A normal fibrotic response to inflammatory stimuli appears to be dependent on the balanced production of a number of stimulatory and inhibitory fibroblast-regulatory mediators by activated mononuclear cells (MNL). To investigate whether altered mediator production contributes to the fibrosis observed in progressive systemic sclerosis (PSS), we stimulated human peripheral blood MNL with concanavalin A (Con A) and lipopolysaccharide (LPS) to produce macrophage mediators that inhibit the proliferation and the collagen production of cultured normal human fibroblasts. The two Con A-induced mediators were lymphokines (LK) as they were exclusively produced by activated T cells and they coloeluted with a Sephaeryl S-200 column with a $M_r$ of 50,000. In contrast, the two LPS-induced mediators were monokines (MK) as they were exclusively produced by activated monocytes, and they coloeluted in the $M_r$ 20,000 range. Each pair of inhibitory LK and MK may also be distinct as inhibition of collagen production still occurred in proliferatively quiescent cultures. A quantitative comparison of the levels of fibroblast-inhibitory LK/MK produced by normal volunteers and long-term PSS patients revealed that although PSS MNL produced normal levels of both collagen production inhibitory mediators, they were aberrant producers of both proliferation inhibitory mediators, being hyperproducers (−49%) of the LK and hyperproducers (+196%) of the MK. These results suggest that reduced production of proliferation inhibitory LK or MK may allow stimulatory mediators to induce the unrestricted fibroblast proliferation observed in early active PSS, which then may be stabilized in long-term PSS by the increased production of proliferation inhibitory MK.

It has long been recognized that chronic inflammatory lesions that are characterized by infiltrating lymphocytes and monoocytes are also associated with an increased fibroblast population and the subsequent formation of fibrotic tissue [1–7]. This pathologic association of mononuclear cells (MNL) and fibroblasts in vivo has recently led to the in vivo investigation of the role activated MNL play in producing a normal fibrotic response. A compilation of these studies suggests that a normal fibrotic response is the result of the carefully balanced production by activated MNL of a number of T cell-derived lymphokines (LK) and monocyte-derived monokines (MK) with antagonistic fibroblast-regulatory activities. To date, both LK and MK preparations have been described with activities both as stimulators and as inhibitors of fibroblast movement [8–10], proliferation [11–16], and protein production, including the fibrosis forming collagens and glycosaminoglycans [17–22]. How the normal balance of stimulatory and inhibitory activities is altered to produce pathologic fibrosis is currently under investigation in a number of inflammation-associated disorders [23,24].

Progressive systemic sclerosis (PSS, scleroderma) is a connective tissue disease of unknown etiology characterized by cutaneous and visceral fibrosis, which during its early stages is associated with extensive MNL infiltration of the tissue sites that later become fibrotic [6,25–27]. The MNL infiltrates are composed primarily of T cells and monocytes/macrophages, along with smaller numbers of B and plasma cells, and the MNL are accompanied by an increased fibroblast population, a portion of which are hyperproducers of collagen [28,29]. How LK/MK-mediated mechanisms contribute to increasing fibroblast number and function in vivo in PSS is unknown at present, but it may involve the unbalanced production of stimulatory and inhibitory LK/MK by the T cell and monocyte/macrophage populations infiltrating the PSS tissue site. While the T cells and monocytes/macrophages present in PSS loci have yet to be functionally classified, studies employing peripheral blood MNL indicate that PSS is associated both with reduced T suppressor cell numbers and with increased suppressor monocyte activity [30–34]. Since aberrant suppressor cell number/function should logically be accompanied by the aberrant production of LK/MK with suppressive or inhibitory activities, we in this report have investigated the production of the LK and MK inhibiting fibroblast proliferation and collagen synthesis by peripheral blood MNL from patients with PSS. The reduced production of any of these inhibitory LK/MK would result in a relative excess of the corresponding stimulatory mediator being present in the PSS loci, which could induce the increased fibroblast proliferation and unregulated collagen production observed in PSS.

In order to be able to discriminatingly assess the production of fibroblast-inhibitory LK/MK in PSS we first optimized the culture conditions necessary for the production and assay of LK/MK supernatants. This enabled us to assay MNL supernatants for fibroblast-inhibitory activities without interference from fibroblast-stimulatory activities, as well as to assay LK activity separately from MK activity, and collagen synthesis inhibitory activity separately from fibroblast proliferation inhibitory activity. Our results suggest that the LK and MK inhibiting fibroblast proliferation and collagen production may be 4 distinct factors, and moreover, in the long-term patients studied, PSS is associated not only with a deficient production
of the LK inhibiting fibroblast proliferation but also with the elevated production of the analogous MK.

