

## Pulmonary sarcoidosis: patterns of cytokine release *in vitro*

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*Pulmonary sarcoidosis: patterns of cytokine release in vitro.* C. Prior, R.A. Knight, M. Herold, G. Ott, M.A. Spiteri. ©ERS Journals Ltd 1996.

**ABSTRACT:** This study was designed to investigate the ability of bronchoalveolar and blood mononuclear cells to produce inflammatory mediators *in vitro* in pulmonary sarcoidosis. Seventeen patients with pulmonary sarcoidosis (stage I n=8; stage II/III n=9) and 10 normal controls were investigated. Bronchoalveolar and peripheral blood mononuclear cells were cultured in serum-free medium, without stimulant, for 24 h, and the supernatants analysed for concentrations of interleukin (IL)-1 $\beta$  (IL-1 $\beta$ ), IL-2, IL-6, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- $\gamma$  (IFN- $\gamma$ ) and neopterin.

Bronchoalveolar lavage cells (BALC) of sarcoid patients released significantly higher amounts of TNF- $\alpha$ , IL-6, IFN- $\gamma$  and neopterin in comparison to normal controls. When smokers were excluded, there was also an increased release of IL-1 $\beta$  and GM-CSF. In the sarcoid group, the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF showed highly significant correlations between each other, but not with IL-2, IFN- $\gamma$  or neopterin. Sarcoid patients whose BALC released more TNF- $\alpha$  or GM-CSF had higher percentage counts of alveolar macrophages but fewer lavage lymphocytes. In sarcoid patients, peripheral blood mononuclear cells (PBMNC) also released higher amounts of IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and GM-CSF but less neopterin than normal controls. Patients whose PBMNC produced more IL-1 $\beta$ , IL-6 and GM-CSF had higher absolute and relative lavage neutrophil counts. No relationships were observed between cytokine release and radiographic or physiological markers of disease severity.

We conclude from this study that sarcoid inflammation is associated with an increased and concerted release of monocyte/macrophage-derived cytokines not only in the lung but also in the peripheral blood. We speculate that the lymphokines, IFN- $\gamma$  and IL-2, are not the primary triggers.

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Although the triggering agent of sarcoidosis is still being sought, there has been considerable progress in understanding the phenomena of local immune activation and granuloma formation [1]. In the lungs, which are most commonly involved, the inflammatory process includes not only the formation of noncaseating granulomata but also the activation of a variety of immune and mesenchymal cells. The alveolar macrophage has been shown to play a pivotal role in orchestrating inflammatory cell accumulation, granuloma formation and fibrogenesis [1, 2]. This activation process is largely effected by the release of proinflammatory mediators, such as interleukin (IL)-1 (IL-1) [3–5], tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [5–7], IL-6 [7, 8], and granulocyte-macrophage colony-stimulating factor (GM-CSF) [9].

The trigger for alveolar macrophage activation in sarcoidosis is unknown. We have speculated [10, 11] that lymphokines, such as interferon- $\gamma$  (IFN- $\gamma$ ) could be primarily involved. Indeed, lung lymphocytes from sarcoid patients produce excessive amounts of IFN- $\gamma$  [12, 13] and also IL-2 [14]. We hypothesized that the enhanced cytokine release by alveolar macrophages might be due

to stimulation *via* IFN- $\gamma$  or IL-2. Similar mechanisms could also play a role in the peripheral circulation. Up to the present time, the interrelationships between the production of interferon- $\gamma$  and monocyte/macrophage-derived mediators in sarcoidosis have never been investigated.

This study was, therefore, designed to determine, simultaneously, the *in vitro* production both of monocyte/macrophage- and lymphocyte-derived cytokines by lung and blood mononuclear cells of patients with pulmonary sarcoidosis. Since it has been recognized that smoking exerts a profound effect on pulmonary [15] and also peripheral [16] leucocytes, special attention has been paid to smoking habits when data were analysed.

