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# Secretion of Monocyte Chemotactic Activity by Alveolar Macrophages

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***The purpose of this study was to determine if alveolar macrophages (AMs) are a source of monocyte chemoattractants and the role bleomycin interaction with AMs may play in the recruitment of monocytes to the lung in a rodent model of bleomycin-induced pulmonary fibrosis. AMs isolated from rats with bleomycin-induced fibrosis secreted significantly greater amounts of monocyte chemoattractants than those isolated from normal rats. When AMs from normal rats were stimulated with bleomycin*** in vitro, ***monocyte chemotactic activity was secreted into the medium. Chemotactic activity secretion by AM stimulated with 0.01 to 0.1 μg/ml bleomycin was significantly higher than that of cells incubated in medium alone. This activity was truly chemotactic for monocytes, but caused only minimal migration of normal AMs. Bleomycin itself at concentrations of 1 pg/ml to 10 μg/ml had no monocyte chemoattractant activity. Characterization of the chemotactic activity in conditioned media (CM) from bleomycin-stimulated AM demonstrated that the major portion of the activity bound to gelatin, was heterogeneous, with estimated molecular weights of 20 to 60 kd, and was inactivated by specific antifibronectin antibody. These findings suggest that fibronectin fragments are primarily responsible for the monocyte chemotactic activity secreted by AMs. Through increased secretion of such chemotactic substances, AMs could play a key role in the recruitment of peripheral blood monocytes into the lung in inflammatory lung disease and fibrosis. (Am J Pathol 1989, 135:571–580)***

In the normal lung AMs are the predominant cell population in the alveoli and remain so in fibrosis, despite the immigration of other leukocytes.[1–4] The AM is considered to have dual origins, arising both from peripheral blood monocytes that migrate into the lung and from the proliferation of resident macrophages.[5–7] In normal human and murine lungs, monocyte influx appears to play a more important role because local proliferation is minimal.[6,8,9] During the early phases of lung inflammation, monocyte influx has been shown to dramatically increase,[4,10] suggesting that the normal mechanisms controlling monocyte migration have been amplified and/or an additional signal has been induced. Depending on the causative agent, the influx of monocytes may result in a transient or more chronic increase in the lung macrophage population.[2–4,11,12] Regardless of its temporal nature, the rise in macrophage numbers appears to be a fundamental element of the lung's response to injury and fibrosis.

Although this influx of blood leukocytes into the lung in the early stages of fibrosis is well documented, the initiating factors are not well understood. The identification of chemotactic factors for monocytes, and the source of such factors, is important in terms of establishing the mechanism by which the lung macrophage population is maintained under normal conditions and by which it may be increased during lung injury and fibrosis. This control of the lung macrophage population may be central to the fibrotic response, in view of the large body of evidence implicating the macrophage as a source of factors influencing fibroblast growth, collagen synthesis, and chemotaxis.[1,13–19] Although both neutrophils and lymphocytes are also found in the lung in fibrosis, these cells represent a relatively minor component when compared with macrophages,[2,9,11] and the roles of these cells in fibrogenesis are less well established.[1,16] Although AMs isolated from fibrotic animals and macrophages stimulated with fibrogenic agents *in vitro* have been shown to secrete chemoattractants for polymorphonuclear neutrophils,[17,20–22] lymphocytes,[22] peritoneal macrophages, and AMs,[23,24] the chemoattractants produced by these fibrotic macrophages have not been directly shown to attract peripheral blood monocytes.

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In the experiments described here the secretion of monocyte chemotactic activity by AMs was analyzed, and the nature of the activity characterized with the aim of identifying the source(s) of the activity. Cells from normal and bleomycin-induced fibrotic animals were compared with regard to their ability to secrete such activity. The *in vitro* stimulation of AMs with the fibrotic agent, bleomycin, has also been studied to determine if direct stimulation of macrophages could provoke the secretion of monocyte chemotactic factors, which could further increase macrophage numbers in the lung during fibrosis.

## Materials and Methods

### Reagents

The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): Histopaque-1077, cycloheximide, trypan blue, toluidine blue, antifibronectin antibody, p-nitrophenyl phosphate, and zymosan A. RPMI-1640 and penicillin-streptomycin were from GIBCO (Grand Island Biological, Grand Island, NY). BW755c was from Wellcome Research Laboratories (Beckenham, England). Sepracell-MN was from Sepratech Corp. (Oklahoma City, OK). BSA (Bovuminar-low endotoxin) was from Armour Pharmaceutical Co. (Kankakee, IL), and heparin was from Elkins-Sinn Inc (Cherry Hill, NJ). Outdated bleomycin (Blenoxane) was a gift of Bristol Laboratories (Syracuse, NY). Ketamine (100 mg/ml) was purchased from Parke-Davis (Detroit, MI). All other chemicals, unless otherwise specified, were reagent grade or better.

### Animals

Male Fischer 344 rats, which were specific-pathogen free, and weighed 150 to 200 g, were used for all studies, and obtained from Charles River (Portage, MI). These rats arrived in filtered cages and were maintained in clean animal quarters separate from other laboratory animals.

### Macrophages and Preparation of CM

AMs were obtained essentially as described.[2] Rats were given a lethal intraperitoneal injection of ketamine (0.75 ml/rat); the lungs were then perfused with 30 to 40 ml PBS (140 mM NaCl, 10 mM $NaH_2PO_4$, and 8 mM NaOH) in distilled water (pH 7.2) through the right ventricle to flush blood from the lungs. The lungs were lavaged *in situ* with ten, 10-ml washes of sterile (37 C) PBS containing 1 mM EDTA and 0.1% (w/v) glucose. Lavage fluid was pooled and centrifuged (400*g*, 5 minutes), the supernatant was discarded, and cells were resuspended in RPMI containing 2 mg/ml BSA and 1% antibiotics (RPMI-BSA). After 2 hours at 37 C, the nonadherent cells were removed and adherent cells washed twice with RPMI. RPMI-BSA with or without the indicated concentrations of bleomycin was then added and incubation continued for 18 hours. In some experiments macrophages were preincubated for 30 minutes with cycloheximide (1 $\mu$g/ml) before the addition of bleomycin. Only CM prepared for molecular weight determination experiments were made with BSA-free medium. At the end of the incubation period, CM were collected and dialyzed exhaustively using 6 to 8 kd molecular weight cutoff dialysis membranes (Spectrum Medical Industries, Los Angeles, CA) against two changes of RPMI at 4 C. Macrophages were scraped from the wells in the presence of 0.5% trypsin and 5 mM EDTA, counted, and viability was determined by exclusion of trypan blue. The final volume of medium/well was adjusted with RPMI-BSA so that the final equivalent concentration was $2 \times 10^5$ cells/ml for all CM.

