FIBROBLASTS (Fb) from patients with sarcoidosis (SA) and hypersensitivity pneumonitis (HP) exhibited a lower proliferative capacity compared with Fb obtained from control (CO) and diffuse interstitial fibrosis patients (DIF). Proliferation of Fb from SA or HP patients was suppressed by autologous LPS-stimulated alveolar macrophages (AM) supernatants but not by those from CO patients. Similarly, alveolar macrophages (AM) derived supernatant, obtained from CO, did not suppress the proliferation of SA and HP Fb. AM from SA and HP patients secreted higher amounts of IL-1α and β compared with controls and compared with Fb from SA and HP patients. Steady levels of IL-1α and β mRNA were expressed in unstimulated and stimulated cultures. Fb from SA and HP patients could be stimulated by LPS to secrete significantly higher levels of PGE₂, than those detected in supernatants from LPS stimulated Fb of DIF patients. Only the proliferation of Fb from SA and HP patients was sensitive to amounts of IL-1 equivalent to those detected in the lung of these diseases. As SA and HP are two diseases where irreversible deterioration occurs in only 20% of the patients, we hypothesize that mediators in the lung may modulate Fb proliferation. IL-1α of AM origin and PGE₂ of Fb origin secreted at high levels, may be candidates for this suppression because it was abrogated by anti IL-1β and indomethacin.

Key words: Alveolar fibroblasts, Bronchoalveolar lavage, Interleukin-1, Interstitial lung diseases, Prostaglandin E₂

Introduction

One of the most prominent histologic features of granulomatous and interstitial diseases in the lung is the close proximity of mononuclear cells with fibroblasts or the matrix secreted by them. Several studies characterized mononuclear cell-derived factors that stimulate or inhibit fibroblast growth and secretory functions in vitro. Other researchers have shown that cytokines are released spontaneously by macrophages isolated from lungs of animals exposed to a variety of stimulants, including cytotoxic drugs and mineral dusts, as well as from patients with idiopathic pulmonary fibrosis (IPF) and sarcoidosis (SA). Furthermore, alveolar lining fluid collected by pulmonary lavage from patients with pulmonary fibrosis has been shown to contain fibrogenic cytokines, such as TGFβ and TNFα, pointing to the role of cytokines in vivo. These data suggest that cytokines are present in situ and may mediate the pathological manifestations of interstitial lung diseases. Lately, efforts have been directed towards the identification of macrophage-derived factors which affect fibroblast growth-function, while fewer studies were oriented towards the investigation of the immunoregulatory and pro-inflammatory role of fibroblasts. Recently, it was shown that murine fibroblasts, stimulated by cytokines and LPS, are able to generate IL-1β activity and that rIL-1 and TNF stimulate normal adult human lung fibroblast to accumulate not to secrete IL-1. Moreover, other studies demonstrated that fibroblast strains secrete inflammatory mediators, such as prostaglandin E₂ (PGE₂), interleukin 6 (IL-6), interleukin 8 (IL-8), monocyte chemotactic peptide (MCP-1) and colony-stimulating factor. As we have previously shown that alveolar fibroblasts can be obtained from long-term cultures of bronchoalveolar cells recovered from patients with SA, we decided to further characterize the interactions between alveolar macrophages and alveolar fibroblasts in interstitial lung diseases in an autologous system. Thus, in the present study we assessed the differential secretion of IL-1 and PGE₂ by these cells, in comparison with alveolar macrophages and the possible role of these mediators as suppressive agents of fibroplasia and fibrosis in these diseases.
Patients and Methods

Study population. Eighteen patients, belonging to three groups were included in this study.

Pulmonary sarcoidosis (SA). Diagnosis was made in six untreated patients (three males and three females, mean age 37 ± 7 years), by clinical and roentgenological presentation, a positive Kveim test, or a positive biopsy of non-caseating granuloma. All patients were in Stage II sarcoidosis. None of them was a smoker.