MATERIALS AND METHODS

Patient and Normal Populations

The patient group consisted of 13 females ranging in age from 20-75 years (mean, 52.3 years). All patients met the criteria for diagnosis of PSS and had a disease duration ranging from 3-20 years (mean, 9.6 years). The patient group included both those who displayed PSS with diffuse scleroderma and those with PSS with the CREST syndrome [35]. None of the patients was receiving corticosteroids, n-penicillamine, or cytotoxic drugs at the time of venipuncture. Informed consent was signed by all patients and these studies were approved by the Mount Sinai School of Medicine Human Subjects Committee. The normal control group consisted of 14 healthy volunteers and hospital personnel, and included 10 females and 4 males ranging in age from 24-58 years (mean, 36.2 years).

Preparation of MNL

All procedures used to prepare MNL and to generate LK/MK supernatants were performed with media and reagents judged to contain less than 0.07 ng/ml endotoxin by the Limulus amebocyte lysate assay (MA Bioproducts, Walkersville, Maryland). Human peripheral blood MNL were recovered by Ficoll-Hypaque fractionation (Ficoll-Paque, Pharmacia, Piscataway, New Jersey) from buffy coat fractions of fresh heparinized blood [36]. Platelets were routinely removed by centrifugation at 60 g in Hanks’ balanced salt solutions (HBSS) (MA Bioproducts) or in some cases by sucrose gradient centrifugation [37]. The resultant MNL pellet was resuspended in Dulbecco’s minimum essential medium containing 25 mm HEPES and 2 mm glutamine (DMEM-HEPES) (MA Bioproducts) plus other supplements as described below.

Preparation of Adherent Monocytes and Enriched T Cells

These enriched MNL fractions were prepared as previously described [38]. Briefly, suspensions of 1 x 10⁷ MNL/ml in DMEM-HEPES plus 10% human AB serum (MA Bioproducts) were incubated at 37°C, for 1 h in tissue culture dishes or flasks (Falcon, Oxnard, California) in a humidified atmosphere of 5% CO₂. Nonadherent cells were removed by washing the culture vessels 6 times with 37°C DMEM-HEPES containing 5% human AB serum. The resultant adherent monolayers consisted of >95% monocytes as determined by α-naphthyl-esterase staining [39]. Enriched T cells were prepared from the nonadherent fraction by E-rosetting with sheep erythrocytes by a previously described rapid E-rosetting technique [40]. Lysis of sheep erythrocytes by hypotonic saline shock followed by centrifugation of the enriched cell population yielded rosette-positive T-cell populations containing <2% monocytes.

Production of LK/MK Containing Supernatants

Fresly prepared total MNL or enriched T cells were incubated at a concentration of 1 x 10⁶ cells/ml in DMEM-HEPES supplemented with 0.5% human serum albumin (HSA) (New York Blood Center, New York, New York) in the presence of (a) no stimulant, (b) 12.5 μg/ml concanavalin A (Con A) (Sigma, St. Louis, Missouri), or (c) 20 μg or 20 μg/ml lipopolysaccharide (LPS) (E. coli 0128:B12, Sigma) at 37°C in a 5% CO₂ atmosphere. Cell-free supernatants were collected by centrifugation after 24 and 72 h of incubation. MNL viability at these times was always greater than 80% as assessed by trypan blue exclusion. Large-scale production was done in 40-ml volumes in 75-cm² culture flasks, while supernatants used in quantitative comparisons were generated in 5-ml volumes in 35-mm tissue culture dishes (cell density of 5 x 10⁴/cm² surface area). Monocyte monolayers used to produce supernatants were prepared by adhering total MNL at a cell density of 5 x 10⁴/cm² to the same culture vessels and incubating as described above. Appropriate concentrations of Con A and LPS were added to unstimulated culture supernatants immediately after harvesting and then after storage of the control supernatants. Harvested supernatants were then placed in boiled dialysis tubing (12,000 - M, cut off) and dialyzed at 4°C against a total of 100 volumes of distilled water and a subsequent 10 volumes of fresh DMEM before being collected, membrane filtered, and stored at −70°C until assayed for activity. Because the concentration of Con A in the generated supernatants was found to have an inhibitory effect on all 3 assays of fibroblast function, all Con A-containing supernatants were assayed in the presence of 25 mM α-methyl-D-mannoside (Sigma), which selectively blocked the inhibitory effect of Con A while having no effect on control cultures. LPS by itself had no effect on the assayed fibroblast functions. However, certain lots of HSA were found to stimulate fibroblast proliferation, and thus the lots of HSA were screened prior to use and only lots without proliferative activity were used in the generation of supernatants.

Primary Fibroblast Cultures

Primary cell cultures were established from sectioned skin explants taken from the forearms of normal adult volunteers as previously described [29]. The fibroblasts were grown to confluence in Ham’s F-10 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), sodium bicarbonate (2 mm), NaHCO₃ (1.87 mmol/ml), and 10% inactivated fetal calf serum (FCS) (Flow Labs, McLean, Virginia) and subcultured after trypsinization. The fibroblasts used to assess LK/MK activity in this study were grown from the papillary section of the dermis and were used between the 4th and 15th passages.