### Methods

#### *Patients and control subjects*

Seventeen patients with pulmonary sarcoidosis (12 males and 5 females; 2 smokers and 15 nonsmokers;

mean age 33 yrs, range 22–51 yrs; radiographic stage I n=8 and stage II/III n=9) were studied. Nine patients were seen at Innsbruck University Hospital, Austria, and another eight at the Department of Respiratory Medicine, North Staffordshire Hospital, UK. The diagnosis was established on compatible clinical and radiographic findings, histological evidence of noncaseating granulomata on one or more organ biopsies, and the exclusion of other granulomatous lung diseases. No distinctions were made between "acute" and "chronic" or "active" and "inactive" disease, since the true duration of the disease is unknown in most cases, and there is no uniformly accepted definition of disease activity. All patients were referred to either hospital for initial assessment, and bronchoalveolar lavage (BAL) was performed as part of their routine clinical work-up. None of the patients was on corticosteroid or other immunosuppressive treatment at the time of the study or had had such treatment within the previous 6 months. Apart from one patient with concomitant anterior uveitis, none had clinical evidence of extrathoracic disease.

Ten healthy normal subjects (7 males and 3 females; 4 smokers and 6 nonsmokers; mean age 23 yrs, range 20–72 yrs) were also investigated.

Detailed lung function tests, including body plethysmography and transfer factor measurement, were performed using standard equipment. For the English patients, the reference values were those described by COTES [17]; for the Austrian patients, Austrian reference values as published by the Austrian Society for Pulmonary Diseases and Tuberculosis [18] were used.

The study was approved by the Ethics Committees of the North Staffordshire Hospital, UK, and of the Faculty of Medicine, University of Innsbruck, Austria. A common protocol was established to ensure that uniform techniques were applied in both centres throughout the study.

#### *Bronchoalveolar lavage (BAL)*

Bronchoalveolar lavage was performed during fiberoptic bronchoscopy under local anaesthesia. A 6 mm fiberoptic bronchoscope (Olympus model BF-1T20D) was wedged into a segmental bronchus of the right middle lobe, which was then lavaged with successive 20 mL aliquots of sterile buffered 0.9% saline up to a total of 180 mL. The lavage fluid was aspirated using gentle suction after each aliquot, collected into a sterilized siliconized glass bottle and kept at 4°C.

#### *Sample processing*

The fluid retrieved was centrifuged at 300×g at 4°C for 10 min, the cell pellet was washed twice in RPMI 1640 medium and the cells were counted in a modified Neubauer haemocytometer. The viability as assessed by cellular exclusion of trypan blue always exceeded 90%. The cell suspension was then adjusted to a concentration of  $1 \times 10^6$  cells·mL<sup>-1</sup> using RPMI 1640 supplemented

with 100 µg·mL<sup>-1</sup> streptomycin and 100 IU·mL<sup>-1</sup> penicillin (but no serum). This cell population will be referred to as bronchoalveolar lavage cells (BALC).

#### *Cytospin preparations*

Cytopreparations were made on a Shandon Cytospin II using 100 µL aliquots of the above cell suspension. They were stained for morphology in Diff Quik (Dade Diagnostics, UK) differential white cell stain. Three hundred cells were counted on each slide.

#### *Preparation of peripheral blood mononuclear cells (PBMNC)*

In a subgroup of nine patients (stage I n=7, stage II/III n=2; 2 smokers and 7 nonsmokers) and in all controls, 20 mL of blood were retrieved by venepuncture at the same time as lavage. The blood was collected in a heparinized syringe, then diluted 1:1 in phosphate-buffered saline and layered over Lymphoprep separation medium (Nycomed Pharma AS, Oslo, Norway). After spinning the tubes for 20 min at 400×g and 20°C, the mononuclear cell fraction was aspirated from the interface. The cells were then processed in the same way as the BALC.

