JCI The Journal of Clinical Investigation

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J Clin Invest. 1984;73(5):1462-1472. https://doi.org/10.1172/JCI111350.

Research Article

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Silica-stimulated Monocytes Release Fibroblast Proliferation Factors Identical to Interleukin 1

A Potential Role for Interleukin 1 in the Pathogenesis of Silicosis

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Abstract. Previous study strongly suggests that silicotic fibrosis is mediated by macrophages and their soluble mediators. The biochemical properties of the mediators involved in silicotic fibrosis, however, are as yet ill defined. The current study, therefore, determined whether human monocyte-macrophages treated with fibrogenic silica dust released factors capable of activating fibroblasts as measured by an increase in fibroblast proliferation. Silica, but not nonfibrogenic diamond dust, stimulated the release of fibroblast proliferation factors. Moreover, the level of fibroblast proliferation activity was comparable with the level of thymocyte proliferation (interleukin-1) activity in the same culture supernatants. The factors responsible for these seemingly diverse activities were found to behave identically when analyzed by gel filtration chromatography, size exclusion chromatography, isoelectrofocusing, ion exchange chromatography, and hydrophobic chromatography. Moreover, the response of these factors to four different proteases and heat (56°C) was also identical, which shows that their comigration on various separation media could not be explained by noncovalent interaction between otherwise unrelated species. The data demonstrate that a monocyte-derived thymocyte proliferation factor having the molecular properties of interleukin 1 is capable of regulating fibroblast proliferation. In silicosis and other fibrotic diseases, the local release of interleukin 1 may contribute to abnormal connective tissue deposition by stimulating fibroblast proliferation, and thereby, amplifying other signals stimulating the synthesis of connective tissue components.

Introduction

The fact that chronic occupational exposure to silica leads to pulmonary fibrosis has been appreciated for more than a century (1). Early histologic studies showed that fibrosis occurred in the vicinity of nodules containing large numbers of silica-laden macrophages. In vitro experiments showing, first, that macrophages die within hours of ingesting silica, and second, that silica particles released by death of macrophages have undiminished cytotoxicity when added to fresh cultures of macrophages suggested that repeated cycles of macrophage killing in vivo leads to the activation of fibroblasts and the deposition of excess connective tissue (2). Consistent with this view, a good correlation was observed between the relative toxicity of various forms of silica and other mineral dusts, as determined on cultured macrophages, and the ability of the dusts to cause fibrosis in vivo (3, 4). Subsequent studies demonstrated that silica-damaged macrophages released soluble factors which stimulated collagen production by cultured fibroblasts (5). Nontoxic, nonfibrogenic mineral dust caused the release of such factors to a significantly lesser extent, suggesting that the fibrosis observed with toxic mineral dusts might be mediated by macrophage-derived factors released as a consequence of cell damage and/or death (5). In confirmation of this hypothesis, experiments using implanted diffusion chambers showed that chambers containing both macrophages and fibrogenic silica caused impressive peritoneal fibrosis, whereas chambers containing macrophages or silica alone caused relatively little fibrogenic reaction (6).

Since fibroblast proliferation is an important feature of certain fibrotic diseases (7, 8) and also appears to be important in

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Received for publication 9 August 1983 and in revised form 12 January 1984.

The Journal of Clinical Investigation, Inc.
Volume 73, May 1984, 1462–1472

the development of silicotic fibrosis (9), we compared the ability of fibrogenic silica and nonfibrogenic diamond dust to stimulate the release of fibroblast proliferation factors by human peripheral blood monocytes. We demonstrate that silica but not diamond dust leads to the release of fibroblast proliferation factors and that the level of fibroblast proliferation activity closely parallels the level of interleukin-1 (IL-1)[1] bioactivity in the same culture supernatants. In addition, we extensively compare the biochemical properties of the fibroblast proliferation factors and IL-1 using a variety of techniques. Evidence is presented that the silica-induced fibroblast proliferation factors and IL-1 behave identically on several different chromatographic separation media, in response to heat, and in response to several proteolytic enzymes having different substrate specificities.

Methods

Mineral dusts. Quartz silica (particle size < 5 μm) was the generous gift of Dr. P. Davies (Merck Institute for Therapeutic Research, Rahway, NJ). This material was fibrogenic as previously reported (6). Diamond dusts (particle sizes < 0.5 μm and 1–2 μm) were kindly provided by The Warren Diamond Powder Co., College Point, NY. Although this material was not tested directly for fibrogenicity, similar preparations have been found by others not to induce a fibrogenic reaction (2, 10). All mineral particulates were washed three times in phosphate buffer saline (PBS), pH 7.2, containing 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate · $2H_2O$ (CHAPS), a zwitterionic detergent (Calbiochem-Behring Corp., La Jolla, CA), and then washed three times in PBS alone. The particles were resuspended after each wash with the aid of an ultrasonic device. After the final wash, each suspension was X-irradiated with 5,000 R to sterilize the particles.

Monocyte and culture supernatant preparation. Peripheral blood mononuclear cells were isolated from the heparinized venous blood of normal volunteers using Isolymph discontinuous gradients (Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, NY). The interface cells were washed three times in RPMI-1640 and suspended at a concentration of 5×10^6 cells/ml in RPMI-1640 containing 2 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% autologous serum. 1 ml aliquots of the cell suspension were placed in 24-mm wells (Linbro, Hamden, CT) and incubated at 37°C in a humidified atmosphere of 5% CO_2:air. After 2 h, the nonadherent cells were removed with five 2 ml washes of warm Hanks' balanced salt solution. The remaining adherent population consisted of >99% latex positive and 98% esterase positive cells, and had the typical morphology of monocytes after Giemsa staining. After the last wash, 1 ml of the RPMI-1640 medium containing 1% autologous serum and 50 μg/ml quartz silica particles was added to each well. After incubating the cultures for an additional 24 h, the supernatants were removed, spun at 2,000 rpm for 10 min, filtered through a 0.22-μm Millex filter (Millipore, Bedford, MA), and frozen at −80°C. Crude supernatants prepared in this manner were the starting material for the fractionation studies described below. In duplicate determinations, cells remaining after 24 h of incubation in the presence of silica were 82 and 84% viable by trypan blue exclusion. 96 and 98% of cells cultured under identical conditions but in the absence of silica were viable.

