

## QUANTITATIVE ANALYSIS OF PROPIONIBACTERIAL DNA IN BRONCHOALVEOLAR LAVAGE CELLS FROM PATIENTS WITH SARCOIDOSIS

H. Ichikawa<sup>1</sup>, M. Kataoka<sup>2</sup>, J. Hiramatsu<sup>1</sup>, M. Ohmori<sup>1</sup>, Y. Tanimoto<sup>1</sup>, A. Kanehiro<sup>1</sup>, Y. Nakata<sup>2</sup>, M. Tanimoto<sup>1</sup>

<sup>1</sup> Department of Hematology, Oncology and Respiratory Medicine, <sup>2</sup> Faculty of Health Sciences, Okayama University Medical School, Okayama city, Okayama, Japan

**ABSTRACT.** *Background and aim of the work:* The causes of sarcoidosis are still unknown. Propionibacterial sub-species are thought to be one of the most likely sources of antigens. Here we attempted to measure the amount of propionibacterial DNA in bronchoalveolar lavage (BAL) cell samples from patients with sarcoidosis and other pulmonary diseases. *Methods:* We examined BAL cells from 42 patients with sarcoidosis and 30 controls. Using quantitative polymerase chain reaction (PCR) for 16S rRNA of *Propionibacterium acnes* (*P. acnes*) and *Propionibacterium granulosum* (*P. granulosum*), we measured the amount of propionibacterial DNA in 500 ng of total DNA extracted from BAL cells from patients with sarcoidosis or other lung diseases. The correlation between clinical findings and the results of quantitative PCR were analyzed. *Results:* The mean level of *P. acnes* DNA from patients with sarcoidosis was 59.9 genomes per 500 ng of total DNA, which was significantly higher than that in controls (20.7 genomes,  $p < 0.0001$ ). The mean level of *P. granulosum* DNA from patients with sarcoidosis was 1.2 genomes, which was similar to that in controls ( $1.0 \pm 1.6$  genomes,  $p = 0.52$ ). The number of genomes of *P. acnes* in BAL cells was correlated with the serum angiotensin-converting enzyme (ACE) level and the percentage of macrophages in BAL fluid from patients with sarcoidosis. *Conclusions:* The amount of *P. acnes* DNA in BAL cells from patients with sarcoidosis was significantly higher than that in BAL cells from patients with other pulmonary diseases. *P. acnes* may be involved in the pathogenesis of sarcoidosis. (*Sarcoidosis Vasc Diffuse Lung Dis* 2008; 25: 15-20)

**KEY WORDS:** Sarcoidosis, *P. acnes*, *P. granulosum*, Bronchoalveolar lavage cells, Quantitative PCR

### INTRODUCTION

Although more than 100 years have passed since sarcoid lesions were reported for the first time, the causes of this systemic granulomatous disease are still unknown.

Sarcoidosis occurs in people all over the world, affecting both sexes and all races and ages, so the etiology of sarcoidosis has been thought to be the same throughout the world. Sarcoidosis seems to result from exposure of a genetically susceptible subject to specific environmental agents (1). Two agents, mycobacteria and propionibacteria, have been regarded as probable causative agents (2). Because of their clinical and immunological features, mycobacteria were thought to be the most likely causative infective agents of sarcoidosis. Some investigators detected mycobacterial DNA by using PCR in tissue samples from patients with sarcoidosis, but others did not. Using PCR, Gazouli showed the existence of

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Correspondence: Hirohisa Ichikawa, MD

2-5-1 Shikata-cho Okayama city,

Okayama prefecture 700-8558, Japan

Tel. ++81-86-235-7227

Fax: ++81-86-232-8226

E-mail: ichihiro1003@yahoo.co.jp

genomes of *P. granulorum* (43.8%) and *M. tuberculosis* (71.7%) in tissue samples (lung and lymph nodes) from patients with sarcoidosis (3). The positivity rates varied greatly (from 0% to 89%) among the published studies (2). No one has isolated *M. tuberculosis* in culture from sarcoid lesions. It is controversial whether there is correlation between mycobacteria and pathogenesis of sarcoidosis.