#### *Cell cultures for cytokine release*

Separate cultures of unstimulated unfractionated BALC and PBMNC ( $1 \times 10^6$  cells·well<sup>-1</sup>) were cultured in triplicate in 35 mm wells in a volume of 2 mL of serum-free, endotoxin-free RPMI 1640 medium in a CO<sub>2</sub>-incubator at 37°C for 24 h. Pilot experiments performed to check the time kinetics of cytokine release showed this to be the optimal incubation time.

After incubation, the contents of each well were harvested and centrifuged; the cell-free supernatants were stored at -70°C until cytokine analysis. Cell viability after the 24 h incubation always exceeded 90%.

#### *Biochemical analysis of culture supernatants*

The supernatants were analysed for cytokine concentrations using the following commercially available kits: enzyme amplified sensitivity immunoassay (EASIA), Medgenix, Fleurus, Belgium: IL-1β, TNF-α, IL-6, GM-CSF, IFN-γ; radioimmunoassay (RIA), Medgenix: IL-2; RIA; Henning, Berlin, Germany: neopterin. The assays were carried out as recommended by the manufacturers. The reported intra-assay variations for all of these assay systems ranged 2.2–6.0%, the interassay variations are indicated as 2.8–10.7%. The lower limits of detection are as follows: IL-1β 2 pg·mL<sup>-1</sup>; TNF-α 5 pg·mL<sup>-1</sup>; IL-6 3 pg·mL<sup>-1</sup>; GM-CSF 3 pg·mL<sup>-1</sup>; IL-2 0.2 U·mL<sup>-1</sup>; IFN-γ 0.03 U·mL<sup>-1</sup>; neopterin 3 nmol·L<sup>-1</sup>. Analyses of culture medium (as used in the 24 h culture experiments) were consistently negative for all mediators.

Table 1. – Bronchoalveolar lavage cell counts of patients and controls

	Total BALC 10 <sup>3</sup> ·mL	Differential count		
		AM %	Lymph %	Neut %
Sarcoids	220±82	71±18	27±18	1.2±1.7
Controls	140±84 *	94±3 **	4±3 **	1.2±1.9

Values are presented as mean±sd. AM: alveolar macrophage; Lymph: lymphocyte; Neut: neutrophil; BALC: bronchoalveolar lavage cells. \*: p<0.05; \*\*: p<0.001, compared to sarcoid patients.

Serum levels of angiotensin-converting enzyme (ACE) were determined by a commercially available colorimetric assay (Bühlmann Laboratories AG, Basel, Switzerland).

#### Statistical analysis

Since most of the data were J-shaped a nonparametric test (Mann-Whitney U-test) was used for comparison between groups. Correlations between two sets of data were calculated using Spearman's rank correlation coefficient ( $r_s$ ).

## Results

#### BAL cell counts

Patients with sarcoidosis had significantly higher total cell counts per millilitre of recovered BAL fluid and higher percentage counts of lymphocytes than controls; accordingly, their percentage counts of alveolar macrophages were lower. The data are summarized in table 1. Fluid recovery was not different between sarcoid patients (median 64%) and controls (median 78%).

#### Cytokine generation by BALC

Concentrations of TNF- $\alpha$ , IL-6, IFN- $\gamma$  and neopterin released by unfractionated, unstimulated BALC were significantly higher (p<0.05) in patients than in controls (fig. 1), whilst levels of IL-1 $\beta$  and GM-CSF were not different. When considering nonsmoking individuals only (represented in figure 1 by circles), the amounts of IL-1 $\beta$  and GM-CSF were also significantly higher in the sarcoid group than in the controls (p<0.05), as were those of TNF- $\alpha$  (p<0.01) and IL-6 (p<0.01). However, the levels of IFN- $\gamma$  and neopterin were not different.

