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Correlation of GM-CSF mRNA in bronchoalveolar fluid with indices of clinical activity in sarcoidosis

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Abstract

***Background*—Granulocyte-macrophage colony stimulating factor (GM-CSF) has several proinflammatory effects, some of which potentially favour granuloma formation. Its mRNA expression by the inflammatory cells recovered from lungs of patients with pulmonary sarcoidosis has been previously reported. In this study an association between GM-CSF expression and manifestations of the disease was explored.**

***Methods*—GM-CSF mRNA was detected by reverse transcription polymerase chain reaction in the cells of bronchoalveolar lavage (BAL) fluid of 20 patients with pulmonary sarcoidosis.**

***Results*—GM-CSF mRNA expression was positive in 15 of 20 patients with sarcoidosis. Fourteen of the 15 patients with positive mRNA expression had worsening or unchanged disease during the year preceding this study, on the basis of radiographic or physical findings, or both, whereas all five "negative" patients were judged to be improving. Similarly, serum levels of angiotensin converting enzyme, the proportion of lymphocytes in BAL fluid, and the CD4+/CD8+ ratio of lymphocytes in BAL fluid were significantly higher in the positive patients.**

***Conclusions*—There was an association between the presence of GM-CSF mRNA in the cells in BAL fluid and other indices of disease activity in sarcoidosis.**

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Sarcoidosis is a systemic granulomatous disorder that commonly involves the lung. Although its cause is still unknown, our knowledge of the mechanism by which the pulmonary lesion develops has increased remarkably since the introduction of bronchoalveolar lavage (BAL). Researchers have elucidated that the inflammatory cells recovered by BAL from the lung of patients with sarcoidosis spontaneously release several cytokines such as interleukin 1 (IL-1), interleukin 2 (IL-2), and interferon γ which could promote granuloma formation by some of their biological effects.[1–3] These data suggest that the interaction of cytokines secreted by the inflammatory cells infiltrating the alveoli favour granuloma formation.

In the search for cytokines that might be involved in sarcoid granuloma formation, we found that a sensitive reverse transcription polymerase chain reaction (RT-PCR) method often detected mRNA of granulocyte-macrophage colony stimulating factor (GM-CSF) in the cells in BAL fluid from patients with pulmonary sarcoidosis but not in the cells in BAL fluid from normal controls.[4] Locally produced GM-CSF could promote granuloma formation via its effects on monocyte macrophage lineage cells.

To evaluate the relationship of GM-CSF with the disease process we compared patients with sarcoidosis whose BAL fluid cells expressed GM-CSF mRNA with those whose BAL fluid cells did not.

Methods

STUDY POPULATION

Twenty untreated Japanese patients with pulmonary sarcoidosis, including the six who had already been reported,[4] were evaluated as to whether or not the cells in their BAL fluid expressed GM-CSF mRNA. The diagnosis of sarcoidosis was made according to previously described criteria including lung biopsy.[5] Table 1 gives the demographic data of these 20 patients, all of whom were being followed at our sarcoidosis clinic. For comparison, five patients with extrinsic allergic alveolitis caused by *Micropolyspora faeni*, *Thermoactinomyces vulgaris* (farmer's lung), or both were studied in a similar fashion. The diagnosis of extrinsic allergic alveolitis was made by (1) exposure to causative antigens, (2) symptomatic acute episodes with cough and breathlessness 4–8 hours after exposure to specific antigens on the farm, (3) chest radiograph showing diffuse ground glass or micronodular shadowing with or without a restrictive pattern of pulmonary function, and (4) positive precipitating antibody to *Micropolyspora faeni*, *Thermoactinomyces vulgaris*, or both. In addition, transbronchial biopsy specimens were obtained in three patients. Granuloma formation was observed in two, and an interstitial mononuclear cell infiltrate compatible with chronic extrinsic allergic alveolitis was observed in the other one. They had all been separated from the causative antigen for a few weeks before BAL was performed.

CLINICAL EVALUATION

The patients were seen every three or six months depending on their disease status.

