \text{hydroxyproline production}/\mu\text{g DNA})}{\text{prolyl-tRNA specific radioactivity}}. \quad (4)$$

Collagen production (percentage of total protein production)

$$\begin{aligned} &= \frac{([\text{H}] \text{hydroxyproline production})}{\text{procollagen prolyl hydroxylation} \div 3.71} \\ & \div \frac{([\text{H}] \text{hydroxyproline production})}{\text{procollagen prolyl hydroxylation} \div 3.71} \\ & + (\text{noncollagen protein production}), \quad (5) \end{aligned}$$

where 3.71 is a factor to account for the relative proportion of proline in collagen compared with other proteins (16).

**Fibroblast  $\text{PGE}_2$  production and cAMP levels.**  $\text{PGE}_2$  in fibroblast culture medium and intracellular cAMP were measured by radioimmunoassays previously described (7).

**Physical properties of CM.** Heat stability of suppressive activity in CM was assessed by incubating CM at  $56^\circ$  or  $80^\circ\text{C}$  for 1 h before incubation with cultured fibroblasts.

CM was fractionated on a  $60 \times 2.5\text{-cm}$  column of Sephacryl 200 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 150 mM NaCl, 0.01 mM Tris, pH 7.5, and calibrated with molecular weight standards. CM or basal medium (3 ml) was chromatographed, and pooled fractions were lyophilized and resuspended in 3 ml of basal medium. Aliquots (500  $\mu\text{l}$ ) of the pooled fractions were then added to fibroblast cultures, and suppression of [ $^3\text{H}$ ]dThd incorporation was measured.

**Statistical evaluation.** Significance of differences between means was determined by  $t$  test.

## RESULTS

**CM-mediated suppression of fibroblast proliferation and collagen production.** Incubation of lung fibroblasts with CM from normal hamster macrophages resulted in a dose-related decrease in fibroblast [ $^3\text{H}$ ]dThd incorporation (Fig. 1). Lower CM concentrations (0.1 and 1.0%) did not affect fibroblast [ $^3\text{H}$ ]dThd incorporation. Fibroblast viability (>90%) was not altered by CM. Although [ $^3\text{H}$ ]dThd incorporation is a more sensitive test of fibroblast proliferation, cell counts were lower in fibroblast cultures exposed to CM. For example, nonconfluent cultures exposed to 20% CM for 24 h contained  $1.51 \pm 0.10 \times 10^5$  cells, while cultures in basal medium contained  $2.61 \pm 0.32 \times 10^5$  cells (mean  $\pm$  SEM,  $n = 3$ ).

Collagen production by fibroblasts was also suppressed by macrophage CM. Nondialyzable [ $^3\text{H}$ ]hydroxyproline in fibroblast culture medium decreased in a dose-related manner as indicated in Fig. 1. No change in [ $^3\text{H}$ ]hydroxyproline was noted at CM concentrations of 0.1 or 1.0%. Table I shows that [ $^3\text{H}$ ]hydroxyproline production per microgram of cell layer DNA decreased after CM exposure while pro-

