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Inflammatory markers and pulmonary granuloma infiltration in sarcoidosis

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ABSTRACT

Background and objective: **Previous studies have demonstrated increases of inflammatory mediators in sarcoidosis while epidemiological studies have also demonstrated an association with increased fungi exposure. This study measured the level of β-glucan in the lungs and of inflammatory mediators in serum, and correlated both with the extent of pulmonary granuloma infiltration.**

Methods: **This is a cross-sectional study of 98 patients with sarcoidosis and 26 controls. β-glucan, a cell wall constituent of fungi, was measured in bronchoalveolar lavage. Inflammatory mediator levels were determined in serum. The extent of granuloma infiltration was estimated on the chest X-ray. Exposure to fungi at home was determined by taking air samples in bedrooms and analysing for the presence of β-N-acetylhexosaminidase.**

Results: **Significantly, higher levels of β-glucan were found in broncho-alveolar lavage in subjects with sarcoidosis as compared with controls. There were significant positive relationships between the extent of granuloma infiltration and the levels of the different inflammatory mediators, except for interleukin-10. Domestic fungal exposure was higher among subjects with sarcoidosis.**

Conclusions: **This is the first time that a specific agent, previously suspected to be related to the risk of sarcoidosis, has been detected in the lung of subjects with sarcoidosis and related to the levels of inflammatory mediators and the degree of home exposure to fungi. The results suggest that exposure to fungi should be explored when investigating patients with sarcoidosis.**

Key words: β-N-acetylhexosaminidase, β-glucan, fungi, inflammation, sarcoidosis.

SUMMARY AT A GLANCE

This study demonstrated β-glucan—a fungal cell wall agent—in the lungs of sarcoidosis patients. It also confirmed a positive relationship between β-glucan and domestic fungi exposure. Lung granuloma infiltration correlated inflammatory cytokines except IL-10, an anti-granuloma cytokine.

Abbreviations: BAL, bronchoalveolar lavage; CTO, chitotriosidase; IL, interleukin; NAHA, β-N-acetylhexosaminidase; TNFα, tumour necrosis factor alpha.

INTRODUCTION

Sarcoidosis is mostly a pulmonary disease, accompanied by an increase in inflammatory mediators, which may develop into pulmonary granuloma infiltration.[1–3] The disease may also be present in other organs of the body such as skin, eyes and kidneys. The severity of the pulmonary disease can be evaluated by chest X-rays to measure the extent of granuloma infiltration.[3,4]

There is no general agreement regarding causal agent(s) for sarcoidosis. Several studies support an association between exposure to fungi and the risk of sarcoidosis. Epidemiological studies have identified specific occupations at risk of developing sarcoidosis; several of these involve exposure to fungi.[5] Inhabitants of mould-infested buildings are at higher risk of sarcoidosis.[6] A higher exposure to fungi was found in homes of patients with sarcoidosis as compared with controls, particularly for those with recurrent disease.[7] Treatment with antifungal medication was in some instances as efficient as treatment with corticosteroids.[4,8]

The inflammatory response in sarcoidosis is characterized by an increase in several inflammatory mediators such as interleukin (IL)-10, IL-12 and tumour necrosis factor (TNF)α.[9–12] Chitotriosidase

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(CTO) is of particular interest as it is a major defence enzyme against fungi.[13] Increased levels of CTO have been reported in sarcoidosis, and CTO has been suggested as a marker of the disease severity.[3,14,15] Angiotensin-converting enzyme is also often used as a marker of disease severity.[16]

Of the several inflammatory mediators in sarcoidosis, IL-10 is of particular interest in relation to fungal exposure. It is an anti-inflammatory mediator and suppresses the formation of granulomas.[17,18] Deficiencies in the production of IL-10 could thus be a contributing factor for granuloma formation in the lungs in sarcoidosis.

If fungi were to play a role in the pathogenesis of sarcoidosis, it is not through infection. It has been suggested that the underlying mechanism is an inflammatory reaction to fungi antigens.[19] One potential antigens is β-glucan which is present in the cell wall of fungi. β-glucan has immuno-modulating effects, including the ability to induce granulomas.[20] The effects depend on the physical characteristics, solubility, dose and route of administration. In previous *in vitro* experiments, particulate β-glucan was found to be a strong inducer of cytokine secretion (IL-6, TNFα, IL-10, IL-12) from peripheral blood mononuclear cells.[21] The reaction was more pronounced among subjects with sarcoidosis. There was also a significant relationship between the domestic exposure to fungi and the spontaneous secretion of IL-6, IL-10 and IL-12 from peripheral blood mononuclear cells.

