Fibroblast-alveolar cell interactions in sarcoidosis and idiopathic pulmonary fibrosis: evidence for stimulatory and inhibitory cytokine production by alveolar cells

B. de Rochemonteix-Galve*, J-M. Dayer*, A.F. Junod**


ABSTRACT: To better understand how the activity of inflammatory cells collected by bronchoalveolar lavage (BAL) could affect the outcome of granulomatous and fibrotic pulmonary diseases, we studied secretory products and messenger ribonucleic acid (mRNA) expression for certain cytokines of BAL cells in 10 controls, 14 patients with interstitial pulmonary fibrosis (IPF) and 22 patients with sarcoidosis. We assessed the activity of 48 h conditioned media for: 1) their biological action on fibroblast proliferation and prostaglandin E, (PGE), collagenase and collagen production by fibroblasts; 2) TNF, levels by bioassay and radioimmunoassay; 3) Interleukin 1 (IL-1) α and β levels and β levels by solid phase enzyme Immunoassay (EIA); 4) tumour necrosis factor (TNF) and IL-1 Inhibitory activity. We also measured, in freshly isolated BAL cells: 1) mRNA levels for IL-1α and β and TNF, 2) cell-associated IL-1α and β by EIA. The only difference found in the assessment of the biological activity of BAL cells conditioned medium was an increase in fibroblast proliferation in sarcoidosis vs IPF patients. The IL-1α and β, and TNF, contents of conditioned media were similar in the three groups. Inhibitory activity against IL-1 and TNF, was found in a few patients. Further analysis revealed two peaks of inhibitory activity against IL-1 (20-25 kD and 35-40 kD), as well as a distinct TNF, Inhibitory activity which could be retained on a TNF,-binding affinity column. No mRNA expression for TNF, was found in freshly isolated BAL cells, whereas very variable levels of IL-1α and β mRNA levels were detected in the three groups. Because of these variable results of differences In functional state between freshly isolated and cultured BAL cells, and of the presence of inhibitory substances against IL-1 and TNF, it is unlikely that the development of fibrosis could be ascribed to a single disorder or abnormality.


With the recent development of bronchoalveolar lavage (BAL), studies on the pathogenesis of granulomatous and interstitial lung disorders have become more and more orientated towards the investigation of the nature and function of inflammatory alveolar cells. Several reports have already indicated that the cell differential bears some relationship with the type of disease and its outcome. Thus, the number of lymphocytes, neutrophils or eosinophils may, for a group of subjects, carry some predictive value, especially in the case of idiopathic pulmonary fibrosis (IPF) [1-3]. For sarcoidosis patients, an increase in the number of T-lymphocytes and in the ratio of CD4/CD8 cells has been found in the various stages of this disease [4], but the relationship between these indices and evolution towards fibrosis remains uncertain [5]. It soon became obvious that another approach was necessary to try to explain the development of fibrosis. A vast number of studies have therefore explored some of the factors involved in the control of fibroblast proliferation and collagen deposition, as well as in the state of activation of the inflammatory cells present in alveolar spaces. Based initially on the assessment of the biological activity of media conditioned by alveolar cells maintained in culture [6-9], these investigations tend now to rely on the measurement of the immunological activity of certain cytokines and of the expression of their messenger ribonucleic acid (mRNA) [10-15]. In most of these studies, however, only one aspect or a few elements were investigated at one particular time for a given disease. In the present report, we present a series of investigations in patients with sarcoidosis and IPF to assess alveolar cell function in a more comprehensive manner and to try to understand the pathogenesis of these
Table 1. — Pulmonary functions and BAL cells differential in controls, sarcoidosis and IPF patients

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>Controls (n=10)</th>
<th>Sarcoidosis (n=23)</th>
<th>IPF (n=14)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Vital capacity*</td>
<td>Total lung capacity*</td>
<td>FEV₁/FVC*</td>
</tr>
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<td></td>
<td>±SD</td>
<td>15</td>
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<td>17</td>
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<tr>
<td>Sarcoidosis</td>
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<td></td>
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<tr>
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<td>96</td>
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<td>12</td>
<td>8</td>
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</tbody>
</table>

*: % of predicted; **: median and range; FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; PaO₂: arterial oxygen tension; PMNs: polymorphonuclear cells; IPF: idiopathic pulmonary fibrosis.

Patients, material and methods

Patients and clinical analysis

Three groups of subjects were studied and their main characteristics are summarized in table 1.

1) Because, for ethical reasons, we could not use normal volunteers, our so-called "control" group (table 1) consisted of 10 patients (8 smokers) who underwent a bronchoscopy and a bronchoalveolar lavage (BAL) in a radiographically normal area for various conditions unrelated to any fibrotic or granulomatous process (n=4) or in whom a lobectomy or pneumonectomy was performed because of bronchial carcinoma (CA) (n=6). The resected specimen was lavaged as previously described [9], great care being taken to exclude the tumour from the lavaged region.

