The Relationship Between Alveolar Macrophage TNF, IL-1, and PGE_2 Release, Alveolitis, and Disease Severity in Sarcoidosis*

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A mononuclear cell alveolitis, comprised in part of activated macrophages, is thought to precede granuloma formation and fibrosis in pulmonary sarcoidosis. Tumor necrosis factor-α (TNF), interleukin 1-β (IL-1), and prostaglandin E_2 (PGE_2) are potent mediators released by activated alveolar macrophages. To determine if alveolar macrophage TNF, IL-1, and PGE_2 release was associated with clinically progressive pulmonary sarcoidosis, we obtained alveolar macrophages from bronchoalveolar lavage of 68 patients with biopsy specimen-confirmed sarcoidosis, cultured the macrophages in the presence and absence of lipopolysaccharide (10 mg/L) for 24 h, and measured TNF (enzyme-linked immunosorbent assay), IL-1 (enzyme-linked immunosorbent assay), and PGE_2 (radioimmunoassay) release. Alveolar macrophages from most patients with sarcoidosis spontaneously released TNF, IL-1, and PGE_2. The amounts of these mediators released (either spontaneously or following lipopolysaccharide stimulation) did not positively correlate with the numbers of any of the cells in bronchoalveolar lavage fluid, the clinical status of disease (stable vs deterioration), steroid usage, or cigarette smoking. The relative release of each of the individual mediators, however, was highly correlated with the release of the other mediators. The studies suggest that these markers of alveolar macrophage activation from a single bronchoalveolar lavage are poor indicators of clinically progressive disease. *(Chest 1993; 103:832-38)*

ELISA = enzyme-linked immunosorbent assay; FBS = fetal bovine serum; IL-1 = interleukin 1; LPS = lipopolysaccharide; NHLBI = National Heart, Lung, and Blood Institute; PGE_2 = prostaglandin E_2; RIA = radioimmunoassay; SCOR = Specialized Center of Research; TLC = total lung capacity; TNF = tumor necrosis factor

Pulmonary sarcoidosis is a disorder of unknown etiology characterized, pathologically, by the presence of granulomas in the lung.[1,2] Temporally, granulomas appear to be preceded by an interstitial pneumonitis (alveolitis) in the lung.[3] In addition, fibrotic areas of lung appear to develop in association with the granulomas.[1-4] Irreversible functional deterioration of the lung in these patients appears to be due to progressive destruction of the underlying lung by these pathologic processes. The factors that regulate the progression of this disease are unknown.[4]

Activated mononuclear phagocytes are an important cellular constituent of both the sarcoid granuloma and the alveolitis.[2] Activated mononuclear phagocytes can release a variety of potent inflammatory mediators, such as interleukin 1-β (IL-1), tumor necrosis factor-α (TNF), and prostaglandin E_2 (PGE_2) that regulate granuloma formation and the fibrotic process in the lung.[5-16] In animal models of granuloma formation, both IL-1 and TNF increase, whereas PGE_2 decreases granuloma size.[17-23] This functional duality suggests that the macrophage, potentially, can upregulate the inflammatory response (by releasing IL-1 and TNF) or turn off the inflammatory response (by releasing PGE_2).

The purpose of the present study was to determine if alveolar macrophages from patients with sarcoidosis preferentially release TNF, IL-1, and/or PGE_2 and to determine if the release of these mediators was related to the clinical status of the disease. Our results indicate that the release of alveolar macrophage TNF, IL-1, or PGE_2 was not associated with clinically deteriorating disease in patients with sarcoidosis. Furthermore, we observed that the release of all three mediators occurred in a parallel fashion from cultured cells.

Methods

Patient Population

All the patients had a diagnosis of sarcoidosis using the following standard criteria[1,2]: (1) a biopsy specimen demonstrating noncaseating epithelioid granulomas and coexistent morphologic features compatible with sarcoidosis; (2) no history of microbiologic evidence of mycobacterial, fungal, or parasitic infection; (3) no history of exposure to inorganic or organic materials known to cause granulomatous lung disease; and (4) consistent clinical, radiographic, and physiologic characteristics. The patients were part of an ongoing

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National Heart, Lung, and Blood Institute (NHLBI)-sponsored Specialized Center of Research (SCOR) program and met the requirements of the Institutional Review Board at the University of Iowa College of Medicine.