### Induction of Bleomycin-Induced Fibrosis

Rats were anesthetized with an intraperitoneal injections of ketamine and given a single intratracheal injection of 1.5 units (mg) of bleomycin in 200 $\mu$l of sterile saline.[2] Control rats received 200 $\mu$l of sterile saline. Animals were killed at the indicated times after intratracheal injections and AMs were obtained as described above. After the 2-hour adherence step, cells were given fresh RPMI-BSA and incubation was continued for 18 hours. CM were collected as described above.

### Monocytes

Blood from normal healthy volunteers or from normal rats was collected in EDTA (2.7% w/v; 1 ml/10 ml blood) by venipuncture, mixed 1:1 (v/v) with PBS containing 1 mg/ml BSA, layered over Hypaque-1077 (three parts blood and two parts Hypaque), and centrifuged (500*g*) at 25 C for 30 minutes. The mononuclear cells collected from the plasma/Hypaque interface were washed once in PBS + BSA, mixed with Sepracell-MN (1v cells: 2v Sepracell), and centrifuged at 25 C for 20 minutes at 1200*g*. Monocytes (band on top of the Sepracell) were collected, washed once in PBS + BSA, and resuspended in RPMI-BSA to a final concentration of $1 \times 10^6$ cells/ml for use in chemotaxis assays. The average yield of monocytes was $2.5 \times 10^5$/ml blood. These cells were more than 95% viable as determined by exclusion of trypan blue. The purity of human monocytes ranged from 60% to 90% of cells as determined by differential counts of Wright's stained cytocentrifuge preparations (Shandon Inc., Pittsburgh, PA) and by staining for nonspecific esterase. Esterase

stains were done using the $\alpha$-naphthyl acetate esterase kit (Sigma) according to the manufacturer's instructions. In monocyte preparations, the contaminating cells were almost exclusively lymphocytes. Polymorphonuclear leukocytes were consistently less than or equal to 0.5% of the total cell population and were not counted when seen on stained filters for chemotactic assays.

Although initially rat monocytes were used in the chemotaxis assays, the total yield ($8 \times 10^4$ cells/ml blood) and purity (≤50%) of monocytes from rat blood was poor when compared with that from human blood. Monocytes from both species were found to respond similarly to CM from control and bleomycin-treated macrophages; in four experiments twofold to threefold as many human and rat monocytes migrated towards the bleomycin CM (0.1 $\mu$g/ml) *versus* the control (no bleomycin) CM. Because there were no evident species differences in the monocyte response to rat AM CM, human monocytes were thereafter used routinely for these assays.

## Chemotaxis Assays

Assays of chemotactic activity were carried out essentially as described previously[25] using 48-well chambers (Neuroprobe Inc., Cabin John, MD) and polycarbonate filters (PVP-free) with 5 $\mu$m pores. All dilutions of CM or other test substances were assayed in triplicate; RPMI + BSA and 1% zymosan-activated serum (ZAS) served as the negative and positive controls, respectively. ZAS was prepared from either human or rat serum.[25] After a 2-hour incubation at 37 C, filters were fixed and stained as before.[25] Cells that migrated to the bottom of the filters were quantitated by counting ten oil immersion fields per filter. Chemotactic activity was standardized and expressed in units as follows:

$$1 \text{ UNIT} = 100 \times (C - C_0)/(C_z - C_0)$$

where

- $C$ = the number of cells migrating to CM or other test substance;
- $C_0$ = the number of cells migrating to RPMI + BSA (negative control); and
- $C_z$ = the number of cells migrating to ZAS (positive control).

Therefore, cell response or activity to ZAS equaled 100 units, whereas that to RPMI + BSA equaled 0 units. The average numbers of monocytes/ten fields representing 100 and 0 units were 226 ± 20 and 50 ± 8 (N = 20), respectively. In assays using AMs, cells were isolated from normal rats as described above and resuspended in RPMI-BSA to a concentration of $2 \times 10^6$/ml and allowed to migrate for 3 hours at 37 C.

## Protease Treatment

CM were incubated with pronase or trypsin for 30 minutes at 37 C before being assayed for chemotactic activity. Pronase immobilized on agarose beads (Pierce Chemical Co., Rockford, IL) was used as described previously;[25] the final concentration of enzyme was 0.2 to 1 mg/ml of CM. Trypsin (Sigma) was used at a final concentration of 0.1 to 5 $\mu$g (1 to 50 units)/ml CM; and at the end of the incubation period soybean trypsin inhibitor was added in five-fold excess to inactivate the trypsin. RPMI + BSA was also incubated with the proteases parallel to CM to serve as a control.

## Molecular Weight Estimation

Macrophage CM prepared in RPMI without BSA was concentrated tenfold using Centricon-10 disposable microconcentrators (Amicon, Danvers, MA) with 10 kd molecular weight cutoff membranes before analysis. Molecular weight estimates of the active components in CM were made by HPLC using a TSK 3000SW gel filtration column ($1 \times 45$ cm) (Varian Instruments, Palo Alto, CA) fitted with a guard column. Isocratic elution was undertaken with PBS at a flow rate of 0.8 ml/minute. Detection was at 220 nm; 1-minute fractions were collected for chemotaxis assays. Before assay, BSA was added to each fraction to a final concentration of 2 mg/ml.

## Gelatin Adsorption

One hundred $\mu$l of CM was adsorbed to 700 $\mu$l of gelatin Sepharose 4B (Pharmacia) for 2 hours at 4 C with gentle agitation. Gelatin-Sepharose was then pelleted by centrifugation (16000*g*) and the supernatant assayed for chemotactic activity. Controls used were unadsorbed CM, CM adsorbed to unmodified Sepharose 4B, and RPMI + BSA and ZAS, which were adsorbed with gelatin-sepharose.