2) The "sarcoidosis" group (table 1) consisted of 22 patients (one of them had two BAL) in whom the diagnosis could be made on pathological evidence, i.e. noncaseating granulomata, or on clinical grounds, namely bilateral hilar adenopathy whether accompanied by erythema nodosum or totally asymptomatic [17]. The various stages are defined as follows: stage 1 = bilateral hilar adenopathy (BHL); stage 2 = BHL + parenchymal interstitial pattern; stage 3a = parenchymal interstitial pattern; stage 3b = parenchymal interstitial pattern + evidence of scarring or retraction. One patient was studied first when in stage 2 and then, two years later, while in stage 3a, receiving 20 mg prednisone every 2 days. All of the other patients were free of medication at the time of their evaluation. Three were smokers.

3) The "idiopathic pulmonary fibrosis" (IPF) group (table 1) consisted of 14 patients among whom 3 were smokers. Diagnosis was based on the presence of dry crackles on auscultation, an interstitial, reticulonodular pattern on the chest radiograph, a restrictive pattern on pulmonary functions studies, and on the exclusion of any known aetiological factor such as exposure to drug, organic or inorganic material [18]. Transbronchial biopsy was performed in 4 patients. In three other
patients the diagnosis was subsequently confirmed at autopsy. Three patients of this group suffered from an associated autoimmune disease (1 dermatomyositis, 1 polymyositis and 1 unclassified collagenosis). None of these patients received corticosteroid or immunosuppressive treatment at the time of the study. The poor response to a subsequent corticosteroid treatment and the fact that with a 2–4 yr follow-up none of the investigated cases turned out to have a different disease also support the diagnosis of IPF as defined by Turner-Warwick et al. [18].

Pulmonary functions were measured according to standard procedures and the predicted values were those established by Quanjer [19].

**BAL cell preparation**

BAL was carried out during fibreoptic bronchoscopy under local anaesthesia. A volume of 200–250 mL of sterile NaCl was instilled into a segmental bronchus, usually in the right middle lobe, by the sequential injection and aspiration of 50 mL aliquots. The recovered fractions (58±10% of instilled volume, mean±SD) were pooled and cells obtained by centrifugation at 800 g for 10 min at 4°C. Cells were washed twice at 4°C in a 1:10 dilution of Balanced Salt Solution (BSS, Millipore, Bedford, MA, USA) and stored at -20°C.

The cell lysate was resuspended in 1 mL of RPMI-1640 containing 100 U·mol·1 penicillin, 100 μg·mL·1 streptomycin, 1% glutamine and 5% foetal calf serum (FCS). All reagents were from Gibco.

**Cell lysates preparation.** The BAL cells freshly isolated (referred to as Tₐ) were resuspended at 2×10⁶ cells·mL⁻¹ in PBS and disrupted by two cycles of freeze-thawing in N₂ and by two sonication on ice using 50 W for 2 min. The cell lysate was centrifuged (40,000 g, 10 min, 4°C), filtered through a 0.22 μm membrane filter (no. SLG S 025 BS, Millipore, Bedford, MA, USA) and stored at -20°C. For enzyme immunoassay (EIA) determination, the cell lysate was resuspended in 1 mL of RPMI-1640 containing 100 U·mol⁻¹ penicillin, 100 μg·mL⁻¹ streptomycin, 1% glutamine and 5% foetal calf serum (FCS). All reagents were from Gibco.

**RNA preparation.** For RNA isolation, 10x10⁶ BAL cells were immediately lysed, after two washes in PBS, in a sterile solution containing 4.5 M guanidinium thiocyanate (International Biogenics Inc., New Haven, Conn., USA), 50 mM ethylenediaminetetra-acetic acid (EDTA), 25 mM sodium citrate, 0.1 M 2-β-mercaptoethanol (Sigma, St. Louis, MO, USA) and 2% sodium N-laurylsarcosine (Sigma), and frozen at -80°C until extraction of mRNA.

**BAL cell cultures.** BAL cells were plated at 2×10⁶ cells·mL⁻¹-well⁻¹ into 24 flat-bottomed, 16 mm diameter wells (Costar 3506. Data Packaging Corp., Cambridge, MA, USA) in RPMI-1640 medium containing 5% FCS, 100 U·mol⁻¹ penicillin and streptomycin. After 48 h, cell-free supernatants, referred to as Tₐ, were harvested, centrifuged and frozen at -20°C for further analysis.

**Bioassays on fibroblasts**

**Fibroblast proliferation induced by BAL cell supernatant.** Dermal fibroblasts were obtained by proteolytic dispersion of human infant foreskin tissues [20], and were plated in flat-bottomed, 96 well culture plates (Costar) at 2×10⁶ cells in 100 μl modified Eagle’s Medium (MEM, Gibco), containing 10% heat inactivated (HI) FCS, penicillin, streptomycin, 10 mM HEPES and 0.1 mM nonessential amino acids (Gibco).

After 24 h in 5% CO₂/95% air in a humified incubator at 37°C, the fibroblast monolayer cultures were washed once with PBS. FCS concentration of BAL cells conditioned medium was then adjusted to 2% FCS with MEM. Further dilutions were done with MEM containing 2% FCS. The assay on fibroblasts was done in quadruplicate. After 48 h, subconfluent cells were pulsed for 16 h with 3H-thymidine (3H-TdR) (0.5 μCi·well⁻¹) (specific activity 2.0 Ci·mmol⁻¹; NEN, Boston, MA, USA). Cells were then removed with trypsin EDTA (Gibco), harvested on glass fibre filter papers (Skatron, Norway) and the incorporated 3H-TdR was counted. 3H-TdR incorporation into DNA of fibroblasts cultured in the presence of 2% FCS was 56±105 cpm·well⁻¹ (mean±SEM).