*P. acnes* is so far the only bacterium to be isolated in culture from biopsy samples of lymph nodes from patients with sarcoidosis (4, 5). Eishi and colleagues detected the genomes of propionibacteria in lymph nodes from patients with sarcoidosis and showed an etiological link between sarcoidosis and Propionibacterium subspecies (6-8). Nishiwaki and colleagues showed that extrapulmonary sensitization by *P. acnes* induced pulmonary Th-1 granulomas, and the eradication of indigenous *P. acnes* by the use of antibiotics decreased the lung granulomas (9). We previously reported that the rate of detection of *P. acnes* DNA in BAL cells using nested PCR was significantly elevated in patients with sarcoidosis (10). We also reported that the blastogenesis, the production of interleukin-2 (IL-2), and the expression of IL-2 receptor of alveolar lymphocytes stimulated by *P. acnes* were greater in patients with sarcoidosis than in patients with other lung diseases and in healthy controls (11, 12). Recently McCaskill et al. showed that *P. acnes* caused a Th-1 dominant immune response and peribronchovascular granulomatous inflammation in experimental models (13). Because of these studies, Propionibacterium subspecies were thought to be involved not only in sarcoid lymph nodes but also in granulomatous inflammation of lungs affected by sarcoidosis.

In this study, we attempted to measure the number of *P. acnes* and *P. granulorum* genomes in BAL cells from patients with and without sarcoidosis by quantitative (Taq Man) PCR, and compared the results with the clinical findings.

## MATERIALS AND METHODS

### Subjects

Samples were collected from patients who were fully informed and gave their consent. This study was approved by the ethics committee of Okayama University Hospital. Bronchoscopy was done for diagnostic purposes between June 1997 and April 2005 at Okayama University Hospital. We studied 42 patients with sarcoidosis, consisting of 4 patients with stage 0, 15 patients with stage I, 16 patients with stage II, and 7 patients with stage III, and 30 patients with non-sarcoidosis lung diseases, consisting of 12 patients with interstitial pneumonia, 10 patients with lung cancer, and 8 patients with other lung diseases. Clinical findings and characteristics of the patients with sarcoidosis and other lung diseases are shown in Table 1. The diagnosis of sarcoidosis was established by the histological findings compatible with sarcoidosis and the clinical findings (increased serum angiotensin converting enzyme (ACE) level, negative tuberculin test, elevation of percentage of lymphocytes and CD4/8 ratio in bronchoalveolar lavage fluid, and no evidence of current infection by *M. tuberculosis* or other organisms known to produce granulomatous lesions).

### SAMPLES

BAL was done in the right middle lobe by instilling 200 ml of physiological saline (50 ml, four times). Recovered BAL fluid was filtered through sterile gauze to remove tissue residue, and was then centrifuged at 400 g for 5 minutes.

### DNA EXTRACTION, AMPLIFICATION, AND QUANTIFICATION

DNA was extracted from BAL cells with QIAamp

**Table 1.** Patients' characteristics.

	No. of cases	M/F	Age (yr)	Smokers/ non-smokers	BAL cells			
					Total cells	Lym (%)	Mac (%)	CD4/CD8
Sarcoidosis	42	21/21*	47.8±17.9 <sup>†</sup>	18/24	21.5±13.8	35.6±22.5 <sup>‡</sup>	62.4±23.42	5.61±5.42 <sup>†</sup>
Non-sarcoidosis lung diseases	30	24/6	59.8±15.8	19/11	21.4±18.8	17.8±18.3	70.6±26.6	1.84±1.94

BAL: bronchoalveolar lavage

Data are shown as mean ± SD. \* p < 0.02, <sup>†</sup> p < 0.05, <sup>‡</sup> p < 0.01, <sup>††</sup> p < 0.001.

DNA mini kits (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol. After extraction, DNA was purified with a mixture of phenol, chloroform and isoamyl alcohol. We measured the concentration of DNA using a spectrophotometer. Approximately 500 ng of DNA from each extract was used for quantitative PCR. We used primers for real-time (TaqMan) PCR to amplify 16S rRNA of *P. acnes* (PA) and *P. granulosum* (PG). Primers PA-F (5'-AACCGCTTTCGCCTGTGA-3') and PA-R (5'-ACGCTCAGGGTTAAGCCC-3') were designed to amplify a 182-bp portion of 16S rRNA of PA. Primers PG-F (5'-ACATGGATCCGGGAGCTTC-3') and PG-R (5'-ACCCAACATCTCACGACACG-3') were designed to amplify a 102-bp portion of 16S rRNA of PG. TaqMan probes PA-TAQ (5'-AGC-GTTGTCCGGATTTATTGGGCG-3'), PG-TAQ (5'-CGGTTACAGGTGGTGCATTGGC-3') were designed to hybridize with the PCR product of *P. acnes* and *P. granulosum*, respectively. These probes contained the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5'-end and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3'-end. Primers and probes were aliquoted to avoid loss of stability by freezing and thawing and stored at -80°C. PCR was done in 20 µl of a mixture containing 500 ng of a DNA sample, 4 pmol of each primer needed, 2 pmol of a TaqMan probe, 60 nmol of MgCl<sub>2</sub>, 4 pmol of Light Cycler Fast-start DNA Master Hybridization Probes® (Roche Diagnostics GmbH, Mannheim, Germany) in pure water (PCR grade). Amplification and detection were performed using a Light Cycler (Roche Diagnostics). The PCR protocol consisted of an initial step at 95°C for 10 min and 50 cycles of 95°C for 10 s and 62°C for 20 s for *P. acnes* and with the profile of 95°C for 10 min and 50 cycles of 95°C for 10 s and 60°C for 20 s for *P. granulosum*. The TAMRA signal was used for standardization of the results. The number of bacterial genomes in BALF samples was estimated with an internal standard curve for each species, prepared in duplicate at the concentrations of 500, 50, and 5 ng/µl for *P. acnes* and 50, 5, and 0.5 ng/µl for *P. granulosum*. Before extracting DNA of *P. acnes* and *P. granulosum* from bacterial cultures, we used achromopeptidase according to the manufacturer's protocol to digest the bacterial walls. DNA was extracted from these bacteria using QIAamp DNA mini kits (QIAGEN GmbH, Hilden, Germany). The concentration of DNA was

