Sarcoidosis is a chronic inflammatory disease in which there is a systemic granulomatous process often involving the lungs and thoracic lymph nodes [1]. Although the pathogenesis of sarcoidosis is far from clear, there is increasing evidence associating sarcoidosis with abnormalities in immune function. In particular, it has been suggested that activated T-cells and macrophages play a crucial role in orchestrating the inflammatory process. Despite evidence for the involvement of these cells in pulmonary sarcoidosis, the mechanisms by which they contribute to the pathogenesis of the disorder and its activity are largely unknown.

Resident macrophages and T-lymphocytes may regulate immune responses via the production of proinflammatory cytokines. A number of studies have recently demonstrated the presence of cytokine messenger ribonucleic acid (mRNA) and immunoreactivity in bronchoalveolar lavage (BAL) from patients with active pulmonary sarcoidosis [2–10]. BAL cells of individuals with granulomatous sarcoid lesions show a marked increase in the numbers of CD4+ T-lymphocytes [11, 12] and elevated levels of T-helper type 1 (Th-1) cytokines, such as interleukin (IL)-2, IL-12 and interferon-γ (IFN-γ) [3, 5, 7, 13]. Alveolar macrophages from these patients also spontaneously release more IL-1, IL-12, tumour necrosis factor-α (TNF-α), granulocyte/macrophage colony-stimulating factor (GM-CSF), IFN-γ and transforming growth factor-β (TGF-β) than those from normal individuals [2, 4–10], suggesting local inflammatory events occurring within the lungs. In addition, an enhanced secretion of IL-6, a cytokine which acts synergistically with IL-1 to promote T-cell proliferation, has recently been described in active pulmonary sarcoidosis [14].

The clinical presentation of pulmonary sarcoidosis can be divided into active or nonactive stages [11, 15]. Whilst there is evidence to suggest that cytokines play a role in the aetiology of pulmonary sarcoidosis, the profile of inflammatory mediators that contribute towards the expression of disease activity remains to be elucidated. Most of the previous studies have investigated cytokines in active pulmonary sarcoidosis [2–10], and little information is available concerning the cytokine profile of nonactive disease. Thus, it is not clear whether cytokine expression is an indication of sarcoidosis in general, or is linked to disease activity. Moreover, so far there has been no comprehensive study of the expression of a large range of cytokines in the same particular population.

We tested the hypothesis that pulmonary sarcoidosis is associated with predominant expression of Th1 cytokines, and that the presence of these cytokines in BAL
cells may be related to disease activity, as defined by conventional clinical and radiological data. Therefore, we examined a large range of cytokines (Th1 and Th-2 helper type-2 (Th2)) in BAL cells of patients with active pulmonary sarcoidosis, and compared their mRNA expression to patients with nonactive pulmonary sarcoidosis and normal controls. To detect cytokine mRNA in these cells, we have used in situ hybridization with complementary ribonucleic acid (cRNA) probes coding for IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-γ.

Materials and methods

Study subjects

Twenty three patients with pulmonary sarcoidosis (13 males and 10 females), with a mean age of 41 yrs (range 24–58 yrs), were investigated. All patients were newly diagnosed with pulmonary sarcoidosis, except for four individuals in whom the disease onset had occurred more than 2 yrs previously. In two of these patients, there was recent evidence of an acute episode (erythema nodosum, cough and dyspnoea). None of the patients were smokers, had any evidence of an atomic nature as defined by the absence of clinical history and negative skin-prick tests to common allergens, or had taken corticosteroids within the past year. The diagnosis of pulmonary sarcoidosis was based on previously described criteria, including lung or mediastinal lymph node biopsy exhibiting evidence of noncaseating epithelioid cell granulomas.

All patients included in this study had a confirmed histological diagnosis of sarcoidosis. By chest radiographic staging, eight of the patients had stage I, 13 had stage II and one had stage III disease. One patient with nonactive pulmonary sarcoidosis had a normal chest radiograph at the time of evaluation, which had been abnormal (stage I) at the time of discovery. According to conventional clinical and radiological data [11], the disease was classified either as active (15 patients) or nonactive (eight patients). The criteria used to determine disease activity were: 1) recently developed or increasing cough or dyspnoea; and/or 2) systemic symptoms, such as cutaneous lesions, weakness, fever, arthralgia; and and/or 3) increasing opacities on chest radiography.