In those experiments in which the effects of silica and diamond dust were compared, adherent cells were incubated with 1 ml of medium containing 5% autologous serum and 0, 25, 50, or 100 μg/ml of either silica or diamond dust. 5%, instead of 1% autologous serum (see above), was used in order to maximize the generation of thymocyte and fibroblast proliferation activity by the cultured cells. The supernatants were collected as described above and tested for biological activity. Phagocytosis of the various mineral particulates was determined microscopically on living or stained cells using phase contrast or polarized light.

Fibroblast proliferation assay. As previously described (11), normal adult human dermal fibroblasts (CRL 1424) were obtained from the American Type Culture Collection, Rockville, MD, and cultured in Dulbecco's Modified Eagle Medium containing 4 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (fibroblast culture medium). In preparation for a fibroblast proliferation assay, confluent cultures (passages 1–5 in our laboratory) were treated with a solution containing 0.5 mg/ml trypsin and 0.2 mg/ml EDTA. The resultant single cell suspension was diluted in fibroblast culture medium to a final concentration of 1×10^5 cells/ml. 100 μl aliquots of this cell suspension were dispensed into flat bottom microtiter wells (#3596; Costar, Cambridge, MA) and incubated overnight at 37°C in a humidified atmosphere of 5% CO_2:air. On the next day, the medium was gently aspirated from the attached fibroblasts and replaced with 150 μl of the test material to be assayed for fibroblast proliferation activity. Samples to be tested for fibroblast proliferation activity had been serially diluted in fibroblast culture medium containing 10% fetal calf serum prior to assay (see below). This concentration of serum and density of fibroblasts (10^4/well) were chosen since, in preliminary experiments, these conditions supported optimal mitogenic stimulation in the presence of monocyte-derived factors. 48 h later, 1 μCi of [^{3}H]thymidine (6.7 Ci/mmol, New England Nuclear, Boston, MA) was added to the wells. After incubation for an additional 16–18 h, the medium was gently aspirated from each well and replaced with 250 μl of trypsin/EDTA solution, and the cultures were further incubated at 37°C for 10 min. The cells were harvested using an automated harvester and the radioactivity was counted in a liquid scintillation counter. Activity was expressed in units per milliliter using a standard preparation described below.

As an independent measure of fibroblast growth, fibroblasts were plated in 16-mm wells (No. 3524, Costar) at a concentration of 6×10^4 cells/well. After incubation for 6 d in fibroblast culture medium with or without supernatant-derived fibroblast proliferation factors, the cells were treated with trypsin/EDTA as above, and counted in a Coulter Counter (Model ZBI; Coulter Electronics Inc., Hialeah, FL), at an amplification setting of 2 and an aperture setting of 0.5.

Thymocyte proliferation assay. IL-1 activity was determined by its capacity to stimulate thymocyte proliferation in the presence of phytohemagglutinin, as previously described in detail (12). Activity was expressed in units per milliliter.

Determination of bioactivity units. Units of biological activity were determined in both the fibroblast and thymocyte proliferation assays by making reference to two standard preparations, each of which had been arbitrarily assigned a value of 100 units/ml. The standard preparations were the supernatants of human monocyte cultures stimulated with silica particles (50 μg/ml) for 24 h. Aliquots of each standard preparation were kept at −70°C until further use. In the experiment shown in Fig. 2, the same standard preparation was used in both the fibroblast and thymocyte proliferation assays in order to compare the amounts of fibroblast and thymocyte proliferation activity obtained for a given supernatant. In subsequent experiments, one of the standard

1. *Abbreviations used in this paper:* IL-1, interleukin-1; pI, isoelectric pH.

preparations was consistently used in the fibroblast proliferation assay while the other standard preparation was used in the thymocyte assay. Samples to be assayed and an aliquot of the standard preparation were serially diluted in culture medium containing 10% fetal calf serum and tested for their capability to stimulate fibroblasts or thymocytes, as described above. The [^{3}H]thymidine incorporation levels were plotted as a function of dilution for both the samples and the standard preparation (Fig. 1). The unit values of the samples were determined by comparing their response curves with that of the standard preparation at 50% of the response given by a 1:10 dilution of the standard preparation. For example, as shown in Fig. 1 for the fibroblast proliferation assay, by dividing the dilution of the standard preparation giving a 50% response (D 50/Standard) by the dilution of the sample giving a 50% response (D 50/sample) and multiplying the quotient by 100, the number of units per milliliter in the sample was determined.

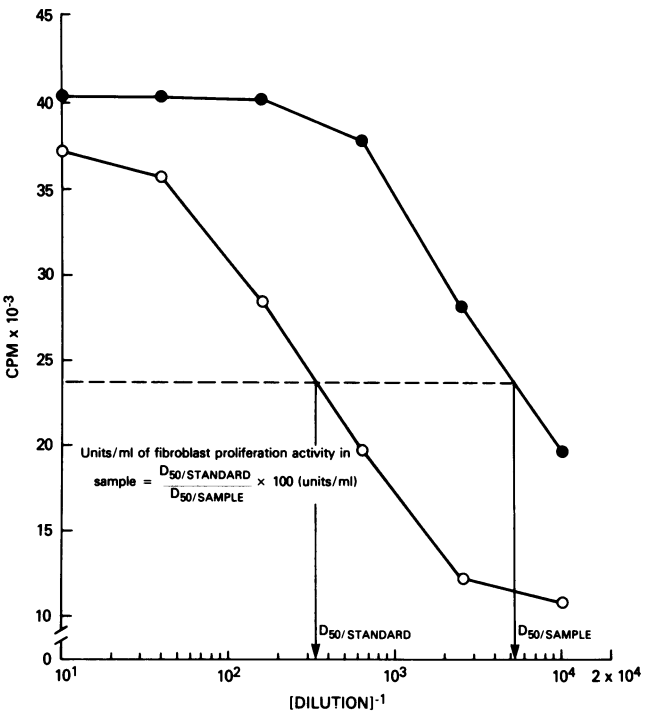

Figure 1. Determination of units of fibroblast proliferation activity. A standard preparation of crude culture supernatant, arbitrarily assigned an activity of 100 units/ml (open circles), and a fraction obtained from gel filtration (closed circles) were both serially diluted and assayed in the fibroblast proliferation assay as described in Methods. Each point is the mean of duplicate determinations. The background level of fibroblast proliferation obtained in the presence of assay medium alone was 9952±460 (SEM) cpm. The dose-response curves were compared at a level (dotted line) equal to one half of the response given by a 1:10 dilution of the standard, i.e., 0.5 (37,273–9,952) + 9,952 = 23,612 cpm. The dilutions of the standard and sample giving this level of [^{3}H]thymidine incorporation (D50/standard and D50/sample, respectively) were then determined by drawing vertical lines (arrows) to the horizontal axis. The number of units per milliliter (in this case 1,575) of fibroblast proliferation activity in the sample was then calculated as shown.