Assay for LK/MK Activity Stimulating Fibroblast Proliferation

Assay of fibroblast-proliferative activity was performed in the absence of FCS using subconfluent fibroblast microcultures (Microtest III—Falcon, 3072) in order to optimize assay sensitivity to proliferation-stimulating signals. Freshly trypsinized fibroblasts were first plated at 3000 fibroblasts per microwell in 100 μl of DMEM + 10% FCS for 18 h at 37°C in a 5% CO₂ atmosphere to permit adherence to microwell bottoms. Cultures were then washed with HBSS and incubated without FCS for 48 h in 100 μl of fresh DMEM + 0.5% bovine serum albumin (BSA) (Sigma) to allow the fibroblasts to achieve proliferative quiescence. The media was then removed and replaced with 200 μl of fresh DMEM + 0.5% BSA containing various dilutions of MNL supernatants or control fractions and incubated for an additional 48 h. Cultures were pulsed with 0.5 μCi [3H]thymidine (20 Ci/mmol, New England Nuclear, Boston, Massachusetts) for the last 24 h of incubation, and the incorporation of [3H]thymidine into DNA was assessed using methods similar to those previously reported [11-14]. Briefly, following media aspiration, cultures were trypsinized, harvested onto glass fiber filters using an automated harvest, lysed by washing with distilled water, and the [3H]thymidine-labeled DNA trapped by the glass fiber filters counted using liquid scintillation spectrometry. All sample dilutions were assayed in triplicate and media control cultures containing no MNL supernatant characteristically gave mean values for [3H]thymidine incorporation of 800-1200 cpm.

Assay for LK/MK Activity inhibiting Fibroblast Proliferation

Assay of fibroblast-growth inhibitory activity was performed using microculture techniques as described for the stimulation of proliferation assay. However, after 18 h adherence, media was removed and then dilutions of MNL supernatants or control fractions in 200 μl fresh DMEM + 10% FCS were added to the fibroblast cultures for 24 h along with 0.5 μCi of [3H]thymidine. After 24 h incubation fibroblasts were harvested and [3H]thymidine incorporation quantitated as before. Media control cultures containing no MNL supernatant gave means of approximately 10,000 cpm (fibroblast growth induced by FCS).

Assay for LK/MK Activity Inhibiting Fibroblast Collagen Production

Assay of collagen production was performed using fibroblast microcultures of identical condition and duration as those used to measure stimulation of proliferation except that 12,000 fibroblasts were plated per well in 100 μl DMEM. The collagenase used in plating and culturing these monolayers was additionally supplemented with 50 μg/ml ascorbic acid and the cultures were pulsed with 1.0 μCi of [3H] proline (5 Ci/mmol, New England Nuclear) in the presence of 50 μg/ml of β-aminopropionitrile for the terminal 24 h of culture. The inclusion of β-aminopropionitrile ensures that the major portion of collagen produced will be secreted into the culture media [41]. Determination of [3H]proline incorporation into collagen was performed using methods similar to those previously described [42]. Briefly, the extracellular [3H]proline-labeled collagen was extracted by the addition of acetic acid to the culture media in each microwell and purified by successive salt precipitations at acid and neutral pH. The final wash precipitate was solubilized in 0.5 M acetic acid, incorporated into a scintillation cocktail, and counted in a liquid scintillation counter. Media control cultures containing no MNL supernatant gave means of 1000-1500 cpm. This assay was done in 0.5% BSA instead of FCS and at confluence in order to be able to assess the effect of LK/MK containing samples on collagen production in the absence of their effects on fibroblast proliferation.
Quantitative Comparison of LK/MK Containing Supernatants

Log-linear dilution analysis was used to evaluate the LK/MK activity produced under different culture conditions and to quantitatively compare the levels of LK/MK produced by normal volunteers and PSS patients. Linear regression lines were generated for each LK/MK supernatant by first assaying in triplicate the [3H]thyminidine or [3H]proline incorporation induced by a series of 3-fold dilutions (1:1.7 to 1:135) of each supernatant and then plotting the mean [3H]thyminidine/proline data against the log10 of each of the 5 dilutions. For the inhibition assays supernatant activity was compared at the 10:1 causing 50% inhibition (ID50) of the counts obtained in media control cultures without supernatant, while LK/MK supernatants stimulating proliferation were compared at the dilution causing a 500% stimulation (SD500) over media controls, and results are presented as the ID50 or SD500. The comparison of the LK/MK levels produced by a series of normal volunteers and PSS patients was further standardized by comparing the regression line for each LK/MK supernatant to that generated by a standard supernatant obtained from the MNL of 1 normal donor. The standard supernatants were always produced, processed, and assayed simultaneously with each group of normal or PSS supernatants tested. Based on the ID50 and SD500, the standard supernatants were arbitrarily assigned a value of 1.0 unit/ml and the levels of LK/MK in the other tested supernatants are expressed as relative units/ml.

