Analysis of BAL fluid in *M. avium-intracellulare* infection in individuals without predisposing lung disease

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Analysis of BAL fluid in M. avium-intracellulare infection in individuals without predisposing lung disease. Y. Yamazaki, K. Kubo, M. Sekiguchi, T. Honda. ©ERS Journals Ltd 1998.
ABSTRACT: The purpose of this study was to investigate the characteristics of pulmonary inflammation caused by *Mycobacterium avium-intracellulare* (MAI) in individuals with neither predisposing lung disease nor immunodeficiency.

We reviewed the records of 20 patients with pulmonary MAI infection (including 19 female patients) whose past history and previous chest radiographs revealed no predisposing lung disease. We analysed the bronchoalveolar lavage fluid (BALF) from these 20 patients and from six normal female controls. The BALF was recovered directly from the relevant segment that was identified with chest-computed tomography.

The BALF cell profiles showed significantly elevated counts for total cells, lymphocytes and neutrophils, but the macrophage cell count was not elevated. The CD4+ lymphocyte count and CD4+/CD8+ ratio were significantly increased compared with those in the controls. The lymphocytes demonstrated phenotypical evidence of activation, with increased expression of human leukocyte antigen-D-related antigen (HLA-DR). The tumour necrosis factor- α , interleukin (IL)-1 β , IL-6 and IL-8 concentrations were significantly increased. The neutrophil elastase concentration was also increased, and it was significantly correlated with the neutrophil cell count in the BALF.

These findings suggest that the increased counts of activated CD4+ lymphocytes and neutrophils and the elevated concentrations of proinflammatory cytokines and neutrophil elastase appear to be common characteristics in *Mycobacterium avium-intracellulare* infection.

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Pulmonary infection with *Mycobacterium avium-intracellulare* (MAI) usually develops in patients with damaged lungs as found in chronic obstructive pulmonary disease (COPD), pulmonary tuberculosis, bronchiectasis, or pneumoconiosis [1–3], or in immunocompromised hosts such as those with acquired immunodeficiency syndrome (AIDS) [4]. Recently, pulmonary MAI infection unaccompanied by any predisposing lung disease or AIDS was recognized [5–7]. These reports have described that MAI infection without predisposing lung disease occurs predominantly in older females and nonsmokers. Using chest-computed tomography (CT), several researchers have found that the coexistence of multiple small nodules and bronchiectasis affecting mainly the upper lobes, middle lobe and lingula segment is characteristic of MAI infection [8–10]. SWENSON *et al.* [11] have described that MAI infection leads to bronchiectasis.

MAI is widely distributed in the environment, especially in water sources [12, 13]. Infection can be contracted by inhalation or ingestion. MAI is recognized as an increasingly encountered and important respiratory pathogen [5–7]. There are several unresolved issues as to the characteristics and development of MAI infection in individuals without predisposing lung disease or immunodeficiency, including the cause of its prevalence in older females who

are nonsmokers, and the reason as to why the upper lobe, middle lobe and lingula segment are predominantly affected.

In the present study, we investigated the inflammatory process of MAI infection using a bronchoalveolar lavage (BAL) study in patients with MAI infection with neither predisposing lung disease nor immunodeficiency. We performed the BAL study using BAL fluid (BALF) obtained directly from the affected segment as identified on the chest CT scans. We analysed the cell counts and populations, chemical contents and concentrations of inflammatory cytokines in the BALF.

Methods

Patients

We studied 20 (including 19 female) patients who visited Shinshu University Hospital for evaluation of respiratory diseases between January 1995 and January 1997. They were deemed to be free from other distinct lung diseases based on their past history and the findings of the previous chest radiography and/or chest CT, and they showed a

positive culture for MAI in their sputum on at least two separate occasions or positive culture in samples obtained from the affected lesion using a sterile bronchoscope. The mean age of the 20 patients was 59 ± 11 yrs. All subjects were free of human immunodeficiency virus (HIV) infection. The clinical characteristics were also reviewed, including body height, weight, tuberculin skin test and routine laboratory test results, chest radiographs and chest CT scans. No patient had received treatment for MAI or anti-inflammatory regimen including corticosteroids.