Gel filtration. A 2.5 × 85-cm bed of Sephacryl S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) was equilibrated at 4°C with column buffer consisting of 100 mM NaCl, 50 mM Tris, pH 7.8 (4°C), and calibrated with the following molecular weight markers: bovine serum albumin, 67,000 mol wt; ovalbumin, 43,000 mol wt; ribonuclease, 13,700 mol wt; myoglobin, 18,000 mol wt; and cytochrome c, 12,500 mol wt. All of the fibroblast and thymocyte proliferation activity in a crude culture supernatant (40 ml) was recovered in a 45–85% ammonium sulfate precipitate. The precipitate was dissolved in 4 ml of column buffer, dialyzed vs. column buffer, and chromatographed at a flow rate of 40 ml/h. 5 ml fractions were collected. Aliquots from every other fraction were assayed for fibroblast and thymocyte proliferation activities and units determined as explained above.

Size exclusion high performance liquid chromatography. 40 ml of crude culture supernatant was concentrated by ammonium sulfate precipitation (see above) and the precipitate was dissolved in 4 ml of column buffer. After dialysis vs. column buffer, a 200 μl sample was chromatographed on a 7.5 × 600 mm TSK G-3000 SW column (LKB, Gaithersburg, MD) by a Hewlett-Packard 1084 B high performance liquid chromatograph at ambient temperature and a flow rate of 1.0 ml/min. The column buffer was 50 mM sodium phosphate, pH 7.0. Absorbance at 214 nm was monitored continuously. 0.5-ml fractions were collected and assayed for bioactivity.

Preparative isoelectric focusing. A 45–85% ammonium sulfate precipitate of crude culture supernatant (40 ml) was dialyzed extensively against 1% glycine. After the addition of 10 ml of Pharmalyte (pH 3.5–10) (Pharmacia Fine Chemicals), the sample was diluted to a volume of 190 ml with distilled water. 8 g of Ultradex (LKB) was then slowly added. The slurry was degassed, poured into a Pharmacia 217 × 217 × 5-mm gel trough, and dried with an overhead hair dryer. Electrode wicks soaked in 0.1 M H_2SO_4 and 1 M ethylenediamine served as the anode and cathode, respectively. The gel trough was then mounted on a Pharmacia FBE 3000 flat bed apparatus, connected to a coolant supply (4°C), and run at a constant power of 30 watts for 6 h. Afterwards, a fractionation grid was inserted and the pH of each fraction was measured by means of a surface electrode. Each gel fraction was then transferred to a small column and eluted with 4 ml of 150 mM NaCl. The fractions were dialyzed extensively in a MD 1200 dialysis apparatus (Bethesda Research Laboratories, Bethesda, MD) against phosphate-buffered saline, pH 7.4, and assayed for bioactivity.

Cation exchange chromatography. A 0.4 × 3.0-cm bed of SP-Sephadex (Pharmacia Fine Chemicals) was equilibrated at 25°C with 10 mM sodium acetate buffer, pH 4.0 (25°C). 2.5 ml of crude culture supernatant was dialyzed against the same buffer and applied to the column. The column was then washed with 15 ml of buffer. A gradient of increasing ionic strength and pH was produced by a Pharmacia gradient maker (Pharmacia Fine Chemicals) loaded with 20 ml of 10 mM acetate buffer and 20 ml of 400 mM acetate buffer, pH 6.3 (25°C). The flow rate was 4 ml/h. 1-ml fractions were collected and each was assayed for bioactivity. Conductivity was measured after 0.1 ml of each fraction had been diluted in 3.9 ml of distilled H_2O with a conductivity meter (type CDM2c, Radiometer, Copenhagen, Denmark).

Anion exchange chromatography. A 1 × 10-cm bed of DEAE Sephacel (Pharmacia Fine Chemicals) was equilibrated at 4°C with 10 mM sodium phosphate buffer, pH 7.5. 10 ml of crude culture supernatant was dialyzed against the same buffer and applied to the column. After washing the column with 20 ml of buffer, a gradient of increasing ionic strength was produced by a gradient maker loaded with 60 ml of 10 mM sodium phosphate buffer and 60 ml of the same buffer containing 1 M NaCl. The flow rate was 6 ml/h. 2-ml fractions were collected and every other

fraction was assayed for bioactivity. The conductivity of each fraction was measured as described above.

Hydrophobic chromatography. A 0.4 × 2.0-cm bed of phenyl-Sepharose (Pharmacia Fine Chemicals) was equilibrated at 25°C with 0.8 M ammonium sulfate, 10 mM sodium phosphate buffer, pH 6.8 (25°C). 3.0 ml of crude culture supernatant was dialyzed against the same buffer and applied to the column. The column was then washed with 4 ml of the buffer. A gradient of decreasing ionic strength and increasing ethylene glycol concentration was produced by a gradient maker loaded with 12 ml of 0.8 M ammonium sulfate, 10 mM phosphate, pH 6.8 (25°C), and 12 ml of 50% (vol/vol) ethylene glycol, 10 mM phosphate, pH 6.8 (25°C). The flow rate was 6 ml/h. 1-ml fractions were collected and each was assayed for bioactivity. Conductivity was measured as described above.