Gel Filtration Chromatography of LK/MK Supernatants

Fifty millilitors of exhaustively dialyzed LK/MK supernatant was concentrated to dryness by lyophilization and redissolved in 2 ml of sterile 0.01 M sodium phosphate-buffered saline, pH 7.4 (PBS). This concentrated sample was applied to a column (diameter 1.6 cm, length 95 cm) that was packed with Sephadex S-200 (Pharmacia). The samples were eluted with PBS containing 0.1% polyethylene glycol 6000 against gravity at a flow rate of 10 ml/h. Fractions of 5.0 ml were collected and tested for activity without further processing. All chromatographic procedures were performed at 4°C.

RESULTS

Stimulation of MNL to Produce Fibroblast-Inhibitory LK/MK Supernatants

In order to be able to assess the effect of fibroblast-inhibitory LK/MK on fibroblast function without interference from the presence of contaminating fibroblast-stimulatory factors, we attempted to establish MNL culture conditions that would allow the production of LK/MK supernatants that exclusively inhibited fibroblast proliferation and collagen production. Preliminary investigations indicated this could best be done by stimulating MNL with LPS to produce MK supernatants and with Con A to produce LK supernatants using the culture conditions described in Materials and Methods. HSA was chosen as the media supplement for LK/MK production instead of serum because HSA maintained the metabolic integrity of the MNL while having no effect on fibroblast proliferation or collagen production, which greatly simplified the interpretation of the subsequent assays of supernatant activity on fibroblast functions. The results as presented in Table I show that MNL stimulated with either 20 μg/ml LPS or 12.5 μg/ml Con A produced supernatants that inhibited both FCS-driven fibroblast proliferation and the extracellular collagen production occurring in confluent proliferatively quiescent fibroblast cultures. The production of both inhibitory factors was time-dependent and was at a maximum after 72 h of culture. In general the activity of LPS-induced supernatants was greater than Con A-induced supernatants and the inhibition of proliferation caused by both factors was generally greater than their inhibition of collagen production. Under the assay conditions employed, no LPS- or Con A-induced supernatants stimulated collagen production nor did Con A-induced supernatants stimulate fibroblast proliferation significantly more than did control supernatants. However LPS-induced supernatants did significantly stimulate fibroblast proliferation, with 24-h supernatants being more potent than supernatant generated for 72 h, which is likely due to the increased production of inhibitory factors after 72 h of culture. Thus we were able to generate Con A-induced LK supernatants that exclusively inhibited fibroblast proliferation and collagen production but our LPS-induced MK supernatants were contaminated with activity stimulating fibroblast proliferation. However, further preliminary investigations revealed that MNL stimulated with low concentrations of LPS (Table I—20 ng/ml LPS) produced supernatants that stimulated fibroblast proliferation but did not inhibit fibroblast proliferation or collagen production. Such supernatants contained maximum proliferation-stimulating activity after only 24 h of culture and moreover the amount of activity was similar to that found in 24-h supernatants induced with 20 μg/ml LPS. This observation made it possible to independently assess the proliferation-stimulating component contaminating our LPS-induced fibroblast-inhibitory MK supernatants.

Table I also illustrates that supernatants from unstimulated MNL contain only minimal activity in all 3 of our fibroblast assays. It should be noted that this is true only if endotoxin-free media and supplements are used, as endotoxin contamination as low as 0.1 ng/ml induced the production of supernatants that stimulated fibroblast proliferation (data not shown). Such endotoxin contamination may be responsible for the previously reported spontaneous production of some fibroblast-regulatory factors [13,16,21,41] as has been reported for other MK [43]. Minor platelet contamination had no effect on the activity of generated supernatants as supernatants produced by MNL totally depleted of platelets by sucrose gradient centrifugation had the same levels of activity as did supernatants produced by MNL depleted of platelets by centrifugation in HBSS at 60 g (not shown).