Patients with active disease also exhibited an increased T-lymphocytosis in BAL (mean range) for active disease 47 (30–71)% (n=15); and for nonactive disease, 13 (8–18)% (n=8); p<0.05) and an elevated level of serum angiotensin converting enzyme (mean±SEM for active sarcoidosis 68.1±6.4 U·mL-1; and for nonactive sarcoidosis 42.8±2.8 U·mL-1; p<0.05 (normal values <55 U·mL-1)). There was no difference in lymphocyte numbers in the BAL fluid between individuals with nonactive sarcoidosis and normal controls (mean (range) for controls 12 (3–26)% (n=9); p>0.05)). In addition, neither group of sarcoidosis patients exhibited airflow limitation or restrictive lung disease (mean±SEM forced expiratory volume in one second (FEV1) for active sarcoidosis 101±4% of predicted value (n=15), and for nonactive sarcoidosis 97±5% pred (n=8); forced vital capacity (FVC) for active sarcoidosis 104±5% pred (n=15), and for nonactive sarcoidosis 100±5% pred (n=8)). Transfer factor of the lungs for carbon monoxide (Tl,CO) for active sarcoidosis was 103±6% pred (n=15), and for nonactive sarcoidosis 94±4% pred (n=8).

As control subjects, nine individuals (five males and four females) with a mean age of 45 yrs (range 32–58 yrs), volunteered to participate in the study. None of the control subjects were smokers, had any evidence of an atomic nature or had taken corticosteroids in the year preceding the study. The patients and control subjects were recruited through the Dept of Pneumonimunoallergy, Calmette Hospital, Lille, France. The study was approved by the Calmette Hospital Ethics Committee and subjects signed a consent form.

Study design

To examine the expression of cytokine mRNA in cells recovered by BAL, fiberoptic bronchoscopy was performed under local anaesthesia on patients with active and nonactive sarcoidosis and normal controls. A 6 mm fiberoptic bronchoscope was wedged into a segmental bronchus of the right middle lobe, which was then lavaged with successive 20 mL aliquots of sterile buffered saline (0.9%) up to a total of 180 mL. The lavage fluid was aspirated using gentle suction after each aliquot, collected into sterile polypropylene tubes, and centrifuged at 300×g for 7 min at 4°C. The cell pellet was suspended in RPMI 1640 at a concentration of 1×10⁶ cells·mL⁻¹ and used to make cytopsin. The cytopsin were fixed for in situ hybridization using 4% paraformaldehyde for 30 min, before being washed in phosphate-buffered saline (PBS), dried, frozen at -80°C and shipped on dry ice to Montreal.

In situ hybridization

In situ hybridization was performed as described previously [16]. Cytopsins were hybridized with 35S-labelled antisense (cRNA) and sense (having an identical sequence to mRNA) riboprobes coding for IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40 subunit) and IFN-γ. The probes were generated from complementary deoxyribonucleic acid (cDNA) and transcribed in the presence of 35S-labelled uridine triphosphate (UTP) and the appropriate SP6 (for the sense probe) or T7 (for the antisense probe) ribonucleic acid (RNA) polymerases. Prior to the application of the probe, the cytopsins were permeabilized with proteinase K (1 µg·mL⁻¹), and then prehybridized with 50% formamide and 2× standard saline citrate (SSC). For hybridization, antisense or sense probes (10⁶ counts per minute (cpm)-section⁻¹) diluted in hybridization buffer were used. Nonspecific binding was removed by posthybridization washing under high stringency conditions in decreasing concentrations of SSC (4–0.05 × SSC). Unhybridized single-stranded RNAs were removed by subsequent treatment with ribonuclease (RNase) A (20 µg·mL⁻¹). The autoradiographs were developed in Kodak D-19 and counterstained with haematoxylin.

Quantification and data analysis

Cytopsin sections were coded and the number of positive cells for mRNA were calculated. A minimum of
1,000 BAL cells were counted, and the number of positive cells are presented as a percentage of the total cells. Statistical differences in the number of positive cells between groups was assessed using a nonparametric Kruskal-Wallis analysis of variance (ANOVA), and subsequent post hoc analyses were performed using a Mann-Whitney U-test. A p-value less than 0.05 was considered statistically significant.

## Results

Positive hybridization cytokine mRNA was demonstrated in most of the samples by specific deposition of silver grains in emulsion overlying individual cells. There were no significant hybridization signals observed when sections were treated with RNase prior to hybridization nor when sections were treated with a sense probe identical to the cytokine mRNA.