Protease susceptibility. The fractions containing both thymocyte and fibroblast proliferation activity after Sephacryl S-200 gel filtration chromatography were pooled, concentrated 10-fold on a YM-10 ultrafiltration membrane (Amicon Corp., Lexington, MA), and then dialyzed against 0.1 M $CaCl_2$, 0.08 M Tris, pH 7.8 (24°C) (13). In experiments designed to test the suscpetibility of these activities to a variety of proteases having different substrate specificities, aliquots of the concentrate were mixed with trypsin (Boehringer Mannheim, West Germany), α-chymotrypsin (Millipore Corp., Bedford, MA), *Streptomyces griseus* protease (Sigma Chemical Co., St. Louis, MO), or subtilisin (Boehringer Mannheim) at a final concentration of 200 μg/ml. After incubation at 37°C for 24 h, each mixture was assayed for both fibroblast and thymocyte proliferation activity and compared with a control aliquot incubated in the absence of enzyme. To determine if the enzymes themselves had any effect on the determination of bioactivity, two types of control experiments were performed. First, each enzyme was incubated for 24 h at a concentration of 200 μg/ml in buffer, and then, assayed in the fibroblast and thymocyte assays at the same dilutions used for the aliquots containing bioactive material. None of the enzymes tested contained any fibroblast or thymocyte proliferation activity and none appeared toxic at the dilutions tested as judged by the lack of suppression of background [^{3}H]thymidine incorporation in each assay. Second, in some experiments, aliquots of the active concentrate incubated alone or in the presence of trypsin or α-chymotrypsin were treated after 24 h of incubation at 37°C with soybean trypsin inhibitor (2 mg/ml final concentration; Sigma Chemical Co.), and then, assayed for fibroblast and thymocyte proliferation activity. This concentration of soybean trypsin inhibitor was demonstrated to eliminate the degradation of *N*-benzoyl-L-tyrosine ethyl ester (Sigma Chemical Co.) by α-chymotrypsin (13) and the degradation of *p*-toluenesulphonyl-L-arginine methyl ester (Sigma Chemical Co.) by trypsin when these enzymes were present at a concentration of 200 μg/ml (14).

Heat inactivation experiments. Replicate aliquots of crude culture supernatant were heated in a 56°C water bath. At various time intervals, single tubes were removed from the water bath and refrigerated at 4°C. Each aliquot was then assayed for thymocyte and fibroblast proliferation activity.

Protein determination. Protein concentration was determined by directly measuring the absorbance at 280 nm, directly measuring the absorbance at 214 nm, or by measuring the absorbance at 590 nm after the addition of Coomassie Brilliant Blue G reagent (Bio-Rad Laboratories, Richmond, CA) (15).

Results

Stimulation of fibroblast and thymocyte proliferation factors by silica and diamond dust. Initially, a silica preparation (particle size < 5 μm) known to be capable of producing pulmonary fibrosis was added to highly purified monocytes at a concentration of 0, 25, 50, or 100 μg/ml. Two different sizes of diamond dust (particle sizes of <0.5 μm and 1–2 μm), a material which is reported to be nonfibrogenic (2, 10), were also added to monocytes at the same concentrations used for the silica dust. Each type of dust was rapidly phagocytized by the monocytes. After 24 h of incubation, the culture supernatants were removed, processed as detailed in Methods, and tested for both fibroblast and thymocyte proliferation activity using the same standard preparation in both assays. As shown in Fig. 2, left, the silica stimulated a 5–10-fold increase in the amount of fibroblast and thymocyte proliferation activity released by the cells as the concentration of silica dust was increased from 0 to 100 μg/ml. Diamond dust having a particle size < 0.5 μm (Fig. 2, middle) did not stimulate the release of thymocyte proliferation activity and stimulated the release of fibroblast proliferation activity only 1–3-fold. Diamond dust having a particle size of 1–2 μm (Fig. 2, right) inhibited the release of thymocyte proliferation activity at all doses tested and likewise inhibited the release of fibroblast proliferation activity except at a dose of 25 μg/ml where a slight stimulation (1.9-fold) was observed. When supernatants generated in the presence of 50 μg/ml silica or diamond dust (particle size < 0.5 μm or 1–2 μm) were mixed

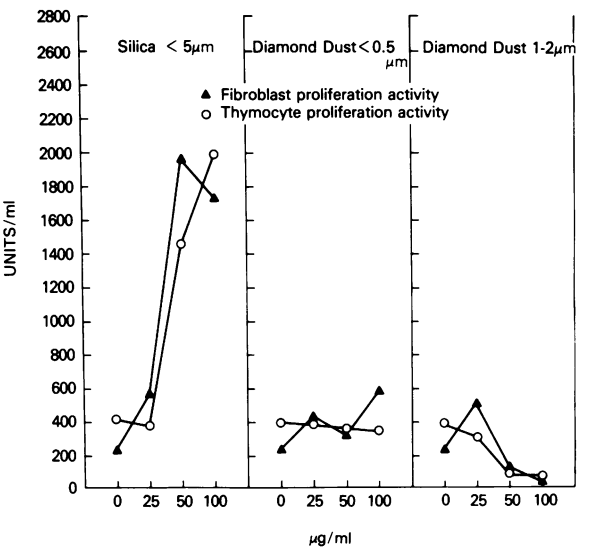

Figure 2. Comparison of the ability of silica and two different sizes of diamond dust to stimulate the release of fibroblast and thymocyte proliferation activities by cultured human monocytes. Monocytes were incubated with silica having a particle size of <5 μm (left), diamond dust having a particle size <0.5 μm (center), or diamond dust having a particle size of 1–2 μm (right) at concentrations of 0, 25, 50, or 100 μg/ml for 24 h. Afterwards, the supernatants were removed, processed as described in Methods, and tested for both fibroblast and thymocyte proliferation activity. Culture medium containing 100 μg/ml of mineral dust and incubated in the absence of cells contained no fibroblast or thymocyte proliferation activity.

together in equal proportions, no inhibition of fibroblast and thymocyte proliferation activity was observed over and above that expected on the basis of dilution alone, i.e., supernatants from diamond-stimulated cells did not appear to contain inhibitors capable of obscuring the proliferative response of fibroblasts and thymocytes to stimulatory factors. Culture medium supplemented with 100 μg/ml of silica or either size of diamond dust and incubated in the absence of cells contained no fibroblast or thymocyte proliferation activity. In general, the level of fibroblast proliferation activity in a given supernatant closely paralleled the level of thymocyte proliferation activity in the same supernatant (Fig. 2). These results demonstrate that silica is capable of stimulating the release of fibroblast and thymocyte proliferation activity from human monocytes. The latter effect has been previously described (16). The increased levels of thymocyte proliferation activity were similar to those stimulated by agents, such as lipopolysaccharide or zymosan (reference 16 and unpublished data). The control experiments with diamond dust suggest that the silica-macrophage interaction is specific and that further study of the molecules responsible for the fibroblast and thymocyte proliferation activities might be relevant to the understanding of silicotic fibrosis.