Diffuse interstitial fibrosis (DIF). Three patients (two males and one female, mean age 61 ± 10 years). Diagnosis of DIF was made by roentgenological evidence of reticular infiltration and different degrees of interstitial fibrosis, demonstrated by transbronchial biopsy. None of them was a smoker.

Hypersensitivity pneumonitis (HP). Three patients belonged to this group (two males, one female, mean age 48 ± 6 years). Roentgenological evidence (X-rays and CT scan) showed reticular nodular pattern with predominant upper zone involvement. Bronchoalveolar lavage (BAL) analysis demonstrated features of a cell-mediated immune response with lymphocytosis. Lung histology was compatible with HP but no attempt was made to characterize the sensitizing antigens. None of them was a smoker.

Control. Six patients (three males and three females, mean age 44 ± 18 years) were admitted for investigation due to persistent cough. All of them presented chest roentgenograms within normal limits. None of them was a smoker.

None of the patients received any medicaments prior to the study. Written consent was obtained from each subject before bronchoscopy. Characterization of cell population present in BAL and pulmonary function test parameters of all patients examined are summarized in Table 1.

Methods:

Bronchoalveolar lavage (BAL). BAL was performed using a flexible fibre optic bronchoscope (BF-B2; Olympus Optical Co., Ltd, Tokyo, Japan) as previously described. The cells were recovered by gentle aspiration. The percentage of lavage fluid (±SD) recovered from each group of patients was as follows: 67 ± 10% from CO and 58 ± 8% from ILD cases. The average total cells recovered was 5 ± 1 x 10⁶ cells from CO and 17 ± 7 x 10⁶ cells from ILD patients.

Preparation of AM and AM supernatants. AM were prepared as previously described. Differential counts were performed on a Giemsa stained cytospincte preparation (Cytospin; Shandon, Southern Products Ltd, Runcorn, Cheshire, UK), by counting a minimum of 500 cells. Cells were adjusted to a final concentration of 10⁶ cells/ml in RPMI 1640 medium, supplemented with 10% heat inactivated FCS, 1% l-glutamine, and 1% streptomycin, penicillin, mycostatin complete medium (Biological Industries, Beit Haemek, Israel). The AM were purified by adherence in a 5% CO₂ humidified atmosphere for 1 h at 37°C. Identification of macrophages was done by morphology and nonspecific esterase staining and counted with an objective micrometer (Olympus Optical Co., Ltd, Tokyo, Japan). The adherent cell population contained more than 90% AM.

AM were cultured in 3-cm diameter plastic Petri dishes (Sterilin, Hounslow, Middlesex, UK) for either 24 h or 72 h. The 24 h period was found to be optimal for testing the production if IL-1 in the presence of lipopolysaccharide (LPS–Escherichia coli 055:B5; Difco Laboratories, Detroit, USA; 10 μg/ml) stimulated cultures. The 72 h period was chosen as the optimal time for release of PGE₂ in unstimulated cultures. Supernatants were recovered, filtered (Acrodisc 0.2 μ; Gelman Sciences) and stored at −70°C until determination for IL-1, and not longer than 2 weeks for PGE₂.

Table 1. BAL cells characteristics and pulmonary function tests (PFT) in all patients