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Table 1 Characteristics of patients with sarcoidosis*

No	Age	Sex	Disease duration (years)	Extrathoracic disease	Clinical course	Smoking index (pack years)†	Radiological stage	Serum angiotensin converting enzyme (IU/l)‡
1	26	M	0	+	Worse	2·25	2	21·6
2	20	F	3	+	Unchanged	0	1	25·2
3	39	M	15	–	Unchanged	12·5	2	30·2
4	42	F	11	–	Unchanged	0	1	20·5
5	26	F	0	+	Worse	0·3	2	25·8
6	29	M	2	+	Unchanged	0	2	27·2
7	32	M	9	+	Worse	2	1	24·2
8	61	F	1	+	Unchanged	0	2	5·9
9	52	F	6	+	Unchanged	0	1	34·7
10	55	F	6	+	Better	0	1	27·8
11	26	F	0	+	Worse	0·65	1	11·3
12	30	F	3	–	Worse	0	2	20·4
13	53	M	1	+	Unchanged	60	1	21·2
14	22	F	1	+	Unchanged	1·5	1	11·0
15	64	F	10	–	Unchanged	0	0	13·7
16	33	M	2	–	Better	12·75	0	9·9
17	21	F	1	–	Better	1·5	1	18·0
18	53	M	6	–	Better	0	1	12·0
19	54	F	3	–	Better	37·5	0	15·7
20	25	M	1	–	Better	5	0	7·0

*Patients 1, 2, 3, 4, 15, and 16 were previously reported.[4]
†Patients 3, 7, 11, 13, and 16 were ex-smokers at the time of the study.
‡Normal value = 8–21 IU/l.

Chest radiography and pulmonary function tests (spirometry and diffusing capacity by carbon monoxide single breath method) were performed annually, followed by BAL within two weeks. Disease duration, presence or absence of extrathoracic disease, smoking status, and serum angiotensin converting enzyme levels were collected from the case notes (table 1).

The clinical course was classified as worse, unchanged, or better by comparing physical findings, chest radiographs, and the results of pulmonary function tests just before BAL and a year earlier. These three criteria were not contradictory in any subject. Radiological changes were evaluated by two independent respiratory physicians without knowledge of other findings. In the pulmonary function tests a 10% or greater change from the previous value was considered significant.

BAL

After informed consent was given by the subject, BAL was performed as previously described.[6] Briefly, three to four 50 ml aliquots of sterile saline were infused into a lingular segment and then recovered by gentle suction immediately after each infusion. Cells were collected by centrifugation and washed once with cold Hanks' balanced salt solution (HBSS, GIBCO, Grand Island, USA).

DETECTION OF GM-CSF mRNA

RNA was immediately extracted from the cells in the BAL fluid by the guanidium isothiocyanate caesium chloride method, except in patient 3 whose alveolar macrophages were enriched.[4] RT-PCR followed by Southern blot analysis was used to detect GM-CSF mRNA as previously described.[4] Briefly, 1 μg of total RNA obtained from BAL fluid cells was reverse transcribed and the resulting cDNA was then amplified by 30 cycles of polymerase chain reaction. The sequences of the primer oligonucleotides are given below. This set of oligonucleotides spans a 268 bp region (nucleotides 144–411) of GM–CSF cDNA[7]: (5' (sense) primer, 5'-AGTAGAGACACTGCTGCTGA-3'; 3' (anti-sense) primer, 5'-GGATGACAAGCAGAAAGTCC-3').

After PCR, 1/20 of the reaction mixture was analysed by Southern blotting. Peripheral blood mononuclear cells stimulated with 0·1% phytohaemagglutinin (PHA-P, Difco, Detroit, USA) and 5 ng/ml phorbol myristate acetate (Sigma, St. Louis, USA) for 24 hours in RPMI1640 (GIBCO) with 10% fetal calf serum (GIBCO) were used as a positive control. GM-CSF cDNA probe was a purified fragment of the pHTB194b plasmid, which was a generous gift of Genetics Institute Inc. (Cambridge, USA).

STATISTICS

Representative values were given as mean (SE) and comparisons between groups were made with the Student's two tailed t test unless otherwise indicated. A p value $<0·05$ was considered significant.