Gel filtration. To determine the approximate size of the molecules responsible for these bioactivities, a 45–85% ammonium sulfate precipitate of a crude culture supernatant derived from silica-stimulated monocytes was chromatographed on a column of Sephacryl S-200. The absorbance of each fraction at 280 nm was determined (Fig. 3, bottom). The elution profile is typical of serum proteins. Every other fraction was then assayed in the thymocyte and fibroblast proliferation assays, and the units of each activity determined. In this and all subsequent experiments, different standard preparations were used in each of the bioassays as described in Methods. As can be seen in the upper panel of Fig. 3, both activities eluted as single sharp symmetrical peaks at a molecular weight of ~13,000.

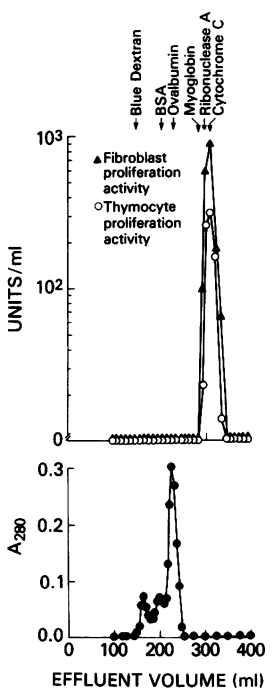

Figure 3. Coelution of fibroblast and thymocyte proliferation activities (units per milliliter) on Sephacryl S-200 gel filtration chromatography. A 45–85% ammonium sulphate precipitate of 40 ml of crude supernatant was dissolved in 4 ml of column buffer and applied to the column. 5 ml fractions were collected. Beginning at an effluent volume of 100 ml, the absorbance of each fraction at 280 nm was determined (bottom). An aliquot of every other fraction was assayed for thymocyte proliferation activity and fibroblast proliferation activity as detailed in Methods (top). The molecular weight markers used to calibrate the column are shown at the top of the figure.

To confirm that the [^{3}H]thymidine incorporation observed in the microwell fibroblast proliferation assay was indeed a measure of fibroblast growth, the peak (effluent volume: 305–310 ml) and flanking (effluent volumes: 275–280 and 345–350 ml) fractions shown in Fig. 3 were tested for their ability to stimulate fibroblast growth as measured by an increase in cell number. Specifically, an aliquot from each of these fractions was diluted 1:10 in fibroblast culture medium and added in triplicate to 6 × 10^4 fibroblasts cultured in 16-mm wells (see Methods). Replicate cultures of fibroblasts cultured in fibroblast culture medium alone served as a control. After 6 d, the fibroblasts in each well were removed and counted in a Coulter counter. The wells stimulated with material from the peak fraction contained 29.5 (±0.5) × 10^4 cells/well (±1SEM), whereas wells stimulated with material from the flanking fractions contained 11.8 (±0.5) × 10^4 and 12.2 (±0.3) × 10^4 cells/well. Wells stimulated with fibroblast culture medium alone contained 11.8 (±0.4) × 10^4 cells/well. Thus, [^{3}H]thymidine incorporation in the microwell fibroblast proliferation assay appeared to be a valid measure of fibroblast growth.

Size exclusion high performance liquid chromatography. The sizes of the molecules responsible for the fibroblast and thymocyte proliferation activities were further compared by using size exclusion high performance liquid chromatography. This technique was used in addition to conventional gel filtration chromatography because of its known ability to often resolve molecules of very similar molecular weights (17). As shown on the upper panel of Fig. 4, the fibroblast and thymocyte proliferation activity profiles were again observed to be superimposable. Each had a retention time corresponding to a molecular weight of 11,500. The absorbance of the column effluent was monitored continuously at 214 nm and is shown in the lower panel of Fig. 4. Good separation of the bioactive materials from the bulk of serum proteins has been achieved.

Isoelectric focusing. To determine the isoelectric points of these mediators, a crude culture supernatant was electrofocused on a flat bed apparatus using granulated gel (Fig. 5). The absorbance of each fraction at 280 nm is plotted on the lower panel. The large peak (isoelectric pH [pI] 4.5–5.0, bottom) is due to human serum albumin which typically migrates as a broad band with this technique. Each fraction was assayed for fibroblast proliferation activity and thymocyte proliferation activity and the units of each activity determined. As shown in the upper panel of Fig. 5, three coinciding peaks of fibroblast and thymocyte proliferation activity were observed at pIs of ~6.8, 5.8, and 5.2.

Ion exchange chromatography. Isoelectric focusing separates molecules according to their net charge. In an attempt to resolve

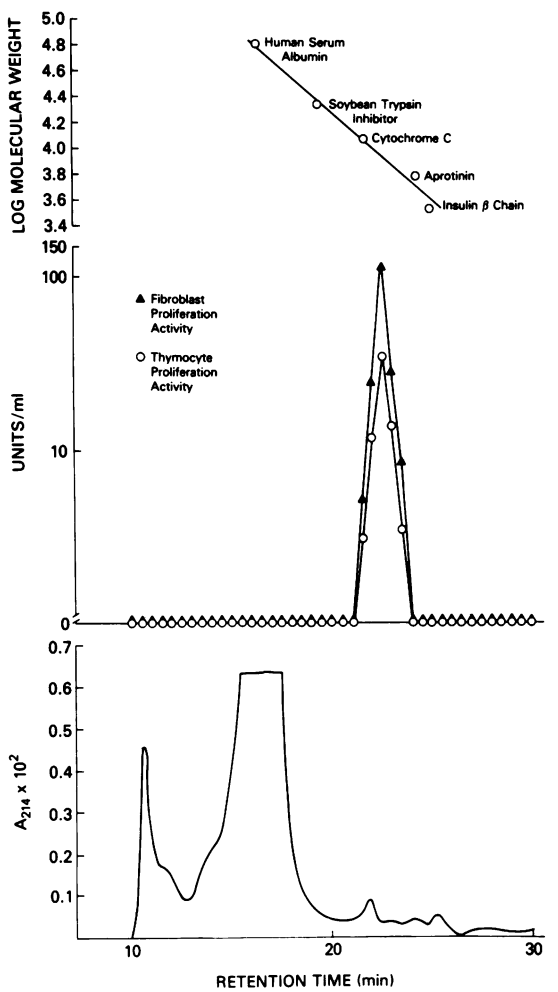

Figure 4. Coelution of fibroblast and thymocyte proliferation activity on size exclusion high performance liquid chromatography. 200 μl of a crude culture supernatant concentrated by ammonium sulphate and dissolved in column buffer was applied to the column. 30-s fractions were collected. Absorbance of the column effluent at 214 nm was monitored continuously (bottom). An aliquot of each fraction was assayed for fibroblast and thymocyte proliferation activity (middle). The log molecular weights of the standards used to calibrate the column are plotted vs. their corresponding elution times in the top panel.