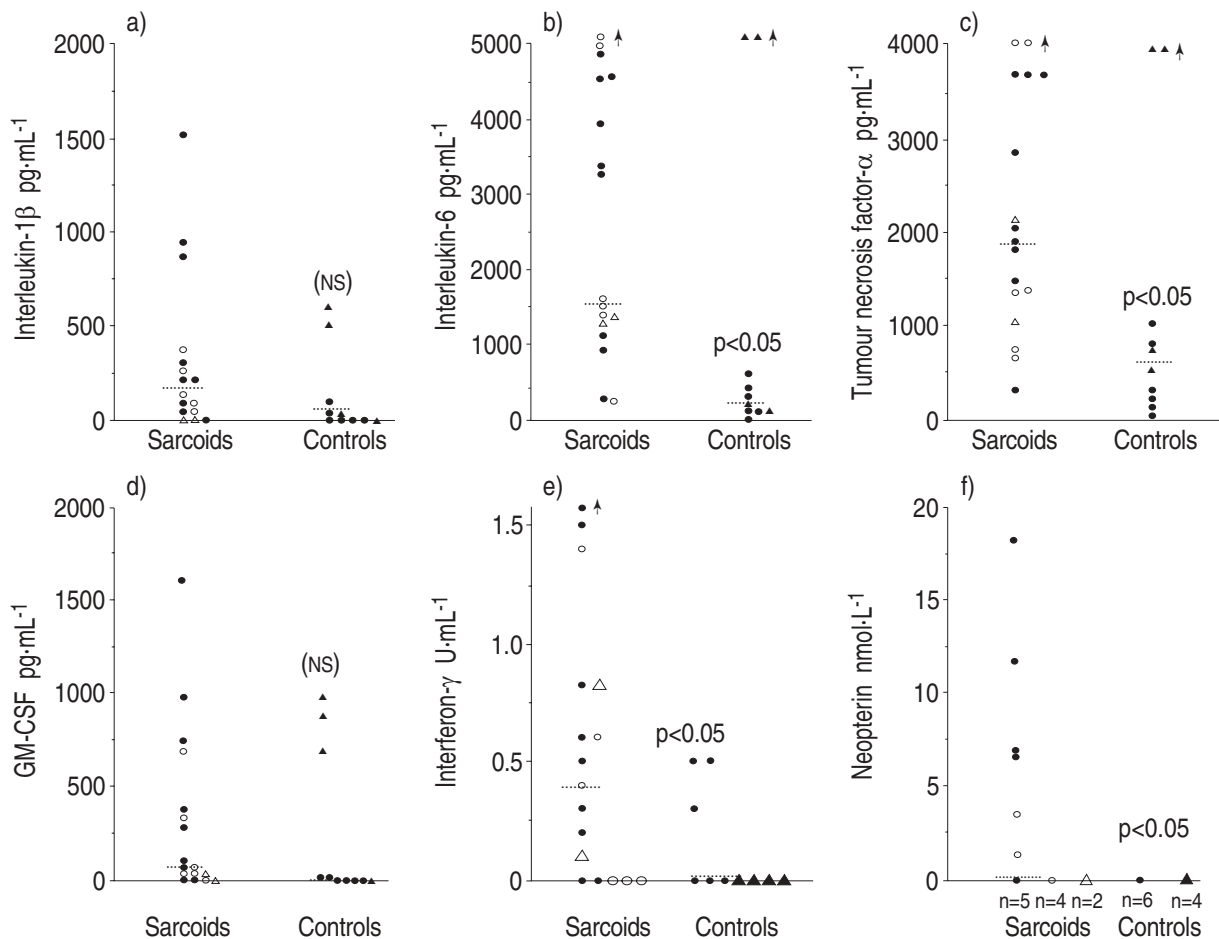


Fig. 1. – Concentrations of: a) IL-1 $\beta$ ; b) IL-6; c) TNF- $\alpha$ ; d) GM-CSF; e) IFN- $\gamma$ ; and f) neopterin in 24 h culture supernatants of unstimulated, unfractionated bronchoalveolar lavage cells from sarcoid patients and controls. Circles denote nonsmokers, triangles denote smokers. In the sarcoid group, open symbols denote stage I, filled symbols denote stage II/III. Dotted bars denote median values. IL: interleukin; TNF- $\alpha$ : tumour necrosis factor- $\alpha$ ; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN- $\gamma$ : interferon- $\gamma$ . NS: nonsignificant.