## ELISA Assays

Before use in the treatment of CM, the goat anti-human fibronectin antibody was tested for cross-reactivity to rat fibronectin with an indirect ELISA assay done essentially according to the method of Engvall and Perlmann,[26] using an alkaline phosphatase second antibody (rabbit anti-goat IgG, Sigma). Human (Sigma) and rat fibronectin (Calbiochem) at concentrations of 1 ng to 10 $\mu$g/ml, and normal human and rat plasma at dilutions of 1:10 and 1:1000 were assayed using the antifibronectin antibody (above). Absorbance of the enzyme product at 410 nm was deter-

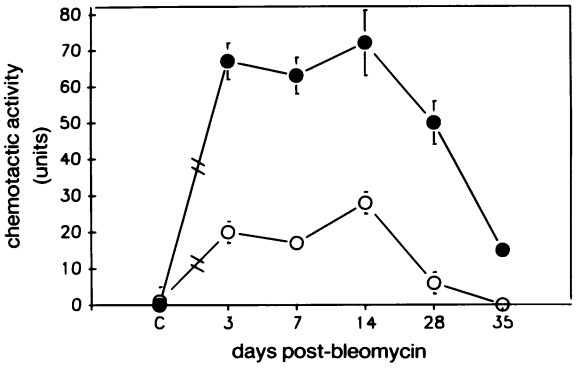


Figure 1. *AM secretion of monocyte chemoattractant activity after intratracheal administration of bleomycin. CM from macrophages of normal (C) and fibrotic animals were prepared and assayed as described in Materials and Methods. For each time point (days after bleomycin administration), data represent the mean ± SEM of CM from three separate rats assayed at 25% (●) and 5% (○) CM in triplicate.*

mined using a TiterTek Multiscan MC (Flow Labs). Using this method it was determined that the anti-human fibronectin antibody bound to both human and rat fibronectin and plasma; the absorbance of 10 μg/ml rat fibronectin had an absorbance reading equal to that of 8 μg/ml of human fibronectin (N = 3). Cross-reactivity of antifibronectin antibodies between rat and human preparations has been previously reported by Molinar et al.[27]

### Antibody Treatment

CM was incubated with the indicated antibody for 1 hour at 25 C and assayed for chemotactic activity as described above. The antibodies used were all IgG fractions of antisera raised in goats against human fibronectin (Sigma), human PDGF (Collaborative Research), and mouse IgG (Cappel Laboratories, West Chester, PA). CM alone and RPMI + BSA and ZAS with the antibodies were incubated in parallel to serve as controls. In some experiments rabbit anti-goat IgG immobilized on Sepharose beads (Sigma) was used to remove antibodies before assay. This treatment did not cause any significant effects on the results. The anti-human PDGF antibody (Collaborative) has been shown to cross-react with a PDGF-like molecule produced by rat AMs.[28]

### Statistical Analysis

The Student's *t*-test was used for comparison of experimental and control groups, with $P < 0.05$ considered to be statistically different. When multiple comparisons were made between treatment groups, analysis of variance was used along with Scheffé's test for making internal comparisons.

## Results

### Secretion of Chemoattractants by AMs from Fibrotic Animals

To determine if AMs are a source of chemoattractants that may be responsible for recruiting monocytes to the lung during fibrosis, CM were prepared using macrophages isolated from rats with bleomycin-induced pulmonary fibrosis, and the secretion of chemotactic activity was compared with that of cells from normal (saline-treated) rats. Macrophages obtained from fibrotic rats 3-to-35 days after the administration of bleomycin secreted significantly greater amounts of monocyte chemoattractant activity than did macrophages from normal (C) animals (Figure 1). As indicated in Figure 1, this activity depended on the concentration of macrophage CM assayed.

### *Effects of* In Vitro *Treatment of AMs with Bleomycin*

To test the hypothesis that the enhanced secretion of chemoattractants by macrophages from fibrotic rats might be the result of a direct interaction between bleomycin and these cells, AMs were isolated from normal rats and placed in culture in the presence or absence of bleomycin for 18 hours. When the resulting macrophage CM were assayed for monocyte chemoattractant activity, it was evident that the secretion of this activity depended on the concentration of bleomycin used to stimulate the cells (Figure 2). In four separate preparations of this kind, optimal activity was found in CM from macrophages given 0.01 to 0.1 μg/ml bleomycin; activity in CM at these con-

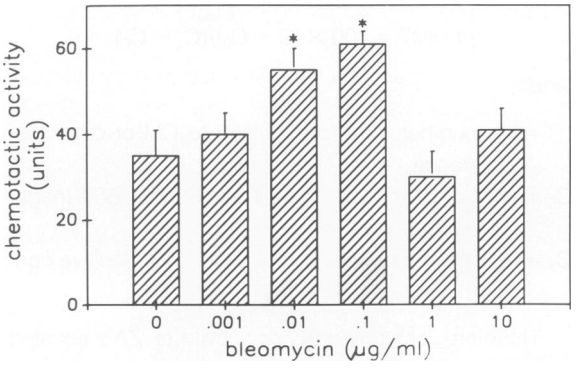


Figure 2. *Effects of bleomycin on monocyte chemotactic activity secretion by AMs* in vitro. *Macrophages were incubated with the indicated concentrations of bleomycin for 18 hours after which CM were assayed for activity. Chemotactic activity was expressed as units as defined in Materials and Methods. Data represent chemotactic activity of 50% CM (mean ± SEM of five separate experiments, each of which was performed in triplicate). Asterisks indicate activity significantly greater than control CM at* $P < 0.05$.

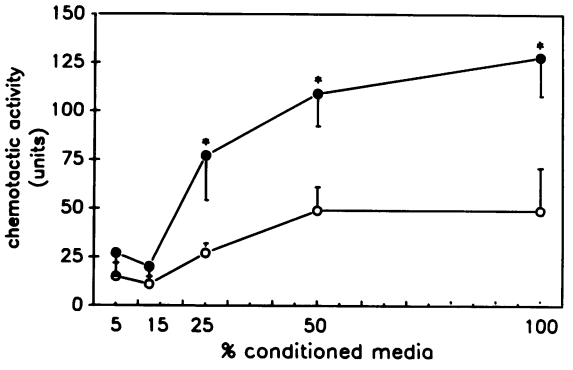


Figure 3. *Dose dependence of chemotactic activity in CM. Macrophages were incubated in ± 0.1 μg/ml bleomycin for 18 hours after which CM was tested for chemotactic activity, which was expressed as in Figure 1. Data are expressed as the mean ± SEM of five experiments. Asterisks indicate activity in bleomycin CM (●) significantly greater than control CM (○); P < 0.05.*

centrations of bleomycin was significantly greater than that in CM from control macrophages incubated in RPMI-BSA alone ($P < 0.05$). Unstimulated cells (no bleomycin) secreted detectable but lower levels of chemotactic activity. Higher concentrations of bleomycin (1 and 10 μg/ml) did not elicit secretion of chemoattractants greater than that of controls. This lack of response at these concentrations was not due to overt toxicity of bleomycin to the cells because macrophage viability as assessed by exclusion of trypan blue and release of the cytosolic enzyme lactate dehydrogenase were unaffected by any of the concentrations of bleomycin.