the thymocyte and fibroblast proliferation factors on the basis of their surface charge characteristics, crude culture supernatant was analyzed by ion exchange chromatography. The results obtained with SP-Sephadex, a cation exchanger, are shown in Fig. 6 *A*. The elution profile of Coomassie Blue reactive material is shown in the lower panel. As shown in the upper panel, single sharp overlapping peaks of fibroblast and thymocyte proliferative activity were eluted as the concentration of counterion in the column buffer was increased. The results obtained with DEAE-Sephacel anion exchange chromatography are shown in Fig. 6 *B*. The elution profile of Coomassie Blue reactive material is shown in the lower panel. As shown in the upper panel, two peaks of fibroblast proliferation activity were observed. One peak eluted in the column wash and the other was eluted by a gradient of increasing ionic strength. When the same fractions were assayed for thymocyte proliferation activity, a similar elution pattern was observed.

Hydrophobic chromatography. Thus far, the thymocyte and fibroblast proliferation factors derived from silica-stimulated monocytes have been compared on the basis of their size and charge properties and have been found to be similar. In a further attempt to resolve these mediators, crude culture supernatant was analyzed by phenyl-Sepharose hydrophobic chromatography (Fig. 7). The elution profile of Coomassie Blue reactive material is shown in the lower panel. As shown in the upper panel, superimposable peaks of fibroblast proliferation activity and thymocyte proliferation activity were eluted as the salt concentration in the column buffer was decreased.

Protease digestion. To determine if both the fibroblast and thymocyte proliferation activities were equally susceptible to digestion by various proteolytic enzymes, replicate aliquots of partially purified factors (Methods) were incubated at 37°C with 200 μg/ml of trypsin, α-chymotrypsin, *S. griseus* protease, or subtilisin for 24 h. Partially purified factors incubated in the absence of proteases and proteases incubated alone served as

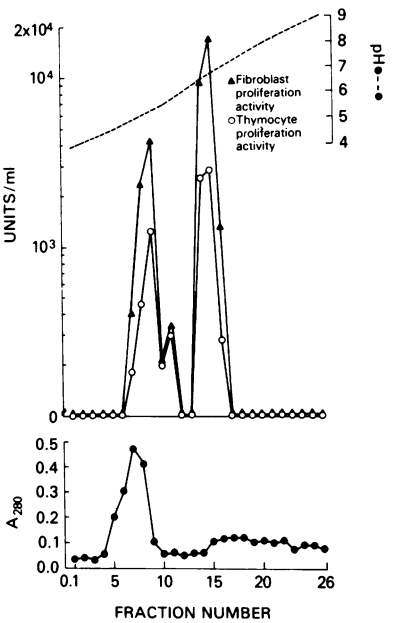

Figure 5. Comigration of fibroblast and thymocyte proliferation activities on preparative isoelectrofocusing. 50 ml of crude culture supernatant was electrofocused as described in Methods. The absorbance of each fraction at 280 nm was determined (bottom). An aliquot of each fraction was assayed for fibroblast and thymocyte proliferation activity (top). The pH of each fraction was measured and is plotted at the top of the figure.

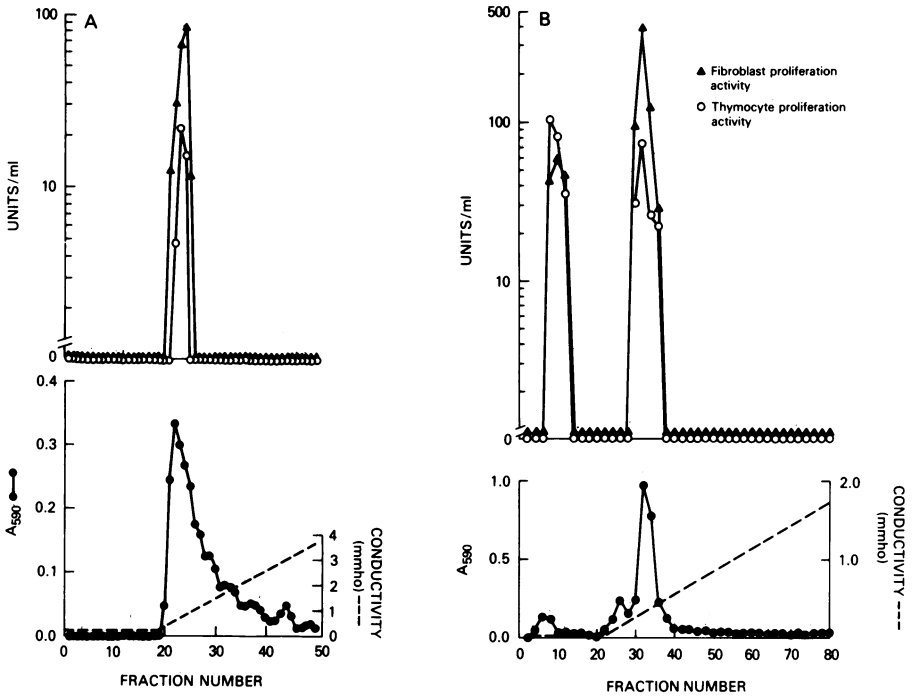

Figure 6. Coelution of fibroblast and thymocyte proliferation activity on SP-Sephadex cation exchange chromatography and DEAE-Sephacel anion exchange chromatography. (*A*) 2.5 ml of crude culture supernatant was dialyzed against 10 mM sodium acetate buffer, pH 4.0 (25°C), and applied to the column. The column was washed with the same buffer and then eluted with a gradient of increasing ionic strength and pH (see Methods). An aliquot of each fraction was reacted with Coomassie Brilliant Blue G and the absorbance at 590 nm was determined (bottom). An aliquot of each fraction was assayed for fibroblast and thymocyte proliferation activity (top). (*B*) 10 ml of crude culture supernatant was dialyzed against 10 mM sodium phosphate buffer, pH 7.5, and applied to a 1 × 10-cm column of DEAE-Sephacel. The column was washed with the same buffer and then eluted with a gradient of increasing ionic strength (see Methods). An aliquot of every other fraction was reacted with Coomassie Brilliant Blue G and the absorbance at 590 nm was determined (bottom). An aliquot of every other fraction was assayed for fibroblast and thymocyte proliferation activity (top).