Table 2. – Correlations between cytokines released by unstimulated sarcoid bronchoalveolar cells (Spearman rank correlation coefficient with p values below)

	IL-1 $\beta$	IL-6	TNF- $\alpha$	GM-CSF	IFN- $\gamma$
IL-6	0.86 <0.001				
TNF- $\alpha$	0.74 <0.01	0.82 <0.001			
GM-CSF	0.73 <0.01	0.77 <0.01	0.80 <0.001		
IFN- $\gamma$	(0.48) (<0.1)	NS	(0.48) (<0.1)	NS	
Neopterin	NS	NS	NS	NS	0.56 <0.05

p-values in parentheses denote trends. IL: interleukin; TNF- $\alpha$ : tumour necrosis factor- $\alpha$ ; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN- $\gamma$ : interferon- $\gamma$ ; NS: non-significant.

In the sarcoid group, there were significant correlations between IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF but no correlations with IFN- $\gamma$ , neopterin (table 2) or IL-2 (data not shown). Levels of neopterin were correlated to those of IFN- $\gamma$  (table 2).

The levels of IL-2 released by BALC were low both in the patients (median 0.5 U·mL<sup>-1</sup>, range 0–1.2 U·mL<sup>-1</sup>) and in the controls (median 0.75 U·mL<sup>-1</sup>, range 0.2–1.1 U·mL<sup>-1</sup>).

#### Cytokine generation by PBMNC

Unstimulated sarcoid PBMNC produced more IL-1 $\beta$ , TNF- $\alpha$ , IL-6 and GM-CSF but less neopterin than PBMNC from controls (table 3). These differences were similar when smokers were excluded from the analysis (data not shown). Only low IL-2 levels (0–1.1 U·mL<sup>-1</sup>) were found in patients and controls (data not shown).

As in the BALC supernatants, there were significant correlations between the levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF released by sarcoid PBMNC (table 4). No

Table 3. – Concentrations of cytokines in 24 h culture supernatants of unstimulated PBMNC from sarcoid patients and controls

	Sarcoids	Controls	p-value
IL-1 $\beta$ pg·mL <sup>-1</sup>	489 (40–1357)	44 (21–432)	<0.01
IL-6 pg·mL <sup>-1</sup>	5640 (454–14500)	441 (24–8300)	<0.01
TNF- $\alpha$ pg·mL <sup>-1</sup>	968 (97–5020)	125 (0–620)	<0.05
GM-CSF pg·mL <sup>-1</sup>	70 (14–215)	12 (0–73)	<0.05
IFN- $\gamma$ U·mL <sup>-1</sup>	0 (0–0.2)	0.1 (0–0.6)	NS
Neopterin nmol·L <sup>-1</sup>	0 (0–0.3)	0.45 (0–2.4)	<0.05

Values are presented as mean, and range in parenthesis. PBMNC: peripheral blood mononuclear cells. For further abbreviations see legend to table 2.

Table 4. – Correlations between cytokines released by unstimulated sarcoid peripheral blood mononuclear cells (Spearman rank correlation coefficient with p values below)

	IL-1 $\beta$	IL-6	TNF- $\alpha$
IL-6	0.78 <0.05		
TNF- $\alpha$	0.83 <0.05	0.67 (<0.1)	
GM-CSF	0.86 <0.05	0.76 <0.05	0.83 <0.05

p-values in parentheses denote trends.

correlations with either IFN- $\gamma$ , IL-2 or neopterin were observed (data not shown).

#### Correlations of cytokine levels with BAL cellularity and serum ACE in sarcoidosis

**BALC supernatants.** In sarcoid lavage samples, the concentrations of TNF- $\alpha$  and GM-CSF showed a positive correlation with percentage counts of alveolar macrophages ( $r_s=0.57$  and  $r_s=0.50$ , respectively), and a negative correlation with percentage ( $r_s=-0.57$  and  $r_s=-0.50$ , respectively) and absolute counts ( $r_s=-0.62$  and  $r_s=-0.62$ , respectively) of BAL lymphocytes ( $p<0.05$  for all correlations). There were no correlations between BALC cytokine generation and lavage neutrophil counts.