expressed in terms of the number of bacterial genomes using the conversion factor  $2.5 \times 10^9$  Da per genome (7). Finally, the result of quantitative PCR for each sample was expressed as the number of bacterial genomes in 500 ng of total DNA extracted from the BAL cells. Negative controls without bacterial DNA were included in every PCR. Assays were done in duplicate for each sample, and the mean number of genomes was calculated. The specificities of primers and probes for the bacterial DNA tested were examined with the same procedures as for BAL cell samples. TaqMan analysis with each set of primers and probes for *P. acnes* or *P. granulosum* was done with 10 ng of bacterial DNA extracted from the strains of *P. acnes* (ATCC 6919) and *P. granulosum* (ATCC 11829). The sensitivity and reproducibility of TaqMan PCR were evaluated from duplicate analyses of 10-fold serial dilutions of each bacterial DNA in five independent experiments.

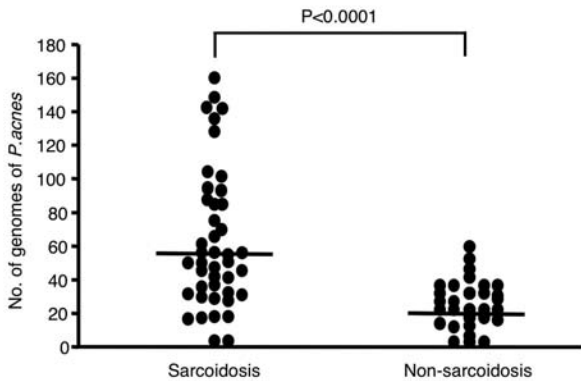
#### STATISTICAL ANALYSIS

Data are expressed as means  $\pm$  SD. The chi-square test was used for evaluation of differences between the two groups regarding sex and smoking habits. The Mann-Whitney U test was used for evaluation of differences between the two groups. Correlations were obtained by Spearman's rank correlation coefficient. Statistical significance was established at a p value of <0.05. We used Stat View 5.0 (SAS Institute, Clog, NC) for statistical analysis.

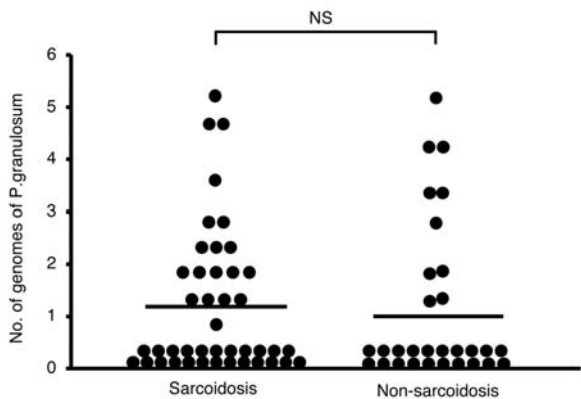
#### RESULTS

There were differences between the patient groups with and without sarcoidosis regarding age and gender, but not smoking habit (Table 1). Regarding BAL findings, the percentage of lymphocytes and CD4/CD8 ratio were higher in patients with sarcoidosis than in those without sarcoidosis. No differences were found between the two patient groups regarding total cells or the percentage of macrophages in BAL. Using the primers and probes for *P. acnes* and *P. granulosum* designed for TaqMan PCR, a single band was seen at the expected size of 182 bp in PCR in DNA amplified from a strain of *P. acnes* (ATCC 6919) and similarly a single band at