### Table I. Stimulation of MNL to produce lymphokine/monokine (LK/MK) supernatants*  

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Hours of stimulation</th>
<th>Stimulation of proliferation</th>
<th>Inhibition of proliferation</th>
<th>Inhibition of collagen production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SD50*</td>
<td>Slmax*</td>
<td>ID50*</td>
</tr>
<tr>
<td>None</td>
<td>24</td>
<td>&lt;1:1.7</td>
<td>1.8</td>
<td>&lt;1:1.7</td>
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<tr>
<td></td>
<td>72</td>
<td>&lt;1:1.7</td>
<td>1.6</td>
<td>&lt;1:1.7</td>
</tr>
<tr>
<td>LPS—20 ng/ml</td>
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<td>1:19.2</td>
<td>9.8</td>
<td>&lt;1:1.7</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1:20.8</td>
<td>10.7</td>
<td>&lt;1:1.7</td>
</tr>
<tr>
<td>LPS—20 μg/ml</td>
<td>72</td>
<td>1:14.9</td>
<td>9.0</td>
<td>&lt;1:1.7</td>
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<tr>
<td>Con A—12.5 μg/ml</td>
<td>72</td>
<td>1:18.5</td>
<td>5.2</td>
<td>&lt;1:1.7</td>
</tr>
</tbody>
</table>

* Representative experiment showing the effects of LK/MK supernatants produced by total MNL stimulated with LPS or Con A for 24 or 72 h on 3 independently assayed fibroblast functions.

* Supernatant dilution inducing a 500% increase over media controls.

* Maximum stimulation index (cpm supernatant/cpm media control) induced by a 1:1.7 supernatant dilution.

* Supernatant dilution inducing a 50% decrease from media controls.

* Maximum inhibition index (cpm supernatant/cpm media control) induced by a 1:1.7 supernatant dilution.
In order to insure that \( [\text{H}]\) thymidine incorporation is a true correlate of fibroblast growth as has been reported by others [11,13,14], we studied the effect of LK/MK supernatants on fibroblast proliferation by continuously culturing subconfluent fibroblasts in fresh-daily 1:2 dilutions of supernatant for 6 days and then counting the resultant cell population. Such experiments showed that fibroblasts grown in 72-h LPS (20 \( \mu \)g/ml) or Con A-induced supernatants contained only one-half to one-quarter as many cells as cultures grown in 10% FCS. The LK/MK-treated fibroblasts were still viable as judged by trypan blue exclusion and thus at these concentrations fibroblast inhibitory LK/MK appear to be cytostatic rather than cytotoxic. Fibroblasts cultured in 24-h LPS (20 \( \mu \)g/ml)-induced supernatants for 6 days contained twice as many cells as those cultured in DMEM-BSA alone (data not shown). Thus our \( [\text{H}]\) thymidine incorporation assay is a true correlate of fibroblast growth.

While the \( [\text{H}]\) proline incorporation assay specifically measures the newly formed collagen in fibroblast microcultures [42], it does not reveal how the LK/MK supernatants are inhibiting collagen production. The LK/MK supernatants may actually be inhibiting collagen synthesis as suggested by others [21,22] or, alternatively, they may be stimulating intracellular degradation of newly formed collagen or simply decreasing the intracellular proline pool size.

### Production of Fibroblast-Inhibitory LK/MK Supernatants by Monocytes and T Cells

In order to confirm that total MNL produce supernatants containing predominately fibroblast-active MK when stimulated with LPS and LK when stimulated with Con A, it was necessary to fractionate MNL into adherent monocytes and E-rosette-positive T cells. The enriched monocytes and T cells were then stimulated with LPS and Con A and the supernatants generated assayed for activity as described in Materials and Methods. The results reported in Table II indicate that active supernatants were generated only by LPS-stimulated monocytes or by Con A-stimulated T cells. Stimulation of T cells with LPS or monocytes with Con A produced supernatants with only minimal activity. Consistent with previous reports of endotoxin contamination of some preparations of T-cell mitogens [44], we found some lots of Con A contained endotoxin contamination and the use of such lots erroneously demonstrated stimulation of MK production by Con A (data not shown). Such endotoxin contamination may also be responsible for previously reported production of fibroblast-proliferation regulatory factors by monocytes after PHA or Con A stimulation [16,45]. Table II also shows that Con A-stimulated T cells produced supernatants that exclusively inhibited fibroblast proliferation and collagen production at the same times as Con A-stimulated total MNL. Likewise, monocytes stimulated with either high or low concentrations of LPS produced supernatants with the same activities and at the same times as did the

### Table II. Production of fibroblast-inhibitory lymphokine/monokine (LK/MK) supernatants by enriched monocytes and T cells

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Hours of stimulation</th>
<th>Fibroblast assay</th>
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</thead>
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<tr>
<td>None</td>
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<td>LPS-20 ng/ml</td>
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<tr>
<td>LPS-20 ( \mu )g/ml</td>
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</tr>
<tr>
<td>Con A-12.5 ( \mu )g/ml</td>
<td>24</td>
<td>&lt;1:1.7</td>
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<table>
<thead>
<tr>
<th>Stimulant</th>
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<th>Fibroblast assay</th>
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<tbody>
<tr>
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<td>T Cell</td>
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<td>LPS-20 ng/ml</td>
<td>24</td>
<td>&lt;1:1.7</td>
</tr>
<tr>
<td>LPS-20 ( \mu )g/ml</td>
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</tr>
<tr>
<td>Con A-12.5 ( \mu )g/ml</td>
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<th>Stimulant</th>
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<td>LPS-20 ( \mu )g/ml</td>
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<tr>
<td>Con A-12.5 ( \mu )g/ml</td>
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<td>&lt;1:1.7</td>
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<tr>
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<td>Collagen</td>
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<td>LPS-20 ng/ml</td>
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<td>&lt;1:1.7</td>
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<tr>
<td>LPS-20 ( \mu )g/ml</td>
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<td>Con A-12.5 ( \mu )g/ml</td>
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<td>&lt;1:1.7</td>
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### Table III. Inhibition of collagen production in the absence of inhibition of proliferation

