

Acta Medica Okayama

Volume 64, Issue 2

2010

Article 1

APRIL 2010

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Abstract

Propionibacterium acnes has been implicated as an etiologic agent of sarcoidosis since the isolation of this bacterium from sarcoid lesions. We experimentally produced a murine pulmonary granuloma model using *P. acnes* with several features that simulate sarcoidosis. Mice were sensitized with heat-killed *P. acnes* and complete Freund's adjuvant and were subsequently challenged with heat-killed *P. acnes* at 2-week intervals. *P. acnes*-challenged mice developed epitheloid cell granulomas in the lungs. These mice showed a pulmonary immune response characterized by an increased number of T-lymphocytes, especially CD4 cells, and the ratio of CD4/CD8 in bronchoalveolar lavage (BAL) fluid also increased. Furthermore, significant elevations in both angiotensin-converting enzyme (ACE) serum levels and antibody titers against *P. acnes* were observed. Mice sensitized with *P. acnes* without complete Freund's adjuvant were capable of forming pulmonary granulomas, which appeared to be caused by indigenous *P. acnes*. The genome of *P. acnes* was found in the lungs, BAL cells, hilar lymph nodes, liver, and spleen in non-sensitized mice, which were thought to be germ-free. These results suggest that the immune response against indigenous *P. acnes* may play an important role in the pathogenesis of granuloma formation in a murine model.

KEYWORDS: *Propionibacterium acnes*, experimental granuloma, sarcoidosis

Original Article

Experimental Pulmonary Granuloma Mimicking Sarcoidosis Induced by *Propionibacterium acnes* in Mice

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Propionibacterium acnes has been implicated as an etiologic agent of sarcoidosis since the isolation of this bacterium from sarcoid lesions. We experimentally produced a murine pulmonary granuloma model using *P. acnes* with several features that simulate sarcoidosis. Mice were sensitized with heat-killed *P. acnes* and complete Freund's adjuvant and were subsequently challenged with heat-killed *P. acnes* at 2-week intervals. *P. acnes*-challenged mice developed epithelioid cell granulomas in the lungs. These mice showed a pulmonary immune response characterized by an increased number of T-lymphocytes, especially CD4⁺ cells, and the ratio of CD4⁺/CD8⁺ in bronchoalveolar lavage (BAL) fluid also increased. Furthermore, significant elevations in both angiotensin-converting enzyme (ACE) serum levels and antibody titers against *P. acnes* were observed. Mice sensitized with *P. acnes* without complete Freund's adjuvant were capable of forming pulmonary granulomas, which appeared to be caused by indigenous *P. acnes*. The genome of *P. acnes* was found in the lungs, BAL cells, hilar lymph nodes, liver, and spleen in non-sensitized mice, which were thought to be germ-free. These results suggest that the immune response against indigenous *P. acnes* may play an important role in the pathogenesis of granuloma formation in a murine model.

Key words: *Propionibacterium acnes*, experimental granuloma, sarcoidosis

Sarcoidosis is a systemic disease characterized by the presence of noncaseating epithelioid cell granuloma; it commonly involves the lungs, eyes, lymph nodes, and skin. Although the pathogenesis of sarcoidosis has been widely investigated, its etiology remains unknown [1-3]. Noncaseating granuloma is a typical histological feature of sarcoidosis; several types of bacteria, fungi, chemical, and non-immunological stimuli can induce granuloma formation. In fact,

the process of granuloma formation is thought to be a physiologic step in the delayed immune response that prevents the spread of noxious and infectious microorganisms [4].

Frequent isolation of *Propionibacterium acnes* from sarcoid lymph nodes has been reported by Homma *et al.* [5, 6]. Recently, *P. acnes* was implicated as the etiologic agent of sarcoidosis, based on the isolation of this bacterium from sarcoid lesions using bacteriological, immunohistochemical, and molecular approaches [7-11]. Therefore, we experimentally produced a murine pulmonary granuloma model with *P. acnes* that shares several features with those of sarcoidosis. We

Received August 21, 2009; accepted October 8, 2009.

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then investigated the immune status in response to sarcoidosis in experimental mice.

Materials and Methods

Animals. This experiment complied with the guidelines of the Institutional Animal Care and Use Committee of Okayama University. C57BL/6J mice (females, 6–8 weeks of age) were kept under specific pathogen-free conditions at the Advanced Science Research Center of Okayama University. Mice were divided into 4 groups (n=10 per group).