**PGE₂, and collagenase production by fibroblasts induced by BAL cell supernatant.** BAL cell culture supernatants diluted in Dulbecco’s MEM (DMEM) containing 10% FCS were added at various dilutions to human dermal fibroblasts [9]. After 72 h, the fibroblast culture supernatant was removed and assayed for prostaglandin E₂ (PGE₂) and collagenase activity as an index of IL-1 like-activity, previously referred to in the literature as interleukin-1-like activity (IL-1L). For enzyme immunoassay, the index of IL-1L was determined by a radioimmunoassay using an antiserum to PGE₂, kindly provided by Dr L. Levine (Brandeis University, Waltham, MA, USA). PGE₂ levels were determined by a radioimmunoassay using an antiserum to PGE₂, kindly provided by Dr L. Levine (Brandwijk University, Waltham, MA, USA). PGE₂ was measured by the lysis of collagen using as substrate 3H-acetylated reconstituted collagen fibrils [21]. One unit represents the lysis of 1 μg·min⁻¹ of labelled collagen at 37°C. Collagenase activity released by fibroblasts exposed to 2% FCS alone was 0.84±0.77 U·mol⁻¹ (mean±SEM).

For the standardization of the bioassays, the index of fibroblast proliferation was calculated from the ratio of the values obtained with proliferation induced by a 1:10 dilution of BAL cell supernatants to those obtained with MEM containing 2% FCS alone. For PGE₂, and collagenase, the stimulation index was calculated from the ratio of the values obtained with a 1:10 dilution of BAL culture supernatants to those obtained with a standard concentration (250 μg·mL⁻¹) of recombinant human IL-1β (rh IL-1β) (Biogen SA, Geneva, Switzerland) [21].
Collagen synthesis by fibroblasts induced by BAL cell supernatants. Fibroblasts were plated in flat-bottomed, 24 well culture plates (Costar) at 3×10^5 in 1 ml MEM, 10% HI FCS, penicillin and streptomycin, and 10 mM HEPES for 48 h. The confluent cells were washed twice with MEM containing 1% bovine serum albumin (BSA) (Sigma). BAL cell conditioned media were adjusted with MEM to a final 2% HI FCS and diluted to 1:10 with MEM (500 µl) containing 2% HI FCS, 50 µg·ml⁻¹ β-aminopropanitrile fumarate, 50 µg·ml⁻¹ ascorbate (Merck, Darmstadt, W. Germany); 20 µlCl of L-(5-³H) proline (sp. act. 13.6 Ci·mmol⁻¹; NEN) was then added. After 24 h, cells were trypsinized and counted. The supernatant (500 µl) was dialysed for 48 h against tap water, hydrolysed by the addition of 500 µl of 6 N HCl for 24 h at 120°C and evaporated under vacuum. The hydrolysates were resuspended in 200 µl 0.05 N Na citrate buffer and after filtration, injected into an anion sulphonic polystyrene resin column (Shimadzu Scientific Instruments, ISC-07, Japan) connected to a Waters chromatographic equipment. The ³H-proline and ³H-hydroxyproline were eluted by 0.05 N Na citrate buffer, pH 3.0, at 55°C, in fractions of 0.5 ml·min⁻¹. ³H-proline and ³H-hydroxyproline were counted and the ³H-hydroxyproline/(³H-hydroxyproline + ³H-proline) ratio (HP/H⁺HP) determined [22].

Production of tumour necrosis factor (TNFα) by BAL cells

TNF activity in BAL cell free supernatants was measured in a cytotoxicity assay using a murine TNF-susceptible cell line L929 in the presence of actinomycin D (1 µg·ml⁻¹) (Sigma) [23]. The specificity of TNFα was ascertained using a specific blocking monoclonal rabbit antibody against rh TNFα (a gift from R. Ulevich, Scripps Clinic, La Jolla, CA, USA). TNFα was also measured using an RIA kit (Medgenic I.R.E., Fleurus, Belgium). The rh TNFα was a gift from Biogen SA.

Production of IL-1α and IL-1β by BAL cells

IL-1α and IL-1β were measured in BAL cell culture supernatants by an EIA [24]. In short, a mouse monoclonal antibody against IL-1α or β (2 µg·200 µl⁻¹) was immobilized on 96 well microtitre plates (Nunc, Gilbe). After an overnight incubation at 4°C, the plates were saturated with 100 µl of a solution containing 3 g·l⁻¹ BSA, 0.1 M phosphate buffer, pH 7.4, 0.01% NaNO₃, 0.4 M NaCl, 1 mM EDTA. The plates were stored at 4°C prior to use. 100 µl aliquots of cell lysates or supernatant culture were added, washed with 0.1 M phosphate buffer, pH 7.4, containing 0.1% Tween. The second monoclonal antibody against IL-1 and coupled to acetylcholinesterase (100 µl) was then added. After an overnight incubation at 4°C, the plates were washed three times and 200 µl of Ellman’s reagent was dispensed into each well. After 30 min the absorbance at 405 nm was measured using automatic equipment (Dynatech Produkte AG, Embrach, Zürich, Switzerland).