the expected size of 102 bp in DNA amplified from *P. granulorum* (ATCC 11829). No amplified band was found in DNA samples from bacteria other than *P. acnes* or *P. granulorum* in multiple experiments. The number of genomes of *P. acnes* in patients with sarcoidosis was  $59.9 \pm 42.0$  copies per 500 ng of total DNA, which was significantly higher than that in patients with non-sarcoidosis lung diseases ( $20.7 \pm 12.6$  copies,  $p < 0.0001$ , Fig. 1). There were no differences in the number of genomes of *P. granulorum* between patients with sarcoidosis ( $1.2 \pm 1.5$  copies per 500 ng of total DNA) and without sarcoidosis ( $1.0 \pm 1.6$  copies,  $p = 0.52$ , Fig. 2). The number of genomes



**Fig. 1.** Real-time PCR measurement of *P. acnes* DNA extracted from BAL cells in 42 patients with sarcoidosis and 30 patients with non-sarcoidosis lung diseases. The y axis shows the number of genomes of *P. acnes* in 500 ng of total DNA from BAL cells. The horizontal line in each group represents the mean expression. A significant difference was found.

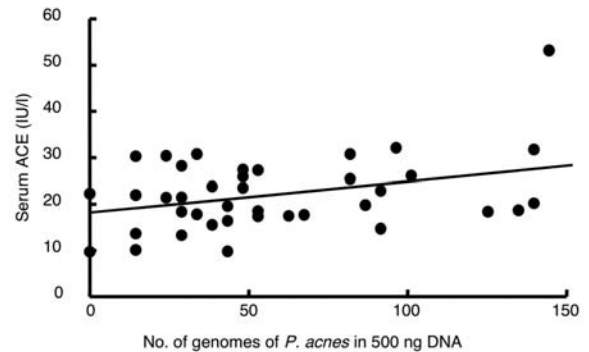


**Fig. 2.** Real-time PCR measurement of *P. granulorum* DNA extracted from BAL cells in 42 patients with sarcoidosis and 30 patients with non-sarcoidosis lung diseases. NS: not significant. The y axis shows the numbers of genomes of *P. granulorum* in 500 ng of total DNA from BAL cells. The horizontal line in each group represents the mean expression. No significant difference was found.

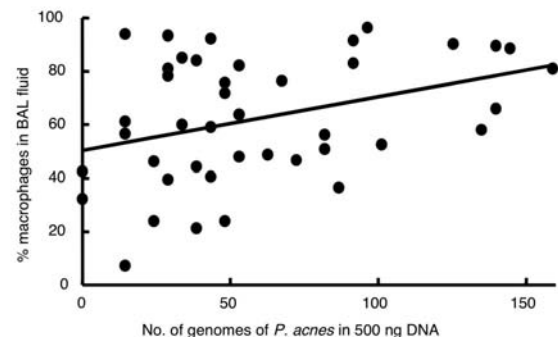
of *P. acnes* in BAL cells from patients with sarcoidosis was 50-fold higher than that of *P. granulorum*. There was no significant correlation between the amount of *P. acnes* DNA and the CD4/8 ratio in BAL lymphocytes. However, the number of genomes of *P. acnes* in BAL cells was significantly correlated with the serum ACE level ( $r = 0.361$ ,  $p < 0.05$ , Fig. 3) as well as with the percentage of macrophages in BAL cells ( $r = 0.360$ ,  $p < 0.05$ , Fig. 4). There was no significant difference in the mean level of *P. acnes* DNA according to either chest X ray stage (I, II, III and IV), or Ga-67 scintigram findings (positive vs negative uptake in lung fields).

## DISCUSSION

Although sarcoidosis seems to result from an exposure of genetically susceptible subjects to a specific environmental agent(s), its etiology is still un-



**Fig. 3.** Correlation between quantitative measurements of *P. acnes* DNA in BAL cells and serum ACE levels in patients with sarcoidosis at the time samples were taken ( $r = 0.361$ ,  $p < 0.05$ ).