<table>
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<th>Supernatant</th>
<th>Inhibition of collagen production</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>([\text{H}])Thymidine incorporation*</td>
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<tr>
<td>Media control</td>
<td>949 ± 84</td>
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<tr>
<td>Control 24 h</td>
<td>1023 ± 135</td>
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<tr>
<td>Control 72 h</td>
<td>1081 ± 220</td>
</tr>
<tr>
<td>LPS 24 h</td>
<td>1263 ± 224</td>
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<tr>
<td>LPS 72 h</td>
<td>1166 ± 72</td>
</tr>
<tr>
<td>Con A 24 h</td>
<td>996 ± 65</td>
</tr>
<tr>
<td>Con A 72 h</td>
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### Fibroblast-Inhibitory LK/MK Supernatants Inhibit Collagen Production Without Inhibiting Fibroblast Proliferation

Since our collagen production assay employed the use of quiescent confluent fibroblast cultures we were able to assess the effect of fibroblast-inhibitory LK/MK supernatants on collagen production in the absence of their effects on fibroblast proliferation. Data in Table III illustrate that both LPS-induced inhibitory MK supernatants and Con A-induced inhibitory LK supernatants inhibited collagen production independently of inhibition of fibroblast proliferation. Thus the MK and LK inhibiting collagen production likely act through mechanisms unrelated to fibroblast proliferation, which suggests that the factors inhibiting collagen production may be distinct molecules from those inhibiting proliferation.

### Gel Filtration Chromatography of Fibroblast-Inhibitory LK/MK Supernatants

To obtain estimates of the \( M_\text{o} \) of the monocyte- and T cell-derived factors inhibiting fibroblast proliferation and collagen production, LK/MK supernatants were chromatographed on a Sephacryl S-200 column. Chromatography of LK supernatant produced by total MNL stimulated for 72 h with 12.5 \( \mu \)g/ml Con A eluted a broad peak of activity inhibiting fibroblast proliferation in the \( M_\text{o} \) range centering around 50,000 (Fig 1). In contrast, the MK inhibiting fibroblast proliferation obtained by 72-h stimulation of total MNL with 20 \( \mu \)g/ml of LPS, eluted in a peak centered around \( M_\text{o} \), 20,000 (Fig 1). The elution of

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*Representative experiments showing the effect of LK/MK supernatants produced by enriched monocytes and T cells stimulated with LPS or Con A for 24 or 72 h on 3 independently assayed fibroblast functions.

\*Values listed are the supernatant dilutions inducing a 500% increase over media controls (SDm). The values listed are the supernatant dilutions inducing a 50% decrease from media controls (IDm).
Fibroblast-Inhibitory Lymphokines/Monokines and PSS

Aberrant Production of the LK and MK Inhibiting Fibroblast Proliferation by MNL of PSS Patients

Since LK/MK production is known to involve the interaction of different MNL subsets through a LK/MK cascade mechanism [46], we chose to investigate the levels of fibroblast-inhibitory LK and MK produced by PSS patients using total MNL rather than enriched populations in order to optimize our chances of detecting a defect in LK/MK production. Total MNL from a series of normal volunteers and PSS patients were stimulated with: (a) 20 ng/ml LPS for 24 h to produce supernatants containing exclusively proliferation stimulatory MK; (b) 20 µg/ml LPS for 72 h to produce proliferation/collagen production inhibitory MK; and (c) 12.5 µg/ml Con A for 72 h to produce exclusively proliferation/collagen production inhibitory LK. The potency of each generated supernatant was comparatively quantitated and expressed as units/ml as described in Materials and Methods. The results presented in Fig 2 show that MNL from the series of PSS patients produced significantly less than normal amounts of the Con A-induced LK that inhibits fibroblast proliferation. The 14 normal volunteers produced 1.01 ± .22 (SD) units/ml of the factor while the 13 PSS patients produced only 0.51 ± .29 units/ml (p < .001 by two-tailed Student's t-test). If only the 11 of 13 PSS patients outside 2 SD of the normal mean are considered, the average is 0.41 ± .16 units/ml (p < .001). It is evident that PSS patients produced fibroblast proliferation inhibitory LK in levels markedly lower than controls. This, however, was not true for the collagen production inhibitory LK as Fig 3 shows that the levels of this factor produced by normal volunteers (1.02 ± .36 units/ml) and PSS patients (1.06 ± .30 units/ml) to be similar. This observation further suggests that the 2 fibroblast-inhibitory LK are separate molecules.