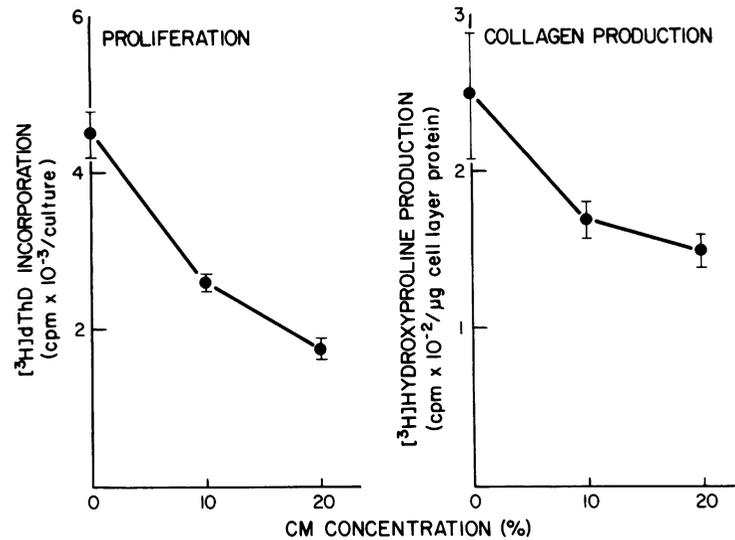


FIGURE 1 CM-mediated suppression of fibroblast proliferation and collagen production. Fibroblasts were cultured in the presence of BAM-CM in varying concentration for 18 h. To measure fibroblast proliferation, [ $^3\text{H}$ ]dThd ( $1\ \mu\text{Ci}$ ) was added for 4 h, and [ $^3\text{H}$ ]dThd incorporation was measured (*left*). To measure collagen production, [ $^3\text{H}$ ]proline ( $10\ \mu\text{Ci}$ ),  $\beta$ -aminopropionitrile ( $20\ \mu\text{g/ml}$ ), and ascorbic acid ( $50\ \mu\text{g/ml}$ ) were added for 6 h, and nondialyzable [ $^3\text{H}$ ]hydroxyproline was measured in the medium (*right*). Values are mean  $\pm$  SEM ( $n = 4$ ). CM-exposed cultures are significantly different from cultures in basal medium ( $P < 0.05$ ).

TABLE I  
Effects of CM on Parameters of Fibroblast Collagen Production

Parameter	Fibroblast culture conditions*		Percent change
	Basal medium	CM	
[ $^3\text{H}$ ]hydroxyproline production ( $dpm \times 10^{-4}/\mu\text{g DNA}$ )	$3.21 \pm 0.26$ (8)	$1.86 \pm 0.14^{\text{  }}$ (9)	42.2
[ $^3\text{H}$ ]proline incorporation ( $dpm \times 10^{-4}/\mu\text{g DNA}$ )	$10.72 \pm 0.51$ (8)	$8.00 \pm 0.13^{\text{  }}$ (9)	25.4
Procollagen prolyl hydroxylation (%)	$40.5 \pm 0.3$ (4)	$41.0 \pm 2.1$ (3)	(1.2)
Collagen production (% of total soluble protein production)	$18.6 \pm 1.1$ (6)	$14.2 \pm 1.1^{\text{§}}$ (9)	23.6
Prolyl-tRNA specific activity ( $dpm/\text{unit weight proline}$ )	$1.07 \pm 0.13$ (5)	$0.97 \pm 0.07$ (9)	(9.3)
Intracellular free proline specific activity ( $dpm/\text{unit}$ weight proline)	$1.56 \pm 0.05$ (6)	$1.31 \pm 0.01^{\text{  }}$ (6)	16.0
Intracellular free proline ( $\text{pg proline}/\mu\text{g DNA}$ )	$237 \pm 11$ (8)	$270 \pm 15^{\text{§}}$ (7)	13.9

\* Fibroblasts were cultured in basal medium or CM (30% concentration) for 18 h. Ascorbic acid and [ $^3\text{H}$ ]proline were added; analyses and calculations were performed as described in Methods. Values are mean  $\pm$  SEM ( $n$ ).

† Significantly different from basal medium,  $P < 0.05$ .

§ Significantly different from basal medium,  $P < 0.01$ .

|| Significantly different from basal medium,  $P < 0.001$ .

collagen prolyl hydroxylation was not reduced by CM. Although intracellular free proline was increased and intracellular free proline-specific activity decreased after CM exposure, prolyl-tRNA-specific activity was not significantly different. Total soluble protein production and hydroxyproline production were significantly decreased in CM-exposed cultures when calculated on the basis of either proline pool. Moreover, collagen production was selectively decreased by CM since the proportion of total soluble protein synthesis directed toward collagen was less in CM-exposed cultures compared with cultures in basal medium (Fig. 2).

Similar suppression of [ $^3\text{H}$ ]dThd incorporation and [ $^3\text{H}$ ]hydroxyproline production was observed when hamster lung fibroblasts were exposed to CM (Table II).