There were considerable differences between the various groups studied in the percentage of cells positive for cytokine mRNA. The most prominently expressed cytokine mRNA in BAL cells of pulmonary sarcoidosis were IL-1β, IL-2, IL-6, IL-10, IL-12 and IFN-γ. Interestingly, considerable percentages of BAL cells from normal individuals also expressed mRNA for IL-1β, IL-10 and IL-12. In comparing disease activity with cytokine mRNA expression, individuals with active sarcoidosis demonstrated higher percentages of cells expressing cytokine mRNA for IL-2, IFN-γ, IL-10 and IL-12 than the individuals with the nonactive form of the disease (figs. 1 and 2) (p<0.003). There was no significant difference in the percentage of cells expressing cytokine mRNA for IL-1β and IL-6 between active and nonactive sarcoidosis (fig. 3) (p<0.005). There were also no significant differences in the percentage of BAL cells expressing mRNA for IL-3, IL-4 and IL-5 between active and nonactive sarcoidosis (mean±SEM value for IL-3 in active sarcoidosis 1.8±0.4% (n=15), and in nonactive sarcoidosis 1.9±0.4% (n=8) (p>0.05); for IL-4 in active sarcoidosis 0.6±0.2% (n=15), and in nonactive sarcoidosis 0.8±0.2% (n=8) (p>0.05); and for IL-5 in active sarcoidosis 1.3±0.3% (n=15), and in nonactive sarcoidosis 1.0±0.3% (n=8) (p>0.05)).

Individuals with active sarcoidosis had a greater percentage of cells expressing cytokine mRNA for IL-2, IL-10, IL-12 and IFN-γ (figs. 1 and 2) (p<0.001) and IL-1β and IL-6 (fig. 3) (p<0.002) than normal controls. There were also significantly more cells exhibiting cytokine mRNA expression for IL-2 and IFN-γ (fig. 1) (p<0.004) and IL-1β and IL-6 (fig. 3) (p<0.003) in nonactive sarcoidosis compared to normal controls. No significant difference in the percentages of BAL cells expressing mRNA for IL-10 and IL-12 (fig. 2) (p>0.05) and IL-3, IL-4 and IL-5 were detected between patients with nonactive sarcoidosis and normal controls (normal mean value±SEM: IL-3 1.4±0.4%; IL-4 0.8±0.2%; IL-5 1.1±0.2% (n=9) (p>0.05)).

To determine whether the increase in lymphocyte-derived cytokines (IL-2 and IFN-γ) in pulmonary sarcoidosis could be attributed to a selective recruitment

### Table 1

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Normal Controls</th>
<th>Active Sarcoidosis</th>
<th>p-value</th>
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<tbody>
<tr>
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<tr>
<td>IL-2</td>
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<td>IFN-γ</td>
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</tbody>
</table>

### Figure 1

Number of interleukin (IL)-2 and interferon-γ (IFN-γ) mRNA-positive cells in BAL fluid from active and nonactive sarcoidosis patients and normal controls. ●: normal controls (n=9); ▲: individuals with nonactive sarcoidosis (n=8); ■: individuals with active sarcoidosis (n=15). mRNA: messenger ribonucleic acid; BAL: bronchoalveolar lavage.

### Figure 2

Number of interleukin (IL)-10 and IL-12 mRNA-positive cells in BAL fluid from active and nonactive sarcoidosis patients and normal controls. ●: normal controls (n=9); ▲: individuals with nonactive sarcoidosis (n=8); ■: individuals with active sarcoidosis (n=15). mRNA: messenger ribonucleic acid; BAL: bronchoalveolar lavage.

### Figure 3

Distribution of interleukin (IL)-1β and IL-6 mRNA-positive cells in BAL fluid from active and nonactive sarcoidosis and in normal controls. ●: normal controls (n=9); ▲: individuals with nonactive sarcoidosis (n=8); ■: individuals with active sarcoidosis (n=15). mRNA: messenger ribonucleic acid; BAL: bronchoalveolar lavage.
Results are expressed as mean±SEM for the individual groups. *: p<0.05 compared to normal controls; †: p<0.05 compared to active sarcoidosis.