Although CM was extensively dialyzed, it was possible that very small amounts of bleomycin remaining in the medium after dialysis might affect monocyte response to CM; bleomycin alone, therefore, was also assayed for chemotactic activity. Concentrations of 1 pg to 10 μg/ml bleomycin failed to promote or inhibit monocyte migration (data not shown), indicating that the chemotactic activity in CM was due to macrophage product(s) and not to bleomycin itself.

Although the amount of activity secreted by the macrophages was highly variable, ranging from 0 to 50 units for the control cells and 50 to 130 units for bleomycin-stimulated cells, control activity was always significantly less ($P < 0.05$) than that secreted by macrophages from fibrotic animals (Figure 1) or stimulated *in vitro* with bleomycin (Figures 2 and 3). For experiments comparing activity secreted by normal and fibrotic macrophages, animals were all from the same shipment; for all experiments for which macrophages were stimulated *in vitro*, bleomycin and control CM were always prepared from the same pool of cells. In this manner any variability in secreted activity caused by differences in animals or culture conditions (lots of BSA, medium, or plates) would have an equal effect on both control and stimulated cells so that any differences in activity detected between the bleomycin and control cells were due to the stimulation by bleomycin.

## Effects of CM on Migration of AM

CM from macrophages of control and fibrotic rats (14 days after bleomycin-administration) and from macrophages stimulated with 0.1 μg/ml bleomycin *in vitro* were assayed for chemotactic activity for AMs. Freshly isolated AMs responded well to ZAS, with an average of 779 ± 36 cells per ten fields (by definition 100 units). In contrast, macrophage migration towards CM from any of the above sources was minimal whether bleomycin or control CM were being tested. Using normal AMs as target cells, CM from fibrotic and control rats contained 9 ± 3 and 12 ± 2 units (N = 4) of activity, respectively, whereas CM from corresponding *in vitro* bleomycin-stimulated macrophages and controls contained a maximum of 8 ± 3 and 9 ± 3 units (N = 8), respectively. Similarly negative results were obtained with doses of CM that were as high as 100%, indicating that there was no detectable response to the CM by the AMs and that the lack of migration at the higher concentrations was not due to an inhibitory effect.

## Characterization of Chemotactic Activity

To determine if the activity in CM was chemotactic and/or chemokinetic in nature, standard checkerboard assays[25] using varying concentrations of CM above and below the filter were carried out. Checkerboard assays of three different CM preparations from bleomycin-stimulated macrophages demonstrated that this activity was truly chemotactic for monocytes (Figure 4), as evidenced by the increased migration of cells in BSA to increasing concentrations of CM in positive gradients (below diagonal)

| Bottom wells: | Top wells: monocytes + indicated %CM | | | |
|---|---|---|---|---|
| %CM | 0% | 5% | 45% | 90% |
| 0% | 0 | 5(4) | 0(0) | 0(0) |
| 5% | 32(16) | 6(5) | 0(0) | 0(0) |
| 45% | 65(7) | 22(12) | 10(6) | 1(1) |
| 90% | 72(1) | 32(1) | 21(7) | 0(0) |

Figure 4. *Checkerboard assay of CM. The percentages indicate the concentration of CM placed either in the bottom or upper well with the cells. Chemotactic activity was expressed in units that were calculated as described in Materials and Methods. Data represent the means (±SEM) of three experiments, each of which were performed in triplicate. The numbers in parentheses equal SEM; N = 3.*

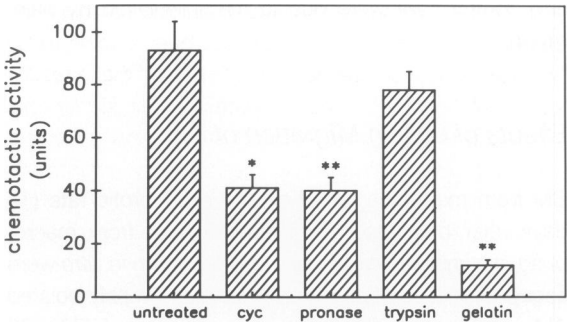


Figure 5. *Characterization of the proteinaceous nature of the monocyte chemotactic activity in CM. Macrophages were incubated in 0.1 μg/ml bleomycin for 18 hours; chemotactic activity in CM was tested for sensitivity to pronase and trypsin digestion. Gelatin: residual activity after adsorption of CM with gelatin Sepharose 4B. Cyc: activity in CM of cells pretreated with 1 μg/ml of cycloheximide. The data represent the mean ± SEM of five experiments. * and **: Indicate activity significantly less than the untreated CM at* $P < 0.05$ *and* $P < 0.001$, *respectively.*

and the absence of an effect on random migration (above diagonal).

As an initial step in the characterization of this chemotactic activity, macrophages were preincubated with or without 1 μg/ml cycloheximide before stimulation with 0.1 μg/ml bleomycin. Activity in CM from cycloheximide-treated macrophages was 45% ± 5% of that in CM from macrophages given bleomycin alone (Figure 5), demonstrating that protein synthesis was necessary for full expression of chemotactic activity and suggesting that the chemotactic activity secreted by macrophages on bleomycin stimulation may be a combination of newly synthesized proteins and release of preformed product. The activity secreted by control macrophages (no bleomycin) was unaffected by incubation with cycloheximide and cell viability was unchanged, indicating that 1 μg/ml cycloheximide was not toxic to these cells.

The proteinaceous nature of activity in CM was confirmed by the effect of pronase, which reduced CM activity by 57% ± 5% (Figure 5). In contrast, although trypsin caused a 16% ± 7% reduction in chemotactic activity, this was not statistically different from that of the untreated CM ($P > 0.05$), suggesting that the proteolytic site(s) did not harbor susceptible basic amino acid residues.