Results

The results of BAL are shown in table 2. In all subjects more than 90% of the lymphocytes recovered by BAL were found to be CD3+ T lymphocytes by flow cytometric

Table 2 Mean (SE) results of bronchoalveolar lavage

	Sarcoidosis (n = 20)	Extrinsic allergic alveolitis (n = 5)
Total cell no. ($\times 10^{-6}$)	21·2 (3·0)	31·4 (8·1)
Differential cell counts (%)		
Macrophages	63·9 (5·9)	33·0 (2·4)
Lymphocytes	35·3 (5·9)	64·8 (2·6)
Neutrophils	0·5 (0·2)	1·8 (1·2)
Eosinophils	0·3 (0·1)	0·1 (0·1)
Lymphocyte subsets*		
CD4+ (%)	68·1 (3·5)	86·2 (2·1)
CD8+ (%)	24·8 (3·8)	10·7 (1·6)
CD4+/CD8+ ratio	4·0 (0·6)	9·8 (2·9)

*Lymphocyte subsets were not determined in patients 4 and 15.

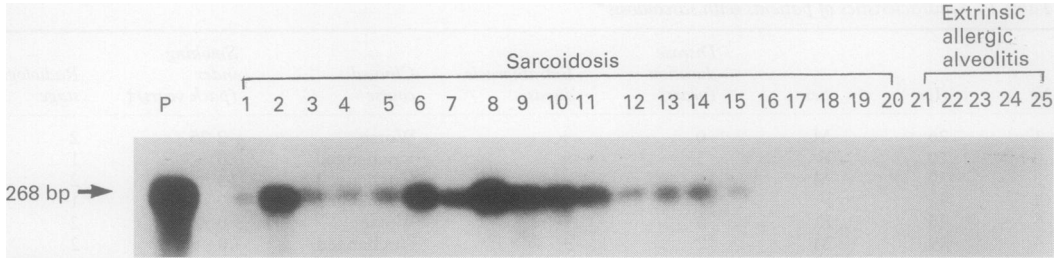

Figure 1 Detection of GM-CSF mRNA by a combination of reverse transcription polymerase chain reaction (RT-PCR) and Southern blotting. Hybridisation bands of 268 bp on lanes 1–15 demonstrate GM–CSF mRNA expression. Lanes 1–20 represent patients 1–20 with sarcoidosis shown in table 1; lanes 21–25 represent patients with extrinsic allergic alveolitis; lane P: 10 times dilution of the RT-PCR product of RNA from peripheral blood mononuclear cells stimulated with 0·1% phytohaemagglutinin and 5 ng/ml phorbol myristate acetate for 24 hours.

analysis (data not shown). Patients with sarcoidosis and extrinsic allergic alveolitis had increased percentages of lymphocytes, and an increased CD4+/CD8+ ratio which indicated that helper T cells were dominant. The increase in CD4+ T cells was higher in the patients with extrinsic allergic alveolitis. For some unknown reason CD4+ dominance in cells of BAL fluid is a common finding in Japanese patients with farmer's lung,[8] whereas it is less frequently observed in other regions.[9]

The RNA samples obtained from patients 1–15 in table 1 contained GM–CSF mRNA as shown by the 268 bp hybridisation bands (fig 1). These bands were faint compared with the positive control (lane P in fig 1, 10 times dilution of the RT-PCR product of RNA from peripheral blood mononuclear cells stimulated with phytohaemagglutinin and phorbol myristate acetate), showing the low copy number of GM-CSF mRNA in BAL fluid cells. No patient with extrinsic allergic alveolitis gave a positive band.

Five patients were judged as having "worse" disease than one year previously. Three (nos. 1, 5, and 11) had newly diagnosed sarcoidosis and were judged as such because they unequivocally noted the onset of extrathoracic symptoms (visual disturbance in all, Heerfordt's syndrome in patient 5, and superficial node swelling in patient 11). Earlier normal chest radiographs were also available in patients 1 and 5. Of the other two, patient 7 developed skin granuloma and a 10% decrease of diffusing capacity, and patient 12 developed diffuse pulmonary infiltrates on the chest radiograph. In all six patients in whom the disease was judged to be "better", shrinkage of bilateral hilar lymphadenopathy was observed without development of parenchymal shadows or deterioration in pulmonary function.