<table>
<thead>
<tr>
<th>Patients</th>
<th>Mac</th>
<th>Ly</th>
<th>Bas</th>
<th>Eos</th>
<th>Neut</th>
<th>DLCO</th>
<th>FVC</th>
<th>FEV₁</th>
<th>FEV₁/FVC</th>
<th>TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>SA (6)</td>
<td>51 ± 15</td>
<td>48 ± 15*</td>
<td>0.6 ± 0.4</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.6</td>
<td>99 ± 14**</td>
<td>99 ± 9</td>
<td>87 ± 10</td>
<td>102 ± 6</td>
<td>97 ± 11</td>
</tr>
<tr>
<td>HP (3)</td>
<td>40 ± 8</td>
<td>58 ± 12*</td>
<td>1.9 ± 1</td>
<td>0.9 ± 1.3</td>
<td>0.8 ± 0.4</td>
<td>114 ± 22**</td>
<td>103 ± 14</td>
<td>104 ± 10</td>
<td>106 ± 3</td>
<td>103 ± 15</td>
</tr>
<tr>
<td>DIF (3)</td>
<td>70 ± 8</td>
<td>13 ± 13</td>
<td>–</td>
<td>8.4 ± 8***</td>
<td>12 ± 8***</td>
<td>69 ± 9</td>
<td>76 ± 8</td>
<td>77 ± 9</td>
<td>106 ± 6</td>
<td>86 ± 7</td>
</tr>
<tr>
<td>CO (6)</td>
<td>86 ± 5</td>
<td>13 ± 5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>103 ± 12</td>
<td>99 ± 3</td>
<td>101 ± 12</td>
<td>109 ± 5</td>
<td>102 ± 5</td>
</tr>
</tbody>
</table>

*p < 0.0001 compared with CO group.  **p < 0.001 compared with DIF group.  ***p < 0.001 compared with CO group.

PFT were performed in all patients prior to the BAL.

Differential counts were performed by counting 500 cells of a Giemsa stain cytoprep as described in Patients and Methods.

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Preparation of alveolar fibroblasts. The fibroblast line was derived from the bronchoalveolar cells as previously described. First clones of proliferating was derived from the bronchoalveolar cells as pre-viously described. First clones of proliferating was derived from the bronchoalveolar cells as previously described. After reaching confluence, usually 5–6 weeks later, the explant tissue was removed with trypsin-EDTA Type B (Biological Industries, Beit Haemek, Israel) for 10 min and cells were split 1:2 at confluence in 25 cm² tissue plastic culture flasks (Sterlin, Hounslow, Middlesex, UK). In all experiments the cells used were from passages 4–7.

Preparation of pulmonary fibroblasts. Stable lines of human pulmonary fibroblasts were used as control cells. Lung specimens from pneumonectomy specimens from patients with thoracic malignancies or benign lesions were minced into pieces of 2–4 mm and incubated in 1 x 5 cm Petri dishes (Sterlin, Hounslow, Middlesex, UK) containing 3 ml of complete DMEM. Every 72 h the non-adherent cells were removed by washing with PBS and fresh media was added. After 2 weeks, cultures reached confluence and the cells were split and used as described above.

Preparation of Fb supernatants and Fb proliferation test. Fb derived supernatants were obtained from 24 h LPS-stimulated Fb cultures cultured for 24 h with or without LPS. Aliquots of the supernatants were frozen at −70°C until used. Proliferation test was performed as previously described. Briefly, 100 µl of Fbs suspended at 10² Fb/ml were allowed to attach for 1–2 h. Aliquots (50 µl) of supernatants of LPS-stimulated AM were added. Proliferation was assessed after 72 h, and pulsed with 1 µCi °H-Tdr (48 µCi/nmol specific activity, Rotem Industries Ltd, Beer-Sheva, Israel) for the last 16 h of culture. The proliferation of Fb in the presence of AM supernatants was compared with that of Fbs DMEM with a final concentration of 100 µg/ml LPS.

Assay of prostaglandin and IL-1 production. Aliquots of AM and Fb supernatants (24 h production) were assayed for PGE₂ by a radioimmunoassay (Advanced Magnetic Inc., MA, USA) and IL-1α and IL-1β production by a RIA Kit (Amerlex-M™ Magnetic separation, Amersham International PLC, Amersham, UK).