## *Molecular Weight Estimation*

To further investigate the identity of the chemotactic components in bleomycin-stimulated macrophage CM, the molecular weights of the active components in bleomycin macrophage CM were estimated. CM was concentrated tenfold using Centricon concentrators (Amicon) that retained material of greater than or equal to 10 kd. This concentrated sample of CM was fractionated by HPLC using a gel filtration column. Assays of column fractions from three separate preparations of CM consistently showed two to three major peaks of activity between 20 and 60 kd (Figure 6). Material less than 10 kd collected during concentration of CM before application over the columns showed no activity above that of RPMI + BSA alone. Macrophages are known to secrete several chemotactic proteins within the MW range of the activity reported here.[18,19,28-31] Of these proteins, PDGF (platelet-derived growth factor) and fibronectin were considered likely candidates for the chemotactic activity in CM because AMs isolated from patients with idiopathic pulmonary fibrosis were shown to secrete increased amounts of both of these proteins.[18,19,32] The relative insensitivity of CM activity to trypsin suggested that PDGF was not a major component of this activity because it has been shown to be inactivated by the concentration of trypsin (5 μg/ml) used here.[33] Although intact fibronectin (220 kd) is inactive as a monocyte chemotactic factor, fragments of this molecule within the molecular weight range of the chemotactic activity recovered from the gel filtration column that bind to gelatin were demonstrated to be active for monocytes.[34]

## *Effects of Gelatin-Sepharose Adsorption*

To determine if fibronectin was responsible for this activity, CM was first subjected to adsorption with gelatin, the rationale being that the fibronectin molecule contains a gelatin binding domain so that the intact molecule and any fragments containing this domain would be removed from the medium by such adsorption. Gelatin treatment removed up to 87% of the chemotactic activity originally present in CM of bleomycin-treated AMs (untreated, Figure 5), whereas adsorption of CM to unmodified Sepharose 4B had no effect on this activity (data not shown). This adsorption of most of the activity by gelatin, coupled with the estimated molecular weights of the active factors,

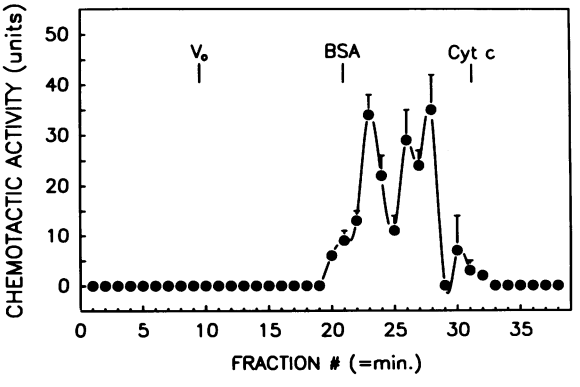


Figure 6. *Estimation of the molecular size of the monocyte chemotactic activity in CM by gel filtration HPLC. CM was prepared, concentrated, and fractionated as described in Materials and Methods. Each fraction was assayed in triplicate for chemotactic activity. Data represent the mean ± SEM of a representative experiment.*

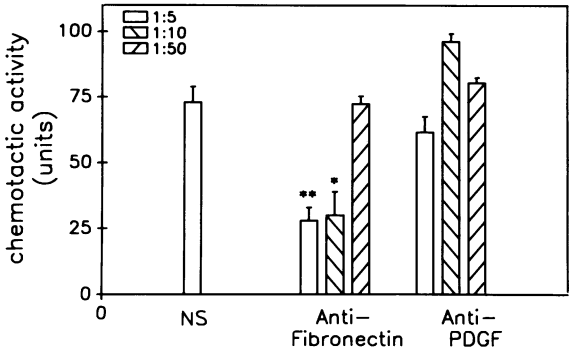


Figure 7. *Effects of antibody treatment on the monocyte chemotactic activity in CM. CM from macrophages incubated 18 hours with 0.1 μg/ml bleomycin were treated with the indicated dilutions of antifibronectin, antiPDGF, or as a control nonspecific IgG (NS). Chemotactic activity was calculated as described in Materials and Methods. Data represent the mean ± SEM of four experiments. *: Indicates activity significantly less than CM treated with NS IgG (P < 0.05).*

strongly implicated fibronectin as a major source of the chemotactic activity in CM. Because other gelatin binding proteins have been reported,[35] it was necessary to confirm this conclusion.

### *Effects of Antibodies on Chemotactic Activity*

To verify the identity of the active factors, CM was treated with specific antibodies against fibronectin and PDGF. Significant reduction ($P < 0.05$) in activity was seen only with antifibronectin antibody which caused a 62% ± 2% decrease when used with three separate CM preparations (Figure 7). Antibodies to PDGF and nonimmune IgG did not significantly affect chemotactic activity. The combined results of the treatment of CM with antibody, gelatin, and pronase, along with the molecular weight estimates, demonstrated that the monocyte chemotactic activity in CM from bleomycin-stimulated macrophages was primarily due to fragments of fibronectin between 20 to 60 kd in size.

### *Comparison of* In Vivo *Versus* In Vitro *Activity*

CM (*in vitro* CM) of macrophages exposed to 0.1 μg/ml bleomycin *in vitro* for 18 hours was tested for their ability to inhibit the migration of monocytes towards CM (*in vivo* CM) of macrophages from rats treated with bleomycin 14 days before killing. Monocytes were suspended in 0%, 5%, 25%, or 100% *in vitro* CM and assayed for their ability to migrate towards 100% *in vivo* CM. The addition of increasing doses of *in vitro* CM above the filter progressively inhibited this chemotactic activity towards the *in vivo* CM placed below the filter, with total inhibition achieved when 100% *in vitro* CM was present above the filter (Table 1). Because this type of inhibition of migration of leukocytes or desensitization has been shown to be specific for a given chemotactic stimulus,[29] these results imply that the CM from both sources contain similar chemoattractant(s). Monocyte response to RPMI + BSA or ZAS was not significantly affected by prior suspension of monocytes in *in vivo* or *in vitro* CM (data not shown), indicating that the inhibition of monocyte migration towards the *in vitro* CM was the result of specific desensitization and not the result of chemotactic inhibitors in the *in vivo* CM.

## *Discussion*

A number of agents such as drugs, asbestos, silica, chemicals, and viruses can provoke a fibrotic and inflammatory reaction in the lung.[1] Although the morphologic and some of the biochemical events occurring in fibrosis have been described in animal models[1-3,8,11,12,17] and in humans,[1,9,18,19] it is unclear what the initiating factors are.