Induction of granuloma. *P. acnes* (ATCC 6919) were cultured on thioglycollate medium II (Dainippon Pharmaceutical, Tokyo, Japan) supplemented with L-cysteine in an anaerobic chamber, and then were harvested, the samples washed, and a portion of the culture was counted in a Fuchs-Rosenthal counting chamber. Then, *P. acnes* were heat-killed for 30 min at 65°C. The mice were divided into 4 groups (n=10 per group). Group 1 was a control group that had been sensitized by subcutaneous injection of 0.2ml saline on day 1 into the footpad, and on days 14 and 28 in the dorsal region. Group 2 was sensitized by

subcutaneous injection of a mixture (0.2ml) of complete Freund's adjuvant (CFA, Cappel, Cochranville, PA, USA) and *P. acnes* (2µg/ml) on day 1 into the footpad, and were challenged with 0.2ml of *P. acnes* suspension (2µg/ml) injected into the dorsal region on days 14 and 28. Group 3 mice were sensitized by subcutaneous injection of a mixture (0.2ml) of CFA and *P. acnes* (2µg/ml) into the footpad on day 1, and were challenged with a subcutaneous injection of *P. acnes* suspension (0.2ml of 2µg/ml) on days 14, 28, and 42 into the dorsal region. Group 4 was sensitized by subcutaneous injection of *P. acnes* suspension (0.2ml of 2µg/ml) on days 1, 14, and 28 into the dorsal region. From groups 1, 2, and 4, samples were collected 6 weeks after sensitization; samples from group 3 were collected 8 weeks after sensitization (Fig. 1).

Bronchoalveolar lavage (BAL) fluid and blood. Mice were anesthetized by an intraperitoneal injection of pentobarbital sodium, and were then fixed to a board. First, blood samples were collected from the abdominal space by excising the vena cava. Blood was centrifuged at 3,000 rpm for 20 min. After centrifugation, the serum was stored at –80°C.

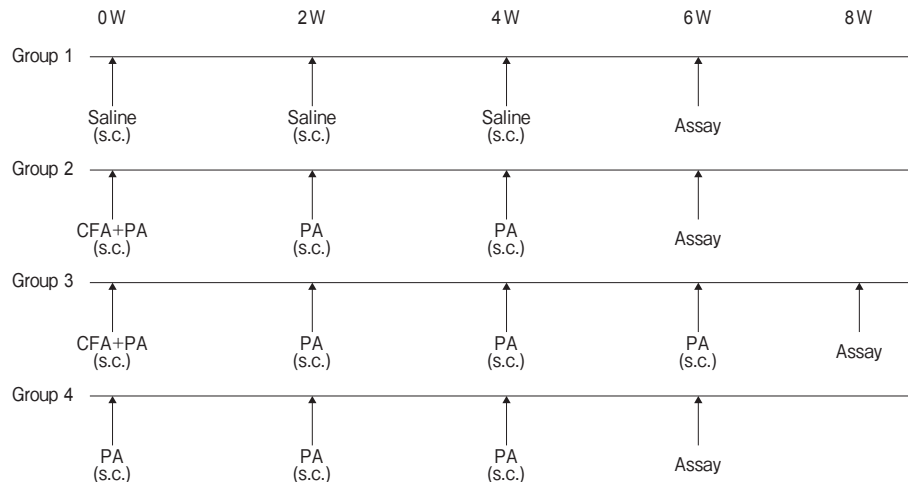


Fig. 1 Experimental protocols. Mice were divided into 4 groups of 10 mice each. Group 1 was the control group, which was sensitized by subcutaneous (s.c.) injection of 0.2ml saline into the footpad on day 1, and into the dorsal region on days 14 and 28. Group 2 mice were sensitized by subcutaneous injection of a mixture (0.2ml) of complete Freund's adjuvant (CFA, Cappel, Cochranville, PA, USA) and *P. acnes* (PA) (2µg/ml) into the footpad on day 1, and were challenged with 0.2ml of *P. acnes* suspension (2µg/ml), injected into the dorsal region on days 14 and 28. Group 3 mice were sensitized by subcutaneous injection of a mixture (0.2ml) of CFA and *P. acnes* (2µg/ml) into the footpad on day 1, and were challenged with subcutaneous injection of a *P. acnes* suspension (0.2ml of 2µg/ml) into the dorsal region on days 14, 28, and 42. Group 4 was sensitized by subcutaneous injection of *P. acnes* suspension (0.2ml of 2µg/ml) into the dorsal region on days 1, 14, and 28. In groups 1, 2, and 4, samples were collected 6 weeks after sensitization; in group 3, samples were collected 8 weeks after sensitization.

Subsequently, the lungs were lavaged with saline (2×1 ml, 37°C) via a trachea tube. The volume of BAL fluid collected was measured for each sample, and the number of cells was counted. The BAL fluid was centrifuged at 1,800 rpm for 15 min at 4°C . After centrifugation, the supernatant was stored at -80°C . The sediment was resuspended in 1 ml of phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), and smear preparations were prepared by an automatic smear system (1,500 rpm, 5 min). The preparations were stained with Giemsa, and differential cell counts were performed. The remaining BAL cells were analyzed in a flow cytometer. The BAL cells were stained with PE-conjugated anti-CD3 antibody (rat; Immunotech, Marseilles, France) and FITC-conjugated anti-CD4 antibody (rat; Immunotech) or anti-CD8 antibody (rat; Immunotech), and were measured using FACScan (BD, Franklin, NJ, USA). Finally, the lungs, hilar lymph nodes, liver, and spleen were removed and stored at -80°C , and the removed portions were fixed in formalin.