The effects of culture conditions on the appearance and/or detection of macrophage-derived fibroblast suppressive activity were tested by varying serum concentrations in both macrophage and fibroblast cultures. The results (Table III) indicate that fibroblasts grown in low serum concentration (0.04%) or serum-free medium are quiescent. Under these conditions, macrophage-derived suppressive activity was not detectable. In fact, stimulation of [ $^3\text{H}$ ]dThd incorporation was observed. Cultures of adherent macrophages contained essentially the same level of fibroblast suppressive activity as suspension cultures.

The appearance of fibroblast suppressive activity after intratracheal bleomycin was studied in normal hamsters

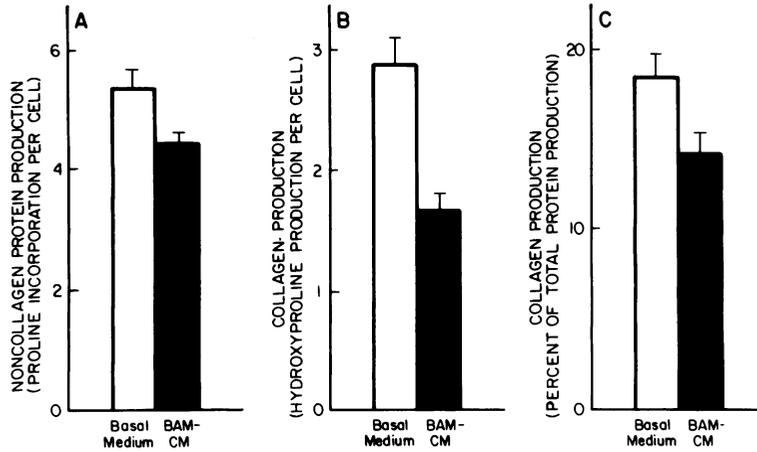


FIGURE 2 Effect of CM on fibroblast soluble protein production. Fibroblasts were cultured for 18 h in basal medium (□) or CM (■; 30% concentration). Ascorbic acid,  $\beta$ -aminopropionitrile, and [ $^3$ H]proline were added, and incubation continued for an additional 6 h. [ $^3$ H]proline incorporation and [ $^3$ H]hydroxyproline production were measured in culture medium; prolyl-tRNA-specific radioactivity and DNA were measured in the cell layer. Noncollagen protein production per cell (A), collagen production per cell (B), and collagen production as a percent of total soluble protein production (C) were calculated as described in Methods. Units of noncollagen protein production and collagen production are comparable but arbitrary because the specific activity of [ $^{14}$ C]dansyl chloride which was used to determine prolyl-tRNA-specific radioactivity was not known with certainty. Values are mean $\pm$ SEM ( $n = 5-9$ ). CM exposure resulted in significant suppression of noncollagen protein production ( $P < 0.001$ ), collagen production ( $P < 0.001$ ), and percent collagen production ( $P < 0.01$ ).

and hamsters treated 1, 2, or 3 wk before bronchoalveolar lavage. The results (Table IV) indicate that suppressive activity derived from cultures containing equal numbers of macrophages increased over the 3-wk period of observation. The change was accompanied by a concomitant increase in the total number of lavage macrophages. Macrophages that were obtained from individual animals 2 wk after bleomycin instillation were cultured, and their effects on fibroblasts were studied in more detail. These macrophages suppressed both fibroblast proliferation and collagen production to a

TABLE II  
Effects of CM on Cultured Hamster Lung Fibroblasts\*

CM concentration	[ $^3$ H]dThd incorporation	[ $^3$ H]hydroxyproline production
%	cpm $\times 10^{-3}$ /culture	cpm $\times 10^{-2}$ / $\mu$ g cell layer protein
0 (Basal medium)	3.65 $\pm$ 0.20	2.53 $\pm$ 0.51
10	2.58 $\pm$ 0.18†	2.12 $\pm$ 0.32
25	2.12 $\pm$ 0.15†	1.81 $\pm$ 0.23†

\* Fibroblasts were cultured as described in Fig. 1. Values are mean $\pm$ SEM,  $n = 4$ .