We recruited six normal volunteers, all female non-smokers (mean age, 52 ± 3 yrs) as controls. None had any previous disease or HIV risk factor, and the findings of chest radiography, chest CT and pulmonary function tests were normal in all volunteers.

The patients and normal female volunteers gave written consent to participate in the study after they had been informed regarding all procedures and the possible risks of the study. The BAL study was approved by the Research Committee of Shinshu University School of Medicine. No complication occurred during or after the study.

BAL study

Subcutaneous injections of atropine (0.5 mg) and pethidine hydrochloride ($0.5 \text{ mg} \cdot \text{kg}^{-1}$) were given. After the oral pharynx and upper airway were anaesthetized with 4% lidocaine, a bronchovideoscope (Olympus BF type P200, Olympus Co., Tokyo, Japan) was wedged in the affected segmental bronchus identified on the chest CT scan. Three 50 mL aliquots of sterile normal saline at 37°C were instilled, and each was removed by gentle suction. The recovered fluid was immediately filtered through sterilized gauze. The lavage fluid was spun in a cytometer (KN-70, Kubota Ltd, Tokyo, Japan) at $44 \times g$ for 5 min and stained with May-Grünwald-Giemsa stain to identify cell populations. Five hundred cells, excluding epithelial cells, were identified per slide to establish differential cell counts, and the counts were expressed as percentages and total numbers of cell types. The rest of the BALF was centrifuged at $300 \times g$ for 10 min at 4°C , and the supernatant was removed. The BALF pellets were analysed for lymphocyte subsets. The subsets of lymphocytes were analysed by flow cytometry using CD4, CD8, and human leukocyte antigen-D-related antigen (HLA-DR) monoclonal antibodies (Becton Dickinson Co., Mountain View, CA, USA). The remaining fluid was stored at -70°C for later biochemical analysis with an assay for the BALF constituents.

One millilitre of recovered lavage fluid was processed for quantitative bacterial and fungal culture by routine methods. The number of organisms present in undiluted specimens was determined by colony counts and expressed as colony-forming units (cfu) $\cdot \text{mL}^{-1}$ fluid $^{-1}$. Bacterial identification and sensitivity tests were performed using standard methods [14], and when more than 10^3 cfu $\cdot \text{mL}^{-1}$ were present, the bacteria were considered to be a pathogen of infection [15]. Routine mycobacterial culture and identification of mycobacterial isolates were also performed [14] using the BALF. For polymerase chain reaction (PCR) identification, 5 mL of recovered BALF was extracted and identified using the PCR-based Roche Amplicore Mycobacterium System (Roche, Basel, Switzerland) [16].

Analysis of cytokines, neutrophil elastase, $\alpha 1$ -antitrypsin and chemical contents

We measured the cytokines in the BALF, previously reported [17], using the following enzyme-linked immunosorbent assay (ELISA) kits: tumour necrosis factor (TNF)- α , interleukin (IL)- 1β and IL-6, R&D Systems Co. (Minneapolis, MN, USA); IL-8, (Toray Fuji Bionics, Tokyo, Japan); neutrophil elastase (NE), Merck Co. (Darmstadt, Germany); $\alpha 1$ -antitrypsin (AT), Behring (Rueil Malmaison, France). The $\alpha 1$ -AT concentration was determined using a Behring Nephelometer. The albumin concentration in the BALF was determined with an automated specific immunoturbidimetric assay.

Statistical analysis

The values are expressed as mean $\pm$ SD in the text, tables and figures. For statistical evaluation of differences between the two groups, Student's unpaired t-test was used. As TNF- α and $\alpha 1$ -AT were not normally distributed, the Mann-Whitney U-test was used. We used linear regression analysis to determine the correlations between variables. A p-value of <0.05 was considered to be statistically significant.