Histological studies. Fixed tissues were paraffin-embedded and cut into $4\text{-}\mu\text{m}$ sections, which were then stained with hematoxylin and eosin (HE) for histological evaluation.

Serum angiotensin-converting enzyme (ACE) measurement. The ACE activity was measured using ACE color (Fuji Rebio, Tokyo, Japan), in which the colorimetric analysis of quinoneimine dye produced by the substrate p-hydroxybenzoyl glycyl-L-His-L-leu was carried out at 405 nm.

Measurement of *P. acnes* antibody in serum. Levels of *P. acnes* antibody in the serum were measured by whole bacterial enzyme-linked immunosorbent assay (ELISA) [12]. Each well of a 96-well microtiter plate (Nunc, Roskilde, Denmark) was coated with $100\mu\text{l}$ of Poly-L-lysine (Sigma, St. Louis, MO, USA) and kept overnight at 4°C . The plate was washed 3 times with washing buffer (0.1M PBS pH7.4 with 0.05% Tween20, PBST). The *P. acnes* suspension ($10\mu\text{g/ml}$) was added to the plate ($50\text{-}\mu\text{l}$ each well), which was then coated with heat-killed *P. acnes* overnight at 4°C . After having been washed, the plate was blocked by $150\mu\text{l}$ PBST with BSA at room temperature for 30 min. After the plate had been washed, serum diluted in PBST with BSA ($50\mu\text{l}$) was added, and the plate was then left at room temperature for 2 h. After a subsequent wash, the plate was

incubated with $50\mu\text{l}$ of horseradish-peroxidase (HRP)-conjugated anti mouse-IgG goat antibody (Sigma) at room temperature for 1 h. The plate was again washed, and enzyme substrate (ABTS; Sigma) with 0.05% H_2O_2 solution was added to yield a total volume of $100\mu\text{l}$. Absorbance was read at 405 nm on a microplate reader.

P. acnes DNA analysis by quantitative real-time polymer chain reaction (PCR)

1. *P. acnes* DNA preparation. We compared the efficacy of 2 approaches to DNA extraction, i.e., the Achropeptidase (WAKO, Osaka, Japan) and BACTOZOL (Molecular Research Center Inc., Cincinnati, OH, USA) methods.

a) Achropeptidase extraction

P. acnes was washed with Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0, TE buffer) and centrifuged at 3,000 rpm for 5 min. The precipitation containing *P. acnes* was suspended in $200\mu\text{l}$ of achropeptidase solution (250 U/ml, WAKO, Osaka, Japan), and was incubated at 55°C for 10 min. Twenty-five microliters of Proteinase K (Qiagen, Tokyo, Japan) and 200 microliters of solution buffer (Buffer AL, Qiagen) were added to the solution, which was mixed by vortexing and then incubated at 70°C for 30 min. Two hundred microliters of ethanol were added to the sample, which was then mixed by vortexing. The mixture was transferred to a QIAamp Spin Column (Qiagen) in a 2-ml collection tube, and the sample was centrifuged at $6,000 \times g$ for 1 min. Five hundred microliters of washing buffer 1 (Buffer AW1, Qiagen) was added to the column, and the sample was centrifuged at $6,000 \times g$ for 1 min. Furthermore, washing buffer 2 (Buffer AW2, Qiagen) was added and centrifuged at $10,000 \times g$ for 3 min. A total of $200\mu\text{l}$ of elution buffer (Buffer AE, Qiagen) was added to the sample, which was then incubated at room temperature for 5 min. After centrifugation of the sample at $6,000 \times g$ for 1 min, the DNA was eluted.

b) BACTOZOL extraction

Lysis solution containing Bactozyme (Molecular Research Center, Inc., Cincinnati, OH, USA), TE buffer, and DNAzol were prepared. *P. acnes* was washed with TE buffer and centrifuged at 3,000 rpm for 5 min. The precipitation containing *P. acnes* was

suspended in 200 μ l of lysis solution. The suspension was then mixed by vortexing and incubated at 50 $^{\circ}$ C for 90 min. The methods of DNA extraction and collection were similar to those used in the Achromopeptidase approach.