Isolation of mRNA transcripts. RNA was extracted by 100 µl of 4.0 M guanidium thiocyanate (GuSCN Sigma Chemical Co., St Louis, USA) from adherent 10⁶ cells/ml stimulated (10 µg LPS + 10 ng/ml IL-1α and β for 24 h) and non-stimulated AFb. The mixture was overlaid on to 100 ml of 5.7 M CsCl and RNA was isolated after overnight ultracentrifugation at 35000 r.p.m. (Kontron Institute, Zurich, Switzerland) at 15°C. The pellet recovered by centrifugation was resuspended in 100 µl DEPC water, 300 µl pure ethanol and 30 µl 3.0 M sodium acetate. The RNA pellet (30' at 15000 r.p.m.) was washed (100 µl 80% ethanol) and amplified using the reverse transcription-mix [RT mix: 3 µl 200 U/µl MMLV, moloney murine leukaemia virus-RT (BRL, Bethesda Research Laboratory, Gaithersburg, MD, USA), 1 µl 40 U/µl RNAsin, 6 µl 5XMMVL buffer, 3 µl random primers (Promega CA, Madison, USA), 3 µl of 1 mg/ml BSA (Sigma Chemicals Co., St Louis, USA) 1.5 µl of 10 mM dNTP mix (Pharmacia, Fine Chemicals AB, Uppsala, Sweden). Each sample contained 17.5 µl and the reaction was performed for 1 h at 42°C.

**PCR assay.** cDNA fragments were amplified (GeneAmp-Clontech Laboratories Inc., Palo Alto, CA, USA) in a polymerase chain reaction (PCR) mix containing 8 µl dNTPs mix 1.25 mM (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and 0.25 µl Taq polymerase, 5 U/µl (Promega CA, Madison, USA) using thermal cycler cells (PT-100 MJ Research Inc., OSP, Cambridge MA, USA). β actin mRNA was evaluated concurrent with IL-1α and β mRNA as an internal control. Products of the combined reverse transcription-polymerase reaction (8 µl PCR product and 2 µl of gel loading buffer) were fractionated by electrophoresis in agarose stained with ethidium bromide and validated by matching predicted size 174/HaelII digest (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Statistics. Student’s t-test was used for statistical evaluations using the Epistat Software, © 1984, T.L. Gustafson. The results are expressed as mean ± SD and values less than 0.05 were considered to be significant.

Results

Effect of AM supernatants on the proliferation of fibroblasts: The basic proliferation rate of Fb from SA and HP patients was shown to be significantly lower than that of Fb in the CO group (Table 2).

AM-derived supernatants were tested for effects on the proliferation of Fb in an autologous culture set-up and in presence of AM supernatant of CO patients (Fig. 1) and on normal Fb lines (Fig. 2). The AM supernatants of SA and HP suppressed the prolifera-

<table>
<thead>
<tr>
<th>Patient</th>
<th>SA-Fb</th>
<th>HP-Fb</th>
<th>CO-Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7753 ± 1007</td>
<td>32677 ± 5257</td>
<td>62466 ± 5528</td>
</tr>
<tr>
<td>2</td>
<td>9399 ± 297</td>
<td>10509 ± 1256</td>
<td>28437 ± 2687</td>
</tr>
<tr>
<td>3</td>
<td>1801 ± 437</td>
<td>9182 ± 1609</td>
<td>25347 ± 5630</td>
</tr>
<tr>
<td>4</td>
<td>7613 ± 1007</td>
<td>30271 ± 982</td>
<td>30271 ± 232</td>
</tr>
<tr>
<td>5</td>
<td>6047 ± 268</td>
<td>46776 ± 2286</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2633 ± 853</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>5874 ± 2771</td>
<td>17459 ± 10776</td>
<td>38759 ± 14033</td>
</tr>
</tbody>
</table>

*p<0.001 compared with CO.