**PBMNC supernatants.** There were significant positive correlations between the concentrations of IL-1 $\beta$  and GM-CSF and the relative ( $r_s=0.72$  and  $r_s=0.77$ , respectively) and absolute ( $r_s=0.88$  and  $r_s=0.76$ , respectively) neutrophil counts in BAL. IL-6 levels correlated with absolute neutrophil counts ( $r_s=0.72$ ;  $p<0.05$  for all correlations). No relationships with alveolar macrophage or lavage lymphocyte counts were recorded.

The only cytokine showing a significant correlation with serum ACE was IL-1 $\beta$  released from sarcoid PBMNC ( $r_s=0.73$ ;  $p<0.05$ ).

#### Relationships of cytokine levels with clinical findings

No differences in cytokine release by BALC between stage I and II/III cases could be detected. PBMNC of stage II/III patients released more IL-1 $\beta$  ( $p<0.05$ ). There were no correlations between cytokine release from either BALC or PBMNC with lung function measurements (forced vital capacity (FVC), forced expiratory volume in one second (FEV<sub>1</sub>), total lung capacity (TLC), transfer factor of the lungs for carbon monoxide (TLCO), carbon monoxide transfer coefficient ( $K_{CO}$ )) at the time of BAL.

#### Discussion

The aim of this study was to determine interrelationships and "patterns" of *in vitro* cytokine release by BALC



and PBMNC in pulmonary sarcoidosis. The results show that, in comparison to normal controls, unstimulated BALC and PBMNC from nonsmoking sarcoid patients produce increased amounts of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF. These four cytokines are released "in concert", whilst there is no relationship between the release of these mediators and the production of the lymphokine IFN- $\gamma$ .

Previous studies on "spontaneous" cytokine release from BALC in pulmonary sarcoidosis have yielded contradictory results. Although the release of IL-1 by alveolar macrophages has been reported to be increased [3, 4], there appear to be no differences in IL-1 messenger ribonucleic acid (mRNA) expression between sarcoid patients and normal subjects [19–21]. Likewise, the production of TNF- $\alpha$  protein and mRNA by sarcoid BALC has been reported to be increased [7, 21], but SPATAFORA *et al.* [22] failed to detect increased baseline TNF- $\alpha$  release. Similar discrepancies exist between studies on cytokine production by sarcoid PBMNC. Some authors have found a decreased production of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 by sarcoid PBMNC and have interpreted this as a consequence of the postulated "anergic state" [22–25]. Others have detected an enhanced IL-6 generation by sarcoid PBMNC [9, 26], which would be in line with the view that these cells are activated [27].

In some of the studies that failed to detect enhanced cytokine production by sarcoid BALC, bioassays were used for supernatant analysis [19, 22], allowing specific inhibitors to interfere with cytokine activity [21, 24]. Another bias could result from differences in patient populations regarding disease severity and smoking behaviour. In our study, the statistics on BALC cytokine generation were strongly influenced by two smoking controls, who showed an exceedingly high release of IL-1 $\beta$  and GM-CSF. One might also argue that examination of cytokine immunoreactivity inside cells might circumvent culture artifacts. Studies in our laboratory have shown that sarcoid alveolar macrophages contain increased amounts of immunoreactive IL-1 $\beta$  [28], yet it is uncertain whether this correlates better with *in vivo* release than "spontaneous" *in vitro* production.

Whilst it has been recognized that IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are concomitantly released by sarcoid BALC [25, 29], we have shown here, for the first time, that GM-CSF release both from lung and peripheral mononuclear cells is also associated with these other three cytokines. Pilot studies using purified cell populations have confirmed that all four mediators are macrophage products (data not shown).