2. Extraction of DNA from mouse organs. DNA was extracted from BAL cells, lungs, lymph nodes, liver, and spleen using a QIAamp DNA Mini Kit (Qiagen). Lungs, lymph nodes, liver, and spleen were homogenized in a blender; BAL cells were used without homogenization. Then, 180 μ l of Buffer AL and 2 μ l of Protease K were added to a microtube containing homogenized tissue or BAL cells, and the samples were vortexed for 15 sec. After the solution was mixed, the microtubes were incubated at 56 $^{\circ}$ C until the tissues had completely dissolved. Then, 200 μ l of Buffer AL was added to the samples, which were then incubated at 70 $^{\circ}$ C for 10 min. Two hundred microliters of 100% ethanol were added to the samples, which were then vortexed for 15 sec. After the solution was mixed, the DNA was collected using a QIAamp Spin Column (Qiagen). DNA concentrations were obtained based on the absorbance, which was read at 260 nm using a spectrophotometer.

3. Quantitative measurement of *P. acnes* DNA by real-time PCR. PCR was performed using a LightCycler (Roche, Basel, Switzerland). Primers PA-F (5'-AACCGCTTTCGCCTGTGA-3'), PA-R (5'-ACGCTCAGGGTTAAGCCC-3'), and TaqMan Probe (5'-FAM-AGCGTTGTCCGGATTTATTGGGCG-TAMRA-3') were designed to amplify the *P. acnes* 16S rRNA. *P. acnes* DNA used as the control was suspended in a 10-fold serial dilution of 100 μ g/ml. DNA extracted from the mouse organs was prepared in amounts of 100 ng/ μ l. The PCR was carried out in a mixture containing 1.6 μ l of 2.5 mM MgCl₂, 0.4 μ l of 10 μ M Primer-F, 0.4 μ l of 10 μ M primer-R, 1.0 μ l of 2 μ M TaqMan Probe (Japanese Gene Institute, Sendai, Japan), 2 μ l of LC Faststart DNA Master Hybridization probe (Roche), 1.0 μ l of sample DNA (100 ng/ μ l), and distilled water, to yield a 20- μ l mixture. The PCR was performed in a LightCycler, with a first step of 10 min at 95 $^{\circ}$ C, 50 cycles of 95 $^{\circ}$ C for 10 sec, and 62 $^{\circ}$ C for 20 sec. The concentration of DNA was expressed in terms of the number of bacterial genomes using the conversion factor 2.5×10^9 Da per genome [9].

Statistical analysis. All results are expressed

as mean \pm SEM. Analysis of variance (ANOVA) was used to determine the level of difference among groups. Significance was defined as a *p* value of less than 0.05.

Results

BAL cell findings. Two milliliters of PBS were instilled into the lungs, and the BAL fluid was collected. The total collected cell counts in groups 1, 2, 3, and 4 were $3.7 \pm 0.8 \times 10^4$ /ml, $6.3 \pm 0.7 \times 10^4$ /ml, $6.8 \pm 0.6 \times 10^4$ /ml, and $6.1 \pm 0.9 \times 10^4$ /ml, respectively. The total cell count for group 3 was significantly higher than that of group 1 (*p* < 0.05). Cell counts in groups 2 and 4 were increased, but the differences between the cell counts of these groups and that of group 1 were not significant. There were no significant differences among groups in terms of the density of macrophages. The density of lymphocytes in group 3 was significantly higher than that of group 1 (*p* < 0.01). The density of BAL lymphocytes in groups 2 and 4 was higher than that of the other 2 groups, but the difference from group 1 was not significant (Fig. 2). As regards the lymphocyte subset in BAL cells determined by flow-cytometric analysis, no significant differences were observed in the percentage of CD3+ cells among groups, although the percentage of CD4+ cells in group 3 was significantly higher than that of group 1 (*p* < 0.05), and the percentage of CD8+ cells in group 3 (*p* < 0.05) was also significantly lower than that of group 1 (Fig. 3). As regards

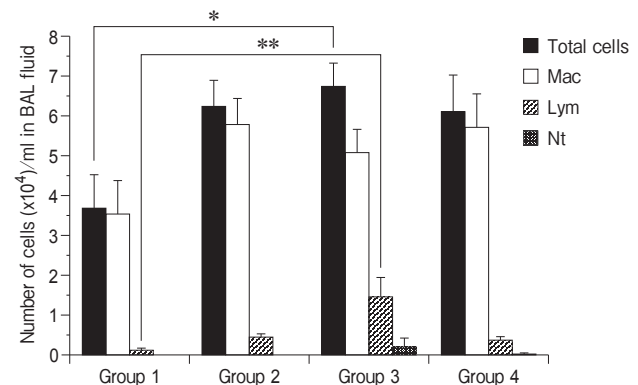


Fig. 2 Cell composition in the bronchoalveolar lavage (BAL) fluid of each group after sensitization and challenge with *P. acnes*. Mac, macrophages; Lym, lymphocytes; Nt, neutrophils. Values are mean \pm SEM. **p* < 0.05, ***p* < 0.01 versus group 1 (control).

the density of lymphocytes in the BAL fluid, the density of CD3+ cells in group 3 was significantly higher than that of group 1, and the density of CD4+ cells was higher in groups 2, 3, and 4 than in group 1, with a significant difference between groups 3 and 1 ($p < 0.01$). The density of CD8+ cells in groups 2 and 3 was significantly higher than that of group 1 ($p < 0.01$) (Fig. 4). The CD4+/CD8+ ratio in groups 2 ($p < 0.05$) and 3 ($p < 0.01$) were significantly higher than that of group 1 (Fig. 5).