Patient Evaluation

The patients were closely followed up in the Clinical Research Center at the University of Iowa by a group of four pulmonologists. At each evaluation, the patients were assessed clinically and by pulmonary function testing. The patients were separated into three groups on the basis of clinical status and prednisone usage during the three months preceding bronchoscopy. The three groups included the following: (1) stable group (patients were clinically stable in the absence of prednisone therapy); (2) deterioration group (patients had evidence of clinical deterioration in the absence of prednisone therapy); and (3) prednisone group (patients were clinically stable in the presence of prednisone therapy).

Deterioration was defined, prospectively, as a significant decrease in two of the pulmonary functions listed below or a significant decrease in one pulmonary function accompanied by an increase in the patient's dyspnea level. Stability was defined as either (1) no change or an improvement in the patient's dyspnea level accompanied by no change in objective pulmonary functions or (2) a decrease in the dyspnea level without a significant change in any pulmonary function. Improvement was defined either (1) as no change in the dyspnea classification accompanied by a significant improvement in two or more objective pulmonary functional criteria or (2) a significant improvement in one objective pulmonary function parameter accompanied by a comparable improvement in the patient's dyspnea level.

The dyspnea level was assigned according to the recommendations of the American Medical Association and the American Thoracic Society[24,25]: (1) class 1 dyspnea, when it occurs, is consistent with the circumstances of the activity; (2) class 2 dyspnea does not occur at rest and seldom occurs during the performance of usual activities of daily living; the individual(s) can keep pace with persons of the same age and body build on the level without breathlessness, but not on hills or stairs; (3) class 3 dyspnea does not occur at rest, but does occur during the usual activities of daily living; the patients can walk a mile at their own pace without dyspnea, although they cannot keep pace on the level with others of the same age and body build; (4) class 4 dyspnea occurs during such activities as climbing one flight of stairs or walking 100 yards on the level; and (5) class 5 dyspnea is present on slight exertion, such as dressing, talking, and at rest.

A statistically significant change in pulmonary function for our pulmonary function laboratory was defined as follows[26,27]: (1) total lung capacity (TLC) ± 10 percent of baseline value; (2) forced vital capacity (FVC) ± 15 percent of baseline value; (3) forced expiratory volume in 1 s (FEV_1) ± 15 percent of baseline value; (4) PaO_2 ± 15 mm Hg if baseline value >80 mm Hg; 10 mm Hg if baseline value is 55 to 80; and 5 mm Hg if baseline value <55 mm Hg; and (5) single breath carbon monoxide diffusing capacity (Dco) ± 10 percent of baseline value.

Pulmonary Function Testing

Pulmonary function tests consisted of spirometry and single breath Dco (Medical Graphics 1070 system, Medical Graphics, St. Paul, Minn) and lung volumes were measured by body plethysmography (Medical Graphics 1085 system). The measurements were performed according to the American Thoracic Society's specifications.[28,29] Predicted normal values were those of Morris et al[30] for spirometry, Goldman and Becklake[31] for lung volumes, and Van Ganse et al[32] for the Dco.

Isolation of Bronchoalveolar Cells

Bronchoalveolar cells were obtained by bronchoalveolar lavage (BAL) at the time of entry into the study, as previously described.[33] The subjects were premedicated with morphine and atropine sulphate. The upper airways were anesthetized with 4 percent lidocaine and the fiberoptic bronchoscope was inserted, transorally, into the tracheobronchial tree and wedged into a subsegmental bronchus of the lingula or the right middle lobe. The BAL consisted of six separate 20-ml aliquots of sterile saline solution infused and retrieved by low-pressure suction. The first 20-ml lavage from each subsegment was discarded. The lavage fluid was filtered through two layers of gauze and centrifuged at 250 g for 5 min. The cell pellet was washed twice in Roswell Park Memorial Institute tissue culture medium (RPMI 1640) containing 5 percent endotoxin free fetal bovine serum (FBS, Hyclone Laboratories, Logan, Utah), 0.3 mg/ml of glutamine, and 100 mg/L of gentamicin. Cell counts and differential cell counts were determined with a hemocytometer and Wright-Giemsa-stained cytocentrifuge preparations, respectively.