Clinical features of the patients who gave positive GM-CSF mRNA expression by the cells in their BAL fluid (patients 1–15, positive group) were compared with those of the patients who did not (patients 16–20, negative group). All patients in the negative group were judged as being clinically better than one year previously, whereas 14 of the 15 patients in the positive group had no improvement ($p < 0·001$, Fisher's exact probability test). These two groups were not statistically different in disease duration and smoking index.

The laboratory findings of the positive and negative groups were compared. Serum levels of angiotensin converting enzyme were higher in the positive group (21·4(2·1) *v* 12·5(2·0) IU/l, $p < 0·05$) (fig 2). The percentage of lymphocytes and CD4+/CD8+ ratio of lymphocytes in BAL fluid were higher in the positive group (44·3(6·2)% *v* 8·2(2·7)%, and 5·0(0·6) *v* 1·5(0·4), respectively, $p < 0·01$ for both) (fig 3).

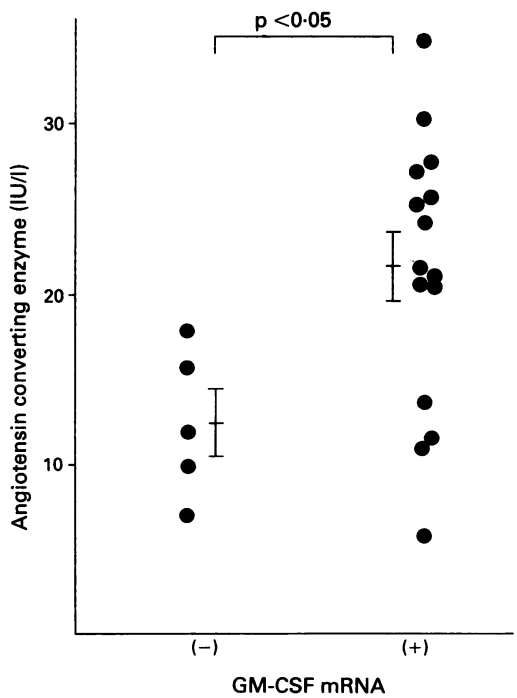

Figure 2 Comparison of serum levels of angiotensin converting enzyme between sarcoidosis patients with positive (+) and negative (−) GM-CSF mRNA expression by BAL cells. Bars indicate mean (SE).

Discussion

Pulmonary sarcoidosis is characterised by an intense cellular immune response localised within the lung. The inflammatory cells recovered from the sarcoid lung by BAL spontaneously release cytokines including IL-1, IL-2, and interferon γ,[1–3] which are considered to mediate the inflammatory reaction. In this report we have investigated the possible involvement of GM-CSF as another inflammatory mediator.

Because GM-CSF is usually produced in very small quantities and its mRNA cannot be detected in cells in BAL fluid by conven-

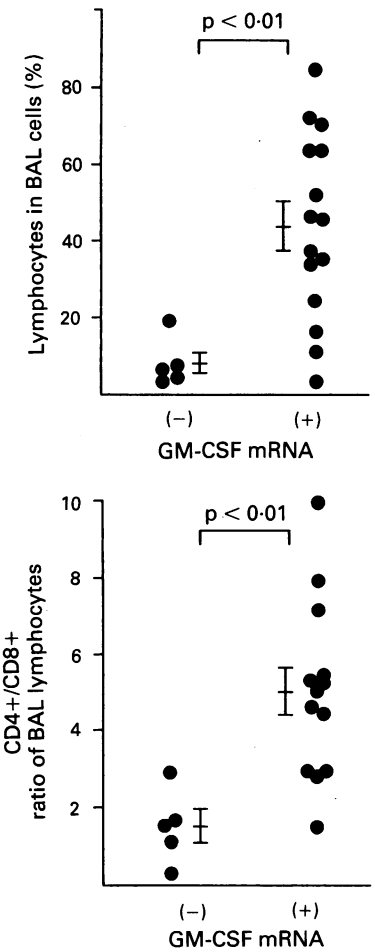

Figure 3 Comparisons of findings in cells in bronchoalveolar lavage (BAL) fluid between sarcoidosis patients with positive (+) and negative (-) GM-SCF mRNA expression by BAL cells. Bars indicate mean (SE). Analysis of lymphocyte subsets was not done in patients 4 and 15.