Histological findings. There were no granulomatous changes seen in the lungs of group 1 mice, and no abnormal findings were observed (Fig. 6A). The formation of an epithelioid cell granuloma was seen

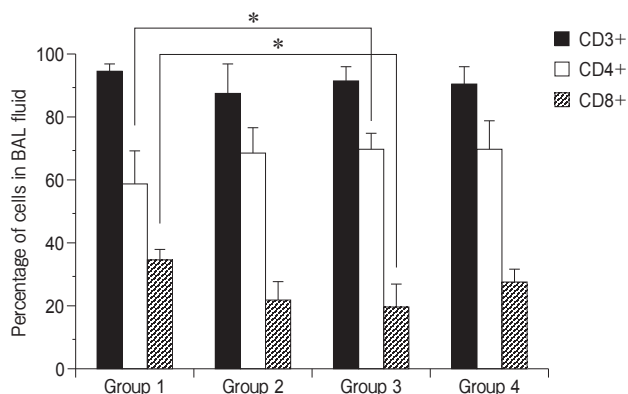


Fig. 3 Percentage of CD3+, CD4+, and CD8+ cells in bronchoalveolar (BAL) fluid. Values are mean \pm SEM. * $p < 0.05$ versus group 1 (control).

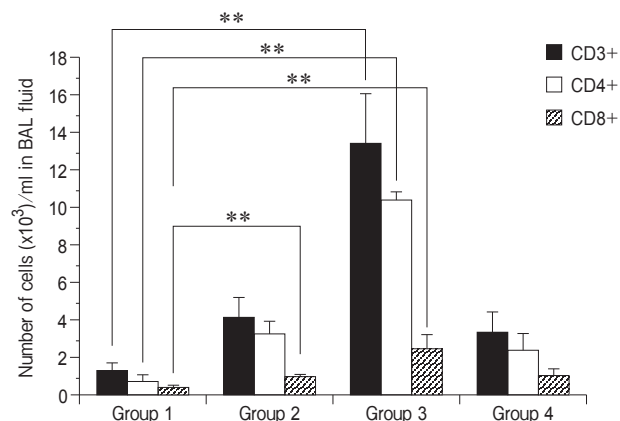


Fig. 4 Density of CD3+, CD4+, and CD8+ cells in bronchoalveolar (BAL) fluid. Values are mean \pm SEM. ** $p < 0.01$ versus group 1 (control).

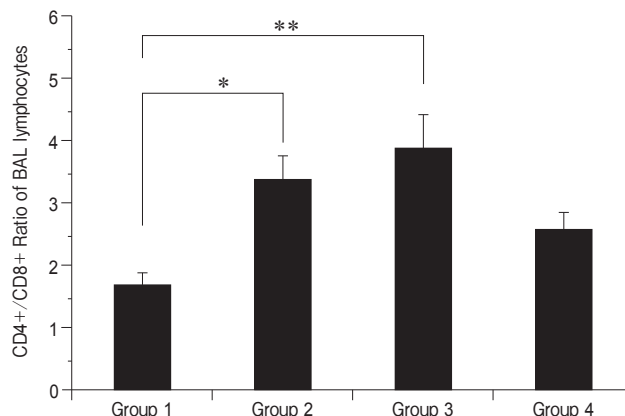


Fig. 5 CD4+/CD8+ ratio of lymphocytes in bronchoalveolar (BAL) fluid. Values are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ versus group 1 (control).

in group 2, and lymphocytic infiltrations into the alveolar walls were also noted. These granulomatous lesions appeared to be immature (Fig. 6B). In group 3, epithelioid cell granulomas with giant cells were present in the lungs, and the alveolar walls were thickened by macrophage and lymphocyte infiltration (Fig. 6C). In group 4, lymphocyte infiltration and an accumulation of mononuclear cells on the alveolar walls were seen, and loose granulomas were observed in the lungs (Fig. 6D).

Angiotensin converting enzyme levels in serum. Angiotensin converting enzyme activity in untreated mice (group 1) was 250 ± 40 IU/l/37°C. However, ACE activity was significantly higher in all other groups than in group 1 (group 2, 310 ± 25 IU/l/37°C, $p < 0.05$; group 3, 301 ± 30 IU/l/37°C, $p < 0.01$; and group 4, 330 ± 26 IU/l/37°C, $p < 0.01$) (Fig. 7).