Cell Culture

The BAL cells were cultured at a density of 1×10^6 cells per milliliter of RPMI-1640 supplemented with 0.3 mg/ml of glutamine, 100 mg/L of gentamicin, and 5 percent FBS in 12-well flat plate culture dishes (Costar, Cambridge, Mass) in an atmosphere of 95 percent humidified air and 5 percent CO_2 at 37°C in the presence or absence of lipopolysaccharide (LPS) (*Escherichia coli* 026:B6; Sigma, St. Louis, Mo; 10 mg/L). Twenty-four hours after stimulation, the supernatants were harvested and frozen at −70°C for later use.

Tumor Necrosis Factor-α Determination

The amounts of TNF in the harvested supernatants were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Endogen, Boston, Mass). The ELISA is specific for TNF and does not cross react with IL-1, IL-2, IL-6, or other cytokines.

Interleukin 1-β Determination

The amounts of IL-1-β were measured in the harvested supernatants using a commercially available ELISA kit (Cistron, Pine Brook, NJ). The ELISA is specific for IL-1-β and does not cross react with IL-1-α, TNF, IL-2, or interferon-γ.

PGE_2 Determination

The amounts of PGE_2 in the supernatants were determined using a commercially available radioimmunoassay (RIA) kit (Advanced Magnetics, Cambridge, Mass). The RIA is specific for PGE with only 1.3 percent cross reactivity with PGF_2 and <1 percent cross reactivity with leukotrienes and thromboxane B_2. Although the assay detects both PGE_2 and PGE_1, we have previously shown that all PGE released by human alveolar macrophages is PGE_2.[34] Thus, all results are reported as PGE_2.

Statistical Analysis

Concentrations of mediator release were corrected for alveolar macrophage number. Since our results were not normally distributed, nonparametric statistics were used to evaluate all of our comparisons. The Mann-Whitney *U* test and Kruskal Wallis one-way analysis of variance were used to evaluate the relationship between mediator release and categoric variables. Spearman's correlation coefficient was used to evaluate the relationship between mediator release and continuous variables.[15]

Results

The study population consisted of 68 patients, of which 37 were female and 31 were male, with the diagnosis of sarcoidosis. On initial evaluation, 34 (50 percent) of the patients entered into the study were

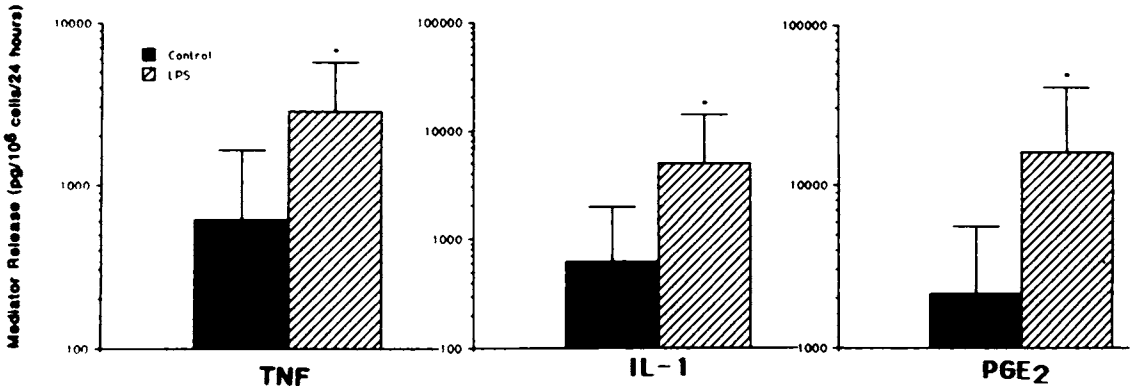

FIGURE 1. Alveolar macrophage TNF, IL-1, and PGE_2 release in patients with sarcoidosis. Alveolar macrophages obtained from patients with sarcoidosis by BAL were cultured for 24 h in the presence or absence of LPS. Supernatant mediator release was measured by ELISA (TNF and IL-1) or RIA (PGE_2). In the absence of LPS stimulation, alveolar macrophages spontaneously released TNF, IL-1, and PGE_2. Following LPS stimulation for 24 h, the release of these mediators was increased significantly. Results are expressed as mean ± SD; n = 68; asterisk = $p<0.05$.