Northern-blot analysis of BAL cells

Within 1 h after the BAL, the cell pellet was frozen (-80°C) in 4.5 M guanidium thiocyanate buffer. The human monocytoid U937 cell line was used as a control. RNA was purified by ultracentrifugation through a cushion of CsCl using a Beckman SW 60 rotor at 150,000 g for 15 h at 20°C [25]. Pellets were resuspended in 200 µl 10 mM Tris HCl, pH 7.4, containing 0.5% sodium dodecyl sulphate (SDS) and 1 mM EDTA. The suspensions were purified by phenol chloroform. After an overnight precipitation in ethanol and 0.3 M Na acetate at -20°C, the RNA contained in the pellet was dissolved in sterile water and its content determined by spectrometry at 260 nm. RNA was denatured with 6 M glyoxal, electrophoresed (5 µl·lane⁻¹) on 1.2% agarose gels, and stained for RNA integrity with ethidium bromide (0.5 µg·ml⁻¹) prior to transfer on a nylon membrane (Biodyne PALL™, Ultrafine filtration Corp., Glencore, NY, USA). After blotting, filters were baked under vacuum for 2 h at 80°C, prehybridized for 4 h at 58°C with 200 µl·cm⁻² of the hybridization mixture containing 50% (v/v) deionized formamide, 50 mM Na-piperazine estrone sulphate, pH 6.8, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 2.5 × Denhardt’s solution and 100 µg·ml⁻¹ denatured salmon sperm DNA [26], then hybridized overnight at 58°C with 50 µl·cm⁻² of fresh hybridization mixture containing the IL-1α and IL-1β RNA probes and/or TNFα cDNA probe. The hybridized membranes were washed up to a stringency of 15 mM sodium chloride and 1.5 mM sodium citrate (0.1xSSC), 0.1% SDS at 70°C. Membranes were sequentially hybridized with TNFα, IL-1α and IL-1β and dehybridized by dipping the membrane in water at 80°C until the water had reached room temperature.

The IL-1α cDNA was a 1764 base pair (bp) clone inserted in the Hind III site of pUC8. The IL-1ß cDNA was a 1200 bp clone inserted in the Pst I site of pAT 153. A 500 bp fragment of IL-1α clone, isolated between Pst I-Hind III, and a 570 bp fragment of IL-1ß clone, isolated between Hind II-Hind III, were both subcloned in pSP 65. The pSP 65 plasmids were transcribed with bacteriophage SP6 RNA polymerase using the method of Maxam et al. [27]. The human TNFα cDNA probe (a gift from Biogenet, Belgium) was a 1600 bp Eco R1 fragment of pAT 153 [28].

Slot blot

The initial slot contained 4 µg·ml⁻¹ of total RNA, the subsequent slots were obtained from sequential dilutions of 1:2 [27]. After transfer to nitrocellulose, using a slot blot apparatus (Schleicher and Schuell Minifold II Inc., Keene, NH, USA), the filters were first prehybridized for 4 h at 42°C in 500 µl·cm⁻² of a solution containing
50% formamide (Fluka AG, Buchs, Switzerland), 2× SSC, 0.1 ng·mL⁻¹ denatured salmon sperm DNA, 5× Denhardt’s solution. The prehybridized filters were then hybridized at 1.5×10⁶ dpm·mL⁻¹ for 18 h at 42°C in 50 μl·cm⁻² of the same solution.

The cDNA plasmid of TNFα probe was labelled with (³²P) dCTP (300 Ci·mM⁻¹; Amersham Corp., Arlington Heights, IL, USA) to a specific activity of 2–5×10⁸ dpm·μg⁻¹ by nick translation. After hybridization, the filters were washed twice for 30 min at room temperature in 2× SSC, 0.1% SDS and twice for 1 h at 65°C in 0.2× SSC, 0.1% SDS and finally in 0.1× SSC. Membranes were exposed to Kodak XAR film at -70°C for 48 h.

IL-1 and TNFα inhibitory activity in BAL cell culture supernatants

BAL cell supernatants were tested (1:5 dilution) for PGE₂ production by dermal fibroblasts (20,000 cells·200 μl⁻¹, well⁻¹) in the presence of rh IL-1β (125 pg·mL⁻¹) or TNFα (1 ng·mL⁻¹). After 72 h in DMEM containing 10% FCS, penicillin and streptomycin, cell free supernatants were assayed for PGE₂ by radioimmunoassay. For further characterization of the inhibitory activities, a 0.2 mg·ml⁻¹ of the 48 h supernatant of a patient in the IPF group was concentrated at 4°C by Aquacide ACA 54 column (La Jolla, CA) down to 500 μL, dialysed against column buffer (cut-off of 3.5 kD membrane, Spectrum Medical Industries Inc., Los Angeles) and applied to an Ultrogel ACA 54 column (60×0.5 cm) (LKB, Bromma, Sweden) equilibrated with a 10 mM Tris-Cl buffer, pH 7.4, containing 165 mM NaCl and 5 mM CaCl₂. To test for the presence of an IL-1 inhibitory activity, eluted fractions (1 ml·fraction⁻¹) were diluted 1:5 in DMEM, 10% FCS and assayed in the presence of 125 pg·mL⁻¹ rh IL-1β for PGE₂ production by fibroblasts as described above. Inhibitory activity was defined by a PGE₂ production value lower that elicited by rh IL-1β alone.