Antibody levels in response to *P. acnes* in the serum. The antibody levels generated in response to *P. acnes* in mouse serum, read 5 min after the start of the reaction at an optical density (O.D.) of $\times 1,000$, were as follows: 60.0 ± 10.0 in group 1, 110.0 ± 4.0 in group 2, 190.0 ± 90.0 in group 3, and 100.0 ± 20.0 in group 4. The levels of *P. acnes* antibody were significantly elevated in groups 2 ($p < 0.05$) and 3 ($p < 0.01$) compared with that of group 1 (Fig. 8).

Quantitative real-time PCR of *P. acnes* DNA. After PCR was carried out with primers R and F, designed to amplify *P. acnes*, electrophoresis was performed in 2% agarose gel. The primers revealed

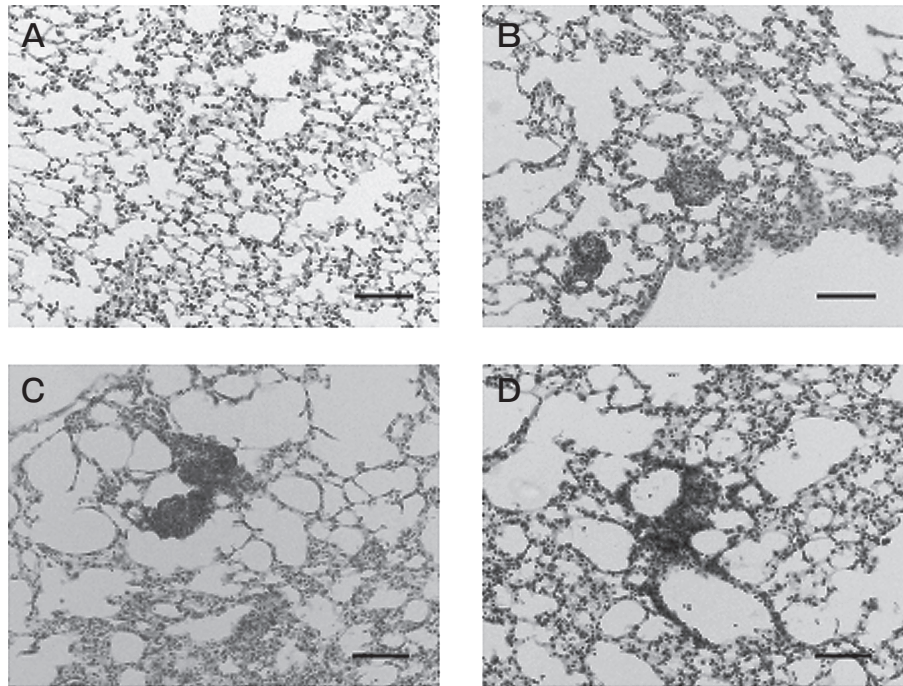


Fig. 6 Histological findings of lungs after sensitization and challenge with *P. acnes* in group 1 (A), 2 (B), 3 (C), and 4 (D). Hematoxylin and eosin (HE)-stained sections from each group are shown. Scale bar, 50 μm.

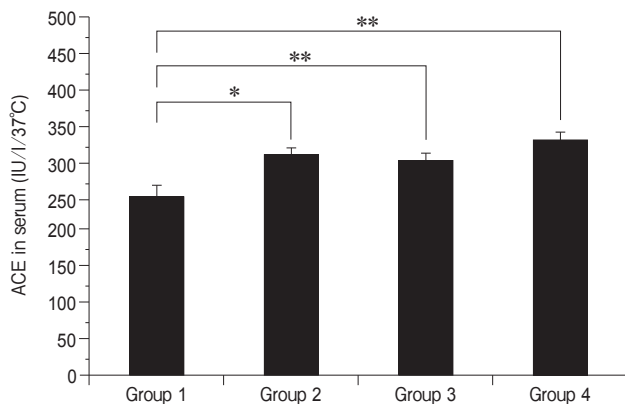


Fig. 7 Angiotensin-converting enzyme (ACE) levels in serum after sensitization and challenge with *P. acnes*. Values are mean ± SEM. * $p < 0.05$, ** $p < 0.01$ versus group 1 as a control.

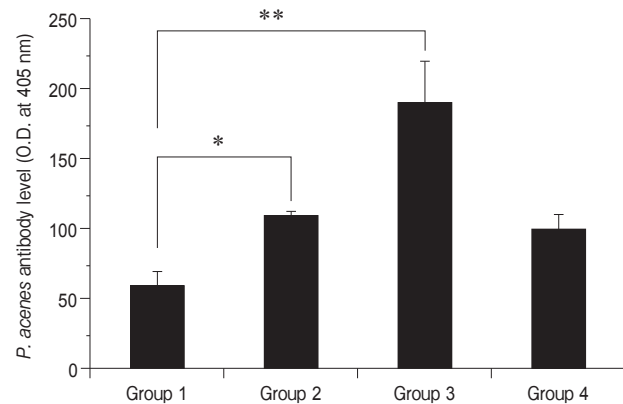


Fig. 8 Antibody level to *P. acnes* in serum after sensitization and challenge with *P. acnes*. Values (Optical Density [O.D.] × 1,000) are mean ± SEM. * $p < 0.05$, ** $p < 0.01$ versus group 1 (control).

a band in the vicinity of 180bp, as expected according to the study design. The achromopeptidase and BACTZOL methods were compared in terms of their relative levels of extraction of *P. acnes* DNA from the tissue. The DNA concentration extracted using the Achromopeptidase approach ($0.87 \pm 0.07 \mu\text{M}$, $n=5$) was significantly higher ($p < 0.02$) than that extracted by the BACTZOL method ($0.55 \pm 0.07 \mu\text{M}$, $n=5$).