† Significantly different from basal medium,  $P < 0.01$ .

TABLE III  
Effect of Culture Conditions on Detection of Macrophage-derived Suppressive Activity\*

Macrophage culture conditions	Fibroblast culture conditions	[ $^3$ H]dThd incorporation
		cpm $\times 10^{-3}$ /culture
No BAM/10% serum	10% serum	5.79 $\pm$ 0.38
Adherent BAM/10% serum	10% serum	4.99 $\pm$ 0.50‡
No BAM/0.4% serum	0.4% serum	0.49 $\pm$ 0.06
Adherent BAM/0.4% serum	0.4% serum	0.79 $\pm$ 0.06‡
No BAM/no serum	no serum	0.09 $\pm$ 0.01
Adherent BAM/no serum	no serum	0.16 $\pm$ 0.01‡
No BAM/10% serum	10% serum	5.67 $\pm$ 0.33
Suspension BAM/10% serum	10% serum	4.68 $\pm$ 0.25‡§

\* Macrophages were cultured in medium containing fetal calf serum concentrations as indicated. An equal number of BAM were cultured in polypropylene tubes ("suspension BAM"). Fibroblasts were cultured in basal medium. 2 d before addition of BAM-CM, the fibroblast medium was changed to contain the serum concentration indicated. After incubation with macrophage CM, fibroblast [ $^3$ H]dThd incorporation was measured. Values are mean $\pm$ SEM,  $n = 4$ .

‡ Significantly different from control culture (No BAM),  $P < 0.05$ .

§ No significant difference from adherent BAM in 10% serum.

TABLE IV  
Appearance of Macrophage-derived Fibroblast Suppressive Activity after Intratracheal Bleomycin\*

Source of macrophages	Lavage macrophages	[ <sup>3</sup> H]dThd incorporation
	%/number × 10 <sup>-6</sup> per lung	cpm × 10 <sup>-3</sup> /culture
Basal medium (no macrophages)	—	5.70±0.16
Control hamster (no bleomycin)	98/1.77	3.72±0.07†
Hamster, 1 wk after bleomycin	65/1.70	2.48±0.10‡
Hamster, 2 wk after bleomycin	80/2.92	2.66±0.22‡
Hamster, 3 wk after bleomycin	80/3.43	1.46±0.10‡§

\* Macrophages were obtained from control hamsters and hamsters previously given intratracheal bleomycin. Lavage cells from each group of four to six animals were pooled. The cells were counted, and differential counts obtained from hematoxylin-eosin-stained cytocentrifuge preparations. Macrophage cultures contained equal numbers of macrophages. Fibroblasts were exposed to CM (10% concentration), and fibroblast [<sup>3</sup>H]dThd incorporation was measured. Values are mean±SEM (n = 4 cultures).

† Significantly different from basal medium, P < 0.01.

‡ Significantly different from control hamster, P < 0.01.

§ Significantly different from 1 or 2 wk after bleomycin, P < 0.01.

greater degree than normal hamster macrophages. Fig. 3 indicates that CM from normal hamster macrophages suppressed [<sup>3</sup>H]dThd incorporation by 18%, while bleomycin-treated hamster macrophage CM resulted