stable during the preceding three months by physiologic and clinical criteria (see "Methods") and were not receiving prednisone therapy. Seven patients not previously receiving prednisone therapy had evidence of clinical deterioration in the preceding three months that necessitated the beginning of prednisone therapy following BAL and clinical assessment. The remaining 27 patients entered into the study were receiving prednisone therapy at the beginning of the study and were clinically stable during the preceding three months. Patients with evidence for clinical deterioration of their pulmonary sarcoidosis were not significantly different than the patients with stable pulmonary disease on the basis of age, sex, baseline pulmonary function studies, or smoking status (Table 1).

To determine if alveolar macrophages from patients with sarcoidosis were activated, we measured TNF, IL-1, and PGE_2 released by alveolar macrophages cultured for 24 h both in the presence and absence of LPS. Alveolar macrophages from patients with sarcoidosis spontaneously released TNF, IL-1, and PGE_2 (Fig 1). Following stimulation with LPS, the release of these mediators was increased several fold. This suggested that the alveolar macrophages from patients with sarcoidosis were activated at baseline and had the capacity to increase mediator release with further stimulation.

To evaluate the relationship between *in vitro* alveolar macrophage mediator release and the presence of *in vivo* alveolitis, we compared the BAL concentration of inflammatory cells with cultured alveolar macrophage TNF, IL-1, and PGE_2 release both in the presence and absence of LPS for 24 h. The release of these mediators by alveolar macrophages *in vitro*, either in the presence or absence of LPS stimulation,

Table 1—*Demographics and Clinical Characteristics

	All	Stable	Deteriorate	Stable With Prednisone
No.	68	34	7	27
Age, yr	46.7 ± 11.9	45.9 ± 10.9	41.3 ± 16.2	49.3 ± 12.4
Female, No. (%)	37 (54)	16 (47)	3 (43)	18 (67)
Smoking, No. (%)				
Never	48 (70)	20 (59)	5 (71)	23 (85)
Former	16 (24)	11 (32)	2 (29)	3 (11)
Current	4 (6)	3 (9)	0	1 (4)
Dyspnea level, No. (%)				
1	22 (32)	11 (32)	1 (14)	10 (37)
2	14 (21)	7 (21)	1 (14)	6 (22)
3	8 (12)	3 (9)	1 (14)	4 (15)
4	18 (26)	10 (29)	2 (29)	6 (23)
5	6 (9)	3 (9)	2 (29)	1 (4)
FEV_1, % pred	84.9 ± 20.6	85.2 ± 26.3	84.0 ± 17.9	87.9 ± 21.3
FVC, % pred	86.2 ± 18.0	90.2 ± 18.8	82.3 ± 13.1	85.9 ± 17.5
DCO, % pred	89.4 ± 20.6	96.6 ± 21.3	90.3 ± 19.7	85.6 ± 16.6
PaO_2, mm Hg	83.1 ± 10.4	83.9 ± 10.7	81.0 ± 8.8	82.7 ± 11.3

*Values are expressed as the mean ± the SD for continuous variables and the frequency (percent) for categoric variables.

Table 2—*The Relationship Between Alveolar Macrophage Mediator Release and Bronchoalveolar Lavage Cellularity**

	Bronchoalveolar Lavage Cellularity, Cells × 10^4/ml			
	Macrophages	Lymphocytes	Neutrophils	Eosinophils
TNF, control	−0.15	−0.28†	−0.11	−0.01
TNF, stimulated	−0.05	−0.31†	−0.13	−0.31†
IL-1, control	0.05	−0.07	−0.10	0.07
IL-1, stimulated	0.16	−0.04	−0.14	−0.14
PGE_2, control	−0.06	−0.22	−0.10	0.16
PGE_2, stimulated	0.09	−0.05	−0.12	−0.03