If β-glucan were to play a role in the inflammatory response and granuloma formation in sarcoidosis, one would expect that higher levels than normal would be present in the lung of patients with sarcoidosis. The aim of the present study was to investigate whether β-glucan is present in the lungs of subjects with sarcoidosis and controls, to measure the levels of different inflammatory mediators in relation to the different degrees of granuloma infiltration in sarcoidosis, and to measure domestic exposure to fungi.

METHODS

Subjects

Patients with newly diagnosed sarcoidosis according to the American Thoracic Society criteria[24] were recruited from the clinic of respiratory diseases at the University Medical Centre, Ljubljana, Slovenia from May 2009 to October 2012. The study included non-smoking subjects with pulmonary sarcoidosis ($n = 98$) and controls without symptoms of respiratory disease ($n = 26$). The demographic characteristics are given in Table 1. The study was approved by the Ethical Committee at the University Medical Centre, Ljubljana (198/05/04). Written consent was obtained.

There were no infectious organisms identified in bronchoalveolar lavage (BAL) except in two patients with *Streptococcus epidermis*, one with *Staphylococcus aureus*, and one with *Enterococcus epidermis*. One control subject had *S. epidermis*.

Table 1 Demographic characteristics of subjects with sarcoidosis and controls

	Sarcoidosis	Controls
Number	98	26
Age, mean/SEM	48.2/1.3	38.8/2.4
Females, *n*/%	46/47	17/65
BAL CD4/CD8, mean/SEM	6.4/0.5	—
Stage of disease		
0, *n*	26	—
I, *n*	4	—
II, *n*	79	—
III, *n*	15	—
Extrapulmonary, *n*	35	—

—, no data; SEM, standard error of the mean.

BAL was performed, and samples were taken for fungal, mycobacterial and other microbial analysis. BAL could only be performed on 10 of the control subjects. Ten millilitre BAL fluid was centrifuged at 1000 *g*, frozen and stored at –70°C. As controls, 20 mL of the liquid used for BAL was passed through a bronchoscope and prepared similarly to the samples. Five control samples were collected.

Chest X-ray evaluation

Chest X-rays were read by two experienced radiologists, blinded to the status of the patient. A score for the presence of granuloma in the lung parenchyma was allotted according to the following classification: 0 = normal, 1 = 25% of lung parenchyma involved, granuloma about 1–2 mm, 2 = 50% involved, granuloma about 2–4 mm, 3 = 75% involved, granuloma, about 4–6 mm, and 4 = 100% involved, granuloma throughout lung parenchyma. Repeat evaluations on two successive occasions showed only minor deviations in the score classification. Control subjects were given score 0.

Inflammatory markers

Inflammatory markers were determined in serum. Angiotensin-converting enzyme was determined using a colorimetric method and expressed as μkat/L.[25] CTO activity was determined using 22 μM 4-methylumbelliferyl-β-D-N,N′,N″-chitotriosidase (Sigma, Maribor, Slovenia) in citrate phosphate buffer (pH 5.2) and expressed as nmol/h/mL.[3,14] IL-6, IL-10, IL-12 and TNFα were measured using commercial ELISA kits (Milenia Biotec, Giessen, Germany and Thermo Scientific, Waltham, MA, USA).

Analysis of β-glucan

Samples of the BAL fluid were mixed with 20 mL of 0.15 mol/L KOH, 0.3 mol/L KCl and 0.1% polybrene and incubated at 37°C for 10 min. For the analysis of β-glucan, a commercially available method based on the reactivity of a Limulus extract was used. The BAL preparation was diluted ×5 in endotoxin-free water, kept in boiling water for 2 min and further diluted ×2

in a protein blocking buffer (Bio dispersing agent, Charles River, Charleston, SC, USA). Thereafter 25 uL was added to each of four wells in a plate pre-prepared with a Limulus reagent specific for β-glucan and read in an automatic analyser (Endosafe PTS, Charles River). The lower limit for detection is 1 pg/mL. Samples yielding a readout value of <10 pg/mL were given the uniform value of 90 to save on reagents. This analysis was performed on 8 controls and 72 subjects with sarcoidosis.