One of the earliest events occurring in an inflammatory reaction in the lung is the influx of peripheral blood leukocytes, most notably neutrophils and later predominantly monocytes. The experiments described here, and those from other laboratories, suggest that the direct or indirect stimulation of AMs by a fibrotic agent may be an important cause of both acute and chronic leukocyte infiltration. Although other investigators have demonstrated that AMs stimulated with silica,[12,21,25,37] asbestos,[20,23,27] paraquat,[17] and bleomycin[22] release chemotactic factors for neutrophils, the present study showed that AMs stimulated with bleomycin either *in vivo* (fibrotic rats) or *in vitro* secrete significantly greater amounts of monocyte chemotactic activity than control unstimulated AMs.

Our finding of substantial monocyte chemotactic activity 3 to 28 days after bleomycin administration suggests that the monocyte chemotactic activity follows a different time course than that for neutrophils because neutrophil

Table 1. *Effect of* In Vitro *CM on Monocyte Migration to* In Vivo *CM*

| *In vitro* CM above filter (%) | Monocyte chemotaxis (units)* |
|---|---|
| 0 | 100 ± 14 |
| 5 | 33 ± 9 |
| 25 | 11 ± 6 |
| 100 | 0 ± 1 |

* Monocytes were prepared as described in Materials and Methods and suspended in the indicated concentrations (%) of *in vitro* CM (obtained from normal AMs treated *in vitro* with 0.1 μg/ml bleomycin). These monocyte suspensions were then placed above the filter and allowed to migrate to *in vivo* CM (obtained from AMs of rats given intratracheal bleomycin 14 days before obtaining AMs) placed below the filter at a concentration of 100%. Data (means of triplicates ± SEM) were expressed in chemotactic units calculated as described in Materials and Methods.

chemotactic activity in fibrotic hamsters[22] was maximal 4 days after animals were given bleomycin, and only slightly elevated by 15 days. This chronologic difference in chemotactic activities for neutrophils and monocytes correlates well with the composition of these leukocytes in lavage fluid after lung injury[2,3,11,12] and likely reflects the respective roles of these cells in acute and chronic inflammation.

The monocyte chemotactic activity secreted by the *in vitro* stimulation of AMs appears to be due to substances similar to those produced by macrophages isolated from fibrotic rats. For the *in vivo* studies, rats were given 1.5 mg (units) of bleomycin intratracheally; with an approximate bronchiolar and intraalveolar lung volume of 10 ml, an initial concentration of 150 $\mu$g/ml could easily be achieved. Although nearly all the bleomycin is cleared from the lung in 24 hours (<1% remaining),[39] it seems likely that the effective *in vitro* concentrations (0.01 to 0.1 $\mu$g/ml) may be found in the lung for a length of time sufficient to stimulate macrophage secretion of chemotactic factors. Although characterization of the chemotactic activity in the *in vivo* CM has not been completed, initial desensitization experiments suggest that similar active factor(s) are produced whether macrophages are exposed to bleomycin *in vivo* or in culture.

The results of the experiments in which the response of AMs to CM were tested, suggest that AM-derived monocyte chemotactic factors in bleomycin-induced fibrosis have a role in increasing macrophage numbers through promoting the influx of peripheral blood monocytes into the lungs but do not attract macrophages already present in the lung to sites of injury. This lack of AM response to CM from stimulated alveolar macrophages has also been described in studies of silicosis.[12,21] The particular role of macrophage-derived chemoattractants in inflammation and fibrosis may depend, however, on the fibrogenic stimulus because in contrast to our findings and those of others just cited,[12,21] AMs from quartz[24] and asbestos-treated animals have been shown to secrete substances that attract other AMs.

Macrophages produce a variety of peptide chemotactic factors for monocytes including PDGF, thrombin (as prothrombin), fibronectin, transforming growth factor-$\beta$ (TGF-$\beta$), and granulocyte macrophage colony-stimulating factor (GM-CSF).[18,19,30-32] Of these, fibronectin is one of the major secretory proteins of AMs.[40] In terms of pulmonary fibrosis, PDGF and fibronectin appear to be likely candidates responsible for the monocyte chemotactic activity in the bleomycin-macrophage CM because AMs isolated from patients with idiopathic pulmonary fibrosis (IPF) secrete significantly higher amounts of both of these proteins than do macrophages from normal individuals.[18,19,31] AMs from monkeys with paraquat-induced fibrosis also secrete greater amounts of fibronectin than do normal monkeys.[17]

Based on the combined results of the characterization studies, the predominant monocyte chemotactic activity in CM from macrophages stimulated with bleomycin *in vitro* has been characterized as a mixture of pronase-sensitive, trypsin-resistant proteins with a molecular weight range of 20 to 60 kd. Although several of the known macrophage-derived monocyte chemotactic proteins are within this size range, the cumulative data from the gelatin-adsorption and antibody experiments support the contention that fibronectin fragments are responsible for the major portion of the activity in CM. Although it is known that TGF-$\beta$ can bind to plasma fibronectin[45] and thus be removed by gelatin adsorption as well, the combined data of the experiments presented here make it seem an unlikely candidate for the primary active protein in the CM. This is based on the fact that trypsin failed to inactivate monocyte chemotactic activity in CM despite its known ability to inactivate TGF-$\beta$;[49] and, more importantly, there is currently no evidence that TGF-$\beta$ activity can be inhibited by antifibronectin antibody. The data from this study, however, do not totally exclude the possibility that tightly bound TGF-$\beta$ (to fibronectin fragments) is partially responsible for the activity described here.

Studies by other investigators showed that fibronectin fragments having a wide molecular size range possess monocyte chemotactic activity; these include fragments ranging in size from 5 to 220 kd.[35,41]

Fibronectin is a relatively large molecule with a molecular weight of 440 kd that represents a dimer of 220 kd subunits; the molecule is made up of regions or domains that are capable of binding to gelatin, heparin, collagen, fibrin, or glycosaminoglycans.[41] Fragments of fibronectin can be generated by treatment with proteases;[41-43] these fragments retain their substrate binding qualities and can be separated and partially identified on this basis. The residual 13% of chemotactic activity remaining in CM after gelatin adsorption may indicate 1) that the adsorptive capacity of the gelatin-Sepharose was exceeded; 2) that some of the activity was due to fibronectin fragments that did not contain the gelatin-binding domain; and/or 3) that some of the total activity was due to factors other than fibronectin (as discussed above). The second possibility is partly support by data from a previous study showing that enzymatic digestion of fibronectin produces a 120 kd active fragment containing the cell attachment domain that is chemotactic for monocytes.[42]

The activity remaining in the CM after pronase and cycloheximide treatment of CM and macrophages suggests that there may also be nonproteinaceous active factors present. Chemotactically active lipids such as leukotriene $B_4$ and platelet-activating factor are secreted by activated macrophages[15,30] and could possibly bind to the BSA, fibronectin, or other proteins in the medium. If activity remaining after protease treatment were due to lipids, the binding of such small lipids to carrier proteins could

account for the lack of activity in the less than 10 kd filtrate of the concentrated CM applied to columns (Figure 5).