*Values represent Spearman's correlation coefficients.
†$p<0.05$.

was not positively related to the concentration of macrophages, lymphocytes, neutrophils, or eosinophils in the recovered BAL fluid (Table 2). In fact, the release of TNF from alveolar macrophages was inversely related to the lymphocyte and eosinophil concentration of the BAL fluid. Likewise, alveolar macrophage TNF (LPS stimulated), IL-1, and PGE_2 release was not consistently related to the smoking status of the patient nor baseline pulmonary function (data not shown). Finally, we were unable to detect a difference in the release of either IL-1, TNF, or PGE_2 from alveolar macrophages in the presence or absence of current prednisone therapy (Fig 2).

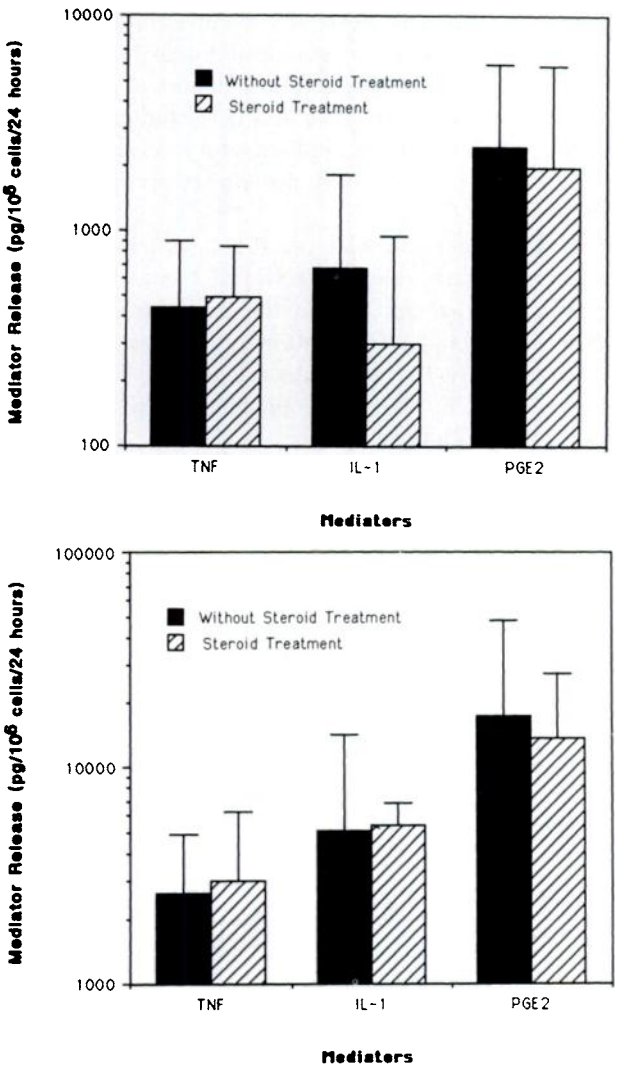

FIGURE 2. Alveolar macrophage TNF, IL-1, and PGE_2 release was similar in patients with sarcoidosis treated with or without glucocorticoids. Alveolar macrophages were obtained from patients who were treated with or without glucocorticoids for three months prior to BAL TNF (ELISA), IL-1 (ELISA), and PGE_2 (RIA) release was measured in the supernatants of alveolar macrophages cultured for 24 h in the absence *(top)* or presence *(bottom)* of LPS. There was no detectable difference in the release of the mediators between patients treated with or without glucocorticoids. Results are expressed as mean ± SD.

To determine if the alveolar macrophage activation in sarcoidosis resulted in a nonselective release of TNF, IL-1, and PGE_2 in a parallel fashion, we compared the release of each of these mediators with their counterparts. In the absence of stimulation for 24 h, the alveolar macrophages from patients with sarcoidosis released TNF, IL-1, and PGE_2 in a parallel fashion (Table 3). Likewise, following LPS stimulation the relative release of these mediators from alveolar macrophages rose in parallel (Table 3).