Results

Patient characteristics

The chief symptoms were cough in 15 patients, sputum in seven, haemoptysis in three, and chest pain in three. The mycobacterial culture yielded a positive result for the sputum from two patients and for the BALF in all patients. Body weight loss, night sweats, and fever were not present. All patients were nonsmokers. The mean body mass index was $19.0 \pm 2.3 \text{ kg} \cdot \text{m}^{-2}$, and the diameter of the induration for the tuberculin skin test was 12 ± 5 mm. The chest CT revealed small nodules in all patients and ectasia of peripheral bronchi and/or bronchioles in 19 patients. The findings were noted mainly in the right upper lobe, middle lobe and the lingula.

Bacterial, mycobacterial culture and PCR identification in the BALF

The number of organisms did not exceed 1×10^3 cfu $\cdot \text{mL}^{-1}$, which is recognized as being clinically relevant for any known pathogen. Three patients showed number of organisms of 1×10^2 cfu $\cdot \text{mL}^{-1}$: one patient for *Klebsiella pneumoniae*, one for both *Streptococcus pneumoniae* and *Haemophilus influenzae*, and the other for *Staphylococcus aureus*. Fungal cultures were all negative. All BALF samples yielded a positive MAI culture (<100 colony: 12 patients, ≥ 100 colony: eight patients). All samples yielded PCR-positive results (*M. avium* in 17 patients and *M. intracellulare* in three patients).

Cell profiles in the BALF

As shown in tables 1 and 2, the total cell, lymphocyte, and neutrophil counts and subpopulations were significantly increased in the patients with MAI infection compared to

Table 1. – Cell number and cell population in the bronchoalveolar lavage from patients with *Mycobacterium avium-intracellulare* (MAI) infection

	Total number of cells	Macrophages	Lymphocytes	Neutrophils	Eosinophils
MAI (n=20) %		49.3±22.0***	28.8±18.5***	21.7±20.5***	0.2±0.4*
×10 ³ .mL ⁻¹	293±237**	115±93	75±66***	102±141***	0.1±0.3*
Controls (n=6) %		92.4±3.7	6.7±3.1	1.0±1.3	0.0
×10 ³ .mL ⁻¹	89±49	83±50	5±2	1±1	0.0

Data shown are mean±SD. *: p<0.05; **: p<0.01; ***: p<0.001, compared with the normal controls.

Table 2. – Lymphocyte subpopulations in the bronchoalveolar lavage from patients with *Mycobacterium avium-intracellulare* (MAI) infection

	CD4+	CD8+	CD4+/CD8+	HLA-DR
MAI (n=20) %	64.3±15.8*	14.8±5.7	5.3±3.0***	55.1±11.2***
×10 ³ .mL ⁻¹	54±50**	11±10**	5.3±2.6*	47±40**
Controls (n=6) %	23.4±13.0	14.2±6.5	1.8±0.8	16.0±11.8
×10 ³ .mL ⁻¹	1.2±0.4	0.6±0.4	2.7±1.5	0.7±0.7

Data shown are mean±SD. *: p<0.05; **: p<0.01; ***: p<0.001, compared with the normal controls.

Fig. 1. – Tumour necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and IL-8 concentrations in the bronchoalveolar lavage fluid in the 20 patients with *Mycobacterium avium-intracellulare* (MAI) infection and in the six normal controls. The minimum detectable value for TNF- α is 0.5 pg·mL⁻¹. Bars show the mean value in each group. **: p<0.01; ***: p<0.001.

the controls, but the alveolar macrophage counts were not significantly different in the two groups. In the analysis of the subsets of lymphocytes, the numbers of CD4+ and HLA-DR+ cells were significantly increased in the patients. The percentage of the CD8+ cells was not significantly increased, although the CD8+ cell count was. The CD4+/CD8+ ratio of the patients (5.3±2.6) was markedly elevated compared with that (2.7±1.5) in the controls.