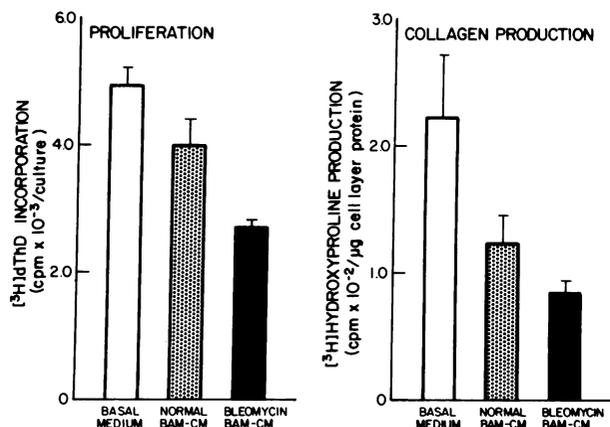


FIGURE 3 Suppression of fibroblast proliferation and collagen production by CM from normal and bleomycin-treated hamster BAM. Fibroblasts were exposed for 24 h to CM (10% concentration in proliferation assay, and 20% in collagen production assay) from normal hamster macrophages or macrophages of bleomycin-exposed hamsters. The macrophage cultures from normal hamsters and from bleomycin-exposed hamsters contained equal numbers of cells. Fibroblast proliferation (left) and collagen production (right) were measured as described in Fig. 1. Values are mean±SD (n = 6 animals). BAM-CM from bleomycin-exposed hamsters suppressed both proliferation and collagen synthesis significantly more (P < 0.01) than BAM-CM from normal hamsters.

in 57% suppression. Similarly, [<sup>3</sup>H]hydroxyproline production was suppressed 44 and 60% by normal and bleomycin-treated hamster macrophage CM, respectively.

CM-mediated stimulation of fibroblast endogenous PGE<sub>2</sub> production and intracellular cAMP. CM from macrophages resulted in striking increases in fibroblast PGE<sub>2</sub> production (Fig. 4). Medium from fibroblasts incubated under basal conditions for 24 h contained only 12 pg PGE<sub>2</sub>/culture. After incubation with CM, fibroblast PGE<sub>2</sub> increased several orders of magnitude in a dose-related manner. Intracellular fibroblast cAMP was also increased after 24 h exposure to CM at 20% concentration. It should be noted, however, that the values of cAMP presented in Fig. 4 do not represent the maximum cAMP levels during this period since peak values occur after 8 h of incubation with CM (Amill-Acosta, S. A., and Clark, J. G., unpublished observations).

Ablation of CM-mediated effects in indomethacin pretreated fibroblast cultures. To establish that CM-mediated stimulation of PG results in increased cAMP and decreased proliferation and collagen production, indomethacin (1 μg/ml), a cyclooxygenase inhibitor, was added to fibroblast cultures 2 h before addition of CM. The results are shown in Fig. 5. Indomethacin completely blocked CM-mediated increases in PGE<sub>2</sub> and cAMP, and suppression of fibroblast proliferation and collagen production was not observed. The CM-mediated increase in [<sup>3</sup>H]dThd incorporation observed

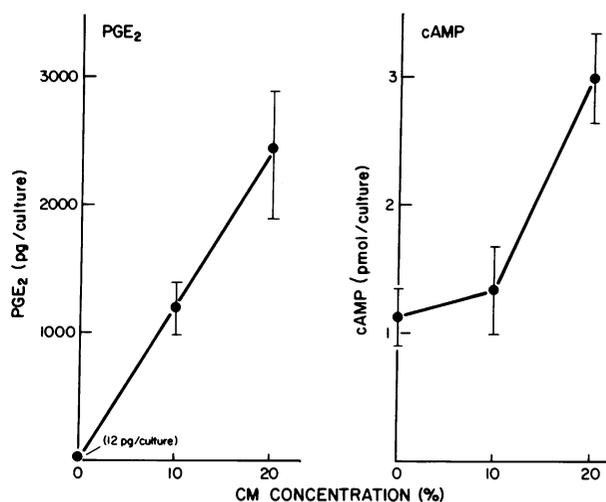


FIGURE 4 CM-mediated stimulation of fibroblast PGE<sub>2</sub> production and intracellular cAMP. Fibroblasts were cultured in the presence of BAM-CM in varying concentration for 24 h. PGE<sub>2</sub> was measured in culture medium (left) and cAMP was measured in cell layers (right) by radioimmunoassay. Values are mean±SEM (n = 4). CM-exposed fibroblast cultures are significantly different from cultures in basal medium (P < 0.05) except for cAMP in 10% CM.