We next wanted to assess whether the patients with sarcoidosis whose conditions deteriorated while not receiving prednisone therapy had greater evidence of alveolar macrophage activation, measured by the ability of cultured alveolar macrophages to release TNF,

Table 3—*Relationship Between TNF, IL-1, and PGE_2 Release From Control and LPS Stimulated Alveolar Macrophages**

	Control			LPS		
	TNF	IL-1	PGE_2	TNF	IL-1	PGE_2
Control						
TNF		0.64†	0.63†	0.34†	0.20	0.05
IL-1			0.70†	0.11	0.41†	0.21
PGE_2				0.04	0.19	0.27†
LPS						
TNF					0.61†	0.43†
IL-1						0.62†
PGE_2						

*Values represent Spearman's correlation coefficients between mediator concentrations released per unstimulated (control) and stimulated (LPS) alveolar macrophage.
†$p<0.05$.

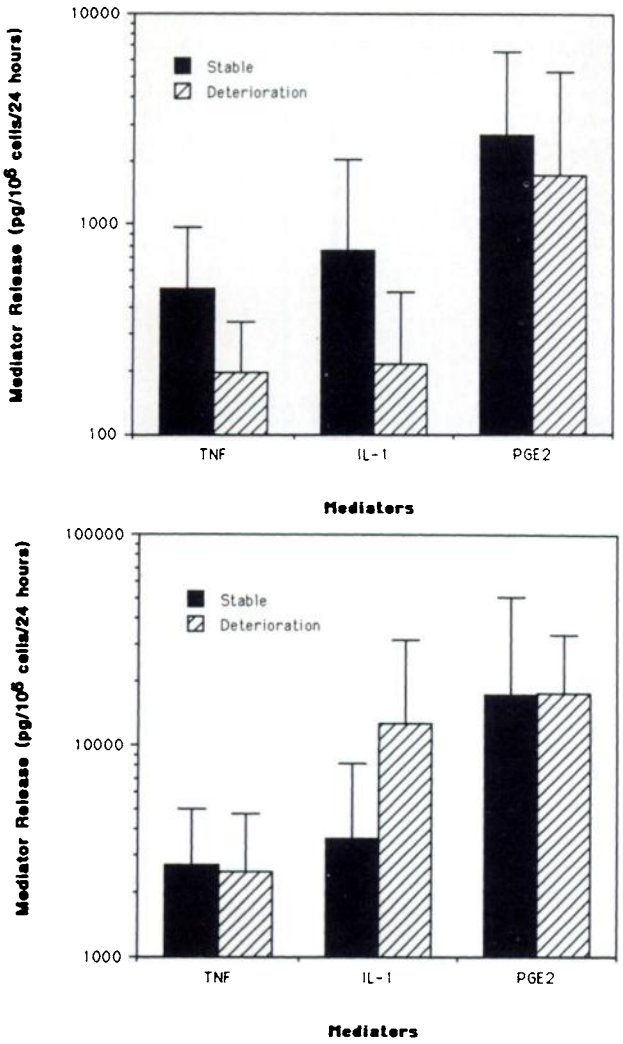

FIGURE 3. Alveolar macrophage TNF, IL-1, and PGE_2 release was not different between patients with clinically stable sarcoidosis and patients with clinical deterioration. Alveolar macrophages were obtained from patients with clinically stable or unstable sarcoidosis by BAL. TNF (ELISA), IL-1 (ELISA), and PGE_2 (RIA) release was measured in the supernatants from alveolar macrophages cultured in the absence *(top)* or presence *(bottom)* of LPS for 24 h. There was no detectable difference in the release of TNF, IL-1, or PGE_2 in patients who had clinically stable vs deteriorating pulmonary sarcoidosis. Results are expressed as mean ± SD.

IL-1, and/or PGE_2, compared with patients with stable disease not receiving prednisone. There was no significant difference in the release of either TNF, IL-1, or PGE_2 (in the presence or absence of LPS stimulation for 24 h) from alveolar macrophages obtained from either the stable or unstable patients with sarcoidosis not receiving prednisone therapy (Fig 3). This suggests that the release of TNF, IL-1, and/or PGE_2 from alveolar macrophages was not associated with prior functional deterioration in patients with sarcoidosis not receiving steroid therapy.