Table 1. – Cytokine mRNA-positive cells expressed as the percentage of total lymphocytes in the BAL fluid for active and nonactive sarcoidosis and normal controls

<table>
<thead>
<tr>
<th>Cytokine</th>
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<th>Nonactive sarcoidosis</th>
<th>Normal controls</th>
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<tr>
<td></td>
<td>(n=15)</td>
<td>(n=8)</td>
<td>(n=9)</td>
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<td>IL-2</td>
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<td>IL-5</td>
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Discussion

These results confirm previous reports suggesting that sarcoidosis is associated with the predominant expression of lymphocyte Th1 cytokines (IL-2 and IFN-γ), as well as cytokines derived from alveolar macrophages, in particular IL-6, IL-10 and IL-12 [3, 5–7, 13]. Furthermore, the present data show that proinflammatory cytokine production (IL-1β, IL-2, IL-6 and IFN-γ) is evident even in nonactive sarcoidosis, and that the active disease is associated with an increase in T-cell and macrophage-derived cytokine mRNA synthesis within the lungs.

Using in situ hybridization techniques, the present study examined cytokine mRNA production in BAL as an indication of ongoing cytokine synthesis within the lungs of individuals with pulmonary sarcoidosis. As such, we did not quantify protein levels or bioactivity in BAL, or from in vitro culture of BAL cells. Cytokine mRNA and protein levels do not always correlate, due to nontranscriptional regulation and intracellular storage. However, a number of reports have demonstrated cytokine immunoreactivity and/or bioactivity in pulmonary sarcoidosis, in particular for IL-1β, IL-2, IL-6, IL-12 and IFN-γ [5–7, 13, 14, 17]. Therefore, whilst we anticipate that our results extend to the production of the bioactive protein, in the interpretation of our findings, we cannot preclude factors which modulate cytokine translation and activity.

Lymphocytic alveolitis is an early initiating event in the formation of granulomatous lesions [12, 18], and it is well established that there is a compartmentalization of T-lymphocytes, especially CD4+ cells, within the lungs of individuals with pulmonary sarcoidosis [13, 18–22]. Our data indicate that there is an increase in the number of BAL cells expressing IL-2 and IFN-γ mRNA in sarcoidosis. Although we did not attempt to phenotype the cellular sources of these cytokines, T-lymphocytes have previously been shown to release IL-2 and IFN-γ in pulmonary sarcoidosis [5, 13], and they are the major cellular source of IL-2 and IFN-γ mRNA in pulmonary tuberculosis [23]. The low expression of IL-4 and IL-5 mRNA in the present study and the increase in cells positive for IL-2, IL-12 and IFN-γ mRNA support previous findings [7] in suggesting that Th1 lymphocytes comprise the majority of T cells found within the lungs. Interestingly, the number of IL-3 mRNA-positive cells was not increased in pulmonary sarcoidosis and although this cytokine has been reported to be a product of Th1 lymphocytes, it is possible that it is regulated concomitantly with other cytokines situated on chromosome 5q, namely IL-4 and IL-5.

The contribution of T-cell-derived cytokines to the pathogenesis of pulmonary sarcoidosis remains to be determined. However, it has been suggested that the release of IL-2 within the lung, in conjunction with other cytokines, such as IL-1 and IL-6, is responsible for the enhanced T-lymphocyte proliferation seen in this disease [6, 13, 14]. Moreover, the secretion of IFN-γ can direct the phenotype of uncommitted T-cells into a Th1-like profile and, via its actions on macrophages [24], contribute to granuloma formation [25]. This profile of Th1 cytokine expression is evident in other granulomatous disorders, notably pulmonary tuberculosis [23]. Indeed, although the aetiology of pulmonary sarcoidosis remains to be established, infection with mycobacterial agents similar to that found in tuberculosis (Mycobacterium tuberculosis) have attracted considerable attention [1].

Alveolar macrophage T-cell activation is hypothesized to be critical in the initiation and maintenance of pulmonary inflammation in sarcoidosis [1]. Whether as a result of the local cytokine milieu or persistent antigen presentation, the activation of alveolar macrophages is a characteristic feature of the sarcoidosis patient. As such, elevated levels of macrophage-derived cytokines, such as IL-1, IL-6, IL-12, TGF-β, GM-CSF and TNF-α [2, 4–10, 26], have previously been demonstrated in this disease. In our study, we did not identify the cell types responsible for cytokine mRNA production. In pulmonary tuberculosis, however, a significant percentage of the cells expressing IFN-γ mRNA are alveolar macrophages [23]. The present results have confirmed the increase in macrophage-derived cytokine expression in sarcoidosis and suggest they occur concomitantly with a Th1 cytokine profile.