To test for the presence of a specific TNFα inhibitory activity, a pool of 120 μL culture supernatant obtained from 13 patients (4 in the control group, 5 in the sarcoidosis group, and 4 in the IPF group) was ten-fold concentrated by Aquacide II and 1 ml was applied to a TNFα-binding affinity column, where rh TNFα is cross-linked to activated mini-leak agarose beads and eluted with 0.2 M glycine-HCl pH 3.2 [29]. Column fractions (500 μL) were sterilized and tested at 1:5 dilution against rh TNFα (0.2 ng·mL⁻¹) in the presence of actinomycin D (1 μg·mL⁻¹) in the L929 cytotoxicity assay.

Statistical analysis

Comparison between several samples was carried out using analysis of variance. Parametric or nonparametric tests (Kruskal-Wallis) were used according to the distribution of the data. Comparison between two means was made using the unpaired t-test or the Mann-Whitney U-test. Unless otherwise specified, values are reported as mean±so.

Results

Clinical and functional characteristics and cellular analysis of BAL

The control group had virtually normal pulmonary functions. Most of them were chronic smokers and 7 out of 10 had bronchial carcinoma (table 1). The cell differential of BAL was within the normal values reported in the literature [30]. Because the majority of BAL were done in resected lobes and lungs, the absolute number of cells recovered cannot be compared to that of the other groups.

The sarcoidosis group subjects, with an average age of 35 yrs, were nearly evenly distributed amongst the three stages, 1, 2 and 3a (table 1). Their pulmonary function values were close to, or within, normal limits. The cellularity of BAL was also the greatest in stage 3a, with an average cell content of 40×10⁶ vs 25×10⁶ and 30×10⁶ in stages 1 and 2, respectively. On the other hand, the cell distribution was similar in the three stages, with an average percentage of lymphocytes of 36%.

In the IPF group, with an average age of 57 yrs, the pulmonary functions were indicative of a slight to moderate degree of restrictive lung disease. The cellularity of BAL amounted to 33×10⁶ cells. In 4 patients, the percentage of lymphocytes was higher than 15%. An increased percentage of neutrophils (≥2%) was observed in 9 patients, with one extreme value of 68%. An increased percentage of eosinophils was seen in 4 subjects.

Simulatary biological activities and immunoreactivities in BAL cell culture supernatants

In the three groups of subjects tested, the BAL cell culture supernatant exhibited similar biological properties on cultured fibroblasts (table 2). The only difference was the capacity of BAL cell culture supernatants of sarcoidosis patients to induce an increased fibroblast proliferation (1.99±0.73) when compared to the IPF group (1.26±0.41) (p<0.01). Otherwise, the ability of fibroblasts to produce PGE₂ and collagenase, in response to BAL cell supernatant, was found to be identical in the three groups tested. With respect to the assessment of collagen production, both ³H-proline incorporation into newly secreted proteins (557±1800, 3496±1832 and 4782±1879 cpm·well⁻¹ for the control, IPF and sarcoidosis groups, respectively, mean of 7–9 samples ±sa), and the percentage of proline hydroxylation (table 2) were similar. It appears, therefore, unlikely that there are significant differences in the rate of collagen synthesis between our groups.
Table 2. — Biological activities and IL-1α, IL-1β and TNF levels of 48 h conditioned media in controls, sarcoidosis and IPF patients

<table>
<thead>
<tr>
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<th>3H-Tdr</th>
<th>PGE₂</th>
<th>Collagenase</th>
<th>Collagen</th>
<th>IL-1α</th>
<th>IL-1β</th>
<th>TNF</th>
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<tr>
<td></td>
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<td>production index</td>
<td>production index</td>
<td>synthesis</td>
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<td>EIA ng·ml⁻¹</td>
<td>bioassay ng·ml⁻¹</td>
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<td>(n=9–23)</td>
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<td>±SD</td>
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<td></td>
<td>±SD</td>
<td>0.41</td>
<td>0.60</td>
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</table>

*: the results in the various groups corresponding to the three stages have been pooled (n=9); PGE₂: prostaglandin E₂; HP/HP+P: ³H-hydroxyproline +³H-proline; IL-1α: interleukin 1α; IL-1β: interleukin 1β; TNF: tumour necrosis factor; EIA: electroimmunoassay.

Within each group of patients, no special correlation could be found between the various biological activities tested and either the percentage or the absolute number of any of the cell types present in the BAL suspension.

The IL-1α and IL-1β related cell activity was also measured by EIA in several BAL cell supernatants. Biological activity on fibroblasts of these supernatants was also assessed. For both IL-1α and IL-1β, we found levels higher than 2 ng·ml⁻¹ in 3 patients with sarcoidosis and 1 patient with IPF. No clear correlation could be seen between these high levels of immunoreactivity and the equivalent biological activity. In general (27 out of 31 measurements), there was a trend for IL-1β concentrations to be lower than those of IL-1α.