**p<0.001 compared with CO.
tion of autologous Fb by 38 ± 7% (Fig. 1a, patients 1-6, left panel) and 31 ± 10% (Fig. 1b, patients 1-3, left panel), respectively. In contrast, CO AM-derived supernatants suppressed proliferation by only 13 ± 5% in four cases (Fig. 1a, patients 1, 3, 5 and 6, right panel) and enhanced by 30 ± 1% in two cases (Fig. 1a, patients 2 and 4, right panel) when tested on Fb of SA. When CO AM supernatants were tested on HP-Fb, 3% suppression in two cases (Fig. 1b, patients 1 and 2, right panel) and 20% enhancement in the third case (p < 0.001 between patients groups and controls). The SA-AM derived supernatants exerted a differential effect when tested on normal Fb: suppression of 34 ± 22% in three cases (Fig. 2a, patients 1, 3 and 6, left panel) and enhancement in three others 29 ± 18% (Fig. 2a, patients 2, 4 and 5, left panel). A similar pattern was seen in the HP group. Supernatants derived from DIF patients enhanced the proliferation of Fb in all cases tested.

Thus, in the autologous culture set-up, AM-derived supernatants from SA and HP patients exerted significant suppression on the proliferative capacity of alveolar Fb, which was much less pronounced when tested on normal Fb. In addition, AM-derived supernatants from control or DIF patients, did not exert a suppressive effect on Fb proliferation.

Analysis of IL-1α, IL-1β and PGE₂ levels in AM and Fb supernatants: LPS-induced supernatants from AM and Fb were tested for their ability to secrete IL-1α, β and PGE₂. As shown in Tables 3 and 4, the levels of IL-1α and β of stimulated SA and HP AM,
Table 4. IL-1β* levels in AM and Fb supernatants

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>AM</th>
<th>Fb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-LPS</td>
<td>+LPS</td>
</tr>
<tr>
<td>SA (6)</td>
<td>0.09 ± 0.04***</td>
<td>2.1 ± 2.0*</td>
</tr>
<tr>
<td>HP (3)</td>
<td>0.08 ± 0.03**</td>
<td>1 ± 0.6**</td>
</tr>
<tr>
<td>DIF (3)</td>
<td>0.08 ± 0.02</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>CO (6)</td>
<td>0.07 ± 0.02</td>
<td>0.17 ± 0.08</td>
</tr>
</tbody>
</table>

* IL-1β was measured in Fb supernatants by RIA as described in Patients and Methods. Results are expressed as concentration of IL-1 (ng/ml).
** p < 0.05 compared with CO and DIF.
*** p < 0.001 compared with CO and DIF.

were significantly higher than those of CO cultures (2.1 ± 2.0 and 1 ± 0.6 ng/ml, compared with 0.17 ± 0.08 ng/ml for IL-1β, and 0.8 ± 0.7 and 0.5 ± 0.2 ng/ml compared with 0.2 ± 0.04 ng/ml for IL-1α, p < 0.05 compared with CO).

LPS did not affect the secretion of IL-1α and IL-1β and only baseline levels of those cytokines were produced by Fb irrelevant of their source. In contrast, when PGE2 secretion was assessed (Table 5) it was demonstrated that LPS-stimulated Fb of SA and HP patients generated significantly higher levels of PGE2 (0.36 ± 0.24 and 0.59 ± 0.27 ng/ml in stimulated supernatants of SA and HP patients compared with 0.16 ± 0.12 and 0.23 ± 0.19 ng/ml in unstimulated cultures). The high levels of PGE2 secreted by stimulated Fb in SA and HP were significantly higher than those found in Fb recovered from DIF patients (0.36 ± 0.24 and 0.59 ± 0.27 ng/ml in SA and HP, compared with 0.06 ± 0.03 ng/ml in DIF, p < 0.05).

Detection of IL-1α and IL-1β mRNA transcripts in Fb: In order to assess whether the IL-1α and IL-1β genes are expressed in Fb of these diseases, we assessed the mRNA transcripts by PCR. IL-1α and IL-1β transcripts were constitutively found in stimulated, as well as non-stimulated, Fbs (Fig. 3a, b, c and d).