Therefore, DNA was extracted using the Achromopeptidase method for the remainder of the experiments. The genome number of *P. acnes* DNA was $14.2 \pm 5.2 \times 10^3$ per 500 ng of total DNA from the lungs of group 1, $21.1 \pm 10.1 \times 10^3$ from group 2, $18.3 \pm 6.3 \times 10^3$ from group 3, and $19.0 \pm 3.8 \times 10^3$ from group 4. The genome of *P. acnes* DNA was detected in the

lungs of all groups; however, there were no significant differences between groups (Fig. 9). The *P. acnes* DNA genome was detected in the lung tissue from non-treated mice (group 1). Thus, the *P. acnes* genome from each organ in group 1 was investigated. The *P. acnes* DNA number was $14.9 \pm 3.5 \times 10^3$ in the lung, $9.1 \pm 3.1 \times 10^3$ in the BAL cells, $7.0 \pm 8.0 \times 10^3$ in the lymph nodes, $5.2 \pm 1.9 \times 10^3$ in the liver, and $3.2 \pm 3.7 \times 10^3$ in the spleen (Fig. 10). The genome number of *P. acnes* was higher in the lungs, BAL cells, and lymph nodes than in the liver and spleen; moreover,

the number of genomes in the lungs was significantly higher than that in the liver ($p < 0.05$) and in the spleen ($p < 0.01$).

Discussion

Although sarcoidosis is thought to result from an exposure of genetically susceptible subjects to a specific environmental agent(s), its etiology remains unknown. Sarcoidosis is a systemic disease characterized by the presence of noncaseating epitheloid cell granulomas. It commonly involves the lungs, eyes, lymph nodes, and skin. A causative agent appears to activate alveolar lymphocytes, which leads to alveolitis and the development of epitheloid cell granulomas [13]. This process then results in an increase in the T-lymphocyte concentration and the CD4+/CD8+ ratio in the BAL fluid, as well as leads to the release of various cytokines [14]. Although many putative causative agents have been investigated, no single agent has been identified [1-3]. *P. acnes*, an anaerobic gram-positive rod, is the only bacterium to be isolated in cultures from biopsy samples of lymph nodes from patients with sarcoidosis [5]. In this study, we experimentally produced a murine pulmonary granuloma model using *P. acnes* with several features that simulate sarcoidosis.

Mice were immunized with heat-killed *P. acnes* suspended in FCA, and then received a boost of heat-killed *P. acnes* alone at 2-week intervals. The formation of epitheloid cell granulomas occurred after the second boost. Moreover, tight epitheloid granulomas with giant cells were found in the group that had received 3 boosts. We performed a series of examinations to elucidate the pathological conditions in granulomatous mice. Initially, the BAL cell profiles in granulomatous mice showed elevated total cell and lymphocyte counts. The CD3+ and CD4+ lymphocyte count and the CD4+/CD8+ ratio were higher than those of the controls. In particular, there were significantly higher levels of these factors in group 3, which received 3 boosts. Furthermore, serum ACE activity was significantly elevated in the sensitized groups. In addition, the antibody activities in response to *P. acnes* in the serum were significantly elevated in groups 2 and 3. Based on these results, it was determined that *P. acnes* sensitization could produce experimental granulomatous inflammation in the

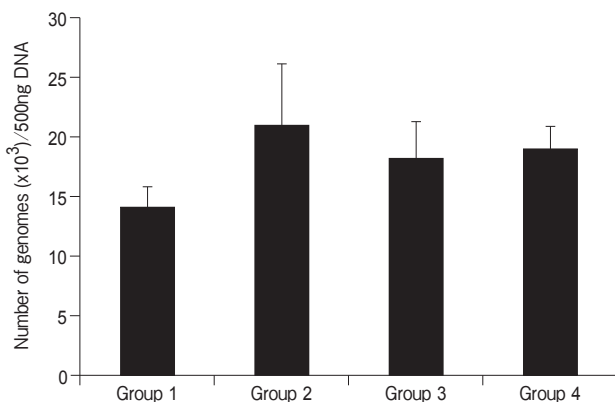


Fig. 9 Number *P. acnes* DNA genomes of from lungs in each group, as determined by quantitative real-time polymer chain reaction. Values (/500 ng DNA) are mean \pm SEM.