Because of volume limitation of cell conditioned media, the biological activity and immunoreactivity of TNF, could be determined in a few samples only. However, TNFα levels of 48 h conditioned media, whether measured by bioassay or RIA, did not differ significantly between the three groups of subjects. In 7 out of 8 combined measurements, RIA levels were higher than bioassay levels (fig. 1).
Inhibitory biological activities in BAL cell culture supernatants

From the analysis of the parallel bioassays and immunoassays for IL-1α and IL-1β we found that some BAL cell supernatants contained high IL-1α or IL-1β immunoreactive levels that could not be correlated with any marked increase in the four biological activities tested on fibroblasts. Because of the lack of sufficient material it was not possible to do a retrospective analysis on all specimens. But, in the available specimens, we tested for the presence of possible inhibitory activities in conditioned media, defined by a decrease in the PGE₂ production when induced by IL-1 or TNFα alone. Out of 11 supernatants from different groups of patients (5 with IPF, 5 with sarcoidosis and 1 control), we found in 3 cases a 20% decrease in PGE₂ production by fibroblasts when exposed to exogenous rh IL-1β and in 2 cases a decrease in PGE₂ production when exposed to exogenous rh TNFα (fig. 2). Of special interest is the fact that all these patients belonged to the IPF group. To further characterize the nature of this IL-1 inhibitory activity, the concentrated BAL cell supernatant of an IPF patient was chromatographed on a molecular size chromatography column. Analysis of the different fractions for an inhibitory effect on PGE₂ production in the presence of a constant amount of rh IL-1β (125 pg·ml⁻¹) revealed a major inhibitory peak with an apparent molecular weight of 20–23 kD and a minor peak around 35–40 kD (fig. 3).

Fig. 2. – Stimulatory and inhibitory activities induced by IL-1 or TNF in 48 h conditioned media. Supernatant of 1 control, 5 sarcoidosis and 5 IPF patients were assayed (dilution 1:40 in DMEM, 10% FCS) in the absence or in the presence of 1 ng·ml⁻¹ rh TNFα. PGE₂ production was tested by RIA. IL: interleukin; TNF: tumour necrosis factor; IPF: idiopathic pulmonary fibrosis.

Preliminary characterization of the TNFα inhibitory activity was performed using a TNFα-binding affinity column, since previous work has shown that such an inhibitor may bind to TNFα [29]. Clear evidence for inhibitory activity can be seen in fractions 9 and 10 (fig. 4).

Levels of mRNA expression for monokine in freshly isolated BAL cells (Tₐ)

The expression of mRNA for TNFα, IL-1α and IL-1β was assessed in 4 controls, 9 sarcoidosis patients (samples 13 and 19 came from the same patient) and 6 IPF patients. The Northern blot was performed on RNA extracts of AM obtained within 1 h after the BAL procedure was completed. No mRNA expression for TNFα was detected in any of the samples tested (data not shown). IL-1α mRNA expression was seen in 1 out of 6 patients with IPF (300 densitometric units), 4 out of 9 patients with sarcoidosis (10–70 units) and 2 out of 4 controls (10 units), whereas mRNA expression for IL-1β was detected in 4 out of 6 IPF patients (10–800 units), 6 out of 9 sarcoidosis patients (10–300 units) and 3 out of 4 controls (10 units) (fig. 5). Densitometric values higher than 10 were observed only in sarcoidosis and IPF patients. On the other hand, the mRNA expression for IL-1α and β of phorbol myristate acetate (50 ng·ml⁻¹) stimulated U937 cells was always present (data not shown).

No correlation appears to exist between the detected level of biological activity or immunoreactivity of the BAL cell culture supernatants and the expression of mRNA.
The presence of TNF sub biological activity and immunoreactivity in 48 h conditioned media, in spite of the fact that mRNA for TNF sub at T sub was hardly detectable, could be explained by a culture-related induction of mRNA for TNF sub (fig. 6).

**Discussion**

Our study differs from most of the previous ones in several features. Firstly, BAL cell function was studied under conditions of nonstimulation, i.e. in the absence of added agents such as phorbol myristate acetate (PMA) or lipopolysaccharides [10, 13, 14]. Secondly, our analysis of BAL cell functions was global, since it included all the cell types recovered by BAL. It is obvious that this procedure makes the quantitative assessment and comparison of AM function in the various groups of patients more difficult; on the other hand, because of the
close interaction between the various inflammatory cells in alveolar spaces, we obtain a more representative picture of the functions of AM. The quantitation of BAL cell function remains, nevertheless, a difficult problem to solve. Tests are usually based on a standard cell plating of 1-2×10^6 cells. Because it is likely that BAL samples in most cases the same alveolar volume, the more cellular BAL specimens, as generally found in sarcoidosis and IPF patients [30], may be more active only by virtue of the increased number of cells present.

Our analysis of the modulation of fibroblast function by BAL cells conditioned medium (T_c) is also original. It represents a more global assessment of fibroblast functions in as much as it considers not only fibroblast proliferation, but also its PGE_2 production, a potent inhibitory factor of its replication rate [22, 31], as well as its collagenase production and its collagen synthesis [22], both factors being involved in collagen deposition. The results obtained, not corrected for the actual number of cells, showed no major difference in our three groups of subjects. There was only a slight but significant increase in [3H]-Tdr incorporation into DNA in fibroblasts exposed to media conditioned by sarcoidosis BAL cells as opposed to that measured in IPF patients. Whether this increase is related to the high percentage of lymphocytes in BAL remains uncertain.