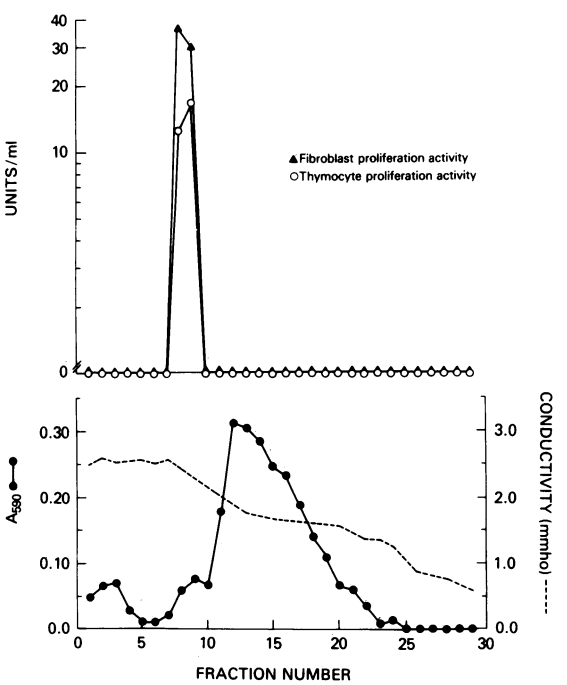

Figure 7. Coelution of fibroblast and thymocyte proliferation activity on phenyl sepharose hydrophobic chromatography. 3 ml of a crude culture supernatant was dialyzed against 0.8 M ammonium sulfate, 10 mM sodium phosphate buffer, pH 6.8, and applied to the column. The column was washed with 4 ml of the same buffer and then eluted with a gradient of decreasing ionic strength and increasing ethylene glycol concentration (see Methods). An aliquot of each fraction was reacted with Coomassie Brilliant Blue G and the absorbance at 590 nm determined (bottom). Each fraction was assayed for fibroblast and thymocyte proliferation activity (top).

controls. Each reaction mixture was then assayed in both the fibroblast and thymocyte proliferation assays. As shown in Table I, neither activity was affected by trypsin whereas both activities were largely destroyed by α-chymotrypsin. In addition, both activities were entirely destroyed by *S. griseus* protease and subtilisin. When the solutions containing only proteases were tested at the same dilutions as the reaction mixtures containing proteases and active factors, no stimulation or inhibition of fibroblast or thymocyte proliferation was observed. As a further test of potential interference by the proteases in the determination of bioactivity, aliquots of active factors incubated alone or in the presence of trypsin or α-chymotrypsin were treated at the end of the 24-h incubation period with soybean trypsin inhibitor in amounts sufficient to entirely quench the activity of these enzymes on synthetic substrates (see Methods). Results identical to those obtained without the addition of soybean trypsin inhibitor (Table I) were observed.

Heat inactivation. The thymocyte and fibroblast proliferation factors were compared by studying their respective rates of inactivation at 56°C. Since the rate at which a protein molecule loses its activity at a given temperature is often a highly characteristic feature of that molecule (18), this approach provided

*Table I. Susceptibility of Silica-induced Fibroblast and Thymocyte Proliferation Factors to Inactivation by Various Proteolytic Enzymes**

Enzyme (*200 μg/ml*)	Percent activity remaining after 24 h	
	Fibroblast proliferation assay	Thymocyte proliferation assay
—	100	100
Trypsin	105	100
α-Chymotrypsin	14	18
S. griseus protease	0	0
Subtilisin	0	0

* The results are those of a representative experiment in which single tubes containing partially purified factors±enzyme were each assayed for fibroblast and thymocyte proliferation activity after incubation for 24 h at 37°C.

a sensitive means of determining whether different mediators were responsible for these activities. As shown in Fig. 8, the rates of decay of the two activities at 56°C were nearly identical. The half-life, as calculated from the initial portion of the decay curve, was 12 min for the fibroblast proliferation activity and 12.5 min for the thymocyte proliferation activity.

Discussion

The observation that monocytes and macrophages, cultured alone or in the presence of various agents, release fibroblast proliferation factors has been confirmed in a number of laboratories (19–21) since this observation was first made by Alexis Carrel in 1923 (22). In the current study, we have shown that silica, an agent known to cause pulmonary fibrosis in vivo, also stimulates the release of fibroblast proliferation factors by monocytes, and that diamond dust, a nonfibrogenic material, does so only slightly or inhibits the release of such factors. These results parallel those of Heppleston and Stiles (5) who showed that silica but not titanium dioxide, a nonfibrogenic material, stimulated macrophages to generate factors which enhanced collagen production by cultured fibroblasts.

Previous studies have shown that macrophages cultured in the presence of silica release IL-1 (16), chemoattractants (23), and factors that regulate fibroblast connective tissue biosynthesis (24). Whether the release of these factors depends in part on the well-known cytotoxic effect of silica on macrophages (2) remains unclear. Data comparing the fibrogenicity of various dusts with their cytotoxicity for macrophages in vitro (3, 4) suggest that macrophage damage may be an important element in the pathogenesis of pulmonary fibrosis caused by mineral dusts. Nevertheless, it should be emphasized that the fibroblast and thymocyte proliferation activity found in the culture supernatants of silica-stimulated monocytes is not simply the result of cell lysis. Macrophages that were incubated in the absence of silica and then lysed released 10-fold less fibroblast and thymocyte proliferation activity than macrophages lysed after incubation in the presence of silica (J. Schmidt and I. Gery, unpublished observations). Heppleston and Stiles (5), likewise, found that macrophage lysates later supplemented with silica contained less fibrogenic activity than macrophages lysed after exposure to silica. Thus, it appears that silica damages macrophages, and at the same time, leads to increased production of factors.