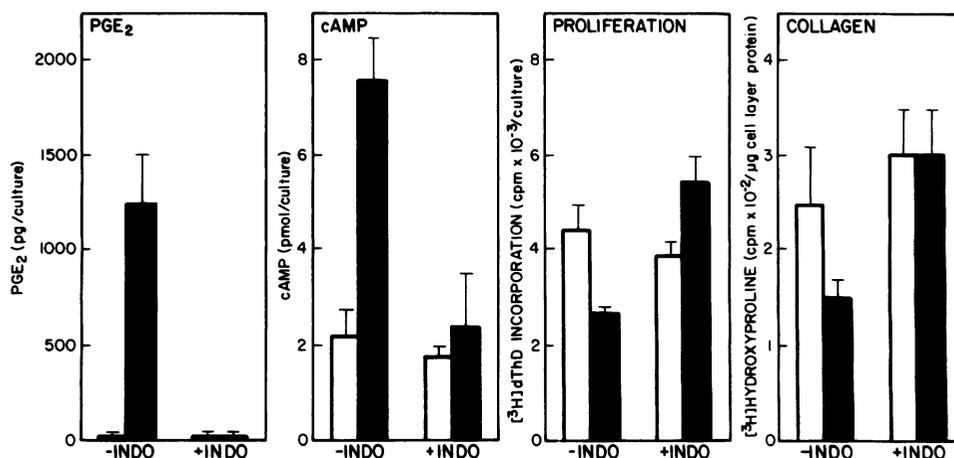


FIGURE 5 Ablation of CM-mediated effects on fibroblasts by indomethacin. Fibroblasts were exposed to basal medium (□) or BAM-CM (■) (10% concentration in proliferation assay and 20% in other assays) as described in Fig. 1 (-INDO). Indomethacin (1.0  $\mu\text{g}/\text{ml}$ ) was added to parallel fibroblast cultures 2 h before exposure to basal medium or BAM-CM (+INDO). In the absence of indomethacin, BAM-CM exposure resulted in stimulation of fibroblast endogenous PGE<sub>2</sub> and intracellular cAMP, and suppression of fibroblast proliferation and collagen production ( $P < 0.01$ ). These CM-mediated effects were absent when fibroblasts were cultured with indomethacin. Values are mean  $\pm$  SD ( $n = 4$ ).

after CM exposure in indomethacin pretreated cultures seen in Fig. 5 was not a consistently reproducible observation.

**Characterization of BAM-derived suppressive activity.** Macrophage-derived fibroblast suppressive factor has an apparent molecular weight of 20,000–30,000 by molecular sieve chromatography. Heating at 56°C for 1 h did not reduce the activity of CM, but heating at 80°C for 1 h resulted in a 50% reduction of suppressive activity.

BAM lysates contained suppressive activity that indicated that the factor is preformed in the cells (Table V). Cycloheximide reduced macrophage <sup>3</sup>H-protein synthesis to <5% of control values, but suppressive activity in CM was not reduced. In fact, suppression of [<sup>3</sup>H]dThd incorporation was greater in fibroblast cultures exposed to CM from cycloheximide-treated macrophages. Macrophage release of suppressive activity was stimulated by Sepharose 4B to levels greater than present in lysates; this suggests that enhanced synthesis and release of suppressive activity is induced by Sepharose 4B.

## DISCUSSION

Lung collagen content is determined at least in part by fibroblast population size and collagen production rates, parameters which are potentially regulated by stimulatory and inhibitory influences within the alveolus. This study demonstrates that alveolar macrophages are capable of releasing a product that sup-