tional methods,[7 10 11] we used a sensitive RT-PCR method to detect GM-CSF mRNA in BAL fluid cells. In contrast to the absence of detectable GM-CSF mRNA in normal controls even by this method,[4] GM-CSF mRNA was detected in 15 of the 20 patients with pulmonary sarcoidosis including those six who had previously been reported.[4] Five with pulmonary sarcoidosis failed to express GM-CSF mRNA, all of whom had improving disease, while most of the patients with GM-CSF mRNA expression had unchanged or worsening disease. Patients with GM-CSF expression also had higher values of such "activity" markers as serum levels of angiotensin converting enzyme, percentage of lymphocytes in BAL fluid cells, and CD4+/CD8+ ratio of lymphocytes in BAL fluid.[12–14] These results indicated that GM-CSF expression roughly paralleled the clinical spectrum of the disease. Since serum levels of angiotensin converting enzyme may reflect the extent and activity of granulomas in the body, and the cells in BAL fluid represent the inflammation in the alveolar structures, our findings suggest that GM-CSF released from the inflammatory cells within the lung promotes granuloma formation and, ultimately, leads to worse disease. However, a prospective study enrolling more patients controlled for such clinical parameters as smoking habit and radiographic stage would be needed to clarify the role of GM-CSF in disease manifestation or prognosis.

One might argue that the small amount of GM-CSF mRNA simply reflected the BAL T lymphocytosis, as the T cell is one of the major sources of GM-CSF.[7 10] We feel this possibility unlikely from the following observations. Firstly, GM-CSF expression was seen in patients with a normal percentage of lymphocytes in the BAL fluid. Secondly, we failed to detect GM-CSF mRNA in several samples of unstimulated peripheral blood mononuclear cells which are usually rich in T cells (data not shown). Thirdly, BAL fluid cells of the patients with extrinsic allergic alveolitis did not contain GM-CSF mRNA though the percentage of lymphocytes in the BAL fluid and CD4+/CD8+ ratio of lymphocytes in BAL fluid were higher in these patients than in the sarcoidosis patients with GM-CSF mRNA expression (64·8(2·6)% *v* 44·3(6·2)%, $0{\cdot}05 < p\ 0{\cdot}1$, and 9·8(2·9) *v* 5·0(0·6), $p < 0{\cdot}05$, respectively). GM-CSF mRNA expression by the cells in BAL fluid may therefore be a manifestation of their functional activation in sarcoidosis.

We did not separate the cells in the BAL fluid into T lymphocytes and alveolar macrophages because the mRNA expression of the cells in the BAL fluid. might have been altered by the time required or by exogenous agents such as antibodies and plastic surface. Consequently, the cell type which expressed GM-CSF mRNA was not established. While T cells were reported to be the main producer of GM-CSF in peripheral blood,[15] monocytes and alveolar macrophages also have been shown to be capable of producing GM-CSF.[16 17] It is conceivable that the nature of the exogenous stimulus, presumed to be present in the sarcoid lung, affects which cell type expresses GM-CSF as is the case in human peripheral blood mononuclear cells.[16] In situ hybridisation experiments are currently under way to determine which cell expresses GM-CSF.

GM-CSF stimulates such activities of monocyte/macrophage lineage cells as proliferation, differentiation, antigen presentation, and production of effector molecules.[10 18] Although the effects of GM-CSF on human monocyte/macrophage lineage cells in the lung have not been fully examined, Nakata *et al* recently reported that GM-CSF stimulates the proliferation of human alveolar macrophages in vitro.[19] On the other hand, several reports have shown that the alveolar macrophages in the sarcoid lung have undergone similar activation to those mentioned above—for example, proliferation, antigen presentation, and cytokine release.[1 20–22] It would be interesting to see how GM-CSF is involved in such activation.

Molecular cloning and recombinant protein production of GM-CSF have facilitated studies of its properties. Its diverse in vitro effects on various types of cells have been elucidated, but little is known about what it actually does in pathological states in vivo. The results presented here could be a clue to the role of GM-CSF in pulmonary sarcoidosis.

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