Although AMs have been identified as a source of the increased amounts of fibronectin in the lung during fibrosis[17-19,33,44] and fibronectin fragments have been shown to be chemotactic for monocytes,[35,42] it has not been previously established that macrophage-derived fibronectin is the predominant monocyte chemotactic factor secreted by AMs in pulmonary disease. The data presented here support the hypothesis that, on challenge with a fibrotic stimulus, AMs produce chemotactically active fragments of fibronectin that may play a key role in promoting monocyte immigration into the lung in fibrosis. In support of this hypothesis, Rennard et al[18] reported that macrophages from patients with IPF secrete ng/ml quantities of fibronectin and that bronchoalveolar lavage (BAL) fluid from such patients contains fragmented fibronectin.[44] Villigers et al[40] reported proteolysis of fibronectin secreted by alveolar macrophages cultured in serum-free medium, and leukocyte elastase has been shown to be capable of fragmenting fibronectin.[48] Such protease degradation of secreted fibronectin may be the mechanism by which bleomycin-stimulated macrophages produce fibronectin that is chemotactically active for monocytes. Despite the presence of numerous endogenous protease inhibitors *in vivo,* the local concentrations of proteases released by macrophages, other cells, or both may be sufficient to overcome or overwhelm these inhibitors to generate these active fragments. Active proteases have been identified *in vivo* in inflammatory reactions.[46,47]

Finally, our results along with those of others[12,17,20,21,23,24,37,38] emphasize the important role of the AM in modulating the response of the lung to fibrogenic agents. The demonstration of *in vitro* stimulation of macrophage secretion of monocyte chemotactic factors, in conjunction with our previous finding[16] that macrophage secretion of growth factor activity for fibroblasts can also be similarly stimulated, suggests that the direct interaction between AMs and fibrogenic stimuli may be a key initiating factor in pulmonary fibrosis. It should be apparent however that the response of the lung to fibrogenic stimuli is not simply a response of the AMs. The lung is an exceedingly complex organ, composed of many different types of cells, all or many of which may play roles in this process.

## References

1. Phan SH. Fibrotic mechanisms in lung diseases, Immunology of Inflammation. Edited by PA Ward. New York, Elsevier 1983, pp 121-147
2. Thrall RS, Barton RW, D'Amato DA, Sulavik SB. Differential cellular analysis of bronchoalveolar lavage fluid obtained at various stages during the development of bleomycin-induced pulmonary fibrosis in the rat. Am Rev Respir Dis 1982, 126:488-492
3. Lemaire I, Beaudoin H, Masse S, Grondin C. Alveolar macrophage stimulation of fibroblast growth in asbestos-induced pulmonary fibrosis. Am J Pathol 1986, 122:205-211
4. van Oud Alblas AB, van Der Linden-Screver B, van Furth R. Origin and kinetics of pulmonary macrophages during an inflammatory reaction induced by intra-alveolar administration of aerosolized heat-killed BCG. Am Rev Respir Dis 1983, 128:276-281
5. Bowden DH, Adamson IYR. Adaptive responses of the pulmonary macrophagic system to carbon. I. Kinetic studies. Lab Invest 1978, 38:422-429
6. van Oud Alblas AB, Mattie H, van Furth R. A quantitative evaluation of pulmonary macrophage kinetics. Cell Tissue Kinet 1983, 16:211-219
7. Shellito J, Esparza C, Armstrong C. Maintenance of the normal AM population: The roles of monocyte influx and AM proliferation *in situ.* Am Rev Respir Dis 1987, 135:78-82
8. Bitterman PB, Saltzman LE, Adelberg S, Ferrans VJ, Crystal RG. AM replication: One mechanism of expansion of the macrophage phagocyte population in the chronically inflamed lung. J Clin Invest 1984, 74:460-469
9. Spurzem JR, Saltini C, Rom W, Winchester RJ, Crystal RG. Mechanism of macrophage accumulation in the lungs of asbestos-exposed subjects. Am Rev Respir Dis 1987, 136: 276-280
10. Adamson IYR, Bowden DH. Role of monocytes and interstitial cells in the generation of AMs: II. Kinetic studies after carbon loading. Lab Invest 1980, 42:518-531
11. Chandler DB, Hyde DM, Giri SH. Morphometric estimates of infiltrative cellular changes during the development of bleomycin-induced pulmonary fibrosis in hamsters. Am J Pathol 1983, 112:170-177
12. Lugano EM, Dauber JH, Daniele RP. Acute experimental silicosis: Lung morphology, histology and macrophage chemotaxin secretion. Am J Pathol 1982, 109:27-36
13. Phan SH, Kunkel SL. Inhibition of Bleomycin-induced pulmonary fibrosis by nordihydroguiaretic acid: The role of alveolar macrophage activation and mediator production. Am J Pathol 1986, 124:343-352
14. Phan SH, McGarry BM, Loeffler KM, Kunkel SL. Binding of leukotriene $C_4$ to rat lung fibroblasts and stimulation of collagen synthesis *in vitro.* Biochemistry 1988, 27:2846-2853
15. Mensing H, Czarnetzki BM. Leukotriene $B_4$ induces *in vitro* fibroblasts chemotaxis. J Invest Dermatol 1984, 82:9-12
16. Denholm EM, Phan SH. 1989. Effects of bleomycin on alveolar macrophage growth factor secretion. Am J Pathol 1989, 134:355-363
17. Scheonberger CI, Rennard SI, Bitterman PB, Fukuda Y, Ferrans VJ, Crystal RG. Paraquat-induce pulmonary fibrosis: Role of alveolitis in modulating the development of fibrosis. Am Rev Respir Dis 1984, 129:168-173
18. Rennard SI, Hunninghake GW, Bitterman PB, Crystal RG. Production of fibronectin by the human AM: Mechanism of the recruitment of fibroblasts to sites of tissue injury during interstitial lung diseases. Proc Natl Acad Sci USA 1981, 78: 7147-7151
19. Martinet Y, Rom WN, Grotendorst GR, Martin GR, Crystal RG. Exaggerated spontaneous release of platelet-derived

growth factor by AMs from patients with idiopathic pulmonary fibrosis. New Engl J Med 1987, 317:202–209