Fungal exposure at home

The exposure to fungi in the homes was determined by analysing the amount of airborne β-N-acetylhexosaminidase (NAHA) as a marker of fungal cell biomass.[26,27] Air samples (around 2000 L) were taken in the subject's bedroom using a filter and a fluorogenic enzyme substrate (4-methylumbelliferyl N-acetyl-β-D-glucosaminide, Mycometer A/S, Copenhagen, Denmark) was added to the filter. After an incubation period of around 30 min, set by room temperature, a developer was added, and the fluorescence of the liquid was read in a fluorometer (Picofluor, Turner Designs, Sunnyvale, CA, USA). The units read were divided by 10 to reduce methodological scatter and expressed as NAHA Units/m^3.

Statistical analysis

The statistical evaluations were made with SPSS v18 (SPSS, Inc., Armonk, NY, USA). Group data were reported as mean and standard error of the mean. Differences between groups were evaluated using the Mann–Whitney test and correlations using Spearman's test. A *P*-value of 0.05 was considered as the level for significance.

RESULTS

β-Glucan in BAL

The control preparations all had β-glucan values of less than 90 pg/mL. Values in the eight healthy controls ranged from 90 to 189 (mean 123, standard error of the mean 16.1) and in subjects with sarcoidosis from 90 to 1554 (mean 483, standard error of the mean 38.3). The difference between controls and sarcoidosis was significant ($P < 0.001$). There was a positive relationship between β-glucan levels and the X-ray scores ($P = 0.001$). There were significant positive relationships between β-glucan and TNFα, IL-2R and IL-6 levels ($P = 0.008$, 0.015 and 0.015 respectively).

Granuloma infiltration and inflammatory markers

Of the subjects investigated, 26 scored 0, 22: 1, 37: 2, 31: 3 and 4: 4 on X-ray reading. The relationship between the different cytokines and the X-ray scores are illustrated in Figure 1.

Chest X-ray scores and TNFα, IL-6 and IL-12 ($P = 0.001$ for all) levels were positively correlated. The values for IL-10 were not linearly related to the X-ray scores $R^2 = 0.007$. CTO and angiotensin-converting enzyme showed a significant correlation with the X-ray scores (both $P = 0.001$). The numerical range was much larger for CTO.

Fungal exposure at home

The NAHA values in the homes of controls ranged from 6 to 76 U/m^3 (mean 15.1, standard error of the mean 2.7) and for sarcoidosis from 2 to 126 U/m^3 (mean 35.4, standard error of the mean 4.1). The difference was statistically significant ($P = 0.001$). There was a positive relationship between NAHA values and TNFα, IL-12 and CTO in serum ($P = 0.038$, 0.016 and 0.008 respectively) and X-ray scores ($P = 0.001$). Higher NAHA scores correlated with higher β-glucan in BAL ($P = 0.001$) as illustrated in Figure 2. Although data are scattered, a higher level of fungal exposure at home was related to a higher amount of β-glucan in BAL.

DISCUSSION

The three main findings are: high level of β-glucan in the lung of patients with sarcoidosis, a positive relationship between β-glucan level and fungal exposure at home and a linear correlation between the granulomatous infiltration and inflammatory cytokines, except for IL-10.

The finding that β-glucan levels were higher in patients with sarcoidosis is similar to the findings in previous studies on acute eosinophilic pneumonia and hypersensitivity pneumonitis.[22,23] Our results suggest that the degree of β-glucan in the lungs reflects the fungal exposure at home. Among those with high levels of cytokines but a low level of fungal exposure at home, exposure at the workplace may play a role.

The relation between increased levels of inflammatory mediators and an increased granuloma score was not unexpected. Previous studies have reported higher levels of IL-12, TNFα and IL-6 in blood and BAL of subjects with sarcoidosis.[2,9–12] The fungal cell wall agents β-glucan and chitin induce the production of cytokines from human monocytes *in vitro* with a stronger reaction from monocytes from subjects with sarcoidosis.[21]

Apart from fungi, other microorganisms, *Propionibacterium acnes* have been suggested to be related to sarcoidosis with a potential causative role. In one study *P. acnes* antibodies were detected in 50% of sarcoid and 15% of non-sarcoid lymph node samples.[28] In another study, immune responses against both Mycobacterial and *P. acnes* were present in BAL from patients with sarcoidosis.[29] In an *in vitro* study, it was reported that *Mycobacterium tuberculosis* peptides induced more INFγ producing T-cells and activated IL-2, IL-6 and TNFα.[30] Similar findings were reported in a study on sarcoidosis patients from two clinical centres.[31] These results illustrate that the inflammatory response in this study is not specific for fungi and could be induced by other microbial agents.