TNF- α , IL-1 β , IL-6, and IL-8 concentrations in the BALF

As shown in figure 1, the IL-1 β , IL-6 and IL-8 concentrations were significantly increased in the patients with MAI infection. The TNF- α concentration was increased in 11 patients (55%), but it was below the detection limit in the other nine patients (45%). TNF- α was not detectable (<0.5 pg·mL⁻¹) in the controls.

NE, α 1-AT and albumin concentrations in the BALF

In all of the patients with MAI infection, as shown in figure 2, the NE and α 1-AT concentrations were significantly increased compared with those in the controls. The α 1-AT concentration was increased in 16 patients (80%) and below the detectable level in four patients. The albumin concentration in the patients (82.9±37.0 μ g·mL⁻¹) was significantly increased compared to that (45.2±7.5 μ g·mL⁻¹) in the controls.

Correlations between the variables in the BALF

There were significant correlations between the NE concentration and the percentage of neutrophils ($r=0.66$, $p<0.01$), between the NE concentration and the neutrophil count ($r=0.62$, $p<0.05$), and IL-8 concentration and the percentage of neutrophils ($r=0.50$, $p<0.05$). There were no other correlations between the concentrations of cytokines and the cell populations.

Fig. 2. – Neutrophil elastase (NE) and α 1-antitrypsin (AT) concentrations in the bronchoalveolar lavage fluid in the 20 patients with *Mycobacterium avium-intracellulare* (MAI) infection and in the six normal controls. The minimum detectable value is 66.3 μ g·L⁻¹ for NE, and 0.34 mg·dL⁻¹ for α 1-AT. Bars show the mean value in each group. ***: $p < 0.001$.

Discussion

Previous reports have described the susceptibility to, and clinical manifestations of MAI infection in individuals with neither predisposing lung disease nor AIDS [5–7]. The disorder is predominant in older females and non-smokers. Most of the patients in our study complained of mild respiratory symptom, 19 of them were female, and their mean age was 59 yrs. All were nonsmokers. The chest CT findings were similar to those previously reported [8–11].

It is difficult to clarify the role of bacterial infection in the development of MAI infection, especially in the formation of bronchiectasis. We could not identify any feature in the three patients who showed positive bacterial culture in the BALF, including the bronchiectatic changes, which could separate them from that in the other 17 patients. TANAKA *et al.* [18] reported that *S. aureus* and *Pseudomonas aeruginosa* were cultured in the bronchial washing specimens from one patient each among 13 patients with MAI infection. Because the bacterial count in our three patients was $<10^3$ cfu·mL⁻¹ in the quantitative culture, we think that these bacteria were not clinically relevant and could not alter the cell numbers and populations, cytokines, or soluble factor in the BALF.

The counts for total cells, neutrophils, and lymphocytes were increased about threefold, 140-fold, and 30-fold, respectively, compared with those in the controls. However, the alveolar macrophage count was no different. In MAI infection, therefore, the main inflammatory cells seem to be neutrophils and lymphocytes. HOEISEL *et al.* [19] noted that the BALF neutrophil count was 3-fold greater in pulmonary tuberculosis than in normal controls. ZHANG *et al.* [20] described that patients with pulmonary tuberculosis showed a 37-fold greater neutrophil cell count compared to their controls. Thus, neutrophils may play a role in MAI infection as well as in pulmonary tuberculosis. In the study by MARTIN *et al.* [21], 48 male patients with chronic bronchitis presented elevated percentages of neutrophils and showed a correlation with the proportion of neutrophils in the BALF for chronic cough and/or phlegm production and chronic airflow obstruction.

Neutrophils may also be related to bronchial inflammation and airway function.