Secondly, our assay, with respect to growth stimulating activity of conditioned medium, was also remarkable in the sense that our cellular substrate was cultured in a medium containing 2% FCS. This FCS concentration allowed us to record a negative as well as a positive effect on fibroblast proliferation. Other groups having used medium either without FCS or with a 10% FCS concentration could measure, respectively, only a positive or negative effect of the conditioned medium [7, 8]. This point is important since, although most investigators have concentrated on the study of stimulating agents on fibroblast growth, several inhibitory agents have been studied and characterized [32]. This is why we have tried to obtain evidence for the presence of inhibitory activity against IL-1 or IL-1-like action as well as against TNF_a [23, 29]. The occurrence of such a phenomenon could help in the understanding of the apparent discrepancies we observed in some of our results, depending on whether they were obtained by bioassay or RIA/EIA. Furthermore, the presence of variable amounts of inhibitory substances of proteic nature, rather than derived from arachidonic acid metabolites, might be a very important factor in the pathogenesis of lung fibrosis or in the spontaneous recovery from sarcoidosis. Increased fibroblast proliferation and collagen production could result from lack of inhibitors rather than from an excess of stimulatory substances. The biological activities of 48 h conditioned media are certainly consistent with this hypothesis, since no major difference emerged inspite of marked differences in clinical outcome. But, although we have found evidence for inhibitory activities in a few samples and some information on the number and molecular weight of inhibitors involved, we cannot, so far, support this assumption by showing that the development of fibrosis is necessarily associated with a decreased secretion of inhibitors.

The biological evidence for the presence of a protein molecule with an IL-1β inhibitory effect deserves a few comments. Preliminary biochemical characterization of this activity (in a patient with IPF) reveals the presence of a major inhibitory factor with an apparent molecular weight of 20-25 kD. This inhibition may be similar to that observed with the 23 kD IL-1 inhibitor (IL-1 INH), originally described in urine from patients with monocytic leukaemia [32]. This factor appears to block IL-1α and β biological activities by competitive binding at the receptor level [33]. It is specific for IL-1 and inactive against TNF [34]. Whether the BAL cell IL-1 INH also competes at the receptor level has yet to be established since many other "nonspecific" inhibitors have been reported in the literature [35]. Because of differences in the experimental procedure and in the recruitment of patients, it is also difficult to compare the inhibitors we found with those characterized by Gossert et al. [36].

Finally, we also observed evidence for an inhibitory activity directed against TNF_a. Its initial characterization has been done using a binding affinity column as previously described for the TNF_a inhibitor (33 kD) found in urine, which blocks TNF_a activity in the L929 cytotoxic assay [23]. Such a finding suggests that the BAL cell TNF_a inhibitor blocks TNF_a activity by binding to the ligand itself [29]. However, further purification is necessary to determine if this inhibitor is distinct from the one described in urine. This inhibitor also blocks PGE_2 induction by TNF_a. The dissociation observed between the RIA and the bioassay for TNF_a in some of the BAL cell culture supernatants may be due to an identical factor.

BAL cells can produce cytokines other than IL-1α, IL-1β or TNF_a, such as, for example, insulin growth factor I and II, interferon, platelet-derived growth factor, transforming growth factor β and others [11, 12, 15, 37]. We have not specifically assayed these cytokines in the present study and, thus, we cannot exclude that modulation of fibroblast proliferation might be due to the presence of cytokines other than IL-1 or TNF. Fibroblast proliferation and the other functions tested on fibroblasts may also be altered in part by synergism or antagonism with other cytokines. However, even though other cytokines may be produced and exert strong actions, the net biological effect of all the cytokines released was not quantitatively different in the three different groups of patients, at least when expressed per unit of cell number.

Since biological results obtained with the 48 h culture supernatants showed little difference in terms of BAL cell action on fibroblast functions between control, sarcoidosis and IPF patients, we assessed whether freshly isolated cells (T_c) expressed differences in the level of mRNA for monokines such as IL-1α, IL-1β and TNF_a. Furthermore, since some monokines are known to be cell-associated (mainly IL-1α) [38], it was also possible to examine the possibility of correlation with the immunoreactive level of monokines in freshly isolated...
cells, which may better reflect the in vivo activity at the site of inflammatory lesion. Our measurements of mRNA levels for IL-1α and IL-1β made in a certain number of subjects in whom the biological activity of conditioned medium was also tested, indicate that there is a marked heterogeneity among the subjects in the three groups. A few patients only showed measurable expression of mRNA for IL-1β, and to a lesser extent IL-1α. There does not seem to be any trend as to a correlation with the stage of the disease, the nature of the inflammatory process or the biological activity of the conditioned medium. However, an important point is the presence of spontaneous mRNA expression for certain monokines in some subjects, an observation which differs from the absence of expression of mRNA for IL-1 in freshly isolated blood monocytes from normal subjects [39]. In contrast to the presence of IL-1 mRNA observed in some subjects, the mRNA expression for TNFα was always negative in all the cases tested in the three groups of patients. This fact suggests that BAL cells can express mRNA for TNFα in our culture conditions after only 3 h and cognate proteins are clearly detected in culture supernatant after 48 h. This finding points to one potential weakness of the in vitro approach, namely the possibility of culture artefacts that might modify the functional state of the BAL cells. The LPS content of our FCS (<0.1 ng·ml⁻¹) was too low to account for this increase in TNFα mRNA expression. It could be that adherence to plastic, the FCS concentration or other unidentified factors, condition BAL cells to synthesize TNFα. On the other hand, mRNA expression for a given monokine, although an absolute prerequisite for the synthesis of this protein, does not necessarily mean that this protein is secreted. Many events take place before a cytokine is secreted, and analysis by Northern blot gives only limited information. It remains, however, interesting that mRNA for TNFα was always negative in these groups of patients with chronic diseases and this may be different in subjects with acute infection or inflammation. It is also possible that the steady-state level of TNFα mRNA is very low and not detectable by our techniques.