**Fig. 4.** Correlation between quantitative measurement of *P. acnes* DNA in BAL cells and % macrophages in BAL fluid in patients with sarcoidosis ( $r = 0.360$ ,  $p < 0.05$ ).

known. Although many possible causative agents, especially infectious agents including mycobacteria, herpes virus, and *Borrelia burgdorferi*, have been extensively studied, no causative agent has been identified. *P. acnes*, an anaerobic gram-positive rod bacteria existing indigenously on the skin or mucosal surfaces (14), is the only bacteria isolated so far from sarcoid lesions (4). This bacterium has been regarded as one of the most probable candidate causative organisms. Indeed, in experimental mice models, *P. acnes* induced pulmonary granulomas (9, 13, 15, 16) and elevation of Th-1 cytokines and chemokines (13). Intratracheal challenge of nonsensitized C57BL/6 mice with *P. acnes* did not induce the development of granulomatous changes (9, 17). On the other hand, Nishiwaki reported that challenging C57BL/6 mice with *P. acnes*-primed helper T cells intravenously after immunization by subcutaneous injection of heat-killed *P. acnes* induced pulmonary granulomatous changes (9). Intratracheal challenge after intraperitoneal injection of heat-killed *P. acnes* led to lung granulomatosis (13). Yi reported granulomatous inflammation after intratracheal challenge with heat-killed *P. acnes* in intravenously sensitized rats (15). Challenging with *P. acnes* intravenously has also been reported to produce pulmonary granulomatous inflammation in the lungs of sensitized rabbits and rats (15, 16). Presensitization with *P. acnes* seems to be essential to cause *P. acnes* related pulmonary granulomatous formations in experimental animal models.

In this study, we examined BAL cells using quantitative PCR for *P. acnes* DNA and *P. granulosum* DNA. *P. acnes* DNA was detected in greater numbers in patients with sarcoidosis compared to those with non-sarcoidosis lung diseases. However, there was no difference in the number of genomes of *P. granulosum* between these two groups, and moreover, the number of genomes of *P. granulosum* was in much smaller amount than that of *P. acnes* in patients with sarcoidosis. The results of this study were in agreement with those of our previous study (10), in which we detected *P. acnes* DNA in BAL cells using nested-PCR.

A Japanese group showed an etiological correlation between *P. acnes* and sarcoidosis (6-8). They reported that the number of genomes of *P. acnes* in lymph nodes from patients with sarcoidosis was much higher than that in lymph nodes from patients

with other pulmonary diseases, as shown by quantitative PCR (6, 7), and also reported the accumulation of genomes of *P. acnes* in and around sarcoid granulomas in lymph nodes by signal amplification with catalysed reporter deposition (8). They also reported that all the patients with sarcoidosis without *P. acnes* in lymph nodes had *P. granulosum* DNA (6). In this regard, their results were different from ours, because we could not find a significant number of genomes of *P. granulosum*. This may result from the difference of samples (lymph nodes vs BAL cells) or the sensitivity of real time PCR. In either case, our results imply that *P. granulosum* might not be involved in the pathogenesis of sarcoidosis.

We previously reported the immunological response to *P. acnes* of BAL cells from sarcoidosis patients. When stimulated with a crude extract of *P. acnes*, the proliferative response of BAL cells from patients with sarcoidosis was higher than that of BAL cells from patients with non-sarcoidosis lung diseases or healthy controls (11). IL-2 production and the expression of IL-2 receptor of BAL cells stimulated by *P. acnes* antigens were greater in patients with sarcoidosis than in normal subjects (12). Moreover Nishiwaki et al. also showed *P. acnes*-specific proliferation of lymphocytes from pulmonary regional lymph nodes in mouse models (9). These data suggest that alveolar lymphocytes and lymphocytes from regional lymph nodes had already been exposed to *P. acnes*.

Although it has been believed for a long time that the lower respiratory tract is germ free, recent studies revealed the existence of *P. acnes* in the peripheral lung. *P. acnes* was detected in culture from the lungs of 8 (33%) of 24 untreated mice (18); moreover, immunostaining showed the existence of *P. acnes* (live bacteria or components of phagocytosed bacteria) in the cytoplasm of alveolar macrophages in normal murine lungs (9) and sarcoid lungs (10). In our study, the number of genomes of *P. acnes* in patients with sarcoidosis was related to the percentage of macrophages in BAL cells. This result supports our previous findings (10). Additionally, the level of serum angiotensin-converting enzyme (ACE), which was elevated in sarcoidosis patients, was clearly associated with the number of genomes of *P. acnes*. This indicates that there may be a relationship between the number of sarcoid granulomas or alveolar macrophages and the number of genomes of *P. acnes*,

because epithelioid cells and macrophages produce ACE in sarcoidosis (19).

The possibility of contamination by *P. acnes* during bronchoscopy or the course of the experiments was excluded, because a significant difference was found between sarcoidosis and non-sarcoidosis samples.

Our observations suggest that *P. acnes* may reside indigenously and proliferate in the lower respiratory tract. Pulmonary granulomas in patients with sarcoidosis may result from delayed hypersensitivity to *P. acnes*, which may play a central role in the antigen-driven immune response in the lungs of patients with sarcoidosis.

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