presses fibroblast proliferation and collagen production. Several lines of evidence indicate that this suppression is mediated by endogenous fibroblast PG production and cAMP formation. First, fibroblast PGE<sub>2</sub> production and cAMP levels are increased in proportion to the concentration of BAM-CM and the degree of CM-mediated suppression. Second, when PGE<sub>2</sub> production is blocked with indomethacin, a cyclooxygenase inhibitor, CM-mediated increases in cAMP and suppression of fibroblast proliferation and collagen production are eliminated. Third, exogenous PGE<sub>2</sub>, in amounts similar to quantities produced by fibroblasts after exposure to CM, results in increased intracellular cAMP and decreased fibroblast proliferation and collagen production (7, 17). It has also previously been shown that PG can stimulate adenylate cyclase with resultant increases in intracellular cAMP (18), and that increases in fibroblast cAMP induced by adrenergic agonists result in a selective decrease in collagen production similar to that demonstrated in this study (19, 20). In addition, dibutyryl cAMP, an analog of cAMP, enters the cell and suppresses fibroblast proliferation and collagen production (7, 19). We, therefore, conclude that a macrophage-derived factor increases endogenous fibroblast PG production with subsequent cAMP formation that results in decreased fibroblast proliferation and collagen production.

Fibroblast collagen production after exposure to macrophage CM was further examined in experiments in which we measured proline-specific radioactivity in the cultured fibroblasts. We observed, as has previously

TABLE V  
Macrophage Production of Fibroblast Suppressive Factor\*

Culture conditions	[ <sup>3</sup> H]dThd incorporation	Percent suppression
	<i>cpm × 10<sup>-4</sup>/culture</i>	
Basal medium	4.69±0.31	
CM (no additions)	3.07±0.16†	34.7
CM (cycloheximide added prior to dialysis)	3.38±0.30†	27.9
CM (cycloheximide)	2.62±0.12†§	44.2
CM (Sephacel)	2.10±0.13†§	55.3
CM (Sephacel + cycloheximide)	1.86±0.11†	60.5
Macrophage lysate	2.77±0.11†	41.0

\* Macrophages were cultured in the presence of cycloheximide (100 µg/ml) and/or Sephadex 4B (10% wt/vol). Fibroblasts were then exposed to dialyzed basal medium or macrophage CM (10% concentration), and fibroblast [<sup>3</sup>H]dThd incorporation was measured. Values are mean±SD (n = 3).

† Significantly different from basal medium, *P* < 0.01.

§ Significantly different from CM (no additions), *P* < 0.01.

|| Significantly different from CM (Sephacel), *P* < 0.05.

been reported (14), that the specific radioactivity of the intracellular free proline pool differs from that of the prolyl-tRNA pool. Also, the specific radioactivity in the intracellular free pool decreased after CM exposure. The data suggest that the fractional decrease may not be equal in the intracellular and prolyl-tRNA pools; this data emphasizes the importance of using an appropriate precursor pool specific activity to calculate actual collagen production rate. Our results also indicate that the change in proline-specific radioactivity after CM exposure is associated with increased intracellular free proline. A possible mechanism for this increase is an increase in endogenously derived proline. It has previously been shown that increased fibroblast cAMP after exposure to adrenergic agonists results in decreased procollagen production due at least in part to increased intracellular degradation of procollagen (20). Increased cAMP and subsequent increased intracellular degradation in CM-exposed cultures could contribute proline to the intracellular free pool, with the resultant decrease in specific radioactivity which we observed. We also found that the decrease in collagen production was greater than that of other soluble proteins produced in culture; this indicates that CM-mediated suppression is selective for collagen. This study did not determine whether the selective decrease in collagen production occurs as the result of increased intracellular degradation of collagen and/or decreased levels of procollagen messenger RNA or procollagen translation rate.

The active product released from alveolar macrophages is nondialyzable, heat stable to 56°C, and has an apparent molecular weight of 20,000–30,000. It does not appear to be species restricted since both hamster

and human fibroblasts are similarly suppressed. The biological and biochemical characteristics of this macrophage product resemble those of the fibroblast-suppressive factor derived from hamster lung explant cultures which we previously reported (7, see below). Interleukin 1 also similarly stimulates PG production and suppresses proliferation of rheumatoid synovial cells, but macrophage-derived fibroblast suppressive factor is of greater apparent molecular weight (21, 22). Further biochemical characterization will be necessary to determine the relationship, if any, to interleukin 1 derived and characterized from murine and human mononuclear cells (23).