Effects of exogenous IL-1β on Fb proliferation: Exogenous IL-1β (concentrations in the range of 0.3–1000 ng/ml), were added to Fb cultures and proliferation was assessed by the tritiated thymidine incorporation. IL-1β, at concentrations of 0.35–62.5 ng/ml (Fig. 4a) and 0.35–125 ng/ml (Fig. 4b) significantly suppressed the basic proliferation rate of Fb by 46 ± 1.4% and 39 ± 0.9% (p < 0.001) compared with proliferation of Fb in complete medium (Fig. 4a and b). These concentrations include the range of IL-1β by AM of SA and HP patients (4–0.8 ng/ml). As a general trend in Fbs of the CO group, no suppressive activity was observed at minute concentrations of IL-1β (Fig. 4c). The modulatory effects of IL-1β were abrogated by anti-IL-1 antibodies or indomethacin (Table 6). A clear reversion of the suppression was achieved in the SA and HP group, but not in the CO and DIF groups.

Discussion

AM are present within the alveoli and bronchioli while the fibrosis occurs in the interstitium. However, it is feasible to assume that AM affect the process of fibrosis because of their close proximity. The outcome of a number of interstitial lung diseases (ILD) results in severe pulmonary fibrosis. In contrast, patients with SA or HP, manifesting inflammatory or granulomatous diseases, usually heal without excessive scarring. In the present study, we assessed the fibroblast–macrophage interactions in ILD, in an attempt to understand the differential outcome of the diseases. We assessed the proliferative capacity of Fb and the secretion of cytokines/inflammatory products by AM and Fb, the latter being the target cells in these diseases.

We showed (Table 2) that Fb recovered from SA and HP, display significantly lower rates of background proliferation, compared with Fb recovered from normal tissue specimens even after serial passages for up to 2 months. The existence of Fb populations from fibrotic lungs which retain enhanced proliferative potential in culture, have already been reported22,23 together with reports on decreased24 or increased25 cytokine production by fibroblasts in chronic GVHD (graft versus host dis-
FIG. 3. Detection of IL-1α and IL-1β mRNA transcripts in Fb. mRNA was isolated as described in Patients and Methods and reverse transcribed in a RT reaction. cDNA fragments were amplified in a PCR reaction. (a,b) IL-1α and IL-1β transcripts of Fb in SA; (c,d) IL-1α and β transcripts of Fb in CO. Each experiment is compared with positive transcription of β-actin.

FIG. 4. Effect of exogenous IL-β on the proliferation of Fb. (a) SA Fb (basic proliferation of Fb = 6600 ± 545 cpm). (b) HP Fb (basic proliferation of Fb = 5429 ± 345 cpm). (c) CO Fb (basic proliferation of Fb = 54173 ± 1327 cpm).
Table 6. Effect of indomethacin and anti-IL-1β antibodies on IL-1β induced suppression on Fb proliferation

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>SA</th>
<th>HP</th>
<th>DIF</th>
<th>CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (1.5 ng/ml)</td>
<td>3561 ± 257</td>
<td>3404 ± 8</td>
<td>12500 ± 500</td>
<td>47908 ± 1866</td>
<td></td>
</tr>
<tr>
<td>+ Ind (1 μg/ml)</td>
<td>7516 ± 354</td>
<td>5062 ± 92</td>
<td>8306 ± 914</td>
<td>48977 ± 9849</td>
<td></td>
</tr>
<tr>
<td>IL-1β (1.5 ng/ml) + anti-IL-1 (20 μg/ml)</td>
<td>5508 ± 142</td>
<td>10782 ± 1052</td>
<td>2648 ± 108</td>
<td>46689 ± 780</td>
<td></td>
</tr>
</tbody>
</table>

* cpns of [%H] thymidine incorporation of fibroblasts. Indomethacin and anti-IL-1 were added and cells were incubated for 72 h. The experiment was repeated three times with similar results.
References
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29. Huliel M, Douvdevani A, Segal S, Apte RN. Different regulatory levels involved in the generation of hematopoietic cytokines (IL-2 and IL-6) in fibroblasts stimulated with inflammatory products. Cytokine 1993; 5: 47-56.