When cell lysates (T≤) were analysed for IL-1α, IL-1β and TNFα by immunoreactivity, we found mostly IL-1α, barely detectable IL-1β and no TNFα (not shown) which correlates with the absence of a detectable level of mRNA for TNFα. Dissociation between the levels of mRNA and the cell-associated immunoreactivity for IL-1α and β was seen in only a few cases. Differences in kinetics may account for these discrepancies. The observation of a rather high IL-1α to IL-1β ratio, when measured by EIA in the cell lysate (T≤) and in the conditioned media (T≥), is of interest since we have demonstrated that freshly isolated peripheral blood monocytes expressed more IL-1β than IL-1α [39]. This ratio may vary depending upon the type of stimulation [40] and the stage of maturation from monocytes to macrophages. These observations were based on studies on monocyte cell lines or on undifferentiated monocytes. The fact that our results on BAL cells are very different, once again suggests caution when, for a given disease, blood monocytes are studied instead of monocye-macrophages from the site of the inflammatory lesion.

In summary, the main lesson that can be drawn from this study and the use of a number of experimental approaches and techniques is that BAL cell function is difficult to assess and may vary considerably within one group of patients. Differences do exist between freshly collected and culture maintained cells. The use of monoclonal antibodies and of nucleotide probes may represent an important technological breakthrough, but biological assays are still needed if new substances, such as inhibitors, are to be discovered. Strong experimental evidence is presented here for the existence of new, still unreported inhibitors. The precise role of such potentially important biological compounds will await the purification and characterization of these molecules and the use of specific probes to assess their expression under various conditions.

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Interactions entre fibroblastes et cellules alvéolaires dans la sarcoidose et dans la fibrose pulmonaire idiopathique : preuve de la production de cytokines stimulantes et inhibitrices par les cellules alvéolaires. B. de Rochemonteix-Galve, J-M. Dayer, A.F. Junod.

RÉSUMÉ : Afin de mieux comprendre comment l’activité des cellules inflammatoires obtenues par lavage bronchoalvéolaire (LBA) peut affecter le devenir des maladies pulmonaires granulomateuses et fibrotiques, nous avons étudié les produits de sécrétion et l’expression de l’ARNm pour certaines cytokines dans les cellules du LBA chez 10 contrôles, 14 patients avec fibrose pulmonaire interstitielle (FPI), et 22 patients avec sarcoidose. Nous avons mesuré l’activité des milieux conditionnés pendant 48 h. pour : 1) leur activité biologique sur la prolifération des fibroblastes ainsi que la production par les fibroblastes de PGE\textsubscript{2}, collagène et collagènes; 2) les taux de TNF\textsubscript{a} par bioassay et radioimmunoassay; 3) les taux d’IL-1\textalpha et \beta par EIA; 4) l’activité inhibitrice contre le TNF et l’IL-1.

Nous avons aussi mesuré dans les cellules de LBA fraîchement isolées les taux d’ARNm pour IL-1\textalpha, \beta et TNF\textsubscript{a}, ainsi que le contenu cellulaire en IL-1\textalpha et \beta par EIA. La seule différence dans les mesures d’activité biologique des milieux conditionnés par les cellules du LBA a été une augmentation de la prolifération des fibroblastes chez les patients atteints de sarcoidose par rapport aux patients avec FPI. Le contenu en IL-1\textalpha et \beta et en TNF\textsubscript{a} des milieux conditionnés était similaire dans les trois groupes. Des activités inhibitrices distinctes, deux contre l’IL-1 et une contre le TNF\textsubscript{a}, furent détectées chez quelques patients. Une analyse ultérieure a révélé la présence de deux peps avec activité inhibitrice contre l’IL-1, correspondant à des poids moléculaires de 20 à 25 kD et 35 à 40 kD, ainsi qu’une activité inhibitrice contre le TNF\textsubscript{a}. Il n’y a pas d’expression d’ARNm pour TNF\textsubscript{a} dans les cellules du LBA fraîchement isolées alors que des taux très variables d’ARNm pour l’IL-1\textalpha et \beta ont été détectés dans les trois groupes. A cause de la variabilité des résultats et des différences observées dans l’état fonctionnel entre les cellules du LBA fraîchement isolées et celles en culture et de la présence de substances inhibitrices contre l’IL-1 et le TNF\textsubscript{a}, il paraît peu probable que le développement de la fibrose puisse être attribué à une anomalie isolée. 