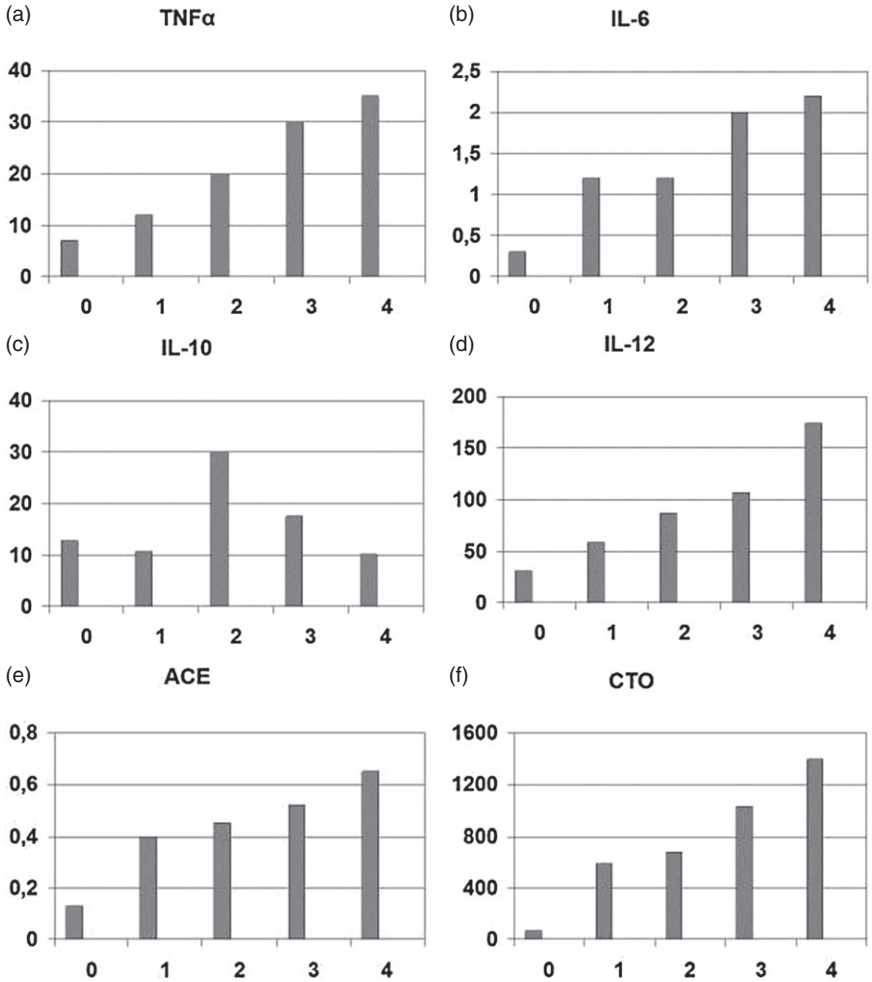

Figure 1 Granulomatous infiltration and cytokine levels.
Relationship between X-ray scores for granulomatous infiltration and inflammatory cytokine values (a) (tumour necrosis factor (TNF)α pg/mL, $P = 0.001$; (b) interleukin (IL)-6 pg/mL, $P = 0.001$; (c) IL-10 pg/mL, $P = 0.013$; (d) IL-12 pg/mL, $P = 0.001$; (e) angiotensin-converting enzyme (ACE) μkat/L, $P = 0.001$; (f) chitotriosidase (CTO) nmol/h/mL, $P = 0.001$).