Since lysates of lavage macrophages contain suppressive activity and cycloheximide did not reduce production by cultured macrophages, adherent alveolar macrophages may release primarily preformed suppressive factor during 24 h in culture. However, increased suppressive activity induced by Sephadex 4B exceeded that in lysates; this indicated that production of macrophage-derived suppressive factor can be stimulated in vitro. Suppressive activity was also greater in macrophage cultures exposed to cycloheximide compared with control macrophage cultures. This may represent superinduction such as is observed after actinomycin D (24). An alternative explanation for this observation is that synthesis and/or release of a macrophage-derived fibroblast stimulatory (growth) factor is reduced by cycloheximide, while release of the suppressive factor is independent of new protein synthesis. A macrophage-derived stimulatory factor whose production is inhibited by cycloheximide has recently been described in human cells (25), and we have also detected

fibroblast stimulatory activity in freshly adherent hamster macrophages (7); this suggests that the production of soluble macrophage-derived products may be subject to different control mechanisms.

The rate of synthesis and/or release of fibroblast-suppressive activity from macrophages could potentially modulate lung collagen accumulation in vivo. Alveolar macrophages obtained from animals after bleomycin treatment released more fibroblast-suppressive activity into culture medium than macrophages obtained from normal hamsters; this suggests that macrophages might limit collagen accumulation after bleomycin-induced injury. This role is further facilitated by the appearance of increased numbers of macrophages at 2 and 3 wk. "Negative regulation" of collagen production as a component of the response to fibrogenic injury in lung has been suggested by previous studies. Phan and Thrall (26) detected a soluble factor in lung tissue which selectively suppressed collagen production in lung explant cultures. They found that suppressive activity was diminished in the first week after bleomycin administration. We recently described a factor present in lung explants that increased endogenous fibroblast PG production and intracellular cAMP level and suppressed fibroblast proliferation and collagen production (7). We found that animals treated with bleomycin initially had decreased levels of this suppressive activity. Relatively few lavage macrophages are present at this time when polymorphonuclear cell exudation predominates (8). Subsequently, levels of suppressive activity exceeding normal values were detected at times when lung collagen synthesis rate was declining (i.e., 1-2 wk after bleomycin administration). Although these observations do not preclude direct stimulation of collagen production during the development of pulmonary fibrosis, they suggest that suppression of collagen synthesis is important in regulating collagen production and in limiting collagen accumulation in response to injury. Failure to appropriately inhibit collagen production after injury could contribute to excessive accumulation of collagen, which is characteristic of pulmonary fibrosis.

Our results indicate that alveolar macrophages, prominent cells in the subacute phase of bleomycin-induced pulmonary fibrosis as well as in pulmonary fibrosis in man (1, 8), are a cellular source of suppressive activity for fibroblasts in the lung. In other studies, mononuclear phagocytes also emerge as secretory cells that may influence the formation of collagen by products that modulate fibroblast proliferation and collagen production (21, 25, 27-31). In addition, release of fibronectin (32, 33), proteases such as collagenase (34) and elastase (35), and PG (36, 37) by macrophages may further modify the production and deposition of connective tissue. However, in vivo interactions between mononuclear phagocyte products and fibroblasts remain

uncertain, in part because regulation of these macrophage secretory products is not well understood. In this study, we included serum in fibroblast cultures so that we could examine dividing fibroblasts, a model system that in this respect may be appropriate for studies related to injured lung in which fibroblasts proliferate. When serum was omitted from the cultures, fibroblasts became quiescent and an increase in fibroblast [<sup>3</sup>H]dThd incorporation was observed after exposure to macrophage CM; this suggests that a fibroblast-stimulating factor is also present. The precise role of serum in the cultures is not known, but it is evident that mononuclear cells and macrophages can release a number of products in vitro, of which some have opposing effects on fibroblasts (38). The result in vivo will depend on control of their release as well as fibroblast "receptivity" to mononuclear cell products. Further characterization of macrophage products, regulation of their release, and putative fibroblast receptors may be useful in elucidating the role of macrophages in pulmonary fibrosis.

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