Data illustrated in Fig 2 also show that MNL from PSS patients produced markedly elevated levels of the LPS-induced MK that inhibits fibroblast proliferation. Normal volunteers produced an average of 1.15 ± .31 units/ml while PSS patients produced 2.96 ± 1.46 units/ml (p < .001). The 9 of 13 PSS patients producing levels greater than 2 normal SD averaged 3.83 ± .66 units/ml (p < .001) and all 9 of these patients were
also hypoproducers of the proliferation inhibitory LK. The increased fibroblast proliferation inhibitory capacity of PSS MNL supernatant was not due to the presence of decreased amounts of contaminating proliferation-stimulating MK as PSS MNL stimulated with 20 ng/ml LPS for 24 h produced normal levels of the proliferation-stimulatory MK as shown in Fig 3 (normal 0.99 ± .32 units/ml; PSS 1.11 ± .33 units/ml). Thus PSS patients truly produced increased levels of the fibroblast-proliferation-inhibitory MK. They did so while producing normal amounts of the collagen production inhibitory MK, as detailed in Fig 3 (normal 1.12 ± .29 units/ml; PSS 1.10 ± .21 units/ml). This latter observation suggests that the MK inhibiting fibroblast proliferation may be different from the MK inhibiting collagen production.

DISCUSSION

The studies described here were undertaken to evaluate the capacity of normal peripheral blood MNL to produced LK and MK inhibiting fibroblast proliferation and collagen production, and to subsequently compare the normal levels produced with those generated by MNL from PSS patients. Our results indicate that normal MNL stimulated with Con A produce T cell-derived LK supernatants that exclusively inhibit fibroblast proliferation and collagen production (Tables I, II). The proliferation-inhibitory LK may be a distinct molecule from that inhibiting collagen production as inhibition of collagen production was demonstrated in the absence of effects on proliferation (Table III). However both the proliferation- and collagen-inhibiting activities coeluted in the M, 50,000 range on Sephacryl S-200 chromatography (Fig 1). This M, estimate is in agreement with those previously published for both LK-inhibitory activities [15,21]. Why MK activity stimulating fibroblast proliferation or collagen production could not be detected in our Con A-induced supernatants was not investigated. The absence, however, may be due to the fact that only minimal production of stimulatory LK occurs at the low MNL density (5 × 10^6/cm^2 surface area) used in our generation cultures, as those reporting generation of stimulatory LK have used either high density surface cultures (4 × 10^6/cm^2) or the even higher density gravity sedimented pellets that form during tube culture generation [11,12,17].

Supernatants inhibiting both fibroblast proliferation and collagen production were also produced by stimulating normal MNL with 20 μg/ml LPS (Table I). While these supernatants were shown to contain exclusively monocyte-derived products (Table II), they also contained a fibroblast proliferation stimulatory MK which could also be produced in the absence of inhibitory MK by stimulating MNL with only 20 ng/ml LPS (Tables I, II). The collagen production inhibitory MK was active in the absence of effects on proliferation (Table III) and both MK inhibitory activities coeluted from a Sephacryl S-200 column in the M, 20,000 range, further suggesting that the inhibitory MK are molecules distinct from the inhibitory LK. This report represents the first estimation of the M, of the LPS-induced MK inhibiting fibroblast proliferation and collagen production, although 2 previous studies have estimated the M, of spontaneously produced (endotoxin contamination induced?) MK that inhibit growth and/or collagen production by inducing prostaglandin synthesis at 12,000–20,000 [16] and 20,000–30,000 [22].

However, the mechanism of action of the fibroblast-inhibitory LK and MK described in this report, and their relation to known immunoregulatory LK/MK remain open to speculation until they have been more extensively characterized as to their cellular sources and biochemical properties, although several immunosuppressive LK/MK with M, similar to our fibroblast-inhibitory factors, including the interferons [47] and the soluble immune response suppressors [48] exhibit antiproliferative activity on cells of nonimmune origin. However, in some other instances the coidentity of fibroblast-regulatory MK/LK and immunoregulatory LK/MK has been proved. Evidence has been presented identifying fibronectin as the macrophage-derived fibroblast chemotactic factor and human monocyte-derived fibroblast proliferation stimulatory activity can be attributed to interleukin 1 [9,14]. Conversely, studies in guinea pigs indicate that T cell-derived fibroblast proliferation stimulating activity is distinct from interleukin 1 [12] and human T cell-derived fibroblast proliferation inhibition factor appears to be distinct from α-lymphotixin [15].