DISCUSSION

These studies were designed to evaluate the relationship between *in vitro* alveolar macrophage TNF, IL-1, and PGE_2 release and *in vivo* clinical status of patients with sarcoidosis. Similar to previous studies, we observed that alveolar macrophages from patients with sarcoidosis *in vitro* spontaneously released TNF, IL-1, and PGE_2.[7,9,12-14] Furthermore, we observed that the release of each of the individual mediators paralleled the release of the other mediators *in vitro* and this release of mediators appeared to be independent of cigarette exposure or steroid usage. We were unable to correlate the *in vitro* release of these mediators to the *in vivo* markers of either alveolitis or clinical deterioration.

Activated mononuclear phagocytes are major cellular elements of the lung in sarcoidosis and have the potential to regulate the cellular inflammatory response.[35] In this respect, alveolar macrophages from patients with "active" sarcoidosis might be expected to be actively releasing greater amounts of proinflammatory mediators, *eg*, TNF and IL-1, and/or releasing lesser amounts of anti-inflammatory mediators, *eg*, PGE_2. Our findings did not directly support this hypothesis.

In this study, alveolar macrophages from patients with sarcoidosis released TNF, IL-1, and PGE_2 in a parallel fashion and not in the preferential manner described above. This pattern of mediator release contrasts with the sequential release of TNF, IL-1, and then PGE_2 from macrophages in experimentally induced granulomatous disease.[17,18] Our studies, of course, do not exclude this type of sequential release of these mediators in sarcoidosis. In the experimental models, it is possible to define the points in time when the disease is initiated and to sequentially follow peaks of activity for each of these mediators. Even in these models, there is some release of all of these mediators at later points in time. The major finding of this study is that it is not possible to study early events in granuloma formation in patients with sarcoidosis utilizing BAL, in the same manner, because few patients are seen until granuloma formation is well developed in the lung. Moreover, our culture techniques prevented us from identifying which specific cell produced or modulated the release of these cytokines.

Secondly, we were also unable to find a relationship between *in vitro* alveolar macrophage mediator release and steroid usage, other *in vivo* markers of alveolitis, or clinical status. This could be due to a number of factors, most notable of which is the possibility that macrophages might be altered by culture conditions and that their *in vitro* activity might not reflect their *in vivo* activity. For example, it is well known that simple cellular adherence can alter alveolar macrophage activity and its ability to release mediators, including prostaglandins.[36] For studies related to pathogenesis of the disease or as a marker of disease activity, this observation suggests that it might be best

to study these cells immediately after they are isolated from the lung, rather than after they have been altered in culture.

Glucocorticoids and cigarette smoking alter TNF, IL-1, and PGE_2 release *in vitro* and *in vivo*.[37-41] However, in our study, we did not observe a difference in mediator release from patients in relation to their steroid or cigarette usage. A simple explanation for the latter may be that the individuals who were receiving glucocorticoids or who were smoking had alveolar macrophages that at baseline released greater amounts of mediators. Following the institution of therapy, these individuals then released similar amounts of TNF, IL-1, and PGE_2 as other nonsteroid-treated or nonsmoking patients. In this respect, some authors have believed that cigarette exposure may have a protective effect in patients with sarcoidosis.[42]

It is becoming increasingly clear, with the broader application of high-resolution computed tomographic (CT) scanning in these patients, that sarcoidosis is not a diffuse, but rather a patchy parenchymal disease.[43] With this in mind, it is not surprising that alveolar macrophages obtained by random sampling of bronchoalveolar structures may not be representative of the activity of the whole lung (reviewed by Gilbert and Hunninghake).[44] This may also explain why these random samples do not reliably predict clinical deterioration. A more relevant study might be to study the activity of cells from specific areas of the lung that are defined by CT scanning and to correlate this activity with clinical status.

In summary, we were able to confirm findings from other studies that have demonstrated activation of alveolar macrophages from patients with sarcoidosis. We were unable, however, to correlate the ability of these cells to release mediators with the clinical status of disease. Further work focusing on cellular activity at compartmentalized sites of disease may provide more insight into the factors that regulate the progression of pulmonary sarcoidosis.

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