20. Glassroth JL, Bernardo J, Lucey EC, Center DM, Jung-Legg A, Snider GL. Interstitial pulmonary fibrosis induced in hamsters by intratracheal administered chrysotile asbestos. Am Rev Respir Dis 1984, 130:242–248
21. Lugano EM, Dauber JH, Daniele RP. Silica stimulation of chemotactic factor release by guinea pig AMs. J Reticuloendothel Soc 1981, 30:381–390
22. Kaelin RM, Center DM, Bernardo J, Grant M, Snider GL. The role of macrophage-derived chemoattractants in the early inflammatory events of bleomycin-induce pulmonary fibrosis. Am Rev Respir Dis 1983, 128:132–137
23. Kagan E, Oghiso Y, Hartmann D. Enhanced release of a chemoattractant for alveolar macrophages after asbestos inhalation. Am Rev Respir Dis 1983, 128:680–687
24. Miller K, Calverley A, Kagan E. Evidence of quartz-induced chemotactic factor for guinea pig AMs. Environ Res 1980, 22:31–39
25. Denholm EM, Lewis JC. Monocyte chemoattractants in pigeon aortic atherosclerosis. Am J Pathol 1987, 126:464–475
26. Engvall E, Perlmann P. Enzyme-linked immunoadsorbent assay; ELISA: III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 1972, 109:129–135
27. Molnar J, Gelder FB, Zong Lai M, Siefring GE, Credo RB, Lorand L. Purification of opsonically active human and rat cold-insoluble globulin (Plasma Fibronectin). Biochemistry 1979, 18:3909–3916
28. Kumar RK, Bennet RA, Brody AR. A homologue of platelet-derived growth factor produced by rat AMs. FASEB J 1980, 2:2272–2277
29. Falk W, Leonard EJ. Specificity and reversibility of chemotactic deactivation of human monocytes. Infect Immunol 1981, 32:464–468
30. Cohn ZA. The macrophage-versatile element of inflammation, The Harvey Lectures Series, Vol. 77. (J. P. Changeux ed) New York Academic Press 1982, pp 63–80
31. Wang JM, Colella S, Allavena P, Mantovani A. Chemotactic activity of human recombinant granulocyte-macrophage colony-stimulating factor. Immunology 1987, 60:439–444
32. Wahl SM, Hunt DA, Wakefield LM, McCartney-Francis N, Wahl L, Roberts AB, Sporn MB. Transforming growth factor type $\beta$ induces monocyte chemotaxis and growth factor production. Proc Natl Acad Sci USA 1987, 84:5788–5792
33. Yamauchi K, Martinet Y, Crystal RG. Modulation of fibronectin gene expression in human mononuclear phagocytes. J Clin Invest 1987, 1720–1727
34. Senior RM, Huang JS, Griffin GL, Deuel TF. Dissociation of the chemotactic and mitogenic activities of platelet-derived growth factor by human neutrophil elastase. J Cell Biol 1985, 100:351–356
35. Norris DA, Clark RAF, Swigart LM, Clark Huff J, Weston WL, Howell SE. Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes. J Immunol 1982, 129: 1612–1616
36. Williams HR, Lin TY. Human polymorphonuclear leukocyte collagenase and gelatinase: Comparison of certain enzymatic properties. Int J Biochem 1984, 16:1321–1327
37. Martin TM, Ayars G, Butler J, Altman LC. The comparative toxicity of volcanic ash and quartz: Effects on cells derived from the human lung. Am Rev Respir Dis 1984, 130:778–782
38. Schoenberger CI, Hunninghake GW, Kawanami O, Ferrans VJ, Crystal RG. Role of AMs in asbestosis: Modulation of neutrophil migration to the lung after acute asbestos exposure. Thorax 1982, 37:803–809
39. Lazo JS, Pham ET. Pulmonary fate of [$^3$H]bleomycin $A_2$ in mice. J Pharmacol Exp Ther 1984, 228:13–18
40. Villiger B, Kelley DG, Engleman W, Kuhn C, McDonald JA. Human alveolar macrophage fibronectin: Synthesis, secretion and ultrastructural localization during gelatin-coated latex particle binding. J Cell Biol 1981, 90:711–720
41. Click EM, Balian G. Domain structure of human plasma and cellular fibronectin. Use of a monoclonal antibody and heparin affinity to identify three different subunit chains. Biochemistry 1985, 24:6685–6696
42. Clark RAF, Wilkner NE, Doherty DE, Norris DA, Howell SE. Cryptic chemotactic activity of fibronectin for human monocytes resides in the 120-kDa cell-binding fragment. J Biol Chem 1985, 263:12115–12123
43. Pierschbacher MD, Hayman EG, Ruoslahti E. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. Cell 1981, 26: 259–267
44. Rennard SI, Crystal RG. Fibronectin in human bronchopulmonary lavage fluid: Elevations in patients with interstitial lung disease. J Clin Invest 1981, 69:113–122
45. Fava RA, McClure DB. Fibronectin-associated transforming growth factor. J Cell Physiol 1987, 131:184–189
46. Gadek J, Kelman JA, Fells GA, Weinberger SE, Horwitz AL, Reynolds HY, Fulmer JD, Crystal RG. Collagenase in the lower respiratory tract of patients with idiopathic pulmonary fibrosis. N Engl J Med 1979, 301:737–742
47. Nakamura T, Yamamoto T, Ishimatsu T, Kambara T. A high molecular-weight trypsin-like protease in the skin sites of delayed hypersensitivity in guinea pigs. Am J Pathol 1984, 114: 250–263
48. McDonald JA, Kelley DG. Degradation of fibronectin by human leukocyte elastase: Release of biologically active fragments. J Biol Chem 1980, 255:8848–8858
49. Frolik CA, Dart LL, Meyers Smith DM, Sporn MB. Purification and initial characterization of a type $\beta$ transforming growth factor from human placenta. Proc Natl Acad Sci USA 1983, 80:3676–3680