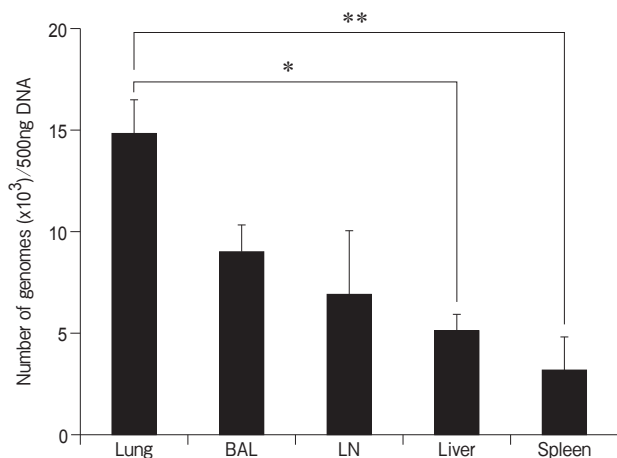


Fig. 10 Number of genomes of *P. acnes* DNA genomes from lungs, bronchoalveolar (BAL) fluid, lymph nodes (LN), liver, and spleen of control mice (group 1) was determined by quantitative real-time polymer chain reaction. Values (/500 ng DNA) are mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ versus lungs.

lungs of mice. Furthermore, this model imitates the clinical features of sarcoidosis in humans.

Ejiri and co-workers [15] reported that an experimental granuloma model in guinea pigs could be produced by an intratracheal challenge with *P. acnes*; however, no detailed analysis (e.g., of lymphocyte subsets of BAL cells) was performed. *P. acnes* has been recognized as a potent immunostimulant capable of producing granulomatous inflammation in the liver in experimental animals after intravenous injection with heat-killed organisms [16]. Although the mechanisms of hepatic granuloma formation in response to *P. acnes* have been extensively investigated, little has been reported on the pulmonary immune response to this anaerobic bacterium [17, 18]. Though sensitization and boosters were applied at extrapulmonary sites in the present model, granuloma formation occurred in the lungs. On the basis of our observations, we reasoned that a target antigen for inducing granulomatous inflammation might have already been present in the lungs. We thus attempted to detect the *P. acnes* genome in lung tissues using real-time PCR, and we confirmed that the genome of *P. acnes* was indeed present in all groups. In addition, the genomes of *P. acnes* DNA were present in the lungs, BAL cells, hilar lymph nodes, liver, and spleen of control mice. Furthermore, granuloma formation was found in group 4, in which sensitization was performed only using *P. acnes*, i.e., without complete Freund's adjuvant. These granulomas appeared to be caused by pre-existing *P. acnes* in normal mice. McCaskill *et al.* [19] produced pulmonary granulomas in C57BL/6 mice by intraperitoneal sensitization and intratracheal challenge with heat-killed *P. acnes*. Although in that study, the routes of sensitization and challenge differed from those used here, the pulmonary immune response in their series supported our results. In group 4, mice were only challenged using heat-killed *P. acnes*, without sensitization by a mixture of CFA and *P. acnes*. The findings indicate that pre-existing *P. acnes* in mice, which had been thought to be germ-free, caused the development of pulmonary granulomas. Nishiwaki *et al.* [20] successfully produced pulmonary granulomas via the transfer of *P. acnes*-primed T-lymphocytes to non-sensitized mice. They proposed an indigenous mechanism in the pulmonary immune response using *P. acnes*. It is already known that *P. acnes* is an indigenous bacterium in the skin, airway,

and bowel of humans [21]. *P. acnes* is also thought to be an indigenous bacterium in the lower respiratory tract and regional lymph nodes, via the airway, and also appears in the liver and spleen, via the bowel, in mice. In our model, we examined the lungs, hilar lymph nodes, liver, and spleen histologically, but granulomatous formations were seen only in the lungs, where the genome number of *P. acnes* was highest among those of all organs examined. Because tightly-formed granulomas were found, with density increasing in proportion to the time of challenge, and as antigen levels were relatively high in areas of granuloma formation, indigenous *P. acnes* is thought to be important for granuloma formation in this murine model of sarcoidosis. However, it should be noted that the pathogenesis of sarcoidosis in human is also dependent on genetic factors such as HLA and immunity-related genes [13], and the etiological mechanisms of this disease remain unclear.

The present results suggested that *P. acnes* sensitization could cause pulmonary inflammation, including granuloma formation, similar to the clinical features of sarcoidosis. It is therefore reasonable to expect that *P. acnes* plays an important etiological role in the pathogenesis of sarcoidosis. The present mouse model may be eventually used to develop new treatments for sarcoidosis.

Acknowledgments. This work was supported in part by funding from a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (grant No. 15590489 to M.K.).

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