Although the ability of macrophages to produce fibroblast proliferation factors has been recognized for some time, only recently have attempts been made to characterize these factors in biochemical terms (25–29). In a previous study (11), we examined the fibroblast proliferation factors generated in secondary human allogeneic mixed lymphocyte reactions and found that they had molecular weights and isoelectric points similar to human IL-1 and that they had IL-1 functional activity. In the present study, fibroblast and thymocyte proliferation factors generated by highly enriched populations of monocytes were analyzed by a variety of biochemical techniques. Both conventional gel filtration chromatography and size exclusion high performance liquid chromatography showed that the apparent molecular weights of the fibroblast proliferation factors and thymocyte proliferation factors were the same. When examined for their net charge characteristics by isoelectrofocusing, both factors had major charge species with pIs of 6.8, 5.8, and 5.2, respectively. The distribution of surface charge on both the fibroblast and thymocyte proliferation factors also appeared to be the same on the basis of anion and cation exchange chromatography. The result obtained with DEAE anion exchange

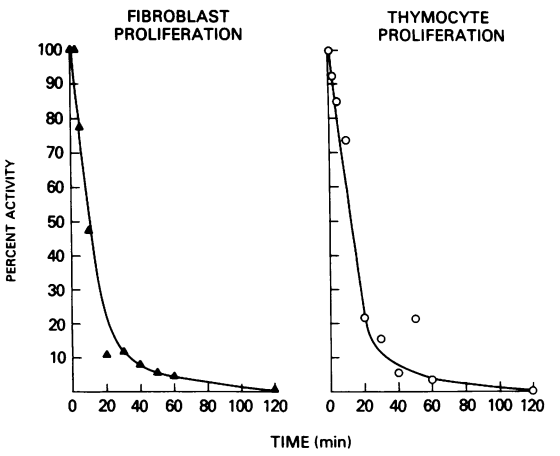

Figure 8. Kinetics of inactivation of the thymocyte and fibroblast proliferation activities at 56°C. Replicate aliquots of crude culture supernatant were incubated at 56°C for the indicated amounts of time and then refrigerated at 4°C. Each aliquot was subsequently assayed for thymocyte and fibroblast proliferation activity. The data is expressed as the percent of that amount of activity found in control aliquots kept at 4°C.

chromatography was particularly important since previous studies have reported resolving macrophage-derived fibroblast proliferation factors from IL-1 by using this chromatographic technique (25–28). We then attempted to resolve the factors on the basis of their ability to interact with a hydrophobic resin, namely, phenyl sepharose. Once again, superimposable activity profiles were obtained. The results up to this point in the study have not ruled out the possibility that the fibroblast and thymocyte proliferation factors are distinct but noncovalently linked, resulting in comigration on a variety of separation media. This possibility was made less likely by both the heat inactivation and enzyme degradation experiments. If the mediators had been distinct but noncovalently bound together, different rates of inactivation at 56°C and differential susceptibility to treatment with various proteolytic enzymes should have been observed. Nonetheless, the half-life of each activity at 56°C was virtually identical. Moreover, each activity behaved identically when treated with trypsin, α-chymotrypsin, *S. griseus* protease, or subtilisin, enzymes having very different substrate specificities. The properties of the thymocyte proliferation factors as defined by gel filtration chromatography (30), isoelectric focusing (31), DEAE anion exchange chromatography (32), and treatment with heat (33), trypsin, and α-chymotrypsin (34) are in agreement with those reported for human IL-1. All these data provide strong evidence that the thymocyte proliferation factor produced by silica-treated monocytes, which has all the biochemical properties of IL-1, is also capable of stimulating fibroblast proliferation.

Results published by other groups, while showing that IL-1 is capable of regulating collagenase production by both dermal and synovial fibroblasts (35, 36), suggest that other macrophage-derived factors distinct from IL-1 may be responsible for the regulation of fibroblast growth (25–29). We suggest, however, that the inability of other groups to detect the fibroblast proliferation activity associated with IL-1, and likewise, our inability to detect other types of macrophage-derived fibroblast proliferation factors is due to important differences in the fibroblast proliferation assays used in these various studies. For example, in our assay, as in the thymocyte proliferation assay, target cell density and serum concentration are important in detecting an IL-1 mediated response (J. Schmidt, unpublished observations). Studies using low serum concentrations (26–28), or sparsely plated target cells (26, 29), or other types of fibroblastic cells (e.g., 3T3 cells [25, 29]) may therefore not detect an IL-1 effect on fibroblasts. The IL-1 effect on fibroblasts may be complex requiring, as suggested by Stiles (37), the presence of other "competence" and/or "progression" factors provided by serum and perhaps fibroblasts themselves. In a fashion analagous to the roles of IL-1 and interleukin-2 in the thymocyte assay (38), IL-1 may stimulate fibroblasts to produce a second factor(s) which is more proximal to the events triggering mitosis. Studies are underway to investigate this hypothesis.

The results of the current study are important because they demonstrate that IL-1, a thymocyte proliferation factor, is capable of regulating the growth of dermal fibroblasts as well. Taken together with the data of others showing that IL-1 may regulate target cells in the hypothalamus (39, 40), liver (41, 42), and synovium (43), these data provide strong confirmatory evidence that IL-1 may play a central hormonal role in regulating a variety of target cells involved in chronic inflammatory processes. Moreover, in light of findings that keratinocytes are also capable of secreting a thymocyte activating factor closely related to IL-1 (44), these findings support the possibility that factors similar to IL-1 may be involved in epidermal-dermal cell interactions. Consistant with this hypothesis, partially purified preparations of epidermal cell-derived thymocyte-activating factor have recently been found to stimulate fibroblast proliferation in vitro (45).

The mechanisms which underlie the development of tissue fibrosis are now being elucidated. It now appears clear that this is a multifaceted, cascading process involving the chemotaxis of fibroblasts (46) into the inflammatory site followed by fibroblast proliferation and secretion of connective tissue components (47). The mediators involved in these various steps appear to be distinct and are derived from macrophages as well as lymphocytes (48). The current study demonstrates that silica, a substance which clearly leads to macrophage-mediated fibrosis in vivo, stimulates the release of IL-1 and that IL-1 has identical properties to the factor which modulates the proliferation of fibroblasts. IL-1 may therefore be responsible for the fibroblast proliferation that has been observed in certain fibrotic diseases (7, 8). Although these studies did not employ cells derived from lung tissues, IL-1 produced by alveolar macrophages (40) may similarly stimulate the proliferation of lung fibroblasts that is observed in silicosis (9). An IL-1-mediated increase in fibroblast number would amplify the effects of other mediators stimulating the synthesis of connective tissue components. The identification of this and other mediators involved in the pathogenesis of tissue fibrosis will provide a rational basis for the design of specific agents to interrupt the development of excess connective tissue deposition.

Acknowledgments

The authors thank Mr. Edwin Warren for providing us with diamond dust, Dr. Philip Davies for providing us with silica dust, Ms. Julia Derr and Mr. Charles Hoes for technical assistance, and Ms. Shirley Starnes for preparation of the manuscript.

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