The lymphocytes in the BALF examined in the present study were mainly the T-cells of the helper/inducer subset (CD4+) expressing HLA-DR (class II major histocompatibility complex antigen), suggesting early T-cell activation. The count of CD8+ cells (suppressor-cytotoxic T-cells) was significantly increased, although the percentage of CD8+ cells did not differ from that in the controls. The role of CD8+ cells in the development of MAI infection was not clarified in the present study. The CD4+/CD8+ ratio was significantly increased in the patients with MAI infection compared with the controls. An elevation of this ratio is well known to occur in sarcoidosis, in which granuloma formation is associated with increased number of activated T lymphocytes. TANAKA *et al.* [18] demonstrated the presence of epithelioid granuloma in eight out of 13 patients with MAI infection by transbronchial lung biopsy. However, LAW *et al.* [22] performed open lung biopsy in HIV-negative patients with pulmonary tuberculosis, and noted that the CD4+/CD8+ ratio in the tissue granuloma (5.1) was similar to that (5.6) in the BALF. They [22] hypothesized that the findings in the BALF may reflect the lymphocyte populations at the site of disease. It is, therefore, conceivable that the elevation of the count of activated CD4+ cells in MAI infection may be related to granuloma formation. Furthermore, MAI infection in normal individuals should be included in the differential diagnosis of patients with increased CD4+ lymphocyte count.

The role of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6 in MAI infection was not clarified in the present study, although it seems likely that these cytokines are important in the pathophysiology in MAI infection. LAW *et al.* [23] reported the increased release of TNF- α , IL-1 β and IL-6 by bronchoalveolar cells lavaged from the radiographically involved site in patients with pulmonary tuberculosis, in comparison to those from an uninvolved site or in normal controls. Experimental studies have demonstrated that TNF- α and IL-1 β play an important role in regulating the microbicidal activity of macrophages in response to *M. avium* [24, 25] and may also play a role in host resistance to *M. avium* in mice [26, 27]. However, the mechanism by which macrophages are involved in the resistance to, and killing of, MAI is unclear in the present study.

Our patients showed significant elevations of the IL-8, NE, α 1-AT concentrations in the BALF. There were significant correlations between the percentage of neutrophils and each of the IL-8 and NE concentrations. IL-8 has been implicated as a chemotactic factor for neutrophils. ZHANG *et al.* [20] reported the elevation of IL-8 concentration in the BALF from patients with pulmonary tuberculosis and demonstrated that *M. tuberculosis* and its components induce release of IL-8 from alveolar macrophages. They [20] have suggested that IL-8 synthesis and release constitute an early response of alveolar macrophages after phagocytosis of *M. tuberculosis*. CHANEZ *et al.* [28] reported an elevated IL-8 concentration and percentage of neutrophils in the BALF in chronic bronchitis. NE, which is released from neutrophils, and α 1-AT are closely connected, as α 1-AT physiologically neutralizes NE. NAKAMURA *et al.* [29] have described that a respiratory epithelial lining fluid in cystic fibrosis containing NE at a high level directly damages epithelial cells in the airway by overwhelming the normal hosts defence. The increased IL-8 concentration in the BALF in MAI infection may lead to a

persistent increase in the neutrophil count and cause bronchial wall inflammation, although the source of IL-8 was not clarified in the present study. An increase in the NE concentration might be related to the inflammation of the airway and may lead to bronchiectasis.

In summary, we performed a bronchoalveolar lavage study in patients with *Mycobacterium avium-intracellulare* infection, predominantly older females and all nonsmokers, with neither predisposing lung disease nor immunodeficiency. In the bronchoalveolar lavage fluid, neutrophils and lymphocytes were the main cellular constituents. Increased numbers of activated T-cells including CD4+ cells may be related to granuloma formation in *Mycobacterium avium-intracellulare* infection. The elevated numbers of neutrophils and concentrations of pro-inflammatory cytokines and neutrophil elastase may lead to chronic inflammation in this disease.

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