Our findings of enhanced monokine release by sarcoid PBMNC are in line with recent studies employing enzyme-linked immunosorbent assay (ELISA) kits rather than bioassays [9, 16]. Our study is the first to show a relationship between the production of IL-1 $\beta$ , IL-6 and GM-CSF by sarcoid PBMNC and BAL neutrophil counts; and we speculate that these mediators are involved in the migration and sequestration of neutrophils to the alveolar space: GM-CSF upregulates neutrophil adhesion molecule expression [30], and induces neutrophil sequestration to the lung [31], whilst IL-1 $\beta$  increases the

expression of adhesion molecules by endothelial cells [32].

The failure to detect IL-2 data might be due to the fact that unseparated cell populations rather than purified lymphocytes [14] were used, so that macrophages might have exerted a suppressive effect [11]. In order to clarify whether methodological reasons could account for our findings, BALC were also cultured in the presence of phytohaemagglutinin (PHA) (data not shown). The levels of IL-2 in supernatants of PHA-stimulated cells were significantly higher ( $p < 0.01$ ) than in those of unstimulated cells, demonstrating that culture, storage, and assay conditions would have allowed the detection of elevated IL-2 levels had they been present.

The excellent correlations between the amounts of monokines point not only to a common trigger but also to mutual stimulation in the generation of these mediators. IL-1 $\beta$  can induce the expression of IL-6, TNF- $\alpha$  and GM-CSF [33], while GM-CSF can, in turn, stimulate IL-1 $\beta$  and TNF- $\alpha$  secretion [34]. This might explain that the same pattern of exaggerated monokine release is also observed in other inflammatory respiratory disorders, such as asthma [35].

There is ample evidence that IL-1 $\beta$ , TNF- $\alpha$  and IL-6 are key cytokines in the formation of granulomas [36, 37], and IL-1 $\beta$  and TNF- $\alpha$  can each induce proliferation of fibroblasts [38]. The role of GM-CSF in the sarcoid lung is now being explored but it is most likely to be involved in the accumulation and activation of mononuclear phagocytes [39].

The trigger which sets off the concerted release of monokines in sarcoidosis is unknown. According to the classical conception of cell-mediated immunity, one would expect mononuclear phagocytes to be stimulated primarily by IFN- $\gamma$ . This is released by activated T-lymphocytes, which are known to be present in high numbers at sites of disease activity [1]. However, we could show no correlation of monokine release with the production of IFN- $\gamma$ . Neopterin, which is a mononuclear phagocyte product specifically released upon stimulation with IFN- $\gamma$  [40], was used as an "internal control" and proved to be correlated with IFN- $\gamma$  production in our assay system, whilst the other four cytokines were not. It seems, therefore, that an exogenous factor, maybe the unknown "sarcoid agent", might directly induce the excessive monokine production by lung and blood mononuclear cells. Indeed, it has already been speculated by another group that a Kveim-like granulomagenic factor isolated from sarcoid BAL fluid might directly trigger the enhanced release of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 [41].

In agreement with earlier work [29], no correlations between *in vitro* cytokine release by BALC or PBMNC and the clinical indices of disease severity measured here were found. However, other clinical variables that were not assessed in this study, such as disease progression or response to therapy, may well be related to cytokine production [10, 42]. Given the high variability in the clinical course of sarcoidosis, the number of patients investigated here is small, and no firm conclusions can be drawn in this respect. Furthermore, there

is no uniformly accepted marker of disease activity [43] to which one could relate the mediator release data.

In conclusion, we have shown a high and concerted release of cytokines with presumed monocyte/macrophage origin in pulmonary sarcoidosis. The production of these mediators - IL-1 $\beta$ , IL-6, TNF- $\alpha$  and GM-CSF - is likely to be mutually stimulatory. The primary drive for the release of these proinflammatory factors is unknown but appears not to be related to IFN- $\gamma$  or IL-2. In addition, the study highlights the potential role of peripheral blood mononuclear cells in contributing to inflammatory cell migration to sites of organ involvement.

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