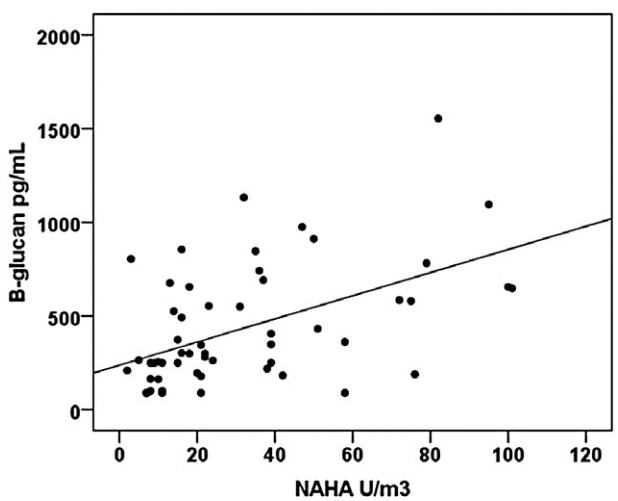

Figure 2 β-Glucan in bronchoalveolar lavage (BAL) and β-N-acetylhexosaminidase (NAHA) in homes.
Relationship between β-glucan in BAL and the amount of NAHA in the homes of subjects with sarcoidosis and controls (correlation coefficient = 0.440, $P = 0.001$).

However, none of these studies evaluated the relationship between the presence of the microbial agents in the environment and cytokine levels or the clinical severity of the disease.

The relationship in the present study between fungal exposure, clinical severity of the disease, and the inflammatory responses suggest that β-glucan might be one causative agent for sarcoidosis. Support for this hypothesis is found in a study on human immunodeficiency virus-infected subjects where β-glucan levels in serum were associated with immunosupression, inflammation and cardiopulmonary function, akin to the findings from the present study.[32]

It would have been desirable to have an equal number of controls with β-glucan levels in BAL. As BAL always involves a certain risk of complications such as bleeding, we could justify only a few control subjects. As there was a relation between clinical parameters expressing disease severity and β-glucan, the lack of controls is not a serious drawback from a methodological point of view. For practical reasons, the samples for cytokines and BAL had to be taken at

different times than the sampling of NAHA. This certainly contributed to the dispersion of values in the dose–response relationships. A further weakness of the study is the relatively small number of subjects with the two highest granuloma scores ($n = 4$). In spite of this, the results demonstrated that the shape of the IL-10 response curve was different from that of the other mediators. IL-10 had a peak value at score 2, and the values were lower at further increases in granuloma invasion.

An inverse relationship in relation to the severity of sarcoidosis has previously been reported for cathelicidin.[33] Cathelicidin is a cationic host defence peptide that is present in macrophages, neutrophils and epithelial cells.[34] It has a broad spectrum of anti-inflammatory activities similar to IL-10.[17] Cathelicidin mRNA is depressed after TNFα exposure. If the high levels of TNFα at the higher granuloma scores as demonstrated here induce a decrease in IL-10, akin to the effect on cathelicidin, the lack of one or both of these inflammation inhibitors would induce an increased risk of granuloma formation. This finding could open a new possibility for understanding the mechanism behind the granuloma development in sarcoidosis, but further work is required to assess the validity of the hypothesis.

Inflammatory mediators have previously been used to diagnose and to assess sarcoidosis. The most widely used are angiotensin-converting enzyme and CTO. Both of these were significantly related to the granuloma scores in this study. The range of values was, however, much larger for CTO. CTO might be a more sensitive marker of the activity of sarcoidosis than angiotensin-converting enzyme. However CTO levels are increased also in other inflammatory lung diseases.[3] This limits the usefulness of these measures for diagnostic purposes. On the other hand, it has been found that CTO is useful to follow the clinical development of sarcoidosis and to evaluate the effect of treatment.[15]

The exposure to β-glucan as such is not sufficient for the development of sarcoidosis because family members of the patients, living in the same environment, do not develop sarcoidosis in spite of a similar home exposure to fungi. This supports the concept that factors related to the individual are also responsible for the development of sarcoidosis such as multiple exposures, infection simultaneously with fungal exposure or genetic predisposition.

The presence of β-glucan in BAL of many patients with sarcoidosis supports the hypothesis that fungi may play a role in the pathogenesis of the disease. Our results demonstrate a relationship between inflammatory mediators and granuloma formation. IL-10 had a different relationship with lower values at the highest degree of granuloma formation. Our results also suggest that CTO may be an appropriate marker of disease activity and may become a tool to follow the effect of treatment in sarcoidosis.

The exposure to high levels of fungi should be explored when diagnosing new cases of sarcoidosis and those with recurrent disease. Measurements of β-glucan in BAL could be used as a diagnostic tool and to follow the effect of treatment.

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