Recent studies indicated that pathologic fibrosis is accompanied by LK/MK production in vivo, as activated monocytes/macrophages and activated T cells that spontaneously produce fibroblast growth factors have been isolated from the fibrotic synovium of rheumatoid arthritis patients and from the lungs of patients with idiopathic pulmonary fibrosis [23,24]. In vivo LK/MK production in PSS is suggested by the presence of T-cell blasts among the infiltrating MNL in affected tissue sites [6] and by the presence of factors with LK/MK activity (fibroblast proliferation stimulation, collagen production stimulation, and proliferation inhibition) in the serum of PSS patients [49–51]. While these studies verify that in vivo “auto-antigen” activation of T cells and monocytes/macrophages results in the production of fibroblast-regulatory LK/MK, they do not indicate whether the normal balance of stimulatory and inhibitory LK/MK is altered in cases of pathologic fibrosis.

In contrast, the results of our in vitro study reveal that peripheral blood MNL from patients with PSS in addition to having a diminished capacity to produce the LK inhibiting fibroblast proliferation, also possess the enhanced capacity to produce the analogus MK (Fig 2). The aberrant production of these 2 fibroblast proliferation inhibitory factors occurred in 9 of 13 PSS patients studied. This characteristic PSS LK/MK production was independent of age as patients of all ages, including one 20-year-old and one 75-year-old, exhibited this aberrant production pattern. Whether this aberrant LK/MK production occurs only in PSS or is also characteristic of other chronic fibrotic diseases remains to be investigated. However, in PSS the aberrant production of these 2 fibroblast proliferation inhibitory factors did occur in the presence of the production of normal levels of both the MK stimulating fibroblast proliferation and the LK and MK inhibiting collagen production (Fig 3).
While the mechanisms responsible for the observed aberrant production of both T cell-derived and monocyte-derived proliferation inhibitory factors likely involve the complex interaction of cells and mediators regulating the LK/MK cascade, recent reports suggest 2 plausible, although possibly simplified, explanations. First, a number of studies suggest that the increased B-cell function associated with autoantibody formation in PSS may be the result of a deficiency of circulating T suppressor cells [30–32]. Since T suppressor cells are a likely source of our proliferation inhibitory LK, then a deficiency of T suppressor cells could explain why we have observed a deficient production of this LK in PSS patients. Second, other studies have suggested that the depressed T-cell functions associated with PSS may be the result of the increased activity of suppressor monocytes [33,34]. These hyperactive suppressor monocytes may be the source of the increased levels of the proliferation inhibitory MK produced by a portion of our PSS patients. Such suppressor monocytes have also been described in murine models of graft-versus-host disease, where they are associated only with the late stages of the disease [52]. Whether suppressor monocytes are present only in PSS patients of long duration has not been investigated, but this may be the case if our results are indicative of increased suppressor monocyte activity, as all the PSS patients we studied had a disease duration of 3 years or more with a group mean of 9.6 years.

Assuming that the production of LK/MK in PSS tissue sites parallels that which we have observed with peripheral blood MN1, then our results suggest that the increased fibroblast population seen in early PSS tissue sites is due to the deficient production of a fibroblast proliferation inhibitory LK, allowing excess fibroblast-growth stimulatory signals to unrestrictedly expand the resident fibroblast population. In vivo, the growth-stimulating signals may include not only stimulatory LK and MK, but also platelet-derived growth factor (PDGF) as studies suggest that the vascular endothelial damage occurring in PSS results in the perivascular aggregation of platelets and the subsequent discharge of PDGF into PSS tissue sites [53]. Our results further suggest that the increased fibroblast growth caused by this relative excess of fibroblast-proliferation stimulatory signals is moderated in long-term PSS patients by the increased production of a fibroblast-growth inhibitory MK. Such a mechanism would play a role in limiting previously initiated fibrosis and could be responsible for the more stabi- lized forms of PSS often seen in long-term patients. Proof of this theory, however, requires investigation of the levels of fibroblast proliferation inhibitory LK/MK produced by patients with newly acquired PSS.

Our results also show that the production of the LK and MK inhibiting fibroblast collagen production is normal and thus these 2 factors cannot be implicated in the induction of the portion of PSS fibroblasts which possess a hyper-collagen-producing phenotype. However, other studies suggest that this phenotypic change may be caused by the excessive production of a collagen production stimulatory LK by PSS MN1 [54,55] or, alternatively, those fibroblasts that have been induced to proliferate by the relative excess of PDGF may innately possess the hyper-collagen-producing phenotype [56]. Thus both the increased fibroblast number and the increased fibroblast function observed in PSS tissue sites appear to be the result of systemic immunologic abnormalities exacerbating an in situ inflammatory response by causing the unbalanced production of stimulatory and inhibitory fibroblast-regulatory LK/MK.

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REFERENCES