In contrast to our findings, several studies have reported no increase either in the expression of IL-10 mRNA or IL-10 immunoreactivity in BAL cells and fluid of individuals with active pulmonary sarcoidosis [7, 8]. Apart from the technical differences in methodology [7] and detection of mRNA versus protein [7, 8], differences in the sarcoidosis patient populations may explain these apparent discrepancies. IL-10 is produced both by cells of the monocyte lineage and CD4+ uncommitted...
T-helper (Th0) and Th2 lymphocytes [27]. As opposed to the previous studies, the individuals with active sarcoidosis in the present study were newly diagnosed and had extremely elevated levels of lymphocytes in their BAL fluid (47%). Furthermore, although exhibiting similar radiographic patterns to the previous study [7], they exhibited no significant alterations in pulmonary function which may be indicative of a more chronic disease. It remains to be determined whether the presence of IL-10 as a potent inhibitor of monocyte and macrophage activation may be a prognostic indicator of spontaneous remission in sarcoidosis.

Considerable difficulty exists in the classification of active versus nonactive pulmonary sarcoidosis. Although a variety of clinical, radiological and serological tests have been proposed as markers of disease activity [15], no single marker has proved to be satisfactory. We chose to define the present patients as active or nonactive on the basis both of chest radiology and clinical status, and therefore our choice of activity criteria must be taken into consideration when interpreting these results. Our findings may also have been influenced by the experimental approach used, in which we examined cytokine gene expression within the local microenvironment. Pulmonary sarcoidosis is a disease associated with compartmentalization of the underlying histopathology. Indeed, numerous studies have shown that analysis of peripheral blood fails to give a true picture of the character and magnitude of immune processes within the lung [3, 5, 12, 13, 18–22]. This has been particularly evident when comparing the profile of cytokine expression within the peripheral blood and BAL fluid of individuals with sarcoidosis [3, 5, 13, 22]. Since cells recovered by BAL have been shown to accurately reflect the alveolaris of lung diseases, including sarcoidosis [17], we chose to examine a wide range of cytokines in the local milieu as a means of investigating the histopathology of clinical activity.

Few previous studies have attempted to differentiate active and nonactive forms of the disease with respect to the cytokine profile within the lungs [4, 9, 14, 21]. The evidence so far suggests that activation both of T-cells and macrophages mediates disease activity in sarcoidosis [1, 5–7, 11–13, 19], and that this process is associated with the production of IL-2 and increased levels of soluble serum IL-2 receptors [7, 21]. From the profile of cytokine mRNA expression observed, the present data indicate that the active disease reflects a preferential activation of tissue-resident macrophages. Indeed, numbers of IFN-γ mRNA-positive cells were significantly less in subjects with the nonactive compared to the active disease, suggesting that the increased recruitment of T-cells producing Th1 cytokines is possibly the primary stimulus in determining the levels of macrophage activation and disease activity.

To determine whether the increase in lymphocyte-derived cytokine mRNA (IL-2 and IFN-γ) could be explained by the increased number of lymphocytes in BAL fluid from patients with active sarcoidosis, we expressed numbers of lymphokine mRNA positive cells as a percentage of the total T-lymphocyte population in BAL. These results demonstrated that the increase in IFN-γ mRNA-positive cells in active and nonactive sarcoidosis could not be entirely explained by the increased numbers of T-lymphocytes within the lung. This suggests either preferential recruitment of IFN-γ mRNA-positive cells from the peripheral circulation, or the increased expression of this cytokine from other cell types, such as macrophages. Local switching to a Th1 phenotype within the lungs may also be occurring. The numbers of IL-3, and IL-5 mRNA-positive cells when expressed as a percentage of the total T-lymphocytes in BAL were markedly decreased in active sarcoidosis compared to nonactive and normal controls. Numbers of IL-4 mRNA-positive cells were additionally decreased in active compared to nonactive sarcoidosis. Since the percentage of IL-3, IL-4 and IL-5 mRNA-positive cells did not differ between active and nonactive sarcoidosis and normal controls, the reduction in Th2 cytokine mRNA-positive cells may be attributed to the recruitment of T-lymphocytes exhibiting either a Th0 or a Th1 profile of cytokine expression.

In summary, we have investigated a wide range of cytokine messenger ribonucleic acid expression in pulmonary sarcoidosis, and that this process is most prominent in the active disease. Further research in this area is needed to determine the factors promoting macrophage activation in active sarcoidosis, and those which serve to limit the production of cytokines in non-active sarcoidosis. Moreover, future studies are required to address the relationship between cytokine expression, disease activity and the progression of sarcoidosis. These results will provide a rational basis for the design of drug treatment and help elucidate the efficacy of new and existing disease entities in clinical trials.

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